A model system for mitochondrial biogenesis reveals evolutionary rewiring of protein import and membrane assembly pathways

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The controlled biogenesis of mitochondria is a key cellular system coordinated with the cell division cycle, and major efforts in systems biology currently are directed toward understanding of the control points at which this coordination is achieved. Here we present insights into the function, evolution, and regulation of mitochondrial biogenesis through the study of the protein import machinery in the human fungal pathogen, Candida albicans. Features that distinguish C. albicans from baker's yeast (Saccharomyces cerevisiae) include the stringency of metabolic control at the level of oxygen consumption, the potential for ATP exchange through the porin in the outer membrane, and components and domains in the sorting and assembling machinery complex, a molecular machine that drives the assembly of proteins in the outer mitochondrial membrane. Analysis of targeting sequences and assays of mitochondrial protein import show that components of the electron transport chain are imported by distinct pathways in C. albicans and S. cerevisiae, representing an evolutionary rewiring of mitochondrial import pathways. We suggest that studies using this pathogen as a model system for mitochondrial biogenesis will greatly enhance our knowledge of how mitochondria are made and controlled through the course of the cell-division cycle.

SAM complex | Omp85 | stop-transfer pathway | Sam51

Mitochondrial biogenesis is an essential aspect of the cell-division cycle and requires replication of the mitochondrial genome, synthesis of lipids to build new membranes, and the import of around 1,000 different proteins encoded in the nuclear genome (1, 2). The pathways for protein import have been defined and are mediated by a series of molecular machines in the outer and inner mitochondrial membranes, along with soluble factors in the intermembrane space and matrix (3, 4). One of these molecular machines, the translocase of the outer membrane (TOM) complex, forms a channel in the outer mitochondrial membrane that serves as the gateway for protein import into mitochondria. Recent work in the model yeast Saccharomyces cerevisiae has shown that phosphorylation of specific subunits of the TOM complex regulates the activity of the protein import channel. An elegant coordination of mitochondrial biogenesis with cellular metabolic needs was seen when glucose levels were high, with phosphorylation of TOM complex subunits coinciding with repression of mitochondrial activity (5, 6). S. cerevisiae responds to the presence of glucose through metabolic cycling, oscillating cycles of aerobic respiration and glycolytic activity. This process requires tight regulatory control of mitochondrial biogenesis cordonned into specific states of metabolic activity that are coordinated with the cell-division cycle (7–9).

Although S. cerevisiae has provided an excellent, genetically tractable model organism to determine how cellular metabolism is integrated with signaling pathways and networks, it is somewhat peculiar with regards to regulation of mitochondrial biogenesis. This yeast is subject to the Crabtree effect: It ferments glucose anaerobically, whether oxygen is available or not (10). Many of the genes required for anaerobiosis arose through a whole-genome-duplication (WGD) event, with duplicated genes modified to provide new functions or new signaling switches (10–13). During optimal, rapid growth in glucose, mitochondrial function is repressed, and transcriptional networks have been reorganized in S. cerevisiae to enable regulation of mitochondrial biogenesis by carbon source (14, 15).

In anticipation of work that soon will enable genetic and biochemical investigations of the integration of metabolic control globally with every cellular pathway, we sought to develop a yeast model in which mitochondrial function is not repressed during optimal growth and which does not show metabolic cycling. This model would provide a means to distinguish those control points that are linked specifically to the switching events in metabolic control. Candida albicans is a budding yeast that diverged from the lineage that gave rise to S. cerevisiae around 300 million years ago (16). The genome of C. albicans has been sequenced (17), and various tools have been developed for genetic deletion, epitope tagging, and genetic studies in this organism (18–21). Unlike S. cerevisiae, C. albicans grows aerobically, and mitochondrial respiration is active during optimal growth in glucose (15, 22, 23). Thus, although mitochondrial activity is regulated through the course of the cell cycle in C. albicans, it is not subject to changes depending on the available carbon source (15, 23, 24).

Here we demonstrate that C. albicans does not undergo metabolic cycling and that mitochondria can be isolated from C. albicans and assayed for the import of mitochondrial proteins in vitro. Using this model system, we find several differences in the import reactions relative to S. cerevisiae, uncovering functional aspects relating to the mitochondrial sorting and assembly machinery (SAM) complex, an essential molecular machine that assembles β-barrel proteins into the mitochondrial outer membrane. We show that in C. albicans the core SAM complex comprises at least three integral membrane proteins: Sam35, Sam50, and a protein which we refer to as “Sam51.” We demonstrate that Sam50 and Sam51 play similar roles in the assembly of the TOM complex, a process that is much more rapid in C. albicans than in S. cerevisiae. Moreover, consideration of the protein import pathways for components of the electron transport chain in C. albicans provides examples of evolutionary rewiring of mitochondrial import pathways in related species, showing that the particular pathway taken into mitochondria was not hard-wired early in evolution; instead, rewiring is open

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to organisms to take advantage of new metabolic or gene-regulatory opportunities.

**Results**

**Protein Import Machinery in Mitochondria from C. albicans.** The components of the protein import pathways as discovered in *S. cerevisiae* are highly conserved in *C. albicans*, and BLAST searches are sufficient to identify candidate homologs in most cases (Table S1). Proteins of overall similar size and domain structures were detected, as detailed in Table S1. Several distinguishing features of *C. albicans* also were observed, e.g., in respect to the TOM complex: *C. albicans* has no Tom70 and has extensions to the cytosolic domains of Tom6 and Tom7 (Table S1). With recent studies in *S. cerevisiae* showing that phosphorylation in the cytosolic domains of TOM subunits is crucial for regulation of TOM complex activity in response to changes in metabolic activity (5, 6), future analysis of sequence variations in *S. cerevisiae* and *C. albicans* could help show how these phosphorylation sites affect cell cycle and metabolism. In some cases hidden Markov models (HMM) proved essential for identifying homologs; for example, an ORF encoding a Tom5-related sequence previously had been unidentified and unannotated because of its small size and sequence divergence. The Mia40 homolog in *C. albicans* previously was known only as a Hap43-repressed gene, because it lacks the characteristic N-terminal transmembrane signature of the Mia40 from *S. cerevisiae*. Using an HMM search, we also found a Sm50-related protein in *C. albicans* with no homolog in *S. cerevisiae* (Table S1). In *S. cerevisiae*, Sam50, Sam35, Sam37, and Mdm10 form the SAM complex, a molecular machine required for the assembly of β-barrel proteins into the mitochondrial outer membrane (3, 4).

