Rewriting the Genome of the Model Eukaryote *Saccharomyces cerevisiae**

Vijayan Sambasivam, Desirazu N Rao, and Srinivasan Chandrasegaran

An international consortium of scientists has embarked on the total design and synthesis of all the 16 yeast chromosomes of the laboratory organism, *Saccharomyces cerevisiae*. Once constructed, the 16 synthetic chromosomes will be consolidated into a single yeast strain along with a new 17th yeast chromosome called the "neochromosome" which contains all the tRNA genes, to generate a designer eukaryotic genome, Sc2.0. The key criterion for the stream-lined yeast (Sc2.0) is that it should retain the same cell fitness and phenotype of the wild-type (Sc1.0), but show increased genetic stability and flexibility to enable future studies. All the 16 synthetic yeast chromosomes have been designed using *BioStudio*, an open-source framework that was developed specifically to design and construct chromosome-size fragments *in silico*. The completely redesigned Sc2.0 genome is a highly modified version of the *S. cerevisiae* genome, with a reduction in the size of ~1.1 million base pairs, which is about 8% of the native genome. In 2017, the Sc2.0 consortium reported the complete synthesis and assembly of 6.5 individual yeast chromosomes in discrete strains and showed consolidation of 2.5 synthetic chromosomes (synIII/synVI/synIXR) into a single yeast strain that bodes well for the successful completion of the Sc2.0 genome.

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Keywords
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Introduction

An organism's genome encodes its form and function. Humans have long been altering the genomes of plants and animals by selective breeding for desirable traits. With the development of

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recombinant technology and molecular biology, scientists have successfully manipulated the genomes of organisms by transferring useful traits (genes) from one to another for beneficial purposes. Thus, genetic engineering has enabled scientists to speed up the process of developing new breeds of plants and animals.

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With recent advances in DNA synthesis technology and the advent of powerful computers, scientists have attained the capability to design and synthesize genes from scratch. This new discipline, called synthetic biology, combines the principles of engineering and biology and has enabled scientists to use computers and chemicals to design organisms with novel functions, by first altering the genetic code *in silico* and then synthesizing and incorporating the designed genes into the genome of cells, to produce, for example, biofuels and pharmaceutical drugs. Synthetic biology can thus be defined as 'the design and construction of novel artificial biological pathways, organisms or devices, or the redesign of existing natural biological systems'. Synthetic biology combines the chemical synthesis of DNA with growing knowledge of genomics to enable researchers to quickly manufacture catalogued DNA sequences and assemble them into new biological pathways and novel genomes.

Scientists have approached synthetic biology broadly in two ways: (1) the top-down approach; and (2) the bottom-up approach. The former uses metabolic and genetic engineering to impart new functions to living cells, while the latter creates new biological systems *in vitro* by bringing together all non-living biomolecular components that lead to the creation of an artificial cell. The top-down approach has been used to (1) build novel biological systems; (2) develop new protein design for production, and (3) metabolic pathways to synthesize natural products and pharmaceuticals that require multiple steps, including all essential enzymes needed for producing the secondary metabolites. The bottom-up approach uses synthetic genomics to design and construct artificial living systems of bacteria and model eukaryotes.

The first breakthrough in DNA synthesis came from Khorana's lab in the 1970s, with the creation of the alanine transfer RNA (tRNA) gene from nucleotides [1]. Subsequent advances in DNA synthesis technology and lower cost of DNA synthesis have enabled the construction of viral and bacterial genomes from oligonucleotides [2]. In 2014, the total synthesis of the first functional designer synthetic eukaryotic (yeast) chromosome, was reported by Chandrasegaran's lab in collaboration with Jef Boeke's lab at the Johns Hopkins University (JHU) [3]. Since then, an international team of scientists (led by Jef Boeke), have embarked on the creation of a designer yeast genome, Sc2.0.

Why create a synthetic yeast genome? Yeasts are economically important microorganisms in the baking and brewing industry. They are also used extensively in the pharmaceutical industry to produce many important drugs. At least 1500 different yeast species have been identified, and several of their genomes have been sequenced [4]. *S. cerevisiae* strain, S288C, which is used extensively in laboratory research, was the first eukaryote organism whose complete genome was deciphered [5]. Its genome is ~12 Mb in size, distributed among 16 linear chromosomes of different lengths. We possess extensive knowledge of yeast physiology, metabolic networks and pathways, as well as, horizontal gene transfers that are readily available. The availability of yeast genome sequences makes *S. cerevisiae* an excellent model eukaryote for designing and constructing a synthetic genome [4, 6].

