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# RNA Polymerase I and Fob1 contributions to transcriptional silencing at the yeast rDNA locus

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## ABSTRACT

**RNA polymerase II (Pol II)-transcribed genes embedded within the yeast rDNA locus are repressed through a Sir2-dependent process called 'rDNA silencing'. Sir2 is recruited to the rDNA promoter through interactions with RNA polymerase I (Pol I), and to a pair of DNA replication fork block sites (Ter1 and Ter2) through interaction with Fob1. We utilized a reporter gene (*mURA3*) integrated adjacent to the leftmost rDNA gene to investigate localized Pol I and Fob1 functions in silencing. Silencing was attenuated by loss of Pol I subunits or insertion of an ectopic Pol I terminator within the adjacent rDNA gene. Silencing left of the rDNA array is naturally attenuated by the presence of only one intact Fob1 binding site (Ter2). Repair of the 2nd Fob1 binding site (Ter1) dramatically strengthens silencing such that it is no longer impacted by local Pol I transcription defects. Global loss of Pol I activity, however, negatively affects Fob1 association with the rDNA. Loss of Ter2 almost completely eliminates localized silencing, but is restored by artificially targeting Fob1 or Sir2 as Gal4 DNA binding domain fusions. We conclude that Fob1 and Pol I make independent contributions to establishment of silencing, though Pol I also reinforces Fob1-dependent silencing.**

## INTRODUCTION

Transcriptional silencing in the budding yeast, *Saccharomyces cerevisiae*, occurs at several distinct genomic regions, including the *HML* and *HMR* silent mating-type cassettes, telomeres and ribosomal DNA (rDNA) (1). The specialized chromatin structures assembled at these loci result in strong position effects on transcription, such that genes located within or very close to the silenced domains are repressed more efficiently than when positioned further away (2–4). Silencing requires the highly conserved NAD$^+$-dependent histone deacetylase Sir2, which comprises the catalytic subunit of two separate silencing complexes, SIR (silent information regulator) and RENT (regulator of nucleolar silencing and telophase) (5). SIR, consisting of Sir2, Sir3 and Sir4, is targeted to specific cis-acting 'silencer' elements flanking *HML* and *HMR* (6), or to telomeric $TG_{1-3}$ repeats through interactions with the telomere binding protein Rap1 (6–8). Localized H4-K16 deacetylation by Sir2 then promotes further SIR recruitment and spreading to form an extended silenced domain (9). RENT consists of Sir2, Net1 and Cdc14 subunits, and specifically silences RNA polymerase II (Pol II) transcription within the rDNA tandem array through its histone deacetylation activity (10,11). RENT also promotes RNA polymerase I (Pol I) transcription of rDNA genes and regulates the exit from mitosis (10,12). Active Pol I transcription is actually critical for silencing of Pol II transcription within the rDNA (2,13), but there is currently no evidence that Pol II transcription from the rDNA reciprocally suppresses Pol I transcription.

The rDNA locus is organized as a tandem array of ∼150 rDNA genes on the right arm of chromosome XII. Each rDNA gene is 9.1 kb and oriented in a right to left orientation as annotated in the *Saccharomyces* Genome Database (SGD). The Pol I-transcribed regions of adjacent repeats are separated by non-transcribed spacers (NTS) that are further divided into NTS1 and NTS2 by the Pol III-transcribed 5S rRNA gene (Figure 1). Silencing of Pol II transcription within the rDNA was originally identified using reporter genes (14,15), but it is now known that endogenous non-coding RNAs are transcribed by Pol II from NTS1 and NTS2, and that Sir2 limits their expression (16,17). NTS1 and NTS2 are also observed in the literature as intergenic spacers IGS1 and IGS2, respectively. We use NTS1 and NTS2 throughout this current study to match SGD annotation. At NTS1, Sir2 (as part of the RENT complex) is recruited to DNA replication fork block (RFB) sites known as Ter1 and Ter2 via interactions with the RFB protein Fob1 (18) (Figure 1). Deleting *FOB1* results in loss of Sir2 binding and reduced silencing activity at NTS1, but not at NTS2, where RENT is instead recruited by interac-

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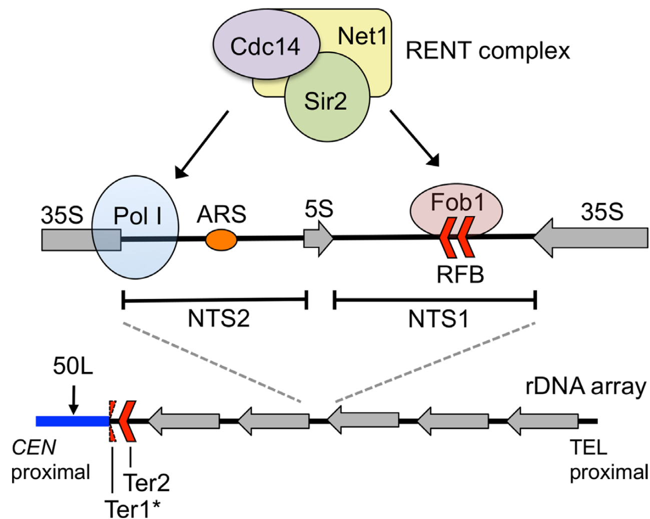


**Figure 1.** Schematic representation of key rDNA silencing features, including the RENT complex and its recruitment to NTS1 via association with Fob1 at a RFB site consisting of Ter1 and Ter2 Fob1 binding sites. RENT is also recruited to NTS2 through association with RNA Pol I at the rDNA 35S promoter region in NTS2. The Ter1 site at the border between leftmost rDNA repeat sequence and unique chromosome XII sequence (blue line) is truncated and inactive (Ter1*). The rDNA array is located on the right arm or chromosome XII, so the centromere (CEN) proximal and telomere (TEL) proximal sides of the array are indicated.

tions with RNA polymerase I at the 35S rDNA promoter (12,18) (Figure 1). Sir2 appears to physically spread beyond NTS2 in the direction of Pol I transcription, resulting in detectable levels of Sir2 enrichment and transcriptional silencing across the entire rDNA gene (2,18–19).

At the centromere-proximal (left) end of the tandem array, silencing modestly spreads outward from the terminal repeat into flanking non-rDNA sequence (2). Overexpression of Sir2 induces additional leftward spreading that is ultimately blocked by a $tRNA^{Gln}$ gene acting as a barrier element ~3 kb away from the array (20). Reporter genes positioned adjacent to the rDNA array are mitotically stable (2), probably because they are not subject to the unequal crossover and gene conversion that often occurs between internal rDNA repeats (21,22). This makes the left-flank reporters very useful for genetic dissection of rDNA silencing mechanisms. In this study we have exploited this left-flank reporter system to further define the characteristics of rDNA silencing adjacent to the tandem array, and determine the relative contributions of Fob1 and Pol I transcription. We demonstrate that Fob1 has a strong localized effect on silencing at the left-flank, and that the RFB sites Ter1 and Ter2 are both required for maximum silencing levels. Upstream contributions from Pol I transcription are critical for maintaining functional silencing levels when Fob1-dependent recruitment of Sir2 is weakened by a naturally occurring disruption of the Ter1 site at the rDNA-unique sequence junction.

