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Genome-Wide Synthetic Genetic Screening by Transposon Mutagenesis in *Candida albicans*

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Abstract

Transposon-based mutagenesis is an effective method for genetic screening on a genome-wide scale, with particular applicability in organisms possessing compact genomes where transforming DNA tends to integrate by homologous recombination. Methods for transposon mutagenesis have been applied with great success in the budding yeast *Saccharomyces cerevisiae* and in the related pathogenic yeast *Candida albicans*. In *C. albicans*, we have implemented transposon mutagenesis to generate heterozygous mutations for the analysis of complex haploinsufficiency, a type of synthetic genetic interaction wherein a pair of non-complementing heterozygous mutations results in a stronger phenotype than either individual mutation in isolation. Genes exhibiting complex haploinsufficiency typically function within a regulatory pathway, in parallel pathways, or in parallel branches within a single pathway. Here, we present protocols to implement transposon mutagenesis for complex haploinsufficiency screening in *C. albicans*, indicating methods for transposon construction, mutagenesis, phenotypic screening, and identification of insertion sites in strains of interest. In total, the approach is a useful means to implement large-scale synthetic genetic screening in the diploid *C. albicans*.

Keywords

Transposon; Insertional mutagenesis; Transposon mutagenesis; Shuttle mutagenesis; Complex haploinsufficiency; Synthetic genetic analysis; Hyphal development; *Candida albicans*

1 Introduction

Candida albicans is the principal opportunistic human fungal pathogen, and its ability to transition between a non-filamentous yeast-like state and filamentous pseudohyphal and hyphal states is required for its pathogenicity [1, 2]. This process of hyphal development has been studied intensely, and genomic approaches have been applied with growing frequency to the study of hyphal development in *C. albicans*. Targeted gene replacement approaches have been implemented to generate heterozygous and homozygous diploid null mutants for sets of *Candida* genes. As the result of numerous independent projects, roughly half of the predicted genes in the *C. albicans* genome have been disrupted through either heterozygous or homozygous mutations [3–6]. Ongoing studies will likely expand this mutant collection significantly in the near future.

In *S. cerevisiae*, pairs of deletion mutants have been analyzed informatively through synthetic genetic studies, most commonly in the form of synthetic lethality screens. Synthetic lethality occurs when pairs of mutations in nonessential genes yield a cumulative loss of cell viability. The Boone laboratory has pioneered large-scale synthetic lethality screening in budding yeast [7, 8], implementing a clever approach for constructing haploid strains with loss-of-function mutations in pairs of genes by mating pairs of single-gene deletion strains and, after sporulation of the mated strains, selecting for meiotic progeny carrying both mutations. Synthetic genetic screening with this approach, termed synthetic genetic analysis (SGA), has been used to dissect regulatory networks and signaling pathways essential for yeast cell growth [7].

While SGA-based approaches have demonstrated the power in synthetic genetic screening, this exact methodology cannot be easily implemented in *C. albicans*, as the fungus lacks a complete sexual cycle with a viable haploid stage. Pairs of independent homozygous loss-of-function mutations can be constructed in diploid *C. albicans*; however, the workload is substantial, as transforming DNA tends to integrate by homologous recombination less efficiently in *C. albicans*. Whereas, 40 bases of homology is sufficient to direct transforming DNA for integration at native loci in *S. cerevisiae*, flanking regions of 80 bases or more are typically required for targeted gene replacement in *C. albicans* [9].

A modified form of synthetic genetic screening, complex haploinsufficiency (CHI), does lend itself well to the analysis of synthetic phenotypes in *C. albicans*. CHI occurs when a strain containing two heterozygous mutations at distinct loci exhibit a synthetic phenotype more severe than the phenotype of either single mutation in isolation (Fig. 1). Pairs of genes identified by CHI are likely components of a shared pathway or are components of interacting/parallel pathways. CHI studies have been implemented previously as dominant modifier screens in *D. melanogaster* and as screens for unlinked non-complementation in *S. cerevisiae* [10, 11]. In pioneering work, Haarer et al. [12] utilized CHI to identify 208 genes that exhibited deleterious synthetic genetic interaction with yeast actin. Relevant to analysis of *C. albicans* biology, double heterozygous mutants can be constructed easily for CHI analysis without the requirement for a viable haploid.

