Fjrs.futa.edu.ng
FUTA Journal of Research in Sciences
ISSN: 2315 – 8239 (Print); E-ISSN: 2489 - 0413
FUTA Journal of Research in Sciences, Vol. 15(2), October, 2019: 212-219

EUKARYOTIC INITIATION FACTOR 2B COMPLEX'S PURIFICATION FROM A PREDOMINANTLY DIPLOID ORGANISM, *C. albicans* BY FLAG-EPITOPE TAGGING OF THE TWO *GCDI* ALLELES

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ABSTRACT

To study protein interaction and expression in cells, the cells are usually tagged to enable location of proteins within cells and for isolation and purification of the protein complexes of interest. This study was carried out to tag the two copies of *GCDI* gene in *Candida albicans* with FLAG epitopes to enable maximal protein expression of the tagged allele as well as purification of the tagged gene product by affinity chromatography. To achieve this, we designed a tagging strategy with four FLAG epitopes making use of a mini *URA3* blaster (*URA3-dpl200*) cassette. This tagging cassette was commercially synthesized and a standard PCR based epitope tagging strategy was taken to tag the *GCD1* genes with the 4X-FLAG-*URA3dpl* cassette. Agarose gel electrophoresis and Western blot analysis were used to confirm the success of the epitope tagging. Purification of the protein product complex of the tagged *GCDI* genes was done by affinity chromatography using ANTI-FLAG M2 Magnetic beads. This strategy enabled the epitope tagging of the two alleles of *GCD1*, which code for a subunit of the eukaryotic initiation factor 2B (eIF2B), a heteropentameric guanine nucleotide exchange factor that functions during translation initiation. The purified tagged protein complex yielded the expected sized bands on SDS PAGE gel. The sub-complex of the protein subunits was enriched in the purified sample detected by mass spectrometry. Therefore, these data show that the tagging of the two *GCD1* alleles with 4xFLAG epitope has successfully allowed the eIF2B to be purified.

KEY WORDS: FLAG epitope tag, *GCD1*, *Candida albicans*, Affinity chromatography, Mass Spectrometry

INTRODUCTION

Candida albicans is the most prevalent and the most pathogenic of the *Candida species*, and is ranked as the fourth leading cause of nosocomial blood-stream infections (Ashman *et al*., 2004).
Candida albicans is the cause of life-threatening infections in immunocompromised patients as well as a variety of surface infections in generally healthy individuals.
The genus *Candida* is the form genus consisting of an array of yeast species in which no complete sexual cycle has been observed (Kwon-Chung and Bennett, 1992; Forche *et al*., 2008). Although an elaborate mechanism for mating can still be operational (Kim and Sudbery, 2011). In *C. albicans*, mating occurs between diploid mating type-like (MTL) a and α strains to generate an a/α tetraploid strain. The resulting tetraploids then undergo efficient, random chromosome loss resulting in diploid cells. (Forche *et al*., 2008) and transient haploid cells with reduced fitness (Hickman *et al*., 2013).

Because of its importance as a human pathogen, many laboratories have undertaken molecular and

genetic studies of this predominantly diploid dimorphic yeast. The development of new drug therapies specific for this yeast will probably require a better understanding of its physiology through biochemical studies and genetic manipulation. Unfortunately, genetic analysis of *C. albicans* is difficult, since this organism has a diploid genome (Kurtz *et al*., 1986), and autonomous plasmid systems developed for routine use are unstable in this organism (Goshorn *et al*., 1992).

To study protein interaction and expression in cells, the cells are usually tagged to enable location of proteins within cells; for isolation and purification of the protein complexes of interest and the interacting partners (Einhauer and Jungbauer, 2001). Epitope tags are useful for affinity purification and immunochemistry

Candida albicans is a predominant diploid, therefore, tagging just a single copy of a gene will not permit maximal protein expression of the tagged allele, as the organism will also be expressing the untagged allele of the gene of interest.

