Human Molecular Genetics, 2010, Vol. 19, No. 6 **1098–1107**
doi:10.1093/hmg/ddp581
Advance Access published on December 30, 2009

Sym1, the yeast ortholog of the MPV17 human disease protein, is a stress-induced bioenergetic and morphogenetic mitochondrial modulator

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Received September 15, 2009; Revised and Accepted December 23, 2009

A peculiar form of hepatocerebral mtDNA depletion syndrome is caused by mutations in the *MPV17* gene, which encodes a small hydrophobic protein of unknown function located in the mitochondrial inner membrane. In order to define the molecular basis of *MPV17* variants associated with the human disorder, we have previously taken advantage of *S. cerevisiae* as a model system thanks to the presence of an *MPV17* ortholog gene, *SYM1*. We demonstrate here that the *SYM1* gene product is essential to maintain OXPHOS, glycogen storage, mitochondrial morphology and mtDNA stability in stressing conditions such as high temperature and ethanol-dependent growth. To gain insight into the molecular basis of the Sym1-less phenotype, we identified and characterized multicopy suppressor genes and metabolic suppressor compounds. Our results suggest that (i) metabolic impairment and mtDNA instability occur independently from each other as a consequence of *SYM1* ablation; (ii) ablation of Sym1 causes depletion of glycogen storage, possibly due to defective anaplerotic flux of tricarboxylic acid (TCA) cycle intermediates to the cytosol; (iii) flattening of mitochondrial *cristae* in Sym1-defective organelles suggests a role for Sym1 in the structural preservation of the inner mitochondrial membrane, which could in turn control mtDNA maintenance and stability.

INTRODUCTION

A peculiar form of hepatocerebral mtDNA depletion syndrome (MDS) is caused by mutations in the MPV17 gene, which encodes a small hydrophobic protein located in the inner mitochondrial membrane (1–4).

Although the function of Mpv17 is unknown, its yeast ortholog, termed Sym1, is a heat-induced gene product, with a role in ethanol metabolism and tolerance (5). The *sym1Δ* strain fails to grow on 2% ethanol at 37°C, whereas growth is normal at 28°C. In addition, both deletion and point mutations of the *SYM1* gene, equivalent to those found in human MDS, cause mtDNA instability (1), leading to increased accumulation of mitochondrial respiratory-deficient *'petite'* mutants (6).

Here we demonstrate that the *SYM1* deletion leads to impairment of mitochondrial bioenergetic functions and morphological features during stress conditions. We also demonstrate that *SYM1* ablation determines a defect in glycogen storage, the source of energy in respiratory adaptation, starvation and stress (7,8).

RESULTS

Phenotype analysis

To evaluate the substrate specificity of the thermosensitive respiratory growth phenotype associated with the ablation of *SYM1*, we exposed a *sym1Δ* haploid mutant strain to an

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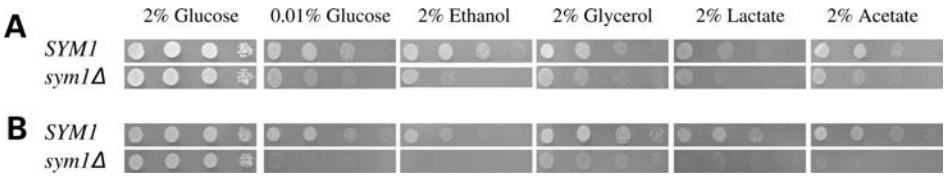

Figure 1. Oxidative growth phenotype at 37°C. Equal amounts of serial dilutions of cells from exponentially grown cultures (10^5, 10^4, 10^3, 10^2 cells) were spotted onto YP plates (**A**) or YNB plates (**B**) supplemented with the indicated carbon sources. Growth was scored after 5 days of incubation at 37°C.

array of aerobic carbon sources. Growth at 37°C was reduced or impaired with all tested substrates, including ethanol (5), acetate, lactate, glycerol and a limited concentration (0.01%) of glucose on a rich medium (YP) (Fig. 1A). This phenotype worsened when these carbon sources were added to a minimal medium (YNB) (Fig. 1B). Growth was normal at 28°C in all conditions (data not shown).

In yeast, faulty OXPHOS can be caused by high mtDNA instability (mtDNA depletion or accumulation of mtDNA deletions) associated with *petite* colonies. When cells were grown in glucose first, then shifted overnight in an obligate aerobic substrate, ethanol (medium YPE), we found that the percentage of *petite* colonies in the *sym1Δ* mutant strain was comparable to that of the parental *SYM1* strain (4.5 versus 3.7%). However, when cells were grown in more permissive condition that allow OXPHOS-defective cells to proliferate (medium A), the number of *petite* colonies significantly increased in mutant versus parental wild-type strain (1). Taken together, these results demonstrate that faulty OXPHOS and higher mtDNA mutability are both present but can occur independently in the *sym1Δ* mutant strain. Interestingly, in both stringent and permissive growth conditions, the morphology of *sym1Δ* mutant mitochondria was profoundly altered, with organellar ballooning, flattening of *cristae* and accumulation of electron-dense particles (Fig. 2).

Chemical suppressors

We observed that the addition of amino acid drop-out (9) was able to rescue the *sym1Δ* OXPHOS phenotype (data not shown). This result prompted us to evaluate which amino acid/s acted as a chemical suppressor. Interestingly, supplementation with either glutamate, aspartate, glutamine or asparagine did partially restore aerobic oxidative growth (Fig. 3), whereas other non-essential amino acids had no effect (data not shown).

