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Genome-Wide Transposon Mutagenesis in *Saccharomyces cerevisiae* and *Candida albicans*

Tao Xu, Nikë Bharucha, and Anuj Kumar

Abstract

Transposon mutagenesis is an effective method for generating large sets of random mutations in target DNA, with applicability toward numerous types of genetic screens in prokaryotes, single-celled eukaryotes, and metazoans alike. Relative to methods of random mutagenesis by chemical/UV treatment, transposon insertions can be easily identified in mutants with phenotypes of interest. The construction of transposon insertion mutants is also less labor-intensive on a genome-wide scale than methods for targeted gene replacement, although transposon insertions are not precisely targeted to a specific residue, and thus coverage of the target DNA can be problematic. The collective advantages of transposon mutagenesis have been well demonstrated in studies of the budding yeast *Saccharomyces cerevisiae* and the related pathogenic yeast *Candida albicans*, as transposon mutagenesis has been used extensively for phenotypic screens in both yeasts. Consequently, we present here protocols for the generation and utilization of transposon-insertion DNA libraries in *S. cerevisiae* and *C. albicans*. Specifically, we present methods for the large-scale introduction of transposon insertion alleles in a desired strain of *S. cerevisiae*. Methods are also presented for transposon mutagenesis of *C. albicans*, encompassing both the construction of the plasmid-based transposon-mutagenized DNA library and its introduction into a desired strain of *Candida*. In total, these methods provide the necessary information to implement transposon mutagenesis in yeast, enabling the construction of large sets of identifiable gene disruption mutations, with particular utility for phenotypic screening in nonstandard genetic backgrounds.

Keywords

Transposon; Gene disruption; Insertional mutant; Genomics; Screen; Yeast; *Candida albicans*; *Saccharomyces cerevisiae*

1. Introduction

Transposons have long been utilized in the laboratory as a tool for the generation of random chromosomal mutations in a broad array of prokaryotes and eukaryotes (1–10). For these purposes, the transposable elements are routinely modified such that the central genes within the transposon are replaced with appropriate markers and functional tags; the ends of the transposon are maintained to direct transposition. In this way, transposons can be used to generate gene disruptions, identify exons/introns, and construct misexpression alleles (11–13). As compared with other methods of mutagenesis, transposon-based approaches hold

numerous advantages. Transposon mutagenesis is an excellent means to generate large numbers of mutations without an excess of labor. Also, the transposon insertions can be easily identified in mutants of interest by inverse PCR and other approaches, eliminating much of the difficulty associated with identifying causative mutations resulting from EMS mutagenesis or UV irradiation. Transposon mutagenesis, however, is not without its drawbacks. In particular, transposition is not strictly random, making it difficult to achieve saturating coverage of target DNA by transposon-based approaches (14). Also, as an obvious byproduct of transposition, the approach is not applicable for the generation of precisely directed mutations, as compared with PCR-based approaches for targeted start codon–stop codon gene replacement (15). On balance, however, transposon mutagenesis is a viable and effective method for many types of large-scale studies, particularly when directed approaches may be cost-prohibitive.

Transposon mutagenesis has been employed very successfully in a range of model organisms; here, we focus on the budding yeast *Saccharomyces cerevisiae* and the pathogenic yeast *Candida albicans* – two lower eukaryotes that have been studied extensively using transposon-based approaches. In these related yeasts, transposon mutagenesis is typically carried out with bacterial transposons (16–21), although the yeast transposable element Ty1 has also been used (22). Bacterial transposons exhibit less insertional bias than Ty1, enabling greater coverage of the target genome. Using bacterial transposons, mutagenesis of a plasmid-based yeast genomic DNA library is typically performed in vitro or in vivo in *Escherichia coli* (4). The resulting insertion alleles are subsequently shuttled into yeast, and the mutagenized DNA integrates into the genome by homologous recombination. As presented in Ross-Macdonald et al. (12) and Kumar et al. (13), Michael Snyder's group pioneered the large-scale application of this approach to generate extensive transposon insertion libraries of *S. cerevisiae* genomic DNA. These libraries were constructed using a specially modified bacterial transposon, such that a single chromosomal transposon insertion could yield a gene disruption allele, reporter-fusion construct, or epitope-tagged allele. Transposon insertion alleles of interest were sequenced, and in total, these plasmid-based collections encompass over 35,000 defined insertion alleles affecting approximately 60% of all annotated yeast genes. Plasmid-based insertion libraries can easily be prepared and introduced into a strain of interest; this is particularly useful for the analysis of gene function in nonstandard strains of *S. cerevisiae* (i.e., any strain for which targeted gene deletion collections are unavailable).

