Canadian Journal of Microbiology
Revue canadienne de microbiologie

**Functional characterization of an N-terminally truncated
mitochondrial porin expressed in *Neurospora crassa***

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2016-0764.R1
Manuscript Type:	Note
Date Submitted by the Author:	11-Mar-2017
Complete List of Authors:	Shuvo, Sabbir Rahman; Dept. of Microbiology Kovaltchouk, Uliana ; Dept. of Microbiology Zubaer, Abdullah; Dept. of Microbiology Kumar, Ayush; Dept. of Microbiology Summers, William A.T.; Dept. of Microbiology Donald, Lynda J.; Dept. of Microbiology Hausner, George; Dept. of Microbiology Court, Deborah A.; Dept. of Microbiology
Keyword:	mitochondrial porin, VDAC, <i>Neurospora crassa</i> , outer membrane, N-terminus

SCHOLARONE™
Manuscripts

Functional characterization of an N-terminally truncated mitochondrial porin expressed in *Neurospora crassa*

Sabbir R. Shuvo, Uliana Kovaltchouk, Abdullah Zubaer, Ayush Kumar, William A.T. Summers, Lynda J. Donald, Georg Hausner, Deborah A. Court*

Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2

Draft

*corresponding author
Department of Microbiology
University of Manitoba
Winnipeg, MB Canada R3T 2N2
Phone (204) 474 8263
Fax (204) 474-7603

ABSTRACT

Mitochondrial porin, which forms voltage-dependent anion-selective channels (VDAC) in the outer membrane, can be folded into a 19 β -stranded barrel. The N-terminus of the protein is external to the barrel, and contains α -helical structure. Targeted modifications of the N-terminal region have been assessed in artificial membranes, leading to different models for gating *in vitro*. However, the *in vivo* requirements for gating and the N-terminal segment of porin are less well-understood. Using *Neurospora crassa* porin as a model, the effects of a partial deletion of the N-terminal segment were investigated. The protein, Δ N2-12porin, is assembled into the outer membrane, albeit at lower levels than the wild-type protein. The resulting strain displays electron transport chain deficiencies, concomitant expression of alternative oxidase, and decreased growth rates. Nonetheless, its mitochondrial genome does not contain any significant mutations. Most of the genes that are expressed in high levels in porin-less *N. crassa* are expressed at levels similar to wild-type or are slightly increased in Δ N2-12porin strains. Thus, although the N-terminal segment of VDAC is required for complete function *in vivo*, low levels of a protein lacking part of the N-terminus are able to rescue some of the defects associated with the absence of porin.

Key words: mitochondrial porin, VDAC, N-terminus, *Neurospora crassa*, alternative oxidase

INTRODUCTION

Voltage-dependent anion-channels (VDAC, mitochondrial porins) reside in the outer membrane (MOM) of mitochondria, where they allow the flow of small solutes across the lipid bilayer. Incorporation of isolated or recombinant mitochondrial porins into artificial bilayers reveals features common to porins from a wide range of organisms: slight anion selectivity, conductances of 3-5 nS in the open state, and susceptibility to voltage-dependent gating to form a slightly cation-selective pore of reduced conductance ("closed state", see Young et al. 2007 for review).

Analysis of refolded, recombinant VDAC1 and VDAC2 from vertebrates has revealed a common structure, comprised of a 19-stranded β -barrel, and an N-terminal segment of about 25 residues that does not form part of the barrel (Hiller et al. 2008, Bayrhuber et al. 2008, Ujwal et al. 2008, Gattin et al. 2015, Schredelseker et al. 2014). The relatively uniform nature of gating by the VDACS characterized to date suggests either a conserved process driven by voltage potentials *in vivo*, or a conserved structure and charge distribution that lead to similar effects on channels analyzed *in vitro*. Several studies using variants of fungal and human porins lacking most or part of the N-terminal segment indicate that these residues are involved in gating (Koppel et al. 1998, De Pinto et al. 2008). Other studies (Popp et al. 1996) have suggested that the N-terminus is not required for gating, but the molecules used in these studies carried N-terminal hexa-histidine tags, which may have partially compensated for the shortened N-termini (see Shuvo et al. 2016 for discussion).

One aspect of the role of N-terminus that has been studied to a lesser extent is its essentiality *in vivo*. Mitochondrial porins lacking either 11 or 22 N-terminal residues are imported into isolated mitochondria of *N. crassa*, although they are assembled into a more protease-sensitive form (Court et al. 1996). Similarly, human VDAC1 (hVDAC1) variants lacking 7 or 19 N-terminal residues co-localize with mitochondria when expressed in transformed monkey

kidney fibroblast (Cos-7) cells (De Pinto et al. 2007). In these cells, overexpression of the truncated hVDAC1 molecules abolished the pro-apoptotic effect associated with overexpression of the wild-type protein, indicating a direct or indirect role for the N-terminus in apoptosis. However, the native monkey VDACs were also expressed in those cells, precluding analysis of the participation of the N-terminally deleted hVDAC in key cellular processes. The importance of this region *in vivo* is further suggested by experiments in which the N-terminal sequence of hVDAC3 was replaced with that of hVDAC1, resulting in a pore that complemented *Saccharomyces cerevisiae* Δ por1 mutant that lacked the main porin isoform (Reina et al. 2010). This yeast is a facultative aerobe, and although these experiments indicate an important role for the N-terminus of VDAC in cellular function, they do not directly test the question of whether a porin lacking part of the N-terminus can complement a VDAC-less strain of an obligate aerobe.

To address this question, the *N. crassa* model system was used; in this organism porin is not essential, but its absence results in severe mitochondrial defects (Summers et al. 2012). *N. crassa* possesses a single VDAC isoform, allowing for more straightforward interpretation of results. Electrophysiological analysis of variants of this porin revealed that the absence of part (Δ 2-12porin) or all (Δ 3-21porin) of the N-terminal segments did not preclude the formation of gated channels (Popp et al. 1996). However, Δ 3-21porin produced noisy channels and, following import into isolated mitochondria, and was more sensitive to externally added protease than wild-type and Δ 2-12porin (Court et al. 1996). Therefore, the latter protein was chosen for analysis and a strain of *N. crassa* was generated in which the wild-type porin gene was replaced with one encoding Δ 2-12porin. The mitochondrial function of the strain was assessed, and the relative levels of mRNA encoding several proteins that are up-regulated in porin-less cells were examined.

