Video Article

CRISPR-mediated Genome Editing of the Human Fungal Pathogen *Candida albicans*

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Abstract

This method describes the efficient CRISPR mediated genome editing of the diploid human fungal pathogen *Candida albicans*. CRISPR-mediated genome editing in *C. albicans* requires Cas9, guide RNA, and repair template. A plasmid expressing a yeast codon optimized Cas9 (CaCas9) has been generated. Guide sequences directly upstream from a PAM site (NGG) are cloned into the Cas9 expression vector. A repair template is then made by primer extension *in vitro*. Cotransformation of the repair template and vector into *C. albicans* leads to genome editing. Depending on the repair template used, the investigator can introduce nucleotide changes, insertions, or deletions. As *C. albicans* is a diploid, mutations are made in both alleles of a gene, provided that the A and B alleles do not harbor SNPs that interfere with guide targeting or repair template incorporation. Multimember gene families can be edited in parallel if suitable conserved sequences exist in all family members. The *C. albicans* CRISPR system described is flanked by FRT sites and encodes flippase. Upon induction of flippase, the antibiotic marker (CaCas9) and guide RNA are removed from the genome. This allows the investigator to perform subsequent edits to the genome. *C. albicans* CRISPR is a powerful fungal genetic engineering tool, and minor alterations to the described protocols permit the modification of other fungal species including *C. glabrata, N. castellii,* and *S. cerevisiae*.

Video Link

The video component of this article can be found at https://www.jove.com/video/58764/

Introduction

Candida albicans is the most prevalent human fungal pathogen[1,2,3]. Understanding differences between *C. albicans* and mammalian molecular biology is critical to development of the next generation of antifungal therapeutics. This requires investigators to be able to quickly and accurately genetically manipulate *C. albicans*.

Genetic manipulation of *C. albicans* has historically been challenging. *C. albicans* does not maintain plasmids, thus all constructs must be incorporated into the genome. Furthermore, *C. albicans* is diploid; therefore, when knocking out a gene or introducing a mutation, it is important to ensure that both copies have been changed[4]. In addition, some *C. albicans* loci are heterozygous, further complicating genetic interrogation[5]. To genetically manipulate *C. albicans*, it is typical to perform multiple rounds of homologous recombination[6]. However, the diploid nature of the genome and laborious construct development have made this a potentially tedious process, especially if multiple changes are required. These limitations and the medical importance of *C. albicans* demand the development of new technologies that enable investigators to more easily manipulate the *C. albicans* genome.

Clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing is a powerful tool that allows researchers to change the sequence of a genome. CRISPR requires three components: 1) the Cas9 nuclease that cleaves the target DNA, 2) a 20 base guide RNA that targets Cas9 to the sequence of interest, and 3) repair template DNA that repairs the cleavage site and incorporates the intended change[7,8]. Once the guide brings Cas9 to the target genome sequence, Cas9 requires a protospacer adjacent motif (PAM) sequence (NGG) directly upstream of the guide sequence to cleave the DNA[9]. The requirement for both the 20 base guide and PAM sequences provides a high degree of targeting specificity and limits off-target cleavage.

CRISPR systems have been designed to edit the genomes of a diverse set of organisms and tackle a wide variety of problems[10]. Described here is a flexible, efficient CRISPR protocol for editing a *C. albicans* gene of interest. The experiment introduces a stop codon to a gene, causing translation termination. Other edits can be made depending on the repair template that is introduced. A fragment marked with nourseothricin (Nat^r) containing yeast codon-optimized Cas9 (CaCas9) and a guide RNA is incorporated into the *C. albicans* genome at a neutral site. Cotransformation with the repair template encoding the desired mutation leads to repair of the cleavage by homologous recombination and efficient genome editing. Described below is the editing of *TPK2*, but all *C. albicans* open reading frames can be targeted multiple times by

CRISPR. The CRISPR system is flanked by FRT sites and can be removed from the *C. albicans* genome by induction of flippase encoded on the CaCas9 expression plasmid. The *C. albicans* CRISPR system enables investigators to accurately and quickly edit the *C. albicans* genome[11,12].