In *Candida albicans*, transposon mutagenesis has served as a powerful method for the generation of large sets of gene disruption mutants, as targeted PCR-based approaches (23) can be challenging to implement in *C. albicans* because of the decreased efficiency of DNA integration by homologous recombination relative to the efficiency observed in *S. cerevisiae*. Shuttle mutagenesis has been applied in *C. albicans* using modified bacterial transposons, as described in several significant studies (19–21). As *C. albicans* is diploid, many forward genetic screens for loss-of-function phenotypes employ heterozygous diploid insertions to screen for haploinsufficiency; this approach has proven effective in identifying genes that contribute to a given cell process and/or function within a signaling pathway. Although growing collections of targeted gene deletion constructs are now available in *C. albicans* (23–25), transposon mutagenesis remains an effective and informative means to probe gene

function on a large scale. As also evident in *S. cerevisiae*, this is particularly true for loss-of-function screens in *C. albicans* that need to be performed in nonstandard or complex genetic backgrounds, in which targeted deletion mutants may be unavailable.

In this chapter, we present protocols for the implementation of transposon-based mutagenesis in *S. cerevisiae* and *C. albicans*. For studies in baker's yeast, protocols are provided detailing (1) the large-scale introduction of transposon-based insertion alleles in a desired strain of yeast; (2) the screening of transposon-mutagenized yeast strains for in-frame insertions, and (3) the subsequent identification of insertion sites in mutant strains of interest by inverse PCR. The availability of transposon insertion libraries in *S. cerevisiae* makes it unlikely that researchers will need to remutagenize yeast genomic DNA; consequently, the above protocols begin with available transposon insertion libraries as a starting point and indicate methods by which the library DNA can be introduced into a desired yeast strain. For the analysis of *C. albicans*, however, researchers may indeed need to mutagenize genomic DNA for a given screen, and thus, we provide here methods describing the following: (1) the construction of an appropriate bacterial transposon for mutagenesis, (2) its application toward the in vitro mutagenesis of a plasmid-based *C. albicans* genomic DNA library, (3) the introduction of transposon insertion alleles into *C. albicans*, and (4) a sample phenotypic screen. Collectively, these protocols outline the necessary steps in applying transposon mutagenesis for genetic screens in yeast, with particular utility for the rapid generation of large sets of identifiable loss-of-function and misexpression alleles in strains for which targeted deletion mutants may be unavailable (26).

2. Materials

2.1. Insertional Mutagenesis of *S. cerevisiae*

1. LB medium, sterile: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1N NaOH, add water to 1 l. Autoclave.
2. 25 mg/ml Kanamycin: Store at 4°C for up to 3 months.
3. 5 mg/ml Tetracycline: In 50% ethanol. Store at -20°C for up to 1 year.
4. TE buffer, sterile: 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0. Autoclave.
5. LB agar plates containing tetracycline and kanamycin: Prepare LB medium containing 15 g/l agar. After autoclaving cool to 45–50°C and add tetracycline to 3 mg/l and kanamycin to 40 mg/l final concentration and pour plates.
6. *Not* I and *Afu* I restriction endonucleases (New England Biolabs, Ipswich, MA or equivalent).
7. One-Step buffer: 0.2 M lithium acetate, 40% (wt/vol) polyethylene glycol (PEG 4000), 100 mM 2-mercaptoethanol.
8. DNA: Sonicated salmon sperm DNA, 2 mg/ml.

