RESEARCH ARTICLE SUMMARY

SYNTHETIC BIOLOGY

Engineering the ribosomal DNA in a megabase synthetic chromosome

Weimin Zhang,* Guanghou Zhao,* Zhouqing Luo,* Yicong Lin, Lihui Wang, Yakun Guo, Ann Wang, Shuangying Jiang, Qingwen Jiang, Jianhui Gong, Yun Wang, Sha Hou, Jing Huang, Tianyi Li, Yiran Qin, Junkai Dong, Qin Qin, Jiaying Zhang, Xinzhi Zou, Xi He, Li Zhao, Yibo Xiao, Meng Xu, Erchao Cheng, Ning Huang, Tong Zhou, Yue Shen, Roy Walker, Yisha Luo, Zheng Kuang, Leslie A. Mitchell, Kun Yang, Sarah M. Richardson, Yi Wu, Bing-Zhi Li, Ying-Jin Yuan, Huanming Yang, Jiwei Lin, Guo-Qiang Chen, Qingyu Wu, Joel S. Bader, Yizhi Cai, Jef D. Boeke, Junbiao Dai†

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.

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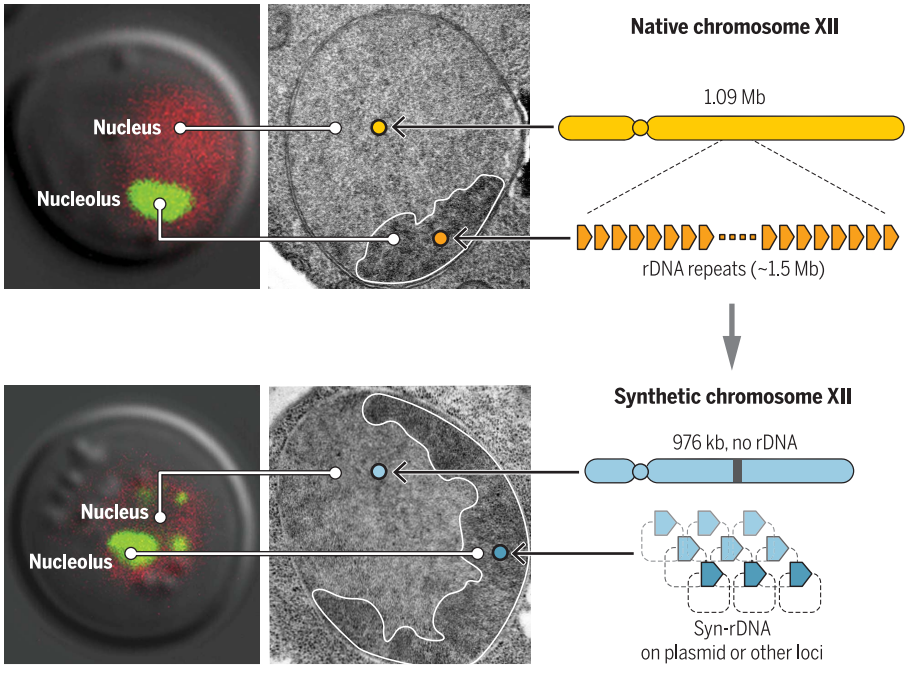
CONCLUSION: The rDNA locus of synXII is highly plastic; not only can it be moved to other chromosomal loci, it can also be altered in its ITS region to masquerade as a distinct species as defined by DNA barcoding, used widely in taxonomy. The ability to perform "species morphing" reported here presumably 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. ■



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.

