SYNTHETIC BIOLOGY

“Perfect” designer chromosome V and behavior of a ring derivative

Ze-Xiong Xie,* Bing-Zhi Li,* Leslie A. Mitchell, Yi Wu, Xin Qi, Zhu Jin, Bin Jia, Xia Wang, Bo-Xuan Zeng, Hui-Min Liu, Xiao-Le Wu, Qi Feng, Wen-Zheng Zhang, Wei Liu, Ming-Zhu Ding, Xia Li, Guang-Rong Zhao, Jian-Jun Qiao, Jing-Sheng Cheng, Meng Zhao, Zheng Kuang, Xuya Wang, J. Andrew Martin, Giovanni Straquadiano, Kun Yang, Xue Bai, Juan Zhao, Meng-Long Hu, Qiu-Hui Lin, Wen-Qian Zhang, Ming-Hua Shen, Si Chen, Wan Su, En-Xu Wang, Rui Guo, Fang Zhai, Yue-Jiao Guo, Hao-Xing Du, Jia-Qing Zhu, Tian-Qing Song, Jun-Jun Dai, Fei-Fli Li, Guo-Zhen Jiang, Shi-Lei Han, Shi-Yang Liu, Zhi-Chao Yu, Xiao-Na Yang, Ken Chen, Cheng Hu, Da-Shuai Li, Nan Jia, Yue Liu, Lin-Ting Wang, Su Wang, Xiao-Tong Wei, Mei-Qing Fu, Lan-Meng Qu, Si-Yu Xin, Ting Liu, Kai-Ren Tian, Xue-Nan Li, Jin-Hua Zhang, Li-Xiang Song, Jin-Gui Liu, Jia-Fei Lv, Hang Xu, Run Tao, Yan Wang, Ting-Ting Zhang, Ye-Xuan Deng, Yi-Ran Wang, Ting Li, Guang-Xin Ye, Xiao-Ran Xu, Zheng-Bao Xia, Wei Zhang, Shi-Lan Yang, Yi-Lin Liu, Wen-Qi Ding, Zhen-Ning Liu, Jun-Qi Zhu, Ning-Zhi Liu, Roy Walker, Yisha Luo, Yun Wang, Yue Shen, Huanming Yang, Yizhi Cai, Ping-Sheng Ma, Chun-Ting Zhang, Joel S. Bader, Jef D. Boeke, Ying-Jin Yuan†, Juan Zhao, Meng-Long Hu, Qiu-Hui Lin, Wen-Qian Zhang, Ming-Hua Shen, Si Chen, Wan Su, En-Xu Wang, Rui Guo, Fang Zhai, Yue-Jiao Guo, Hao-Xing Du, Jia-Qing Zhu, Tian-Qing Song, Jun-Jun Dai, Fei-Fli Li, Guo-Zhen Jiang, Shi-Lei Han, Shi-Yang Liu, Zhi-Chao Yu, Xiao-Na Yang, Ken Chen, Cheng Hu, Da-Shuai Li, Nan Jia, Yue Liu, Lin-Ting Wang, Su Wang, Xiao-Tong Wei, Mei-Qing Fu, Lan-Meng Qu, Si-Yu Xin, Ting Liu, Kai-Ren Tian, Xue-Nan Li, Jin-Hua Zhang, Li-Xiang Song, Jin-Gui Liu, Jia-Fei Lv, Hang Xu, Run Tao, Yan Wang, Ting-Ting Zhang, Ye-Xuan Deng, Yi-Ran Wang, Ting Li, Guang-Xin Ye, Xiao-Ran Xu, Zheng-Bao Xia, Wei Zhang, Shi-Lan Yang, Yi-Lin Liu, Wen-Qi Ding, Zhen-Ning Liu, Jun-Qi Zhu, Ning-Zhi Liu, Roy Walker, Yisha Luo, Yun Wang, Yue Shen, Huanming Yang, Yizhi Cai, Ping-Sheng Ma, Chun-Ting Zhang, Joel S. Bader, Jef D. Boeke, Ying-Jin Yuan†

INTRODUCTION: The Saccharomyces cerevisiae 2.0 project (Sc2.0) aims to modify the yeast genome with a series of densely spaced designer changes. Both a synthetic yeast chromosome arm (synXLR) and the entirely synthetic chromosome (synIII) function with high fitness in yeast. For designer genome synthesis projects, precise engineering of the physical sequence to match the specified design is important for the systematic evaluation of underlying design principles. Yeast can maintain nuclear chromosomes as rings, occurring by chance at repeated sequences, although the cyclized form is unfavorable in meiosis given the possibility of dis- centric chromosome formation from meiotic recombination. Here, we describe the de novo synthesis of synthetic yeast chromosome V (synV) in the “Build-A-Genome China” course, perfectly matching the designer sequence and bearing loxPsym sites, distinguishable watermarks, and all the other features of the synthetic genome. We generated a ring synV derivative with user-specified cyclization coordinates and characterized its performance in mitosis and meiosis.

RATIONAL: Systematic evaluation of underlying Sc2.0 design principles requires that the final assembled synthetic genome perfectly match the designed sequence. Given the size of yeast chromosomes, synthetic chromosome construction is performed iteratively, and new mutations and unpredictable events may occur during synthesis; even a very small number of unintentional nucleotide changes across the genome could have substantial effects on phenotype. Therefore, precisely matching the physical sequence to the designed sequence is crucial for verification of the design principles in genome synthesis. Ring chromosomes can extend those design principles to provide a model for genomic rearrangement, ring chromosome evolution, and human ring chromosome disorders.