9. Synthetic Complete (SC) dropout medium, sterile: Per liter, 1.3 g dropout powder, 1.7 g Yeast Nitrogen Base without Amino Acids/Ammonium Sulfate, 5 g Ammonium Sulfate, 20 g dextrose. Autoclave.
10. YPAD medium, sterile: 1% yeast extract, 2% peptone, 2% dextrose, 80 mg/l adenine. Autoclave.
11. 3-MM filter paper, cut to 4-in diameter discs (Whatman Inc., Clifton, NJ).
12. Glass petri dishes, 9-cm and 15-cm.
13. Chloroform.
14. 0.7 M Potassium phosphate, pH 7.0: Prepare 0.7 M solution of K_2HPO_4 and adjust pH to 7 with HCl.
15. 20 mg/ml X-gal: In 100% *N,N*-dimethylformamide.
16. X-gal plates, sterile: Per liter, 1.7 g Yeast Nitrogen Base without Amino Acids/Ammonium Sulfate, 5 g Ammonium Sulfate, 20 g dextrose, 20 g agar, 0.8 g dropout powder, NaOH pellet. Add water to 900 ml and autoclave. Cool to 45–50°C then add 100 ml 0.7 M potassium phosphate, pH 7.0, and 2 ml X-gal solution.
17. SC-Ura medium, sterile: Per liter, 1.3 g SC-Ura dropout powder, 1.7 g Yeast Nitrogen Base without Amino Acids/Ammonium Sulfate, 5 g Ammonium Sulfate, 20 g dextrose. Autoclave.
18. Clinical tabletop centrifuge.
19. 45°C water bath.
20. 96-well plate reader (optional).
21. Thermal cycler.
22. Blunt-end restriction enzyme and buffer (e.g., *A*/ul).
23. Oligo ABP1:
GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGA-
GAGAAGGGAGAG.
24. Oligo ABP2:
GACTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGT
CCTCTCCCTTC.
25. Universal Vectorette (UV) Oligo:
CGAATCGTAACCGTTCGTACGAGAATCGCT.
26. Primer PRSQZ: CGACGGGATCCCCCTTAACG.
27. Annealing buffer for inverse/vectorette PCR: 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl.
28. 10 mM rATP.

29. Primer M13(–47): CGCCAGGGTTTTCCCAGTCACGAC.
30. Taq DNA polymerase.
31. 80 mM dNTP mix: 20 mM with respect to each dNTP.

2.2. Insertional Mutagenesis of *C. albicans*

1. pGPS3 transposon donor plasmid (New England Biolabs, Ipswich, MA), customized.
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-Sce* I (VDE I), *Pvu* II with supplied buffers (New England Biolabs, Ipswich, MA or alternative).
9. ElectroMAX™ Stbl4™ 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 10XTE, 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 K₂PO₄, 13.5 g agar in 1 l water, pH 7.2 after autoclaving.
26. RNA extraction kit (Qiagen, Valencia, CA).
27. Ribopure Yeast kit (Ambion, Austin TX).
28. 0.1 M DTT (Dithiothreitol).
29. 100 mM dNTP mix: 25 mM with respect to each dNTP.
30. M-MLV reverse transcriptase with supplied First-Strand buffer (5×) (Invitrogen Corp., Carlsbad, CA).
31. Ribonuclease Inhibitor (40 U/μl).
32. Standard lab equipment: tabletop centrifuge, microcentrifuge, 45°C water bath, 25°C and 30°C shaking incubator, 30°C incubator, 65 and 75°C heat block, agarose gel equipment.
33. Oligonucleotide sequences:

3' RACE adapter: 5'
GCGAGCACAGAATTAATACGACTCACTATAGGT₁₂ 3'
(Ambion Inc., Austin TX)

3' RACE outer primer: 5'
GCGAGCACAGAATTAATACGACT 3'

URA3 Forward Primer: 5' GACCTATAGTGAGAGAGCAG 3'
34. Phenol–chloroform (1:1): Equilibrated with 0.1 M Tris–HCl (pH 7.6).

