Candida albicans Gene Deletion with a Transient CRISPR-Cas9 System

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ABSTRACT Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated gene 9 (CRISPR-Cas9) systems are used for a wide array of genome-editing applications in organisms ranging from fungi to plants and animals. Recently, a CRISPR-Cas9 system has been developed for the diploid fungal pathogen Candida albicans; the system accelerates genetic manipulation dramatically [V. K. Vyas, M. I. Barrasa, and G. R. Fink, Sci Adv 1(3):e1500248, 2015, http://dx.doi.org/10.1126/sciadv.1500248]. We show here that the CRISPR-Cas9 genetic elements can function transiently, without stable integration into the genome, to enable the introduction of a gene deletion construct. We describe a transient CRISPR-Cas9 system for efficient gene deletion in C. albicans. Our observations suggest that there are two mechanisms that lead to homozygous deletions: (i) independent recombination of transforming DNA into each allele and (ii) recombination of transforming DNA into one allele, followed by gene conversion of the second allele. Our approach will streamline gene function analysis in C. albicans, and our results indicate that DNA can function transiently after transformation of this organism.

IMPORTANCE The fungus Candida albicans is a major pathogen. Genetic analysis of this organism has revealed determinants of pathogenicity, drug resistance, and other unique biological features, as well as the identities of prospective drug targets. The creation of targeted mutations has been greatly accelerated recently through the implementation of CRISPR genome-editing technology by Vyas et al. [Sci Adv 1(3):e1500248, 2015, http://dx.doi.org/10.1126/sciadv.1500248]. In this study, we find that CRISPR elements can be expressed from genes that are present only transiently, and we develop a transient CRISPR system that further accelerates C. albicans genetic manipulation.

KEYWORDS: Candida albicans, genetics

Candida albicans is a human commensal that lives on mucosal surfaces. It is thus poised to proliferate when conditions are permissive, which can lead to mucosal and invasive infections (1, 2). Molecular genetics of C. albicans can facilitate the discovery of antifungal agents and elucidation of pathogenesis mechanisms. However, the creation of homozygous deletion mutants in this diploid organism remains a slow step in gene function analysis.

Recently, Vyas and colleagues greatly accelerated C. albicans genetic analysis by adapting a clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated gene 9 (CRISPR-Cas9) system to rapidly create C. albicans homozygous mutants (3). CRISPR-Cas9 systems are adaptive immune systems in bacteria (4). The RNA-guided endonuclease activity of Cas9 has been harnessed for an array of genome-editing applications in organisms ranging from fungi to plants and animals (5, 6). The Vyas system consists of a Candida codon-optimized Cas9 nuclease gene (CaCAS9) and a single guide RNA (sgRNA) gene whose product directs Cas9 to cleave a specific site in the genome. The 20-base guide sequence from the sgRNA hybridizes to a genomic target, enabling CaCas9 to produce a double-strand break when the
A genomic target is followed by the protospacer-adjacent motif sequence, NGG. A CRISPR-induced double-strand break triggers homology-directed repair with mutagenic donor DNA so the genomic target can be precisely edited.

A potential rate-limiting step of the Vyas CRISPR system is the reliance upon genomic integration of the vector encoding CaCas9 and the sgRNA. In essence, the desired genome editing frequency may have been limited to the integration frequency of the CaCas9-sgRNA expression construct. We report here our finding that the CaCas9-sgRNA expression construct does not require genomic integration for functional activity. This observation led us to hypothesize that transient introduction of CaCAS9 and the sgRNA gene might simplify C. albicans genome editing. We report here the evidence in support of this hypothesis and describe our modifications of the Vyas constructs that enable a transient introduction approach.

