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Dual CRISPR-Cas9 Cleavage Mediated Gene Excision and Targeted Integration in *Yarrowia lipolytica*

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CRISPR-Cas9 technology has been successfully applied in *Yarrowia lipolytica* for targeted genomic editing including gene disruption and integration; however, disruptions by existing methods typically result from small frameshift mutations caused by indels within the coding region, which usually resulted in unnatural protein. In this study, a dual cleavage strategy directed by paired sgRNAs is developed for gene knockout. This method allows fast and robust gene excision, demonstrated on six genes of interest. The targeted regions for excision vary in length from 0.3 kb up to 3.5 kb and contain both non-coding and coding regions. The majority of the gene excisions are repaired by perfect nonhomologous end-joining without indel. Based on this dual cleavage system, two targeted markerless integration methods are developed by providing repair templates. While both strategies are effective, homology mediated end joining (HMEJ) based method are twice as efficient as homology recombination (HR) based method. In both cases, dual cleavage leads to similar or improved gene integration efficiencies compared to gene excision without integration. This dual cleavage strategy will be useful for not only generating more predictable and robust gene knockout, but also for efficient targeted markerless integration, and simultaneous knockout and integration in *Y. lipolytica*.

1. Introduction

The oleaginous yeast *Yarrowia lipolytica* has been explored as a platform for the production of lipid-based fuels and chemicals.[1–6] Strain engineering has been aided by recent developments in CRISPR-Cas9 technology that provide powerful tools for targeted genome editing in *Y. lipolytica*.[7–9] The genomic DNA double-strand break (DBS) introduced by sgRNA guided Cas9 can be either repaired through error-prone pathways such as non-homologous ending joining (NHEJ) and microhomology-mediated end joining (MMEJ), or through the more accurate homologous recombination (HR). Both NHEJ and HR have been used in *Y. lipolytica* for genome editing to enable loss of function genetic studies and metabolic engineering.[8,10] Targeted gene knockout achieved by NHEJ or MMEJ creates indels in the coding region frequently causing frameshift or loss of key amino acids. Indels that occur in core promoter region can also cause loss of function[11]; however, frameshift mutations may produce protein fragments that can be toxic, have unexpected functions[12] or result in exon skipping and alternative splicing.[13] Furthermore, indels leave the majority of the original genomic sequence intact, making HR repair of indels significantly more likely.[14] Although DSB repair mediated by HR through a donor DNA template offers precise integration of a target DNA fragment, the activity of HR in *Y. lipolytica* is relatively low[15,16] and typically requires the use of selectable markers that make multiplexing difficult. Furthermore, the requirement for construction of long homology arms in donor DNA is somewhat time-consuming and labor intensive. Recently, HR at a single Cas9-induced DNA DSBs showed 40–60% efficiency for markerless integration; however, efficiency varied across genetic loci and only a small number of standard sites have been identified.[8,9]

Recently, several studies in multicellular eukaryotes have used CRISPR-Cas9 to make excisions or deletions to large regions of genomic DNA. This method involves creating a pair of DSBs that are repaired by NHEJ together, thus removing any DNA between the target sites. This method has been demonstrated in *C. elegans*, mice, rabbits, and in mouse and human cell lines resulting in deletions of over 100 kb segments.[17–23] Whole genes and exons have been targeted in many instances.[18,20,23,24] There is also great interest in using paired sgRNA to target large regions of long-non-coding DNA.[17,19] DNA excision has been accomplished through injection of purified CRISPR-Cas9 complexes, as well as transformation with genetic constructs, indicating the broad applicability of these methods.[25,26] Removing large segments of genomic DNA through CRISPR-Cas9 using paired sgRNA sequences has not yet been demonstrated in yeast or fungi.

NHEJ is the preferred mechanism for repairing DSBs of DNA in *Y. lipolytica* compared to HR and MMEJ.[27] *Y. lipolytica* typically requires 0.5–1 kb homologous regions flanking the

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targeted locus to only achieve a relatively low integration rate with selective markers.[15] CRISPR-Cas9 based markerless targeted integration has been demonstrated in *Y. lipolytica* only through HR.[8] Among several tested sites, only a few had relatively high integration efficiency using 1 kb homology arms. Recently, a new homology mediated end joining (HMEJ) based strategy was demonstrated for targeted integration in animal embryo and tissue cells, including non-dividing cells. This strategy used CRISPR-Cas9 induced cleavage at a single genomic DNA site and two sites on template donor vector containing sgRNA target sequences flanking 800 bp homology arms, resulting in much higher integration efficiency than HR based method[28,29]; however, this HMEJ based integration strategy has not been demonstrated in yeast or fungi, nor combined with dual cleavage of genomic DNA.

