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Homology-Integrated CRISPR–Cas (HI-CRISPR) System for One-Step Multigene Disruption in *Saccharomyces cerevisiae*

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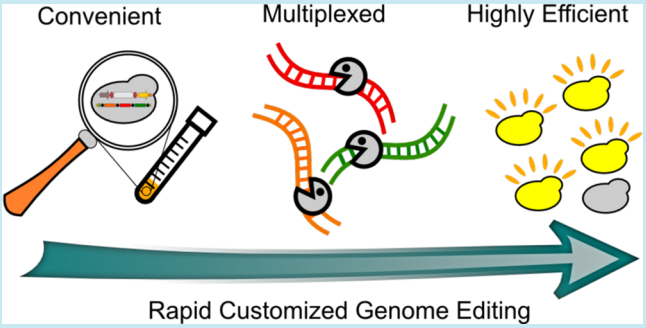
Supporting Information

ABSTRACT: One-step multiple gene disruption in the model organism *Saccharomyces cerevisiae* is a highly useful tool for both basic and applied research, but it remains a challenge. Here, we report a rapid, efficient, and potentially scalable strategy based on the type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–CRISPR associated proteins (Cas) system to generate multiple gene disruptions simultaneously in *S. cerevisiae*. A 100 bp dsDNA mutagenizing homologous recombination donor is inserted between two direct repeats for each target gene in a CRISPR array consisting of multiple donor and guide sequence pairs. An ultrahigh copy number plasmid carrying iCas9, a variant of wild-type Cas9, trans-encoded RNA (tracrRNA), and a homology-integrated crRNA cassette is designed to greatly increase the gene disruption efficiency. As proof of concept, three genes, *CAN1*, *ADE2*, and *LYP1*, were simultaneously disrupted in 4 days with an efficiency ranging from 27 to 87%. Another three genes involved in an artificial hydrocortisone biosynthetic pathway, *ATF2*, *GCY1*, and *YPR1*, were simultaneously disrupted in 6 days with 100% efficiency. This homology-integrated CRISPR (HI-CRISPR) strategy represents a powerful tool for creating yeast strains with multiple gene knockouts.



KEYWORDS: *CRISPR–Cas, Saccharomyces cerevisiae, multiple gene disruption, gene knockout, genome editing*

Multiple gene disruption is a widely used strategy for the characterization of specific gene functions within complicated genetic pathways.[1] It also facilitates the construction of versatile model hosts for heterologous gene expression,[2] identification of multidrug-resistant gene sets,[3] and investigation of disease mechanisms.[4] In addition, multiple gene disruption is also considered to be an effective strategy for improving biofuels and biochemicals production in metabolic engineering.[5,6]

As a model organism, *Saccharomyces cerevisiae* has been widely used for genetic research,[7] exploration of disease mechanisms,[8] and metabolic engineering.[9] Single gene disruption in *S. cerevisiae* is typically achieved by substituting the target gene with a selectable marker through homologous recombination (HR), which is highly efficient in *S. cerevisiae*.[10] Therefore, multiple gene disruption is traditionally achieved in a sequential manner,[11] by recycling the limited number of selectable markers. Unfortunately, challenges inevitably arise during this process. Not only is this process slow and tedious, but also marker removal can frequently cause mistargeting events.[12,13] Very few strategies exist today to perform simultaneous multiple gene disruption in yeast. A recently developed tool, Green Monster, allows deletion of multiple genes in *S. cerevisiae* via cycles of sexual assortment and fluorescence activated cell sorting (FACS).[14] However, each deletion target has to be first replaced by a reporter gene, and iterative rounds of mating and sporulation are required, which is very laborious and time-consuming.[14]

The type II bacterial Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins (CRISPR–Cas) system was recently exploited as an efficient gene-targeting technology in several prokaryotes and eukaryotes.[15−18] A trans-activating crRNA (tracrRNA):crRNA duplex (or a chimeric guide RNA (gRNA)) directs the Cas9 protein to cleave a target DNA sequence with a required protospacer adjacent motif (PAM), which is composed of any of the four bases followed by two guanines.[19] Simultaneous delivery of multiple gRNAs opens up the possibility for targeting multiple genes at the same time.[15] In a recent work, a near 100% single gene replacement efficiency was reached after cotransformation of the CRISPR–Cas system and a linearized HR donor DNA in *S. cerevisiae*.[20] However, there are several limitations for multiple gene targeting using this strategy. First, either 5 μg of marker cassette (1.4 kb) or 1 nmol (~58.5 μg) of double-stranded oligonucleotide donor (90 bp) is required to achieve a 100% single gene replacement efficiency.[20] It is hard to ensure that this amount of DNA will all be accepted by *S.*

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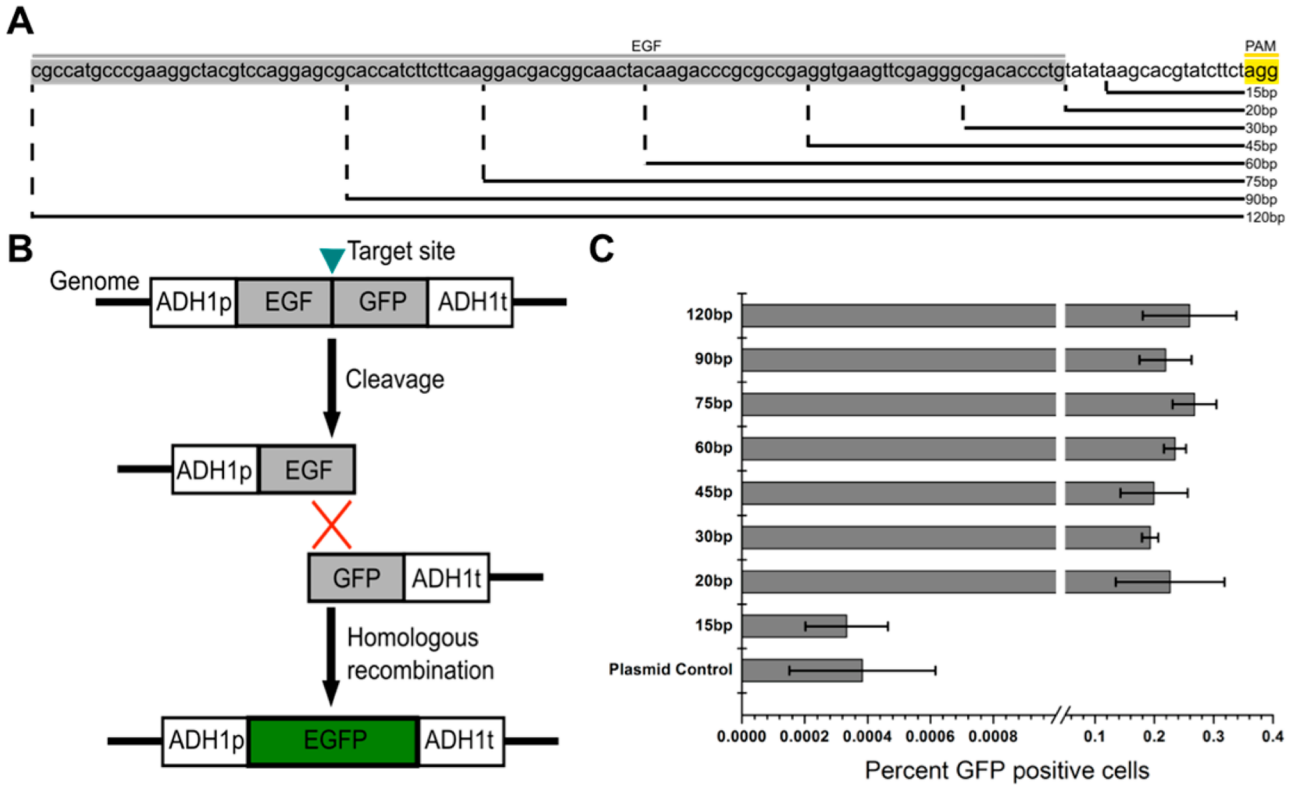

