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Creating functional chromosome fusions in yeast with CRISPR–Cas9

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CRISPR–Cas9-facilitated functional chromosome fusion allows the generation of a series of yeast strains with progressively reduced chromosome numbers that are valuable resources for the study of fundamental concepts in chromosome biology, including replication, recombination and segregation. We created a new yeast strain with a single chromosome by using the protocol for chromosome fusion described herein. To ensure the accuracy of chromosome fusions in yeast, the long redundant repetitive sequences near linear chromosomal ends are deleted, and the fusion orders are correspondingly determined. Possible influence on gene expression is minimized to retain gene functionality. This protocol provides experimentally derived guidelines for the generation of functional chromosome fusions in yeast, especially for the deletion of repetitive sequences, the determination of the fusion order and cleavage sites, and primary evaluation of the functionality of chromosome fusions. Beginning with design, one round of typical chromosome fusion and functional verifications can be accomplished within 18 d.

Introduction

Chromosome numbers vary in eukaryotic species during evolution. For example, the number of chromosomes in different yeast species ranges between 6 and 16 (ref. [1]). Chromosome fusion events occur occasionally but usually result in dicentric chromosomes, leading to chromosomal abnormalities and genomic instability due to problems associated with chromosome segregation during cell division[2–4]. Recently, we developed an efficient CRISPR–Cas9-facilitated chromosome fusion method in the eukaryotic model organism *Saccharomyces cerevisiae*. The RNA-guided nuclease Cas9[5,6] cleaves chromosomal double-stranded DNA specifically at designed sites, which initiates the repair of damaged DNA ends by ligation via homologous recombination, thereby greatly increasing the efficiency of chromosome fusion. By sequential chromosome fusions, we generated a functional single-chromosome yeast strain from the 16 natural chromosomes[7], as well as a series of intermediate yeast strains with reduced chromosome numbers. These engineered organisms are valuable resources for the study of fundamental concepts in chromosome biology, including replication, recombination and segregation. Furthermore, these engineered chromosomes provide a tractable system for the study of eukaryotic chromosome evolution and biological functions. This protocol provides guidelines for experimental design, as well as a step-by-step procedure for the generation and validation of functional chromosome fusions in yeast. Given its simplicity, efficiency and portability, we expect that this method can be easily adapted for chromosome fusions in other organisms.

Overview of the generation of a functional single-chromosome yeast strain

S. cerevisiae haploid cells contain 16 natural chromosomes, ranging from 230 kb to 1.5 Mb (ref. [8]) in size. The 16 chromosomes can be artificially fused into one (Fig. 1) by 15 rounds of sequential pairwise chromosome fusions, with the deletion of 15 centromeres, 30 telomeres and 19 telomere-associated repeat sequences (RSs)[7]. The long (e.g., >2 kb) RSs proximal to different chromosomal ends, instead of our designed DNA-targeting cassettes, will be preferentially recognized by the host (in this case *S. cerevisiae*) as homologously recombined segments when the corresponding chromosome ends are cleaved by CRISPR–Cas9, which can strongly interfere with the designed chromosome fusions. In addition, the deletion of two telomeres during chromosome fusion will lead to the formation of the same group of long RSs adjacent to telomeres to form a long palindromic structure that is prone to genetic instability[9–11]. The homology of the DNA sequences within 35 kb of

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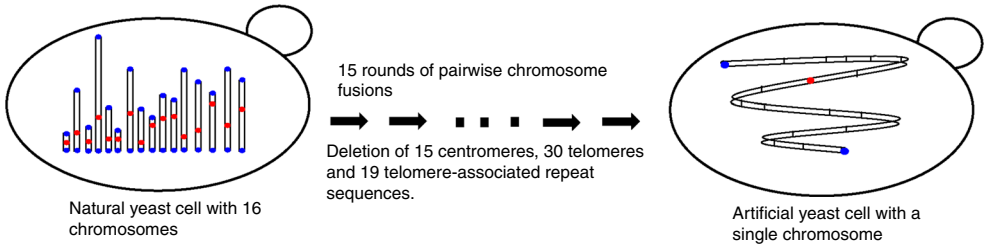

Fig. 1 | Overview of the generation of a functional single-chromosome yeast strain. The centromere and telomere are indicated by red and blue dots, respectively.

all 32 chromosome ends is checked with software from the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the long (>2 kb) sequences with identity of >90% to each other are considered as the same group of RSs. There are protein-coding genes within the telomere-associated RSs; therefore, to minimize the potential influence on gene functionality, one copy of each group of long repeats should be retained. All of the redundant copies of telomere-associated RSs should be deleted.

Generation of functional chromosome fusions in yeast

A pilot experiment was performed to rapidly evaluate the technical feasibility of chromosome fusions. The 16 natural chromosomes of the haploid *S. cerevisiae* strain BY4742 were divided into eight groups (Table 1) on the basis of the criterion that the disappearance of two natural chromosomes or appearance of one newly fused larger chromosome should be easy to distinguish by size via pulsed-field gel electrophoresis (PFGE) separation. Although budding yeast has a 'point centromere'[12] that is ~125 bp in length in the 16 natural chromosomes, the centromere sequences from different chromosomes are not identical. In seven of the eight groups of pairwise chromosome fusions (Table 1), the centromeres from the seven large chromosomes were retained, and chromosome fusion strains were successfully obtained. To test whether the centromeres of small chromosomes can also maintain proper activity, we designed one group of chromosome fusions that retained the centromere of the smaller chromosome (chr. I, 230 kb) instead of the larger one (chr. II, 810 kb). The corresponding fused chromosome with a size of ~1 Mb could use the centromere from chromosome I for segregation. The above results indicate that centromeres from both large and small chromosomes could maintain proper segregation of the fused chromosome with relatively large sizes.

The sub-telomeric regions within 35 kb of each chromosome end exhibited a high degree of sequence redundancy. The 15 groups of long RSs (RS1–15; >2 kb, sequence identity >90%) at the chromosome ends are shown in Fig. 2. Each group of repeat sequences had two to three copies dispersed on different chromosomes. To preserve as many functional genes as possible while avoiding potential interference effects of long repeats on homologous recombination during chromosome fusion, we preserved only one copy each of RS1–15 and deleted the redundant copies. Distal copies cannot be conveniently deleted during chromosome fusion; therefore, these copies (indicated by the red arrowheads in Fig. 2) were deleted separately by the CRISPR–Cas9 system before fusion. For example, as shown in Fig. 2a, the repeat sequence RS1 has three copies (at the left end of chr. I (I-L), the right end of chr. I (I-R), and the right end of chr. VIII (VIII-R)), RS2 has two copies (at I-R and VIII-R). RS1 and RS2 at I-R can be deleted during fusion of chr. I with the other chromosome. Deletion of RS1 at VIII-R requires a separate deletion before chromosome fusions. In the end, only one copy of RS1 and RS2 is retained. The scheme for deleting the redundant copies of other RSs is shown in Fig. 2b. For technical feasibility, the adjacent copies of the four RSs (RS14, -10, -15 and -9 in Fig. 2b) located in the left end of chromosome IX (IX-L) were retained, and the remaining redundant copies were deleted.

The order of chromosome fusions is determined largely by the deletion of the redundant copies of telomere-associated RSs. As shown in Fig. 3a,b, one copy each of RS1 and RS2 adjacent to the telomere at I-R can be deleted together during end-to-end fusion of I-R and II-L, which determines the chromosome fusion order of chromosomes I and II (Fig. 3a). The other copies of RS1 at I-L and RS3 at II-R are retained in the fusion of chromosomes I and II. Moreover, as the copy of RS2 at II-R was deleted during I–II fusion, the only remaining copy of RS2 at VIII-R has to be retained, which determines the chromosome fusion order of VII–VIII (Fig. 3b). The copy of RS3 at VII-R is deleted

Table 1 | Details of eight groups of pairwise chromosome fusions

Chromosomes for fusion (chr. length in kb)	Fused chromosome (chr. length in kb)[a]	Centromere retained	Left arm of fused chromosome (kb)	Right arm of fused chromosome (kb)	Centromeric index[b]	Positive rate of chromosome fusion[c]
I (230), II (810)	I + II (1,006)	I	151	855	0.15	5/5
III (320), IV (1,530)	III + IV (1,826)	IV	743	1,083	0.41	5/5
XVI (950), V (580)	XVI + V (1,510)	XVI	556	954	0.37	3/5
VI (270), XIV (780)	VI + XIV (1,036)	XIV	880	156	0.15	2/3
VII (1,090), VIII (560)	VII + VIII (1,609)	VII	493	1,116	0.31	1/3
IX (440), X (750)	IX + X (1,160)	X	850	310	0.27	3/5
XIII (920), XII (1,080 + 1,500)	XIII + XII (1,980 + 1,500)[d]	XII	1,053	927 + 1,500[d]	0.30	3/4
XV (1,090), XI (670)	XV + XI (1,737)	XV	327	1,410	0.19	2/3

[a]Some RSs at the chromosome ends were deleted during chromosome fusion. [b]Centromeric index indicates the position of the centromere and is calculated by dividing the length of the short arm by the total chromosome length. [c]For each transformant, dozens to hundreds of colonies were usually obtained. The positive colonies were PCR-verified and sequenced. For example, 3/5 means three positive colonies out of the five randomly selected colonies. [d]Chromosome XII has 100–200 copies of rDNA repeats (~9.1 kb) and an estimated length of ~1.5 Mb.

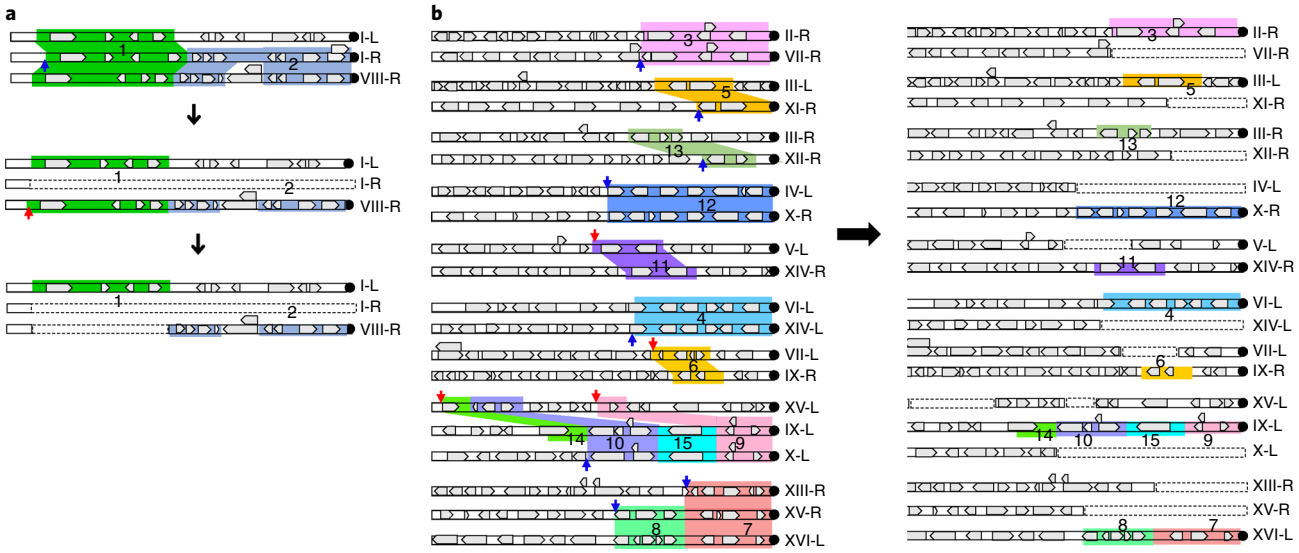

Fig. 2 | Repetitive sequences near the chromosome ends. The sequences that share >90% identity within the 35-kb regions of the chromosome ends are shown as clusters shaded in the same color. The light gray arrowheads represent the coding sequences of genes, and the direction of the arrows indicates the direction of gene transcription. Different groups of RSs are marked with different colors and numbered from 1 to 15. '-L' and '-R' indicate the left and right ends of the chromosome, respectively. The redundant copies of RSs deleted together with the corresponding telomeres in chromosome fusions are marked by blue arrows (sites of Cas9 cleavage) and shown as dashed lines. The copies deleted separately before chromosome fusions are marked by red arrows. **a**, Specific example illustrating deletion of the redundant copies of RS1 and RS2. **b**, The full set of the other chromosome ends with RSs highlighted.

during the fusion of VII–VIII. There is one more copy of RS1 at VIII-R, which is not adjacent to telomeres and must be deleted before chromosome fusion. There are alternative schemes that can achieve the same outcome. As shown in Fig. 3c,d, one possibility is to delete the two copies of RS1 at I-L and VIII-R separately before chromosome fusion and retain the copies of RS1 and RS2 at I-R, which determine the chromosome fusion order of II–I. Fusion of II–I deletes one copy of RS3. Therefore, the other copy of RS3 at VII-R has to be retained, which determines the fusion order of VIII–VII. The redundant copy of RS2 at VIII-R can be deleted during chromosome fusion of VIII–VII. However, the alternative scheme requires an additional separate deletion event, which would increase the workload for the entire project; therefore, it is not preferred for the purposes of this project.

