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Inhibition of homologous recombination by a cohesin-associated clamp complex recruited to the rDNA recombination enhancer

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Silencing within the yeast ribosomal DNA (rDNA) repeats protects the integrity of this highly repetitive array by inhibiting hyperrecombination and repressing transcription from foreign promoters. Using affinity purification combined with highly sensitive mixture mass spectrometry, we have analyzed the protein interaction network involved in suppressing homologous recombination within the rDNA locus. We show that the Net1 and Sir2 subunits of the RENT (regulator of nucleolar silencing and telophase exit) silencing complex, and Fob1, which recruits RENT to the nontranscribed spacer I (NTS1) region of rDNA, are physically associated with Tof2. In addition to RENT components and Fob1, Tof2 copurified with a two-subunit complex composed of Lrs4 and Csm1. Tof2, Lrs4, and Csm1 are recruited to the NTS1 region by Fob1 and are specifically required for silencing at this rDNA region. Moreover, Lrs4 and Csm1 act synergistically with Sir2 to suppress unequal crossover at the rDNA and are released from the nucleolus during anaphase. Together with previous observations showing that Csm1 physically associates with cohesin, these findings suggest a possible model in which RENT, Tof2, and Lrs4/Csm1 physically clamp rDNA to the cohesin ring, thereby restricting the movement of rDNA sister chromatids relative to each other to inhibit unequal exchange.

[*Keywords*: rDNA silencing; rDNA recombination; Lrs4; Csm1; cohesin; Sir2]

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Eukaryotic genomes contain a wide variety of repetitive DNA, including arrays of essential genes, transposons, and retroelements. Such repetitive sequences are attractive substrates for homologous recombination events, some of which may lead to unwanted chromosomal rearrangements or repeat instability due to unequal crossover between sister chromatids. Cells have therefore evolved mechanisms that protect regions such as the ribosomal DNA (rDNA) locus, a prime example of a highly repetitious segment of the genome whose stability is absolutely critical for growth and survival.

In all eukaryotes, rDNA is organized as one or more arrays containing anywhere from <100 to >10,000 repeating units, which can form one or more nucleoli where rRNA synthesis, processing, and assembly into ribosomes occur (Nomura 2001). How the integrity of rDNA is maintained is not well understood, but has been extensively studied in the budding yeast *Saccharomyces cerevisiae*, which has 100–200 copies of rDNA tandemly repeated (Petes and Botstein 1977). However, rDNA recombination rates in budding yeast are significantly lower than would be expected for such a large, repetitive locus, indicating that recombination within the array is negatively regulated (Petes 1980). Such regulation is important for suppression of unequal recombination events that cause loss of repeats or generate extrachromosomal rDNA circles, the latter of which leads to premature cellular senescence in this organism (Sinclair and Guarente 1997).

Several lines of evidence indicate that suppression of recombination at rDNA is related to gene silencing, a form of transcriptional repression analogous to the heterochromatin found at repetitive regions in larger eukaryotes. First, rDNA recombination levels in *S. cerevisiae* are down-regulated by *SIR2* (Gottlieb and Esposito 1989), an NAD^+-dependent deacetylase (Imai et al. 2000;

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Landry et al. 2000) originally identified for its roles in transcriptional silencing at the mating-type cassettes and at telomeres (Ivy et al. 1986; Rine and Herskowitz 1987; Gottschling et al. 1990). Furthermore, *SIR2* is also required for "rDNA silencing," the suppression of both Ty1 transposition and RNA polymerase II (Pol II)-dependent transcription within rDNA (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). These and other observations, such as the altered sensitivity of rDNA to micrococcal nuclease, *dam* methylation, and psoralen cross-linking in *sir2*Δ mutants (Fritze et al. 1997; Smith and Boeke 1997), support a model in which silencing mechanisms create an altered chromatin structure at this locus that is also refractory to recombination.

The mechanism of silencing is best understood at the mating-type cassettes and at telomeres. Silencing initiates via the association of DNA-binding proteins that recruit the SIR complex, consisting of Sir2, Sir3, and Sir4 proteins (Rusche et al. 2003), with chromatin. Sir2 likely deacetylates the N-terminal tails of histones, while Sir3 and Sir4 are structural components of silent chromatin that bind to these deacetylated tails (Hecht et al. 1995). Repeated rounds of deacetylation and histone binding result in stepwise spreading of SIR complexes along the chromatin fiber (Moazed 2001; Rusche et al. 2003). Of the Sir proteins, only Sir2 is required for rDNA silencing (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). Sir2 is part of a separate complex that regulates rDNA-specific silencing and cell cycle progression, called RENT (regulator of nucleolar silencing and telophase exit), which contains two other subunits, Net1 (also known as Cfi1) and Cdc14. Net1 is located in the nucleolus and recruits Sir2 to rDNA (Straight et al. 1999), and specific alleles of Sir2 selectively abolish binding to Net1 (Cuperus et al. 2000). Net1 also sequesters the protein phosphatase Cdc14 in the nucleolus until the RENT complex disassembles during late anaphase (Shou et al. 1999; Visintin et al. 1999). The resulting release of Cdc14 permits cells to exit from mitosis (Visintin et al. 1998; Shou et al. 1999).

Chromatin immunoprecipitation experiments show that RENT subunits are primarily associated with two regions of the rDNA unit (Huang and Moazed 2003; Stegmeier et al. 2004). In budding yeast, each 9.1-kb repeat yields a 35S precursor rRNA (transcribed by RNA Pol I) and a 5S rRNA (transcribed by RNA Pol III), separated by two nontranscribed spacers, NTS1 and NTS2 (Petes and Botstein 1977). RENT preferentially cross-links with DNA sequences within NTS1 and a region spanning the RNA Pol I promoter (TIR, transcription initiation region) and the 5′ end of the 35S coding region (Fig. 2A, below; Huang and Moazed 2003; Stegmeier et al. 2004). Silencing factors are recruited to these regions using distinct pathways, suggesting that silencing at these regions may regulate different biological functions (Huang and Moazed 2003).

NTS1 contains several sequence elements that stimulate recombination and also establish a polar replication fork block (RFB) (Keil and Roeder 1984; Brewer and Fangman 1988). Both the recombination and fork-blocking activities require the fork-blocking protein Fob1, which can directly bind to sequences in this region (Kobayashi and Horiuchi 1996; Kobayashi 2003; Mohanty and Bastia 2004). Deletion of *FOB1* results in both the suppression of recombination and a complete loss of rDNA silencing specifically at NTS1, and cross-linking studies indicate that association of Sir2 with this region is abolished (Huang and Moazed 2003). Furthermore, Fob1 physically interacts with the RENT complex in vivo, indicating that Fob1 not only stimulates recombination but also represses it through recruitment of the RENT complex to NTS1 (Huang and Moazed 2003). Factors responsible for recruiting the RENT complex to the NTS2 region have not been identified, but Pol I is an attractive candidate since it is required for silencing of Pol II promoters (Buck et al. 2002; Cioci et al. 2003) and physically interacts with RENT in vivo and in vitro (Shou et al. 2001; Huang and Moazed 2003).

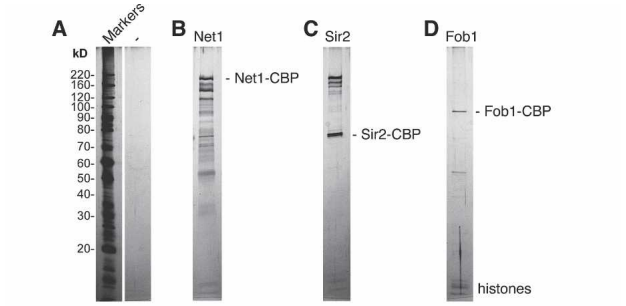
Once silencing factors are recruited to rDNA, it is unknown how they prevent recombination. Some observations are not fully consistent with models in which silencing renders rDNA chromatin less accessible to recombination (and transcriptional) machinery. For example, rDNA is highly active (60% of total transcription in rapidly growing yeast cells) (Woolford and Warner 1991), and Pol I transcription is not dramatically affected by *SIR2*-dependent silencing mechanisms (Shou et al. 2001; Sandmeier et al. 2002). In addition, the rate of mitotic recombination within individual rDNA units on the same chromatid is unchanged despite the deletion of *SIR2*, whereas unequal recombination between sister chromatids increases (Kobayashi et al. 2004). Rather than preventing access to DNA in a nonspecific manner, which is a general property of heterochromatic silencing mechanisms, rDNA silencing complexes may alter chromatin in ways that allow the assembly of specialized structures that regulate recombination.

Here we have analyzed the composition of core rDNA silencing complexes in budding yeast using a combination of native protein affinity purifications and highly sensitive mixture mass spectrometry analysis. We find that known rDNA silencing factors interact with Tof2, a nucleolar protein that is required for silencing specifically at the NTS1 region of rDNA. Tof2 predominantly cross-links to NTS1 sequences that overlap the binding site of the fork-blocking protein Fob1, and requires Fob1 for its association with NTS1. Purification of Tof2 shows that it interacts with Lrs4 and Csm1, which have previously been shown to play critical roles in chromosome segregation during meiosis I as subunits of the monopolin complex, which also contains the meiotic-specific protein Mam1. Lrs4 and Csm1 are both required for silencing at NTS1 and associate with the NTS1 region in a Fob1- and Tof2-dependent, but Sir2-independent, manner. Moreover, Lrs4/Csm1 are required for suppression of unequal recombination within rDNA and act synergistically with Sir2 to suppress recombination in rDNA. Finally, the mitotic exit network is required for the release of Lrs4 and Csm1 from the nucleolus during anaphase.

