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A deletion variant partially complements a porin-less strain of *Neurospora crassa*.

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Abstract

Mitochondrial porin, the voltage-dependent anion channel, plays an important role in metabolism and other cellular functions within eukaryotic cells. To further the understanding of porin structure and function, *Neurospora crassa* wild-type porin was replaced with a deletion variant lacking residues 238-242 (238porin). 238porin was assembled in the mitochondrial outer membrane, but the steady state levels were only about 3% of those of the wild-type protein. The strain harbouring 238porin displayed cytochrome deficiencies and expressed alternative oxidase. Nonetheless, it exhibited an almost normal linear growth rate. Analysis of mitochondrial proteomes from a wild-type strain FGSC9718, a strain lacking porin (Δ Por-1) and that expressing 238porin revealed that the major differences between the variant strains were in the levels of subunits of the NADH:ubiquinone oxidoreductase (complex I) of the electron transport chain, which were reduced only in Δ Por-1 strain. These, and other proteins related to electron flow and mitochondrial biogenesis, are differentially affected by relative porin levels.

1. Introduction

Mitochondrial porin forms a highly abundant, voltage-dependent anion-selective channel (VDAC) in the outer membrane of mitochondria. The aqueous channel formed by the 19-stranded β -barrel (Hiller et al. 2008, Bayrhuber et al. 2008, Ujwal et al. 2008, Gattin et al. 2015, Schredelseker et al. 2014) allows for the passage of small metabolites, thereby mediating efficient introduction of substrates and release of products to and from mitochondria (reviewed by Summers and Court 2010). In artificial membranes, porin exhibits voltage-dependent gating that shifts the pore between a slightly anion-selective open state and a slightly cation-selective, semi-closed state (Benz 1994, Homblé et al. 2012).

Mitochondrial porin has been shown to interact with many other cellular proteins (for example see Roman et al. 2005), including those involved in the regulation of apoptosis in mammalian cells (reviewed by Shoshan-Barmatz and Mizrachi 2012), and as a result the structure and function of porin are of great interest. Human VDAC1 (hVDAC1; Hiller et al. 2008, Bayrhuber et al. 2008), and VDAC2 (hVDAC2, Gattin et al. 2015), mouse VDAC1 (Ujwal et al. 2008) and zebrafish VDAC2 (Schredelseker et al. 2014) each form a novel barrel consisting of 19 anti-parallel transmembrane β -strands. The structure also contains a N-terminal α -helix, which in the solved structures resides within the pore, although electrophysiological data using native VDAC have been interpreted to indicate that the N-terminal helix forms part of the barrel (see Colombini 2009 for discussion). Although structural data for non-vertebrate VDAC are not currently available, a 19- β -strand barrel was suggested for *Neurospora crassa* porin through secondary structure prediction analyses (Young et al. 2007) that align well with the structures of vertebrate VDAC (Summers and Court 2010, Fig. 1).

Recent work has shown that porin is non-essential for mitochondrial function in *N. crassa* (Summers et al. 2012). This haploid organism is an obligate aerobe that expresses only one porin isoform, allowing straightforward assessment of the effects of a porin variant on the cell.

Although strains lacking porin (Δ Por) are viable, they exhibit extremely inhibited growth rates and mitochondrial dysfunction, demonstrating the necessity of porin for normal mitochondrial and cellular function (Summers et al. 2012). In this study, to further the understanding of the structure and functions of mitochondrial porin, a deletion variant, 238porin, was examined *in vivo*.

The 238porin variant contains a five-amino acid deletion (residues: NDRGV, Runke et al. 2006) at positions 238-242 (highlighted region in Fig. 1B), which according to predictions based on the solved structures encompasses the loop or turn between β -strands 16 and 17. The corresponding sequence in hVDAC1 and hVDAC2 is NNSSL, and is well conserved in vertebrate VDAC isoforms 1 and 2 (see Schredelseker et al. 2014 for examples). These residues encompass the link between β -strands 16 and 17 in all models published to date (see above for references). A previous study (Runke et al. 2006) examined various biophysical aspects of *N. crassa* porin deletion variants including 238porin, which inserted into and formed pores in artificial lipid bilayers. This variant was found to have pore properties, such as aqueous channel conductance and gating behaviour, similar to those of the wild-type porin. Therefore, it has the potential to form pores in mitochondria, and was chosen as a starting point for the analysis of the roles of specific segments of VDAC *in vivo*.

2. Materials and Methods

2.1 2.2 Strains and Growth Conditions

The strains of *Neurospora crassa* used in this study are detailed in Table 1. Strains with FGSC designations were obtained from the Fungal Genetics Stock Center, Kansas City, KS (McCluskey et al. 2010). The generation of the cDNA encoding *N. crassa* 238porin is described in Runke et al. (2006) and the nomenclature used in therein has been used in the current work. A recombination-based method derived from that used by Colot et al. (2006) was used to introduce

the 238porin coding sequence into the *Neurospora* genome. In brief, segments of DNA flanking the native porin gene (NCU04304) in strain FGSC 9720 (Colot et al. 2006), the 238porin cDNA (Runke et al. 2006) and the hygromycin-resistance cassette (Staben et al. 1989) were amplified by PCR using the primers listed in Supplementary Table 1, and used to construct the plasmid shown in Fig. 1A. The transformation cassette was removed from the plasmid by digestion with *PvuII*, purified and transformed into FGSC 9718 as described in Colot et al. (2006). A hygromycin-resistant isolate was obtained and purified by repeated subculturing. The gene replacement in the resulting 238Por strain was confirmed by PCR and DNA sequence analyses of the junctions and internal segments of the 238porin coding sequence. *Neurospora* cultures were maintained on Vogel's minimal medium (VMM) and linear growth rates were determined in race tubes as described by Davis and De Serres (1970); race tubes were obtained from the FGSC.

