**RESEARCH ARTICLE SUMMARY**

**SYNTHETIC BIOLOGY**

**Engineering the ribosomal DNA in a megabase synthetic chromosome**

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**INTRODUCTION:** It has long been an interesting question whether a living cell can be constructed from scratch in the lab, a goal that may not be realized anytime soon. Nonetheless, with advances in DNA synthesis technology, the complete genetic material of an organism can now be synthesized chemically. Hitherto, genomes of several organisms including viruses, phages, and bacteria have been designed and constructed. These synthetic genomes are able to direct all normal biological functions, capable of self-replication and production of offspring. Several years ago, a group of scientists worldwide formed an international consortium to reconstruct the genome of budding yeast, *Saccharomyces cerevisiae*.

**RATIONALE:** The synthetic yeast genome, designated Sc2.0, was designed according to a set of arbitrary rules, including the elimination of transposable elements and incorporation of specific DNA elements to facilitate further genome manipulation. Among the 16 *S. cerevisiae* chromosomes, chromosome XII is unique as one of the longest yeast chromosomes (~1 million base pairs) and additionally encodes the highly repetitive ribosomal DNA locus, which forms the well-organized nucleolus. We report on the design, construction, and characterization of chromosome XII, the physically largest chromosome in *S. cerevisiae*.

**RESULTS:** A 976,067-base pair linear chromosome, synXII, was designed, based on the native chromosome XII sequence of *S. cerevisiae*, and chemically synthesized. SynXII was assembled using a two-step method involving successive megachunk integration to produce six semisynthetic strains, followed by meiotic recombination-mediated assembly, yielding a full-length functional chromosome in *S. cerevisiae*. Minor growth defect “bugs” detected in synXII were caused by deletion of tRNA genes and were corrected by introducing an ectopic copy of a single tRNA gene. The ribosomal gene cluster (rDNA) on synXII was left intact during the assembly process and subsequently replaced by a modified rDNA unit. The same synthetic rDNA unit was also used to regenerate rDNA at three distinct chromosomal locations. The rDNA signature sequences of the internal transcribed spacer (ITS), often used to determine species identity by standard DNA barcoding procedures, were swapped to generate a *Saccharomyces* synXII strain that would be identified as *S. bayanus*. Remarkably, these substantial DNA changes had no detectable phenotypic consequences under various laboratory conditions.

**CONCLUSION:** The rDNA locus of synXII is highly plastic; not only can it be moved to other chromosomal loci, it can also be altered in its ITS region to masquerade as a distinct species as defined by DNA barcoding, used widely in taxonomy. The ability to perform “species morphing” reported here presumably reflects the degree of evolutionary flexibility by which these ITS regions change. However, this barcoding region is clearly not infinitely flexible, as only relatively modest intragenus base changes were tolerated. More severe intergenus differences in ITS sequence did not result in functional rDNAs, probably because of defects in rRNA processing. The ability to design, build, and debug a megabase-sized chromosome, together with the flexibility in rDNA locus position, speaks to the remarkable overall flexibility of the yeast genome.

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**Hierarchical assembly and subsequent restructuring of synXII.** SynXII was assembled in two steps: First, six semisynthetic synXII strains were built in which segments of native XII DNA were replaced with the corresponding designer sequences. Next, the semisynthetic strains were combined with multiple rounds of mating/sporulation, eventually generating a single strain encoding full-length synXII. The rDNA repeats were removed, modified, and subsequently regenerated at distinct chromosomal locations for species morphing and genome restructuring.
We designed and synthesized a 976,067–base pair linear chromosome, synXII, based on native chromosome XII in *Saccharomyces cerevisiae*. SynXII was assembled using a two-step method, specified by successive megachunk integration and meiotic recombination-mediated assembly, producing a functional chromosome in *S. cerevisiae*. Minor growth defect “bugs” detected in synXII, caused by deletion of tRNA genes, were rescued by introducing an ectopic copy of a single tRNA gene. The ribosomal gene cluster (rDNA) on synXII was left intact during the assembly process and subsequently replaced by a modified rDNA unit used to regenerate rDNA at three distinct chromosomal locations. The signature sequences within rDNA, which can be used to determine species identity, were swapped to generate a *Saccharomyces* synXII strain that would be identified as *Saccharomyces bayanus* by standard DNA barcoding procedures.