Results

Affinity purification of rDNA silencing complexes

To better understand the mechanisms that regulate recombination at the rDNA, we affinity-purified factors that both stimulate and suppress rDNA recombination from budding yeast extracts using the tandem affinity purification (TAP) method (Rigaut et al. 1999). The endogenous *NET1*, *SIR2*, and *FOB1* genes were modified to produce proteins with C-terminal TAP tags, each of which was fully functional for silencing (Huang and Moazed 2003; Tanny et al. 2004). The TAP tag is a dual epitope tag consisting of a calmodulin-binding peptide (CBP) separated from two Protein A repeats by a TEV-protease cleavage site (Rigaut et al. 1999). Following purification, the complexes were visualized by SDS-PAGE followed by silver staining (Fig. 1A–D). Additionally, the complexes were TCA-precipitated, digested with trypsin, and analyzed by liquid chromatography coupled with tandem mass spectrometry analysis (LC-MS/MS) (Peng and Gygi 2001; Tanny et al. 2004). Analysis of the entire mixture permits the identification of peptides from proteins that are not highly abundant and/or not readily visible on Coomassie- or silver-stained polyacrylamide gels and would therefore not be selected for individual band analysis, making it possible to identify factors that may interact transiently or peripherally with core complex components. The lists of specific proteins presented for each of the purifications in this study exclude proteins that were also found in parallel untagged/mock purifications as well as peptides from likely contaminants, such as ribosomal subunits or heat-shock proteins, which copurify with many different types of complexes (Supplementary Table 1). In general, we observe a correlation between the number of peptides that are identified by LC-MS/MS and the relative abundance of each protein in the purified mixtures, so that stoichiometric components of complexes are represented by peptides that correspond to a similar percentage of protein length (referred to as percent coverage).



E LC-MS/MS of total mixtures
Protein (#unique peptides, coverage)

Net1-TAP (I)	Sir2-TAP (I)	Sir2-TAP (II)	Fob1-TAP (I)	Fob1-TAP (II)
Net1 (98, 38%)	Sir4 (77, 52%)	Sir4 (105, 64%)	**Fob1** (32, 41%)	**Fob1** (36, 42%)
Sir2 (16, 34%)	Net1 (45, 41%)	Net1 (68, 49%)	Net1 (6, 8%)	Tof2 (9, 18%)
Tof2 (22, 34%)	**Sir2** (27, 38%)	**Sir2** (28, 47%)	Tof2 (5, 9%)	H2B (3, 15%)
Cdc14 (27, 58%)	Sir1 (8, 15%)	Sir1 (15, 27%)	H2B (2, 8%)	Net1 (2, 2%)
5 pol I subunits	Cdc14 (6, 16%)	Cdc14 (12, 27%)	Cdc14 (1, 2%)	Cdc14 (2, 4%)
	Tof2 (4, 6%)	Tof2 (9, 14%)	H1 (1, 4%)	
Net1-TAP (II)	H2B (1, 7%)	Sir3 (6, 8%)	H2A (1, 5%)	
Net1 (109, 57%)	Sir3 (1, 1%)		H4 (1, 12%)	
Sir2 (21, 37%)				
Tof2 (11, 22%)	Kap95 (16, 19%)	Kap95 (14, 18%)	Top1 (9, 14%)	Top1 (13, 18%)
Cdc14 (10, 27%)	Srp1 (6, 15%)	Srp1 (8, 23%)	Reb1 (5, 10%)	Nog1 (4, 7%)
	Rfm1 (3, 10%)	Rfm1 (2, 7%)	Nog1 (1, 2%)	Reb1 (1, 1%)
Csm1 (2, 22%)		Rtn1 (1, 4%)		

F

Figure 1. Affinity purifications of rDNA silencing complexes. Silver-stained SDS-PAGE gels of complexes purified from untagged (*A*), Net1-TAP (*B*), Sir2-TAP (*C*), and Fob1-TAP (*D*) cells; 2.5% of the total purified material is shown. (*E*) The results of the total mixture analysis by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). (*F*) The protein sequence alignment of the N termini of Tof2 and Net1 indicates 30% identity and 53% similarity within the N-terminal 250 amino acids (shaded area). (CBP) Calmodulin-binding protein.

As expected, Net1 complexes consisted primarily of Net1, Sir2, and Cdc14 (Fig. 1B,E; Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999; Tanny et al. 2004). One purification also contained several subunits of Pol I, while in the second, Pol I subunits appeared in mock purifications from untagged strains, likely due to sheer abundance. However, the interaction between Net1 and Pol I is supported by other evidence. First, Pol I subunits do not copurify with the other complexes studied here. Second, Pol I is required for rDNA silencing (Buck et al. 2002; Cioci et al. 2003). Finally, we and others have observed that Net1 physically interacts with Pol I in vivo and in vitro (Shou et al. 2001; Huang and Moazed 2003).

Net1 and Sir2 purifications also contained a large number of peptides from Tof2. In particular, in Net1 purifications, Tof2 peptides were represented with similar abundance to Sir2 and Net1 (Fig. 1E). Tof2 was originally identified in a yeast two-hybrid screen for topoisomerase I-interacting factors (Park and Sternglanz 1999), and a previous purification of RENT had also identified a small number of peptides from Tof2 (Tanny et al. 2004). However, no physiological function has been ascribed to Tof2. Tof2 and Net1 share 22% identity (40% similarity) (Park and Sternglanz 1999; Shou et al. 1999; Straight et al. 1999), with the region of highest conservation (30% identity, 53% similarity) located within the first 250 amino acids of both proteins (Fig. 1F).

Supporting the finding that Tof2 copurifies with both Net1 and Sir2 complexes, immunoprecipitation of either Net1-GFP or Sir2 results in the coprecipitation of Tof2-HA3 (Supplementary Fig. 1A). Consistent with previous results, Sir2 purifications also yielded interacting subunits from both the RENT complex (Net1 and Cdc14) and the SIR mating-type/telomeric silencing complex (Sir3, Sir4) (Fig. 1C,E). Surprisingly, we also found a significant number of peptides from Sir1 (15%–27% amino acid sequence coverage), which binds to DNA-binding factors and recruits the other Sir proteins to mating-type silencers (Rusche et al. 2003). Sir1 has not previously been shown to copurify with the entire SIR complex, although an interaction between Sir1 and overexpressed Sir3 has been detected (Ho et al. 2002). Our findings suggest the existence of a soluble form of the SIR complex that includes Sir1.

Purification of Sir2 also reproducibly yielded many peptides from the α and β subunits of the importin family, Srp1 (Ima1) and Kap95 (Imb1). Importin-α and importin-β form a heterodimer that mediates bidirectional translocation of hundreds of proteins across the nuclear envelope, but each protein also participates in cellular processes unrelated to transport (Goldfarb et al. 2004). Interestingly, screens for genes that bypass the requirement for the kinase Cdc15 to exit from mitosis have identified alleles of not only *CDC14* and *NET1* (Shou et al. 1999; Visintin et al. 1999), but also of several karyopherins (*SRP1*, *MTR10*, and *KAP104*) (Asakawa and Toh-e 2002; Shou and Deshaies 2002). Finally, Sir2 purifications contained two to three peptides of Rfm1 (7%–10% amino acid coverage), a DNA-binding protein that is required for the recruitment of Sum1 and Hst1, a yeast Sir2 homolog, to middle sporulation genes (Xie et al. 1999; McCord et al. 2003).

Purification of Fob1 yielded low amounts of Net1, Cdc14, and Tof2 peptides, consistent with previously observed physical interactions between Fob1 and RENT (Fig. 1D, E; Huang and Moazed 2003) and Net1 and Tof2 (Fig. 1E). In addition, Fob1 purifications contained a significant number of Top1 (topoisomerase I) peptides. The copurification of Tof2 and Top1 with Fob1 is consistent with the previous identification of Tof2 as a Top1-interacting protein in a yeast two-hybrid screen (Park and Sternglanz 1999). Furthermore, Top1 is required for rDNA silencing at NTS2 (Smith et al. 1999) and NTS1 (Supplementary Fig. 4), prevents Pol II transcription-dependent Ty1 transposition (Bryk et al. 1997), suppresses mitotic recombination within rDNA, and modulates rDNA chromatin structure (Christman et al. 1988, 1993; Cavalli et al. 1996). Fob1 purifications also contained a small number of peptides from Reb1, the 35S rRNA transcription termination factor (Lang et al. 1994), and Nog1, a nucleolar GTPase that is involved in ribosome biogenesis and nuclear transport (Park et al. 2001; Jensen et al. 2003; Kallstrom et al. 2003; Saveanu et al. 2003). These results are consistent with the observation that the RFB region overlaps the site of Pol I transcription termination, where Reb1 binds (Morrow et al. 1989), and with previous two-hybrid interactions detected between Fob1 and Nog1 (Ito et al. 2001).

Since Tof2 peptides were present in Fob1 complexes, it seemed likely that physical interactions, direct or indirect, between these proteins is responsible for recruitment of Tof2. To confirm this, we performed coimmunoprecipitation experiments from extracts prepared from yeast strains in which the endogenous copies of both *FOB1* and *TOF2* were modified to express Fob1-Myc13 and Tof2-HA3. Immunoprecipitation of Fob1-Myc13 resulted in the coprecipitation of both Tof2 and Sir2 (Supplementary Fig. 1B). In contrast, Fob1-Myc13 did not interact with actin, an abundant cytoskeletal protein.

