



Published in final edited form as:

Cell Microbiol. 2015 September ; 17(9): 1350–1364. doi:10.1111/cmi.12438.

Fungal-specific subunits of the *Candida albicans* mitochondrial complex I drive diverse cell functions including cell wall synthesis

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Summary

Our published research has focused upon the role of Goa1p, an apparent regulator of the *Candida albicans* mitochondrial complex I (CI). Lack of Goa1p effects optimum cell growth, CI activity, and virulence. Eukaryotic CI is composed of a core of 14 alpha-proteobacterial subunit proteins and a variable number of supernumerary subunit proteins. Of the latter group of proteins, one (NUZM) is fungal-specific, and a second (NUXM) is found in fungi, algae and plants but is not a mammalian CI subunit protein. We have established that NUXM is orf19.6607 and NUZM is orf19.287 in *C. albicans*. Herein, we validate both subunit proteins as NADH:ubiquinone oxidoreductases (NUO) and annotate their gene functions. To accomplish these objectives, we compared null mutants of each with WT and gene-reconstituted strains. Genetic mutants of genes *NUO1* (19.6607) and *NUO2* (19.287), not surprisingly, each had reduced oxygen consumption, decreased mitochondrial redox potential, decreased CI activity, increased reactive oxidant species (ROS), and a decrease in chronological aging *in vitro*. Loss of either gene results in a disassembly of CI. Transcriptional profiling of both mutants indicated significant down regulation of genes of carbon metabolism, as well as upregulation of mitochondrial-associated gene families which may occur to compensate for the loss of CI activity. Profiling of both mutants also demonstrated a loss of cell wall β -mannosylation but not in a conserved CI subunit (*ndh51*). The profiling data may indicate specific functions driven by the enzymatic activity of Nuo1p and Nuo2p. Of importance, each mutant is also avirulent in a murine blood-borne, invasive model of candidiasis associated with their reduced colonization of tissues. Based upon their fungal-specificity and roles in virulence, we suggest both as drug targets for antifungal drug discovery.

Keywords

virulence; mitochondria; tissue colonization; *Candida albicans*

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Competing interests.

The authors have no conflict of interests.

Introduction

Candida albicans is one of the most common opportunistic fungal pathogens of humans. This species as well as other *Candida* species are part of the mucosal and skin microbiome; from these sites superficial or invasive candidiasis can occur in patients with risk factors such as HIV/AIDS (oral candidiasis), or nosocomial, blood-borne infections following surgery, in premature infants, and neutropenic patients undergoing cancer chemotherapy or allogeneic bone marrow transplantation (Calderone *et al.*, 2014; Calderone *et al.*, 2012).

Survival of *C. albicans* within anatomically distinct host niches highlights the fact that the organism must have unique strategies for adaptation to a range of physiological extremes (Bambach *et al.*, 2009; Barelle, *et al.*, 2006; Brown *et al.*, 2014a; Brown *et al.*, 2014b; Ramirez and Lorenz, 2007; Enie *et al.*, 2014; Enie *et al.*, 2012; Fradin, *et al.*, 2003; Askew *et al.*, 2000; Prieto, *et al.*, 2014). For example, on mucosal surfaces, nutrient limitation and oxidative stress cause selective pressures which impact survival of pathogens and commensals (Brown *et al.*, 2014b; Fradin *et al.*, 2003). Signal transduction pathways support adaptation to stress conditions, illustrating the importance of cell communication in the survival of *C. albicans* (Prieto *et al.*, 2014).

Underlying these complexities, it is also clear that energy production of pathogens like *C. albicans* is critically important to survival (Enie *et al.*, 2014, 2012). The concept of “flexible metabolism” in *C. albicans* has been coined to reflect two levels of regulation (Enie *et al.*, 2014): 1) flexibility to utilize diverse carbon sources such as fatty acids and amino acids in the absence of sustainable glucose; 2) switching from anaerobic to aerobic (mitochondrial oxidation) to provide energy for growth at specific host niches. Flexible metabolism also depends on proper energy-yielding metabolic events. In this regard, the mitochondrial impact on cell growth and energy production of *Candida spp in vitro* has been studied but less so in the context of virulence and pathogenesis. Without question, the lack of energy production should be a major weakness for most pathogens.

Our criteria for the study of Complex I (CI) subunit proteins is that they must be conserved in fungi and be required for virulence. To this end, in the current study, we have identified CI proteins that fulfill these criteria. We also note our published data on the Goa1p and its role in CI activities in *C. albicans* (Bambach *et al.*, 2009; Sun *et al.*, 2013; Li *et al.*, 2011; Chen *et al.*, 2012; She *et al.*, 2013; Khamooshi *et al.*, 2014). Goa1p, is required for CI enzyme activity but is not a CI subunit (Bambach *et al.*, 2009). *GOA1* is only found in the CTG clade of *Candida spp*, which includes most of the pathogenic *Candida* species except *C. glabrata* (Bambach *et al.*, 2009). Because of its importance in growth and virulence described above, Goa1p might be considered a potential target for antifungal drug therapy. Its highly conserved nature among only species of the CTG clade suggests that an inhibitor may be of narrow specificity but still a potentially useful therapeutic.

Other virulence factors such as phase transition are also regulated by cell respiratory pathways (McDonough *et al.*, 2002; Velluci *et al.*, 2007). Recently, cell levels of glutathione were deemed critical to the yeast → hyphae transition as a reduction in glutathione, CI activity, and ATP, as well as an increase in the alternative oxidant pathway (AOX)

accompanied the loss of phase transition (Gredouari, *et al.*, 2014). Loss of other genes results in mitochondrial deficiencies that likewise affect drug resistance, antifungal susceptibility, drug tolerance, and virulence in *C. albicans* (Shingu-Vazquez and Traven, 2011; Dagley *et al.*, 2011). Others have suggested that the common denominator of these effects on cells is a reduction of membrane phospholipid homeostasis (Dagley, *et al.*, 2011). With respect to cell wall synthesis, the *C. albicans* Ccr4-Pop2 mRNA deadenylase is a regulator of mRNA stability and translation. Mutants lacking this gene are compromised in cell wall β -glucan synthesis, resulting in an increased sensitivity to caspofungin, a decrease in filamentation, and a decrease in virulence (Shingu-Vazquez and Traven, 2011). Underlying these cell wall changes are dysfunctional mitochondria and a phospholipid imbalance. In this regard, a mitochondrial outer membrane complex protein (Sam37) of the Sorting and Assembly Machinery complex is critical to phospholipid synthesis and other properties of mitochondria. The *sam37* is phenotypically similar to that described for the Ccr4-Pop2 mRNA deadenylase (Dagley *et al.*, 2011; Qu *et al.*, 2012). Thus, as the major source of energy, mitochondria are crucial for growth, response to the environment, reproduction, and decreased virulence.

The supposed underrepresentation of mitochondrial studies in the field of fungal pathogens may be due to the presumed conserved nature of the mitochondria-encoded and imported nuclear proteins. To address this presumption in this study, we focus on two non-mammalian subunits of *C. albicans* mitochondrial CI to confirm their importance in cell growth and virulence in a murine model. Both subunits were first denoted as NUXM and NUZM (Gabaldon *et al.*, 2005). NUXM is broadly conserved in *C. albicans*, *Neurospora crassa*, *Yarrowia lipolytica*, *Arabidopsis thaliana*, and *Chlamydomonas reinhardtii*. On the other hand, *NUZM* is fungal-specific (Gabaldon *et al.*, 2005; Abdrakhmanova *et al.*, 2004; Bridges, Feamley and Hirst, 2010; Bridges *et al.*, 2009; Pereira *et al.*, 2013; Videira and Duarte, 2002; Videira, 1998). We have identified these as orf19.6607 (NUXM) and orf19.287 (NUZM), and now report the phenotypes of gene-deleted strains in comparison to parental and gene-reconstituted controls. Based upon their non-conservation in mammals and the loss of important activities in mutants (such as virulence), we suggest that both may be attractive targets for therapeutic intervention.

