

## Original Research Article

# Enhancement and mapping of tolerance to salt stress and 5-fluorocytosine in synthetic yeast strains via SCRaMble

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## ABSTRACT

Varied environmental stress can affect cell growth and activity of the cellular catalyst. Traditional path of adaptive evolution generally takes a long time to achieve a tolerance phenotype, meanwhile, it is a challenge to dissect the underlying genetic mechanism. Here, using SCRaMble, a genome scale tool to generate random structural variations, a total of 222 evolved yeast strains with enhanced environmental tolerances were obtained in haploid or diploid yeasts containing six synthetic chromosomes. Whole genome sequencing of the evolved strains revealed that these strains generated different structural variants. Notably, by phenotypic-genotypic analysis of the SCRaMbled strains, we find that a deletion of gene YFR009W (*GCN20*) can improve salt tolerance of *Saccharomyces cerevisiae*, and a deletion of gene YER056C can improve 5-fluorocytosine tolerance of *Saccharomyces cerevisiae*. This study shows applications of SCRaMble to accelerate phenotypic evolution for varied environmental stress and to explore relationships between structural variations and evolved phenotypes.

## 1. Introduction

*Saccharomyces cerevisiae* is a widely used cell factory that has been engineered to produce fuels, chemicals and other bioproducts [1,2]. The fermentation of *Saccharomyces cerevisiae* can be impacted by a variety of stresses, such as high temperature, osmotic pressure and toxic byproducts [3,4]. Biological evolution is a continuous process of mutation and natural selection in nature [5]. Among varied scale of genomic mutations, the structural variation plays a key factor in promoting species diversity and speciation [6–8]. Traditional genomic evolution approaches have been used in yeast, which always focus on small-scale variants [9,10]. The chemically synthesized *Saccharomyces cerevisiae* genome was encoded an inducible genomic rearrangement system - SCRaMble (synthetic chromosome rearrangement and modification by loxPsym-mediated evolution) in the Synthetic Yeast Genome Project (Sc 2.0 project), which enables large-scale genomic structural variations [11–14]. SCRaMble has been used to evolve yeast harboring one or two synthetic chromosomes to obtain different evolutionary phenotypes [15–18]. Synthetic haploid yeast harboring one or two chromosomes

was mated to generate heterozygous diploids, which can reduce lethality due to deletion of essential genes by SCRaMble [19]. However, the occurrence of the rearrangement was limited to only one or two synthetic chromosomes. Recently, a haploid yeast yZSJ025 harboring six synthetic chromosomes (synII [20], synIII [12], synV [21], synVI [22], synIXR [11], synX [23]) was constructed and SCRaMbled, and more than 260,000 rearrangement events were detected in a single SCRaMble experiment, suggesting that the power of using SCRaMble to generate a library of structural variations [24].

Here, haploid yeasts harboring the six synthetic chromosomes were used to SCRaMble and the evolved strains were screened under five environments (temperature, alkalinity, sodium chloride, metallic ion and drug). A total of 222 evolved haploid and diploid strains were generated by a single round of SCRaMble. Whole-genome sequencing revealed that a large number of structural variants were generated in both SCRaMbled haploid and diploid strains. A common deletion region of YER056C was detected in two of the flucytosine-tolerant SCRaMbled strains, then deletion of YER056C was further confirmed to contribute to the phenotypical adaptation of 5-

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fluorocytosine resistance in wild type BY4742. In addition, deletion of YFR009W was detected in three of salt-tolerant SCRaMbled strains and was verified to enhance growth in sodium chloride environment in BY4742. Our work suggests that evolved strains can be obtained using SCRaMbled under different environmental stresses in a short time and phenotype-related genes can be rapidly targeted through a standardized loxPsym analysis method.

## 2. Materials and methods

### 2.1. Strains, plasmids and media

The plasmid, yeasts and primers used in the study are listed in [Supplementary Table 1](#). The plasmid pYW180 (pRS416-pCLB2-Cre-EBD) was available in the laboratory. The strain yZSJ025 harboring six synthetic chromosomes (*MATa his3Δ1 leu2Δ0 ura3Δ0 IXL synLXR SYNX HO::tR(CCU)J synII, III, V, VI, IXR and X*), wild type strain BY4742 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) and the heat-resistant strain Y12 (*MATa ura3::KanMX ho::Hyg*) were available in our laboratory. SCRaMbled was induced using SC-Ura (synthetic medium lacking uracil, 0.67% Yeast Nitrogen Base, an amino acid mix minus, 2% (w/v) glucose) medium containing 1 μM β-estradiol (Sigma-Aldrich). Yeast strains were grown at 30 °C in YPD (yeast extract peptone dextrose) medium containing 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract. All tolerant strains were finally verified by the serial dilution assay after loss of plasmid. Except for the alkaline medium, all were in synthetic medium for the serial dilution assay. 5-fluorocytosine and cadmium nitrate were added to the medium after sterilization, and other selective medium were added directly together with sterilization. The concentrations were selected on the basis of our pre-experiments.