**Assembly**

To construct synXII, we designed a total of 33 megachunks with sizes ranging from 26 to 39 kb using BioStudio (6); each megachunk was synthesized as 16 to 26 “minichunks” of ~1.6 kb (www.syntheticyeast.org; fig. S1). Through-out synXII, 123 TAG stop codons were converted to TAA and 299 loxpSym sites were inserted.

A standard workflow was established to assemble and screen synthetic strains displaying high fitness (fig. SIB). The strategy to replace the native chromosome involves alternating each megachunk with a *LEU2* or *URA3* selective marker (3, 4) (fig. SIC). This strategy worked well when the number of replacements was small (11 for synIII) but became less efficient once the number of rounds was extended. Therefore, an alternative approach (designated as meiotic recombination-mediated assembly [MRA]) was adopted, in which six initial strains were used for chromosome replacement simultaneously, each harboring six or seven megachunks of synXII (Fig. 1, step I). Subsequently, these intermediate strains were crossed, sporulated, and screened for spores in which the synthetic sequences were combined. After four or five rounds of successive MRA, multiple strains containing the entire synXII were obtained (Fig. 1). In these strains, we intentionally left the rDNA locus intact. The MRA method provided a diagnostic tool to identify synthetic fitness defects (or synthetic lethality) between different synthetic regions. During the process of assembling synXII, several such regions were identified, which led to a severe fitness defect when changed to synthetic sequences for either known or unknown reasons. These “buggy” regions were modified or restored to native sequences to meet fitness requirements (fig. S3).

**Characterization**

The synXII strains were subjected to polymerase chain reaction tag (PCRTag) analyses that revealed the presence of synthetic PCRTags and the absence of native PCRTags at expected loci (Fig. 2A and figs. S4 and S5). Seven pairs of native PCRTags produced amplicons in the synthetic background; we attribute this to the presence of homologous genes, as deleting the homologous copy removed the background (fig. S6). Unlike other reported synthetic yeast chromosomes (9–11), synXII does not show any obvious size difference from XII because of the large size of this particular chromosome under the conditions used. Only after removal of the entire rDNA locus in both synthetic and wild-type strains could synXII be distinguished from XII by the 100-kb reduction in size (see below).

We found growth of the synXII strain (yeast chr12_9_02) to be largely indistinguishable from the isogenic wild type in rich medium (Fig. 2B), except for a minor slow-growth phenotype, observed only during short incubation times (fig. S7) or in synthetic medium (Fig. 2C, compare first two rows). These growth defects might be due to a slight delay during the G2-M transition in the synXII strain (Fig. 2D). To map the origin of this growth defect, we examined all intermediate strains and found that it was inherited from strains harboring synthetic megachunk S-Z (fig. S8, A and B). Further analysis revealed that the defect was introduced when megachunks S-U and V-Z were combined. Through sequence comparison, we identified two leucine tRNA genes (*tL(UAG)L1* and *tL(UAG)L2*) in megachunk S and *tL(UAG)L1* in megachunk W; both were removed in the design phase. Given that there are only three *tL(UAG)L1* genes in the yeast genome, we hypothesized that the copy number reduction might cause the growth defect. We therefore designed two arrays (tRNA array A and B) to include all tRNA genes within chromosome XII. We found that the presence of either array (A contains *tL(UAG)L1* and B contains *tL(UAG)L2*) could rescue the growth defect. Furthermore, the presence of a single copy of one
of the leucine tRNA genes completely restored normal growth and cell cycle progression (Fig. 2, B and D).

Two synXII strains were subjected to whole-genome sequencing, which revealed the presence of fully designed synthetic sequences except for a handful of expected regions, which were restored to the native form to overcome thefitness defects. All sequence variations from the design are listed in table S2, including nine missense defects. All sequence variations from thegenome sequencing, which revealed the presence of an ectopic copy of the rDNA, because their expression was restored in the presence of an ectopic copy of the TLL(UAG)L2 gene (fig. S9). In addition, the expression of three genes on chromosome XII was substantially altered. CTR3 resides at a locus near a Ty-insertion hotspot and is often mutated in many lab strains by transposon insertion (12, 13). The increased expression of CTR3 in synXII may be caused by the removal of the transposon elements near its 5’terminal region (UTR). Two other genes, G4S2 and AHP1, were down-regulated, potentially resulting from the incorporation of PCRTags orloxPsym sites (9, 10).

