Doctoral Dissertation

Development of a new genome engineering technology, CRISPR-PCDup, to create segmental aneuploid and exploration of synthetic lethality in yeast

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Contents

Chapter 1

General Introduction

1.1 Genome engineering in *Saccharomyces cerevisiae*

Genome engineering is defined as a technique to create particular alteration of genome in both prokaryotes and eukaryotes. This technology is one of the most powerful technologies to manipulate strains with desired characters and understand genome function. Chromosome engineering is a subset of genome engineering which facilitates targeted chromosomal modifications such as small deletion, insertion, duplication, inversion or translocation of chromosome. Genome function could be investigated through chromosome manipulation. By chromosome engineering, scientists have tried to renovate, redesign or synthesize genomic information on chromosome for many years. Some of interesting achievements are described here which deals with various unique chromosome modifications.

An ancient tool used for engineering yeast cells on the chromosomal level is yeast artificial chromosomes (YACs) (Burke et al. 1987). These are chromosome arms containing telomeres, yeast auxotrophic markers, and yeast elements for replication and segregation. YACs were successfully applied for optimizing metabolic pathways using random assembly of pathway genes and connected promoters. A flavonoid pathway comprising seven different genes was hereby successfully reconstructed (Naesby et al. 2009). Bridge-induced translocation (BIT) allows us to generate the translocation event at desired chromosomal regions by transformation with a DNA cassette containing a selectable marker flanked by two homologous sequences corresponding to two different chromosome location (Tosato et al. 2005). PCR-mediated chromosome spitting (PCS) technology was developed to split native chromosome at any desired location at a single transformation in *Saccharomyces cerevisiae* (Sugiyama et al. 2005). One of applications of PCS is shuffling method which allows swapping selected chromosomal regions with the corresponding region of other strains (Sugiyama et al. 2006). Another application of PCS was PCR-mediated chromosome deletion (PCD) technology. PCD enables to delete any chromosomal region at a single transformation

(Sugiyama et al. 2008). Genome reorganization technology was also another application of PCS which generates a large array of genome composition through combinatorial loss of mini-chromosomes in yeast cells (Ueda et al. 2012). PCR-mediated chromosome duplication technology called PCDup (Natesuntorn et al. 2015) was a technology which was further developed to duplicate any desired chromosomal regions as an independent chromosome. PCDup was able to duplicate any regions with lengths from 50 kb to 300 kb. In that study, PCDup was used to produce a series of 200 kb segmental duplication that covers whole genome of *S. cerevisiae*. These segmental duplications of some chromosomal regions produced enhanced or decreased resistance phenotypes or growth enhancement or retardation when cells were grown under particular stress and even nutrient rich conditions. Therefore, PCDup could be considered as a powerful breeding tool to generate superior strains because segmental aneuploids are occasionally found in industrial yeast strains such as those used for fermentation of wine and beer (Borneman et al. 2011; Dunn et al. 2012). Although PCS and PCD technology were improved to target multiple chromosomal regions simultaneously, PCDup was critically limited to target only one chromosomal region at a time and lag behind to target multiple regions.

DiCarlo et al. (2013) first introduced Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) associated protein (Cas9) in yeast successfully and studies in several organisms have shown that multiple genomic targets are possible with the Cas9 system (Mali et al. 2013; Cong et al. 2013; Jiang et al 2013). Recently, by taking advantage of CRISPR/Cas9, previous PCS technology was improved to target at least four chromosomal regions to split at a single transformation (Sasano et al. 2016). By combining CRISPR/Cas9 system with simple PCR-generated DNA modules harboring marker gene, deletion of large chromosomal region (500 kb) and targeting multiple chromosomal regions for deletion became possible (Easmin et al. 2019a; Easmin et al. 2019b).

These technologies uncover the efficiency of CRISPR/Cas9 system on splitting, deletion, and replacement of multiple chromosomal regions. Therefore, it can be said that genome engineering has entered into the CRISPR/Cas9 era to improve previous novel technologies to target not only multiple chromosomal regions very efficiently but also develop advanced genome engineering technologies which were just an imagination in the past.

1.2 Genome editing is improved by CRISPR-Cas9

Scientific community hashave great interest CRISPR and CRISPR-associated (Cas) immune system of bacteria for RNA-guided endonuclease activity (Carroll 2012; Jinek et al. 2012). CRISPR/Cas module is a part of an adaptive immune system of bacteria that recognizes and cleaves foreign invading DNA (Horvath and Barrangou 2010; Marraffini and Sontheimer 2010). The Cas9 gene, from the type II bacterial CRISPR system of *Streptococcus pyogenes*, complexes with a designer genome targeting CRISPR guide RNA (gRNA) to determine the site specificity of the double-stranded DNA cutting activity (Jinek et al. 2012; Bhaya et al. 211). CRISPR systems offer an advantage to zinc finger (Hirayama et al. 2003) and transcription activator-like effector DNA binding proteins (Gersbach and Perez-Pinera 2014) because CRISPR/Cas9 system is cost effective and time saving. On the other hand, the simplicity of these RNA-guided nucleases has allowed scientists to repurpose the CRISPR/Cas9 system to create site-specific double-strand DNA breaks (DSBs) in a variety of eukaryotic cells (Cong et al. 2013; Mali et al. 2013). Since DeCarlo et al. (2013) first successfully introduced CRISPR/Cas9 system in haploid *S. cerevisiae*, this system also allowed engineering of diploid and polyploid industrial yeast strains (Ryan et al. 2014; Zhang et al. 2014; Stovicek et al. 2015), which were challenging issue to manipulate genetically due to the difficulties for modifying multiple sites and the lack of many selection markers (Le Borgne 2012). Additionally, by combining several gRNAs, multiple sites can be targeted

simultaneously, allowing the unprecedented speed of genetic editing of multiple sites and regions in the genome (Ryan et al. 2014; Bao et al. 2015; Jakočiūnas et al. 2015). For increasing diversity of genomes and speeding up genome engineering, CRISPR/Cas9 system is becoming one of the major choices of modern biotechnologists.

1.3 Revealing function of genes and genome propelled by genome engineering

Elucidation of gene function has become the main task of genome engineering. Apart from unraveling direct physical interactions between gene products, the discovery of functional interactions carries the potential of revealing novel functions of genes and of assigning novel genes to the appropriate cellular mechanisms. A powerful genetic method for assigning a function to a gene is to identify additional genes that become essential for cell survival in the absence of particular gene of interest. These genes are referred to as synthetic lethal genes with each other. Synthetic lethality between two genes may imply that their products carry out the same or similar functions by alternative pathways (Koren et al. 2003). Defects in one gene are compensated by the activity of the pathway represented by the other gene and lack of both genes results in cell death, due to complete loss of the function carried out by both pathways. Synthetic lethality, however, is also often seen between two genes whose products directly interact with each other and operate in a common pathway (Koren et al. 2003).

Previously, synthetic lethality screening in *S. cerevisiae* was basically conducted by a plasmid dependence assay. Cells transformed with a plasmid carrying the gene of interest in a background of a chromosomal deletion or mutation in that gene are mutagenized and screened for mutants that have become unable to survive in the absence of the plasmid (Basson et al. 1987). Later synthetic lethal interaction was systematically analyzed by "Synthetic genetic array" (SGA) analysis (Tong et al. 2001). SGA involves a series of replica-pinning procedures in which mating followed by meiotic recombination is used to convert an input array of single mutants into an output array of double mutants (Tong et al. 2001). SGA has been used

extensively for synthetic lethal screening of non-essential genes involved in many cellular functions. The genome-wide study by SGA analysis revealed that approximately 10,000 gene combinations are synthetically lethal for the growth of *S. cerevisiae* (Costanzo et al. 2010). On the other hand, it has been predicted that the complete genetic network of *S. cerevisiae* contains over 200,000 synthetic lethal combinations (Baryshnikova et al. 2013). Verification of this prediction might not be possible by using SGA because construction of a double disruptant is not possible if the two genes to be disrupted are tightly linked on the same chromosome. Because of this situation, synthetic lethal interactions between linked gene-pairs remains largely unknown. To facilitate an investigation of the lethal interactions of linked gene-pairs that escape detection by the SGA method and in order to provide a complete genetic interaction map of *S. cerevisiae*, Kaboli et al. (2014) performed genome-wide chromosomal segmental deletion and subsequent mini-chromosome loss assay by employing PCR-mediated chromosome deletion (PCD) (Sugiyama et al. 2008) and PCS (PCR-mediated chromosome splitting technology (Sugiyama et al. 2005). The final outcome of these results showed that 49 among 110 regions were undeletable and these 49 regions were not previously described by SGA method for harboring synthetic lethal combinations of genes. This result indicates that there might be unknown synthetic lethal combinations of gene-pair present in those 49 undeletable regions and gives an opportunity to narrow down these regions to pinpoint gene-pairs having synthetic lethal interaction.

1.4 Objective of this study

Targeting multiple chromosomal regions at a single transformation helps to create genomes with a great diversity and saves both labor and time. In Chapter 1, it is already described that it became possible to manipulate multiples sites in the genome by the advent of CRISPR/Cas9 system. On the other hand, the purpose of genome engineering is not only destined to develop techniques for manipulating genome, but also find out interesting genome

functions like synthetic lethal interactions among genes as described here. Based upon the above idea, in Chapter 2 I tried to duplicate multiple chromosomal regions simultaneously as one of the objectives of this study. To achieve this objective, I incorporated CRISPR/Cas9 system with our previously developed PCDup method and tried to duplicate single chromosomal region more efficiently than conventional PCDup, duplicate two larger chromosomal regions simultaneously and extend the length of duplicated chromosomal region. In Chapter 3, as the second objective of this study, I attempted to identify synthetic lethal combinations among gene-pairs in previously described 49 undeletable chromosomal regions. To achieve this objective, I chose four of the smallest undeletable chromosomal regions among the 49 and performed replacement analyses by using DNA module harboring only marker gene. Interestingly, all the regions were replaceable. The implications of these interesting outcome are discussed later. In chapter 3, I also did deep analysis of the replaced regions by employing two novel genome engineering technologies, i.e., PCS and PCDup to see whether the target regions are essential or nonessential for viability. In Chapter 4, I discussed the reason why I used CRISPR/Cas9 system instead of other technologies like Zinc Figure Nucleases (ZFNs) or Transcription Activator-Like Effector Nucleases (TALENs) to induce DSB and two plasmid system for CRISPR-PCDup technology to target multiple chromosomal regions. I also discussed possible reasons for getting viable transformants in search of synthetic lethality in undeletable regions. Finally, I concluded that my study demonstrated the effectiveness of novel genome editing technology, i.e., CRISPR-PCDup and exploited genome functions by revealing intrinsic essentiality of the undeletable regions. Thus, my study will be helpful to comprehend analysis of genomic mystery to modern scientific community for genome science and biotechnology.

Chapter 2

A novel approach for simultaneous segmental chromosomal duplication in *Saccharomyces cerevisiae*

2.1 Introduction

Saccharomyces cerevisiae is a model organism of immense industrial interest. It is known that many of the characteristics essential for the industrial application of *S. cerevisiae*, such as stress tolerance, are controlled by more than one gene (Swinnen et al. 2012). Consequently, genome engineering technologies are required for the rapid and effective exploitation of multiple genetic loci. Among various technologies, chromosome engineering is promising because it facilitates large scale genomic manipulation by altering chromosomes, thereby offering a powerful means of elucidating chromosome and genome function. Additionally, chromosome engineering can be used to generate useful yeast strains through the creation of a wide array of genetic diversity followed by a screening procedure to isolate the desired strains under defined culture conditions. However, a major limitation of chromosome engineering is the simultaneous manipulation of multiple chromosomal sites and regions.

Previously, a variety of new chromosome engineering technologies was developed in *S. cerevisiae.* One such method, named PCR-mediated chromosome duplication (PCDup), enables the duplication of any desired chromosomal region as an independent chromosome (Natesuntorn et al. 2015). PCDup is able to duplicate chromosomal regions with lengths from 50 kb to 300 kb. Using PCDup, we discovered that segmental duplication of some chromosome regions leads to an enhanced resistant phenotype when the cells are grown under stress conditions. However, the PCDup method has limitations because duplication is restricted to a single region at each transformation step. Simultaneous duplication of two or more target regions in the genome of an organism, even in the yeast genome, has not been achieved. Time is also a major consideration when conducting genome engineering. For example, one round of duplication takes at least 11 days including confirmatory analysis and if the results are in failure, constructing stains by successive multiple chromosome duplications is both time consuming and laborious.

PCDup technology is based on the mechanism of homologous recombination. I reasoned that an improvement of homologous recombination activity might be the key to enhancing chromosome duplication efficiency. It has previously been shown that induction of double-strand breaks (DSBs) can increase recombination efficiency near the site of the DSB by as much as 4000-fold (Storici et al. 2003). Recently, RNA-guided programmable CRISPR/Cas systems have played a major role in facilitating precision genome engineering by sequence-specific introduction of double-strand breaks (DSBs) (Cong et al. 2013; Jinek et al. 2012; Sander and Joung 2014). Moreover, the CRISPR/Cas9 system has been shown to be functional in *S. cerevisiae* (DiCarlo et al. 2013). Thus, this method permits induction of site specific DSBs using an appropriate gRNA.

In this chapter, I introduced DSBs into the genome of *S. cerevisiae* using the CRISPR/Cas9 system before attempting a chromosome duplication. I showed that the integration of CRISPR/Cas9 into PCDup system, which I called CRISPR-PCDup, produces an effective genome engineering technology that enhances chromosomal duplication efficiency with a high level of fidelity and is capable of simultaneously targeting multiple chromosomal regions.

2.2 Materials and Methods

2.2.1 Strains and media

Strains used in this study are listed in Table 1. FY833 and FY834 cells containing plasmid p414-TEF1p-Cas9-CYC1t were used as a host strain (SJY415 and SJY30, respectively) for the CRISPR-PCDup experiments. *Escherichia coli* DH5α was used for plasmid construction and propagation. *E. coli* recombinant strains were grown in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin (Nacalai Tesque, Kyoto, Japan). Yeast cells are grown in YPDA medium containing 1% Bacto-Yeast Extract (BD Bioscience, San Jose, CA),

2% Bacto-Peptone (Difco, Detroit, MI), 2% glucose (Wako, Tokyo, Japan), 2% agar (Wako) and 0.004% adenine sulfate (Wako) and in Synthetic Complete (SC) medium containing 0.67% Yeast Nitrogen Base without Amino Acids (Difco), 0.2% dropout mix of amino acids and nucleic acid bases and 2% glucose. SC medium lacking specific amino acids was used for the selection of transformants. For sporulation, diploid strain was cultivated in sporulation medium containing 1% potassium acetate (Wako), 0.1% bacto-yeast extract and 0.05% glucose. Agar (2% w/v) was included for solid medium.

Table 1. Strains and plasmids used in the CRISPR-PCDup experiments

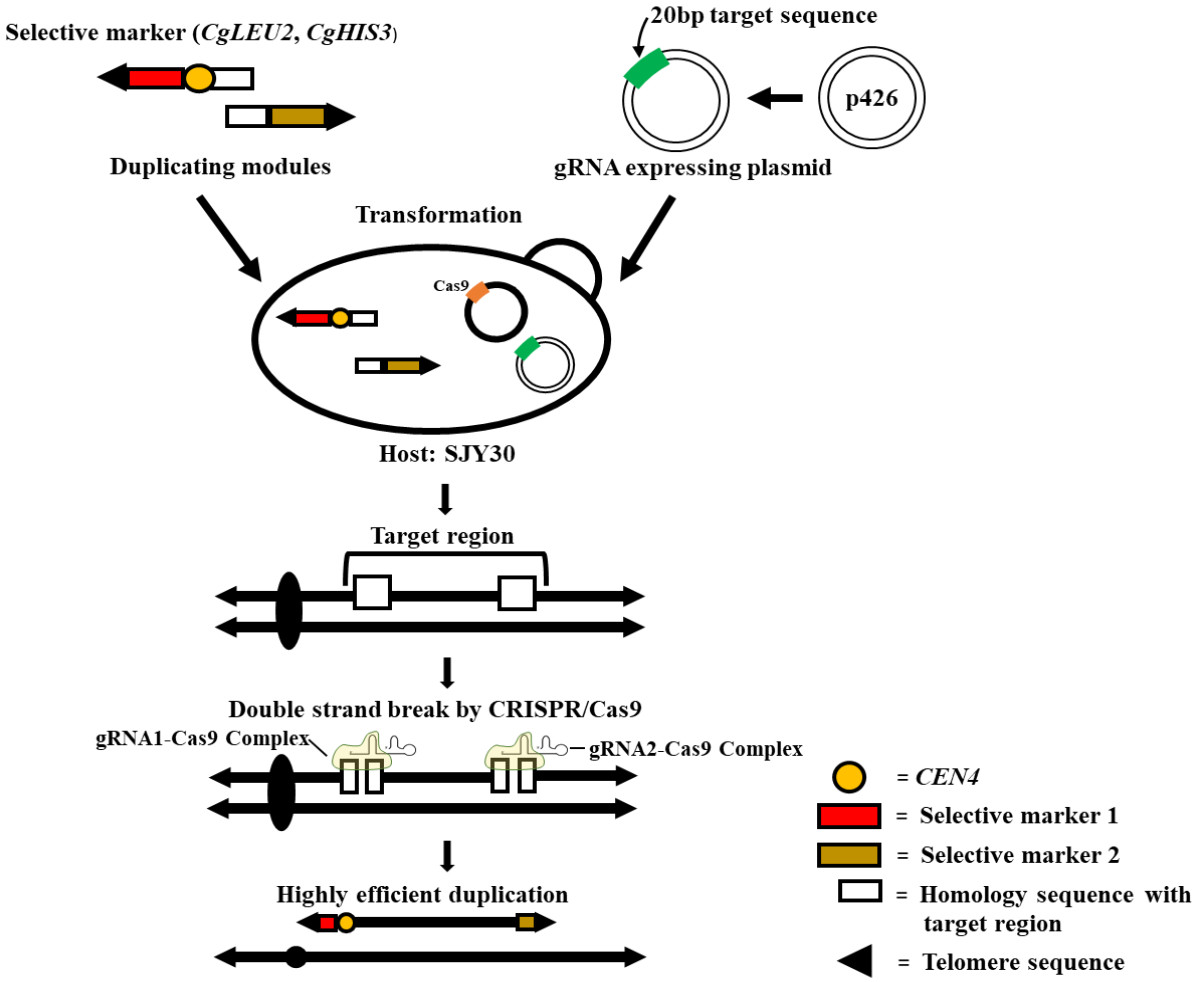
Strain or plasmid	Description	Remarks
Strain		
FY833	*MATa ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	(Winston et al. 1995)
SJY415	Trp+ transformants of FY833 harboring plasmid p414-TEF1p-Cas9-CYC1t	This study
FY834	*MATα ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	(Winston et al. 1995)
SJY30	Trp+ transformants of FY834 harboring plasmid p414-TEF1p-Cas9-CYC1t	(Sasano et al. 2016)
Plasmid		
pUG6	Containing loxP-flanked marker gene deletion cassette: loxP-pAgTEF1-kanMX-tAgTEF1-loxP	(Güldener et al. 1996)
p3009	The *loxP-CgHIS3-loxP* module containing plasmid constructed by modifying pUG6	(Sugiyama et al. 2005)
p3121	The *CEN4* module containing plasmid constructed by modifying pUG6	(Sugiyama et al. 2005)
p3122	The *CEN4-loxP-CgLEU2-loxP* module	(Sugiyama et al.