MATERIALS AND METHODS

Strains and growth conditions – The strains used in this study are listed in Table 1 and were maintained according to Davis and De Serres (1970). Strains harboring the VDAC (porin) variant genes were created using the homologous recombination strategy developed by Colot et al. (2006) and Ninomiya et al. (2004) and the wild-type strain FGSC 9718. For Δ N2-12porin, the wild-type VDAC gene was replaced by that encoding a variant lacking residues 2-12. Conidia were transformed with a linear DNA fragment containing base pairs (bp) -1000 to -320 upstream of the native porin start codon (amplified from FSGC 9720), the hygromycin resistance cassette from pCNS44 (Staben et al. 1989), bp -320 to -1 upstream of the porin gene, the cDNA sequence encoding Δ 2-12porin (Popp et al. 1996), and about 1400 bp of sequence downstream of the porin stop codon. The first and last sequences listed were designed to provide homologous sequences for the double recombination event that would replace the wild-type porin coding sequence with the Δ 2-12porin coding sequence flagged with an adjacent hygromycin-resistance marker. DNA sequence analysis of the integrated sequences revealed that the recombination event to the 3' side of the ORF occurred between exon 4 in the genome and the transformed cDNA. Thus, intron 4 is retained in the Δ 2-12porin strain.

A strain expressing a relatively low amount of VDAC (LoPo) was constructed from FGSC 9718 by replacing the native VDAC promoter region with that of the *N. crassa tom40* gene. Wild-type conidia were transformed with a linear DNA fragment containing bp -1000 to -320 upstream of the native porin start codon (amplified from FSGC 9720), the hygromycin resistance cassette from pCNS44 (Staben et al. 1989), bp -300 to -1 upstream of the *tom40* gene (from FGSC 9718), and the cDNA sequence encoding the wild-type VDAC (Popp et al. 1996). Following confirmation of the correct insertion by DNA sequencing, homokaryotic strains were generated by 7 rounds of repeated subculturing and confirmed by PCR. Levels of VDAC were determined using an antibody against residues 7-20 (R. Lill and W. Neupert, Universität

München) or residues 195-210 (GenScript, Piscataway, NJ).

Mitochondrial analyses

Mitochondria were isolated according to Harkness et al. (1994), following growth in Vogel's Medium (VM). The respiration control rate (RCR) was used to assess integrity as described in Bahn et al. (2016) and Kuznetsov et al. (2008). Mitochondrial integrity was more than 70% for all experiments.

Growth rate, cytochrome spectra and O₂ consumption measurements were collected as described in Summers et al. (2012), with the exception that an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) was used. Microscopy was performed following the protocol described in Ferens et al., in press.

RNA extraction and cDNA synthesis

Cultures were grown for 21-25 hours at 30°C in VM. One gram (wet weight) of hyphae was flash frozen with liquid nitrogen and was ground with RNase-free sand in a mortar and pestle. One fifth of each sample was used for whole cell RNA extraction using a Plant RNA kit (Qiagen, Mississauga, ON, Canada) following manufacturer's instructions except that the initial volume of RLC buffer was 650 µl to which 6.5 µl of 2-mercaptoethanol were added. To remove the genomic DNA, the samples were treated with 1.5 Kunitz units of DNase I (Qiagen) for 30 min at 37°C, followed by heat inactivation at 65°C for 5 min. Next, 1 µg of total RNA was used to synthesize cDNA using the Bio-Rad iScript reverse transcriptase kit (Bio-Rad, Mississauga, ON, Canada). To ensure that all genomic DNA was removed by DNase treatment, a control reaction lacking reverse transcriptase (NRT) was set up using all the reaction components except the reverse transcriptase enzyme.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

Analysis was carried out with the primers listed in Table 2. The efficiency of the primers was tested with 10-fold serial dilutions of genomic DNA and ranged from 88-103%. The expression of genes was determined by quantitative PCR by using SsoFast Evagreen Supermix (Bio-Rad). The NRT control described above and a reaction lacking template (NTC) were used to control for the presence of genomic DNA in the sample, and for nucleic acid contamination in the reaction mixture, respectively. Reactions were set up using primer concentrations of 300 nM and 1.6 µl of cDNA (diluted 1:2) in 8-µl reaction mixtures. Actin (NCU04173) was used as a reference gene. Relative expression levels were determined using the cycle threshold method ($\Delta\Delta C_t$) on an Eco Real-Time PCR System (San Diego, California, USA). Relative expression levels were calculated using the following equations:

$$\Delta C_q = C_q \text{ target} - C_q \text{ reference}$$

$$\Delta\Delta C_q = \Delta C_q \text{ sample} - \Delta C_q \text{ control}$$

$$R = 2^{-\Delta\Delta C_q}$$

where “C_q” is the PCR cycle in which fluorescence is detected, “target” to the mRNA under study, “reference” to actin, “sample” to the data obtained with $\Delta 2$ -12Porin cDNA and “control” to that from the wild type (FGS9718). Each measurement was carried out in triplicate, data analysis was performed using EcoStudy Software v 5.0.

Mitochondrial genome analysis

Mitochondrial DNA (mtDNA) was isolated following the protocol described in Bertrand et al. (1985). Approximately 100 ng of DNA in 75 µl of H₂O were supplied to Genome Quebec (Innovation Centre, McGill University, Quebec, Canada) for Illumina sequencing using the MiSeq platform. DNA samples were barcoded and combined on a MiSeq run. Average size

reads were 250 bp, average quality was 35 and quality offset was 33, reconfirmed by FastQC. Paired-end reads were assembled *de novo* with the A5-MiSeq pipeline (Coil et al., 2015), which was updated for Illumina MiSeq from the A5 pipeline (Tritt et al., 2012). The scaffolds were sorted and joined to prepare the complete genome with the aid of our own Python script as well as manual inspection. The mtDNA sequence was annotated with the program Artemis (Rutherford et al., 2000) on the basis of the gene, intron, rRNA and tRNA coordinates predicted with the MFannot and RNAweasel online programs (Gautheret and Lambert, 2001; Lang et al., 2007; <http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl>). The comparison of the genome sequences of the wild type (GenBank KY213951), the Δ N2-12porin (KY498477) and Δ Por-1 (KY498478) mutants and the previously published mitochondrial genome of *N. crassa* (NC_026614.1) was done in MAFFT (multiple sequence alignment server selecting the progressive method of alignment; Katoh et al., 2002).