## MATERIALS AND METHODS

### Yeast strains and plasmids

Yeast strains were derived from the JB740 derivative (*MATα his3Δ200 leu2Δ1 ura3-167*) of GRF167 (23), and the previously described rDNA silencing reporter cassette *mURA3-HIS3* (15), which was integrated into unique chromosome XII sequence adjacent to the leftmost rDNA gene. All strains were grown at 30°C. Integration of the cassette 50 bp left of the rDNA (50L, Figure 1) to produce YSB348 was previously described (2). Strains deleted for *RPA135* harbor pNOY199, a *TRP1 2μ* plasmid that expresses 35S rRNA precursor from a *GAL7* promoter (24). Such strains are only viable on galactose-containing media. Matching *RPA135*$^+$ control strains were also transformed with the pNOY199 plasmid and maintained on galactose media. Several manipulations of the leftmost rDNA gene were performed throughout the study and are described below, with genotypes provided in Supplementary Table S1. Genomic manipulations were confirmed by colony PCR, and silencing phenotypes were initially analyzed with at least two colony isolates to ensure uniformity among clones. Plasmids used in the study are listed in Supplementary Table S2, and oligonucleotide sequences are listed in Supplementary Table S3.

*Premature termination constructs.* A 419 bp portion of NTS1 containing the Pol I termination sequences was PCR amplified from genomic DNA with primers JS640 and JS641 (Chromosome XII SGD coordinates 460495–460914), while a 419 bp portion of ΦX174 DNA was amplified with primers JS642 and JS643. The NTS1 fragment contains all known Pol I cis-acting termination sequences, including the Reb1 binding site, poly T (T2) region and Ter1/Ter2. Both fragments were cloned into the TA cloning vector pCRII (Invitrogen). Fragments were excised from pCRII with *Spe*I and *Xba*I and then ligated into the *Nhe*I site of pSB830, a plasmid with the 5′ *Bgl*II rDNA fragment ligated into pNEB193 (New England Biolabs). *Not*I sites of the pSB830 derivatives were filled-in with Klenow enzyme and then used for the final integrating constructions in a manner similar to the previously described construct pSB735 (2). Structures of the integrating plasmids (reading left to right) consist of 1200 bp of the left-flanking unique sequence, the *mURA3-HIS3* reporter cassette integrated at the *Swa*I site located 61 bp left of the rDNA sequence (2) and an entire modified rDNA repeat (Figure 3A). The termination and ΦX174 insertions are 1.98 kb downstream from the Pol I start site. *Not*I releases the fragments, which are then transformed into JB740 to replace the endogenous leftmost rDNA gene. Integrants were selected on SC-his plates. pSB858 harbors the ΦX174 insertion, pSB854 has the Pol I terminator in a forward direction and pSB856 has the terminator in the reverse orientation.

*BglII-flip.* The orientation of a large *Bgl*II DNA fragment that spans most of the 35S-coding region was reversed in pSB692 by cutting and re-ligating to produce pSB783. The *mURA3-HIS3* reporter cassette was ligated into the *Asc*I site of pSB783 (61 bp away from the rDNA) to make pSB820. The pSB820 construct was digested with *Not*I and

transformed into JB740. *Bgl*II-flip strains were selected on SC-his plates.

*Ter1-repair and Ter2-delete.* The Ter1 repair construct was generated by first PCR amplifying 1200 bp of the left flanking (L-F) DNA from the 1200 L-F reporter strain YSB364 with primers JS39 and JS731. Primers JS61 and JS732 were used to amplify NTS1 sequence (including Ter1) to the *Bsr*GI site in the 35S coding region from internal rDNA repeats of YSB364. JS731 introduces an *Asc*I site into the rDNA-proximal end of the unique flanking DNA fragment, while JS732 introduces an *Asc*I site adjacent to the repaired Ter1 site in NTS1 of the rDNA fragment. The unique rDNA-flanking *Bam*HI-*Asc*I fragment and the rDNA *Asc*I-*Bsr*GI fragment were then directionally ligated into Litmus 38 vector (New England Biolabs) to produce pSB863. The *mURA3-HIS3* reporter was isolated as an *Asc*I fragment from pSB735 and ligated into the *Asc*I site of pSB863 to produce pSB865, which was then cut with *Not*I and *Bsr*GI to integrate the replacement cassette into JB740. The Ter2 delete construct (pSB893) was generated with the same strategy, except that primer JS732 was substituted with JS870, which introduces an *Asc*I site, but amplifies NTS1 while excluding the Ter1 and Ter2 sites. Positions and sequences of Ter1 and Ter2 for the various constructs were based on previous mapping (25). The left terminal 10 bp are CTCATGTTTG for JB740 (WT), GGCATGCCCT for the Ter1 repair and CCTTCTCTTT for the Ter2 delete. The Ter1 repair and premature terminator sequences were combined by ligating pSB865/*Spe*I, *BsrG*I; pSB735/*BsrG*I, *Mlu*I; and pSB849/*Mlu*I, *Not*I fragments into the pSB873/*Spe*I, *Not*I digested vector to make pSB912. The Ter1 repair and 35S promoter deletion sequences were combined with the same strategy except the pSB707/*Mlu*I, *Not*I fragment replaced the pSB849/ *Mlu*I, *Not*I fragment to make pSB902. Both constructs were integrated by transformation of JB740 with *Not*I digestions, and selection for $His^+$ colonies.

*targeting.* The *mURA3-HIS3* cassette was isolated as an *Asc*I fragment from pSB735 and ligated into the *Asc*I site of pASC to make pSB890. pASC was first generated by ligating an *Asc*I linker into LITMUS-38 cut with *Sna*B1 and *Stu*I. A double-stranded oligonucleotide (JS1432 and JS1433) containing 2 $UAS_{Gal}$ sites was phosphorylated and ligated into the *Bgl*II site adjacent to the *mURA3* promoter in pSB890. A clone containing 4 copies of $UAS_{Gal}$ was identified (pNM99-4) and the *mURA3-HIS3* cassette was then released from pNM99-4 as an *Asc*I fragment and used to replace the *mURA3-HIS3* cassette in the Ter2Δ vector pSB893, generating pJSS106-1. A *Not*I-*Bsr*GI fragment from pJSS106-1 was then transformed into YSB619 to produce the $4xUAS_{Gal}$ targeting strain JS1352. YSB619 is a *gal4Δ::natMX* version of JB740. The *mURA3-HIS3* cassette with or without $4x\text{-}UAS_{Gal}$ sites was also PCR amplified from pNM99-4 and pSB890 with primers JS2676 and JS2677, respectively, and integrated into the *TRP1* locus as a non-rDNA control location.

*GBD-Fob1 hybrid and FOB1 overexpression.* The *FOB1* open reading frame was amplified from a *FOB1*-containing pNEB193 vector (pSB776) by PCR with primers JS334 and JS335. The PCR product was fused to the *GAL4* DNA binding domain with the following three-piece ligation; pRS313 cut with *Eco*RI and *Sal*I, pSB362 cut with *Eco*RI and *Bam*HI, and the *FOB1* PCR product cut with *Bam*HI and *Sal*I to make pSB785. pSB362 is a plasmid containing *GAL4* DNA binding domain driven by the *RAP1* promoter. The structure of pSB785 is the *RAP1* promoter followed by *GBD-FOB1* in the *HIS3 CEN* plasmid pRS313. pSB822 was made by ligating the *Not*I-*Sal*I insert from pSB785 into the *LEU2 CEN* pRS415 plasmid.

The *FOB1 2μ* vector pEG3 was constructed by PCR amplifying a 2405 bp segment of genomic DNA (chrIV 675733–678137) containing the *FOB1* gene into the *Bam*HI site of pNEB193. The *FOB1* gene insert was confirmed by Sanger DNA sequencing and then subcloned into the *Bam*HI site of pRS425 to generate pEG3.