Tof2 is an NTS1-specific rDNA silencing factor

The presence of Tof2 in Fob1, Net1, and Sir2 complexes strongly suggested that Tof2 might play a role in rDNA silencing. To test this possibility, we deleted *TOF2* in strains carrying an *mURA3* reporter gene integrated into one of three sites: outside the rDNA array at the *LEU2* gene and within the rDNA unit at two locations exhibiting strong silencing (NTS1 and NTS2 reporters) (Fig. 2A; Huang and Moazed 2003). Cells were 10-fold serially diluted and spotted on complete medium as a plating control and on medium lacking uracil to monitor expression of the reporter genes.

Consistent with previous observations, the reporter gene was strongly silenced at either the NTS1 or NTS2 sites, as indicated by poor growth on –URA medium compared with the same reporter inserted at a euchromatic locus (Fig. 2B, cf. rows 1 and 2,3; Huang and Moazed 2003). As expected, silencing was dependent on the presence of the *SIR2* gene (Fig. 2B, cf. rows 2,3 and 8,9). Similar to the region-specific silencing gene *FOB1*, deletion of *TOF2* resulted in complete derepression of the NTS1 reporter (Fig. 2B, cf. rows 2 and 5) but had no effect on silencing of the NTS2 reporter (Fig. 2B, cf. rows 3 and 6). Loss of NTS1 silencing was specific to *TOF2*, since addition of a single-copy plasmid containing the *TOF2* gene under the control of its native promoter restored silencing to *tof2*Δ cells (Supplementary Fig. 2A). Furthermore, Western blot analysis of whole-cell extracts (WCEs) showed that the amount of endogenous Sir2 was unaffected by deletion of *TOF2*, arguing that the loss of silencing at NTS1 was not due to changes in Sir2 protein levels (Supplementary Fig. 2B).

We next tested whether Tof2 is required for silencing at other heterochromatic regions, such as telomeres. *TOF2* was deleted in strains in which a *URA3* reporter gene was integrated within the telomeric repeats of Chromosome VIIL or ~15 kb away at the *ADH4* locus. Cells were spotted onto complete medium as a plating and growth control or onto medium supplemented with 5-fluoroorotic acid (5-FOA), which is toxic to cells expressing *URA3*. As expected, wild-type cells fully express *URA3* at the *ADH4* locus and were unable to grow on 5-FOA medium (Fig. 2C, row 1). In contrast, the telomeric *URA3* reporter was silenced efficiently, permitting growth on 5-FOA medium (Fig. 2C, row 2) in a *SIR2*-dependent manner (Fig. 2C, cf. rows 2 and 4; Aparicio et al. 1991). However, the absence of either *TOF2* or *FOB1* had no effect on telomeric silencing (Fig. 2C, cf. rows 2 and 6,8). Therefore, Tof2, like Fob1, is an NTS1-specific rDNA silencing factor.

To examine the subcellular location of Tof2, we performed immunofluorescence microscopy on cells in which the endogenous *TOF2* gene was modified to produce a protein with a C-terminal HA3 epitope tag (Tof2-HA3). Cells expressing Tof2-HA3 exhibited wild-type levels of silencing, suggesting that the tagged protein was functional (Supplementary Fig. 3A). For comparison, we also analyzed the localization of Net1-GFP, a well-characterized nucleolar marker (Straight et al. 1999). Similar to Net1-GFP, Tof2-HA3 formed compact and crescent-shaped structures and did not colocalize with the DNA-specific dye DAPI, which is typically excluded from the nucleolus (Fig. 2D). Merged images showed

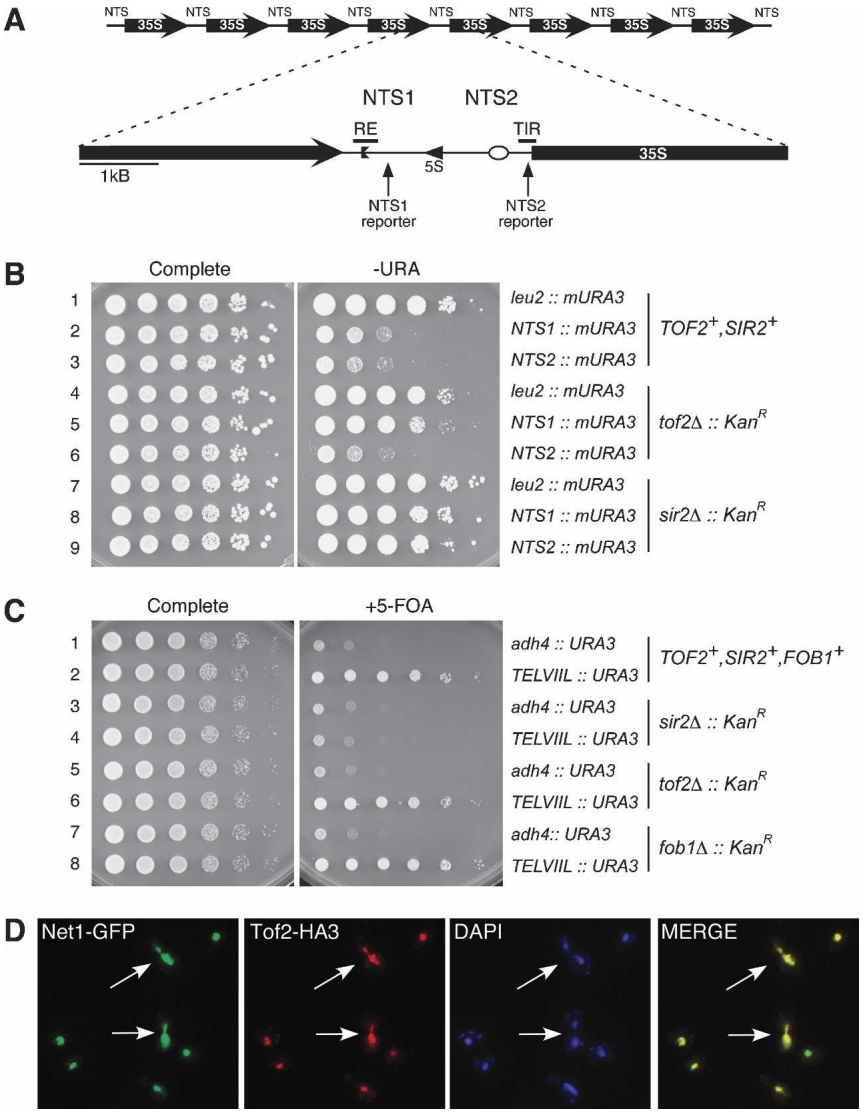


Figure 2. Tof2 is a nucleolar protein required specifically for rDNA silencing at NTS1. (*A*) The physical structure of the tandemly repeating *RDN1* locus of *S. cerevisiae* is shown *above*, and a single 9.1-kb rDNA unit is shown expanded *below*. Each repeat yields a Pol I-transcribed 35S precursor rRNA (shown as a divided thick arrow) and a Pol III-transcribed 5S rRNA (arrowhead). The 35S coding regions are separated by an NTS, which is divided by the 5S gene into NTS1 and NTS2. Solid bars indicate the recombination enhancer (RE) region and the Pol I TIR. The locations of the RFB (◤) and autonomously replicating sequences (⬭) are indicated. Vertical arrows indicate insertion sites of the NTS1 and NTS2/TIR silencing reporters. (*B*) *TOF2* is required for rDNA silencing at NTS1 but not at NTS2/TIR. Silencing within rDNA was assessed by monitoring the growth of 10-fold serial dilutions of cells plated on –URA medium. SC medium was used as a plating control. *TOF2* is required specifically for silencing only at NTS1, unlike *SIR2*, which is required for silencing at both NTS1 and NTS2. Locations of rDNA reporter genes are as indicated in Figure 2A. (*C*) *TOF2* and *FOB1* are not required for telomeric silencing. Silencing was assessed by monitoring the growth of 10-fold serial dilutions of cells on SC (synthetic complete) medium supplemented with 5-FOA. SC medium was used as a plating control. The *URA3* reporter gene was integrated either adjacent to the telomeric repeats of Chromosome VIIL (*TELVIIL*) or ~15 kb away, at the *ADH4* locus. (*D*) Tof2 colocalizes with nucleolar marker Net1. Immunofluorescence images show the subcellular localization of Net1-GFP (green), Tof2-HA3 (red), and DAPI-stained DNA (blue). The merged image shows that Net1-GFP and Tof2-HA3 colocalize to nucleolar domains that are nonoverlapping with the rest of the genome (yellow). Arrows indicate dividing cells.

strong colocalization of Tof2-HA3 and Net1-GFP, even in cells actively undergoing mitosis (Fig. 2D, arrows), demonstrating that Tof2 is a bona fide nucleolar protein.