Determination of relative protein level by mass spectroscopy

Mitochondria were washed with 1 M sodium carbonate (pH 10) as described in Court et al. (1996) to enrich for membrane proteins and proteins were separated via SDS-PAGE. Regions of the gel containing both VDAC (30 kDa) and adenine nucleotide translocator (ANT, 34 kDa) were excised from SDS-PAGE gels, and prepared for mass spectrometry analysis, essentially as described by Shevchenko et al. (2006). Mass spectra were acquired on a prototype MALDI QqTOF instrument (Loboda et al. 2000). Relative porin levels were estimated by normalizing total ion counts (TIC) for porin to the TIC for ANT obtained for each strain, and then comparing to the same value from the wild-type samples (see Supplementary Table 1).

RESULTS

Porin lacking the N-terminal 12 amino acids is assembled poorly into the outer membrane

In vitro-synthesized Δ 2-12porin can form pores in artificial membranes (Popp et al. 1996) and can be imported into isolated mitochondria, where it assembles in a membrane-embedded form that is more accessible to externally added protease than the wild-type protein (Court et al. 1996). To investigate the function of this truncated porin *in vivo*, the gene for the wild-type porin was replaced with that encoding Δ N2-12porin. To determine if Δ N2-12porin was assembled into the mitochondria of the resulting strain, western blot analysis was performed (Fig. 1). The truncated porin was present in significantly lower levels than in the wild-type. The relative level of Δ N2-12porin, normalized to that of the inner membrane adenine nucleotide translocator (ANT), was about one-tenth of that in the wild-type strain, as estimated by mass spectroscopy (Supplementary Table 1) and quantitation of signals on the western blots.

One possible explanation for the low levels of Δ N2-12porin is a reduction in steady state levels of the corresponding mRNA. To investigate this possibility, porin (NCU04304) mRNA levels were analyzed using qRT-PCR (Fig. 2). In Δ N2-12porin strain mRNA levels were not reduced compared to wild-type. Thus, the low protein levels likely reflect suboptimal post-transcriptional processes.

The low levels of the variant protein complicate the analysis of the mutant strains, because phenotypic changes could result from reduced protein levels, or the partial truncation of the N-terminus, or both. In order to separate these factors, a control strain was generated in which the transcription of porin is driven by the *tom40* promoter. Tom40 is significantly less abundant in the MOM than is porin (Schmitt et al. 2006). The resulting strain, LoPo, harbours about half as much porin as does the wild-type strain (Fig. 1, and Supplementary Table 1).

Strains expressing porin lacking the N-terminal 12 amino acids display electron transport chain deficiencies and altered staining with MitoTracker

Strains of *Neurospora crassa* lacking mitochondrial porin (Δpor) exhibit deficiencies in cytochromes *b* and aa_3 , and express alternative oxidase, indicating reduced mitochondrial function (Summers et al. 2012). To determine if the $\Delta 2$ -12porin strain possesses similar characteristics, cytochrome spectra (Fig. 3A) and oxygen consumption (Fig. 3B) were analyzed. Levels of cytochromes *b* and aa_3 were reduced in $\Delta N2$ -12porin strain and enhanced expression of cytochrome *c* was observed, which is common in *Neurospora* strains with deficiencies in cytochromes *b* and aa_3 (for example see Infanger and Bertrand 1986). As expected from these results, the $\Delta 2$ -12porin strain expresses cyanide-insensitive, salicylhydroxamic acid (SHAM)-sensitive oxygen consumption, indicative of expression of alternative oxidase (Lambowitz and Slayman 1971). To exclude the possibility that the defects in cytochromes aa_3 and *b* resulted from spontaneous mutations in the mitochondrial genes encoding some of the proteins they associate with, this DNA was sequenced from the wild-type, ΔPor -1 and $\Delta 2$ -12porin strains. The only mutation detected was in $\Delta 2$ -12porin, and was a short deletion in a segment containing a partial duplication of the ND2 gene (bp 59634-59638 of the FGSC9718 mtDNA sequence). This duplication does not encode a functional ND2 polypeptide (Agsteribbe et al. 1989; McCluskey 2012). Mutations in nuclear genes may also be responsible for these mutations, but analysis of these genomes are beyond the scope of the current study.

LoPo, the strain harbouring a lower level of wild-type porin (50% of wild-type) is similar to wild-type in terms of growth (Table 3) and electron transport chain (Fig. 3A). Thus, while it cannot be excluded that the level of porin is insufficient in $\Delta N2$ -12porin variant, the data

obtained with LoPo suggest that the defects in Δ N2-12porin are due to the truncated porin rather than reduced porin levels. xxxxx

Hyphae were stained with MitoTracker to assess the overall abundance and distribution of mitochondria in the strain harbouring Δ N2-12porin. Compared to the wild-type and LoPo strains, the variant strain displayed lower numbers of fluorescent mitochondrial punctae (Fig. 4).

Gene expression associated with Δ 2-12porin

Analysis of the mitochondrial proteome of the Δ Por-1 strain revealed a subset of proteins and corresponding mRNAs that were overexpressed in that strain compared to the wild-type FGSC 9718 (Summers et al. 2012, Supplementary Table 2). To evaluate whether Δ N2-12porin can compensate for the loss of porin, quantitative PCR of cDNA derived from Δ N2-12porin RNA was used to determine if the same set of genes are upregulated in this strain. RNA levels corresponding to proteins present in lower relative amounts in Δ Por-1 were similar for both wild-type and Δ Por-1 strains (Summers et al. 2012), and were therefore excluded from the current analysis. These proteins are constituents of the intermembrane space, suggesting that damage to the MOM during mitochondrial preparation resulted in their loss.

The results of the RT-qPCR analysis for alternative oxidase in Δ N2-12porin was similar to wild-type (Fig. 2). In contrast to the previous study (Summers et al. 2012), alternative oxidase (*aod-1*) mRNA was detected in wild-type RNA samples (Supplementary Table 2), likely due to the use of a more sensitive qPCR detection system. This observation agrees with the detection of mRNA for *aod-1* in wild-type strains using RNAseq (Desaulniers 2013), even though alternative oxidase activity, and mRNA and protein are not readily detectable in wild-type by northern and western blot methods, respectively (Tanton et al. 2003). Given the apparent similarities in *aod-1* mRNA levels in wild-type and Δ N2-12porin strains, it is unexpected that cyanide-insensitive respiration is detected only Δ N2-12porin. Similarly, incongruous results were

obtained with the 238porin strain, which expresses low levels of porin lacking residues 238-242. In this strain, alternative oxidase activity was detected although the protein was not (Ferens et al. in press). In contrast, both *aod-1* mRNA and protein were observed in the Δ Por-1 strain. The reasons for these observations are not clear, but may include regulation at the post-transcriptional level.