rDNA transplantation, nucleolar organization, and species identity swap

A unique feature of chromosome XII is the existence of more than 100 copies of rDNA repeats, which form a well-organized nucleolus similar to that present in all eukaryotic cells (14). We removed the entire rDNA region from synXII after having introduced a high-copy plasmid harboring a single ectopic rDNA unit (15) (yeast_chr12_9_04). The size of synXII was reduced to ~1 Mb and was easily distinguished from the native XI without rDNA (Fig. 3A and fig. S10). The nucleolar morphology was examined using Nop10-GFP (green fluorescent protein) to label the nucleolus and Nic96-mCherry to mark the nuclear envelope. We found that removal of the rDNA region resulted in the disruption of the previously well-organized nucleolar structure and a GFP “mini-nucleolus signal” became dispersed within the nucleolus (16) (Fig. 3B). There is no obvious difference between synXII and wild-type strains both in the presence and absence of an rDNA region. Similar results were obtained using electron microscopy (fig. S11).

Next, we tested whether an engineered rDNA unit could be used to regenerate a normal nucleolus and at the same time alter the internal transcribed spacer (ITS) region, a “DNA barcode” used widely in species identification. We constructed several chimeric rDNA units by swapping the ITS regions (from S. bayanus to S. pombe) for corresponding sequences from Saccharomyces bayanus, Schizosaccharomyces pombe, or Candida albicans (fig. S12). A plasmid shuffling assay indicated that the ITS regions from S. pombe or C. albicans could not support cell viability (Fig. 3C). In contrast, replacing the ITS regions with those from S. bayanus resulted in normal cell growth, even in the presence of a point mutation in the 18S rRNA coding region that confers hygromycin resistance (15) (Fig. 3C). We then tested whether this chimeric rDNA unit could be integrated back into the chromosomal locus and function normally after regenerating a new rDNA cluster. Several genomic locations were chosen as potential new homes for the rDNA including the original location from which the rDNA had been removed on synXII, the right arm of chromosome XV (ChrXV), which is similar in size to chromosome XIIIR, and the right arm of chromosome III (ChrIIIIR), which is a much shorter
Fig. 2. Characterization of synXII. (A) PCRTag analysis of synXII and BY4741. Only one pair of tags from each megachunk was selected as representative. The complete PCRTag analysis is shown in figs. S4 and S5. (B) Fitness analysis of synXII strain with or without the ectopic tL(UAG)L2 gene on various growth conditions. The types of media, growth temperature, and period were as indicated. YPD, yeast extract peptone-glycerol-ethanol; DTT, dithiothreitol; CPT, camptothecin. (C) Removing all tRNA genes from synXII led to a growth defect. After 10-fold serial dilution, cells (10^3 for each spot) were dropped onto synthetic complete medium lacking leucine, uracil, and lysine (SC-Leu-Ura-Lys). The tRNA array A included tL(UAG)L1, tL(UAG)L2, and tV(AAC)L on a centromeric plasmid. Array B included tA(AGC)L, tL(CAU)L, tL(UAU)L1, tQ(UU)L1, tD(GUC)L1, tR(AGC)L, tA(AGC)L, tR(UUG)L, and tP(UGG)L. The three tRNA genes were tL(UAG)L1, tL(UAG)L2, and tL(UAG)L2. (D) Ectopically expressed tL(UAG)L2 could rescue the cell cycle delay of synXII. The yeast strains were synchronized with hydroxyurea, stained by propidium iodide, and analyzed by flow cytometry. (E) Transcript profiling of wild-type (BY4741) and synXII containing tL(UAG)L2 using a volcano plot. Genes deleted from synXII are labeled in brown. Genes with significant expression difference are shown in blue (for genes located on chromosome XII) and black (for genes on other chromosomes). Marker genes are labeled in cyan. The dashed line denotes the family-wise error rate threshold at 10^{-4} (threshold = 1.4 × 10^{-4}).
to interrupt a nonessential gene, but in many cases this led to severely impaired mitochondrial function or stress resistance. Theoretically, the defect should have been restored once the next megachunk was integrated (fig. S18). However, the mitochondrial defects commonly resulted in a substantially slower growth rate, greatly prolonging the replacement cycle (fig. S16A). Third, synonymous recoding within an open reading frame is generally well tolerated. We found at least one case during the process of replacing megachunk E, in which the function of MMM1 was impaired as a result of recoding to generate a PCRTag (YLL006W.1F) (9, 10). Fourth, deletion of a hypothetical intron within the 5′ UTR of COQ9 led to a transcriptional block (fig. S16). Because we paid close attention to cell growth in YPGE medium throughout the assembly process, most of the detected defects are related to mitochondrial function. There might be other minor defects in the synthetic genome, which went unnoticed under the conditions tested. However, given that the final strains display nearly wild-type fitness once a tRNA is supplied, synXII is the largest synthetic linear chromosome synthesized and it is fully functional.