Analysis of mitochondria

Mitochondria were isolated from mycelia grown at 30°C in liquid VMM with shaking for 18 h (FGSC 9718a) or 21 h (238Por), using the procedure of Harkness et al. (1994). Protein concentrations were determined by a Bradford assay (BioShop, Burlington, ON).

Proteins were analyzed by SDS-PAGE using 12% acrylamide gels, and transferred to nitrocellulose, according to the manufacturer's instructions (Bio-Rad, Mississauga, ON). Primary antibodies against *N. crassa* porin, Tom40 and Tom70 were kindly provided by Drs. Roland Lill and Walter Neupert, Universität München. The secondary antibody used was mouse α -rabbit-IgG-alkaline phosphatase (Sigma-Aldrich, Oakville, ON). Membrane bound complexes were isolated from membranes by resuspending mitochondria (100 μ g protein) in 50 μ l 1% (w/v) n-Dodecyl β -D-maltoside (DDM) and incubating on ice for 30 min followed by microcentrifugation at 13,000 rpm for 30 min to remove insoluble material (Summers et al. 2012) and the TOM complex was detected using antibodies against TOM40 (Sherman et al. 2006). Complexes were analyzed by Red-Native-PAGE in the presence of Ponceau S (Drab et al. 2011)

using a 6%-13% acrylamide gradient and western blotting. An in-gel NADH dehydrogenase activity assay was performed as described (Calvaruso et al. 2008).

Cytochrome spectra were determined as previously described (Summers et al. 2012); the reduced minus oxidized difference spectrum is presented (Rifkin and Luck 1971). Expression of alternative oxidase was confirmed as described by Summers et al. (2012), using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria).

For imaging of mitochondria, *N. crassa* cultures were grown on solid VMM for 12-16 hours at 30°C. Staining with 0.1 ng/ml of MitoTracker Green FM (Life Technologies, Burlington ON) was performed as described by (Wideman et al. 2010), except that hyphae were grown directly on the microscope slide. Images were collected with a Zeiss Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

Proteomic Analysis of Mitochondria

Crude mitochondrial samples were prepared from *N. crassa* strains as above. To avoid any damage to the mitochondrial outer membranes and loss of intermembrane space proteins caused by further purification of mitochondria, the samples were utilized directly in this state. The proteomic analysis was conducted as described (Gungormusler-Yilmaz et al. 2014), using liquid chromatography (LC) and tandem mass spectroscopy (MS/MS). The resulting data sets contained many non-mitochondrial protein contaminants due to the use of crude mitochondrial preparations. These contaminant proteins inherently vary in abundance between samples and therefore were removed from the data sets prior to analysis to avoid skewing the data. To identify mitochondrial proteins in the data sets a reference set of mitochondrial proteins was constructed as follows: i) proteins predicted from genes annotated as “mitochondrial” in the Broad Institute’s *N. crassa* genome database (<http://www.broadinstitute.org/annotation/genome/neurospora/GenomesIndex.htm>), ii) *N. crassa* proteins annotated as mitochondrial in the UniProt database (<http://www.uniprot.org/>), iii)

proteins known to reside in the mitochondrial outer membrane of *N. crassa* (Schmitt et al. 2006) and iv) any detected protein sequence scoring a mitochondrial probability ≥ 0.9 via the TargetP subcellular localization prediction server (Emanuelsson et al. 2000).

The resulting data sets of mitochondrial proteins were used to identify differential expression of proteins in the *N. crassa* VDAC mutants. Log₂ difference values were calculated for each pair of proteins and each set of values was normalized to generate a mean near zero and standard deviation near one. *z*-scores were calculated for proteins detected in both the wild-type and mutant strains. A minimum of 95% confidence was used to identify proteins that have differences in expression between two strains ($-1.96 > z > 1.96$, Gungormusler-Yilmaz et al. 2014). These data are presented in Supplementary Tables 2 (Δ Por-1 vs. wild-type); 3 (238Por vs. wild-type) and 4 (Δ Por-1 vs. 238Por). This approach focuses on the population of differences in expression, rather than the absolute degrees of difference, and is used because the sensitivity of cells to the levels of each protein is unknown. Protein functional data was extracted from the Clusters of Orthologous Groups database (COG; <http://www.ncbi.nlm.nih.gov/COG/>) and the Kyoto Encyclopedia of Genes and Genomes databases (KEGG; <http://www.genome.jp/kegg/>).