### Silencing assays

Strains were patched onto synthetic complete (SC) plates or SC-leu plates when selecting for *LEU2* containing plasmids, and allowed to grow for ∼15 to 20 h. Cells were scraped from the patches with a wooden applicator stick and resuspended in sterile water. The cell suspensions were normalized to an $OD_{600}$ of 1.0, serially diluted in 5-fold increments in a 96-well plate and then 5 μl of each dilution spotted onto the SC agar plates indicated in each figure. Photos were taken after 2 days for SC or SC-leu control plates. Silencing indicator plates were incubated for 3 to 4 days. For the *rpa135Δ* experiments, each strain was maintained on SC-trp galactose plates to select for pNOY199 and support growth.

### Chromatin immunoprecipitations

Log-phase yeast cultures (50 ml) in Yeast-extract Peptone Dextrose (YPD) medium were cross-linked with 1% formaldehyde for 20 min at 30°C. Cells were pelleted by centrifugation and washed 2 times with cold Tris-buffered saline. The cells, in 0.6 ml of FA-140 lysis buffer (50 mM HEPES, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 1x protease inhibitor cocktail; Sigma), were lysed with acid-washed glass beads (425–600 μm, Sigma) and a Mini-BeadBeater (Biospec Products). The cell lysates were sonicated with a Biorupter 300 (Diagenode) for 60 cycles (30 s 'ON' and 30 s 'OFF'). Equivalent amounts of lysate (2.5 mg protein) were incubated overnight at 4°C with 5 μl of αSir2 antibody yN-19 or αFob1 antibody yL-18, both from Santa Cruz Biotechnology, Inc. Next, 60 μl of salmon sperm DNA blocked protein G-agarose beads were added for 2 h at 4°C and then washed once with 1 ml of FA-140, twice with 1 ml of FA-500 (same as FA-140 except NaCl was increased to 500 mM) and twice with 1 ml LiCl solution (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium dodecyl sulfate, 1 mM EDTA). DNA was eluted from the beads 2 times with 75 μl of elution buffer (5x TE plus 1% SDS). The combined DNA solution was incubated at 65°C overnight to reverse cross-linking. Purified DNA samples were analyzed

by quantitative real-time PCR, and the results normalized with the input DNA PCR signal, and indicated by relative IP in the graphs. Standard deviations were calculated from the results for three independent biological replicates. The oligonucleotide sequences used are provided in Supplementary Table S3.

### Quantitative reverse transcriptase-PCR

Total RNA was extracted as described previously (26). cDNA was made for each strain using the master antisense rRNA primer JS2689. Quantitative real-time PCR (qRT-PCR) was then performed with combinations of JS2689 and the following template specific primers: total RNA control (JS2690), ΦX174 (JS2693), Ter1 forward (JS2692) and Ter1 reverse (JS2691). Oligonucleotide sequences are listed in Supplementary Table S3. SYBR-green RT-qPCR signal was quantified using the $2^{(-\Delta\Delta C_T)}$ method where the signal of a given sample is normalized to the unspecific signal from WT and to an internal control of total transcribed rRNA (27). A representative equation is as follows: $2\wedge((Ct_{Wt} - Ct_{sample}) - (Ct_{Wt_Total_Rna} - Ct_{Sample_Total_RNA}))$. For strain YRH4 and its derivatives, qRT-PCR was performed as previously described (28), with cDNA primers JS765 and JS766 for the read-through rRNA or primers JS769 and JS770 for *ACT1* mRNA.

### Western blotting

A trichloroacetic acid (TCA) precipitation method was used to prepare cell lysates from WT and *rpa14Δ* strains. Briefly, 10 ml of log-phase cells were harvested and washed once with 1 ml of 20% TCA. The pellets were resuspended with 0.5 ml of 20% TCA and transferred to 1.5 ml microfuge tubes which already contained 0.5 ml of glass beads. The tubes were vortexed 4 times for 30 s and then allowed to settle for 5 min on the bench. The lysates were transferred to new microfuge tubes. Glass beads in the first tube were washed twice with 0.5 ml of 5% TCA, with each wash being added to the new microfuge tube. The lysates were precipitated at 3000 rpm (800 *xg*) for 10 min at 4°C. The pellets were resuspended in 200 μl of 1x SDS sample buffer and neutralized by adding 50 μl of 2M Tris base. The SDS samples were boiled for 5 min and 10 μl loaded onto a 9% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes (Millipore) using a BioRad semi-dry transfer apparatus. Membranes were incubated with 1:3000 dilutions of α-tubulin monoclonal antibody (B-5-1-2, Sigma), polyclonal α-Fob1 antibody (yL-18, Santa Cruz) or polyclonal α-Sir2 antibody (YN-19, Santa Cruz). HRP-conjugated secondary antibodies from Promega were used at 1:5000 dilutions, and images developed with HyGLO (Denville Scientific).

## RESULTS

### RNA polymerase I activity impacts Sir2 protein levels and association with rDNA

Strains lacking the essential Pol I subunit Rpa135 are defective for rDNA silencing of the *mURA3* reporter gene located either within or adjacent to the array (2) (Figure 2A,

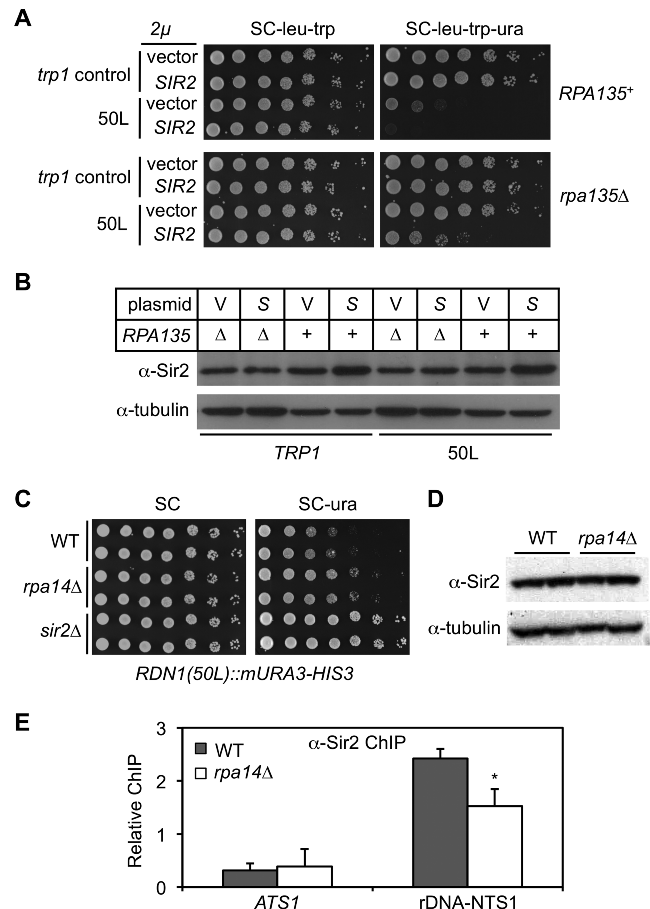


**Figure 2.** Loss of Pol I subunits causes rDNA silencing defects flanking the array. (**A**) Silencing of the *mURA3* reporter gene integrated in unique chromosome XII sequence 50 bp left of the rDNA array (50L). As a control, the *mURA3-HIS3* cassette was integrated at the *TRP1* locus through gene replacement. An empty vector or a *2μ SIR2* plasmid was transformed into each strain background. The 5-fold serial dilutions derived from two independent colonies for each strain were plated. Photos for the *RPA135*$^+$ strains were taken at day 3, while *rpa135Δ* photos were taken at day 4. (**B**) Western blot for native Sir2 and α-tubulin from whole cell extracts derived from WT and *rpa135Δ* strains. V indicates empty vector, and S indicates *2μ SIR2*. (**C**) Effect of deleting *RPA14* on rDNA silencing at the 50L position. The *sir2Δ* strain is a control for full loss of silencing. SC plate photo is from day 2, and SC-ura photo from day 3. (**D**) Sir2 protein Western blot from WT and *rpa14Δ* strains used in panel C. (**E**) Quantitative chromatin IP (ChIP) assay for native Sir2 protein at the *ATS1* promoter (negative control, primers JS1164-JS1165) and at NTS1 in the rDNA (primers JS1100-JS1101). **P*-value of decreased Sir2 binding at NTS1 was <0.01 from a two-tailed *t*-test.