Protocol

1. Identification and Cloning of Guide RNA Sequence

1. **Identification of guide RNA sequence**
   1. Identify a 5'-NGG-3' PAM sequence close to where the stop codon will be inserted. (**Figure 1A**) Labeled are all PAM sequences found in the first 100 base pairs of *TPK2* (**Figure 1A**).
      NOTE: Guide sequences targeting each *C. albicans* open reading frame can be found at http://osf.io/ARDTX [11,12].
   2. Identify the Forward Guide Primer_3 sequence, which will be the 20 bases directly upstream of a NGG PAM site and will not contain more than 5 Ts in a row. Left-click on the base directly upstream of the NGG and drag the cursor 20 bases, then left-click on the primer tab to add the primer.
   3. Identify the Reverse Guide Primer_3 sequence, which will be the complement to the Forward Guide sequence.
      NOTE: Shown are guides that use PAM_3 (**Figure 1B**).
   4. Right-click the primer and select "copy primer data". Paste the sequences into a text editing program.

2. **Add overhang sequences to Forward and Reverse Guide oligos to facilitate cloning (Table 1, Figure 1B).**
   1. Add the nucleotide sequence ATTTG to the 5' end of the Forward Guide Primer_3 before purchasing.
   2. Add G to the 3' end of the Forward Guide Primer_3 before purchasing.
   3. Add the nucleotide sequence AAAAC to the 5' end of the Reverse Guide Primer_3 before purchasing.
   4. Add C to the 3' end of the Reverse Guide Primer_3 before purchasing.

3. **Digest CaCas9 expression vector pV1524 with BsmBI.**
   NOTE: pV1524 contains an ampicillin (Amp^r) and nourseothricin (Nat^r) markers. Cas9 has been codon-optimized for *C. albicans*.
   1. Digest the plasmid by adding: 2 µg of pv1524, 5 µL of 10x Buffer, 1 µL of BsmBI, and H_2O to 50 µL in a 1.5 mL tube. Incubate at 55 °C for 20 min. (Alternatively, digest pv1524 for 15 min with Esp3I, an isoschizomer of BsmBI, at 37 °C.)
   2. Cool to room temperature (RT) and spin for 30 s at 2,348 x *g* to bring condensation to the bottom of the tube. Proceed to step 1.4 or store the digested plasmid at -20 °C.

4. **Phosphatase-treat the digested backbone.**
   1. Add 1 µL of calf intestinal phosphatase (CIP) to the digestion mixture and incubate at 37 °C for 1 h.
   2. Purify the digested plasmid using a commercially available polymerase chain reaction (PCR) purification kit (instructions provided with kit) and elute it in 30 µL of elution buffer (EB).

5. **Phosphorylate and anneal Forward Guide Primer_3 and Reverse Guide Primer_3.**
   1. Add 0.5 µL of 100 µM Forward Guide Primer_3, 0.5 µL of 100 µM Reverse Guide Primer_3, 5 µL of 10x T4 ligase buffer, 1 µL of T4 polynucleotide kinase, and 43 µL of H_2O to a PCR tube.
   2. Add 5 µL of 10x T4 ligase buffer, 1 µL of T4 polynucleotide kinase, and 44 µL of molecular biology-grade H_2O in a second PCR tube.
      NOTE: This will serve as the negative control.
   3. Incubate the reaction mixtures in a thermocycler at 37 °C for 30 min, then at 95 °C for 5 min.
   4. Cool the mixture at the slowest ramp rate to 16 °C to anneal the ,oligos. Then place the annealed oligo mixture at 4 °C.