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Engineering the ribosomal DNA in a megabase synthetic chromosome

Weimin Zhang,[1,2]* Guanghou Zhao,[1,3]* Zhouqing Luo,[1]* Yicong Lin,[1] Lihui Wang,[1] Yakun Guo,[1] Ann Wang,[1]† Shuangying Jiang,[1] Qingwen Jiang,[1] Jianhui Gong,[4] Yun Wang,[4] Sha Hou,[1] Jing Huang,[1] Tianyi Li,[1,2] Yiran Qin,[1] Junkai Dong,[1] Qin Qin,[1] Jiaying Zhang,[1,2] Xinzhi Zou,[1] Xi He,[1] Li Zhao,[1] Yibo Xiao,[1] Meng Xu,[1] Erchao Cheng,[1] Ning Huang,[1] Tong Zhou,[1] Yue Shen,[4,5,6] Roy Walker,[5] Yisha Luo,[5] Zheng Kuang,[7] Leslie A. Mitchell,[7] Kun Yang,[8] Sarah M. Richardson,[8] Yi Wu,[9] Bing-Zhi Li,[9] Ying-Jin Yuan,[9] Huanming Yang,[4,10] Jiwei Lin,[11] Guo-Qiang Chen,[1] Qingyu Wu,[1] Joel S. Bader,[8] Yizhi Cai,[5] Jef D. Boeke,[7] Junbiao Dai[1]‡

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.

Chromosome XII, the largest chromosome in *Saccharomyces cerevisiae* (~2.5 Mb, accounting for about 20% of the 12.5-Mb yeast genome) includes approximately 1.5 Mb of repetitive DNA encoding the ribosome RNA (*1*, *2*). SynXII was designed according to the general scheme of the entire Sc2.0 project (*3*, *4*), producing a 976,067–base pair (bp) "designer" chromosome. The ribosomal cluster was completely eliminated in the design with a plan to provide the rDNA on multicopy plasmids or regenerate it (Fig. 1); 21 tRNA genes, 15 annotated repeat clusters, and 28 introns were removed. However, one essential tRNA gene, *TRR4* (*tR(CCG)L*), was restored at its original locus, as were all introns within ribosomal proteins, as they can impair cell fitness when removed (*5*) (table S1). Throughout synXII, 123 TAG stop codons were converted to TAA and 299 loxPsym sites were inserted.

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. S1A). We divided each megachunk into three to eight chunks that overlapped one another by one minichunk to facilitate "one-pot" in vitro assembly (*7*, *8*), followed by transformation and in vivo recombination (fig. S1A). In addition, we performed assembly procedures to obtain plasmids containing the cloned chunks in parallel, which could be used to replace the corresponding megachunk with increased efficiency (fig. S2).

A standard workflow was established to assemble and screen synthetic strains displaying high fitness (fig. S1B). The strategy to replace the native chromosome involves alternating each megachunk with a *LEU2* or *URA3* selective marker (*3*, *4*) (fig. S1C). 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 G_2-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* in megachunk S and *tL(UAG)L2* in megachunk W]; both were removed in the design phase. Given that there are only three *tL(UAG)L* 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

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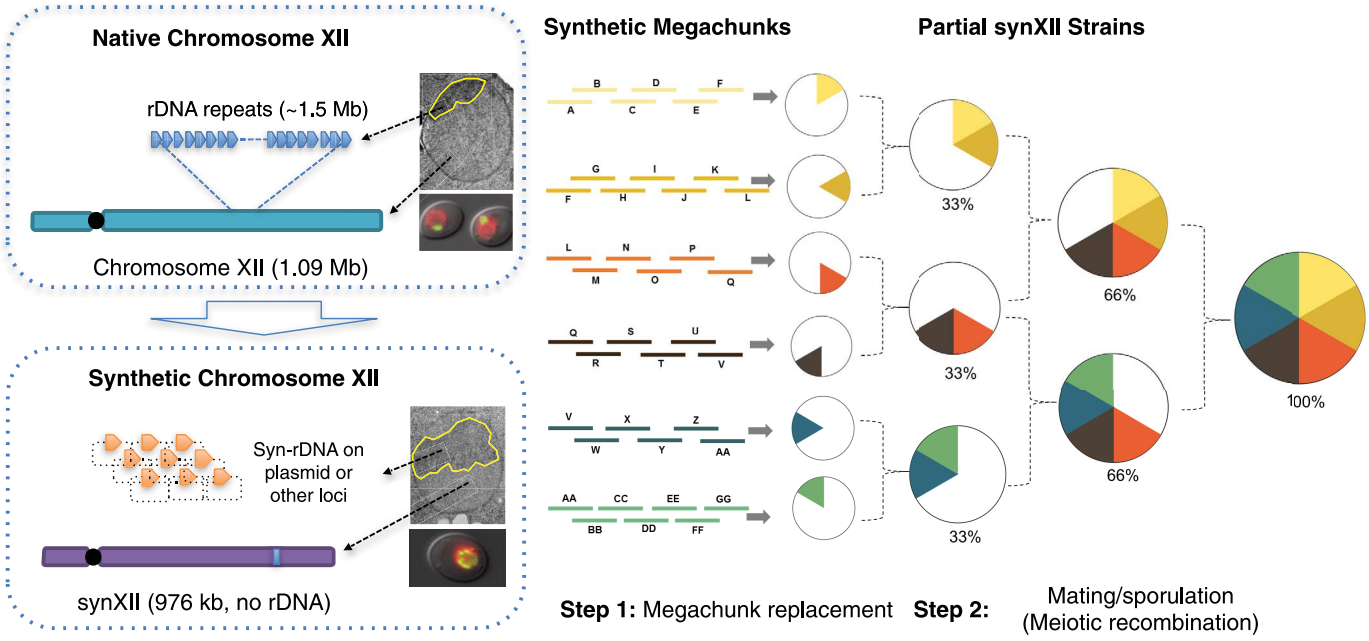