50L position). Endogenous rDNA genes in these mutants are inactive, but cells remain viable (though slower growing) because they express 35S rRNA off a plasmid (pNOY199) using the galactose-inducible *GAL7* promoter (24). To test whether the *rpa135Δ* mutant retained any silencing potential in the absence of Pol I transcription, we overexpressed *SIR2* from a high copy *2μ* plasmid and measured silencing at the 50L position. Silencing in an *RPA135*$^+$ control strain was strengthened by the *SIR2* plasmid, as expected (2), but was also partially restored to the *rpa135Δ* mutant (Figure 2A). Sir2 protein levels relative to tubulin loading were slightly reduced in the *rpa135Δ* strains compared to

*RPA135*$^{+}$, and surprisingly were not increased by the 2$\mu$ *SIR2* plasmid in the mutant strain (Figure 2B), despite the improved silencing. These results suggest the steady state Sir2 protein level is tightly regulated in the absence of Pol I transcription.

We next tested whether deleting a non-essential Pol I subunit would also cause an rDNA silencing defect, but without any changes in Sir2 protein levels. Rpa14 is located in the stalk region of Pol I, a structure that interacts with the essential initiation factor Rrn3 and may facilitate polymerase processivity (29–32). A Pol I transcription defect from the leftmost rDNA repeat was confirmed for *rpa135Δ* and *rpa14Δ* mutants using a previously described and validated strain background, YRH4, in which Pol I termination sequences were removed from the leftmost rDNA gene (Supplementary Figure S1A), allowing RT-PCR detection of a unique read-through rRNA (28) (Supplementary Figure S1A and B). Compared to a *sir2Δ* control, deleting *RPA14* caused a moderate rDNA silencing defect of *mURA3* at the 50L position, even though the overall growth rate was close to WT levels on non-selective SC medium (Figure 2C). Steady state Sir2 protein levels were normal in the *rpa14Δ* mutant (Figure 2D), but Sir2 association with NTS1 using quantitative chromatin immunoprecipitation (ChIP) assays was reduced (Figure 2E). Pol I subunit defects, therefore, negatively impact rDNA silencing at the 50L position independently of growth rate and Sir2 protein levels.

Eliminating transcription of the leftmost rDNA gene by deleting its promoter also causes a silencing defect downstream of the mutated repeat (2). Deleting the promoter prevents Pol I from being recruited, essentially making the gene inert. Alternatively, we asked whether allowing Pol I transcription to initiate, but then stopping the polymerase before it approached the downstream *mURA3* reporter, would impact silencing. A 419 bp portion of NTS1 containing all Pol I termination sequences was inserted into an *Nhe*I site located 1.98 kb downstream of the Pol I transcriptional start site (Figure 3A). An equal sized stuffer fragment of unrelated ΦX174 phage DNA was inserted as a control. Transcriptional termination with both orientations of the NTS1 sequence was confirmed by RT-PCR specific to the uniquely tagged leftmost rDNA gene (Figure 3B). Repression of *mURA3* was clearly maintained with the ΦX174 control, but was lost when the termination sequences were inserted in either orientation (Figure 3C). This result suggested that progression of Pol I through the rDNA gene was important for establishing silencing downstream.

We next asked whether there was anything special about the rRNA sequence transcribed from the leftmost repeat that locally contributes to silencing downstream at the 50L position. This was done by simply flipping the orientation of a *Bgl*II restriction fragment encoding most of the rRNA sequence and then reintegrating the silencing cassette back into the left flank (Figure 3D, Flip). Local Pol I transcription from the left repeat will then produce an RNA with antisense rRNA sequence between the *Bgl*II sites. Since orientation of the *Bgl*II fragment had no effect on silencing strength (Figure 3E), we conclude that while transcription through the leftmost repeat is required for efficient silencing adjacent to the array (Figure 3B), the actual sequence of the RNA has no bearing on silencing activity (Figure 3E).

### Ter sites function in silencing at the left-flank

The edge of the leftmost rDNA gene that abuts unique chromosome XII sequence is not annotated as NTS1 in the SGD. This is because the rDNA/flanking sequence junction occurs within the middle of the DNA replication fork block site Ter1 (ChrXII coordinate 45148), thus disrupting a Fob1 binding site (Figure 1). The left-flank from our wild-type lab strain (JB740) was cloned and sequenced and found to precisely match the sequence in SGD (data not shown). NTS1 sequences normally distal of Ter1 are absent, but the major Pol I termination sequences remain intact and are functional (28). Silencing of *mURA3* near this disrupted Fob1 binding site is weaker than when it is positioned at an intact NTS1 sequence within the tandem array (2). A second RFB/Fob1 binding site known as Ter2 remains intact at the end of the leftmost repeat (Figures 1 and 4A), so we hypothesized this Ter2 site was sufficient to establish silencing, but the Ter1 site would be necessary for full silencing activity. To directly test this idea, we repaired the full Ter1 sequence (Figure 4A; see Materials and Methods for construction) and assayed again for silencing. Silencing of *mURA3* at the normal 50L position without Ter1 was insufficient for growth on 5-FOA, but repairing Ter1 (Ter1-R) strengthened silencing of *mURA3* enough to induce moderate growth on 5-FOA (Figure 4B). Importantly, this enhanced silencing phenotype remained fully dependent on *SIR2* and *FOB1* (Figure 4B), suggesting that Ter1 reinforces silent rDNA chromatin structure at the left flank.

As anticipated from previous studies (2,33), ectopic expression of *SIR2* from a high copy vector dramatically improved rDNA silencing of the 50L and Ter1-repair strains, demonstrated by weaker growth on SC-leu-ura and stronger growth on SC-leu + FOA (Figure 4C). We next asked whether *FOB1* was also limiting for rDNA silencing by overexpressing *FOB1* from a high copy vector and measuring silencing with the robustly repressive Ter1-repair strain and the moderately repressive 50L strain. Surprisingly, the high copy *FOB1* plasmid instead weakened silencing in both reporter strains, as indicated by strong Ura$^{+}$ growth (Figure 4C). Fob1 overexpression was confirmed by western blotting (Figure 4D). Interestingly, the Ter1-R strain overexpressing Fob1 remained partially FOA resistant while strongly Ura$^{+}$ (Figure 4C), reminiscent of telomeric silencing when *URA3* is integrated next to a telomere (4). The same phenotype was also observed with *mURA3-HIS3* integrated at NTS1 within the array (Supplementary Figure S2). We hypothesize that Ter1 repair or the complete NTS1 sequence confers some epigenetic inheritance to rDNA silencing that is being uncovered by *FOB1* overexpression, perhaps through inappropriate interactions with RENT that affect its recruitment. Alternatively, Fob1 overexpression has a non-specific effect on 5-FOA resistance.