All pairwise chromosome fusions were successful, and difficulties were seldom encountered, except with the fusion of chromosomes XV and XI. We tried three different trial experiments to delete the XV centromere during chromosome fusions; however, we always obtained slow-growing cells (Fig. 4).

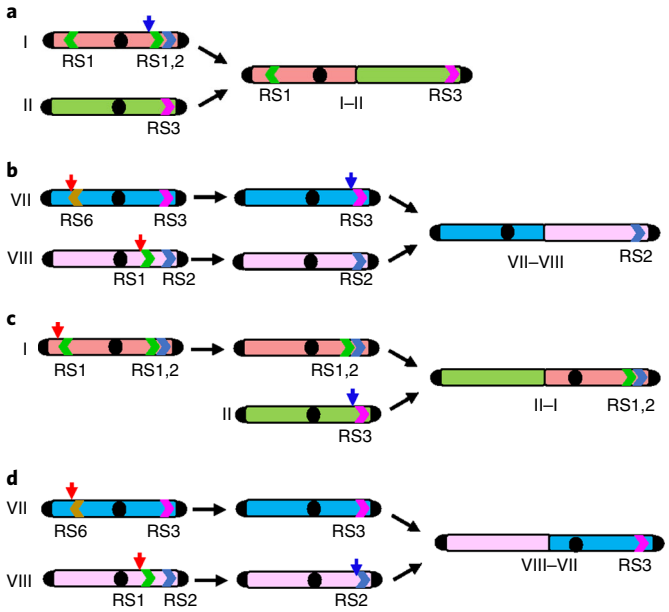

Fig. 3 | Diagram of different options for deletion of telomere-associated RSs and determination of the order of chromosome fusion. The centromeres and telomeres are marked by black dots and semicircles, respectively. RS1, -2, -3 and -6 are marked by green, blue, purple and brown arrowheads, respectively. The redundant copies of RSs deleted before chromosome fusion are indicated by red arrows. The redundant copies of RSs deleted together with the corresponding telomeres during chromosome fusion are indicated by blue arrows. The choice of copies of RSs to be deleted determines the chromosome fusion orders. **a**,**b**, As one option, the deletion of RS1,2 at I-R leads to I–II fusion (**a**) and further determines VII–VIII fusion (**b**). **c**,**d**, An alternative solution in which RS1 is deleted at I-L but RS1,2 is retained at I-R leads to II–I fusion (**c**) and further determines VIII–VII fusion (**d**).

Fortunately, the fourth attempt, in which the XI centromere was deleted but the XV centromere was retained during chromosome fusion, resulted in a healthy strain that grew robustly.

For all eight groups of pairwise chromosome fusions, we observed no obvious growth defect of the fusion strains (Fig. 5), indicating that robust yeast strains can be generated with proper design and testing.

Workflow for one round of chromosome fusion

Each round of chromosome fusion involves four stages (Fig. 6):

- *Design and construction*. For two chromosomes to be fused into one, one centromere and two telomeres need to be deleted. Three specific cleavage sites near the to-be-deleted centromere and telomeres are carefully selected to minimize the possible influence on the expression of adjacent genes. The corresponding guide RNAs (gRNAs) targeting the above three cleavage sites are generated by fusion PCR amplifications and cloned in the plasmid pgRNA (gRNA expression plasmid). The two donor DNA cassettes that contain homologous sequences (i.e., R1 and R2) to the chromosome cleavage ends are also constructed via overlapped PCR amplification.
- *Chromosome fusion*. Plasmid pgRNA and two donor DNA cassettes are co-introduced by transformation into yeast cells that constitutively express the nuclease Cas9. Expression of the three gRNAs leads to cleavage at the designed sites on two chromosomes by Cas9. The three cleaved chromosome segments are sealed by the two donor DNA cassettes both containing the marker (*URA3*). Theoretically, one marker may be enough for selection of a fusion event. However, we found that, to a certain extent, using two *URA3* markers increased the rate of successful chromosome fusions compared with that obtained when we used only one marker.
- *Marker removal*. For the next round of chromosome fusion, the selection marker *URA3* on the fused chromosome must be deleted. The Cas9 expression plasmid contains a galactose-inducible gRNA that targets a site proximal to *URA3*. Therefore, simply transferring the chromosome-fusion-harboring yeast cells into galactose-containing medium can induce gRNA expression, which leads to cleavage by Cas9 at sites proximal to the two *URA3* markers. The three resulting chromosome segments are repaired and ligated via homologous recombination at R1 and R2 separately, which deletes the two

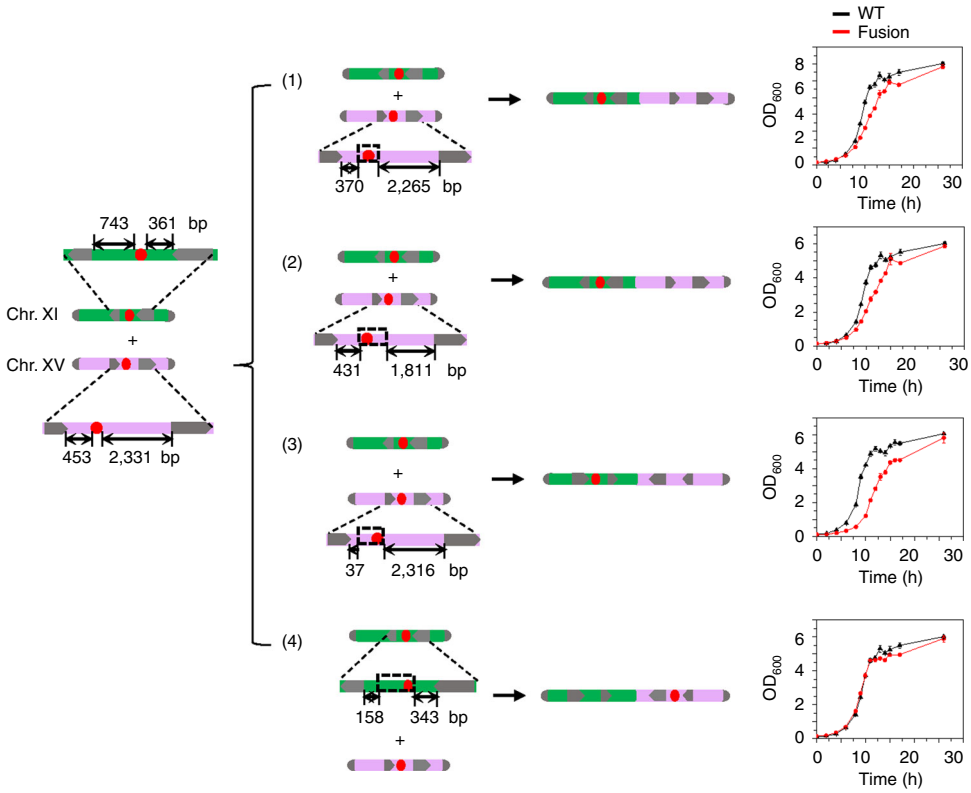

Fig. 4 | Different trial experiments for the fusion of chromosomes XV and XI. Four trial experiments for the fusion of chromosomes XV and XI were performed. Experiments 1–3 deleted the XV centromere and preserved the XI centromere. Experiment 4 deleted the XI centromere and preserved the XV centromere. The deleted region is marked by a dashed rectangle. The centromeres are marked by red dots. The genes adjacent to the centromeres are marked by gray arrowheads. The telomeres are marked by gray semicircles. The numbers (e.g., 370, 2,265, 431, 1,811) next to the deleted centromeres indicate the distances from the deleted region to the coding sequence of the nearest gene in base pairs (bp). The growth curves of the corresponding chromosome fusion strains are shown on the right. Three biological replicates were assayed, and the mean values and s.d. are shown.

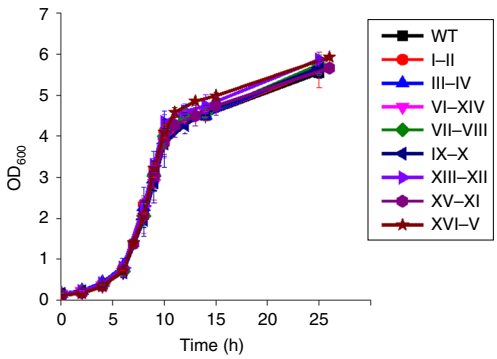

Fig. 5 | Growth curves of yeast strains with pairwise chromosome fusions. Cells in the exponential growth phase were diluted in fresh YPAD medium to an OD_{600} of 0.1, and cell growth was measured hourly. Data from three biological replicates are presented (mean ± s.e.m.).

URA3 selection markers. In addition, the same cleavage site also exists in the backbone of the gRNA expression plasmid pgRNA, and induction of the gRNA deletes pgRNA. Deletion of the *URA3* selection marker and the gRNA expression plasmid pgRNA will allow the reintroduction of new donor DNA cassettes and new gRNA expression plasmid in the next round of chromosome fusion.