Fig. 1. The design and hierarchical assembly of synXII. The native chromosome XII consists of a 1.09-Mb chromosome plus a cluster of 9.1-kb ribosomal DNA (rDNA) repeats, which form the well-shaped nucleolar structure within the nucleus. For synXII, a high-copy rDNA plasmid was introduced, allowing the rDNA cluster to be deleted; the result was a disruption of nucleolus structure. SynXII was assembled in two steps. During the first step of megachunk replacement, synthetic megachunks were integrated into six strains respectively through homologous recombination and marker swapping as described (*3*, *4*). In the second step, the semisynthetic chromosomes XII in the six strains were combined with multiple rounds of MRA, resulting in several intermediate strains with partial synXII and eventually the complete synXII. The nucleolus is highlighted in a yellow circle in the electron microscopy images and in green using Nop10-GFP in the confocal image. NIC96-mCherry (red) was used to identify the nucleus.

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 the fitness defects. All sequence variations from the design are listed in table S2, including nine missing loxPsym sites, six 1-bp insertions or deletions (indels), and 16 new single-nucleotide polymorphisms (SNPs). Three of the six indels were located within the polyA regions outside coding sequences (*AYT1*, *COQ9*, and *NMA1*). Among the 16 SNPs, 6 corresponded to the wild-type nucleotides at these positions (asterisks in table S2) and others were single amino acid mutations. In addition, three sequence variations were discovered: one 9-bp deletion, one 9-bp substitution, and one 5-bp substitution. The 9-bp deletion within *RRN5* resulted in the loss of three amino acids (E-N-Q), which were tandemly repeated four times originally. The two sequence substitutions corresponded to a "patch" of native DNA, presumably due to homologous recombination between the native and synthetic DNA.

Transcript profiling

Transcript profiling of the initial isolate of synXII (Fig. 2E) revealed that the expression of the majority of genes was not affected relative to the wild type. We found several genes in arginine metabolism (*ARG1*, *ARG4*, *ARG7*, and *CPA2*) that were significantly increased in expression, which was apparently related to the deficiency of leucine tRNA, because their expression was restored in the presence of an ectopic copy of the *tL(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′ untranslated region (UTR). Two other genes, *GAS2* and *AHP1*, were down-regulated, potentially resulting from the incorporation of PCRTags or loxPsym 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 XII 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-nucleoli signal" became dispersed within the nucleus (*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 18*S* to 25*S*) 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 18*S* 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 (ChrXVR), which is similar in size to chromosome XIIR, and the right arm of chromosome III (ChrIIIR), which is a much shorter