Previous ChIP assays showed that Fob1 predominantly binds to NTS1, but there was also significant binding that overlaps with Sir2 at NTS2 (18). Deleting *FOB1* surprisingly had no effect on silencing the *mURA3* reporter gene when located within the 5′ end of the 35S rRNA transcribed

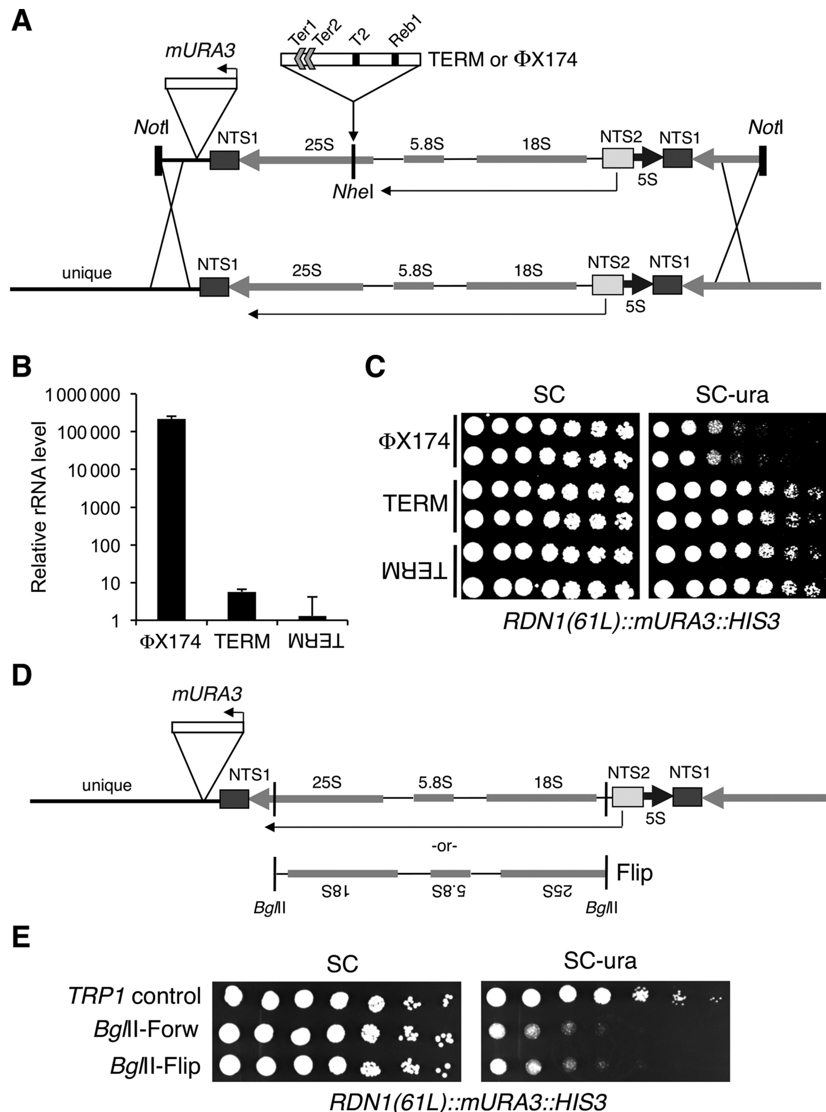


**Figure 3.** Effects of manipulating the leftmost rDNA gene structure on rDNA silencing. (**A**) A *Not*I fragment containing the leftmost rDNA gene and a portion of the adjacent gene with either the Pol I termination sequences (TERM) or an equal sized fragment of ΦX174 DNA (ΦX174) was integrated into the genome by replacing the endogenous leftmost rDNA gene. Pol I termination cis-acting sequences are indicated for the TERM insertion. Bent horizontal arrow indicates the direction of Pol I transcription. (**B**) RT-PCR quantitation of Pol I transcription from the leftmost rDNA gene, relative to total rRNA levels. (**C**) Specific loss of silencing when the TERM sequence was integrated within the 25S rDNA sequence in either orientation. (**D**) Schematic diagram showing structure of a *Bgl*II rDNA gene fragment from the leftmost rDNA gene that was flipped in orientation. (**E**) Silencing assay showing no effect of flipping orientation of the *Bgl*II rDNA fragment. *Bgl*II-Forw indicates the strain constructed with a normal rRNA orientation, and *Bgl*II-Flip indicates the strain constructed with orientation of the *Bgl*II fragment reversed. In the *TRP1* control strain, YSB519, *mURA3-HIS3* replaced the *TRP1* gene on chromosome IV.

region (18). We considered the possibility that another reporter gene (*MET15*) located in NTS2, but outside the transcribed region, would be silenced in a Fob1-dependent manner. As shown in Figure 4E, silencing of the *MET15* gene integrated within NTS2 was clearly weakened in a *fob1Δ* strain (white colony color), but unlike a *sir2Δ* control, the loss of silencing was not accompanied by an increase in recombination-induced *MET15* loss (dark brown sectoring).

The Ter1-repaired silencing observed in Figure 4B suggested that Sir2 recruitment to NTS1 via Fob1 had a strong localized effect on silencing at the left flank. We predicted that removing Ter2 from the leftmost rDNA repeat would dramatically weaken silencing because of a complete loss of Fob1 binding and an inability to locally recruit Sir2. Ter2 was specifically removed only from the leftmost repeat (Figure 5A, schematic), and silencing of the adjacent *mURA3* reporter analyzed. Importantly, other Pol I termination sequences (the Reb1-binding site and the poly T (T2) site) were left intact to prevent read-through transcription. As shown in Figure 5B, silencing measured on SC-ura plates was significantly weakened in the Ter2Δ strain as compared to the normal 50L strain that contains the Ter2 site. Deleting *FOB1* from the Ter2Δ strain did not make silencing any worse, confirming that Fob1 functions in silencing at the left flank through Ter1 and Ter2 sequences. However, deleting