**Oxidative Phosphorylation and Protein Import into C. albicans Mitochondria.** During growth in glucose, *S. cerevisiae* represses mitochondrial function through changes in gene expression and metabolite levels via the yeast metabolic cycle (8). Chemostat cultures of *S. cerevisiae* revealed a cyclic change in oxygen consumption (Fig. 1A), consistent with that seen previously (8, 9). Metabolic cycling was not observed with cultures of *C. albicans*: These grew at comparably high cell density but had a constant requirement for oxygen consumption (Fig. 1A). We isolated mitochondria from cultures of *C. albicans* to test their capacity for precursor import (Fig. 1B). The model precursor protein Su9-DHFR is destined to the mitochondrial matrix and is translocated across the outer and inner membranes to be processed in two steps, thereby generating an intermediate (i) form and then a mature (m) form of the imported protein (25). The intermediate and mature forms were protected from trypsin by the intact mitochondrial form of the imported protein (25). The intermediate and mature forms were detected, as detailed in Table S1. Several distinguishing features of *C. albicans* also were observed, e.g., in respect to the TOM complex: *C. albicans* has no Tom70 and has extensions to the cytosolic domains of Tom6 and Tom7 (Table S1). With recent studies in *S. cerevisiae* showing that phosphorylation in the cytosolic domains of TOM subunits is crucial for regulation of TOM complex activity in response to changes in metabolic activity (5, 6), future analysis of sequence variations in *S. cerevisiae* and *C. albicans* could help show how these phosphorylation sites affect cell cycle and metabolism. In some cases hidden Markov models (HMM) proved essential for identifying homologs; for example, an ORF encoding a Tom5-related sequence previously had been unidentified and unannotated because of its small size and sequence divergence. The Mia40 homolog in *C. albicans* previously was known only as a Hap43-repressed gene, because it lacks the characteristic N-terminal transmembrane signature of the Mia40 from *S. cerevisiae*. Using an HMM search, we also found a Sm50-related protein in *C. albicans* with no homolog in *S. cerevisiae* (Table S1). In *S. cerevisiae*, Sam50, Sam35, Sam37, and Mdm10 form the SAM complex, a molecular machine required for the assembly of β-barrel proteins into the mitochondrial outer membrane (3, 4).

**Stop-Transfer Pathway: Rewiring the Import of Cytochromes.** A distinguishing feature of mitochondria isolated from *C. albicans* is the inefficiency with which they import Cyb2, a heme-containing dehydrogenase (1-lactate cytochrome-c oxidoreductase) essential for the utilization of l-lactate as a carbon source. Fig. 2A describes the pathway for Cyb2 import via the stop-transfer sorting pathway as it occurs in *S. cerevisiae*. The import of ScCyb2 depends on active unfolding of the heme-binding domain by the action of matrix-located Hsp70 (26–30). All the necessary components for the

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**Fig. 1.** Protein import into mitochondria from *C. albicans*. (A) Chemostat culture of *S. cerevisiae* CEN.PK and *C. albicans* DAY185 strains. (B) Mitochondria isolated from wild-type *C. albicans* (50 μg protein per lane) were incubated with the indicated proteins at 25 °C for the indicated times (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis. The precursor (p), intermediate (i), and mature (m) forms are indicated where relevant. (C) Mitochondria isolated from *C. albicans* (50 μg protein per lane) were incubated with the indicated proteins at either 25 °C or 4 °C for the indicated times (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis.
stop-transfer pathway are present in *C. albicans* (Table S1), but, as compared with *S. cerevisiae*, import of ScCyb2 is highly inefficient in mitochondria from *C. albicans*, with a greatly diminished processing to produce the intermediate ("iCyb2") and mature ("mCyb2") forms (Fig. 2B). Similarly, the import of cytochrome c1 from *S. cerevisiae* (ScCyt1) also follows the stop-transfer pathway (31), and the transformed strains were monitored for growth in semisynthetic medium with either L-lactate or glucose as a carbon source. Wild type, blue; Δcyb2, red; Δcyb2/ScCYB2, green; Δcyb2/CaCYB2, purple. All strains grew to comparable density in glucose medium. (D) Sequence alignment of the N-terminal 28 residues of Cyb2 from *C. albicans* (CaCyb2), *S. cerevisiae* (ScCyb2), and related yeasts. Sequence identity and similarity are indicated by asterisks and dots, respectively. The processing site that generates the mature form of ScCyb2 is the peptidyl bond between residues L84 and D85, four residues upstream from Q89.

Fig. 2. The stop-transfer pathway for protein import into mitochondria. (A) The stop-transfer pathway occurs by the sequential interaction with the TOM complex (white rectangles) and the TIM23 complex (white triangles), and the sequential processing by the mitochondrial processing peptidase (MPP) in the mitochondrial matrix and the inner membrane peptidase (IMP) in the inner membrane. (B) Mitochondria isolated from *C. albicans* (50 μg protein per lane) were incubated with the precursor (p) forms of ScAdh3 or ScCyb2 or ScCyt1 at 25 °C for the indicated times (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis. The intermediate (i) and mature (m) forms are indicated where relevant. (C) The CYB2 genes from *S. cerevisiae* and *C. albicans* were amplified and cloned into a plasmid for expression in a Δcyb2 strain of *S. cerevisiae*, and the transformed strains were monitored for growth in semisynthetic medium with either L-lactate or glucose as a carbon source. Wild type, blue; Δcyb2, red; Δcyb2/ScCYB2, green; Δcyb2/CaCYB2, purple. All strains grew to comparable density in glucose medium. (D) Sequence alignment of the N-terminal 28 residues of Cyb2 from *C. albicans* (CaCyb2), *S. cerevisiae* (ScCyb2), and related yeasts. Sequence identity and similarity are indicated by asterisks and dots, respectively. The processing site that generates the mature form of ScCyb2 is the peptidyl bond between residues L84 and D85, four residues upstream from Q89.

