Yeast
Yeast (2012)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/yea.2910

Research Article

Analysis of protein function in clinical *C. albicans* isolates

Maryam Gerami-Nejad[1], Anja Forche[2], Mark McClellan[1] and Judith Berman[1]*
[1]*Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*
[2]*Department of Biology, Bowdoin College, Brunswick, ME, USA*

**Correspondence to:*
J. Berman, Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455, USA.
E-mail: jberman@umn.edu

Received: 10 April 2012
Accepted: 30 May 2012

Abstract

Clinical isolates are prototrophic and hence are not amenable to genetic manipulation using nutritional markers. Here we describe a new set of plasmids carrying the *NAT1* (nourseothricin) drug resistance marker (Shen *et al*., 2005), which can be used both in clinical isolates and in laboratory strains. We constructed novel plasmids containing HA–*NAT1* or MYC–*NAT1* cassettes to facilitate PCR-mediated construction of strains with C-terminal epitope-tagged proteins and a *NAT1*–pMet3–*GFP* plasmid to enable conditional expression of proteins with or without the green fluorescent protein fused at the N-terminus. Furthermore, for proteins that require both the endogenous N- and C-termini for function, we have constructed a *GF–NAT1–FP* cassette carrying truncated alleles that facilitate insertion of an intact, single copy of *GFP* internal to the coding sequence. In addition, *GFP–NAT1*, *RFP–NAT1* and M-Cherry–*NAT1* plasmids were constructed, expressing two differently labelled gene products for the study of protein co-expression and co-localization *in vivo*. Together, these vectors provide a useful set of genetic tools for studying diverse aspects of gene function in both clinical and laboratory strains of *C. albicans*.

Keywords: ***Candida albicans*; clinical isolates; plasmids; *NAT1*; fluorescent tags**

Introduction

Candida albicans is a commensal fungus residing in the oral cavity, the gastrointestinal tract and the genitourinary tract of humans. It is also an opportunistic pathogen with a disease spectrum ranging from mild superficial infections, such as oral thrush, to life-threatening infections, such as disseminated candidiasis in patients with severely compromised immune systems.

Molecular genetic analysis of clinical isolates, which are generally prototrophic, has been hindered by the lack of available selectable markers. Recently, several markers, including drug resistance markers such as *IMH3* (resistance to mycophenolic acid) (Beckerman *et al*., 2001), *SAT1* and *NAT1* (resistance to norceothricin) (Reuss *et al*., 2004; Sasse and Morschhaeuser, 2012; Shen *et al*., 2005) and fluorescent epitopes including *GFP*, *YFP*, *CFP* and *RFP*, have allowed the manipulation of clinical isolates and have expanded the transformation tools available for laboratory strains as well (Gerami-Nejad *et al*., 2001, 2004, 2009; Milne *et al*., 2011). However, to date, only a limited number of plasmids are available specifically to study conditional repression or induction of gene expression in clinical strains. To extend the range of available tools, we have constructed a variety of plasmids using the *NAT1* marker in combination with epitope and fluorescent protein tags, for labelling proteins at the N- and C-termini as well as at a position internal to the protein. Importantly, all plasmids can be used to study gene function in clinical isolates of *C. albicans*.

Materials and methods

Strains and media

All strains were derived from SC5314 (Table 1) and were grown at 30 °C in rich medium (YEPD; 1% yeast extract, 1% bactopeptone, 2% glucose