Figure 1. CRISPR–Cas targeting EGFP reporter with guide sequence GFP0. (A) Scheme showing the design of GFP0 spacers with different lengths. The PAM sequence is highlighted in yellow. The 3′ end of EGF sequence is highlighted in gray. (B) Scheme showing the design of the EGFP reporter system. EGFP function will be restored after Cas9 cleavage and homologous recombination. (C) Percentage of GFP positive cells 48 h after transformation of the CRISPR–Cas system with different GFP0 spacers. Error bars indicate standard deviation of three biological repeats.

cerevisiae when multiple donors need to be introduced.[21,22] Second, as observed with other genome editing tools (e.g., transcription activator-like effector nucleases (TALENs)),[23,24] the targeting efficiency mediated by CRISPR–Cas is also site-dependent.[25,26] The 100% gene replacement efficiency has been reported only for targeting CRISPR-susceptible sites.[20] Third, several RNA polymerase III regulatory elements are needed to express gRNAs targeting multiple genes,[18,19] which is inconvenient for standardized plasmid construction. Therefore, it is not surprising that this strategy has not been used to generate multiple gene disruptions in *S. cerevisiae*.

Using the CRIPSR–Cas from *Streptococcus pyogenes*,[27] we developed a HI-CRIPSR system to address these limitations. To the best of our knowledge, this is the first example of using CRISPR–Cas for multiple gene disruption in *S. cerevisiae*. As proof of concept, three endogenous genes, *CAN1*, *LYP1*, and *ADE2*, were simultaneously disrupted. The overall efficiency ranged from 27 to 87% when different sets of targeting sites were used. Furthermore, another three genes involved in an artificial hydrocortisone biosynthetic pathway, *ATF2*, *GCY1*, and *YPR1*, were simultaneously disrupted in 6 days with 100% efficiency, which was determined by random genotyping.

RESULTS AND DISCUSSION

Identification of the Minimum Length of a Targeting Sequence. During the process of crRNA maturation, the 5′-end region of the spacer is cleaved by RNase III and unknown endogenous nuclease(s), which means that this region does not contribute to crRNA function.[27,28] Therefore, we sought to exploit this region to harbor HR disruption donors in such a way that multiple crRNAs and HR disruption donors can be assembled in one step. To this end, the minimal length of a functional spacer was identified using an enhanced green fluorescent protein (EGFP)-based single-strand annealing reporter system (Figure 1).[29] Repair of the two truncated EGFP DNA fragments can be triggered by a double strand break (DSB) generated by CRISPR–Cas, resulting in the expression of a functional EGFP (Figure 1B). A spacer with 20 bp upstream of the PAM sequence successfully restored the function of EGFP, and comparable expression of functional EGFP was also observed when the spacer length was increased to 120 bp (Figure 1C).

Design of the HI-CRISPR System. The core design principle of the HI-CRISPR system is to achieve efficient gene disruption in *S. cerevisiae* with simple manipulations. The HR disruption donor containing an 8 bp deletion in the middle is designed to recombine with the target genomic locus (Figure 2A). The 8 bp deletion includes the PAM sequence and the last 3 bp of the protospacer, which prevents continuous recognition and cleavage by CRISPR–Cas after recombination. To avoid repeated introduction of dsDNA fragments as repairing templates, the HR disruption donor was maintained under selective pressure by integrating the donor between two direct repeats as a 5′ extension of the guide sequence (Figure 2B), which enables one-step construction of all required elements (Figure S1). In the CRISPR array, expression of multiple spacers flanked by direct repeats is driven by a single promoter, where the resultant transcript can be processed into multiple crRNAs.[30] To take advantage of natural polycistronic CRISPR arrays for multiplexed targeting, crRNA is used instead of gRNA (Figure 3A). The transcribed pre-crRNA can be processed into mature crRNA by host RNase III and unknown

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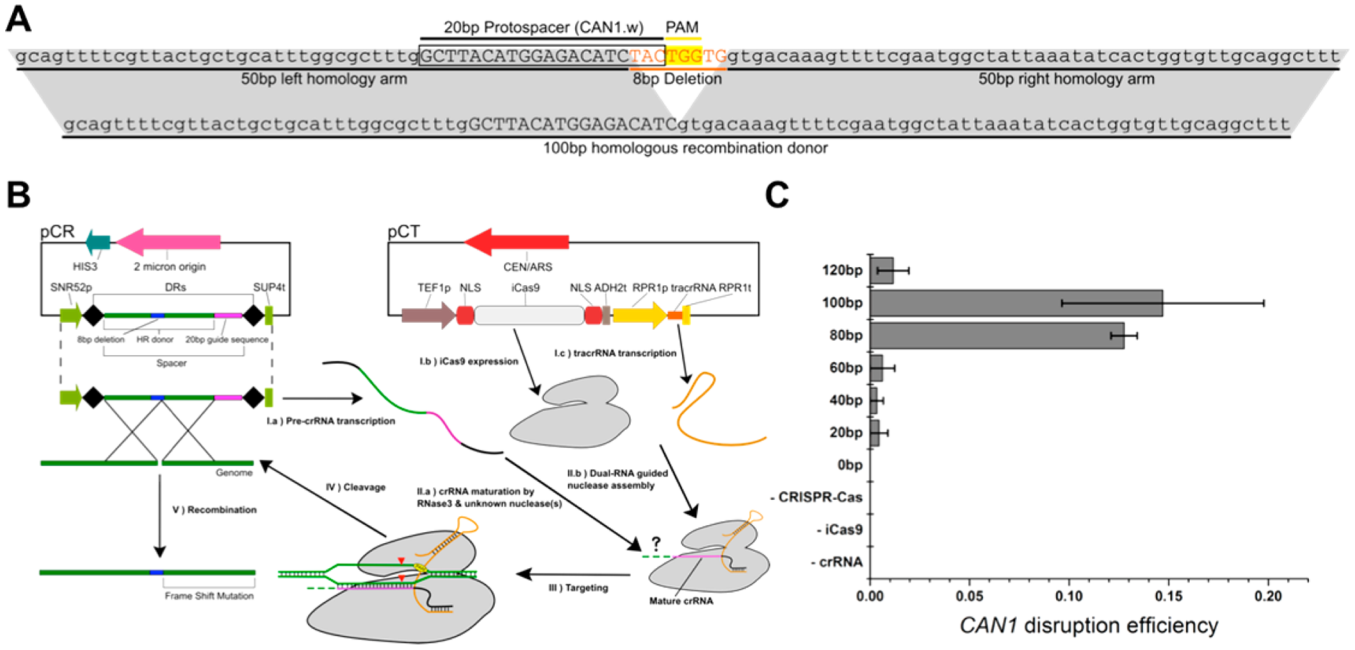

Figure 2. Determination of the optimal HR donor length for efficient single gene disruption. (A) Scheme showing the design of a 100 bp HR donor for the CAN1.w site. The 8 bp deletion includes the PAM sequence and the last 3 bp of the guide sequence. HR donors with different lengths were obtained by simultaneously changing the 5′ end of left homology arm and the 3′ end of right homology arm by 10 bp. (B) Schematic of the two-plasmid HI-CRISPR system for single gene disruption. The crRNA array was constructed using the pRS423 backbone to generate plasmid pCR. iCas9 and tracrRNA were constructed using the pRS415 backbone to generate plasmid pCT. The pre-crRNA is transcribed from the pCR plasmid and processed into mature crRNA by RNase III and unknown nuclease(s). The green dashes at the 5′ end of mature crRNA and the question mark indicate that it is unknown whether the HR donor is fully cleaved. iCas9 and tracrRNA are expressed from the pCT plasmid and complexed with mature crRNA to form the dual-RNA-guided nuclease. The nuclease is then guided to target the genomic locus. The target gene is cleaved by iCas9, generating a DSB. The DSB is repaired through recombination with the HR disruption donor harbored in the spacer of the crRNA array on plasmid pCR. The repaired gene function is disrupted by a small deletion in the donor sequence. (C) *CAN1* disruption efficiency obtained 2 days after transformation. Error bars indicate standard deviation of two biological repeats.

nuclease(s). Cas9 and tracrRNA are expressed separately and then assembled with crRNA to form the dual-RNA-guided endonuclease. The nuclease is then guided by crRNA to target and cleave the genomic locus to generate a DSB. The DSB is repaired through recombination with the HR disruption donor. Once the HR disruption donor is recombined with the target locus, a frame shift will be generated to abolish the normal protein translation (Figure 2B). In most cases, a premature stop codon was consequently introduced.