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Sc2.0 Project

The original idea to design and synthesize a eukaryotic chromosome (*Figure* 1) was conceived by Chandrasegaran and Jef Boeke in 2005 at JHU, USA. The initial proof-of-principle experiments were performed in Chandrasegaran's lab, by replacing ~30 kb wild-type segment of yeast with the corresponding designed synthetic segment. By 2007, the research on synthetic yeast chromosomes had morphed into an ambitious project aiming to rewrite the *S. cerevisiae* genome (Sc1.0) into a designer synthetic yeast

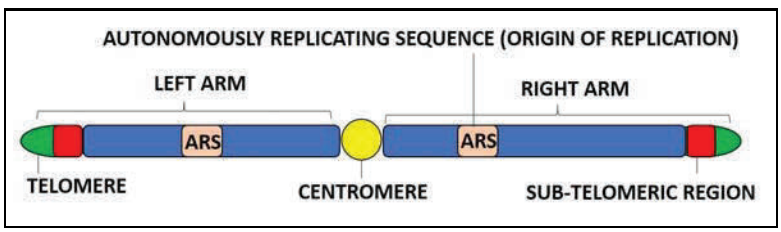

Figure 1. Schematic of a typical yeast chromosome and its parts.

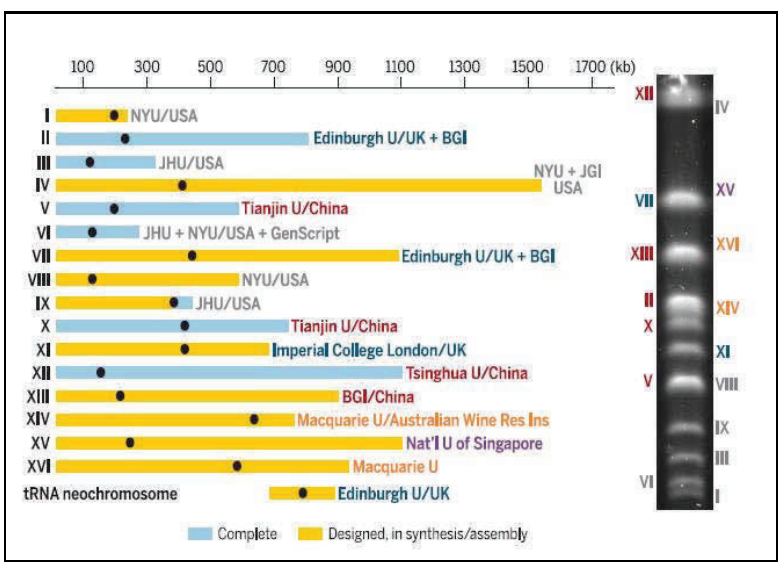

Figure 2. Sc2.0 Consortium chromosome assignments (adapted from [7]).

version (Sc2.0). Joel Bader was brought into the team a few years later, to develop the software needed to design and construct chromosome-sized fragments *in silico*.

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A global effort is currently underway to build the Sc2.0 genome. Individual synthetic yeast chromosomes are being assembled in several laboratories around the world (*Figure* 2). The Sc2.0 genome has been designed using the open-source framework called *BioStudio* that enables the design, synthesis, and assembly of chromosome-sized fragments, starting with oligonucleotides [7].

Three principles guided the design of the Sc2.0 genome: (1) it should result in a (near) wild-type phenotype and fitness; (2) it should lack destabilizing elements to avoid being unstable or undergo rearrangements; and (3) it should have genetic flexibility

to facilitate future studies. In other words, the synthetic Sc2.0 yeast strain should retain the near wild-type (Sc1.0) cell fitness and phenotype after the introduction of all the contemplated design changes and genome alterations. But, how does one go about designing a Sc2.0 genome that will facilitate future studies? Yeast contains about 6000 genes, and almost 5000 of these are non-essential when disrupted individually. We decided to flank all non-essential genes with loxPsym sites. Once the Sc2.0 genome is built, the synthetic Sc2.0 strain could be treated with Cre recombinase for various time intervals and screened for survivors. PCRTag analysis (see below) and sequencing the genomes of survivors, would reveal what combinations of the non-essential genes have been deleted from the starting Sc2.0 genome, and thereby, enable us to infer the minimal set of genes (minimal genome) required for yeast viability.