3. Results and Discussion

In order to assess the *in vivo* function of 238porin, a cDNA coding sequence (Runke et al. 2006), linked to an upstream hygromycin-resistance cassette (Fig. 1A) was used to replace the wild-type porin gene in *N. crassa*. The resulting strain, 238Por, harbours a very low level of the variant (Fig. 2 and Table 4), indicating that the molecule either is not expressed efficiently at the transcriptional or translational levels, and/or is not effectively targeted to or assembled in the mitochondrial outer membrane (MOM). In mitochondria, 238porin is resistant to externally added protease (data not shown), suggesting that the small amount present is correctly assembled (see Court et al. 1996).

Unexpectedly, the low levels of 238porin in mitochondria were not linked to significant growth defects (Table 2). The 238Por strain grew at more than 85% of the wild-type (FGSC 9718) rate at both 22°C and 30°C. This is in contrast to the severe reduction in growth rates observed in Δ Por mutants, particularly at lower temperatures (Summers et al. 2012, Table 2). Thus, the very low levels of 238porin variant are able to partially complement the lack of wild-type porin.

One characteristic of the porin-less strains of *N. crassa* is a deficiency in the cytochromes assembled with mitochondrially-encoded polypeptides (Summers et al. 2012); these types of defects also arise when mitochondrial translation is blocked (Descheneau et al. 2005), and in other nuclear and mitochondrial mutants of *N. crassa* (for example see Rifkin and Luck 1971). Similarly, the 238Por strain displays deficiencies in cytochromes *aa₃* and *b*, and an increased level of cytochrome *c* (Fig. 3). It also displays cyanide-resistant, SHAM-sensitive oxygen consumption (Table 2), characteristic of strains expressing alternative oxidase (Lambowitz and Slayman 1971).

In principle, these differences between wild-type and 238Por cells could reflect low levels of porin, expression of a mutant porin, or a combination of the two. Attempts were made to generate a strain expression low levels of wild-type porin. Reduction of porin to 30-60% of wild-type levels was not associated with any detectable changes in growth rate, cytochrome profile, or expression of alternative oxidase (S. Shuvo and D. Court, personal communication), but very low levels of porin corresponding to those in 238Por (<5%) were not achieved. Given the ability of 238Por to generate an apparently wild-type pore in artificial membranes, the defects in 238Por may be most reflective of the low amount of porin. Nonetheless, it was predicted that further analysis of 238Por variant would reveal cellular changes responsible for almost wild-type growth rates in the absence of a normal mitochondrial bioenergetics. To that end, the proteomes of 238Por, Δ Por-1 and Por-WT strains were assessed.

1-Dimensional LC MS/MS analysis of the mitochondrial proteomes

Proteins were extracted from partially purified mitochondria and subjected to LC MS/MS. Peptides from a total of 1102 different proteins were detected, and slightly more than half of the proteins identified from each strain were predicted to reside in mitochondria (summarized in Table 3). The proteomic approach used in the current study detected a similar number of proteins (509) as was detected from *N. crassa* mitochondria through the use of iTRAQ from sucrose-gradient purified organelles (260, Keeping et al. 2011 and 488, Summers et al. 2012). In the latter study, there were a number of intermembrane space proteins that were present in significantly reduced amounts in Δ Por-1, in spite of relatively normal levels of the corresponding mRNA (Summers et al. 2012). These proteins include TIM8, ketol-acid reductoisomerase, nucleoside diphosphate kinase-1, adenylate kinase, and ubiquinone cytochrome *c* reductase hinge. They were not reduced in the Δ Por-1 organelles used in the current study (Supplementary Table 2). Thus it appears that the outer membranes of Δ Por-1 organelles were disrupted during the sucrose-gradient purification step in the iTRAQ studies.

The outer membrane proteome was well represented in the current study; 29 of the 30 proteins identified by Schmitt et al. (2006) in purified outer membranes were detected in the current study in wild-type mitochondria (Table 3). The exception was a mitochondrial import protein 1 (MIM1, NCU01101), a 130-amino acid protein required for assembly of TOM20 into the outer membrane (Popov-Celeketic et al. 2008). In contrast to the previous iTRAQ study (Summers et al. 2012), TOB55, a β -barrel in the topogenesis of mitochondrial outer membrane β -barrels (TOB) complex was detected in the current work, and there was a non-significant reduction its levels in Δ Por-1 and 238Por ($\log_2$ ratios of -0.3 and -0.5, respectively). Thus, as for the case for another β -barrel, TOM44 of the translocase of the outer membrane (see Summers et al. 2012 and Fig. 2), upregulation of the TOB55 β -barrel was not increased as a mechanism for

compensating for the absence of VDAC. The remaining predicted MOM β -barrel, MDM10 has yet to be detected in any large scale proteomic analysis.