In Bharucha et al. [13], we implemented the first CHI screen in *C. albicans*, utilizing complex haploinsufficiency to identify genetic interactions with the Regulation of Ace2p and Morphogenesis (RAM) pathway, a signaling network required for wild-type hyphal development. For this analysis, we constructed double heterozygous mutants by random transposon mutagenesis of a strain already heterozygous for deletion of *CBK1*. The *CBK1* gene encodes a kinase of the Ndr/Lats family that is a central component of the RAM pathway [14, 15]. The *cbk1* /*CBK1* mutant is haploinsufficient for hyphal development; on solid Spider medium, which is nitrogen- and carbon-poor, *cbk1* /*CBK1* colonies exhibit decreased hyphae, decreased central wrinkling, and an expanded zone of peripheral pseudohyphae [14, 16]. Following transposon mutagenesis, *cbk1* /*CBK1* double heterozygotes were screened for decreased central colony wrinkling and a decreased zone of peripheral filamentation. By this approach, we identified 41 unique genes that interacted by CHI with *CBK1* [13].

Here, we present methods for the implementation of a CHI screen in *C. albicans*. An overview of the steps in this process is presented in Fig. 2. To apply CHI, we constructed a transposon suitable for mutagenesis of the *C. albicans* genome through modification of a Tn7-derived bacterial transposon. This modified transposon was used for shuttle mutagenesis of a plasmid-based *C. albicans* genomic DNA library. Resulting transposon-mutagenized genomic DNA inserts were excised by restriction enzyme digestion, and the insertion alleles were introduced into the desired *C. albicans* mutant strain by DNA transformation. Double heterozygous mutants were screened for phenotypes of interest, and the site of insertion was identified in selected strains. In total, the protocols here provide the necessary information to apply CHI screening for the analysis of synthetic genetic interactions with essential or nonessential *C. albicans* genes.

2 Materials

1. pGPS3 transposon donor plasmid (New England Biolabs, Ipswich, MA), customized, or available by request.
2. Genomic DNA library for mutagenesis.
3. 10× Tn7 mutagenesis buffer: 250 mM Tris-HCl (pH 8.0), 20 mM ATP, 20 mM DTT (Dithiothreitol).
4. 300 mM magnesium acetate.
5. 3 M sodium acetate.
6. 1 M lithium acetate, sterile, (autoclaved).
7. TnsABC* Transposase: 7 µg/ml TnsA*, 10 µg/ml TnsB*, 20 µg/ml TnsC* in buffer containing 25 mM Tris-HCl (pH 7.9), 500 mM NaCl, 2 mM MgCl₂, 1 mM ATP, 0.5 mM DTT, 0.8 mM EDTA, and 50 % Glycerol (obtained from Nancy Craig's lab, Johns Hopkins University).
8. Restriction endonucleases: *Spe* I, *Pi*-SceI (VDE I), *Pvu* II with supplied buffers (New England Biolabs, Ipswich, MA or alternative).
9. ElectroMAXTM Stbl4TM cells (Invitrogen, Carlsbad, CA) or any library-efficient competent cells.
10. 95–100 % ethanol; 70 % ethanol.
11. 10× TE: 100 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0.
12. TE: 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0.
13. 15 % glycerol, sterile (autoclaved).
14. Maxiprep plasmid isolation kit (Qiagen, Valencia, CA).
15. 10 mg/ml sonicated salmon sperm DNA; 10 mg/ml yeast tRNA.
16. 1 M LiAc.

17. TE-LiAC mix, sterile: 1 volume 10× TE, 1 volume 1 M LiAc, 8 volumes water.
18. 50 % PEG (Polyethylene glycol, MW 3350): Filter-sterilized.
19. PEG-LiAC-TE mix: 8 volumes 50%PEG, 1 volume 10XTE, 1 volume 1 M LiAc.
20. 25 mg/ml kanamycin: Store at 4 °C for up to 3 months.
21. 50 mg/ml ampicillin: Store at 4 °C for up to 3 months.
22. LB plates with 40 mg/l kanamycin and 50 mg/l ampicillin.
23. YPD + uridine medium, sterile: 10 g yeast extract, 20 g Bacto peptone, 20 g dextrose, 80 mg uridine in 1 l water. Autoclave.
24. YPD + uridine plates, sterile: YPD + uridine medium containing 15 g agar in 1 l water. Autoclave.
25. Spider medium, sterile: 10 g nutrient broth, 10 g mannitol, 2 g K₂ PO₄, 13.5 g agar in 1 l water, pH 7.2 after autoclaving.
26. RNA extraction kit (Qiagen, Valencia, CA).
27. 0.1 M DTT (Dithiothreitol).
28. 100 mM dNTP mix: 25 mM with respect to each dNTP.
29. Ribonuclease inhibitor (40 U/μl).
30. Standard lab equipment: tabletop centrifuge, microcentrifuge, 45 °C water bath, 25 and 30 °C shaking incubator, 30 °C incubator, 65 and 75 °C heat block, agarose gel equipment.
31. Phenol–chloroform (1:1): Equilibrated with 0.1 M Tris–HCl (pH 7.6).
32. Breaking buffer: 2 % Triton X-100; 1 % SDS; 100 mM NaCl; 100 mM Tris–HCl; 1 mM EDTA, pH 8 (filter sterilized).
33. 10 mg/mL RNaseA: store at –20 °C for up to 6 months.

