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Assessment of Cre-lox and CRISPR-Cas9 as tools for recycling of multiple-integrated selection markers in *Saccharomyces cerevisiae*[§]

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We evaluated the Cre-lox and CRISPR-Cas9 systems as marker-recycling tools in *Saccharomyces cerevisiae* recombinants containing multiple-integrated expression cassettes. As an initial trial, we constructed rDNA-nontranscribed spacer- or Ty4-based multiple integration vectors containing the *URA3* marker flanked by the loxP sequence. Integrants harboring multiple copies of *tHMG1* and *NNV-CP* expression cassettes were obtained and subsequently transformed with the Cre plasmid. However, the simultaneous pop-out of the expression cassettes along with the *URA3* marker hampered the use of Cre-lox as a marker-recycling tool in multiple integrants. As an alternative, we constructed a set of CRISPR-Cas9-gRNA vectors containing gRNA targeted to auxotrophic marker genes. Transformation of multiple integrants of *tHMG1* and *NNV-CP* cassettes by the Cas9-gRNA vector in the presence of the *URA3* (stop) donor DNA fragments generated the Ura[-] transformants retaining multiple copies of the expression cassettes. CRISPR-Cas9-based inactivation led to the recycling of the other markers, *HIS3, LEU2,* and *TRP1*, without loss of expression cassettes in the recombinants containing multiple copies of *tHMG1*, *NNV-CP*, and *SfBGL1* cassettes, respectively. Reuse of the same selection marker in marker-inactivated *S. cerevisiae* was validated by multiple integrations of the *TrEGL2* cassette into the *S. cerevisiae* strain expressing *SfBGL1*. These results demonstrate that introducing stop codons into selection marker genes using the CRISPR-Cas9 system with donor DNA fragments is an efficient strategy for marker-recycling in multiple integrants. In particular, the continual reuse of auxotrophic markers would facilitate the construction of a yeast cell factory containing multiple copies of expression cassettes without antibiotic resistance genes.

Keywords: *Saccharomyces cerevisiae*, multiple integration, marker-recycling, Cre-lox, CRISPR-Cas9

Introduction

The traditional baker's yeast, *Saccharomyces cerevisiae*, has been widely used as a host to produce a variety of valuable industrial compounds and heterologous proteins, ranging from small metabolites, such as ethanol, to recombinant therapeutic proteins like insulin (Hong and Nielsen, 2012; Kim *et al*., 2015). *S. cerevisiae* has also been used as a host for the production of advanced biofuels and fine chemicals that include farnesene, isobutanol (Borodina and Nielsen, 2014), resveratrol (Li *et al*., 2015), and opioids (Galanie *et al*., 2015). Recently, *S. cerevisiae* has also been exploited as a delivery system for cancer vaccines and oral vaccines, resulting in humoral and cellular immune responses without negative side effects (Franzusoff *et al*., 2005; Kim *et al*., 2014; Patterson *et al*., 2015). The rapidly expanding molecular genetic toolboxes based on synthetic biology approaches have further extended the scope of *S. cerevisiae* as microbial cell factories for the production of diverse endogenous and heterologous products of industrial interest (Krivoruchko *et al*., 2011; Redden *et al*., 2015; Chen *et al*., 2018; Thak *et al*., 2020).

Construction of recombinant yeast strains capable of producing metabolites of commercial value requires several rounds of genetic interventions. These include the introduction of heterologous genes and assembly of whole metabolic pathways, and removal of endogenous genes to guide the metabolic flux towards the products of interest. To implement heterologous metabolic pathways, a set of different expression cassettes of foreign genes should be introduced into host cells. The copy number of expression cassettes is one of the major factors to be considered in achieving high expression of heterologous genes (Chen *et al*., 2012). Although 2 μ-based vectors with high copy numbers are generally used for high-level expression in *S. cerevisiae*, these plasmids are not stably maintained, leading to heterogeneous gene expression in a population of cells (Jensen *et al*., 2014). In contrast, integration of a designed expression cassette into loci on host chromosomes by homologous recombination is beneficial because it allows for the stable maintenance of an expression cassette. Therefore, chromosomal integration of expression cassettes in multiple copies can maintain stable expression of the introduced genes at high levels for many generations. Several strategies for multiple integration using homologous recombination into multiple target loci by transformation of a single construct have been developed utilizing repetitive multiple target sequences. These include delta elements of the retrotransposon Ty elements (Sakai *et al*., 1990; Lee and Da Silva, 1997; Maury *et al*., 2016) and ribosomal DNA clusters (Lopes *et al*., 1989; Fujii *et al*., 1990; Moon *et al*., 2016).

Genetic manipulation generally requires selection of markers

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Table 1. Strains used in this study

Strain	Genotype	Source/Reference
Y2805	*MATa pep:HIS3 prb-Δ1.6 R can1 his3-20 ura3-52*	Kang *et al.* (2000)
Y2806	*MATa pep:HIS3 prb-Δ1.6 R can1 his3-20 ura3-52 leu2::tc*	Moon *et al.* (2016)
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1 MAL2-8C SUC2*	Entian and Kötter (2007)
CEN.PK2-1C-erg7	CEN.PK2-1C carrying pMET3-*ERG7*	Jung *et al.* (2014)
Y/NTS-NNV(16U_loxP)	Y2805 harboring multiple-integrated NNV(16U_loxP) cassettes at rDNA-NTS locus	This study
Y/Ty-NNV(16U_loxP)	Y2805 harboring multiple-integrated NNV(16U_loxP) cassettes at Ty4 locus	This study
e/NTS-tHMG1(16U_loxP)	CEN.PK2-1C-erg7 harboring multiple-integrated tHMG1 (16U_loxP) cassettes at rDNA-NTS locus	This study
C/NTS- NNV(16U_loxP)	CEN.PK2-1C harboring multiple-integrated NNV(16U_loxP) cassettes at rDNA-NTS locus	This study
Y6/NTS-NNV(50L)	Y2806 harboring multiple-integrated NNV(50L) cassettes at rDNA-NTS locus	Moon *et al.* (2016)
C/NTS-tHMG1(101H)	CEN.PK2-1C harboring multiple-integrated tHMG1 (101H) cassettes at rDNA-NTS locus	Moon *et al.* (2016)
C/NTS-SfBGL1(86T)	CEN.PK2-1C harboring multiple-integrated SfBGL1 (86T) cassettes at rDNA-NTS locus	This study
C/NTS-SfBGL1-TrEGL2(86T)	CEN.PK2-1C harboring multiple-integrated SfBGL1 and TrEGL2 (86T) cassettes at rDNA-NTS locus	This study

to screen transformants. Recycling of the selection markers allows the repeated use of the same selection marker and could facilitate the construction of recombinant yeast strains that require multiple expression cassettes of heterologous genes into host cells. In particular, the repeated use of multiple-integrated markers for genetic constructs that are integrated stably in the genome is required to develop an industrial strain in which multiple copies of expression cassettes are stably integrated into the host genome.

In this study, we evaluated the potential of Cre-lox and CRISPR-Cas9 systems as marker-recycling tools in *S. cerevisiae* recombinants containing multiple-integrated expression cassettes. As an initial trial, we combined the Cre-lox system with multiple integration systems based on ribosomal DNA-nontranscribed spacer (rDNA-NTS) and Ty elements. As the second strategy, we applied the CRISPR-Cas9 system as a tool for the recycling multiple-integrated markers, such as *URA3*, *HIS3*, *LEU2*, and *TRP1*. The findings demonstrate that introducing stop codons into selection marker genes by the Cas9-gRNA vectors with the donor DNA fragments is an efficient strategy to recycle multiple-integrated selection markers while maintaining the multiple-integrated expression cassettes.