Promoter and northern blot analysis

In cells grown at 28°C, *SYM1* expression was repressed by glucose and robustly induced by aerobic oxidative carbon sources, including ethanol (5) (Fig. 4A), as typically observed for genes associated with respiratory metabolism. However, in agreement with previous reports (5), glucose repression was abolished at 37°C, suggesting for *SYM1* a role in the heat response, independent of its role in aerobic oxidative metabolism. The highest expression occurred in cells grown on ethanol at 37°C, suggesting an additive effect of two stressing conditions, ethanol and temperature, in *SYM1* induction.

By *in silico* analysis of the *SYM1* genomic region (http://www.yeastract.com/; (10)), we found a number of potential transcription factor binding sites (Supplementary Material, Table SI), including a GTCAC motif specific to Rtg1/Rtg3 (11) located 693 bp upstream from the *SYM1* ORF start codon. Rtg1/Rtg3 are transcription factors involved in the mitochondrion-nucleus retrograde response (RTG) (12), a homeostatic control that induces adaptive responses in nuclear gene expression when mitochondrial activities, such as respiration or biogenesis, are failing. In support of a retrograde regulation of *SYM1*, we also showed that this gene is overexpressed in ρ^0 derivatives of BY4741 cells and not induced in ρ^0 derivatives of *rtg1Δ* and *rtg3Δ* mutants (Fig. 4B).

Multicopy suppressors of the *sym1Δ* null allele

To gain further insight into the function of Sym1, we then transformed the *sym1Δ* strain with a multicopy DNA library and selected clones able to grow on 2% ethanol at 37°C. This assay showed that, besides *SYM1* itself, another gene, *YMC1*, was able to rescue the *sym1Δ* OXPHOS growth phenotype (Fig. 5A). Ymc1 is a mitochondrial carrier protein involved in the coordination of metabolite trafficking between peroxisomes and mitochondria, with a probable role in the transport of tricarboxylic acid (TCA) cycle intermediates (13). Not only was *YMC1* able to suppress the OXPHOS growth phenotype, but also completely abolished mtDNA instability (Fig. 5B).

We then compared the phenotypes of *sym1Δ* and *ymc1Δ* single mutants with that of a *sym1Δ ymc1Δ* double mutant strain. The *ymc1Δ* mutant was indistinguishable from the wild-type strain, whereas the *sym1Δ ymc1Δ* double mutant displayed higher sensitivity to ethanol, more severe OXPHOS-growth phenotype and higher mtDNA instability, than the *sym1Δ* mutant (Fig. 6). These results indicate that *YMC1* becomes crucial for aerobic growth and mtDNA integrity when *SYM1* is lacking, and that *SYM1* is likely to act on a metabolic pathway that compensates the absence of Ymc1.

Ymc1p shares structural homology with the carnitine/acylcarnitine translocator, encoded by the *CRC1* gene (14), and its activity overlaps that of other transporters including Ymc2, Odc1 and Odc2, which are involved in peroxisome-mitochondrial coordination for bioenergetic utilization of fatty acids (13). Hardly any metabolic rescue was obtained by overexpressing *CRC1, ODC2* or *YMC2* in the *sym1Δ* strain, whereas *ODC1* had an effect similar to *YMC1* (Fig. 5A). *ODC1* encodes an oxodicarboxylate carrier which transports α-ketoadipate, α-ketoglutarate and other TCA cycle intermediates such as citrate, by a counter-exchange mechanism through the inner mitochondrial membrane (15).