Discovery of a Cas9 Variant. A Cas9 variant was accidentally obtained during the plasmid construction process. This gene harbors two point mutations (Figure S2), resulting in a double mutant variant (D147Y, P411T). Gene disruption efficiency mediated by the variant was higher than that of wild-type Cas9 (Figure S3). This gene was named iCas9 (improved Cas9) and therefore adopted for all subsequent experiments.

Determination of the Optimal Length of an HR Disruption Donor. The optimal length of an HR disruption donor contained in a spacer was further determined for single gene disruption by CRISPR–Cas. The endogenous negative selectable gene *CAN1*, encoding plasma membrane arginine permease, was chosen as the target. The disruption mutant of *CAN1* grew in the presence of toxic arginine analogue canavanine, enabling a quick readout of disruption efficiency.[31] HR disruption donors with 20 bp increments were designed by simultaneously changing the 5′ end of the left homology arm and the 3′ end of the right homology arm by 10 bp (Figure 2A). A two-plasmid system was used for CRISPR–Cas expression (Figure 2B). The iCas9 gene and tracrRNA, driven by the TEF1p and RPR1p promoters, respectively, were cloned into the centromeric plasmid pCT (Table S1). Together with the 20 bp guide sequence targeting 1331 bp downstream of the start codon of *CAN1* (site CAN1.w), the 20, 40, 60, 80, 100, and 120 bp HR disruption donors were constructed on the 2 μ plasmid pCR (Table S1) for *CAN1* disruption, achieving gene disruption efficiencies of 0.44, 0.33, 0.62, 12.76, 14.71, and 1.16% on day 2, respectively (Figure 2C). *CAN1* gene disruption was not detectable in the absence of an HR disruption donor and a crRNA (Figure 2C). Similarly, no gene disruption was detectable when only the HR donor and crRNA were present (Figure 2C). We sequenced 16 randomly selected canavanine-resistant colonies and verified the expected 8 bp deletion at the targeted locus in all clones (Figure S4), indicating that the loss of *CAN1* function was caused by CRISPR–Cas assisted homologous recombination.

The highest *CAN1* disruption efficiency (14.71%) was obtained using a 100 bp HR disruption donor. Therefore, this donor length was chosen for subsequent gene disruptions. However, this efficiency was not yet high enough for multiplex gene targeting. We speculate that suboptimal cleavage or low HR efficiency can be improved by giving the cells more time to grow. Thus, we sought to improve the gene disruption efficiency by growing the cells for a prolonged period of time in liquid SC medium, which prevented the loss of CRISPR–Cas plasmids. As shown in Figure S5, *CAN1* disruption efficiency was steadily improved through serial transfers in liquid SC-LH medium. After 12 days of culture in liquid medium, a 100% *CAN1* disruption efficiency was obtained.

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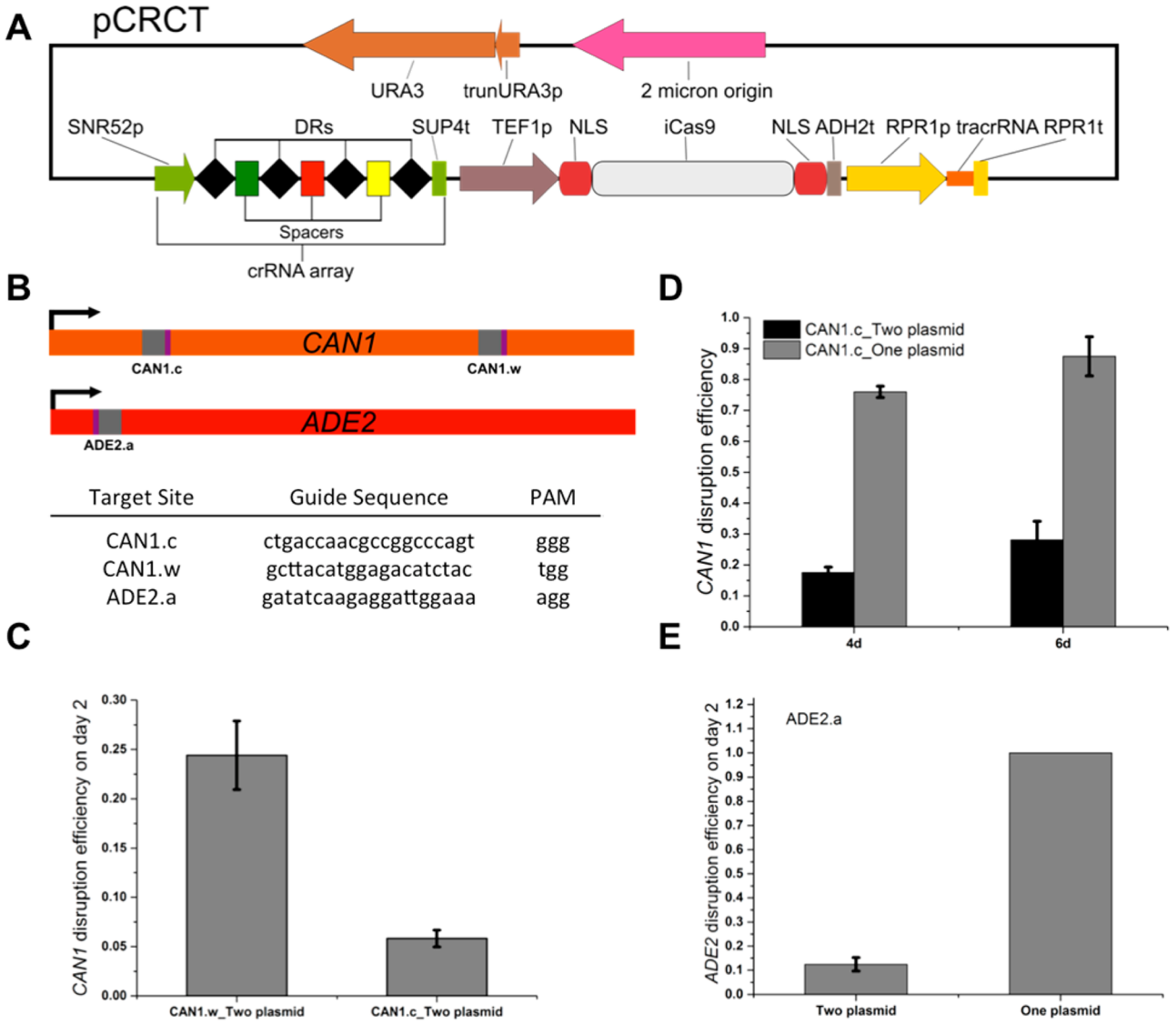

Figure 3. Gene disruption efficiency by the one-plasmid HI-CRISPR system. (A) Scheme showing the design of one-plasmid HI-CRISPR system. (B) The upper scheme shows the relative positions of the CAN1.w, CAN1.c, and ADE2.a target sites within each gene. The gray box represents the guide sequence, and the purple bar represents the PAM sequence. A PAM sequence on the left indicates a site on the negative DNA strand. The table summarizes the guide sequence and PAM sequence of each target site. (C) *CAN1* disruption efficiencies were assessed 2 days after transformation of the two-plasmid system targeting either CAN1.w or CAN1.c. (D) *CAN1* disruption efficiencies were assessed 4 or 6 days after transformation of either two-plasmid or one-plasmid CRISPR−Cas systems targeting the CAN1.c site. (E) *ADE2* disruption efficiency was assessed 2 days after transformation of the one-plasmid system targeting at the ADE2.a site. All error bars indicate standard deviations of three biological repeats.