Materials and Methods

Strains, plasmids, and culture conditions

The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Yeast cells were grown at 30°C in complete medium, either YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or YPG (1% yeast extract, 2% Bacto peptone, 2% galactose). For selection of transformants, fol-

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source
YEGα-MCS-NNV-CP	YEGα-MCS: P_{GAL10}-NNV	Choi *et al.* (2013)
pT-NTS-P(16)URA3	pGEM-T easy-5' NTS2-p(16)URA3-3' NTS2	Moon *et al.* (2016)
pT-loxP-16URA3	pGEM-T easy-loxP-16URA3-loxP	This study
pT-NTS-16U-loxP	pGEM-T easy-5' NTS2- loxP-16URA3-loxP -3' NTS2	This study
pT-NTS-NNV(16U_loxP)	pGEM-T easy-5' NTS2-P_{GAL10}-NNV-loxP-16 URA3-loxP-3' NTS2	This study
YEpU-tHMG1	YEGα-MCS containing P_{GAP1}-tHMG1 T_{GAL7}	This study
pT-NTS-tHMG1(16U_loxP)	pGEM-T Easy-5' NTS2-P_{GAP1}-tHMG1-loxP-p(16)URA3-loxP-3' NTS2	This study
pT-Ty4(5'HR-3'HR)	pGEM-T Easy-5'HR-Ty4/3'HR-Ty4	This study
pT-Ty4-NNV(16U_loxP)	pGEM-T Easy-5'HR-Ty4-P_{GAL10}-NNV-loxP-p(16)URA3-loxP-3'HR-Ty4	This study
pSH69	Expression vector of Cre recombinase under the *GAL10* promoter	EUROSCARF
pT-NTS-50L-NNV	pGEM-T easy-5' NTS2- P_{GAL10}-NNV-50LEU2-3'NTS2	Moon *et al.* (2016)
pT-NTS-101H-tHMG1	pGEM-T easy-5' NTS2- P_{GAP1}-tHMG1-101HIS3-3'NTS2	Moon *et al.* (2016)
Y2pU-SfBGL1-Flag	YEGα-P_{GAL10}-MFα-KEX2:FLAG-BGL1A-6xHIS:T_{GAL7}-ScURA3	Luu *et al.* (2019)
pT-NTS-86T-tHMG1	pGEM-T easy-5' NTS2-P_{GAP1}-tHMG1-86TRP1-3' NTS2	Moon *et al.* (2016)
pT-NTS-SfBGL1(86T)	pGEM-T easy-5' NTS2- P_{GAL10}- MFα KEX2:FLAG-BGL1A-6xHIS:T_{GAL7}-86TRP1-3' NTS2	This study
pT-NTS-TrEGL2(86T)	pGEM-T easy-5' NTS2-P_{GAL10}- MFα KEX2:HA-TrEGL2-6xHIS:T_{GAL7}-86TRP1-3' NTS2	This study
Coex 413-Cas9-ucc1 gRNA	Coex 413-Cas9 vector containing *UCC1* targeting gRNA with *HIS3* selection marker	Hong *et al.* (2019)
YCpH-C9U3g	Coex 413-Cas9 containing *URA3* targeting gRNA	This study
YCpH-C9T1g	Coex 413-Cas9 containing *TRP1* targeting gRNA	This study
CAS9-NAT	CEN-NAT vector expressing P_{TEF1}-Cas9-T_{CYC1}	Addgene
YCpU-C9L2g	CAS9-NAT containing *LEU2* targeting gRNA with *URA3* selection marker	This study
YCpU-C9H3g	CAS9-NAT containing *HIS3* targeting gRNA with *URA3* selection marker	This study

lowing synthetic complete media were used: SC-U, SC-H, SC-L, or SC-T (0.67% yeast nitrogen base [YNB] without amino acids, 2% glucose, drop-out amino acid mixture without uracil, histidine, leucine, or tryptophan), or SCH-U (0.67% YNB without amino acids, 2% glucose, drop-out amino acid mixture without uracil, 1.4% hygromycin B). For Cre recombinase expression, YPG containing 1.4% hygromycin B was used. Transformation of *S. cerevisiae* was performed according to the modified lithium acetate-dimethyl sulfoxide (DMSO) method (Gietz and Woods, 2002). *Escherichia coli* transformants were cultured in LB medium (0.5% yeast extract, 1% Bacto tryptone, 1% NaCl) supplemented with 100 μg/ml ampicillin.

Construction of rDNA-NTS/Cre-lox integration vectors for the expression of *NNV-CP* and *tHMG*1

The rDNA-NTS-based multiple integration vectors combined with the Cre-lox system were constructed as follows. The loxP-16*URA3*-loxP fragment, which consists of the *URA3* gene with a 16 bp truncated promoter (16*URA3*) flanked by loxP sequences in the same direction, was amplified by fusion PCR using pT-NTS-P(16)URA3 as a template with two sets of primers: URA3(SmaI) fw1/URA3(SmaI) rv1 and URA3 (SmaI) fw2/URA3(SmaI) rv2 (Supplementary data Table S1). After digestion with SmaI, the loxP-16*URA3*-loxP fragment was cloned into the pGEM-T-Easy vector (Promega) to generate pT-loxP-16URA3. The loxP-16*URA3*-loxP fragment was obtained by XbaI and NotI digestion from pT-loxP-16URA3 and subcloned into pT-NTS-P(16)URA3 to generate pT-NTS-16U-loxP. To construct the multiple integration vector for *NNV-CP* expression, pT-NTS-NNV(16U_loxP), the *NNV-CP* expression cassette under the control of the *GAL10* promoter, was obtained by BamHI/XbaI digestion from YEGa-MCS-NNV-CP and ligated with pT-NTS-16U-loxP.

To construct the pT-NTS-tHMG1(16U_loxP) multiple integration vector for *tHMG1* expression, the *tHMG1* expression cassette under the control of the *GAP1* promoter was obtained from YEpU-tHMG1 by BamHI/XbaI digestion and ligated with pT-NTS-16U-loxP. For targeted integration of each expression cassette into the rDNA-NTS locus of host chromosomes, pT-NTS-NNV(16U_loxP) was digested with SphI and MluI, and pT-NTS-tHMG1(16U_loxP) was digested with SpeI and XhoI, respectively, before transformation.

Construction of Ty4/Cre-lox integration vector for *NNV-CP* expression

The Ty4/Cre-lox multiple integration vector for *NNV-CP* expression, pT-Ty4-NNV(16U_loxP), was constructed as follows. The 5' upstream region of Ty4 long terminal repeats (LTRs) was amplified by PCR using the primers Ty4 5'HR fw and Ty4 5'HR rv, and 3' downstream region of Ty4 LTRs was amplified by PCR using the primers Ty4 3'HR fw and Ty4 3'HR rv from the genomic DNA obtained from the CEN.PK2-1C strain. Using fusion PCR, the two PCR products obtained were combined, leading to Ty4(5'HR-3'HR) PCR fragments. This fragment was cloned using the pGEM-T Easy vector, resulting in pT-Ty4(5'HR-3'HR). The 5'HR-3'HR fragment was generated by BamHI/NotI digestion of pT-Ty4 (5'HR-3'HR) and ligated with pT-NTS-NNV(16U_loxP), leading to pT-Ty4-NNV(16U_loxP). For targeted integration of the *NNV-CP* expression cassettes into the Ty4 locus, pT-Ty4-NNV(16U_loxP) was digested with SphI and MluI before transformation of yeast cells.

Construction of rDNA-NTS-based integration vectors for expression of *SfBGL1* and *TrEGL2*

To construct pT-NTS-SfBGL1(86T), the rDNA-NNS-based integration vector for *SfBGL1A* expression using *TRP1* as a selection marker, the *SfBGL1* expression cassette under the control of the *GAL10* promoter was obtained from Y2pU-SfBGL1-Flag (Luu *et al.*, 2019) by BamHI/SalI digestion and ligated with BamHI/SalI-digested pT-NTS-86T-tHMG1 (Moon *et al.*, 2016) to replace the *tHMG1* expression cassette, generating pT-NTS-SfBGL1(86T). To construct an rDNA-NNS-based integration vector for *TrEGL2* expression, the *S. cerevisiae* codon-optimized *TrEGL2* gene was synthesized (Cosmogentech) and fused with the DNA fragment encoding the C-terminal part of MFα by PCR with the hemagglutinin (HA) epitope amplified from pT-NTS-SfBGL1(86T) using two sets of primers, MFα KEX2 fw/MFα KEX2 rv and TrEGL2 fw/TrEGL2 rv (Supplementary data Table S1). The obtained fusion PCR product, MFα-KEX2-TrEGL2, was digested with EcoRI/XhoI and ligated with EcoRI/SalI-digested pT-NTS-SfBGL1(86T), resulting in pT-NTS-TrEGL2(86T). For targeted integration into the rDNA-NTS locus, pT-NTS-SfBGL1 (86T) and pT-NTS-TrEGL2(86T) were digested with SpeI and NsiI, respectively, before the transformation of yeast cells.