The expression levels of several other genes that are upregulated in Δ Por-1 were not distinguishable from wild-type ($P > 0.05$; Fig. 2). The only exception was the RNA for *coq4* (NCU02157), which is involved in ubiquinone biosynthesis, and was present in much lower levels than in wild-type ($P < 0.05$).

DISCUSSION

The N-terminus may contribute to several aspects of porin function, including gating and/or changes to barrel shape that may influence ion selectivity and pore size (see Shuvo et al. 2016) and interactions with metabolic enzymes such as hexokinase (Arzoin et al. 2009). The current study indicates that porin lacking part of the N-terminal segment is not assembled at normal levels in the membrane, and the associated mitochondria do not maintain a normal electron transport chain.

In spite of these low levels of porin, the growth rate of the Δ N2-12porin strain is over 85% of the wild-type rate (Table 2), in comparison to less than 30% for the porin-less strain (Summers et al. 2012). Similarly, a variant expressing 238porin at levels less than 5% of wild-type porin, also grows at more than 85% of the wild-type rate (Ferens et al., in press). Thus, low levels of two different porin variants partially rescue the growth rate defect associated with the absence of porin. Taken together, these results suggest that Δ N2-12porin is at least partially functional, as 238porin contains a wild-type N-terminus and is associated with a similar

phenotype when expressed at low levels. Both proteins are capable of generating wild-type sized pores in artificial membranes (Popp et al. 1996).

Nonetheless, the reduced levels of $\Delta 2$ -12porin complicate the interpretation of the phenotypes associated with the mutant. In an attempt to separate the effects of the N-terminal truncation and the low level of porin, a strain expressing low levels of wild-type porin was generated by replacing the porin promoter with that of Tom40. The resulting strain expressed ~50% of wild-type levels of porin and is phenotypically wild-type, although it cannot be excluded that a further reduction in wild-type porin levels would lead to mitochondrial defects.

$\Delta N2$ -12porin is present at lower levels in mitochondria than the wild-type protein, although the amounts of mRNA are not lower than in wild-type. Therefore, reduced levels of $\Delta 2$ -12porin are most likely linked to one or more defects in translation, nascent protein stability and subsequent targeting and assembly of the protein in the outer membrane. *In vitro*-synthesized $\Delta N2$ -12porin can be imported and assembled into isolated mitochondria (Court et al. 1996), indicating that the molecule retains a mitochondrial targeting signal. Once it has crossed the MOM through the TOM complex, assembly of porin into the membrane requires the sorting and assembly complex (SAM, reviewed in Dukanovic and Rapaport 2011). The “ β -signal” recognized by the SAM complex is located in the C-terminal β -strand of the protein (see Bay et al. (2012) for the predicted *N. crassa* signal), so it is unlikely that targeting to the SAM complex is defective. Nonetheless, inefficient release from the TOM or assembly via the SAM complex cannot be ruled out.

Sampling of mRNA for genes overexpressed in Δ Por-1 strains revealed levels closer to wild-type in the $\Delta N2$ -12porin-expressing strain. This result suggests that low levels of $\Delta N2$ -12porin can partially compensate for the absence of wild-type porin. The only under-expressed mRNA in $\Delta N2$ -12por was for *coq4* (NCU02157), involved in ubiquinone biosynthesis. The reason for this reduction is not clear. Although statistical support was not strong, the mRNA

putatively encoded by NCU05850 was increased two-fold. Although this gene is annotated as a rubredoxin-NAD(+) reductase, recent work has identified it as a homologue of the apoptosis-related oxidoreductase (*aif-1*, Carneiro et al. 2012). However, unlike its mammalian counterpart, AIF-1 does not appear to be required for assembly of complex I and therefore the reason for its abundance in Δ Por-1 is not clear.

CONCLUSIONS

The N-terminus of porin is required for the efficient assembly of the protein in mitochondria, and maintenance of the standard electron transport chain and lower levels of mitochondria are observed in the Δ N2-12por strain. The expression of low levels of Δ N2-12porin is associated with only some of the changes in gene expression observed in the Δ Por-1 strain.

ACKNOWLEDGEMENTS

This work was funded through Discovery Grants from NSERC to DAC, AK and GH. UK received a URSA from NSERC and SRS and WATS were supported in part by the Faculty of Science, University of Manitoba. SRS was the recipient of a University of Manitoba Graduate Fellowship and SRS and AZ were supported in part by the GETS program of the Faculty of Graduate Studies. Initial analyses of the strains were completed as part of the BSc Honours thesis of Justine Gallardo. We thank Ameet Bharaj for excellent technical assistance, and Dr. Jason Treberg and Mr. Andre Dufresne, Department of Biological Sciences, for use of the Oxygraph and for assistance with fluorescence microscopy, respectively. We acknowledge Drs. W. Ens and K.G. Standing for use of the instruments in the Department of Physics and Astronomy and we appreciate the assistance from Dr. Neil J. Holliday, Department of Entomology, with statistical analysis of the MS data. We also thank Mr. Manu Singh and Mr. Malaka de Silva (Department of Microbiology) for their help with RT-qPCR.

REFERENCES

Agsteribbe, E., Hartog, M. and de Vries, Hans. 1989. Duplication of the tRNAMet and tRNACys genes and of fragments of a gene encoding a subunit of the NADH dehydrogenase complex in *Neurospora crassa* mitochondrial DNA. *Curr. Genet.* **15**: 57-62.

Arzoine, L., Zilberberg, N., Ben-Romano, R. and Shoshan-Barmatz, V. 2009. Voltage-dependent anion channel 1-based peptides interact with hexokinase to prevent its anti-apoptotic activity. *J. Biol. Chem.* **284**(6): 3946-3955.

Banh, S., Wiens, L., Sotiria, E., and Treberg, J.R. 2016. Mitochondrial reactive oxygen species production by fish muscle mitochondria: Potential role in acute heat-induced oxidative stress. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **191**:99-107.