Electron transport chain

Both Δ Por-1 and 238Por display defects in cytochromes associated with mitochondrially encoded polypeptides and express alternative oxidase (Fig. 3 and Table 2). Proteomic analysis further revealed defects in NADH:Ubiquinone oxidoreductase (complex I) in Δ Por-1 (Table 4), which are supported by changes in the high molecular weight complexes detected by in-gel NDAH dehydrogenase assays (Fig. 4)

One of the main differences between the proteomes of Δ Por-1 and 238Por mitochondria is in the levels of subunits of complex I, which oxidizes NADH and translocates hydrogen ions into the intermembrane space. None of the 27 subunits of complex I detected were present in significantly reduced levels ($\log_2$ difference < -1.6) in 238Por (Supplementary Table 3), suggesting that this complex was functional. In contrast, in Δ Por-1 mitochondria, the levels of the 9.8- and 20.1-kDa subunits (NCU04781 and NCU09460) were significantly lower than in wild-type (Table 4), and levels of these proteins and the 40-kDa subunit were significantly different when the data from the two mutant strains were compared (Supplementary Table 4). A nuo9.8 mutant lacking the former subunit fails to assemble the membrane-bound and peripheral arms of complex I (Marques et al. 2003), suggesting that assembly of this complex would be impaired in Δ Por-1. In fact, fifteen of the 27 other complex I subunits detected were present at levels 2.5- to 5-fold lower than in wild-type, although these differences were statistically insignificant (see Supplementary Table 2). The differences in complex I activity are supported by the NADH dehydrogenase in-gel assay (Fig. 4). *Neurospora* expresses four alternative NAD(P)H dehydrogenases allowing bypass of complex I, oxidizing NAD(P)H without proton translocation. Of these, NDE2 (Carneiro et al. 2004) and NDI1 (Duarte et al. 2003) are essential for the viability in the absence of functional complex I. The relative levels of these proteins were not significantly

changed in the Δ Por-1 mitochondria. Another alternative enzyme, NDE1, was unchanged and NDE3 (Carneiro et al. 2007) was not detected. Thus, the reduced levels of several subunits of the NADH dehydrogenase complex are not associated with overexpression of the alternative dehydrogenases in Δ Por-1 mitochondria and the defects in complex I may contribute to the very slow growth of Δ Por-1 in comparison to 238Por (Table 2).

The reduction in complex I subunits in Δ Por-1, and the reduced levels of cytochromes *b* and *aa₃* in both variant strains might contribute to suboptimal ratios of NAD(P)H/NAD(P)⁺, leading to changes in expression of proteins that impact these ratios. The increased levels of an NAD(P)H transhydrogenase (NCU01140, Gameiro et al. 2013), and a short chain dehydrogenase (NCU02097) in both strains (Tables 4 and 5) may be involved in optimizing NAD⁺/NADH levels, particularly in Δ Por-1. Both variant strains harbour relatively high levels of an annotated rubredoxin reductase (NCU05850, Table 5). It has been identified as a homologue of apoptosis-inducing factor (Carneiro et al. 2012), an oxidoreductase that may be expressed in response to abnormal NAD⁺/NADH ratios.

There are no obvious differences between the variant and wild-type strains in terms of the nuclear-encoded co-enzyme Q: cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) proteins that were detected, and the mitochondrially encoded proteins associated with cytochromes *aa₃* in complex IV and *b* in complex III were not detected in any mutant strain in this and other studies (Summers et al. 2012, Keeping et al. 2011). Two proteins related to biogenesis of the electron transport chain, OMS1 (NCU04764, Lemaire et al. 2004) and OXA2 (Funes et al. 2004) are increased in Δ Por-1 (Table 4 and Supplementary Table 2), indicating a response at the level of the proteome are related to the assembly of these complexes. As expected in cells with defective electron transport chains (Lambowitz and Slayman 1971), cyanide-resistant alternative oxidase (AOD) activity was detected in both variant strains (Table 2) and both AOD-1 and AOD-3 proteins were detected in the Δ Por-1 proteome but not that of the

wild-type (Supplementary Table 2). However, transcription of *aod-1* is induced in *N. crassa* treated with chloramphenicol, while that of *aod-3* is not (Tanton et al. 2003), suggesting that the two genes are regulated independently, and the inducers for both are present in Δ Por-1. However, alternative oxidase activity is not observed in *aod-1* mutants (discussed in Tanton et al. 2003), suggesting that the AOX-3 polypeptide detected in this study is non-functional. In spite of the cyanide-resistant respiration observed, neither alternative oxidase protein was detected in 238Por, suggesting that their levels were sufficient for biological activity but were below detection by the methods used.

In terms of the F_1F_o -ATP synthase (complex V), in Δ Por-1 (Tables 4 and 7), there was a higher level of a protein (NCU03558) that is similar to the ϵ -subunit (Atp15p, Tetaud et al. 2014) and an inner membrane protease involved in maturation of subunit 6 (NCU00107, ATP-23, Zeng et al. 2007) was less abundant in 238Por (Table 5) and not detected in Δ Por-1. In both variant strains, there was a significant deficit in the putative e-subunit of the F_o subcomplex (NCU09143, Tables 4 and 5), which will be discussed below.