In this study, a dual cleavage strategy mediated by paired sgRNAs was developed for *Y. lipolytica* to create complete gene knockouts via gene excision in a more predicable manner than error prone indels. This method was demonstrated on six target genes of interests including genes controlling lipid metabolism (*FAA1, PEX10, MFE, POX3*) as well as partially truncated markers frequently complemented in *Y. lipolytica* auxotrophic strains (*LEU2* and *URA3*). This dual-cleavage strategy proves to be robust and efficient towards both coding and noncoding region of genes with varied length of up to 3.5 kb. Furthermore, the feasibility of DNA excision assisted integration based on either HR or HMEJ was demonstrated at the *PEX10* locus. HMEJ based integration was nearly twice as efficient as HR. Thus, this gene excision method can be combined with the HMEJ based gene integration method to consolidate and speed up metabolic engineering efforts.

2. Experimental Section

2.1. Chemicals and Enzymes

Chemicals used in this study were obtained from Sigma–Aldrich unless otherwise stated. All enzymes were purchased from New England Biolabs (NEB). Plasmid mini-preps were performed using the Zyppy™ Plasmid Miniprep Kit (Zymo Research). PCR clean-ups were performed using the DNA Clean & Concentrator™ (Zymo Research). Oligonucleotides were purchased from either IDTDNA or Eurofins.

2.2. Strains and Culture Conditions

Plasmid propagation was performed using Escherichia coli DH10β competent cells (NEB). Transformations in *E. coli* were performed using standard methods.[30] *Y. lipolytica* strain PO1f (ATCC MYA-2613; MATa leu2−270 ura3−302 xpr2−322 axp) was purchased from ATCC. *Y. lipolytica* transformations were done using the lithium acetate method as previously described with a minor modification for cell propagation after the transformation.[10] All transformations were done in biological triplicate. All *Y. lipolytica* liquid cultures were grown at 215 rpm and 28 °C. *Y. lipolytica* was grown in selective dropout Yeast Synthetic Complete (YSC) liquid or solid media (1.5% agar) comprised of yeast nitrogen base (YNB) without amino acids (Difco) and selective complete supplement mixture (Sunrise Science Products). In all media, 2% (w/v) D-glucose was used as carbon source.

2.3. Plasmid Construction and gRNA Design

All plasmids used in this study are listed in Table S1, Supporting Information. Unless otherwise stated, sequence and ligation independent cl6oning (SLIC)[31] was used for cloning. Parent pCas9-LEU or URA plasmids that were used for creating targeting plasmids were generated by modifying pCRISPRyl plasmid from previous work.[8] The Pol III–tRNA hybrid promoters was used to drive the expression of sgRNA. To make parent pCas9-Leu plasmid, a gBlock containing a unique NsiI site upstream to trcRNA and a mutation to the NsiI site in the LEU2 promoter were inserted into pCRISPRyl digested with AatII and NsiI (Figure S4, Supporting Information). The parent pCas9-Ura plasmid was generated by replacing the LEU2 marker with URA3 marker. All targeting pCas9 plasmids were generated by insertion of two annealed oligonucleotides containing target sequences of designed gRNAs and SLIC overhangs into parent plasmid digested with NsiI. The oligonucleotides used to create single sgRNA targeting plasmids in this study are presented in Table S2, Supporting Information. The dual sgRNA targeting plasmid pCas9-LEU-ΔPEX10-sgRNA1&2 was constructed by introducing the PEX10 sgRNA2 expression cassette into pCas9-LEU-ΔPEX10-sgRNA1 digested with XmaI.

In order to construct HR and HMEJ donor plasmids for *PEX10* locus, we first constructed plasmid pTEF-intron-GFP-CYC1, followed by replacing GFP with the hygromycin (*hph*) gene to make pTEF-intron-hph-CYC1. Then, 1 kb homology arms flanking *PEX10* cleavage sites were PCRed from PO1f genomic DNA. The backbone vector for constructing HR and HMEJ donor plasmid containing URA marker was PCRed from plasmid pTEF-URA-UAS1B8-GFP-CYC1. Finally, the HR and HMEJ donor plasmids were constructed by cloning the hygromycin cassette, homology arms, and vector together. For HMEJ donor plasmids, upcut and downcut recognition sites that are identical to upstream and downstream target sequence of *PEX10* gene were cloned to flank the homology arms. All oligonucleotide sequences for constructing dual sgRNA, HR, and HMEJ plasmids are listed in Table S3, Supporting Information. All sgRNA sequences were designed using the Chop–Chop online program (http://chopchop.cbu.uib.no) based on the target sequence (Table S1, Supporting Information) of each gene and the ranking of target sequences.[32,33] The designed sgRNA sequences for each of the loci tested are listed in Table S4, Supporting Information.