Table 1. Strains and plasmids used in this study

ID	Genotype	Reference
Strains		
YJB2348(SC5314)	wt	(Yagishita *et al.*, 1981)
YJB7617(RM1000#2)	*ura3*Δ::λ*imm*434/*ura3*Δ::*imm*434 *his1*::*his*G/*his1*::*his*G	(Negredo *et al.*, 1997)
YJB3731(BWP17)	*ura3*Δ::λ*imm*434/*ura3*Δ::λ*imm*434, *his1*::*his*G/*his1*::*his*G, *arg4*::*his*G/*arg4*::*his*G	(Wilson *et al.*, 1999)
YMG9024	BWP17; *CDC3*/*CDC3–GFP–NAT1*	This study
YMG9282	*ENO1*::*GF–NAT1–FP*	This study
YMG9296	*ENO1*::*GFP*	This study
YMG11306	*ENO1*/*ENO1–RFP–NAT1*	This study
YJB11986	BWP17; pMet–*NAT1–DAD2*/*DAD2*	(Burrack *et al.*, 2011)
YJB11987	BWP17; pMet–*NAT1–DAD2*/*dad2*::*HIS1*	(Burrack *et al.*, 2011)
YMG12409	RM1000; *NOP1*/*NOP1*–M-Cherry–*NAT1*	This study
YMG12466	RM1000; *SNF2*/*SNF2*–HA–*NAT1*	This study
YMG12511	*SNF2*/*SNF2*–MYC–*NAT1*	This study
Plasmids		
p1372	pGEM–*URA3*	(Wilson *et al.*, 1999)
p2105	pJK796	(Shen *et al.*, 2005)
pMG1886	p*URA3*–Met3–*GFP*	(Gerami-Nejad *et al.*, 2004)
pMG1874	pHA–*URA3*	(Gerami-Nejad *et al.*, 2009)
pMG1905	pMYC–*URA3*	(Gerami-Nejad *et al.*, 2009)
pMG2162	pCa*RFP*	(Gerami-Nejad *et al.*, 2009)
pMG2120	p*GFP–NAT1*	This study
pMG2137	p*GF–NAT1–FP*	This study
pMG2190	p*NAT1*–Met3–*GFP*	This study
pMG2261	p*RFP–NAT1*	This study
pMG2343	pM–Cherry–*NAT1*	This study
pMG2352	pHA–*NAT1*	This study
pMG2353	pMYC–*NAT1*	This study

and 1.5% agar), synthetic complete medium (SDC; 0.5% ammonium sulphate, 0.17% yeast nitrogen base without amino acids, 2% glucose, 2% agar and amino acids) or synthetic complete medium lacking specific amino acids (Sherman, 1991). For *NAT1* selection, 400 μg/ml nourseothricin (Jena Bioscience, Germany) was added to the medium. *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA, USA) and standard media and methods were used for plasmid manipulation (Ausubel *et al.*, 1995). Yeast genomic DNA was isolated according to the method of Hoffman and Winston (1987).

Construction of plasmids

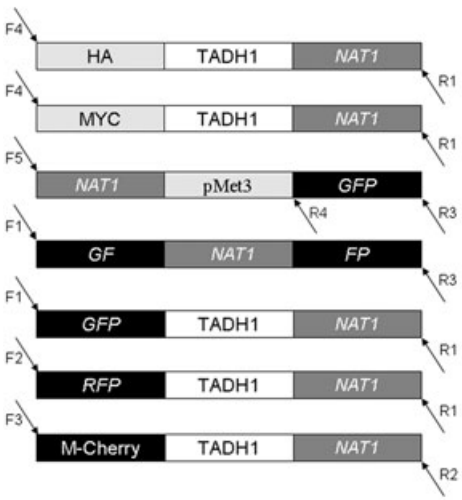
pMG2120 (p*GFP–NAT1*)

Plasmid pMG871 was cut with *Sac*I and *Sal*I. Plasmid pJK795 (Shen *et al.*, 2005) was cut with *Sac*I and *Sal*I to liberate the *NAT1* marker. The *NAT1* fragment was gel purified and ligated into pMG871 to generate plasmid pMG2109. To introduce the *C. albicans ADH1* terminator after *GFP*, the *ADH1* terminator from pMG1602 (Gerami-Nejad *et al.*, 2001) was isolated as an *Eco*RI–*Pst*I fragment, filled with Klenow and ligated to the *Sal*I cut site between *GFP* and *NAT1* to generate pMG2120 (Figure 1; see also supporting information, Figure S1).

pMG2261 (p*RFP–NAT1*)

Plasmid pMG2162 (Ca*RFP*; synthesized by Celtek Genes, Nashville, TN, USA) was cut with *Bam*HI and *Stu*I, run out on an agarose gel and the fragment containing Ca*RFP* was gel-purified. Plasmid pMG2120 (see above) was first cut with *Pac*I and treated with mung bean nuclease (NEB), then cut with *Bgl*II and treated with alkaline phosphatase (NEB). Finally, Ca*RFP* was ligated into cut pMG2120 to yield plasmid pMG2261 (Figure 1; see also supporting information, Figure S1).