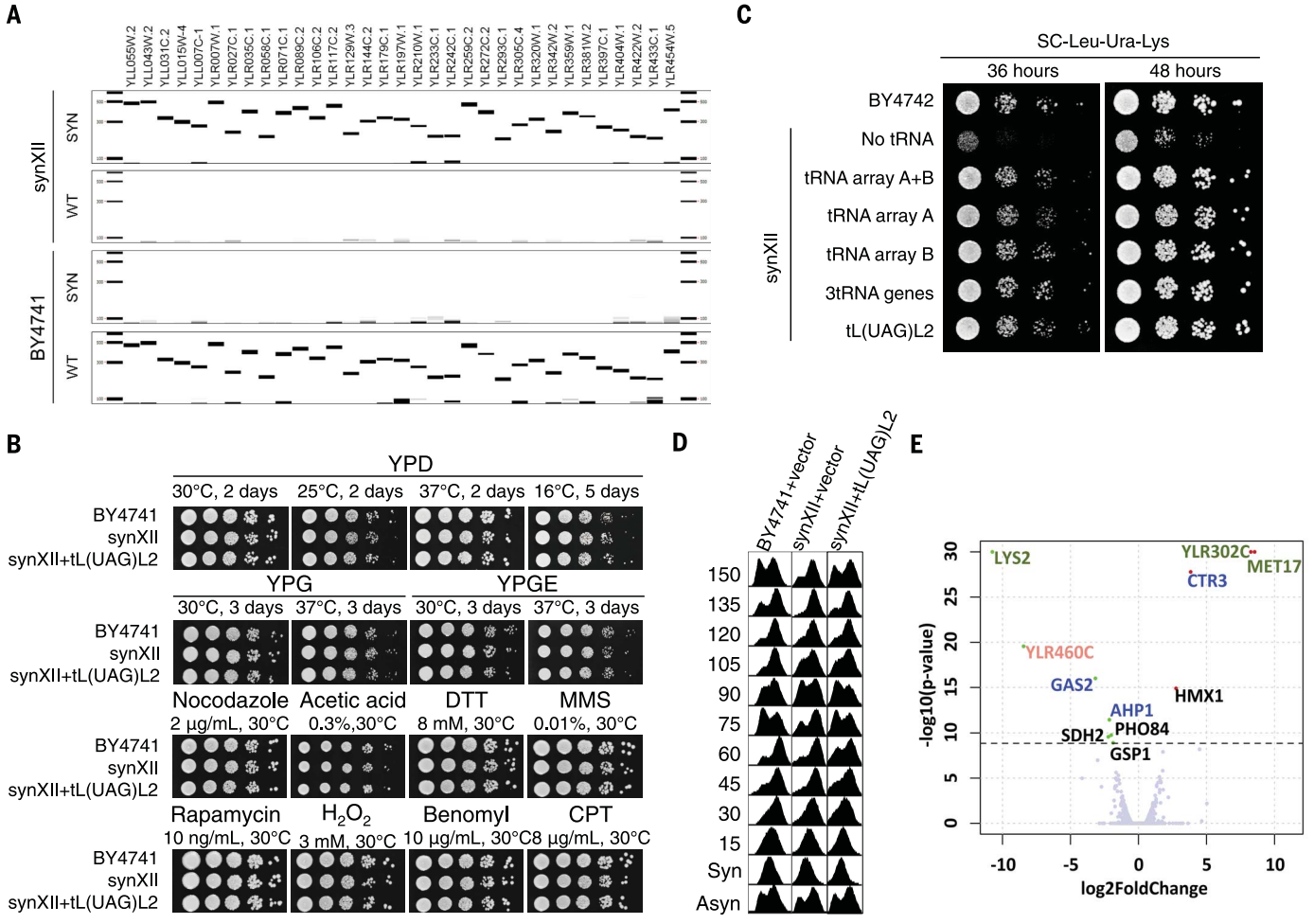

Fig. 2. Characterization of synXII. (**A**) PCRTag analysis of synXII and BY4741. Only one pair of tags from each megachunk was selected as representative. The complete PCRTag analysis is shown in figs. S4 and S5. (**B**) Fitness analysis of synXII strain with or without the ectopic *tL(UAG)L2* gene on various growth conditions. The types of media, growth temperature, and period were as indicated. YPD, yeast extract–peptone-dextrose; YPGE, 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 µl for each spot) were dropped onto synthetic complete medium lacking leucine, uracil, and lysine (SC −Leu −Ura −Lys). The tRNA array A included *tI(AAU)L2*, *tN(GUU)L*, *tL(UAA)L*, *tK(UUU)L*, *tR(CCG)L*, *tE(UUC)L*, *tD(GUC)L2*, *tX(XXX)L* [a predicted tRNA gene with undetermined specificity (*26*)], *tI(AAU)L1*, *tL(UAG)L2*, and *tV(AAC)L* on a centromeric plasmid. Array B included *tA(AGC)L*, *tL(CAA)L*, *tI(UAU)L*, *tL(UAG)L1*, *tQ(UUG)L*, *tD(GUC)L1*, *tR(ACG)L*, *tA(UGC)L*, *tS(AGA)L*, and *tP(UGG)L*. The three tRNA genes were *tL(UAG)L1*, *tI(UAU)L*, 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^{-5} (threshold = 1.4×10^{-9}).

arm (fig. S13). At each locus, intergenic regions between a nonessential gene and LTRs were used as integration sites to avoid potential deleterious effects (Fig. 3D). A DNA fragment containing 1.2 or 2 copies of the rDNA unit carrying a *hyg1* mutation was inserted at each target locus at first (fig. S13). The resulting strains were propagated in medium containing increasing amounts of hygromycin B to allow the amplification of rDNA repeats (see materials and methods for details). After