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The ORFs encoding CaCyb2 and ScCyb2 were cloned for expression from a plasmid, and Δcyb2 mutants of *S. cerevisiae* were transformed. On minimal growth medium with L-lactate as a carbon source, the Δcyb2 mutants depend on ScCyb2 activity to feed electrons from L-lactate into the electron transport chain (Fig. 2C). CaCyb2 could not support growth of the Δcyb2 mutant on L-lactate, although the transcript was expressed at high levels (Fig. S1A), and the functional domains of Cyb2 are highly homologous (Fig. S1B), suggesting that the *Candida* protein was not imported sufficiently well into the mitochondrial intermembrane space in *S. cerevisiae*. The import signals for delivery of the cytochromes Cyb2 and Cyt1 to the intermembrane space in *S. cerevisiae* are well characterized N-terminal extensions, and comparative sequence analysis shows that CaCyb2 and the Cyb2 homologs from related yeasts entirely lack this N-terminal stop-transfer sorting sequence (Fig. 2D). Similarly, CaCyt1 lacks a large segment from within the N-terminal stop-transfer sorting sequence found on ScCyt1 (Fig. S1B).

Sam35, Sam37, and the Assembly of Voltage-Dependent Anion-Selective Channel Oligomers in *C. albicans*. The assembly of β-barrel proteins into mitochondria can be measured readily in vitro using blue-native PAGE (BN-PAGE), and two model proteins
have been used as substrates in these assays: Tom40 and the voltage-dependent anion-selective channel (VDAC), also known as “porin.” In the case of Tom40, the assembly reaction measures both the assembly of the Tom40 β-barrel and the recruitment of other TOM subunits to form the mature TOM complex. When mitochondria isolated from C. albicans are used, the assembly of Tom40 from S. cerevisiae occurs at a rate similar to that of the assembly of Tom40 from C. albicans, demonstrating that there is sufficient sequence similarity in the interactive surfaces so that the heterologous Tom40 can be assembled into the oligomeric TOM complex (Fig. 3A).

The major β-barrel protein in the outer mitochondrial membrane is the hetero-oligomeric VDAC. Newly imported VDAC monomers assemble into oligomers with preexisting VDAC (32). ScVDAC is inserted into the outer membrane in a time-dependent manner forming numerous oligomers, including a major 200-kDa form, which are resolved by BN-PAGE (Fig. 3B). In contrast, the oligomers of ScVDAC in C. albicans mitochondria are of uniform stoichiometry and/or are less dynamic in nature: The imported ScVDAC showed the majority of the protein in stable oligomers of ∼400 and ∼440 kDa (Fig. 3B). In S. cerevisiae, the import and assembly of VDAC depends largely on the SAM complex subunit Sam37 (33). In both S. cerevisiae and C. albicans, deletion of Sam37 leads to a concomitant reduction in levels of Sam35, and this reduction can be suppressed by overexpression of the SAM35 gene (34, 35). With a sam37Δ strain of C. albicans engineered to have one copy of the SAM25 gene under the control of the strong TEF1 promoter, a 9.5-fold up-regulation of SAM35 transcript levels was measured in the mutant (sam37ΔΔsam35)† (Fig. 3C). That Sam35 then is assembled into the SAM complex in the sam37ΔΔsam35† strain is evident by the change in the size of Tom40 assembly intermediate I compared with the sam37Δ strain (Fig. 3D). Only a minor defect in VDAC assembly is seen in mitochondria from the C. albicans sam37Δ strain, and the increased levels of SAM35 did not rescue this defect (Fig. 3E). The very mild phenotype seen in C. albicans is in contrast to the strong requirement for Sam37 in VDAC assembly measured in S. cerevisiae (33). For quality control, we show that mitochondria from the sam37ΔΔsam35† strain import ScFp, ScAdh3, and ScAac1 with efficiency comparable to that of mitochondria from the wild type strain under conditions where protein import was linear with respect to time and dependent on the membrane potential (ΔΨm) (Fig. 3F).

**TOM Complex Assembly in C. albicans.** In S. cerevisiae, the β-barrel core of the TOM complex, Tom40, is assembled via two intermediate forms into the mature TOM complex (Fig. 4A) in a time-dependent manner that, in vitro, takes more than 60 min to complete (34, 36). Mitochondria from C. albicans assemble Tom40 into the mature TOM complex much more rapidly: When [35S]-labeled CaTom40 was imported into mitochondria isolated from C. albicans, Intermediate I (representing [35S]-CaTom40 bound in the SAM complex) forms within 2 min and is transferred into the mature, ∼400-kDa form of the TOM complex within 10 min (Fig. 4B). The loss of Sam37 has a major impact on the assembly of the TOM complex, including a decrease in the amount of [35S]-CaTom40 entering the intermembrane space as judged by SDS/PAGE and BN-PAGE. These effects are suppressed partially by overexpression of SAM35 (Fig. 4B). These results in C. albicans are consistent with the functions of Sam35 and Sam37 observed in the S. cerevisiae model system (34, 37).

The Sam35 subunit of the SAM complex is enigmatic, because it interacts selectively with substrate β-barrel proteins in the intermembrane space but behaves as if it were a peripheral membrane protein on the outer surface of mitochondria: It is largely accessible to proteolysis and is readily extracted from mitochondrial membranes using sodium carbonate treatment (37). Hydrophobicity analysis of CaSam35 suggested that it has potential membrane-spanning segments that are more hydrophobic than the amphipathic segments detected in ScSam35 (Fig. 4C). We sought to take advantage of this difference and use the more hydrophobic C. albicans protein to determine whether Sam35 has a transmembrane segment. Mitochondria isolated from a strain expressing N-terminally epitope-tagged HA-CaSam35 and from a strain expressing C-terminally epitope-tagged CaSam35-HA were treated with trypsin, and in both cases CaSam35 behaves as an outer membrane protein, exposed on the mitochondrial surface and sensitive there to protease (Fig. 4C). Incubation of mitochondria with sodium carbonate to strip peripheral membrane proteins revealed that both HA-CaSam35 (Fig. 4D) and CaSam35-HA (Fig. 4E) behave as an integral membrane protein.