Construction of CRISPR-Cas9 vectors targeting *URA3*, *LEU2*, *HIS3*, and *TRP1*

The CEN-based yeast vector expressing Cas9 and guide RNA (gRNA) targeting *URA3,* YCpH-C9U3g, was constructed as follows. The 452 bp DNA fragment containing the 20-mer *URA3*-targeting gRNA (Zhang *et al.*, 2014) flanked with the partial *SNR52* promoter and the *SUP4* terminator was amplified by fusion PCR with two primer sets: Total gRNA fw/URA3gRNA rv and URA3gRNA fw/Total gRNA rv (Supplementary data Table S1) using Coex 413-Cas9-ucc1 gRNA as a template. The amplified P_{SNR52}-gRNA_URA3-T_{SUP4} fragment was subcloned into a T-blunt vector, resulting in pTB-gRNA_URA3. After sequencing confirmation, pTB-gRNA_URA3 was digested with XbaI/SalI and ligated with XbaI/SalI-treated Coex 413-Cas9-ucc1 gRNA to replace the DNA fragment encoding *UCC1*-tarteting gRNA, resulting in YCpH-C9U3g, a Coex 413-Cas9 vector containing *URA3* gRNA (Supplementary data Fig. S1A). The vectors YCpH-C9L2g, YCpH-C9T1g (Supplementary data Fig. S1B), and YCpH-C9H3g, which express Cas9 and the 20-mer gRNA targeting *LEU2*, *TRP1*, and *HIS3* (Zhang *et al.*, 2014), respectively, were constructed using the same strategy as described for the construction of YCpH-C9U3g using the gene-specific primer sets, as listed in Supplementary data Table S1. YCpU-C9H3g, a vector expressing Cas9 and *HIS3* gRNA with *URA3* selection marker, was constructed by three-piece ligation of the PSNR52-gRNA_HIS3-TSUP4 PCR fragments amplified by gene-specific primer sets from YCpH-C9H3g using the pri-

mer set Total gRNA fw2 and Total gRNA rv2, the *URA3* gene fragment amplified from the *S. cerevisiae* genomic DNA using the primer set URA3(SM)fw and URA3(SM)rv, and the NcoI/MluI-digested CAS9-NAT (Supplementary data Fig. S1C). To construct YCpU-C9L2g, a vector expressing Cas9 and *LEU2* gRNA with selection marker *URA3,* the MunI-digested P_{SNR52}-gRNA_LEU2-T_{SUP4} fragment was obtained from YCpH-C9L2g and ligated with MunI-digested YCpU-C9H3g to replace the *HIS3* gRNA fragment with the *LEU2* gRNA fragment (Supplementary data Fig. S1D). The 93 mer donor DNA(stop) fragments of *URA3, LEU2*, *HIS3,* and *TRP1* containing two stop codons were prepared by PCR using each primer (Supplementary data Table S1).

Western blot analysis

To detect NNV-CP protein or FLAG-tagged tHMG1 protein, intracellular protein extracts from yeast cells cultivated in YPG or YPD for 24 h, respectively, were prepared by bead-beating cells with TOMY in lysis buffer TNE (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation, the obtained supernatants were separated by SDS-PAGE and subjected to immunoblotting using mouse anti-Red-spotted grouper nervous necrosis virus coat protein (RGNNV-CP) serum (Kim *et al*., 2014) or anti-FLAG antibody (Roche), and subsequent detection using the alkaline phosphatase (AP)-substrate kit (Bio-Rad). For western blotting of recombinant SfBGL1 and TrEGL2 proteins secreted extracellularly, the cell culture supernatants were obtained after cultivation in YPG, separated by SDS-PAGE, and analyzed using anti-FLAG antibody (Sigma-Aldrich) and anti-HA antibody (Roche), respectively. Western blot signals were detected using horseradish peroxidase (Sigma-Aldrich).

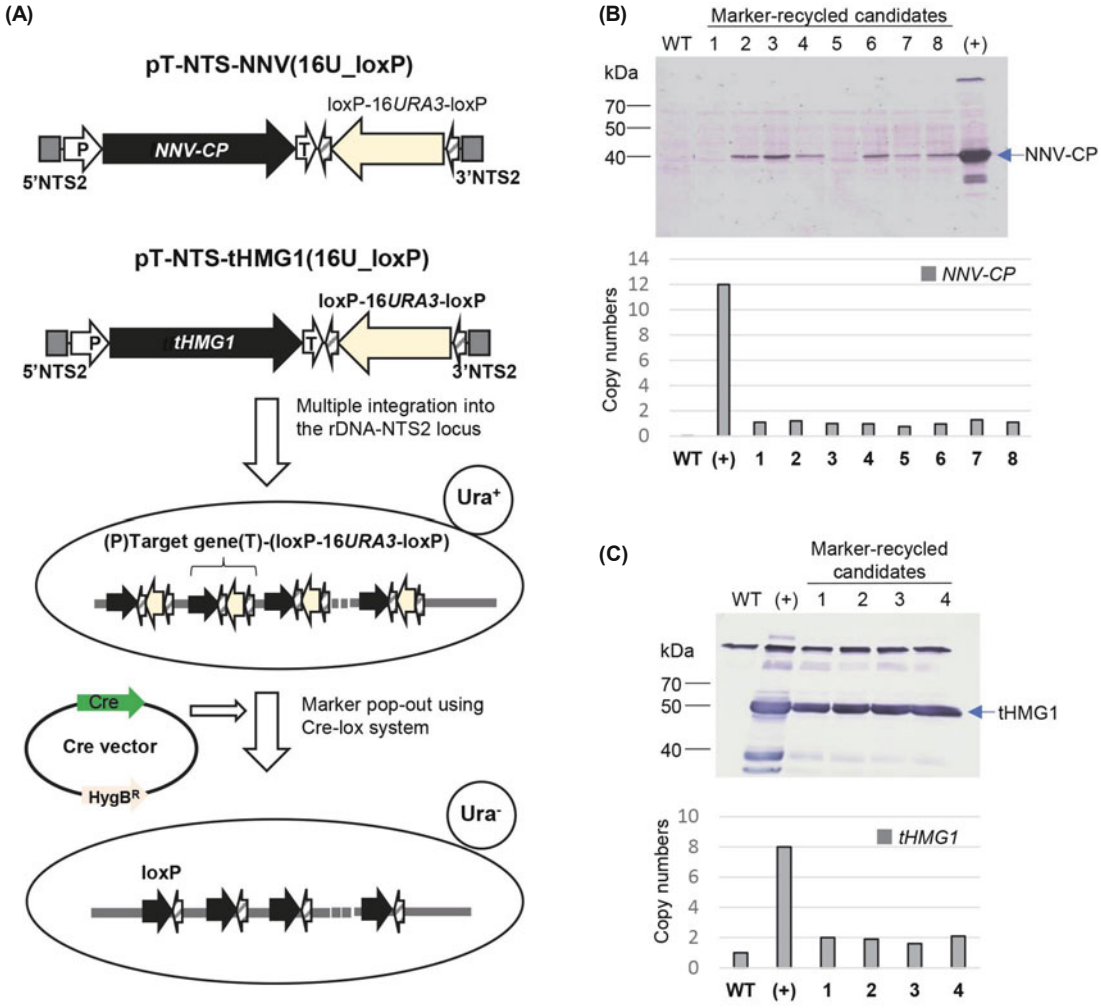

Fig. 1. Combination of Cre-lox system with rDNA-NTS-based multiple integration system for marker-recycling. (A) Scheme of the generation of recombinant strains harboring multiple integration of *NNV-CP* expression cassette at the locus of rDNA-NTS of host chromosome using pT-NTS-NNV(16U_loxP) vector (Top) and *tHMG1* expression cassette using pT-NTS-tHMG1(16U_loxP) (Bottom), respectively, and subsequent auxotrophic marker-recycling by Cre-lox. (B and C) Analysis of protein expression levels and copy numbers in the Ura$^-$ derivatives of the parental strains (+), Y/NTS-NNV(16U-loxP) #12 and e/NTS-tHMG1(16U-loxP) #10, containing multiple copies of *NNV-CP* (B) and *tHMG1* (C) expression cassettes, respectively, after expression of Cre recombinase. The NNV-CP protein was detected by mouse anti-RGNNV-CP serum and tHMG1 protein tagged with FLAG at its C-terminus was detected using anti-FLAG antibody. The copy numbers were analyzed by qRT-PCR and normalized by *ACT1*.

Copy number determination by quantitative real-time PCR (qRT-PCR) analysis

For genomic DNA extraction, yeast cells were grown in the appropriate drop-out medium at 30°C, suspended in STES lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS), and vortexed vigorously with glass beads using TOMY for 5 min. After addition of 200 µl phenol:chloroform:isoamylalcohol (25:24:1, v/v) and 200 µl 1X TE buffer, followed by vortexing using TOMY for 2 min, the yeast cell extract was centrifuged. The supernatant was precipitated by adding ethanol. The precipitate was re-dissolved and incubated with 50 µg/ml RNase. The obtained DNA samples were used as templates in the qRT-PCR cocktail, which contained 12.5 µl Maxima SYBR Green qPCR Master Mix (Fermentas) and 10 pmol of each set of forward and reverse oligonucleotide primers listed in Supplementary data Table S1. The PCR reaction was monitored using a CFX96 real-time PCR detection system (Bio-Rad). The copy numbers of *NNV-CP*, *HMG1*, *SfBGL1*, and *TrEGL2* integrated into host chromosomes were estimated by comparison with *ACT1* in the host genome as an internal reference gene (one copy in the genome). A standard curve for each target was prepared using pure plasmids or genomic DNA samples.

Cellulase activity assay

The culture supernatants of yeast cells, cultivated in YPG for 24 h, were concentrated using an amicon tube (30 kDa cut-off, Sigma-Aldrich) and reacted with the substrate 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-cellopentaoside (BPNPG5), provided by the cellulase assay kit (CellG5 Method, Megazyme), at 40°C for 10 min. The reaction was terminated by adding a stopping reagent (2% [w/v] Tris buffer [pH 10]) and the absorbance of 4-nitrophenol at 400 nm was measured. The cellulase activity (CellG5 Units/ml) was calculated as indicated in the cellulase assay kit.