	containing plasmid constructed by modifying pUG6	2008)
p3123	The *CEN4-loxP-CgHIS3-loxP* module containing plasmid constructed by modifying pUG6	(Sugiyama et al. 2008)
pSJ23	A derivative of pUG6 carrying *URA3*	(Easmin et al. 2019b)
pSJ69	loxP site-deleted p3008	(Easmin et al. 2019a)
pSJ70	loxP site deleted p3009	(Easmin et al. 2019b)
p414-TEF1p-Cas9-CYC1t	*TEF1*p-Cas9-*CYC1*t module containing YCp type plasmid	(DiCarlo et al. 2013)
p426-SNR52p-gRNA.CAN1.Y-SUP4t	SNR52p-gRNA.*CAN1* Y-*SUP4*t module containing YEp type plasmid	(DiCarlo et al. 2013)

2.2.2 CRISPR-PCDup

Details of the conventional PCDup technology for chromosome duplication has been described previously (Natesuntorn et al. 2015). Briefly, two DNA modules necessary for duplication were prepared as follows. Each DNA module has 50 bp homologous sequence with the target and additionally contains either a selective marker (*Candida glabrata LEU2* [*CgLEU2*] or *CgHIS3* or *URA3*) along with a telomere seed sequence (six copies of a 5′-CCCCAA-3′) or a centromere along with or without selective marker (*CgLEU2* or *CgHIS3*) and telomere seed sequence. p3121 (Sugiyama et al. 2005) was used as a template to add only centromere. p3122 (Sugiyama et al. 2008) was used to add centromere along with *CgLEU2.* p3123 (Sugiyama et al. 2008) was used to add centromere along with *CgHIS3* to the module. p3009 (Sugiyama et al. 2005) was used as a template to prepare the duplication module containing *CgHIS3*. *CEN4* sequence was added to one of the DNA modules so that the resulting new chromosomes possessed one centromere. Template plasmids used for targeting chromosomal regions are listed in Table 1. Primers for constructing the DNA module are listed

in Table S1. gRNA expression plasmids were constructed according to Sasano et al. (2016) and the software CRISPRdirect (https://crispr.dbcls.jp/) was used to select the 20 bp target sequences. Oligonucleotide primers used for the construction of gRNA expression plasmids are listed in Table S2. For targeting each chromosomal region, two gRNA expressing plasmids (Sasano et al. 2016) were introduced (7.5 μg each) along with the corresponding duplicating DNA modules into the transformation mixture. An outline of the CRISPR-PCDup method is



shown in Fig. 1.

Fig. 1. Outline of the CRISPR-PCDup method. gRNA expressing plasmid and duplicating modules are introduced into the SJY30 strain, which harbors a Cas9-expressing plasmid. In transformed cells, CRISPR/Cas9 mediated double-strand breaks (DSBs) are induced near the target site. Chromosome duplication is then facilitated by PCDup. This new technology is named CRISPR-PCDup.

2.2.3 Yeast and *E. coli* transformation

S. cerevisiae was transformed using the lithium acetate method (Gietz and Schiestl 2007). After transformation, SC medium lacking the appropriate amino acids was used for selection of transformants having the marker gene from the duplicating module. *E. coli* was transformed according to the method described by Easmin et al. (2019a).

2.2.4 Colony PCR, pulse-field gel electrophoresis (PFGE) and Southern hybridization

Colony PCR was performed according to the method described by Easmin et al. (2019a). All PCR amplifications were carried out on an Astec PC-320 Program Temp Control System (Astec, Fukuoka, Japan). Pulse field gel electrophoresis and Southern hybridization were performed according to Sasano et al. (2016). PFGE was carried out in CHEF-DR III pulse-field gel electrophoresis system (Bio-Rad) on 1% gel in 0.5 x TBE (Tris-borate-EDTA) buffer at 14°C. After ethidium bromide staining, DNA was transferred onto a Hybond^{TM}-N+ membrane (GE Helthcare) by capillary blotting. Probe labeling, hybridization, and signal detection were carried out by using an ECL^{TM} nucleic acid labeling and detection system (GE Healthcare). The oligonucleotide primers used for amplifying DNA fragments for probes in Southern hybridization are shown in Table S3.

2.2.5 Tetrad analysis

Tetrad analysis was done according to Sugiyama et al. (2006) by using Singer Instruments MSM dissection microscope (Somerset, UK).

2.3 Results

2.3.1 Increasing duplication efficiency by CRISPR-PCDup

PCDup is previously developed technology, which allows duplication of any desired chromosomal region of the *S. cerevisiae* genome (Natesuntorn et al. 2015). In this chapter, I developed CRISPR-PCDup technology, which is an integration of PCDup with CRISPR/Cas9 that facilitates simultaneous and multiple duplication of chromosome segments in *S. cerevisiae*. I reasoned that integration of the CRISPR/Cas9 system with PCDup might increase the frequency of homologous recombination, thereby enabling simultaneous duplication. Initially, I examined whether the CRISPR-PCDup method works more efficiently compared with the previous PCDup technology. Cas9-expressing strain (SJY30) was used as a host strain for chromosome segmental duplication. SJY30 showed no significant growth defect, which suggested that Cas9 expression is not toxic in this strain. I designed gRNA targeting sequences located just outside of the duplicating region and near both edges. The genomic positions chosen for duplication in this study and the gRNA targeting sequences are shown in Table 2 and Table S4, respectively. Initially, I attempted to produce duplication of the Chr3-1 region (1-158020) and Chr3-2 region (157543-316620) on Chromosome 3 separately (Fig. 2). SJY30 strain was transformed with gRNA expressing plasmids and two kinds of duplication modules marked with *CgHIS3* for Chr3-1 and *CEN4*+*CgHIS3* for Chr3-2. Target sequences on these gRNA-expressing plasmids were located near the edge of the Chr3-1 and Chr3-2 regions (Table S4). When the CRISPR-PCDup system was employed, a total of 62 and 1316 His^+ transformants were obtained for the Chr3-1 and Chr3-2 region, respectively (Table 3). By contrast, using 50 bp homology sequence with the target, conventional PCDup gave only 2 and 51 His+ transformants under the same transformation conditions for the Chr3-1 and Chr3-2 region, respectively. I chose 8 transformants at random from those obtained by CRISPR-PCDup for the Chr3-1 and Chr3-2 region and subjected them to pulsefield gel electrophoresis

(PFGE) and subsequent Southern blot analysis to determine whether the duplication event had occurred at the expected locus. The results of this analysis showed that all 8 transformants for duplication of the Chr3-1 and Chr3-2 regions had the expected duplicated chromosome (Fig. 2; i.e. 158 kb band in panel A and 160 kb band in panel B) in addition to intact Chromosome 3 (317 kb band). However, using conventional PCDup, 2 and 4 transformants were analyzed for the Chr3-1 and Chr3-2 region, respectively, but none had the expected duplicated chromosome (Fig. 2A and 2B). Based on these observations, I concluded that CRISPR-PCDup efficiently enhanced segmental duplication of a single chromosomal region.

Table 2. Duplication of various chromosomal regions

Duplication event	**Name of target regions**	**Size and coordinate number of the target region**	**Plasmids used for preparation of the duplicating module**
Single duplication	Chr3-1	Chr3 (1 - 158020) (158 kb)	p3009
	Chr3-2	Chr3 (157543 - 316620) (160 kb)	p3123
	Chr5-3	Chr5 (398496 - 576874) (177 kb)	pSJ70
	Chr15-L1	Chr15 (569775 - 969009) (400 kb)	p3009, p3122
	Chr15-L2	Chr15 (618914 - 969009) (350 kb)	p3009, p3122
	Chr15-L3	Chr15 (670548 - 969009) (300 kb)	p3009, p3122
	Chr15-L4	Chr15 (718509 - 969009) (250 kb)	p3009, p3122
	Chr15-L5	Chr15 (767986 - 969009) (200 kb)	p3009, p3122
Simultaneous double duplication	Chr3-2 and Chr15-L5	Chr3 (157543 - 316620) (160 kb) and Chr15 (767986 - 969009) (200 kb)	p3122 and p3009, p3121
Sequential duplication	Chr3-1	Chr3 (1 - 158020) (158 kb)	pSJ69
	Chr8-1	Chr8 (1 - 202241) (200 kb)	pSJ70
	Chr14-4	Chr14-4 (597394 - 784333)	pSJ23

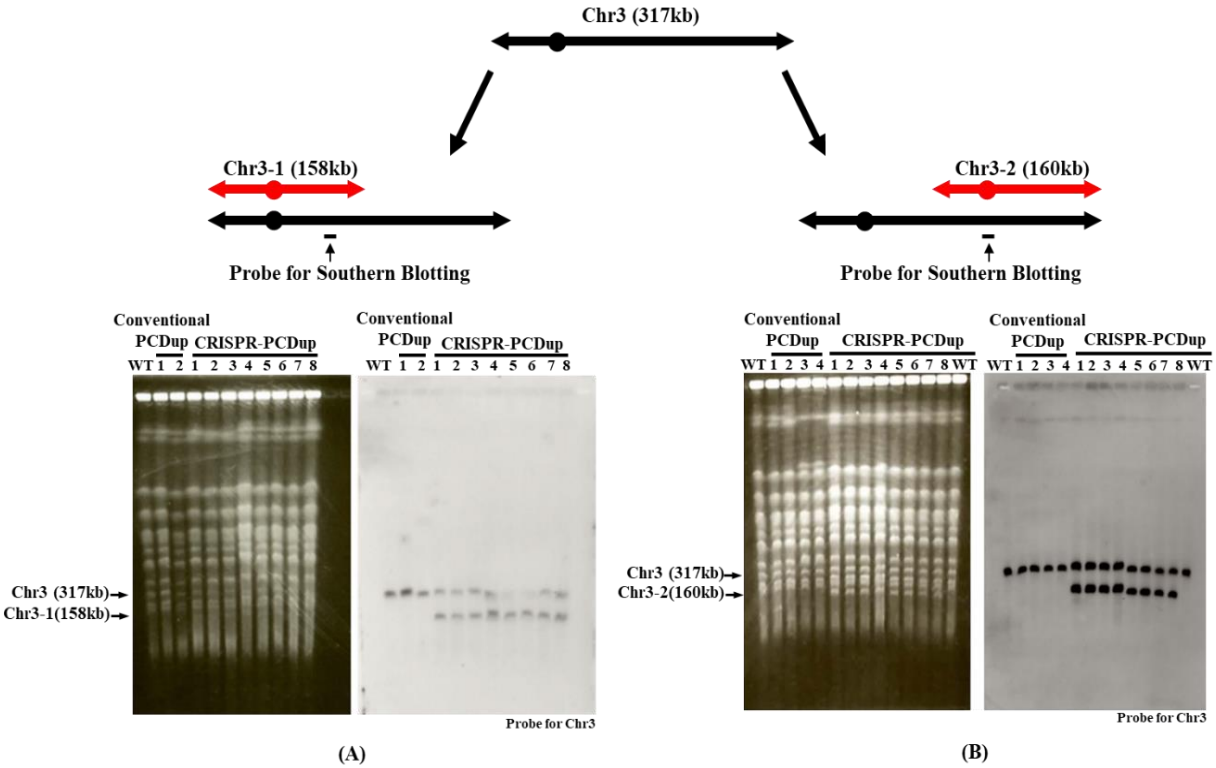


Fig. 2. Duplication of the Chr3-1 and Chr3-2 region. The Chr3-1 and Chr3-2 regions of Chromosome 3 were chosen for the initial experiments. Both duplicating modules were prepared so as to be marked with *CgHIS3* and *CEN4+CgHIS3*, respectively. After transformation, two chromosomes of 158 kb (Fig. 2A) and 160 kb (Fig. 2B) were expected to be generated from Chr3-1 and Chr3-2, respectively. The left and right panel of Fig. 2A and Fig. 2B are PFGE along with the corresponding Southern blot analysis of wild type SJY30; 2 and 4 transformants selected from the conventional PCDup experiment for the Chr3-1 and for the Chr3-2 region, respectively and 8 transformants randomly selected from CRISPR-PCDup for both the Chr3-1 and Chr3-2 regions. The right panel of Fig. 2A and Fig. 2B shows the results of Southern blot analysis for detecting the 317 kb Chromosome 3 and newly duplicated 158 kb and 160 kb chromosomes, respectively.

2.3.2 Simultaneous double duplication by CRISPR-PCDup

Despite numerous attempts, simultaneous duplication of two different genomic regions by conventional PCDup has never been achieved. The results in the previous section revealed that the duplication of a single chromosomal region was possible at high frequency. Next, I attempted to induce a simultaneous duplication of two genomic regions on different chromosomes, namely Chr3-2 (160 kb) and Chr15-L5 (200 kb) (Fig. 3), using our new CRISPR-PCDup approach. I obtained 75 His^+ Leu^+ transformants using CRISPR-PCDup whereas no transformants were obtained using conventional PCDup (Table 3). Of the 75 His^+

Leu$^+$ transformants obtained by CRISPR-PCDup, 25 were randomly selected and analyzed using PFGE and Southern blot analysis to verify whether or not the anticipated double duplication had occurred. Ten of the transformants showed double duplication as evidenced by the presence of a 200 kb and 160 kb band. Fig. 3A, 3B and 3C show 10 out of 25 candidate transformants analyzed by PFGE and Southern blotting. Results show that five out of 10 transformants had a double duplication. When we analyzed the remaining 15 transformants by PFGE and Southern blotting, we found that five transformants had also undergone a double duplication while the other 10 transformants had either a single duplication or no duplication event (data not shown). Therefore, we conclude that simultaneous double duplication is possible by using CRISPR-PCDup.

Table 3. CRISPR-PCDup increases duplication efficiency and induces simultaneous double duplication

Duplicated regions	Method	Number of transformants(n)	Karyotype analysis(n)	Correct transformants(n)
Chr3-1 (158 kb)	CRISPR-PCDup	62	8	8 (100%)
	Conventional PCDup	2	2	0 (0%)
Chr3-2 (160 kb)	CRISPR-PCDup	1316	8	8 (100%)
	Conventional PCDup	51	4	0 (0%)
Chr3-2 (160 kb) and Chr15-L5 (200 kb)	CRISPR-PCDup	75	25	10 (40%)
	Conventional PCDup	0	-	-

Symbol "-" indicates that karyotype analysis and counting the number of correct transformants were not applicable.

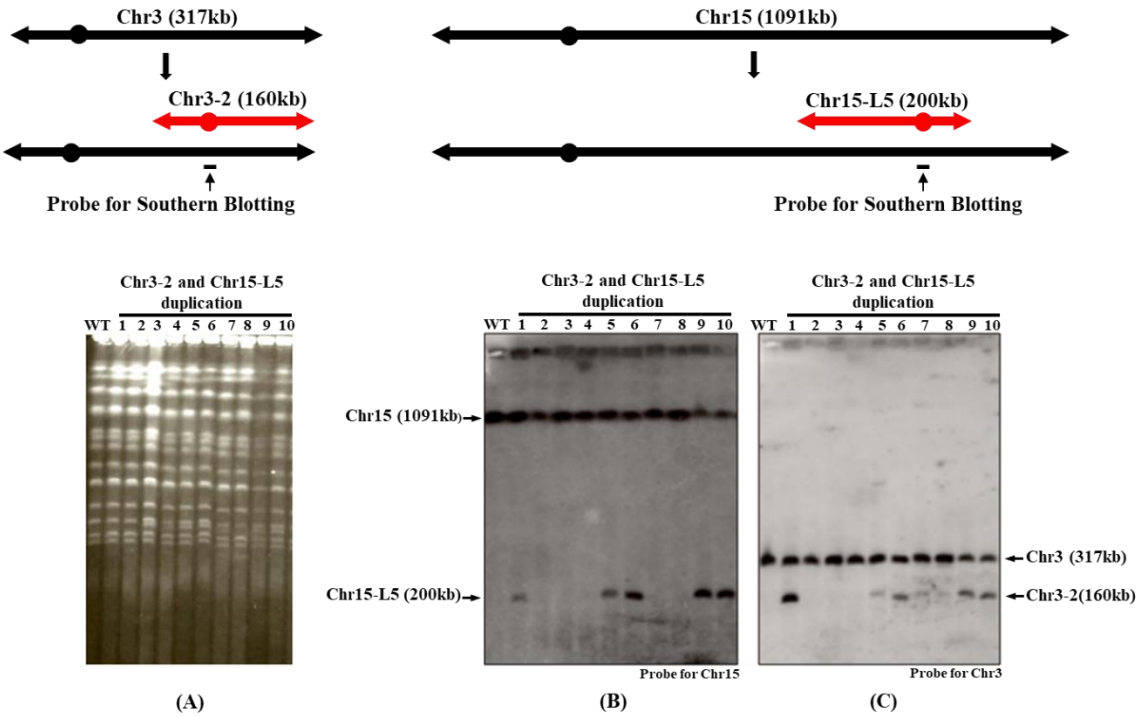


Fig. 3. Simultaneous double duplication in the Chr3-2 and Chr15-L5 regions. The Chr3-2 region of Chr3 and the Chr15-L5 region of Chr15 were simultaneously duplicated by CRISPR-PCDup. The duplicating module of Chr3-2 was marked with *CEN4* and *CgLEU2*; C15-L5 was marked with *CEN4* and *CgHIS3*. After duplication, 2 derived chromosomes are expected to be generated; 160 kb from the C3-2 region and 200 kb from the C15-L5 region. Fig. 3A represents PFGE analysis of the wild-type strain, SJY30 and 10 randomly chosen transformants. Fig 3B and 3C show the results of Southern blot analysis for detecting Chr15 (1091 kb) and the newly duplicated 200 kb chromosome along with Chr3 (317 kb) and the newly duplicated 160 kb chromosome, respectively.