**Fig. 3. Morphing species identity with chimeric rDNA repeats.** (A) Karyotype analysis of native chromosome XII and synXII with or without chromosomal rDNA repeats by pulsed-field gel electrophoresis. The identity of each chromosome is indicated at the right. The black arrowhead points to both native XII and synXII containing the rDNA cluster. After removing the entire rDNA, the native chromosome XII was indicated with the open arrowhead and synXII with red dots. (B) Removal of chromosomal rDNA array disrupted nuclear structure. The nucleus was visualized with Nop10-GFP (green) and NIC96-mCherry (red) used to position the nucleus. (C) Swapping the internal transcribed spacer (ITS) with that of S. bayanus enabled cells to survive. The native ITS was replaced with its corresponding sequences from other species to construct the chimeric rDNA unit, which was tested in a plasmid shuffling assay. Sb-ITS, Ca-ITS, and Sp-ITS represent chimeric rDNA containing ITS sequences from *Saccharomyces bayanus*, *Candida albicans*, and *Schizosaccharomyces pombe*, respectively. An asterisk indicates the presence of the T1758C substitution in 18S rDNA, which confers hygromycin B resistance. (D) Locations of rDNA array on chromosomes XV and III. The red arrows point to the position where the seed sequence was integrated. The numbers are the chromosome coordinates from the *Saccharomyces* Genome Database. (E) Karyotype analysis of yeast strains containing Sb-ITS chimeric rDNA array at different chromosomes by PFGE and Southern blot. P denotes the yeast strain with rDNA locus removed from native chromosome XII and put onto the plasmid. The black arrowhead shows the synXII without chromosomal rDNA region; the red arrowheads denote the chromosomes containing the chimeric rDNA array. EtBr, ethidium bromide. (F) Restriction enzyme digestion to ensure the absence of native ITS sequence in chimeric rDNA strains. A DNA fragment within ITS was amplified by PCR, which contained an Apal recognition site only existing in *S. cerevisiae*. The PCR products were treated with (top) or without (bottom) Apal. (G) Growth analysis of strains with reconstructed chimeric rDNA array. (H) Nucleolar morphology analysis of strains with reconstructed chimeric rDNA array using Nop10-GFP (green) and NIC96-mCherry (red) as described in Fig. 1. (I) Nucleolar morphology analysis of strains with reconstructed chimeric rDNA array using electron microscopy. Scale bars, 500 nm.
pelleted, washed with 1 mL 5 mM CaCl2 and heat-shocked at 42°C for 15 min. The cells were

The colonies appeared on the selective plates after overnight incubation at 30°C, the colonies can grow on one type of medium but not the other were identified and subjected to PCR analysis to verify the incorporation of the entire synthetic megachunk.

**Genomic DNA preparation for PCRTag analysis**

Yeast cells were washed with 500 μL sterile water and resuspended into 100 μL breaking buffer [30 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0, 2% (v/v) Triton X-100, 1% (w/v) SDS]. 100 μL of 0.5 mm Glass beads (Biospec, I079105) and 200 μL of phenol/chloroform/isoamyl alcohol (25:24:1) were added into the tube and vortexed at 2000 rpm for 10 min. Add 100 μL sterile water into the tube, and mix briefly. Centrifuge the tube at 12,000 rpm for 10 min. Transfer the top layer into a new tube and add 500 μL 100% ethanol and chill the tube at −20°C for 15 min before centrifugation (13,000 rpm, 5 min at 4°C). The pellet was washed with 500 μL 75% ethanol and dried in vacuum pump (Eppendorf AG 22331 Hamburg, 45°C, 3 min). The genomic DNA was dissolved in 50 μL sterile water and stored at −20°C.