3. Methods

3.1. Transposon Mutagenesis of *S. cerevisiae*

Protocols for the construction of plasmid-based transposon insertion libraries of *S. cerevisiae* genomic DNA have been described previously (4, 16), and insertional libraries (12, 13) are available upon request from the authors free of charge. The library consists of yeast genomic DNA derived from a strain lacking both its mitochondrial genome [ρ^-] as well as 2-micron DNA [*cir*⁰]. Ten pools of this genomic DNA library were individually mutagenized using a modified tetracycline resistant Tn7-derived transposon (13, 27) (Fig. 1a). The transposon supports the generation of gene disruption alleles, reporter fusions, and epitope-tagged alleles as described more fully in Ross-Macdonald et al. (16) and Kumar et al. (13). Thus, each plasmid in the library contains an insert of yeast genomic DNA and a single Tn7 transposon insertion; since Tn7 exhibits transposition immunity (28), plasmids with multiple

insertions will be rare. The kanamycin-resistant vector backbone of this library is small to minimize the possibility that a transposon insertion occurs in the plasmid rather than in the genomic DNA insert. Each mutagenized pool contains five genome equivalents of DNA, encompassing in excess of 300,000 independent insertions.

To utilize the transposon insertion library for functional analysis, the insertion alleles must be introduced into a desired strain of yeast by DNA transformation. Subsequently, mutagenized strains of interest can be selected, and the site of transposon insertion in a strain can be identified by inverse PCR. Protocols for these procedures are provided below. An overview of these steps is provided in Fig. 1b.

3.1.1. Amplifying Library DNA in *E. coli* for Transformation of Yeast

1. Introduce a suitable amount of mutagenized library DNA into any tetracycline- and kanamycin-sensitive *E. coli* strain by standard transformation procedures. Select transformants on LB plates supplemented with tetracycline and kanamycin (see Note 1).
2. Elute transformant colonies as follows: place 6 ml of LB medium onto each plate and scrape cells into a homogeneous suspension. Dilute an aliquot of this eluate into LB medium supplemented with tetracycline (3 µg/ml) and kanamycin (40 µg/ml) to yield a culture of nearly saturated cell density. Incubate at 37°C with aeration for 2–3 h.
3. Isolate plasmid DNA by any standard miniprep or large-scale protocol.

3.1.2. Transforming Yeast with the Mutagenized Library DNA

1. Digest a small aliquot of plasmid DNA (e.g., 1 µg) with *Not*I. Subsequently, analyze a portion of the reaction mixture by agarose gel electrophoresis to ensure release of transposon-mutagenized yeast DNA from the plasmid vector (see Note 2). Store the remaining reaction mixture for later use in step 4 below.
2. Grow a 10-ml culture of any desired *ura3* yeast strain to mid-log phase (a density of 10⁷ cells/ml) maintaining appropriate selection if applicable; if no plasmids are present in the strain, then use YPD medium. To screen for disruption phenotypes, a haploid strain is often used; from previous experience, we estimate that 10% of transposon insertions in essential genes are viable. For the eventual analysis of hypomorphic mutants (e.g., for the study of essential genes), choose a *ura3 leu2* strain (see Note 3).

¹Approximately 10,000 transformants should be obtained per pool following overnight growth at 37°C. Electroporation may be useful if insufficient numbers of transformants are obtained by chemical transformation.

²Upon electrophoresis, a distinct 2.1-kb band (corresponding to the vector) and broad 8-kb band should be visible: the broad 8-kb band consists of 2–3-kb inserts of yeast genomic DNA carrying the 6-kb transposon mutagenized construct.

³Essential genes can be screened for haploinsufficiency by introducing the insertion library into a diploid strain of yeast. The Tn7 transposon used for mutagenesis carries internal *lox* sites such that the majority of the transposon can be removed by Cre-*lox* recombination. Following Cre-mediated excision of transposon sequence, residual sequence encoding an epitope tag remains. In many cases, this small residual epitope insertion sequence can result in a conditional or hypomorphic allele, of particular utility for the analysis of essential genes. For this purpose, choose a *ura3 leu2* strain, as the pGAL-Cre plasmid (for Cre-*lox* recombination), which carries the *LEU2* marker, will need to be introduced into the background strain.