3 Methods

The methods presented here describe the (1) design and application of multipurpose bacterial transposons for mutagenesis of *C. albicans* DNA, (2) introduction of transposon insertion libraries into *C. albicans* for functional analysis, (3) selection of an appropriate *C. albicans* background strain for complex haploinsufficiency screening, (4) phenotypic screening of mutagenized *C. albicans*, and (5) identification of transposon insertion sites in mutagenized *C. albicans* strains.

3.1 Generating a Transposon Mutagenized *C. albicans* Genomic DNA Library

Transposon-mediated gene disruption is an advantageous method for large-scale mutagenesis in *C. albicans*. Typically, genome-wide transposon insertions are generated via

shuttle mutagenesis using a modified bacterial transposon; by this approach, a *C. albicans* genomic DNA library is mutagenized in vitro or in *E. coli*, and insertion alleles are subsequently introduced into *C. albicans* by DNA transformation. We have previously constructed transposons for mutagenesis of *S. cerevisiae* and *C. albicans* DNA from the bacterial transposons Tn3 and Tn7 [17–21]. The Tn7 system has been adapted for use *in vitro* by Nancy Craig's group at Johns Hopkins University and provides better genomic coverage. Statistical analysis of the Tn3 and Tn7 insertion sites indicates that Tn7 has a less-prominent bias in target site selection than Tn3 [22]. For this reason, this chapter presents protocols for the use of a Tn7-based library.

The Tn7 transposon construct contains functional elements that aid in both mutagenesis and downstream applications. In this protocol a modified pGPS3 plasmid carrying Tn7 is used (see Note 1). The transposable element is bounded by end sequences which act as substrates for the recombination proteins mediating Tn7 transposition. The Tn7 transposon carries the kanamycin and *URA3* genes to enable selection in *E. coli* and *C. albicans*, respectively. Though both homozygous and heterozygous mutants can be constructed, the latter is described here for the purpose of discerning genetic interactions in a CHI screen.

The Tn7 transposon system can be used to generate a library of mutants covering the majority of the *C. albicans* genome. Three transposase proteins, TnsA, TnsB, and modified TnsC, facilitate this transposition reaction. TnsB binds to the Tn7 sequence; TnsC binds the target DNA, and TnsA binds the TnsB-DNA complex [23, 24]. The modification to the TnsC protein greatly diminishes sequence specificity, allowing for broader coverage of the genome. Additionally, only one transposon insertion occurs within a single DNA molecule, making double insertions within a single DNA fragment in the genomic library unlikely [25].

The modified Tn7 transposon contains the *C. albicans* *URA3-dpl200* cassette, which enables gene replacement via homologous recombination and counterselection with 5-FOA [26]. The *C. albicans* WO-1 pEMBly23 genomic library was mutagenized with the customized donor plasmid containing the Tn7 transposon. This library was generated by insertion of the partial BamHI–HindII digestion of WO-1 into pEMBly23 (see Note 2). The protocol for transposon mutagenesis of the *C. albicans* genome follows.

1. 80 ng of a genomic DNA library derived from the *Candida albicans* strain WO-1 in pEMBLY23 is combined with 20 ng of the customized donor plasmid in a total reaction volume of 20 µl containing 1× TN7 mutagenesis buffer and 1 µl of the transposase TnsABC. The mixture is incubated at 37 °C for 10 min.
2. 1 µl of magnesium acetate (300 mM stock concentration) is then added, and the mixture is further incubated for 1 h at 37 °C, followed by heat inactivation for 10 min at 75 °C.