2.4. Analysis of Gene Excision and Integration

The analysis of gene excision and integration was done using colony PCR and confirmed with DNA sequencing (Eurofins). Primers used for colony PCR analysis are listed in Table S5, Supporting Information. Q5® High-Fidelity DNA Polymerase

(NEB) was used for colony PCR. Colonies from plates were picked and resuspended in the PCR reaction mixture. A 10-min cell lysis at 95 °C followed by standard PCR reaction was used for colony PCR. A three-primer set method was used to identify mutants with successful gene excision events. 3-primer set including out-5′, in-5′, and out-3′ was used to identify "no excision" and "excision" in single step. If there is no excision, in-5′, and out-3′ can amplify 710 bp from wide type. If there is excision event, out-5′ and out-3′ can amplify 500 bp from mutant. Sanger sequencing was used for genotyping verification. For identifying indels at upstream and downstream target regions of *PEX10*, a 2-primer set method was used for Sanger sequencing. For identifying integration events, 2 primer sets were used to amplify 5′ junction and 3′ junction around homology regions. As each PCR uses one primer complementary to the genome and the other to the *hph* gene, amplification identifies site-specific integration. It should be noted that although we integrated the *hph* gene, we did not use its hygromycin resistant phenotype in any part of our analysis, as we wish to keep this method and its analysis markerless and general.

3. Results

3.1. Gene Excision is Accomplished by Use of CRISPR-Cas9 With Two sgRNA Target Sequences

Given that two DNA DSBs can be repaired together by end-joining in mammalian cells, we designed two plasmids to drive the expression of individual CRISPR-Cas9 and sgRNA. Each plasmid has one Cas9 gene driven by the strong hybrid promoter TEF-UAS1B8 and one SCRp'-tRNA driven sgRNA cassette (**Figure 1**A). Each plasmid has its own selectable marker, either the *LEU2* or *URA3* gene, that complements the auxotrophic phenotype of the PO1f strain. Two DSBs can be created at the two target sites designed to flank the excised DNA fragment. These breaks must occur simultaneously and the remaining genomic pieces end-joined in order for DNA excision to occur. The general design for two sgRNAs is to target both upstream of the start codon and downstream of stop codon, respectively (Figure 1B). These target sites were chosen based on the availability of PAM sequences and the absence of predicted off-target effects. Furthermore, targeting in transcriptional initiation and termination regions has the added benefit of likely being more unoccupied by nucleosomes,[34–36] making the DNA more accessible for Cas9 cleavage.[37,38] The predicted cleavage site is 3 or 4 bp upstream of the PAM sequence. The *Y. lipolytica* PO1f strain was co-transformed with pCas9-Leu and pCas9-Ura plasmids and grown on selective dropout YSC solid media for 2 days. The first colony PCR analysis allows us to pick colonies with less heterogeneity. To generate homogenous colonies, single colonies were streaked on solid YPD media for another day before analysis for gene excision by colony PCR. One final step of streaking on YPD and spot testing on YPD, YSC-Leu, and YSC-Ura plates is performed to ensure curing and homogeneity (Figure 1C). This experimental protocol allows for the simple deletion of genes in 5 days.