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In Silico Design of the Sc2.0 Genome Using *BioStudio*

Suggestions for the types of changes to be incorporated into the Sc2.0 genome were obtained by Jef Boeke from the community of yeast researchers. Only conservative changes were included, as more drastic changes could result in 'dead' yeast. Thus, the synthetic yeast should have the same cell fitness as the wild-type and grow normally, is an obvious minimal requirement for the Sc2.0 strain. The design changes include: (1) Replacement of all stop codons from TAG into TAA; (2) Introduction of loxPsym sites at 3' end of all the non-essential genes and large deletions; (3) Removal of long terminal repeats (LTRs), introns, transposons, retrotransposons and sub-telomeric regions; (4) Relocation of all tRNA genes into a new 17th yeast chromosome called the "neochromosome"; (5) Introduction of PCRTags, which are used to distinguish the wild-type sequence from the synthetic sequence, is done by incorporating recoded sequences into ORFs using degenerate codons; and (6) Introduction or deletion of restriction enzyme sites by base substitutions within ORFs to facilitate assembly of the synthetic chromosomes (*Box* 1).

Box 1. Design Changes Incorporated into the Sc2.0 Genome*

Elements removed	Transfer RNA (tRNA) genes, Transposons (Ty), long terminal repeats (LTRs), introns, and sub-telomeric sequences.
Elements relocated to extrachromosomal array	tRNA genes are translocated on to a 17th yeast chromosome, called a neochromosome.
Elements replaced	(1) TAG stop codons were replaced by TAA. Removal of the TAG stop codons from the synthetic genome allows for future genetic code manipulation. (2) The telomeres were specified by a minimal 'universal telomere cap' comprising 305 bp of repeated $T(G)_{1-3}$ sequence. (3) Single synonymous codons were used to incorporate unique restriction sites (or delete sites) to facilitate synthetic chromosome assembly. (4) Short stretches of synonymous codons (fewer than ten codons) were recoded to generate 'PCR-Tags' that serve as the basis for PCR primer design. PCR-Tags are used to quickly distinguish wild-type from synthetic sequence by selective PCR amplifications.
Elements introduced	Symmetrical loxP (loxPsym) sites were inserted in the 3′ UTR of all non-essential genes as well as at synthetic landmarks such as sites of LTR and tRNA deletion or flanking the centromere. LoxPsym sites lack the directionality of canonical loxP Cre recombinase sites and can align in two orientations. Therefore, both inversions and deletions are possible during SCRaMbLE using Cre recombinase.
Elements not changed	Gene order was preserved in Sc2.0 to prevent incorporation of a non-permissible configuration. Induction of SCRaMbLE results in changes in gene order and chromosome/genome structure. All recovered SCRaMbLEd yeasts will have viable genome structures.

*Changes made to the Sc2.0 genome are same as those incorporated into synIII chromosome [3].

The completely redesigned Sc2.0 genome is a highly modified version of the *S. cerevisiae* genome, with a reduction in the size

of ~1.1 million base pairs that are about 8% of the native genome. Deletions, insertions, and other alterations of Sc2.0 chromosome design were implemented using *BioStudio*. The software was specifically developed for eukaryotic genome design. It incorporates design modifications from nucleotides to genome-scale and enforces version control to systematically track edits.

Workflow for the Construction of Sc2.0 Chromosomes

Conceptually, the hierarchical synthesis of the designer Sc2.0 genome is quite simple. First, the synthetic chromosomes are designed by incorporating all the desired changes based on the native *S. cerevisiae* chromosome sequences. Second, the designed chromosomes are compiled into overlapping 750 bp building blocks (BBs), which are then assembled in yeast into ~2–4 kb minichunks with one BB overlap with its neighbours. Alternatively, overlapping 10 kb chunks could be purchased from commercial vendors. Third, an iterative strategy with alternating genetic markers is used to replace the wild-type sequences of native chromosomes with corresponding synthetic minichunks (or chunks) by SwAP-In (switching auxotrophies progressively for integration) method that employs homologous recombination (*Figures* 3 and 4) [7]. This works well in yeast because it is a highly recombinogenic organism. Fourth, to achieve complete Sc2.0 genome synthesis, individual synthetic chromosomes that are being built by the Sc2.0 Consortium teams around the world (16 synthetic yeast chromosomes and the neochromosome), will be consolidated into a single yeast strain in a final step.