Results

Combining the Cre-lox system with the rDNA-NTS-based multiple integration system

As an initial trial to evaluate the potential of Cre-lox as a marker-recycling tool in *S. cerevisiae* recombinants containing multiple-integrated expression cassettes, we combined the Cre-lox system with a previously developed rDNA-NTS-based multiple integration system (Moon *et al.*, 2016). The pT-NTS-tHMG1(16U_loxP) and pT-NTS-NNV(16U_loxP) multiple integration vectors were designed to contain the defective *URA3* marker flanked by the same directional loxP sequences (Fig. 1A). The constructed vectors, pT-NTS-tHMG1 (16U_loxP) and pT-NTS-NNV(16U_loxP), contain the expression cassettes for a truncated 3-hydroxyl-3-methylglutaryl-CoA reductase (tHMG1) and a capsid protein of red-spotted grouper necrosis virus (NNV-CP), respectively. To ensure the targeted integration of *tHMG1* or *NNV-CP* expression cassettes into the rDNA-NTS2 locus of the host genome, pT-NTS-tHMG1 (16U_loxP) and pT-NTS-NNV(16U_loxP) were digested with SpeI/XhoI and SphI/MluI, respectively, and introduced into *S. cerevisiae* Y2805 and CEN.PK2-1C-erg7 strain (Table 1), respectively. To obtain transformants containing multiple copies of the *tHMG1* and *NNV-CP* cassettes integrated with rDNA-NTS sites, qRT-PCR analysis using genomic DNA from each transformant as a template was conducted. Among the obtained *S. cerevisiae* transformants with various integration copy numbers, we chose the integrant Y/NTS-NNV (16U_loxP) #12, harboring 12 copies of the *NNV-CP* cassette, and the integrant e/NTS-tHMG1(16U_loxP) #10, harboring approximately eight copies of the *tHMG1* cassette, respectively, for further study.

To direct the pop-out of the loxP-*URA3*-loxP selection marker from the obtained multiple integrants, the Cre expression vector that expresses Cre recombinase under the *GAL10* promoter was introduced into Y/NTS-NNV(16U_loxP) #12 and e/NTS-tHMG1(16U_loxP) #10 integrants. The transformants selected on the SCH-U plate were subjected to prolonged incubation on YPG agar containing 2% galactose and supplemented with hygromycin B. The Ura3$^-$ auxotrophic phenotype was screened for by replica plating. Most of the Cre transformants showed the pop-out of the integrated *URA3* selection markers. However, they exhibited dramatically decreased expression of NNV-CP and tHMG1 proteins when analyzed by western blotting, implying the loss of these expression cassettes along with selection markers (Fig. 1B and C, top). We analyzed the copy numbers of NNV-CP in the transformants (1–8) derived from the Y/NTS-NNV(16U_loxP) #12 through

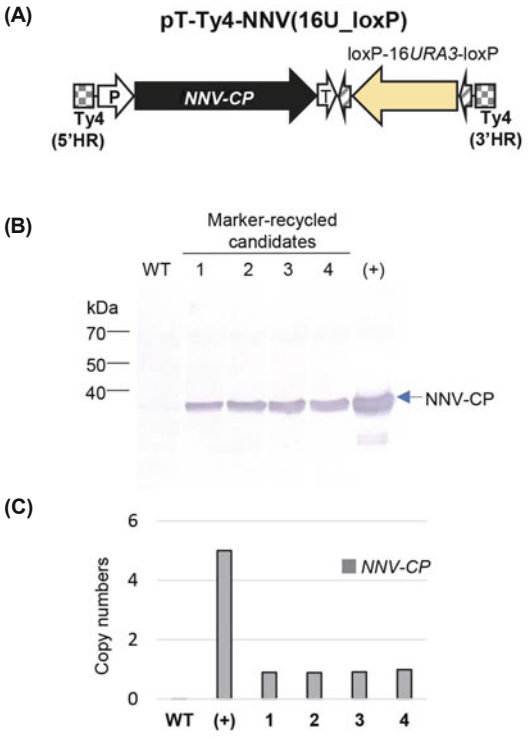

Fig. 2. Combination of Cre-lox system with Ty4-based multiple integration system for marker-recycling. (A) Scheme of the generation of recombinant strains harboring multiple integration of *NNV-CP* expression cassette at the locus of Ty4 elements of the host chromosome using pT-Ty4-NNV (16U_loxP) vector. (B and C) Analysis of protein expression levels (B) and copy numbers (C) of *NNV-CP* in the Ura$^-$ derivatives of the parental strain (+), Y/Ty-NNV(16U_loxP) #4, after expression of Cre recombinase.

qRT-PCR. All the tested transformants retained only one or two copies of the *NNV-CP* expression cassette after Cre expression by cultivation on YPG (Fig. 1B, bottom). The results strongly indicated that although the pop-out efficiency of the multiple-integrated selection marker was pronounced, the removal of whole expression cassettes was caused simultaneously with marker pop-out, leaving only one or two copies of *NNV-CP* expression cassettes. The loss of *tHMG1* expression cassette by Cre expression was also confirmed by qRT-PCR analysis of four transformants derived from e/NTS-tHMG1 (16U_loxP) #10 (Fig. 1C, bottom). The collective rsults strongly indicate that using the Cre-lox system as a marker-recycling tool was hampered in the rDNA-NTS-based multiple integrants by the simultaneous pop-out of the whole expression cassette tandem integrated at the rDNA cluster, probably by Cre-mediated direct repeat recombination.

Combining the Cre-lox system with the Ty4-based multiple integration system

We further tested the Cre-lox system as a recycling tool for selection markers in another multiple integration system based on Ty elements, in which the integration sites are dispersed and remote. To direct stable integration of expression cassettes without tandem repeats, we employed the Ty4 element, which exists in much lower copies than the Ty1 family (Stucka *et al.*, 1989), and which is spread on all chromosomes except chromosomes I and XI (Kim *et al.*, 1998). The rDNA-NTS sequences flanking the *NNV-CP* expression cassettes and the loxP-*URA3*-loxP selection marker were replaced with the 5'-upstream and 3'-downstream fragments corresponding to the (LTRs) of Ty4, resulting in the final vector pT-Ty4-NNV (16U_loxP) (Fig. 2A). For targeted integration into the Ty4 locus, pT-Ty4-NNV(16U_loxP) was digested with SphI and MluI before transformation of *S. cerevisiae* Y2805. By screening copy numbers by qPCR, integrant #4 containing five copies of *NNV-CP* cassettes was chosen for further analysis. After introducing the Cre recombinase vector into the Y/Ty-NNV(16U_loxP) #4 strain, the obtained transformants were plated on YPG plates containing hygromycin B to induce the expression of Cre recombinase. Western blot analysis of the four transformants revealed significantly decreased expression levels of NNV-CP protein, indicating looping out of the whole expression cassettes integrated at the dispersed locus of Ty4 sites by the action of Cre (Fig. 2B). The qRT-PCR analysis further confirmed that the transformants after Cre expression lost all but one copy of the *NNV-CP* expression cassette (Fig. 2C).