Tof2 primarily associates with the NTS1 region of rDNA in a Fob1-dependent but Sir2-independent manner

The silencing phenotype, nucleolar localization, and physical interaction with Fob1 and RENT subunits suggested that Tof2 should be associated with rDNA. To test this possibility, we mapped the association of Tof2 with rDNA by chromatin immunoprecipitation (ChIP). The endogenous *TOF2* gene was modified to produce a protein with a C-terminal TAP tag (Tof2-TAP), which was fully functional for rDNA-NTS1 silencing (Supplementary Fig. 3B). Cells were cross-linked with formaldehyde, and Tof2-TAP was immunoprecipitated using IgG-Sepharose from WCEs containing sheared chromatin. WCE and immunoprecipitated (IP) chromatin from untagged and Tof2-TAP strains served as template DNA in quantitative PCR. rDNA was amplified to produce the PCR products schematically diagrammed in Figure 3B and as described previously (Huang and Moazed 2003). In parallel, Net1-TAP and Fob1-TAP, whose profiles of rDNA association have already been characterized, were also immunoprecipitated (Huang and Moazed 2003).

The overall association profile of Tof2-TAP with rDNA closely resembled that of Fob1-TAP (Fig. 3A,B; Huang and Moazed 2003). Tof2 primarily associated with the region of rDNA that precisely overlaps the RFB of NTS1, consistent with its specific requirement in NTS1 silencing (Fig. 2B). Also similar to what has been observed for Fob1, we observed a much smaller peak of Tof2 association with the TIR, although neither protein

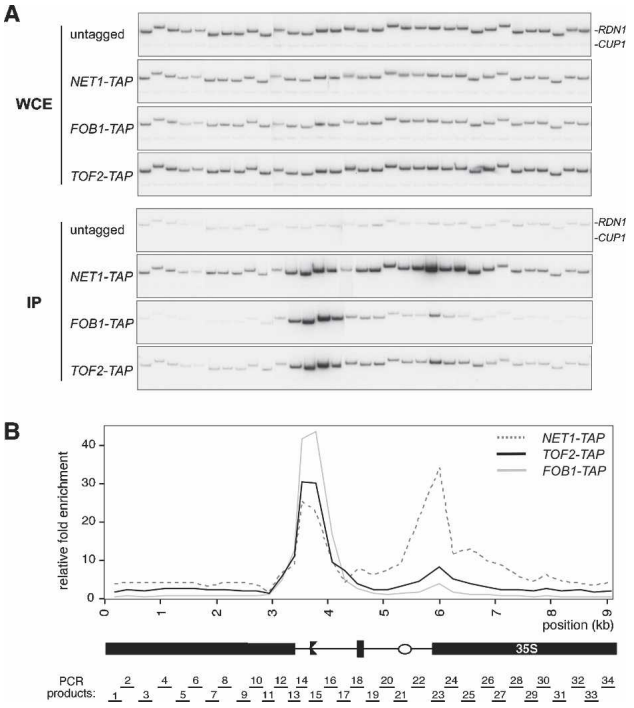


Figure 3. Tof2 associates primarily with the NTS1 region of rDNA. (*A*) PCR products amplified from WCE (*upper* panels) and IP (*lower* panels) chromatin. Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 1–4 and 6–34 are shown. (*B*) Representative graph showing the relative fold association of Tof2-TAP (solid black line), Net1-TAP (dashed gray line), and Fob1-TAP (solid gray line) across the rDNA repeat. A schematic representation of the rDNA unit is shown *below* the graph, with significant features shown as in Figure 2A and PCR products depicted *below*. Most of the Tof2-TAP is concentrated within NTS1, with a smaller peak observed near the border of NTS2 and the 35S rRNA coding region.

was required for silencing at this location (Fig. 2B; Huang and Moazed 2003). In contrast, Net1 associates with both NTS1 and NTS2/TIR regions (Fig. 3A,B; Huang and Moazed 2003).

To better understand the assembly of silencing complexes at NTS1, we examined the association of Tof2 with rDNA in the absence of both Fob1 and Sir2. Data from a representative experiment are shown in Figure 4A with quantification of relative fold enrichment of rDNA shown in Figure 4B. In the absence of *FOB1*, the association of Tof2 with the NTS1 region was completely abolished, while association with the NTS2/TIR region was unaffected. We conclude that, similar to what was observed for Net1 and Sir2 (Huang and Moazed 2003), *FOB1* is absolutely required for the localization of Tof2 specifically to NTS1, but there exists a *FOB1*-independent mechanism for localization of Tof2 to the TIR. However, in contrast to NTS1, the association for Tof2 with the TIR region is not required for silencing since deletion of *TOF2* has no effect on the silencing of a reporter gene inserted at NTS2 (Fig. 2B). Notably, the physical interaction detected between Sir2 and Tof2 is not required for the recruitment of Tof2 to rDNA. In the absence of *SIR2*, the association of Tof2-TAP with rDNA was completely unaffected (Fig. 4A,B). Thus, Tof2 is recruited to NTS1 primarily by Fob1.

Role of Tof2 in the association of Sir2 with NTS1

The localization of Tof2 primarily to NTS1 and its ability to localize to rDNA in the absence of Sir2 led us to ask whether Tof2 influences Sir2 assembly at NTS1. A representative example of these data is shown in Figure

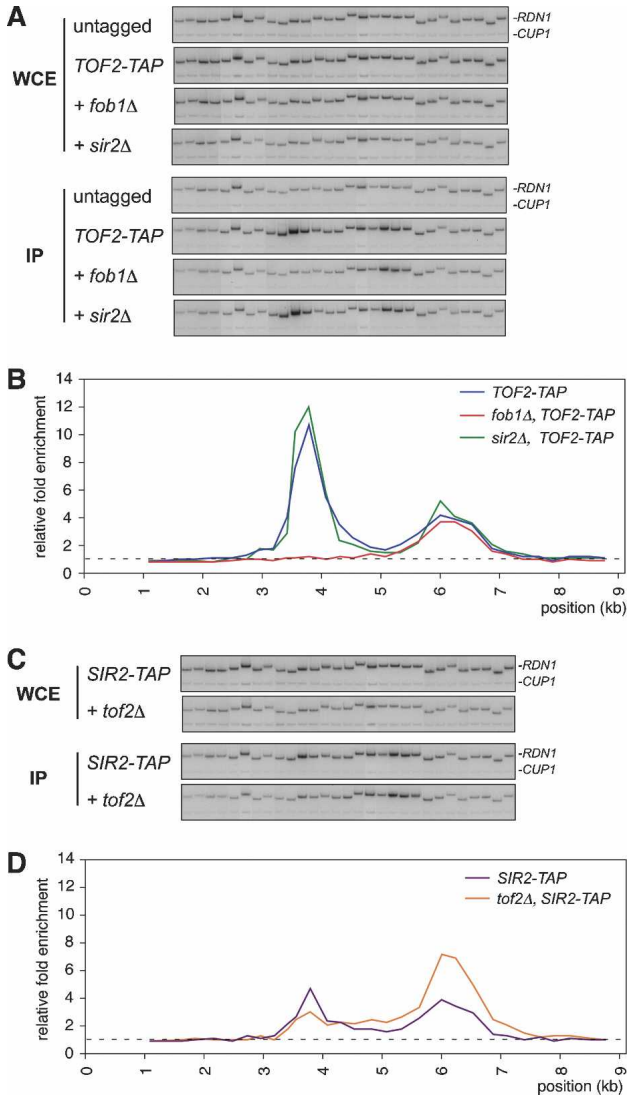


Figure 4. Tof2-TAP requires *FOB1* but not *SIR2* for association with NTS1. (*A*,*C*) Examples of ChIP data showing PCR products amplified from WCE and IP DNA. Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 4 and 6–33 are shown. (*B*) Representative graphs showing the association of Tof2-TAP across an rDNA repeat in wild-type (blue), *fob1*Δ (red), or *sir2*Δ (green) cells. (*D*) Sir2-TAP association with rDNA is largely Tof2 independent.

4C with quantification in Figure 4D. As previously observed, the mapping profile of Sir2-TAP displayed two regions of enrichment, located at NTS1 and NTS2/TIR (Fig. 4C,D; Huang and Moazed 2003). Deletion of the *TOF2* gene resulted in a partial loss of Sir2-TAP from NTS1 (approximately twofold reduced relative fold enrichment), while at NTS2/TIR, the association of Sir2 slightly increased (approximately twofold increased relative fold enrichment). These findings suggest that Sir2 does not require Tof2 for association with rDNA. Furthermore, the deletion of *TOF2* did not noticeably affect the amount of Net1-HA3 or Sir2 that associates with Fob1-Myc13, suggesting that Tof2 is also not required for the stability of RENT-Fob1 interactions (Supplementary Fig. 1C). We conclude that Tof2 represents a new NTS1-specific rDNA silencing factor that is recruited to NTS1 via Fob1 and contributes to rDNA silencing independently of Sir2 localization.

Tof2 is associated with subunits of the monopolin complex

Purification of Tof2-TAP complexes followed by mixture mass spectrometry analysis yielded peptides from Net1, Cdc14, and Sir2 (Fig. 5A,C). In addition, consistent with the observation that Fob1 physically interacts with Tof2 in vivo, we recovered several Fob1 peptides in Tof2 purifications. However, Top1, which has previously been shown to interact with Tof2 in a two-hybrid assay, did not copurify with Tof2 (Fig. 5C), raising the possibility that Tof2 and Top1 interact transiently or via Fob1 (see Fig. 1D,E). When we tested the requirement for *TOP1* in transcriptional silencing of the *mURA3* reporter gene located at either NTS1 or NTS2, we found that *TOP1* was required for silencing at NTS1 to the same extent as *SIR2* and, consistent with previous results (Bryk et al. 1997; Smith et al. 1999), loss of *TOP1* partially derepressed the silencing marker at NTS2 (Supplementary Fig. 4).