Following their now established functions as NADH:Ubiquinone Oxidoreductases, we named orf19.6607 (*NUO1*) and orf19.287 (*NUO2*) and use this gene nomenclature throughout the manuscript.

Results

Identification of the *C. albicans* NUXM and NUZM

The gene sequences of NUXM and NUZM were first reported for *Neurospora crassa* (Azevedo *et al.*, 1992; Videira *et al.*, 1990). Those sequences were identified as orf19.6607 and orf19.287 from the *Candida* Genome Database by protein BLAST analysis of protein sequences (<http://www.candidagenome.org>).

Growth on non-glucose carbon sources and oxygen consumption are reduced in the CI mutants

The generation times (GT) in YPD broth of both mutants were 109 min (*nuo1*) and 133 min (*nuo2*) compared to 87 min in WT cells (Noble *et al.*, 2010). Since *NUO1* (19.6607) and *NUO2* (orf19.287) are putative NADH:Ubiquinone oxidoreductases, we determined if null mutants of each (*nuo1* and *nuo2*) have phenotypes suggestive of typical defects of other CI mutants. Our assays included growth on mitochondrial substrates such as glycerol or oleic acid, oxygen consumption, mitochondrial membrane potential, reactive oxidant production (ROS), chronological life span (CLS), and CI enzyme activity. Both the *nuo1* and *nuo2* mutants had reduced growth on YPD agar (Fig. 1A, **left panel**) as well as on mitochondrial substrates compared to WT and gene reconstituted strains (Nuo1R and Nuo2R) (Fig. 1A, **middle and right panels**). *nuo2* displayed a greater growth defect than *nuo1* on YP-glycerol. Both mutants were equally unable to grow on oleic acid (Fig. 1A, **right panel**).

Likewise, *nuo1* and *nuo2* had reduced oxygen consumption rates (OCR) when compared to WT or to each gene-reconstituted strain (Fig. 1B). A statistically significant lower oxygen consumption rate (OCR) was noted with the *nuo1* versus *nuo2* ($p < 0.05$). The OCR is ~36% and 53% for *nuo1* and *nuo2* mutants, respectively, compared to WT cells. The OCRs of the reconstituted strains Nuo1R and Nuo2R are 75% and 86% of WT levels. OCR was lowered substantially in all strains following treatment with the CI-inhibitor rotenone (Fig. 1B, CI-IHR-OCR), demonstrating that the reduction in oxygen consumption is associated with CI. The remaining oxygen consumption is probably due to other alternative NADH-Q oxidoreductases that are rotenone-insensitive.

Mitochondrial membrane potential is decreased in *nuo1* and *nuo2*

The measurement of mitochondrial membrane potential (ψ_m) is a valuable indicator of the energy status of cells. Using the JC-1 fluorescent dye, we found higher numbers of nonpolarized cells in both *nuo1* and *nuo2* mutants, respectively (Fig. 1C, **compare the null and gene reconstituted strains, upper 2 panels with WT cells, lower panel**). The reduction in membrane potential (ψ_m) in *nuo2* was greater than in *nuo1* (Fig. 1C, Table insert). The membrane potential of the gene reconstituted strains was higher than the null mutants. These data support a correlation of reduced growth with a reduction in membrane potential (ATP synthesis) and respiration.

Chronological life span (CLS) is shorter in both mutants

To determine if CLS is affected in the null strains (*nuo1* and *nuo2*), growth in minimal medium plus amino acids and glucose was compared to WT and gene reconstituted strains. CLS is a measure of the length of time cells remain in stationary phase. Cells of each strain were collected daily for 9 days from SD medium supplemented with 2% glucose, standardized to equal cell numbers, and plated on YPD agar for 48 h to determine % survival. CLS was affected in both null strains, while the gene reconstituted strains were intermediate in CLS compared to WT cells (Fig. 2A). In comparing WT and reconstituted strains, both mutants had nearly similar percentages of surviving cells by the day 9. At day 2, % survival of *nuo1* and *nuo2* decreased to 30~40%. Following this sharp decrease, the

survival rate of *nuo1* increased to 60% from days 4–6 much like WT and reconstituted strains. But, survival decreased again to about 10% by day 9. On the other hand, the decrease in survival of *nuo2* in % was more gradual than *nuo1* from days 4–8 (~42% ~ 46%). The slower decrease in viability of *nuo2* may suggest that a difference of mitochondrial quality control or retrograde response between two null mutants. Likely, Nuo1p is engaged in such compensatory pathway due to imperfect of ETC and damaging ROS level.

The longer generation times and decline in cell survival (CLS) of *nuo1* and *nuo2* are likely due to decreased CI activity and a loss in mitochondrial membrane potential so that cells produce less total ATP. Also, a consequence of reduced CI enzyme activity (see section below) is an increase in reactive oxidant species (ROS) that is known to contribute to a shortened CLS and a decrease in cell viability (Chen *et al.*, 2012). As we expected, the reduction in CLS in both mutants is accompanied by an increase in ROS (Fig. 2B). Mitochondria are potent producers of cellular superoxide then was detected by DHE in this study. Meanwhile the cellular peroxide (H₂O₂) was measured by DC-FDA fluorescent dye. As Figure 2B,C shown, both superoxide and peroxide are increased significantly in null mutants when compared to each control strain. We found the superoxide levels in *nuo1* and *nuo2* are 6.9-fold and 6.1-fold higher than WT, indicating that electron leakage occurred in the null mutants. At same time, the cellular peroxide levels were > 3-fold higher in both mutants compared to reconstituted strains Nuo1R, Nuo2R and WT with DC-FDA measurement. The ROS levels of *nuo1* and *nuo2* mutants in fact are lower than *goal* described previously (Li et al, 2011), in which 7-fold higher ROS was observed by DC-FDA fluorescent that corresponded prior to death of cells at day 7. Nevertheless, these data correlate with a greater loss in CLS with ETC mutants.

CI enzyme activity is reduced in both *nuo1* and *nuo2*

Mitochondrial NADH dehydrogenase activity was also evaluated in both CI mutant strains and *ndh51*. As shown in Table 1, CI activity in *nuo1* and *nuo2* mutants was reduced by 62 and 55%, respectively, while CI enzyme activity was reduced by 87% in the *ndh51* compared to WT. The large loss of CI activity in *ndh51* is probably related to the fact that the Ndh51p subunit is a flavoprotein and responsible for NADH binding to CI (McDonough *et al.*, 2002).

By contrast, CII activities of *nuo1* and *nuo2* mutants were similar to that of WT cells (Table 1), but higher than *ndh51* cells (succinate oxidation), suggesting a major role for Ndh51p in CII activity. These data indicate that *NUO1*, *NUO2* and *NDH51* are all critical for CI activity and that CII enzyme activity may require Ndh51p as well. CIV activity is much lower in *nuo1* (23% of WT cells) but higher in *nuo2* (80% of WT cells, while about 50% of control in the *ndh51*). Likely, *NUO1* is also associated with maintenance of CIV enzymatic activity.