2.3.3 Synthetic lethality is not caused by simultaneous double duplication

After successfully duplicating two chromosomal regions simultaneously using CRISPR-PCDup technology, I next attempted to duplicate other chromosomal regions in two different chromosomes, namely Chr3-1 (158 kb) (1-158020) and Chr8-1 (200 kb) (1-202241). I used DNA modules harboring the *CgLEU2* marker to duplicate the Chr3-1 region and the *CgHIS3* marker to duplicate the Chr8-1 region. Leucine- and histidine-minus (-Leu-His) medium was used to subsequently select His^+ Leu^+ transformants. 15 His^+ Leu^+ transformants were obtained and these transformants were checked by colony PCR. None of the 15 transformants showed simultaneous duplication of the Chr3-1 and Chr8-1 regions. Next, another combination comprising the Chr3-1 and Chr14-4 (184 kb) (597394-784333) regions

were chosen for simultaneous duplication mediated by CRISPR-PCDup. I used DNA modules harboring the *CgLEU2* marker to duplicate the Chr3-1 region and the *URA3* marker to duplicate the Chr14-4 region. Leucine- and uracil- minus (-Leu-Ura) medium was used to select Leu^+Ura^+ transformants. 66 Leu^+Ura^+ transformants were isolated and 15 transformants subsequently checked by colony PCR. The results revealed that none of the 15 transformants showed simultaneous duplication of the Chr3-1 and Chr14-4 regions.

Unsuccessful double duplication may be due to synthetic lethality caused by simultaneously duplicating these two sets of chromosomal regions. To investigate this possibility, I attempted to construct the double duplication in a sequential manner. Initially, the Chr3-1 region was duplicated and an attempt was made to duplicate the Chr8-1 region. In all, 155 His^+Leu^+ transformants were obtained and 5 transformants were arbitrarily picked for colony PCR analysis. The colony PCR revealed that of the 5 transformants, 1 gave the expected result (Fig. 4A and 4B). I also attempted to sequentially duplicate the Chr14-4 region in the Chr3-1 duplicated transformants. In total, 796 Leu^+Ura^+ transformants were isolated and 15 were arbitrarily picked for colony PCR. Of these, 7 transformants had the expected double duplication (Fig. 4C and 4D). Primers used for colony PCR are listed in Table S5.

These findings indicated that sequential double duplication of the Chr3-1 and Chr8-1 regions as well as the Chr3-1 and Chr14-4 regions is possible. I also used an alternative approach to further confirm synthetic lethality is not caused by simultaneous double duplication. Tetrad analysis of diploids was conducted to investigate whether double duplication causes synthetic lethality or not by mating transformants harboring two single duplicated regions. For this purpose, mating type α host SJY30 was chosen and the Chr3-1 region was duplicated using the *CgLEU2* harboring DNA module. In a separate experiment, I took mating type **a** host SJY415 and duplicated the Chr5-3 (177 kb) region with the *CgHIS3* harboring DNA module (data not shown). A diploid is then constructed by mating two

transformants harboring either the Chr3-1 or Chr5-3 duplicated regions. After making diploids, tetrad analysis was performed. This analysis revealed that $Leu^{+}His^{+}$ spores were viable, confirming that double duplication of these two regions is not lethal. Overall, these observations suggest that unsuccessful obtaining of double duplication is not due to synthetic lethality caused by double duplication. I will discuss the possible reasons for this observation in Discussion section.

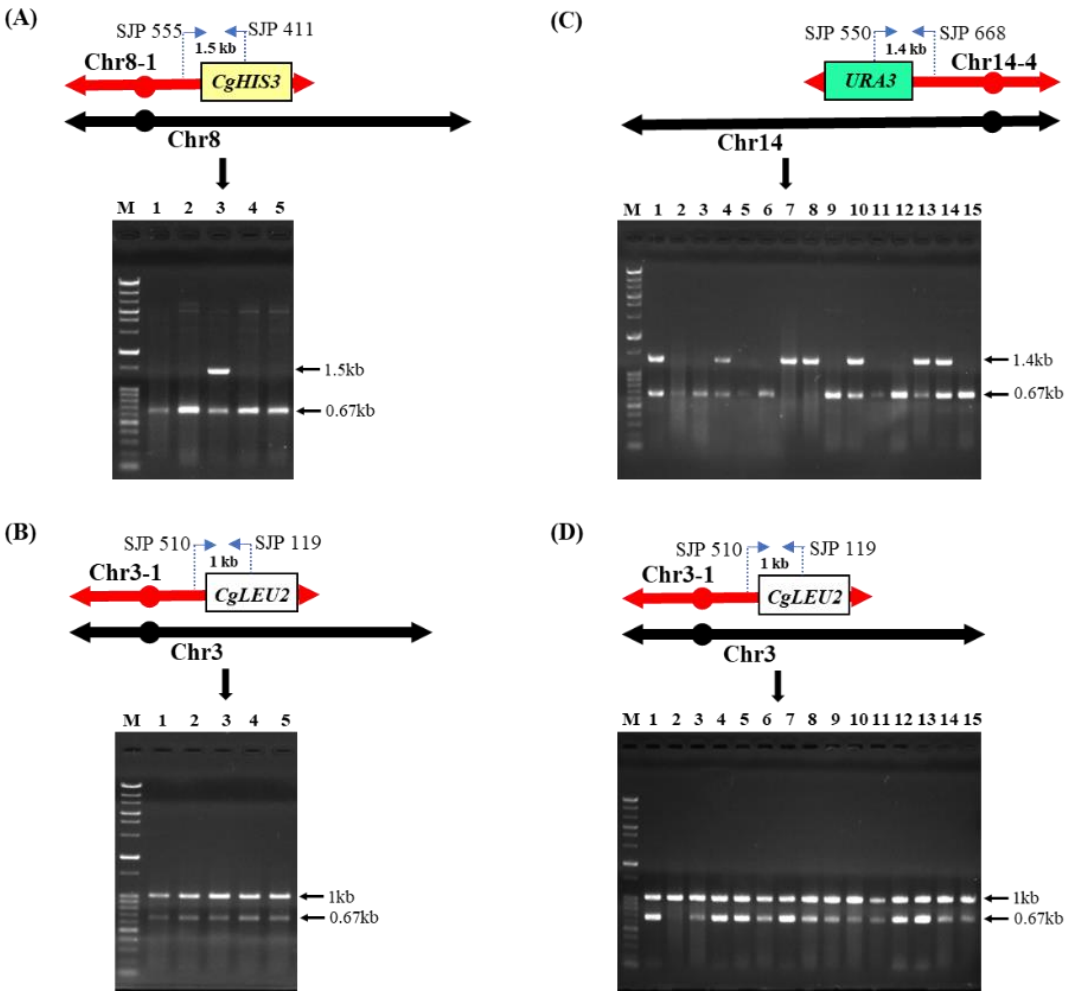


Fig. 4. Colony PCR analysis of Chr3-1 and Chr8-1 as well as Chr3-1 and Chr14-4 sequentially duplicated transformants. In the colony PCR, each lane represents independent-transformants. Fig. 4A and 4B represents the sequential duplication of the Chr3-1 and Chr8-1 regions, respectively. In Fig. 4A, primers SJP 555 and SJP 411 were used to amplify the 1.5 kb band from the duplicated Chr8-1 region and in Fig. 4B, primers SJP 510 and SJP 119 were used to amplify the 1 kb band from the duplicated Chr3-1 region. Fig. 4C and 4D represent the sequential duplication of the Chr3-1 and Chr14-4 regions, respectively. In Fig. 4C, primers SJP 550 and SJP 668 were used to amplify the 1.4 kb band

from the duplicated Chr14-4 region and in Fig. 4D, primers SJP 510 and SJP 119 were used to amplify the 1 kb band from the duplicated Chr3-1 region. In all PCR analyses the 0.67kb *CNE1* gene on chromosome 1 was also amplified as an internal control by a common set of primers SJP 121 and SJP 242.

2.3.4 Upper size limit of duplication by CRISPR-PCDup

It was previously reported that up to 300 kb of chromosomal region could be duplicated by conventional PCDup (Natesuntorn et al. 2015). However, in this study using CRISPR-PCDup I successfully duplicated single chromosomal regions more efficiently than using the conventional PCDup procedure. Thus, I examined whether the upper size limit of the duplicated regions is increased using CRISPR-PCDup technology. For this purpose, I attempted to construct a series of segmentally duplicated chromosomes of increasing size (200 kb, 250 kb, 300 kb, 350 kb and 400 kb of Chr15), (Table 4). I found that all the regions could be duplicated (Fig. 5A and 5B). In the case of conventional PCDup, I did not get any transformants for the duplication of 200 kb to 400 kb. Previously, Natesuntorn et al. (2015) were able to duplicate up to 300 kb using conventional PCDup by employing 400 bp homology sequence in the DNA module for homologous recombination. By contrast, in this study, I used a 50 bp homology sequence in the DNA module along with CRISPR/Cas9. Despite this much shorter homology sequence, I achieved duplication of up to 400 kb. I believe that introduction of a DSB mediated by CRISPR/Cas9 enabled at least 400 kb duplication even when a relatively short 50 bp homology sequence was employed. Thus, in the absence of CRISPR/Cas9, a 50 bp homology might be insufficient for successful homologous recombination to occur.

Table 4. CRISPR-PCDup can duplicate up to 400kb of chromosomal region

Size of the duplication (Name of region)	gRNA	No. of transformants	Proportion of correct transformants
400 kb	+	25	20% (2/10)
(Chr15-L1)	-	0	-
350 kb	+	40	20% (2/10)
(Chr15-L2)	-	0	-
300 kb	+	733	3% (1/35)
(Chr15-L3)	-	0	-
250 kb	+	114	20% (2/10)
(Chr15-L4)	-	0	-
200 kb	+	120	90% (9/10)
(Chr15-L5)	-	0	-

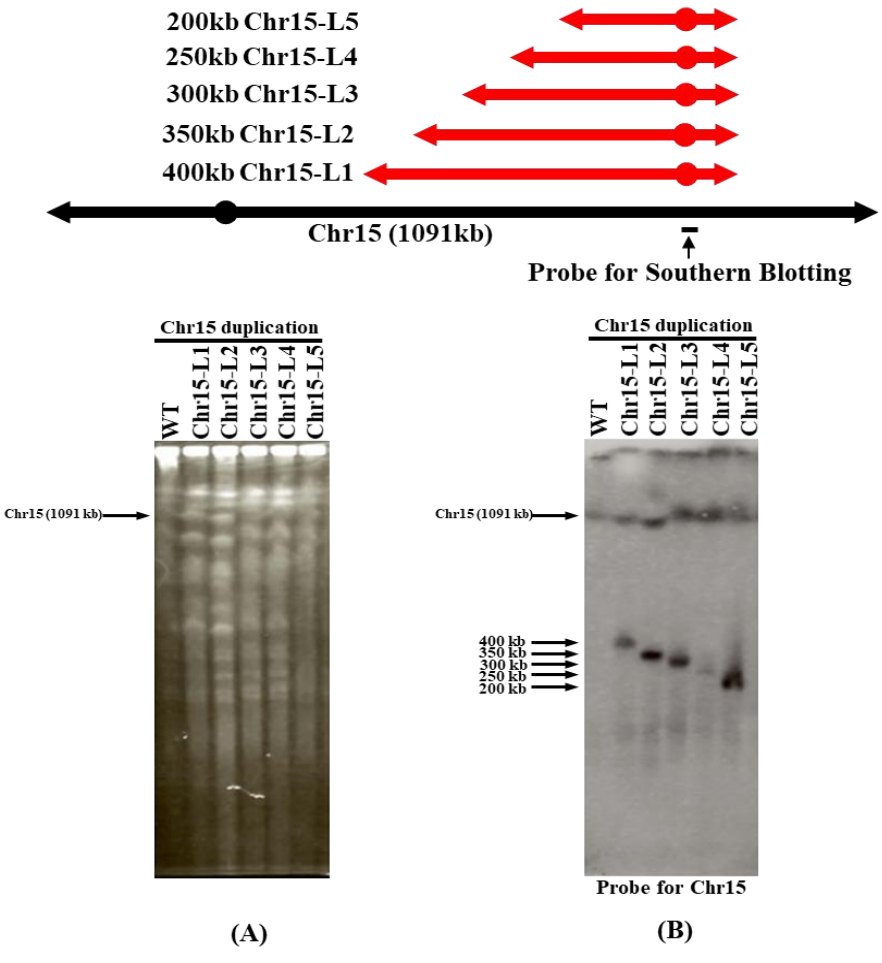


Fig. 5. Duplication of the 200 kb to 400 kb chromosomal region in Chr15. Region Chr15-L1 to Chr15-L5 of Chr15 was selected. All the duplicating modules were marked with *CgHIS3* along with *CEN4* and *CgLEU2*. Fig. 5A represents PFGE analysis of wild type SJY30 and transformants obtained from the duplication experiments for the Chr15-L1, Chr15-L2, Chr15-L3, Chr15-L4 and Chr15-L5 regions by CRISPR-PCDup. Fig. 5B shows the results of Southern blot analysis after PFGE for the detection of the newly generated 400 kb, 350 kb, 300 kb, 250 kb, and 200 kb chromosomes, respectively.

2.4 Discussion

In this chapter, I have developed a novel chromosome engineering technology by combining CRISPR/Cas9 system with PCDup technology which I called CRISPR-PCDup. Since integration of CRISPR/Cas9 system into PCDup method may increase homologous recombination frequency, I expected that CRISPR-PCDup enables targeting multiple chromosomal regions to be duplicated by a single transformation. Previously, DiCarlo et al. (2013) reported that foreign donor DNA was integrated with nearly 100% frequency at the target site when a DSB is induced by CRISPR/Cas9 in *S. cerevisiae*. Indeed, in this study, I found that duplication efficiency was increased approximately 25 to 30 fold when targeting a single site with the help of CRISPR/Cas9 (Table 3). In addition, the proportion of transformants analyzed with the desired karyotype by conventional PCDup was 0%. By contrast, 100% of randomly selected transformants obtained using the CRISPR-PCDup method possessed the anticipated chromosomal changes (Table 3). Although this technology has not yet been tested for other chromosomal regions, I suppose that CRISPR-PCDup may duplicate any chromosomal regions with significantly greater efficiency than conventional PCDup. Besides, a significant increase in the efficiency of a single duplication event is probably the reason for the success of simultaneous double duplications of at least two large chromosomal regions. I believe the enhanced efficiency of this new method arises from the DSBs induced by CRISPR/Cas9 that stimulate an increased rate of homologous recombination.

Next, I attempted simultaneous duplication of two sets of chromosomal regions named as Chr3-1 and Chr8-1 as well as Chr3-1 and Chr14-4 but I did not get simultaneous double duplications in these two cases. Natesuntorn et al. (2015) proposed that duplicating

chromosomal regions requires chromosome nondisjunction as one of possible mechanisms. It is likely that incorporation of CRISPR/Cas9 has an influence on homologous recombination but not that on chromosome nondisjunction. Therefore, I think that the frequency of chromosome nondisjunction is the same even if I incorporated CRISPR/Cas9 system into PCDup method. In this case, if the frequency of homologous recombination is not so high in two target sites, I may not get double duplication. By contrast, since sequential duplication needs only one homologous recombination for each transformation followed by possible chromosome nondisjunction, I think that this may be the reason by which I got duplication of Chr3-1 and Chr8-1 as well as Chr3-1 and Chr14-4 regions in a sequential manner but not simultaneously. On the other hand, I got success to duplicate Chr3-2 and Chr15-L5 regions simultaneously when I incorporated CRISPR/Cas9 system. Therefore, I think that possible reason for getting this success of obtaining double duplication simultaneously is that the frequency of homologous recombination became significantly higher by using CRISPR/Cas9 system compared with that of conventional PCDup method. Although it is not so easy to directly estimate the frequency of homologous recombination which is needed to duplicate multiple chromosomal regions simultaneously, I suggest that increased frequency of homologous recombination may contribute to the success of getting simultaneous duplication of Chr3-2 and Chr15-L5 regions.

In this study, I was able to lengthen the regions to be duplicated to 400 kb which is 100 kb larger than the longest duplication (300 kb) by conventional PCDup. In a previous study Natesuntorn et al. (2015) proposed that the upper-size limitation of chromosome duplication might be controlled by the frequency of chromosome nondisjunction because the rate of chromosome nondisjunction decreases as the length of the chromosome increases (Hieter et al. 1985). According to this data, larger duplicated chromosomes give rise to decreased rate of chromosome nondisjunction, but I believe that the number of resultant duplicated regions was

increased significantly by CRISPR/Cas9 before chromosome nondisjunction occurs. As a result, chance of obtaining a longer duplicated chromosome might be increased and I think that this is the reason why I got viable transformants harboring 400 kb duplicated chromosome.

Simultaneous segmental duplication of multiple chromosomal regions is not reported in any organism. Here, I have demonstrated for the first time that it is possible to simultaneously duplicate two large segments of chromosomal regions (160 kb and 200 kb) using newly developed CRISPR-PCDup technology. Since segmental aneuploidy are occasionally found in industrial yeast strains displaying robustness (Borneman et al. 2011, Dunn et al. 2012) and it was also previously revealed that duplicating several chromosomal regions gives rise to stress resistance against ethanol, high temperature, acetic and sulfuric acid (Natesuntorn et al. 2015), I think that CRISPR-PCDup technology should contribute to investigating combinatorial effect of segmental aneuploidy in an efficient way. Moreover, since duplicated chromosomes act as independent chromosomes comprising extra-copies of many genes, those chromosomes may be suitable for studying the effect of over-expression of many genes on cell physiology. In conclusion, CRISPR-PCDup is a promising tool not only for generating yeast strains that exhibit desired industrial traits but also for studying the fundamentals of genome function.

2.5 Summary and conclusion of Chapter 2

Previously, PCR-mediated chromosome duplication (PCDup), was developed in *Saccharomyces cerevisiae* that enabled the duplication of any desired chromosomal region, resulting in a segmental aneuploid. From one round of transformation, PCDup can duplicate a single chromosomal region efficiently. However, simultaneous duplication of multiple chromosomal regions is not possible using PCDup technology, which is a serious drawback. Sequential duplication is possible, but this approach requires significantly more time and effort.

Because PCDup depends upon homologous recombination, I reasoned that it might be possible to simultaneously create duplications of multiple chromosomal regions if I could increase the frequency of these events. Double-strand breaks have been shown to increase the frequency of homologous recombination around the breakpoint. Thus, I aimed to integrate the genome editing tool CRISPR/Cas9 system, which induces double-strand breaks, with conventional PCDup. The new method, which I named CRISPR-PCDup increased the efficiency of a single duplication by up to 30 fold. CRISPR-PCDup enabled the simultaneous duplication of long chromosomal segments (160 kb and 200 kb regions). Moreover, I was also able to increase the length of the duplicated chromosome by up to at least 400 kb, whereas conventional PCDup can duplicate up to a maximum of 300 kb. Given the enhanced efficiency of chromosomal segmental duplication and the saving in both labor and time, I propose that CRISPR-PCDup will be an invaluable technology for generating novel yeast strains with desirable traits for specific industrial applications and for investigating genome function in segmental aneuploid.