RESULTS

Candida CRISPR system function without stable integration. We set out to use the CRISPR system for deletion and replacement of gene-sized DNA regions in C. albicans. The ADE2 gene was targeted because homozygous ade2 mutations result in a visible red phenotype, thus simplifying their identification. We used the CRISPR system vector pADE2-sgRNA, which has a functional CaCAS9 and an sgRNA gene directed against ADE2 (3). It also contains a NAT selection marker and 2-kb arms to enable homologous integration at the ENO1 locus. Using Arg^- BWP17 cells, an ADE2 gene deletion construct containing an ARG4 cassette (the ade2::ARG4 template) was transformed with or without linearized pADE2-sgRNA DNA. We selected for Arg^- transformants, which selects for the ade2::ARG4 deletion rather than the CRISPR system vector, in order to assay the effects of the CRISPR system on integration. Transformation of the ade2::ARG4 template together with linearized pADE2-sgRNA DNA produced red Arg^- colonies at a high frequency, whereas transformation of the ade2::ARG4 template alone produced only white Arg^+ colonies (Fig. 1). These results suggested that the Candida CRISPR system induced the homozygous deletion of the ADE2 open reading frame (ORF) (1.7 kb) and integration of the ARG4 cassette. We then tested for integration of the pADE2-sgRNA plasmid segment by testing colonies for the NAT marker. Among thirty red and thirty white Arg^- colonies tested, none were nourseothricin resistant (NatR phenotype), indicating that they did not express the NAT marker. However, the abundance of red Arg^- transformants from the cotransformation samples argued that functional expression of CaCas9 and the sgRNA gene had occurred upon transformation.

Transient CRISPR system for C. albicans. In view of the results described above, we tested a C. albicans transient CRISPR system that targets the ADE2 gene. This system consisted of separate CaCas9 and sgRNA expression cassettes (Fig. 2A). The CaCas9 expression cassette was PCR amplified from the plasmid pV1093 (3). The sgRNA expression cassette was constructed through single-joint PCR (Fig. 3). We used the
sgRNA ADE2.1 guide RNA sequence (as designed and used by Vyas et al. [3]), which directs CaCas9 activity to a site 41 bp downstream from the ADE2 start codon. An ade2/H9004::NAT construct served as a template to create a deletion of the ADE2 target gene (Fig. 2B). The ade2/H9004::NAT construct had 80-bp arms homologous to sequences upstream or downstream from the ADE2 coding region, and the site targeted by the sgRNA ADE2.1 guide RNA was immediately adjacent to one ade2/H9004::NAT deletion endpoint and 1.7 kbp from the other. The ade2/H9004::NAT construct was cotransformed into

FIG 2 Transient CRISPR-Cas9 system. (A) Structures of CaCas9 and sgRNA expression cassettes. The CaCas9 gene was the Vyas codon-optimized version, expressed from the ENO1 promoter and flanked by a CYC1 terminator (3). The sgRNA was expressed under the SNR52 promoter and contained the ENO1 terminator (3). The guide sequence is 20 bp long and designed for each target sequence. (B) Schematic diagram of ADE2 deletion strategy. The CRISPR system produces double-strand breaks (DSB) at target sequences. The double-strand breaks can be resolved by homology-directed repair with the gene deletion templates, which have 80-bp arms homologous to the target gene, to create a homozygous deletion of ADE2.

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FIG 3 Construction of sgRNA expression cassette. Three DNA synthesis steps fuse DNA fragments comprising the SNR52 promoter and the sgRNA scaffold. Chimeric primers 2 and 3 carry 20 complementary bases of guide sequence. In the first step, PCR is used to create two segments of the sgRNA gene. The SNR52 promoter is amplified with primers 1 and 2; the sgRNA scaffold is amplified with primers 3 and 4. In the second step, primer extension is used to fuse the two PCR products, with chimeric extensions acting as primers. In the third step, PCR with nested primers 5 and 6 is used to amplify the final sgRNA expression cassette.
strain SC5314 with PCR products for CaCAS9 and the sgRNA ADE2.1 gene, and NatR transformants were selected. Transformants were recovered at a frequency of $4.7 \times 10^{-5}$ (Table 1). This version of the CRISPR system produced red transformants at high frequency (Fig. 4; Table 1), and PCR genotyping of 10 such colonies verified that they carried only ade2Δ::NAT alleles (Fig. 5A and B). The production of red colonies among the transformants required both CaCAS9 and sgRNA (Table 1). In the absence of either CRISPR component, the frequency of NatR transformants was reduced more than 10-fold and only white transformants were recovered (Table 1). We used PCR to determine whether CaCAS9 or the sgRNA expression cassette was detectable in genomic DNA of the transformants. Among 10 transformants, we detected no signal from CaCAS9 with internal primers and no signal from the sgRNA expression cassette (Fig. 5B). These results indicated that intact CRISPR component cassettes had not integrated into the genome. The effect of CaCAS9 and the sgRNA expression cassette on the transformation outcome argued that the CRISPR system had functioned at a critical time during transformation. Overall, our results indicate that a transient presence of intact CRISPR component genes is sufficient to promote recombination events that result in gene deletion.