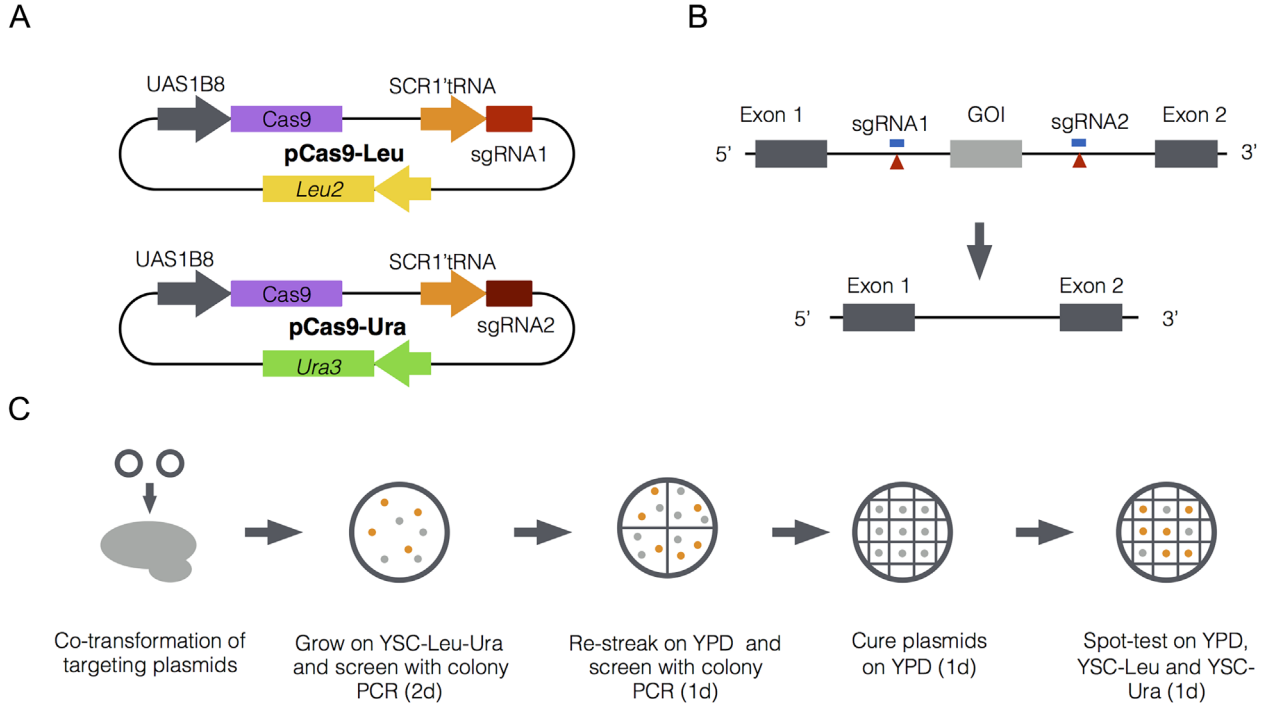

Figure 1. Gene excision using dual cleavage strategy mediated by paired sgRNAs. A) Schematic design of targeting plasmids containing Cas9, sgRNAs, and selective markers. B) Schematic representation of the dual-cleavage strategy. The sgNRA are shown in blue, and cleavage sites are noted by the red arrows. C) Workflow for screening mutants with excision requiring 5 days from start to finish.

3.2. Efficient Screening of Gene Excision Enables Identification of the *PEX10* Deletion

As a demonstration of the dual CRISPR-Cas9 gene excision in *Y. lipolytica*, we chose to knockout the *PEX10* gene that encodes a protein required for peroxisome matrix protein import and peroxisome biogenesis.[39] The knockout of *PEX10* has been used to disrupt β-oxidation and facilitate higher lipid accumulation.[4,40] To create DSBs approximately 200 bp upstream of the transcription start site (TSS) and 80 bp downstream of transcription end site (TES), we cloned PEX10-sgRNA1 and PEX10-sgRNA2 into pCas9-LEU and pCas9-URA, respectively. These plasmids were transformed into *Y. lipolytica* PO1f and screened for gene deletion events. The distance between the two cleavage sites is 1475 bp. The targeted region was amplified for genomic DNA PCR analysis using a 3-primer set (**Figure 2**A). A partial region of *PEX10* with length of 816 bp amplified by primers out-5′ and in-3′ indicated colonies lacking a gene deletion, while a fragment with length of 500 bp amplified by PCR primers out-5′ and out-3′ represented a *bona fide PEX10* deletion (Figure 2A and B). In total, 48 colonies were screened by this 3-primer PCR analysis and 10 colonies (10/48, 20.8%) showed the targeted dual site induced deletion of *PEX10*. Sanger sequencing for 8 of 10 DNA fragments showed that the cleaved blunt-ended genomic DNAs were ligated by NHEJ with precise end-joining, and with small deletions for 2 of 10 (Figure 2C).

Deletion of *PEX10* requires synchronized cutting of both cleavage sites directed by two separate sgRNAs during the same cell cycle. Non-synchronized DNA cleavage may lead to indel formation at a single site, preventing future cutting and gene excision events. To check the cutting efficiency of each sgRNA, six colonies were randomly picked from the 38 colonies that showed no *PEX10* deletion. Both 5′ and 3′ junctions were PCRed with 2-primer set (out-5′ and in-3′ for 5′ junction, and in-5′ and out-3′ for 3′-junction) and sequenced. All six clones showed between 1 and 4 bp deletions around 5′ junction, while four of the clones had a 1 bp insertion, one clone had a 1 bp deletion, and one clone showed no mutation around 3′ junction (Figure S1, Supporting Information). The results indicate that high efficiency cleavage was achieved for both sgRNAs and that indel formation was the major competition for gene excision.