Stimulation of Gene Disruption by Increasing the Plasmid Copy Number. Although a high efficiency of single gene disruption was achieved, it was time-consuming. In order to shorten the time needed for efficient gene disruption, we attempted to stimulate gene disruption by increasing the copy number of the plasmid harboring the HI-CRISPR system. Previous studies showed that the *URA3* gene with a truncated promoter on a plasmid was poorly expressed in yeast cells. To survive under the selection pressure, the cell needs to maintain a much higher copy number (~200 copies per cell) than that of a typical 2 μ-based plasmid (10−40 copies per cell).[32,33] Therefore, the ultrahigh copy number receiver plasmid pCRCT was constructed for expressing the CRISPR−Cas gene and harboring the HR disruption donor (Figure 3A). As expected, a 100% *CAN1* disruption efficiency was obtained after only 4 days of cultivation in selective medium when the CAN1.w site was targeted. To further demonstrate the efficacy of this one-plasmid-based HI-CRISPR system, a poorly targeted site of the *CAN1* gene (site CAN1.c), 362 bp downstream of the start codon (Figure 3B), was also chosen for analysis. Under the two-plasmid system, the *CAN1* disruption efficiency with the CAN1.c target was 5.82% after 2 days of cultivation in liquid culture, which was 76% lower than that with the CAN1.w target (Figure 3C). Under the single plasmid system, the *CAN1* disruption efficiencies were 76 and 87% after 4 and 6 days of cultivation in liquid medium, respectively. In comparison, the corresponding disruption efficiencies of the CAN1.c target were only 18 and 28%, respectively, using the two-plasmid system (Figure 3D).

To investigate whether the improved gene disruption efficiency was generally applicable, disruption of another gene, *ADE2*, was performed. The *ADE2* gene encodes a

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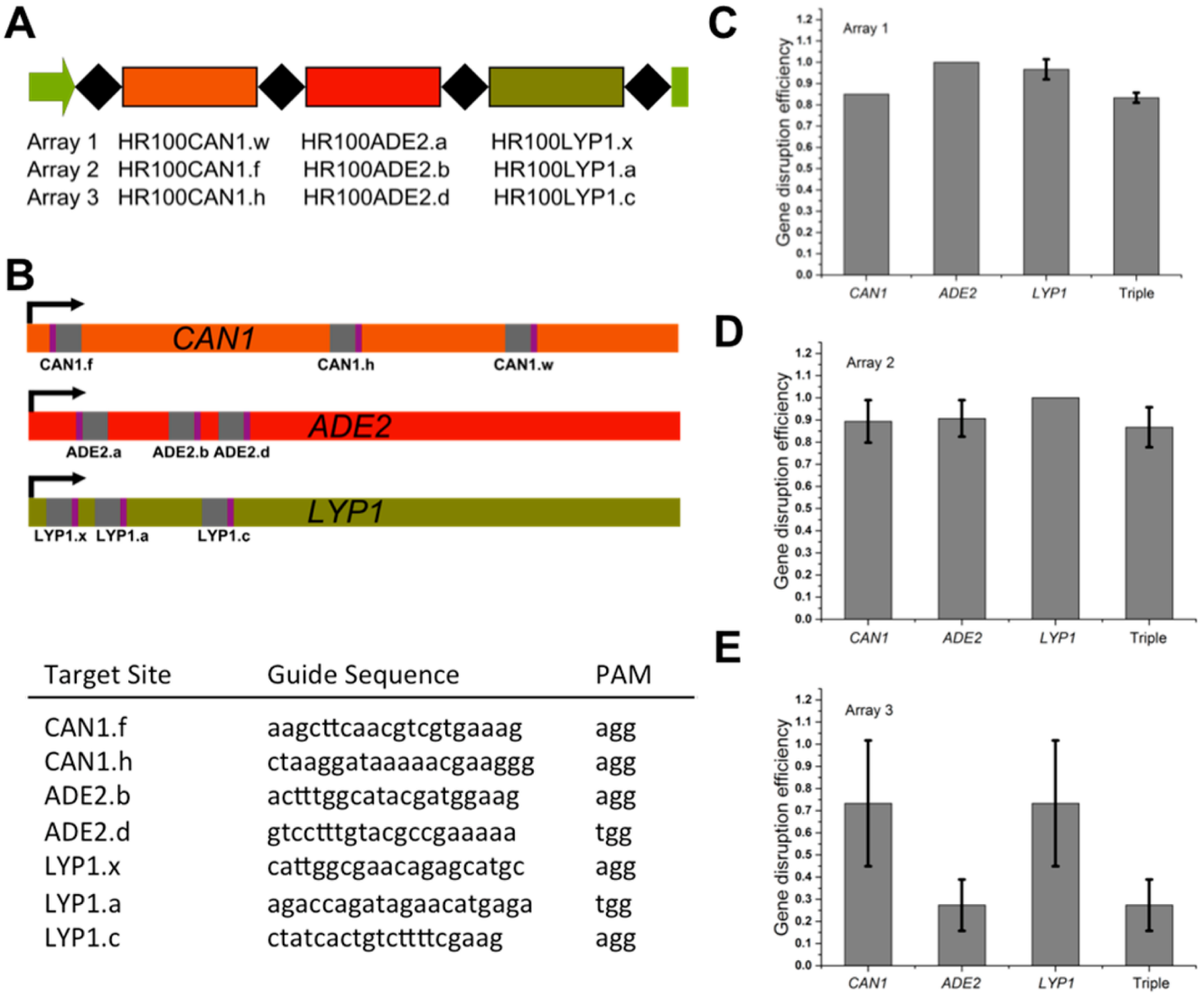

Target Site	Guide Sequence	PAM
CAN1.f	aagcttcaacgtcgtgaaag	agg
CAN1.h	ctaaggataaaaacgaaggg	agg
ADE2.b	actttggcatacgatggaag	agg
ADE2.d	gtcctttgtacgccgaaaaa	tgg
LYP1.x	cattggcgaacagagcatgc	agg
LYP1.a	agaccagatagaacatgaga	tgg
LYP1.c	ctatcactgtcttttcgaag	agg

Figure 4. Simultaneous triple gene disruption using the one-plasmid HI-CRISPR system. (A) Schematic illustration of three different HI-CRISPR arrays. (B) Scheme showing the relative positions of target sites within each gene used in triple gene disruption experiments. The guide and PAM sequences of each site are summarized in the table. (C–E) Single gene and triple gene disruption efficiencies assessed 4 days after transformation of the corresponding CRISPR arrays using the one-plasmid system. Error bars indicate standard deviation of three biological repeats.

phosphoribosylaminoimidazole carboxylase required for adenine biosynthesis. Red pigment will accumulate in the *ADE2* nonsense mutant cell when adenine is absent from the medium.[34] When targeting 154 bp downstream of the start codon of the *ADE2* gene (site ADE2.a, Figure 3B), a 100% disruption efficiency was achieved with the plasmid pCRCT-ADE2a after 2 days in liquid culture, whereas only a 12.5% *ADE2* disruption efficiency was obtained with the two-plasmid system consisting of pCT and pCR-ADE2a at the same time point (Figure 3E). Taken together, these results demonstrated that increasing the plasmid copy number was an effective strategy for improving gene disruption efficiency mediated by CRISPR–Cas.