We sought to determine whether the guide RNA target site could be distant from both deletion endpoints. To that end, we tested the sgRNA ADE2.2 genomic target site, which was located 912 bp downstream from the start codon, a site that is 0.9 kbp from each ade2Δ::NAT deletion endpoint. We observed that ade2Δ::NAT deletion events were stimulated to a comparable extent by sgRNA ADE2.2 and ADE2.1 guide RNA genes (Table 1; Fig. 4). PCR genotyping of red transformants made with the ADE2.2 sgRNA gene verified that 9 of 10 carried only ade2Δ::NAT alleles and failed to detect the presence of CaCAS9 or the sgRNA expression cassette (Fig. 5A and B). These results indicate that recombination sites can lie far from the site of CRISPR-directed double-

### TABLE 1 Transformation frequencies with the transient CRISPR system

| CaCAS9 expression cassette | sgRNA expression cassette | ade2Δ::NAT deletion construct | NAT resistance frequency | Homozygous ADE2 deletion frequency |
|---------------------------|---------------------------|--------------------------------|--------------------------|-----------------------------------|
| −                         | −                         | +                              | $1.4 \times 10^{-6}$     | 0.0                               |
| +                         | −                         | +                              | $8.3 \times 10^{-7}$     | 0.0                               |
| −                         | ADE2.1                    | +                              | $4.7 \times 10^{-5}$     | 2.1 $\times 10^{-5}$              |
| +                         | ADE2.1                    | +                              | 0.0                      | 0.0                               |
| −                         | ADE2.2                    | +                              | $1.3 \times 10^{-6}$     | 0.0                               |
| +                         | ADE2.2                    | +                              | $2.1 \times 10^{-5}$     | 1.4 $\times 10^{-5}$              |
| +                         | ADE2.2                    | −                              | 0.0                      | 0.0                               |
| −                         | ADE2.2                    | −                              | 0.0                      | 0.0                               |

*Transformation frequency was calculated as the ratio of the number of colonies on YPD+NAT medium divided by the number on nonselective YPD medium. Each transformation experiment was repeated four times.

### FIG 4 Functional assay for the Candida transient CRISPR system via recovery of ade2Δ/ade2Δ mutants. Strain SC5314 was transformed with the ADE2 deletion template and CaCAS9 expression cassette without an sgRNA cassette (A), with the ADE2.1 sgRNA cassette (B), or with the ADE2.2 sgRNA cassette (C) and plated on YPD+NAT. (D) A 1/10,000 dilution of the negative control (no transforming DNA control) was plated on nonselective YPD plates.
strand break formation in C. albicans, as has been found previously in yeast and mouse (7, 8).