**C. albicans has a Second Member of the Omp85 Protein Family.** The feature distinguishing SAM complex components in C. albicans from those in S. cerevisiae is a second protein sharing 22.8% sequence identity and predicted domain composition with ScSam50 (Fig. S2A). We refer to this protein as “Sam51.” This protein is not simply a result of gene duplication from the well-characterized WGD event, because we found homologs of Sam51 in most clades of yeast, including species prior and subsequent to the WGD event (Fig. S2B). Sam51 is found throughout the Ascomycota, indicating that its absence in S. cerevisiae is the result of a secondary loss in this particular species (Fig. S2C and Fig. 5). Reconstruction of the ancestral lineage at the point of genome duplication predicts that the ancestor had both SAM51 and SAM50 genes, with data for the ancestor derived from the Yeast Gene Order Browser (38). In the post-WGD species, there are various permutations in the observed losses of the SAM51 and SAM50 genes (Fig. S2C). The Sam51 proteins are as similar to bacterial BamA proteins as to the Sam50 sequences, as shown by the clear branching pattern forming three independent subgroups of the Omp85 protein family (Fig. 5). This phylogenetic analysis demonstrates that the Sam51 group of sequences forms a monophyletic branch with the exclusion of all Sam50 sequences and therefore represents a separate subgroup of Omp85 proteins (Fig. 5).

RT-PCR shows that under standard growth conditions the SAM51 gene is expressed at higher levels than SAM50 in wild-type C. albicans (Fig. 5B). We raised antibodies to recombinant Sam50 and Sam51 and used a semiquantitative immunoblot, calibrated with titrations of purified Sam50 and Sam51, to determine that the steady-state protein levels of Sam50 and Sam51 are similar in C. albicans mitochondria (Fig. 5C).

SAM51 is not essential, and a homozygous diploid mutant sam51ΔΔ was created from which mitochondria were isolated. Tom40 is imported and assembled rapidly in mitochondria from C. albicans (Figs. 3A and 4B). To analyze the kinetics of assembly of the TOM complex in the absence of Sam51, [35S]-CaTom40 was bound to mitochondria from wild-type C. albicans or the sam51ΔΔ mutant and was chased into the TOM complex (Fig. 6A) or monitored in standard assembly assays run at 16°C (Fig. 6B). Both assay systems showed that Sam51 plays a role in assembly of the TOM complex. Although the [35S]-Tom40 substrate occupied the “assembly intermediate I” stage in mitochondria from wild-type cells, the assembly intermediate is somewhat more transient in mitochondria lacking Sam51 (Fig. 6A). The assembly of VDAC proceeded so rapidly, even at 16°C, that no defect was observed in the kinetics of assembly (Fig. 6C), but a small decrease in the steady-state level of VDAC was seen in mitochondria from the sam51ΔΔ mutant (Fig. 6D). Thus, although Sam51 plays a role in β-barrel assembly, deletion of Sam51 results in only a mild defect of the assembly process.

Attempts to recover a homozygous diploid mutant sam50ΔΔ were unsuccessful, consistent with an essential role for the SAM50 gene in viability of C. albicans. A strain was engineered with SAM50 gene expression under the control of a repressible promoter, and growth on agar under repressive conditions showed a nearly total loss of viability (Fig. 6E). After only 3 h in liquid culture, gene...
expression was shut down in this sam50ΔΔSAM50ΔΔ strain, with transcriptional repression confirmed by quantitative PCR (39), so that expression was below the level of detection (Fig. 6D). Mitochondria were isolated after growth under repressive conditions for 3 h, and Western blots using antisera specific for either Sam50 or Sam51 show there is no compensatory up-regulation of Sam51 levels in the sam50ΔΔSAM50ΔΔ strain (Fig. 6D). Mitochondria isolated from the sam50ΔΔSAM50ΔΔ strain and the sam51ΔΔ strain grown on the equivalent minimal medium were assayed for TOM complex assembly and showed that the sam51ΔΔ mutant and the sam50ΔΔSAM50ΔΔ mutants have equivalent rates and extent of assembly of the [35S]-Tom40 subunit into the mature TOM complex (Fig. 6F). We conclude that both Sam50 and Sam51 participate in the assembly of Tom40 and have overlapping roles in the entry and exit of substrate proteins through the SAM complex.

**Discussion**

Characterization of mitochondrial protein import in *C. albicans* opened the way to using this yeast as a model for mitochondrial biogenesis. Distinctions in the protein import pathway in *C. albicans* include an evolutionary rewiring of mitochondrial protein import pathways between related species. Furthermore, the *C. albicans* system revealed aspects of the function of the mitochondrial SAM complex that can be exploited in experiments directed at understanding the role that each component plays in the assembly of the TOM complex and other proteins in the mitochondrial outer membrane.

**Evolutionary Rewiring of Targeting Routes.** It is well documented that *S. cerevisiae* has coevolved a forceful import motor and bipartite targeting sequences to deliver cytochromes into the intermembrane space via the stop-transfer pathway (reviewed in refs. 3 and 4). *C. albicans* has the capacity for import by the stop-transfer pathway, as evidenced by cytochrome *c1* from *S. cerevisiae* reaching the intermembrane space in *C. albicans* mitochondria and being processed there (Fig. 2B). Surprisingly, the cytochromes *c1* and *b2* in *C. albicans* and related yeasts lack the necessary targeting sequences to take the stop-transfer route into the mitochondrial intermembrane space, suggesting an evolutionary rewiring of targeting routes. Consistent with this model, the *C. albicans CYB2* gene could not rescue the *S. cerevisiae* Δcyb2 mutation, although it was expressed at high levels and the functional domains of Cyb2 are highly conserved between these two yeasts. How then do the cytochromes enter the mitochondrial intermembrane space in *C. albicans*? Three potential routes are available: (i) the disulfide-relay pathway, mediated by Mia40; (ii) the cytochrome *c* pathway, mediated by heme lyases; or (iii) another route, not previously described.

Both CaCyb2 and CaCyt1 have several cysteine residues that in principle could allow for interaction with Mia40. Although these residues are not the CX2C or CX3C motifs characteristic of most MIA substrates (3, 4), emerging data indicate that substrates that have CX2C and other arrangements of cysteine residues (e.g., Erv1, Csa1) use the MIA pathway for import into the intermembrane space (40–42). An alternative pathway would be driven by cytochrome heme lyases (e.g., Cyt2 and Cyc3), by analogy with the pathway for cytochrome *c* that is driven by the heme lyase Cyc3. In this import pathway the attachment of heme can serve to drive completion of polypeptide transfer across the outer membrane.