3.3. Gene Excision by Dual CRISPR-Cas9 Cleavage Efficiently Targets Several Genes

To further test the capability of the dual CRISPR-Cas9 cleavage system in *Y. lipolytica*, five additional genes were chosen for gene deletion. *FAA1* encodes the cytoplasmic fatty acid synthetase that activates free fatty acid to fatty acyl-CoA.[41] The *FAA1* knockout has been used to improve free fatty acid production in *Y. lipolytica*.[5] *FAA1* deletion was accomplished using two sgRNAs designed to delete a small 350 bp fragment within the protein-coding region of *FAA1*, resulting in 10 out of 31 (32.6%) screened colonies that generated deletions (**Table 1**).

MFE encodes a multi-functional enzyme that catalyzes two steps of the β-oxidation cycle within the peroxisome.[42] Knockout of *MFE* was shown to significantly increase the lipid accumulation in *Y. lipolytica*.[3] *MFE* deletion was accomplished using two sgRNAs designed to delete a large 3161 bp fragment containing core promoter region, ORF and a part of terminator, resulting in 3 out of 21 (12.5%) screened colonies that generated deletions (Table 1).

The *POX3* gene is involved in the first step of β-oxidation of fatty acid, and encodes a short chain fatty acid specific acyl-CoA oxidase converting acyl-CoA to 2-trans-enoyl-CoA. Knockout of *POX3* decreases the metabolism of short chain fatty acids.[43] Two sgRNAs targeting coding region near TSS and TES, respectively, were designed to delete 2090 bp fragment of the coding region, leading to 11 out of 48 (22.9%) screened colonies with excision (Table 1).

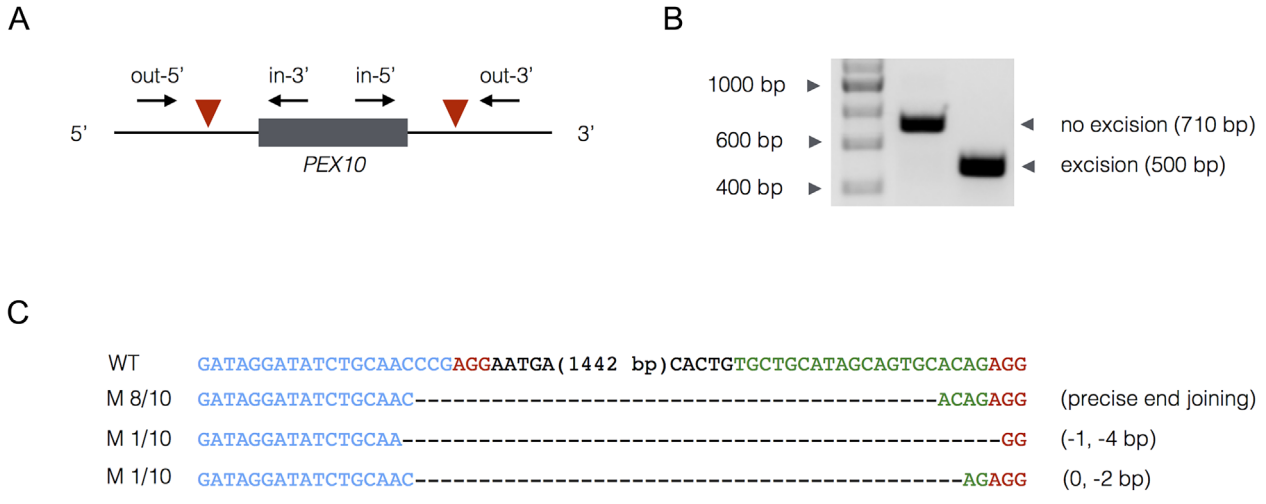

Figure 2. Knockout of *PEX10* through gene excision. A) Primer sets for colony PCR verification of *PEX10* excision. 3-primer set including out-5′, in-5′, and out-3′ is used for identify excision event. 2-primer sets (out-5′ and in-3′, in-5′ and out-3′) are used for amplification of upstream and downstream targeting regions with indels. Red arrow indicates cleavage sites. B) PCR analysis of 10 representative colonies with *PEX10* excision. C) Sequences showing colonies with gene excision are repaired by NHEJ with precise end joining or indels. WT, wild type. M, mutants with excision.

Table 1. Summary of gene excision targeting six genes with different lengths.

