Cassettes for PCR-mediated gene tagging in *Candida albicans* utilising nourseothricin resistance

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Abstract

In recent years a number of molecular tools have been reported for use in the human fungal pathogen *Candida albicans*, including PCR-mediated approaches for gene disruption, conditional expression and epitope tagging. Traditionally these methods have utilised auxotrophic markers, however the availability of auxotrophic markers can be limiting and in some instances their use may also impact on the interpretation of results. As a result the use of positive selection markers has now become more commonplace. Here we report the development and validation of a set of cassettes for PCR-mediated gene tagging and over-expression studies utilising the nourseothricin resistance (*CaNAT1*) positive selection marker. In particular we have produced cassettes containing yeast enhanced *GFP*, *YFP*, *CFP*, *RFP* and a combined V5-6xHis epitope tag. The cassettes are engineered for use in PCR-mediated gene tagging strategies where insertion is targeted to the 3′ end of the gene of interest. In addition, to facilitate protein functional analysis and genetic suppression studies through the use of over-expression, we have also constructed a promoter replacement cassette containing the *ENO1* promoter which is known to be expressed at a high level. These cassettes expand on the range of molecular tools available for working with *C. albicans* and may also be used in other *Candida* species that display sensitivity to nourseothricin.

Keywords: *Candida albicans*; Positive selection; Nourseothricin resistance; Fluorescent protein; Epitope tagging; Over-expression

Introduction

Candida albicans, the most common fungal pathogen of humans, causes a wide range of clinical diseases ranging from superficial infections to life-threatening systemic disease in the immunocompromised (Calderone, 2002; Odds, 1988; Perlroth *et al*., 2007). Traditionally the molecular analysis of this important pathogen has been hampered by a combination of its obligate diploid nature, lack of exploitable sexual cycle, low transformation efficiency and atypical codon usage (Berman and Sudbery, 2002; Noble and Johnson, 2007). However, with the development of rapid PCR-mediated strategies for gene targeting (Gerami-Nejad *et al*., 2001; Wilson *et al*., 1999; Wilson *et al*., 2000) efficient genetic tools have now been developed, including methods for epitope tagging. Epitope tags are essential molecular tools enabling protein detection, localisation and purification, and those available in *C. albicans* to date include the fluorescent protein markers GFP, YFP, CFP, RFP, mCherry and Venus (Gerami-Nejad *et al.,* 2001; Gerami-Nejad *et al.,* 2009, Reijnst *et al*., 2011), alongside the small epitopes FLAG, Myc, HA, V5, GST and poly-His (Gerami-Nejad *et al.,* 2009; Hernday *et al.,* 2010; Lavoie *et al.,* 2008; Schaub *et al.,* 2006).

Genetic manipulation in *C. albicans* has traditionally used auxotrophic markers. The first to be developed, and the most commonly used, is *URA3* (Fonzi and Irwin, 1993). However, the expression of *URA3* from heterologous loci is now known to impact on virulence and virulence-associated attributes, therefore bringing its use into question (Bain *et al.,* 2001; Brand *et al.,* 2004; Cheng *et al.,* 2003; Staab and Sundstrom, 2003). Further strains have been developed that allow the use of additional auxotrophic markers such as *ADE2*, *LEU2*, *ARG4* and *HIS1* (Fonzi and Irwin, 1993; Negredo *et al.,* 1997; Noble and Johnson, 2005; Wilson *et al.,* 1999). However, in general, the range of auxotrophic markers available in common laboratory strains can still be limiting, and the use of auxotrophic markers also precludes the direct analysis of clinical isolates of *C. albicans* as these strains are not auxotrophic. A potential route around this limitation is the use of positive selection markers, and some are now available for use in *C. albicans*. The first successfully developed positive selection marker provided resistance to mycophenolic acid (Wirsching *et al*., 2000a, Wirsching *et al*., 2000b, Beckerman *et al.,* 2001). However, the resistance marker is a mutant derivative of the native *IMH3* gene, and consequently its homology to the chromosomal copy of *IMH3* negatively impacts upon its use in gene targeting approaches. Nourseothricin has since been successfully developed as a positive selection marker, with resistance encoded by the heterologous markers *CaNAT1* or *SAT1* (Reuss *et al.,* 2004; Roemer *et al.,* 2003; Shen *et al.,* 2005), and more recently a synthetic hygromycin resistance gene has also been reported to function in *C. albicans* (Basso *et al.,* 2010).