Oxidative stress

Oxidative stress is a predictable outcome in cells lacking complete electron transport chains and several of the differences between the proteomes of the wild-type and variants indicate direct or indirect responses to oxidative stress. Both variants express higher levels of alternative oxidase, which acts on reduced ubiquinone (Maxwell et al. 1999) and a Zn-dependent alcohol dehydrogenase (NCU09285, MIG-6, Tables 4 and 5, Summers et al. 2012). Expression of this open reading frame is induced by menadione. Only 238Por showed an increased levels of the protein predicted to encode an atypical 2-Cys peroxiredoxin (NCU06880), associated with resistance to hydrogen peroxide in mammalian cells (Seo et al. 2000).

In contrast, several proteins linked to reducing oxidative stress are in lower levels in one or both of the variant strains, including proline oxidase (NCU02936, Goncalves et al. 2014) and

the thiamine thiazole synthase (Chatterjee et al. 2011). Perhaps unexpectedly, there is over 8-fold less of the latter enzyme (NCU06110; CyPBP37) in Δ Por-1 than in the wild-type and 238Por strains, respectively (Table 4 and Supplementary Table 4). Another thiamine biosynthetic enzyme, NMT-1 (NCU09345) is drastically reduced only in Δ Por-1 (Table 4) and has been implicated in protection against oxidative stress in yeast (Franken et al. 2008). Finally, lipoic acid has antioxidant activity (discussed in Petersen Shay et al. 2008) and lipoic acid synthase (NCU00565) is reduced only in 238Por only (Table 5). Other impacts of reduced lipoic acid levels are discussed with respect to the TCA cycle and heme biosynthesis below (Fig. 7).

Both strains show evidence of increased responses to misfolded proteins, which could result from the effects of reduced ATP levels on chaperone activity. Δ Por-1 and 238Por have low-level overexpression of the proteolytic subunit of the Clp protease (NCU4578, Tables 4 and 5), which participates in the mitochondrial unfolded protein response (reviewed by Hamon et al. 2015). Aspartyl aminopeptidase (NCU00122) also is present in higher levels in both variant strains, and aminopeptidases may “moonlight” as chaperones (Malki et al. 2005, Lee and Zhang 2009, Scranton et al. 2012). Three other proteases, carboxypeptidase Y (NCU00477), the mitochondrial AAA ATPase (NCU05459), and the intermembrane space AAA protease IAP-1 (NCU03359) are present in elevated levels in Δ Por-1 compared to 238Por (Supplementary Table 4). The latter protease is part of the mitochondrial protein quality control system (Klanner et al. 2001), again suggesting increased protein misfolding in Δ Por-1.

Several of the differentially expressed proteins are linked to the TCA cycle, through the generation of cofactors or substrates, or utilization of TCA intermediates as substrates (Fig. 7). In mitochondria, the pyruvate dehydrogenase complex (PDC) converts pyruvate into acetyl-CoA, which can then enter the TCA cycle (Fig. 7A). Lipoic acid is a PDC cofactor (Morikawa et al. 2001). Increased levels of pyruvate dehydrogenase kinase (PDK, NCU03796), which inactivates PDC (Zhang et al. 2014), and reduced lipoic acid synthase (LAS, NCU00565) in 238Por (Table

5) suggest lower levels of PDC activity in 238Por, which might optimize ratios of its substrates (pyruvate, NAD^+ and coenzyme A) and products (acetyl-CoA and NADH^+ and H^+). A second potential link to acetyl-CoA production is through the 3-ketoacyl CoA thiolase (NCU05558), which participates in both fatty acid degradation and fatty acid elongation. The slightly higher levels of acyl-CoA synthase (NCU03929) might drive higher levels of fatty acid biosynthesis and ensure release of acetyl-CoA. Finally, the carnitine shuttle is involved in movement of acetyl groups between peroxisomes and mitochondria (reviewed by Strijbis et al. 2008). In $\Delta\text{Por-1}$, the lower levels of trimethyllysine dioxygenase (TMLD, NCU03802), involved in carnitine biosynthesis, could potentially influence the shuttle and the acetyl-CoA levels in the organelle.

Several proteins present in lower levels only in $\Delta\text{Por-1}$ are linked to intermediates of the TCA cycle (Fig. 7B). TMLD consumes α -ketoglutarate and generates succinate, bypassing a step that reduces NAD^+ . The lower levels of a putative succinate/fumarate mitochondrial transporter (Sfc1p homologue, NCU08561) would potentially drive matrix succinate levels lower, without increasing levels of ROS via a backlog of reduced ubiquinone. The yeast homologue of the transporter, Sfc1p (or Acr1p) is required for acetyl-CoA synthetase activity (Fernandez et al. 1994) again linking changes in $\Delta\text{Por-1}$ with reduced acetyl-CoA levels.

Both mutant strains harbour lower levels of 5-aminolevulinate synthase (ALAS, NCU006189, Tables 4 and 5), which is involved in heme biosynthesis (reviewed by Hunter and Ferreira 2011) and may be a response to reduced heme requirements due to reduced levels of cytochromes *aa₃* and *b* in the porin variants. Reduction in ALAS activity would also prevent diversion of succinyl-CoA from the TCA cycle (Fig. 7B). Conversely, α -ketoglutarate dehydrogenase (KGD), which generates succinyl-CoA, requires lipoic acid ((Schonauer et al. 2009)) and lipoic acid synthase is reduced in 238Por.