Chapter 3

Systematic approach for assessing whether a particular chromosomal region is essential in *Saccharomyces cerevisiae* for cell viability

3.1 Introduction

Discovering genetic interaction networks is required for identifying novel genes and pathways and for predicting similar networks in genomes. Baker's yeast *Saccharomyces cerevisiae* is largely used and best characterized single-celled eukaryotic model for the study of a variety of biological processes (Karathia et al. 2011). More than 80% of the genes in *S. cerevisiae* are not required for cell proliferation in nutrient medium. This makes *S. cerevisiae* an useful experimental organism to reveal the function of non-essential genes (Winzeler et al. 1999; Giaever et al. 2002). The inactivation of some non-essential genes in specific combinations can have a lethal effect (Novick et al. 1989; Guarente 1993). This property also makes the yeast genome resistant to engineering and could be problematic for generating new strains. Synthetic lethal genetic interactions have been extensively studied in *S. cerevisiae* using synthetic genetic array (SGA) analysis, in which a query mutation is systematically crossed with almost all viable deletion mutants to obtain double-mutant meiotic progeny (Tong et al. 2001, 2004; Giaever et al. 2002). However, formation of double mutants in SGA analysis depends on meiotic recombination. Double mutant construction is not possible if the two genes to be combined are tightly linked on the same chromosome. As a consequence, numerous linked gene-pairs that form small colonies of double mutants have been overlooked in SGA (Kaboli et al. 2014).

To overcome the limitation of constructing double mutants of two tightly linked genes on the same chromosome, PCR-mediated chromosome deletion technology (PCD) was developed. Deletion of all regions harboring only non-essential genes throughout the genome led to the interesting discovery that 49 chromosomal regions were undeletable (Kaboli et al. 2014). This result indicates that there might be unknown lethal combinations of non-essential genes present in these 49 undeletable regions, which were not reported by SGA. This finding motivated me to identify the genes responsible for the synthetic lethality in all of the 49

undeletable regions. In this study, I chose four of the smallest undeletable regions from these 49 regions and attempted to narrow down the genes responsible for the synthetic lethality by replacing the sub-regions with DNA modules harboring markers in various combinations.

3.2 Materials and methods

3.2.1 Strains, plasmids, and media

The strains and the plasmids used in this study are listed in Table 5. *S. cerevisiae* strain SJY4 was used as a parental strain for the replacement of chromosomal regions. The strain SJY576, where the Chr2-6 (Chromosome 2: 318749-330960) region was replaced by a DNA module harboring *CgLEU2*, was used as a host strain for splitting the left edge of *CgLEU2*. The strain SJY577, transformants of SJY576 where the left edge of *CgLEU2* was split, was used as a host strain for splitting the right edge of *CgLEU2*. I used the loxP site-deleted plasmid pSJ69 (Easmin et al. 2019a) and pSJ70 (Easmin et al. 2019b) derived from p3008 and p3009, respectively (Sugiyama et al. 2005) as templates in which loxP-flanked DNA sequences were deleted to avoid undesired site-specific recombination. The plasmid pSJ69 harboring selective marker *CgLEU2* was used as a template to synthesize a DNA module for replacement of a particular chromosomal region. The plasmid pSJ70 harboring *CgHIS3*, loxP site-deleted plasmid pSJ23 (Easmin et al. 2019b) harboring a *URA3* marker and the plasmid p3121 (Sugiyama et al. 2005) harboring *CEN4* were used to split the left and right edge of the DNA module-replaced chromosomal region. The plasmids p3121 and pSJ23 were used to construct DNA modules to duplicate target chromosomal regions.

Yeast strains were grown at 30˚C in YPDA medium (See Chapter 2). Supplemented minimal medium (0.67% yeast nitrogen base without amino acids [Difco, Sparks, MD, USA], 0.2% to 1% amino acids [L-Leucine (Wako), L-Histidine (Wako), L-Lysine HCL(Wako), L-Methionine (Wako) and L-Tryptophan (Wako)] or nucleic acid bases [Adenine HCL (Wako),

Uracil (Kishida, Osaka, Japan)] and 2% glucose) lacking specific amino acids or nucleic acid bases were used to select transformants to examine auxotrophic phenotypes. 5-Fluoroorotic acid (5-FOA) medium, prepared according to Kaboli et al. (2014), was used to screen clones for the presence of the *URA3* marker gene. For plate assays, agar (2% w/v) was added to solidify the medium.

3.2.2 Preparation of DNA modules

Several types of DNA modules to replace, split or duplicate target regions were prepared by PCR. To construct DNA modules for replacing target regions, the forward primer was designed by choosing a 50 bp sequence just prior to the target region using the Saccharomyces Genome Database (SGD: www.yeastgenome.org) and an additional 20 bp sequence homologous to the 5'-GGCCGCCAGCTGAAGCTTCG-3' sequence (7–26th nucleotide position) of plasmid pSJ69. Likewise, the reverse primer was also designed by choosing a 50 bp reverse sequence just after the respective target region using SGD and an additional 20 bp reverse sequence homologous to the 5'- AGGCCACTAGTGGATCTGAT-3' sequence (1602–1621th nucleotide position) of plasmid pSJ69 (Fig. 6). Splitting modules were prepared according to Sasano et al. (2016) by using pSJ70, pSJ23, and p3121 as template plasmids. The duplication module was prepared according to Natesuntorn et al. (2015) with slight modification. Specifically, rather than a 400 bp homology region used by Natesuntorn et al. (2015), I used a 50 bp homology sequence to duplicate the target regions. Primers used for making DNA modules for replacement, splitting or duplicating target regions are listed in Table S6.

Table 5. Strains and plasmids used in this study

Strain or Plasmid	Description	Remarks/Reference
Strains		
SJY4	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Winston et al. 1995
SJY576	Leu^+ Transformant of SJY4, Chr2-6 region replaced with *CgLEU2*	This study
SJY577	His^+ Transformants of SJY576 for left edge splitting of *CgLEU2* module with *CgHIS3*	This study
SH30072	Ura^+ Transformant of SJY4, harboring Chr1-2 region in the mini-chromosome	Kaboli et al. 2014
SH30075	Ura^+ Transformant of SJY4, harboring Chr2-2 region in the mini-chromosome	Kaboli et al. 2014
SH30077	Ura^+ Transformant of SJY4, harboring Chr2-4 region in the mini-chromosome	Kaboli et al. 2014
SH30079	Ura^+ Transformant of SJY4, harboring Chr2-6 region in the mini-chromosome	Kaboli et al. 2014
SH30080	Ura^+ Transformant of SJY4, harboring Chr2-7 region in the mini-chromosome	Kaboli et al. 2014
SH30084	Ura^+ Transformant of SJY4, harboring Chr3-2 region in the mini-chromosome	Kaboli et al. 2014
Plasmids		
pSJ23	A derivative of pUG6 carrying *URA3*	Easmin et al. 2019b
pSJ69	loxP site-deleted p3008	Easmin et al. 2019a
pSJ70	loxP site deleted p3009	Easmin et al. 2019b
p3121	The *CEN4* module containing plasmid constructed by modifying pUG6	Sugiyama et al. 2005

3.2.3 Yeast transformation, colony PCR

Yeast transformation and colony PCR were performed according to Chapter 2. Primers used for colony PCR used to check replacement, splitting and duplication are listed in Table S7. Spot assay was performed according to Kaboli et al. (2014)

3.2.4 Mini-chromosome loss assay and spot assay

Transformants to be tested were cultivated overnight in YPDA liquid medium and after serial dilution, cells were plated on YPDA plates (master plate) and incubated at 30°C for 48 hours. Colonies were replica-plated on SMM (Synthetic Minimal Media) plates without uracil (Ura minus), without leucine (Leu minus), without leucine and uracil (Leu minus and Ura minus), 5-FOA and fresh YPDA plates and incubated at 30°C for 24 to 72 hours. Spot assay was performed according to Kaboli et al. (2014)

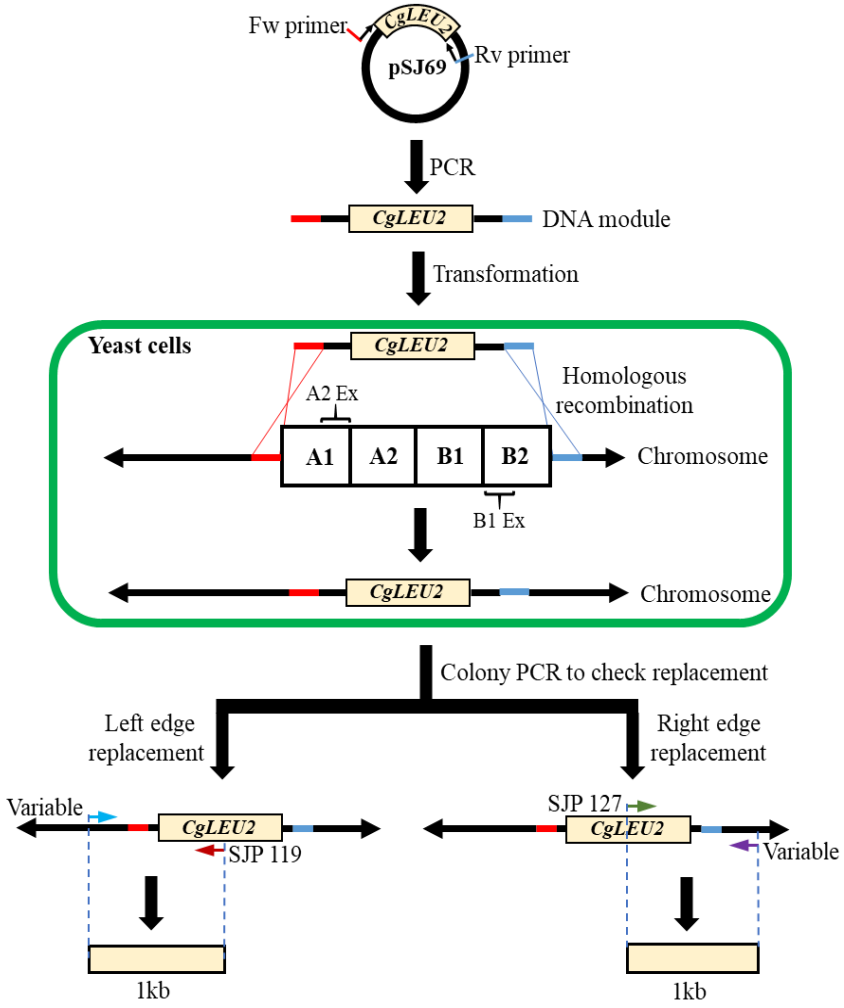


Fig. 6. Overview of replacement analysis of target region. The target region is replaced by DNA module harboring *CgLEU2*. For amplification of DNA module, forward and reverse primer was designed to anneal with the plasmid pSJ69 and DNA module was amplified by PCR. DNA module has 50 bp homology sequence with the target region (harboring A1, A2, A2 (Ex), B1, B1 (Ex) and B2 sub-regions) in both edges and after transformation in yeast cell, target region was replaced by the DNA module through homologous recombination. Then, transformants were checked by colony PCR; replacement of left edge of target region was checked with forward primer depending upon the target region and reverse primer SJP 119 which leads to production of 1kb band. On the other hand, replacement of right

edge of target region was checked with forward primer SJP 127 and reverse primer depending upon respective region which also amplify 1kb band.

3.3 Results

3.3.1 Identification of non-essential genes responsible for synthetic lethality of undeletable chromosomal regions

According to the previous study (Kaboli et al. 2014), 49 chromosomal regions containing only non-essential genes were identified to be undeletable from the *S. cerevisiae* genome. These observations indicate that yeast cells cannot survive if these regions are deleted and further suggests that the regions are likely to harbor genes responsible for synthetic lethality. To identify genes responsible for the synthetic lethality, I embarked on a systematic study of all 49 regions with the aim of pinpointing gene-pairs in the respective undeletable regions. As a part of this comprehensive project, I selected four of the smallest chromosomal regions, i.e., Chr2-6 (Chromosome 2: 318749-330960; 12.2 kb), Chr9-2 (Chromosome 9: 87850-102249; 14.4 kb), Chr2-2 (Chromosome 2: 21866-37346; 15.5 kb) and Chr11-2 (Chromosome 11: 188434-204755; 16.3 kb) (Saccharomyces Genome Database: www.yeastgenome.org). Here, I employed an approach to narrow down the regions responsible for synthetic lethality by using genome engineering technology. For this purpose, I divided each region into 6 sub-regions called A1, A2, A2 Extension (Ex), B1, B1 Extension (Ex) and B2 (Fig. 6). Then, I attempted to delete these sub-regions through replacement of DNA modules in various combinations for all regions. The combinations that we tested were A1+A2, B1+B2, A1+A2+B1, A2+B1+B2, A1+A2+B1 (Ex) and A2 (Ex)+B1+B2. Results of the replacement by transformation experiments of these regions are shown in Table 6. If replacement of a particular sub-region results in lethality, transformants should not be obtained. By contrast, if deletion of the same sub-region does not lead to lethality, viable transformants will be obtained. In all cases a substantial number of viable transformants were isolated (Table

6). Transformants were verified by randomly picking two to six of them and performing structural analysis of the chromosomes by colony PCR. In colony PCR, the replacement of the left and right edge of the respective chromosomal sub-regions were checked (Fig. 7A to 7F; Fig. S1, S2 and S3). Results of colony PCR revealed that most of the transformants had the expected chromosomal structure, indicating that each targeted chromosomal sub-region was replaced by a DNA module harboring *CgLEU2* (Table 6). From these observations, I concluded that none of the sub-regions harbor genes responsible for synthetic lethality.

These observations motivated me to check whether the whole region could be replaced by the *CgLEU2* marker. First, transformation experiments were performed to replace the entire Chr2-6 region. In this experiment, I obtained 759 transformants for the replacement of the Chr2-6 region (Table 6) and subsequent analysis by colony PCR showed that six out of six transformants had the expected structural alteration (Fig. 7G), indicating that the entire Chr2-6 region could be replaced with *CgLEU2*. I also conducted a similar experiment for the other three chromosomal regions, Chr2-2, Chr9-2, and Chr11-2. These studies showed the other three chromosomal regions could also be replaced by the *CgLEU2* marker without causing lethality (Fig.8). These results are inconsistent with previous findings (Kaboli et al. 2014), which showed that these regions cannot be deleted. However, the methodology in the previous work was different. Thus, I performed additional experiments described in the next section to explore the apparent inconsistency.

3.3.2 Transformants harboring a mini-chromosome comprising only genetic markers are viable

I noted the following difference in methodology between this study and the previous study might explain the apparently contradictory results. In the previous study, Kaboli et al. (2014) constructed a mini-chromosome comprising target regions marked with the *URA3* gene

by PCR-mediated one-step splitting (PCS) technology. Mini-chromosome loss assays were then performed to analyze whether a particular region was essential for cell viability. In all, 49 regions were found to be undeletable. This conclusion was based upon the observation that they did not see colony formation by transformants harboring the mini-chromosome on 5-FOA medium. By contrast, in this study, deletion through replacement of four out of the 49 regions with a DNA module did not result in lethality. To investigate why deletion through replacement of these chromosomal regions rather than simple deletion gave viable transformants, I split the left and right edge of one of the replaced regions (Chr2-6) by PCS technology (Sasano et al. 2016). Then, I constructed a mini-chromosome consisting of *CgLEU2* and *URA3* marker for performing mini-chromosome loss assays by the 5-FOA method.

For generating a mini-chromosome, I first split (between nucleotide no. 318748 of Chromosome 2 and nucleotide no. 1 of *CgLEU2* sequence) the left edge of the Chr2-6 region, which had been replaced by a DNA module containing the *CgLEU2* marker. This experiment was done by using two kinds of splitting modules, one of which contained the *CgHIS3* marker and the other contained *CEN4* (Fig. 9A). Transformants were selected on SMM medium without leucine and histidine. In all, 827 Leu^+ His^+ transformants were obtained (Table 7), ten of which were arbitrarily picked for analysis by colony PCR. Eight of the ten transformants had the anticipated splitting at the left edge of the Chr2-6 replaced region (Fig. 9A). Among these eight transformants, one (called SJY577) was selected for subsequent splitting (between nucleotide no. 1685 of *CgLEU2* sequence and nucleotide no. 330961 of Chromosome 2) at the right edge of the *CgLEU2* marker of a newly generated split chromosome. In this transformation experiment, I used two splitting DNA modules; one module contained the *URA3* marker and the other module contained *CEN4* (Fig. 9B). I selected transformants on SMM medium without leucine, histidine, and uracil. Five out of 917 Leu^+ His^+ Ura^+ transformants obtained (Table 7) were arbitrarily picked and checked by colony PCR. Two

out of five transformants had the expected splitting at the right edge of the Chr2-6 replaced region (Fig. 7B). In this way, Chromosome 2 was split into three parts to generate a mini-chromosome comprising only *CgLEU2* (DNA module) and the *URA3* marker. These cells, like those harboring unsplit Chromosome 2, were viable despite the entire Chr2-6 chromosomal region being deleted from the genome.

3.3.3 Assessing whether the newly generated mini-chromosome is essential for cell viability

Cells harboring a mini-chromosome were cultivated in liquid YPDA medium, plated on YPDA plate (treated as a master plate for replica plating) and replica-plated on Ura minus and Leu minus medium, 5-FOA along with YPDA medium (as a control) and incubated for 24 hours (Fig. 10). Two kinds of colonies were observed on the YPDA master plate (Fig. 10). One type of colony (Type 1) showed growth on 5-FOA and YPDA control media but no growth on Ura minus and Leu minus media (i.e. Ura$^-$ and Leu$^-$ colonies). The second type of colony (Type 2) showed growth on Ura minus and Leu minus media and YPDA control plates (i.e. Ura$^+$ and Leu$^+$ colonies) but no growth on 5-FOA medium. Because Ura$^-$ (and Leu$^-$) cells are considered to have lost the mini-chromosome, growth of these cells on YPDA and 5-FOA medium indicates that the mini-chromosome is not required for viability. This result confirmed the findings described in the previous section. However, there remains an apparent inconsistency with the results obtained by Kaboli et al. (2014), which showed that loss of the Chr2-6 region was lethal to the cells. I reasoned that there might be an unknown suppressor mutation somewhere in the 16 chromosomes that suppresses lethality. Indeed, there is intrinsic selection pressure to isolate suppressor mutations that suppress lethality caused by deletion of an essential region of the chromosome. To explore this hypothesis, I performed further experiments described in the next section.