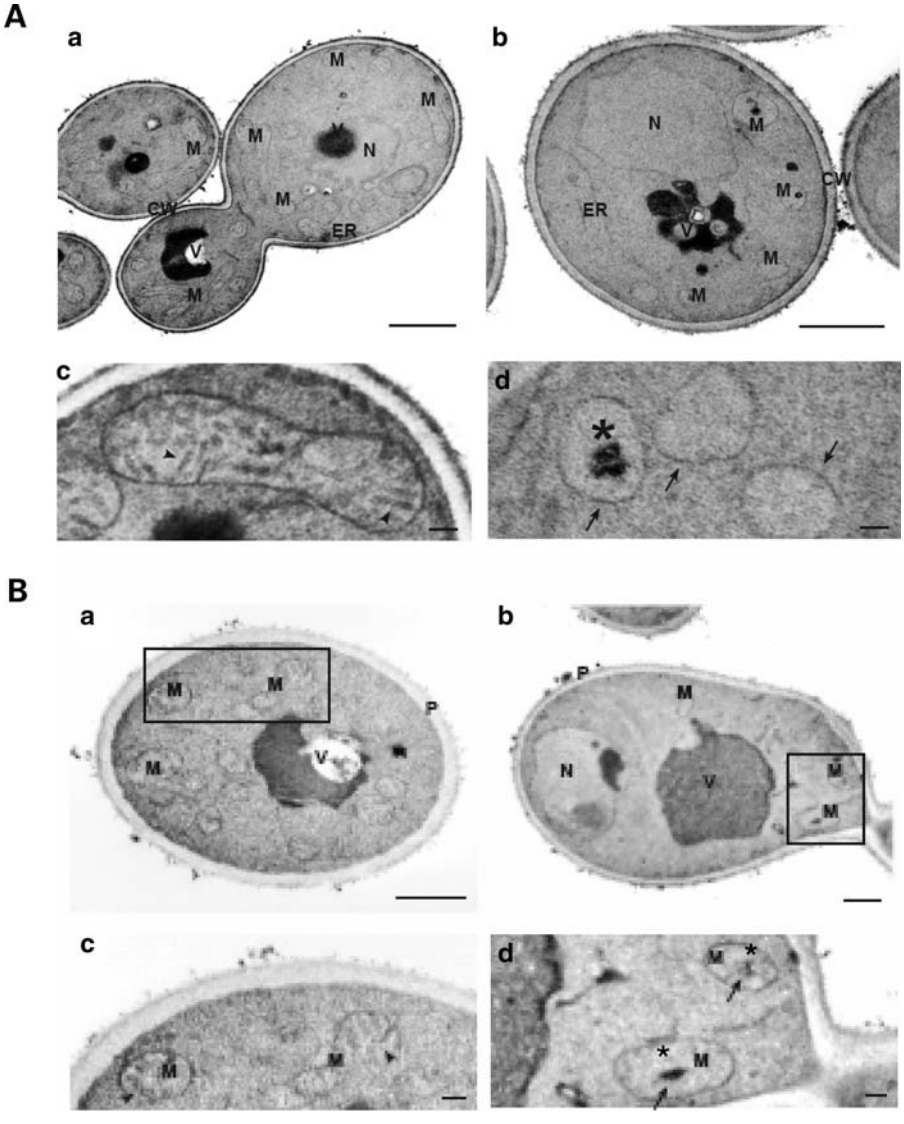

Figure 2. Transmission electron micrographs of BY4741 and *sym1Δ* strains cultured in medium A (**A**) or shifted in medium YPE overnight (**B**). (a) Wild-type cells and (b) mutant cells. N, nucleus; ER, endoplasmic reticulum; M, mitochondrion; CW, cell wall; V, vacuole. *Bars*, 1 μm. Ultrastructural morphology of individual representative mitochondria from BY4741 (c) and *sym1Δ* (d) cells. The arrowheads denote *cristae* (c). The arrows point to mitochondria and asterisk indicates a mitochondrion with an electron-dense body (d). Bars, 0.1 μm.

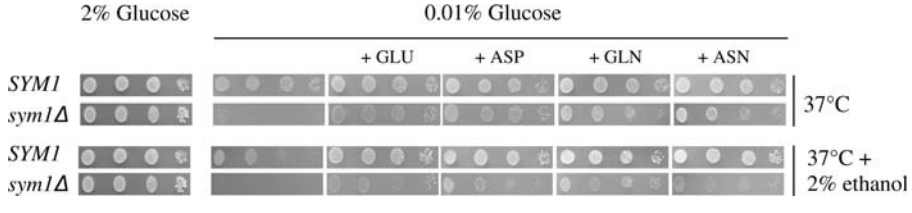

Figure 3. Chemical rescue. Equal amounts of serial dilutions of cells from exponentially grown cultures (10^5, 10^4, 10^3, 10^2 cells) were spotted onto YNB plates supplemented with 0.01% glucose with or without 2% ethanol and 2% glucose, as control. Growth was scored after 5 days of incubation at 37°C. GLU, glutamate; GLN, glutamine; ASP, aspartate; ASN, asparagine. Amino acids concentration, 1 mM.

As for mtDNA maintenance, overexpression of either *ODC1* or *ODC2* reduced the percentage of *petite sym1Δ* colonies by ∼50%, while *YMC2* had a much lesser effect (Fig. 5B).

In contrast with the *ymc1Δ* mutant, the *odc1Δ* mutant strain displayed impaired aerobic growth at 37°C, stress sensitivity and increased mtDNA instability (Fig. 6), suggesting that Odc1 plays a major role in cell functioning independently from the *SYM1* allele. The *sym1Δ odc1Δ* double mutant showed OXPHOS defective growth that was undistinguishable from that of each single mutant, but growth in ethanol and *petite* production were worsened, suggesting that *ODC1* and *SYM1* are both necessary for ethanol tolerance and mtDNA stability (Fig. 6).