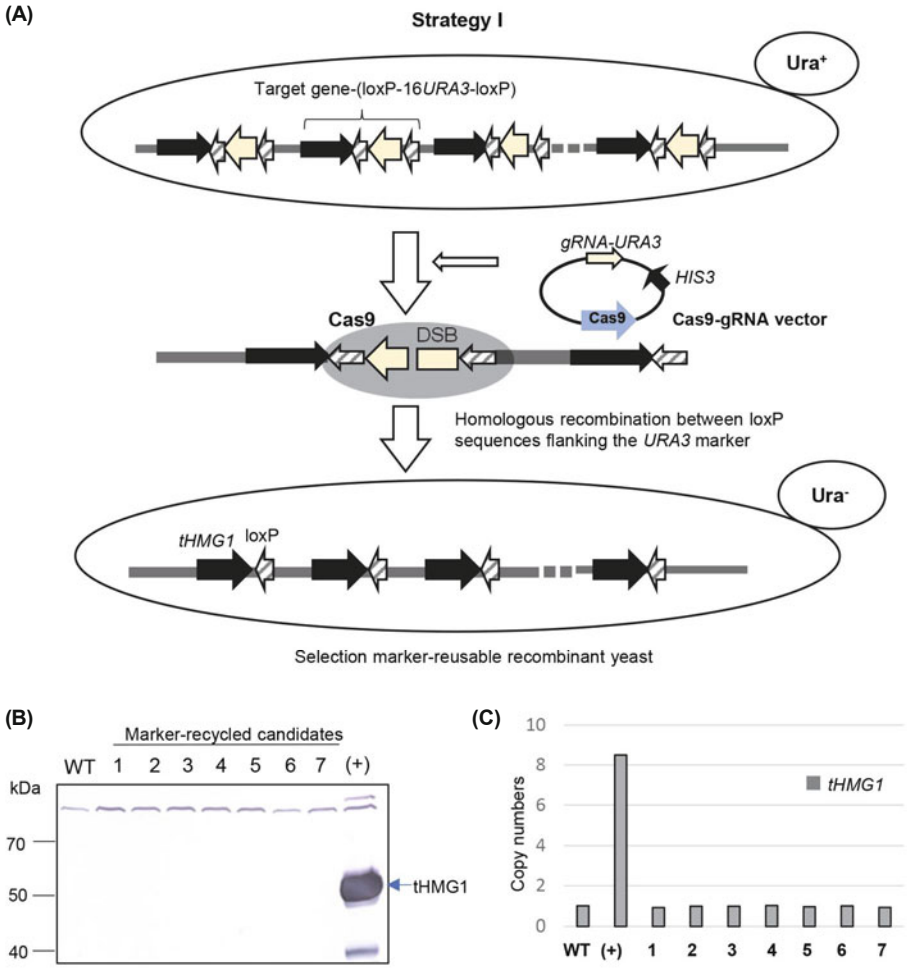

Fig. 3. Recycling multiple-integrated *URA3* auxotrophic selection markers using a Cas9-gRNA vector without a donor DNA fragment. (A) Scheme of strategy I to induce pop-out of the multiple-integrated selection markers by homology-directed repair (HDR) between the repeated lox-sequences flanking the auxotrophic marker. (B and C) Western blot analysis (B) and copy number quantitation (C) of *tHMG1* expression in Ura$^-$ derivatives of the parental strain (+), e/NTS-tHMG1(16U-loxP) #10, after transformation of Cas9-gRNA vector.

Coupling the CRISPR-Cas9 system without donor DNA to the rDNA-NTS multiple integration system

To reduce the risk of losing expression cassettes by homologous recombination between directed repeats, we examined the feasibility of the CRISPR-Cas9 system as a recycling tool for multiple-integrated auxotrophic selection markers. We constructed YCpH-C9U3g to express Cas9 and *URA3*-targeted guide RNA (gRNA) in one vector backbone (Supplementary data Fig. S1). As the first strategy in exploiting the CRISPR-Cas9 system, we attempted to recycle multiple-integrated selection markers without a donor DNA fragment. In a previous study on CRISPR-Cas9-induced marker excision in *Candida albicans*, the selection markers flanked by direct repeats were efficiently excised by homologous recombination enhanced by Cas9-mediated DNA double-stranded breaks (DSBs) (Huang and Mitchell, 2017). Because the integrant e/NTS-tHMG1 (16U_loxP) #10 harbors the *URA3* selection markers flanked by two loxP sequences, we expected that the DSBs on *URA3*, generated by the gRNA-Cas9 complex, could activate the homologous recombination between the loxP flanking sequences, leading to excision of each *URA3* (Fig. 3A). The integ-

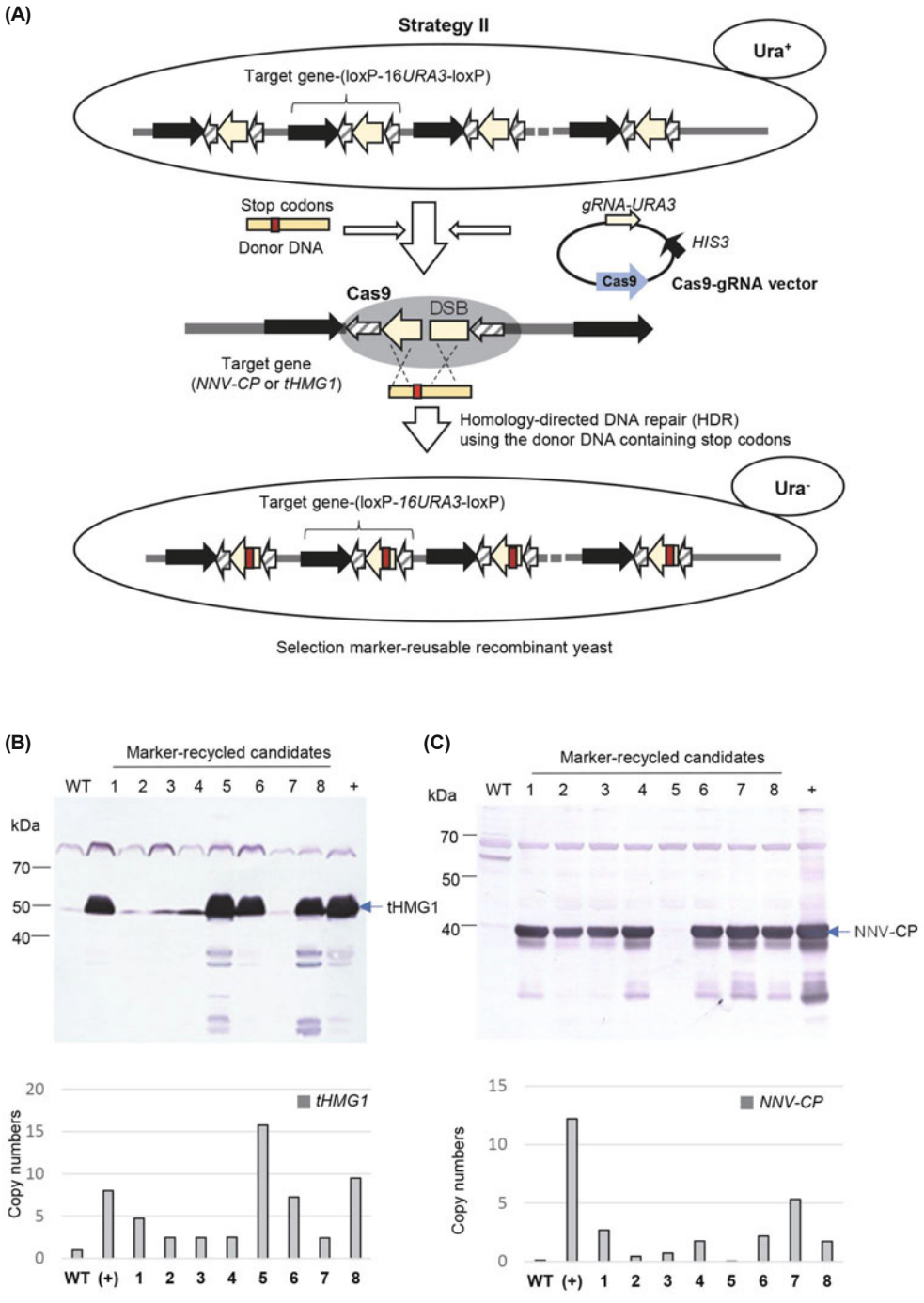

Fig. 4. Recycling of multiple-integrated *URA3* auxotrophic selection markers using Cas9-gRNA vector with a donor DNA(stop) fragment. (A) Scheme of strategy II to introduce stop codons into the multiple-integrated selection markers. DNA repair based on HDR with donor DNA fragments results in the exchange with donor DNA fragment containing stop codons to inactivate auxotrophic selection markers. (B and C) Analysis of western blot and copy numbers of *tHMG1* (B) and *NNV-CP* (C) expression in the *URA3*-inactivated derivatives of the parental strains (+), Y/NTS-NNV(16U-loxP) #12 and e/NTS-tHMG1 (16U-loxP) #10.

rant e/NTS-tHMG1(16U_loxP) #10 was transformed with YCpH-C9U3g only without donor DNA, and the transformants were obtained by plating on SC-H. The His^+ transformants were subjected to prolonged incubation on YPD and screened for Ura^- auxotrophic phenotype by replica plating. Western blotting and copy number analysis revealed the complete loss of *tHMG1* expression cassette in all the tested Ura^- transformants, indicating the simultaneous pop-out of both *tHMG1* expression cassettes and *URA3* selection markers (Fig. 3B and C). Homology-directed repair (HDR) between *URA3*, integrated in tandem, might occur more actively than HDR between the loxP sequences flanking *URA3*. These results suggest that the CRISPR-Cas9 system without donor DNA is not suitable for the recycling multiple-integrated selection markers.