The eukaryotic initiation factor 2B (eIF2B) is a heteropentameric guanine nucleotide exchange factor that functions during translation initiation and consists of α, β, γ, δ, and ε subunits, encoded by *GCN3, GCD7, GCD1, GCD2* and *GCD6* respectively. The eIF2B catalyzes the otherwise slow exchange of GDP for GTP on its substrate eIF2. This is a rate-limiting and regulated step in protein synthesis and the target of many regulatory processes often resulting in a process known as integrated stress response (Kenner *et al*,. 2019). Previously a combined V5-6xHis epitope tag (Milne *et al*., 2011) was used to tag the gene of interest (*GCD1*), however, repeated attempts made to purify the tagged protein using the V5 purification method and also the 6xHis tag purification did not yield the expected sized bands, nor was any of the protein subunits detected by mass spectrometry from the purified samples.
In an attempt to circumvent some of these potential bottlenecks, a tagging strategy was designed making use of a mini *URA3* blaster (*URA3-dpl200*) cassette and a FLAG epitope tag. We epitope tag two alleles of *GCD1*, which codes for a subunit of the eIF2B and used a single affinity chromatography step to purify the complex.

MATERIALS AND METHODS

Media and growth conditions

The Candida strains used in this study are listed in Table 1. The CAI4 background was used throughout the study (Fonzi and Irwin, 1993). The CAI4 strain is a *URA3* auxotroph. The strains were grown and maintained as described previously (Egbe *et al.*, 2015). For FLAG-tagged studies, strains were grown in synthetic complete dextrose medium lacking uridine (SCD-ura).

Table 1: **Yeast strains used in this study**

Candida strains	Genotype	Source
CAI4	*ura3/ura3::λimm434/ura3::λimm434*	(Fonzi and Irwin, 1993)
YMK 2311	*ura3/ura3::λimm434/ura3::λimm434-GCD1-4XFLAG-dpl200::GCD1*	This study
YMK 2312	*ura3/ura3::λimm434/ura3::λimm434-GCD1-4XFLAG-dpl200::GCD1-4XFLAG-dpl200.*	This study

Table 2: Identification of the eIF2B subunits by mass spectrometry (MS) analysis

Identified proteins	Mol. Weight (kDa)	NE1 (Untagged)	NE2 (Tagged)
Orf19.407 *GCD6*	82	3	50
Orf19.481 *GCD1*	54	2	31
Orf19.6776 *GCD2*	56	3	7
Orf19.6904 *GCN3*	35	4	3
Orf19.825 *GCD7*	41	-	4

The values shown are the exclusive peptide count indicating the confidence of the identification. It can be seen that there are significantly more peptides identified in the tagged sample NE2 for *GCD6* and *GCD1* indicating these proteins are enriched in sample NE2 relative to the untagged sample NE1.

Construction of the 4X-FLAG-*URA3dpl* tagging cassette

A cassette was designed with four FLAG epitopes and *URA3* as the selectable marker. Four copies of the purifying epitope were used in order to increase the binding of the Flag antibody to the gene of interest. The *URA3* marker was flanked by a 200bp segment of *C. albicans URA3* 3′ sequences to allow PCR amplification of the cassette for transformation as well as marker removal via homologous integration between the repeats, such that reuse is possible. The cassette, 4X-FLAG-*URA3dpl200*, was synthesized commercially (by Biomatik, USA) and cloned into a standard vector (pBMH).

Tagging of the *GCD1* gene with 4X-FLAG-*URA3*dpl cassette

A standard PCR based epitope tagging strategy was taken to tag the *GCD1* gene with the 4X-FLAG-*URA3dpl* cassette. In short, primers were designed with regions complementary to an appropriate region of the *GCD1* gene, as well as a region that allows amplification of the 4X-FLAG-*URA3*dpl cassette. An overview of the strategy for tagging both alleles of the *GCD1* gene is shown in Figure 1 while the precise site of integration for the PCR products is shown in Figure 2. For the first *GCD1* allele, correct integration of the Flag-tag into the genome was verified using PCR reactions on genomic DNA purified from potential transformants (Figure 2). In addition, Western blotting was used to verify that a tagged protein of the correct size (60 kDa) is present in protein extracts from the transformed strain but not in extracts from the parental strain (Figure 3).