**Fig. 3.** Sam37 is a major determinant of SAM function in *C. albicans*. (A) Mitochondria (50 μg protein per lane) from *C. albicans* were isolated and assayed for import of [35S]-CaTom40 and [35S]-ScTom40, and were monitored by BN-PAGE and phosphorimage analysis. (B) Mitochondria were isolated from *S. cerevisiae* and *C. albicans*, assayed for import of ScVDAC (Por2), and analyzed by BN-PAGE (Upper) or SDS/PAGE (Lower), followed by phosphorimage analysis. (C) The expression level of SAM35 in the indicated strains was monitored by quantitative PCR. Shown are averages plus SE of three independent biological repeats assayed in duplicate (39). (D) Mitochondria from the same strains of *C. albicans* were isolated and assayed for import of [35S]-CaTom40 for 30 min, visualized by BN-PAGE and phosphorimage. The relative size of the SAM complex can be inferred from the migration of “intermediate II” (IntII), indicated by the asterisks. (E) Mitochondria from *C. albicans* wild-type, sam37ΔΔ, or sam37ΔΔSAM35ΔΔ strains (50 μg protein per lane) were incubated with VDAC at 25 °C for the indicated times (min). Then samples were analyzed by BN-PAGE (Upper) or SDS/PAGE (Lower), followed by phosphorimage analysis. (F) Mitochondria from the indicated strains of *C. albicans* (50 μg protein per lane) were incubated with ScF1, β or ScAdh3 at 4 °C or with ScAac1 at 25 °C for the indicated time (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis.
(43, 44). The proposition that evolution has rewired targeting routes might provide a broad explanation to some curious past examples in mitochondrial protein import in diverse organisms. For example, the cytochrome c1 found in the protozoan Trypanosoma brucei has no recognizable stop-transfer sequence, and in protist mitochondria TBCy1 is imported into mitochondria in the absence of a membrane potential, leading to the suggestion that a route other than stop-transfer is used for its entry into the intermembrane space (45). Our results also shed light on differences observed between S. cerevisiae and humans. In S. cerevisiae the Mia40 protein is imported via the stop-transfer pathway, but in humans the homolog of Mia40 is imported via the Mia40 pathway (3, 4). This difference had been puzzling, initially calling into question the functional homology between the yeast and human Mia40 proteins. Based on our data for the C. albicans cytochromes, for which the functional and structural conservation with S. cerevisiae cannot be doubted, we suggest that the distinct import pathways are not an inevitable consequence of an event in the earliest eukaryotes that has been hard-wired into mitochondrial biogenesis but instead reflect rewiring options open to organisms to take advantage of new evolutionary opportunities.

Assembly of VDAC and Assembly of the TOM Complex. VDAC is required for metabolite exchange between the cytosolic and mitochondrial pools. In S. cerevisiae VDAC exists in numerous packing densities and oligomeric forms highly sensitive to metabolic conditions in the cell, ranging from monomers and trimers to regions of membrane where the packing density is 80% VDAC, functioning as a voltage-dependent molecular sieve (46). In S. cerevisiae the assembly of these dynamic VDAC oligomers is highly sensitive to the presence of Sam37 (33). The situation in C. albicans is quite distinct, with an apparently more regular arrangement of VDAC in a predominant oligomeric form and with little effect observed for Sam37 in the assembly of this VDAC oligomer.

Sam35 is necessary for newly imported [35S]-Tom40 to engage productively with the Sam complex (34, 37) and functions as the receptor for the targeting signal present at the C terminus of Tom40, VDAC, and other β-barrel proteins (37). Until now, it has been difficult to rationalize how Sam35, thought to be a peripheral protein on the outer surface of the outer membrane, could perform this function on the inner surface of the outer membrane. The topology of CzSam35 explains this apparent contradiction: Although most of Sam35 is exposed to the cytosol, at least one transmembrane span is integrated into the outer membrane, leaving some part of Sam35 exposed in the intermembrane space and thus explaining how it can function as a receptor for β-barrel proteins. Sodium carbonate extraction is an empirical method that depends on the hydrophobicity of a transmembrane span to resist chaotropic forces at pH ∼11 (47), and the relatively amphipathic nature of ScSam35 makes this integral membrane protein in this species prone to alkali extraction from the mitochondrial outer membrane.

Although at present Sam51 has been described only in C. albicans, molecular phylogenetics (Fig. S2B) and comparative genome analysis (Fig. S2C) demonstrate that the presence of genes encoding both Sam50 and Sam51 is the ancestral yeast condition. In the course of evolutionary time, through the WGD event, sam51 genes were lost from some yeasts, such as S. cerevisiae. Given that Sam51 seems to be dispensable, it is of great interest that none of the yeasts in the same category as C. albicans have, in fact, dispensed with its function. In C. albicans, assembly of newly imported [35S]-Tom40 into the TOM complex is much faster than in S. cerevisiae and is driven by both Sam50 and Sam51. Mitochondria with a nearly complete depletion of Sam50 or complete depletion of Sam51 have similarly mild defects in TOM complex assembly. This result is consistent with Sam50 and Sam51 playing equivalent roles in β-barrel assembly but is in sharp contrast to the growth phenotypes of the two

Fig. 4. Sam35 is an integral membrane protein functioning in substrate entry into the SAM complex. (A) Schematic of the assembly pathway for the Tom40 precursor, showing its incorporation into assembly intermediate I, assembly intermediate II, and the mature TOM complex. (B) Mitochondria (50 μg protein per lane) from the indicated strains of C. albicans were isolated and assayed for the import of CaTom40. Equal samples were withdrawn for analysis by BD-PAGE (Upper) or SDS/PAGE (Lower) and phosphorimaging. (C) Hydrophobicity analysis of CaSam35 with dense alignment surface method (DAS) showed it to have a more hydrophobic character than the prototypical ScSam35 and a conserved DUF2731 domain (35) indicated by the pink shape, with the gray bar representing the protein drawn to scale with the x axis. Schematics of HA-tagged versions of Sam35 are shown also. Mitochondria from C. albicans HA-SAM35 strain and SAM35-HA were isolated, incubated with protease K for 20 min at 4 °C, and then analyzed by SDS/PAGE and immunoblotting. Tom71 is an outer membrane protein. Tim23 is an inner membrane protein. (D) Mitochondria from C. albicans HA-SAM35 were isolated and incubated with 0.1 M Na2CO3 (pH 11.5), and the extracted membranes were resolubilized by centrifugation. The extract supernatant (“extract”) and the extracted membranes (“membrane”) were analyzed by SDS/PAGE and immunoblotting. Tim10 is a soluble protein in the intermembrane space. (E) Mitochondria from C. albicans SAM35-HA were isolated and incubated with 0.1 M Na2CO3 (pH 11.5), and the extracted membranes were resolubilized by centrifugation and analyzed by SDS/PAGE and immunoblotting.
Materials and Methods