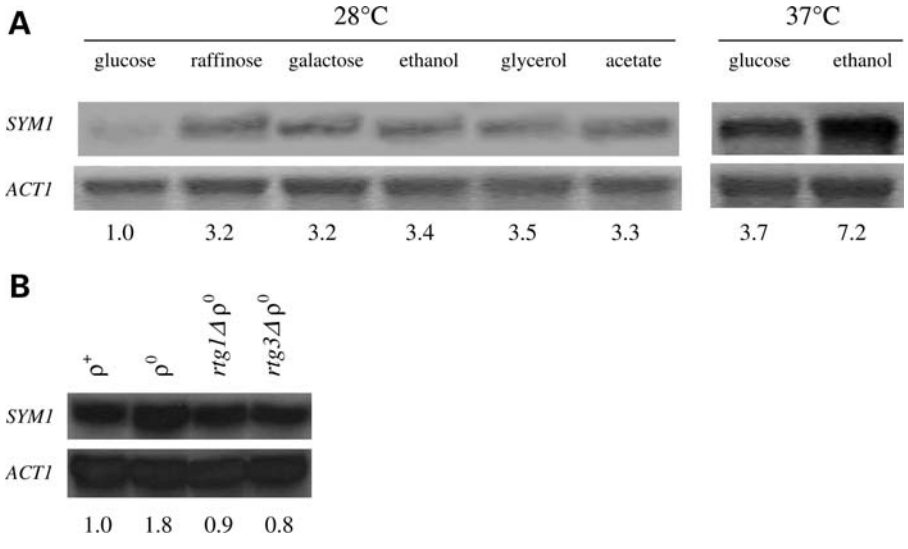

Figure 4. (**A**) Transcription of *SYM1* in BY4741. Total RNA was prepared from cells grown on the indicated temperatures and carbon sources (at 2%). (**B**) Transcription of *SYM1* in ρ^+ and ρ^0 derivatives of BY4741, *rtg1Δ* and *rtg3Δ* cells. Total RNA was prepared from cells grown at 28°C on 0.6% glucose. Northern blot hybridization was carried out using probes of labelled *SYM1* and *ACT1* as a reference. Each lane contained 30 μg of total RNA. Signal quantitation and normalization were carried out as described in Materials and Methods, and the value 1.0 in each panel indicates the reference of normalized intensity with which other signals were compared.

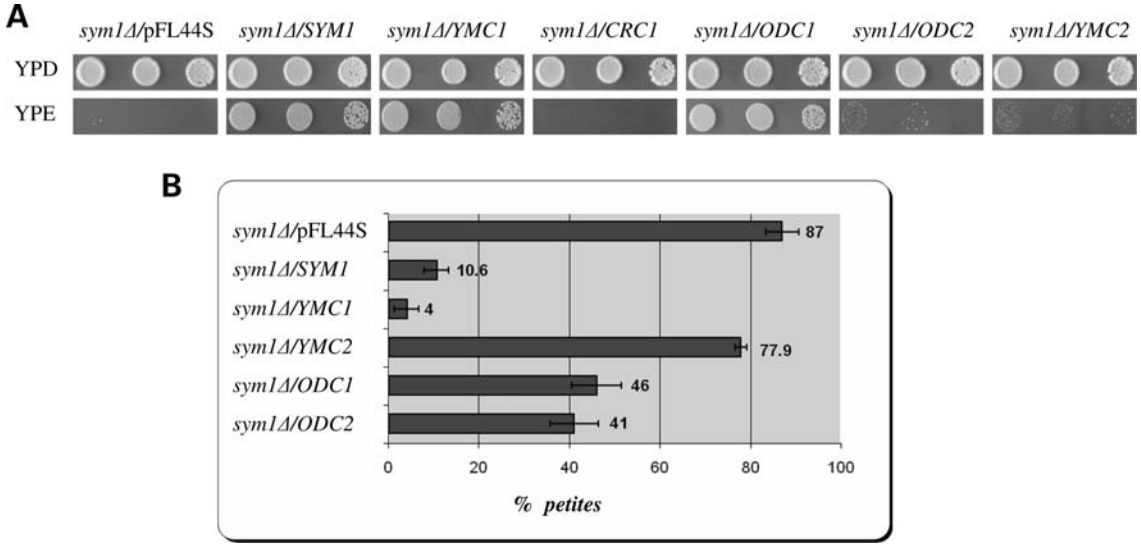

Figure 5. Complementation studies in *sym1Δ* strain. The mutant was transformed with the multicopy pFL44S plasmid and pFL44S carrying *SYM1, YMC1*, *CRC1, ODC1, ODC2* or *YMC2*. (**A**) Equal amounts of serial dilutions of cells from exponentially grown cultures (10^5, 10^4, 10^3 cells) were spotted onto plates supplemented with 2% ethanol (YPE) or 2% glucose (YPD). Growth was scored after 3 days of incubation at 37°C. (**B**) Respiratory-deficient mutant (*petite*) accumulation after 15 generations of growth at 37°C. Black bars represent the percentage of *petites*. More than 4000 colonies per strain were scored. All values are means of three independent experiments.

Growth characteristics of yeast strains *cit1Δ sym1Δ* and *cit2Δ sym1Δ*

We then asked whether suppression by *ODC1*, and possibly *YMC1* as well, were due to increase transport of citrate into mitochondria. In yeast, citrate is produced by two citrate synthase isoforms: mitochondrial, Cit1, and peroxisomal, Cit2. In yeast ρ^0 cells, *CIT2* expression increases 10-fold or more, so that peroxisomes become the main source of citrate to feed the mitochondrial TCA cycle (16). We compared oxidative growth phenotype and tolerance to ethanol of *cit1Δ* and *cit2Δ* single mutants and *cit1Δ sym1Δ* and *cit2Δ sym1Δ* double mutants. The phenotype of the single *cit1Δ* and *cit2Δ* mutants was undistinguishable from that of the wild-type strain (Fig. 6). This result was expected since one single citrate synthase activity is sufficient to support oxidative metabolism (17). The *sym1Δ cit1Δ* double mutant showed a phenotype more severe than that of *sym1Δ*, indicating that when *SYM1* is lacking, the citrate produced by Cit2 is unable to sustain growth. This is not due to defective entry of citrate from peroxisomes to mitochondria because the *sym1Δ cit2Δ* strain had also a phenotype worse than that of the *sym1Δ* single mutant, suggesting that Sym1 is