Table 6. Replacement of various chromosomal regions

Name of the region (Co-ordinate number)	Replaced Sub regions (Co-ordinate number)	Number of transformants	Number of transformants analyzed	Transformants with expected replacement	Replaceable/ Non-replaceable
Chr2-2 (21866-37346)	A1+A2 (21866-29606)	771	6	6	Replaceable
	B1+B2 (29606-37346)	1001	6	5	Replaceable
	A1+A2+B1 (21866-33476)	200	6	4	Replaceable
	A2+B1+B2 (25737-37346)	611	6	6	Replaceable
	A1+A2+B1 (Ex) (21866-36036)	314	6	4	Replaceable
	A2 (Ex)+B1+B2 (24473-37346)	289	6	6	Replaceable
	A1+A2+B1+B2 (21866-37346)	494	6	4	Replaceable
Chr2-6 (318749-330960)	A1+A2 (318749-324853)	930	6	6	Replaceable
	B1+B2 (324856-330960)	1171	5	5	Replaceable
	A1+A2+B1 (318749-327908)	411	6	6	Replaceable
	A2+B1+B2 (321802-330960)	626	6	6	Replaceable
	A1+A2+B1 (Ex) (318749-329133)	316	6	5	Replaceable
	A2 (Ex)+B1+B2 (321064-330960)	256	6	6	Replaceable
	A1+A2+B1+B2 (318749-330960)	769	6	6	Replaceable
Chr9-2	A1+A2 (87850-95050)	731	5	5	Replaceable
	B1+B2 (95050-102249)	703	2	1	Replaceable

(87850 102249)	A1+A2+B1 (87850-98650)	400	6	4	Replaceable
	A2+B1+B2 (91451-102249)	452	6	3	Replaceable
	A1+A2+B1 (Ex) (87850-100181)	313	6	6	Replaceable
	A2 (Ex)+B1+B2 (89524-102249)	211	6	6	Replaceable
	A1+A2+B1+B2 (87850-102249)	445	6	3	Replaceable
Chr11-2 (188434-204755)	A1+A2 (188434-196595)	922	6	6	Replaceable
	B1+B2 (196595-204755)	965	6	6	Replaceable
	A1+A2+B1 (188434-200675)	443	6	2	Replaceable
	A2+B1+B2 (192515-204755)	627	6	6	Replaceable
	A1+A2+B1 (Ex) (188434-201328)	364	6	6	Replaceable
	A2 (Ex)+B1+B2 (190334-204755)	217	6	4	Replaceable
	A1+A2+B1+B2 (188434-204755)	515	6	4	Replaceable

Table 7. Splitting left and right edge of replaced Chr2-6 region

Region replaced by *CgLEU2*	**Splitting point**	**Number of transformants**	**Number of transformants analyzed**	**Transformants with expected splitting**
Chr2-6	Left edge	827	10	8
	Right edge of replaced Chr2-6 left split transformant	917	5	2

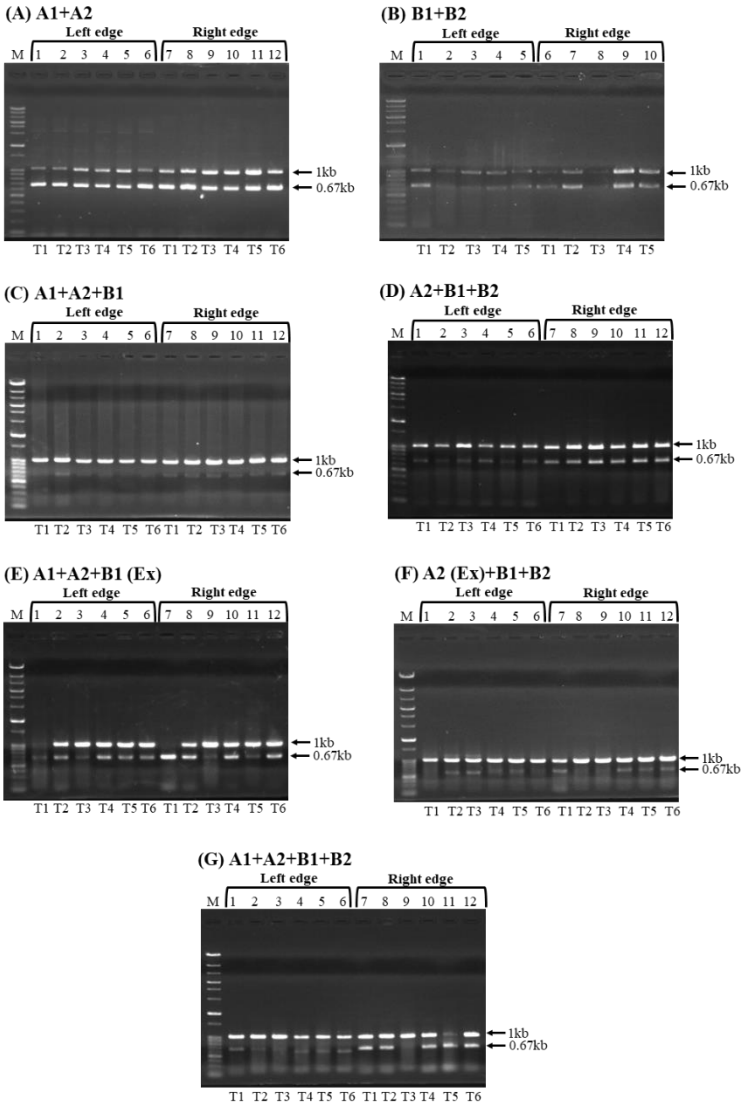


Fig. 7. Colony PCR analysis of replaced sub-regions of Chr2-6 region. Each lane represents checking of left or right edge replacement of Chr2-6 sub-regions in individual transformants (T1, T2, T3, T4, T5 and T6). M represents markers (Gene Ladder Wide 2, Nippon Gene, Toyama, Japan). A common set of primers (SJP 121 and SJP 242) was used in all PCR verification experiments to amplify the 0.67 kb CNE1 gene on Chromosome 1 as an internal control. 1 kb band was the expected band for replacement of either left or right edge of sub-regions. (A) SJP 118 and SJP 119 were used for checking the left edge whereas SJP 127 and SJP 384 were used for checking the right edge replacement of A1+A2 sub-regions, respectfully. (B) SJP 390 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 385 were used for checking right edge replacement of B1+B2 sub-regions, respectively. (C) SJP 118 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 457 were used for checking right edge replacement of A1+A2+B1 sub-regions, respectively. (D) SJP 427 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 385 were used for checking right edge replacement of A2+B1+B2 sub-regions, respectively. (E) SJP 118 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 479 were used for checking right edge replacement of A1+A2+B1 (Ex) sub-regions, respectively. (F) SJP 483 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 385 were used for checking right edge replacement of A2 (Ex)+B1+B2 sub-regions, respectively. (G) SJP 118 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 385 were used for checking right edge replacement of entire Chr2-6 region, respectively.

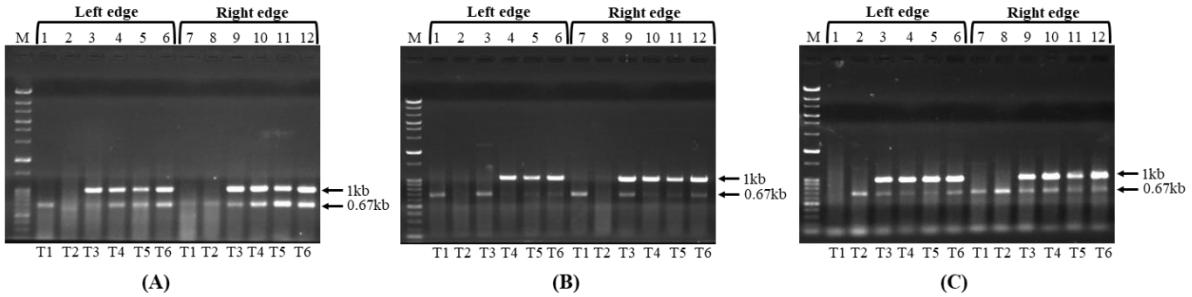


Fig. 8. Colony PCR analysis of replaced Chr2-2, Chr9-2 and Chr11-2 regions. Each lane represents checking of left or right edge replacement of entire Chr2-2, Chr9-2 and Chr11-2 regions in individual transformants (T1, T2, T3, T4, T5, and T6), respectively. 1 kb band was the expected band for replacement of either left or right edge of entire regions. (A) SJP 217 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 383 were used for checking right edge replacement of Chr2-2 region, respectively; (B) SJP 215 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 369 were used for checking right edge replacement of Chr9-2 region, respectively; (C) SJP 219 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 387 were used for checking right edge replacement of Chr11-2 region, respectively.

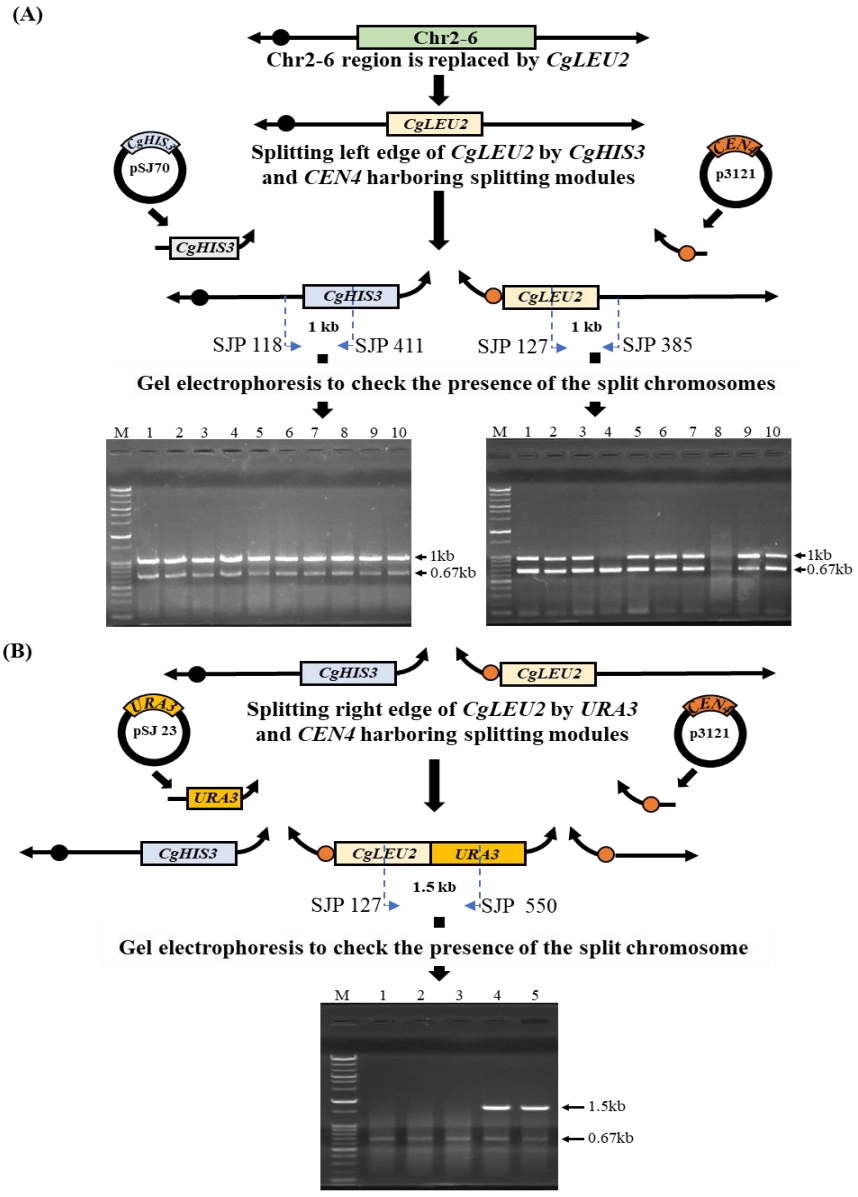


Fig. 9. (A) Two splitting modules (One module synthesized from the plasmid pSJ70 contain *CgHIS3* and the other module synthesized from the plasmid p3121 contain *CEN4* as a centromere) were introduced into the host strain SJY 576 (Chr2-6 region replaced transformants) to split left edge of *CgLEU2*. Bottom part of Fig. 9A represents gel electrophoresis of colony PCR and Lane 1 to 10 represents 10 individual transformants were checked to amplify 1 kb band denoting the left edge of *CgHIS3* and right edge of *CgLEU2*. Primers used for colony PCR are illustrated in Fig.9A. (B) After splitting left edge of *CgLEU2* in Chr2-6 region replaced transformants (SJY 577), I tried to split sequentially the right edge of *CgLEU2*. 2 splitting modules (One module synthesized from the plasmid pSJ23 contain *URA3* and another module synthesized from the plasmid p3121 contain *CEN4* as a centromere) were introduced into SJY 577 to split right edge of *CgLEU2*. Bottom part of Fig. 9B represents gel electrophoresis of colony PCR and 5 individual transformants were checked for amplifying 1.5 kb band denoting the right edge of *CgLEU2* and left edge of *URA3*. Primers used for colony PCR are illustrated in Fig. 9B

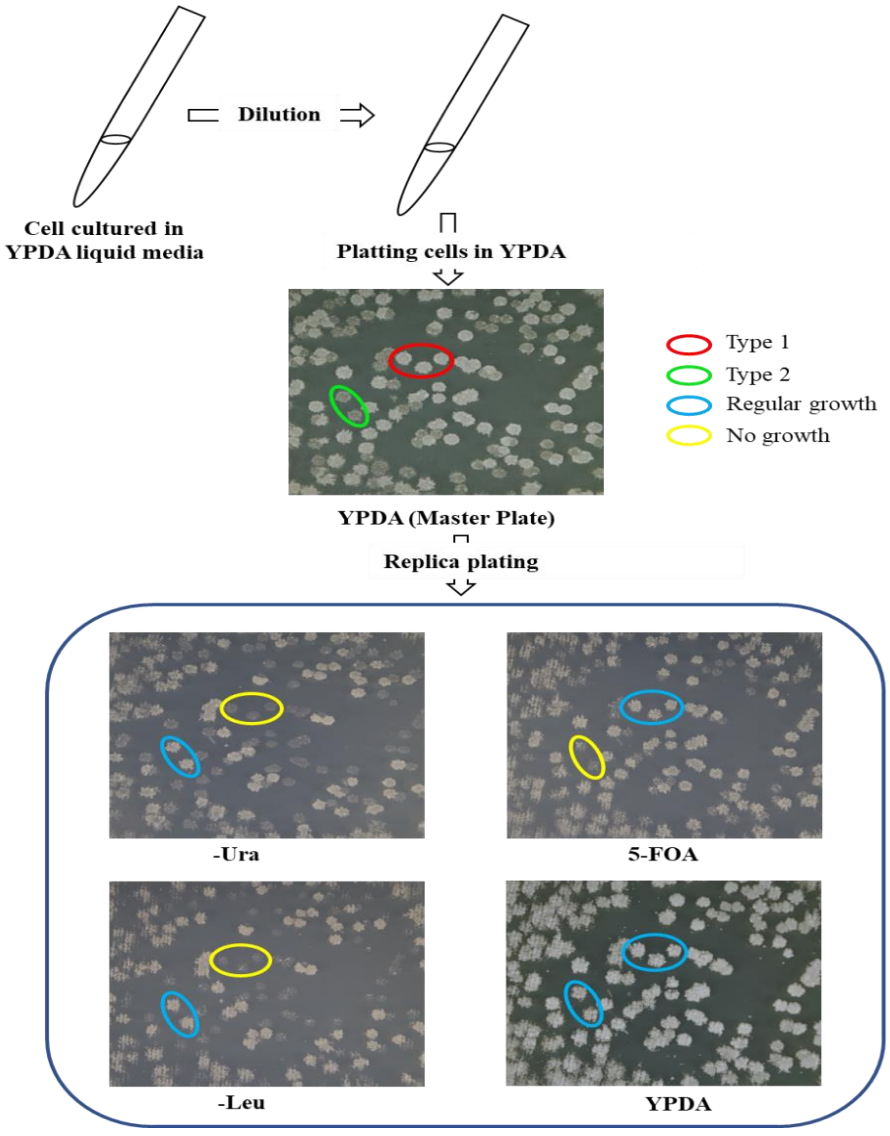


Fig. 10. Mini-chromosome loss assay. Transformants (constructed by splitting left and right edge of *CgLEU2* replaced Chr2-6 region) harboring mini-chromosome consisting of only *CgLEU2* and *URA3* markers were cultured in liquid YPDA medium and subjected to dispense on YPDA plate after appropriate dilution. After colony formation, this plate was used as a master plate to replica plating on

Ura minus, Leu minus, 5-FOA and YPDA media (as a control). After replica plating, two types colonies were appeared in YPDA master plate, we named them as Type 1 and Type 2. To distinguish them, three Type 1 colonies were circled by red color and two Type 2 colonies were circled by green color. In other replica-plated plates, colonies that showed regular growth were circled by blue color and colonies that showed no growth were circled by yellow color. Type 1 colonies could not grow on Ura minus, Leu minus media but can grow on 5-FOA and YPDA media. On the other hand, Type 2 colonies could grow on Ura minus, Leu minus and YPDA media but could not grow on 5-FOA medium.

3.3.4 Checking the suppressor mutation hypothesis

If I duplicate target region first and then replace target region of intact chromosome with *CgLEU2*, it would be possible to avoid the occurrence of a suppressor mutation because second copy of target region on duplicated chromosome should work to escape the chance of getting suppressor mutation. To, investigate this suppressor mutation hypothesis, I arbitrarily chose the Chr2-6 and Chr11-2 regions and duplicated each region of strain SJY4 using a DNA module harboring *URA3* and *CEN4* by PCDup technology. These experiments generated transformants harboring either the Chr2-6 or Chr11-2 regions on a mini-chromosome. In all, 1208 and 892 transformants were obtained for duplication of the Chr2-6 and Chr11-2 regions, respectively. Ten transformants from the two separate experiments were picked at random (Fig. 11A and 12B). In each case, one of the ten transformants had the expected duplication. Next, the Chr2-6 or Chr11-2 region of intact chromosome was replaced by a DNA module harboring *CgLEU2*. In all, 130 and 33 transformants were obtained for the replacement of the Chr2-6 and Chr11-2 regions, respectively. Ten transformants from each experiment were subsequently picked up at random and analyzed by colony PCR. Six and ten transformants were found to have the expected structure for the replacement of Chr2-6 and Chr11-2 regions on intact chromosome (Fig. 12A and 12B). I named these transformants Chr2-6 (dup + rep) and Chr11-2 (dup + rep).

Along with Chr2-6 (dup + rep) and Chr11-2 (dup + rep) transformants, I also tested some of the transformants constructed in previous study (Kaboli et al. 2014) harboring Chr1-2, Chr2-2, Chr2-4, Chr2-6, Chr2-7 and Chr3-2 regions in the mini-chromosome. I cultivated

all of those transformants in YPD liquid medium overnight. Cells were then spotted onto YPD, Ura minus and 5-FOA plate (Fig. 13). I incubated those plates and each day I observed the growth of colonies and took the photos as shown in Fig. 13. I found 3 kinds of phenotypes on the colonies originated from those transformants. I categorized them as Class I, Class II and Class III. Class I transformants did not show growth even after a long period of incubation in 5-FOA medium. Thus, the target region is considered to be essential for viability. Class II transformants showed regular growth in 5-FOA medium even after day 1. Therefore, the chromosomal region that was deleted from this transformants was considered to be non-essential. On the hand, Class III transformants did not show growth after day 1, but they gradually formed so called papillae colonies in 5-FOA medium within day 3. I defined that the chromosomal region deleted from Class III transformants is intrinsically essential but lethality could be compensated and consequently adaptable cells appeared during a longer incubation. I will discuss this interesting issue in Discussion section.