RESULTS: We chemically synthesized, assembled, and incor- porated designer chromosome synV (536,024 base pairs) of S. cerevisiae according to Sc2.0 principles, based on the complete nucleotide sequence of native yeast chromosome V (576,874 base pairs). This work was performed as part of the “Build-A-Genome China” course in Tianjin University. We corrected all mutations found—including duplications, substitutions, and indels—in the initial synV strain by using integrative cotransformation of the precise desired changes and by means of a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-based method. Altogether, 3331 corrected base pairs were required to match to the designed sequence. We generated a strain that exactly matches all designer sequence changes that displays high fitness under a variety of culture conditions. All corrections were verified with whole-genome sequencing; RNA sequencing revealed only minor changes in gene expression—most notably, decreases in expression of genes relocated near synthetic telomeres as a result of design. We constructed a functional circular synV (ring_synV) derivative in yeast by precisely joining both chromosome ends (telomeres) at specified coordinates. The ring chromosome showed restoration of subtelomeric gene expression levels. The ring_synV strain exhibited fitness comparable with that of the linear synV strain, revealed no change in sporation frequency, but notably reduced spore viability. In meiosis, heterozygous or homozygous diploid ring_wtV and ring_synV chromosomes behaved similarly, exhibiting substantially higher frequency of the formation of zero-spore tetrads, a type that was not seen in the rod chromosome diplods. Rod synV chromosomes went through meiosis with high spore viability, despite no effort having been made to preserve meiotic competency in the design of synV.

CONCLUSION: The perfect designer-matched synthetic chromosome V provides strategies to edit sequence variants and correct unpredictable events, such as off-target integration of extra copies of synthetic DNA elsewhere in the genome. We also constructed a ring synthetic chromosome derivative and evaluated its fitness and stability in yeast. Both synV and synVI can be circularized and can power yeast cell growth without affecting fitness when gene content is maintained. These fitness and stability phenotypes of the ring synthetic chromosome in yeast provide a model system with which to probe the mechanism of human ring chromosome disorders.

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“Perfect” designer chromosome V and behavior of a ring derivative

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Perfect matching of an assembled physical sequence to a specified designed sequence is crucial to verify design principles in genome synthesis. We designed and de novo synthesized 536,024–base pair chromosome synV in the “Build-A-Genome China” course. We corrected an initial isolate of synV to perfectly match the designed sequence using integrative cotransformation and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)–mediated editing in 22 steps: synV strains exhibit high fitness under a variety of culture conditions, compared with that of wild-type V strains. A ring synV derivative was constructed, which is fully functional in Saccharomyces cerevisiae under all conditions tested and exhibits lower spore viability during meiosis. Ring synV chromosome can extend Sc2.0 design principles and provides a model with which to study genomic rearrangement, ring chromosome evolution, and human ring chromosome disorders.

SYNTHETIC YEAST GENOME

Perfect matching of an assembled physical sequence to a specified designed sequence is crucial to verify design principles in genome synthesis. We designed and de novo synthesized 536,024–base pair chromosome synV in the “Build-A-Genome China” course. We corrected an initial isolate of synV to perfectly match the designed sequence using integrative cotransformation and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)–mediated editing in 22 steps: synV strains exhibit high fitness under a variety of culture conditions, compared with that of wild-type V strains. A ring synV derivative was constructed, which is fully functional in Saccharomyces cerevisiae under all conditions tested and exhibits lower spore viability during meiosis. Ring synV chromosome can extend Sc2.0 design principles and provides a model with which to study genomic rearrangement, ring chromosome evolution, and human ring chromosome disorders.

S
ynthesis of several viral and transposon genomes has laid the groundwork for the field of de novo genome engineering (1–5). The Saccharomyces cerevisiae 2.0 project (Sc2.0), taking a similar de novo approach, aims to modify the yeast genome with a series of densely spaced designer changes. Several synthetic chromosomes have been shown to function in

yeast, including the synthetic yeast chromosome arm (synXK), the entirely synthetic chromosome (synIII) (6, 7), and four additional synthetic chromosomes described in this issue (8–11). For designer genome synthesis projects, precisely matching the physical sequence to the specified design is important for the systematic evaluation of underlying design principles. Even a very small number of unintentional nucleotide changes across the genome could, in principle, have effects on phenotype (7, 12). The synthetic chromosomes constructed to date encode a number of new mutations and very short regions of native sequence that can be missed with polymerase chain reaction tag (PCRTag) analysis. Although there is no evidence that any variants are responsible for specific phenotypes, it is important to develop methods with which to make the sequence a perfect match to the design.