Defects in CI assembly in the absence of CI subunits and *Goa1*

In order to understand how *NUO1* and *NUO2* affect CI activity, the ETC complexes of mitochondrial extracts were electrophoresed in blue native polyacrylamide gels (BN-

PAGE). The CI protein (~1MD molecular mass) is absent or minimally apparent in *nuo1*, *nuo2*, and *ndh51* compared to WT, Nuo1R, and Nuo2R (Fig. 3A). CIII and CV are largely unaffected in each mutant. This observation may point to a role of both genes in stability or assembly of the CI complex.

To quantify the loss of CI in each mutant, we also measured the gel density of CI for each strain by using ratios of CI/CIII and CI/CV since CIII and CV are largely unaffected in each mutant (Fig. 3B). We found that the CI of *nuo1* is reduced by ~60 to 70% compared to WT or Nuo1R cells when data are expressed as gel density ratios of CI/CIII and CI/CV. Similar to *nuo1*, a reduction of CI subunit is also found in *nuo2*. An ~80% reduction of CI in this mutant is seen using both CI/CIII and CI/CV ratios, and a 75% reduction of CI staining is apparent in the *ndh51*. The Ndh51p is one of core members of CI that is responsible NADH binding and oxidation as stated above. These results suggest that loss of CI enzyme activity in all mutants may reflect a disassembly or instability of CI, similar to published data with *GOA1* (Li *et al.*, 2011).

Transcriptional changes in *nuo1*, *nuo2*, *ndh51*, and *goa1*

Transcriptional profiles of each mutant were compared to the corresponding reconstituted and WT strains. A total of 586 genes are down-regulated, and 808 genes are up-regulated in both *nuo1* and *nuo2* mutants. The overall transcriptional changes are similar for *nuo1* and *nuo2* in regard to metabolic pathways, ribosomal biogenesis, signal transduction, oxidative responses, and DNA repair mechanisms. The similarities of *nuo1* and *nuo2* may suggest redundant roles for both genes. Transcriptional profiling of genes with changes 3-fold greater or less that are involved in carbon metabolic pathways is indicated in Fig. 4. For broad comparisons, we have also included the transcription profiles of *ndh51* and *goa1*. Since Nuo1p, Nuo2p, and Ndp51p are CI subunits, we focused on genes, among others, that are related to carbon metabolism and mitochondria functions (Fig. 4).

In regard to the carbon metabolism, a large number of hexose transporters including glucose (*HGTs*) and other sugar transporters (*HXTs*) as well as genes of the major facilitator superfamily are significantly down-regulated in both *nuo1* and *nuo2* when compared to WT (-3.4 to -45.8-fold) (Supplementary Table S2 and Fig. 4A). Transcriptional levels of glucokinase *GLK1* and *GLK4* decreased by -7.0 to -9.2-fold; genes of alternative carbon pathways are also down regulated, for example, GALs (galactose utilization, -3.5 to -5.8-fold) and glycogen catabolic processes (*GPH1*, -6.3 to -8.3-fold, Fig. 4A,C and Supplementary Table S2). Gene changes associated with carbon metabolism are similar to those of *goa1*. In addition, several ARO genes (*ARO3*, *ARO8*, *ARO10*) are also decreased significantly in both CI *nuo* mutants, similar to *goa1* and *ndh51*. These genes encode aromatic decarboxylases of the Ehrlich fusel oil pathway which leads to the formation of aromatic alcohol and products of central carbon metabolism. These results suggest that *NUO1* and *NUO2* may be associated directly or indirectly with non-glucose carbon metabolism.

In *goa1*, genes encoding β -oxidation of fatty acids and other peroxisomal activities are significantly down regulated (Fig. 4,B,C). For example, genes of the TCA cycle (*CIT1*, *PDK2*), fatty acid transporters of peroxisomes (*PEXs*) for fatty acid metabolism (*POX*, *POT*,

PXP), and the glyoxylate cycle (*ICL1* and *MLS1*) are down regulated. However, these genes are not changed or have minor changes in transcriptional levels in both *nuo1* and *nuo2* (Figure 4,B,C). Instead, gluconeogenesis is decreased in both NUO mutants more than *goal*, as demonstrated by *PDC11* (−5.8 fold), *PGI11* (−7.11 to −7.55-fold) and *FBA1* (−2.79 to −2.92-fold) but not in *goal* and *ndh51*. The products of these genes are the key enzymes to direct three irreversible steps of gluconeogenesis. As *NUO1* and *NUO2* have less influence on peroxisomal functions, *GOA1* may provide functions related to the integrity of both mitochondria and peroxisomes.

In regard to mitochondrial function, we found that the gene changes are very different in *nuo1* and *nuo2* from *goal* except for mitochondrial rRNA biogenesis and protein translation (Fig. 4D and Supplementary Table S2). In the absence of *NUO1* and *NUO2*, the genes encoding mitochondrial ETC proteins of CI, CIII, CIV and CV subunit proteins, ETC protein complex assembly, transporters of several intermembrane (TIMs) proteins are up-regulated, opposite that of *goal*. Upregulation of mitochondrial rRNA biogenesis is consistent with cytoplasmic rRNA machinery shown in all four mutants and suggests a likely universal regulatory network to govern the ribosome biogenesis system based on cell energetic status (data not shown). Down regulation of several CI encoding genes occurred in the *goal* only (Fig. 4D).

Genes encoding cell wall synthases and cell surface proteins in *nuo1* and *nuo2* differ from the *ndh51*

We note that gene changes in several categories of cell wall mannosyltransferases, chitin synthases and chitinases, exo/endo glucanases, and adhesins/cell surface proteins are much more highly represented in *nuo1* and *nuo2* than in the *ndh51* subunit mutant (Fig. 5, A–D). As many as 15 adhesin-encoding genes are down regulated in *nuo1* and *nuo2* (Fig. 5D). Especially down regulated genes among *nuo1* and *nuo2* are adhesins and other cell wall surface proteins. These changes are not seen in *ndh51*.

Deletion of NUO1 and NUO2 results in avirulence and reduced fungal growth in a mouse model of systemic candidiasis

C. albicans mutants with slow growth *in vitro* usually have defects in virulence but, not always (Noble *et al.*, 2010). To address the role of these genes in causing candidiasis, we used a murine, blood-borne invasive candidiasis model. Experiments were done to measure survival of mice and tissue loads of each strain. Data represent two biological replicates. Both *nuo1* and *nuo2* are avirulent (Fig. 6A). A decrease in survival in mice infected with WT cells of *C. albicans* began on day 2; all mice were moribund by day 6. For the gene reconstituted strains, partial virulence was seen by day 6 (40–55 % mortality) and all mice infected with Nuo1R and Nuo2R inoculation were moribund by day 11 and 13, respectively. On the other hand, mice survived infection when infected with *nuo1* and *nuo2* (Fig. 6A). These data indicate that both genes are required for *C. albicans* virulence *in vivo*.

To correlate the avirulence of null mutants with their growth in tissues, the fungal load (CFU/g tissue) was measured from kidney, spleen, and liver of infected mice at 1, 24, 48, and 72 h post-infection. After 1 h, tissue loads were the same for all strains (Fig. 6, B–E).

Compared to the high tissue load (3-log_{10}) in WT, Nuo1R and Nuo2R at 48 h post infection, colonization of both mutants was reduced by at least 2 logs_{10} in all tissues (Fig. 6B). The difference in colonization among organs is seen in all strains during disease, but fungal clearance is more affected in *nuo1* and *nuo2* infected mice. CFU in kidneys decreased 2-logs by 48 h and 3-logs by 72 h and the rapid clearance is even more apparent in spleen and liver. By 24 h, mutant strains are cleared totally or have scant growth (Fig. 6). These results imply that each CI I subunit is required by *C. albicans* for colonization and tissue invasion.