A

37°C							
% ethanol	0.5	1	1.5	2	3	4	5
SYM1	+	+	+	+	+	-	-
sym1Δ	+	+	-	-	-	-	-
ymc1Δ	+	+	+	+	+	+	-
sym1Δ ymc1Δ	+	-	-	-	-	-	-
odc1Δ	+	+	+	-	-	-	-
sym1Δ odc1Δ	+	-	-	-	-	-	-
cit1Δ	+	+	+	+	+	-	-
sym1Δ cit1Δ	-	-	-	-	-	-	-
cit2Δ	+	+	+	+	+	-	-
sym1Δ cit2Δ	+	-	-	-	-	-	-

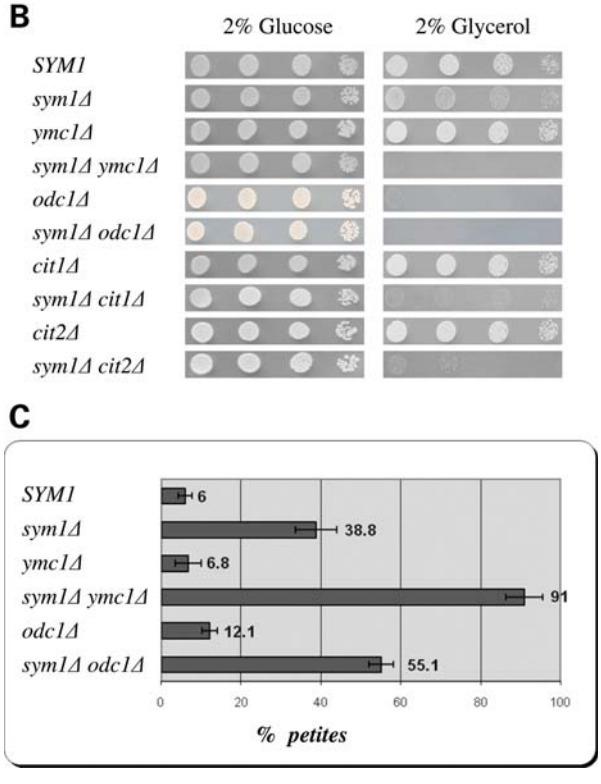

Figure 6. Comparison of phenotypes of *sym1Δ*, *ymc1Δ*, *sym1Δ ymc1Δ*, *odc1Δ*, *sym1Δodc1Δ*, *cit1Δ*, *cit2Δ*, *sym1Δ cit1Δ* and *sym1Δ cit2Δ* strains. (**A**) Ethanol resistance profile. 10^6 cell/ml from exponentially grown cultures was inoculated at 37°C in YP at different ethanol concentrations. Growth was evaluated after 4 days. (**B**) Equal amounts of serial dilutions of cells from exponentially grown cultures (10^5, 10^4, 10^3, 10^2 cells) were spotted onto YNB plates supplemented with the indicated carbon sources. Growth was scored after 6 days of incubation at 37°C. (**C**) Respiratory-deficient mutant (*petite*) accumulation after seven generations of growth at 37°C. Black bars represent the percentage of *petites*. More than 4000 colonies per strain were scored. All values are means of three independent experiments.

likely to play a role in the utilization/biosynthesis rather than transport of citrate.

TCA cycle and respiratory enzymes

We then measured four TCA cycle enzymes in *sym1Δ* and *SYM1* strains: citrate synthase, and isocitric, succinate and malate dehydrogenases. To avoid interference by mtDNA instability, the assays were performed in mitochondria isolated from cells grown in conditions in which the percentage of *petite* colonies was comparable between the *sym1Δ* mutant and the parental *SYM1* strain (medium B). None of these enzymatic activities was significantly affected, with the exception of succinate dehydrogenase (SDH), which was reduced by 60% (Fig. 7). We also observed mild reduction of enzymatic activities of complex III, IV and V (Fig. 7). In agreement with the reduction of SDH activity, we found a drastic reduction in the amount of SDH subunit 2 by western blot (WB) of SDS–polyacrylamide gel electrophoresis (Fig. 8A) as well as of fully assembled SDH (complex II) on two-dimensional blue-native gel electrophoresis, 2D-BNGE (Fig. 8B). To determine whether Sym1 establish stable physical contact with complex II, we carried out 2D-BNGE WB analysis using a strain expressing an HA-tagged Sym1 recombinant variant. We showed that HA-tagged Sym1 takes part in a high molecular–weight complex (>600 kDa) which is clearly distinct from complex II (Fig. 8C).

Finally, iodine staining (18) showed marked reduction of glycogen content in *sym1Δ* versus wild-type *SYM1* cells (Fig. 9), possibly due to shortage of gluconeogenetic TCA cycle intermediates.