In order to facilitate tagging of the second allele of the *GCD1* gene, the *URA3* marker gene was first excised through homologous recombination between the 200bp flanking. *URA3* is a counter-selectable marker in that its loss can be selected for by using growth media supplemented with 5-fluoro-orotic acid (5FOA). Therefore, single colonies where the *URA3* gene has potentially been excised were selected on 5FOA plates to yield Ura^- strains and *URA3* loss was verified using PCR on genomic DNA.

The resulting strain was used in a second transformation with the 4X-FLAG-*URA3*dpl200 cassette. Selection of transformants and verification of the correct integration of the Flag-tag in the second allele of the gene was again performed using PCR on genomic DNA preparations from potential transformants.

Western blot analysis

A 50 ml OD_{600} 0.7 of culture grown in YPD was harvested in a clinical centrifuge at 5000 rpm for 5 min. The cells were lysed, and protein samples were prepared as described previously (Ashe *et al.*, 2000). Immunoblots were probed using anti FLAG antibodies and antibodies raised against yeast translation elongation factor 1 (Tef1p) as a loading control.

Preparation of yeast cell extracts for FLAG-Affinity purification

Yeast cultures were grown to A_{600} = ~0.6, pelleted, snap frozen in liquid nitrogen and ground in lysis buffer (30mM HEPES, 800mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 1mM PMSF, 5mM β-mercaptoethanol, 1% (v/v) Calbiochem Set IV Protease Inhibitor Cocktail, 1µg/ml pepstatin, 1µg/ml leupeptin, 1µg/ml aprotinin in dH_2O pH 7.5) using liquid nitrogen in a freezer mill (Spec 6870, SPEX, UK). Lysates were cleared through two centrifugation steps (15,000 x *g* at 4°C) and quantified using Bradford Reagent.

FLAG Affinity purification of proteins

Protein extracts were made from cultures of the Flag-tagged strain and an untagged strain and affinity purified using a modification of the FLAG affinity purification method (Mohammed-Qureshi *et al*. 2007). A 400µl of ANTI-FLAG M2 Magnetic beads (Sigma Aldrich Company Ltd, Dorset, UK) was pre-washed twice with 1ml lysis buffer to remove the storage buffer. Whole cell extract (10mg of total protein) was added to the beads and incubated with rotation for 2 h at 4°C. The supernatant (flowthrough) was removed from the beads, which were further washed three times in 1ml lysis buffer for 1 min and twice for 15 min in 1ml low salt buffer (30mM HEPES, 100mM KCl, 10% glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 1mM PMSF, 5mM β-mercaptoethanol, 1% (v/v) Calbiochem Set IV Protease Inhibitor Cocktail, 1µg/ml pepstatin, 1µg/ml leupeptin, 1µg/ml aprotinin in dH_2O pH 7.5). Bound proteins were eluted by incubation with 500µl elution buffer (30mM HEPES, 100mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 1mM PMSF, 5mM β-mercaptoethanol, 1% (v/v) Calbiochem Set IV Protease Inhibitor Cocktail, 1µg/ml pepstatin, 1µg/ml leupeptin, 1µg/ml aprotinin in dH_2O pH 7.5, 0.1mg/ml 3X FLAG peptide (Sigma) for 2 h at 4°C with constant rotation and the resulting eluate was collected. Protein eluates were concentrated to 20 µl using Amicon Ultra 0.5mL Filters with a 3 KDa cut off (Millipore, Billerica, MA, USA) and loaded onto a 12% SDS-PAGE resolving gel. Samples were electrophoresed into the top 1 cm of the gel and stained by Instant Blue Stain (Expedeon Limited, Cambridge, UK). The immunoprecipitated proteins were excised from the gel, and processed by the Michael Smith Biological Mass Spectrometry Facility.