3. Pellet cells in a clinical tabletop centrifuge at $1,100 \times g$ for 5 min. Wash once with 5 volumes of One-Step Buffer.
4. Resuspend cells in 1 ml One-Step Buffer supplemented with 1 ml of 2 mg/ml denatured salmon sperm DNA. Add 100- μ l aliquots from this suspension to 0.1–1 μ g *Not*I-digested plasmid DNA from step 1 (see Note 4). Vortex, and incubate at 45°C for 30 min.
5. Pellet cells and subsequently suspend in 400 μ l SC-Ura medium. Spread 200- μ l aliquots onto SC-Ura plates and incubate at 30°C for 3–4 days. Up to 1,000 transformants may be recovered per μ g of transforming DNA (see Note 5).

3.1.3. Screening Yeast Transformants for β -Galactosidase Activity

1. The transposon used to generate this library contains a *lacZ*-reporter gene trap, such that if the transposon lands in-frame with surrounding gene coding sequence, a β -galactosidase protein chimera will be produced. To maximize detection of *lacZ* fusions expressed at low levels, patch transformant colonies onto YPD plates (supplemented with 80 μ g/ml adenine if using an *ade2* host strain) at a density of up to 100 colonies per plate.
2. Place a sterile disc of Whatman 3-MM filter paper onto a plate of SC-Ura medium; repeat for as many plates as needed. Replicate transformant cells onto filter-covered plates using a multichannel pipettor or hand-pinning tool and incubate overnight at 30°C. Alternative growth conditions (e.g., growth on sporulation medium) may be substituted as desired.
3. Following overnight growth, lift filters from plates and place in the lid of a 9-cm glass petri dish. Place this lid inside a closed 15-cm petri dish containing chloroform to permeabilize the cells. Incubate for 10–30 min.
4. Place filters with colony side up onto fresh X-gal plates (5-bromo-4-chloro-3-indolyl- β D-galactopyranoside). Incubate inverted at 30°C for up to 3 days. After several days of growth, β -galactosidase levels can be reliably estimated from the observed intensity of blue staining (see Note 6).
5. Transformants containing in-frame *lacZ*-fusions may be screened for mutant phenotypes simply by incubating these mutants under desired growth conditions (e.g., in the presence and absence of a particular drug of interest).

⁴Use a small quantity of transforming DNA to minimize generation of transformants containing more than one insertion.

⁵To ensure 95% coverage of the genome (without regards to in-frame reporter activity), screen 30,000–50,000 colonies. To identify in-frame insertions within at least 95% of all yeast genes, screen approximately 180,000–200,000 transformants for β -gal activity.

⁶We typically observe β -gal activity in 12–16% of transformants.

3.1.4. Identifying Transposon Insertion Sites by Inverse PCR—Several methods are available for the identification of transposon insertion sites in mutants of interest, including direct sequencing of mutants and inverse or vectorette PCR-based approaches (18, 29, 30) (Fig. 2). In inverse PCR, genomic DNA is digested with a blunt-end restriction endonuclease possessing a 4–6 base pair recognition sequence. Digested DNA fragments are ligated to a pair of annealed primers containing a nonhomologous central region; these primer pairs form “anchor bubbles” flanking each genomic fragment. PCR is then performed using a primer complementary to the transposon and a primer identical to sequence within the anchor bubble. During the initial round of amplification, only the transposon primer can bind its template; however, during subsequent cycles, the anchor bubble primer can anneal to the extended transposon primer, resulting in selective amplification of DNA sequence adjacent to the point of transposon insertion. Protocols for this procedure are as follows.