In addition to the above proteins, Tof2 copurified with Lrs4 and Csm1, two of three subunits previously identified as the monopolin complex, which co-orients sister chromatids during meiosis I (Fig. 5C; Toth et al. 2000; Rabitsch et al. 2003). The third subunit of monopolin, Mam1, is expressed only during meiosis I (Toth et al. 2000) and therefore would be absent from our mitotic extracts. Consistent with copurification with Tof2, both Lrs4 and Csm1 localize to the nucleolus during mitosis and most of meiosis (Rabitsch et al. 2003). Furthermore, each has been identified previously as required for rDNA silencing (Smith et al. 1999; Rabitsch et al. 2003). In addition, two-hybrid analysis detected an interaction be-

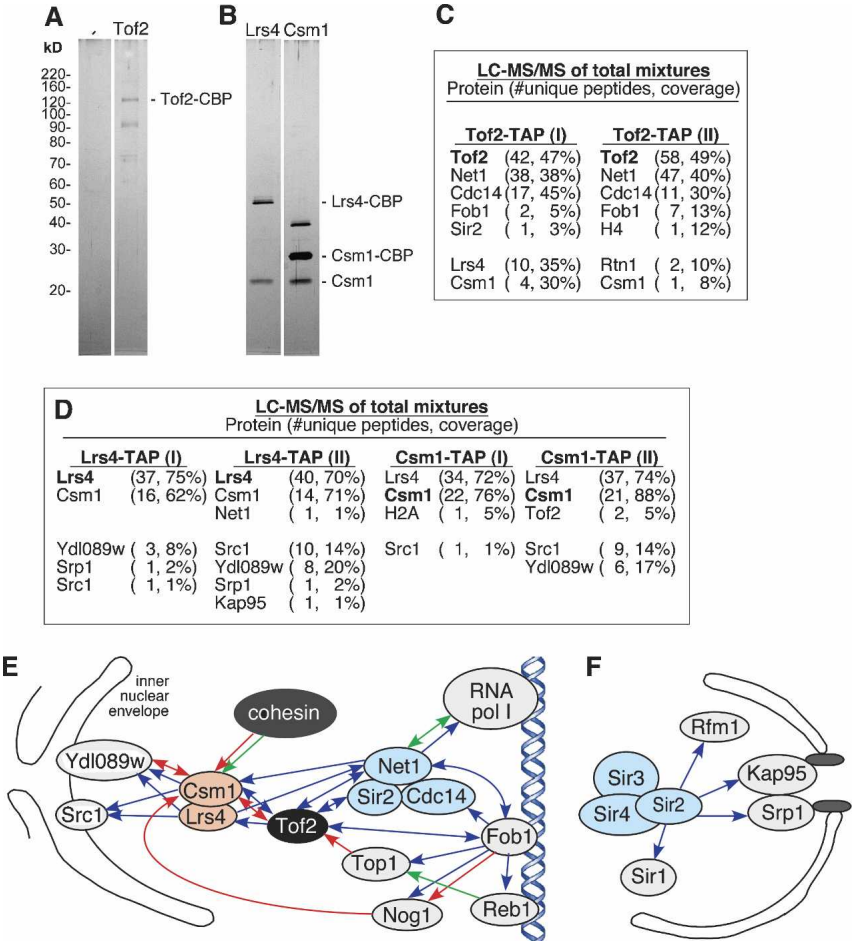


Figure 5. Affinity purifications of native Tof2, Lrs4, and Csm1 complexes. Silver-stained SDS-PAGE gels of native complexes purified from Tof2-TAP (*A*) and Lrs4-TAP and Csm1-TAP (*B*); 2.5% of total purified material is shown. (*C*,*D*) The results of the total mixture analysis by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). (*E*,*F*) Summaries of the protein–protein interaction network of silencing factors. Arrows indicate physical interactions determined by affinity purifications from this work (blue) or by others (green), or by yeast two-hybrid (red). Direction of the arrowheads is from bait toward interactor.

tween Tof2 and Csm1 (Ito et al. 2001; Wysocka et al. 2004), and one of our Net1 purifications yielded trace amounts of Csm1 (Fig. 1E).

We next purified the Lrs4 and Csm1 proteins via the TAP tag. While deletion of either gene results in slow growth on glucose (Smith et al. 1999; Rabitsch et al. 2003), modification of either protein with the TAP tag did not result in any obvious growth defects (J. Huang, unpubl.), and cells expressing either modified protein were competent for transcriptional silencing at rDNA (Supplementary Fig. 5), suggesting that these proteins were functional. Purification of either protein resulted in a complex consisting primarily of Lrs4 and Csm1 in stoichiometric amounts as shown by silver staining (Fig. 5B). Notably, the Csm1-TAP purification yielded not only the tagged form of Csm1, but also a faster migrating form that was identified by band analysis to be Csm1 lacking the C-terminal epitope tag (Fig. 5B). This observation suggests the presence of at least two Csm1 protomers in the Lrs4/Csm1 complex. The truncated form of Csm1-CBP is likely to result from proteolytic cleavage during purification. The Lrs4 and Csm1 purifications yielded trace amounts of both Net1 and Tof2 (Fig. 5D), further supporting the existence of a network of protein–protein interactions that link these proteins to the RENT complex. Lrs4-TAP purifications also yielded minor amounts of the importins Srp1 and Kap95 as well as significant numbers of peptides from two putative inner nuclear envelope proteins: Src1, which shares homology with the human Lap2 protein (Supplementary Fig. 6) and Ydl089w (Fig. 5D; Huh et al. 2003). The significance of these interactions is discussed in the Supplemental Material. A summary of the interactions uncovered by our purifications and those described in the literature is presented in Figure 5, E and F.

Lrs4 and Csm1 associate primarily with NTS1 in a Tof2- and Fob1-dependent manner and are required for silencing at NTS1

We next tested whether the previously observed presence of Lrs4/Csm1 in the nucleolus (Rabitsch et al. 2003) was due to their association with rDNA by performing ChIP assays using Lrs4-TAP and Csm1-TAP. Examples of PCR products amplified from WCE or IP material and quantifications of these data are shown in Figure 6. The results showed that Lrs4-TAP and Csm1-TAP IP sequences primarily from the NTS1 region, overlapping the RFB. A slight enrichment was also observed at the NTS2/TIR region, similar to what has been observed for Fob1 and Tof2 proteins. In the absence of *TOF2*, neither protein precipitated NTS1 sequences significantly above background, suggesting that their recruitment to this region may require physical interactions with Tof2 (Fig. 6). Furthermore, *FOB1* was also required for the localization of both subunits to NTS1, either through additional physical interactions or indirectly through the requirement for Fob1 to properly localize Tof2 to the region (Fig. 6). However, in the absence of Sir2, both proteins still associated with the NTS1 region, although Csm1 associated with NTS1 less efficiently. These findings suggest that Sir2 may help stabilize Lrs4/Csm1 at this region but is not absolutely required.

Previously, *LRS4* and *CSM1* were shown to be required for silencing using *mURA3* (in NTS2) or *MET15* (in 35S) reporter genes, respectively (Smith et al. 1999; Rabitsch et al. 2003). We tested whether *LRS4* and *CSM1* were required for silencing of the *mURA3* reporter gene integrated at either NTS1 or NTS2. We found that the NTS1 reporter was completely derepressed in both deletion

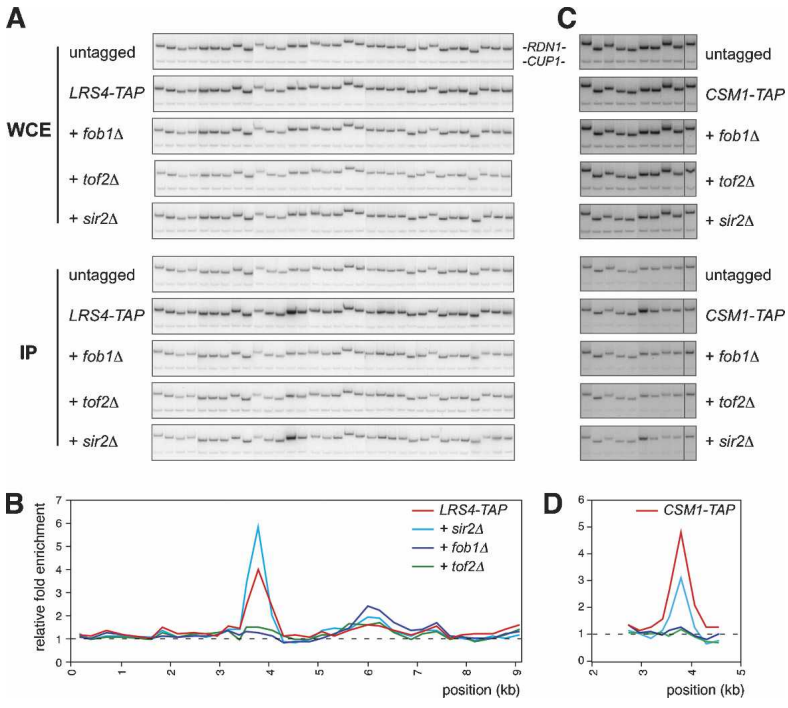


Figure 6. Lrs4-TAP and Csm1-TAP associate with NTS1 in an *FOB1*- and *TOF2*-dependent manner. Examples of ChIP data showing PCR products amplified from WCE and IP DNA associated with Lrs4-TAP (*A*) and Csm1-TAP (*C*). Multiplex PCR was performed to amplify *RDN1* and *CUP1* sequences as indicated. PCR products 2–4, 6–34, and 1 (*A*) and 10–18 and 23 (*C*) are shown. Quantifications of these data are shown in *B* and *D*. Both Lrs4-TAP and Csm1-TAP associate significantly with NTS1 but not with the NTS2/35S region.

