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Functional diversity of complex I subunits in *Candida albicans* mitochondria

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Abstract

Our interest in the mitochondria of *Candida albicans* has progressed to the identification of several proteins that are critical to complex I (CI) activity. We speculated that there should be major functional differences at the protein level between mammalian and fungal mitochondria CI. In our pursuit of this idea, we were helped by published data of CI subunit proteins from a broad diversity of species that included two subunit proteins that are not found in mammals. These subunit proteins have been designated as Nuo1p and Nuo2p (NADH-ubiquinone oxidoreductases). Since functional assignments of both *C. albicans* proteins were unknown, other than having a putative NADH-oxidoreductase activity, we constructed knock-out strains that could be compared to parental cells. The relevance of our research relates to the critical roles of both proteins in cell biology and pathogenesis and their absence in mammals. These features suggest they may be exploited in antifungal drug discovery.

Initially, we characterized Goa1p that apparently regulates CI activity but is not a CI subunit protein. We have used the *goa1*Δ for comparisons to Nuo1p and Nuo2p. We have demonstrated the critical role of these proteins in maintaining CI activities, virulence, and prolonging life span. More recently, transcriptional profiling of the three mutants and an *ndh51*Δ (protein is a highly conserved CI subunit) has revealed that there are overlapping yet also different functional assignments that suggest subunit specificity. The differences and similarities of each are described below along with our hypotheses to explain these data. Our conclusion and perspective is that the *C. albicans* CI subunit proteins are highly conserved except for two that define non-mammalian functions.

Keywords

accessory subunits; mitochondria; cristae formation; *Candida albicans*

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1. Introduction

The scope of this review is focused upon a knowledge base of complex I (CI) subunit proteins from *C. albicans*. As there are 39 subunit proteins in the *C. albicans* CI, the task of characterizing all would be immense. So, our research emphasis is primarily on two fungal-specific subunit proteins (Nuo1p and Nuo2p) and one other (Goa1p) that regulates CI activities. In assigning functions to these proteins, we have demonstrated their critical roles in cell biology that transcend their activity as only sources of cell energy. There would appear to be a significant level of subunit specificity in their cell activities, discussed below. This concept has incentivized our effort to explore subunit-specific activities further. Our data are also relevant to the disease process and antifungal susceptibility.

Structural and comparative genomic studies of nuclear and mitochondrial genomes of yeast have shown that roughly 10% of nuclear-encoded genes are involved in mitochondrial biogenesis and function (Marcotte et al. 2000). Also, the percentage of organism-specific mitochondrial genes is very high in yeast (22%) relative to, for example, worm (1%) (Marcotte et al. 2000). These genomic differences suggest that mitochondria carry species-specific functions that probably reflect their survival in their environmental niches. With regard to the contribution of mitochondria proteins to antifungal drug discovery, concerns are that the broad similarity of fungal and human mitochondria precludes their development as fungal-specific drug targets. However, our published data demonstrate the presence of two fungal-specific mitochondrial CI subunit proteins, Nuo1p and Nuo2p (She et al. 2015) and a third mitochondrial regulator of CI, Goa1p (Bambach et al. 2009).

The annotation of CI subunits has not been completed in all eukaryotes, which is particularly true when the focus shifts to accessory subunits (Gabaldón et al. 2005; Brandt 2006; Vinothkumar et al. 2014). Some accessory subunits may play a role in preventing high energy electron leakage during the electron transfer between ETC (electron transport chain) complexes as they physically surround the functional core, stabilizing CI redox groups (Guénebaut et al. 1998), regulating CI assembly (Ugalde et al. 2004; Marques et al. 2005), as observed in Nuo1p, Nuo2p (She et al. 2015) and Goa1p (Bambach et al. 2009), and mediating signal transduction when fungi are challenged with cell death inducers (reviewed in Kmita and Zickermann 2013; Gonçalves et al. 2015). Other functions have been assigned to CI subunits as an acyl carrier protein (Sackmann et al. 1991; Schneider et al. 1995; Dobrynin et al. 2010). In fact, two acyl carrier proteins (ACPM1 and ACPM2) were identified in *C. albicans* when compared to paralogs from mammals or *Neurospora crassa* (Runswick et al. 1991). The mitochondrial acyl carrier proteins are proposed either to participate in prosthetic group synthesis for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes or direct long chain fatty acid synthesis for repair of mitochondrial membrane damages (Kmita and Zickermann 2013).

2. Results and Discussion

2.1 Mitochondrial CI composition in *C. albicans*

Mitochondria in part are derived from α-proteobacteria in eukaryotic cells to meet their energy demands for cellular activities and survival (Gray et al. 2001). Mitochondrial

complex I (CI) – the first protein complex of the ETC – is needed to provide a proton motor force for complex V (CV) to synthesize ATP. However, CI is absent in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Schatz et al. 1966). In these organisms, NADH oxidation is performed by the alternative mitochondrial NADH:ubiquinone oxidoreductase NDI1 (Maas et al. 2010), which is present in *C. albicans (NDE1 and YMX6)* as well. These monomer enzymes face the matrix side of the mitochondrial inner membrane and do not transfer protons across the mitochondrial inner membrane. Such genetic differences reduce the value of *S. cerevisiae* as model to study CI. The absence of mitochondrial CI is believed to provide an advantage to fermentative yeast species for their ecological adaptation (Merico et al. 2007). This critical evolutionary event is believed to have occurred when an ancestor of *Saccharomyces* diverged around 100 million years ago from other yeasts such as *Candida* (reviewed in Chou and Leu, 2010), when fruit-bearing species emerged (Merico et al. 2007). Intriguingly, unlike other pathogenic *Candida* spp. such as *C. parapsilosis*, *C. tropicalis*, CI does not exist in *C. glabrata* as well, yet this species has become the second most common cause of candidiasis (Koszul et al. 2003).