In this work we have developed a set of PCR-mediated gene tagging cassettes for *C. albicans* utilising the nourseothricin resistance marker *CaNAT1*. In particular we have combined the *GFP*, *YFP*, *CFP* and *RFP* fluorescent protein tags and a V5-

6xHis epitope tag with the *CaNAT1* marker. In addition we have developed a constitutive expression system utilising the promoter of the highly expressed enolase gene, *ENO1*. This will allow the application of over-expression studies for genetic suppression and protein functional analysis. These cassettes increase the range of molecular tools available for working with *C. albicans* and may potentially be used in other *Candida* species that display sensitively to nourseothricin.

Materials and Methods

Strains, media and culture conditions

All strains constructed and used in the present study are detailed in Table 1. *C. albicans* strains were grown in either YEPD medium (1% yeast extract, 2% mycological peptone, 2% glucose) or SC medium (0.67% yeast nitrogen base, 2% glucose, 0.079% complete supplement mixture [Formedium, Hunstanton, UK]) at 30°C. Uridine (50 µg/ml) was added to media as required. To select for nourseothricin resistance strains were plated on to Sabouraud dextrose agar (1% mycological peptone, 4% glucose, 1.5% Agar) containing 200 µg/ml nourseothricin (Werner BioAgents, Jena, Germany). To induce β-N-acetylhexosaminidase activity strains were grown in SC-GlcNAc (0.67% yeast nitrogen base, 0.079% complete supplement mixture, 25 mM N-acetylglucosamine).

Cassette construction

Fusion PCR (Wach, 1996) was used in the construction of our tagging and over-expression cassettes that incorporate the nourseothricin resistance marker. For the carboxy terminus fluorescent protein tagging cassettes *GFP*, *YFP*, *CFP, RFP* coupled to the *CaADH1* terminator were PCR amplified from pGFP-URA3, pYFP-URA3, pCFP-URA3 and pRFP-URA3 (Gerami-Nejad *et al.,* 2001; Gerami-Nejad *et al.,* 2009) respectively with primer pair GFP-F and FP-R-FUS for *GFP*, *YFP* and *CFP,* and primer pair RFP-F and FP-R-FUS for *RFP* (Table 2). The *CaNAT1* resistance marker, under the control of the *Ashbya gossypii TEF1* promoter and terminator, was PCR amplified from pJK795 (Shen *et al.,* 2005) using primers NAT1-F-FUS and NAT1-R. To allow for fusion PCR the GFP-R-FUS and NAT-F-FUS oligonucleotides incorporated complementary sequences at the 5′ end. For fusion PCR to generate the full length cassettes the first round PCR products were purified, combined and PCR amplified using primer pairs GFP-F and NAT1-R for the *GFP*, *YFP* and *CFP* cassettes, and RFP-F with NAT1-R for the RFP cassette. All PCR reactions for cassette construction were carried out using Extensor High Fidelity PCR master mix (Thermo Scientific, Epsom, UK). The *GFP-NAT1* cassette was cloned into the TOPO TA vector pCR2.1 (Invitrogen, Paisley, UK) and all other cassettes were cloned into StrataClone pSC-A-amp/kan (Agilent Technologies, Stockport, UK) to generate pGFP-NAT1, pYFP-NAT1, pCFP-NAT1 and pRFP-NAT1 (Fig. 1).

For the over-expression cassette the *CaENO1* promoter was amplified from *C. albicans* genomic DNA using the ENO1-F-FUS and ENO1-R primers. The purified PCR product was combined with the *Ca*NAT1 marker (amplified with NAT1-F-FUS and NAT1-R as previously) by fusion PCR using primers ENO1-R and NAT1-R. To generate the *V5-6xHis-NAT1* cassette the *V5-6xHis* coding region coupled with the *ScCYC1* terminator was amplified from pYES2.1 (Invitrogen, Paisley, UK) with primer pair YES-V5-F and CYC-T-R-FUS. The purified product was then fused to the Ca*NAT1* marker by fusion PCR using the YES-V5-F and NAT1-R primers. The *NAT1-ENO1p* and *V5-6xHis-NAT1* cassettes were then cloned into the TOPO TA vector pCR2.1 (Invitrogen, Paisley, UK) to generate pNAT1-ENO1p and pV5-NAT1.

In addition to the *V5-6xHis-NAT1* cassette we also developed a *V5-6xHis-URA3* cassette. Briefly, the *V5-6xHis* coding region and *ScCYC1* terminator was amplified from pYES2.1 (Invitrogen, Paisley, UK) with primer pair YES-V5-F and CYC-T-R, and the product cloned into pGEM-T Easy (Promega, Southampton, UK). The insert was then released by NotI digestion and subcloned into the NotI site of CIp10 (Murad *et al.,* 2000) to generate pV5-URA3. The orientation of the *V5-6xHis* sequence relative to the *URA3* marker in CIp10 was confirmed by PCR to allow this vector to act as either an integrative vector or as a template for PCR-mediated gene tagging strategies.