### 2.2. Yeast transformation

The yeast colonies were incubated in 5 mL YPD overnight at 30 °C with shaking at 220 rpm and transferred to fresh medium the next day. The cultures were incubated at 30 °C and 220 rpm until the OD<sub>600</sub> reached 0.5–0.8. Cells were washed once with sterile H<sub>2</sub>O, resuspended in 0.1 M LiAc solution and placed on ice until needed. The yeast transformation system contained 620 μL of 50% polyethylene glycol (PEG-3350), 40 μL of salmon sperm DNA (100 mg/mL), 90 μL of 1 M LiAc and 150 μL of a mixture of plasmids or fragments and cells. The system was incubated at 30 °C for 30 min, followed by the addition of 90 μL of DMSO and then heat shock at 42 °C for 18 min. The cells were resuspended in 5 mM CaCl<sub>2</sub> for 5 min and incubated on solid SC-Ura mediums for 2–4 days.

### 2.3. SCRaMbled of haploid and diploid strains

Haploid and diploid strains were transformed with pRS416-pCLB2-CreEBD. The yeast colonies were inoculated in 5 mL liquid SC-Ura media at 30 °C overnight with shaking at 220 rpm. Cultures were diluted to a starting OD<sub>600</sub> of 0.5 in 5 mL of fresh liquid SC-Ura media. 1 μM β-estradiol was added to liquid SC-Ura media. Haploid and diploid yeast were induced for 8 h and 6 h at 30 °C with shaking at 220 rpm, respectively. After induction, cultures were washed twice with water to wash out β-estradiol, and cells were plated onto different selective solid mediums. The plates were incubated at 30 °C for 2–4 days.

### 2.4. Yeast mating and verification

Yeasts with different mating types were incubated overnight in selective media. 1 mL of yeasts with different mating types was harvested and washed twice with ddH<sub>2</sub>O. Then 200 μL of each culture was added to 5 mL of YPD and incubated at 30 °C at 220 rpm for 8 h. 1 mL of the mating solution was centrifuged to obtain cells and washed twice with ddH<sub>2</sub>O. Cells were incubated onto different selective solid medium at

30 °C for 2 days. Diploidy was verified by mating type primers (tester a and tester alpha).

### 2.5. Screening and verification of different condition-tolerant strains

An automation equipment of colony picker (Qpix) was used for the initial screening, and big clones were picked into selective solid medium by setting parameters. Big clones were selected for serial dilution assays. After the initial screening, single clones were incubated in YPD for 24 h to lose plasmid to stabilize the phenotype. The plasmid-losing strains were screened with solid medium (Sigma-Aldrich) containing 5-FOA. Serial dilution assays were again performed to screen enhanced strains under different conditions.

### 2.6. Serial dilution assays for selections

Yeasts were inoculated in 5 mL of YPD overnight at 30 °C with shaking at 220 rpm. Then, 200 μL of the culture was transferred to 5 mL of YPD at 30 °C with shaking at 220 rpm and incubated to an OD<sub>600</sub> of 1. The solution was serially diluted in 10-fold increments in ddH<sub>2</sub>O five times, and 2–4 μL of the diluted solution was plated from lowest to highest concentration on the corresponding media. The media were maintained for 2–4 days under the same conditions and then photographed to observe growth.

### 2.7. Growth curve assay

Single colonies were cultured to saturation in 5 mL SC medium at 30 °C. The cultures were inoculated into a 250 mL shake flask containing 50 mL of SC medium with 0.4 M NaCl and 1.5 M NaCl with an initial OD<sub>600</sub> of 0.2, and cultured at 30 °C and 220 rpm. Three parallel assays were set up for each strain. The OD value was measured at appropriate intervals. Growth curves were plotted using GraphPad Prism software.