Bay, D.C., Hafez, M., Young, M.J. and Court, D.A. 2012. Phylogenetic and coevolutionary analysis of the beta-barrel protein family comprised of mitochondrial porin (VDAC) and Tom40. *Biochim. Biophys. Acta* **1818**(6): 1502-1519.

Bayrhuber, M., Meins, T., Habeck, M., Becker, S., Giller, K., Villinger, S., Vonrhein, C., Griesinger, C., Zweckstetter, M. and Zeth, K. 2008. Structure of the human voltage-dependent anion channel. *Proc. Natl. Acad. Sci. (U.S.A.)* **105**(40): 15370-15375.

Bertrand H., Chan B.S, Griffiths A.J. 1985. Insertion of a foreign nucleotide sequence into mitochondrial DNA causes senescence in *Neurospora intermedia*. *Cell* **41**(3):877-884.

Carneiro, P., Duarte, M. and Videira, A. 2012. Characterization of apoptosis-related oxidoreductases from *Neurospora crassa*. PLoS One **7**(3): e34270.

Coil, D., Jospin G., and Darling, A.E. 2015. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics **31**(4):587-589.

Colot, H.V., Park, G., Turner, G.E., Ringelberg, C., Crew, C.M., Litvinkova, L., Weiss, R.L., Borkovich, K.A. and Dunlap, J.C. 2006. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. (U.S.A.) **103**(27): 10352-10357.

Court, D.A., Kleene, R., Neupert, W. and Lill, R. 1996. Role of the N- and C-termini of porin in import into the outer membrane of *Neurospora* mitochondria. FEBS Lett. **390**(1): 73-77.

Davis, R.H. and De Serres, F.J. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17**: 79-143.

De Pinto, V., Reina, S., Guarino, F. and Messina, A. 2008. Structure of the voltage dependent anion channel: state of the art. J. Bioenerg. Biomembr. **40**(3): 139-147.

De Pinto, V., Tomasello, F., Messina, A., Guarino, F., Benz, R., La Mendola, D., Magri, A., Milardi, D. and Pappalardo, G. 2007. Determination of the conformation of the human VDAC1 N-terminal peptide, a protein moiety essential for the functional properties of the pore. Chembiochem. **8**(7): 744-756.

Desaulniers, A.B. 2013. The role of YVH1, a dual specificity phosphatase, in the production of alternative oxidase in the filamentous fungus *Neurospora crassa*. M.Sc., University of Alberta.

Dukanovic, J. and Rapaport, D. 2011. Multiple pathways in the integration of proteins into the mitochondrial outer membrane. *Biochim. Biophys. Acta* **1808**(3): 971-980.

Ferens, F. G., Spicer, V., Krokhin, O.V., Motnenko, A., Summers, W.A.T., and Court, D.A. in press. A deletion variant partially complements a porin-less strain of *Neurospora crassa*. *Biochem. Cell Biol.* Published on the web 2 November 2016, 10.1139/bcb-2016-0166

Gattin, Z., Schneider, R., Laukat, Y., Giller, K., Maier, E., Zweckstetter, M., Griesinger, C., Benz, R., Becker, S. and Lange, A. 2015. Solid-state NMR, electrophysiology and molecular dynamics characterization of human VDAC2. *J. Biomol. NMR* **61**(3-4): 311-320.

Harkness, T.A., Nargang, F.E., van der Klei, I., Neupert, W. and Lill, R. 1994. A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria. *J. Cell Biol.* **124**(5): 637-648.

Hawltischek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.U. and Neupert, W. 1988. Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. *Cell* **53**(5): 795-806.

Hiller, S., Garces, R.G., Malia, T.J., Orekhov, V.Y., Colombini, M. and Wagner, G. 2008. Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. *Science* **321**(5893): 1206-1210.

Infanger, A. and Bertrand, H. 1986. Inversions and recombinations in mitochondrial DNA of the (SG-1) cytoplasmic mutant in two *Neurospora* species. *Curr. Genet.* **10**(8): 607-617.

Koppel, D.A., Kinnally, K.W., Masters, P., Forte, M., Blachly-Dyson, E. and Mannella, C.A. 1998. Bacterial expression and characterization of the mitochondrial outer membrane channel. Effects of n-terminal modifications. *J. Biol. Chem.* **273**(22): 13794-13800.

Kuznetsov, A.V., Veksler, V., Gellerich, F.N., Saks, V., Margreiter, R., and Kunz, W.S. 2008. Analysis of mitochondrial function *in situ* in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* **3**(6):965-976.

Lambowitz, A.M. and Slayman, C.W. 1971. Cyanide-resistant respiration in *Neurospora crassa*. *J. Bacteriol.* **108**(3): 1087-1096.

Loboda, A.V., Krutchinsky, A.N., Bromirski, M., Ens, W. and Standing, K.G. 2000. A tandem quadrupole/time-of-flight mass spectrometer with a matrix-assisted laser desorption/ionization source: design and performance. *Rapid Commun. Mass Spectrom.* **14**(12): 1047-1057.

McCluskey, K. (2012) Variation in mitochondrial genome primary sequence among whole-genome-sequenced strains of *Neurospora crassa*. *IMA fungus* **3**(1): 93-98.

McCluskey, K., Wiest, A. and Plamann, M. 2010. The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. *J. Biosci.* **35**(1): 119-126.

Mertins, B., Psakis, G., Grosse, W., Back, K.C., Salisowski, A., Reiss, P., Koert, U. and Essen, L.O. 2012. Flexibility of the N-terminal mVDAC1 segment controls the channel's gating behavior. PLoS One **7**(10): e47938.

Nargang, F.E., Adames, K., Rub, C., Cheung, S., Easton, N., Nargang, C.E. and Chae, M.S. 2012. Identification of genes required for alternative oxidase production in the *Neurospora crassa* gene knockout library. G3 (Bethesda) **2**(11): 1345-1356.

Ninomiya, Y., Suzuki, K., Ishii, C. and Inoue, H. 2004. Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc. Natl. Acad. Sci. (U.S.A.) **101**(33): 12248-12253.

Popp, B., Court, D.A., Benz, R., Neupert, W. and Lill, R. 1996. The role of the N and C termini of recombinant *Neurospora* mitochondrial porin in channel formation and voltage-dependent gating. J. Biol. Chem. **271**(23): 13593-13599.