Mitochondrial biogenesis

The translocase of the outer membrane (TOM complex) recognizes signals in mitochondrial pre-proteins and translocates the polypeptides across the outer membrane (reviewed in Dukanovic and Rapaport 2011). The stability of the TOM complex is reduced in Δ Por mitochondria, as indicated by the increased migration rate of Tom40-containing complexes during non-denaturing PAGE (Summers et al. 2012, Fig. 5). In contrast, the complex in 238Por migrates similarly to that of the wild-type strain, indicating that the small amount of 238Porin is sufficient for maintenance of the complex.

Depletion of mitochondrial porin also is associated with altered mitochondrial morphology (Summers et al. 2012). Staining of whole mitochondria with MitoTracker revealed similar morphology for 238Por and wild-type organelles (Fig. 6). However, there appeared to be a lower number of well-stained organelles per hyphal tip, and those observed generally were larger in the 238Por cells. Under the staining conditions used, long string-like mitochondria were not observed in hyphal tips in the wild-type.

With respect to different morphology, a potential Mdm33p homologue (NCU06546) is required for normal morphology and distribution of mitochondria (mdm; Messerschmitt et al. 2003) is relatively more abundant in Δ Por-1 and 238Por strains (Tables 4 and 5). FZO1 encoded by uvs-5 (NCU00436) is a mitofusin homologue in the outer membrane (Schmitt et al. 2006) is more than 8-fold reduced in Δ Por-1 mitochondria (Table 4), but was present in almost normal levels in 238Por organelles. This defect in Δ Por-1 may contribute to the small mitochondria seen Δ Por-1 strains (Summers et al. 2012), as uvs-5 strains have fragmented mitochondria (Kurashima et al. 2013).

In both strains, there was a significant deficit in the putative e-subunit (NCU09143) of the F_0 ATPase synthase subcomplex (Tables 4 and 5). This subunit, Atp21p in *S. cerevisiae*, is involved in the formation of monomers of the F_1F_0 -ATP synthase that are competent for dimerization (Wagner et al. 2009) and the dimers in turn appear to contribute to the curvature of the inner membrane (Davies et al. 2012). The mitochondrial organizing structure (MitOS) of the

inner membrane is proposed to interact with the ATP synthase dimers and contribute to the development of normal morphology. The putative homologue (NCU01024) of one component of the yeast MitOS, Aim5p, is expressed at lower levels in Δ Por-1 mitochondria (Table 4), perhaps contributing to the abnormal morphology. Interestingly, porin is linked to MitOS through genetic interactions (Hoppins et al. 2011) although the nature of the interaction is unknown.

Two variably expressed proteins participate in synthesis of the non-protein components of the outer membrane. The sterol 24-C methyltransferase (NCU03006) is involved in ergosterol biosynthesis, and is present at 3.0- to 4.6-fold higher levels in both strains. The full extent of the impact of porin defects on sterol biosynthesis is not known because the other enzymes in the pathway from zymosterol to ergosterol (ERG-2, ERG-3, ERG-5) were not detected in any strain, and ERG-6 was detected only in the wild-type strain. An enzyme involved in fatty acid biosynthesis, 3-oxoacyl-(acyl-carrier-protein) reductase (NCU01092) was not detected in the wild-type strain, but was present in almost 8-fold higher levels in Δ Por-1 (Table 4).

Conclusions

238porin is present in relatively low levels in the outer membrane, but nonetheless partially complements a porin-less strain of *Neurospora*. Its presence is associated with almost wild-type growth rate, although 238Por expresses alternative oxidase and suffers defects in the electron transport chain. The proteomic studies revealed links to altered electron flow, NADH metabolism and mitochondrial morphology in both Δ Por-1 and 238Por strains. The strain expressing 238Por appears to contain higher levels of complex I of the ETC, and the carbon flow through the TCA cycle in these cells may be different from that in Δ Por-1 organelles. Thus, the cellular response to the presence of a low level of 238Por allows more rapid hyphal growth.

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Figure Legends

Figure 1. Porin variant used in this study. **A)** Schematic diagram of the construct used to replace the wild-type porin gene to create the 238porin strain. The hygromycin-resistance cassette (HygB^R) and the flanking promoter and transcription termination regions were obtained from pCNS44 (Staben et al. 1989). The 238por cDNA was amplified from cloned DNA described in Runke et al. (2006). **B)** Model of wild-type and 238porin. The structures of wild-type *N. crassa* porin (left panel, P07144) and 238porin (right panel, Runke et al. 2006) were predicted using Phyre² (Kelley and Sternberg 2009) and the human VDAC1 structure (left panel, Hiller et al. 2008, PDB 2K4T). The residues absent from 238porin are indicated in red in the wild-type structure, and the shortened β -strands resulting from the absence of residues 238-242 are indicated by the arrow in the 238por structure. Images were created in the PyMOL Molecular Graphics System, Version 1.7.4.

Figure 2. Western blot analysis of wild-type (Por-WT) and 238por-containing mitochondria. 30 μ g of mitochondrial protein from each strain were analyzed by western blotting with antibodies against the proteins indicated below each panel.