Utilisation of cassettes in strain construction

To utilise the cassettes for PCR-directed gene tagging they were amplified by PCR with primers containing the appropriate gene-specific sequences to drive homologous recombination (Fig. 1, Tables 2 and 3). The standard amplification protocol used Thermoprime Master Mix (Thermo Scientific, Epsom, UK) and cycling conditions of an initial denaturation of 3 min at 94°C, followed by 30 cycles of 94°C 1 min, 55°C 1min and 72°C 3 min, and a final extension at 72°C for 5 min. Typically 50 µl of unpurified PCR product was then used to transform *C. albicans* using standard methodologies (Gietz and Woods, 2002). Following transformation cells were incubated in YEPD at 30°C for 4 h to allow expression of the *CaNAT1* marker before plating onto selective media. Correct integration of the cassettes was confirmed by colony PCR (Linder *et al.,* 1996) using a primer within the cassette and a second targeting the chromosomal integration site outside the region included in the original targeting primer.

To confirm function of the *GFP*, *YFP*, *CFP* and *RFP* cassettes they were amplified with gene-specific sequences to target them to the *CaACT1* locus immediately downstream of its promoter (primers ACT1-GFP-F and ACT1-FP-R for *GFP*, *YFP* and *CFP*; ACT1-RFP-F and ACT1-FP-R for *RFP*; Tables 2 and 3). The amplified cassettes were transformed into the Ura^+ strain NGY152 and correct integration was confirmed by colony PCR (primers ACT1-S and GFP-UP for *GFP*, *YFP* and *CFP*; ACT1-S and RFP-UP for *RFP*; Tables 2 and 3). To tag *CDC3* the GFP-NAT1 cassette was amplified with CDC3-GFP-F and CDC3-FP-R and transformed into the

clinical isolate SC5314. Correct integration was confirmed by PCR with primer pair GFP-UP and CDC3-S.

To test the *ENO1p* over-expression and *V5-6xHis* cassettes the *HEX1* gene encoding β-N-acetylhexosaminidase was targeted. For over-expression the *NAT1-ENO1p* cassette was amplified using HEX-ENO-F and HEX-ENO-R primers and transformed into strain NGY152, to replace the native promoter of one allele with the *ENO1* promoter. Correct integration was confirmed by PCR screening using the ENO-SF and HEX-ENO-S primers. To introduce the C-terminal V5-6xHis epitope tag into Hex1p the *V5-6xHis-NAT1* and *V5-6xHis-URA3* cassettes were amplified with Hex-V5-F and either HEX1-V5-NAT1-R or HEX1-V5-URA3-R respectively. The *CaNAT1*-containing cassette was transformed into NGY152 and the *URA3*-containing cassette into the ura$^-$ strains CAI-4 or NGY98 (Δpmr1/Δpmr1). Correct genomic integration was determined using primer pair HEX-V5-S and V5-S.

Protein detection methods

For microscopy studies strains were grown to logarithmic phase, briefly washed in PBS and viewed either live or following a short (10 min) fixation in 70% ethanol. Epifluorescence and differential interference microscopy was carried out using a motorised inverted IX81 microscope (Olympus, Southend-on-Sea, UK) with epifluorescence illumination for GFP (Ex460/40, BS495, Em525/50), YFP (Ex500/24, BS520, Em542/27), CFP (Ex436/20, BS455, Em480/40) and RFP (Ex562/40, BS593, Em624/40). Filter sets were from Chroma (Rockinham, USA) or Semrock (Rochester, USA), and digital images were captured using a CoolSnap HQ2 camera (Roper Scientific, Germany) and processed using Metamorph software (version X; Molecular Devices, Wokingham, UK).