1. Prepare genomic DNA by any standard protocol (see Note 7). Digest 5 µg of yeast genomic DNA with a blunt-end restriction endonuclease (such as *A*/*u* I) in a total volume of 20 µl. After overnight digestion, the enzyme is heat-inactivated by incubating 20 min at 65°C.
2. Primers ABP1 and ABP2 are annealed to each other to form the adaptor anchors by mixing 1 pmole of each primer in 200 µl of annealing buffer. The primer mixture is heated for 5 min at 95°C and allowed to slowly cool to 37°C.
3. The adaptors are ligated to the DNA fragments by adding 1 µl of the annealed primers, 0.25 µl of 10 mM ATP, 3 µl of 10× restriction buffer used in the digest (Buffers 1,2,3, and 4 from New England BioLabs are appropriate for use), and 24.25 µl H₂O to the 20 µl restriction digest mixture from Step 1. The ligation reaction is incubated overnight at 16°C.
4. A standard 100 µl PCR reaction is set up using 5 µl from the ligation mixture, 2.5 µl each of primers UV and M13(–47) at 20 µM, 5 U of Taq polymerase and 1 µl of 80 mM dNTP mix in a final volume of 100 µl. The PCR program consist of one cycle of 2 min at 92°C, followed by 35 cycles of 20 s at 92°C, 30 s at 67°C and 45–180 s at 72°C with a final extension of 90 s at 72°C. A 50 µl volume reaction can be used if preferred.
5. Analyze PCR products by gel electrophoresis. Each PCR product is gel-purified using standard protocols into a final volume of 30 µl TE. 10 µl of the purified product is sufficient for one sequencing reaction with primer PRSQZ (see Note 8).

3.2. Transposon Mutagenesis of *C. albicans*

Transposon-mediated gene disruption is one of the few widely available methods applicable for large-scale mutagenesis in the important human pathogen, *Candida albicans*. Various

⁷Care should be taken to obtain high-quality DNA, as this is critical to successful PCR amplification.

⁸The PCR protocol provided here should yield approximately 200–400 ng of product, constituting sufficient template for at least 2–3 DNA sequencing reactions.

groups have used transposon mutagenesis to study gene function in this organism over the past few years by creating both heterozygous and homozygous mutants (19, 20, 31). In this text, we describe details of transposon mutagenesis of a *C. albicans* genomic DNA library to create heterozygous mutants for the study of a developmental program leading to hyphal growth. The first part of this section illustrates the use of the bacterial transposon Tn7 (32) in an in vitro mutagenesis reaction to create insertion mutants in a *Candida albicans* genomic DNA library. In this reaction, three transposase proteins act together to facilitate transposition – TnsA, TnsB, and TnsC* (33, 34). TnsB binds to the Tn7 sequences in the transprimer; TnsC* binds to the target DNA, and TnsA binds to the TnsB–DNA complex. The three proteins then enable the insertion of the transprimer into the target DNA molecules. Transposons insert randomly into target DNA; by virtue of transposition immunity, only one transposon insertion occurs within a single DNA molecule, so double insertions into a DNA fragment in the genomic library are unlikely (28).

For mutagenesis, the bacterial Tn7 transposon transprimer region was modified to include the *Candida albicans* *URA3*-based cassette, *URA3-dpl200* (35, 36) (see Note 9), which enables gene replacement by homologous recombination and counter-selection by 5' FOA. This *URA3* cassette was inserted adjacent to the kanamycin-resistance marker in the pGPS3 plasmid carrying Tn7. The following subsections describe protocols for introducing a genomic DNA library mutagenized with this Tn7-based transposon into the desired *C. albicans* strain as well as a sample phenotypic screen. The steps are summarized in Fig. 3.