### 2.8. Whole genome sequencing

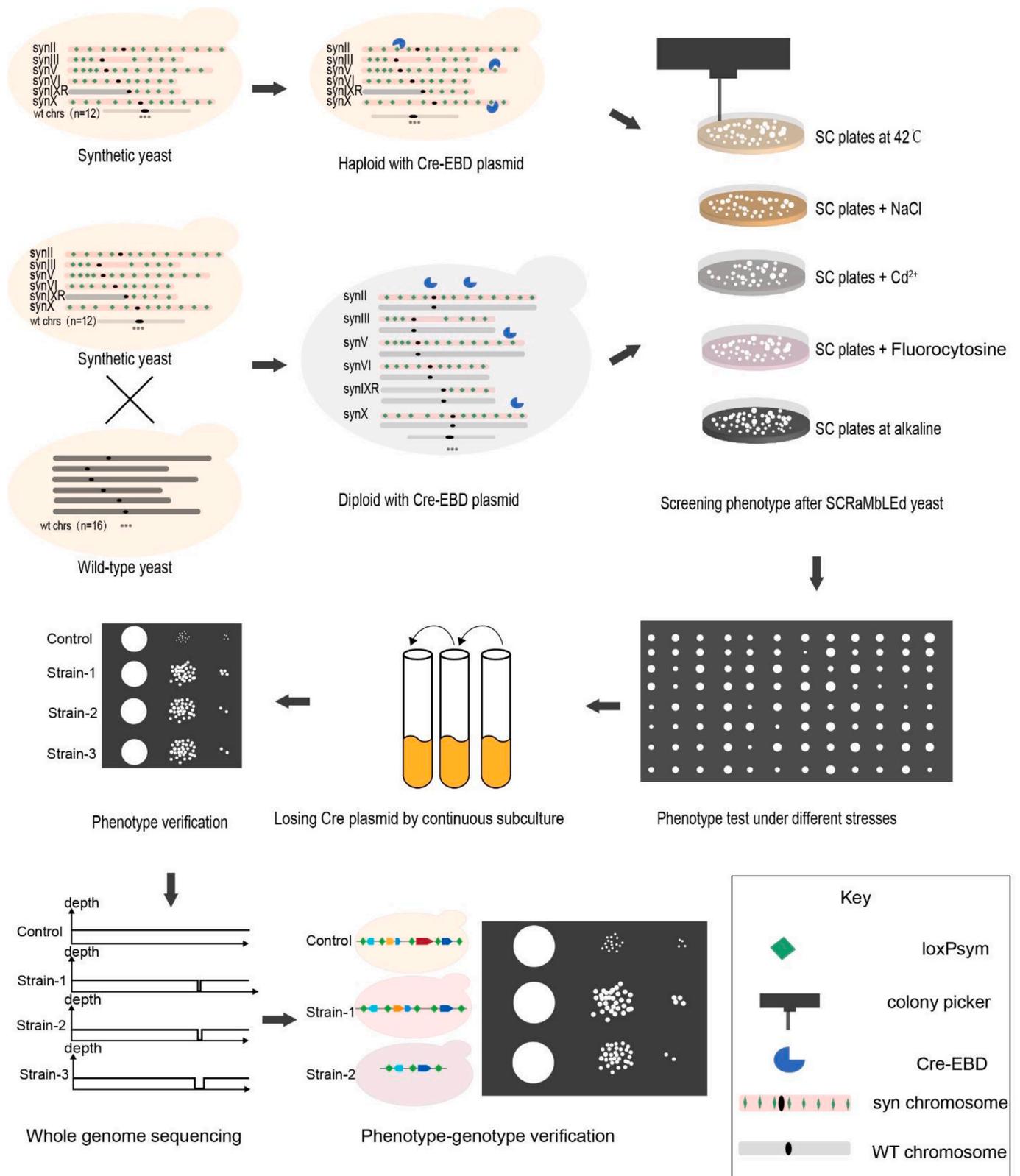
Cells were harvested at exponential phase and sent to BGI (the Wuhan Genomics Institute) for whole genome sequencing. The strain samples were prepared according to BGI's standard preparation protocol. The samples were sent on dry ice to BGI in Wuhan. The sequencing data were analyzed and inductively charted for easy analysis. Structural variations (SVs), including insertion, deletion, inversion, intra-chromosomal translocation and interchromosomal translocation, were analyzed.

### 2.9. Detection of SCRaMbled events

Referring to the analytical approach [24], structural variations in the synthetic chromosomes were identified by the alignment of loxPsym sites and neighboring sequences to the yZSJ025 genome. Sequences containing 877 loxPsym sites in the yZSJ025 genome and their extensions by 116 bp on both sides were extracted as references. The following criteria was used to identify and screen rearrangements for further studies: (1) reads containing all the entire 34 bp loxPsym site sequences with flanking sequences belonging to two loxPsym sites of the reference; (2) reads with one end less than 4 bp apart from loxPsym were excluded; (3) reads containing two or more mismatched bases were excluded. Identical reads were considered to be a result of a single rearrangement event.