Protein extracts were prepared in 100 mM Tris-HCl pH 7.5, 0.01% SDS, 1 mM dithiothreitol, 10% glycerol containing protease inhibitors (Roche Applied Science, Burgess Hill, UK) by glass bead disruption in a FastPrep machine (Qbiogene, Cambridge, UK). The resulting lysate was clarified by centrifugation at 21500 *g* for 10 min. Prior to western blotting, 50 µg of protein extracts were separated on a 10% NuSep Tri-HEPES-SDS gel (Generon, Maidenhead, UK) before blotting on to a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin in TBS-T (Tris-buffered saline containing 0.01% Tween 20) for 2 h, before probing with the mouse anti-V5 primary antibody (Invitrogen, Paisley, UK) at a 1:5000 dilution for 1 h. The membrane was then washed three times in TBS-T prior to incubation with the secondary antibody, anti-mouse IgG-horseradish peroxidase (Invitrogen, Paisley, UK), at 1:10000 dilution for 1 h. Following a final four washes in TBS-T, proteins were detected using Lumiglo (New England Biolabs, Hitchin, UK) according to manufacturer's instructions.

For protein purification, using the 6xHis tag, protein extracts were prepared in 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Tween-20, 1 mM β-mercaptoethanol, 10%

Glycerol, 10 mM Imidazole by glass bead disruption. Hex1-V5-6xHis was then batch purified from 20 mg of protein extract using Ni-NTA agarose beads as per manufacturer's instructions (QIAGEN, Crawley, UK) with proteins eluted in 50 mM Tris-HCl pH 7.5, 300 mM Imidazole.

Hex1p activity was determined by a modified version of the assay used by Cannon *et al.,* (1994). Briefly, protein extracts were incubated with the substrate pNP-GlcNAc (1.25 mM; Glycosynth, Warrington, UK) in 0.1 M citrate/KOH buffer pH 4 for 30 min at 37 °C. Reactions were stopped by the addition of 1 M Na_2CO_3 and absorbance at 420 nm determined. Hex1p activity was expressed as nmol p-nitrophenol released per min per mg protein.

Results and Discussion

Construction of PCR tagging cassettes

To facilitate the analysis of gene function in *C. albicans* we constructed gene tagging and over-expression cassettes incorporating the *CaNAT1* positive selection marker for nourseothricin resistance (Shen *et al.,* 2005). These cassettes complement those currently available for work in this important pathogen. To generate carboxy-terminus fluorescent protein tagging cassettes we combined the previously developed *GFP*, *YFP*, *CFP* and *RFP* variants (Gerami-Nejad *et al.,* 2001; Gerami-Nejad *et al.,* 2009) with the nourseothricin resistance marker, *CaNAT1*, by fusion PCR (Fig. 1). In addition we also generated cassettes containing a C-terminal V5-6xHis epitope tag for use in protein analysis, and a cassette containing the enolase promoter (*ENO1p*) for driving constitutive over-expression of the target gene (Fig. 1). Full details of the cassette construction are given in the Materials and Methods. The nourseothricin resistance marker used in this work (Shen *et al.,* 2005) is under the control of the heterologous *Ashbya gossypii TEF1* promoter and terminator, thereby avoiding problems associated with the misdirection of gene targeting when native control sequences are used.

Gene tagging using these cassettes follows standard protocols; cassettes are PCR amplified using oligonucleotide primers containing 70 bp of homology to the target gene to direct integration (Table 3). This level of homology produces a reasonable proportion of transformants in which integration has occurred at the desired target site. All the gene tagging cassettes use a common reverse primer, and the *GFP*, *YFP* and *CFP* cassettes also share a common forward primer. For selection of nourseothricin resistant transformants we found that a minimum of four hours growth in non-selective medium was required post transformation to allow expression of the *CaNAT1* marker, similar to previous reports on the use of the *SAT1* marker (Reuss *et al.,* 2004). However, in our hands selection on Sabouraud dextrose medium with 200 µg/ml nourseothricin proved the most reliable, and required the use of lower concentrations of nourseothricin than other media.

Validation of fluorescent protein fusion cassettes

To confirm the four fluorescent protein tagging cassettes (*GFP*, *YFP*, *CFP* and *RFP*; Fig. 1) were functional we integrated each cassette immediately downstream of the start codon of the constitutively expressed *ACT1* gene. Cassettes were amplified and transformed into the prototrophic strain NGY152. Nourseothricin resistant colonies appeared 1-2 days post transformation and correct genomic integration at the *ACT1* locus was confirmed by colony PCR. Fluorescence microscopy clearly demonstrated that all four cassettes were functional, with cells displaying bright fluorescence throughout the cytoplasm with some exclusion from the vacuole (Fig. 2A). No bleed-through between filter sets was apparent, consistent with the potential use of these fluorescent proteins for co-localisation and co-expression studies.