One-Step Multiple Gene Disruption by the HI-CRISPR System. Next, multiple simultaneous gene disruptions mediated by the HI-CRISPR system was investigated. In addition to *CAN1* and *ADE2*, the *LYP1* gene, encoding lysine permease, was chosen as the third target for multiple disruption. Similar to that for the *CAN1* disruption mutant, the *LYP1* disruption mutant can grow in the presence of thialysine, a toxic lysine analogue.[35] Three sites, including CAN1.w (1331 bp downstream of the start codon of *CAN1*), ADE2.a (154 bp downstream of the start codon of *ADE2*), and LYP1.x (79 bp downstream of the start codon of *LYP1*), which showed a high efficiency for corresponding single gene disruption (Figures 3 and S6), were combined together as array 1 for multiple gene disruption (Figure 4A). After 4 days of culture in SC-U medium, an 83% triple gene disruption efficiency was achieved using array 1 (Figure 4C and Table S2).

To evaluate the general applicability of this HI-CRISPR system for simultaneous multigene disruption, another two CRISPR arrays were designed for these three target genes without prescreening the target sites. To minimize the possibility of choosing a low-efficiency target site and to ensure the targeting specificity of the CRISPR–Cas system, several criteria were adopted: (i) An earlier region of the gene is preferred for targeting to ensure disruption of gene function. (ii) According to a previous study, one base pair mismatch at the last 12 bp of the guide sequence abolished the cleavage activity of CRISPR–Cas.[18] Therefore, the last 12 bp of the

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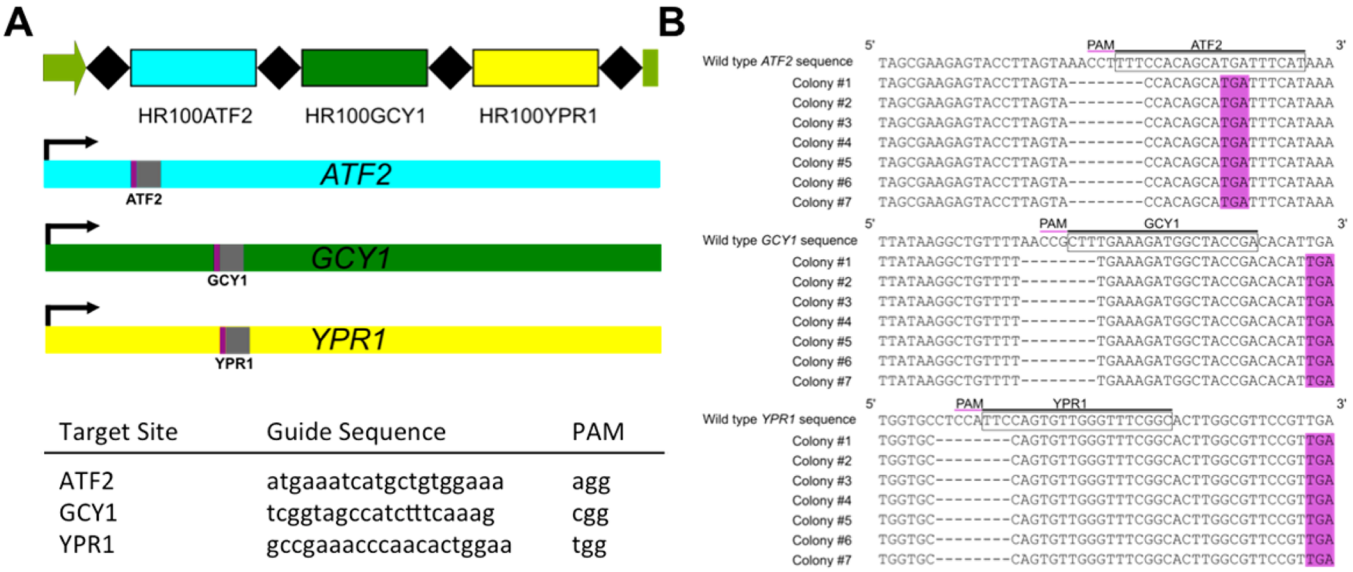

Target Site	Guide Sequence	PAM
ATF2	atgaaatcatgctgtggaaa	agg
GCY1	tcggtagccatctttcaaag	cgg
YPR1	gccgaaacccaacactggaa	tgg

Figure 5. Simultaneous disruption of three genes involved in a hydrocortisone biosynthetic pathway using the one-plasmid HI-CRISPR system. (A) Schematic of the HI-CRISPR array and relative positions of target sites within each gene. The guide and PAM sequences of each site are summarized in the table. (B) Alignment of gene sequences from seven randomly picked colonies against the wild-type sequences. All seven colonies were triple disruption mutants.

guide sequence should be unique in the genome of *S. cerevisiae*, minimizing off-target activity. On the other hand, a genetic screen study using CRISPR−Cas showed that the gRNA efficiency is associated with specific sequence characteristics.[26] These specific characteristics are also included in the design criteria: (iii) purines are preferred in the last 4 bp of spacer, (iv) the antisense strand is preferred for targeting, and (v) the GC content of the 20 bp guide sequence should be between 20 and 80%. In addition, type III RNA polymerase terminates transcription at a small polyT stretch.[36] So, (vi) polyT (more than 4 T's) sequences are avoided in both the HR disruption donor and the crRNA guide sequence.

As a result, array 2 was designed to target 65 bp downstream of the start codon of *CAN1* (site CAN1.f), 473 bp downstream of the start codon of *ADE2* (site ADE2.b), and 112 bp downstream of the start codon of *LYP1* (site LYP1.a). Using array 2 for multiple gene targeting, a triple gene disruption efficiency (87%) was achieved in 4 days, similar to that using array 1 (Figure 4C−D and Table S2). Array 3 was designed to target 846 bp downstream of the start codon of *CAN1* (site CAN1.h), 555 bp downstream of the start codon of *ADE2* (site ADE2.d), and 543 bp downstream of the start codon of *LYP1* (site LYP1.c). After 4 days culture in SC-U medium, the triple gene disruption efficiency reached 27% (Figure 4E and Table S2).

To demonstrate that the HI-CRISPR system can be used to disrupt genes whose mutations do not lead to clear phenotypes, another three yeast endogenous genes involved in an artificial hydrocortisone biosynthetic pathway, *ATF2*, *GCY1*, and *YPR1*,[37] were chosen as candidates for disruption. Three target sites, ATF2, GCY1, and YPR1, were designed according to the criteria and assembled together with corresponding HR disruption donors (Table S4) into a single crRNA array (Figure 5A). After 6 days of culture in SC-U medium, single colonies were isolated by diluted plating, and seven of them were randomly picked and screened directly by PCR and sequencing. All seven colonies were shown to be triple disruption mutants at the defined target sites (Figure 5B).