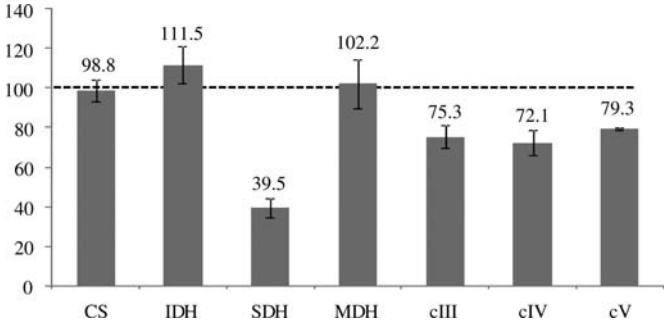

Figure 7. Biochemical activities of TCA cycle enzymes (CS, citrate synthase; IDH, isocitric dehydrogenase; SDH, succinate dehydrogenase; MDH, malate dehydrogenase) and respiratory chain complexes (cIII, complex III; cIV, complex IV; cV, complex V). Cells exponentially grown at 28°C in YPD were shifted at 37°C in YPE supplemented with 0.6% glucose (medium B) and harvested after 13 h. The values of the *sym1Δ* mutant are expressed as percentage of the activities obtained in the *SYM1* strain.

DISCUSSION

The *sym1Δ* mutant showed severe OXPHOS growth defects when grown in stressing conditions (i.e. high temperature, 37°C, and high ethanol) indicating for Sym1 a role in stress tolerance, as previously suggested (5). *SYM1* was also induced in mtDNA defective cells but not in *rtg1Δ* and *rtg3Δ* mutants, indicating for this protein a role in the RTG response (12). In both yeast and humans, RTG senses mitochondrial dysfunction and induces adaptive changes in the expression of several genes involved in energy metabolism, nutrient sensing, transport and stress pathways (19,20). Accordingly, analysis of the *SYM1* promoter region revealed a number of transcription factor binding sites involved in the stress response.

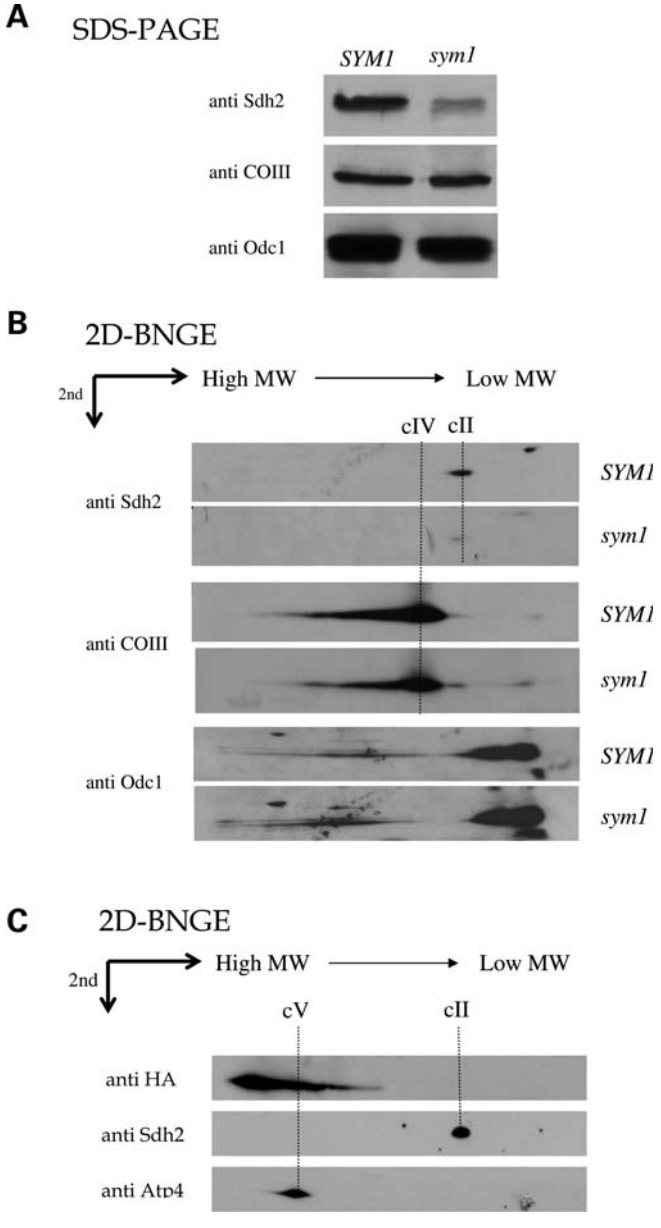

Figure 8. Western blot of denaturing SDS–PAGE (**A**) and second dimension (2D) blue-native gel electrophoresis (**B**) of mitochondria from *SYM1* and *sym1Δ* strains. Western blot of second dimension (2D) blue-native gel electrophoresis (**C**) of mitochondria from *sym1Δ* strain expressing an HA-tagged Sym1 recombinant variant. An antibody against Sdh2 was used to detect complex II (cII); an antibody against COIII (cytochrome c oxidase, subunit III) was used for complex IV (cIV); an antibody against Atp4 (ATP synthase, subunit 4) was used for complex V; an antibody against HA-tag was used for tagged Sym1 and an antibody against Odc1 was used as a loading control.