The *CaNAT1* and *SAT1* markers have previously been shown to function in clinical isolates of *C. albicans* (Reuss *et al.,* 2004; Shen *et al.,* 2005). We targeted the *GFP-NAT1* cassette to tag the septin Cdc3 in the SC5314 clinical isolate. Fluorescence imaging of the tagged strain clearly demonstrated the characteristic cell cycle-dependent localisation of Cdc3 (Gerami-Nejad *et al.,* 2001; Warenda and Konopka, 2002) to the mother-bud neck (Fig. 2B). In addition the *GFP-NAT1* cassette has also been successfully used to visualise spindle pole body proteins that are expressed at a low level (Milne, Cheetham, Bates in preparation).

Validation of epitope tagging and constitutive over expression cassettes

The epitope tagging cassettes contain both a V5 and C-terminal 6xHis epitope tag, combined with either the *CaNAT1* nourseothricin resistance marker or the *URA3* auxotrophic marker. This combination of epitopes only increases protein size by 3.7 kDa and offers the benefits of efficient detection through the V5 epitope and protein purification using the 6xHis epitope. To test the function of both the *V5-6xHis-NAT1* and *V5-6xHis-URA3* cassettes they were targeted to tag *HEX1* in the NGY152 and CAI-4 strains respectively. *HEX1* encodes β-N-acetylhexosaminidase, a well characterised hydrolytic enzyme whose expression is induced in medium containing GlcNAc as the sole carbon source (Cannon *et al.,* 1994; Niimi *et al.,* 1997). Tagged strains were grown in inducing and non-inducing conditions, and proteins were extracted and analysed by western blotting using an anti-V5 antibody. As expected, only when grown on GlcNAc was Hex1 expressed and it was clearly detectable as both an unmodified (67 kDa) and heavily glycosylated form (~125 kDa) in soluble protein extracts (Fig. 3A). We also tested the *V5-6xHis-URA3* cassette by tagging Hex1 in a *Capmr1*Δ mutant which is known to demonstrate glycosylation defects (Bates *et al.,* 2005). In this mutant the glycosylated form of the tagged Hex1 protein displayed a clear increase in its electrophoretic mobility, a defining characteristic of the mutant's gross defect in glycosylation (Fig. 3B). In addition to the V5 tag for protein detection the cassettes also contain the 6xHis epitope which can be employed in protein purification using standard methods. To confirm this epitope is

functional we used it to successfully purify Hex1p-V5-6xHis from soluble protein extracts (Fig. 3C).

The *HEX1* gene was also targeted to validate the *NAT1-ENO1p* constitutive over-expression cassette. The *ENO1* promoter was chosen as it has been shown to be expressed at high levels, and it has previously been demonstrated to be suitable for gene-overexpression studies (Bates *et al.,* 2007; Staab *et al.,* 2003). The cassette was targeted to replace the native promoter of one copy of the *HEX1* gene, and expression monitored through determining Hex1 enzyme activity. The assay results confirmed that *HEX1* is only expressed in wild type cells in the presence of GlcNAc, consistent with previous observations. Replacing the endogenous *HEX1* promoter with the *NAT1-ENO1* promoter cassette resulted in the clear over-expression of Hex1 under both inducing and non-inducing conditions (Fig. 3D). Therefore the *NAT1-ENO1p* cassette is capable of driving high level gene expression which may be useful for both the functional analysis of proteins and in studying genetic interactions through epistasis-based approaches.

Conclusion

In summary we have developed a set of cassettes for tagging and over-expression studies in *C. albicans* utilising the positive selection marker *CaNAT1* for nourseothricin resistance. These cassettes complement and extend the range of molecular cell biology tools currently available for use in this important pathogen. In addition these cassettes will be of potential use in other *Candida* species, or indeed in other fungi, that display sensitivity to nourseothricin.

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Table 1.

C. albicans strains

Strain	Parent Strain	Genotype	Reference
SC5314	-	Clinical isolate	(Gillum *et al*., 1984)
CAI-4	-	*ura3Δ::imm434/ura3Δ::imm434*	(Fonzi and Irwin, 1993)
NGY152	CAI-4	As CAI-4 but *RPS1*/*rps1Δ*::CIp10	(Brand *et al*., 2004)
SBC153	NGY152	As NGY152 but *ACT1/ACT1p-GFP-NAT1*	This study
SBC154	NGY152	As NGY152 but *ACT1/ACT1p-YFP-NAT1*	This study
SBC155	NGY152	As NGY152 but *ACT1/ACT1p-CFP-NAT1*	This study
SBC156	NGY152	As NGY152 but *ACT1/ACT1p-RFP-NAT1*	This study
SBC157	SC5314	*CDC3/CDC3-GFP-NAT1*	This study
SBC158	NGY152	As NGY152 but *HEX1/HEX1-V5-6xHis-NAT1*	This study
SBC152	CAI-4	As CAI-4 but *HEX1/HEX1-V5-6xHis-URA3*	This study
NGY98	NGY97	As CAI-4 but *pmr1Δ::hisG*/*pmr1Δ::hisG*	(Bates *et al*., 2005)
SBC159	NGY98	As NGY98 but *HEX1/HEX1-V5-6xHis-URA3*	This study
SBC160	NGY152	As NGY152 but *NAT1-ENO1p-HEX1/HEX1*	This study

Table 2.