- *Functional validation*. Positive colonies are verified by PCR amplification and DNA sequencing. The chromosomal DNA of the positive colonies is further separated by PFGE according to size. The fusion of two chromosomes will result in the disappearance of two DNA bands at the corresponding sizes and the

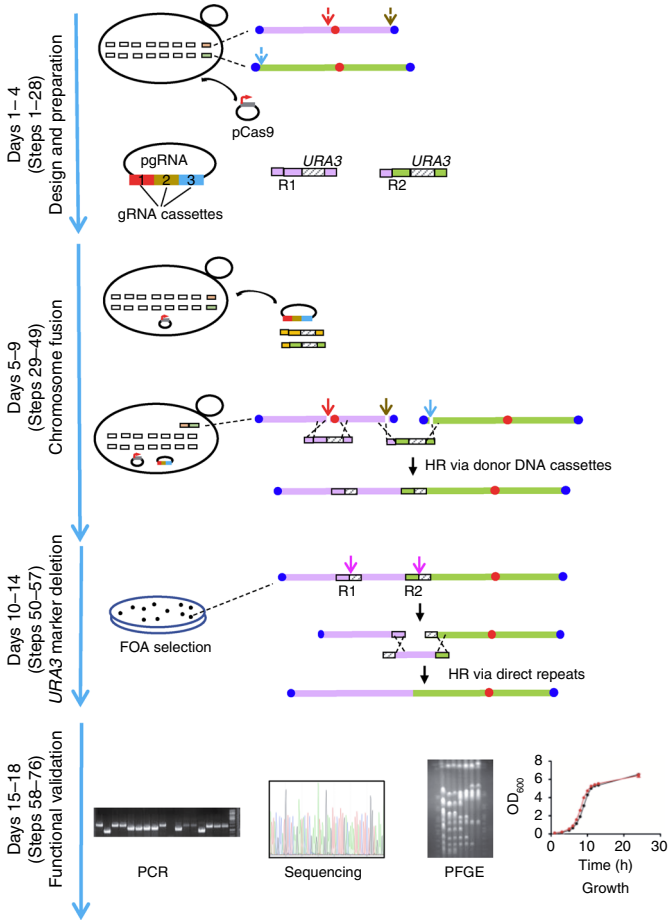

Fig. 6 | Timeline and overview of one round of chromosome fusion. Steps for design and construction, chromosome fusion, marker removal and functional validation are depicted. The three cutting sites near the centromere and two telomeres are marked by red, dark gold and light blue arrows. The three corresponding gRNAs are marked by red, dark gold and light blue blocks. Cas9 should be expressed before chromosome fusion. To save time, the transformation of pCas9 into the yeast cell can be done at the stage of design and preparation (days 1–4) before chromosome fusion (days 5–9). After confirmation of successful chromosome fusion and centromere deletion, 5-FOA selection is used to isolate colonies in which the *URA3* marker was deleted (days 10–14). For functional validation (days 15–18), PCR amplification and DNA sequencing confirm the deletion of the centromere and telomeres in pairwise chromosome fusion, as well as the correct ligation of the resulting three chromosome segments. PFGE confirms the deletion of two natural chromosomes and the appearance of the fused chromosome with an expected size. HR, homologous recombination.

appearance of one new DNA band with a size corresponding to the fused chromosome. The functionality of the fusion strain is primarily evaluated by the growth of the yeast cells harboring the fused chromosome.

Advantages of the CRISPR–Cas9-facilitated chromosome fusion method

Traditional methods[13,14] that rely only on homologous recombination for chromosome fusions usually generate dicentric chromosomes, which are not stable unless one of the two centromeres is inactivated. With conditional inactivation of the centromere of a chosen chromosome and provision of a homologous recombination cassette that targets two ends of two chromosomes, a temporary stable chromosome fusion can be achieved, albeit with very low efficiency[15]. In comparison, the CRISPR–Cas9-facilitated chromosome fusion has several advantages:

- *Fast and efficient*. The utilization of CRISPR–Cas9 to cleave chromosomal DNAs at multiple sites and the high performance of yeast homologous recombination allow fast and efficient ligation of chromosome segments.
- *Allows cumulative chromosome fusions*. The selection marker and the gRNA plasmid removal are easily accomplished by one step of galactose induction, which allows cumulative chromosome fusions because the markers can be reused in the next round of fusion.

- *Portable*. Because it requires only functional CRISPR–Cas9 and the host's homologous recombination machinery, this method can be easily adapted for chromosome fusions in other yeast species (e.g., fission yeast *Schizosaccharomyces pombe*) or perhaps in complex eukaryotic organisms (e.g., plants and animals).

Comparison with other methods

A very similar CRISPR–Cas9-facilitated chromosome fusion method by Luo et al.[16] was published simultaneously with our work[7] and describes the generation of a two-chromosome yeast. Despite the similarities, our method differs in four aspects: (i) we delete the redundant copies of long repetitive sequences near chromosomal ends to ensure the accuracy of chromosome fusion in yeast. We speculate that removal of all these long repeats is important for the high success rate of chromosome fusion (20–100%) in our reported work[7]. (ii) We use the *URA3* marker for easy selection of positive chromosome fusions and remove the marker in a subsequent step. In comparison, chromosome fusion without any selection marker can result in a low success rate of chromosome fusion in some cases. (iii) We clone three gRNAs into one plasmid, which increases the efficiency of cotransformation with target cassettes and is convenient for gRNA plasmid removal before the next round of chromosome fusion. In comparison, Luo et al.[16] used two plasmids to deliver three gRNAs (one carrying one gRNA and the other carrying two gRNAs), which makes plasmid elimination before the next round of chromosome fusion more difficult. (iv) The chromosome fusion orders are different in the two works[7,16], although we believe the orders might be flexible.

Limitations of the CRISPR–Cas9-facilitated chromosome fusion method

The long repetitive sequences at the chromosome ends can strongly interfere with homologous recombination and therefore decrease the accuracy of chromosome fusion. If only one homologous region is exactly matched while another homologous region has more than one matched site, then the accuracy of chromosome fusion drops to 1–2% as reported by Luo et al.[16], which is at least ten times lower than our reported efficiency (20–100%)[7]. Therefore, the repetitive sequences at the chromosome ends can be problematic during chromosome fusion; the experiment should be carefully designed to delete these sequences.

The yeast strain needs to have at least three auxotrophies (Leu, His and Ura) to allow for proper maintenance of the two plasmids (i.e., pCas9 and pHIS426 for the CRISPR–Cas9 system) and for the selection of *URA3* marker. *URA3* is a perfect choice because integration of this marker can be used as positive selection for chromosome fusion, and deletion of *URA3* allows cell growth on a 5-fluoroorotic acid (5-FOA) plate, which can be used as negative selection for marker deletion. If the above three auxotrophies are not available in other species, then the selection strategy must be changed.

There are some potential limitations to the extension of this method to other species such as plants and animals, which contain more complex chromosome structures, including a megabase-long centromere and extensive repetitive sequences. Ligation of the cleaved chromosome segments after deletion of the extremely long centromere would be difficult. Moreover, the long repetitive sequences in these genomes, especially near the telomeres, may interfere with homologous recombination during chromosome fusions.

Materials

Reagents

! CAUTION When using reagents that can cause skin and eye irritation, please wear gloves and goggles or use a fume hood

Biological materials

- *S. cerevisiae* BY4742 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; Euroscarf, cat. no. Y10000) was used as a host to create chromosome fusions. Genomic DNA was isolated and used as the template for amplifying homologous arms of donor DNA cassettes
- *S. cerevisiae* S288C (ATCC, cat. no. 204508) genomic DNA was used as a template for PCR amplification of selection marker genes
- *Escherichia coli* DH10B (Invitrogen, cat. no. 18297010) was used as a host for plasmid cloning

Guide RNA preparation

- *Plasmids*. pCas9[17] (for constitutive expression of Cas9) was derived from p415-GalL-Cas9-CYC1t (Addgene, plasmid no. 43804); p426-SNR52p-gRNA.CAN1.Y-SUP4t (Addgene, plasmid no. 43803)

Table 2 | Primer sequences for PCR amplification of three gRNA-expressing cassettes

Primer	Sequences (5′– 3′)
Primer sequences for gRNA for chr. VII and chr. VIII fusion	
gRNA1-P1	ACTGAATTCTCTTTGAAAAGATAATGTATGATTATGCTTTC
gRNA1-P2	GCTCTAAAACAATCTCTTGCCGATCTAAAGGATCATTTATCTTTCACTGCGGAGAAG
gRNA1-P3	ATGATCCTTTAGATCGGCAAGAGATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
gRNA1-P4	AGAGGATCCAGACATAAAAAACAAAAAAAGCACCACCG
gRNA2-P1	TCTGGATCCTCTTTGAAAAGATAATGTATGATTATGCTTTC
gRNA2-P2	GCTCTAAAACTAGCCATAGATTCTACTCGGGATCATTTATCTTTCACTGCGGAGAAG
gRNA2-P3	ATGATCCCGAGTAGAATCTATGGCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
gRNA2-P4	AGACCATGGAGACATAAAAAACAAAAAAAGCACCACCG
gRNA3-P1	TCTCCATGGTCTTTGAAAAGATAATGTATGATTATGCTTTC
gRNA3-P2	GCTCTAAAACTGGCAGTATTCTTACCCCAAGATCATTTATCTTTCACTGCGGAGAAG
gRNA3-P3	ATGATCTTGGGGTAAGAATACTGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
gRNA3-P4	AATGCGGCCGCAGACATAAAAAACAAAAAAAGCACCACCG
Primer sequences for gRNA for RS1 and RS6 deletion	
RS6-P2	GCTCTAAAACTGGATTATTTGCGAACCAACGATCATTTATCTTTCACTGCGGAGAAG
RS6-P3	ATGATCGTTGGTTCGCAAATAATCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
RS1-P2	GCTCTAAAACTGATGAAGCACTTTTGCTCAGATCATTTATCTTTCACTGCGGAGAAG
RS1-P3	ATGATCTGAGCAAAAGTGCTTCATCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG

The orange text indicates restriction enzyme digestion sites. Red text indicates 20-bp targeting sequences. Underlined text indicates overlaps between fragments.

Table 3 | Primer sequences for gRNA expression vector construction

Primer	Sequence (5′–3′)
p426-P1	CAAAAGCTGGAGAATTCTCTTCTGCGGCCGCGTCATGTAATTAGTTATGTCACGCTTAC
p426-P2	TCCTAGCGCTCACCAAGCTCACCCTATGCGGTGTGAAATACCGC
p426-P3	TCTATACGTGTCATTCTGAAATTGAAAAGCTGTGGTATGGTGCAC
p426-P4	ATTACATGACGCGGCCGCAGAAGAGAATTCTCCAGCTTTTGTTCCCTTTAGTGAGG
p426-P5	TATTTCACACCGCATAGGGTGAGCTTGGTGAGCGCTAGGAGTCAC
p426-P6	CCATACCACAGCTTTTCAATTTCAGAATGACACGTATAGAATGATGC
p426-F	TGTGGAATTGTGAGCGGATAAC
p426-R	GGGACCTAGACTTCAGGTTGTC

was used as a template for PCR amplification of gRNA expression cassettes; pXX11[18] was used as a template for PCR amplification of *URA3*

- PCR primers (Jie Li Biology; Tables 2–5)
- Phanta Max super-fidelity DNA polymerase (Vazyme Technologies, cat. no. P505-D3)
- Phanta 2× Taq Plus master mix (Vazyme Technologies, cat. no. P111-W3)
- TAE buffer (Tris–acetic acid–EDTA, 50×; Yeasen, cat. no. 60116ES76)
- Agarose (Sigma-Aldrich, cat. no. V900510)
- UltraPure ethidium bromide (Thermo Fisher Scientific, cat. no. 15585011) **! CAUTION** UltraPure ethidium bromide is suspected to cause genetic defects. Avoid contact with skin.
- 1-kb Plus DNA ladder (Thermo Fisher Scientific, cat. no. 10787018)