Gene	Excised length (kb)	Cut position (TSS/TES)	Total colony	Colony with deletion	Excision efficiency (%)
FAA1	0.37	+334/−703	31	10	32.6
PEX10	1.48	−242/+99	48	10	20.8
POX3	2.09	−77/+82	48	11	22.9
MFE	3.16	−200/+176	21	3	14.3
LEU2	2.11	−822 /+752	36	6	16.7
URA3	3.56	−404/+510	44	7	15.9

The excision efficiency is presented as the percentage of colonies with excision in total colonies screened that were pooled together from biological triplicates.

LEU2 and *URA3* were partially disrupted in the auxotrophic PO1f strain with long homology to *LEU2* and *URA3* markers on several common plasmids.[44] These regions may allow gene conversion from the plasmid markers that repair the *LEU2* and *URA3* genes.[14] Complete deletion of *LEU2* and *URA3*, including regions of the promoter and terminator, can eliminate this possibility of gene conversion. Paired sgRNAs were designed to cleave outside the promoter and terminator regions of *LEU2* and *URA3* so that all homology to the plasmid markers could be deleted. For *LEU2*, the 2110 bp deletion was observed in 11 out of 36 (16.7%) screened colonies, and for *URA3*, the 3560 bp deletion was observed in 7 out of 44 (15.9%) screened colonies (Table 1). DNA sequence analysis of one randomly picked mutant for each target gene showed that most deletions were precisely ligated without indels (Figure S2, Supporting Information).

3.4. Targeted Integration Accomplished by Excision Assisted HR and HMEJ Repair

To perform targeted integration using dual cleavage strategy, a CRISPR-Cas9 plasmid that has both sgRNA expression cassettes targeting *PEX10* locus, and two types of donor plasmids bearing the *hph* gene were constructed (**Figure 3**A and B). HR donor plasmid contains 1 kb homology flanking the cleavage sites and HMEJ donor plasmid has identical 1 kb homology with two identical sgRNA1 target sequences flanking the homology arms (Figure 3B). The HR based method uses a circular donor plasmid as template to mediate targeted integration at dual cleavage sites through the HR pathway. In contrast, the HMEJ based method provides a linear donor template released by dual cleavage at sgRNA1 target sites on the HMEJ donor plasmid, and mediate targeted integration through both HR and HMEJ pathways

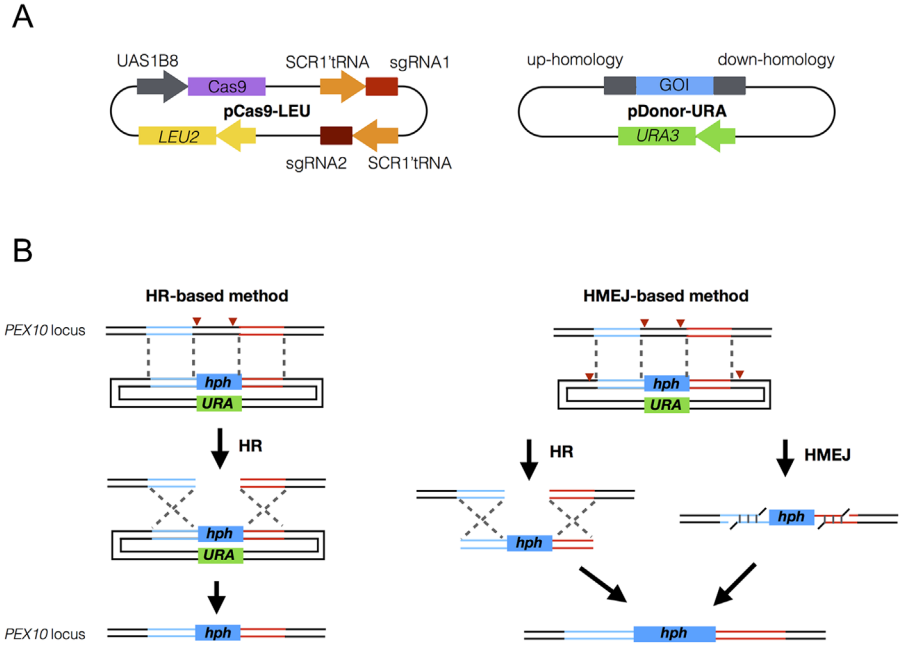

Figure 3. Schematic overview of gene integration through excision assisted HR and HMEJ mediated repairs. A) General plasmid design for excision assisted integration. Donor plasmid can be either HR donor with only homology arms, or HMEJ donor with homology arms and sgRNA target sequences flanking homology arms. B) Schematic representation of integration of hygromycin expression cassette into *PEX10* locus through HR and HMEJ based methods. Red arrow indicates sgRNA target site.