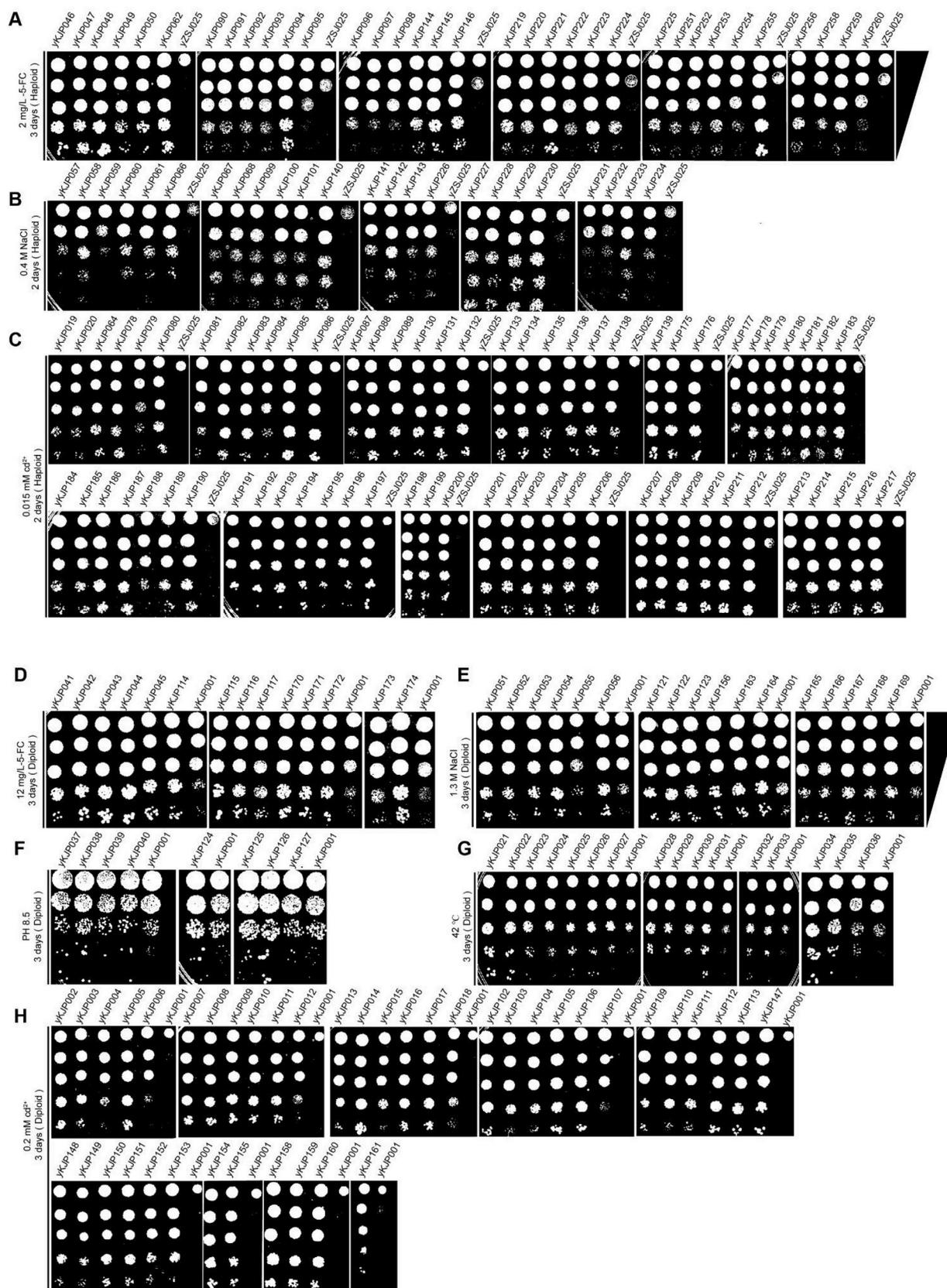
### 2.10. Deletion of target genes

Genes were deleted by homologous recombination. Homologous arms upstream and downstream of target gene were amplified from the genomes of the synthetic strain yZSJ025 and wild type BY4742. The selective marker *URA3* was amplified from pRS416. The three parts

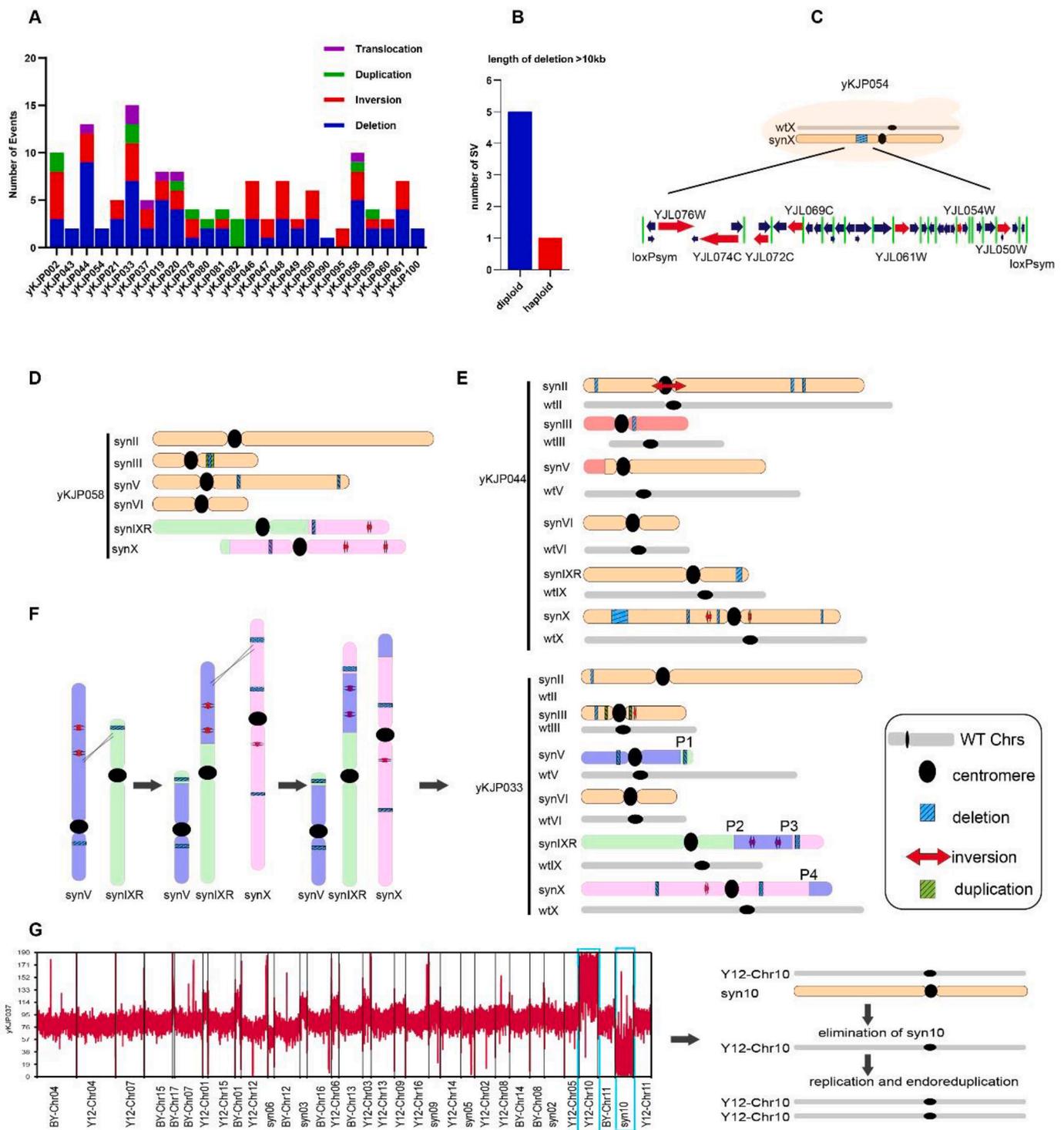


**Fig. 1.** The workflow of SCRaMBLE to improve tolerance in synthetic haploid and diploid yeasts and analyze the association between genotypes and phenotypes.

were then ligated together by overlap PCR, and then gel purified DNA fragments were directly transformed into yZSJ025 and BY4742 on solid SC-Ura medium. Strain BY4742 was inserted with marker *URA3* into the HO site, which had not improved tolerance.