Table 4 | Primer sequences for generation of donor DNA cassettes

Primer	Sequences (5′ – 3′)
Primer sequences for Chr. VII and Chr. VIII fusion donor DNA	
P1	CAAAGGCACTATCCTTTTTCCCTTCTTC
P2	TTACCATGTATATGTTTAATTCTATCTATCATGTAACACTCCGTTG
P3	GAGTGTTACATGATAGATAGAATTAAACATATACATGGTAACAAATATTACTATC
P4	TACAATCTGCCTGATTACTAGCGAAGCTGCGGGAACCAAGGAAGATGATCCTATCAATG
P5	CTTGGTTCCCGCAGCTTCGCTAGTAATCAGGCAGATTGTACTGAGAGTGCACC
P6	GAACTTTCATGTTAATGACAGTTTTGCTGGCCGCATCTTCTC
P7	GAAGATGCGGCCAGCAAAACTGTCATTAACATGAAAGTTCAATAATGTTTTCATAC
P8	GTGATAACACAAAAGCGTAATATGAGTAATG
P9	GATACACTGTCAAGAAGGCTTTGGCT
P10	TTAGTTTCCTATTTCTAAATAATAATCGTAAGAAAGAATTTACGGAG
P11	AATTCTTTCTTACGATTATTATTTAGAAATAGGAAACTAACCAGTAAATTAG
P12	TACAATCTGCCTGATTACTAGCGAAGCTGCGGGCAACCTGGCTTCAGCTGGGAAATAAT
P13	CAGGTTGCCCGCAGCTTCGCTAGTAATCAGGCAGATTGTACTGAGAGTGCACC
P14	AGTATAATAAGTAGGATGGTGTTTTGCTGGCCGCATCTTCTC
P15	GAAGATGCGGCCAGCAAAACACCATCCTACTTATTATACTAAATCGTTTTG
P16	GAAAGAAGGAGGAATCTTTCCATTTTG
Primer sequences for RS1 and RS6 deletion fusion donor DNA	
RS1-P1	AACACAGCGTGTTTAACAGATAATGTTC
RS1-P2	GGTGCTTATTCAAGCAATGTAGCAAAAGTGCTTCATCATGGCTG
RS1-P3	CATGATGAAGCACTTTTGCTACATTGCTTGAATAAGCACCTCACAGAG
RS1-P4	TACAATCTGCCTGATTACTAGCGAAGCTGCGGGTGCAGACCAACACATAAGCAATTGG
RS1-P5	GTCTGCACCCGCAGCTTCGCTAGTAATCAGGCAGATTGTACTGAGAGTGCACC
RS1-P6	TAAACTTCTCGCAATAAATGGTTTTGCTGGCCGCATCTTCTC
RS1-P7	GAAGATGCGGCCAGCAAAACCATTTATTGCGAGAAGTTTAATAAGTAG
RS1-P8	CCCTTTTGTTGCACAACAGCGGGT
RS6-P1	CTGCAATCAACACCACCATGCAAACAG
RS6-P2	CACTATCGGAGATAAACTCAGCGAACCAACCGAACATGCATTGA
RS6-P3	TGCATGTTCGGTTGGTTCGCTGAGTTTATCTCCGATAGTGTCCCAG
RS6-P4	TACAATCTGCCTGATTACTAGCGAAGCTGCGGGAGGGGCCATTTTGCTAGAACTGC
RS6-P5	GGCCCCTCCCGCAGCTTCGCTAGTAATCAGGCAGATTGTACTGAGAGTGCACC
RS6-P6	ATTTCCACCCTGGATTATTTGTTTTGCTGGCCGCATCTTCTC
RS6-P7	GAAGATGCGGCCAGCAAAACAAATAATCCAGGGTGGAAATATTACTGG
RS6-P8	TCGTCAACAGAAGCTTTTTCCTAAGTC

Red text indicates 20-bp targeting sequences.

- Supercoiled DNA ladder (TaKaRa, cat. no. 3585A)
- Wizard SV gel and PCR clean-up system (PCR Purification Kit; Promega, cat. no. A9281)
- FastDigest BamHI (Fermentas/Thermo Fisher Scientific, cat. no. FD0054)
- FastDigest EcoRI (Fermentas/Thermo Fisher Scientific, cat. no. FD0275)
- FastDigest NcoI (Fermentas/Thermo Fisher Scientific, cat. no. FD0573)
- FastDigest NotI (Fermentas/Thermo Fisher Scientific, cat. no. FD0593)
- FastDigest DpnI (Fermentas/Thermo Fisher Scientific, cat. no. FD1703)

Table 5 | Primer sequences for chromosome fusion PCR verification

Primer	Sequences (5′–3′)
Primer sequences for PCR verification of chromosome fusion	
P1	CAGGGTAGTAGACACTAATATGGACC
P2	CCGCGAAAATTTCCGGATAAATCCT
P3	CAAGAAAGTGGTGCGAATGGATGG
P4	CAATCCCTGGGGTACTCCAGTTAGGT
P5	TGACCTTTGCTCTACCAAGAGGTGC
P6	TAACGAACCTTTGCAGCCCGTCTTTATTG
Primer sequences for PCR verification of RS1 and RS6 deletion	
RS1-P1	ATGATTACACATTCTAAGACTTCACA
RS1-P2	CCAGAACCGTCCAGTGATTCAAAACG
RS1-P3	GATCCAGTGACAATGAAGCATATTGAAGTACG
RS6-P4	TCATGCTGGACTGGAGCTGTAGTTACA
RS6-P5	TCCAGTGCTCTATCCAAGGACGGT
RS6-P6	CCACTATAGCTCCTCTTGTTGTCTATC

- T4 DNA ligase (New England Biolabs, cat. no. M0202)
- Tryptone (Oxoid, cat. no. LP0042)
- Yeast extract (Oxoid, cat. no. LP0021)
- Sodium chloride (NaCl; Sinopharm Chemical Reagent, cat. no. 10019308)
- Agar (Sigma-Aldrich, cat. no. A7002)
- Ampicillin (100 mg ml^{-1}, sterile filtered; Sigma-Aldrich, cat. no. A5354)
- Phenol (Sinopharm Chemical Reagent, cat. no. 10015318) **! CAUTION** Phenol is toxic, corrosive and mutagenic. Avoid contact with skin and eyes and inhalation.
- Chloroform (Sinopharm Chemical Reagent, cat. no. 10006818) **! CAUTION** Chloroform is toxic, corrosive and mutagenic. Avoid contact with skin and eyes and inhalation.
- 8-Hydroxyquinoline (Sigma-Aldrich, cat. no. 252565) **! CAUTION** 8-Hydroxyquinoline is toxic and corrosive. Avoid contact with skin and eyes and inhalation.
- Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. 74255) **! CAUTION** SDS can cause skin and eye irritation.
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045) **! CAUTION** NaOH can cause severe skin burns and eye damage.
- Sucrose (Sigma-Aldrich, cat. no. V900116)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Plasmid Mini Kit I (Omega, cat. no. D6943-02)
- TIANamp Yeast DNA Kit (Tiangen, cat. no. DP307-02)

Yeast cell culture

- D-(+)-Glucose (Sigma-Aldrich, cat. no. G7021)
- Yeast extract (Oxoid, cat. no. LP0021)
- Peptone (Biosciences, cat. no. 211677)
- Adenine hemisulfate salt (Sigma-Aldrich, cat. no. A3159)
- Agar (Biosciences, cat. no. 214010)
- Yeast nitrogen base without amino acids and ammonium sulfate (YNB-AA/AS; Sigma-Aldrich, cat. no. Y1251)
- $(NH_4)_2SO_4$ (Sigma-Aldrich, cat. no. A4418)
- Yeast synthetic drop-out medium supplements without histidine, leucine, tryptophan and uracil (Sigma-Aldrich, cat. no. Y2001)
- Yeast synthetic drop-out medium supplements without leucine (Sigma-Aldrich, cat. no. Y1376)
- Yeast synthetic drop-out medium supplements without uracil (Sigma-Aldrich, cat. no. Y1501)
- Yeast synthetic drop-out medium supplements without histidine (Sigma-Aldrich, cat. no. Y1751)
- L-Tryptophan (Sigma-Aldrich, cat. no. T8941)
- 5-FOA hydrate (Sangon Biotech, cat. no. A601555) **! CAUTION** 5-FOA can cause skin and eye irritation.

- D-(+)-Galactose (Sigma-Aldrich, cat. no. G5388)
- D-(+)-Raffinose pentahydrate (Sigma-Aldrich, cat. no. R0250)
- Lithium acetate dihydrate (Sigma-Aldrich, cat. no. L6883)
- PEG (Sigma-Aldrich, cat. no. P3640)
- Deoxyribonucleic acid sodium salt from salmon testes (salmon sperm DNA) (Sigma-Aldrich, cat. no. D1626)

Karyotyping analysis

- KOD FX DNA polymerase (Toyobo, cat. no. KFX-101) ▲ **CRITICAL** KOD FX DNA polymerase exhibits high activity when yeast cells are used directly as templates in a colony PCR reaction.
- EDTA (Sigma-Aldrich, cat. no. 798681) ! **CAUTION** EDTA can cause eye irritation.
- 10× TBE (Bio-Rad, cat. no. 1610733) ! **CAUTION** 10× TBE can cause skin irritation.
- Pulsed-field certified agarose (Bio-Rad, cat. no. 1620137)
- Certified megabase agarose (Bio-Rad, cat. no. 1613109)
- Low-melting agarose (Sangon Biotech, cat. no. A600015 or Bio-Rad, cat. no. 1703594)
- Zymolyase-20T (MP Biochemicals, cat. no. 320921)
- Proteinase K (Sangon Biotech, cat. no. A600451) ! **CAUTION** Proteinase K can cause skin and eye irritation.
- Tris base (Roche, cat. no. 10708976001)
- Glycerol (Sigma-Aldrich, cat. no. G5516)
- Sodium deoxycholate (Sigma-Aldrich, cat. no. D6750) ! **CAUTION** Sodium deoxycholate is harmful if swallowed.
- *N*-lauroylsarcosine sodium salt (Sigma-Aldrich, cat. no. L9150) ! **CAUTION** *N*-lauroylsarcosine sodium salt can cause skin burns and eye damage. Avoid contact with skin and inhalation.
- Tris-HCl (Thermo Fisher Scientific, cat. nos. 15567027 and 5568025)

Equipment

- Pipette tips (Axygen, cat. no. T-200-Y, T-300, T-1000-B)
- Microcentrifuge tubes (1.5 ml; Axygen, cat. no. MCT-150-C)
- Microcentrifuge tubes (10 ml; Hai Men, cat. no LXG-10ML-L). A 15-ml tube (Corning, cat. no. CLS430791) can be used as a substitute
- PCR tubes (Axygen, cat. no. PCR-02-C)
- Culture plates (Corning, cat. no. 430167)
- Falcon round-bottom polystyrene tubes (Corning, cat. no. 352001)
- Centrifuge tubes (50 ml; Corning, cat. no. 430290)
- 0.22-μm filters (Merck Millipore, cat. no. SLGP033RB)
- Spectrophotometer (DNA concentration measurement; Thermo Fisher Scientific, model no. Nanodrop 2000c)
- ProFlex PCR system (Thermo Fisher Scientific, cat. no. 4484073)
- Desktop microcentrifuges (Eppendorf, cat. nos. 5424, 5430R and 5417R)
- Gel electrophoresis system (Tanon, cat. no. EPS300)
- PFGE CHEF-DR III system (Bio-Rad, cat. no. 1703697)
- PFGE disposable plug molds (Bio-Rad, cat. no. 1703713)
- Vortex mixer (Vortex-Genie 2, Scientific Industries, cat. no. SI-0236)
- Digital gel-imaging system (ProteinSimple, model no. AlphaImager HP)
- Spectrophotometer (cell optical density measurement; Beckman, model no. DU730)

Reagent setup

TAE electrophoresis solution

Dilute TAE buffer in ddH_2O to prepare a 1× working solution and store this solution at room temperature (20–25 °C) for up to 6 months.

TBE electrophoresis solution

Dilute TBE buffer in ddH_2O to prepare a 0.5× working solution and store this solution at room temperature for up to 6 months.

LB medium

For cultivation of *E. coli* cells, combine 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 0.5% (wt/vol) NaCl (for plates, add 15 g l^{-1} agar). Add ampicillin to the autoclaved LB medium at a concentration of

100 μg ml^{-1}. Prepared medium can be stored at 4 °C for up to 1 month. **! CAUTION** The LB medium should be cooled to <50 °C to avoid inactivation of antibiotics.

Plasmid extract solution I
Plasmid extract solution I contains 10.3% (wt/vol) sucrose, 0.25% (wt/vol) bromophenol blue, 25 mM Tris-HCl, pH 8, and 10 mM EDTA, pH 8. Store the solution at room temperature for up to 6 months.