Discussion

Complex I (CI) of eukaryotes

CI of the mitochondrial electron complex chain is a slightly open L-shaped protein, with a hydrophobic “arm” that functions as a proton pump. This arm is located in the mitochondrial inner membrane (P module), and a hydrophilic arm extends into the mitochondrial matrix and is composed of an N module for binding and oxidation of NADH and a Q module which transfers electrons to ubiquinone (Pereira *et al.*, 2013). The mammalian CI contains hydrophobic core subunits of prokaryotic origin that are conserved in all species. This core consists of 14 subunit proteins which include seven hydrophobic subunits (encoded by the mitochondrial genome for proton translocation through the inner membrane arm) and seven other subunits that ligate either FMN to catalyze NADH oxidation or iron-sulfur clusters to transfer electrons from the flavin to the ubiquinone. Besides these 14 core subunits, eukaryotes possess a number of supernumerary protein subunits which are encoded by nuclear or mitochondrial genes. For example, 31 proteins have been described in human CI, of which 21 are thought to be ancestral eukaryotic core subunits (Gabaldon *et al.*, 2015; Kuffner *et al.*, 1998). The majority of subunits in the eukaryotic core group are conserved in fungi such as *C. albicans* and *N. crassa*.

The number of accessory subunits varies among fungi, plants and animals. For example, CAI84 is found in *C. parapsilosis* but not in *C. albicans*. In comparison to the human mitochondrial CI, *C. albicans* lacks 10 subunits (NDUFA4, FA7, FA10, NDUFB1, B2, B5, B6, NDUFC1, FC2 and NDUFV3), but on the other hand has 2 fungal-specific subunits NUXM (*NUO1*) and NUZM (*NUO2*) and also may have one additional accessory protein (ACPM) (Gabaldon *et al.*, 2015). As a result, we conclude that the mitochondrial CI of *C. albicans* is composed of at least 39 subunit proteins (Supplementary Table S3). To date, the function of most of these accessory subunits is not well defined in every species, even though many human diseases have been associated with some CI deficiencies (Mimaki *et al.*, 2012).

The assembly of CI has been investigated in *N. crassa* (Pereira *et al.*, 2013; Kuffner *et al.*, 1998), and at least two findings of CI assembly are thought to explain the inability of cells to form a complete CI. First, mutations in subunits of the matrix arm (modules N and Q) lead to a disassembly of CI and an accumulation of membrane arm (P module) fragments (Mimaki *et al.*, 2012). Second, accessory assembly factors have been suggested, in which NDUFAF2 appears to connect P and Q modules (Pereira *et al.*, 2013; Kuffner *et al.*, 1998). The assembly of the *C. albicans* CI has not been studied, but our data suggest that gene deletions causing a loss of CI subunit proteins (Nuo1p, Nuo2p, Ndh51p) result in an almost

complete loss of the CI protein (Fig 3). Further, the *goal1*, an unlikely CI subunit, also does not assemble a CI. Perhaps Goa1p is an accessory protein for CI structural integrity or stability in *C. albicans* as well (Li et al, 2011).

Mitochondria in model yeast and *C. albicans*

The model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* use a single enzyme for mitochondrial CI activity (Gabaldon *et al.*, 2005; Mimaki *et al.*, 2012). In addition to not having a CI, *S. cerevisiae* lacks an alternative respiratory pathway (AOX) (Helmerhorst *et al.*, 2005). Further, *S. cerevisiae* is Crabtree negative, fermenting glucose and repressing mitochondrial functions when oxygen is available (Shingu-Vazquez and Traven, 2011). In contrast, *C. albicans* is Crabtree negative and mitochondrial respiration is active when optimal glucose is present (Shingu-Vazquez and Traven, 2011). The contrast in glucose utilization is also associated with oxidative stress resistance, being higher in *C. albicans* compared to yeast (Rodacky *et al.*, 2009). New data suggest that *C. albicans* acts differently from *S. cerevisiae* in carbon catabolite control (Sandai *et al.*, 2014). The premise of this data is that alternative carbon assimilation in *C. albicans* continues even after a preferred source (glucose) is available, which allows this pathogen to adapt to a host niche where carbon availability changes over time. This adaptation would require metabolic pathways such as the glyoxylate cycle, gluconeogenesis, and fatty acid degradation, each affected by loss of CI.

Virulence and a functional CI

Our current study supports the idea of others that a loss of mitochondrial function causes reduced virulence or biofilm formation in *C. albicans* (Table 2) (Bambach *et al.*, 2009; Shingu-Vazquez and Traven, 2011; Qu *et al.*, 2012; Noble *et al.*, 2010; Becker *et al.*, 2010; Marchais *et al.*, 2005). The putative or characterized functional assignments of genes that are essential for virulence encode CI subunits, mitochondrial DNA repair, ribosomal activities, cell integrity, and an apparent regulator of CI (Goa1p) that may be required for complex assembly or stabilization, as described above. The orf/genes listed in Table 2 represent functional annotation derived from phenotypes of knockout (null) mutants, or by strain fitness in repressible conditional mutants. Considering the lack of virulence of these mutants and their fungal-specific origins, we suggest they may have high potential as antifungal drug targets. Inactivating mitochondria, as has been reported in at least mutants of *GOA1* and *SAM37*, has profound crippling effects on fungal cell functions (Bambach *et al.*, 2009; Shingu-Vazquez and Traven, 2011).

A comparison of transcriptional profiling of all CI mutants

We compared all mutants by microarray analysis. All mutants had significant down regulation of carbon metabolism genes (Fig. 4). In most of the transcriptional profiling of gene families, such as mitochondrial membrane transporters (TIM/TOMS) and gluconeogenesis, *nuo1* and *nuo2* clustered together, implying some redundancy in direct or indirect functions. In contrast, down regulation of peroxisomal genes of the glyoxylate cycle, β -oxidation, and peroxisomal transporters overlapped substantially in *goal1* and *ndh51*. Diversity among mutants was especially prominent among genes encoding cell

wall functions. Here, *nuo1*, *nuo2* and *goal* were more similar in their profiles than *ndh51* (Fig. 5). These data may imply additional functions (besides CI enzyme activity) associated with *NUO1* and *NUO2* compared to *NDH51*. It would seem, therefore, that the fungal-specific subunits of *C. albicans* are functionally more diverse than the conserved Ndh51p in driving cell activities.

Mitochondrial therapeutics

Mitochondria are at the forefront of therapeutics against cancers and even neurological diseases (Mimaki *et al.*, 2012; Sarosiek *et al.*, 2013; Theodosakis *et al.*, 2014; Sakhrani and Padh, 2013; Swerdlow *et al.*, 2013). Anticancer approaches to target mitochondria include apoptosis induction in tumor cells as well as remodeling cellular energetic status via changes in respiration and metabolic pathways. Cancer cells exhibit a Crabtree-negative, fermentative metabolism (glycolysis) even in the presence of abundant oxygen, like *S. cerevisiae* (Shingu-Vazquez and Traven, 2011; Mimaki *et al.*, 2012). Reversing this activity is one of several scenarios of metabolic reprogramming in cancer cells. Consequently, therapies are sought against glycolytic enzymes such as *PKM2* which is elevated in tumor cells. Other inhibitors of lactate production show promise (Sakhrani and Padh, 2013). Targeting the BCL-2 family of pro- and anti-apoptotic proteins of cancer organelles (like mitochondria) has been developed to induce different levels of mitochondria apoptotic priming in cancers (Phan *et al.*, 2014; Sakhrani and Padh, 2013). Delivery of chemotherapeutics directly to “cancer programmed” cells is underway in drug discovery (Swerdlow *et al.*, 2013). The role of mitochondria in therapy against melanomas is being defined; one approach is the reversal of glycolysis as described above (Theodosakis *et al.*, 2014).