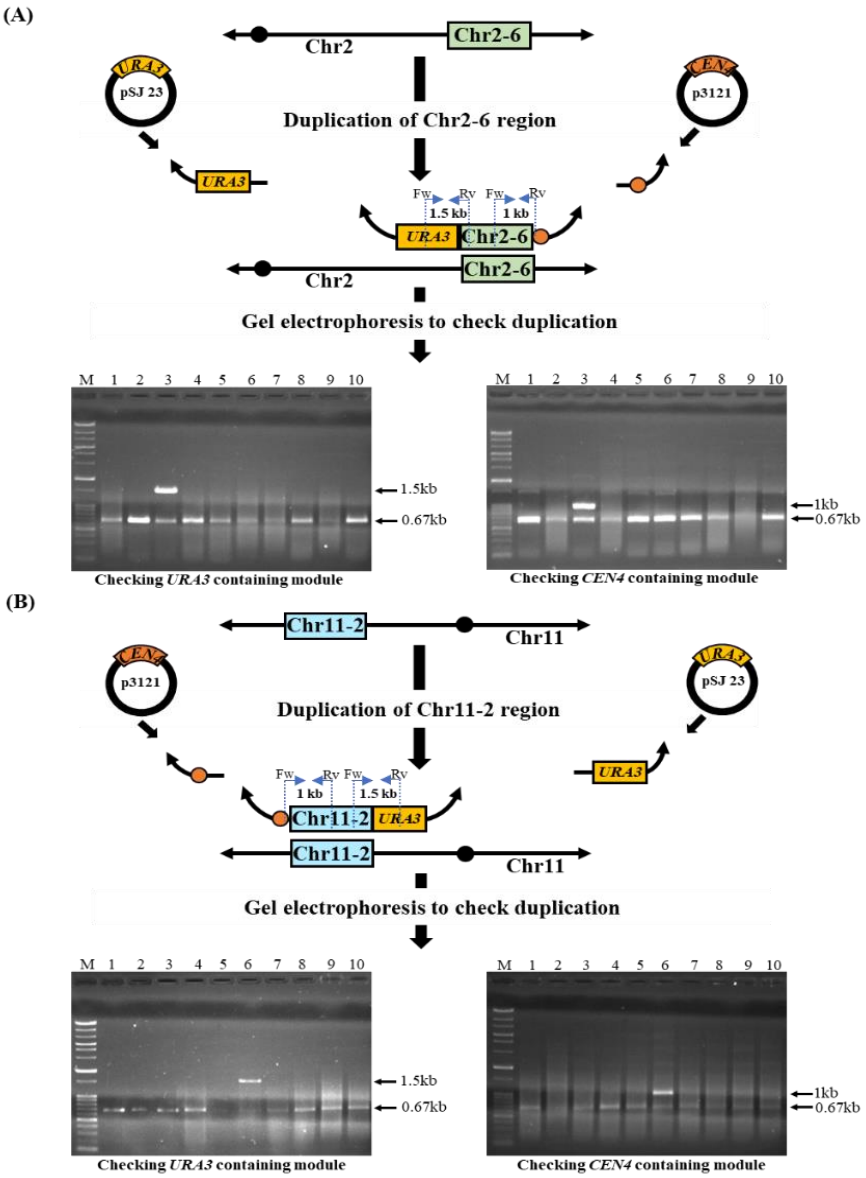


Fig. 11. Duplication of Chr2-6 and Chr11-2 regions and checking transformants by colony PCR. Upper part of Fig. 11A and 11B represents 2 duplication modules (One module synthesized from the plasmid pSJ23 contain *URA3* and another module synthesized from the plasmid p3121 contain *CEN4* as a centromere) were introduced into SJY4 to duplicate Chr2-6 and Chr11-2 regions, separately. Bottom part of Fig 11A and 11B represents gel electrophoresis of colony PCR and each lane represents 1 transformants without the most left lane, which contains marker. In Fig. 11A, SJP 550 and SJP 690 were used to check *URA3* harboring DNA module whereas SJP 694 and SJP 697 were used to check *CEN4* harboring DNA module for the duplication of Chr2-6 region, respectfully. In Fig. 11B, SJP 692 and SJP 550 were used to check *URA3* harboring DNA module whereas SJP 697 and SJP 696 were used to check *CEN4* harboring DNA module for the duplication of Chr11-2 region, respectfully.

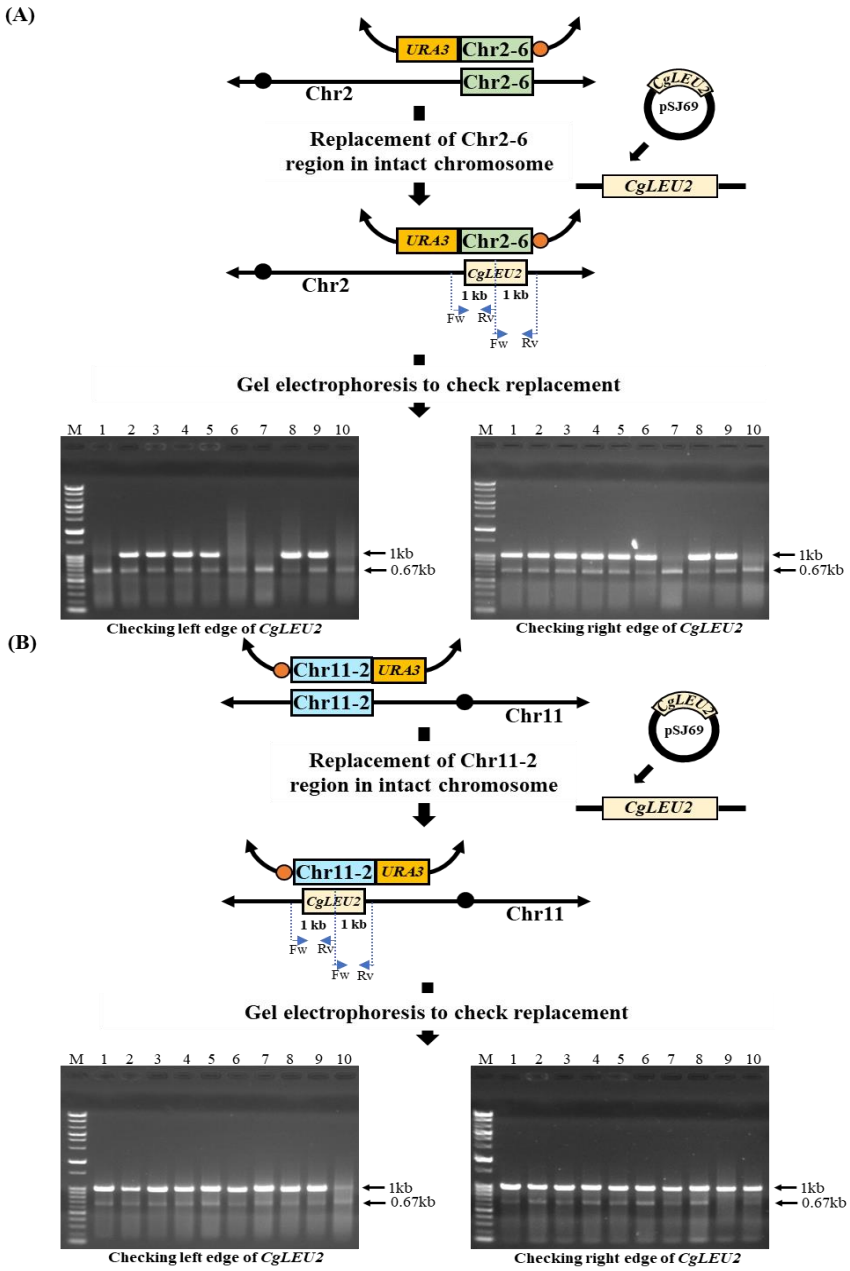


Fig. 12. Checking Chr2-6 (dup + rep) and Chr11-2 (dup + rep) transformants. Upper part of Fig. 12A and 12B represents DNA module (synthesized from the plasmid pS69 as template contain *CgLEU2*) was introduced into Chr2-6 and Chr11-2 duplicated transformants to replace Chr2-6 and Chr11-2 regions from the intact chromosome, respectfully. Bottom part of Fig 12A and 12B represents colony PCR and each lane represents 1 transformants without the most left lane, which contains marker. In Fig. 12A, SJP 217 and SJP 119 were used to check left edge of *CgLEU2* whereas SJP 127 and SJP 383 were used to check right edge of *CgLEU2* for the replacement of Chr2-6 region in Chr2-6 duplicated transformants, respectfully. In Fig. 12B, SJP 219 and SJP 119 were used to check left edge of *CgLEU2* whereas SJP 127 and SJP 387 were used to check right edge of *CgLEU2* for the replacement of Chr11-2 region in Chr11-2 duplicated transformants, respectfully.

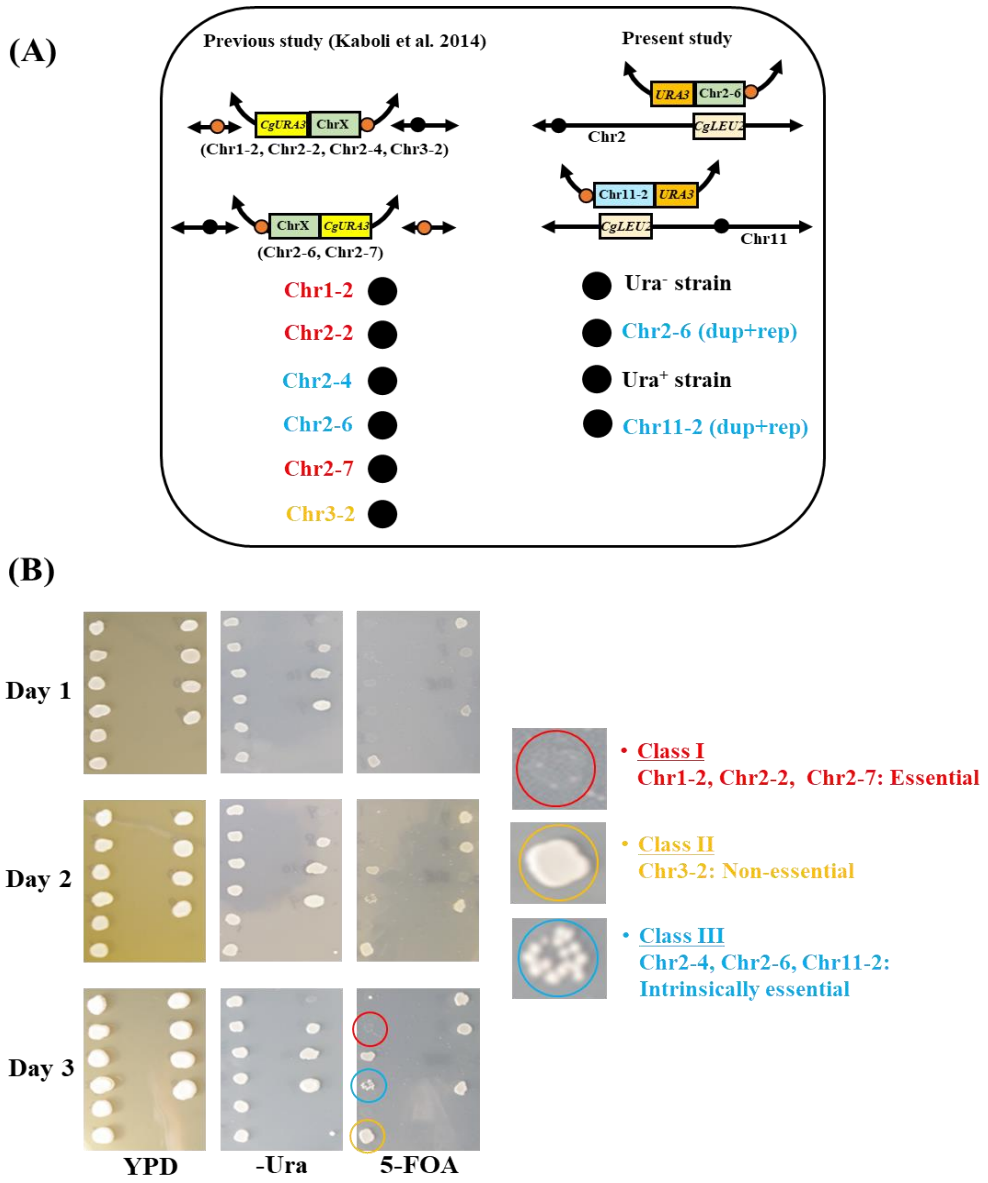


Fig. 13. Spot assay of mini-chromosome harboring strains. Fig. 13A represents position of spots in YPD, -Ura and 5-FOA plate. Left side of Fig 13A indicates the structure of chromosome and spots of transformants harboring Chr1-2, Chr2-2, Chr-2-4, Chr2-6, Chr2-7 and Chr3-2 regions in mini-chromosome constructed by previous study (Kaboli et al. 2014). Right side of Fig 13 indicates the structure of chromosome and spots of Chr2-6 (dup + rep) and Chr11-2 (dup + rep) transformants. Spots of Ura^- and Ura^+ strains were negative and positive control, respectively. Fig. 13B represents the spotting assay of all strains in YPD, -Ura and in 5-FOA plate from day 1 to day 3. Three types of colonies were found according to the growth of transformants and these transformants were categorized as Class I, Class II and Class III. Transformants harboring Chr1-2, Chr2-2 and Chr2-7 regions belong to Class I transformants and regions deleted in Class I transformants were considered to be essential while transformants harboring Chr3-2 region belong to Class II transformants and the region deleted in Class II was treated as non-essential. The third type of transformants harboring Chr2-4, Chr2-6 and Chr11-2 regions belong to Class III and regions deleted in Class III were considered to be intrinsically essential. From each class of transformants, one representative colony was circled. Colony representing Class I transformants (harboring Chr2-2 region) was circled by red color, Class II transformants (harboring Chr3-2 region) was circled by yellow color and Class III transformants (harboring Chr2-6 region) was circled by blue color.

3.4 Discussion

There is an inconsistency between the results of this study and a previous study where mini-chromosomes comprising target regions were constructed by PCS followed by mini-chromosome loss. In previous study, deletion of the 49 target regions led to lethality. On the other hand, in this study, direct deletion through replacement of four target regions so far tested did not lead to the compete lethality. I thought that one possible reason is that splitting of chromosome by PCS might bring telomere repression to genes present in the region close to the artificially added telomere. Therefore, if expression of an essential gene becomes repressed by telomere repression, the cell would die. In order to avoid such telomere repression occurring for an essential gene, Kaboli et al. (2014) split the chromosome at least 1 kb apart from the essential genes. Therefore, after splitting both edges of a particular target region to create a mini-chromosome comprising the target region along with marker, he confirmed that transformants containing mini-chromosome are viable and then, conducted a mini-chromosome loss assay. Because transformants containing the mini-chromosome were viable, he judged that telomere repression does not occur. Thus, a simple explanation for lethality after mini-chromosome loss is that the target region may have a gene-pair that results in synthetic lethality as the target region contains only non-essential genes.

In this study, I deleted the same chromosomal region by a one-step replacement. Thus, I can assume that telomere repression does not occur because the chromosome is continuous and therefore there is no newly added artificial telomeres in the resultant chromosome. In this way, expression of essential genes present in the left and right side close (i.e., within 1 kb) to the replaced region is not be repressed and should remain functional. However, it should be noted that the target regions had been deleted by replacement with the *CgLEU2* marker and further mini-chromosome loss assays of the *CgLEU2* marker also resulted in viable cells. Thus,

reasonable interpretation is needed to explain how the resulting transformed cells could be viable by one-step replacement with a marker gene.

I reasoned that during selection of transformants, suppressor mutations might have occurred that suppress the lethality caused by deletion of the target region. Once a suppressor mutation occurs, transformants would continue to be viable even after further manipulation. To investigate whether this suppressor mutation hypothesis is the case, I constructed strains harboring duplication of each of the two target regions (Chr2-6 and Chr11-2) (Fig. 11) using PCDup technology and subsequently replaced the target region of the intact chromosome by using *CgLEU2* harboring DNA module (Fig. 12). Then, I did spot test in YPD, Ura minus and 5-FOA plate (Fig. 13) by using not only the transformants constructed in present study but also in previous study (Kaboli et al. 2014). Transformants were categorized into three types according to their growth phenotype in 5-FOA medium. Class I and Class II transformants formed no colony and regular-sized colony, respectively. Chromosomal region deleted in these transformants were considered to be essential and non-essential for viability. However, Class III transformants displayed different colony phenotype, i.e., papillae appeared after a long incubation due to most probably adaptable capability against lethality. This observation means that those chromosomal regions of Class III transformants are intrinsically essential but lethality could be compensated during longer incubation. I reasoned that possible gross alteration of gene expression caused by deletion of many genes at a time may affect physiological change, resulting in compensation or adaptation for viability. Therefore, the chromosomal regions which were deleted from these transformants could be considered as intrinsically essential regions. This idea is consistent with the "Mass action of gene" hypothesis (Bonney et al. 2015) for gaining adaptability. Mass action of gene hypothesis was reported as the idea that growth fitness cannot be attributed to specific change of gene expression caused by the deletion of critical genes. Rather this hypothesis proposes an idea that growth fitness is

determined by gross change caused by the deletion of many genes simultaneously. From the overall discussion, I came to a conclusion that specific suppressor mutation might not be responsible for viability in the case of deletion of at least chr2-6 and chr11-2 region (Class III transformant) but rather change of entire gene expression profile may lead those cells to be viable. My study highlights an important caveat to evaluate whether a particular region of the *S. cerevisiae* genome is essential or non-essential or intrinsically essential for cell viability. I believe that prudent approaches such as replacement, splitting and mini-chromosome loss assay with careful observation of growth phenotype are needed for the analysis of essentiality or non-essentiality of a particular chromosomal region to understand precisely genome function in *S. cerevisiae.*

3.5 Summary and Conclusion

Previously it was identified that 49 undeletable chromosomal regions harboring non-essential genes in the genome of *Saccharomyces cerevisiae* by employing two novel genome engineering technologies, i.e., PCR-mediated chromosome deletion (PCD) and PCR- mediated chromosome splitting (PCS) technology. In the previous study, it was proposed that there might be unknown synthetic lethal combinations of genes present in such undeletable regions of the genome. Based upon this idea, in this study, I chose four of the smallest undeletable chromosomal regions among the 49 and performed extensive further analyses to narrow down the gene-pairs responsible for lethality by replacing sub-regions in various combinations with a DNA module comprising the *CgLEU2* marker. However, since the results revealed that not only the sub-regions but the entire region was replaceable, I converted one (Chr2-6) of the four entire regions replaced with marker to a mini-chromosome and then conducted a mini-chromosome loss assay. The results demonstrated that cells which had undergone loss of the mini-chromosome were viable. I reasoned that viable cells may arise via a suppressor mutation

elsewhere in the genome. To explore this hypothesis, two chromosomal regions (chr2-6 and chr11-2) were duplicated to construct a mini-chromosome marked with *URA3* by PCR-mediated chromosome duplication (PCDup). Target regions were replaced in the intact chromosome prior to performing mini-chromosome loss assays. These results confirmed that cells without the mini-chromosome survived, suggesting that viable transformants do not arise by suppressor mutation. From all of these observations, I came to an important conclusion that *S. cerevisiae* chromosomal region harboring only non-essential genes could be categorized into three classes, i.e., Class I, Class II and Class III. Class I region might have a few critical genes responsible for synthetic lethality. Therefore, if this region is deleted, cells would die. Class II region likely harbors genes which have no interaction with each other. As a result, even if this region is deleted, no phenotypic change occurs. Class III region is defined to contain genes which cause gross change of gene expression profile when those genes were deleted simultaneously. When these chromosomal regions are deleted, cells could occasionally survive by gross alteration of cell physiology which could be called compensatable essentiality. More detailed study of 49 chromosomal regions that were identified undeletable in previous study (Kaboli et al, 2014) remains for future study.