Whereas almost all natural eukaryotic chromosomes are linear and flanked by telomeres, prokaryotic genomes are typically circular and lack telomeres. Yeast can maintain nuclear chromosomes as rings (6), although this is unfavorable in meiosis given the possibility of formation of dicentric chromosomes (13). These native ring chromosomes form through chance recombination events templated by repetitive sequences. We converted a linear synV to a ring by design, a process that could potentially enable more precise modeling of ring chromosome disorders if performed in mammalian cells. Circular “ring” chromosomes have been reported in a wide variety of human genetic disorders, including epilepsy (14, 15), intellectual delay (15), various dysmorphic features (16), leukemia (17), and microcephaly (18, 19). Further, therapies for genetic disorders based on chromosome circularization have been proposed (20). Given the complexity of inheritance and pleiotropy associated with human ring chromosomes, model systems for the functional impact of chromosome circularization by design are needed. Yeast-based models arising from Sc2.0, with hundreds of distinguishable PCRTags and loxPsynm sites, could have value for studying breakage-fusion-breakage mechanisms and rearrangement of ring chromosomes during meiosis, as well as for developing methods to reverse growth defects caused by genomic disorders, which may not be available within the wild-type ring chromosomes. Here, we show that a functional circular synV (ring_synV) can be constructed by design in yeast and offer insight into its stability and performance in meiosis.

Design and synthesis

We designed synV according to Sc2.0 principles, on the basis of the complete nucleotide sequence of native yeast chromosome V [576,874 base pairs (bp)] (21, 22). Major edits during synV design included deleting two subtelomere regions, 20 tRNA genes, 30 transposons/Ty elements, and 10 introns and inserting 176 loxPsynm sites; additional base changes included 62 TAG/TAA stop-codon swaps and 339 synonymous recordings to introduce PCRTags derived from native chromosome V (fig. LA and fig. SIA) (21, 22).

All synV building blocks (BBs), ~750-bp primary DNA segments, and minichunks—~2- to 4-kb secondary DNA segments—were built, starting with oligonucleotides, and sequence-verified by students of the “Build-A-Genome (BAG) China” course offered at Tianjin University (TJU) (23). Sixty-one students were organized into five groups to build the synthetic DNA for synV (536,024 bp) in ~4 months (figs. S1B and S2).

The native chromosome V (wtV) was replaced by synV in 17 steps of minichunk incorporation and two rounds of universal telomere cap (UTC) replacement (fig. SIA). During incorporation, both genotypes and phenotypes of intermediate strains were evaluated. Only the isolates exclusively producing synthetic PCRTag ampiclons and exhibiting high fitness under different conditions—including yeast extract, peptone, and dextrose (YPD); yeast extract peptone ethanol (YPE); yeast extract peptone glyceral (YPG); and synthetic complete medium (SC) and synthetic dextrose (SD) at 30° and 37°C, respectively—were used for the next round of incorporation (fig. S3).
Dextrose; SC, synthetic complete medium; SD, synthetic dextrose; YPG, yeast extract peptone glycerol; YPE, and likely reduced in expression because of removal of subtelomeric DNA. YPD, yeast extract, peptone, and in silico from native chromosome V (wtV, 576,874 base pairs) (fig. S1A). (yXZX347, yeast_chr05_9_01) and wtV (BY4741) strains under different conditions (figs. S3 and S6). Genes with significant expression changes are labeled in red.

Fig. 1. Characterization of synV. (A) Synthetic chromosome V (synV, 536,024 base pairs) was designed in silico from native chromosome V (wtV, 576,874 base pairs) (fig. S1A). (B) Phenotypic analysis of synV (yXZX347, yeast_chr05_9_01) and wtV (BY4741) strains under different conditions (figs. S3 and S6). (C) PCRTag analysis (one PCRTag per ~30 kb) of synV (yXZX512, version “yeast_chr05_9_04”) (figs. S8 and S9). (D) Transcriptome analysis of synV (yXZX347, yeast_chr05_9_01) relative to wild-type strain (BY4741). Genes with significant expression changes are labeled in red. YER187W and YER188W are telomere adjacent and likely reduced in expression because of removal of subtelomeric DNA. YPD, yeast extract, peptone, and dextrose; SC, synthetic complete medium; SD, synthetic dextrose; YPG, yeast extract peptone glycerol; YPE, yeast extract peptone ethanol.

Characterization of synV
To evaluate the fitness of the synV strain (yXZX347, yeast_chr05_9_01), we examined colony size, growth curves, and morphology of synV cells under various conditions. No detectable differences were found between synthetic and native strains under most conditions, including high temperature, ethanol, acetic acid, or sorbitol (fig. 1B and figs. S4 to S6). The genotype of synV was characterized by PCR using wild-type and synthetic PCRTag primer pairs (fig. 1C and figs. S7 to S9), and the size reduction of synV was demonstrated by means of pulsed-field gel electrophoresis (PFGE). The transcriptional comparison in rich medium revealed no differential expression for the majority of genes in synV and native strains (Fig. 1D). Only five genes were significantly differentially expressed (adjusted \( P < 10^{-5} \), Benjamini Hochberg method): |log (fold changes)| > 2, three of which (MCM3, YER187W, and YER188W) are located on chromosome V. MCM3 is essential and encodes a subunit of the replicative helicase. The other two genes are subtelomeric; their decreased expression can be attributed to adjacency.

“Perfect” chromosome synV
Whole-genome sequencing (WGS) of the initial synV isolate (yXZX345, yeast_chr05_9_01) revealed 34 differences compared with the in silico-designed synV (yeast_chr05_9_41). The variants included two long segmental duplications, a region with quadruplication of a short segment, and 31 short indels and single-nucleotide variants, most of which represent residual native sequences resulting from incomplete replacement through homologous recombination, or “patchworks” (tables S1 and S2). We developed methods with which to convert every variant nucleotide to perfectly match the designed sequence.