2.1.1 The CI of *C. albicans*—In *C. albicans*, the molecular mass of CI is around 800 kDa according to BN-PAGE electrophoresis data (Li et al. 2011). CI is composed of at least 39 subunit proteins which are either mammalian paralogs or fungal-specific subunits (She et al. 2015). Functionally, the *C. albicans* CI is conserved in mammalian and even prokaryotic cells that couple the TCA cycle with oxidization of NADH, except for the few exceptions mentioned above (reviewed in Friedrich and Scheide, 2000). To date, the CI structure in *C. albicans* remains somewhat unclear, but its L-shape is consistent among eukaryotic species (Guénebaut et al. 1998; Efremov et al. 2010).

Like most eukaryotes, *C. albicans* has 14 core CI genes of a bacterial progenitor that are proton-pumping NADH: ubiquinone oxidoreductases, seven of which are encoded by mitochondrial DNA (*CaalfMP03, CaalfMP09,CaalfMP10,CaalfMP14, CaalfMP13, CaalfMP02,CaalfMP2*); seven other subunit genes are nuclear-encoded [Ndh51 (NDUFV1), orf19.2091(NDUFV2), orf19.7560(NDUFS1), orf19.6531(NDUFS2), orf19.1710(NDUFS3), orf19.4758(NDUFS8), orf19.6794(NDUFS7)]. The first group of mitochondrial synthesized proteins is located in the membrane arm of CI, while the second group of seven proteins is predicted to be on the hydrophilic arm. To better compare the subunit functions of later group, bovine paralogs are indicated in parenthesis. For example, NDUFV1 is a NADH dehydrogenase ubiquinone flavoprotein 1 (for NADH binding) and NDUFS1 is NADH dehydrogenase ubiquinone Fe - S protein (for quinone binding). As expressed here with “orf” numbers, these nuclear-encoded genes in *C. albicans* are not studied yet except for *NDH51* (McDonough et al. 2002; Vellucci et al. 2007). The functions of Ndh51p have been further analyzed in our lab (She et al. 2013; Sun et al. 2013a).

2.1.2 Unique CI subunit proteins—The remaining sections of this review focus upon the activities of Nuo1p, Nuo2p and Goa1p, which are either broadly fungal specific (Nuo1p, Nuo2p) or unique to most *Candida* species (Goa1p). The phenotypes of mutants are listed in Table 1. Ndh51p (mentioned above) is part of the conserved CI core of numerous species

including fungi. The *ndh51*Δ is useful in comparing phenotypes (and functions) to *nuo1*Δ, *nuo2*Δ, and *goa1*Δ.

Eukaryotic CI accessory proteins are assembled by their addition to the prokaryotic minimal structure. Therefore, the functional difference among organisms and species is mostly associated with the non-core proteins. In the case of *C. albicans,* 25 proteins including Nuo1p, Nuo2p belong to this non-core group. Both Nuo1p and Nuo2p have been confirmed as CI subunits in *N. crassa* even through their precise locations in CI are not determined (Azevedo et al. 1992; Marques, et al. 2005). As stated, both proteins are fungal-specific or share specificity with lower eukaryotes but not with mammals.

The mitochondrial respiratory and enzymatic deficiencies of *goa1*Δ suggest that Goa1p is strongly associated with CI functions (Table 1), but apparently, Goa1p is not a CI subunit. This protein translocates to mitochondria during oxidative stress (Bambach et al. 2009). But, how long the protein remains in mitochondria and its binding site are not resolved. *C. albicans* is a member of the CTG clade of the Ascomycetous Saccharomycotina, of which CTG is translated as “serine” instead of universal “leucine.” The CTG clade includes all *Candida* pathogens except *C. glabrata*. We believe Goa1p is a CI regulator. More recently, we have focused on Nuo1p and Nuo2p since they are broadly conserved among pathogenic fungi. Their specificity and functional activities suggest that both proteins are potential drug targets.

2.2 CI integrity depends on CI core and non-core subunits

In the context of ATP synthesis, the mitochondrial ATP pathway is highly efficient even though other alternative respiration and fermentative modes are also important for *C. albicans* to survive in the host (Helmerhorst et al. 2002; Lorenz et al. 2004). CI functionally interacts with other ETC protein complexes by forming super protein complexes. However, super complex formation and regulation are not well understood. Given the apparent amount of energy required to form super complexes, likely, super complex formation is not always an “on” process. We speculate that a signal transduction network may guide this dynamic process.

In our previous studies, we demonstrated that Nuo1p, Nuo2p, Goa1p, and Ndh51p of *C. albicans* are required for CI assembly (Li, et al. 2011; She et al. 2015). However, mechanistically, CI assembly in *Candida* is unstudied. Likely, the CI of *C. albicans* retains an L-shaped structure that consists of a hydrophobic inner membrane arm and a peripheral arm which projects into the mitochondrial matrix. By tracking a single subunit of CI to its structural location, a few assembly models have been proposed for mammalian CI (Antonicka et al. 2003; Ugalde et al. 2004) and *N. crassa* CI (Tuschen G, 1990). The difference among these models is that either the two arms of CI are formed independently then joined or joined at an early stage of assembly. The location of the two fungal-specific CI subunits would be interesting to determine, given their possible roles in CI assembly as observed previously. We hope to understand CI assembly in *C. albicans*.