Mass spectrometric analysis

The SDS PAGE resolving gels were removed from the gel running apparatus and soaked in Simply Blue SafeStain (Invitrogen) for 1 h, then washed in water. Bands stained with Coomassie were excised from SDS PAGE gels and dehydrated using 400µl of 100% acetonitrile followed by vacuum centrifugation. Dried gel pieces were reduced with 10mM dithiothreitol and alkylated with 55mM iodoacetamide. Gel pieces were then washed alternately with 25mM ammonium bicarbonate followed by acetonitrile. This was repeated, and the gel pieces dried by vacuum centrifugation. Then 100ng sequencing grade trypsin (Sigma), diluted in 50mM ammonium bicarbonate, was added and the gel samples were incubated overnight at 37°C. Following digestion, the supernatants were removed to a fresh tube.

Digested samples were analysed by LC-MS/MS using an UltiMate® 3000 Rapid Separation liquid chromatography (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to a LTQ Velos Pro (Thermo Fisher Scientific, Waltham, MA) mass spectrometer.

Peptide mixtures were separated using a gradient from 92% solution A (0.1% formic acid (FA) in water) and 8% solution B (0.1% FA in acetonitrile) to 33% solution B, in 44 min at 300nl min^{-1}, using a 250mm x 75µm i.d 1.7µM BEH (Ethylene Bridged Hybrid) C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data dependent analysis.

Data Analysis

Data produced were searched using Mascot (Matrix Science UK), against the Uniprot database with taxonomy of [*C. albicans*] selected. Data were validated using Scaffold (Proteome Software, Portland, OR).

RESULTS

The expected PCR products generated from strains having two Flag-tagged alleles of *GCD1* were obtained- the lane labelled '1st and 2nd' contains a

DNA band for the first tagged allele, a DNA band for the second tagged allele yet no DNA band where the untagged allele migrates (Figures 2 and 3). Western analysis using an antibody raised against Flag protein also showed approximately 2-fold higher levels of protein from the strain in which the two *GCD1* alleles were tagged relative to the strain where only one allele of the gene is tagged (Figure 3).

FLAG affinity purification of eIF2B for analysis by mass spectrometry

The purified proteins separated by SDS PAGE alongside whole cell extract samples from the tagged and untagged strains and visualized by staining with Coomassie blue are shown in Figure 4. From the gel, some of the protein bands are of the correct sizes (Figure 4) for subunits of eIF2B. In particular, the bands at ~80kDa and ~55kDa are eIF2Bε and eIF2Bγ, especially given that eIF2Bγ is the tagged subunit and work across eukaryotes has shown that this subunit forms a catalytic sub-complex with eIF2Bε (Koonin, 1995).

In order to clarify which eIF2B subunits were present in the samples, immunoprecipitated samples were briefly run into an SDS PAGE gel, stained and protein bands were excised from the gel and analysed by mass spectrometry. Table 2 shows that eIF2B subunits were enriched in the sample, as shown by the number of peptides present in samples generated from the tagged strains relative to untagged. In particular, the eIF2Bγ and ε subunits, which are known to form the catalytic sub-complex, are heavily enriched in the tagged samples. Therefore, these data shows that the double tagging of the two *GCD1* alleles with 4xFLAG epitope has successfully allowed purification of subunits of eIF2B.