**Fig. 2.** Evolutionary haploid and diploid strains were obtained in five environments using SCRAMBLE. (A) A total of 34 fluorocytosine-resistant haploid strains. (B) A total of 24 salt-tolerant haploid strains. (C) A total of 68 cadmium nitrate-tolerant strains haploid strains. (D) A total of 14 fluorocytosine-resistant diploid strains. (E) A total of 17 salt-tolerant diploid strains. (F) A total of 8 alkaline-tolerant diploid strains. (G) A total of 16 heat-resistant diploid strains. (H) A total of 41 cadmium nitrate -resistant diploid strains.



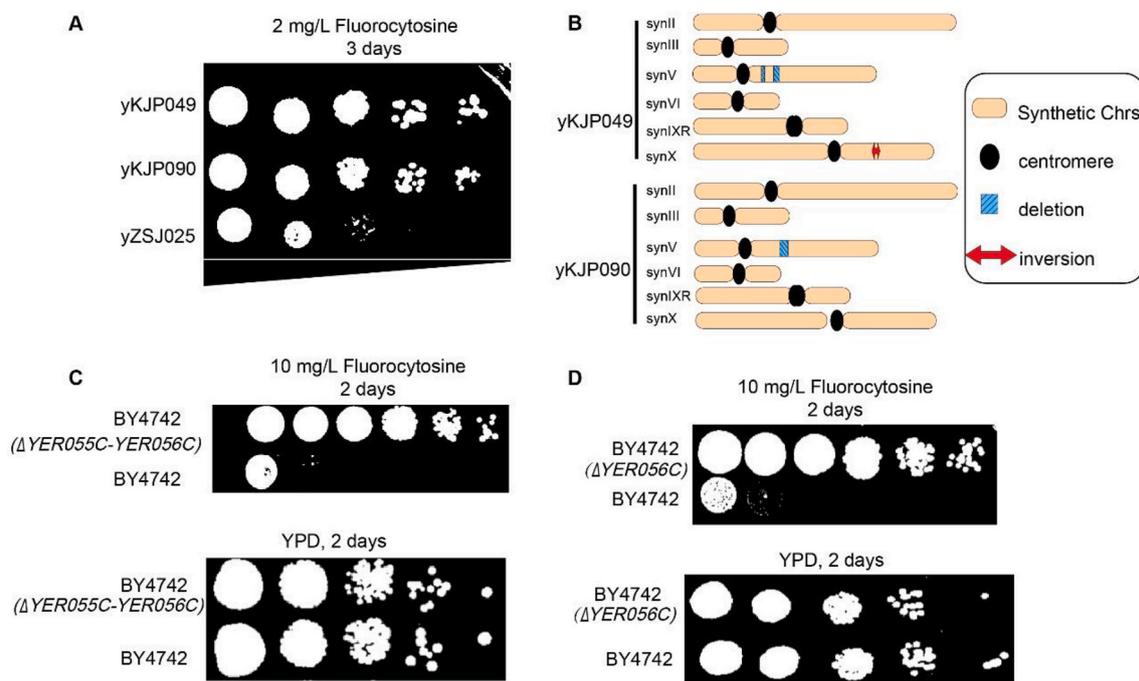
**Fig. 3. Complex structural variations in 25 SCRaMbLED strains.** (A) Statistical analysis of structural variations in 25 SCRaMbLED strains. (B) The length of the deletion exceeds 10 kb in haploid and diploid. (C) Deletion of seven essential genes in diploid strain yKJP054. (D) Information of varied rearrangement events in haploid strain yKJP058. (E) Information of varied rearrangement events in diploid strain yKJP044. (F) Translocation of three synthetic chromosomes in diploid strain yKJP033. (G) Coverage map of diploid strain yKJP037.

### 3. Results

#### 3.1. SCRaMbLE improves various tolerances in haploid and diploid synthetic strains

A synthetic *Saccharomyces cerevisiae* strain yZSJ025 which harbors six synthetic chromosomes was constructed in our lab [24]. The

synthetic *Saccharomyces cerevisiae* comprises 894 loxPsym sites in the 3'UTRs of nonessential genes, and 263,520 rearranged events were detected in a SCRaMbLED pool, showing that the ability of SCRaMbLE to generate a very diverse library of structural variations [24]. To further explore the phenotypically evolutionary ability of SCRaMbLE, the haploid synthetic strain yZSJ025 was SCRaMbLED and screened on various stressful conditions. First, a Cre-EBD plasmid pYW180



**Fig. 4.** Mapping the 5-fluorocytosine resistant gene by analysis of SCRaMbLED strains. (A) SCRaMbLED strains yKJP049 and yKJP090 compared with the control strain yKJP025 on solid medium with 2 mg/L 5-FC. (B) Details of structural variations in synthetic chromosomes of yKJP049 and yKJP090. (C) Growth fitness of YER055C-YER056C knockout strain in BY4742 on solid medium with 10 mg/L 5-FC and nonselective YPD solid medium compared with the control strain BY4742. (D) Growth fitness of YER056C (*FCY2*) knockout strain on solid medium with 10 mg/L 5-FC and nonselective YPD solid medium compared with the control strain BY4742.