JHU 'Build-a-Genome' Course

The Sc2.0 project offered an excellent framework for undergraduate student participation in cutting-edge interdisciplinary research. Construction of eukaryotic genomes requires synthesis that is orders of magnitude higher than the synthesis of entire viral or prokaryotic genomes. The high-throughput Sc2.0 project served as an ideal platform to pursue synthesis of *S. cerevisiae* chromo-

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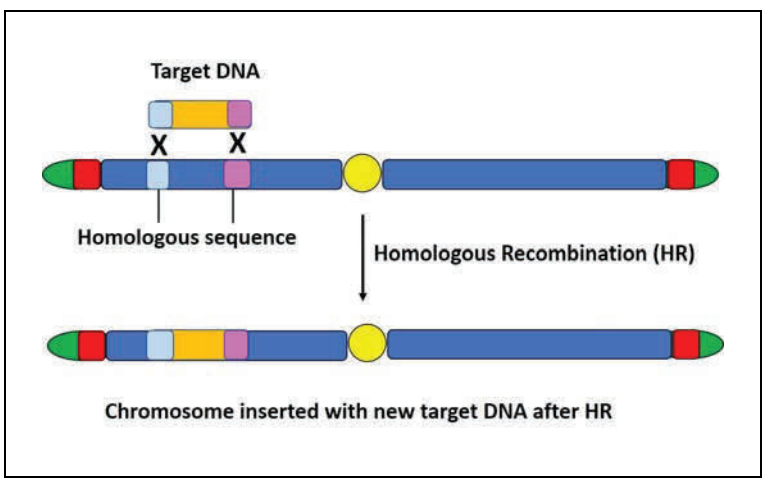

Figure 3. Diagram of a yeast chromosome undergoing homologous recombination with a donor segment.

somes in an undergraduate laboratory setting, in the JHU Build-A-Genome (BAG) course [9]. The students are exposed to the engineering of biology on a genome-wide scale while focusing on a limited region of the genome. SynIII and several other synthetic sequences were designed and ordered from commercial suppliers in the form of oligonucleotides. The undergraduates in BAG course assembled the oligonucleotides into ~750 bp BBs. Once trained in the assembly of such DNA BBs by PCR, the students readily accomplish high-yield gene synthesis and assembly of larger ~10 kb fragments. The undergraduates become technically proficient and are capable of adapting the gained knowledge from BAG course to independent research after their participation. Regular lab meetings in the BAG class help to prepare them in critical thinking and for future roles in laboratory research.

Debugging

Because of our incomplete understanding of yeast genetics and biology, the design changes of the Sc2.0 genome have the potential to introduce unintended consequences in transcription and translation, which could affect cell fitness and genotype, leading to growth defects ("bugs"). In such cases, correction of the bugs is a crucial component of the workflow to restore normal growth