a 2-week period, cells were replica-plated onto medium containing 5-fluoroorotic acid (5-FOA) to cure the strains of the wild-type rDNA plasmids. Subsequently, single colonies were isolated and analyzed. Pulsed-field gel electrophoresis (PFGE) and Southern blot indicated that a new rDNA cluster had been regenerated at each locus (Fig. 3E), consisting solely of the chimeric 9.1-kb rDNA repeat, and the copy number was comparable to that of the wild type (Fig. 3F and fig. S14). Relocation of the rDNA cluster into each location showed little effect on fitness (Fig. 3G) and formed nucleolar structures indistinguishable from that of the wild type (Fig. 3H), which were further verified by electron microscopy (Fig. 3I). Together, our results demonstrate that ITS regions are swappable and a fully functional rDNA unit can be rebuilt with no apparent deleterious effect (fig. S15). Furthermore, the position of the rDNA cluster within the genome is flexible and not limited by the size of its residing arm even when a chimeric sequence is used, consistent with other reports (*15*, *17*).

SynXII debugging

Several hurdles were overcome during the assembly of synXII. First, synXII initially grew slower than the wild type, in part resulting from the complete removal of all tRNA genes on the chromosome, and in particular removal of two of three *tL(UAG)L* genes (Fig. 2 and fig. S8). A similar phenomenon was observed during the synthesis of synX, in which the deletion of *tR(CCU)J*, the only arginine tRNA with the CCU anticodon, led to a severe growth defect in YPGE medium (*10*) (fig. S17). Second, the location of selective markers in each megachunk was designed