Reina, S., Palermo, V., Guarnera, A., Guarino, F., Messina, A., Mazzoni, C. and De Pinto, V. 2010. Swapping of the N-terminus of VDAC1 with VDAC3 restores full activity of the channel and confers anti-aging features to the cell. FEBS Lett. **584**(13): 2837-2844.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.A. and Barrell B. 2000. Artemis: sequence visualization and annotation. Bioinformatics **16**(10):944-945.

Schmitt, S., Prokisch, H., Schlunck, T., Camp, D.G., 2nd, Ahting, U., Waizenegger, T., Scharfe, C., Meitinger, T., Imhof, A., Neupert, W., Oefner, P.J. and Rapaport, D. 2006. Proteome analysis of mitochondrial outer membrane from *Neurospora crassa*. Proteomics **6**(1): 72-80.

Schredelseker, J., Paz, A., Lopez, C.J., Altenbach, C., Leung, C.S., Drexler, M.K., Chen, J.N., Hubbell, W.L. and Abramson, J. 2014. High resolution structure and double electron-electron resonance of the zebrafish voltage-dependent anion channel 2 reveal an oligomeric population. *J. Biol. Chem.* **289**(18): 12566-12577.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V. and Mann, M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**(6): 2856-2860.

Shuvo, S.R., Ferens, F.G. and Court, D.A. 2016. The N-terminus of VDAC: Structure, mutational analysis, and a potential role in regulating barrel shape. *Biochim. Biophys. Acta* **1858**(6): 1350-1361.

Staben, C., Jensen, B., Singer, M., Pollock, J., Schechtman, M., Kinsey, J. and Selker, E.U. 1989. Use of a bacterial Hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. *Fungal Genet. Newsl.* **36**: 79-81.

Summers, W.A. and Court, D.A. 2010. Origami in outer membrane mimetics: correlating the first detailed images of refolded VDAC with over 20 years of biochemical data. *Biochem. Cell Biol.* **88**(3): 425-438.

Summers, W.A., Wilkins, J.A., Dwivedi, R.C., Ezzati, P. and Court, D.A. 2012. Mitochondrial dysfunction resulting from the absence of mitochondrial porin in *Neurospora crassa*. *Mitochondrion* **12**(2): 220-229.

Summers, W.A.T. 2010. An *in vivo* approach to elucidating the function of mitochondrial porin by the characterisation of *Neurospora crassa* strains deficient in porin. Ph.D. thesis, University of Manitoba.

Tanton, L.L., Nargang, C.E., Kessler, K.E., Li, Q. and Nargang, F.E. 2003. Alternative oxidase expression in *Neurospora crassa*. Fungal Genet. Biol. **39**(2): 176-190.

Tritt, A., Eisen, J.A., Facciotti, M.T., and Darling, A.E. 2012. An integrated pipeline for de novo assembly of microbial genomes. PLoS One **7**(9):e42304.

Ujwal, R., Cascio, D., Colletier, J.P., Faham, S., Zhang, J., Toro, L., Ping, P. and Abramson, J. 2008. The crystal structure of mouse VDAC1 at 2.3 Å resolution reveals mechanistic insights into metabolite gating. Proc. Natl. Acad. Sci. (U.S.A.) **105**(46): 17742-17747.

Young, M.J., Bay, D.C., Hausner, G. and Court, D.A. 2007. The evolutionary history of mitochondrial porins. BMC Evol. Biol. **7**: 31.

Figure Legends

Figure 1. Western blot analysis mitochondria purified from wild-type (WT), LoPo and Δ N2-12porin (Δ N2-12) strains. 20 μ g of mitochondrial proteins were loaded in each lane. Blots were immunodecorated with antibodies against residues 195-210 of porin (α VDAC) or α Tom70 (Translocase of the outer membrane 70). The top and middle panels represent 30- and 5-minute exposures of the blot probed with α VDAC, and the lower panel was probed with the loading control, Tom70.

Figure 2. RT-qPCR analysis of selected genes in Δ N2-12porin. The expression profiles of seven genes [(NCU04304, *por* encoding porin), (NCU07953, *aod-1* encoding alternative oxidase-1), (NCU06518, NADH-cytochrome b_5 reductase 2), (NCU05633, stomatin family protein), (NCU08476, conserved hypothetical protein), (NCU02157, *coq4*), (NCU05850, *aif-1*, apoptosis-inducing factor, annotated in the Broad Institute database as rubredoxin-NAD⁺ reductase 2)] were analyzed by RT-qPCR, normalized to actin ($\Delta\Delta$ Cq). These normalized expression values are shown relative to the wild-type, which was set to 1. The data represent an average of at least two biological replicates and the error bars indicate the range of relative expression levels obtained. * indicates $P < 0.05$ based on a students' t-test.

Figure 3. (A) Cytochrome spectra obtained from Δ N2-12porin, wild-type and LoPo. Peaks representing absorbance by cytochromes *c*, *b*, and *aa₃* are indicated. **(B)** Alternative oxidase expression in wild-type, Δ N2-12porin and LoPo strains. Cultures were grown at 30°C for 14 hours for wild-type, and LoPo and 16 hours for Δ N2-12porin. The first bar in each set represents the oxygen consumption in the drug-free state. Second and third bars indicate consumption of

oxygen after addition of inhibitor of the standard electron transport chain (KCN) alone, or in combination with the alternative oxidase pathway (SHAM), respectively.

Figure 4: Morphology of mitochondria in wild-type, LoPo, and Δ N2-12porin strains. Cultures were grown on glass slides on solidified VM medium at 30°C for 12 hours for wild-type, 13 hours for LoPo and 16 hours for Δ N2-12porin, followed by staining with MitoTracker Green^{FM}. Stained material was observed at 1630X magnification. Mitochondria at the hyphal tips (**A**) and distal regions of hyphae (**B**) are shown. Scale bar represents 5 μ m.

Draft

Table 1. *Neurospora crassa* strains used in this study.

Strain name	Genotype/Comments	Reference
FGSC* 9718 (wild-type)	<i>mus-51::bar mat a</i>	(Colot et al. 2006)
ΔPor-1	<i>Δpor::hph mus-51::bar mat a</i> transformant of FGSC 9718	(Summers et al. 2012)
ΔN2-12porin	<i>hph+, porΔ2-12 mus-51::bar mat a</i> transformant of FGSC 9718	this work
LoPo	<i>hph+, tom40prom:por, mat a</i> transformant of FGSC 9718	this work

* Strains bearing FGSC names were obtained from the Fungal Genetics Stock Center (McCluskey et al. 2010)

Table 2. Primers used for qRT-PCR and sequencing reactions.