(pRS416-pCLB2-Cre-EBD) was transformed into yZSJ025. After 8 h' SCRaMbLE induced by  $\beta$ -estradiol, cells were plated onto different selective solid mediums. An automation equipment of colony picker (Qpix) was used to accelerate the process of screening. After nonselective culture for 24 h, the evolved SCRaMbLED strains were phenotypically verified by serial dilution assays on various selective mediums (Fig. 1). A total of 126 SCRaMbLED haploid strains with enhanced tolerance were obtained under 2 mg/L 5-fluorocytosine (34 of strains), 0.4 M NaCl (24 of strains) and 0.015 mM cadmium nitrate (68 of strains) comparing with the initial strain yZSJ025 (Fig. 2A–C).

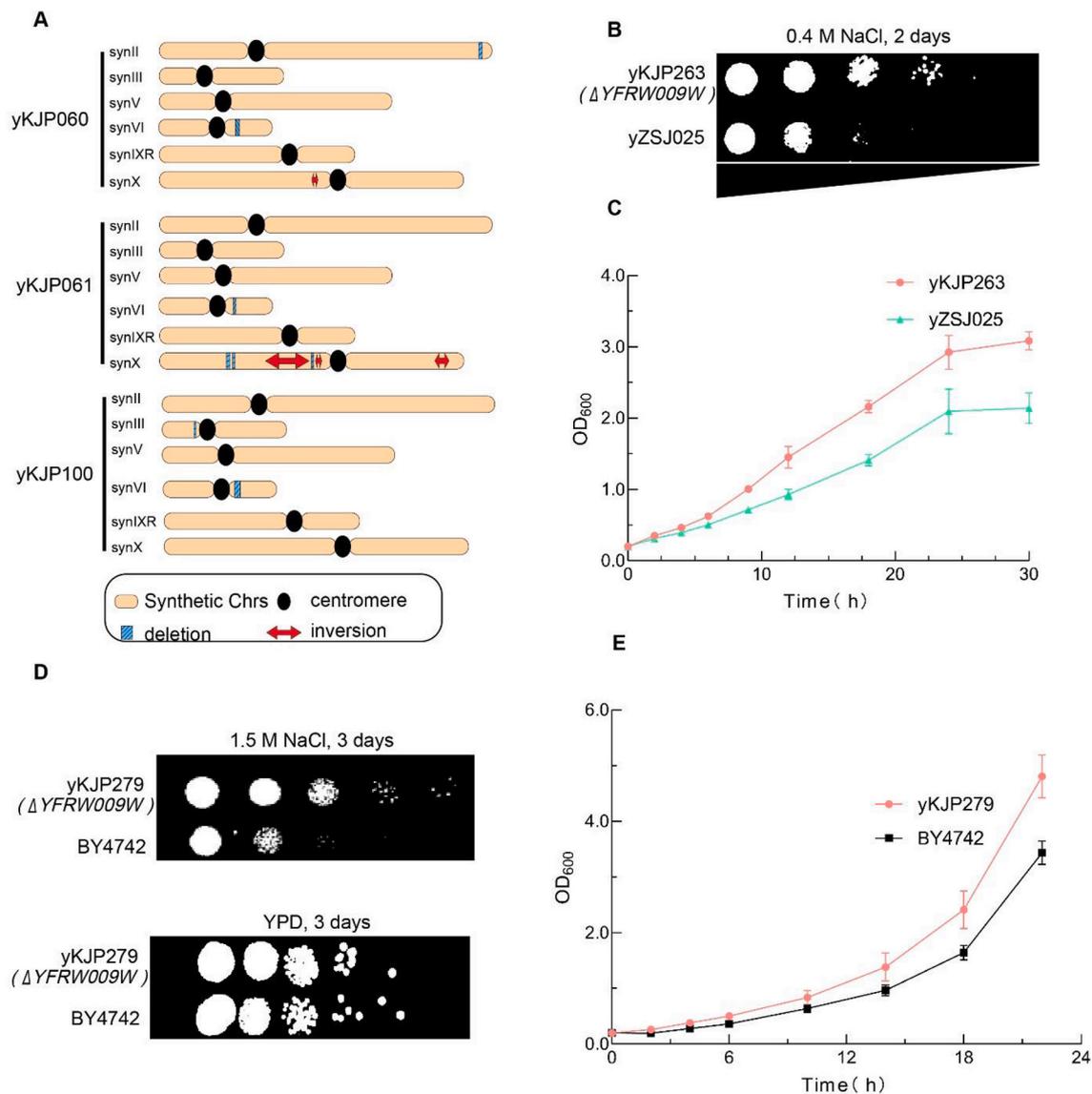
Diploid yeast generally has a growth advantage over haploid under stressful environment and high lethality of SCRaMbLE in haploid has been reported [8,19]. To enhance the robustness and reduce the lethality of SCRaMbLE, industrial strain Y12 was mated to the synthetic strain yZSJ025 to generate a heterozygous synthetic diploid strain yKJP001. Our results show that the diploid strain yKJP001 had better robustness compared with the haploid yZSJ025 under the conditions we tested. Compared with haploid strain, heterozygous diploid strain showed less SCRaMbLE-mediated cell death, while allowing access to the various phenotype of other wild-type strains [19]. For instance, 1.3 M NaCl was used to screen for the diploid while the screening concentration for the haploid was 0.4 M NaCl. A total of 96 SCRaMbLED diploid strains with enhanced tolerance were obtained under 12 mg/L 5-fluorocytosine (14 of strains), 1.3 M NaCl (17 of strains), pH 8.5 (8 of strains), 42 °C (16 of strains) and 0.2 mM cadmium nitrate (41 of strains) comparing with the initial strain yKJP001 (Fig. 2D–H). Our results show that for different conditions of screening, the frequency of SCRaMbLE to generate an enhanced tolerance is varied. We found that the frequency of evolved strains with enhanced fitness under cadmium nitrate was higher than the other conditions. This phenomenon may be caused by varied genotype-phenotype correlations for different phenotypes or varied mutation rates for different genomic locus [25–27]. We also noticed that the degree of enhancement by SCRaMbLE was varied under the same pressure, although the selected evolved strains were all grown better than the control strain. For example, evolved strains yKJP021,