Copy number variation discovered through complete genome sequencing could be the result of integration of the extra copy (or copies) directly on synV or misintegration elsewhere in the genome. An “endoreduplication backcross” strategy was applied to distinguish these possibilities (fig. S10 and tables S2 to S4) (24). This revealed that the large-scale duplications at positions 291,217–299,980 bp and 305,355–325,235 bp were readily outcrossed, and we concluded that they most have integrated into locations on another chromosome(s) (fig. S10, A to C). The extra copies of the 140,356–142,771 bp region, however, segregated with synV (figs. S10, D and E, and S11). This tandemly quadruplicated region (which lacks essential genes) was successfully repaired to single copy in two steps: First, the entire region was replaced with URA3 and, second, a single-copy synthetic fragment was integrated by selecting for loss of URA3 on 5-FOA medium (fig. S10, D and E, and tables S2 to S4).

In order to remove all residual patches of native chromosome V sequence and correct other mutations, we first applied an integrative cotransformation strategy specifying a single selectable integration event and cotransforming a pool of DNA fragments in twofold molar excess, covering all other regions targeted for correction; transformants were screened by means of colony PCR with site-specific primers so as to identify corrections at the selectable site plus all secondary target regions. Repaired sequences were subsequently verified by means of restriction digest and Sanger sequencing (fig. 2, A, B, and C). The transformant with the greatest number of repaired sequences was used in a subsequent round of cotransformation. In total, we grouped 31 mutations into 22 “target regions” for correction based on proximity. Subsequently, target regions 7, 13, and 15 were further subdivided into two or three independent segments for repair (fig. S12A and table S1).

To boost the efficiency of cotransformation, we introduced targeted clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-induced double-strand breaks (DSBs) to change the mutation to the designed sequence (fig. 2, A, D, and E; fig. S12B;
Circularization of synV

We cyclized synV (yXZX538, yeast_chr05_9_05) and simultaneously deleted both chromosome ends (telomeres) without altering the gene content of synV (Fig. 3A and table S6). This was confirmed by the appearance of a PCR amplicon spanning the extreme chromosome termini (Fig. 3B and table S6), and disappearance of the linear synV band in PFGE (Fig. 3C). The synV ring derivative strain, ring_synV (yXZX565), exhibited good fitness compared with the linear synV strain (yXZX538, yeast_chr05_9_05) (Fig. 3, D and E, and fig. S13). We also generated a circular derivative of native chromosome V, ring_wtV (yXZX915) (Fig. 3B and tables S2 and S7), as a control for subsequent experiments. Cyclization was targeted at the same positions with ring_synV, so the gene content of the circular derivatives was identical.

Long-term fitness of ring_synV was tested by serially culturing in YPD medium for ~60 generations, and the persistence of the circular chromosome was demonstrated with PFGE analyses (Fig. 3C and fig. S14). In meiosis, heterozygous or homozygous diploid ring_wtV and ring_synV chromosomes behaved similarly, exhibiting substantially higher frequency of spore inviability, including the formation of zero-spore tetrads, a type that was not seen in the rod chromosome diploids (Fig. 3F). Diploids with two ring chromosomes were more severely affected than those with one. Ring chromosomes are expected to lead to spore lethality because single crossover events would lead to the formation of dicentric chromosomes. Rod chromosomes went through meiosis with high spore viability despite no effort having been made to preserve meiotic competency in the design of synV.

Given that chromosome V is a large chromosome of S. cerevisiae built in 19 iterative steps of integrative transformation, it is not surprising that we identified new mutations and unpredictable events such as off-target integration of extra copies of synthetic DNA into other native chromosomes. We developed several methods to fix these types of sequence alterations, including substantial segmental duplications, short indels, and single-nucleotide mutations. We also constructed a ring synthetic chromosome derivative by design, which directs yeast cell growth without affecting fitness. Unlike previously documented ring chromosomes in S. cerevisiae, ring_synV includes hundreds of distinguishable PCR tags and loxp sites. Further, the chromosome structure, including the order and orientation of genes, is modifiable by means of synthetic chromosome rearrangement and modification by loxP-mediated evolution (SCRaMbLE), and the changes are easily tracked with PCRTag analysis or WGS. Analysis of “SCRaMbLants” could provide insight into human ring chromosome disorders.

Materials and methods

SynV design and nomenclature

Synthetic chromosome V (synV, 536,024 base pairs) was designed in silico from native chromosome V and tables S3 to S5) (25). Donor-mediated repair, which was designed to alter one or more base pairs falling within the 20-bp protospeacer sequence, is sufficient to disrupt guide RNA (gRNA) recognition. Approximately 61% of the Sc2.0 genome sequence falls within 20 bp of a protospeacer adjacent motif (PAM) sequence (nGG) and therefore amenable to alteration by this strategy. In 20 additional steps, we produced strain yXZX846, in which all originally identified variant bases were directed to design, producing version yeast_chr05_9_22. WGS confirmed that all mutations had been corrected; however, two new mutations not associated with the corrected regions were observed; these did not notably affect fitness.
harboring the indicated wtV and synV linear and ring derivatives.

of synV and ring_synV on various media. (~750 bp per BB) which were assembled from

migrates faster than native V (wtV), and the circular chromosome ring_synV is not detected. After 5 days

with gene content identical to linear synV. (~Fig. 3. Construction, characterization, and meiotic stability analysis of ring_synV chromosome.