mutant strains, with a loss of viability seen only for the deletion of the \( \text{SAM50} \) gene. Given the emergence of data from \( S. \text{cerevisiae} \) suggesting the involvement of subunits of the SAM complex in contacts between the mitochondrial outer membrane and endoplasmic reticulum (48, 49) and between the mitochondrial outer membrane and the mitochondrial inner membrane (50), \( C. \text{albicans} \) is a unique system in which to address the essential function of Sam50 that might distinguish it from Sam51.

Concluding Remarks. Taken together, the rewiring of protein import pathways, the less dynamic use of VDAC for metabolite exchange, and the subtle differences in the subunits contributing to the activity of the SAM complex show the utility of studying the mitochondrial import machinery in \( C. \text{albicans} \). We anticipate that parallel investigations into the regulation points of TOM complex assembly and mitochondrial protein import using both \( C. \text{albicans} \) and \( S. \text{cerevisiae} \) will provide a more comprehensive picture of import pathway mechanisms and of the links among metabolism, the cell cycle, and mitochondrial biogenesis.

Materials and Methods

Yeast Strains. The \( C. \text{albicans} \) strains are derivatives of BWP17 (51). The wild-type strains were DAY185 (URA3\(^+\) ARG4\(^+\) HIS1\(^+\)) and DAY286 (URA3\(^+\) ARG4\(^+\)) (52). The \( C. \text{albicans} \) sam37ΔΔ mutant and the sam37ΔΔ mutant overexpressing \( \text{SAM50} \) (sam37ΔΔ \( \text{SAM50} \)) under the strong constitutive promoter \( \text{TEFI} \) have been described previously (35). The \( \text{SAM35} \) mutant was constructed by standard methods using PCR and homologous recombination, with the \( \text{URA3} \) and \( \text{ARG4} \)-based selection cassettes (48). The conditional \( C. \text{albicans} \) \( \text{SAM50} \) mutant \(( \text{sam50Δ} \text{SAM50}ΔΔ) \) was made by deleting the first allele with the \( \text{ARG4} \) marker cassette and placing the other allele under the control of the repressible \( \text{MET3} \) promoter (1,362 bp of the 5′ UTR of \( \text{MET3} \)) by constructing a \( \text{URA3}-\text{MET3} \) promoter fusion cassette. The \( \text{MET3} \) promoter was repressed by the addition of 2.5 mM methionine and 0.5 mM cysteine to synthetic growth medium. The epitope-tagged \( \text{SAM35} \) strain was constructed by fusing a single HA tag to the N terminus of one of the alleles of \( \text{SAM35} \) under the \( \text{TEFI} \) promoter using the plasmid pCJN498 (53). The C-terminal fusion of the HA tag was driven by the \( \text{ADH1} \) promoter. Growth was monitored in synthetic medium lacking uracil at \( 30^\circ C \), with either 2% (wt/vol) glucose or 2% (wt/vol) \( \ell \)-lactate (pH 4.6) as the carbon source.

For metabolic cycling experiments prototrophic strains of yeast were grown as previously described (8). The \( S. \text{cerevisiae} \) strain CEN.PK and the \( C. \text{albicans} \) strain DAY185 (52) were used in these experiments.
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indicated time of chase for analysis by BN-PAGE and phosphorimaging. (sam51

Fig. 6. Sam50 and Sam51 function in outer membrane protein assembly. (A) Mitochondria (50 μg protein per lane) from C. albicans wild-type or the sam51ΔΔ strains were incubated with CaTom40 for 10 min at 25 °C, isolated, and resuspended in import buffer at 25 °C. Samples were taken after the indicated time of chase for analysis by BN-PAGE and phosphorimaging. (B) Mitochondria (50 μg protein per lane) from C. albicans wild-type or sam51ΔΔ strains were isolated and assayed for import of CaTom40 at 16 °C and ana-

Preparation of Mitochondria. For the preparation of mitochondria, cultures of S. cerevisiae strain W303 were grown in rich medium [YPAGal; 2% (wt/vol) yeast extract, 1% peptone, 0.01% adenine, 2% (wt/vol) galactose]. C. albicans strains were grown in rich yeast extract peptone dextrose (YPD) medium [2% (wt/vol) glucose] at 30 °C. Mitochondria were isolated by differential centrifugation based on the method of Daum et al. (58). Homogenization was performed in 0.6 M sorbitol, 20 mM K+ Mes (pH 6.0), 1 mM PMSF. The mitochondrial pellet was resuspended in 0.6 M sorbitol, 20 mM K+ K+ Mes (pH 7.4), 10 mg/mL BSA.

Protein Import Assays and Electrophoresis. In vitro translation and import assays were performed as described (34). When necessary, the membrane potential was dissipated by the addition of a 100x solution of antimycin (8 μM), valinomycin (1 μM), and oligomycin (20 μM) to mitochondrial samples. Proteins were analyzed by SDS-PAGE or by BN-PAGE as previously described (34).