6. **Ligate the annealed oligos into digested pv1524 from step 1.4.3.**
   1. Add the following to a PCR tube: 1 µL of 10x T4 ligase buffer, 0.5 µL of T4 DNA ligase, 0.5 µL of annealed oligo mix, digested CIP-treated purified plasmid (20–40 ng), and H_2O to a 10 µL total volume.
   2. Add the following to a PCR tube: 1 µL of 10x T4 ligase buffer, 0.5 µL of T4 DNA ligase, digested CIP-treated purified vector (20–40 ng), 1 µL of negative control mixture, and H_2O up to a 10 µL total volume.
   3. Incubate both tubes in a thermocycler at 16 °C for 30 min, then at 65 °C for 10 min, then cool to 25 °C.

7. Transform 5 µL of the ligation mixtures into chemically competent *Escherichia coli* DH5α using a standard heat shock transformation protocol. Select on LB Amp/Nat media (200 µg/mL Amp, 50 µg/mL Nat).
   NOTE: Failure to select pV1524 and its derivatives on double selection media will result in loss of Nat/CaCas9/guide module by FLP/FRT excision in bacteria.

8. Purify plasmids from four transformants by miniprep, and sequence the insertion sequence with sequencing primer (**Table 1**).
   Note: Most of the time, sequencing 4 transformants is sufficient to identify at least 1 correct clone.

9. Save plasmids that have the guide RNA sequence cloned a single time into the BsmBI cut site at -20 °C.

2. Designing and Generation of Repair Template

1. Insert a stop codon by left-clicking in the gene sequence and adding nucleotides that encode a stop codon and restriction digestion site (**Figure 1C, Table 1**).
   NOTE: The insertion will disrupt the PAM sequence.
   NOTE: A restriction digestion site will be included in the repair template sequence to facilitate efficient screening of clones (**Figure 1C**).
2. Left-click 10 bases downstream of where the mutation will be made and drag the cursor 60 bases upstream. Left-click on the primer tab to add the primer. This will add Repair Template Forward_3. Left-click 10 bases upstream of where the mutation will be made and drag the cursor 60 bases downstream. Left-click on the primer tab to add the primer. This will add Repair Template Reverse 3. (**Figure 1C**)

3. **Perform primer extension to generate the repair template.**
   1. Add 1.2 µL of 100 µm repair template forward primer, 1.2 µL of 100 µm repair template reverse primer, 6 µL of deoxynucleotide triphosphates (dNTPs) (total concentration 40 mM), 6 µL of buffer, 0.6 µL of Taq polymerase (3 units), and 45 µL of H_2O to each of the 4 PCR tubes.
   2. Perform primer extension by running between 20 and 30 rounds of PCR. Example extension conditions: 2 min at 95 °C, (30 s at 95 °C, 1 min at 50 °C, 1 min at 68 °C) x 34, 10 min at 68 °C.
   3. Combine contents of all 4 PCR tubes in a 1.5 mL tube and use a PCR purification kit to purify products in 50 µL of H_2O.
   4. Quantitate the primer extension products to ensure sufficient DNA by determining the absorbance at 260 nm.
      NOTE: Typical final concentration of the primer extension product is ~200–300 ng/µL.

3. Transformation of *C. albicans* with Repair Template and Plasmid

1. **Make TE/lithium acetate.**
   1. Mix 10 mM Tris-Cl, 1 mM EDTA, 100 mM lithium acetate, and H_2O (all stock solution pH 7.5) to achieve 50 mL total volume.

2. **Make PLATE**
   1. Mix 40% PEG 3350, 100 mM lithium acetate, 10 mM Tris-Cl, 1 mM EDTA, and H_2O (all stock solution pH 7.5) to achieve 50 mL total volume.

3. **Digest correctly-cloned plasmids from step 1.9.**
   1. Add 10 µg of plasmid, 4 µL of 10x buffer, 0.4 µL of 10 mg/mL bovine serum albumin (BSA), 0.5 µL of KpnI, 0.5 µL of SacI, and H_2O to 40 µL total volume in a 1.5 mL tube. Incubate at 37 °C overnight (**Figure 1D**).