Three strategies were implemented in the BAG course to ensure efficiency and speed of produc-

The BB is (~30 kb mega-

14 kb per minichunk) and 942 BBs

3 to 6 BBs and overlapped one BB as a homologous

assembly methods. Each minichunk consisted of

~70-nt long oligonucleotides were synthesized

SynV was synthesized from oligonucleotides. The

progress updates. (iii) A committee consisting of

building blocks synthesis

SynV was synthesized from oligonucleotides. The

oligonucleotide is “o16” and the oligonucleotide number is 16.

“Build-A-Genome (BAG) China” course

“Build-A-Genome (BAG) China” course in TJU

building blocks belonging to one BB were mixed into one

overlapping minichunks between two

oligonucleotides were used as 5’ and 3’ primers to

amplify the diluted T-PCR product to obtain the

full length ~750-bp PCR product. The PCR amplification products, after gel purification, were

ligated into pEASY-Blunt Cloning Vector (TransGen Biotech) followed by Escherichia coli transformation and blue-white screening. Twelve white colonies

were analyzed by colony PCR with the M13F and M13R primers which were available for pEASY-

Blunt Cloning Vector (fig. S2A). Three colonies with full length PCR product were sequenced and the right colonies were stored after sequence aligned with the reference sequence by using Serial Cloner software (7, 23).

All the 942 BBs were synthesized and verified by the students of “Building-A-Genome (BAG) China” course in TJU, China.

Minichunk assembly

All the 942 BBs were assembled into 263 minichunks (~2–4 kb in size, by the students of “Building-A-Genome (BAG) China” course in TJU, using overlap-extension PCR (OE-PCR) and yeast assembly methods. Each minichunk consisted of 3 to 6 BBs and overlapped one BB as a homologous arm between the adjacent minichunks (fig. S2B) (7).

Overlap-extension PCR (OE-PCR)

Equimolar PCR gel recovery products of BBs were mixed and the two primers with restriction enzyme site were used to amplify the minichunk with the method of OE-PCR (26). The full length amplification products, after gel purification, were ligated into the pEASY-Blunt Cloning Vector or pJET Vector (Thermo Fisher Scientific) followed by E. coli transformation and blue-white screening. Twenty-four white colonies were picked for colony PCR analysis, and three colonies with full length product were picked for Sanger DNA sequencing (26).

Yeast assembly

The RADOM yeast recombination assembly method was used to build the minichunks (27). It is efficient to assemble a correct minichunk by co-transforming 3 to 6 BBs and a liner shuttle vector with 40-bp terminal overlaps.
Totally 263 minichunks were made from: OE-PCR (203: pEASY-Blunt vector, 201; pET vector, 2), yeast assembly (60: pRS316 vector, 4; pRS425 vector, 2; pRS426 vector, 54).

**Replacement of native chromosome V with synV minichunks**
The native chromosome V was replaced by synV after 17 steps of minichunk incorporation and two rounds of UTC (7) replacement. In each round of minichunk incorporation, average 15 equimolar (~70 fino) minichunks were co-integrated into the yeast, and an auxotrophic marker (URA3 or LEU2) was fused into the last minichunk as well as ~500 bp of wild-type sequence for homologous recombination. The wild-type telomeres and subtelomere regions were replaced with UTC (Fig. S3). During incorporation, both genotypes and phenotypes of the intermediate strains were evaluated to make sure the correct strain was selected as the starting strain for the next round of incorporation (Fig. S3).

**PCRTag analysis**
PCRTag analysis was the mainstay to make sure the success of synV incorporation (Figs. S7 to S9). The intermediate strains which have the correct auxotroph and a similar fitness with the wild-type strain (BY4741) were picked for genomic DNA (gDNA) extraction and PCRTag analysis. The presence of synthetic PCRTag amplicons (SYN) and the absence of wild-type PCRTag amplicons (WT) revealed the replacement of native chromosome V by synV.

**Yeast genomic DNA extraction for PCRTag analysis**
Yeast genomic DNA extraction method was modified from the previous study (28). Yeast cells were isolated from 1 mL of overnight culture by spinning at 12000 rpm for 1 min in a centrifuge at room temperature (RT). The pellet was washed with 1 mL of sterile water and then resuspended with 400 µL STE/TBE buffer. An equal volume of glass beads and 200 µL of Phenol-Chloroform (25:24) were added and vortexed for 10 min at RT. After 12000 rpm spinning for 5 min, the aqueous layer was transferred into a labeled 1.5 mL tube and 1 mL precooled 100% ethanol and 30 µL of 3 M NaOAc were added. The tube was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was discarded and the DNA pellet was washed by adding 1 mL precooled 70% ethanol, centrifuged 5 min at 4°C. The DNA was air-dried and resuspended with 100 µL of 10 mM TE (pH7.4), stored at -20°C. For each 15 µL PCR reaction, 0.5 µL of gDNA was added as template.