ORF [*]	Forward Primer (5'-3')	Reverse Primer (5'-3')
NCU07953	GGAGCGACGAAAAGTTTGAG	CCCTCGCCCTCCTTATAGTC
NCU06518	TGCACAGTACCGAGCTCATC	TGACCATGAGCTCAACGAAG
NCU02157	AATACCCTGTTGCCGATGAC	TTTACATCCCGCTCCAATC
NCU05850	CCCAAGATCAAGTGCAAGGT	AGCTTGCTGAGATCGGTCAT
NCU05633	ACAGCAGCGACAACACAAAG	CTGCTGTCGAGGAGGAAGTC
NCU04173	TTCTCTCGAGAAGTCCTACG	TGACAATGTTGCCATAGAGA
NCU08476	ATCCCGAGGAACTGATTGTG	AACAGCCCCGTAAACTCTGA
NCU06550	CGAGATTGCTCAGGCTAAGG	ACCCAAACTCCATCATGAGC
NCU11655/ NCU04304	CACAAGGTCAACTCCCAGGT	ATCGAAAGAGGCACCAACAC
Nad2 sequencing primers	GTAGGTTTACATGGAGGTTTA CTTC	CCTGAATTTGCTCATAGTAAACATG
Nad2_whole	GATCGAGTAGGACCAGAG	GCTCCATGAACATTATTAATTC

^{*}ORF numbers are taken from *Neurospora crassa* Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>)

Table 3. Growth rates and relative porin levels of strains expressing wild-type or variants of porin. Growth rates were determined at 22°C on VM.

Strain	Growth rate (cm/24hr)	Porin relative to ANT [*] (%)
FGSC [†] 9718 (wild-type)	7.7 ± 0.4 [‡]	100
ΔPor-1	1.6 ± 0.1 [‡]	0
ΔN2-12porin	6.6 ± 0.3 [§]	10
LoPo	7.1 ± 0.3	46

^{*}based on MS analysis as described in Materials and Methods (see Supplementary Table 1)

[†]FGSC, Fungal Genetics Stock Center

[‡]taken from Summers et al. 2012

[§]taken from Summers 2010 and this work

Draft

Figure 1. Western blot analysis mitochondria purified from wild-type (WT), LoPo and ΔN2-12porin (ΔN2-12) strains. 20 μg of mitochondrial proteins were loaded in each lane. Blots were immunodecorated with antibodies against residues 195-210 of porin (αVDAC) or αTom70 (Translocase of the outer membrane 70). The top and middle panels represent 30- and 5-minute exposures of the blot probed with αVDAC, and the lower panel was probed with the loading control, Tom70.

A

B

Supplementary Table 1. Observed intensity of the MALDI-produced monoisotopic ions from VDAC and ANT. Manual peak pick using the expected ions list as a guide.

STRAIN SAMPLE	WT SR15		LoPo SR16		ΔN2-12-1 SR17		ΔN2-12-2 SR18	
SPECTRUM II expected ions	10986 intensity	11004 intensity	10985 intensity	11006 intensity	10984 intensity	11007 intensity	10983 intensity	11008 intensity
VDAC								
845.402	6462	4905	0	0	0	0	0	0
974.447	7167	4343	3157	0	0	0	0	0
1018.517	6077	4747	4050	3977	0	0	0	0
1091.549	5317	3535	0	0	0	0	0	0
1189.643	10223	6864	7260	7871	5040	4676	4007	6106
1259.748	19277	14967	5403	6027	0	0	0	0
1493.775	42465	29260	14379	15018	0	0	0	0
1758.003	10178	8336	4232	4784	0	0	0	0
2045.042	4685	3895	4657	4746	3153	2986	4115	5089
2302.168	13011	9961	0	5126	0	0	0	0
2319.199	25589	17129	6683	6697	0	0	0	0
2658.316	5422	3669	0	0	0	0	0	0
3257.597	3000	2126	0	0	0	0	0	0
3492.78	4470	2769	916	1001	0	0	0	0
3521.759	3597	2279	1212	1052	0	0	0	0
3839.856	2667	2020	942	775	0	0	0	0
VDAC	169607	120805	52891	57074	8193	7662	8122	11195
ANT								
1045.601	6022	3116	3840	4151	2706	3431	0	0
1210.622	25812	17583	17513	15372	11459	10274	8136	10838
1236.659	28521	20571	23803	16080	11044	10376	6341	6070
1285.636	30298	14043	25430	21954	14553	17641	11907	14847
1321.727	82659	53755	64637	53182	37222	38126	16740	22017
1338.717	41184	27072	30682	23628	22028	18313	14189	20204
1349.652	47574	30205	38645	35374	23277	22449	21069	33846
1358.71	32059	23344	18493	17870	13965	12342	7294	10666
1364.754	14843	10806	10677	8717	5800	5490	4329	5044
1441.737	11933	6655	13173	11049	7891	9660	8173	12120
1474.748	79114	48152	54906	42089	32893	30986	21804	24341
1599.889	25165	18198	15270	14021	11096	10390	11457	14076
1717.87	42844	23849	30256	23125	17830	17054	13268	14513
2193.145	15474	8078	11060	8665	8041	6917	10469	11455
2409.329	7306	5653	5741	5460	4321	4220	5640	6608
ANT	490808	311080	364126	300737	224126	217669	160816	206645
VDAC/ANT	0.35	0.39	0.15	0.19	0.04	0.04	0.05	0.05
% of WT (average of two spectra)	100		46		10		14	

Supplementary Table 1. Observed intensity of the MALDI-produced monoisotopic ions from VDAC and ANT. Manual peak pick using the expected ions list as a guide.