In mammalian cells, a number of assembly factors are involved with CI assembly – namely, Ndufaf1, Ndufaf2, Ndufaf3, Ndufaf4, Ndufaf5, and Ecsit (Vogel et al. 2007; McKenzie and

Ryan, 2010). Each protein has a different role in CI assembly and is active at different stages of assembly. The corresponding paralogs of the *C. albicans* putative assembly factors and their transcriptional profiles in each of the four mutants are listed in Table 2.

According to the McKenzie model (McKenzie and Ryan 2010), the CI Q module (a hydrogenase) forms during an early stage of CI assembly and requires the Ndufaf3, Ndufaf4, and Ndufaf5 assembly factors. Ndufaf3 and Ndufaf4 are not found, but Ndufaf5 (orf19.2541) was identified in *C. albicans* as a mammalian paralog in the *Candida* Genome Database (CGD) (http://www.candidagenome.org/). In mammalian cells, Ndufaf5 is required for insertion of the ND1(mitochondrial encoded CI subunit) into the membrane arm. The Ndufaf5 assembly factor contains an S-adenosylmethionine-dependent methyltransferase (SAM) domain, which is also conserved in the *C. albicans* paralog. The existence of such a catalytic feature suggests that *C. albicans* CI subunits may also be modified by methylation for a full function (Carroll et al. 2005). Each of the four *C. albicans* CI mutants expresses normal levels of Ndufaf5, suggesting that assembly defects likely do not occur at an early stage of CI assembly.

During the middle stage of CI assembly, ND2, ND3, and ND6 are recruited to the early stage CI assembly body to form an intermediate subcomplex by Ndufaf1 (CIA30) and Ecsit in mammalian cells (Küffner et al. 1998; Bourges et al. 2004). In *N. crassa*, two proteins, CIA84 (84kDa) and CIA30 (30kDa), are required for middle stage maturation of CI (Küffner et al. 1998). We did not identify Ecsit and CIA84 paralogs in *C. albicans*. However, CIA30, a mitochondrial CI intermediate-associated protein 30, has been identified (Dunning et al. 2007). The deletion of CIA30 in *N. crassa* resulted in an accumulation of the CI matrix and small membrane arms. The expression levels of *CIA30* in all mutants (*goa1*Δ, *ndh51*Δ, *nuo1*Δ, *nuo2*Δ) are down-regulated (Table 2), with a maximum of -4.66 fold in *goa1*Δ. If a similar assembly process is also present in *C. albicans*, we propose that middle stage assembly may be interrupted in these CI mutants. The detection of intermediate products in each CI mutant with signature antibodies to each CI arm will answer this question. Intriguingly, CIA30 is also present in *S. pombe* which does not contain a CI (Janssen et al. 2002). The broadly conserved CIA protein indicates that it is not directly involved in oxidative phosphorylation of the CI substrate.

In the CI assembly model of mammalian cells, Ndufaf2 is a check-point in the late-stage assembly pathway. Unlike other assembly factors, Ndufaf2 is also a structural component of CI that directs N-module (electron input) binding to Q (electron output) and P (proton translocation) modules for CI maturation. Ndh51p is located in the N-module, which is responsible for NADH binding and oxidization. As shown in Table 2, Ndufaf2 is increased transcriptionally in *ndh51*Δ, *nuo1*Δ and *nuo2*Δ compared to wild type cells. Both *nuo1*Δ and *nuo2*Δ have more than a 3-fold higher Ndufaf2 expression (She et al. 2015).

In filamentous Ascomycetes, pentatricopeptide repeat proteins (PPP) are required for CI assembly (Solotoff et al. 2015). PPP proteins are implicated in mitochondrial RNA metabolism. The deletion of PPP genes affected synthesis and stability of transcripts of mitochondrial-encoded NDs that in turn caused CI disassembly. The biochemical roles of each CI subunit in CI assembly in *C. albicans* need additional characterization.

2.3 Functional differences other than NADH oxidation among CI subunit mutants

The functional phenotypes of each null mutant (*goa1*Δ, *nuo1*Δ, *nuo2*Δ, and *ndh51*Δ) are listed in Table 1. These data summarize the critical roles of their gene products in maintaining mitochondrial CI activities *in vitro*. Except for *ndh51*Δ which has not been tested, each of the other three mutants is avirulent in a disseminated candidiasis murine model (Bambach et al. 2009; She et al. 2013; She et al. 2015). The reduction of CI enzymatic activity in each mutant and CI disassembly prevents optimal ATP production for cellular activities, and elevates ROS levels that further damage the mitochondria and reduce cell life span (Bambach et al. 2009; Li et al. 2011; Chen et al. 2012; She et al. 2015).

2.3.1 Glycerol utilization—The growth phenotypes in a glycerol medium have been used to evaluate a respiratory stress response. Non-fermentable carbon sources force *C. albicans* cells to utilize a mitochondrial oxidation pathway for ATP production. Therefore, growth arrest occurs when the mitochondrial respiratory pathway is inhibited in the mitochondrial mutants. In our previous findings, most but not all CI mutants (11/13) failed to grow in glycerol (Sun et al. 2013a). These genes either evolved from a prokaryotic ancestor or are of eukaryotic origin (Gabaldón et al. 2005; Friedrich and Scheide 2000). Two exceptions for the glycerol growth phenotype are mutants in *orf19.3611* (*NDUFS6*) and *orf19.3290* (*NDUFS4*); both gene products are derived from eukaryotic origin. The precise roles of these two proteins in CI are unknown but likely have minor roles in oxidative respiration. In contrast, the other 11 CI subunits, including the four subunits we mentioned above, have critical roles in NADH oxidation.