Figure 3. The reduced minus oxidized difference spectra obtained from wild-type (Por-WT), 238por, and Δ Por-1 mitochondria. Peaks representing absorbance by cytochromes *c*, *b*, and *a/a₃* are indicated.

Figure 4. Red-Native PAGE of DDM-solubilized mitochondrial membrane complexes. A) In-gel NADH dehydrogenase activity assay. B) The same gel stained with Coomassie Brilliant Blue. Arrows indicate bands corresponding to complex I. The intensity of the lower bands of the Coomassie-stained gel were used to confirm that similar amounts of DDM-solubilized material were present in each lane of the gel.

Figure 5. Western blot detection of the Translocase of the Outer Membrane (TOM) complex from Red-Native PAGE of DDM-solubilized mitochondrial membrane complexes. Antibodies against the TOM complex 40-kDa subunit (TOM40) were used to identify the TOM complex.

Figure 6. Fluorescence microscopy. Hyphae of wild-type (left) and 238Por (middle) and Δ Por-1 (right) were stained with MitoTracker and observed at 1000x magnification.

Figure 7. Connections among proteins present in different relative amounts in 238por and Δ Por-1 mitochondria. **A)** Mitochondrial proteins. Proteins present in relatively high or low amounts are indicated by arrows directed upward or downward, respectively, in split boxes referring to 238Por (left) and Δ Por-1 (right). In the case of enzymes, the reactions they catalyze are represented by thin or thick arrows if the proteins are in low or high levels, respectively. The blunt arrow between PDK and PDC indicates an inhibitory interaction; all others are positive effects. The dotted line linking TMLD and carnitine in peroxisomes indicates that the mitochondrial enzyme TMLD is required for synthesis of carnitine, which participates in the carnitine shuttle between mitochondria and peroxisomes. The dotted arrow denotes an effect of Sfc1p on the activity of Ac-CoA synthetase as described in the text. PDC, pyruvate decarboxylase; PDK, PDC kinase; Ac-CoA synthase, acetyl-coenzyme A synthase; Carn, carnitine; ka-CoA, 3-ketoacetyl-CoA; TMLD, trimethyllysine dioxygenase.

B) Links between differentially expressed proteins and the TCA cycle in 238porin. The TCA cycle is indicated by double arrows linking substrates/products (boxes). Reduction in lipoic acid synthase (LAS) levels, could reduce the amounts of lipoic acid (grey diamond), a cofactor of α -ketoglutarate dehydrogenase (KGD, speckled circle), potentially decreasing levels of succinyl-CoA. ALAS, 5-Aminolevulinate Synthase. For references, see text.

Tables

Table 1. *Neurospora crassa* strains utilized and their respective genotypes

Strain	Relevant Genotypes	Reference
FGSC 9718	<i>mus-51::bar mat a</i>	Colot et al. 2006
FGSC 9720	<i>mus-52::bar his-3 mat A</i>	Colot et al. 2006
238Por (FGSC 9718 background)	<i>238por hph⁺</i>	This work
ΔPor-1 (FGSC 9718 background)	<i>Δpor1::hph⁺</i>	Summers et al. 2012

Table 2. Linear growth rates and cyanide-resistant respiration of *Neurospora crassa* strains on Vogel's minimal medium

Strain	Linear Growth Rates		Relative O ₂ consumption (%)		
	22°C (cm/day) [percentage of wild-type rate]	30°C (cm/day) [percentage of wild-type rate]	No additions	With KCN	With SHAM ^a
FGSC 9718	7.8 ± 0.4 [100]	11.4 ± 0.3 [100]	100	10.2	n/d ^b
238Por	6.9 ± 0.1 [88]	9.8 ± 0.2 [86]	100	90.3	3.2
ΔPor-1	1.3 ± 0.3 [17]	3.2 ± 1.4 [28]	100	95.6	12.0

^a SHAM, Salicylhydroxamic acid

^b n/d, not detected

Table 3. Protein identification by LC-MS/MS. See Supplementary Tables 2-4 for details

Strain	Total proteins	Predicted mitochondrial proteins	OM proteins (of 30) ^a
FGSC 9718	965	546	29
238Por	977	548	28
ΔPor-1	922	542	28

^aOf the proteins detected in isolated outer membranes of *N. crassa* (Schmitt et al. 2006)