DOI: 10.1002/yea



Plasmid name	Plasmid number	Plasmid size in kbp
pHA-*NAT1*	pMG2352	3.7
pMYC-*NAT1*	pMG2353	3.7
p*NAT1*-Met3-*GFP*	pMG2190	3.5
p*GF-NAT1-FP*	pMG2137	3.7
p*GFP-NAT1*	pMG2120	3.7
p*RFP-NAT1*	pMG2261	3.2
pM-Cherry-*NAT1*	pMG2343	3.2

Figure 1. Transformation cassettes for use in *C. albicans*. Cassettes including name, number and size of amplification product illustrate the region of each plasmid to be amplified by PCR, using primers with homology to the integration site of interest (arrows). Detailed depictions of each cassette are provided in Materials and methods and Figure S1 (see supporting information); F1–F6 and R1–R4 are primers for amplification of the transformation cassettes from plasmids (for specific sequences, see supporting information, Table S1). kbp, kilobase pairs. Sequences of each cassette are provided at: http://www.cbs.umn.edu/labs/berman/NAT1-tags.html

pMG2343 (pM-Cherry–*NAT1*)

First, pMG2254 containing M-Cherry (Gerami-Nejad *et al*., 2009) was digested with *Eco*RI and *Not*I. Then pGEM–*URA3* (Wilson *et al*., 1999) was cut with *Eco*RI and *Not*I and the 811 bp insert was ligated into cut pMG2254 to generate pMG2338. pMG2338 was then cut with *Not*I and filled with Klenow to generate blunt ends. Plasmid pJK795 (Shen *et al*., 2005) was cut with *Hin*dII and *Eco*RV to liberate the 1.2 kbp *NAT1* marker, which was ligated into the *Not*I-cut pMG2338, generating pMG2342. pMG2342 contains M-Cherry together with the *URA3* and *NAT1* markers. To remove *URA3* from this plasmid, it was cut with *Spe*I and *Bcl*I and ligated together to generate pMG2343 (Figure 1; see also supporting information, Figure S1).

pMG2352 (pHA–*NAT1*)

pMG2120 (p*GFP–NAT1*) was digested with *Nde*I (filled with Klenow) and *Nsi*I to remove the *GFP* sequence. Plasmid pMG1874 (pHA–*URA3*) (Gerami-Nejad *et al*., 2009) was digested with *Hin*dIII (filled with Klenow) and *Nsi*I to remove the HA sequence. The fragment containing the HA sequence was gel-purified and ligated into cut pMG2120 to generate pMG2352 (Figure 1; see also supporting information, Figure S1).

pMG2353 (pMYC–*NAT1*)

pMG2120 (p*GFP–NAT1*) was digested with *Nde*I (filled with Klenow) and *Nsi*I to remove the *GFP* sequence. Plasmid pMG1905 (pMYC–*URA3*) (Gerami-Nejad *et al*., 2009) was digested with *Hin*dIII (filled with Klenow) and *Nsi*I to remove the MYC sequence. The fragment containing the MYC sequence was gel-purified and ligated into cut pMG2120 to generate pMG2353 (Figure 1; see also supporting information, Figure S1).

pMG2190 (p*NAT1*–pMet3–*GFP*)

Plasmid pMG1886 (p*URA3*–pMet3–*GFP*) (Gerami-Nejad *et al*., 2004) was cut with *Eco*RI to excise the *URA3* marker, filled with Klenow and then digested with *Xho*I. Plasmid pJK795 was cut with *Eco*RV and *Sal*I to liberate the *NAT1* marker. The *NAT1* fragment was gel-purified and ligated into cut pMG1886 to generate pMG2190 (Figure 1; see also supporting information, Figure S1).

DOI: 10.1002/yea

pMG2137 (p*GF–NAT1–FP*)

Plasmid pMG2120 (p*GFP–NAT1*) was cut with *Hpa*I and *Bgl*II to excise 231 bp of the *GFP* coding region, filled with Klenow and religated to generate pMG2133. This plasmid contains 500 bp of the N-terminal region of *GFP*. pMG2133 was cut with *Sac*II and treated with Klenow. The 460 bp C-terminal sequence of *GFP* was amplified from pMG1602 using primers 1402 and 1403 (see Table 2), cut with *Xba*I and then treated with Klenow. This insert was ligated into cut pMG2133 to generate pMG2137 (Figure 1; see also supporting information, Figure S1).