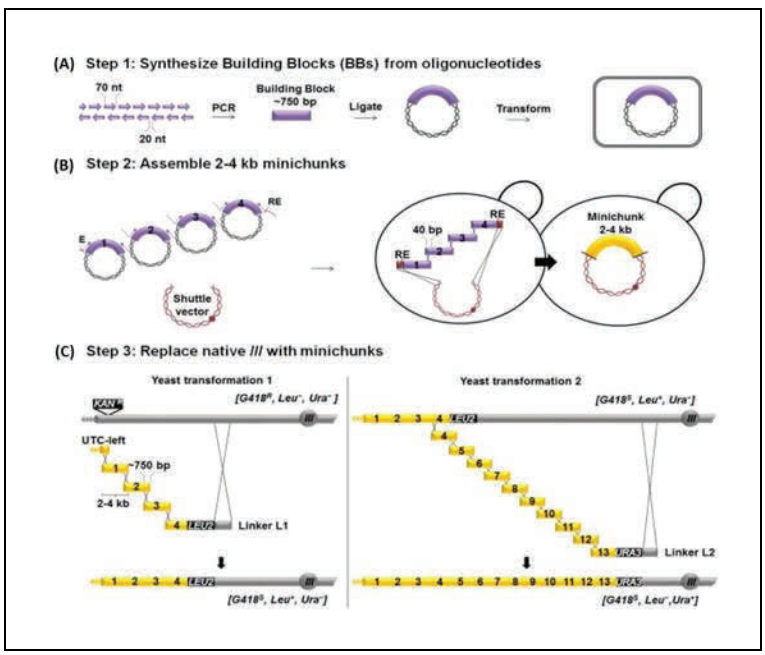

Figure 4. Workflow for the constructing the synIII chromosome. (A) Building block (BB) synthesis. 750 bp BBs (purple) were synthesized from oligonucleotides by JHU students in the BAG course. (B) Assembly of minichunks. 2–4 kb minichunks (yellow) were assembled by HR in S. cerevisiae. Adjacent minichunks were designed to encode overlap of one BB to facilitate downstream assembly steps. Minichunks were flanked by a rare cutting restriction enzyme (RE) site, *Xma*I or *Not*I. (C) Direct replacement of native yeast chromosome III with pools of synthetic minichunks. Eleven iterative one-step assemblies and replacements of native genomic segments of yeast chromosome III were carried out, using pools of overlapping synthetic DNA minichunks and alternating genetic markers (LEU2 or URA3), which enabled complete replacement of native III with synIII (adapted from [3]).

to the synthetic yeast strains. In many instances, debugging could be done by just reverting back to the wild-type sequence within the Sc2.0 genome. In other instances, the construction process itself could introduce sequence changes such as insertion, deletion or silence base-pair changes into Sc2.0; if these mistakes do not result in growth defects, it is not essential to correct them. Thus, debugging is a critical step for the successful construction and assembly of the synthetic yeast chromosomes and the Sc2.0 genome. The debugging performed on individual synthetic yeast chromosomes that were completed by 2017 are listed in *Table* 1.

Consolidating the Synthetic Chromosomes into the Sc2.0 Yeast Strain

For final successful completion of the Sc2.0 genome, all the 16 synthetic chromosomes (synI-synXVI which are constructed in discrete yeast strains), and the neochromosome need to be consolidated into a single yeast strain. To this end, a conditional chromosome destabilization strategy to generate Sc2.0 polysynthetic

Table 1: Completed Sc2.0 chromosomes by laboratories around the world.

Sl. No.	Synthetic Chromosome number	Size (bp)	Laboratory where the work was performed	Remarks	Growth defect	Debugging	Ref
1.	SynIII	272,871	Srinivasan Chandrasegaran JHU, USA	Reported the synthesis of the first functional designer synthetic eukaryotic chromosome	No growth defect	No debugging	[3]
2.	SynII	770,000	Huanming Yang, BGI-Shenzhen, China	-	Recoding of TS10 gene and NCL1 with URA3 marker integration affected the growth.	Normal growth was restored by reintroduction of native sequences.	[4]
3.	SynV	536,024	Ying-Jin Yuan, Tianjin University, China	Mistakes were corrected by CRISPR-mediated editing. A fully functional ring shaped synV derivative was also made by end joining of its telomeres.	YER187W and YER188W that encodes for a replicative helicase and encode for sub-telomeric gene respectively, showed decreased expression.	All variants were corrected by back cross to the wild type.	[10]
4.	SynVI	242,745	Jef Boeke, NYU, USA	-	Growth defect was due to synonymous coding changes in the PRE4 (YFR050C) gene, which encodes an essential proteasome subunit. And alteration of the HIS2 transcription start site due to tRNA deletion and loxPsym site insertion.	Growth defect was corrected by introducing SYN-WT. PRE4 allele in the triple-syn strain. Deleted tRNA genes will be restored by 'neochromosome'	[8]
5.	i) SynIXR	91,000	Jef Boeke, NYU, USA	Reported completion of a synthetic chromosome arm	No defect	No debugging	[11]
	ii) SynIXL	315,000	Srinivasan Chandrasegaran, JHU, and Jef Boeke, NYU, USA.	Ongoing project in the lab	-	-	-
6.	SynX	707,459	Ying-Jin Yuan, Tianjin University, China	Pooled PCRTag mapping (PoPM) was used to identify the growth defect	FIP1 ORF was affected by PCRTag recoding and introduction of a loxPsym site affected the promoter function of nearby gene ATP2	Corrected by reintroduction of native sequences.	[6]
7.	SynXII	976,067	Junbiao Dai, Tsinghua University, China	SynXII was assembled by a two-step method: 1) megachunk integration; and 2) meiotic recombination-mediated assembly	Removal of tRNA gene (TRR4). GAS2 and AHP1 genes were down regulated due to the incorporation of recoded sequence for the PCRTags.	Corrected by reintroduction of the native sequences.	[12]