2.3.2 Azole sensitivity—Studies of individual CI subunits in other species indicate that cell functions other than NADH oxidation may occur. Many of CI subunit proteins have alternative cellular roles in metabolism such as ACPM, mentioned above. On the other hand, in *N. crassa*, some CI mutants have lost CI enzymatic activity completely even through the CI complex structure is intact (Gonçalves, 2015). In the presence of fluconazole, most (11/13) CI mutants of *C. albicans* have growth defects. The hypersusceptibility to fluconazole is correlated with their lack of growth on a glycerol medium (Sun et al. 2013a). Only the *ndufs4*Δ mutant was not hypersusceptible to fluconazole. These data support the idea that CI subunits in *C. albicans* may also regulate or be associated with other cellular activities in a gene-specific manner.

The cause of hypersusceptibility to fluconazole varies among the CI mutants (Sun et al. 2013). For example, genes of ergosterol biosynthesis (ERGs) are remarkably reduced transcriptionally in *ndh51*Δ. We found that 12/19 *ERG* genes are down-regulated with an average fold change of -4.56 (-2.24 ∼ -7.54 fold). Notably, a significant number of *ERGs* are also reduced in *nuo1*Δ (7 *ERGs*) and *nuo2*Δ (6 ERGs) even though the overall fold changes are less on average (-2.5). Meanwhile, *ERG2* and *ERG25* are up-regulated in both *nuo*Δ strains. A correlation of reduced ergosterol levels and mitochondrial energetics was also observed by others in different mitochondrial mutants (Thomas et al. 2013). We predict that Goa1p has a minor role in ergosterol synthesis because *ERG27* (-2.37) is only gene that was down regulated in the ergosterol synthesis pathway. The significance of fluconazole

hypersusceptiblility needs to be extended to studies of *C. albicans* biofilms, since resistance to azoles has been reported in biofilms (Mathe and VanDijck, 2013).

Mechanisms other than ergosterol synthesis may also contribute to azole sensitivity in *goa1*Δ and *nuo*Δ mutants. For example, up regulation of efflux proteins of the MFS (major facilitator superfamily) are correlated with a hypersusceptible phenotype of mitochondrial mutants (Thomas et al. 2013; Sun et al. 2013b). There are a number of nuclear-encoded mitochondria proteins that are fungal-specific and putative efflux proteins (Marcotte et al. 2000). Interestingly, 21-28% of nuclear encoded proteins have unknown functions in mitochondria. A number of antibiotic transporter genes are included. These gene homologs in *C. albicans* are reduced 2.9 ∼ 8.6 fold transcriptionally in *goa1*Δ (orf19.1308, orf19.3395 and *SGE1*) and 3.2 ∼ 6.4 fold in *nuo*Δ mutants (orf19.1308, *TPO3*). The gene product of *TPO3* is a polyamine transporter that belongs to MFS family that is often co-overexpressed with MDR genes in clinical fluconazole resistant strain. The reduction of MFS efflux in CI mutants (*goa1*Δ and *nuo*Δ) perhaps increases intracellular azole concentration.

2.3.3 Cell membrane-related functions—Compositional alterations of phospholipids in the cytoplasmic membrane have a significant impact on *Candida* adherence and biofilm formation (Ghannoum et al. 1986; Lattif et al. 2011). In *C. albicans*, a large number of cell surface adhesin proteins are GPI-anchored in the cell membrane that in turn interacts with host immune cell receptors (Gow and Hube 2012). Moreover, the most striking feature of GPI-anchored proteins is their enrichment in lipid rafts (Simons and Sampaio 2011). Lipid rafts in the yeast cell membrane are enriched by 3 ∼ 5 fold in ergosterol and glycosphingolipids than the surrounding lipid bilayer (Mollinedo, 2012), which presumably serves as an organizing center for signaling and membrane protein trafficking (Pike 2004).

We have observed that the three proteins (Goa1p, Nuo1p, and Nuo2p) are all required for an optimal colonization in host tissue (Bambach et al. 2009; She et al. 2015) and, at least for Goa1p, binding to epithelial cell lines (She et al. 2013). As we discussed above, the reduction of ERG expression in three CI mutants (*ndh51*Δ and *nuo*Δ) likely caused changes in the ergosterol/phospholipid ratio that in turn alter the composition of cell membranes even if the synthesis of phospholipid is not affected. However, it seems that a different strategy occurs in *goa1*Δ. Without *ERG* changes, the entire *ALS* gene family and genes for first step of GPI synthesis are reduced in this mutant. In addition, phospholipids and lipid metabolism are affected more in *goa1*Δ than in the other three CI mutants. Therefore, while the other CI subunits modulate ERG synthesis, Goa1p may regulate cell membrane phospholipid metabolism.

The composition of the mitochondrial inner membrane is quite different from that of the cytoplasmic membrane since the former has abundant cardiolipin content (Hsu et al. 2005; Minkler and Hoppel 2010). Data from mammalian cells indicate that this type of phospholipid is required for ETC functions by assisting complex insertion at cristae and the inner membrane of mitochondria (Böttinger, 2012). Transcriptionally, gene expression for mitochondrial cristae and inner membrane organization varies among the mutants listed in Table 3. In agreement with general phospholipid metabolism, these genes are all down-regulated in *goa1*Δ. For example, *TAZ1* and *orf19.6062.3* are reduced more than 15-fold in

*goa1*Δ. Taz1p is required for cardiolipin synthesis while orf19.6062.3 is required for cristae formation. Both are down regulated in the *ndh51*Δ mutant remarkably but not in the *nuo*Δ mutants.