Investigation of Increased Multiplexing. To investigate how many functional crRNAs can be contained in such a CRISPR array, nine crRNAs with only one functional crRNA targeting the CAN1.w site of the *CAN1* gene in different positions of the CRISPR array were designed (Figure 6A). When the functional crRNA was placed at the first position, the *CAN1* disruption efficiency was similar to that observed using only one crRNA (Figure 6B). However, the *CAN1* disruption

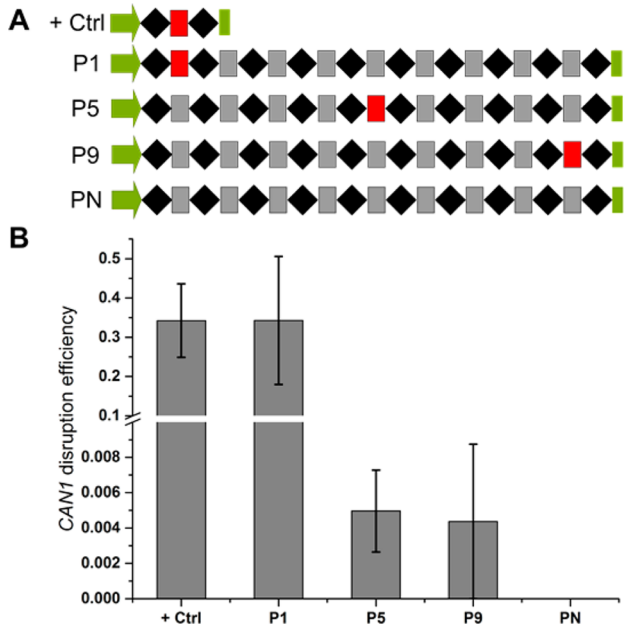

Figure 6. Investigation of the crRNA number tolerance for use in a single crRNA array. (A) Scheme showing the design of P1, P5, P9, and PN crRNA arrays. The red box represents the genome targeting HR80CAN1.w spacer, whereas the gray boxes represent nontargeting spacers with random sequences. (B) *CAN1* disruption efficiencies calculated 6 days after transformation. Error bars indicate standard deviations of two biological repeats.

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Table 1. Comparisons among Different Multiple Simultaneous Gene Disruption Techniques in *S. cerevisiae*

methods	Cre−LoxP[13]	Green Monster[14]	this work
genes disrupted	4	16	3
time spent	35 days	143 days	4 days
average time per gene	8.75 days	8.94 days	1.33 days
selection or screening needed	auxotrophic marker selection	FACS	no
labor	intensive	intensive	low
ectopic sequences introduced	yes	yes	no
plasmid construction	multiple PCR	multiple PCR	one step Golden Gate
other constraints	selectable markers limited	(1) Single gene disruption mutant needed; (2) genotyping after each round; (3) some genes cannot be targeted.	target site dependency

efficiency dropped to near zero when this crRNA was placed at the fifth position (Figure 6B).

By taking advantage of the crRNA maturation mechanism of CRISPR−Cas and physiological properties of *S. cerevisiae*, we developed a new strategy for efficient and user-friendly multiple simultaneous gene disruption in *S. cerevisiae*. Three features distinguish this system from other related methods: (i) customized crRNAs containing HR disruption donors allow easy construction in one step, (ii) gene disruption efficiency was greatly improved using an ultrahigh copy number plasmid, and (iii) multiple gene disruption mutants can be easily obtained by random genotyping without screening or selection. Although only three genes were efficiently disrupted by this system, the plasmid can be removed by counter selection of *URA3* and recycled for the next round of multiple gene disruption. Therefore, this strategy is potentially scalable. As summarized in Table 1, compared to other existing methods, this strategy is more efficient, less time-consuming, and easier to manipulate. However, this strategy also has limitations. It was noted that high gene disruption efficiency was attained after a certain time period of incubation in liquid medium, making the isolation of gene disruption mutants with growth defects problematic.

An interesting finding of this study was iCas9. The two mutations (D147Y, P411T) were in the REC1 domain of Cas9, which is a critical region for DNA cleavage.[38] This may contribute to the improved targeting efficiency by iCas9. Preliminary studies also indicated that no obvious off-target effect was found when using HI-CRISPR for multiple gene disruption (data not shown), suggesting the potential broad applicability of iCas9 for genome editing.

The *CAN1* disruption efficiency with a 120 bp HR disruption donor was much lower than that with a 100 bp HR disruption donor (Figure 2C), which is unexpected. The most likely cause was that the 120 bp HR donors gave rise to a deficient CRISPR−Cas system. In fact, in contrast to the 100 bp HR donor, a poly-T sequence was found in the 120 bp HR donor, which might cause premature transcriptional termination of the crRNA. In addition, it has been reported that the sequence downstream of the promoter, recognized by type III RNA polymerase, affects the promoter strength.[39] Therefore, the sequence of the 120 bp HR donor might also account for the decreased transcriptional strength of crRNA.

The all-in-one HI-CRISPR system using a single ultrahigh copy number plasmid greatly improved the gene disruption efficiency (Figure 3). Compared to nonhomologous end joining, *S. cerevisiae* always prefers HR to repair a DSB.[40] The overall efficiency of CRISPR−Cas mediated gene disruption in *S. cerevisiae* therefore depends on two aspects: the efficiency of DSB introduction by CRISPR−Cas and the efficiency of the subsequent HR repair. The use of an ultrahigh copy number plasmid may have contributed to both processes. First, increasing the copy number of the plasmid is considered to be an effective strategy for increasing enzyme expression in *S. cerevisiae*,[41] which, in our case, may have helped with Cas9 expression and led to higher cleavage efficiency. Second, under the all-in-one system, increasing the copy number of the plasmid also increases the availability of the HR disruption donor. Since we have already optimized the length of the donor based on *CAN1* disruption efficiency using the two-plasmid system (Figure 2C), increasing donor availability may further improve HR efficiency.

Different triple gene disruption efficiencies were observed using different CRIPSR arrays (Figure 4), indicating that the site dependence of CRISPR−Cas is still a challenge for achieving a higher efficiency of multiple simultaneous gene disruption. Although this effect is partially alleviated through the use of the ultrahigh copy number plasmid pCRCT (Figure 3), better rules for choosing efficient CRISPR targeting sites are still needed to obtain multiple gene disruption mutants more reliably. Alternatively, using multiple crRNAs to target each gene may address the site dependence issue. However, our results suggest that the maximum number of functional crRNAs will be no more than four (Figure 6). Possible reasons for this are the incomplete transcription and/or instability of the long CRISPR RNA. Developing regulation elements to enhance the expression and/or improve the stability of CRISPR RNA may address this limitation.

In addition to multiple simultaneous gene disruption, there are other potential applications of this HI-CRISPR system. A comparable gene disruption efficiency was found between the 100 bp and 80 bp HR disruption donors (Figure 2C). Taking advantage of the spared length, an HR disruption donor can be designed to make a modification instead of deletion of the target locus with the assistance of CRISPR−Cas (e.g., point mutations and tag insertions). Moreover, the 120 bp homology-integrated spacer was the only difference among every single gene disruption cassette. Such length of oligos can be synthesized on a CustomArray (CustomArray Inc.) in a complex pool,[26] and a library of gene modification cassettes can be constructed using this system. Given its flexibility and potential applications, this HI-CRISPR system can be a valuable tool to conduct genome-scale studies in *S. cerevisiae*.

METHODS

Strains and Media. The EGFP reporter strain was constructed elsewhere based on the *S. cerevisiae* strain HZ848 (MATα *ade2-1 ade3*Δ*22 ura3*Δ*0 his3-11,15 trp1-1 leu2-3,112*

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and can1-100).[29] The *S. cerevisiae* strain BY4741 (MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was used in this study for assessing *CAN1*, *ADE2*, and *LYP1* gene disruption efficiency. The *S. cerevisiae* strain CEN.PK2-1c (MATa *ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8^{C} SUC2*) was used for disruption of *ATF2*, *GCY1*, and *YPR1*. YPAD media was used to grow yeast cells before transformation. After transformation, cells were grown in appropriate synthetic complete (SC) media minus the auxotrophic compound complemented by the plasmids. SC-LHR (SC-leucine, histidine, and arginine) and SC-UR (SC-uracil and arginine) agar plates with 60 μg/mL L-canavanine (Sigma) were used to select for *CAN1* disrupted cells. SC-LHK (SC-leucine, histidine, and lysine) and SC-UK (SC-uracil and lysine) agar plates with 250 μg/mL thialysine (S-2-aminoethyl-L-cysteine, Sigma) were used to select for *LYP1* disrupted cells. SC-LH (SC-leucine and histidine) and SC-U (SC-uracil) agar plates minus adenine hemisulfate were used to screen for *ADE2* disrupted cells.