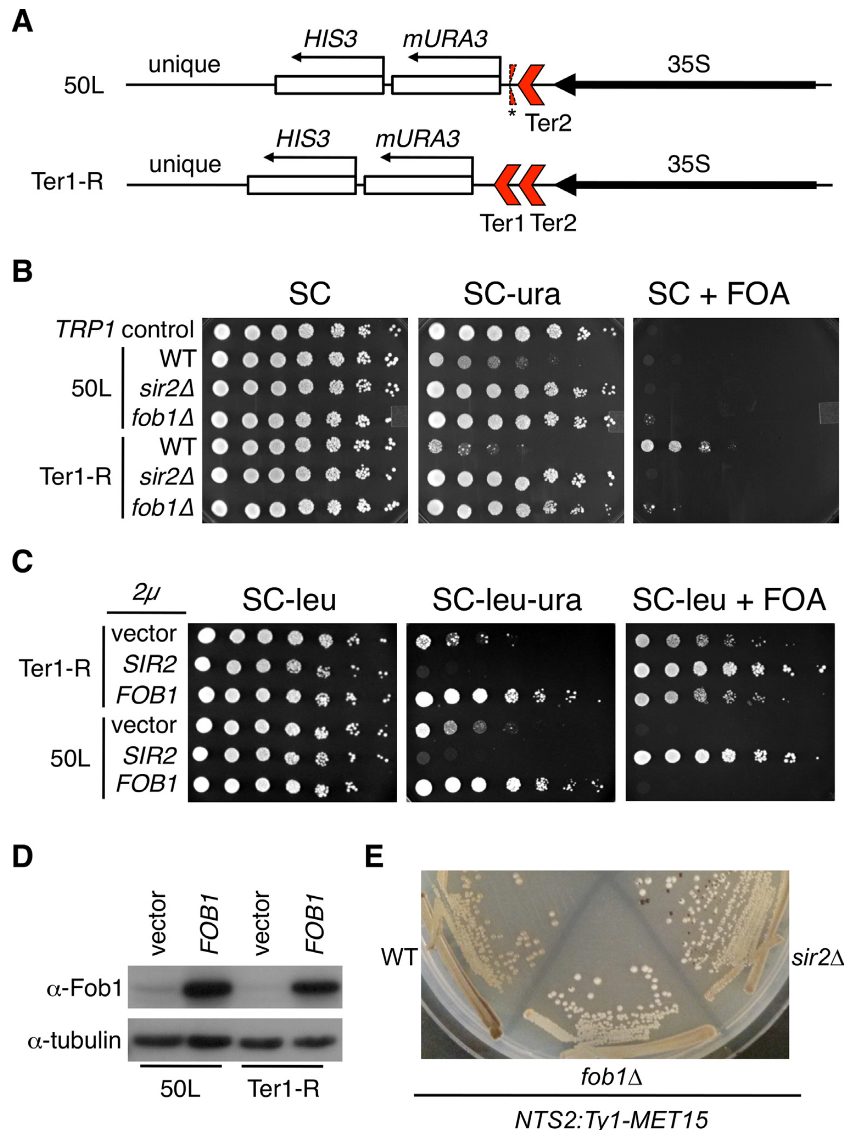


**Figure 4.** Repair of the leftmost Ter1 site improves silencing. (**A**) Schematic representation of repaired Ter1 site adjacent to the flanking *mURA3* reporter gene. The naturally occurring truncated Ter1 site in the 50L strain is indicated by an asterisk. (**B**) Silencing assay showing the effects of repairing the Ter1 site. Improved silencing required *SIR2* and *FOB1*. In the *TRP1* control strain, YSB519, *mURA3-HIS3* replaced the *TRP1* gene on chromosome IV. (**C**) Effects of *SIR2* and *FOB1* overexpression on rDNA silencing in the Ter1-R and normal 50L strains. The *2μ LEU2* plasmids used were pRS425 (empty vector), pSB766 (*SIR2*) and pEG3 (*FOB1*). (**D**) Western blot of Fob1 overexpression in 50L and Ter1-R strains. (**E**) Silencing of a *Ty1-MET15* reporter integrated into NTS2 of WT, *sir2Δ* and *fob1Δ* strains. Strains were grown for 5 days on modified lead acetate (MLA) plates to develop the tan/brown coloring.

*SIR2* from the Ter2Δ strain caused even weaker silencing, suggesting that in the absence of Ter1 and Ter2, low levels of Sir2 may still propagate along with Pol I toward the flanking unique sequence. Supporting this idea, *SIR2* overexpression effectively restored silencing in the Ter2Δ background, even when *FOB1* was deleted (Figure 5C).

## Fob1 and Sir2 targeting

To prove that Fob1 was sufficient to induce silencing at the left flank and to further study silencing function of Fob1, we targeted a Gal4 DNA binding domain (GBD)-Fob1 fusion protein (see Materials and Methods) to the left flank. The targeting strains in this study have Ter1 and Ter2 replaced by 4 tandem Gal4 binding sites (Figure 6A, 4xUAS). Endogenous *GAL4* was also deleted to prevent competition for $UAS_{Gal}$ binding sites with the fusion protein. This targeting approach has been successful in dissecting silencing mechanisms mediated by the DNA binding proteins Rap1 and Abf1, and the Sir proteins (34–39). The 4xUAS and Ter2Δ strains showed no silencing activity when only GBD was expressed (Figure 6B and C, top rows). GBD-Fob1 expression in the 4xUAS strain produced significant silencing activity, as indicated by weaker growth on SC-leu-ura and stronger growth on SC-leu+FOA (Figure 6B, row 2), but had no effect in the Ter2Δ strain that lacked $UAS_{Gal}$ sites (Figure 6C, row 2). Targeted GBD-Fob1 silencing activity was also fully Sir2-dependent (Figure 6B, row 5), suggesting

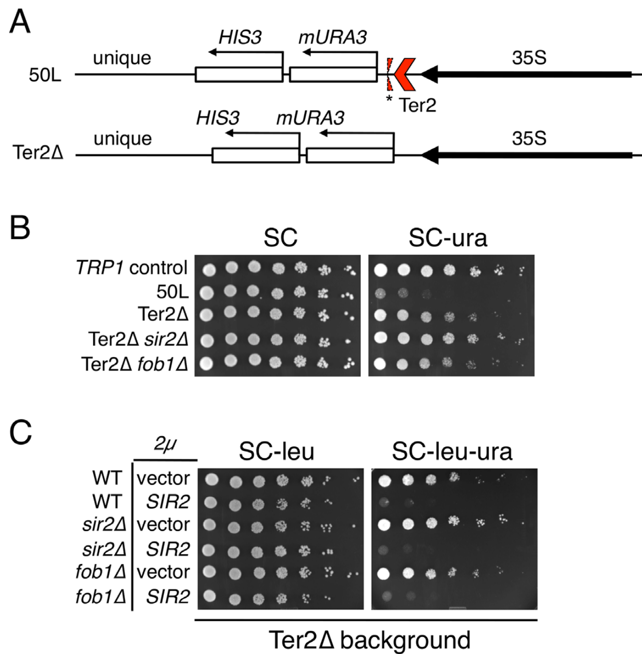


**Figure 5.** Loss of the Ter2 site from leftmost rDNA gene causes localized loss of silencing. (**A**) Schematic indication of Ter2 site removal (Ter2Δ). The truncated, inactive Ter1 site is indicated by an asterisk. (**B**) Silencing assay of *mURA3* showing effect of deleting Ter2. (**C**) *SIR2* overexpression fully suppresses the silencing defect when Ter2 is deleted.

the targeted GBD-Fob1 was capable of recruiting endogenous Sir2. GBD-Sir2 expression was included as a positive control and produced even stronger targeted silencing that completely prevented growth on SC-leu-ura (Figure 6B, row 3), even when endogenous *SIR2* was deleted (Figure 6B, row 6). Untargeted expression of GBD-Sir2 in the Ter2Δ strain was sufficient to induce partial silencing activity that was unaffected by deleting endogenous *SIR2* (Figure 6C, rows 3 and 6). This result supports the hypothesis that Sir2 effects on rDNA silencing at NTS1 and the left flank are not exclusive to localized targeting, but also likely subject to Pol I-dependent propagation.

*FOB1* overexpression has a negative effect on rDNA silencing (Figure 4C), which could partially explain why GBD-Sir2 targeted silencing was more effective than GBD-Fob1. Indeed, GBD-Fob1 expression caused much stronger targeted silencing when endogenous *FOB1* was deleted (Figure 6B, row 8). This could be because significantly less GBD-Fob1 protein is expressed compared to endogenous Fob1 in these strains (Supplementary Figure S3). Importantly, GBD-Fob1 did not induce silencing when targeted to 4xUAS-*mURA3* located at the *TRP1* locus, though GBD-Sir2 targeting did induce some repression that was dependent on the Gal4 binding sites (Figure 6D). This result suggested that Fob1 function in silencing is at least partially dependent on proximity to the rDNA and perhaps cooperating with Pol I. To begin addressing this idea, we used ChIP to test whether Fob1 association with the rDNA showed any Pol I dependency. As shown in Supplementary Figure S4, endogenous Fob1 association with NTS1, and even NTS2, was significantly reduced in an *rpa135Δ* strain, suggesting that Pol I transcription may help stabilize Fob1 association.