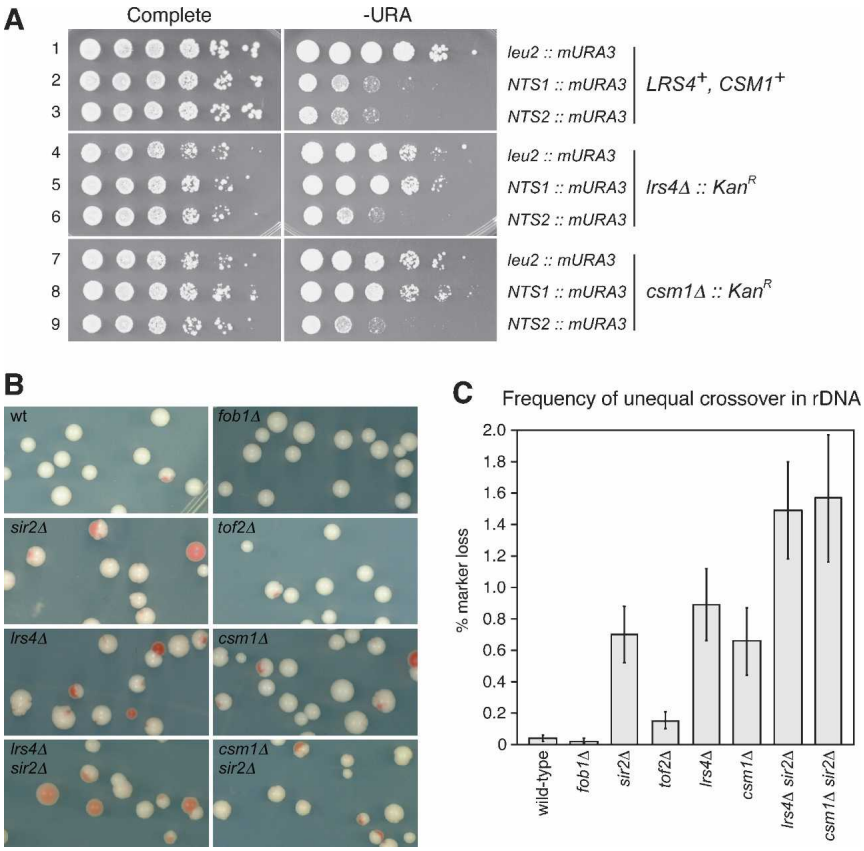


Figure 7. *LRS4* and *CSM1* are required for NTS1 silencing and unequal sister chromatid exchange. (*A*) Silencing was assayed as described in Figure 2B, and locations of reporters are shown in Figure 2A. Cells lacking *LRS4* or *CSM1* exhibit a complete loss of silencing at NTS1 but wild-type levels of silencing at NTS2. (*B*) Unequal sister chromatid exchange is monitored by loss of the *ADE2* gene located within the rDNA array. Cells expressing *ADE2* are white, while cells lacking the *ADE2* gene are red. Half-sectored colonies represent loss of the marker during the first division upon plating. Entirely red colonies are descended from a cell that has lost the marker prior to plating. (*C*) Unequal sister chromatid exchange is represented as percent marker loss, calculated as the ratio of half-sectored colonies to the total number of colonies, excluding entirely red colonies.

mutants (Fig. 7A, cf. rows 5,8 and 4,7, respectively). However, deletion of either *LRS4* or *CSM1* had only a weak effect on the NTS2/TIR reporter, which was silenced ~10^3-fold better than the NTS1 reporter (Fig. 7A, cf. rows 6,9 and 5,8). The difference in silencing defects at NTS1 compared with NTS2/TIR in these mutant backgrounds is consistent with our observations that Tof2, Lrs4, and Csm1 are primarily localized to the NTS1 region of rDNA (Figs. 3, 6).

LRS4 *and* CSM1 *are required for suppression of rDNA recombination*

We hypothesized that Tof2, Lrs4, and Csm1 may assemble at the NTS1 region to cooperate with the RENT complex in generating a chromatin structure that inhibits unequal recombination within the rDNA. To test this, we measured the rate of *ADE2* marker loss from the rDNA array in cells lacking each of the NTS1-specific silencing factors, described above, as well as Sir2. Colonies in which the *ADE2* marker has been lost accumulate a red pigment, while colonies that maintain and express *ADE2*, which is only weakly silenced in rDNA, remain white. The rate of unequal recombination or exchange is determined by the number of half-sectored colonies compared with the total number of colonies present (Kaeberlein et al. 1999). Half-sectored colonies signify a marker loss event during the first division after plating. Entirely red colonies have lost the marker prior to plating and thus were excluded from the total number of colonies. Loop-out events (recombination between repeats on the same chromosome) do not produce half-sectored colonies because the excised *ADE2* marker lacks a centromere and is preferentially retained in the mother cell (Murray and Szostak 1983). Examples of colonies are shown in Figure 7B, and quantification of unequal crossover in rDNA is presented in Figure 7C.

As expected, deletion of *FOB1* decreased the rate of marker loss by half compared with wild-type cells, while deletion of *SIR2* greatly increased it (>20-fold) (Fig. 7C; Kaeberlein et al. 1999). Similarly, deletion of either *LRS4* or *CSM1* caused dramatic increases in marker loss rate (29- and 21-fold, respectively) (Fig. 7C). Combining either the *lrs4* or *csm1* mutation with deletion of *SIR2* had an additive effect, with double mutants exhibiting a nearly 50-fold increase in marker loss compared with wild-type cells (Fig. 7C). Thus, Lrs4/Csm1 and Sir2 may regulate recombination via independent mechanisms. However, although Tof2 was required for the association of Lrs4/Csm1 with rDNA-NTS1, the deletion of *TOF2* caused a smaller increase in recombination rates (less than fivefold) (Fig. 7C), suggesting that, like Fob1, Tof2 may be required for both rDNA recombination and silencing.

Lrs4 and Csm1 are released from the nucleolus during anaphase

The RENT complex disassembles during anaphase, releasing the phosphatase Cdc14 (as well as Sir2) from the nucleolus and promoting exit from mitosis (Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999). To test whether the Lrs4/Csm1 complex is also released from rDNA during mitosis, we examined the subcellular localization of each protein throughout one complete cell cycle. Cells carrying an HA-tagged version of Lrs4 (Lrs4-6HA) were arrested with α-factor in G1 followed by release into medium lacking pheromone. Lrs4-6HA was released from the nucleolus and became dispersed throughout the nucleus during anaphase (Fig. 8A,B). We saw full release of Lrs4 and Csm1 in 40% of cells (Fig. 8E), in contrast to Cdc14, which is released in all anaphase cells (Shou et al. 1999; Visintin et al. 1999). Whether Lrs4/Csm1 are retained in the nucleolus by means other than the RENT complex is not known. However, similar to what is observed for Cdc14, nucleolar localizations of Lrs4-6HA and Csm1-9Myc depended on *NET1*, as both proteins were dispersed throughout the nucleus at every stage of the cell cycle in *net1*Δ cells (Fig. 8F; data not shown).

We next determined whether the dispersal of Lrs4 and Csm1 required the two regulatory networks that promote Cdc14 release from the nucleolus during early and late anaphase: the Cdc14 early-anaphase release network (FEAR network) and the mitotic exit network (MEN), respectively (for review, see Stegmeier and Amon 2004). We did not detect FEAR network-dependent release of Lrs4 and Csm1 during early anaphase (Supplementary Fig. 7A). Furthermore, release of Csm1 and Lrs4 was in-