In order to determine whether the transient CRISPR system would allow homozygous deletion recovery for loci besides ADE2, we used the system to delete the FRP1 gene in the SN152 strain background (9), using frp1Δ::CdARG4 (with ARG4 from Candida dubliniensis) as the template sequence. We used a yhb5ΔΔ strain (10) as a transformation recipient because we hypothesized that Yhb5 and Frp1 may have related functions. Targeting was accomplished with an sgRNA sequence developed by Vyas et al. (3) that targets the 5’-end of the FRP1 coding region, directing cleavage adjacent to one template recombination site and 1.6 kbp from the other. Among 8 Arg+ transformants, three yielded only an frp1Δ::CdARG4 PCR product, as expected for homozygous deletion mutants (Fig. 5C and D). These results indicate that homozygous deletion mutants can be recovered with the transient CRISPR system at two loci, FRP1 and ADE2, and can be employed with the popular SN152 strain background.
Independent integration and allelic gene conversion. We considered two models for the CRISPR-enabled generation of homozygous mutants. One model is that homozygotes arise from independent recombination events that integrate two repair template molecules, one per allele (Fig. 6A). A second model is that homozygotes arise from a recombination event that integrates a repair template into one allele, followed by a gene conversion or crossover event that transfers the mutant allele sequence into the remaining wild-type allele (Fig. 6B). These models are not mutually exclusive; it is possible that both mechanisms occur. We used transformation of two differentially marked repair templates into strain BWP17 to test these models. We mixed ade2Δ::NAT and ade2Δ::ARG4 deletion cassettes in a 1:1 molar ratio and transformed BWP17 cells with the mixture, along with the CaCAS9 and sgRNA ADE2.1 PCR products. Double-strand breaks are represented by lightning bolts. (A) In the independent integration model, homozygotes arise from independent recombination events that integrate two repair template molecules, one per allele. Assuming that ade2Δ::NAT and ade2Δ::ARG4 deletion templates have equal chances of integration into ADE2 alleles (which we designate Ade2A and Ade2B), then we predict equal numbers of red homozygous transformants of genotypes ade2Δ::ARG4/ade2Δ::NAT, ade2Δ::NAT/ade2Δ::ARG4, and ade2Δ::NAT/ade2Δ::NAT. Only the first three classes will be detected among Arg+ transformants, so the ratio of red Arg+ NatR transformants to red Arg+ NatS transformants should be 2:1. (B) In the allelic gene conversion model, homozygotes arise from a recombination event to integrate a repair template into one allele, followed by a gene conversion or crossover event that copies the mutant allele sequence into the wild-type allele. This model predicts that none of the Arg+ red colonies will express the NAT marker, so the ratio of red Arg+ NatR transformants to red Arg+ NatS transformants should be 0:1.

FIG 6 Two models for the CRISPR-enabled generation of a homozygous ADE2 deletion. We depict an experiment in which mixed ade2Δ::NAT and ade2Δ::ARG4 deletion templates are transformed along with the CaCAS9 and sgRNA ADE2.1 PCR products. Double-strand breaks are represented by lightning bolts. (A) In the independent integration model, homozygotes arise from independent recombination events that integrate two repair template molecules, one per allele. Assuming that ade2Δ::NAT and ade2Δ::ARG4 deletion templates have equal chances of integration into ADE2 alleles (which we designate Ade2A and Ade2B), then we predict equal numbers of red homozygous transformants of genotypes ade2Δ::ARG4/ade2Δ::NAT, ade2Δ::NAT/ade2Δ::ARG4, and ade2Δ::NAT/ade2Δ::NAT. Only the first three classes will be detected among Arg+ transformants, so the ratio of red Arg+ NatR transformants to red Arg+ NatS transformants should be 2:1. (B) In the allelic gene conversion model, homozygotes arise from a recombination event to integrate a repair template into one allele, followed by a gene conversion or crossover event that copies the mutant allele sequence into the wild-type allele. This model predicts that none of the Arg+ red colonies will express the NAT marker, so the ratio of red Arg+ NatR transformants to red Arg+ NatS transformants should be 0:1.
The skewed ratio of selected to unselected markers is significantly different from the 1:2 ratio that is expected from model 1, according to a chi-square test (P value = 0.0001). These results indicate that homozygous mutations sometimes arise from two independent integration events, one at each allele (model 1), and sometimes arise from a single integration event at one allele, followed by gene conversion or crossing over to alter the second allele (model 2).