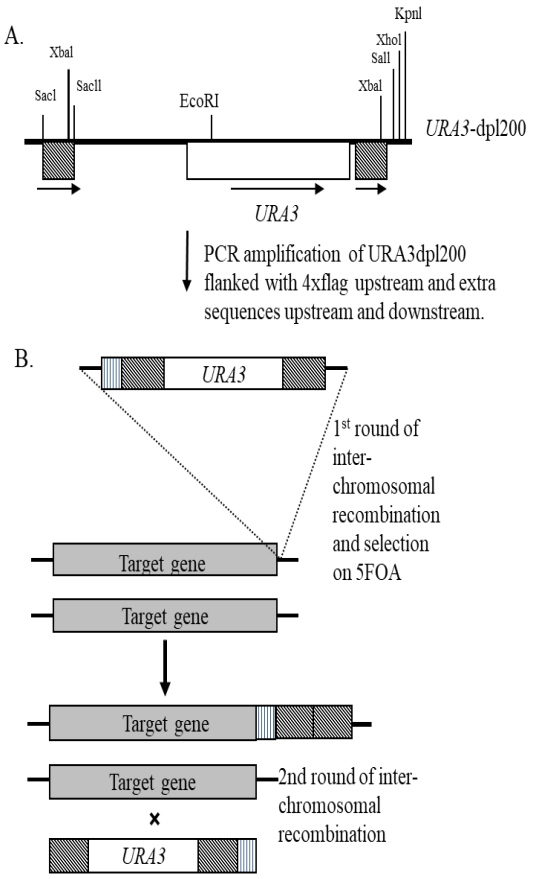


Figure 1: Strategy for tagging the two alleles of *C. albicans* genes using the mini Ura-blaster technique. (A) Restriction map of *URA3*-dpl200 cassette from plasmid pDDB57. The locations of the URA ORF (white box) and duplicated 3′ sequences (hatched boxes) are shown. (B) The schematic of the sequential tagging of the two alleles of a gene, epitope tag (stripped box).

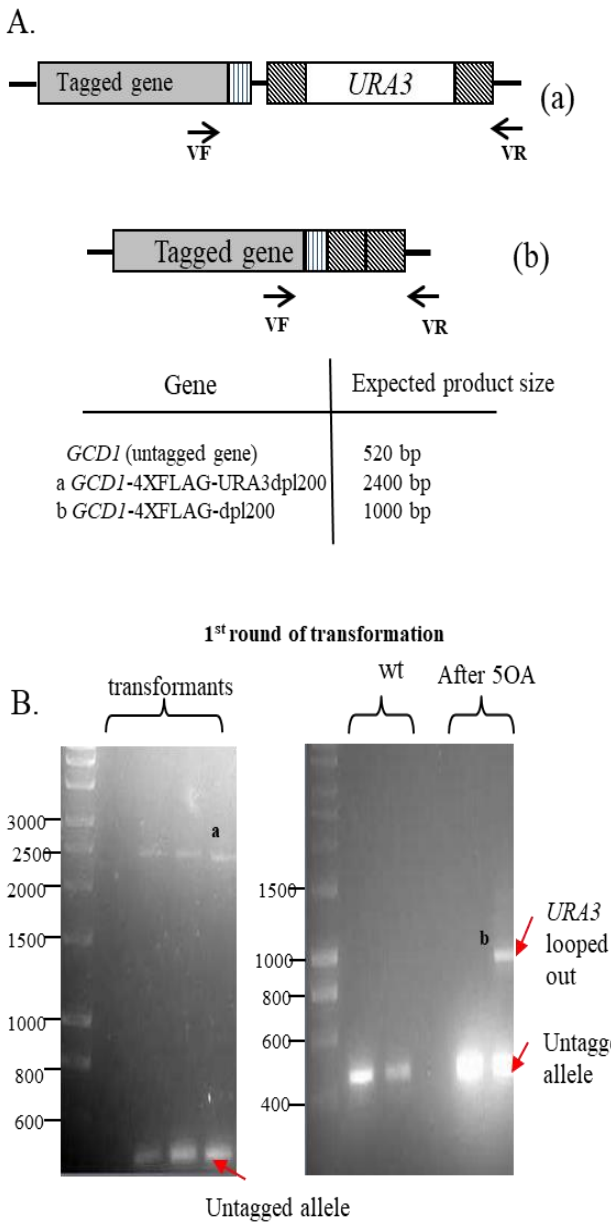


Figure 2: Verification of genomic C-terminal FLAG tagging of the first allele of *GCD1* in CAI4 strain. A. Schematic of PCR strategy used to verify genomic integration of the FLAG-tag and the expected PCR product sizes. B. Agarose DNA electrophoresis of PCR products. (a) refers to the transformants bearing the whole tagging cassette, while (b) refers to transformants that have lost the *URA3* gene.

