Supplemental Data

System for assaying rDNA mutations

To specifically assay the efficacy of turning off chromosomal rDNA transcription and investigate the levels of plasmid-derived pre-rRNA, we used the no rDNA (empty vector) control and the ES3Δ_L mutant that contained a deletion in the sequence assayed by primer extension, such that we could differentiate mutant from endogenous pre-rRNA by their size. We assayed 27S pre-rRNA levels by primer extension at different time points after shifting to the restrictive temperature (Supplemental Fig. 1B). The levels of genome-derived rRNA progressively diminished while the mutant plasmid-derived pre-rRNA was expressed over a 24 hour period. Based on this, we decided to use a 6 hour shift to the restrictive temperature in order to assay phenotypic effects of all further ES deletions. While there is residual pre-rRNA from the endogenous WT rDNA transcription even after the 6 hour shift, we also wanted to minimize secondary effects arising from prolonged shift to the restrictive temperature.

Information about each ES_L

$ES19_L$: This is the only completely dispensable ES among the ones that we studied. We observed near WT levels of 25S rRNA being made in the ES19Δ_L mutant, in agreement with its healthy growth phenotype. (Musters et al. 1991) showed that this region (referred to as V9 in their study) can be replaced by corresponding *E.coli* or mouse rRNA sequences without any discernable effect on ribosome assembly and function. Even though (van Beekvelt et al. 2000) have indicated that deletion of V9 may have slight effects on the biogenesis of 25S rRNA, our deletion more closely resembles the one used by (Musters et al. 1991) and is consistent with their finding that this region is dispensable. $ES19_L$ makes few contacts with r-proteins L25, L8 and L2 in the mature 60S subunit (Ben-Shem et al. 2011). As pointed out

previously (van Beekvelt et al. 2000), it is clear that binding of those r-proteins to this ES is not necessary for their recruitment, since the r-proteins themselves are essential.

ES27h$_L$: This is the largest ES present in *Saccharomyces cerevisiae* rRNA. We assayed only a partial deletion in this study, due to technical limitations we faced in mutagenesis. We found that although this deletion did not strongly inhibit growth, it resulted in a slight accumulation of 7S pre-rRNA. This could partially explain the decrease in levels of 60S ribosomal subunits observed in this mutant by the more sensitive sucrose gradient assay, even though we observed near-WT levels of mature 25S rRNA being made. Processing of 7S pre-rRNA has been shown to be dispensable for the normal function of the ribosome (Rodriguez-Galan et al. 2015), hence it is not surprising that this mutant yields a slight 7S pre-rRNA processing defect while it exhibits no growth defect. This has been a very intriguing expansion segment for various reasons. Apart from serving as a major binding site of the export factor Arx1, which is also non-essential (Bradatsch et al. 2012), this ES has been shown to adopt two conformations, with the helix stem either extending toward the L1 stalk or toward the polypeptide exit tunnel (Beckmann et al. 2001). While several studies have investigated the effects of insertions in this ES, a previous deletion analysis concurred with our results on the dispensability of this part of ES27 (Jeeninga et al. 1997). The same study pointed out that the larger deletions of ES27 were lethal and resulted in defective ribosome biogenesis. This ES is also known to have a putative quadruplex forming sequence, the functions of which are yet unknown (Karen Kormuth, personal communication).

ES39$_L$: This is evidently the earliest-acting ES; its deletion causes accumulation of the early 27SA$_2$ pre-rRNA processing intermediate. This is a large ES occupying a major segment of 25S rRNA domain 6. R-proteins that bind to domain 6, including L33, L16 and L20 and L3, have been previously shown to function in early steps of ribosome assembly. Hence, this ES39$_L$Δ deletion phenotype agrees with the function of r-proteins that bind it.

$ES5_L$: This early-acting ES is located in 25S rRNA domain 1. In accordance with the depletion phenotype of the r-proteins that bind this ES and other regions of domain 1, such as L8 and L36, deletion of $ES5_L$ results in an early phenotype.

$ES7_L$: This is the second largest ES in yeast; its deletion causes an early phenotype. It forms major contacts with early-acting r-proteins, including L4, L6, L7, L33 and L20. Consistent with our results, (Jeeninga et al. 1997) showed that this ES is necessary for growth and for processing of 27SA pre-rRNA.