Transformation of plasmids

To obtain the transformation cassettes from the plasmids, PCR was performed using primers homologous to the plasmid and to the appropriate target-gene sequence integration site (Figure 1; see also supporting information, Table S1). To test the functionality of constructs, *CDC3* was tagged with *GFP*, *ENO1* with *RFP*, *NOP1* with M-Cherry and *SNF2* with HA and MYC epitopes (for primers, see supporting information, Table S1). PCR reactions were performed as described previously (Gerami-Nejad *et al*., 2001). The products from 10 50 µl PCR reactions were pooled, precipitated with ethanol, resuspended in 30 µl water and used to transform *C. albicans* strains SC5314, RM1000 or BWP17 (Table 1; see also supporting information, Table S1), as described elsewhere (Gerami-Nejad *et al*., 2001). Transformants were selected by plating onto YEPD medium containing 400 µg/ml nourseothricin. To identify correct transformants, genomic DNA was prepared as described (Wilson *et al*., 1999) and used as template in diagnostic PCRs, using one primer within the transformation cassette and a second primer outside the targeted locus (see supporting information, Table S1).

To test the functionality of the p*NAT1*–pMet3–*GFP* construct (pMG2190, conditional expression of N-terminally tagged proteins), one copy of *DAD2*, an essential gene in *C. albicans*, was deleted and the construct was inserted into the second copy of *DAD2*. Transformants were selected on YEPD containing 400 µg/ml nourseothricin, and correct transformants were determined by diagnostic PCR with primers 640 and 3695 (see supporting information, Table S1). In addition, correct transformants were directly screened for *GFP*, *RFP* and M-Cherry expression, using fluorescence microscopy. Cells were imaged at × 100 magnification using a Nikon E600 microscope, with *GFP* and Texas red filter sets as appropriate, using constant exposure times and scaling for each strain.

Immunoblot detection of fusion proteins

Western blots were done to detect the fusion proteins. Strains expressing Snf2–HA (YMG12466) or Snf2–MYC (YMG12511) and control strain SC5314 were grown for 5 h in YEPD at 30 °C. The cells were spun down, the pellets resuspended in 300 µl Thorner buffer (40 mM Tris, pH 8, 5% SDS, 8 M urea and 100 µM EDTA) and incubated for 15 min at 95 °C. To lyse the cells, 500 µm glass beads (Bio Spec Products, Bartlesville, OK, USA) were added and the samples were vortexed for 30 min at 4 °C. Electrophoresis buffer (5×), 2-mercaptoethanol and bromophenol blue were added to a total volume of 400 µl and the samples were incubated at 95 °C for 15 min. To pellet insoluble cell debris, cell lysates were centrifuged at 14 000 × *g* for 6 min. Protein amounts of the supernatant were determined spectrophotometrically at A280, and roughly equal amounts of sample were run out on an 8% polyacrylamide gel. The gel was blotted to PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA). One half of the membrane was incubated with mouse monoclonal anti-HA antibody (12CA5, Roche Applied Science, Indianapolis, IN, USA), followed by HRP-anti-mouse antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). The other half was detected with HRP-monoclonal mouse anti-myc antibody (9E10, Roche). Equal sample loading was confirmed by total protein staining of the blot with Indian ink after detection.

Results and discussion

Because *C. albicans* has an unconventional codon usage (CUG encodes serine instead of leucine), functional markers with compatible codon content are required. The *NAT1* marker, which confers resistance to nourseothricin, is the preferred marker for selecting clinical strains, which are generally auxotrophic, and combine it with a series

DOI: 10.1002/yea

of epitope and fluorescent protein tags that have been codon-optimized for use in *C. albicans*. Here, we modified previously constructed sets of plasmids carrying *URA3* or *HIS1* markers (Gerami-Nejad *et al*., 2001, 2004, 2009) by replacing the prototrophic markers with *NAT1*.