In *nuo1*Δ and *nuo2*Δ mutants, mitochondrial inner membrane related genes (Table 3) are less affected; oppositely, other gene expressions are increased including *TAZ1*. Among repressed genes, orf19.6060 that putatively is responsible for assembly of the mitochondrial eisosome rafts is reduced 3 ∼ 4 fold in both *nuo*Δ mutants compared to WT cells. The eisosome cell membrane domain plays a crucial role in reshaping the plasma membrane (Ziółkowska et al. 2011). Consistent with orf19.6060, two eisosome component genes – *LSP1* and *PIL1*, are also reduced transcriptionally in both *nuo*Δ mutants. In combination with down-regulation of ERGs in *nuo*Δ mutants described above, the membrane rafts of cytoplasm and mitochondria membranes may be further disturbed in *nuo*Δ mutants that in turn alter the adhesion properties and colonization of host tissue. Thus, while Goa1p regulates membrane phospholipid metabolism of both the cytoplasm and mitochondria membranes, Nuo1p and Nuo2p play roles in construction of membrane domains. Ndh51p is crucial for NADH binding in CI, but it seems to be involved with mitochondrial inner membrane cardiolipin and cristae formation as well. The large influence on ergosterol synthesis and less effect on cytoplasm phospholipid metabolism distinguishes Ndh51p from Goa1p in regards to cell membrane construction. No matter which mechanism is affected, each of the CI subunit proteins more or less contributes to mitochondrial inner membrane and cristae formation.

2.3.4 Cell wall synthesis—Mitochondrial dysfunction in *C. albicans* is known to be associated with loss of cell wall integrity (Dagley et al. 2011; She et al. 2013). Recently we have shown by transmission electron microscopy that in *goa1*Δ, the cell wall has three major defects: 1). the outer cell wall fibrils are shorter; 2) the inner cell wall is more electron transparent but the diameter of the inner cell wall is equal to WT cells; 3) lack of a typical electron dense layer in juxtaposition to the cell plasma membrane. The quantitative study of cell wall carbohydrates demonstrated a major loss of mannan polysaccharide of larger and intermediate molecular weights in *goa1*Δ when compared to WT. NMR studies demonstrated that β-1,2 linked mannan is reduced in the *goa1*Δ mutant (submitted). Such defects in the outmost cell wall layer resulted in an increased sensitivity to caspofungin, an inefficient binding to Alcian blue (measures phosphomannan), and growth inhibition on medium supplemented with Congo red and Calcofluor white (She et al. 2013). In agreement with these phenotypes and chemical analysis in *goa1*Δ, the genes for cell wall synthesis have two major changes in transcriptional levels, 1) reduced β-mannosyltransferase gene (BMTs) expression, particular *BMT3 - 9*, which are required for β-mannan elongation either in phosphopeptidomannan or in phospholipomannan (Mille et al. 2008; Fabre et al. 2014). 2) Reduced glucanase gene expression, which is required for linkage formation among β-1,6 cell wall glucan (Kollár et al. 1997).

A similar transcriptional profile of cell wall synthesis appears in *nuo1*Δ and *nuo2*Δ mutants in regard to α- and β- mannosyltransferases (She et al. 2015). However, chitin synthase/ chitinase and protein mannosyltransferase reduced more significant in *nuo*Δ mutants than other two CI mutants. A comparison of cell wall composition in *nuo*Δ mutants is planned.

That data may answer a few key questions about how mitochondrial CI regulates cell wall assembly. However, in preliminary phenotype studies, both mutants are inhibited by caspofungin and Alcian blue binding is reduced, suggesting that Nuo1p and Nuo2p also modify the cell wall.

On the other hand, fewer cell wall synthesis genes are changed in *ndh51*Δ even though growth inhibition was also seen in medium containing Congo red and Calcofluor white (She et al. 2013). As described above for other functions, cell wall data of mutants also demonstrate functional differences and similarities in CI subunit proteins as determined in CI mutants.

3. Future studies

The study of *C. albicans* CI subunits has provided important insights into the contribution of mitochondria electron transport chain (ETC) CI in pathogenesis. Of the many phenotypes observed in our previous study, beyond the substantial effects on mitochondrial electron activity, non-core subunits of mitochondrial ETC CI, Nuo1p, Nuo2p and Goa1p, and conserved Ndh51p are all required for maintaining cell-membrane related activities such as ergosterol, phospholipid metabolism and cell wall synthesis. Our data reveal that each subunit may act differently in regard to these activities. To test if these phenotypes depend on an optimum ratio of ergosterol/phospholipid composition that constitutes the membrane raft and GPI anchors for adhesins and cell wall synthases, we will analyze the membrane lipids including mitochondrial membrane and continue cell wall carbohydrates profiling in each mutant.

As for the CI assembly, we hope to gain insights into the functional and structural interaction of accessory and core subunits at the mitochondrial inner membrane and cristae. Additional analysis of mitochondrial cardiolipin in each CI mutant may demonstrate if the phospholipid metabolism (cardiolipin) at the mitochondrial inner membrane guides insertion of ETC CI in this organism.

In conclusion, we have functionally identified and characterized functionally three CI subunit proteins that are unique to *Candida* species of the CTG clade or more broadly specific to fungi and other organisms but not found in mammals. Energy production (ATP) is significantly reduced in cells lacking each of these genes, yet cell functions appear to be defined by each subunit, Nuo1p and Nuo2p being similar but different from Goa1p and the broadly conserved Ndh51p. The importance of the data presented clearly suggests that their functions and specificity require a translational emphasis to their development as targets for antifungal drug discovery. For other fungi, we hope that studies of mitochondria are initiated to determine the concept of CI subunit protein functional specificity (Papon et al. 2013; Hegedusova et al. 2014).