yKJP022 and yKJP034 grew better than others at 42 °C. These varied phenotypes may be due to the different genomic variations caused by SCRaMbLE, which is discussed subsequently.

### 3.2. Complex structural variations of synthetic chromosomes induced by SCRaMbLE

To explore structural variants (SVs) in the evolved strains, a total of 25 SCRaMbLED haploid and diploid strains obtained from different conditions were whole genome sequenced. The novel junctions in the SCRaMbLED genome were detected and the type of rearrangement were determined by a previous method [24]. A total of 137 rearrangement events were detected in the 25 sequenced strains (Fig. 3A). Deletion, inversion, duplication and translocation events were found in both haploid and diploid SCRaMbLED strains [28] (Supplementary Table 2). There were 5 events of deletion with sizes exceeding 10,000 bp in the SCRaMbLED diploid strains, compared with only one in haploids (Fig. 3B). This is consistent with a previous finding that the size of structural variations in SCRaMbLED diploid is much larger than that detected in SCRaMbLED haploid [29]. We also found that the deletions of essential genes occurred in synthetic chromosomes for three SCRaMbLED diploid strains in which the longest length of deletion was over 55,000 base pairs encompassing the seven essential genes (YJL076W (*NET1*), YJL074C (*SMC3*), YJL072C (*PSF2*), YJL069C (*UTP18*), YJL061W (*NUP82*), YJL054W (*TIM54*) and YJL050W (*MTR4*)) in synX of yKJP054 (Fig. 3C and Supplementary Figs. 1–3).

In addition, translocations were detected in synthetic chromosomes for both SCRaMbLED haploid and diploid strains. A translocation occurred between synthetic chromosome IXR (synIXR) and synthetic chromosome X (synX) in a SCRaMbLED haploid strain yKJP058 (Fig. 3D), and for a SCRaMbLED diploid strain yKJP044, a translocation was detected between synthetic chromosome III (synIII) and synthetic chromosome V (synV) (Fig. 3E). Interestingly, we also found a translocation involving three synthetic chromosomes in a SCRaMbLED diploid strain yKJP033, in which synIXR was first translocated with



**Fig. 5. Deletion of YFR009W (*Gcn20*) enhances sodium chloride tolerance.** (A) Details of structural variations in synthetic chromosomes of yKJP060, yKJP061 and yKJP090. (B) Growth fitness of YFR009W knockout strain yKJP263 on solid medium with 0.4 M NaCl compared with control strain yZSJ025. (C) Growth curves of yKJP263 and yZSJ025 in a SC liquid media with 0.4 M NaCl. (D) Growth fitness of YFR009W knockout strain yKJP279 on solid medium with 1.5 M NaCl and nonselective YPD solid medium compared with the strain BY4742. (E) Growth curves of yKJP279 and BY4742 in a SC liquid media with 1.5 M NaCl.

synV, then the variant synIXR was translocated with synX, resulting a neochromosome which consist of gene contents from original chromosomes V, IXR and X. A total of 15 rearrangement events were detected in the yKJP033, including 7 deletions, 4 inversions, 2 duplications and 2 translocations (Fig. 3F and Supplementary Fig. 4). we also found that whole chromosome deletion of synX was detected in a SCRaMbLED diploid yKJP037, in which chrX of Y12 was reduplicated (Fig. 3G and Supplementary Fig. 5). These results show the ability of SCRaMbLE to generate complex structural variations.