4. **Grow an overnight culture of *C. albicans* SC5314, wild-type prototroph, at 25 °C in yeast peptone dextrose supplemented with 0.27 mM Uridine (YPD + Uri), ideally to OD$_{600}$ less than 6.**
   1. Pellet 5 OD$_{600}$ units of cells per transformation by spinning for 5 min at 2,348 x *g* and suspend the 5 OD$_{600}$ of pelleted cells in 100 µL TE/lithium acetate.

5. Add the following to a 1.5 mL tube in the order listed: 1) 100 µL of cells from step 3.4.1, 2) 40 µL of boiled and quick-cooled salmon sperm DNA (10 mg/mL), 3) 10 µg of plasmid digestion from step 3.3.1, 4) 6 µg of purified repair template, and 5) 1 mL of PLATE.
6. Add the following to a 1.5 mL tube in the order listed: 1) 100 µL of cells, 2) 40 µL of boiled and quick-cooled salmon sperm DNA (10 mg/mL), 3) H_2O volume equal to that of transforming DNA in step 3.5, and 4) 1 mL of PLATE.
   Note: This will serve as a negative control.
7. Mix the transformations gently by pipetting and let incubate at 25 °C overnight.
8. Heat shock the cells by placing them in a 44 °C water bath for 25 min.
9. Spin for 5 min at 2,348 x *g* in a benchtop centrifuge and remove the PLATE mixture supernatant. Wash once by adding 1 mL of YPD + Uri and centrifuge again for 5 min at 2,348 x *g*.
10. Suspend the cells in 0.1 mL of YPD + Uri and incubate on a roller drum or shaker at 25 °C overnight.
11. Plate on YPD + Uri with 200 µg/mL nourseothricin. Colonies will appear in 2–5 days.

4. Streaks for Single Colonies

1. **Divide a 100 mm x 15 mm Petri dish into quarters, and label each quadrant.**
   1. Touch one of the colonies from the transformation plate with a sterile toothpick or applicator and streak across the longest side of the quadrant.
   2. Streak for single colonies using an aseptic technique and allow the colonies to grow at 30 °C for 2 days.

5. Colony PCR

1. **Design the forward check primer (FCP) ~200 base pairs upstream of the restriction site that was introduced and the reverse check primer (RCP) ~300 base pairs downstream.**
   1. Add 0.3 µL of FCP, 0.3 µL of RCP, 0.3 µL of thermostable polymerase (ExTaq 1.5 units), 3 µL of dNTPs (total concentration 40 mM), 3 µL of ExTaq Buffer, and 23 µL of H_2O to a 1.5 mL tube.
      NOTE: Addition of 0.5 µL/reaction dimethyl sulfoxide (DMSO) can improve PCR efficiency.
   2. Add 0.25 µL of a single yeast colony from step 4.1.2 to the mixture from step 5.1.1, using a P10 pipette tip and taking care not to disturb the agar.
   3. Amplify DNA by PCR and run 5 µL of the PCR on a gel to ensure amplification is successful, taking care not to disturb the cell pellet at the bottom of the tube.

6. Restriction Digestion of Colony PCR

1. Add 10 µL of PCR product (taking care not to not disturb the cell pellet at the bottom of the tube), 3 µL of buffer, 1 µL of restriction enzyme, and 16 µL of H_2O, then incubate according to manufacturer's instructions and resolve on an agarose gel to identify correct genome edits.
   NOTE: The restriction enzyme used here is the site encoded in the *TPK2* specific repair template.

7. Saving Strains

1. Grow an overnight culture from colonies that have been confirmed by restriction digestion in YPD + Uri at 30 °C.
2. Add 1 mL of culture and 1 mL of 50% glycerol (bringing the final concentration of glycerol to 25%) to a tube suitable for storage at -80 °C.
3. Store the correct clones at -80 °C.
   NOTE: Correct strains can be stored at -80 °C for many years.