(Figure 3B). The HMEJ pathway is proposed to be active in G1/early S phase and has similar mechanism to single-strand annealing pathway.[28,45]

To evaluate the excision assisted targeted integration efficiency, colony PCR analysis was performed using different primer sets (**Figure 4**A). P1 and P2 were used to verify integration at 5′ junction. P3 and P4 allowed verification of integration at 3′ junction. Out-5′ and out-3′ was used to determine gene excision efficiency. The resulting integration efficiency is presented as the percentage of colonies with integration at both 5′ and 3′ junctions from total screened colonies from biological triplicates (Figure 4B). The HR based method resulted in similar efficiency for integration and excision (Figure 4C). On the other hand, the HMEJ based method offered much higher integration efficiency compared to excision. The integration efficiency of HMEJ-based method ($37.5 \pm 8.84\%$) was more than twofold higher than the HR-based method ($16.67 \pm 3.61\%$) (Figure 4C). As important controls to exclude the possibility of false positives, single cleavage of the *PEX10* gene in the presence of HR or HMEJ donor templates was tested, and resulted in lower integration efficiency for HR based method and no integration for HMEJ based method (Figure 4C). Genotyping analysis of one randomly picked clone with an integration event showed that HR and HMEJ based methods resulted in precise in-frame integration at 5′ junction (Figure S3, Supporting Information).

4. Discussion

In this study, a simple and robust dual CRISPR-Cas9 system mediated by paired sgRNAs was first developed for deletions of large chromosomal DNA fragments. The experimental protocol allows the deletion of targeted gene in 5 days. Deletion mutants can be directly and easily identified by PCR and agarose gel electrophoresis, without sequencing DNA fragment or without implementing nuclease-based assays to detect indels. Gene excision enabled deletion of the *PEX10* gene was the first demonstration of CRISPR-Cas9 *PEX10* knockout. Similarly, we efficiently deleted the POX3 gene using gene excision where previously we failed to edit this locus using a single sgRNA.[8]

In order for a gene excision to occur, both sgRNA targeted Cas9s must cleave the genomic DNA during the same cell cycle. Once a single indel occurs, it prevents subsequent cleavage, thereby preventing gene excision. Remarkably, the deletion efficiency for six genes ranged from 14.3% to 32.6%. Since the promoters for both sgRNAs were identical, the strength and dynamics of Cas9 activity were likely comparable, leading to simultaneous cleavage and repair that resulted in gene excision. Sequencing results from the deletion of six genes suggest that the majority of excised sites were precisely ligated by direct end joining of both cleaved ends one base upstream of each PAM sequence. Therefore,