Plasmid extract solution II
Plasmid extract solution II contains 1% (wt/vol) SDS and 0.2 M NaOH. The solution should be freshly prepared.

Unbuffered phenol–chloroform
Mix 50 g of phenol, 50 ml of chloroform, 10 ml of H_2O and 50 mg of 8-hydroxyquinoline; store in a brown glass bottle at 4 °C for up to 6 months.

YPAD (yeast extract, peptone, adenine, glucose) medium
YPAD medium contains 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) glucose and 80 mg l^{-1} adenine hemisulfate salt (for plates, add 18 g l^{-1} agar) mixed in ddH_2O. Autoclave at 110 °C for 15 min. The prepared medium can be stored at 4 °C for up to 6 months.

Selection medium lacking uracil, leucine and histidine (SC-Ura-His-Leu)
For selection of yeast transformants with *URA3*, *HIS3* and *LEU2* marker genes, the medium contains 2% (wt/vol) glucose; 0.17% (wt/vol) YNB-AA/AS; 0.5% (wt/vol) $(NH_4)_2SO_4$; 0.192% (wt/vol) yeast synthetic drop-out medium supplements without histidine, leucine, tryptophan and uracil; and 0.005% (wt/vol) L-tryptophan mixed in ddH_2O. Autoclave at 110 °C for 15 min. Store the medium at 4 °C for up to 6 months.

Selection medium lacking leucine (SC-Leu)
For selection of yeast transformants with the *LEU2* gene, the medium contains 2% (wt/vol) glucose, 0.17% (wt/vol) YNB-AA/AS, 0.5% (wt/vol) $(NH_4)_2SO_4$ and 0.192% (wt/vol) yeast synthetic drop-out medium supplements without leucine mixed in ddH_2O. Autoclave at 110 °C for 15 min. Store the medium at 4 °C for up to 6 months.

Selection medium lacking histidine (SC-His)
For selection of yeast transformants with the *HIS3* gene, the medium contains 2% (wt/vol) glucose, 0.17% (wt/vol) YNB-AA/AS, 0.5% (wt/vol) $(NH_4)_2SO_4$ and 0.192% (wt/vol) yeast synthetic drop-out medium supplements without histidine mixed in ddH_2O. Autoclave at 110 °C for 15 min. Store the medium at 4 °C for up to 6 months.

Selection medium lacking uracil (SC-Ura)
For selection of yeast transformants with the *URA3* gene, the medium contains 2% (wt/vol) glucose, 0.17% (wt/vol) YNB-AA/AS, 0.5% (wt/vol) $(NH4)_2SO_4$ and 0.192% (wt/vol) yeast synthetic drop-out medium supplements without uracil mixed in ddH_2O. Autoclave at 110 °C for 15 min. Store the medium at 4 °C for up to 6 months.

5-FOA solution
Dissolve 200 mg of FOA hydrate in 1 ml of dimethylsulfoxide and store at −20 °C in the dark for up to 6 months. 5-FOA is added into SC-Leu medium with agar that has been cooled to 50 °C at a concentration of 1 mg ml^{-1}. The plates containing 5-FOA should be prepared freshly.

20% (wt/vol) galactose solution
Dissolve 20 g of galactose in 80 ml of ddH_2O, adjust the volume to 100 ml with ddH_2O and filter the solution through a 0.22-μm filter. The solution can be stored at 4 °C for up to 6 months.

30% (wt/vol) raffinose solution
Dissolve 30 g of raffinose in 80 ml of ddH_2O, adjust the volume to 100 ml with ddH_2O and filter the solution through a 0.22-μm membrane. The solution can be stored at 4 °C for up to 6 months.

TE25S buffer
Mix 2.5 ml of 1 M Tris-HCl, pH 8, 5 ml of 0.5 M EDTA, pH 8, and 92.5 ml of ddH_2O. Filter-sterilize the solution, and store for up to 1 year at room temperature.

Lysis buffer
Mix 1 ml of 1 M Tris-HCl, pH 7.5, 10 ml of 0.5 M EDTA, pH 8.0, and 89 ml of ddH_2O. Filter-sterilize the solution, and store for up to 1 year at room temperature.

Wash buffer
Mix 2 ml of 1 M Tris-HCl, pH 8.0, 10 ml of 0.5 M EDTA, pH 8.0, and 88 ml of ddH_2O. Filter-sterilize the solution, and store for up to 1 year at room temperature.

Zymolyase-20T solution
This solution is 20 mg ml^{-1} of Zymolyase-20T in 25% (wt/vol) glycerol. Add 400 mg of Zymolyase-20T, 500 mg of glucose and 0.5 ml of 1 M Tris-HCl, pH 7.5, to 9 ml of ddH_2O. Stir until dissolved. Add 10 ml of 50% (wt/vol) glycerol. Mix thoroughly and transfer 500-µl aliquots to 1.5-ml Eppendorf tubes. Store at −20 °C for up to 6 months.

Proteinase K solution
Combine 100 mM EDTA, 0.2% (wt/vol) sodium deoxycholate, 1% (wt/vol) *N*-lauroylsarcosine sodium salt and 1 mg ml^{-1} proteinase K. Store the solution at −20 °C for up to 6 months.

Yeast genomic DNA extraction
Extract genomic DNA from BY4742 cells using the TIANamp Yeast DNA Kit according to the manufacturer's instructions.

Yeast strain BY4742 (containing pCas9)
BY4742 (pCas9) is constructed by the introduction of 100 ng of pCas9 into 5×10^7 BY4742-competent cells according to a standard LiAc transformation method[19] as described in the 'Procedure' section (Steps 29–42). Plate the transformants on SC-Leu plates and grow at 30 °C for 2–3 d. BY4742 (pCas9) must be maintained on SC-Leu media, as pCas9 contains the *LEU2* marker gene. The BY4742 (pCas9) colonies on the plate can be stored at 4 °C for up to 1 month before subculture.

Procedure

Design of the gRNA and donor DNA cassettes ● Timing 1 d

1 *Determine the to-be-deleted sequences during chromosome fusion(s).* Each chromosome fusion event removes one centromere, two telomeres and telomere-associated RSs. For sequences of the centromere and telomeres, please refer to the *S. cerevisiae* S288C genome annotation (taxonomy ID: 559292) in the National Center for Biotechnology Information (NCBI) Taxonomy Browser database. Telomere-associated RSs are based on information available online (https://www2.le.ac.uk/colleges/medbiopsych/research/gact/resources/yeast-telomeres/general-structure-of-yeast-chromosome-ends), and the exact sequences with >90% similarity are determined via a BLAST search against the S288C genome sequence in the NCBI database. For example, when fusing chr. VII and chr. VIII, we BLAST-searched 35-kb sequences at the end of the chromosomes against the whole genome, and found that sequences 1,076,129–1,084,213 on chr. VII have 95% identity to 804,880–812,968 on chr. II. These two sequences are named RS3. All repeated sequences shown in Fig. 2 were determined in this way.

2 *Design of the targeting components.* Each pairwise chromosome fusion requires three gRNA expression cassettes targeting near one centromere and two telomeres (Fig. 7). Follow these four main considerations in the selection of the 20-nt cleavage site: (i) the 20-nt sequence is manually selected from the approximate deletion region, directly upstream of any 5′-NGG sequence (protospacer adjacent motif (PAM)); (ii) the selected 20-nt sequence should be subjected to a BLAST search against the S288C genome to ensure that the last ten constituent nucleotides are not identical to other regions followed by any 5′-NGG; (iii) the selected 20-nt sequence should contain almost equal AT and GC levels; and (iv) avoid having more than five A/T clusters near the 5′-NGG.

3 *Design the donor DNA cassettes.* Fusion of each pair of chromosomes requires two donor DNA cassettes to ligate the three chromosome segments that result after deletion of two telomeres and one centromere.

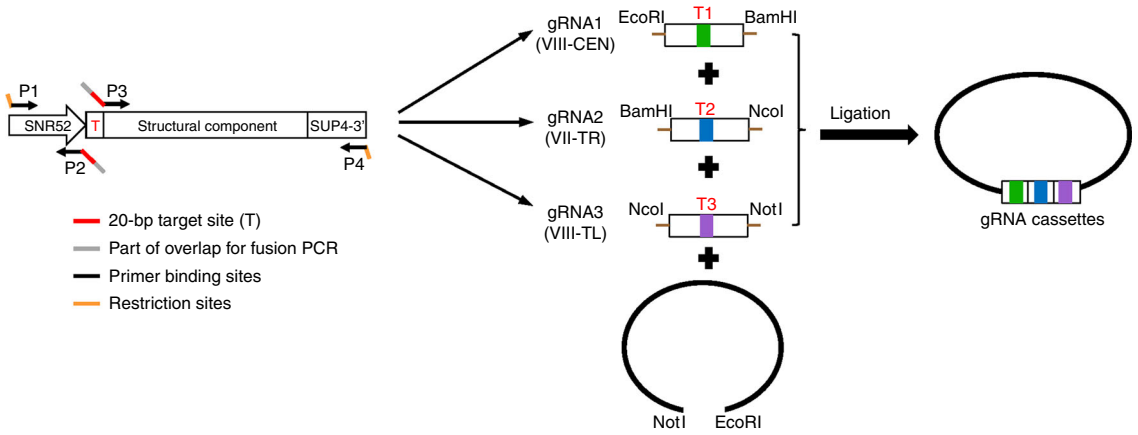

Fig. 7 | Schematic representation of the design and construction of the gRNA expression plasmid. Two segments, namely, the SNR52 promoter and structural component/SUP3′ flanking sequences, are PCR-amplified with primers P1/P2 and P3/P4, respectively. The 20-bp gRNA target sites (red line) are introduced by primers P2 and P3. Each gRNA expression cassette is generated by fusion PCR of the above two segments. The three gRNA expression cassettes containing 20-bp sequences targeting the centromere of chromosome VIII (i.e., VIII-CEN), the right end of chromosome VII (VII-R), and the left end of chromosome VIII (VIII-L) are digested with restriction enzymes and ligated to the EcoRI/NotI-digested vector to generate the gRNA expression plasmid.

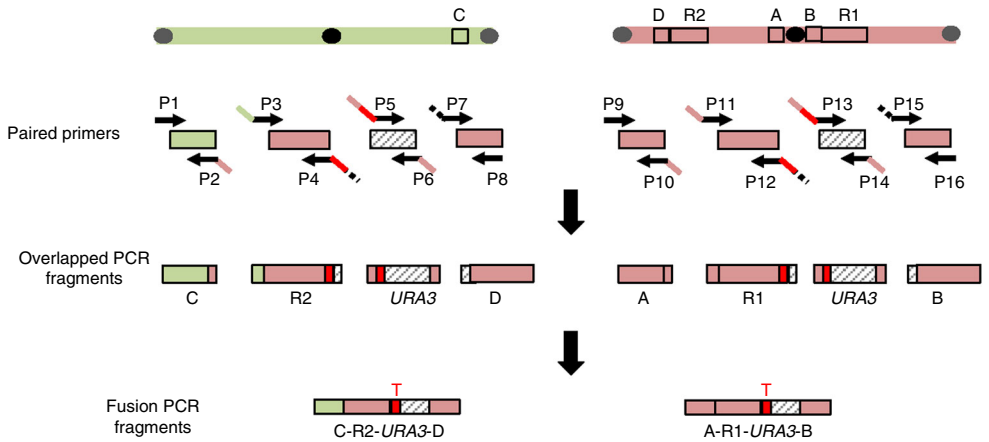

Fig. 8 | Design and construction of donor DNA cassettes for chromosome fusion. Schematic of donor DNA cassette construction. Donor DNA cassettes are generated by fusion PCR. C and D are sequences adjacent to the to-be-deleted telomeres. R2 is next to D. A and B are sequences on both sides of the to-be-deleted centromere, and R1 is next to B. PCR-amplify each segment using BY4742 strain DNA as the template. *URA3* is generated by PCR using pXX11 as a template. A new gRNA target site (T; red block) between the direct repeat and *URA3* is introduced into the cassette by primers. After annealing at the overlap sequences for each fragment, construct the fusion donor cassettes with the outside primers (P1/P7 and P9/P16).