Δ Por-1 SR19		expected ions from to sequence		
10982 intensity	11009 intensity			
VDAC				
0	0	845.402	119	125 QSNFHGR
0	0	974.447	275	283 VGTSFTFES
0	0	1018.517	197	206 VNSQVEAGSK
0	0	1091.549	33	42 SNTPNNVAFK
0	0	1189.643	213	224 TGNTVGLEVATK
0	0	1259.748	241	252 GVAAIAYNVLLR
0	0	1493.775	96	109 AEGIFSFLPATNAR
0	0	1758.003	237	252 INDRGVAAIAYNVLLR
0	0	2045.042	33	51 SNTPNNVAFKVTGKSTHDK
0	0	2302.168	253	274 EGVTLGVGASFDQKLDQATHK
0	0	2319.199	12	32 SANDLLNKDFYHLAAGTIEVK
0	0	2658.316	133	159 GPTANIDAIVGHEGFLAGASAGYDVQK
0	0	3257.597	253	283 EGVTLGVGASFDQKLDQATHKVGTSFTFES
0	0	3492.78	126	159 AFFDLLKGPTANIDAIVGHEGFLAGASAGYDVQK
0	0	3521.759	61	92 FTDKPNGLTVTQTWNTANALETKVEMADNLAK
0	0	3839.856	160	196 AAITGYSAAVGYHAPTYSAAITATDNLVSFASYYHK
0	0			
ANT				
0	0	1045.601	146	155 LANDAKSAKK
8736	8929	1210.622	160	169 QFNGLVDVYR
13035	8068	1236.659	171	182 TIASDGIAGLYR
8658	11943	1285.636	57	66 YNGIIDCFKR
24971	37331	1321.727	183	195 GFGPSVAGIVVYR
16690	17717	1338.717	160	170 QFNGLVDVYRK
14273	23273	1349.652	67	78 TTADEGVMALWR
9494	11688	1358.71	39	49 LLVQNQDEMIR
7409	6375	1364.754	170	182 KTIASDGIAGLYR
5096	6385	1441.737	56	66 RYNGIIDCFKR
21713	26785	1474.748	87	98 YFPTQALNFAFR
9964	10579	1599.889	37	49 IKLLVQNQDEMIR
12019	16745	1717.87	87	100 YFPTQALNFAFRDK
8709	10401	2193.145	7	28 VLGMPFPVADFLMGGVSAAVSK
6228	5900	2409.329	29	49 TAAAPIERIKLLVQNQDEMIR
166995	202119			
0.00	0.00			
0				

Supplementary Table 2. Descriptions of ORFs for which mRNA levels were analyzed by qRT-PCR.

ORF [*]	Annotation	Relative mRNA expression level i) Δ Por-1 ⁺ ii) Δ 2-12porin	Putative function and comments	Reference
NCU07953	Alternative oxidase-1	i) n/d [‡] ii) 0.9	Expressed when electron flow through electron transport chain is prevented	(Li et al. 1996)
NCU06518	NADH-cytochrome b ₅ reductase	i) n/d ii) 0.5	Ergosterol biosynthesis and oxidative stress response	(Lee et al. 2001)
NCU02157	COQ4	i) 3.7 ii) 0.2	Ubiquinone biosynthesis	(Belogradov et al. 2001)
NCU05850	Rubredoxin-NAD(+) reductase	i) 14.9 ii) 2.5	Identified as apoptosis-inducing factor -1 (<i>aif-1</i>)	(Carneiro et al. 2012)
NCU05633	Stomatin family protein	i) 7.0 ii) 1.0	Related to SLP2 of mouse and AtSLP1 of <i>Arabidopsis thaliana</i> , which are involved in the assembly of respiratory super complexes in the inner membrane	(Gehl et al. 2014), (Mitsopoulos et al. 2015)
NCU04173	β -Actin	n/a [§]	Used to normalize qRT-PCR data	(Sun et al. 2012)
NCU08476	Conserved hypothetical protein	i) 9.2 ii) 2.0	Unknown function	
NCU11655/ NCU04304	Porin	i) n/a ii) 2.0	Voltage dependent anion channel	(Kleene et al. 1987)

^{*}ORF numbers are taken from *Neurospora crassa* Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>)

[†]data taken from (Summers et al. 2012)

[‡]not detected in wild-type

[§]not applicable

References for Supplementary Table 2

Belogrudov, G.I., Lee, P.T., Jonassen, T., Hsu, A.Y., Gin, P. and Clarke, C.F. 2001. Yeast COQ4 encodes a mitochondrial protein required for coenzyme Q synthesis. *Arch. Biochem. Biophys.* **392**(1): 48-58.

Braun, E.L., Fuge, E.K., Padilla, P.A. and Werner-Washburne, M. 1996. A stationary-phase gene in *Saccharomyces cerevisiae* is a member of a novel, highly conserved gene family. *J. Bacteriol.* **178**(23): 6865-6872.

Carneiro, P., Duarte, M. and Videira, A. 2012. Characterization of apoptosis-related oxidoreductases from *Neurospora crassa*. *PLoS One* **7**(3): e34270.

Gehl, B., Lee, C.P., Bota, P., Blatt, M.R. and Sweetlove, L.J. 2014. An Arabidopsis stomatin-like protein affects mitochondrial respiratory supercomplex organization. *Plant Physiol.* **164**(3): 1389-1400.

Kleene, R., Pfanner, N., Pfaller, R., Link, T.A., Sebald, W., Neupert, W. and Tropschug, M. 1987. Mitochondrial porin of *Neurospora crassa*: cDNA cloning, in vitro expression and import into mitochondria. *EMBO J.* **6**(9): 2627-2633.

Lee, J.S., Huh, W.K., Lee, B.H., Baek, Y.U., Hwang, C.S., Kim, S.T., Kim, Y.R. and Kang, S.O. 2001. Mitochondrial NADH-cytochrome b(5) reductase plays a crucial role in the reduction of D-erythroascorbyl free radical in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1527**(1-2): 31-38.

Li, Q., Ritzel, R.G., McLean, L.L., McIntosh, L., Ko, T., Bertrand, H. and Nargang, F.E. 1996. Cloning and analysis of the alternative oxidase gene of *Neurospora crassa*. *Genetics* **142**(1): 129-140.

Mitsopoulos, P., Chang, Y.H., Wai, T., Konig, T., Dunn, S.D., Langer, T. and Madrenas, J. 2015. Stomatin-like protein 2 is required for in vivo mitochondrial respiratory chain supercomplex formation and optimal cell function. *Mol. Cell Biol.* **35**(10): 1838-1847.

Sun, J., Tian, C., Diamond, S. and Glass, N.L. 2012. Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in *Neurospora crassa*. *Eukaryot Cell* **11**(4): 482-493.