(poly-syn) chromosome strains, called an "endoreduplication intercross" was developed (*Figure* 5). Briefly, intercross was carried out between two opposite mating type of yeast strains such as such MATa and MATα. Each strain (also called double-syn strain) is with a pair of synthetic chromosomes and a modified native chromosome. MATa strain is with synVI/synIXR and native III, while MATα is with synIII/synVI and native IX. The native chromosomes are integrated with a pGAL1-CENx gene. GAL1 promoter is used to destabilize the adjacent centromere, and the integrated plasmid also carries an adjacent URA3 gene for selection against 5′ fluoroorotic acid (5-FOA). This approach simultaneously disrupts the centromere function of two specified native chromosomes in a doubly heterozygous diploid synthetic strain to generate a "2n–2" strain. In diploid yeast cells, each chromosome can be individually lost, to yield hemizygotes for the destabilized chromosome. Such 2n–2 strains endoredu-

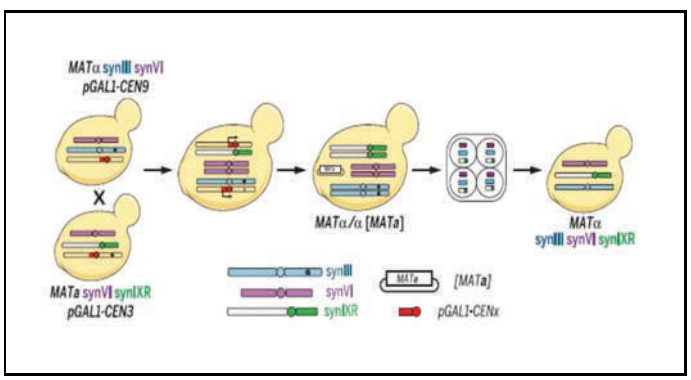

Figure 5. Endoreduplication intercross of the synthetic chromosome containing yeast strains to consolidate all three synthetic chromosomes such as synIII, synVI and synIXR into one strain (synIII/synVI/synIXR). Intercross was carried out between two opposite mating type of yeast strains such as such MATa and MATα. Each strain (also called double-syn strain) is with a pair of synthetic chromosomes and a modified native chromosome. MATa strain is with synVI/synIXR and MATα is with synIII/synVI. After the strains are crossed, destabilization of the native chromosomes yielded hemizygotes (2n-2) for the corresponding synthetic chromosomes. These are capable of endoreduplication to generate a 2n state and can be sporulated. By sporulation and dissection, haploid poly-synchromosome strains were generated (adapted from [7]).

plicate the remaining single destabilized chromosomes to generate the 2n state. After the strains are crossed, induction of the GAL1 promoter by galactose leads to destabilization of the native chromosomes to yield hemizygotes (2n–2) for the corresponding synthetic chromosomes, which after endoreduplication generated the 2n state. The desired haploid poly-synchromosome strains were generated by sporulation of these cells, followed by dissection. Using this method, poly-syn chromosome yeast strains encoding two (synIII/synVI; synIII/synIXR; synVI/synIII) and three synthetic chromosomes (synIII/synVI/synIXR), respectively, were generated from individual synVI, synIII, and synIXR strains. All combinations of synthetic chromosomes were capable of directing the growth of diploid yeast cells in the absence of the corresponding native chromosomes. In principle, using this strategy, any pair of Sc2.0 chromosomes may be consolidated into a single strain without the need for sequence alteration.