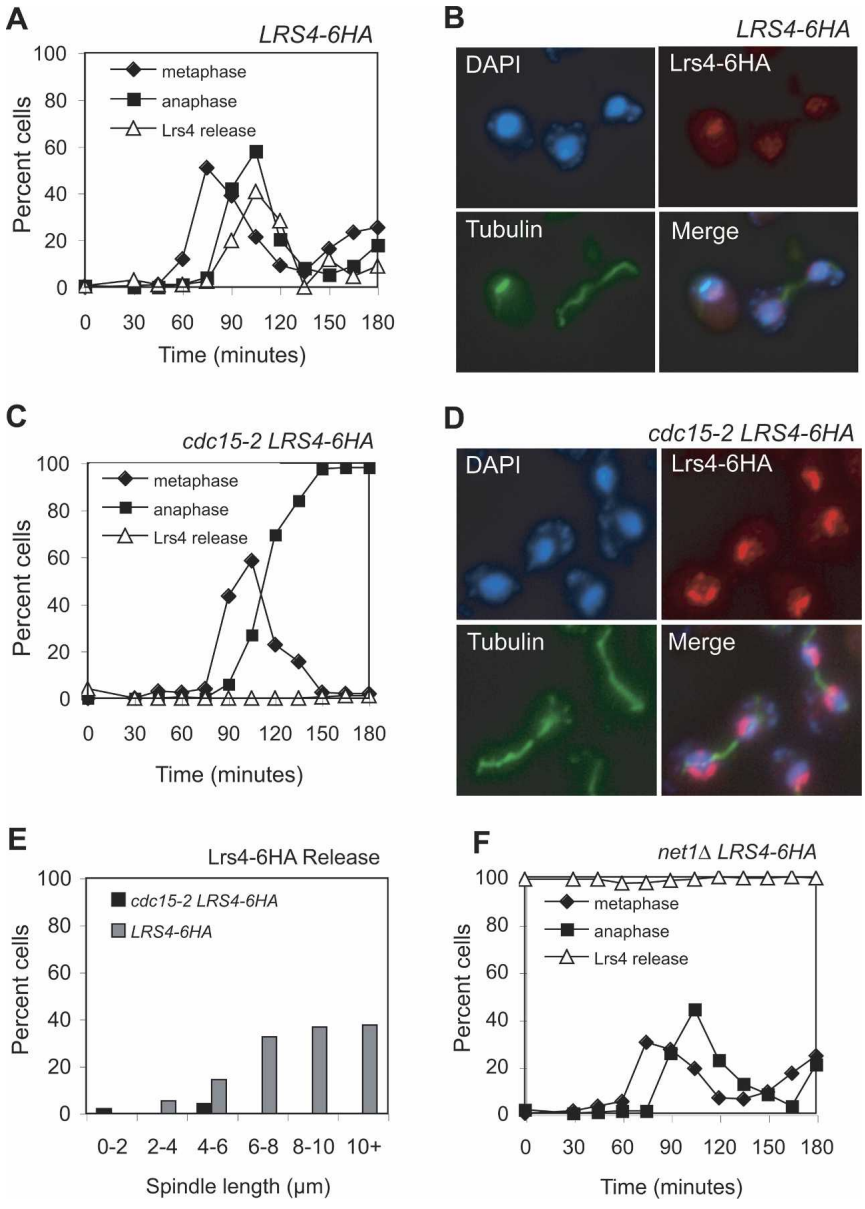


Figure 8. Lrs4-6HA is released from the nucleolus during anaphase. (*A*) Wild-type cells (A13838) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/mL). When arrest was complete, cells were released into YEPD medium lacking pheromone at 23°C. At the indicated times, samples were taken to determine the percentage of cells with metaphase and anaphase spindles, as well as the percentage of cells with Lrs4-6HA released from the nucleolus. (*B*) An example of Lrs4 release in anaphase cells. Lrs4-6HA is shown in red, microtubules in green, and DNA in blue. (*C*) *cdc15-2* cells (A13839) carrying an *LRS4-6HA* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/mL). When arrest was complete, cells were released into YEPD medium lacking pheromone at 37°C. At the indicated times, samples were taken to determine the percentages of cells with metaphase and anaphase spindles and the percentage of cells with Lrs4-6HA released from the nucleolus. (*D*) An example of Lrs4 localization in *cdc15-2* cells. Lrs4 is shown in red, microtubules in green, and DNA in blue. (*E*) Wild-type (A13838) and *cdc15-2* cells (A13839) were grown as described in *A*, and the localization of Lrs4-6HA was determined with respect to the length of the mitotic spindle as described in Stegmeier et al. (2004). (*F*) Wild-type (A13838) and *net1*Δ cells (A14568) were grown as described in *A*. At the indicated times, samples were taken to determine the percentages of cells with metaphase and anaphase spindles, and the percentage of cells with Lrs4-6HA released from the nucleolus.

dependent of Cdc14 (Supplementary Fig. 7B–D; data not shown). Whether this is due to Lrs4 and Csm1 release from the nucleolus being independent of the FEAR network or whether the release is too transient to be detected is at present unknown. We did find that release of Lrs4 and Csm1 depended on Cdc15, which is required for activation of the mitotic exit network (Fig. 8C–E; data not shown). Therefore, we conclude that the Lrs4/Csm1 complex is released from the nucleolus during anaphase and that disassembly is regulated by the mitotic exit network.

Discussion

Our findings reveal an extensive network of protein–protein interactions that regulate rDNA silencing and recombination. In particular, several factors specifically localize to the NTS1 region of rDNA, underscoring the importance of this region as a recombination control center. The requirement for the NTS1-specific factors Tof2 and the Lrs4/Csm1 complex in suppression of rDNA recombination indicates that this region is a key target of *trans*-acting factors that regulate rDNA recombination levels. Furthermore, the association of Lrs4/Csm1 with both chromatin (this study) and the cohesin complex (Newman et al. 2000; Graumann et al. 2004) suggests a novel mechanism for the assembly of a bridge or clamp complex that inhibits unequal sister chromatid exchange by restricting the movement of rDNA repeat units on different chromatids relative to each other (Fig. 9). Below we discuss the implications of these results for the regulation of rDNA recombination and its link to the pathways that regulate exit from mitosis.

A recombination control and pairing center at the NTS1 region of rDNA

Previously, it had been shown that the transcriptional silencing components of the RENT complex, Net1 and Sir2, associate with both the NTS1 and the NTS2 regions (Huang and Moazed 2003). Silencing factors are recruited to the NTS1 region by the replication fork-blocking protein Fob1 (Huang and Moazed 2003), which binds specifically to sequences within NTS1 that constitute a recombination hotspot (Keil and Roeder 1984; Kobayashi 2003; Mohanty and Bastia 2004). Both Fob1 and the sequences bound by it are required for all recombination within rDNA, indicating that this region is influenced by both inhibitory and stimulatory mechanisms. Affinity purifications of these regulatory factors have now identified a large network of interacting proteins that are specifically required for silencing at the NTS1 region, preferentially localize to NTS1, and collaborate to inhibit recombination. One such new factor is Tof2, which is recruited to rDNA-NTS1 via Fob1. Tof2 appears to act as an adaptor that recruits the Lrs4 and Csm1 proteins, strong negative regulators of unequal sister chromatid exchange that are required for NTS1-specific silencing. However, deletion of *TOF2* results in a smaller increase in recombination rates compared with deletion of *SIR2*, *LRS4*, or *CSM1*, suggesting that Tof2, like Fob1, may

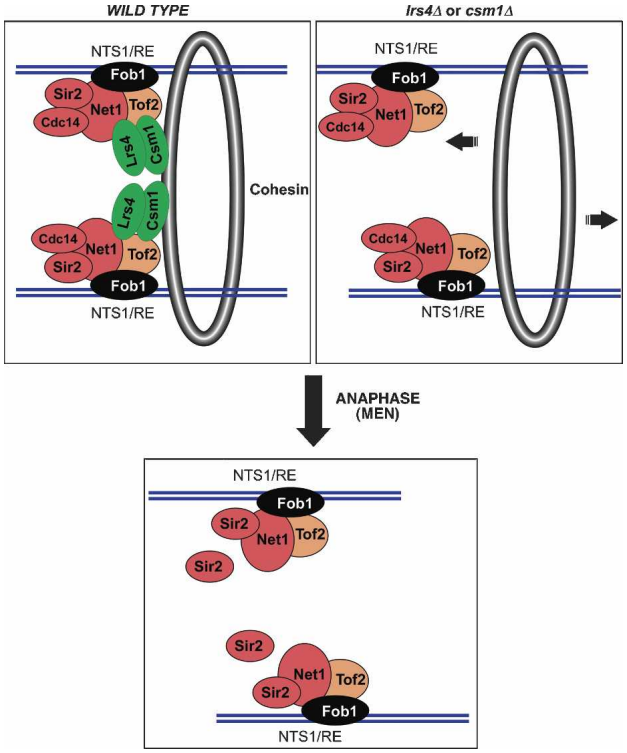


Figure 9. Model for a protein bridge that inhibits recombination by unequal crossover. Within NTS1, RE sequences are bound by Fob1, which is required for the recruitment of the RENT complex, consisting of Net1, Sir2, and Cdc14. Fob1 also recruits Tof2, which is required for the association of Lrs4/Csm1 with RE sequences. Lrs4/Csm1 may form a protein bridge that clamps sister chromatids together, either directly or through association with cohesin. Lrs4/Csm1–cohesin association would clamp rDNA to the cohesin ring, thereby restricting the movement of sister chromatids relative to each other to inhibit unequal exchange. (*Bottom*) The bridge is disassembled during mitosis by the release of cohesin and Lrs4/Csm1. (*Right* side) In *lrs4*Δ and *csm1*Δ mutant cells, cohesin is no longer clamped to rDNA, allowing unrestricted movement of sister chromatids relative to each other.

be required for stimulation of rDNA recombination as well as for the recruitment of inhibitors of recombination.

Prior studies had established a role for Lrs4 and Csm1 in the regulation of chromosome segregation during meiosis, when they form the "monopolin" complex with a meiosis-specific factor, Mam1 (Toth et al. 2000; Rabitsch et al. 2003; Lee et al. 2004). During prophase I and metaphase I, this complex localizes to centromeric regions to form a "sister chromatid clamp" that somehow bridges and co-orients sister chromatids to ensure proper segregation toward the same pole, resulting in a reductional division characteristic of meiosis I (Toth et al. 2000; Rabitsch et al. 2003). Throughout other stages of meiosis as well as mitosis, Lrs4 and Csm1 are found in the nucleolus (Rabitsch et al. 2003), but where these proteins localize within the nucleolus and how they function in rDNA silencing have been unknown. Our data

show that Lrs4 and Csm1 form a stable, two-subunit complex that primarily associates with the NTS1 region. The Lrs4/Csm1 complex is recruited to this site by two NTS1-specific factors, Fob1 and Tof2, and physically associates with Tof2 and NTS1-associated RENT. Here, we have shown that these proteins not only inhibit RNA Pol II-dependent transcription but also suppress mitotic recombination. Since Lrs4/Csm1 are present in the nucleolus throughout most of meiosis, they are likely to collaborate with Sir2 to repress meiotic recombination as well (Gottlieb et al. 1989).