**PCRTags confirmation**

To confirm the strains, two rounds of PCR were performed. At first, four pairs of PCRTags, evenly distributed in synthetic chunk, were chosen to screen the phenotypically desired clones. rTaq DNA polymerase (TaKaRa, R001W) was used together with 300 ng of genomic DNA in a 10 μL reaction containing 1 μM of primer each. The PCR program was as following: 1 cycle of 94°C for 5 min, 30 cycles of 94°C/30 s-55°C/30 s-72°C/30 s, 1 cycle of 72°C for 7 min and 16°C keep. The clones passed the first round of PCR test were subjected to next round of PCRTag PCR analysis using all primers within the megachunk to identify the ones containing the entire synthetic DNA.

**Native chromosome XII replacement**

The entire synXII contains 33 megachunks, which were used to replace the native chromosome iteratively as described previously (3). Six strains, each containing a KanMX4 deleted gene from YKO collection were used as the parental strains to perform the replacement in parallel. 3-4 synthetic chunks with one minichunk overlapping to cover the entire megachunk were introduced followed by clone selection, PCRTag confirmation as described above.

**Megachunk combination by MRA**

Meiotic recombination-mediated assembly was used to combine partial synthetic synXII strains. Two strains with different mating type were mated on the YPD plate for 4–6 hours at 30°C. Then the diploid cells, identified by their characteristic thick zygotic neck or trefoil shape, were isolated with a micromanipulator (Carl Zeiss Microscopy GmbH, Binocular microscope Axio Lab.A1). The isolated diploids were verified and subjected to sporulation using protocol as described (www-sequence.stanford.edu/group/yeast_deletion_project/spo.html). The diamond shaped asc us were dissected onto the YPD plate. After growth at 30°C for 48 hours, the cells were replicated onto various selective media to identify their auxotroph and mating type. The colonies with desired markers were selected for PCR verification. Again, two rounds of PCRTag analysis were performed with selective PCRTags at first followed by all PCRTags. The selective PCRTag analysis used one pair of PCRTag per chunk by colony PCR method (see below). If, in some cases, a completely combined synthetic strain could not be identified, a succeeding round of MRA will be carried out using spores with the most synthetic DNA until at least two clones containing the desired synthetic chromosome were obtained.

**Yeast colony PCR for rapid screening of WT and SYN PCRTags**

Yeast cells in from single spore were resuspended in 30 μL ddH2O and lysed in a thermocycler (Applied Biosystems, GeneAmp PCR System 9700 Dual 96 well) (95°C/10 min, 10°C/1 min, 3 cycles, kept at 10°C). After centrifugation at 3000 rpm for 1 min, 3 μL of supernatant was used as template in 10 μL PCR reaction (see above).

**Mating type switch**

Overnight haploid yeast culture was diluted to OD600 = 0.1 in 5 mL YPD, then cultured for 4–5 hours until OD600 reached 0.4. About 500 ng plasmid carrying HO endonuclease (pBD37 or pD148, plasmid information available at www.syntheticyeast.org) under the control of Gal1 promoter was transformed. The transformed cells were cultured in 5 mL selective medium for the plasmid at 30°C overnight. The overnight culture was diluted to OD600 = 0.1 using 5 mL induction medium containing 2% galactose and cultured at 30°C for 4 hours. The cells were diluted and plated onto YPD plate, and then the single colonies without plasmid but with the desired mating type were chosen for further experiments.

**Sporulation**

A diploid colony was inoculated into 2 mL GNA medium (5% dextrose, 3% Difco Nutrient Broth, 1% Difco Yeast Extract) at 30°C overnight. Then the cells were washed with sterile water four times, diluted to OD600 = 1.0 in sporation medium (1% potassium acetate, 0.005% zinc acetate, supplemented with any amino acids required for the diploids at one-fourth concentration as that in the SC medium). Mixed well and incubated at 25°C for 3–7 days.