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References

Antonicka H, Ogilvie I, Taivassalo T, et al. Identification and characterization of a common set of complex I assembly intermediates in mitochondria from patients with complex I deficiency. J Biol Chem. 2003; 278:43081–43088. [PubMed: 12941961]

Azevedo JE, Nehls U, Eckerskorn C, et al. Primary structure and mitochondrial import in vitro of the 20.9 kDa subunit of complex I from *Neurospora crassa*. Biochem J. 1992; 288:29–34. [PubMed: 1445273]

Bourges I, Ramus C, Mousson de Camaret B, et al. Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. Biochem J. 2004; 383:491–499.10.1042/BJ20040256 [PubMed: 15250827]

Böttinger L, Horvath SE, Kleinschroth T, et al. Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes. J Mol Biol. 2012; 423:677–686.10.1016/j.jmb.2012.09.001 [PubMed: 22971339]

Brandt U. Energy converting NADH: quinone oxidoreductase (Complex I). Annu Rev Biochem. 2006; 75:69–92. [PubMed: 16756485]

Carroll J, Fearnley IM, Skehel JM, et al. The post-translational modifications of the nuclear encoded subunits of complex I from bovine heart mitochondria. Mol Cell Proteomics. 2005; 4:693–699. [PubMed: 15728260]

Chen H, Calderone R, Sun N, et al. Caloric restriction restores the chronological life span of the Goa1 null mutant of *Candida albicans* in spite of high cell levels of ROS. Fungal Genet Biol. 2012; 49:1023–1032.10.1016/j.fgb.2012.09.007 [PubMed: 23063955]

Chou JY, Leu JY. Speciation through cytonuclear incompatibility: insights from yeast and implications for higher eukaryotes. Bioessays. 2010; 32:401–411.10.1002/bies.200900162 [PubMed: 20414898]

Dagley MJ, Gentle IE, Beilharz TH, et al. Cell wall integrity is linked to mitochondria and phospholipid homeostasis in *Candida albicans* through the activity of the post-transcriptional regulator Ccr4-Pop2. Mol Microbiol. 2011; 79:968–989.10.1111/j.1365-2958.2010.07503.x [PubMed: 21299651]

Dobrynin K, Abdrakhmanova A, Richers S, et al. Characterization of two different acyl carrier proteins in complex I from *Yarrowia lipolytica*. Biochim Biophys Acta. 2010; 1797:152–159.10.1016/j.bbabio.2009.09.007 [PubMed: 19766092]

Dunning CJR, McKenzie M, Sugiana C, et al. Human CIA30 is involved in the early assembly of mitochondrial complex I and mutations in its gene cause disease. EMBO J. 2007; 26:3227–3237.10.1038/sj.emboj.7601748 [PubMed: 17557076]

Efremov RG, Baradaran R, Sazanov LA. The architecture of respiratory complex I. Nature. 2010; 465:441–445.10.1038/nature09066 [PubMed: 20505720]

Fabre E, Sfihi-Loualia G, Pourcelot M, et al. Characterization of the recombinant *Candida albicans* β-mannosylation of cell wall phosphopeptidomannan. Biochem J. 2014; 457:347–360. [PubMed: 24138199]

Friedrich T, Scheide D. The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. FEBS Lett. 2000; 479:1–5. [PubMed: 10940377]

Gabaldón T, Rainey D, Huynen MA. Tracing the evolution of a large protein complex in the eukaryotes, NADH:ubiquinone oxidoreductase (Complex I). J Mol Biol. 2005; 348:857–870. [PubMed: 15843018]

Ghannoum MA, Burns GR, Elteen KA, Radwan SS. Experimental evidence for the role of lipids in adherence of *Candida* spp. to human buccal epithelial cells. Infect Immun. 1986; 54:189–193. [PubMed: 3759234]

Gonçalves AP, Cordeiro JM, Monteiro J, et al. Involvement of mitochondrial proteins in calcium signaling and cell death induced by staurosporine in *Neurospora crassa*. Biochim Biophys Acta. 2015; 1847:1064–1074.10.1016/j.bbabio.2015.05.011 [PubMed: 26004627]

Gow NA, Hube B. Importance of the *Candida albicans* cell wall during commensalism and infection. Curr Opin Microbiol. 2012; 15:406–412.10.1016/j.mib.2012.04.005 [PubMed: 22609181]

Gray MW, Burger G, Lang BF. The origin and early evolution of mitochondria. Genome Biology. 2001; 2:reviews1018.1, 1018.5. [PubMed: 11423013]

Guénebaut V, Schlitt A, Weiss H, et al. Consistent structure between bacterial and mitochondrial NADH: ubiquinone oxidoreductase (complex I). J Mol Biol. 1998; 276:105–112. [PubMed: 9514725]

Hegedusova E, Brejova B, Tomaska L, et al. Mitochondria genome of the basidiomycetous yeast *Jaminea angkorensis*. Curr Genet. 2014; 60:49–59. [PubMed: 24071901]

Helmerhorst EJ, Murphy MP, Troxler RF, Oppenheim FG. Characterization of the mitochondrial respiratory pathways in *Candida albicans*. Biochim Biophys Acta. 2002; 1556:73–80. [PubMed: 12351220]

Hsu FF, Turk J, Rhoades ER, et al. Structural characterization of cardiolipin by tandem quadrupole and multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization. J Am Soc Mass Spectrum. 2005; 16:491–504.