Lrs4/Csm1 and Sir2 may represent two independent pathways that suppress recombination. While all three proteins are required for suppression of recombination, our data show that they can localize to the NTS1 region independently. Deletion of either *LRS4* or *CSM1* has little or no effect on the localization of Sir2 to the NTS1 region (Supplementary Fig. 8), suggesting that these proteins function in a step downstream from Sir2 localization to silence rDNA. Similarly, deletion of *SIR2* has little effect on Lrs4/Csm1 or Tof2 localization (Fig. 6). In this model, the Tof2-dependent recruitment of Lrs4/Csm1 to rDNA contributes to the regulation of silencing and recombination independently of Sir2 recruitment.

Our analysis suggests two possible mechanisms by which Lrs4/Csm1 inhibit unequal crossover (Fig. 9). One possibility is that Lrs4 and Csm1 form a bridge across sister chromatids through homotypic and/or heterotypic interactions to restrict the movement of rDNA sister chromatids relative to each other. Both Lrs4 and Csm1 contain extensive coiled-coil regions that may be involved in such interactions (Newman et al. 2000; Rabitsch et al. 2003), and our purifications of Csm1 complexes contain both epitope-tagged and untagged forms of Csm1, suggesting that it can form at least homodimers. However, an Lrs4/Csm1 sister chromatid bridge on its own is unlikely to impede chromosome segregation because both proteins appear to be released from the nucleolus in late anaphase after chromosome segregation is already completed (Fig. 8A; data not shown). A second possibility is that Lrs4/Csm1 physically associate with the cohesin complex to inhibit unequal crossover. The cohesin complex on its own should be unable to restrict the movement of sister chromatids relative to each other, as cohesin forms topological rings that embrace sister chromatids (Haering et al. 2002; Chang et al. 2005; Ivanov and Nasmyth 2005), but allow DNA and chromatin to move freely within the ring (Glynn et al. 2004; Lengronne et al. 2004). These findings demonstrate that cohesin does not interact with DNA or chromatin in a stable manner, and thus, we propose that the association of cohesin with chromatin-bound Lrs4/Csm1 is necessary to "fix" two sister chromatids relative to each other and thereby inhibit unequal exchange (Fig. 9). Notably, purification of the Scc1/Mcd1 subunit of the cohesin complex uncovered multiple peptides of Csm1 (Graumann et al. 2004). Furthermore, an independent two-hybrid study testing pairwise interactions between all predicted coiled-coil motifs in the budding yeast proteome showed that the coiled-coil domain of Csm1 specifically interacts with the coiled-coil region of the Smc1 subunit of the cohesin complex (Newman et al. 2000). We note that our purifications of Lrs4 and Csm1 were devoid of cohesin subunits (Fig. 5D), although in some experiments we observed a weak enrichment of Scc1/Mcd1 in both Csm1 and Lrs4 immunoprecipitations (J. Huang, unpubl.). This discrepancy may be due to the transient or temporally regulated nature of the monopolin–cohesin interaction, which may only occur stably on chromatin.

An Lrs4/Csm1-cohesin clamp model is consistent with the observation that Sir2 is required for maximal association of cohesin with the NTS1 recombination enhancer region (Kobayashi et al. 2004). Previously, Sir2-dependent silencing mechanisms have been proposed to prevent recombination by suppressing transcription from divergent RNA Pol II promoters in this region, which would otherwise promote removal of cohesin rings (Kobayashi and Ganley 2005). However, as mentioned above, the presence of cohesin alone is unlikely to prevent unequal sister chromatid exchange, and therefore, Lrs4/Csm1 may be required to clamp rDNA to the cohesin ring. Recruitment of cohesin may be a feature that the rDNA-NTS1 region shares with other heterochromatin-like domains, such as silent chromatin at the budding yeast mating type loci (Chang et al. 2005) and heterochromatin surrounding fission yeast centromeres (Bernard et al. 2001; Nonaka et al. 2002).

Relationship between rDNA silencing and cell cycle proteins

The first connection between rDNA silencing and the cell cycle came from the discovery that the Net1 protein is responsible for the recruitment of Sir2 to rDNA as well as the sequestration of Cdc14 in the nucleolus to prevent premature exit from anaphase during mitosis or anaphase I during meiosis (Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999; Buonomo et al. 2003; Marston et al. 2003). However, it is unclear why proteins that regulate the cell cycle and rDNA silencing form subunits of the same complex and assemble together on rDNA chromatin. We suggest that clamping of sister chromatids in close alignment from S phase until the onset of anaphase must be coordinated with DNA replication such that, as new daughter DNA strands emerge, corresponding repeat units are held together in register (Fig. 9). In this model, the cohesin component of the clamp is released from chromosomes at the onset of anaphase, while the release of Lrs4/Csm1 occurs later in anaphase after the completion of chromosome segregation. The release of Lrs4/Csm1 during mitosis may be required to allow a resetting of the Lrs4/Csm1-cohesin clamp during S phase.

Although some rDNA silencing and cell cycle factors are preferentially associated with NTS1, nearly all also localize to the NTS2 region. For example, both Fob1 and Tof2 localize to NTS2, although neither is required for silencing there. The requirements for the association of RENT and other factors with these two regions are likely

to be distinct. For example, the association of RENT subunits and other factors with NTS1 is Fob1-dependent, unlike association with NTS2. Moreover, in the absence of *FOB1*, the kinetics of Cdc14 release from the nucleolus is not affected (Stegmeier et al. 2004), suggesting that a Fob1-independent mechanism(s) maintains nucleolar sequestration of Cdc14 and prevents premature exit from mitosis. The NTS2/35S region may serve as a secondary binding site for regulatory factors such as Cdc14 and Lrs4/Csm1 if the NTS1 site is perturbed during either mitosis or meiosis. Since Lrs4/Csm1 ensure a reductional division of chromosomes only during meiosis I by dynamic relocalization to centromeres (Toth et al. 2000; Rabitsch et al. 2003), inappropriate loss of association with rDNA may be catastrophic. Thus, nucleolar sequestration of Lrs4 and Csm1 and meiosis-specific expression of the third monopolin subunit Mam1 may be redundant mechanisms, one spatial and one temporal, to ensure the fidelity of chromosome segregation.

Materials and methods

Yeast strains and plasmids

The yeast strains are listed in Supplementary Table 2. PCR-based construction of epitope-tagged yeast strains (Longtine et al. 1998; Rigaut et al. 1999) and plasmid construction are described in the Supplemental Material.

Purification and identification of native complexes

Protein purification was performed as described (Tanny et al. 2004) with the modifications outlined in the Supplemental Material. Ten percent of the peak fraction was run on a 10%–20% SDS-PAGE gradient gel and silver-stained. For the mass spectrometry analysis of complexes, whole protein mixtures were digested in solution and 10%–20% was analyzed by LC-MS/MS. Peptides were separated across a 50-min gradient ranging from 7% to 30% ACN in 0.1% FA in a microcapillary (125 µm × 18 cm) column packed with C_{18} reverse-phase material (Magic C18AQ, 5-µm particles, 200 Å pore size; Michrom Bioresources) and on-line analyzed on either a LTQ-Orbitrap or a LTQ-FT hybrid mass spectrometer (ThermoElectron). For each cycle, one full MS scan acquired on the Orbitrap or the ICR cell at high mass resolution was followed by 10 MS/MS spectra on the linear ion trap from the 10 most abundant ions. MS^2 spectra were searched using the Sequest algorithm. Peptide matches were filtered to <0.5% false positives using a target-decoy database strategy. The final lists of proteins present in the various complexes were obtained by subtracting protein matches found also in an untagged control sample.

Silencing assays

rDNA silencing assays were performed as described (Huang and Moazed 2003) with the modifications described in the Supplemental Material. Telomeric silencing was assayed by plating cells onto synthetic complete (SC) or SC supplemented with 0.8 g/L 5-FOA.

Immunofluorescence microscopy

Immunofluorescence assays were performed essentially as described (Guthrie and Fink 1991). Additional details are provided in the Supplemental Material. Indirect in situ immunofluorescence methods and antibody concentrations for Lrs4-6HA nucleolar release assays were as previously described (Visintin et al. 1999; Stegmeier et al. 2004).

ChIP assays

ChIP assays and quantification of the data were performed essentially as previously described (Huang and Moazed 2003). Relative fold enrichment was determined by calculating the ratio of rDNA to *CUP1* enrichment in the IP material and comparing this with the ratio of rDNA to *CUP1* enrichment in the WCE material. This is represented in the following calculation: [rDNA(IP)/*CUP1*(IP)/rDNA(WCE)/*CUP1*(WCE)].

Unequal sister chromatid exchange assays

Assays were performed as previously described (Kaeberlein et al. 1999). The unequal sister chromatid crossover rate was calculated by dividing the number of half-red/half-white colonies by the total number of colonies. Red colonies were excluded from all calculations. Details are provided in the Supplemental Material.

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