8. Removal of Nat[r] Marker

1. Streak correct transformant onto yeast peptone maltose (YPMaltose) (2% maltose).
2. Pick a colony from the streaked plate and culture the yeast for 48 h at 30 °C in liquid YPMaltose 20 g/L maltose.
3. Plate 200–400 cells on YPMaltose 20 g/L maltose and incubate at 30 °C for 24 h.
4. Replicate the plate onto YPMaltose and YPMaltose 200 µg/mL Nat.
5. Incubate at 30 °C for 24 h.
   NOTE: Colonies that no longer grow on YPMaltose 200 µg/mL nourseothricin but grow on YPMaltose have lost the Nat[r] marker *(CaCAS9)* and guide RNA.
6. Save the strains that have lost the Nat[r] marker *(CaCAS9)* and guide RNA as done in steps 7.1–7.3.
   NOTE: A similar plasmid, pV1393, uses the *SAP2* as opposed to a *MAL2* promotor. Growth on YCB–BSA will induce flippase and removal of Nat[r] if pV1393 is used for gene editing.

Representative Results

Sequences of guide RNAs and repair templates that target *C. albicans TPK2,* a c-AMP kinase catalytic subunit, were designed according to the guidelines suggested above. Sequences are shown in (**Table 1, Figure 1**). Guide RNAs were cloned into CaCas9 expression vectors and cotransformed with repair template in wild-type *C. albicans*. An *Eco*RI restriction digestion site and stop codons in the repair template disrupt the PAM site and facilitate screening for correct mutants (**Figure 1**). Transformants were streaked for single colonies and screened by colony PCR and restriction digestion for incorporation of the repair template **(Figure 2)**. Restriction digestion quickly distinguishes wild-type from mutant sequences.

Oligonucleotide Name	Oligonucleotide Sequence
Forward Guide Primer_3	**ATTTG**gggtgaactatttgttcgcc**G**
Reverse Guide Primer_3	**AAAAC**ggcgaacaaatagttcaccc**C**
Repair Template Forward_3	tcagcaatatcagcaacaatttcaacaaccgcagcaacaactttat**TAAGAATTC**ggcga
Repair Template Reverse_3	attttgtccagtttgggctgcagcagggtgaactatttgttcgcc**GAATTCTTA**ataaag
Forward Check Primer	ttaaagaaacttcacatcaccaag
Reverse Check Primer	actttgatagcataatatctaccat
Sequencing Primer	ggcatagctgaaacttcggccc