Coupling the CRISPR-Cas9 system in the presence of donor DNA to the rDNA-NTS multiple integration system

As an alternative strategy, we tested the CRISPR-Cas9 system in the presence of a 93 mer *URA3* donor DNA fragment (stop) containing two stop codons (Supplementary data Table S2). This was performed based on the speculation that HDR using the donor DNA results in the exchange with donor DNA fragments, which would introduce stop codons into the multiple-integrated selection markers to prevent the synthesis of the marker proteins (Fig. 4A). The integrant e/NTS-tHMG1 (16U_loxP) #10 was transformed with YCpH-C9U3g in the presence of the *URA3* donor DNA fragment(stop) (Supplementary data Table S2) and selected on SC-H. After prolonged incubation of the obtained His^+ transformants on YPD, the transformants were screened for the $Ura3^-$ auxotrophic phe-

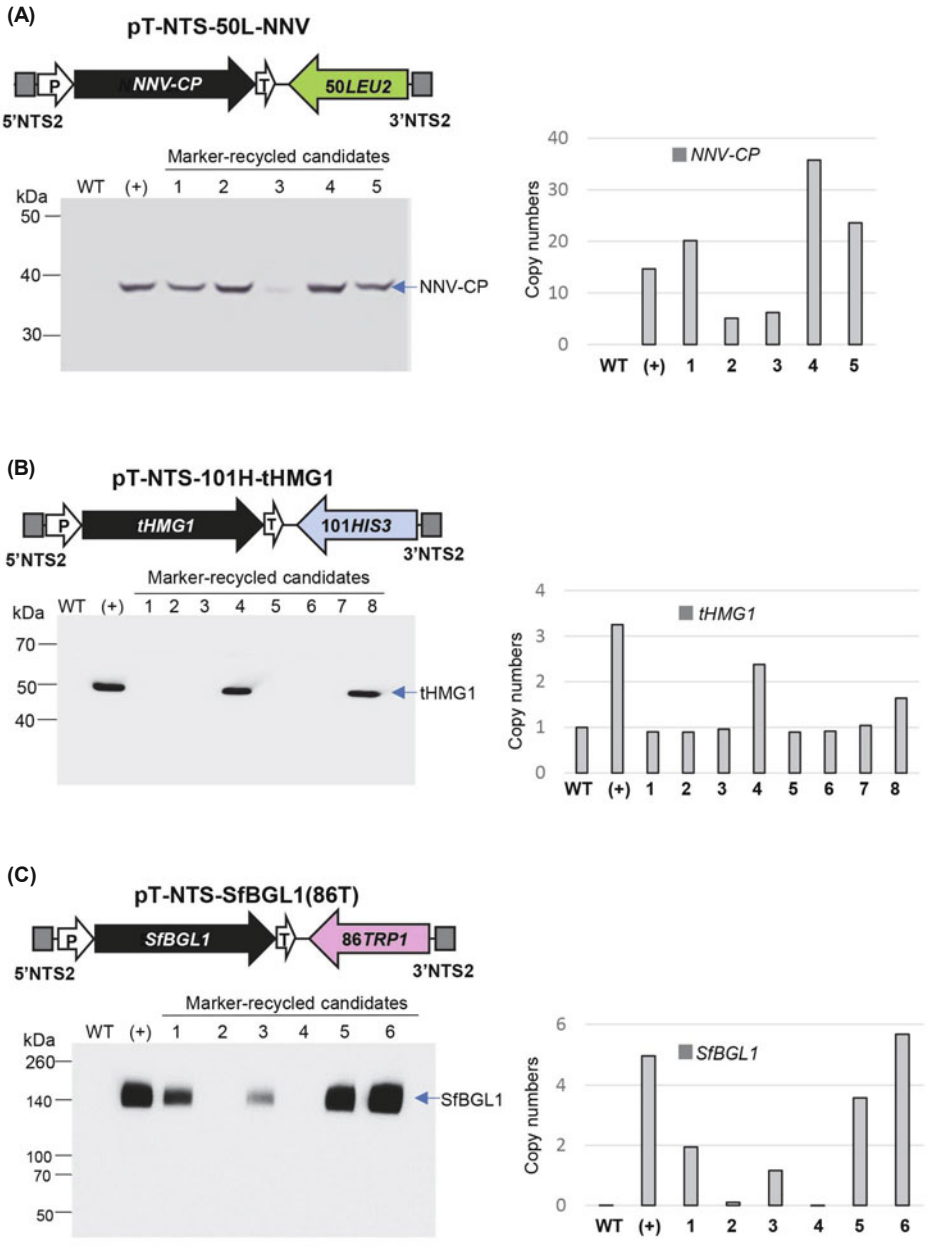

Fig. 5. Recycling of multiple-integrated auxotrophic selection markers *LEU2*, *HIS3*, and *TRP1* using Cas9-gRNA vector with a donor DNA(stop) fragment. Scheme of the expression cassettes for *NNV-CP* (A), *tHMG1* (B), and *SfBGL1* (C) integrated in multiple copies at the locus of rDNA-NTS sites of host chromosome is shown as top panel. Analysis of western blot and copy numbers of the transformants derived from the multicopy-integrant control (+) after introduction of Cas9-gRNA vector with a donor DNA(stop) fragment for each selection marker are presented in the left and right panels, respectively. A. Analysis of the *LEU2*-inactivated derivatives of the parental strain (+), Y6/NTS-NNV(50L). B. Analysis of the *HIS3*-inactivated derivatives of the parental strain (+), C/NTS-tHMG1(101H). (C) Analysis of the *TRP3*-inactivated derivatives of the parental strain (+), C/NTS-SfBGL1(86T).

notype. Western blot analysis revealed that the expression levels of tHMG1 protein in several Ura3$^-$ auxotrophs (50% frequency) were comparable to that of the parental strain before the introduction of the Cas9-*URA3* gRNA vector, indicating that the *tHMG1* cassette was retained in multiple copies in these Ura3$^-$ auxotrophs (Fig. 4B). Although some fraction of the *tHMG1* expression cassette appeared to pop-out by direct repeat recombination, the results strongly indicate that the CRISPR-Cas9 system in the presence of donor DNAs can be used for effective marker-recycling in the