$ES15_L$: This early-acting ES present in rRNA domain 2, is surrounded by L4, L18 and L6, all of which have an early phenotype when depleted.

$ES3_L$: This middle-acting ES is the only ES in 5.8S rRNA. $ES3\Delta_L$ results in accumulation of 27S pre-rRNA that is not accompanied by an evident increase in 27SA pre-rRNA. Consistent with this observation, $ES3_L$ establishes multiple contacts with L25, a protein that has been implicated in the middle processing steps.

$ES20_L$, $ES26_L$: These two ES form loops on opposite sides of helix 54 in domain 3 and are involved in several stacking and other non-canonical interactions (Ben-Shem et al. 2011). We classify them as 'middle'-acting, since they have a strong accumulation of 27S pre-rRNA that is not accounted for by an increase in 27SA pre-rRNA. In the mature ribosome, they make contacts with L27 and L34, depletions of which have been shown to have a middle pre-rRNA processing defect. In addition, assembly factor Nop7 has been shown to crosslink to these sites (Granneman et al. 2011). Although depletion of Nop7 has been shown to have an early phenotype, we believe that Nop7 may have other functions in middle steps, which may be uncovered by mutating its binding site with these ES.

$ES9_L$, $ES10_L$, $ES12_L$: These ES are all located in domain 2 in the secondary structure, near H38, and are located underneath the central protuberance of the ribosome. Proteins that localize near the central

protuberance have been implicated in late nuclear processing steps; this trend is consistent with the late phenotypes exhibited by these ES deletions. ES12Δ_L also results in a slight increase in 27S pre-rRNA, similar to a few other ribosomal assembly mutants deficient in 7S processing (Saveanu et al. 2001; Gamalinda et al. 2014). Also, a recent study showed that the central protuberance undergoes a major structural change prior to export of the assembling ribosome (Leidig et al. 2014), coincident with late pre-rRNA processing steps, which further adds credence to the hypothesis that these ES enable proper construction of the central protuberance and other related events that eventually lead to nucleocytoplasmic export of the assembling ribosome.

ES41$_L$: Deletion of ES41$_L$ located near the 3' end of 25S rRNA, leads to a mild accumulation of 7S pre-rRNA, while also leading to a slight increase in levels of 27S pre-rRNA. The effect of deletion of this ES on production of mature 25S rRNA seems modest, even though the assembly factor Rei1 has been shown to contact ES41$_L$ (Greber et al. 2012).

Supplemental Figure Legends

Supplemental Fig. 1: System used for rDNA mutagenesis. (A) Illustration of the yeast strains and the mechanism of endogenous rDNA transcription shut-off showing both the permissive temperature (left) and the restrictive temperature (right) conditions. Repeats of rDNA transcriptional units are shown as black boxes. (B) Levels of pre-rRNAs after shifting to the restrictive temperature. Levels of endogenous WT pre-rRNA and plasmid-derived mutant pre-rRNA that is shorter in size (right panel, shorter) were measured by primer extension, using total RNA extracted at multiple time points upon shifting to the restrictive temperature. U2 snRNA is the loading control.

Supplemental Fig. 2: The pre-rRNA processing pathway in yeast. Early, middle and late pre-rRNA processing steps, and pre-rRNA intermediates are color-coded in green, purple and orange, respectively, and various pre-rRNA species are indicated.

Supplemental Fig. 3: An alternative representation of the primer extension data in Fig. 3B. Shown here are the mean values from three biological replicates. Error bars represent standard deviation.

Supplemental Table 1: Shown are each of the ES mutations investigated in this study and the contacts of each ES with r-proteins are also shown (Ben-Shem et al. 2011). Both ES and r-proteins (Gamalinda et al. 2014) are color coded according to their deletion/depletion phenotype (Early: Green; Middle: Purple; Late: Orange). The number in brackets denotes the number of atomic contacts made. Lighter shades represent fewer than 5 atomic contacts. Non-essential ES and r-proteins with undetermined function are shown in gray.

Supplemental Table 4 (A-C): Each of the essential ES tested in this study are shown individually along with the r-proteins that form crystal structure contacts with that particular ES, color-coded according to function.

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