Janssen R, Smeitink J, Smeets R, van Den Heuvel L. CIA30 complex I assembly factor: a candidate for human complex I deficiency? Hum Genet. 2002; 110:264–270. [PubMed: 11935339]

Kmita K, Zickermann V. Accessory subunits of mitochondrial complex I. Biochem Soc Trans. 2013; 41:1272–1279.10.1042/BST20130091 [PubMed: 24059519]

Kollár R, Reinhold BB, Petráková E, et al. Architecture of the yeast cell wall. Beta(1→6)-glucan interconnects mannoprotein, beta(1→)3-glucan, and chitin. J Biol Chem. 1997; 272:17762–17775. [PubMed: 9211929]

Koszul R, Malpertuy A, Frangeul L, et al. The complete mitochondrial genome sequence of the pathogenic yeast *Candida* (*Torulopsis*) glabrata. FEBS Lett. 2003; 534:39–48. [PubMed: 12527359]

Küffner R, Rohr A, Schmiede A, et al. Involvement of two novel chaperones in the assembly of mitochondrial NADH:Ubiquinone oxidoreductase (complex I). J Mol Biol. 1998; 283:409–417. [PubMed: 9769214]

Lattif AA, Mukherjee PK, Chandra J, et al. Lipidomics of *Candida albicans* biofilms reveals phase-dependent production of phospholipid molecular classes and role for lipid rafts in biofilm formation. Microbiology. 2011; 157:3232–3242.10.1099/mic.0.051086-0 [PubMed: 21903752]

Li D, Chen H, Florentino A, et al. Enzymatic dysfunction of mitochondrial complex I of the *Candida albicans* goa1 mutant is associated with increased reactive oxidants and cell death. Eukaryot Cell. 2011; 10:672–682.10.1128/EC.00303-10 [PubMed: 21398508]

Lorenz MC, Bender JA, Fink GR. Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot Cell. 2004; 3:1076–1087. [PubMed: 15470236]

Lotte M, van Dijck P. Recent insights into Candida albicans biofilm resistance mechanisms. Curr Genet. 2013; 59:251–264. [PubMed: 23974350]

Maas MF, Sellem CH, Krause F, et al. Molecular gene therapy: overexpression of the alternative NADH dehydrogenase NDI1 restores overall physiology in a fungal model of respiratory complex I deficiency. J Mol Biol. 2010; 399:31–40.10.1016/j.jmb.2010.04.015 [PubMed: 20398675]

Mathe L, Van Dijck P. Recent insights into *Candida albicans* biofilm resistance mechanisms. Curr Genet. 2013; 59:251–264. [PubMed: 23974350]

Marcotte EM, Xenarios I, van Der Bliek AM, Eisenberg D. Localizing proteins in the cell from their phylogenetic profiles. Proc Natl Acad Sci USA. 2000; 97:12115–12120. [PubMed: 11035803]

McDonough JA, Bhattacherjee V, Sadlon T, Hostetter MK. Involvement of *Candida albicans* NADH dehydrogenase complex I in filamentation. Fung Genet Biol. 2002; 36:117–127.

McKenzie M, Ryan MT. Assembly factors of human mitochondrial complex I and their defects in disease. IUBMB Life. 2010; 62:497–502. [PubMed: 20552642]

Merico A, Sulo P, Piskur J, Compagno C. Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. FEBS J. 2007; 274:976–989. [PubMed: 17239085]

Mille C, Bobrowicz P, Trinel P, et al. Identification of a new family of genes involved in β-1,2 mannosylation of glycans in *Pichia pastoris* and *Candida albicans*. J Biol Chem. 2008; 283:9724–9736. [PubMed: 18234669]

Minkler PE, Hoppel CL. Separation and characterization of cardiolipin molecular species by reverse-phase ion pair high-performance liquid chromatography-mass spectrometry. J Lipid Res. 2010; 51:856–865.10.1194/jlr.D002857 [PubMed: 19965604]

Mollinedo F. Lipid raft involvement in yeast cell growth and death. Front Oncol. 2012; 2:140.10.3389/fonc.2012.00140 [PubMed: 23087902]

Papon N, Savini V, Lanoue A, et al. *Candida guilliermondi*: biotechnological applications, perspectives for biological control, emerging importance and recent advances in genetics. Curr Genet. 2013; 59:73–90. [PubMed: 23616192]

Pike LJ. Lipid rafts: heterogeneity on the high seas. Biochem J. 2004; 378:281–292. [PubMed: 14662007]

Runswick MJ, Fearnley IM, Skehel JM, Walker JE. Presence of an acyl carrier protein in NADH:ubiquinone oxidoreductase from bovine heart mitochondria. FEBS Lett. 1991; 286:121–124. [PubMed: 1907568]

Sackmann U, Zensen R, Röhlen D, et al. The acyl-carrier protein in *Neurospora crassa* mitochondria is a subunit of NADH:ubiquinone reductase (complex I). Eur J Biochem. 1991; 200:463–469. [PubMed: 1832379]

Schatz G, Racker E, Tyler DD, et al. Studies of the DPNH-cytochrome b segment of the respiratory chain of baker's yeast. Biochem Biophys Res Commun. 1966; 22:585–590. [PubMed: 4287816]

Schneider R, Massow M, Lisowsky T, Weiss H. Different respiratory-defective phenotypes of *Neurospora crassa* and *Saccharomyces cerevisiae* after inactivation of the gene encoding the mitochondrial acyl carrier protein. Curr Genet. 1995; 29:10–17. [PubMed: 8595652]

She X, Zhang L, Chen H, et al. Cell surface changes in the *Candida albicans* mitochondrial mutant goa1Δ are associated with reduced recognition by innate immune cells. Cell Microbiol. 2013; 15:1572–1584.10.1111/cmi.12135 [PubMed: 23490206]

She X, Khamooshi K, Gao Y, et al. Fungal-specific subunits of the *Candida albicans* mitochondrial complex I drive diverse cell functions including cell wall synthesis. Cell Microbiol. 201510.1111/cmi.12438

Simons K, Sampaio JL. Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol. 2011; 3:a004697.10.1101/cshperspect.a004697 [PubMed: 21628426]

Solotroff V, Moseler R, Schulte U. Two pentatricopeptide repeat domain proteins are required for the synthesis of respiratory complex I. Curr Genet. 2013; 61:19–29.