DISCUSSION

This study presents three conclusions that will be useful for future application and interpretation of CRISPR-promoted mutants of *C. albicans*. First, we have found that the CRISPR components—the *CaCAS9* and sgRNA gene cassettes—can function when introduced transiently and without direct selection. This finding may streamline *Candida* CRISPR usage. The finding also implies that other genes might be introduced into *C. albicans* transiently for a variety of applications. Second, we have found that CRISPR-targeted cleavage can occur far from sites of recombination in *C. albicans*, as has been documented in other organisms. Hence, in *C. albicans*, internal cleavage in a coding region can be used to create a complete gene deletion. Third, we found that CRISPR-generated homozygous mutants can arise through gene conversion events between alleles, an outcome that emphasizes the need for mutant phenotype validation with this system. We discuss each point in turn, and then summarize with a brief overview of *C. albicans* transient CRISPR system usage.

The fact that CRISPR components can be functionally expressed transiently has several useful implications. Because genomic integration is unnecessary for function, sgRNA gene cassettes can be synthesized through the single-joint PCR method (11), thus minimizing investigators’ time and expense. In addition, we envision that multiple sgRNA expression cassettes might be transformed together to enable rapid genomic targeting of several loci at once, as demonstrated for integrated constructs by Vyas et al. (3). We note that transient expression of CRISPR components may minimize off-target cleavage activity and associated toxicity (12, 13).

We suggest that transiently transforming DNA expression may have additional uses. For example, one could screen pooled *C. albicans* mutants for altered green fluorescent protein (GFP) reporter gene expression by cell sorting after transformation of a non-integrating GFP fusion gene. Also, one could assay for transient rescue of phenotypic defects in such properties as adherence, cell type switching, or mating with a suitable nonintegrating effector gene construct. Therefore, transient expression approaches may accelerate future functional analysis in *C. albicans*.

Our data indicate that CRISPR-targeted cleavage can occur far from sites of recombination, a result that is not surprising based on studies in yeast and mouse (7, 8). The finding is useful because it indicates that complete gene deletions are possible with the CRISPR system in *C. albicans*. Vyas et al. have defined unique sgRNA sequences computationally (3), with the goal of minimizing the potential for secondary-site cleavage, but not all of these ideal sgRNA sequences may lie in essential parts of coding regions. A complete gene deletion obviates the concern that a site mutation may cause only partial gene inactivation.

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**TABLE 2** Recovery of unselected markers among Ade⁺ transformants

| Experiment | Transformant selection | No. of red transformants by phenotype | Total no. of transformants |
|------------|-----------------------|--------------------------------------|---------------------------|
| 1          | Arg⁺                  | 17 3 NA 20 20                         | 20                        |
| 2          | Arg⁺                  | 15 8 NA 23 23                         | 23                        |
| 3          | Arg⁺                  | 26 15 NA 41 41                        | 41                        |
| 3          | Nat⁺                  | NA 20 15 5 20                         | 20                        |

*aade2Δ:ARG4 and ade2Δ:NAT deletion templates were mixed in a 1:1 molar ratio. BWP17 cells were transformed with the mixture along with the *CaCAS9* and sgRNA ADE2.1 PCR products. Transformants were selected through either an Arg⁺ or Nat⁺ phenotype, as indicated in the table. Red transformants were purified and scored for the unselected marker. The results of three independent experiments are shown.*

*bNA, not applicable because the selection did not allow recovery of the phenotype.*

NAT. The skewed ratio of selected to unselected markers is significantly different from the 1:2 ratio that is expected from model 1, according to a chi-square test (P value = 0.0001). These results indicate that homozygous mutations sometimes arise from two independent integration events, one at each allele (model 1), and sometimes arise from a single integration event at one allele, followed by gene conversion or crossing over to alter the second allele (model 2).
The fact that CRISPR-promoted homozygous mutations can occur through both independent integration events and single integration followed by allelic gene conversion has several implications for gene function analysis. Most importantly, the occurrence of allelic gene conversion may affect neighboring genes, and it will be prudent to validate homozygous mutant phenotypes. Such validation may be accomplished by complementation by the introduction of a wild-type copy of the gene of interest, as is currently the standard in the field. Complete complementation may not always be feasible, as in the case of multigene mutants. In such cases, the connection between genotype and phenotype is strengthened if several independent mutant isolates give consistent results. Fortunately, the CRISPR system facilitates the isolation of numerous homozygotes, so the construction of independent mutant isolates will be accelerated.