**PCRTag analysis reaction conditions**
Completed PCRTag analysis of synV and WT was carried out using the modified method (7) and the whole PCRtags can be accessed on the Sc2.0 website (www.syntheticyeast.org). GoTag Hot Start Polymersase (Promega, Madison, WI), 200 mM each of forward and reverse primers, and gDNA of BY4741 or synV (yXXZ512, version “yeast_chr05_9_04”) were used to amplify the PCR product in a 4 µL final volume. The following PCR program was used: 95°C/5 min, 30 cycles (95°C/30 s, 53°C/30 s, 72°C/30 s), 72°C/10 min, 4°C/. Detection of PCRTags was performed by diluting samples to 25 µL in H2O and using a Caliper LabChip GXII (Perkin Elmer, Waltham, MA) and the HT DNA 5K LabChip Kit, Version 2. Virtual gel images were generated using LabChip GX software version 4.0.1418.0.

**DNA preparation for genome sequencing**
Yeast cells were harvested from saturated overnight culture by centrifugation and followed by washing with sterile water. Genome sequencing DNA preparation was carried out using the method elsewhere (7).

**Genome sequencing of synV**
Paired-end whole genome sequencing of synV strains yXXZ345 (version “yeast_chr05_9_01”), yXXZ347 (version “yeast_chr05_9_01”) were performed using an Illumina HiSeq 280,320,320 and 60,598,810 raw reads were obtained and used for downstream analysis. Briefly, reads were first mapped using Bowtie2 with default parameters; a reference genome was constructed with the sequence for strain BY4741. Duplication was visualized in IGV browser. Base changes and short indels were detected using the HaslotypeCaller function of the Genome Analysis Toolkit (GATK) with standard parameters. Structural variation was detected using BreakDancer. Native sequence and vector sequence were detected by realigning the reads rejected by Bowtie2 in the first round against a reference genome containing the native chromosome(s) of interest or vector DNA.

Paired-end whole genome sequencing of final synV strains yXXZ846 (version “yeast_chr05_9_22”) was performed using an Illumina HiSeq 4000. The original figure data were transformed into raw sequenced reads (raw data, 5,752 Mb) by CASAVA base calling. The sequenced data were filtered and the sequence of Adapter and low quality data were removed, resulting in the clean data used for subsequent analysis. Mapping the reads to the reference sequence using BWA software, counting the coverage of the reference sequence to the reads and make explanations of the alignment results using the SAMtools software.

**RNA extraction for RNA sequencing**
Yeast cells were harvested by centrifugation and followed by washing in sterile water. Total cell RNA was extracted as described (7).

**RNASeq of synV and ring_synV**
Total cell RNA sequencing was performed according to the previous study (7). FastQC (version 0.10.1) was used for accessing RNAseq data quality. TopHat (version 2.0.9) and Bowtie2 (version 2.1.0) with standard parameters were applied for mapping of pair-end RNA-Seq raw reads to yeast synthetic reference genome. Data format conversion was performed in SAMtools (version 1.2.1). For each gene, feature counts were computed using HTSeq and then were utilized for differential expression analysis in DESeq (one R package, version 1.18.0). For each gene, adjusted P-value was calculated using Benjamini-Hochberg procedure. The relationships between log2 fold change and log2 P-adjusted were indicated in volcano plot, which facilitated the determination of significant expressed genes. The synV RNAseq result was aligned with the wtV, and 5 genes were found with significant changes (adjusted P < 10−5, Benjamini Hochberg method; |log2 fold change| > 2). Three of the 5 genes were on the chromosome V: YEL032W (MC3: protein involved in DNA replication) (P value = 4.61 × 10−39, adjusted = 1.48 × 10−35, log2 fold change = 2.78) increased -6.88-fold, YER187W (uncharacterized gene) (P value = 7.61 × 10−23, adjusted = 9.79 × 10−19, log2 fold change = -5.88) decreased ~58.86-fold, and YER188W (dubious gene) (P value = 3.55 × 10−12, adjusted = 3.81 × 10−16, log2 fold change = -5.95) decreased ~62.00-fold. The ring synV RNAseq result was aligned with the synV, and the telomeric genes were found with significant changes (P < 0.05, and |log2 fold change | > 1): YEL072W (nonessential gene) (P value = 1.58 × 10−7, adjusted = 1.44 × 10−4, log2 fold change = 2.24) increased ~4.72-fold, YER187W (uncharacterized gene) (P value = 5.97 × 10−20, adjusted = 9.51 × 10−17, log2 fold change = 4.21) increased ~18.51-fold and YER188W (dubious gene) (P value = 1.07 × 10−7, adjusted = 1.14 × 10−4, log2 fold change = 2.41) increased ~5.31-fold.

**Cell morphology**
Yeast cells were grown to mid-log phase in SC medium at 30°C. DIC images were collected by using an EVOS Fluorescent Cell Imaging System (60X) (Fig. S4).

**Pulsed field gels**
The method to prepare the chromosome-size DNAs was modified from elsewhere (29). Synthetic chromosomes were identified by running the BY4741 sample on the same gel whose molecular karyotype was known. The samples were run under the following condition: 1.6% low melting agarose gel, 0.5× TBE buffer (pH 8.0), CHEF apparatus, 14°C, a voltage of 6 V/cm, an angle of 60°C, a switch time of 60 s and an electrophoresis time of 24 hours.

**Inverse PCR analysis of the YEL070W1 WT PCRTag amplicon**
Both SYN and WT PCRTags of YEL070W1 yielded amplicons during the PCRTag analysis. To make sure whether the wild-type YEL070W1 gene was replaced by synthetic DNA segments, inverse PCRTag primers (SYN-IPCRTag and WT-IPCRTag) were designed, based on the sequences of SYN and WT PCRTag sequences, and inverse PCR was carried out to analyze the YEL070W1 WT amplicon. First, gDNA was digested by SacI prior to the self-ligation. Second, the self-ligated DNA was amplified by inverse PCRTag primers and the gel purified products were sequenced, followed with alignment and BLAST (30, 31).