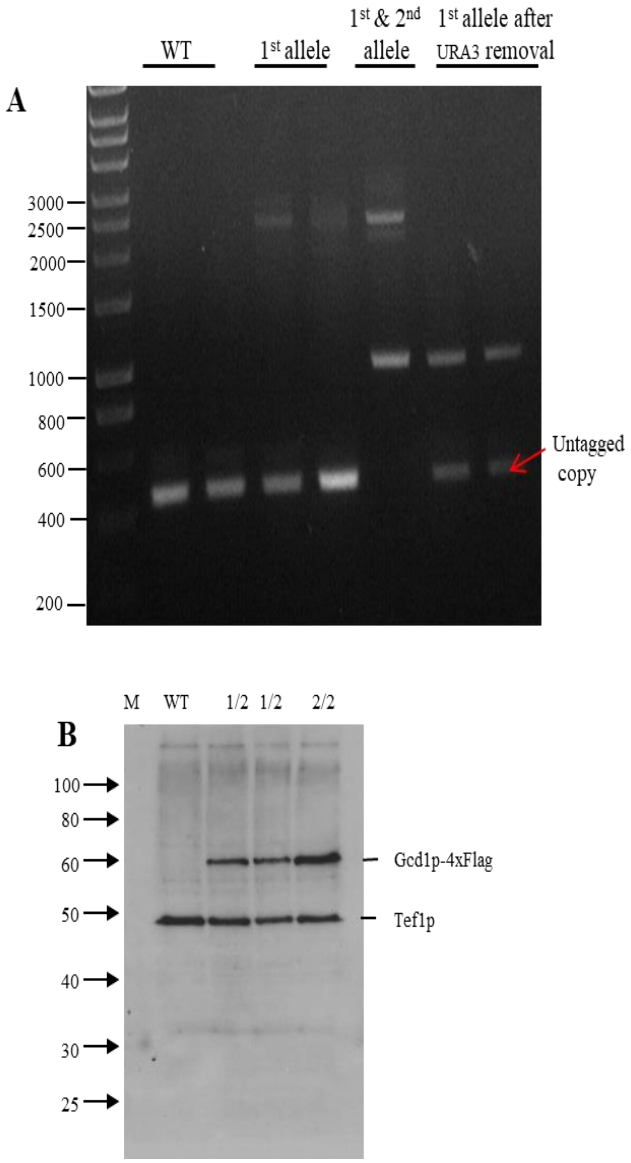
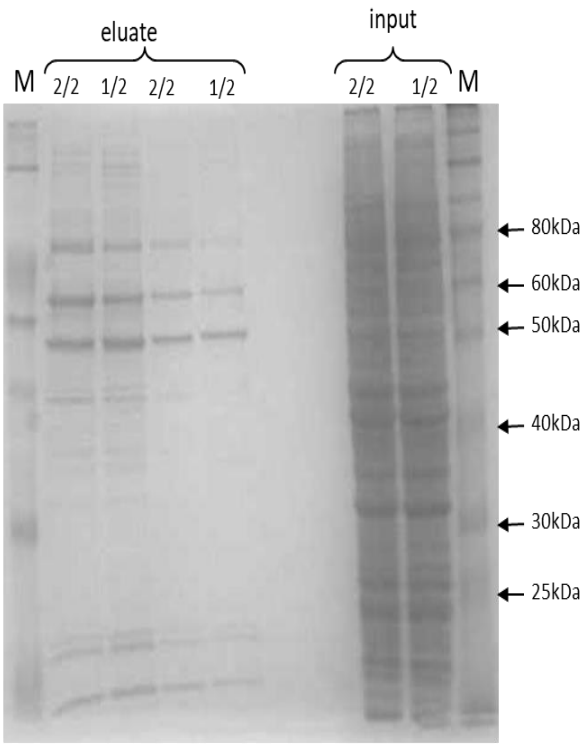