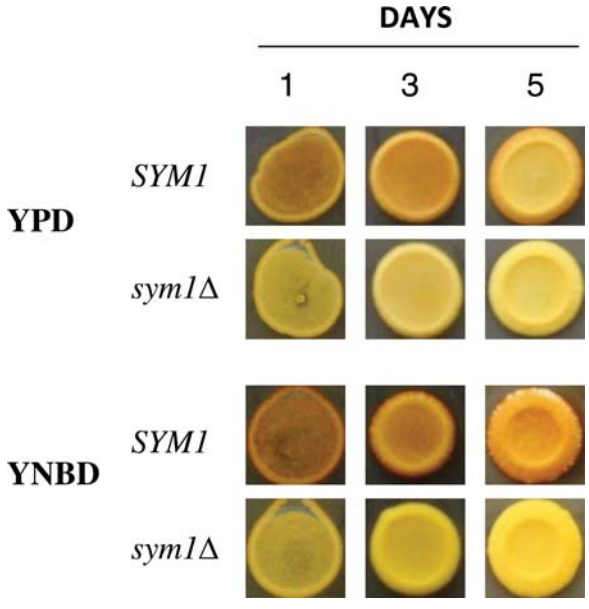

Figure 9. Glycogen staining of *SYM1* and *sym1Δ* cells at different times of growth on agar plates. Strains were grown for 4 days on YPD liquid medium and then 20 μl (4×10^5 cells) were spotted on YPD or YNBD agar plates supplemented with amino acids drop out. Exposure to iodine for 1 min was done after 1, 3 and 5 days growth, using a different plate for each exposure.

At high temperature (37°C), the *sym1Δ* mutant displayed a number of defective phenotypes: variably impaired OXPHOS, consisting in inability/reduced ability to grow on aerobic carbon sources, defective glycogen storage, altered mitochondrial morphology and high mtDNA instability.

The following evidence supports the view that OXPHOS impairment and high mtDNA instability occur independently in the *sym1Δ* mutant. First, OXPHOS impairment is not the consequence of mtDNA instability, since aerobic growth arrest occurred in the *sym1Δ* mutant with no increase of *petite* colonies. Second, overexpression of either *YMC1* or *ODC1* suppressed equally well the growth defect, but only the former gene was able to completely suppress the *petite* phenotype. Third, only overexpression of *ODC1*, but not of *ODC2*, was able to rescue the oxidative growth defect, in spite that both reduced mtDNA instability.

Which role can then be hypothesized for Sym1 in OXPHOS metabolism? The rescue of the oxidative growth defect by glutamine, glutamate, asparagine and aspartate suggests defective production of common derivatives, e.g. alpha-ketoglutarate and/or oxalacetate, supporting the idea that *SYM1* is involved in a homeostatic control of TCA cycle intermediates. We showed that the *sym1Δ* mutant has reduced succinate dehydrogenase activity, which is part of the TCA cycle and could determine reduction or imbalance of TCA intermediates. Mild reduction of SDH activity was also occasionally reported in liver of MPV17 mutant patients (4,21) but neither in the majority of human patients (1,2,22) nor in Mpv17−/− mice (23). This difference may be due to the presence, in mammals but not in yeast, of at least three proteins similar to Mpv17 (M. Zeviani, personal observation), located in either mitochondria, peroxisomes or both, whose expression could alleviate the consequences of the mutant protein.

In yeast, the function of a protein can be investigated by searching for phenotype suppressors. We found two multicopy suppressors for the metabolic defect of *sym1Δ*, namely *YMC1* and *ODC1*, both encoding mitochondrial transporters. While the supposed TCA cycle intermediate substrate of Ymc1 is still unknown, Odc1 is deemed to carry out the counter-exchange of a number of TCA intermediates between the mitochondrial matrix and the cytosol, thus controlling the

alpha-ketoadipate and alpha-ketoglutarate pools (15). Odc1 and Ymc1 can play an anaplerotic role on the TCA cycle, by transferring intermediates from peroxisomes to mitochondria, thus contributing to the utilization of energy substrates, e.g. long-chain fatty acids (13). Interestingly enough, *ODC1* was also identified as a multicopy suppressor of a mutant strain deleted in the *FMC1* gene, which encodes a mitochondrial protein involved in the assembly/stability of the yeast F0F1-ATP synthase at 37°C (24). The rescuing mechanism performed by *ODC1* involved an increase in the flux of TCA cycle intermediates from peroxisomes into mitochondria, resulting in an increase in mitochondrial substrate-level-dependent ATP synthesis. The same mechanism could contribute to the *ODC1*-dependent suppression of the *sym1Δ* phenotype. Taken together, these observations suggest that anaplerotic mitochondrial pathways related to the TCA cycle are defective in the *sym1Δ* mutant.

This idea is also supported by the observation that glycogen storage is indeed reduced in the *sym1Δ* strain. Glycogen amount is dependent on gluconeogenesis, which is in turn regulated by the anaplerotic flux of TCA intermediates from mitochondria to the cytosol (25). The inability of the *sym1Δ* mutant strain to accumulate glycogen may take part in its defective adaptation to stress. Interestingly, patients with Mpv17 mutations suffer of dramatic, often fatal, hypoglycaemic crises, which are likely due to glycogen shortage in liver (1,26).

The observation that the double mutations *ymc1Δ sym1Δ* and *odc1Δ sym1Δ* worsen the phenotype of the *sym1Δ* single mutation suggests that partially impaired TCA cycle, possibly due to reduced SDH activity, is worsened by concomitant reduction in the supply of TCA intermediates. Accordingly, over-expression of Odc1 and/or Ymc1 transporters suppresses the *sym1Δ* phenotype. Likewise, the observation that *sym1Δ cit2Δ* and *sym1Δ cit1Δ* double mutants worsen the phenotype of *sym1Δ* single mutant can be explained by defective mitochondrial citrate utilization in stress conditions.