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**Endoreduplication backcross**

Genome sequencing analysis revealed that there were two regions with duplications of long segments (291,217–299,580 bp and 305,355–325,236 bp, copy number is 2) on initial synV strain (yXXZ346, version "yeast_chr05_9_01"), which showed a notable fitness defect onYPD +6% ethanol medium at 37°C. The duplications on synV could be due to either integration of both copies on synV, or misintegration of one copy into another chromosome(s). An "endoreduplication backcross" strategy was applied to distinguish these possibilities (8, 32) (fig. S10). The synV strain (yXXZ345, version "yeast_chr05_9_01") was backcrossed to a CEN-conditional V strain (yLM657, pGAL-CENS::KL, URA3, MATa) and induced with galactose. The loss of CEN-conditional chromosome V was verified by selecting on 5-FOA (5-fluoroorotic acid) medium and analyzing with PCRTags. Phenotypic characterization was performed on YPD +6% ethanol medium at 37°C after random spore analysis.

**Highly efficient sporulation and random spore analysis**

Highly efficient sporulation was performed according to the method elsewhere (33). The diploid cells were grown to OD_{600} = 1 in 3 mL YPD medium and harvested by centrifugation (5000 rpm, 1 min). The pellets were washed three times with sterile water and incubated in 3 mL YPK medium (20 g/L peptone, 10 g/L yeast extract, 10 g/L potassium phosphate, pH 7.2) to 25 OD_{600}/ml. Zymolyase-20T (20 mg/mL) was added to a concentration of 20 OD_{600}/ml and incubated at 30°C for 24 hours. Subsequently, cells were washed and harvested into 3 mL YPK medium and resuspended in softening plasting buffer (1 M sorbitol, 10 mM potassium phosphate, pH 9.4) at 5 OD_{600}/ml and incubated at 30°C for 30 min. The suspensions were centrifuged at 1000 g for 5 min at RT and resuspended in softening buffer (10 mM dithiothreitol, 100 mM Tris-SO₄, pH 9.4) at 5 OD_{600}/ml and incubated at 37°C for 10 min. The pellets were isolated by centrifugation at 1000 g for 5 min and resuspended with spheroplasting buffer (1 M sorbitol, 100 mM potassium phosphate, pH 7.2) to 25 OD_{600}/ml. Zymolyase-20T (20 mg/mL) was added to a concentration of 0.5 mg/OD_{600} and the spheroplasting reaction was carried out at 30°C for 30 min. The cells were incubated at 55°C for 3 min, and subsequently, the above suspension was centrifuged at 1000 g for 5 min and washed once with 0.5% (v/v) Triton X-100. Finally, the spore pellets were resuspended with 0.5% (v/v) Triton X-100 whose amount was 25% the volume of spheroplasting buffer used above, followed by brief sonication to disperse the spores and stored at 4°C.

**Quadruplication analysis**

Genome sequencing analysis revealed a region with quadruplication of short segment (140,356-142,771 bp), corresponding exactly to minichunk "chr05_3_39_EL01" which had been digested with the restriction enzyme site "NotI", was present on synV (yXXZ347, version "yeast_chr05_9_01"), PCR and qPCR methods were used to confirm the quadruplication structure and copy number (figs. S10 and S11). qPCR analysis revealed the copy number was 4, which was consistent with all the genome sequencing analysis result, and all the 4 copies were tandemly ligated with "GGGCGGCG".

**Integrative cotransformation repair strategy**

Integrative cotransformation strategy was done in two steps: First, one SNV/indel was used as a "target region" and deleted by integrating selective marker at this locus, and second, the marker was removed by cotransferring a pool of DNA fragments in twofold molar excess covering all other regions targeted for correction.

Using target region 2 in the starting synV strain yXXZ473 (version "yeast_chr05_9_02") as an example, URA3 was used to delete this region (fig. 2, A, B, and C, and tables S3 and S4). Next, a verified DNA pool containing all of the mini-chunks for repair of the 22 target regions, including target 2 and 13-2, was cotransferring with selection on 5-FOA. After site-specific PCR and Sanger DNA sequencing analysis, the synV strain yXXZ338 (version "yeast_chr05_9_07") had regions 2 and 13-2 both repaired and was selected as the starting strain for the next repair step.

**CRISPR/Cas9 based cotransformation repair strategy**

The CRISPR/Cas9 based cotransformation strategy was used to correct the SNVs/indels which were contained into the 14-bp target sequence (12 bp from the seed sequence and 2 bp from PAM) (25). First, one "target region" was used for the gRNA plasmid construction. Second, we transformed the Cas9 plasmid into the strain with draft synV. Third, a pool of DNA fragments, covering all other regions targeted for correction, and the gRNA plasmid were cotransferring into that strain. Last, site-specific PCR and Sanger DNA sequencing were used to verify the candidate colonies.

For example, using synV strain yXXZ338 (version "yeast_chr05_9_07"), we targeted region 14 for DSBs, cotransferring the entire pool of mini-chunks for all regions yet to be corrected. In this case, we isolated a strain with two target regions corrected (regions 14 and 16) (yXXZ633, version "yeast_chr05_9_07") (fig. A, 2, A, D, and E and tables S3 and S4).