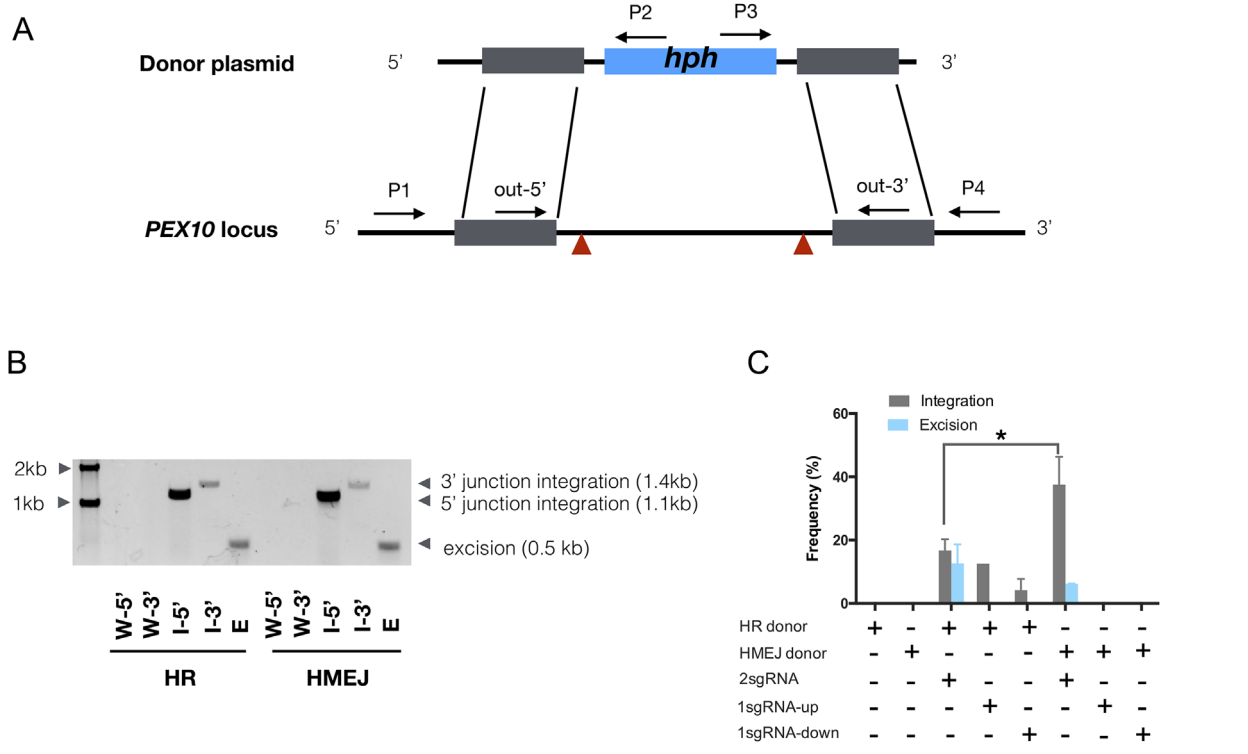

Figure 4. Integration of hygromycin cassette through excision assisted HR and HMEJ based method into *PEX10* locus. A) Primer sets for verification of hygromycin integration at 5′ and 3′ junction, and verification for excision. P1 and P2 for verification of integration at 5′ junction. P3 and P4 for verification of integration at 3′ junction. P1 and P4 for verification of excision. B) PCR analysis for representative colonies with integration or excision through HR and HMEJ. W-5′ and 3′, wild type at 5′ and 3′ junction. I-5′ and 3′, mutant with integration at 5′ and 3′ junction. E, mutants with excision. C) Integration and excision frequency for HR and HMEJ based methods. The results are from three biological replicates and presented as mean ± SD. $^{*}p < 0.05$, Sidak's multiple comparisons test.

dual sgRNA directed knockout strategy is more predictable and does not produce truncated protein fragments that could still be translated.

The genome deletion size can be as small as 300 bp and greater than 3500 bp, suggesting the versatility of dual-cut deletion strategy; however, the exact limits in *Y. lipolytica* of this strategy have yet to be determined as we have not tried to find the upper limit for gene excision size. Analysis of DNA excision in mammalian cells suggests that fragments with megabase size can be excised[46]; however, as the fragment excised increases in length, the efficiency will decrease.[47] A weak correlation between excision fragment size and integration efficiency was observed in *Y. lipolytica* in this study. New methods recently developed for designing paired sgRNA sequences may further enhance the efficiency of our system.[48]

Based on the efficient dual cleavage system, HR and HMEJ based method were later developed for targeted integration at the *PEX10* locus. HMEJ offered higher integration efficiency than the HR based method. Comparison between single and dual cleavage with HR or HMEJ donor templates suggested that only single cleavage on *PEX10* locus was required for HR mediated integration while dual cleavage was necessary for HMEJ mediated integration. Evidence suggests that the HMEJ donor may provide a template for both the HMEJ and the HR repair pathway, thus resulting in higher overall integration efficiency.[28] The HMEJ pathway was shown to be active in G1 and early S phase and could potentially compete with NHEJ in repairing the dual cleavage.[28,45] The ratio of integration to excision efficiency for HMEJ (≈6) was much higher than for HR (≈1.3), indicating that the HMEJ donor is more efficient at providing template for repairing dual cleavage.

The LEU2/URA3 markers were to ensure efficient plasmid retention by *Y. lipolytica* after transformation. The *hph*+ phenotype was not selected for (or even tested) as no hygromycin was used in outgrowth plates following vector transfection into *Y. lipolytica*. Therefore, the successful integration of *hph* into the targeted site was not due to selective pressure. This ensures our test measures the true efficiency of the system not an artificially improved efficiency due to selective conditions.

Overall, these CRISPR-Cas9 methods continue to advance our ability to more rapidly engineering *Y. lipolytica* for useful biotechnological phenotypes. Elucidating the underlying mechanism for gene excision and excision assisted targeted integration may benefit further optimization of this strategy. To determine the versatility of the HMEJ based integration method, additional studies demonstrating HMEJ at different genomic loci are need. Strategies to synchronize and or arrest the cell cycle may also benefit gene excision and excision assisted targeted integration.

Abbreviations

CRISPR, clustered regularly interspaced short palindromic repeats; DBS, double-strand break; HMEJ, homology mediated end joining; HR, homologous recombination; MMEJ, microhomology-mediated end joining; NHEJ, non-homologous end joining.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

Keywords

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