Each donor DNA cassette contains four segments, including two homology arms, the direct repeat and the selection marker gene *URA3*. Generally, 300–400-bp sequences flanking the genomic targeting sites are chosen as homologous arms. The 200–300-bp sequences proximal to one of the homologous arms are PCR-amplified and then used as direct repeats for marker removal (Fig. 8).

▲ **CRITICAL STEP** For centromere deletion and homology-directed repair, we sometimes use 50-bp homology arms to avoid interfering with adjacent gene expression. The homology arm between *URA3* and the direct repeats will be deleted along with *URA3* during the homologous recombination of two direct repeats. Therefore, a shorter homologous arm (50 bp) will be safer and will minimize the potential interference of the adjacent genes' expression.

Preparation of gRNA expression plasmid ● Timing 3 d

4 Generate the gRNA expression construct by ligating the PCR-generated gRNA cassettes into the vector. We use gRNA1 as an example to illustrate the steps involved in gRNA preparation.

You should carry out the same set of steps (Steps 4–12) to prepare gRNA2 and gRNA3. Set up two parallel PCR reactions to amplify fragment 1 (the SNR52 promoter) of gRNA1 with the primers gRNA1-P1/2 and fragment 2 (the gRNA structural component/SUP 3′ flanking sequences) with primers gRNA1-P3/4; the expected sizes of PCR products are 308 and 125 bp, respectively (Fig. 7, Table 2). Set up the PCR reactions as follows:

Component	Amount
Phanta Max buffer, 2×	25 μl
dNTP mix, 10 mM	1 μl
gRNA1-P1 (25 μM; Table 2)	0.8 μl
gRNA1-P2 (25 μM; Table 2)	0.8 μl
Template (p426-SNR52p-gRNA.CAN1.Y-SUP4t)	10 ng
Phanta Max super-fidelity DNA polymerase	1 μl
ddH_2O	Up to 50 μl

▲ **CRITICAL STEP** To minimize error in PCR amplification of the gRNA expression cassettes, use DNA polymerases with high fidelity.

5 Perform PCR amplification by using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–34	95 °C, 20 s	50 °C, 30 s	72 °C, 10 s
35			72 °C 5 min

6 Separate the PCR products on a 1% (wt/vol) agarose gel in 0.5× TAE buffer at 110 V for 40 min. Stain the gel with 1 μg ml^{-1} ethidium bromide for 20 min to check for a single DNA band of the expected size from each of the two PCR reactions.

7 Purify the PCR products with the Promega gel and PCR clean-up system.

■ **PAUSE POINT** The purified PCR products can be stored at –20 °C for 6 months.

8 *Fusion PCR to generate each of the gRNA expression cassettes*. Set up the following first PCR for annealing of the two fragments with overlapping sequences to generate the gRNA expression cassette.

Component	Amount	Final concentration
Phanta Max buffer, 2×	12.5 μl	1×
dNTP mix, 10 mM	0.5 μl	0.2 mM
Fragment 1 (308 bp; Step 7)	30.8 ng	100 ng kb^{-1}
Fragment 2 (125 bp; Step 7)	12.5 ng	100 ng kb^{-1}
Phanta Max super-fidelity DNA polymerase	0.5 μl	0.02 U
ddH_2O	Up to 25 μl	

9 Perform the first PCR amplification by using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–7	95 °C, 20 s	Gradient from 56 °C to 53 °C, 30 s	72 °C, 15 s
8–17	95 °C, 20 s	53 °C, 30 s	72 °C, 15 s
18			72 °C, 5 min

■ **PAUSE POINT** The first PCR product can be stored at 4 °C for several days.

10 Set up the second PCR for amplification of the gRNA expression cassette.

Component	Amount (μl)	Final concentration
Phanta Max buffer, 2×	25	1×
dNTP mix, 10 mM	1	0.2 mM
gRNA1-P1 (25 μM; Table 2)	0.8	20 μM
gRNA1-P4 (25 μM; Table 2)	0.8	20 μM
First PCR product (Step 9)	2.5	
Phanta Max super-fidelity DNA polymerase	1	0.02 U
ddH_2O	Up to 50	

11 Perform the second PCR amplification by using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–34	95 °C, 20 s	50 °C, 30 s	72 °C, 10 s
35			72 °C, 5 min

PAUSE POINT The PCR product can be stored at –20 °C for 6 months.

12 Separate the PCR products on a 1% (wt/vol) agarose gel in TAE buffer at 110 V for 40 min and stain the gel with ethidium bromide. Purify the single 397-bp DNA band with the Promega gel and PCR clean-up system. This DNA band corresponds to gRNA1. The same set of steps (Steps 4–12) should be carried out to generate gRNA2 and gRNA3 with the appropriate primers listed in Table 2.

13 Digest the pHIS426 vector with restriction enzymes at 37 °C for 30 min.

Component	Amount
pHIS426	1 μg
EcoRI	2 μl
NotI	2 μl
10× FastDigest Green buffer	4 μl
ddH_2O	Up to 40 μl

14 Separate the digested product by gel electrophoresis, and purify the expected DNA band with a size of 5.9 kb with the Promega gel and PCR clean-up system.

PAUSE POINT The purified digested vector can be stored at –20 °C for 6 months.

15 For each of the three gRNA expression cassettes, set up a restriction endonuclease digestion reaction as described below. Digest separately the gRNA expression segment gRNA1 with EcoRI and BamHI, gRNA2 with BamHI and NcoI, and gRNA3 with NcoI and NotI. Incubate the digestion mixture at 37 °C for 30 min. Purify the DNA from the digestion mixture with the Promega gel and PCR clean-up system. We show digestion of gRNA1 expression cassette from Step 12 as an example. Digest gRNA2 and gRNA3 with the corresponding enzymes in the same manner.

Component	Amount (μl)
gRNA1	32
EcoRI	2
BamHI	2
10× FastDigest buffer	4
ddH_2O	Up to 40

16 *Cloning of the gRNA expression cassettes into pHIS426*. Ligate the three digested gRNA expression cassettes into the EcoRI/NotI-digested pHIS426 from Step 14 with the following reaction, and incubate the mixture at 25 °C for 30 min.

Component	Amount
pHIS426 (EcoRI//NotI)	60 ng
gRNA1 (EcoRI/BamHI)	10 ng
gRNA2 (BamHI/NcoI)	10 ng
gRNA3 (NcoI/NotI)	10 ng
T4 ligase	1.5 μl
10× T4 ligase buffer	1.5 μl
ddH_2O	Up to 15 μl

17 *Transformation*. Introduce the entire 15 μl of ligation reaction into 100 μl of *E. coli* DH10B competent cells prepared by KCM chemical transformation method[20] as previously described. Spread the cells on LB medium supplemented with 100 μg ml^{-1} ampicillin, and incubate overnight at 37 °C.

18 Verify the colonies from the transformation in Step 17 by a modified procedure of plasmid isolation. Inoculate five colonies to a new plate containing ampicillin with sterile nonfilter pipette tips by drawing 1-cm^2 squares and incubate at 37 °C for overnight. Using a pipette tip, transfer a little bit of bacteria culture from each square into 20 μl of plasmid extract solution I and vortex. Add 10 μl of plasmid extraction solution II, invert and incubate at 50 °C for 10 min to obtain lysates. Add 10 μl of unbuffered phenol–chloroform and vortex thoroughly. Centrifuge at 12,000*g* for 5 min at 4 °C and run 10 μl of the supernatant at 110 V for 40 min to check for the 7.1-kb plasmid DNA band.

? TROUBLESHOOTING

19 *Plasmid isolation*. Inoculate two or three colonies from Step 18 confirmed to contain the correct size plasmid to 5 ml of LB medium containing 100 μg ml^{-1} ampicillin and cultivate at 37 °C, 240 r.p.m. for 16 h. Isolate the plasmid DNA using the Plasmid Mini Kit I according to the manufacturer's instructions.

20 Confirm the sequences of the three inserted gRNA expression cassettes by sequencing with the primers p426-F and p426-R listed in Table 3.

Preparation of donor DNA cassettes by fusion PCR amplification ● Timing 2 d

21 As shown in Fig. 8, set up a parallel set of PCR reactions with the primers listed in Table 4. Use P1/P2 primers to amplify the homologous arm C, P3/P4 for the direct repeat region R2, P5/P6 for *URA3* (for the telomere repair cassette) and P7/P8 for homologous arm D. Construct the telomere deletion/ligation cassette by fusion PCR of fragments C, R2, *URA3* and D with 40-bp overlaps (Steps 21–26). Use P9/P10 primers for amplification of homologous arm A, P11/P12 to amplify direct repeat region R1, P13/P14 for *URA3* (centromere deletion/ligation cassette) and P15/P16 for homologous arm B. Construct the centromere deletion/ligation cassette by fusion PCR of fragments A, R1, *URA3*, B (Step 27). The *URA3* gene is PCR-amplified using pXX11 as a template, and the homologous arms and the direct repeat region are PCR-amplified using BY4742 genomic DNA as a template as described in Steps 4–7.

22 Set up the following first PCR for annealing of the four fragments with overlapping sequences. We use the telomere deletion/ligation cassette as an example.

Component	Amount
Phanta Max buffer, 2×	12.5 μl
dNTP mix, 10 mM	0.5 μl
C (0.4 kb)	40 ng
R2 (0.3 kb)	30 ng
D (0.4 kb)	40 ng
URA3 (1.1 kb)	110 ng
Phanta Max super-fidelity DNA polymerase	0.5 μl
ddH_2O	Up to 25 μl

23 Perform the first PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–7	95 °C, 20 s	Gradient from 56 °C to 53 °C, 30 s	72 °C, 1.5 min
8–17	95 °C, 20 s	53 °C, 30 s	72 °C, 1.5 min
18			72 °C, 7 min

PAUSE POINT The first PCR mix can be stored at 4 °C for several days.

24 Set up the following second PCR to amplify the donor DNA cassette:

Component	Amount (µl)
Phanta Max buffer, 2×	25
dNTP mix, 10 mM	1
P1, 25 µM	0.8
P8, 25 µM	0.8
First product mix (Step 23)	2.5
Phanta Max super-fidelity DNA polymerase	1
ddH_2O	Up to 50

25 Perform the second PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–34	95 °C, 20 s	50 °C, 30 s	72 °C, 1.5 min
35			72 °C, 7 min

PAUSE POINT The first PCR mix can be stored at 4 °C for several days.

26 Separate the second PCR product on a 1% (wt/vol) agarose gel at 110 V for 40 min, and purify the single DNA band of the expected size (in the given example, 2.1 kb) with the Promega Gel and PCR Purification Kit.

CRITICAL STEP We recommend using donor DNA at a final concentration of ~100 ng μl^{-1}.

27 Prepare the centromere deletion/ligation cassette with A (0.4 kb), R1 (0.2 kb), B (0.4 kb) and *URA3* (1.1 kb) referring to Steps 22–26.

28 (Optional) If there are repeats (RS1, 6, 11, 14/10 and 9, marked by red arrowheads in Fig. 2) on the chromosome that need to be deleted in advance, delete these repeats by CRISPR–Cas9-mediated targeting before chromosome fusion. Each deletion needs expression of one gRNA targeting the deletion site and one donor DNA cassette. For example, we delete RS1 and RS6 before chr. VII and chr. VIII fusion by one round of CRISPR–Cas9-mediated targeting with two gRNAs and two donor DNA repair cassettes, generated by primers listed in Tables 2 and 4. Prepare the gRNA expression plasmid as described in Steps 4–20 and prepare the donor DNA cassette as described in Steps 21–26. The corresponding PCR verification primers are listed in Table 5.