**Yeast colony PCR analysis (YCPCR)**

Yeast colony PCR analysis method was modified from the method elsewhere (7). Yeast cells from single colonies were resuspended into 30 μL of 20 mM NaOH and treated with the following program in a thermocycler: 95°C for 5 min and 4°C for 1 min, 3 cycles. 1 μL solution was added to a regular PCR mix and performing PCR cycles.

**Yeast site–specific colony PCR (YS-CPCR)**

Yeast sites specifically colony PCR was used to identify the SNVs/indels correction (35). We designed primers for each SNV/indel: a shared forward (reverse) primer for both the designed sequence and the variant sequence, and two respective reverse (forward) primers for either the designed sequence (syn) or variant sequence (var). The respective primers were identical except for the most 3'-nucleotide(s). Gradient PCRs were performed to determine the specific annealing temperature for both the syn primer pairs and var primer pairs.

Every colony was analyzed via the PCR with both the syn and var primer pairs. Only the colonies with SNVs/indels repaired can yield amplicons with the syn primers vice versa the var primers. The YS-CPCR reactions included 5 μL of 2x GoTaq Green PCR master mix (Promega, Madison, WI), 0.25 μmol of each primer, 1 μL of template, and 4 μL of water.

**Enzyme digestion analysis**

Enzyme digestion was used to confirm the correction of synV/indels. The PCR products, covering the SNVs/indels, were amplified with the amplifying primers followed by enzyme digestion. Restriction fragments corresponding to the correct sizes were indicated on a 1.5% agarose gel (1x TTE buffer).

**Sanger DNA sequencing**

Sanger DNA sequencing was employed to verify the PCR and enzyme digestion results. The amplification products were yielded and sequenced with the amplifying primers. Sanger sequencing was performed in GENEWIZ, and the results were identified by sequence alignment with the designed synV.

**Circularization of synV**

The synV (yXXZ338, version "yeast_chr05_9_06") was cyclized with homologous recombination strategy by deleting the telomere ends to achieve the synthetic ring chromosome V, ring_synV (yXXZ605). The circular chromosome ring_synV encoded all genes present on the parental synV, except for the deletion of 689 bp of the left end of synV and 698 bp of the right end of synV.

The native chromosome V in BY4741 was cyclized in the same location of ring_synV and DSBs were induced by cleaving the two telomeric regions with CRISPR/Cas9, to achieve the wild-type ring chromosome V control with a URA3 selective marker, ring_wtV::URA3 (yXXZ915) (fig. 3B, fig. S13 and table S6, S7).

**Growth curve analysis**

Colonies of BY4741, synV and the intermediate strains were inoculated in 5 mL YPD medium. Moderate overnight culture was added to 200 μL of YPD medium to OD_{600}=0.2. Microplate reader was used to read the OD_{600} every 10 min at 30°C for 24 hours. The growth experiment was repeated for three times.

**Serial dilution assays**

Colonies of BY4741, synV and the intermediate strains were grown overnight in 5 mL of YPD medium at 30°C and then were serially diluted.
in 10-fold increments in water and plated onto YPD, SC, SD, YPG and YPE. BY4741 and synV (yX2X347, version "yeast_chro_09_01") were also plated onto several kinds of media (7, 36). All kinds of plates were incubated at 30°C and 37°C (figs. S3 and S8).

All chemicals (benomydil [Aldrich, 381586; micromutator inhibitor], camptothecin [Sigma, C9911; topoisomerase inhibitor], hydroxyurea [Sigma, H8627; defective DNA replication], sorbitol [Sigma, S1879; osmotic stress], ethanol [Fisher, BP2818], CaCl2, NaCl, SDS, sodium orthovanadate, hydroxy-nymycin B [Calbiochem, 400053] and acetic acid] were added adequate amount into YPD, except for 6-azauracil (6-AC; Sigma, A7575; defective transcription elongation) was mixed into SC medium with 2% dextrose. YPG (respiratory defects) and YPE (respiratory defects) were prepared with 2% glycerol or 2% ethanol. High pH and low pH (9.0 and 4.0), vacuole formation defects were prepared by adding NaOH or HCl in YPD. For hydrogen peroxide (Sigma, H1009; oxidative stress) and cycloheximide (Sigma, C7698; defective protein synthesis), overnight cultures were treated for 2 hours in drug, harvested by centrifugation and resuspended in water prior to plating the serial dilution on YPD plates.

**Stability analysis of ring_synV**

Stability analysis of ring_synV chromosome was carried out in diploid strains.

A series of diploid strains were constructed to test the stability of ring_synV (tables S2 and S3). One single colony of diploid was picked up from SC-Ura (or SC-Leu) plate and inoculated in 5 mL of YPD medium at 30°C overnight and then 2 mL of overnight culture was transferred to 5 mL of fresh YPD medium. After ~60 generations incubation in YPD medium, PFGE was used to analyze the stability of ring chromosome. PFGE analysis revealed that the ring_synV (ring_wtV) chromosome was not linearized in diploid strains during 5-day incubation (Fig. 3c and fig. S14). The loss of the linear wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band as a result of circularization is difficult to assess by PFGE since wtV co-migrates with wtV band.
"Perfect" designer chromosome V and behavior of a ring derivative

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