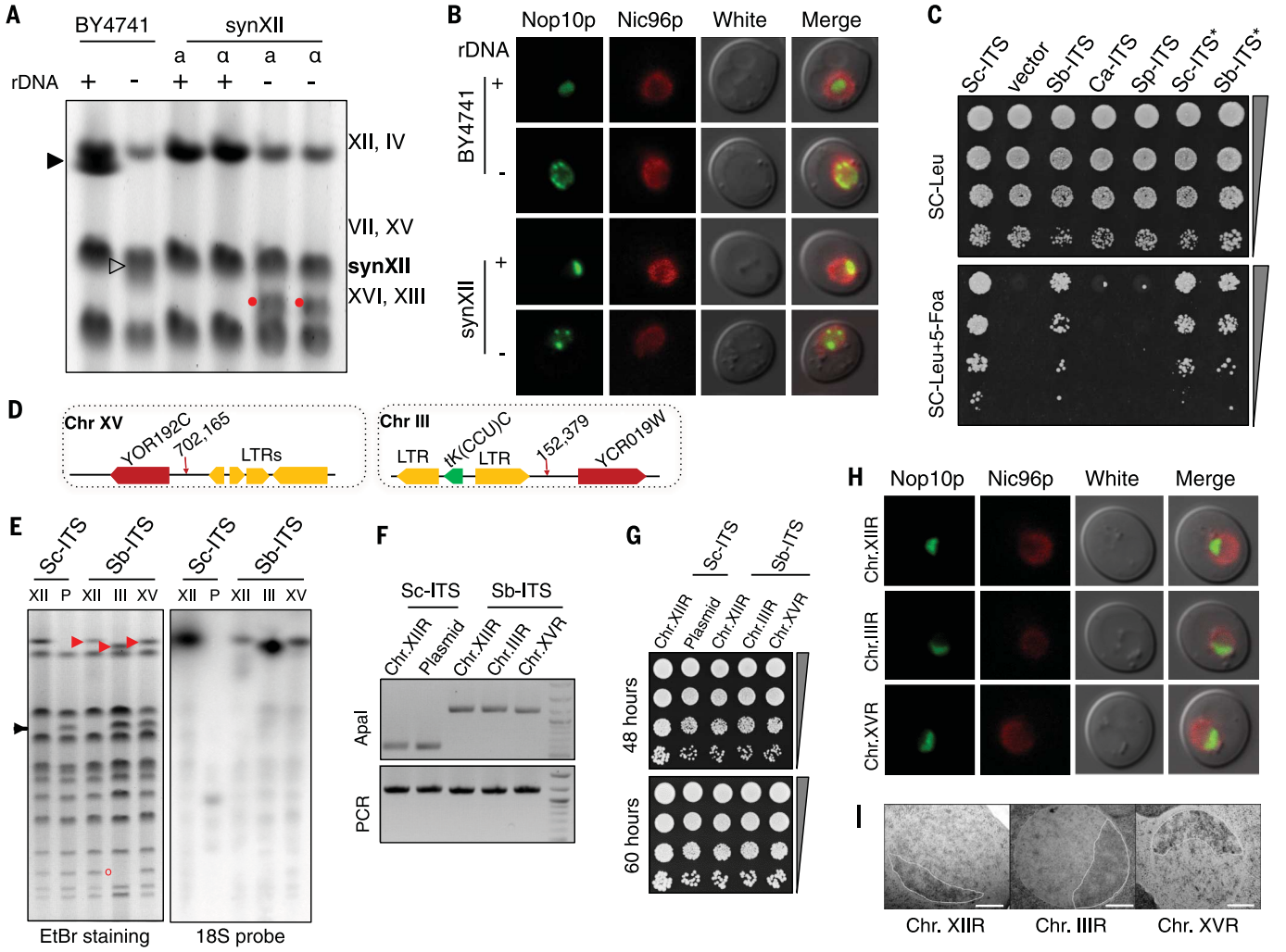

Fig. 3. Morphing species identity with chimeric rDNA repeats. (**A**) Karyotype analysis of native chromosome XII and synXII with or without chromosomal rDNA repeats by pulsed-field gel electrophoresis. The identity of each chromosome is indicated at the right. The black arrowhead points to both native 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 nucleolar structure. The nucleolus was visualized with Nop10-GFP (green) and NIC96-mCherry (red) was 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 *Apa*I recognition site only existing in *S. cerevisiae*. The PCR products were treated with (top) or without (bottom) *Apa*I. (**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.

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.

SynXII has a highly plastic rDNA locus that not only can be moved to other chromosomal

loci, but also can 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. The more severe intergenus differences in ITS sequence did not result in functional rDNAs, probably because of a defect 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.