Chapter 4

General Discussion

The aim of this study was to deal with two important issues of genome engineering. One is to develop a new technology for genome engineering and the other is to reveal unknown genome function. From these points of view, in Chapter 1, I discussed the impact of genome engineering technologies, especially in yeast, by focusing on the effect of CRISPR/Cas9 technology to boost up genome editing. I also emphasized that genome engineering is applied not only to develop further novel techniques but also can be devoted to find out genetic interaction network in the genome. Based upon such discussion about the impact of genome engineering, in Chapter 2, I developed a new genome engineering technology which we named CRISPR-PCDup. After successful application of CRISPR-Cas9 system in yeast genome (DeCarlo et al. 2013), yeast scientists tried to utilize CRISPR/Cas9 system to target multiple chromosomal regions simultaneously (Bao et al. 2014; Ryan et al. 2014; Jakočiūnas et al. 2015; Sasano et al. 2016; Easmin et al. 2019b). This is because CRISPR/Cas9 is advantageous to induce site specific double strand break over ZFNs and TALENs, since both of these technologies require the engineering of specific protein pairs for each target site (Ma et al. 2014) which are generally very time consuming and costly. On the other hand, by redesigning only 20 bp sequence in 5'end of gRNA, CRISPR/Cas9 system can be programmed to target any desired sequence very easily (Jinek et al. 2012). Thus, CRISPR/Cas9 genome engineering has been proven to be fast, versatile and considered to be the most useful genome editing technique (Cong et al. 2013; Zalatan et al. 2014).

For successful CRISPR/Cas9 engineering, design, expression, and delivery of the gRNA components are crucial parameters (Stovicek et al. 2017). In *S. cerevisiae*, the most common strategy has been to express a chimeric gRNA molecule from a high-copy vector to ensure its abundant expression (DiCarlo et al. 2013; Bao et al. 2015; Jakočiūnas et al. 2015a; Jakočiūnas et al. 2015b). Sasano et al. (2016) used gRNA- expression plasmid constructed by SLIC method (Li and Elledge 2012) to split multiple chromosomal regions and they succeeded

in splitting up to four chromosomal regions at a single transformation. Although other simpler methods to deliver gRNA than plasmid base method were developed (Easmin et al. 2019a), in this study, I also constructed and employed gRNA-expressing plasmid according to Sasano et al. (2016) to target chromosomal regions for duplication. By using two plasmid system (gRNA and Cas9 expressing plasmid) it was possible to increase the efficiency of single site duplication up to 30 fold. It was also possible to simultaneously duplicate two large chromosomal regions (160 kb and 200 kb) along with duplication of 400 kb chromosomal regions. As I mentioned in Chapter 2 that CRISPR-PCDup technology may contribute to investigating combinatorial effect of segmental aneuploidy and studying the effect of over-expression of many genes on segmentally duplicated chromosomes, CRISPR-PCDup should be added to the field of genome engineering technology as an efficient new tool for generating industrially useful yeast strains and for studying the fundamentals of genome function.

To further find out unknown genetic interaction network like synthetic lethal interaction among genes, in Chapter 3, I investigated four undeletable chromosomal regions as determined by Kaboli et al. (2014) that harbors only non-essential genes and performed systematic analysis by replacing sub-regions to identify unknown gene-pairs which is responsible for the lethality. My analysis revealed very interesting finding that deletion by replacement of any sub-regions or entire regions do not lead to ultimate lethality. Moreover, when I converted one particular replaced region to mini-chromosome and conducted mini-chromosome loss assay, I got viability. For further confirmation of the essentiality or non-essentiality, I duplicated the target regions before replacement for avoiding so called suppressor mutations from intrinsic selection pressure and subsequently I replaced the target region of intact chromosome by DNA module harboring *CgLEU2* marker. Then, I performed mini-chromosome loss assay by spotting cells of transformants along with those constructed by previous study (Kaboli et al. 2014). I found three types of transformants depending upon the phenotype of colony formation which I called Class I, Class II and Class III transformants. Class I, Class II and Class III transformants shows

no growth, regular growth and intermediate growth like formation of papillae after longer incubation, respectively. As Class I and Class II transformants are considered to harbor essential and non-essential chromosomal regions, respectively and Class III transformants are supposed to harbor intrinsically essential chromosomal regions, this data motivated me to draw an important conclusion that deletion of a single gene and deletion of chromosomal region may lead to same consequence regarding viability or lethality. These observations imply that chromosomal regions harboring non-essential genes could be categorized into three classes. If we delete a particular chromosomal region and found viability or lethality, we could say that the particular chromosomal region is non-essential or essential, respectively. When particular region is deleted, if we found "Intermediate" colony phenotype as observed in Class III transformants, we can assume that compensation might occur. In this case, we further hypothesize that this compensation or adaptation might be caused by the change of entire gene expression profile in Class III transformants. Therefore, such an "Intermediate" colony phenotype observed in "Class III" transformants may give a very important suggestion for considering the essentiality or non-essentiality of specific chromosomal regions in *S. cerevisiae* genome.

I believe that technology described in Chapter 2 add a new methodology to genome engineering field and I would like to emphasize that, CRISPR-PCDup would be helpful to construct useful yeast strains harboring genetic traits of industrial value and understand genome function. As described in Chapter 3, deletion of specific essential chromosomal regions were occasionally compensatable or adaptable towards viability. This class of essential regions can be distinguished from "non-adaptable" essential regions, whose loss could not be overcome. Finally, I want to conclude that my observations should promote basic biologists and modern biotechnologists to re-evaluate the concept of chromosome essentiality not only in terms of cell viability but also in light of cellular adaptability.

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Supplementary Tables

Table S1. Primers used for constructing DNA modules

Primer name	Sequence (5'-3')
gRNA15-l0-Dup	TACTTATTAACGTACTCAAACAACTACACTTCGTTGTATCTCA GAATGAGGGCCGCCAGCTGAAGCTTCG
gRNA15-l1-Dup	TTAGTATTTTGTGTTTTTTACAACAACCTCTCGACTATTGTAT ACCAGTTGGCCGCCAGCTGAAGCTTCG
gRNA15-l2-Dup	GATTTGAACTTTTGTTCTCTCTCTCAACTTTTTCTTTTCTTTGT CCTTGT GGCCGCCAGCTGAAGCTTCG
gRNA15-l3-Dup	TATCTGTAGTTTCCTTCCATTACATAACGCATAATATACTATT TCCATAG GGCCGCCAGCTGAAGCTTCG
gRNA15-l4-Dup	TACGTTAAAAAAACACATGGTCTTATTTTCCAAAATGCCTAT TCCCTATA GGCCGCCAGCTGAAGCTTCG
gRNA15-l5-Dup	GACTATAGAAGAAGCGTTCCAGTCAATTCTACTACATCAGTC TTCCGACAGGCCGCCAGCTGAAGCTTCG
C3-1 Dup 50 bp	TAATACTGCTGTTGAGGTTTTCTTCTTCAGGGCTGCTCACAAC GTGATATGGCCGCCAGCTGAAGCTTCG
C3-2 Dup 50 bp	TGTAAGAATATTTGGTATGGCTAAAGTAAGCAAAGCCATATC CCGATCCCGGCCGCCAGCTGAAGCTTCG
C5-3 Dup 50 bp	TCTCTTCATAGAGCTCGTCGAAGAGGCAATAGGAACACAAC GCCTTACCAGGCCGCCAGCTGAAGCTTCG
C8-1 Dup 50 bp	ATTAAAGCGTTAACTCACTCATTATTGTAGCTTATGCGTTTCT CCTCCTCGGCCGCCAGCTGAAGCTTCG
C14-4 Dup 50 bp	TTCGCTCAAGTATATTCCGCGTTAATCAACCCACCTGACCCA CATTCTAAGGCCGCCAGC TGAAGCTTCG
CA Primer	CCCCAACCCCAACCCCAACCCCAACCCCAACCCCAAAGGCC ACTAGTGATCTGAT

Table S2. Primers used for constructing the gRNA expressing plasmid

Primer name	Sequence (5'-3')
gRNA15-l0 Fw	TTATAACAAAGCGAACAAAAGTTTTAGAGCTAGAAATAGCAAG
gRNA15-l0 Rv	TTTTGTTCGCTTTGTTATAAGATCATTTATCTTTCACTGCGGA
gRNA15-l1 Fw	GTAGCATCTATGCAAGAAACGTTTTAGAGCTAGAAATAGCAAG
gRNA15-l1 Rv	GTTTCTTGCATAGATGCTACGATCATTTATCTTTCACTGCGGA
gRNA15-l2 Fw	TCGTCACAATCTAATCAACAGTTTTAGAGCTAGAAATAGCAAG
gRNA15-l2 Rv	TGTTGATTAGATTGTGACGAGATCATTTATCTTTCACTGCGGA
gRNA15-l3 Fw	AAAAGATGTAAGATAGACTAGTTTTAGAGCTAGAAATAGCAAG
gRNA15-l3 Rv	TAGTCTATCTTACATCTTTTGATCATTTATCTTTCACTGCGGA
gRNA15-l4 Fw	ACATATAGGCAAAGATATATGTTTTAGAGCTAGAAATAGCAAG
gRNA15-l4 Rv	ATATATCTTTGCCTATATGTGATCATTTATCTTTCACTGCGGA
gRNA15-l5 Fw	TAGGATACAATCAGCGATGTGTTTTAGAGCTAGAAATAGCAAG
gRNA15-l5 Rv	ACATCGCTGATTGTATCCTAGATCATTTATCTTTCACTGCGGA
Ch3 Dup P6-Fw	GGACGTATTCAGCGCAGTTGGTTTTAGAGCTAGAAATAGCAAG
Ch3 Dup P6-Rv	CAACTGCGCTGAATACGTCCGATCATTTATCTTTCACTGCGGA
Ch3 Dup P1-Fw	AAGGGATCGGAATAAGAGTCGTTTTAGAGCTAGAAATAGCAAG
Ch3 Dup P1-Rv	GACTCTTATTCCGATCCCTTGATCATTTATCTTTCACTGCGGA
Ch8 Dup P1-Fw	GGATCTTCCACTCCGGTTCGGTTTTAGAGCTAGAAATAGCAAG
Ch8 Dup P1-Rv	CGAACCGGAGTGGAAGATCCGATCATTTATCTTTCACTGCGGA
Ch14 Dup P1-Fw	GTGTTTAGACTAGGTTTGTC GTTTTAGAGCTAGAAATAGCAAG
Ch14 Dup P1-Rv	GACAAACCTAGTCTAAACAC GATCATTTATCTTTCACTGCGGA

Table S3. Primers used for Southern blotting

Primer name	Sequence (5'-3')
C3-1-p-f	GCAAGACTCTGGTCTCTTCT
C3-1-p-r	ACACCTGAGTGGGTCATCAC
C3-2-p-f	CTCTTAGCGGACCGTTTTGG
C3-2-p-r	ATCTCTCCGCAGGGGTAAGC
C15-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCC
	CCAATTCACAATTTGTCGAT
C15-5-L-r	TACAGGTCAATGAAAATGCG

Table S4. Positions of duplication points on different chromosomes

Name of region	Chromosome	Nucleotide position	gRNA targeting sequence (5′-3′)
Chr3-1	Chr3	158050.5	GGACGTATTCAGCGCAGTTG
Chr3-2	Chr3	157533.5	AAGGGATCGGAATAAGAGTC
Chr8-1	Chr8	202256.5	GGATCTTCCACTCCGGTTCG
Chr14-4	Chr14	597353.5	GTGTTTAGACTAGGTTTGTC
Chr15-L0	Chr15	969084.5	TTATAACAAAGCGAACAAAA
Chr15-L1	Chr15	569774.5	GATGCATCTATGCAAGAAAC
Chr15-L2	Chr15	618913.5	TCGTCACAATCTAATCAACA
Chr15-L3	Chr15	670547.5	AAAAGATGTAAGATAGACTA
Chr15-L4	Chr15	718508.5	ACATATAGGCAAAGATATAT
Chr15-L5	Chr15	767985.5	TAGGATACAATCAGCGATGT

Table S5. Primers used for colony PCR

Primer name	Chromosome number (coordinates)	Nucleotide sequence (5'-3')
SJP 119	*CgLEU2* (776-800) Rv	CCCACTAGTTCTCTAACAACGACGA
SJP 121	*CNE1* (211-230) Fw	TCACAGGGTCGATTGCAAGG
SJP 242	*CNE1* (880-861) Rv	CTGGTGGTTCAGTGCCATCT
SJP 411	*CgHIS3* (401-425) Rv	CGCCTCCTTGAACGCTTGGCCCAGC
SJP 510	Chr3-1 (157820-157844) Fw	GCTACATAGCGTTCATTTTT TAGGT
SJP 550	*URA3* check (116545-116569) Rv	GCTTCAAACCGCTAACAATACCTGG
SJP 555	Chr8-1 (201541-201565) Fw	AAAAAATGTGGGATGAAGACTCCCG
SJP 668	Chr14-4 (597970-597994) Rv	ATGGAGAGCACAATCCAGCTTCTTA

Table S6. Primers used to generate DNA modules for replacement, splitting and duplication

Name of the Primer	Description (Chromosome number and co-ordinates)	Nucleotide sequence (5'-3')
Replacement		
SJP 90	Chr2 (318700-*318749*) Fw	TAATAAACCTCTTTTCGTATTTTTATGGCTTTCTTTG TGGAACATTGGGGGGCCGCCAGC TGAAGCTTCG
SJP 91	Chr2 (324854-324903) Rv	TATATAACTCCATTGATGCTGAAGCGATTCCAAATA AAGTTCCGAAATCCAGGCCACTAGTGGATCTGAT
SJP 92	Chr2 (324806-324855) Fw	GATTTCGTTATGTCCACCAATGCTTAAAGTGACCGT ATTTTGGAGGAAGGGGCCGCCAGCTGAAGCTTCG
SJP 93	Chr2 (330960-331009) Rv	TACTCAAGATGAAAGGTGCACATACGATTGCAGTT GCCTCAACTGATGAAAGGCCACTAGTGGATCTGAT
SJP 197	Chr9 (87800-87849) Fw	TTAAGGACATTCACGGACGCATCCCAGAAATGCTG TGATTATACGCAACGGGCCGCCAGCTGAAGCTTCG
SJP 198	Chr9 (95051-95100) Rv	ACAATAACCTCTATGAATCCAGACACAACCAAATA AAGAAAACTGAAGGGAGGCCACTAGTGGATCTGAT
SJP 199	Chr9 (95000-95049) Fw	AAATGAATTTTTAGAGTAGGAGAAGAAGGTTGAAG AAATGAACAATCGCGGGCCGCCAGCTGAAGCTTCG
SJP 200	Chr9 (102250-102299) Rv	TAATAGTGTGTAAATTGTGCGTTCAATTAGCAAAGA AAGGCTTGGAGAGAAGGCCACTAGTGGATCTGAT

SJP 201	Chr2 (21816-21865) Fw	AGTGAATAATTTTAGATTTTGTTACATATAATTCTG
		CTTGCCTATCTCTTGGCCGCCAGCTGAAGCTTCG
SJP 202	Chr2 (29607-29656) Rv	TTTTATTCCAACAATTATATGTGCTTGTATTCAGCTC
		TTTATTGAGTTTGAGGCCACTAGTGGATCTGAT
SJP 203	Chr2 (29556-29605) Fw	TTCAATCACGTAAGGTGGAAGAGAATGACATGAAG
		ATTGAGAAACAGTGAGGCCGCCAGCTGAAGCTTCG
SJP 204	Chr2 (37347-37396) Rv	GAACCGAAAAGAACGATACCGACTTGACCAGGCTC
		CAAGTTCAAAGCCATAGGCCACTAGTGGATCTGAT
SJP 205	Chr11 (188384-188433) Fw	ACATAAAGATAAACCAGTTTTTTTTGTTCAACGTCA
		ATTGTGGCAATGTTGGCCGCCAGCTGAAGCTTCG
SJP 206	Chr11 (196596-196645) Rv	GCTTATATGACTCCTTATAAAGACACAAGAAATAC
		GGTGCCTGTTGCAGCAGGCCACTAGTGGATCTGAT
SJP 207	Chr11 (196545-196594) Fw	TTGTCGATTTGGCTTGATTTCTGATTTGTAACGTCAT
		TCACTGCCCCTGTGGCCGCCAGCTGAAGCTTCG
SJP 208	Chr11 (204756-204805) Rv	ATAGTTTTGATCGAAGCTTCCTTTTCAGGGTTACGC
		CTATGGTAGATAGCAGGCCACTAGTGGATCTGAT
SJP 460	Chr2 (36037-36086) Rv	CTCAGACAATACTGAAGCTGTGTTAAAGACCTATTA
		GTTGAACATGTTATAGGCCACTAGTGGATCTGAT
SJP 461	Chr2 (329134-329183) Rv	TCCGATTATGAAAGTGATAACGAATACAGAAATAT
		GGATGAGGATTCAATAGGCCACTAGTGGATCTGAT