SCRaMbLEing of synV Enables Host Strain Improvements for Metabolic Engineering

SCRaMbLE (Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution) of synthetic yeast strains by exposing to Cre recombinase, enables one to rapidly induce recombination events that lead to extensive rearrangements between specific loxPsym sites (*Figure* 6). SCRaMbLEing of genomes allows one to investigate if and how gross rearrangements lead to

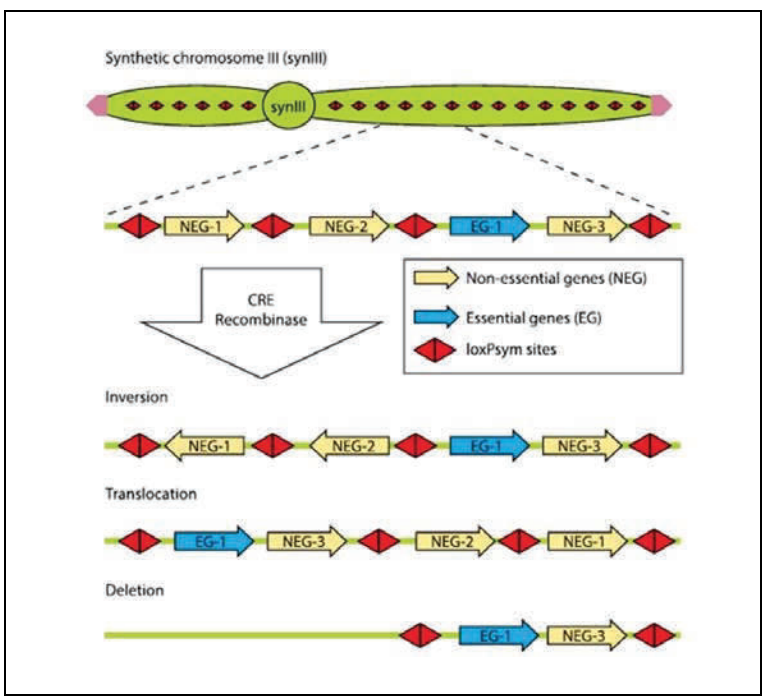

Figure 6. Synthetic chromosome rearrangement and modification by loxP-mediated evolution (SCRaMbLE) of the synIII strain. Examples of inversion, translocation and deletion products resulting from Cre recombinase treatment of synIII strain are shown (adapted from [2]).

phenotypic changes (scramblotypes) (*Figure* 7). It offers a new way to create very efficient microorganisms for metabolic engineering. Ellis group in the UK showed that scrambling of the synV chromosome rapidly generates new, improved host strains for diverse heterologous pathways, such as for violacein and penicillin biosynthesis and for xylose utilization [13]. This research lays the foundation for how synthetic yeast chromosomes and Sc2.0 genome, could be used in metabolic engineering of heterologous pathways to achieve productivity improvements in a fast and simple way.

In 2017, Sc2.0 consortium teams reported complete synthesis and assembly of 6.5 individual yeast chromosomes, each of which is available as discrete yeast strains.

Completed Synthetic Chromosomes

In 2017, Sc2.0 consortium teams reported complete synthesis and assembly of 6.5 individual yeast chromosomes, each of which is available as discrete yeast strains. Furthermore, consolidation of 2.5 synthetic chromosomes (synIII, synVI and synIXR) into a single yeast strain has also been reported by the endoreduplication

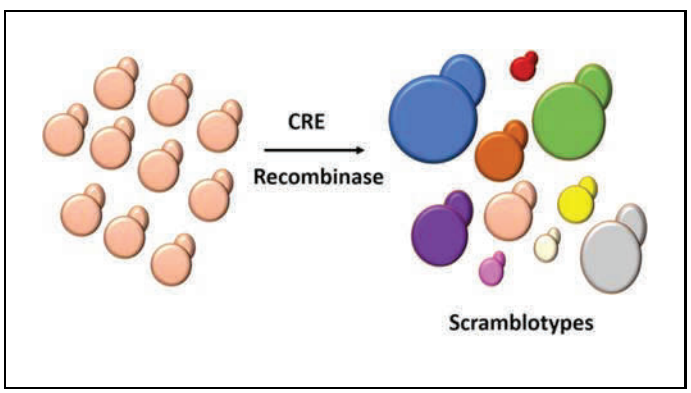

Figure 7. Schematic of new phenotypes (scramblotypes) arising from SCRaMbLEing (after Cre recombinase treatment) due to inversion, translocation and deletion products at loxPsym sites.

cross [8]. The consolidated synthetic yeast strain also shows near wild-type cell fitness and phenotype, indicating high tolerance of the *S. cerevisiae* genome for genetic manipulation and genome plasticity.