Oligonucleotides used for cassette and strain construction

Primer	Sequence (5′-3′) [a]
NAT1-F-FUS	GTCAGCGGCCGCATCCCTGCGATATCAAGCTTGCCTCGTC
NAT1-R	CGTTAGTATCGAATCGACAGC
GFP-F	GGTGGTGGTTCTAAAGGTGAAGAATTATT
RFP-F	GGTGGTGGTGATAACACTGAAGATGTTATT
FP-R-FUS	GCAGGGATGCGGCCGCTGACATATTTCAACGCCTTCCAGC
ENO1-F-FUS	GCAGGGATGCGGCCGCTGACATTTGTATCTTTAGTAGACATG
ENO1-R	TGTTGTAATATTCCTGAATTATC
YES-V5-F	AAGGGCGAGCTTCGAGGTC
CYC-T-R-FUS	GCAGGGATGCGGCCGCTGACTGCAGGGCCGCAGCTTGC
CYC-T-R	TGCAGGGCCGCAGCTTGC
ACT1p-GFP-F	ctggttttctttctttcttagaaacattatctcgatattaatattaaaaaaatataatcattcaaaatgGGTGGTGGTTCT AAAGGTGAAGAATTATT
ACT1p-RFP-F	ctggttttctttctttcttagaaacattatctcgatattaatattaaaaaaatataatcattcaaaatgGGTGGTGGTGAT AACACTGAAGATGTTATT
ACT1p-FP-R	gtgtgtattattaatgtgacagtaacatcccaaacgagaaatattatgtcgacaacaaaaaagtttgatcCGTTAGTA TCGAATCGACAGC
ACT1p-S	caccaagatttattgccaacg
CDC3-GFP-F	acaaaaattattaccacaagacccaccagcacaaccagctccacaaaagagtcgtaaaggatttttacgtGGTGGT GGTTGTAAAGGTGAAGAATTATT
CDC3-FP-R	tactgacaatttttatacatcacaatatcaaattaaacaaacagattaacaaacaaataaactaaattaagttacataCG TTAGTATCGAATCGACAGC
CDC3-S	aagagaatgggtattgaacaag
HEX-ENO-F	gcgttttatggtttaccccacaaaggtccgtgttttcaaaaatttctaaaagatagatctattaatgtggctagtCGTTAGT ATCGAATCGACAGC
HEX-ENO-R	aaccttggcagcgtggacaaccacattgcacaaccaaagaagcaaatgaaagataatcattttatctaacaccatTGT TGTAATATTCCTGAATTATC
HEX-ENO-S	accaagccatgtaatgctcc
HEX-V5-F	acggggtttctcctttggtgccaaaatactgtttgctcaatccacacgcttgtgatttgtacaaaaatccaccagtttatAAG GGCGAGCTTCGAGGTC
HEX-V5-NAT-R	acttccgttccctttgagcacttagatagtgatatcgtatattttctttccaaaccatctattccagacacagatctCGTTA GTATCGAATCGACAGC
HEX-V5-URA-R	acttccgttccctttgagcacttagatagtgatatcgtatattttctttccaaaccatctattccagacacagatctCTAGTT CTAGAAGGACCACC
HEX-V5-S	acaaggattccaacggacac

[a] Sequences to facilitate fusion PCR are underlined. Gene-specific sequences are lowercase

Table 3.
Oligonucleotides for cassette utilisation

Primer	Sequence (5'-3')[a]
(G/Y/C)FP-F	(gene-specific sequence)-**GGT GGT GGT** TCT AAA GGT GAA GAA TTA TT
RFP-F	(gene-specific sequence)-**GGT GGT GGT** GAT AAC ACT GAA GAT GTT ATT
FP-R	(gene-specific sequence)-CGTTAGTATCGAATCGACAGC
GFP-UP	CACCTTCACCGGAGACAG
RFP-UP	ATAATCTGGAATATCAGCTGG
V5-F	(gene-specific sequence)-AAG GGC GAG CTT CGA GGT C
V5-NAT1-R	(gene-specific sequence)-CGTTAGTATCGAATCGACAGC
V5-URA3-R	(gene-specific sequence)- CTAGTTCTAGAAGGACCACC
V56xHIS-S	TGCAGGGCCGCAGCTTGC
ENO1p-F	(gene-specific sequence)- CGTTAGTATCGAATCGACAGC
ENO1p-R	(gene-specific sequence-CAT)- TGTTGTAATATTCCTGAATTATC
ENO1p-S	TTGATAATTCAGGAATATTACAAC