New antifungal therapies against new targets seem to be a useful pursuit due to the limitations of current antifungals. Given the availability of many extensive compound libraries, it would be useful to screen repurposed inhibitors against select reduced fitness mutants, such as those discussed in this paper. Such an approach is ongoing in several labs including ours, summarized in (Calderone *et al.*, 2014). It follows that compounds that target specific mitochondrial proteins should extensively disrupt cell metabolism and growth. In this regard, a recent observation is that cell division is dependent upon mitochondrial protein transport and energy production (Harbauer *et al.*, 2014).

Experimental procedures

Strains and Chemicals

All strains used in this study are listed in Supplemental Table S1. The homozygous mutants of *C. albicans* mitochondrial null mutants orf19.6607 and orf19.287 were originally described by Noble *et al.*, 2010. The gene reconstituted strains of each mutant Nuo1R (*nuo1* /*NUO1*) and Nuo2R (*nuo2* /*NUO2*) were constructed in our lab by introducing one allele of each gene into the corresponding mutant strain lacking the *ARG4*. Cassettes for each transformation were constructed in plasmid pSN69 (Arg4⁺) by insertion of two PCR products flanking *ARG4*. The primers used to amplify the flanking sequences are listed in Supplemental Table S1. At the 5'-end, fragment A corresponds to the full length

gene ORF plus ~1.5kb upstream sequence of the ATG codon. At the 3'-end, fragment B is a ~1.0 kb homologous fragment from the stop codon of each gene. Arg⁴⁺ transformants were selected on yeast nitrogen base-minimal medium lacking arginine but containing histidine. The integration of the transforming DNA was confirmed by PCR analysis of 10 transformants. Phenotypes of null and reconstituted strains (Nuo1R and Nuo2R) were compared to the parental strain WT (SN250) (Arg⁻His⁻Leu⁻). In addition, *ndh51* (JM02) and its reconstituted strain Ndh51R (JM03) were used in some experiments for comparison to the mutants described above (Velluci *et al.*, 2007). Ndh51p is a CI subunit protein but not fungal-specific.

The fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide) (Perelman *et al.*, 2012; Gallagher, 2010) was obtained from Invitrogen, Inc. The other fluorescent dyes DHE (dihydroethidium) and DCFDA (2',7'-dichlorofluorescein diacetate) were purchased from Santa Cruz Biotechnology and Sigma-Aldrich respectively. All the reagents for mitochondrial function studies were acquired from Sigma-Aldrich, Inc. Precast BN-PAGE gradient gels (4 to 16%) and a protein ladder were purchased from Invitrogen, Inc.

Growth in non-glucose media and chronological life span (CLS) determinations

All strains were grown at 30°C in YPD with 2% glucose, or YP medium supplemented with 2% glycerol (YPG) or 3% oleic acid as carbon sources. For CLS experiments, minimal medium (SD) with 2% glucose and 0.67% yeast nitrogen base supplemented with amino acids was used. All strains were routinely grown in 5 ml of YPD broth (Difco Laboratories) at 30°C overnight, washed in phosphate buffered saline (PBS) and then -inoculated in 100 ml of SD with an OD₆₀₀ of 0.2 at day 0. Shake cultures were grown at 30°C (200rpm), and one- ml samples were then removed from each strain daily at 1–9 days. Each aliquot of 400–600 cells was transferred to two YPD agar plates. Colony counts (% survival) were determined after a 48 h incubation. Data are indicated as % survival compared to the original inocula (Chen *et al.*, 2012).

Oxygen consumption, membrane polarization and ROS measurements

Oxygen consumption was measured polarographically using a Hansatech Oxygraph, (Model 910) Cells of each strain were collected from exponential phase growth (3 to 4 h) in YPD broth. The oxygen consumption rate (OCR) was determined at 0–5 min from 2.5×10^8 cells suspended in 1 ml of YPD broth (Li *et al.*, 2011). For determinations of mitochondrial membrane potential, the cyanine dye JC-1 was used (Li *et al.*, 2011; Hao et al 2013; Ding et al, 2014). Two forms of JC-1 (green fluorescence and red fluorescence) are used to discriminate energized and non-energized mitochondria since the red fluorescent aggregation in mitochondrial matrix is depend on the mitochondrial membrane potential. All strains were grown in YPD for 8h at 30°C, washed, and suspended in 1 ml of PBS then incubated with 5 μM JC-1 in 2% DMSO for 30 min at 30°C in the dark. The emission spectra at 488 nm and excitation spectra at 595 nm were determined by FACScan flow cytometer (Becton Dickinson).

Two oxidation-sensitive fluorescent dyes commonly are used for detecting cellular H₂O₂ (dihydrofluorescein diacetate, DCFDA) or superoxide anion (dihydroethidium, DHE) respectively. All yeast cultures were collected at 24 h after inoculation for ROS measurement of each strain and washed once before staining with PBS (pH 7.2). For determination of superoxide, cells (5×10^6) of each strain in 1-ml of PBS were stained with 5mM DHE in PBS (pH 7.2) at 30°C for 15 min and washed with PBS once prior to measurements by FACScan (excitation at 488 and emission at 567 nm). The same number of yeast cells was stained with 20 μ M DCFDA at 30°C for 15 min for measuring intracellular ROS (excitation at 488 and emission at 535 nm). PI (to 10 μ g/ml) was added 1 min before flow cytometry (Li *et al.*, 2011). ROS levels were determined from triplicate samples of each strain.

Extraction of mitochondrial protein; Complex I, II and CIV enzyme assays

Extraction of mitochondrial proteins was done as previously described (Li *et al.*, 2011; Barrientos, 2002). In brief, one liter of the overnight grown cells was collected, washed, and converted to spheroplasts with 100 mg of Zymolyase 20T (Seikagaku Biobusiness, Inc.) in 30 ml of 1 M sorbitol-Tris buffer containing 1 mM DTT. Spheroplasts lysis was carried out by sonication, and mitochondria were obtained using a two-step Percol gradient. 30% Percol in buffer PB1 (0.3 M mannitol, 10 mM TES, 0.1% BSA, pH 7.5) was placed at the bottom of a centrifugation tube and an equal volume of 20% Percol in PB2 buffer (0.3 M sucrose, 10 mM TES, 0.1% BSA, pH 7.5) was layered on top of the PB1 buffer. Crude mitochondria were then overlaid at the top of a centrifuge tube before centrifugation at $40,000 \times g$ for 45 min at 4°C. Purified mitochondria (whitish band) were collected and extracted with 10% w/v N-dodecyl β -D maltoside.

Enzyme activities of the mitochondrial electron transport chain (ETC) complexes CI, CII and CIV were measured spectrophotometrically in 96-well plates (Li *et al.*, 2011). The oxidation rates of CI or CII, and reduced cytochrome C (CIV) substrates were monitored by plotting changes in OD values per min to determine mitochondrial complex activities (see below).

CI assay—The reduction of the electron acceptor DCIP (2,6-dichloroindophenol) was monitored at OD₆₀₀ in a 200 μ l volume containing 25 mM potassium phosphate (pH 7.4), 0.35% BSA, 70 μ M decylubiquinone, 60 μ M DCIP and 4 μ M antimycin A (Li *et al.*, 2011). 10 ~ 50 μ g purified mitochondrial protein was first pre-incubated with 195 μ l of the reaction aliquot at 37°C for 10 min. The decrease in absorbance at OD₆₀₀ was measured at 30 sec intervals for 5 min at 37°C immediately after the addition of 5 μ l of 10 mM NADH. Assays were also measured in the presence of 1mM rotenone CI inhibitor following an additional 5 min incubation.