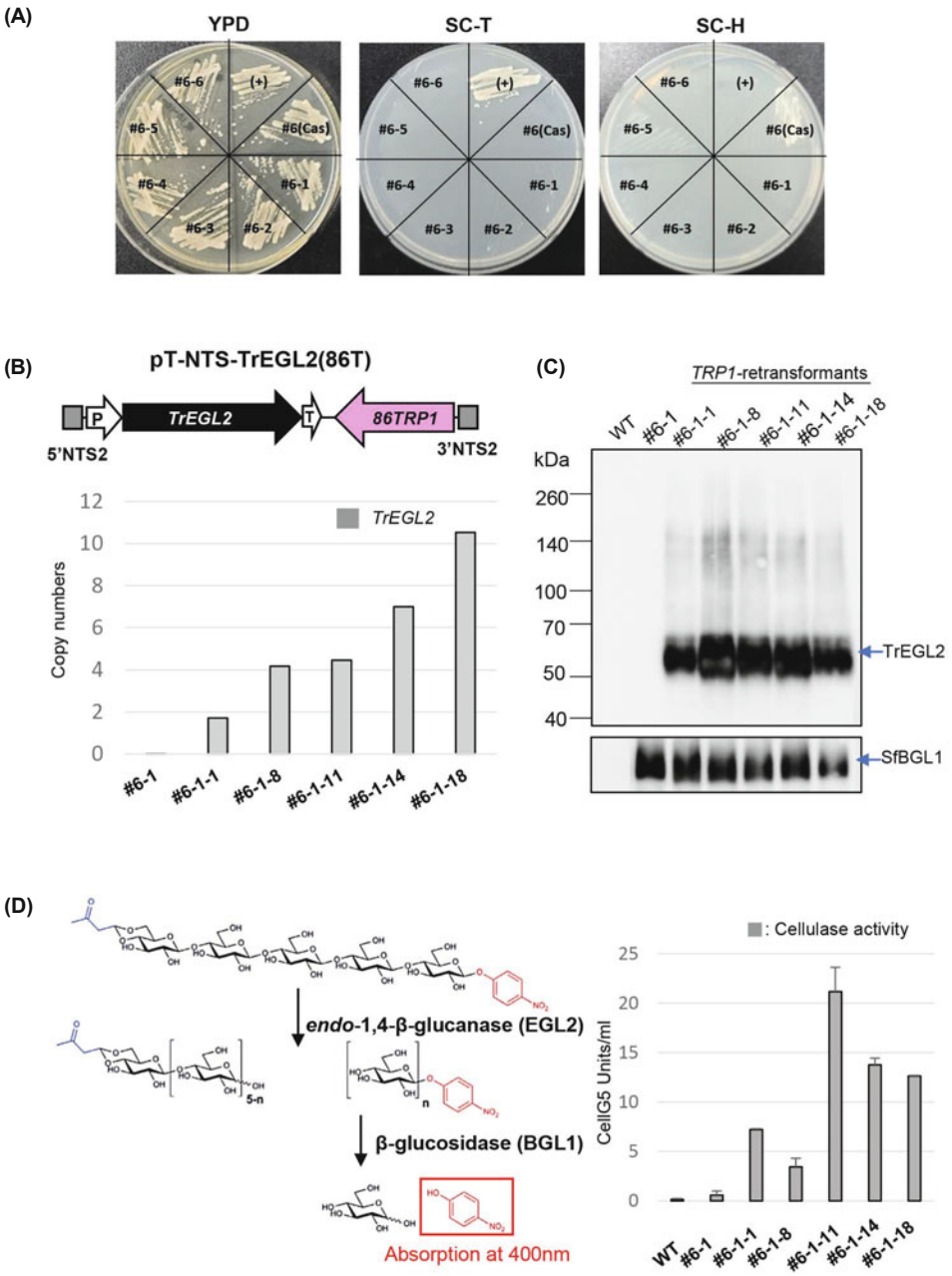

Fig. 6. Reuse of *TRP1* for integration of *TrEGL2* expression cassettes into *TRP1*-recycled *SfBGL1* expressing transformants. (A) Pop-out of the Cas9-gRNA vector from C/NTS-SfBGL1(86T) transformants. (+), the parental strain C/NTS-SfBGL1(86T) harboring five copies of the *SfBGL1* cassette integrated at rDNA-NTS sites before transformation with Cas9-gRNA vector; #6(Cas), *TRP1*-inactivated C/NTS-SfBGL1(86T) by transformation with Cas9-gRNA vector (His$^+$ Trp$^-$), which was presented in Fig. 5C; #6-1~#6-6, derivatives with His$^-$ Trp$^-$ phenotype, generated from the #6(Cas) strain after continuously cultivation on YPD. (B) Reuse of *TRP1* for transformation after pop-out of Cas9-gRNA vector. Scheme of *TrEGL2* expression cassette using *TRP1* as selection marker for multiple integration at the locus of rDNA-NTS sites of host chromosome is shown at top panel. The *TrEGL2* expression cassette was introduced into the #6-1 strain, a *TRP1*-inactivated *SfBGL1* expressing transformant without Cas9-gRNA vector, and Trp$^+$ transformants were selected to generate C/NTS-SfBGL1-TrEGL2(86T) strains. The copy numbers of *TrEGL2* cassette in C/NTS-SfBGL1-TrEGL2(86T) strains, #6-1-1, #6-1-8, #6-1-11, #6-1-14, and #6-1-18, are presented in the bottom panel. (C) Expression levels of TrEGL2 and SfBGL1 proteins in C/NTS-SfBGL1-TrEGL2(86T) strains. (D) Cellulase activity analysis of C/NTS-SfBGL1-TrEGL2(86T) strains. Cellulase activity (CellG5 Units/ml) was defined as the amount of enzyme required to release 1 μmol of 4-nitrophenol/min at 40°C under the defined assay conditions described in the cellulase assay kit, CellG5 Method (Megazyme).

recombinant strains carrying multiple-integrated selection markers. To confirm the feasibility of CRISPR-Cas9 to recover the Ura3$^-$ auxotrophic phenotype in multiple integrants harboring another different expression cassette, the integrant Y/NTS-NNV(16U_loxP) #12 was transformed with YCpH-C9U3g along with the *URA3* donor DNA(stop) fragment. Western blot analysis of the obtained Ura3$^-$ transformants revealed the retention of the *NNV-CP* cassette in comparable copy numbers compared to the parental strain (+) (Fig. 3C). After marker inactivation, the Cas9-gRNA vectors could be efficiently eliminated from the transformants by continuous cultivation on YPD without loss of integrated expression cassettes (Supplementary data Fig. S2A and B). Altogether, these results support that Cas9-induced marker inactivation with donor DNA is an efficient strategy to recycle multiple-integrated selection markers for subsequent genetic manipulation.

Application of the CRISPR-Cas9 system to recycling of other multiple-integrated selection markers

To confirm the feasibility of CRISPR-Cas9 to recover other auxotrophic phenotypes besides *URA3*, we constructed a set of Cas9 vectors containing gRNA targeted to *LEU2*, *HIS3*, and *TRP1* (YCpU-C9L2g, YCpU-C9H3g, and YCpH-C9T1g, respectively; Supplementary data Fig. S1) and evaluated it as a tool for recycling multiple-integrated auxotrophic marker genes (Fig. 5). To recycle the multiple-integrated *LEU2* marker genes, the integrant Y6/NTS-NNV(50L) was transformed by introducing YCpU-C9L2g with the *LEU2* donor DNA fragment(stop). The Y6/NTS-NNV(50L) integrant, obtained by transformation of *S. cerevisiae* Y2806 strain with pT-NTS-50L-NNV in our previous study (Moon *et al*., 2016), harbored 15 copies of the *NNV-CP* cassettes. Western blot and copy number analyses of the Leu$^-$ transformants indicated that all the multiple-integrated *LEU2* genes were exchanged with the inactivated *leu2* allele with two stop codons, while retaining the *NNV-CP* cassette in multiple copies (Fig. 5A).

To test the recycling of the multiple-integrated *HIS3* marker genes, YCpU-C9H3g was introduced in the presence of the *HIS3* donor DNA fragment(stop) into the integrant C/NTS-tHMG1(101H) (Fig. 5B), obtained by transformation of *S. cerevisiae* CEN.PK2-1C with pT-NTS-101H-tHMG1 in our previous study (Moon *et al*., 2016). The integrant carrying 3–4 copies of the *tHMG1* cassette was chosen for the analysis in this study, since overexpression of tHMG1 protein above a certain level generated an inhibitory effect on the production of squalene (Moon *et al*., 2016). Western blotting and copy number analyses revealed that the multi-integrated *HIS3* marker genes were inactivated with conservation of the *tHMG1* expression cassette, although at a lower rate compared to the recycling of other auxotrophic markers *URA3* and *LEU2* (Fig. 5B).

In an effort to expand the scope of tested expression cassettes in marker-recycling analysis, we constructed the pT-NTS-SfBGL1(86T) vector. The vector contains the *Saccharomycopsis fibuligera BGL1* expression cassette under control of the *GAL10* promoter and the defective *TRP1* with an 86 bp promoter as a selection marker. The C/NTS-SfBGL1(86T) integrant harboring six copies of *SfBGL1* was obtained by transformation of *S. cerevisiae* CEN.PK2-1C strain with SphI/MluI-digested pT-NTS-SfBGL1(86T). The Cas9-gRNA vector YCpH-C9T1g was introduced with the *TRP1* donor DNA fragment(stop) into the integrant C/NTS-SfBGL1(86T) for marker-recycling. Western blot analysis of β-glucosidase expression and copy number analysis of *SfBGL1* revealed that several His$^+$ Trp$^-$ transformants retained the *SfBGL1* expression cassette without loss, while the multi-integrated *TRP1* marker genes were inactivated (Fig. 5C). These results demonstrate that introducing stop codons into selection marker genes by the CRISPR-Cas9 system with donor DNA fragments is an efficient strategy to recycle multiple-integrated selection markers regardless of the type of auxotrophic gene.

Reuse of the same selection marker in marker-recycled recombinant *S. cerevisiae*

To validate the feasibility of reuse of the same selection marker in marker-recycled recombinant strains, we attempted to reuse *TRP1* to integrate the second expression cassette in multiple copies into *TRP1*-inactivated *SfBGL1* expressing transformant after pop-out of the Cas9-gRNA vector (Fig. 6A). The C/NTS-SfBGL1(86T) #6 strain described in Fig. 5C, harboring *SfBGL1* expressing cassette and Cas9-gRNA vector, was continuously cultivated on YPD. The #6-1 to #6-6 derivatives with the His$^-$ auxotrophic phenotype due to loss of Cas9-gRNA vector were generated. Western blot analysis confirmed that the expression level of SfBGL1 protein were not altered after the pop-out of the Cas9-gRNA vector (Supplementary data Fig. S2C). The obtained C/NTS-SfBGL1(86T) #6-1 strain was re-transformed with pT-NTS-TrEGL2(86T), which is an rDNA-NTS-based vector containing the expression cassette of an endoglucanase gene (*EGL2*) from *Trichoderma reesei* and the defective *TRP1* with 86 bp promoter as a selection marker (Fig. 6B). The resultant *S. cerevisiae* C/NTS-SfBGL1-TrEGL2(86T) strains were expected to have multiple copies of *SfBGL1* and *TrEGL2* cassettes integrated at the rDNA-NTS locus. Copy number analysis of the *TrEGL2* cassette in the *TRP1*-reused #6-1 transformants showed that *TrEGL2* cassettes were efficiently integrated into host chromosomes in multiple copies. Western blot analysis confirmed that *TrEGL2* expression was proportional to the copy numbers up to four copies, while the expression of *SfBGL1* was maintained without significant change (Fig. 6C).