¹In place of the *URA3-dpl200* cassette, other markers may be used for selection in *C. albicans*, particularly to avoid position-specific effects caused by *URA3*. The customized donor plasmid with the customized *URA3-dpl200* cassette is available upon request.

²This library is available from the NIH AIDS Research and Reference Reagent Program.

3. Digest with the restriction enzyme *PI-SceI* for 3 h at 37 °C to destroy any unreacted donor plasmid that might remain in the mix.
4. The mixture is then subjected to phenol extraction as follows. Add 100 µl phenol–chloroform mix and subject to centrifugation for 5 min at 12,750×*g* in a microfuge. The mixture will separate into two layers. Remove the upper layer carefully and transfer to a clean microcentrifuge tube containing 250 µl 100 % Ethanol, 10 µl NaAc (3 M stock concentration), and 0.5 µl tRNA (10 mg/ml stock concentration). Keep at –80 °C for a minimum of 30 min (or –20 °C for 1 h). Spin at 12,750×*g* for 30 min at 4 °C and subsequently add 50 µl 70–80 % ethanol. Spin for 10 min at 12,750×*g* and resuspend in 30 µl 1× TE.
5. The mixture is then diluted tenfold, and 10 µl of the dilution is transformed by electroporation into ElectroMAX Stbl4 *E. coli* cells (Invitrogen). Transformants are plated on LB + ampicillin + kanamycin plates and incubated at 30 °C for 2 days.
6. Multiple mutagenesis reactions are performed to allow maximum coverage (see Note 3). Cells from each mutagenesis reaction are harvested and stored in 15 % glycerol. Plasmids are recovered using high-efficiency alkaline lysis (Maxiprep kit by Qiagen, Valencia, CA) for subsequent yeast transformation.

3.2 Introducing the Transposon Insertion Alleles into the Desired *C. albicans* Genetic Background

Steps for introducing the mutagenized library into the appropriate strain of *C. albicans* are described below. In order to carry out a CHI screen for genetic interactions with a gene of interest the background strain must be heterozygous for that gene.

1. Digest 6 µg plasmid DNA (recovered from the mutagenesis reaction above) with *Pvu* II to release the genomic DNA fragments (see Note 4). Analyze a small fraction of this digest on an agarose gel to ensure that the digestion is complete. Subject the rest of the digest to phenol extraction and elute in 25 µl TE buffer (see Note 5).
2. Grow a 5 ml culture of the desired *C. albicans* strain in appropriate medium such as YPD + uridine overnight (39). Add 100–500 µl of this overnight culture to 50 ml YPD + uridine to bring the culture to an OD600 of 0.1–0.2. Incubate at 30 °C with shaking for approximately 5 h until the culture reaches mid-log phase (OD600 of approximately 1).
3. Pellet the cells at 3,000 rpm (approximately 1,400×*g*) for 5 min. Wash with 30 ml sterile water. Resuspend the cell pellet in 500 µl TE-LiAc.

³From past experience, we recommend carrying out a total of nine or more independent reactions to improve coverage of the target genomic DNA library.

⁴A larger amount (2–6 µg) of the plasmid library DNA is used for each transformation reaction to ensure sufficient number of transformants per reaction.

⁵This step is essential to purify and concentrate the DNA in a smaller volume for transformation.

4. Add 10 µl of salmon sperm DNA (10 mg/ml stock concentration) to two sterile microcentrifuge tubes. Add the entire volume of phenol-extracted DNA digest (25 µg) to one of the tubes and mix gently with a pipette tip. Add the same volume of sterile water or elution buffer to the other tube and mix; this will serve as a negative control. Add 100 µl of the resuspended cells to the microcentrifuge tubes and mix gently. Incubate the tubes at room temperature for 30 min.
5. Add 700 µl PEG-LiAC-TE mix to each tube and mix by inversion. Incubate at room temperature in a shaking water bath overnight.
6. Heat-shock the cells by incubating in a 42 °C water bath for 1 h (*see* ^{Note 6}).
7. Pellet the cells at 3,000 rpm (1,400×*g*) for 3 min. Add 150 µl sterile water to resuspend the pellet and plate on selective medium (SC-Ura in this case). Incubate the plates at 30 °C for 2–3 days (*see* ^{Note 7}).
8. Freeze transformants in 15 % glycerol in 96-well plates.