CII assay—DCIP was again used as an electron acceptor, and the substrate for the CII assay was succinate. 200 μ l of incubation buffer containing 80 mM potassium phosphate (pH 7.5), 0.1% BSA, 2 mM EDTA, 0.2 mM ATP, 50 μ M decylubiquinone, and 80 μ M DCIP was used for enzyme reactions. 10 ~ 50 μ g of purified mitochondrial protein was added to the reaction buffer at 37°C for 10 min in 96-well plates without substrate. After 10

min, 0.3 mM KCN and 10 mM succinate (5 μ l volume) was added to each assay reaction. The absorbance at OD₆₀₀ was measured at 1 min intervals for 5 min at 37°C. The specificity of CII activity was verified in the presence of 5 mM malonate for an additional 5 min.

Complex IV—Activity was determined at OD₅₅₀ by measuring a decrease in absorbance during the oxidation of reduced cytochrome C (*cytC*). In our previous protocol, the reduced form of *cytC* was obtained by treatment with ascorbic acid followed by a tedious dialysis process (Li *et al.*, 2011). In the current CIV assay, we used 0.1 mM DTT to reduce *cytC* which is measured as a color change from dark orange-red to pale purple-red after a 15 min treatment at RT. 10 ~ 50 μ g of purified mitochondrial protein was dissolved in 190 μ l of reaction buffer in 96-well plates containing 10 mM potassium phosphate (pH 6.5), 0.25 M sucrose, 0.1% BSA, 2 mM EDTA at 37°C, 10 min. The reaction was measured after the addition of 5 μ l of 1 mM lauryl maltoside and 10 μ M of reduced *cytC* in 5 μ l.

Blue native polyacrylamide gel (BN-PAGE) electrophoresis

Mitochondrial protein was prepared as described previously (Li *et al.*, 2011). Prior to loading mitochondrial proteins onto a BN-PAGE gradient gel (4 to 16%), the protein samples derived from mitochondria of each *C. albicans* strain were concentrated and solubilized in 10% DDM (*n*-dodecyl- β -D-maltoside). A total of 20 μ l of each sample (~100 to 150 μ g of protein) was mixed with BN sample buffer (2X). Electrophoresis was performed in an X-Cell SureLock mini-cell system (Invitrogen) with 200 ml of cathode buffer in the upper (inner) buffer chamber and 150 ml of anode buffer in the lower (outer) buffer chamber. The cathode buffer contains 0.02% Serva Blue G-250, supplemented with 0.02% DDM, and the anode buffer consisted of 50 mM bis-Tris, pH 7.0. Electrophoresis was carried out with 65 V for 1 h and then raised to 120 V overnight at 4°C. The blue gel was decolorized using methanol: acetic acid: water (50 : 10 : 40 v/v/v), with shaking, until the appearance of blue bands was noted. Image intensity was measured using ImageJ software (Li *et al.*, 2011). All strains were evaluated for the reduction or loss of ETC complexes.

RNA and microarray analysis

The contribution of mitochondrial CI *NUO1* and *NUO2* to transcriptional events was determined. RNA isolation and microarray experiments were done as described previously (Li *et al.*, 2011; Sun *et al.*, 13, She *et al.*, 2013; Khamooshi *et al.*, 2014). All strains were grown in 20-ml of 2% SD medium at 30°C for either 5 h for WT and reconstituted strains, or 8 h for both mutants. One-color microarray-based gene expression analysis was done using the Agilent format chips containing 6,101 *C. albicans* genes including nuclear and 12 mitochondrial-encoded genes all in duplicate. Hybridization was completed in an Agilent SureHyb hybridization chamber, and scans were processed with an Agilent SCAN G2505C Microarray Scanner System. The image files were analyzed by Agilent Feature Extraction Software, and cyanine 3 intensities were then logarithmically transformed and normalized. The fold-change for each gene in duplicate was calculated by comparisons to WT and the corresponding gene reconstituted strains. A fold-change of 3 was used as a cut off value for determining significant changes.

Murine model of blood-borne disseminated candidiasis

A mouse model of disseminated candidiasis was used in order to measure the virulence of the mitochondrial mutants (Lionakis *et al.*, 2011). Virulence of each null strain was compared to the corresponding gene reconstituted (Nuo1R, Nuo2R) and WT strains in 6–8 week old female BALB/c mice from Yangzhou University (China). Tissue levels of strains were measured as CFU/g in kidney, liver and spleen. The animal experiments were done under the guidance of a protocol approved by the Animal Study Committee of the Institute of Dermatology, CAMS, according to the National Guidelines for Animal Care. Mice were injected via the lateral tail vein with 1×10^6 yeasts of *C. albicans* WT, mutant strains *nuo1* and *nuo2*, and the gene reconstituted strains. All strains were grown in YPD overnight, harvested, washed and suspended in PBS (pH 7.5) prior to injection into mice. For survival experiments, each *C. albicans* strain was used to inoculate 10 mice. Mice were randomly assigned to groups for each strain (WT, *nuo1*, *nuo2*, Nuo1R and Nuo2R) and given food and water *ad libitum*. We observed mice 2-times per day to determine those that were moribund. Concomitantly, 12 mice were also inoculated with each strain for the determination of fungal burden. Three mice from each group were euthanized after 1, 24, 48, and 72 h post-infection. Kidney, spleen, and liver were removed, weighed, and homogenized in PBS. Following serial dilutions, tissue homogenates were plated in duplicate on YPD agar plates containing penicillin and streptomycin to quantitate the CFU (colony forming units) per gram of tissue after 48 h of incubation at 30°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to acknowledge the Georgetown University Medical Center Lombardi Cancer Center for their help with the microarray analysis. The GUMC Biomedical Graduate Research Organization generously provided bridge funds. We are also wish to thank the National Key Basic Research Program of China for funds (2013CB531605). Funds were also provided by the NIH-NIAID, AI090290 to RC. We are indebted to Dr. Suzanne Noble for providing the CI null mutants and Dr. Margaret Hostetter for providing the *ndh51* and gene reconstituted strains.

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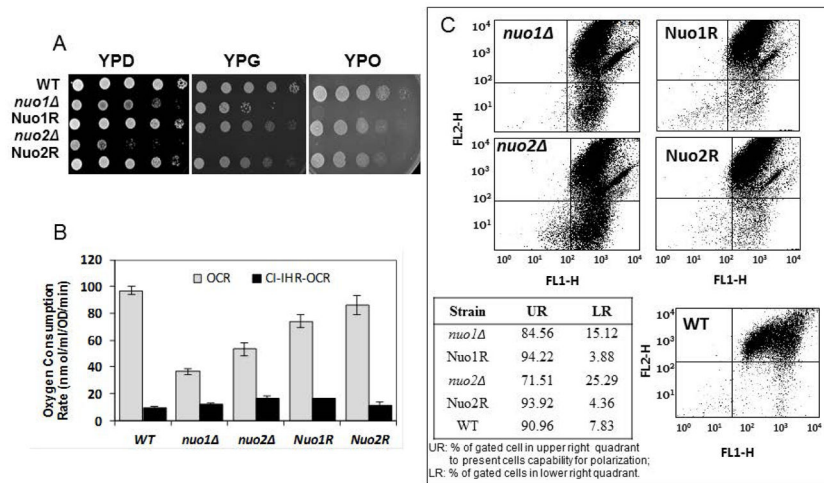


Figure 1.