### 3.3. Mapping the 5-fluorocytosine resistant gene by analysis of SCRaMbLED strains

5-fluorocytosine (5-FC), a transitional antifungal that is commercially used in the clinic [30,31]. However, it is facing challenges on the development of resistance in fungal pathogens [32]. Yeast was used as a model to study molecular mechanisms of 5-FC resistance [33]. In our experiment of SCRaMbLE, a total of 48 flucytosine-resistant SCRaMbLED strains were generated, among them 9 of the strains were used to

analyze structural variations by whole genome sequencing. Structural variations, including deletion, inversion, duplication and translocation, were found among the resistant clones (Supplementary Table 2). Notably, for two SCRaMbLED haploid strains yKJP049 and yKJP090 which show significantly enhanced resistance of 5-FC, a deletion region containing genes YER055C and YER056C was detected in yKJP090, while three rearrangement events occurred for strain yKJP049, including a deletion of YER056C (Fig. 4A and B, Supplementary Fig. 6). We suspected that the deletion region from YER055C to YER056C or YER056C would contribute to the enhanced resistance of 5-FC. We then used homologous recombination to seamlessly knock out the target genes in wild type strain BY4742. The deletion of region YER055C-YER056C obviously increased resistance of 5-FC for BY4742 by serial dilution assays on a solid medium with 10 mg/L 5-FC, while the growth was normal on nonselective YPD medium (Fig. 4C). Furthermore, we separately knocked out YER055C and YER056C, and found that the deletion of YER056C (*FCY2*) alone was able to rapidly improve 5-FC resistance (Fig. 4D), meanwhile, the deletion of YER055C had no significant effect. This finding is consistent with pervious report that

*FCY2* gene encoding a cytosine permease can transport 5-FC into fungal cells to inhibit DNA replication, transcription and protein synthesis [34, 35]. For other 5-FC resistant strains that do not contain the deletion of YER056C, we also carried out experiments in which some selected genes were separately knocked out, but no significant target regions were found. The underlying genetic mechanism of these SCRaMbLEd strains may be contributed by complex SNPs or gene interactions in the evolved genomes which is still a challenge to analyze systematically [ [36–38]].

### 3.4. Deletion of *YFR009W* (*Gcn20*) enhances sodium chloride tolerance

Environmental changes of osmotic pressure, which is usually caused by varied concentrations of sugar or salt, can affect the activity of yeast cells [39,40]. Here, we used sodium chloride as a hyperosmotic stress to screen the SCRaMbLEd strains. Among the salt-tolerant SCRaMbLEd strains, 6 of them were whole genome sequenced to analyze the structural variations. Whole-genome sequencing showed that different structural variants were detected in six salt-tolerant strains (Supplementary Table 2). Among them, we found a deletion in *synII* and an inversion in *synX* for yKJP060, three deletions and three inversions in *synX* for yKJP061 and a small deletion in *synIII* for yKJP100 (Fig. 5A, Supplementary Table 2). Notably, there was a commonly deleted region involving one gene YFR009W (*Gcn20*) for the three SCRaMbLEd strains (Supplementary Fig. 7). We speculate that this deleted region might enhance the tolerance under sodium chloride stress. To test the hypothesis, a knockout strain (yKJP263) was constructed from the initial synthetic yeast yZSJ025, and we find that deletion of gene YFR009W was sufficient to increase tolerance of 0.4 M NaCl by serial dilution assays on solid medium (Fig. 5B). Moreover, growth curves of the strains were also measured in a SC liquid media and it was observed that the knockout strain yKJP263 grew faster than the control strain (Fig. 5C). We further analyzed the effect of this knockout in the wild type strain BY4742. The YFR009W knockout strain (yKJP279) from BY4742 can improve tolerance on solid medium with 1.5 M NaCl, meanwhile it grew same as BY4742 on nonselective YPD medium (Fig. 5D). A similar trend was also observed in a SC liquid media (Fig. 5E).

These results reveal that *Gcn20* is a target gene that is relevant to the tolerance of sodium chloride, and that the deletion of *Gcn20* can improve sodium chloride tolerance in *Saccharomyces cerevisiae*, which has not been reported. *Gcn20* was reported as a positive regulator of Gcn2p kinase, and can form a complex with Gcn1p in amino acid-starved cells [41,42]. The biochemical mechanism of the genotype-phenotype correlation which deletion of *GCN20* enhances sodium chloride tolerance is awkward.

## 4. Discussion

In this study, hundreds of evolved haploid and diploid strains harboring six synthetic chromosomes were obtained under various stressful conditions by SCRaMbLE. Different types and scales of structural variation were detected in the SCRaMbLEd strains. By comparing of the SCRaMbLEd genomes, some related genes were found to be associated with the enhanced tolerance. Notably, we find that deletion of YFR009W (*Gcn20*) can enhance yeast tolerance of sodium chloride. Our study shows the ability of SCRaMbLE to accelerate phenotypical evolution and to map the underlying genetic regions. As the number of synthetic chromosomes increases, the genomic diversity generated by SCRaMbLE will further increase, which poses a challenge with regard to the high-throughput screening of evolved phenotypes, especially for phenotypes that are not dependent on colony color or colony morphology. The development of biosensors or high-throughput screening devices may solve this problem. In addition, random structural variations generated by SCRaMbLE can cause high lethality, so it is need to develop more rational control methods for SCRaMbLE, such as orthogonal chromosomal rearrangement system using orthogonal site-specific recombinase systems [43].

## CRedit authorship contribution statement

**Jianping Kang:** Conceptualization, Methodology, Investigation, Writing – original draft. **Jieyi Li:** Investigation. **Zhou Guo:** Investigation. **Sijie Zhou:** Methodology. **Shuxin Su:** Writing. **Wenhai Xiao:** Supervision. **Yi Wu:** Conceptualization, Supervision, Project administration, Writing – review & editing. **Yingjin Yuan:** Supervision, Project administration.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2022.04.003>.

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