Figure 3: Verification of genomic C-terminal 4X FLAG tagging of the second allele of *GCD1* in CAI4 strain. A. Agarose DNA electrophoresis of PCR products generated by amplification of genome extracts. Product sizes are as presented in Figure 2. **B**. Immunoblots on extracts generated from the indicated strains. (1/2 and 2/2 refer to one and two alleles of genes tagged with 4xFLAG epitope)



eIF2B subunits	Mol. Weight (kDa)
Gcd6 ε	81.9
Gcd2 δ	55.9
Gcd1 γ	54.3
Gcd7 β	41.0
Gcn3 α	35.1

Molecular weights of *Candida albicans* eIF2B subunits protein extracts

Key: M-Protein marker

Figure 4: FLAG affinity purified eIF2B subunits protein extracts. Coomassie stained gels of FLAG affinity purified eIF2B from *Candida* strains bearing one copy and two copies of the 4xFLAG tag.

DISCUSSION

The eukaryotic initiation factor 2B (eIF2B) is a five-subunit guanine nucleotide exchange factor, that functions during translation initiation to catalyze the otherwise slow exchange of GDP for GTP on its substrate eIF2. The study of the effect of various stressful conditions that affect translation initiation requires that eIF2B complex or subcomplex be enriched and purified from the cell lysate. A tagging strategy with four FLAG epitopes making use of a mini *URA3* blaster (*URA3-dpl200*) cassette was utilized to tag two alleles of *GCD1 of C. albicans.*

The results obtained from this work showed that double tagging of the two allelles of the gene of a predominantly diploid organism like *C. albicans* can be achieved using a modification of the mini *URA3* blaster cassette. Although the cassette was initially designed for gene deletions in *C. albicans* (Wilson *et al.*, 2000; Ganguly and Mitchell, 2012), here it was adapted for the sequential FLAG-epitope tagging of two alleles of the *GCD1* gene in *C. albicans.*

Previously a combined V5-6xHis epitope tag (Milne *et al*., 2011) was used to tag the *GCD1* gene, however the proteins could not be purified, nor were they detected by mass spectrometry (Unpublished data). There are a number of possible explanations for the difficulty in purifying the eIF2B protein including insufficient protein levels or inaccessibility of the epitope tag. In *S. cerevisiae*, an eIF2B purification system has been developed, where all five eIF2B subunits are overexpressed from two plasmids, and FLAG epitopes on eIF2Bγ allow purification (Mohammed-Qureshi *et al*., 2007). *C. albicans* is predominantly diploid and autonomous plasmid systems developed for routine use are unstable in this organism (Goshorn *et al*., 1992). Moreover the recently identified haploid phase of *C. albicans* has also been shown to exhibit reduced fitness (Hickman *et al*., 2013) Therefore, to tag the two alleles of the gene that code for eIF2Bγ in this diploid, a different tagging system was designed using the 4xFLAG epitope tag. The FLAG tag system (Sigma-Aldrich) as reported by Mohammed-Qureshi *et al*., (2007) was found to have greater specificity and enabled purification from a cell lysate in a single affinity step. This system enabled the successful purification of a subcomplex of eIF2B.

CONCLUSION

The purification of *C. albicans* eIF2B complex will ovide a greater insight into molecular mechanisms of integrated stress response and also enhance our understanding of the structure and function of eIF2B, a key conserved protein complex that plays critical role in protein translation initiation across eukaryotes. The *URA3* blaster technique previously designed for gene deletion in *C.*

albicans was successfully adapted to epitope tag the two copies of *GCDI* which code for a subunit of the eIF2B and subsequent purification of the protein complex by affinity chromatography. These results pave the way for the generation of endogenously tagged allelic copies of genes in diploid cells that will allow the biochemical isolation of native protein complexes for proteomic studies as well as studies of protein localization in diploid cells.

Acknowledgements
Grateful thanks to David Knight and Emma-Jane Keevil in the Biomolecular Analysis Core Facility, The University of Manchester for their help with mass spectrometry analysis. NEE was funded by the Tertiary education trust fund Nigeria,

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