Materials and methods

Minichunk synthesis and chunk construction

The entire synXII was synthesized as 1.6 kb DNA minichunks in collaboration with WuXi Qinglan Biotechnology Inc. (www.qinglanbiotech.com), and sequenced to be 100% identical to the design (see supplementary materials for DNA sequence of all plasmids). Each minichunks was flanked by two recognition sites of Type IIs restriction enzymes (BsaI or BsmBI) and the sticky ends identical to any internal ones within a megachunk were prohibited. Five or six minichunks were pooled together to perform Golden-Gate Assembly as described (fig. S1). In brief, 250 ng of each plasmid was mixed in a 10 μL reaction volume containing 1 U of enzyme (BsaI, NEB, R3535 or BsmBI, NEB, R0580), 1 U T4 DNA ligase (Thermo, EL0011) and buffer, and then assembled in a thermocycler using the following program: 37°C 1 hour; 50°C 15 min; 80°C 5 min; 4°C hold. The assembled mixture was combined and used directly to transform yeast, or in the other case, by adding an acceptor vector to obtain the new plasmid (the chunk plasmid).

Yeast transformation

The yeast strain was inoculated in 5 mL YPD and incubated overnight at 30°C in a shaker at 220 rpm. In next morning, OD_{600} was measured, diluted to 0.1 in fresh YPD and cultured at the same condition for 4–5 hours until the OD_{600} reached 0.4–0.6. The cells were harvested by centrifugation at 3000 rpm for 5 min, washed with equal volume of sterile water, 20 mL 0.1 M LiOAc respectively and resuspended in 1 mL 0.1 M lithium acetate (LiOAc). Mix 100 μL competent cells with transformation buffer (312 μL 50% PEG3350, 41 μL 1 M LiOAc and 25 μL ssDNA) and synthetic chunks, and incubated at 30°C for 30 min followed by adding 50 μL DMSO before heat-shock at 42°C for 15 min. The cells were pelleted, washed with 1 mL 5 mM $CaCl_2$ and plated onto selective medium.

Selecting clones with expected phenotype

The colonies appeared on the selective plates were replicated onto synthetic complete medium lacking uracil (SC–Ura) or leucine (SC–Leu). After overnight incubation at 30°C, the clones 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 [10 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, 11079105) 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 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 asci 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 succeeded 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 ddH_2O 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 OD_{600} = 0.1 in 5 mL YPD, then cultured for 4–5 hours until OD_{600} reached 0.4. About 500 ng plasmid carrying HO endonuclease (pJD137 or pJD148, 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 OD_{600} = 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 OD_{600} = 1.0 in sporulation 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 OD_{600} = 0.1, and grew until OD_{600} 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-22-1WB) 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, M0303) 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 was analyzed 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. 5×10^8 cells) was collected by spinning at 3000 rpm for 5min, washed once in 1 mL ice cold PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 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 $MgCl_2$, 17% glycerol, 0.5 mM spermine, 0.15 mM spermidine) 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 ddH_2O.

Stress sensitivity assay

Single colonies were cultured in YPD overnight at 30°C. Cells were adjusted to the same OD_{600}, 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 ab1791, 1:5000) were used, followed by ECL detection system (GE Healthcare, RPN2209) 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 nitrocellulose membrane, and hybridized to a radiolabeled complete coding sequence of COQ9 and ACT1, respectively, in ULTRAhyb (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 Liebman'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 resulted 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 T1758C mutation was introduced to generate resistance to hygromycin B, resulting plasmid *pRDN-S-HygR* (*15*). The DNA sequence of ITS1-5.8S-ITS2 region in *S. bayanus*, *C. albicans*, and *Sch. 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 (*23*). 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 on a 1.0% agarose gel in 0.5× TBE (pH8.0) for 38 hours at 14°C on a BioRad CHEF Mapper apparatus. The voltage was 5 V/cm, at an angle of 120° and switch time from initial 46.67 s to final 2 min 49.31 s.

Southern blot

DNA samples were separated by agarose gel, and transferred onto Hybond-N^+ membrane (Amersham). The samples were UV crosslinked and hybridized with ^{32}P-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 (OD_{600} = 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.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/355/6329/eaaf3981/suppl/DC1
Figs. S1 to S18
Tables S1 to S3
Reference (*27*)

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Engineering the ribosomal DNA in a megabase synthetic chromosome

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