? TROUBLESHOOTING

CRISPR-Cas9-facilitated chromosome fusion ● Timing 5 d

29 *Preparation of yeast cells for transformation*. Inoculate a single colony of yeast BY4742 (pCas9) (see 'Reagent setup' section) from a SC-Leu plate into 5 ml of SC-Leu medium and grow at 30 °C with shaking at 240 r.p.m. overnight.

30 Transfer the overnight culture to 25 ml of SC-Leu medium in a 250-ml flask to a starting OD_{600} of 0.2–0.3 and grow at 30 °C, 240 r.p.m. for 5–6 h to a final OD_{600} of 0.8–1.0.

31 Pellet yeast cells in a 50-ml conical tube by centrifugation at 5,000*g*, 4 °C for 5 min.
32 Discard the supernatant, and resuspend the pellet with 25 ml of ddH_2O.
33 Centrifuge at 5,000*g* for 5 min at 4 °C, and wash the pellet with 25 ml of ddH_2O again.
34 Resuspend the yeast cells in 300 µl of ddH_2O, and divide the cell suspensions into three 1.5-ml Eppendorf tubes at room temperature, to be used for three separate transformations: with donor DNA and gRNA plasmid DNA mixture (Step 35), respectively, positive control pXX11 for testing the transformation efficiency, and an equal amount of ddH_2O as negative control.
35 *Introduction of the gRNA expression plasmid (pgRNA) and the donor DNA cassettes into the prepared yeast cells*. Prepare the DNA mixture as follows: combine 1 µg of pgRNA (Step 20), 1 µg of each of the two donor DNA cassettes (from Steps 26 and 27) and ddH_2O in a final volume of 34 µl. In addition, prepare the same transformation mix, but replace the DNA mixture with 100 ng of pXX11 as a positive control for testing transformation efficiency or 34 µl of ddH_2O as a negative control.
36 Prepare the transformation mixture as follows:

Component	Amount (µl)
PEG3350, 50% (wt/vol)	240
LiAc, 1.0 M	36
Salmon sperm DNA, 2.0 mg ml^{-1}	50
DNA mixture (Step 35)	34
Total	360

37 Harvest yeast cells at 12,000*g* for 30 s at room temperature, and discard the supernatant with a micropipettor.
38 Resuspend yeast cells from Step 34 with each of the three tubes containing 360 µl of transformation mixtures thoroughly by vortexing.
39 Incubate the cell suspension at 42 °C for 30 min.
40 Centrifuge at 12,000*g* for 30 s at room temperature. Discard the supernatant with a micropipettor.
41 Wash the pellet with 1 ml of ddH_2O, and resuspend the pellet with 200 µl of ddH_2O.
42 Spread 100 µl of the cell suspension on a SC-Ura-Leu-His plate, and incubate at 30 °C for 2–3 d. We can get several to a few hundred transformants in this step.
PAUSE POINT The plates can be stored at 4 °C for up to 1 month.
? TROUBLESHOOTING
43 *Preparation of the template*. Transfer a small amount of yeast from four transformant colonies (Step 42) with a sterile nonfilter pipette tip to 15 µl of ddH_2O in individual PCR tubes and vortex; use untransformed yeast cells as a negative control. Use 1 µl of the cell suspension for PCR amplifications.
44 Set up the following PCR mixture, which is sufficient for five PCR reactions (to test four transformant colonies and one untransformed negative control from Step 43). Note that we use multiple primers to check the telomere deletion. Primers P1 and P4 are designed to anneal outside the homologous arms C and D, to verify successful chromosome end fusion. Primers P2 and P3 anneal inside the telomere deletion region. Note that primers are paired P1 and P2 (0.8 kb, negative control), P3 and P4 (1.3 kb, negative) or P1 and P4 (2.7 kb, positive).

Component	Amount (µl) (for five reactions)
KOD FX buffer, 2×	50
dNTP mix, 2 mM	2
P1, (25 µM; Table 5)	0.6
P2, (25 µM; Table 5)	0.6
P3, (25 µM; Table 5)	0.6
P4, (25 µM; Table 5)	0.6
KOD FX polymerase	2
ddH_2O	Up to 100

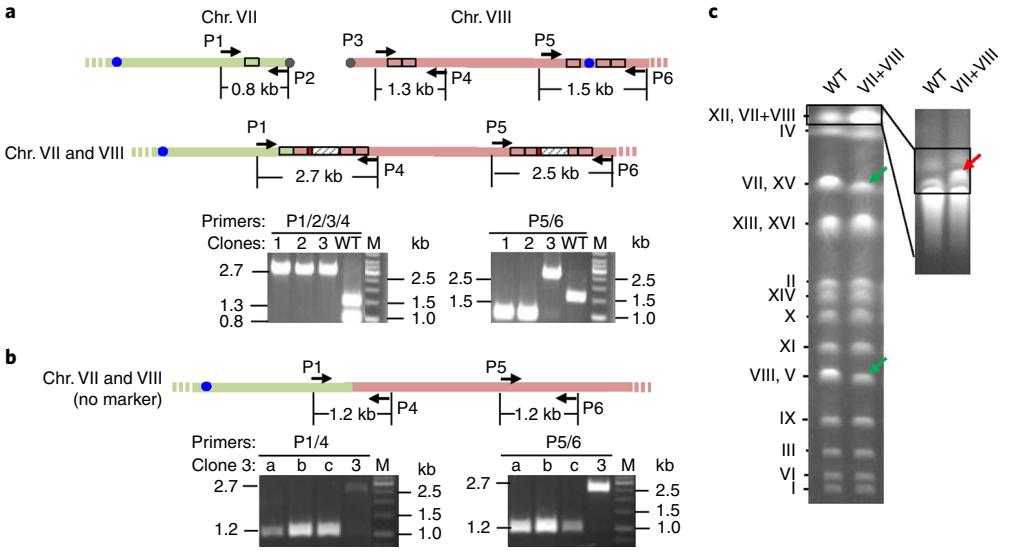

Fig. 9 | Confirmation of chromosome fusion. a, Diagram of PCR primers for verification of the fusions of chr. VII and chr. VIII and agarose gels showing PCR analysis of the fusion of chr. VII and chr. VIII. **b**, Diagram of PCR markers for checking marker removal, and agarose gel electrophoresis showing successful marker removal as assessed by PCR. **c**, Chromosomal DNA analysis by PFGE. The red arrow indicates the newly fused chromosome VII–VIII. The green arrow indicates the disappearance of chromosomes VII and VIII. The sample was electrophoresed under two conditions. The left condition was 1.0% (wt/vol) pulsed field certified agarose in 0.5× TBE, pH 8.0, at 14 °C at 6 V cm^{-1} with an included angle of 120°, a 60-s switch time for 22 h and 90-s switch time for 12 h. The right condition was 0.8% (wt/vol) pulsed field certified megabase agarose in 1× TAE, pH 8.0, at 7 °C at 3 V cm^{-1} with an included angle of 106° and a 500-s switch time for 50 h.

▲ **CRITICAL STEP** *Verification of the chromosome fusion by colony PCR.* We use fusion of chr. VII and VIII as an example (Fig. 9). For PCR confirmation of chromosome fusion, we set up two parallel PCR reactions with primers P1, P2, P3 and P4 for validation of telomere deletion and with P5 and P6 for centromere deletion. As shown in Fig. 9, the expected fusion of VII-R and VIII-L should generate a PCR product with a size of 2.7 kb, whereas the natural nonfused VII-R and VIII-L should generate two PCR products with sizes of 0.8 and 1.3 kb, respectively. Successful deletion of the VIII centromere should result in a PCR product of 2.5 kb, whereas the natural nondeleted VIII centromere will result in a PCR product of 1.5 kb.

45 Divide the mixture into five PCR tubes (20 µl per tube), and add 1 µl of yeast cell suspension (Step 43) as a template.

▲ **CRITICAL STEP** We recommend Toyobo KOD FX polymerase because this enzyme exhibits high activity when yeast cells are used directly as templates in colony PCR reactions.

46 In parallel, set up the following PCR mixture to verify successful deletion of VIII centromere with template from Step 43. The P5 and P6 primers are designed to anneal outside of homologous arms A and B, to verify successful centromere deletion. Primers P5 and P6 generate a 2.5-kb (successful deletion) or 1.2-kb (no deletion) DNA band.

Component	Amount (µl) (for five reactions)
KOD FX buffer, 2×	50
dNTP mix, 2 mM	2
P5, 25 µM	0.6
P6, 25 µM	0.6
KOD FX polymerase	2
ddH_2O	Up to 100

47 Divide the mixture into five PCR tubes (20 µl per tube), and add 1 µl of the yeast cell suspension as a template.

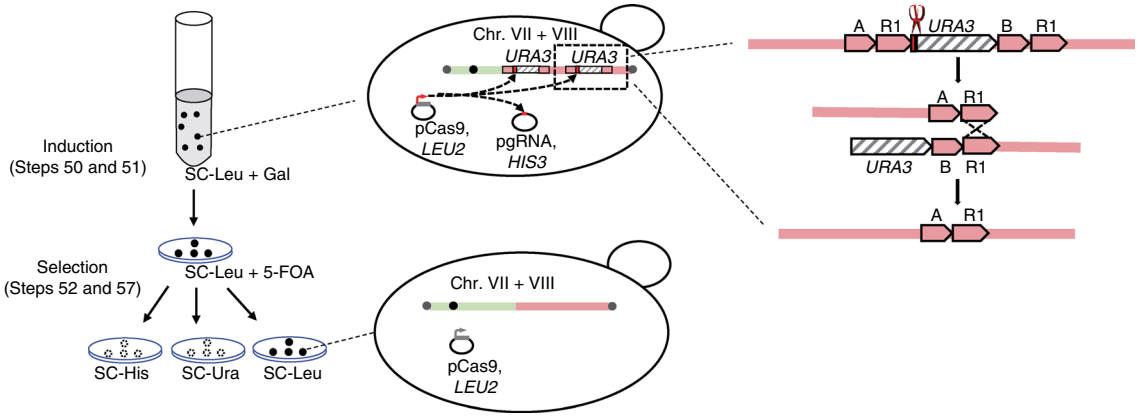

Fig. 10 | Schematic diagram of the removal of the selection marker and gRNA expression plasmid. Transfer of the chromosome fusion yeast cells into galactose-containing medium induces the expression of the gRNA on pCas9 to cut at the target site near the *URA3* gene and on the backbone of pgRNA (with the *HIS3* selection marker). Homologous recombination between direct repeats results in deletion of *URA3*. Yeast cells lacking *URA3* are selected on plates containing 5-FOA. Colonies grown on SC-Leu + 5-FOA plates are further spotted on SC-His, SC-Ura and SC-Leu plates. Deletion of the *URA3* marker gene and pgRNA (with the *HIS3* selection marker gene) results in no cell growth on SC-Ura and SC-His plates. SC-Leu is used to maintain pCas9. The colonies that grow on only SC-Leu plates are considered positive clones.

48 Perform colony PCR reactions set up in Steps 44–47 by using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	94 °C, 4 min		
2–34	98 °C, 10 s	50 °C, 30 s	68 °C, 3 min
35			68 °C, 7 min

49 Separate the PCR products by gel electrophoresis, purify with the Promega Gel and PCR Purification Kit and sequence the expected DNA bands with PCR verification primers (Table 5), P1/P4 for fusion of VII-R and VIII-L, and P5/P6 for VIII-centromere deletion. Store confirmed strains that have successful chromosome fusion and centromere deletion at –70 °C.