Constructs for conditional repression or induction of protein transcription

To construct cassettes for inserting epitope tags at the C-terminus of genes, we modified HA-tagging vectors pMG1874 (pHA–*URA3*) and pMG1905 (pMYC–*URA3*) by inserting *NAT1* in place of *URA3*, while retaining the *ADH1* transcription termination sequence (Figure 1; see also supporting information, Figure S1). To test the functionality of the HA and MYC tags, pMG2352 (pHA–*NAT1*) or pMG2353 (pMYC–*NAT1*) constructs were integrated at the C-terminus of the *SNF2* gene via PCR-mediated fusion (YMG11987 and YMG12511, Table 1). Nourseothricin-resistant colonies appeared after 3 days and correct transformants were checked by diagnostic PCR, using primers 335 and 5157 (see supporting information, Table S1). Western blots with antibodies against the epitopes detected the production of the HA (Figure 2A, lane 2) and MYC-tagged proteins (Figure 2B, lane 2), while no signal was observed for the control strain SC5314 (Figure 2A, B, lane 1).

To construct a *NAT1*–pMet3–*GFP* template for the PCR-mediated insertion of a regulatable promoter with or without an N-terminal *GFP* fusion, we modified the pMG1886 (p*URA3*–pMet3–*GFP*) construct (Gerami-Nejad *et al*., 2004) by replacing the *URA3* with the *NAT1* marker. This construct was used to tag the essential gene *DAD2* in a *DAD2*-heterozygous strain (Figure 3). Under conditions that induce pMet3 (medium lacking methionine and cysteine), control and conditional strains were able to grow (Figure 3, left). Under conditions that repress pMet3 (medium containing methionine and cysteine), the control strain grew while none of the three pMet3-*DAD2* transformants were able to proliferate (Figure 3, right), confirming that the promoter is effective at repressing expression.

Fluorescent protein fusion constructs

To facilitate the study of proteins in clinical isolates, we combined *NAT1* with *GFP*, *RFP* or

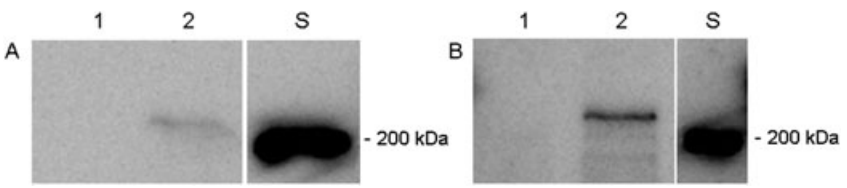


Figure 2. Western blots and antibody detection of epitope-tagged strains: (A) lane 1, wt control (SC5314); lane 2, HA-tagged strain YMG12466 (*SNF2*–HA–*NAT1*): (B) lane 1, wt control (SC5314); lane 2, MYC-tagged strain YMG12511 (*SNF2*–MYC–*NAT1*). Expected sizes of fusion protein products: Snf2–HA, 210 kDa; Snf2–MYC, 223 kDa; S-size standard

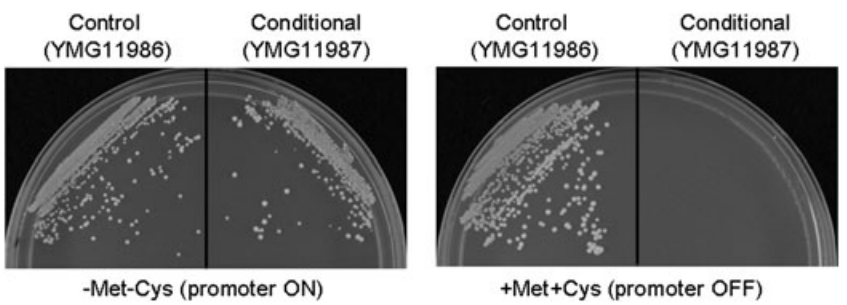


Figure 3. Conditional expression of pMet (*DAD2*/*dad2*::*HIS1*, strain YMG11987) and the control strain pMet–*DAD2*/*DAD2* (YMG11986) exhibited growth on SDC–Met–Cys medium (left), but the conditional strain failed to grow on SDC–Met–Cys (right)