A. Growth defects on glucose and non-glucose carbon media. *C. albicans* strains were grown overnight in YPD medium, washed and serially diluted in PBS. 3 μ L of each serial dilution was plated on the YP medium supplemented with 2% glucose (YPD), 2% glycerol-YP (YPG), or 3% oleic acid YP-agar plates. All cultures were incubated for 2 days at 30°C. Both *nuo1* and *nuo2* grew less than control strains on YPD and had additionally reduced growth on YPG and YPO. The *Nuo1R* and *Nuo2R* gene-reconstituted strains were intermediate in growth compared to WT and null strains. **B.** The oxygen consumption rates (OCR) of *nuo1*, *nuo2*, *Nuo1R*, and *Nuo2R* and WT are shown. Both mutants consume less oxygen than control strains. Oxygen consumption was also measured in the presence of rotenone, a CI inhibitor (CI-IHR-OCR) demonstrating that respiration is mainly due to CI activity. **C.** Mitochondria membrane potential (ψ M) for all strains is shown. Flow cytometric analysis of strains stained with JC-1 indicates a greater proportion of cells with non-polarized mitochondria from *nuo1* and *nuo2* mutants (upper left), reconstituted strains (upper right) and WT cells (lower right). The WT strain and reconstituted strains *Nuo1R* and *Nuo2R* display a typical fluorescent profile of high staining in the upper right quadrants (UR). Non-polarized cells fluoresce approximately 3–5 times more in both mutant cell populations than control strains. The relative proportions of mutant cells (UR, LR) are indicated in the table insert.

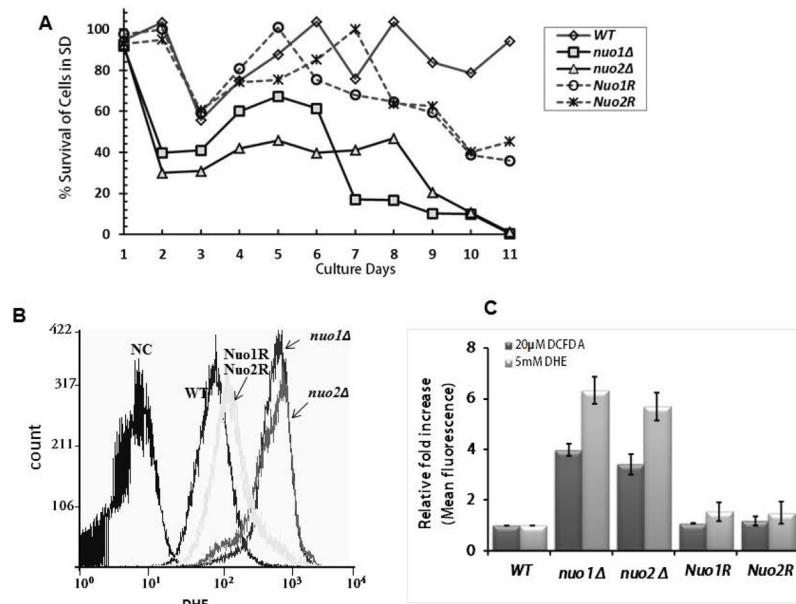


Figure 2.

The chronological life span (CLS) of each strain measures the length of time when cells are viable in stationary phase. **(A)** *C. albicans* strains (OD_{600} of 0.2) were grown at 30°C in 100 ml of SD containing 2% glucose. From 1 to 9 day-old cultures, 1-ml of culture was removed, serially diluted, and spread on YPD agar to determine viability. The % survival are shown (CFU/primary inoculum) as: WT (SN250); *nuo1* ; *nuo2* ; *Nuo1R*; *Nuo2R*. **(B)** DHE staining was done to determine superoxide content. Both null mutants are strongly stained with DHE compared to WT cells; the corresponding reconstituted strains have slightly higher levels of superoxide than WT. **(C)** cellular peroxide of each strain is also estimated with DC-FDA. The relative fold-increase of fluorescence with both DHE and DC-FDA stains in the null mutants *nuo1* and *nuo2* was 6-fold higher in superoxide and 3-times higher in peroxide compared to control strains.

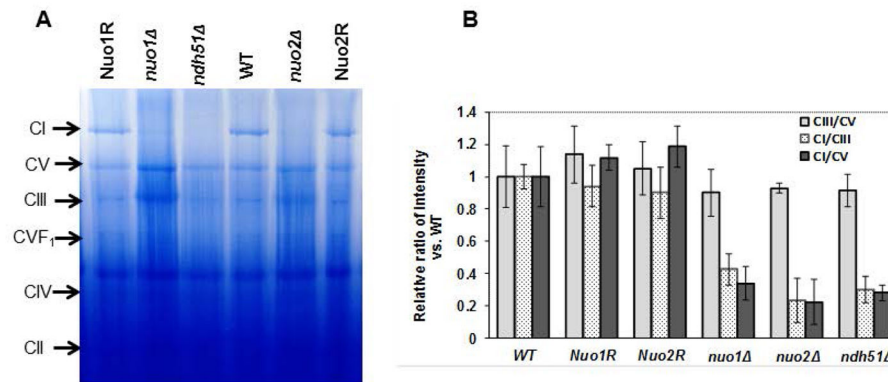


Figure 3.

(A) BN-PAGE electrophoresis of *C. albicans* mitochondrial OXPHOS complexes in WT, *nuo1*⁻, *nuo2*⁻, and *ndh51*⁻, as well as the gene reconstituted strains. Equal concentrations of total mitochondrial proteins from all strains were separated by BN-PAGE. The CI band is markedly reduced in each of the three CI mutants compared to levels in the matched set of control strains, WT and reconstituted strains. (B) The stainable amount of CI from each strain is expressed as relative ratios of the intensity of CI/CIII and CI/CV in each strain versus to WT. Levels of CIII/ CV are minimally changed in all mutants but CI/CIII and CI/CIV are considerably reduced (intensities) suggesting less CI in all null strains.

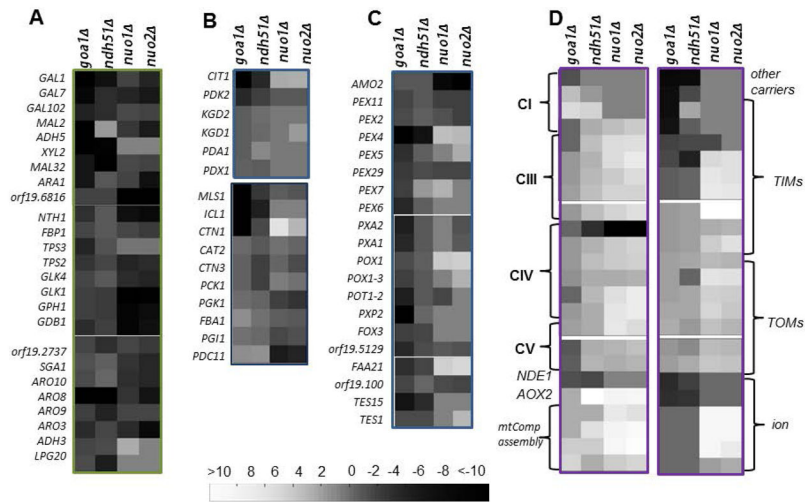


Figure 4.