The transient CRISPR system for *C. albicans* (Fig. 2) may be used in the future as follows. The *CaCAS9* expression cassette may be PCR amplified from plasmid pV1093 (3). sgRNA expression cassettes may be designed for almost any gene with the sequences presented by Vyas et al. (3) and may be constructed through single-joint PCR (Fig. 3). Finally, mutant gene templates may be created through PCR amplification of a deletion-insertion allele (Fig. 2B). The use of auxotrophic *C. albicans* strains, such as BWP17 or SN152, will allow the use of nutritional markers, as well. Finally, transformation may be conducted with the unmarked *CaCAS9* and sgRNA gene PCR products and the deletion-insertion template PCR product, followed by selection for the relevant marker. We believe this transient CRISPR system will streamline gene function analysis in *C. albicans*.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *C. albicans* strains SC5314 (14), BWP17 (ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::hisGRhis1Δ::hisG arg4Δ::hisG/arg4Δ::hisG) (15), and a yh65 mutant derivative of strain SN152 referred to as Noble ORF_NegativeMutantCollection_Mutant no. 643 (Δ::imm434/Δ::imm434 his1Δ::hisGRhis1Δ::hisG arg4Δ::hisG/arg4Δ::hisG) (15), and a yh65 mutant derivative of strain SN152 were grown at 30°C in YPD medium.

**Plasmids/DNA.** To construct the pNAT plasmid, plasmid pCJN542 (16) was cut with SacI and SpeI to remove the *TDH3* promoter. The SacI-SpeI fragment containing the nourseothricin resistance cassette (NatR) was blunt-ended and self-ligated (17) to yield plasmid pNAT. The plasmid pV1093 used in this study was a kind gift from Valmik Vyas (3). We cloned the 20-bp guide sequence for *SNR52* from *C. dubliniensis* and *LEU2* from *Candida maltosa* (10) to yield pADE2-sgRNA. The *CaCAS9* expression cassette containing the *SNR52* promoter, guide sequence, and sgRNA scaffold sequence was assembled by the single-joint PCR method (11). In the first step, the *SNR52* promoter and sgRNA scaffold components were PCR amplified using both flanking primers and internal chimeric primers (Fig. 3). The chimeric primers overlapped by a 20-base segment that specified the guide sequence. In the second step, both components were joined by primer extension, relying upon annealing of the complementary chimeric primer extensions. In the third step, the joined product was PCR amplified with nested primers to yield the sgRNA cassette (Fig. 3). Gene deletion PCR constructs were synthesized using plasmid pNAT or pRS-ARG4 (15) or the *CdARG4* plasmid pSN105 (10), modified slightly, as the template. The primers were designed to include 80 bases with homology to the sequences upstream or downstream from the target gene (Fig. 2B). The oligonucleotides used in this study are listed in Table S1 in the supplemental material. PCR was conducted with Ex Taq in accordance with the manufacturer’s instructions (TaKaRa Bio, Inc.).

**Fungal transformation.** PCR products for transformation were purified and concentrated with the GeneJET PCR purification kit (Thermo Fisher Scientific, Inc.). In the original *C. albicans* CRISPR system, the deletion constructs (8 μg) were cotransformed with the CRISPR expression plasmid (5 μg), which had been linearized by digestion with KpnI and SacI (3). In the transient CRISPR system, the deletion constructs (3 μg) were cotransformed with the *CaCAS9* cassette (1 μg) and sgRNA cassette (1 μg), using the lithium acetate transformation method (18). The transformation frequency was calculated as the ratio of the number of cells that form colonies on selective medium divided by the number on nonselective YPD medium.
SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://dx.doi.org/10.1128/mSphere.00130-16.

Table S1, DOCX file, 0.1 MB.

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