DOI: 10.1002/yea

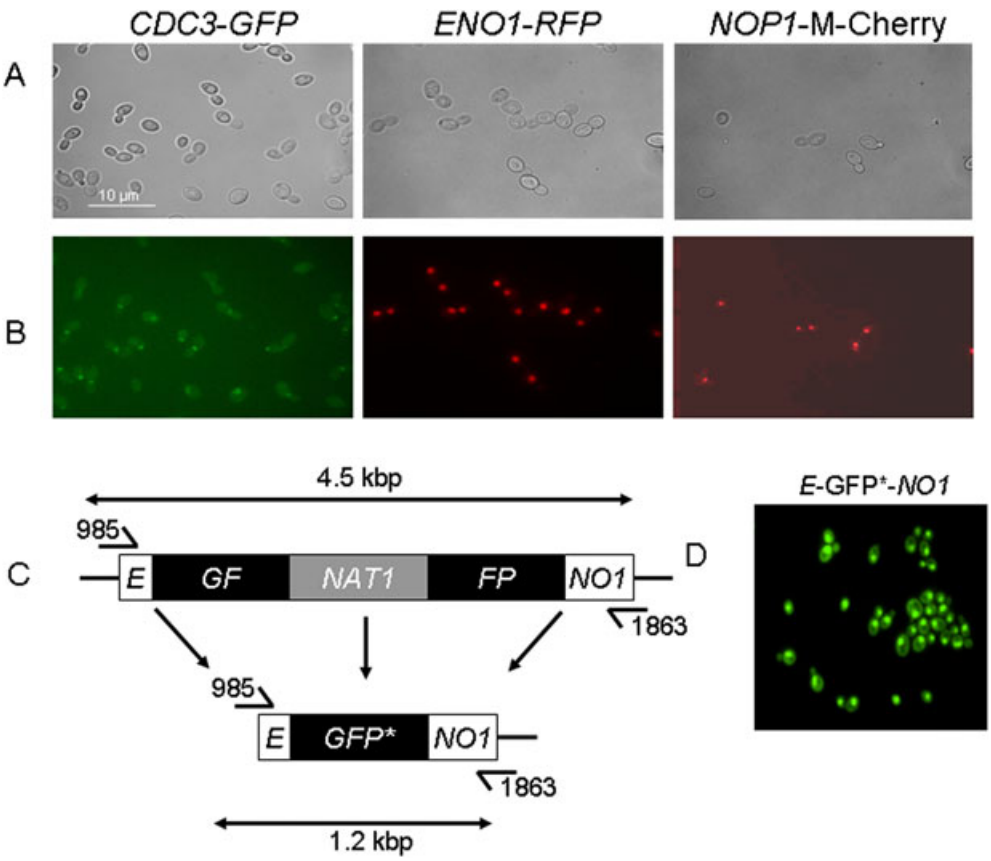


Figure 4. Expression of fluorescent protein fusions in *C. albicans*. (A) DIC images and (B) fluorescent images for auxotrophic strain YMG9024 expressing Cdc3 tagged with *GFP* (left), Eno1 tagged with *RFP* (centre) in auxotrophic strain YMG11306, and Nop1 tagged with M-Cherry (right) in strain YMG12409 (C) Transformation with *pGF–NAT1–FP* cassette before and after *NAT1* loop-out step (D). Expression of *Eno1* internally tagged with *GFP* results in localization of Gfpp to the nucleus (identical to native *ENO1* locus). Microscopy images were taken at × 100 magnification using a Nikon E600 microscope, using *GFP* and Texas red filter sets as appropriate. kbp, kilobase pairs

M-Cherry (Figure 1; see also supporting information, Figure S1) and used them to generate strains expressing fluorescent protein fusions. *GFP–NAT1*, *RFP–NAT1* and M-Cherry–*NAT1* cassettes were amplified from plasmids pMG2120, pMG2261 and pMG2343, respectively, using appropriate primers (see supporting information, Table S1) and were transformed into strains BWP17, SC5314 and RM1000, respectively, immediately following the start codons of *CDC3*, *ENO1* and *NOP1* genes, respectively. Nourseothricin-resistant colonies appeared after 3 days and correct integration of the constructs was confirmed by colony PCR (Gerami-Nejad *et al*., 2001). In addition, fluorescence microscopy was performed to determine whether the fluorescent protein fusions localized as expected (Figure 4A, B). Cdc3–*GFP* signal was accurately observed at the mother bud neck (Figure 4B, left), Eno1–*RFP* localized to the nucleus (Figure 4B, centre) and Nop1–M-Cherry signal was detected in the nucleolus (Figure 4B, right).