Summary

The complete design and synthesis of several synthetic yeast chromosomes have established *S. cerevisiae* as the basis for designer eukaryotic genome biology. Consolidation of multiple synthetic yeast chromosomes in a single yeast strain bodes well for successful completion of the Sc2.0 genome. The usefulness of synthetic chromosomes (and the eventual Sc2.0 genome) has already been proven by scrambling synV synthetic strain to achieve host strain improvements in metabolic engineering. The high tolerance of *S. cerevisiae* genome, and living systems in general, for genetic manipulation, genomic plasticity and genome modification is truly amazing!

The complete design and synthesis of several synthetic yeast chromosomes have established *S. cerevisiae* as the basis for designer eukaryotic genome biology.

Acknowledgement

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of Biochemistry.

Box 2. Glossary

Auxotroph: A mutant organism that requires a particular additional nutrient which the wild-type strain does not.

Conservative changes: Substitution of one amino acid by another, possessing similar biological properties.

Degenerate codons: Multiple codons that specify the same amino acid.

Diploid/haploid cells: Cells containing two sets (2n) of chromosomes are called diploid (2n), and those containing one set (n) are called haploid.

Endoreduplication: Replication event where the mitosis is incomplete, which results in increased nuclear content and chromosomes.

Genotype: Genetic information present within a cell of an organism. It contributes to the physical attributes of the organism.

Heterozygous: A gene having two different types of alleles for a specific trait.

***In silico*:** Experiments that use computational methods to study DNA and protein sequence data, structure and function.

Introns: Non-coding regions of DNA within genes that initially were identified as non-essential; some of which were later shown to have regulatory functions.

loxPsym site: Symmetrical loxP sites are 34 base pairs (bp) in length and consists of two 13 bp inverted repeats separated by an 8 bp spacer region. Cleavage and strand exchange of recombining loxP sites, occurs within the spacer region.

Long terminal repeats (LTRs): Identical long stretches of DNA that are present in hundreds to thousands of copies at the $5'$ and $3'$ ends of retrotransposons and viral DNA Mating alpha(α) and 'a' allele: Haploid yeast contains two mating factors 'a' and 'α', which are needed for successful mating cycles. Alpha allele produces α-factor (pheromones), which makes 'a' allele containing yeast cells to protrude a projection towards α-factor producing cells for mating.

Mb (Megabase) and Kb (kilobase): Measures length of genomic sequences.1Mb = 106 bases;1 Kb = 103 bases

Metabolic engineering: Process of manipulating genes within an organism in order to increase production of the desired compounds.

Open reading frame (ORF): The coding region of genes, which begin with a start codon and ends with a stop codon.

PCRTags: PCRTags are watermarks that are introduced into the synthetic genome in order to easily distinguish it from the wild-type sequence using PCR.

Phenotype: Observable physical characteristics of an organism are referred to as phenotype, which depends on the genotype.

Contd.

Box 2. ***Contd.***

Proteomics: Study of the complete set of proteins produced by a cell.

Retrotransposons: DNA segments that use "copy and paste mechanism" to amplify themselves within a genome. These DNA segments are first transcribed to RNA, then reverse transcribed to DNA, and then inserted into the genome at different target sites.

Silent mating loci: Segment of the chromosome that is responsible for the switching of mating 'a' cells to 'α' cells and vice-versa in yeasts. Yeast cells carry additional silenced copy of both Mat a and Mat α at the silent mating loci.

Silent mutations: Changes in one of the letters of a codon, which does not alter the amino acid.

Transcription: The process of copying genetic information from the DNA template to RNA is called transcription.

Transcriptomics: Study of the complete set of all RNA transcripts produced by a cell.

Translation: The process of protein synthesis from mRNA using ribosomes within a cell.

Transposons: DNA segments that move from one location to another within the genome, also known as "jumping genes".

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