Table 1: List of oligo nucleotides used for this study. Sites added for cloning purposes are capitalized and bolded in the guide primer sequences. Sequences in the repair template that mutate the genomic DNA are capitalized and bolded.
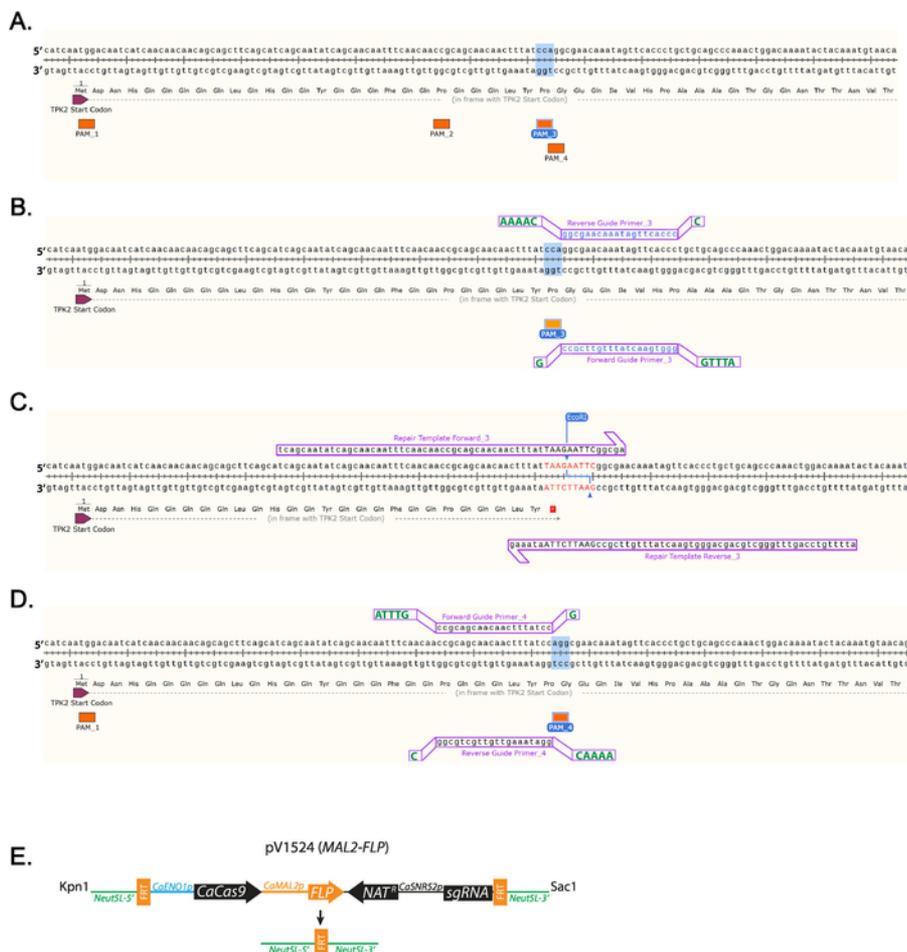
Figure 1: Diagram of guide RNA and repair template design. (**A**) Labeling of all the PAM sequences in the first 100 nucleotides of *TPK2*. PAM sequence 3 (PAM_3) is highlighted, as that is the sequence used in this study. (**B**) Guide RNA design using PAM_3. 20 base primers designed using SnapGene are lowercase and blue. Additional bases required for cloning are uppercase and green shown offset. (**C**) Repair template primers that insert a TAA stop codon and *Eco*RI site are inserted into the *TPK2* reading frame. DNA that differs from the wild-type sequence is red and uppercase. (**D**) Example of how a guide is designed on the positive strand of DNA using PAM_4. (**E**) Schematic diagram of pV1524 after cloning of the guide RNA and digestion with KpnI and SacI. Neut5L-5' and Neut5L-3' target the vector to the Neut5L site