Because GBD-Fob1 targeted silencing was dependent on the *mURA3* reporter being proximal to the rDNA, we hypothesized that the nucleolar RENT complex was mediating silencing, not the SIR complex. If the SIR complex was involved, then deleting *SIR4* would prevent targeted silencing. Instead, we observed that deleting *SIR4* actually enhanced targeted silencing with GBD-Fob1 and had no effect in the fully silenced GBD-Sir2 strain (Figure 7A), consistent with RENT being the relevant Sir2 complex in this context. Fob1 is also involved in recruiting the cohesin clamp complex (Lrs4/Csm1) to NTS1 (40). Lrs4 and Csm1 are both required for silencing at NTS1 within the tandem array (40,41), so we next asked if Lrs4 was required for Fob1-targeted silencing to the left flank. Silencing assays with the *lrs4Δ* mutant were complicated by a general slow growth phenotype (Figure 7B, SC-leu), but the deletion appeared to have little effect on silencing strength with targeted GBD-Fob1 or GBD-Sir2 on the SC-leu-ura or SC-leu+FOA plates. We also tested whether Nsi1, a Pol I termination factor that cooperates with Fob1 to recruit RENT (42), had any effect on silencing at the left flank (50L) position. Surprisingly, little effect was observed in the *nsi1Δ* strain (Supplementary Figure S5), so it was not pursued further in targeting experiments.

The above results indicated there was a strong localized effect of Fob1 on rDNA silencing. But we also demonstrated that Pol I transcription of the leftmost rDNA repeat was required for full silencing at this position (Figures 2C and 3B). To determine which of these two processes was more critical for localized rDNA silencing, the Ter1 sequence was repaired in the context of either the prematurely terminated rDNA repeat (Figure 8A) or the Pol I promoter deletion (Figure 8B), both of which normally impair silencing when Ter1 is missing (Figure 3A and B, (2)). Interestingly, Ter1 repair fully restored silencing of *mURA3* to both mutants, so much so, that the strains became FOA-resistant (Figure 8A and B). Ter1, therefore, has a powerful positive contribution to silencing that overrides the local Pol I transcription defect, and helps explain why rDNA silencing is relatively weak adjacent to the tandem array where Ter1 is disrupted, as compared to internal NTS1 regions. Pol I activity may be significantly more critical for silencing within the array due to its effects on nucleolar organization.

## DISCUSSION

The results from this study have revealed a strong and localized effect of Fob1 on rDNA silencing at the left flanking region of the rDNA locus. Fob1 is known to be required for recruitment of Sir2 (RENT) and the cohibin complex (Lrs4/Csm1) to NTS1, where both complexes independently function in rDNA silencing (18,40). The Ter1 and Ter2 sites appear to be analogous to silencers in that they act to recruit Sir2 through an intermediary DNA binding protein, Fob1. At the left flanking region, recruitment of Sir2 appears to be the major silencing function of Fob1. Targeted silencing with GBD-Fob1 did not require *LRS4*, but was fully dependent on *SIR2*. Furthermore, GBD-Sir2 targeting fully suppressed the silencing defect of a *fob1Δ*

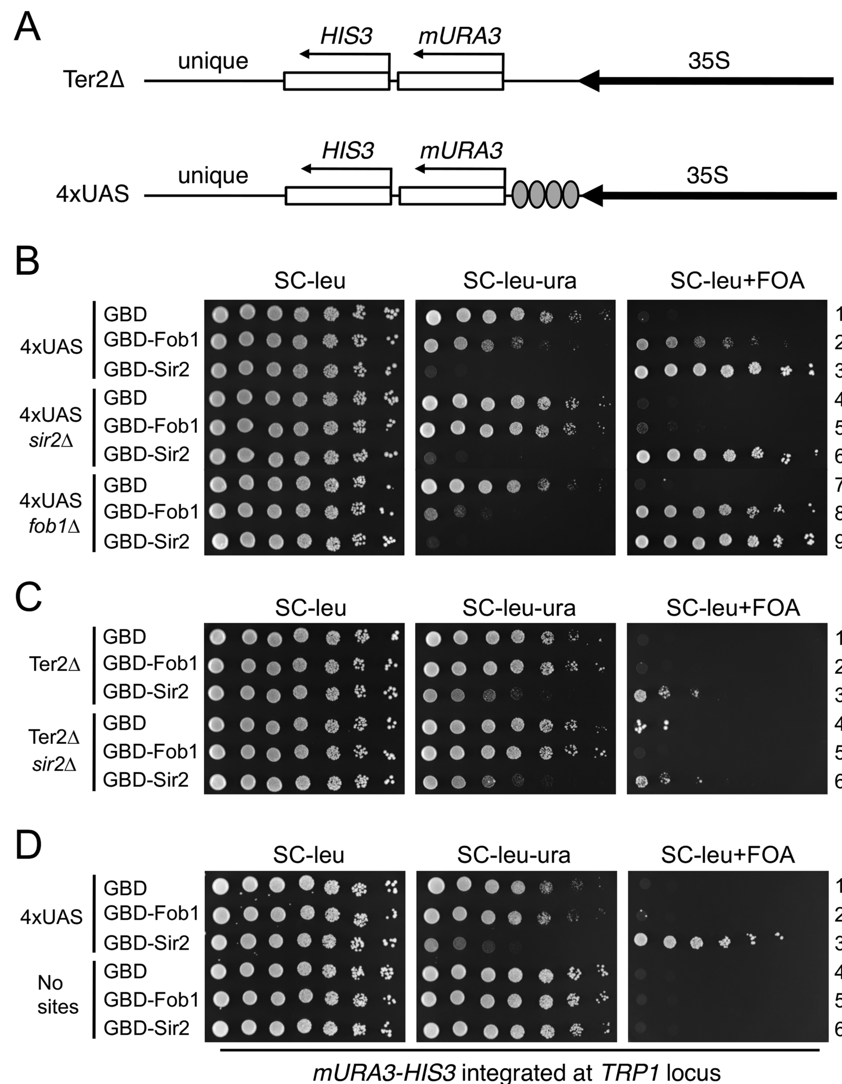


**Figure 6.** Artificial targeting of GBD-Fob1 or GBD-Sir2 restores localized silencing to Ter2 strains. (**A**) Schematic indication of 4 $UAS_{Gal}$ sites (ovals) integrated in place of the normal Ter2 site adjacent to the *mURA3* reporter. (**B**) Effects of expressing GBD alone (pGC280), GBD-Fob1 (pSB822) or GBD-Sir2 (pGC244) in WT, *sir2Δ* or *fob1Δ* strain backgrounds that contain the 4xUAS cassette. (**C**) Effects of GBD-Fob1 or GBD-Sir2 on silencing when the Ter2 is deleted, but the 4xUAS cassette is not inserted. (**D**) Targeting silencing when *mURA3-HIS3* (with or without 4xUAS sites) was integrated at the *TRP1* control locus. Row numbers are indicated to the right of each panel.

mutant. Targeting GBD-Sir2 was more effective at restoring silencing than targeting GBD-Fob1, probably because there was direct recruitment of GBD-Sir2, rather than GBD-Fob1 acting to bridge the interaction of endogenous Sir2 with chromatin. The lack of Fob1 had no impact on GBD-Sir2 targeted rDNA silencing, and GBD-Sir2 was also able to establish localized repression at non-rDNA locations like the *TRP1* locus, while GBD-Fob1 was not. These results point to a Sir2-centric role for Fob1 in silencing at the left flank and are consistent with an earlier study showing that replication fork arrest and rDNA silencing are independent and separable Fob1 functions (43). The results also demonstrate the effectiveness of using GBD targeting of factors for mechanistic studies of rDNA silencing. Targeted silencing in this context has the advantage of isolating generalized effects on nucleolar organization away from localized roles in gene repression.