Antibody Production. Open-reading frames encoding Sam50 and Sam51 were synthesized in codon-optimised form for expression in Escherichia coli (GenScript) and cloned into a pET9 expression vector with an N-terminal 10xHis tag. Recombinant expression in E. coli produced inclusion bodies containing the proteins. This material was purified by washing with 10% (wt/vol) TritonX-100 in PBS and using Ni-NiTA agarose (Qiagen) as per manu-

Fig. 6. Sam50 and Sam51 function in outer membrane protein assembly. (A) Mitochondria (50 μg protein per lane) from C. albicans wild-type or the sam51ΔΔ strains were incubated with CaTom40 for 10 min at 25 °C, isolated, and resuspended in import buffer at 25 °C. Samples were taken after the indicated time of chase for analysis by BN-PAGE and phosphorimaging. (B) Mitochondria (50 μg protein per lane) from C. albicans wild-type or sam51ΔΔ strains were isolated and assayed for import of CaTom40 at 16 °C and ana-

lized as above. (C) Mitochondria (50 μg protein per lane) from C. albicans wild-type or the sam51ΔΔ strains were isolated and assayed for import of VDAC at 25 °C or 16 °C. At the indicated times (min), a sample was removed for analysis by BN-PAGE and phosphorimaging. (D) Complete shutdown of SAM50 expression in the sam50ΔΔ strain was verified by quantitative PCR. RNA was extracted from cells grown for either 3 or 24 h in the presence of 2.5 mM methionine and 0.5 mM cysteine to repress the MET3 promoter. Mitochondria were isolated from the C. albicans sam50ΔΔ strain after 3 h incubation in repressive conditions for comparison with wild-type and the sam51ΔΔ strain using SDS-PAGE and immunoblotting with antibodies re-

ACKNOWLEDGMENTS. We thank Nermin Celik for expert advice on the use of hidden Markov models; Tara Quenault for help with quantitative PCR analysis; Benjamin Tu for his generous gift of the CEN.PK S. cerevisiae strain; and Chaille Webb, Denise Leyton, and Matt Belousoff for critical comments on the manuscript. This work was supported by a Project Grant from the National Health and Medical Research Council (to A.T. and T.L.). V.L.H. was supported by an Australian Postgraduate Award; B.J. was sup-

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Supporting Information

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Fig. S1. Multiple sequence alignment for Cyb2 and Cyt1 from yeast. (A) The expression levels of the CaCYB2 gene were measured by quantitative PCR in the Saccharomyces cerevisiae Δcyb2 mutant strain transformed either with an empty vector or with a vector containing the CaCYB2 gene. The LinReg program (1, 2) was used to calculate the starting concentration (NO) of the CaCYB2 transcript. Levels of the ACT1 transcript in the same samples are shown to demonstrate Legend continued on following page
that the CaCYB2 transcript was expressed at higher levels than ACT1. Data shown are average plus SE of two independent biological repeats assayed in duplicate. (B) Schematic representation of Cyb2 from Fig. 2 is shown, and sequence alignments of yeasts in the group that did not undergo whole-genome duplication (WGD) are compared with the sequence from S. cerevisiae. The species are Candida albicans, Debaryomyces hansenii, Scheffersomyces stipitis, Meyerozyma guilliermondii, Lodderomyces elongisporus, and Clavispora lusitaniae. The alignment was prepared with ClustalW, and sequence identity and similarity are indicated by asterisks and dots, respectively. The N terminus of Cyb2 from C. albicans aligns well with Cyb2 from S. cerevisiae, but starting with residue Q89. The processing site that generates the mature form of ScCyb2 is the peptidyl bond between residues L84 and D85, four residues upstream from Q89. An equivalent analysis for the cytochrome c1 (Cyt1) is also shown. The N terminus of cytochrome c1 from C. albicans aligns well with cytochrome c1 from residue S. cerevisiae, but a region is deleted from within the stop-transfer–targeting region. Note, the 16 N-terminal residues of ScCyt1 are sufficient to target a reporter protein into the mitochondrial matrix (3). A C-terminal region of cytochrome c1 (pink) serves as a transmembrane segment for insertion into the inner membrane (4) and is conserved in C. albicans and related yeasts.

Fig. S2. (Continued)
Fig. S2. (Continued)
Fig. S2.  (Continued)
Material for a figure (Fig. S2) is included in the document. The figure shows phylogenetic analysis of Sam50 and Sam51 sequences with respect to other Sam50 orthologs from different fungal species. The sequences are aligned using the software SeaView, and the phylogenetic tree is constructed using the neighbor-joining method with 1000 bootstrap replicates. The tree is rooted using the Escherichia coli BamA sequence as an outgroup. The sequence accession numbers are provided for all sequences in the figure. The Sam50 sequences are shown in dark blue, and the Sam51 sequences are shown in light blue. The figure also includes a bar graph showing the number of domains in each sequence, with the POTRA domains highlighted in green. The tree is rooted with the E. coli BamA sequence as an outgroup, and the sequences are aligned using the software SeaView. The phylogenetic analysis was conducted using the neighbor-joining method with 1000 bootstrap replicates. The tree is rooted using the Escherichia coli BamA sequence as an outgroup. The sequence accession numbers are provided for all sequences in the figure. The Sam50 sequences are shown in dark blue, and the Sam51 sequences are shown in light blue. The figure also includes a bar graph showing the number of domains in each sequence, with the POTRA domains highlighted in green.
Table S1. Mitochondrial protein import machinery in C. albicans