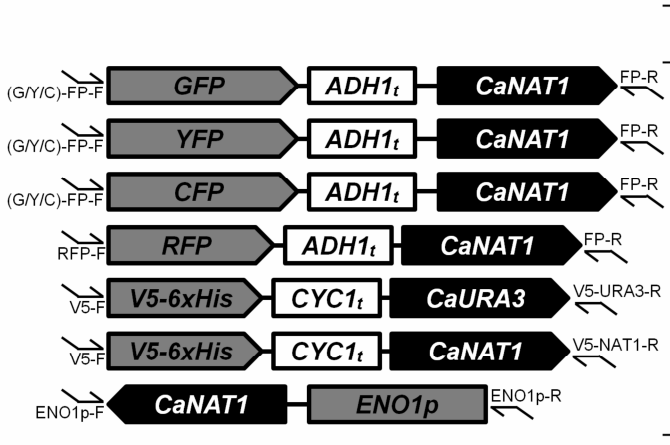
[a] For C-terminal protein tagging the gene-specific sequence in the forward primer is directly upstream of the stop codon; the required reading frame is indicated by spacing in the sequences, with sequences in bold encoding a glycine linker. Underlined nucleotides show the position of the ATG start codon for the *ENO1* promoter.

Figure Legends

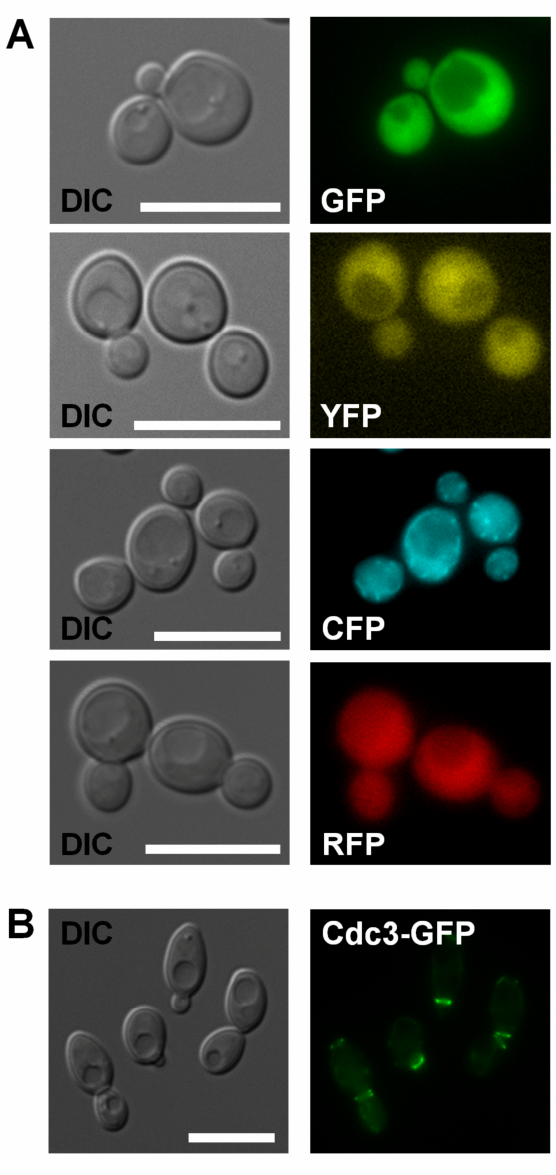
Figure 1. Diagram of PCR-based tagging and over-expression cassettes. Black boxes, selection marker (*CaNAT1* or *CaURA3*); grey boxes, *GFP*, *YFP*, *CFP*, *RFP*, *V5*-6x*His* or *ENO1* promoter (*ENO1p*) sequences; white boxes, terminator sequences ($CaADH1_t$ or $ScCYC1_t$).The *CaNAT1* marker is under the control of the heterologous *Ashbya gossypii TEF1* promoter and terminator as originally described (Shen *et al.,* 2005). The GFP-NAT1, V5-6xHis-NAT1 and NAT1-ENO1p cassettes were cloned into pCR2.1 (Invitrogen); YFP-NAT1, CFP-NAT1, RFP-NAT1 cassettes into pSC-A-amp/kan (Strataclone); and the V5-6xHis-URA3 cassette was cloned into CIp10 (Murad *et al*., 2000). Oligonucleotide primers used to amplify cassettes are described in the Materials and Methods and Tables 2 and 3.