3.2.1. Introducing the Transposon into a *C. albicans* Genomic DNA Library

1. 80 ng of a genomic DNA library derived from the *Candida albicans* strain WO-1 pEMBL23 (NIH AIDS Research and Reference Reagent Program (37, 38)) is combined with 20 ng of the customized donor plasmid in a total reaction volume of 20 μ l containing 1 $\times$ TN7 mutagenesis buffer and 1 μ l of the transposase TnsABC*. The mixture is incubated at 37°C for 10 min.
2. 1 μ l 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.
3. Digest with the restriction enzyme *PI-Sce* I 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 μ l phenol-chloroform mix and subject to centrifugation for 5 min at 12,750 $\times$ *g* in microfuge. The mixture will separate into two layers. Remove the upper layer carefully and transfer to a clean microcentrifuge tube containing 250 μ l 100% Ethanol, 10 μ l NaAc (3 M stock concentration) and 0.5 μ l tRNA (10 mg/ml stock concentration). Keep at –80°C for a

⁹In place of the *URA3-dpl200* cassette, other markers may be used as well (e.g., the nourseothricin-resistance marker), particularly to avoid any position-specific effects from *URA3* insertion. The customized donor plasmid with the *URA3-dpl200* cassette is available upon request.

minimum of 30 min (or -20°C for 1 h). Spin at $12,750\times g$ for 30 min at 4°C and subsequently add $50\text{ }\mu\text{l}$ 70–80% ethanol. Spin for 10 min at $12,750\times g$ and resuspend in $30\text{ }\mu\text{l}$ $1\times\text{ TE}$.

5. The mixture is then diluted tenfold, and $10\text{ }\mu\text{l}$ 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 two days.
6. Multiple mutagenesis reactions are performed to allow maximum coverage (see Note 10). 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.

3.2.2. Creating *C. albicans* Mutant Strains from the Transposon Insertion Library

1. Digest $6\text{ }\mu\text{g}$ plasmid DNA (recovered from the mutagenesis reactions above) with *Pvu* II to release the genomic DNA fragments (see Note 11). 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 $25\text{ }\mu\text{l}$ TE buffer (see Note 12).
2. Grow a 5 ml culture of the desired *C. albicans* strain in appropriate medium such as YPD + uridine overnight (39). Add $100\text{--}500\text{ }\mu\text{l}$ of this overnight culture to 50 ml YPD + uridine to bring the culture to an OD_{600} of 0.1–0.2; incubate at 30°C with shaking for approximately 5 h until the culture reaches mid-log phase (OD_{600} of approximately 1).
3. Pellet the cells at 3,000 rpm (approximately $1,400\times g$) for 5 min. Wash with 30 ml sterile water. Resuspend the cell pellet in $500\text{ }\mu\text{l}$ TE-LiAc.
4. Add $10\text{ }\mu\text{l}$ of salmon sperm DNA (10 mg/ml stock concentration) to two sterile microcentrifuge tubes. Add the entire volume of phenol-extracted DNA digest ($25\text{ }\mu\text{l}$) 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 $100\text{ }\mu\text{l}$ of the resuspended cells to the microcentrifuge tubes and mix gently. Incubate the tubes at room temperature for 30 min.
5. Add $700\text{ }\mu\text{l}$ PEG-LiAc-TE mix to each tube and mix by inversion. Incubate at room temperature in a shaking water bath overnight.

¹⁰From past experience, we recommend carrying out a total of nine or more independent reactions to improve coverage of the target genomic DNA library.

¹¹A larger amount (2–6 μg) of the plasmid library DNA is used for each transformation reaction to ensure sufficient number of transformants per reaction.

¹²This step is essential to purify and concentrate the DNA in a smaller volume for transformation.

6. Heat shock the cells by incubating in a 44°C water bath for 20 min (see Note 13) (40).
7. Pellet the cells at 3,000 rpm ($1,400 \times g$) 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 14).
8. Freeze transformants in 15% glycerol in 96-well plates.

3.2.3. A Sample Phenotypic Screen—The transposon-insertion mutant strains may be used for any phenotypic screen of interest. Here, we describe a protocol to screen the mutants for hyphal growth phenotypes. The hyphal phenotype of the parental strain used for the transformation is compared to that of the mutants, and any alterations in hyphal growth are scored as positive. The screen is carried out in a 96-well format as described here.

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 (obtained as described above in Subheading 3.2.3) 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 Spider medium for the analysis of hyphal growth phenotypes) (41).
4. Incubate the plates at 37°C for 5 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.