SJP 462	Chr9 (100182-100231) Rv	TTATCTATGAATAAAATAAACGCCCAAAGAGGCAC
		TGAAGACGCTGTGACAGGCCACTAGTGGATCTGAT
SJP 463	Chr11 (201329-201378) Rv	CCAATGAGAAGATGTCTCGAAACATTCATTGAGTC
		GTGGACACCAGTGTTAGGCCACTAGTGGATCTGAT
SJP 464	Chr2 (24423-24472) Fw	CAAGAAAGTTTGGTTTACTATGGACAATGGGGTCCC
		TACTATTTGTTCTTGGCCGCCAGCTGAAGCTTCG
SJP 465	Chr2 (321014-321063) Fw	TCCTCAGTTATGCGCTCAGGTGACTTTCCAGCAAGT
		GAGCCGGCGCCCCTGGCCGCCAGCTGAAGCTTCG
SJP 466	Chr9 (89474-89523) Fw	TTATTAGATCTCAAGTTATTGGAGTCTTCAGCCAAT
		TGCTTTGTATCAGAGGCCGCCAGCTGAAGCTTCG
SJP 467	Chr11 (190284-190333) Fw	TTGTAGTCAACGGCTTCTTAAGATCTTTGGCCTTGA
		GTTCAGCTATAAATGGCCGCCAGCTGAAGCTTCG
Splitting		
SJP 13	CA Primer	CCCCAACCCCAACCCCAACCCCAACCCCAACCCCA
		AAGGCCACTAGTGATCTGAT
SJP 519	*CgLEU2* (1-50) Rv	CAAGATAGGGATGATTACAGAGCACACATTTCCGG
		GAAACACAGAATTGGGGCCGCCAGC TGAAGCTTCG
SJP 520	*CgLEU2* (1636-1685) Fw	GCTATATTAGCTTGTGCATTCGCATGTATCGGCAAA
		CGAACTTTACGTAAGGCCGCCAGCTGAAGCTTCG
SJP 522	Chr2 (318699-318748) Fw	CTAATAAACCTCTTTTCGTATTTTTATGGCTTTCTTT
		GTGGAACATTGGGGGCCGCCAGCTGAAGCTTCG

SJP 523	Chr2 (330961-331010) Rv	CTACTCAAGATGAAAGGTGCACATACGATTGCAGT TGCCTCAACTGATGAGGCCGCCAGCTGAAGCTTCG
Duplication		
SJP 671	Chr2 (318749-318798) Rv	TGAACCAGCGGAGTGCCTTTAGTATTATAGTTTAAA AAAGCTGGAATAGCGGCCGCCAGCTGAAGCTTCG
SJP 672	Chr2 (330911-330960) Fw	AAGCGTTGATCAAGTATTCGGCGCCGTATTCCTTCG CTATTTTAAGCTTTGGCCGCCAGCTGAAGCTTCG
SJP 675	Chr11 (188434-188483) Rv	AAATGACGTTGGGAAAAGATGTCTCTTCGCTGTTCC CAGACGTCTTGAAAGGCCGCCAGCTGAAGCTTCG
SJP 676	Chr11 (204706-204755) Fw	TCAGAGAAAAGGACGGTCTATGGGCCATTATTGCTT GGTTAAATATCTTGGGCCGCCAGCTGAAGCTTCG

Table S7. Primers used for colony PCR

Name of the Primer	Chromosome number (coordinates)	Nucleotide sequence (5'-3')
SJP 118	Chr2 (318549-318573) Fw	TTAGTTTACACCCGTCCCATGGCCGA
SJP 119	*CgLEU2* (776-800) Rv	CCCACTAGTTCTCTAACAACGACGA
SJP 121	*CNE1* (211-230) Fw	TCACAGGGTCGATTGCAAGG
SJP 127	*CgLEU2* (776-800) Fw	TCGTCGTTGTTAGAGAACTAGTGGG
SJP 215	Chr9 (87646-87670) Fw	CCCACAACAATGTCAACTTCATCTT
SJP 216	Chr9 (94846-94870) Fw	CATATTCACATGTTTCTCATTTTTT
SJP 217	Chr2 (21686-21710) Fw	ATAATACTAATGCATTTAAATCATA
SJP 218	Chr2 (29426-29450) Fw	ATGATATATAAACAACTTCAATAAA
SJP 219	Chr11 (188256-188280) Fw	CAACTCTTATCATTGACATCGTTCT
SJP 220	Chr11 (196416-196440) Fw	TTTGCTCTTGCTGCCAATGCAGAAG
SJP 242	*CNE1* (880-861) Rv	CTGGTGGTTCAGTGCCATCT
SJP 368	Chr9 (95116-95140) Rv	CACAAACTCGAATCCAAGTTCAAAA
SJP 369	Chr9 (102315-102339) Rv	TTAGATGAATACCGGCTCTATAGAA
SJP 382	Chr2 (29672-29696) Rv	TACTAGTAACGTAAATACTAGTTAG
SJP 383	Chr2 (37412-37436) Rv	GGTTCTCTTGACCAATTCACCTTCT
SJP 384	Chr2 (29672-29696) Rv	AGTATCGAATCCATAAAAGCGACCA
SJP 385	Chr2 (331026-331050) Rv	TTTTGCCGCAGCGGGTGGTGTGGGA
SJP 386	Chr11 (196661-196685) Rv	AAAGCTTCTCGATGGAAGCAAAGAA
SJP 387	Chr11 (204821-204845) Rv	GAAGAAAGTACGGCCATACTCGTTC
SJP 390	Chr2 (324656-324680) Fw	GAAATTGGGTTCATTTGCTTTCAGT
SJP 411	*CgHIS3* (401-425) Rv	CGCCTCCTTGAACGCTTGGCCCAGC
SJP 426	Chr2 (25537-25561) Fw	TTCTTCAAAAGTTGGCGGAGGTGGA
SJP 427	Chr2 (321602-321626) Fw	TGCAGCATCAGCTTATTGACCTCGC
SJP 428	Chr9 (91251-91275) Fw	TGTTGAGTCAATTTTGTTTGCGTTT
SJP 429	Chr11 (192315-192339) Fw	GTAGTCAGGTTTGGATTTACCAATA
SJP 456	Chr2 (33542-33566) Rv	ATGTGTCTCGGGATACCTCAATTTC
SJP 457	Chr2 (327974-327998) Rv	CGCCTGTGCAATTTTTTGCCTATCA
SJP 458	Chr9 (98716-98740) Rv	GAAGCCAGGTAGAAAGTACACCACC
SJP 459	Chr11 (200741-200765) Rv	TTTGAGAAATGGTTGAACCTTTCAC
SJP 478	Chr2 (36102-36126) Rv	TGTGATTGCGCCTATTGCAGAAGGA

SJP 479	Chr2 (329199-329223) Rv	AAAAGTAGATTTTCCCTCTAACAAA
SJP 480	Chr9 (100247-100271) Rv	CGAAGAACTCAGTGCCATAACGGTG
SJP 481	Chr11 (201394-201418) Rv	AAAGCAATTAGGTATGCTACCTCAT
SJP 482	Chr2 (24273-24297) Fw	TATCTAGACAGGACTTGGTGCAAGA
SJP 483	Chr2 (320864-320888) Fw	AATGGCTTTTTGCCTATTTTGGCAG
SJP 484	Chr9 (89324-89348) Fw	TCTCTTTCTTCTTCCAAAGCAACGA
SJP 485	Chr11 (190134-190158) Fw	AATCTGACAAGCCCTGAATGACATT
SJP 550	*URA3* (116545-116569) Rv	GCTTCAAACCGCTAACAATACCTGG
SJP 690	Chr2 (319525-319549) Rv	CAAAAACCATTGAATTATAGTACCA
SJP 692	Chr11 (203955-203979) Fw	GGTGGGCCAGCTCCAGAGAGTGTCA
SJP 694	Chr2 (329960-329984) Fw	CAAAGTCACGCAAATCTAATGTATC
SJP 696	Chr11 (189410-189434) Rv	TAGAACGACGTTTAAAGGTCCTAGT
SJP 697	pUG6 (7-26) Rv	CGAAGCTTCAGCTGGCGGCC

Supplementary Figures

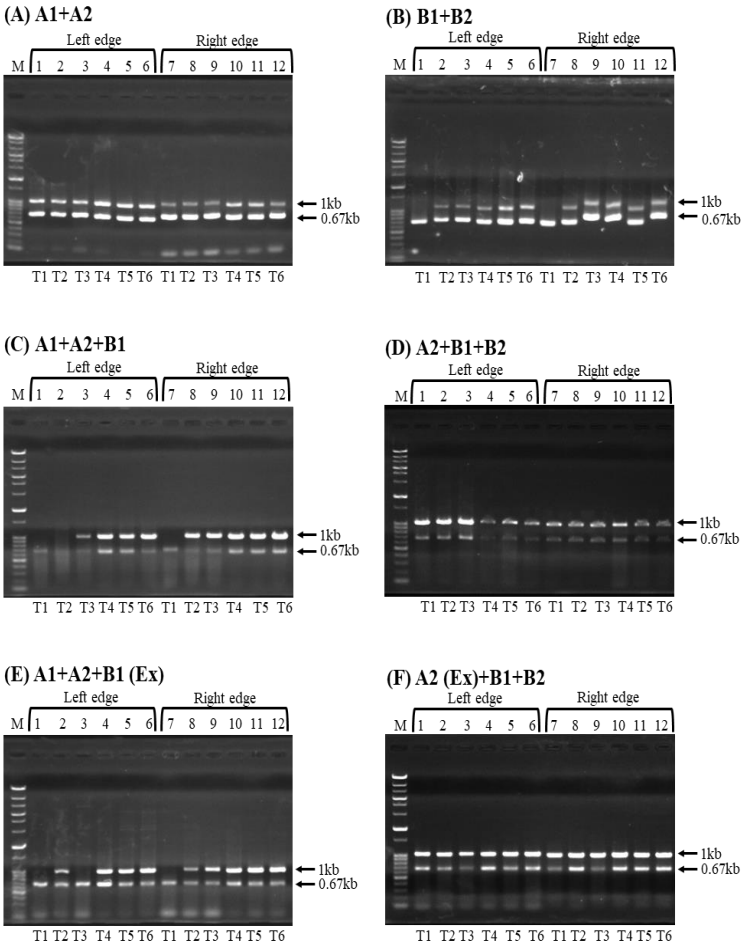

Fig. S1. Colony PCR analysis of replaced sub-regions of Chr2-2 region. Each lane represents checking of left or right edge replacement of Chr2-2 sub-regions in individual transformants (T1, T2, T3, T4, T5 and T6). 1 kb band was the expected band for replacement of either left or right edge of sub-regions. (A) SJP 217 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 382 were used for checking right edge replacement of A1+A2 sub-regions, respectively. (B) SJP 218 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 383 were used for checking right edge replacement of B1+B2 sub-regions, respectively. (C) SJP 217 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 456 were used for checking right edge replacement of A1+A2+B1 sub-regions, respectively. (D) SJP 426 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 383 were used for checking right edge replacement of A2+B1+B2 sub-regions, respectively. (E) SJP 217 (forward primer) and SJP 119 were used for checking left edge whereas SJP 127 and SJP 478 (reverse primer) were used for checking right edge replacement of A1+A2+B1 (Ex) sub-regions, respectively. (F) SJP 482 (forward primer) and SJP 119 were used for checking left edge whereas SJP 127 and SJP 383 (reverse primer) were used for checking right edge replacement of A2 (Ex)+B1+B2 sub-regions, respectively.

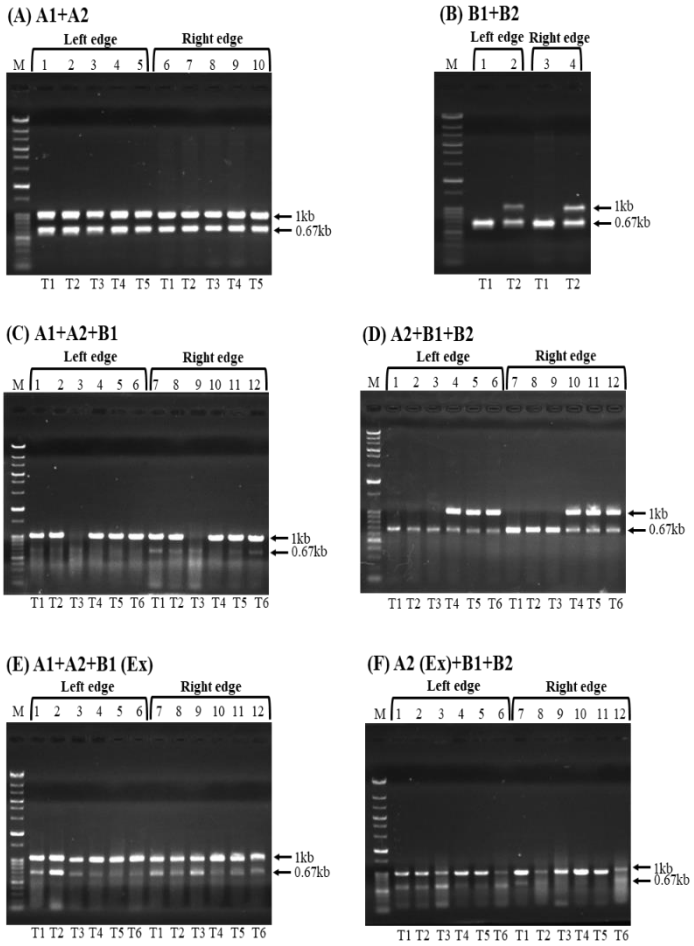

Fig. S2. Colony PCR analysis of replaced sub-regions of Chr9-2 region. Each lane represents checking of left or right edge replacement of Chr9-2 sub-regions in individual transformants (T1, T2, T3, T4, T5 and T6). 1 kb band was the expected band for replacement of either left or right edge of sub-regions. (A) SJP 215 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 368 were used for checking right edge replacement of A1+A2 sub-regions, respectively. (B) SJP 216 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 369 were used for checking right edge replacement of B1+B2 sub-regions, respectively. (C) SJP 215 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 458 were used for checking right edge replacement of A1+A2+B1 sub-regions, respectively. (D) SJP 428 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 369 were used for checking right edge replacement of A2+B1+B2 sub-regions, respectively. (E) SJP 215 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 480 were used for checking right edge replacement of A1+A2+B1 (Ex) sub-regions, respectively. (F) SJP 484 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 369 were used for checking right edge replacement of A2 (Ex) +B1+B2 sub-regions, respectively.

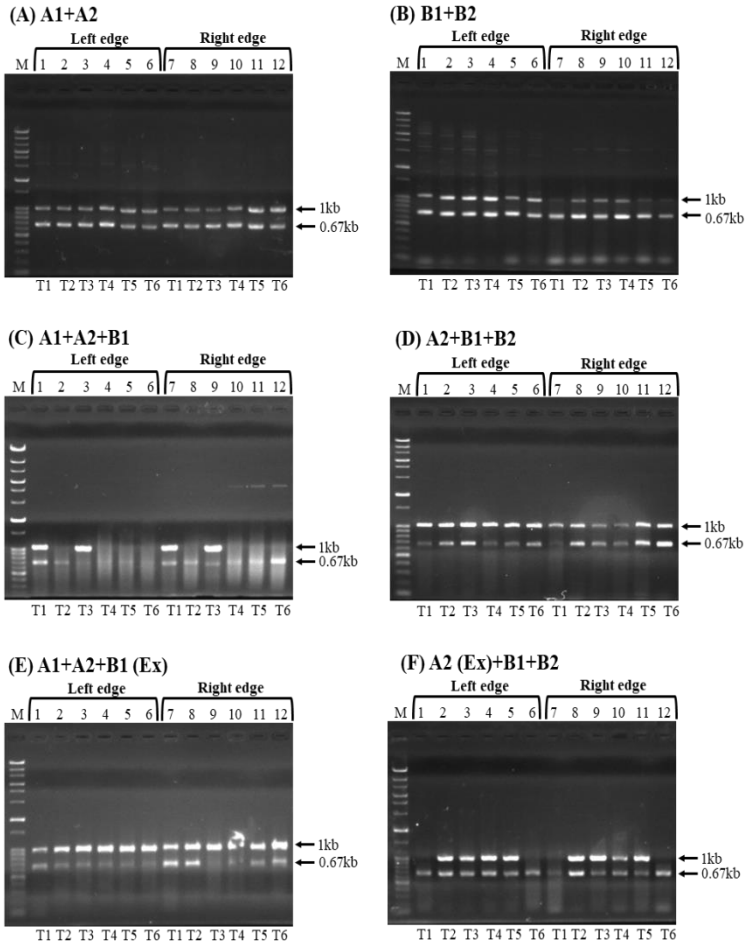

Fig. S3. Colony PCR analysis of replaced sub-regions of Chr11-2 region. Each lane represents checking of left or right edge replacement of Chr11-2 sub-regions in individual transformants (T1, T2, T3, T4, T5 and T6). 1 kb band was the expected band for the replacement of either left or right edge of sub-regions. (A) SJP 219 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 386 were used for checking right edge replacement of A1+A2 sub-regions, respectively. (B) SJP 220 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 387 were used for checking right edge replacement of B1+B2 sub-regions, respectively. (C) SJP 219 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 459 were used for checking right edge replacement of A1+A2+B1 sub-regions, respectively. (D) SJP 429 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 387 were used for checking right edge replacement of A2+B1+B2 sub-regions, respectively. (E) SJP 219 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 481 were used for checking right edge replacement of A1+A2+B1 (Ex) sub-regions, respectively. (F) SJP 485 and SJP 119 were used for checking left edge whereas SJP 127 and SJP 387 were used for checking right edge replacement of A2 (Ex)+B1+B2 sub-regions, respectively.

List of Publication

1. **Hassan N,** Sasano Y, Kimura S, Easmin F, Ekino K, Taguchi H, Harashima S (2020a) CRISPR-PCDup: a novel approach for simultaneous segmental chromosomal duplication in *Saccharomyces cerevisiae*. ***AMB Express*** 10:27. https://doi.org/10.1186/s13568-020-0957-4

2. **Hassan N,** Easmin F, Sasano Y, Ekino K, Taguchi H, Harashima S (2020b) Systematic approach for assessing whether undeletable chromosomal regions in Saccharomyces cerevisiae are required for cell viability. ***AMB Express*** (Manuscript in revison)

3. Easmin F, **Hassan N**, Sasano Y, Ekino K, Taguchi H, Harashima S (2019a) gRNA transient expression system for simplified gRNA delivery in CRISPR/Cas9 genome editing. ***J Biosci Bioeng*** 128(3):373-378

4. Easmin F, Sasano Y, Kimura S, **Hassan N**, Ekino K, Taguchi H, Harashima S (2019b) CRISPR-PCD and CRISPR-PCRep: Two novel technologies for simultaneous multiple segmental chromosomal deletion/replacement in *Saccharomyces cerevisiae*. ***J Biosci Bioeng***. https://doi.org/10.1016/j.jbiosc.2019.08.004

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