**Total RNA extraction, sequencing, and analysis**

The yeast strains were cultured in appropriate medium overnight, diluted to OD600 = 0.1, and grew until OD600 reached about 1.0. Cells (15 OD) was collected by centrifugation at 4°C at 3000 rpm for 10 min and washed with iced water, and resuspended in 600 μL TRIzol (Life Technologies, 15596). 300 μL glass beads were added and the yeast cells were disrupted with Mini-Beadbeater-96 (Biospec, OA60AP-22-1W) for 1 min. Transfer
600 μL liquid mixture into a new microfuge tube and add 300 μL 1-chloro-3-bromopropane. Vigorously shake the tube by hand for 3 min. Centrifuge at 12,000 rpm for 6 min, and then transfer upper aqueous layer (~300 μL) into a new tube. RNA was precipitated by adding an equal volume of isopropanol and pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The pellet was washed with 700 μL 75% ethanol and dissolved in 100 μL RNase-free water. Total RNA was digested with DNaseI (NEB, M0309) and purified with RNA clean kit (TIANGEN, DP412) before sending for high-throughput sequencing. The raw reads were mapped onto the synthetic reference genome using TopHat with standard parameters. Counts of reads overlapping genes were computed in R using the countOverlaps function from the GenomicRanges package. Differential expression analysis was performed by DESeq and volcano plots were generated in R.

**Yeast genomic DNA preparation for DNA sequencing**

The genomic DNA was prepared using the method described by Bespalov et al. (18) with minor modification. 5 mL overnight yeast culture (approx. 5 × 10^6 cells) was collected by spinning at 3000 rpm for 5min, washed once in 1 mL ice cold PBS (37 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), and resuspended in 300 μL of nuclei isolation buffer (NIB: 50 mM MOPS pH 8.0, 150 mM potassium acetate, 2 mM MgCl2, 17% glycerol, 0.5 mM spermidine, 0.15 mM spermine) and transferred into a 2-mL tube containing 300 μL acid-washed glass beads (BioSpec, Cat. No.11079105, 0.5 mm dia.). The samples were vortexed for 30 s, and then cooled on ice for 30 s, which was repeated 8 times to disrupt the cells. 110 μL 10% SDS was added into the lysate, mixed well, and then incubated at 65°C for 3.5 hours. Collected the cell lysate and adjusted to 500 μL in total volume with buffer TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 500 μL saturated NaCl was added. Mixed gently and then the mixture was centrifuged for 30 min at 8000 rpm. 800 μL supernatants were added into equal volume of 100% isopropanol to precipitate DNA. The pellets were dissolved in 200 μL TE buffer, phenol extracted, precipitated in 500 μL 100% ethanol with 70 μL 3 M sodium acetate. The DNA was dissolved in 200 μL TE buffer: 10 μL 10 mg/mL RNase was added and incubated at 37°C for 3 hours before phenol extraction and ethanol precipitation of DNA. Eventually, the genomic DNA was dissolved in 100 μL ddH2O.

**Stress sensitivity assay**

Single colonies were cultured in YPD overnight at 30°C. Cells were adjusted to the same OD600 series diluted by 10-fold and spotted onto various selective plates. YPD medium in 30°C was used as control. For temperature stresses, plates were incubated at 16°C, 25°C, and 37°C for appropriate time, respectively, before photography. Besides these, all other plates with drugs were incubated at 30°C for 2–4 days before photography.

**Protein tagging, Western blot, and microscope imaging**

Proteins were fused to an epitope-tag in vivo using a PCR-based C-terminal tagging cassette as described (19), and in some strain backgrounds, the selective markers were swapped. For Western blot, the total protein extracts (20) were separated on a 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. Mouse monoclonal anti-HA (Sigma H3663, 1:2000) and rabbit polyclonal anti-Histone H3 (Abcam ab791, 1:5000) were used, followed by ECL detection system (GE Healthcare, RPN2200) according to the manufacturer’s instruction. To visualize the tagged proteins, cells were imaged using a Zeiss LSM780 confocal microscope under 100× objective.

**Total RNA extraction and Northern blot**

Total RNA was extracted from cells grown in YPD at log-phase using Trizol (Life Technologies, 15596-026) followed by glass beads disruption with Mini-Beadbeater-96 (Biospec, OA60AP-22-1WB). The RNAs were separated on an agarose gel containing 1.2% formaldehyde and transferred to a nylon membrane complete coding sequence of COQ9 and ACT1, respectively, in ULTRAhyb buffer (Thermo Fisher Scientific, AM8607) according to the manufacturer’s instruction (21).