Sun N, Fonzi W, Chen H, et al. Azole susceptibility and transcriptome profiling in *Candida albicans* mitochondrial electron transport chain complex I mutants. Antimicrob Agents Chemother. 2013a; 57:532–542.10.1128/AAC.01520-12 [PubMed: 23147730]

Sun N, Li D, Fonzi W, et al. Multidrug-resistant transporter mdr1p-mediated uptake of a novel antifungal compound. Antimicrob Agents Chemother. 2013b; 57:5931–5939.10.1128/AAC.01504-13 [PubMed: 24041896]

Thomas E, Roman E, Claypool S, et al. Mitochondria influence CDR1 efflux pump activity, Hog1-mediated oxidative stress pathway, iron homeostasis, and ergosterol levels in *Candida albicans*. Antimicrob Agents Chemother. 2013; 57:5580–5599. [PubMed: 23979757]

Tuschen G, Sackmann U, Nehls U, et al. Assembly of NADH: ubiquinone reductase (complex I) in *Neurospora* mitochondria. Independent pathways of nuclear-encoded and mitochondrially encoded subunits. J Mol Biol. 1990; 213:845–857. [PubMed: 2141652]

Ugalde C, Janssen RJ, van den Heuvel LP, et al. Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency. Hum Mol Genet. 2004; 13:659–667. [PubMed: 14749350]

Vellucci VF, Gygax SE, Hostetter MK. Involvement of *Candida albicans* pyruvate dehydrogenase complex protein X (Pdx1) in filamentation. Fungal Genet Biol. 2007; 44:979–990. [PubMed: 17254815]

Vinothkumar KR, Zhu J, Hirst J. Architecture of mammalian respiratory complex I. Nature. 2014; 515:80–84.10.1038/nature13686 [PubMed: 25209663]

Vogel RO, Janssen RJ, van den Brand MA, et al. Cytosolic signaling protein Ecsit also localizes to mitochondria where it interacts with chaperone NDUFAF1 and functions in complex I assembly. Genes Dev. 2007; 21:615–624. [PubMed: 17344420]

Ziółkowska NE, Karotki L, Rehman M, et al. Eisosome-driven plasma membrane organization is mediated by BAR domains. Nat Struct Mol Biol. 2011; 18:854–856.10.1038/nsmb.2080 [PubMed: 21685922]

Table 1

Phenotypes of *C. albicans* CI mutants

CI mutants	CI activity (down by %)	CI disassembly	glycerol growth	fluconazole hypersusceptible	virulence requirement
*goa1*Δ	80%	80%	no	Yes	Yes
*ndh51*Δ	87%	70%	no	Yes	Yes (unpublished)
*nuo1*Δ	62%	60%	no	Yes	Yes
*nuo2*Δ	55%	80%	no	Yes	Yes

Table 2

Possible mitochondrial CI I assembly factors in *C. albicans*

Mammal. name	*Candida* orthologs	ident. rate[a]	Assembly role	Expression in CI mutant (fold changes)			
				ndh51Δ	***goa1Δ***	***nuo1Δ***	***nuo2Δ***
Ndufaf3	_[b]	-	early stage	-	-	-	-
Ndufaf4	-	-	early stage	-	-	-	-
Ndufaf5	orf19.2541	38%	early stage	<2.0[c]	<2.0	<2.0	<2.0
Ndufaf1	CIA30 (orf19.3353)	32.6%	middle stage	-2.41	-4.66	<2.0	-2.13
Ecsit	-		middle stage	-	-	-	-
Ndufaf2 (NDUFA12)	(orf19.1625)	34.0%	late stage	2.34	1.23	3.35	4.12

[a]: identity rate when comparing with mammalian gene

[b]: not identified in *C. albicans*

[c]: the fold change is less than 2-fold

Table 3

The transcription of gene for mitochondrial cristae and inner membrane organization

	Function	goa1Δ	ndh51Δ	nuo1Δ	nuo2Δ
orf19.3089	mitochondrial cardiolipin metabolic process/cristae formation	-3.99	-7.58	8.11	7.12
orf19.4096	cardiolipin biosynthesis-TAZ1	-15.03	-14.20	9.90	12.23
orf19.4210	Cardiolipin remodeling-PLA2	-3.38	-6.39		
orf19.3449.2	cardiolipin biosynthetic process	-2.03			
orf19.5796	mitochondrial inner membrane organization	-2.37			
orf19.7166	cardiolipin metabolism	2.49		5.15	4.93
orf19.6066	mitochondrial sphingosine metabolism	-2.95	-2.37		
orf19.6639	mitochondrial fusion	-2.41	-2.01		
orf19.3900	mitochondrial fusion	-2.22			
orf19.6790	mitochondrial organization	-3.11	-2.10	-2.57	-2.26
orf19.7619	mitochondrial organization	-2.17	-6.15	5.46	6.13
orf19.6062.3	mitochondrial cristae formation	-271.49	-121.72		
orf19.6160	mitochondrial eisosome assembly & raft	-2.38		-3.76	-4.03
orf19.7386	mitochondrial inner membrane organization	-2.13		10.78	12.02