<table>
<thead>
<tr>
<th>Function</th>
<th>Protein</th>
<th>SC5314 ORF ID</th>
<th>% identity (similarity)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translocase of the outer membrane (TOM) complex in outer membrane for protein translocation into mitochondria</td>
<td>Tom5</td>
<td>None</td>
<td>42 (65)</td>
<td>Hidden Markov model (HMM) search identified an open-reading frame (47aa residues) too small for annotation, in the region Ca21Chr1.1421439–1421582*</td>
</tr>
<tr>
<td></td>
<td>Tom6</td>
<td>ORF19.1650</td>
<td>56 (67)</td>
<td>Annotated as Tom6</td>
</tr>
<tr>
<td></td>
<td>Tom7</td>
<td>ORF19.6531.1</td>
<td>70 (81)</td>
<td>Inappropriately annotated as Tom7†</td>
</tr>
<tr>
<td></td>
<td>Tom20</td>
<td>ORF19.2953</td>
<td>49 (72)</td>
<td>Annotated as Tom20</td>
</tr>
<tr>
<td></td>
<td>Tom22</td>
<td>ORF19.3969</td>
<td>44 (62)</td>
<td>Annotated as Tom22</td>
</tr>
<tr>
<td></td>
<td>Tom40</td>
<td>ORF19.6524</td>
<td>62 (78)</td>
<td>Annotated as Tom40</td>
</tr>
<tr>
<td></td>
<td>Tom70</td>
<td>None</td>
<td>—</td>
<td>Single isoform (Tom71) is present in C. albicans‡</td>
</tr>
<tr>
<td></td>
<td>Tom71</td>
<td>ORF19.3700</td>
<td>43 (64)</td>
<td></td>
</tr>
<tr>
<td>Chaperones in intermembrane space for β-barrel (and other) membrane proteins</td>
<td>Tim9</td>
<td>ORF19.6696</td>
<td>81 (88)</td>
<td>Annotated as Tim9</td>
</tr>
<tr>
<td></td>
<td>Tim10</td>
<td>ORF19.4577.3</td>
<td>69 (81)</td>
<td>Annotated as Tim10</td>
</tr>
<tr>
<td></td>
<td>Tim8</td>
<td>ORF19.6183</td>
<td>57 (72)</td>
<td>Annotated as Tim8</td>
</tr>
<tr>
<td></td>
<td>Tim13</td>
<td>ORF19.2754</td>
<td>64 (75)</td>
<td>Annotated as Tim13</td>
</tr>
<tr>
<td>Sorting and Machinery (SAM) complex for β-barrel protein assembly</td>
<td>Sam35</td>
<td>ORF19.7267</td>
<td>26 (41)</td>
<td>Annotated as Sam35</td>
</tr>
<tr>
<td></td>
<td>Sam37</td>
<td>ORF19.1532</td>
<td>37 (56)</td>
<td>Annotated as Sam37</td>
</tr>
<tr>
<td></td>
<td>Sam50</td>
<td>ORF19.7358</td>
<td>31 (49)</td>
<td>Annotated as Sam50</td>
</tr>
<tr>
<td></td>
<td>Sam51</td>
<td>ORF19.925</td>
<td>25 (40)</td>
<td>Annotated as &quot;uncharacterized ORF&quot;§</td>
</tr>
<tr>
<td>Component of both SAM and ER-mitochondria encounter structure (ERMES) complexes</td>
<td>Mdm10</td>
<td>ORF19.184</td>
<td>35 (53)</td>
<td>Annotated as Mdm10</td>
</tr>
<tr>
<td>α-Helical protein insertion into outer membrane</td>
<td>Mia40</td>
<td>ORF19.2977</td>
<td>50 (72)</td>
<td>Annotated as &quot;Hap43-repressed gene&quot;§</td>
</tr>
<tr>
<td></td>
<td>Erv1</td>
<td>ORF19.2863.1</td>
<td>52 (75)</td>
<td>Annotated as Erv1</td>
</tr>
<tr>
<td>Inner membrane peptidase</td>
<td>Imp1</td>
<td>ORF19.3061</td>
<td>54 (70)</td>
<td>Annotated as Imp1</td>
</tr>
<tr>
<td></td>
<td>Imp2</td>
<td>ORF19.1981</td>
<td>54 (71)</td>
<td>Annotated as Imp2</td>
</tr>
<tr>
<td>Carrier translocase of the inner mitochondrial membrane (TIM22 complex)</td>
<td>Tim18/</td>
<td>ORF19.4022/</td>
<td>44 (58)/</td>
<td>Functional analysis required to distinguish between Tim18 and Sdh4 isoforms†</td>
</tr>
<tr>
<td></td>
<td>Sdh4</td>
<td>ORF19.4468</td>
<td>38 (59)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tim54</td>
<td>ORF19.5143</td>
<td>39 (58)</td>
<td>Annotated as Tim54</td>
</tr>
<tr>
<td></td>
<td>Tim12</td>
<td>ORF19.4620</td>
<td>42 (59)</td>
<td>Annotated as Tim12</td>
</tr>
<tr>
<td>Translocase of the Inner mitochondrial membrane (TIM23 complex)</td>
<td>Tim23</td>
<td>ORF19.1361</td>
<td>61 (77)</td>
<td>Annotated as Tim23</td>
</tr>
<tr>
<td></td>
<td>Tim17</td>
<td>ORF19.150</td>
<td>82 (89)</td>
<td>Annotated as Tim17</td>
</tr>
<tr>
<td></td>
<td>Tim50</td>
<td>ORF19.680</td>
<td>48 (63)</td>
<td>Annotated as Tim50</td>
</tr>
<tr>
<td>Presequence translocase-associated motor (PAM) engagement</td>
<td>Tim21</td>
<td>ORF19.3691</td>
<td>45 (64)</td>
<td>Annotated as Tim21</td>
</tr>
<tr>
<td>PAM</td>
<td>Pam16</td>
<td>ORF19.7222</td>
<td>50 (65)</td>
<td>Annotated as Pam16</td>
</tr>
<tr>
<td></td>
<td>Pam17</td>
<td>ORF19.240</td>
<td>54 (73)</td>
<td>Annotated as Pam17</td>
</tr>
<tr>
<td></td>
<td>Pam18</td>
<td>ORF19.4190</td>
<td>55 (67)</td>
<td>Annotated as Pam18. Has &quot;intermembrane space&quot; domain characteristic of Pam18**</td>
</tr>
<tr>
<td></td>
<td>Mdj2</td>
<td>ORF19.3574</td>
<td>41 (61)</td>
<td>Annotated as Mdj2. Does not have &quot;intermembrane space&quot; domain of Pam18 proteins**</td>
</tr>
<tr>
<td></td>
<td>Ssc1</td>
<td>ORF19.1869</td>
<td>78 (88)</td>
<td>Annotated as Ssc1</td>
</tr>
</tbody>
</table>

*Shading denotes ORFs for which there was initial uncertainty with respect to functional homology.

*The 47-residue ORF is too small to be captured by automated gene assignments but shares high sequence similarity with Tom5 and has been shown to be transcribed (1).

†HMM search identified an ORF (101 aa residues) candidate Tom7. Candida Genome Database (www.candidagenome.org) has been alerted to this misannotation.

‡Tom70 and Tom71 are paralogs in S. cerevisiae, a relic of ancient genome duplication (2).

§Sam51 is characterized in this study.

*The gene encoding Mia40 is one of a collection controlled by the iron-responsive transcription factor Cap2/Hap43 (3).

†Tim18 and Sdh4 are isoforms of a related protein and are both found in the mitochondrial inner membrane (4).

**Although Mdj2 was previously thought to be found only as a result of the ancient genome duplication in the Saccharomycetaeae (5), and of uncertain importance, its presence in C. albicans suggests a fundamental importance of the Mdj2 and an alternate evolutionary history.