The cellulase activity of the *S. cerevisiae* C/NTS-SfBGL1-TrEGL2(86T) strains was subsequently measured by detecting the amount of 4-nitrophenol released from the substrate 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-cellopentaoside (BPNPG5) (Fig. 6D), which was reported as a suitable substrate to determine synergistic action of two enzymes BGL1 and EGL2 (Wightman *et al*., 2020). Whereas the release of 4-nitrophenol from BPNPG5 was hardly detected in the strain #6-1, expressing only *SfBGL1,* the increased release of 4-nitrophenol was apparently observed in the transformants co-expressing *TrEGL2* and *SfBGL1* (#6-1-1, #6-1-8, #6-1-11, #6-1-14, #6-1-18) in a copy number-dependent manner up to four copies of *TrEGL2* (Fig. 6D). As indicated in western blot analysis (Fig. 6C), the higher copy numbers of *TrEGL2* expression cassettes over four copies might cause the saturation of secretion capacity, thus rather decreasing the overall cellulase activity. Altogether, these results strongly support that coupling the CRISPR-Cas9 system to the multiple integration system based on rDNA-NTS allows the ge-

neration of marker-recycled multiple integrants, which can be subsequently transformed by re-using the same selection marker. This facilitates the construction of recombinant yeast cells harboring multiple-integrated copies of several expression cassettes even with a few selection markers.

Discussion

For the construction of yeast cell factories capable of producing metabolites or proteins of commercial value, multiple integration of the expression cassette of a target gene into the host genome is required to achieve a high and stable level of heterologous gene expression. In some cases, multiple integration of different sets of expression cassettes into the host genome is required to implement complex heterologous metabolic pathways or to co-express several subunit proteins. The number of genetic manipulations that can be accomplished might be limited by the number of selection markers that are available. Thus, recycling of selection markers integrated in multiple copies would be necessary for subsequent manipulation. The use of auxotrophic selection markers is advantageous, particularly in constructing antibiotic-marker-free recombinant yeast strains for use as whole cells for oral vaccines or food additives, considering possible risks that include the release of antibiotic resistance genes into the environment and the creation of antibiotic-resistant pathogenic bacteria (Zhang *et al*., 2012). However, only a few auxotrophic genes are available, and the host strains chosen for genetic manipulation are frequently not multiple auxotrophic strains. In this study, we evaluated the Cre-lox and CRISPR-Cas9 systems as tools for recycling auxotrophic markers integrated in multiple copies along with expression cassettes in recombinant *S. cerevisiae* strains.

Our initial trial to exploit the Cre-lox system for recycling selection markers integrated in multiple copies at rDNA-NTS sites of the recombinant strains was hampered by the simultaneous pop-out of whole expression cassettes and selection marker genes (Fig. 1). To examine the possibility that the simultaneous loss of expression cassettes along with selection marker genes might be due to integration into rDNA-NTS sites, which are present in tandem repeated copies on host chromosome XII more than 100 copies in *S. cerevisiae* (Petes, 1980), we further investigated the combination of the Cre-lox system with Ty elements as integration targets, in which the integration sites are dispersed and remote on all chromosomes of *S. cerevisiae* (Fig. 2). We observed the loss of expression cassettes along with pop-out of selection marker genes even with the multiple integration system based on Ty4, a low-copy number element that is present as one copy located in a cluster of Ty elements and tRNA genes (Stucka *et al*., 1989) and spread on all chromosomes in *S. cerevisiae*, except chromosomes I and XI (Kim *et al*., 1998). This suggests that multiple tandem insertions are not the main factor causing the loss of the introduced cassettes. It is speculated that, regardless of insertion sites and patterns, the presence of multiple copies of the same expression cassettes on chromosomes might serve as the spot for homologous recombination, causing chromosomal rearrangements to pop-out the introduced expression cassettes.

We subsequently tested the feasibility of the CRISPR-Cas9 system to recycle selection markers integrated into rDNA-NTS sites as multiple tandem insertions. The introduction of the Cas9 vector containing *URA3*-targeting gRNA into the multiple integrants, e/NTS-tHMG1(16U_loxP) and Y/NTS-NNV(16U_loxP), resulted in the complete pop-out of selection markers and expression cassettes (Fig. 3). In contrast, transformation with the Cas9-gRNA vector along with the donor DNAs containing stop codons successfully generated the Ura$^-$ transformants that stably retained the expression cassettes in multiple copies (Fig. 4). It is likely that the presence of donor DNA might favor homology-directed DNA repair using the donor DNA fragments, resulting in DNA editing of *URA3* using donor DNA. This would prevent recombination between the selection marker genes located on the chromosome. The CRISPR-Cas9-based inactivation with a donor DNA(stop) fragment led to the efficient recycling of multiple-integrated markers besides *URA3*, such as *HIS3*, *LEU2*, and *TRP1,* without significant loss of the expression cassettes (Fig. 5). We further demonstrated the efficient reuse of a selection marker in marker-recycled recombinant *S. cerevisiae* by introducing the second expression cassettes into the *TRP1*-inactivated recombinant strains using the same *TRP1* selection marker (Fig. 6). The findings demonstrated that CRISPR-Cas9-induced marker inactivation is an efficient strategy to recycle multiple-integrated selection markers.

Several approaches have been developed to recycle a selection marker gene, initially using counter-selectable markers, such as the *URA3* and *TRP1* genes flanked with directed repeat sequences, on 5-fluoroorotic acid (5-FOA) and 5-fluoroanthranilic acid (5-FAA) (Boeke *et al*., 1984; Toyn *et al*., 2000). Recently, more advanced techniques based on Cre-lox (Jensen *et al*., 2014), I-SceI-induced double-strand DNA breaks (Solis-Escalante *et al*., 2014), and CRISPR-Cas9 (Huang and Mitchell, 2017) have been reported as efficient strategies for the simultaneous removal of selective markers. Previous studies of marker-recycling have been mostly applied to the selection marker integrated as a single copy in one site or to different selection markers integrated as one copy at different sites. Considering that the construction of recombinant yeast strains often requires multiple integration of expression cassettes into the host genome, which inevitably results in the integration of the same selection markers in multiple copies, development of tools to recycle selection markers integrated as multiple copies into the host genome is necessary for the subsequent introduction of additional expression cassettes to implement heterologous metabolic pathways. On the other hand, by exploiting the CRISPR-Cas9 system, several attempts for multi-copy integration without the use of any selection markers were recently made to facilitate the insertion of expression cassettes into defined genomic loci (Jessop-Fabre *et al*., 2016), Ty element sequences (Tsai *et al*., 2015; Shi *et al*., 2016), or into rDNA clusters (Wang *et al*., 2018) in *S. cerevisiae*. However, such marker-less multi-copy integration approaches are feasible when transformants harboring multiple integrations of expression cassettes can be easily screened by high-performance liquid chromatography of product formation in the culture broth, such as 2,3-butanediol (Shi *et al*., 2016) and 3-hydroxypropionic acid (Jessop-Fabre *et al*., 2016), or by color detection of intracellular pro-

ducts on plates, such as beta-carotene (López *et al*., 2020).

In the present study, we showed that the CRISPR-Cas9 system with donor DNA fragments containing stop codons is a suitable tool for the recycling multiple-integrated selection markers in *S. cerevisiae*. However, even the CRISPR-Cas9-based method could not avoid unpredictable recombination events including interchromosomal recombination or ectopic recombination, leading to loss or amplification of some fraction of integrated expression cassettes, as observed in Figs. 4 and 5. Such random genomic perturbation could be controlled by employing engineered alleles of RAD51 protein, a key recombinase in homologous recombination (Bonilla *et al*., 2020). For example, a K342E mutant of RAD51 was reported to enhance oligonucleotide recombination when compared to wild-type RAD51 (Liu *et al*., 2004). In a previous effort to develop yeast oligo-mediated genome engineering (YOGE) technology, RAD51 (K342E) was overexpressed to construct the yeast strain with highly efficient oligo-mediated recombination capabilities (DiCarlo *et al*., 2013). Thus, it might be expected that co-expression of such engineered RAD51 recombinases with Cas9, a RNA-guided endonuclease, either as a separate protein or as RAD51-Cas9 fusion, can improve the present method by favoring HDR using donor DNAs as template over HDR between homologous sequences on chromosomes. On the other hand, the length of donor fragments, which are 90-mer oligonucleotides in the present study, might be increased to improve the efficiency of homology-directed DNA repair using the donor DNA fragments.

In conclusion, we showed that introducing stop codons into selection marker genes by Cas9-gRNA vectors with donor DNA fragments is an efficient strategy to recycle multiple-integrated selection markers while maintaining the expression cassettes integrated in multiple copies. Coupling of the CRISPR-Cas9 system to rDNA-NTS-based multiple integration for marker-recycling is expected to facilitate the construction of synthetic yeast cells carrying optimal copies of a desired expression cassette by sequential rounds of gene manipulation with limited sets of auxotrophic markers. In particular, the rDNA-NTS-based multiple integration combined with the CRISPR-Cas9 system would be a useful genetic tool for the development of a food or vaccine-grade yeast cell factory by integrating different sets of expression cassettes in multiple copies into host genomes using only auxotrophic markers repeatedly without antibiotic selection markers.

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Conflict of Interest

The authors have no conflict of interest to report.

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