Figure 2. Validation of fluorescent protein fusion cassettes in *C. albicans*. (A) Visualisation of fluorescent protein expression in *ACT1p-GFP*, *ACT1p-YFP*, *ACT1p-CFP and ACT1p-RFP* strains. Direct interference microscopy (DIC) micrographs are to the left of each fluorescence micrograph. Details of the filters used for the detection of each fluorophore are provided in the Materials and Methods. (B) Cdc3 tagged with GFP in the SC5314 clinical isolate. Scale bars are 10 μm throughout.

Figure 3. Validation of epitope tagging and over-expression cassettes. (A and B) Western blot analysis of Hex1-V5-6xHis expression in soluble protein extracts utilising an anti-V5 antibody. Tagged Hex1 is apparent both in an unmodified form (67 kDa as predicted) and in a heavily glycosylated form present as a wide band of an approximate average molecular weight of 125 kDa. (A) Strains were grown in non-inducing (glucose, lanes 1 & 3) or inducing (GlcNAc, lanes 2 & 4) conditions. Lanes 1 and 2, proteins from SBC152 (Hex1p-V5-6xHis-*URA3*); Lanes 3 and 4, proteins from SBC158 (Hex1p-V5-6xHis-*NAT1*). (B) Hex1-V5-6xHis-*URA3* tagging in CAI-4 (lane 1) and NGY98 (*Capmr1*Δ, lane 2) strain backgrounds. (C) Hex1p-V5-6xHis purification using Ni-NTA agarose beads; lane 1 molecular weight marker, lane 2 soluble protein extract, lane 3 non bound proteins, lane 4 bead wash, lane 5 eluted proteins. (D) Hex1 enzyme activity of NGY152 and SBC160 (*ENO1p-HEX1*) strains grown under inducing (GlcNAc) and non-inducing (glucose) conditions, all assays were conducted in triplicate.



Plasmid name	PCR product size (kb)
pGFP-NAT1	2.7
PYFP-NAT1	2.7
pCFP-NAT1	2.7
pRFP-NAT1	2.4
pV5-URA3	1.8
pV5-NAT1	1.6
pNAT1-ENO1	2.2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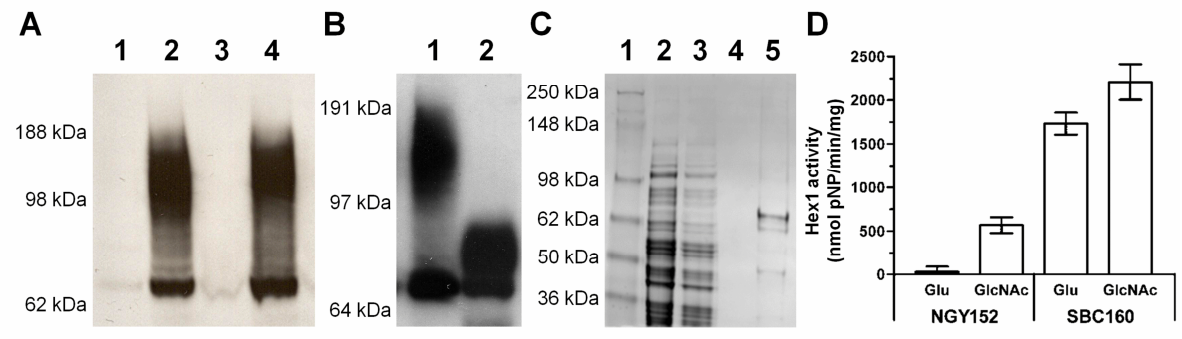
DIC
GFP
DIC
YFP
DIC
CFP
DIC
RFP
B
DIC
Cdc3-GFP


A
1 2 3 4
188 kDa
98 kDa
62 kDa
B
1 2
191 kDa
97 kDa
64 kDa
C
1 2 3 4 5
250 kDa
148 kDa
98 kDa
62 kDa
50 kDa
36 kDa
D
Hex1 activity
(nmol pNP/min/mg)
2500
2000
1500
1000
500
0
Glu
GlcNAc
Glu
GlcNAc
NGY152
SBC160