in the *C. albicans* genome. *CaENO1p* is the promotor that drives expression of the yeast-optimized *CaCas9*. Nat[r] is the nourseothricin resistance cassette. CaSNR52p is the promotor driving guide RNA expression (sgRNA). FRT sites are cleaved and recombined by flippase (FLP) removing the CRISPR cassette upon flippase expression. A schematic similar to (E) was published by Vyas *et al*.[11]. Please click here to view a larger version of this figure.
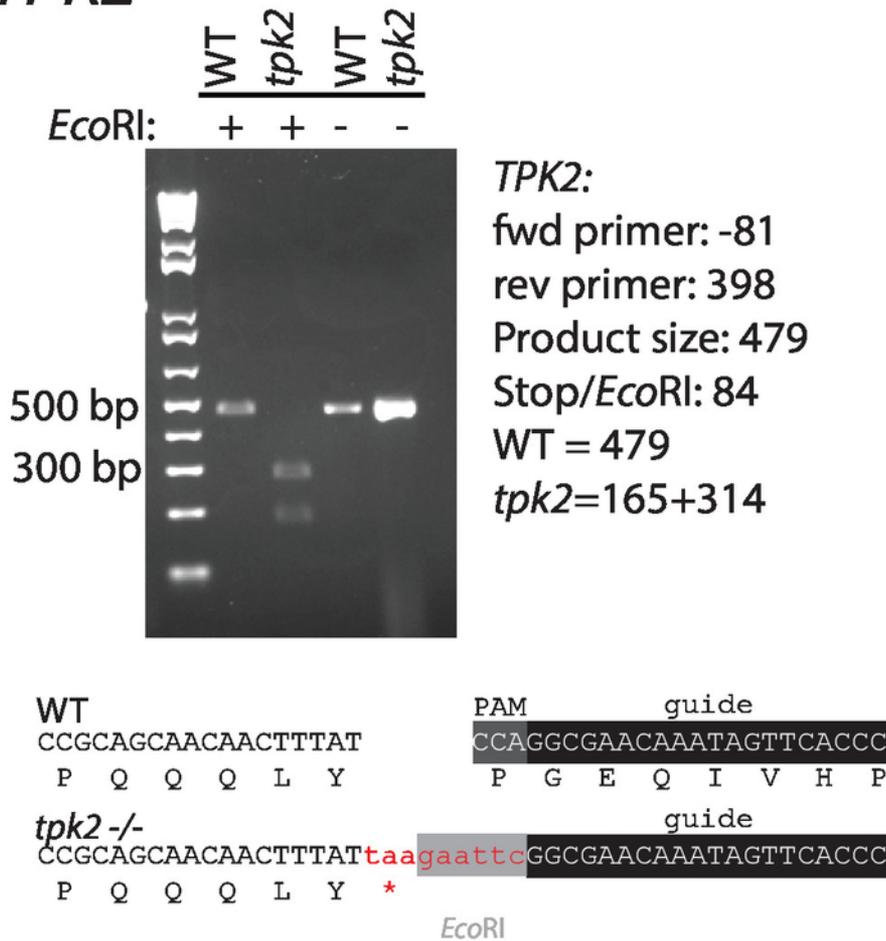
Figure 2: Introduction and confirmation of a stop codon and *Eco*RI restriction site to *TPK2*. Primers used for amplification are listed in **Table 1**. Wild-type and mutant sequences are shown below the gel. This figure has been modified from Vyas et al.[11]. Please click here to view a larger version of this figure.
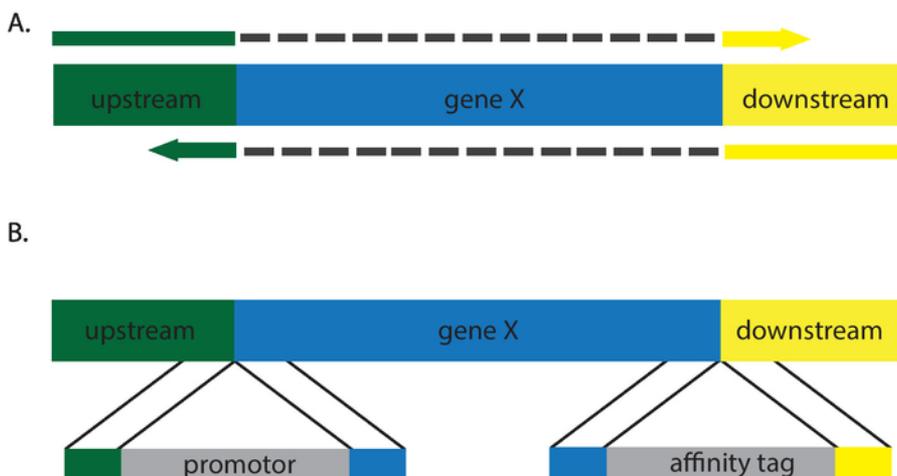
Figure 3: Cartoon description of repair templates that will generate (A) deletions and (B) insertions. Grey dashes in (A) depict intervening sequences not present in the repair template primers. Please click here to view a larger version of this figure.