3.3 Sample Phenotypic Screen

The resulting transposon mutant strains can be used for numerous phenotypic screens. Here we describe a protocol to screen for hyphal growth defects relative to the parent strain chosen for mutagenesis.

1. Dispense 600 µl selective medium (SC-Ura in this case) in 96-well culture plates and inoculate with a small fraction of the pure colony in the individual wells.
2. Allow strains to grow for approximately 24 h in a 30 °C shaking incubator.
3. Using a hand-pinning tool (or multichannel pipettor), dispense a small amount (1–2 µl) onto the desired plates to be used for the phenotypic screen (e.g., plates containing 10 % serum medium for the analysis of hyphal growth phenotypes).
4. Incubate the plates at 37 °C for 3 days.
5. Colonies with altered hyphal growth relative to the starting strain are scored as positive. These strains should be retested to confirm the phenotype (*see* ^{Note 8}).

⁶The heat shock step may be performed at 44 °C for 20 min instead of 42 °C for 1 h. The optimal heat shock length should be determined empirically if initial results are inadequate.

⁷In order to obtain pure colonies of transformants, it is advisable to restreak transformants onto fresh plates before any further analysis is carried out.

⁸Transposon mutagenesis lends itself towards hypomorphs, and not true knockouts. Genetic interactions must be confirmed by the creation of a true knockout.

3.4 Identifying Insertion Sites by DNA Sequencing

Transposon mutagenesis of the *C. albicans* genome is an efficient way to create genetic disruptions; however, the site of disruption is random. To identify the site of insertion for mutant strains of interest we describe the use of Illumina whole genome sequencing (see Note 9). By sequencing strains of interest and finding junctions of the transposon sequence and *C. albicans* genomic sequence, we can identify the disrupted gene or promoter using the Basic Local Alignment Search Tool (BLAST). Below we describe the protocol for obtaining DNA from strains of interest to be sequenced.

1. Grow a 5 ml culture of the desired *C. albicans* strain in appropriate medium such as YPD + uridine overnight.
2. Transfer the culture to centrifuge tube and pellet the cells at 3,000 rpm (1,400×*g*), for 5 min at room temperature. Discard the supernatant and resuspend pellet with sterile water.
3. Flash spin and remove supernatant. Resuspend in 200 µl Breaking Buffer. Add an equal volume of glass beads and 200 µl phenol–chloroform.
4. Vortex for 1 min, place on ice for 1 min (repeat two times).
5. Add 200 µl TE pH 8. Vortex for 1 min and centrifuge at 15,000 rpm (24,000×*g*), for 10 min at 4 °C.
6. Transfer aqueous layer to a clean tube and add 1 mL –20 °C molecular grade ethanol. Mix and centrifuge at 15,000 rpm (24,000×*g*) for 10 min at room temperature.
7. Discard the supernatant and resuspend in 400 µl TE pH 8. Add 3 µl RNaseA (10 mg/mL) and incubate at 37 °C for 5 min.
8. Add 10 µl 3 M sodium acetate and 1 mL –20 °C molecular grade ethanol. Mix well and place on ice for 10 min.
9. Spin at 15,000 rpm (24,000×*g*) for 10 min at room temperature. Wash with 70 % molecular grade ethanol and spin again at 15,000 rpm (24,000×*g*) at room temperature.
10. Discard supernatant and allow the pellet to dry. Resuspend the DNA in 100 µl TE pH 8 or sterile water.

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⁹An alternative method for identifying insertion sites is 3' RACE. Extracting the cellular RNA from the strain of interest and converting it to cDNA by reverse-transcription allows for a template for amplification in a PCR reaction with one primer complementary to an adapter sequence and another complementary to the *URA3* selective marker.

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Fig. 1.

Overview of complex haploinsufficiency. Single heterozygous mutations in *YFG1* (Your Favorite Gene 1) and *YFG2*, respectively, yield wild-type phenotypes, as indicated by the representation of serially diluted cultures. The strain carrying heterozygous mutations in both *YFG1* and *YFG2* yields a more severe phenotype, illustrating complex haploinsufficiency

Fig. 2.

Schematic diagram indicating the Tn7-derived transposon used for mutagenesis of *C. albicans* genomic DNA, with the *URA3* auxotrophic marker shown. Subsequent steps for constructing double heterozygous strains for phenotypic screening of complex haploinsufficiency are outlined. Tn7L, Tn7 *left terminus*; Tn7R, Tn7 *right terminus*