Table 4. Proteins detected in altered levels in ΔPor-1 mitochondria. Data are taken from Supplementary Table 3.				
Elevated in ΔPor-1				
Accession Number	Protein Description	Expression Difference (Log ₂)	COG Group	Additional Descriptions (KEGG)
NCU03558	Hypothetical protein	3.35	None	Eukaryotic F-type ATPase ε-subunit
NCU01140	NAD(P) Transhydrogenase	2.94	Energy production and conversion	None
NCU05850	Rubredoxin-NAD ⁺ Reductase	2.63	General function prediction only	Similar to pyridine nucleotide-disulphide oxidoreductase
NCU06546	Hypothetical Protein	2.36	None	K17983 sensitive to high expression protein 9
NCU05495	CVNH Domain-Containing Protein	2.29	None	None
NCU00865	Oxalate Decarboxylase OxdC	2.12	General function prediction only	None
NCU01650	Hypothetical Protein	1.83	None	Large subunit ribosomal protein MRP49
NCU00122	Aspartyl Aminopeptidase	1.82	Amino acid transport and metabolism	None
NCU04578	ATP-Dependent Clp Protease Proteolytic Subunit	1.65	Intracellular trafficking, secretion, and vesicular transport	None
NCU04764	Hypothetical Protein	1.59	None	Methyltransferase OMS1
Reduced in ΔPor-1				
NCU03802	Trimethyllysine Dioxygenase	-3.05	None	None
NCU05338	Hypothetical Protein	-3.22	None	None
NCU08561	Succinate/Fumarate Mitochondrial Transporter	-3.36	None	None
NCU01633	Hexose Transporter HXT13	-3.4	None	Similar to hexose transporter
NCU02564	Cysteine synthase 2	-3.56	Amino acid transport and metabolism	None
NCU04946	Hypothetical protein	-3.73	None	None
NCU00436	GTPase FZO1	-4.11	None	Hydrolases. Acting on acid anhydrides. Acting on GTP; involved in cellular and subcellular movement.
NCU04781	NADH:Ubiquinone Oxidoreductase 9.8 kDa Subunit	-4.23	None	None
NCU09345	Thiamine Biosynthesis Protein NMT-1, Variant 2	-5.07	Inorganic ion transport and metabolism	Pyrimidine precursor biosynthesis enzyme
NCU06110	Thiazole Biosynthetic Enzyme	-5.39	Coenzyme transport and metabolism	similar to stress-responsive gene product
NCU09143	Hypothetical Protein	-5.72	None	F-Type ATP synthase e-subunit
NCU09460	NADH:Ubiquinone Oxidoreductase 20.1kD Subunit	-5.86	None	None
NCU01024	Hypothetical Protein	-6.45	None	Altered inheritance of mitochondria protein 5

Table 5. Proteins detected in altered levels in 238por mitochondria. Data are taken from Supplementary Table 3.				
Elevated in 238Por				
Accession Number	Protein Description	Expression Difference (Log ₂)	COG Group	Additional Descriptions (KEGG)
NCU01140	NAD(P) Trans Hydrogenase	3.91	Energy production and conversion	None
NCU05495	CVNH Domain-Containing Protein	3.47	None	None
NCU06546	Hypothetical Protein	2.84	None	K17983 sensitive to high expression protein 9
NCU05850	Rubredoxin-NAD ⁺ Reductase	2.79	General function prediction only	Similar to pyridine nucleotide-disulphide oxidoreductase
NCU00122	Aspartyl Aminopeptidase	2.52	Amino acid transport and metabolism	None
NCU09263	Anchored Cell Wall Protein 4 (Hypothetical)	2.51	None	None
NCU07021	Peptide Chain Release Factor 3	2.36	Translation, ribosomal structure and biogenesis	None
NCU06880	AhpC/TSA Family Protein	2.23	Posttranslational modification, protein turnover, chaperones	Atypical 2-Cys peroxiredoxin
NCU03929	Acyl-CoA Synthetase	2.18	Lipid transport and metabolism	Similar to Fum16p
NCU03796	Pyruvate Dehydrogenase Kinase	2.17	None	None
NCU04578	ATP-Dependent Clp Protease Proteolytic Subunit	2.16	Intracellular trafficking, secretion, and vesicular transport	None
NCU09285	Zinc-Containing Alcohol Dehydrogenase	2.12	General function prediction only	None
NCU03006	Sterol 24-C-Methyltransferase	2.12	Coenzyme transport and metabolism	None
NCU16657	Tryptophanyl-tRNA Synthetase	2.11	Translation, ribosomal structure and biogenesis	None
NCU10021	MFS Monosaccharide Transporter	2.11	None	None
NCU05558	3-Ketoacyl-CoA Thiolase	2.1	Lipid transport and metabolism	None
NCU06958	Mito Ribosomal Protein S21	2.03	None	None
NCU02802	Mitochondrial Ornithine Carrier Protein	1.98	None	None
NCU07858	DUF498 Domain-Containing Protein	1.94	Function unknown	None
NCU00264	Hypothetical Protein	1.91	Signal transduction mechanisms	None
Reduced in 238Por				
NCU02936	Proline Oxidase	-1.84	None	None
NCU06189	5-Aminolevulinate Synthase	-1.86	Coenzyme transport and metabolism	None
NCU00107	Mitochondrial Inner Membrane Protease ATP-23	-2.41	None	None
NCU02504	DUF1640 Domain-Containing protein	-2.62	None	None
NCU00565	Lipoic Acid Synthetase	-2.82	Coenzyme transport and metabolism	None

NCU08354	Hypothetical Protein	-2.84	None	None
NCU01024	Hypothetical Protein	-2.9	None	altered inheritance of mitochondria protein 5
NCU09143	Hypothetical Protein	-3.93	None	F-type H ⁺ -transporting ATP synthase e-subunit
NCU04304	Outer Mitochondrial Membrane Protein Porin	-4.96	None	Voltage-dependent anion channel protein 2

Draft

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Draft

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