Discussion

C. albicans CRISPR efficiently edits the *C. albicans* genome. pV1524 encodes a yeast codon-optimized Cas9 and is designed such that investigators can easily clone guide RNA sequences downstream of the *CaSNR52* promoter (**Figure 1**)[11]. It must be ensured that only a single

copy of the guide sequence has been cloned into CaCas9 expression vectors by sequencing, as extra copies will impede genome editing. If multiple copies of the guide are introduced consistently, one should lower the concentration of the annealed guide used in ligation. The vector and protocol described allow targeting of any *C. albicans* gene. Although *C. albicans* is diploid, only a single transformation is required to target both alleles of a gene. Furthermore, the processive nature of CRISPR-CaCas9 genome editing enables researchers to target multiple members of gene families. Many gene families such as the secreted aspartyl proteases (SAPS) and agglutinin-like sequence proteins (ALS) are important for *C. albicans* virulence. CRISPR genome editing will facilitate investigation of these gene families.

The protocols described above introduce a stop codon to an open reading frame, resulting in the phenotypic equivalent of a null (**Figure 2**). A wide variety of genetic alternations can be made by varying the repair template. Nonsense, missense, and silent mutations can be inserted via recombination with an appropriate repair template. Incorporation of a restriction site streamlines transformant screening, as those without must be screened by sequencing[12,13]. In addition, *C. albicans* CRISPR enables researchers to generate insertions and deletions, making it an ideal system to insert affinity tags, perform promotor swaps, and generate knockouts (**Figure 3**). Screening for correct transformants for these mutations is more laborious, as it is necessary to sequence the edits to confirm correct incorporation of the repair templates. Furthermore, Southern blot may be necessary to ensure additional copies of a gene have not been inserted at additional locations in the genome. The requirement of the NGG PAM site places slight limitations on the regions of the genome that can be targeted. The development of alternative CRISPR systems that use alternative nucleases such as Cpf1 or variations on the Cas9 system have/will alleviate many of these limitations[14]. To the investigators' knowledge at this time, these systems have not yet been applied to *C. albicans*.

The CRISPR system described in the above protocol has been developed such that it can be applied in a wide variety of species including *Saccharomyces cerevisiae*, *Naumouozyma castellii*, and the human pathogen *Candida glabrata*[11]. Transformation and efficient editing of these yeast requires slight changes to the described protocol, but the framework for editing these alternate genomes is remarkably similar to that described for *C. albicans*[12]. Furthermore, yeast provide an excellent mechanism to develop genome editing procedures. In yeast, when *ADE2* is mutated, a precursor to the adenine biosynthesis pathway accumulates, turning the cells red. This easily observable phenotype allows investigators to identify edited cells and quickly troubleshoot genome editing protocols. Combined with the extensive molecular biology toolbox available for fungi, protocols for editing numerous yeast species have been developed[15,16]. Such a broad application of genome editing technology in fungi has the potential to significantly impact a wide variety of scientific disciplines.

CRISPR has greatly improved the efficiency of genome engineering in *C. albicans*, but to date CRISPR has not been used to perform genome wide screens in *C. albicans*. Current protocols require a repair template to introduce mutations, as the nonhomologous end joining pathway in *C. albicans* is inefficient[12]. The generation of repair template oligos for every gene is a significant barrier to the execution of genome-wide screens. The confluence of decreased costs of DNA synthesis and advances in CRISPR technologies will make development of deletion libraries more feasible. For instance, expression of a repair template from the CaCas9 vector paves the way for the development of sustainable plasmid libraries that target every gene[11]. Furthermore, transient *Candida* CRISPR protocols that do not require CaCas9 expression vector incorporate into the *C. albicans* genome have been developed[17]. In addition, increased guide expression increases genome editing efficiency[18]. These, and other advances to CRISPR technologies, are crucial to the development of genome-wide screens in *C. albicans*[19,20,21,22].

The *C. albicans* genome is diploid, but A and B alleles are not always identical[5]. Such heterozygosity provides both challenges and opportunities. If one aims to target both alleles, a PAM site, guide sequence, and repair template that will act on both copies of the gene must be used. However, depending upon single nucleotide polymorphisms present in a gene, the *C.albicans* CRISPR system enables investigators to target a single allele. Such precision has the potential to allow investigators to examine functional differences between alleles. Targeting specific alleles must be done carefully, as loss of heterozygosity (LOH) at an allele or of an entire chromosome has been observed. When editing single *C. albicans* alleles, one must examine adjacent DNA sequences to determine if a clone has maintained a diploid SNP profile. In addition, off-target effects are quite low for *C. albicans* CRISPR, but whole genome sequencing can be considered for key strains.

Disclosures

The authors have nothing to disclose.

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