CRISPR–Cas Target Site Selection and Donor Design. A 12 bp seed sequence together with NGG PAM sequence (N_{12}NGG) was searched on both strands of the target gene sequence and blasted against the *S. cerevisiae* S288c genome (NCBI Taxonomy ID: 559292). The unique sequences were selected as target sites to minimize off-target effects.[18] A 100bp donor sequence was designed to have two 50 bp homology arms flanking the Cas9 cutting site and incorporate an 8 bp deletion including the PAM sequence, thus introducing a frame-shift mutation (Figure 2A,B). All donor and guide sequences were examined and did not contain more than 4 T residues in a row to prevent early termination of RNA transcription.

Plasmid Construction. The RPR1p, RPR1t, and SNR52p regulatory elements were PCR-amplified from wild-type BY4741 genomic DNA. SUP4t was directly synthesized into cloning primers. The CRISPR RNA elements DR and tracrRNA were amplified from plasmid pX260, which was a gift from Dr. Feng Zhang.[18] The human codon-optimized Cas9 gene used in the EGFP reporter assay was also amplified from plasmid pX260. The Cas9 gene in pGal10p-Cas9 was originally from *S. pyogenes* SF370 and PCR amplified from plasmid pMJ806, which was a gift from Dr. Jennifer A. Doudna.[42] This gene was tagged with a FLAG tag and an SV40 nuclear localization signal on its N-terminus as well as an SV40 nuclear localization signal on its C-terminus. The two point mutations in iCas9 sequence were accidentally introduced during construction of plasmid pCT (Figures S2 and S7 and Tables S1 and S3). All of the spacer sequences were ordered as gBlocks from Integrated DNA Technologies (Coralville, IA) and assembled into pCR or pCRCT plasmids using the Golden Gate assembly method (Figure S1).[43] All of the sequence information, primers used for plasmid construction, and the iCas9 sequence can be found in Supporting Information (Tables S4–S8 and Figure S2). The plasmid descriptions can be found in Supporting Information Table S1. The plasmid construction processes are summarized in Supporting Information Table S3 and Figures S1 and S7.

Yeast Transformation. Plasmid transformation of BY4741 and CEN.PK2-1c (1 μg of each plasmid per transformation) was carried out using LiAc/SS carrier DNA/PEG method.[44] After transformation, cells were recovered in 1 mL of YPAD at 250 rpm and 30 °C for 1 h, washed with water once, transferred to 2 mL of the appropriate SC media, and cultivated at 250 rpm, 30 °C. Every 48 h, 100 μL of cell culture was transferred into 2 mL of fresh SC media to ensure cell viability. The remaining cell culture was discarded.

Calculation of Single Gene Disruption Efficiency. To determine the optimal HR donor length, *S. cerevisiae* BY4741 cells were transformed with pCT and pCR-CAN1.w with different HR donor lengths. Two-hundred microliters of 10^4-fold diluted cell culture was plated onto selective and nonselective plates on the appropriate days. Cells were allowed to grow for 2 days on the plate, and the *CAN1* single gene disruption efficiency was determined by calculating the ratio of yeast colonies formed on selective and nonselective plates. Experiments were done in duplicate.

To compare the two-plasmid and one-plasmid CRISPR–Cas systems, cells were transformed with either the two-plasmid or one-plasmid CRISPR–Cas system targeting different sites. Two-hundred microliters of 10^4-fold diluted cell culture was plated onto selective and nonselective plates on the appropriate days. Cells were allowed to grow for 2 days, and the *CAN1* single gene disruption efficiency was determined by calculating the ratio of yeast colonies formed on selective and nonselective plates. The *ADE2* single gene disruption efficiency was determined by calculating the percentage of pink colonies on SC plates minus adenine hemisulfate. Experiments were done in triplicate.

Calculation of the Triple Gene Disruption Efficiency. *S. cerevisiae* BY4741 cells were transformed with the one-plasmid CRISPR–Cas system targeting the *CAN1*, *ADE2*, and *LYP1* genes. Two-hundred microliters of 10^4-fold diluted cell culture was plated onto SC-U plates on day 4. After 2 days, a total of 20 (array 1) or 50 (arrays 2 and 3) colonies were randomly selected and streaked onto selective plates. After another 2 days, growth and color of each colony were recorded. The triple gene disruption efficiency was determined by calculating the proportion of colonies with all three genes disrupted. Experiments were done in triplicate.

Yeast Genomic DNA Extraction and Target Site Sequencing. The 16 canavanine-resistant *S. cerevisiae* BY4741 colonies (Figure S4) or 7 randomly picked *S. cerevisiae* CEN.PK2-1c colonies (Figure 5) were each inoculated into 2 mL of YPAD media and cultured overnight at 250 rpm, 30 °C. One milliliter of overnight culture was spun down, and genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, San Luis Obispo, CA). The target sites were PCR-amplified and sequenced by Sanger sequencing (ACGT, Inc., Wheeling, IL).

■ ASSOCIATED CONTENT

Supporting Information

Additional methods, figures, and tables as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

[‖]Z.B. and H.X. contributed equally to this work. H.X., J.L., Z.B., and H.Z. designed the experiments; Z.B. and H.X. performed all of the experiments with the help of L.Z., X.X., N.S., and T.S.; H.X., Z.B., and H.Z. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CRISPR−Cas, Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins; DSB, double strand break; gRNA, guide RNA; HR, homologous recombination; PAM, protospacer adjacent motif; TALENs, transcription activator-like effector nucleases; tracrRNA, trans-encoded RNA

■ REFERENCES

(1) Yu, B. J., Sung, B. H., Koob, M. D., Lee, C. H., Lee, J. H., Lee, W. S., Kim, M. S., and Kim, S. C. (2002) Minimization of the *Escherichia coli* genome using a Tn5-targeted Cre/loxP excision system. *Nat. Biotechnol. 20*, 1018−1023.

(2) Komatsu, M., Uchiyama, T., Omura, S., Cane, D. E., and Ikeda, H. (2010) Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A. 107*, 2646−2651.

(3) Parsons, A. B., Brost, R. L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G. W., Kane, P. M., Hughes, T. R., and Boone, C. (2004) Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat. Biotechnol. 22*, 62−69.

(4) McCarroll, S. A., Bradner, J. E., Turpeinen, H., Volin, L., Martin, P. J., Chilewski, S. D., Antin, J. H., Lee, S. J., Ruutu, T., Storer, B., Warren, E. H., Zhang, B., Zhao, L. P., Ginsburg, D., Soiffer, R. J., Partanen, J., Hansen, J. A., Ritz, J., Palotie, A., and Altshuler, D. (2009) Donor-recipient mismatch for common gene deletion polymorphisms in graft-versus-host disease. *Nat. Genet. 41*, 1341−1344.

(5) Raab, A. M., Gebhardt, G., Bolotina, N., Weuster-Botz, D., and Lang, C. (2010) Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. *Metab. Eng. 12*, 518−525.

(6) Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., Chou, K. J., Hanai, T., and Liao, J. C. (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng. 10*, 305−311.

(7) Bennett, C. B., Lewis, L. K., Karthikeyan, G., Lobachev, K. S., Jin, Y. H., Sterling, J. F., Snipe, J. R., and Resnick, M. A. (2001) Genes required for ionizing radiation resistance in yeast. *Nat. Genet. 29*, 426−434.

(8) Khurana, V., and Lindquist, S. (2010) Modelling neurodegeneration in *Saccharomyces cerevisiae*: why cook with baker's yeast? *Nat. Rev. Neurosci. 11*, 436−449.