Marker removal ● Timing 5 d

50 Inoculate a single colony with successful chromosome fusion and centromere deletion in 5 ml of SC-Leu-Ura-His liquid medium and cultivate at 30 °C and 240 r.p.m. overnight.

51 To induce the expression of gRNA targeting the *URA3* marker and pgRNA (Fig. 10), transfer the overnight yeast culture into 3 ml of SC-Leu medium containing 2% (wt/vol) galactose and 3% (wt/vol) raffinose instead of glucose to a starting OD_{600} of 0.3, and cultivate at 30 °C, 240 r.p.m. for 16 h.

52 Spread 100 µl of liquid culture on SC-Leu medium supplemented with 1 mg ml^{-1} 5-FOA (SC-Leu + 5-FOA), and cultivate at 30 °C for 2–3 d. Usually, 10^2–10^3 colonies grow on the SC-Leu + 5-FOA plate.

? TROUBLESHOOTING

53 For preliminary analysis of marker deletion, select several transformant colonies grown on the SC-Leu + 5-FOA plate and inoculate on three different plates (SC-Ura, SC-His and SC-Leu) with sterile nonfilter pipette tips. Successful removal of *URA3* marker gene and pgRNA (with *HIS3* selection marker gene) results in no cell growth on SC-Ura and SC-His plates. SC-Leu is used to maintain pCas9 to start the next round of chromosome fusion.

54 Transfer a small amount of colony from the SC-Leu plate with a sterile nonfilter pipette tip into 15 µl of ddH_2O in an individual PCR tube and vortex. Take 1 µl of the yeast cell suspension as a template and use with the PCR mixture detailed below. Make the same PCR mixture in parallel with the primer pairs P1/P4 and P5/P6 to check for the removal of the two *URA3* marker genes. Do this for three transformant colonies, and use cells obtained before *URA3* marker removal (from Step 50) as a negative control.

Component	Amount for four PCRs (μl)
KOD FX buffer, 2×	50
dNTP mix, 2 mM	2
P1 (Table 4, 25 μM)	0.6
P4 (Table 4, 25 μM)	0.6
KOD FX polymerase	2
ddH_2O	Up to 100

55 Divide 25 μl of the mixture into each PCR tube, and add 1 μl of yeast cell suspension from Step 54 as a template. Take 1 μl of yeast cell suspension from Step 50 as a negative control.

56 Perform PCR with the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	94 °C, 4 min		
2–34	98 °C, 10 s	50 °C, 30 s	68 °C, 1.5 min
35			68 °C, 7 min

57 Separate the PCR products by gel electrophoresis and purify the DNA bands with the expected size (1.2 kb), and confirm by DNA sequencing (Fig. 9b).

Functional verification ● Timing 4 d

58 *Karyotype analysis of the chromosome fusion strain by PFGE.* Embed the yeast cells with the chromosome fusion in agarose plugs as follows. Inoculate a single successful-fusion colony into 5 ml of YPAD medium, and cultivate at 30 °C and 240 r.p.m. overnight.

59 Transfer the overnight cell culture into 25 ml of YPAD medium in a 250-ml flask to a starting OD_{600} of 0.2–0.3, and cultivate at 30 °C and 240 r.p.m. to a final OD_{600} of 1.0.

60 Harvest yeast cells as described in Step 37 and wash the cells in 25 ml of ddH_2O followed by 50 mM EDTA, pH 8.0, and centrifuge at 5,000*g* at 4 °C for 5 min.

61 Resuspend the yeast cells in 750 μl of 10 mM Tris-HCl, pH 7.5, and transfer the cells to a 1.5-ml Eppendorf tube.

62 Centrifuge the cells at 5,000*g* at 4 °C for 5 min, and discard the supernatant.

63 Resuspend the cells in 150 μl of Zymolyase-20T solution. Immediately combine the cell suspension with an equal volume of 2% (wt/vol) low-melting agarose solution, and mix gently but thoroughly.
▲ **CRITICAL STEP** Equilibrate the agarose solution to 50 °C in a water bath before use.

64 Transfer the mixture to plug molds using a sterile pipette tip, and incubate at 4 °C for 30 min.

65 Push the solidified agarose plugs using the snap-off tool provided with the plug mold into 10-ml centrifuge tubes with 5 ml of lysis buffer and 500 μl of Zymolyase-20T solution. Incubate the tubes at 37 °C in a water bath for 3 h.

66 Remove the lysis buffer with a pipette; wash once in 5 ml of wash buffer by inverting several times, and rinse the plugs in 5 ml of proteinase K solution at 50 °C without agitation for 36 h.

67 Wash the plugs in 5 ml of wash buffer three to four times at room temperature with gentle agitation.
■ **PAUSE POINT** Plugs can be stored at 4 °C for 2 weeks.

68 Assemble the PFGE apparatus according to the manufacturer's instructions.

69 Cut one-third of a plug using a razor blade and place it on the end of each tooth of the comb. Put the comb back into the holder.

70 To separate 0.2–2-Mbp chromosomal DNA, electrophorese the sample from Step 69 in a 1.0% (wt/vol) pulsed-field certified agarose in 0.5× TBE, pH 8.0, at 14 °C at 6 V cm^{-1} with an included angle of 120°, a 60-s switch time for 22 h and 90-s switch time for 12 h. To separate 1–3-Mbp chromosomal DNA, electrophorese the sample in 0.8% (wt/vol) pulsed-field certified megabase agarose in 1× TAE, pH 8.0, at 7 °C at 3 V cm^{-1} with an included angle of 106° and a 500-s switch time for 50 h. To separate 3–12-Mbp DNA fragments, electrophorese the sample in 0.8% (wt/vol) pulsed-field certified megabase agarose in 1× TAE, pH 8.0, at 6 °C at 1.5 V cm^{-1} with an

included angle of 106° and a switch time of 30 min for 27 h, at 2 V cm^{-1} with an included angle of 100° and a switch time of 25 min for 27 h, and at 2.5 V cm^{-1} with an included angle of 96° and a switch time of 20 min for 27 h.

71 Remove and stain the gel with ethidium bromide at 1 μg ml^{-1} for 30 min to check chromosome DNA bands.

72 *Growth analysis of the chromosome fusion strain*. Inoculate three individual colonies of the wild-type strain BY4742 and the chromosome fusion strain from Step 57 into 5 ml of YPAD medium, and cultivate at 30 °C and 240 r.p.m. overnight.

73 Transfer the overnight cell culture into 25 ml of YPAD medium in a 250-ml flask to a starting OD_{600} of 0.1, and cultivate at 30 °C and 240 r.p.m.

74 Measure the OD_{600} hourly until the stationary phase. It takes about 12 h to reach the stationary phase. After reaching the stationary phase, continue to incubate the culture overnight.

75 Measure the OD_{600} of yeast cells after 24 h of cultivation.

76 Store the chromosome fusion yeast strain in 30% (wt/vol) glycerol at –70 °C, or inoculate in SC-Leu medium to start the next round of chromosome fusion.

Troubleshooting

Troubleshooting advice can be found in Table 6.

Table 6 | Troubleshooting table

Step	Problem	Possible reason	Solution
18	Incorrect size of gRNA expression plasmid	Incomplete digestion of the inserts or vector	Increase the amount of restriction enzyme used
28	No DNA bands of the expected size	Poor quality of DNA segments used for fusion PCR	Redesign the primers for PCR amplification to obtain specific and high-quality DNA segments
42	No transformants	Low concentration of DNA for transformation	Obtain a high concentration of DNA through fusion PCR by, for example, optimizing PCR conditions or scaling up the reaction
52	Too many colonies	Inactive 5-FOA	Use freshly prepared 5-FOA for selection

Timing

Steps 1–3, design of the gRNA and donor DNA cassettes: 1 d
Steps 4–20, preparation of gRNA expression plasmid: 3 d
Steps 21–28, preparation of donor DNA cassettes by fusion PCR amplification (can be done in parallel with preparation of the gRNA expression plasmid in Steps 4–20): 2 d
Steps 29–49, CRISPR–Cas9-facilitated chromosome fusion: 5 d
Steps 50–57, marker removal: 5 d
Steps 58–76, functional verification: 4 d

Anticipated results

Each round of chromosome fusion and functional validation requires ~18 d. In general, dozens to hundreds of transformants can be obtained in experiments of chromosome fusions, with a positive rate of 20–100% (defined as the percentage of sequenced colonies containing the correct chromosome fusion) using one selection marker. We compared the positive rates of three pairwise fusions (XVI–V, IX–X, VII–VIII) under the following conditions: (i) using two *URA3* markers for selection of centromere and telomere deletions, (ii) using only one *URA3* marker for selection of telomere deletions and (iii) using only one *URA3* marker for selection of centromere deletions. The positive rates for XVI–V, IX–X and VII–VIII were 75%, 100% and 50%, respectively, under condition (i); 50%, 50% and 75%, respectively, under condition (ii); and 25%, 50% and 50%, respectively, under condition (iii). Therefore, we think that, to a certain extent, using two *URA3* markers increased the positive rate of chromosome fusions compared with that obtained with only one marker.

The positive rates do not seem to decrease with accumulation of chromosome fusion events[7]. Moreover, the positive rate of two-chromosome fusion can be further improved to nearly 100% by the use of two different selection markers[17]. Further improvements even allowed simultaneous fusion of multiple chromosomes with 75% positive rates[17]. The efficiency of marker removal can reach nearly 100%.

For each round of chromosome fusion, the growth rate of positive transformants is evaluated. The expected growth should be as robust as that of wild-type cells. If a growth defect is detected, then it is very possible that deletion of the centromere or telomeres has affected the functions of adjacent genes, leading to slow growth. For example, deletion of the XV centromere caused a modest growth defect in all three trial experiments, and therefore we retained the XV centromere in the final single-chromosome yeast and deleted other centromeres during sequential chromosome fusions.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article

Data availability

The plasmids used in this protocol, including pCas9 (accession number 1.2624), pHIS426 (accession number 1.2623) and pXX11 (accession number 1.2613), can be obtained from the Registry and Database of Bioparts for Synthetic Biology (http://npbiosys.scbit.org/strainOrder) upon reasonable request. All relevant data are reported in the article.

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Author contributions

Z.Q. and X.X. designed and analyzed all the experiments. Y.S. constructed the chromosome fusion yeast strains and performed PCR verification. N.L. conducted the PFGE confirmation experiment and growth assays. X.X. and Y.S. wrote the primary manuscript with a substantial contribution from Z.Q.

Competing interests

The authors declare no competing interests.

Additional information

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Key references using this protocol

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☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

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Data collection	No software was used.
Data analysis	The software OriginPro 2015 was used for generating the growth curves.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	In the study, we started from a haploid cell of budding yeast Saccharomyces cerevisiae BY4742 to create a serial of functional chromosome fusion yeast strains through successive chromosomal end-to-end fusions and centromere deletions.
Data exclusions	The growth assay is used as a primary evaluation of chromosome fusion functionality. If a growth defect occurred , then an alternative design and construction of chromosome fusion should be tested.
Replication	All attempts at replication were successful.
Randomization	The sixteen natural chromosomes of the haploid S. cerevisiae strain BY4742 are divided into eight groups randomly except one consideration, which is that the disappearing natural chromosomes or the appearing fused chromosomes are easy to distinguish by their sizes.
Blinding	For the creating of the single chromosome yeast, the investigators were blinded to the order of chromosome fusions because there were no any reported information for guidance.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☒	☐ Unique biological materials
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Saccharomyces cerevisiae strain BY4742 was used in the study.
Authentication	Saccharomyces cerevisiae strain BY4742 used in the study were bought from Euroscarf.
Mycoplasma contamination	The Saccharomyces cerevisiae strain BY4742 were not tested for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.