Proteins such as those located in the cell wall often require that both the N- and C-termini remain intact to ensure proper protein secretion and localization. In laboratory strains we label such proteins with a p*GF–URA3–FP* cassette (pMG2082; Gerami-Nejad *et al*., 2009). Here we replaced *URA3* with *NAT1* to produce pMG2137 (p*GF–NAT1–FP*), which tags proteins internally by first being inserted in-frame within the coding sequence of interest, and then screening for excision of *NAT1* via recombination between the overlapping regions of *GFP* (Figures 1, 4C). As a proof of principle, *GF–NAT1–FP* was amplified from pMG2137 with appropriate primers (see

DOI: 10.1002/yea

supporting information, Table S1) and inserted between the start codon and the first amino acid of the *ENO1* gene in auxotrophic strain YMG9282. Norceothricin-resistant colonies were screened for accurate integration of the construct by diagnostic PCR. Correct transformants were then grown overnight in YEPD to facilitate recombination between the overlapping flanks of the *GFP* gene, thereby restoring its function (Figure 4D). *GFP* was readily detected in these cells and could be exploited to identify colonies in which the recombination had occurred (Figure 4D). Thus, the p*GF–NAT1–FP* construct enables internal tagging of a protein in a single transformation step.

In summary, the plasmids described here expand the number of tools available for working with *C. albicans* strains. Most importantly, they increase the facility of performing biochemical and cell biological studies of *C. albicans* clinical isolates.

Acknowledgements

We would like to thank Ben Harrison for help with microscopy. This research was supported by the National Institutes of Health (Grant Nos R01AI0624273 and R01AI075096, to J.B.).

Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Detailed maps of plasmids described in this study
Table S1. Primers used in this study

References

Ausubel FM, Brent R, Kingston RE, *et al*. 1995. Current Protocols in Molecular Biology. Wiley: New York.

Beckerman J, Chibana H, Turner J, *et al*. 2001. Single-copy *IMH3* allele is sufficient to confer resistance to mycophenolic acid in *Candida albicans* and to mediate transformation of clinical *Candida* species. *Infect Immun* **69**: 108–114.

Burrack LS, Applen SE, Berman J. 2011. The requirement for the Dam1 complex Is dependent upon the number of kinetochore proteins and microtubules. *Curr Biol* **21**: 889–896.

Gerami-Nejad M, Berman J, Gale CA. 2001. Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in *Candida albicans*. *Yeast* **18**: 859–864.

Gerami-Nejad M, Dulmage K, Berman J. 2009. Additional cassettes for epitope and fluorescent fusion proteins in *Candida albicans*. *Yeast* **26**: 399–406.

Gerami-Nejad M, Hausauer D, McClellan M, *et al*. 2004. Cassettes for the PCR-mediated construction of regulatable alleles in *Candida albicans*. *Yeast* **21**: 429–436.

Hoffman CS, Winston F. 1987. A 10-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**: 267–272.

Milne SW, Cheetham J, Lloyd D, *et al*. 2011. Cassettes for PCR-mediated gene tagging in *Candida albicans* utilizing nourseothricin resistance. *Yeast* **28**: 833–841.

Negredo A, Monteoliva L, Gil C, *et al*. 1997. Cloning, analysis and one-step disruption of the *ARG5,6* gene of *Candida albicans*. *Microbiology* **143**: 297–302.

Reuss O, Vik A, Kolter R, *et al*. 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341**: 119–127.

Sasse C, Morschhaeuser J. 2012. Gene deletion in *Candida albicans* wild-type strains using the *SAT1*-flipping strategy. *Methods Mol Biol* **845**: 3–17.

Shen J, Guo W, Kohler JR. 2005. *CaNAT1*, a heterologous dominant selectable marker for transformation of *C. albicans* and non-*albicans Candida* species. *Infect Immun* **73**: 1239–1242.

Sherman F. 1991. Getting started with yeast. *Methods Enzymol* **194**: 3–21.

Wilson RB, Davis D, Mitchell AP. 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* **181**: 1868–1874.

Yagishita K, Jinnouchi H, Yamamoto H. 1981. Studies on the mechanism of synergistic action with synergisidin and imidazole antimycotics. *Japan J Antibiot* **34**: 1485–1495.

DOI: 10.1002/yea