Transcriptional profiling of carbon metabolism and mitochondria in mutants *goal1*, *ndh51*, *nuo1*, and *nuo2*. Up and down regulated genes of mutants are compared to WT cells. Down regulated and up regulated genes are coded from black to white as indicated. Four functional gene groups are shown: **A.** glycolysis, glycogen metabolism, non-glucose metabolism, and the Ehrlich Pathway of which amino acids are used as carbon sources; **B.** Mitochondrial TCA and peroxisomal glyoxylate cycle and gluconeogenesis; **C.** Peroxisomal transporters, β -oxidation; **D.** Mitochondria CI-CV complexes, transporters of inner membranes (TIMs) and transporters of outer membrane (TOMs) of mitochondria; mitochondrial ETC assembly, and ion channels (magnesium, phosphate). General observations are that: **1)** each of the 4 mutants down regulate numerous genes of carbon metabolism (**A**), **2)** *goal1* is especially affected in genes in categories **B**, **C** and **D**, that includes the glyoxylate cycle (carbon conservation), peroxisomal transporters (PEX genes), and TIM/TOM genes, **3)** *nuo1* and *nuo2* exhibit many ETC Complex and TIM/TOM genes that are upregulated (**D**), and **4)** peroxisomal genes are less associated with *nuo1* and *nuo2* (**C**).

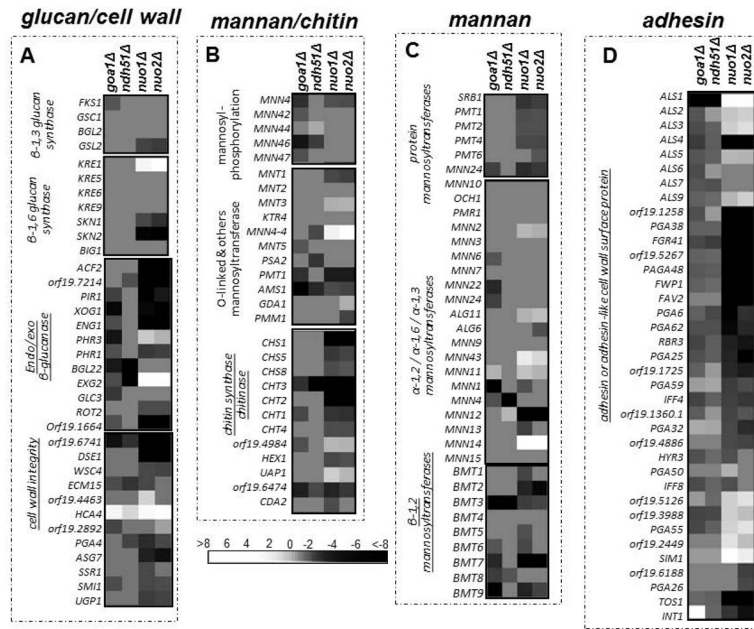


Figure 5. Transcriptional profiling of genes encoding cell wall functions in mutants. Functional annotation for each gene set is indicated (A–D) and displays of upregulated versus down regulated genes are coded in white (up) to black (down). Differences are observed especially among the gene groups that are underlined. Gene changes are similar in the *nuo1* and *nuo2* but strikingly different compared to *ndh51*.

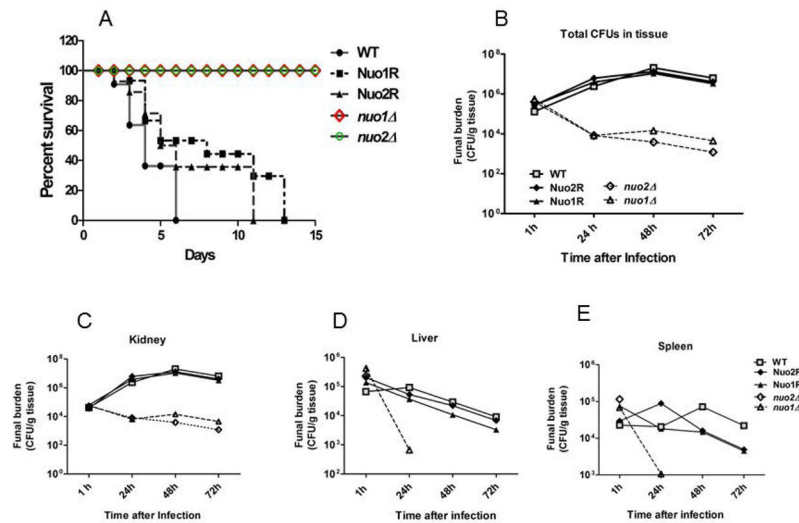


Figure 6.

A. Mouse survival (female BALB/c, 20 g, 10 mice/group) following infection with 1×10^6 yeast cells of the *C. albicans*. WT (solid line), the one-allele reconstituted strain Nuo1R and Nuo2R (dashed lines). The colored lines indicate each mutant strain, *nuo1* (red); *nuo2* (green). Both *nuo1* and *nuo2* are avirulent, while the Nuo1R and Nuo2R are intermediate in virulence compared to the fully virulent WT cells. **B–E.** Tissue counts (CFU/g tissue) of mice infected with null mutants as well as WT and gene reconstituted strains. Following infection, at 1, 24, 48 and 72 h, kidney, liver and spleens were removed, weighed, homogenized and samples plated on YPD agar medium. The total CFUs in tissue for each strain are shown over time. Data are from two experiments with 3 mice per time point for each strain (mean \pm SD). The CFU/gram of tissue of *nuo1* and *nuo2* mutants is 2–3 logs less than control strains from infected kidneys. Both mutants were also readily cleared from liver and spleen; no fungi were recovered from both of organs by 24–48h. Both of mutants are indicated as dashed lines. **B–E.** WT strain and reconstituted strains Nuo1R and Nuo2R tissue counts are represented as CFUs/g tissue. **B.** all tissues; **C.** Kidney; **D.** Liver; **E.** Spleen.

Table 1

Mitochondrial ETC CI, CII, and CIV enzyme activities

	Enzyme activity (nM/min/mg protein)		
	<i>CI</i>	<i>CII</i>	<i>CIV</i>
WT	828.68 ± 24.68	516.15 ± 55.84	301.80 ± 30.45
<i>nuo1</i>	317.63 ± 15.97*	420.31 ± 21.49	70.80 ± 35.97*
<i>nuo2</i>	375.78 ± 27.41*	522.84 ± 19.50	243.47 ± 26.49
<i>ndh51</i>	109.15 ± 5.05*	127.43 ± 49.67*	155.96 ± 22.95*

* : $p < 0.001$, mutant versus the WT

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Table 2

C. albicans null or conditional mutants of mitochondrial genes with reported avirulence or decreased virulence phenotypes.

Orf/gene	Gene function ^a	Other functions ^b	Reference
Orf19.2019*	Putative Mt- Large ribosomal subunit	Protein maturation & Mt-translation, <i>S. cerevisiae</i> <i>MRPL16</i>	Becker <i>et al.</i> , 2010
Orf19.2956*	Putative Mt – genome maintenance	Recombinational repair	Becker <i>et al.</i> , 2010
Orf19.7019	Mt-ribosomal protein	Induced upon adherence to polystyrene	Becker <i>et al.</i> , 2010, Marchais <i>et al.</i> , 2005
<i>GOA1</i> **	Putative Regulator of Mt-CI	Cross-talking with peroxisomes, cell wall synthesis, Chronological life span (CLS)***	Bambach <i>et al.</i> , 2009
Orf19.6607* Orf 19.287	CI subunit	Optimal growth	Noble <i>et al.</i> , 2010; this paper
<i>SAM37</i> *	Sorting and Assembly; Machinery, Mt-outer membrane	Avirulent; fitness, cell wall integrity	Shingu-Vazquez <i>et al.</i> , 2011; Qu <i>et al.</i> , 2012

^a gene function for mitochondria;

^b functions other than mitochondria.

* Fungal-specific;

** CTG clade of Saccharomycotina (subphylum of Ascometes); includes most *Candida* species except *C. glabrata*.

*** CLS, chronological life span.