**Construction of strains with complete deletions of chromosomal rDNA**

To delete the entire chromosomal rDNA repeats, strains were transformed with a high-copy plasmid containing a single rDNA repeat (pRDN-WT; 2micron, URA3, gifted from Susan Lieberman’s laboratory (22) at first. At the same time, two plasmids were constructed, containing a NatMX4 cassette flanked by homologous sequences corresponding to the left and right regions of the rDNA locus for the native and synthetic strains, respectively. These plasmids were linearized and transformed into the strains containing pRDN-WT. The resulting transformants were replicated onto medium with 5-FOA to identify clones failed to grow, which indicated that the cells solely depend on the rDNA on plasmid due to the loss of chromosomal rDNA repeats and were not able to lose the URA3-based plasmids. The candidate strains were confirmed by pulsed-field gel electrophoresis, followed by Southern blot.

**Construction of plasmids containing chimeric rDNA**

The native rDNA unit was PCR amplified and cloned into pRS425 to generate pRDN-S, which was subsequently used to construct the chimeric rDNA. In addition, a T758C mutation was introduced to generate resistance to hygromycin B, resulting plasmid pRDN-S-HygR (25). The DNA sequence of ITS1-5.8S-ITS2 region in S. bayanus, C. albicans, and S. pombe were synthesized from overlapping oligos and used to swap the corresponding sequence in pRDN-S and pRDN-S-HygR to generate the chimeric rDNA constructs. Detailed sequence information is listed in table S5.

**Plasmid shuffling assay**

The yeast strains with deleted chromosomal rDNA region and bearing pRDN-WT were transformed with either wild-type or chimeric rDNA plasmids. The plasmid shuffling assay was performed by growing the cells onto media containing 5-FOA (26). The 5-FOA resistant clones were verified by PCR to ensure the loss of pRDN-WT plasmids using primers specific to both URA3 and ITS regions.

**Reconstruction of the chromosomal rDNA array**

The procedures to regenerate the rDNA array were outlined in fig. S13. In brief, the rDNA repeats (1.2 or 2 copies) containing the hyg1 mutation were integrated at a chromosomal locus by homologous recombination. After confirming the targeted integration, the strains were patched on SC-Leu plate and replicated sequentially onto YPD medium supplied with increasing amount of hygromycin B (50 μg/mL for 1 day, 100 μg/mL for 1 day, 200 μg/mL for 2 days) until at final concentration 300 μg/mL (for 1 day). Finally, the cells were replicated onto medium containing 5-FOA to lose the pRDN-WT plasmid and colonies were streaked onto fresh medium before PCR confirmation and PFGE analysis.

**Pulsed-field gel electrophoresis**

Chromosome-sized DNA was prepared as described previously (24). Identity of the chromosomes was inferred from the known molecular karyotype of WT (BY4741 or BY4742) that was run on the same gel. Samples were resolved using the Random primer DNA Labeling Kit (TaKaRa, 6045), and hybridized using UltraHyb hybridization buffer (Thermo Fisher Scientific, AM8607).

**Southern blot**

DNA samples were separated by agarose gel, and transferred onto Hybond-N membrane (Amer sham). The samples were UV crosslinked and hybridized with 32P-labeled probes. Probes were prepared using the Random primer DNA Labeling Kit (TaKaRa, 6045), and hybridized using UltraHyb hybridization buffer (Thermo Fisher Scientific, AM8607).

**Transmission electron microscopy**

Cells were cultured in YPD overnight and transferred into fresh medium until mid-log phase (OD600 = 0.4–0.6). Cells were collected and subjected to sample preparation for electron microscopy using a method described elsewhere (25). The samples were examined with a transmission electron microscope H-7650 at an acceleration voltage of 80 kV.

**REFERENCES AND NOTES**


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Supplementary Materials

www.sciencemag.org/content/355/6329/eaaf3981/suppl/DC1

Figs. S1 to S18
Tables S1 to S3
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Engineering the ribosomal DNA in a megabase synthetic chromosome

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