(9) Ostergaard, S., Olsson, L., and Nielsen, J. (2000) Metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev. 64*, 34−50.

(10) Lorenz, M. C., Muir, R. S., Lim, E., McElver, J., Weber, S. C., and Heitman, J. (1995) Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene 158*, 113−117.

(11) Akada, R., Kitagawa, T., Kaneko, S., Toyonaga, D., Ito, S., Kakihara, Y., Hoshida, H., Morimura, S., Kondo, A., and Kida, K. (2006) PCR-mediated seamless gene deletion and marker recycling in *Saccharomyces cerevisiae*. *Yeast 23*, 399−405.

(12) Davidson, J. F., and Schiestl, R. H. (2000) Mis-targeting of multiple gene disruption constructs containing hisG. *Curr. Genet. 38*, 188−190.

(13) Delneri, D., Tomlin, G. C., Wixon, J. L., Hutter, A., Sefton, M., Louis, E. J., and Oliver, S. G. (2000) Exploring redundancy in the yeast genome: an improved strategy for use of the cre−loxP system. *Gene 252*, 127−135.

(14) Suzuki, Y., St Onge, R. P., Mani, R., King, O. D., Heilbut, A., Labunskyy, V. M., Chen, W., Pham, L., Zhang, L. V., Tong, A. H., Nislow, C., Giaever, G., Gladyshev, V. N., Vidal, M., Schow, P., Lehar, J., and Roth, F. P. (2011) Knocking out multigene redundancies via cycles of sexual assortment and fluorescence selection. *Nat. Methods 8*, 159−164.

(15) Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., and Jaenisch, R. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell 153*, 910−918.

(16) Jiang, W. Y., Bikard, D., Cox, D., Zhang, F., and Marraffini, L. A. (2013) RNA-guided editing of bacterial genomes using CRISPR−Cas systems. *Nat. Biotechnol. 31*, 233−239.

(17) Hwang, W. Y., Fu, Y. F., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J. R. J., and Joung, J. K. (2013) Efficient genome editing in zebrafish using a CRISPR−Cas system. *Nat. Biotechnol. 31*, 227−229.

(18) Cong, L., Ran, F. A., Cox, D., Lin, S. L., Barretto, R., Habib, N., Hsu, P. D., Wu, X. B., Jiang, W. Y., Marraffini, L. A., and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science 339*, 819−823.

(19) Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., and Church, G. M. (2013) RNA-guided human genome engineering via Cas9. *Science 339*, 823−826.

(20) Dicarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., and Church, G. M. (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR−Cas systems. *Nucleic Acids Res. 41*, 4336−4343.

(21) Moerschell, R. P., Tsunasawa, S., and Sherman, F. (1988) Transformation of yeast with synthetic oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A. 85*, 524−528.

(22) Yamamoto, T., Moerschell, R. P., Wakem, L. P., Ferguson, D., and Sherman, F. (1992) Parameters affecting the frequencies of transformation and co-transformation with synthetic oligonucleotides in yeast. *Yeast 8*, 935−948.

(23) Doyle, E. L., Hummel, A. W., Demorest, Z. L., Starker, C. G., Voytas, D. F., Bradley, P., and Bogdanove, A. J. (2013) TAL effector specificity for base 0 of the DNA target is altered in a complex, effector- and assay-dependent manner by substitutions for the tryptophan in cryptic repeat-1. *PLoS One 8*, e82120.

(24) Aouida, M., Piatek, M. J., Bangarusamy, D. K., and Mahfouz, M. M. (2014) Activities and specificities of homodimeric TALENs in *Saccharomyces cerevisiae*. *Curr. Genet. 60*, 61−74.

(25) Gasiunas, G., and Siksnys, V. (2013) RNA-dependent DNA endonuclease Cas9 of the CRISPR system: holy grail of genome editing? *Trends Microbiol. 21*, 562−567.

(26) Wang, T., Wei, J. J., Sabatini, D. M., and Lander, E. S. (2014) Genetic screens in human cells using the CRISPR−Cas9 system. *Science 343*, 80−84.

(27) Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., and Charpentier, E. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature 471*, 602−607.

(28) Karvelis, T., Gasiunas, G., Miksys, A., Barrangou, R., Horvath, P., and Siksnys, V. (2013) crRNA and tracrRNA guide Cas9-mediated DNA interference in *Streptococcus thermophilus*. *RNA Biol. 10*, 841−851.
(29) Sun, N., Bao, Z. H., Xiong, X., and Zhao, H. M. (2014) SunnyTALEN: a second-generation TALEN system for human genome editing. *Biotechnol. Bioeng. 111*, 683−691.
(30) Deveau, H., Garneau, J. E., and Moineau, S. (2010) CRISPR/Cas system and its role in phage-bacteria interactions. *Annu. Rev. Microbiol. 64*, 475−493.
(31) Whelan, W. L., Gocke, E., and Manney, T. R. (1979) The CAN1 locus of *Saccharomyces cerevisiae*: fine-structure analysis and forward mutation rates. *Genetics 91*, 35−51.
(32) Romanos, M. A., Scorer, C. A., and Clare, J. J. (1992) Foreign gene expression in yeast: a review. *Yeast 8*, 423−488.
(33) Loison, G., Vidal, A., Findeli, A., Roitsch, C., Balloul, J. M., and Lemoine, Y. (1989) High level of expression of a protective antigen of schistosomes in *Saccharomyces cerevisiae*. *Yeast 5*, 497−507.
(34) Dorfman, B. Z. (1969) The isolation of adenylosuccinate synthetase mutants in yeast by selection for constitutive behavior in pigmented strains. *Genetics 61*, 377−389.
(35) Zwolshen, J. H., and Bhattacharjee, J. K. (1981) Genetic and biochemical properties of thialysine-resistant mutants of *Saccharomyces cerevisiae*. *J. Gen. Microbiol. 122*, 281−287.
(36) Pagano, A., Castelnuovo, M., Tortelli, F., Ferrari, R., Dieci, G., and Cancedda, R. (2007) New small nuclear RNA gene-like transcriptional units as sources of regulatory transcripts. *PLoS Genet. 3*, 174−184.
(37) Szczebara, F. M., Chandelier, C., Villeret, C., Masurel, A., Bourot, S., Duport, C., Blanchard, S., Groisillier, A., Testet, E., Costaglioli, P., Cauet, G., Degryse, E., Balbuena, D., Winter, J., Achstetter, T., Spagnoli, R., Pompon, D., and Dumas, B. (2003) Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat. Biotechnol. 21*, 143−149.
(38) Nishimasu, H., Ran, F. A., Hsu, P. D., Konermann, S., Shehata, S. I., Dohmae, N., Ishitani, R., Zhang, F., and Nureki, O. (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell 156*, 935−949.
(39) Young, L. S., Rivier, D. H., and Sprague, K. U. (1991) Sequences far downstream from the classical tRNA promoter elements bind RNA polymerase III transcription factors. *Mol. Cell. Biol. 11*, 1382−1392.
(40) Agmon, N., Pur, S., Liefshitz, B., and Kupiec, M. (2009) Analysis of repair mechanism choice during homologous recombination. *Nucleic Acids Res. 37*, 5081−5092.
(41) Chen, Y., Partow, S., Scalcinati, G., Siewers, V., and Nielsen, J. (2012) Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production. *FEMS Yeast Res. 12*, 598−607.
(42) Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science 337*, 816−821.
(43) Liang, J., Chao, R., Abil, Z., Bao, Z., and Zhao, H. (2014) FairyTALE: a high-throughput TAL effector synthesis platform. *ACS Synth. Biol. 3*, 67−73.
(44) Gietz, R. D., and Schiestl, R. H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc. 2*, 31−34.