3.2.4. Identification of Transposon Insertions of Interest—This section describes the use of 3' RACE to identify transposon insertion sites in mutants of interest. The procedure begins with extraction of cellular RNA from the strain using a standard extraction protocol and its subsequent conversion by reverse-transcription into cDNA. The cDNA is used as a template for amplification in a PCR reaction with one primer complementary to an adapter sequence and another complementary to the *URA3* selective marker. The details of the protocol are described here.

1. Inoculate 50 µl of the frozen stock of the desired strain in 4 ml SC-Ura and incubate overnight in a 30°C shaking incubator.
2. Extract total cellular RNA from the entire overnight culture (Ribopure Yeast kit).

¹³The heat shock step may be performed at 42°C for 1 h instead of 44°C for 20 min. The optimal heat shock length should be determined empirically if initial results are inadequate.

¹⁴In order to obtain pure colonies of transformants, it is advisable to restreak transformants onto fresh plates before any further analysis is carried out.

3. Add 1–5 µg of extracted RNA to a nuclease-free microcentrifuge tube together with 1 µl 100 mM dNTP mix, 1 µl 3' RACE adapter (500 µg/ml stock concentration) and sterile water for a total of 12 µl. Incubate at 65°C for 5 min and then keep on ice for 2 min.
4. Add 4 µl 5× First-Strand buffer, 2 µl DTT (0.1 M stock concentration) and 1 µl ribonuclease inhibitor (40 U/µl stock concentration). Mix gently and incubate at 37°C for 2 min.
5. Add 1 µl M-MLV reverse transcriptase (200 U) and mix gently. Incubate for 50 min at 37°C. Heat-inactivate the reaction for 15 min at 70°C.
6. Use 2–5 µl of the cDNA obtained in a standard PCR amplification reaction with primers complementary to the 3' RACE adapter (3' RACE outer primer) and the 3' end of the *URA3* gene (*URA3* Forward Primer). The PCR product should comprise a fragment of the *URA3* marker along with the Tn7-insertion site in the genome. This PCR product can be sequenced directly using primers complementary to the *URA3* gene (see Note 15).

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¹⁵Alternatively, the PCR product can be cloned into a TA vector using standard cloning procedures. The vector DNA is then extracted using standard alkaline lysis; the DNA is sequenced, and BLASTN analysis can be used to identify the disrupted gene.

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Fig. 1.

Overview of the steps involved in applying transposon insertion libraries for mutagenesis in *Saccharomyces cerevisiae*. **(a)** Diagrammatic representation of the bacterial transposon used in the insertional library; the Tn7 transposon is shown here. An insertion in a sample gene (*YFG1*) is shown; brackets represent codons in the hypothetical gene sequence, and an in-frame insertion is represented here. The 3 $\times$ HA sequence encodes three copies of the hemagglutinin (HA) epitope. Cre-mediated recombination at the transposon-encoded *lox* sites results in a 99-codon insertion element appropriate for epitope-tagging and the possible generation of hypomorphic alleles. **(b)** To utilize the transposon insertion library for mutagenesis, the library is amplified in *E. coli* and introduced into yeast by DNA transformation. Yeast transformants are screened for a desired phenotype, and insertion sites in mutants of interest are identified by DNA sequencing or inverse/vectorette PCR.

Fig. 2. Inverse or vectorette PCR for the identification of insertion sites in *S. cerevisiae* mutants of interest. The major steps are indicated. *Alu I* is indicated as an example restriction enzyme for this application; primer sequences are included in the Materials list.

Transposon mutagenesis of *C. albicans* genomic DNA library:

Introduce mTn-mutagenized genomic DNA into *C. albicans*:

Screen for desired phenotypes (e.g., hyphal growth):

Fig. 3.

Overview of steps involved in the transposon mutagenesis of *Candida albicans*. This diagram indicates use of the WO-1 genomic DNA library as described in the Methods text and the *URA3-dpl200* cassette incorporated in a modified Tn7 transposon. Specific media suggestions are included for the analysis of hyphal phenotypes in disruption mutants; alternative growth conditions can be used based on the chosen screen.