In addition to Sir2 (RENT), Fob1 recruits Tof2 and the cohibin complex (Lrs4/Csm1) to NTS1, which in conjunction with cohesin, aligns the rDNA array to prevent unequal sister chromatid exchange (40). Tof2 associates with NTS1 and silences at this domain independently of Sir2, and deleting *TOF2* has no effect on silencing at NTS2 (40). Even though Fob1 does not ChIP as strongly to NTS2 ((39) and Supplementary Figure S4), we were able to detect a modest silencing defect for the *fob1Δ* mutant using a sensitive colorimetric reporter gene (*MET15*) at NTS2 (Figure 3D). Similarly, *lrs4Δ* mutants have previously been shown to have silencing defects with *MET15* integrated within NTS2 (41), even though a defect was not noticeable with *mURA3* (40). The *mURA3* reporter used in the Huang *et al.* study is located within NTS2, but downstream of the 35S transcriptional start site. Reporter genes positioned within the Pol I transcribed region can be repressed independent of

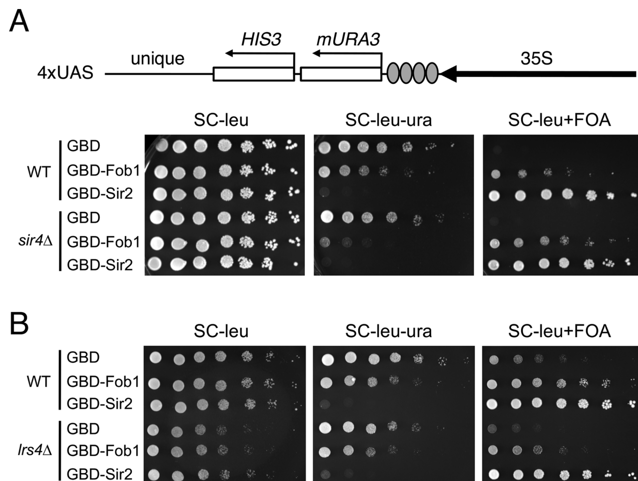


**Figure 7.** Targeted GBD-Fob1 and GBD-Sir2 silencing in (**A**) *sir4Δ* and (**B**) *lrs4Δ* strain backgrounds.

Sir2 because of interference from Pol I reading into the Pol II promoter (28), so one possible reason for differences in Fob1-dependent silencing between these studies is that the *MET15* reporter used here is located in NTS2, but outside the 35S transcribed region.

It is possible that Fob1 has an additional role internally within the rDNA array to promote higher ordered chromatin structure that contributes to silencing of Pol II transcription. Indeed, a recent study found that Fob1 mediates 'chromosome kissing' between the Ter1/2 sites from different rDNA repeats (44). These interactions were dependent on Fob1 oligomerization and regulated by intramolecular inhibitory interactions between the N- and C-terminal domains of Fob1. Under this scenario, loss of Fob1 would alter 3D chromatin architecture in such a way that disrupts silencing of internal reporter genes within the tandem array. Targeted silencing at the left flanking region would be less susceptible to such architectural chromatin changes. This could also help explain why repairing the Ter1 site, and thus improving localized Fob1/Sir2 recruitment, overrides the negative effects of Pol I transcription defects that are predicted to alter overall nucleolar organization. Chromosome kissing could also contribute to the putative epigenetic inheritance of rDNA silencing (stable FOA-resistance) we observe when Ter1 is repaired, thus allowing for possible association of the left flanking region with an internal rDNA repeat.

## The relationship between RNA Pol I and Pol II transcription within the rDNA locus

Sir2-dependent silencing of Pol II transcription within the *S. cerevisiae* rDNA locus was initially discovered through the use of Ty1 elements and reporter genes such as *mURA3* and *ADE2* (14–15,45). However, it is now well established there is native Pol II transcription occurring within the rDNA that is under Sir2 control, including non-coding RNAs derived from NTS1 and NTS2 (16,17). Functionally, the silencing/pausing of these Pol II transcripts within NTS1 and NTS2 was shown to be required for chromatin

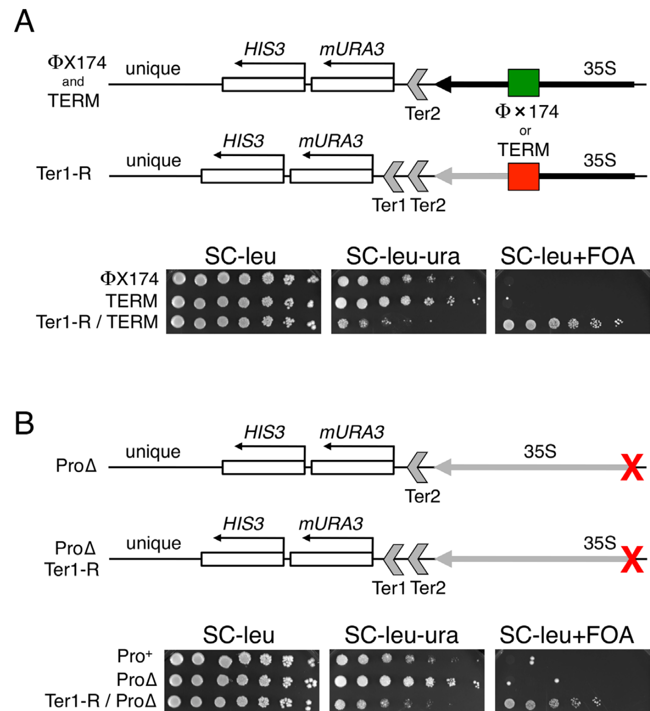


**Figure 8.** Ter1 site repair super-suppresses the localized silencing defects caused by (**A**) premature termination of Pol I transcription from the leftmost rDNA gene, or (**B**) deletion of the Pol I promoter (ProΔ) from the leftmost rDNA gene (2). In panel A, the ΦX174 sequence insertion is used as a control that does not terminate transcription or inhibit silencing, as compared to the TERM sequence insertion.

looping that functionally separates Pol I from Pol III transcription (46). Fob1 recruitment of Sir2 to NTS1, and the resultant silencing of non-coding RNAs could therefore be making significant contributions to the formation of higher ordered chromatin organization within the nucleolus.

Sir2 has the capacity to modestly spread beyond the leftmost rDNA repeat (2), but when overexpressed, can repress an *mURA3* reporter gene positioned up to ~2700 bp away from the disrupted Ter1 site (2,20), a distance that corresponds to the position of a $tRNA^{Gln}$ boundary element (20). In between, there are no genetic elements annotated in SGD other than a Ty1 LTR next to the tRNA gene. However, tiling array and RNA-seq studies have identified divergent transcription of two stable non-coding RNAs from this otherwise barren region of the genome (47,48). Their expression could potentially also be under Sir2 control during specific growth conditions that induce Sir2 overproduction. Lack of full Fob1 targeting, and therefore RENT targeting due to the disrupted Ter1 site, makes silencing at the 50L position highly sensitive to upstream Pol I transcription activity. Changes in rRNA expression could, therefore, also potentially impact the expression of these flanking non-coding RNAs, just as the non-coding RNAs derived from the intergenic spacers are susceptible to silencing in a Pol I-dependent manner (49). The mechanism of how Pol I functions in the silencing of Pol II transcription at the rDNA remains mysterious, but our new results suggest that Pol I progression to the 3′ end of the rDNA gene is important for localized silencing of Pol II downstream of

that gene. Perhaps supercoiling ahead of the numerous Pol I molecules transcribing the rDNA genes contributes to stabilization of Fob1 association with RENT at NTS1. Taken together, we conclude that Fob1 plays a dominant role in establishing silent chromatin downstream of rDNA repeats, and propose that Pol I transcription is required to stabilize Fob1/RENT recruitment.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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