Our results on mitochondrial morphology suggest that Sym1 is involved in the maintenance of mitochondrial *cristae* irrespective of the increase of *petite* colonies, indicating that the morphogenetic effects of Sym1 are likely to precede and possibly determine its effects on mtDNA stability. Interestingly, abnormal morphology of the *cristae* is believed to cause mtDNA instability in several yeast mutants (27,28). The disappearance of mitochondrial *cristae*, electron-dense inclusions and proliferation of spherical, membrane-surrounded organelles were consistently observed also in mouse Mpv17−/− liver cells (23). MtDNA is contained within a protein-DNA nucleoid complex physically associated with the mitochondrial inner membrane; hence, the disorganization of the latter could lead to mtDNA defective replication. Moreover, the integrity of the mitochondrial genome can be compromised by the absence of proteins located in the inner mitochondrial membrane, which are not directly involved in mtDNA synthesis (27,29,30).

The *sym1Δ* strain showed low SDH activity and amount, but SDH deficiency is unlikely to explain all the *sym1Δ* phenotypes. For instance, SDH deficiency is not, or only marginally, associated with mtDNA instability in yeast (30,31), and there is no evidence of a morphogenetic role of SDH in mitochondrial biogenesis. In addition, we showed that, *in vivo*, Sym1 is part of a high molecular−weight complex that is much bigger than, and clearly distinct from, the SDH complex, suggesting that the effects of Sym1 on SDH assembly/maintenance are unlikely to be the only or primary function of this protein. While further work is warranted to identify the primary role of Sym1, including the molecular dissection and characterization of the Sym1-containing protein complex, our results indicate that Sym1 is involved in the structural and functional stability of the inner mitochondrial membrane, thus controlling crucial mechanisms related to this compartment, including the activity of respiratory chain complexes, the morphology of mitochondria and the maintenance and integrity of the nucleoid structures.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study were BY4741 (*MATa*; *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) and their isogenic *sym1:kanMX4, ymc1:kanMX4, odc1:kanMX4, cit1:kanMX4, cit2:kanMX rtgt1:kanMX4 and rtg3:kanMX4* mutants. The double mutants *sym1Δ ymc1Δ*, *sym1Δ odc1Δ*, *sym1Δ cit1Δ* and *sym1Δ cit2Δ* were obtained disrupting the *SYM1* gene in the single mutants *ymc1:kanMX4, odc1:kanMX4, cit1:kanMX4, cit2:kanMX* by replacing *SYM1* ORF with Hyg^R deletion cassette. The Hyg^R cassette was obtained by PCR using as template pAG32 and the primers SYM-HYGF and SYM-HYGR (see Supplementary Material, Table SII). The ρ^0 derivative of the BY4741, *rtgt1Δ and rtg3Δ* strains were obtained by growing cells for ∼30 generations in YPD medium containing 20 μg/ml of ethidium bromide. Isolates were verified as ρ^0 by 4,6-diamidino-2-phenylindole (DAPI) staining. Cells were cultured in yeast nitrogen base (YNB) medium [0.67% YNB without amino acids (ForMedium™, UK)] supplemented with appropriate amino acids and bases for auxotrophy or, where indicated, with 1 g/l of drop-out powder (9) containing all amino acids. Various carbon sources (Carlo Erba Reagents, Italy) were added at the indicated concentration. YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (ForMedium™). Media were solidified with 20 g/l agar (ForMedium™). YNB supplemented with 2% ethanol (YNBE); YNB supplemented with 2% glucose (YNBD). YP supplemented with 2% ethanol (YPE); YP supplemented with 2% glucose (YPD), medium A: YP supplemented with 2% ethanol and 2% glucose, medium B: YP supplemented with 2% ethanol and 0.6% glucose.

Multicopy suppressor screen

The *sym1:kanMX4* strain was transformed with a pSEY8 *URA3* yeast genomic library and grown on YNB supplemented with 2% glucose (YNBD) plates. About 100000 URA^+ transformants were recovered. These colonies were then replica-plated into YP supplemented with 2% ethanol (YPE). The YPE plates were incubated for 4–5 days at 37°C, to identify cells able to grow at the non-permissive temperature. Suppressor colonies were re-streaked on YPE at 37°C, and plasmid DNA recovered from these clones.

Genomic inserts in each plasmid were sequenced at both ends and compared with the *S. cerevisiae* genome using NCBI BLAST search.

Cloning procedures and plasmids vectors

SYM1, *YMC1*, *ODC1*, *YMC2*, *ODC2* and *CRC1* genes were PCR amplified using BY4741 genomic DNA as template and the appropriate forward and reverse primers containing restriction sites at their 5′ ends listed in Supplementary Material, Table SII and next cloned in the multicopy vector pFL44S (32) carrying the *URA3* marker. The HA-tagged *SYM1* was obtained by PCR amplification of the *SYM1* open reading frame (ORF) using BY4741 genomic DNA as template and SYM1-FW and SYM1-HA as primers (see Supplementary Material, Table SII). The reverse primer used contained the sequence information to encode the haemagglutinin (HA) epitope and a translational stop codon, in addition to a *SYM1* specific sequence for priming. The recombinant PCR product was cloned into the yeast centromeric vector pFL38 (32) carrying the *URA3* marker. All plasmid clones obtained were completely sequenced on both strands.

Mitochondrial DNA mutation frequency

Strains were pregrown at 28°C in YNB medium supplemented with 2% ethanol in order to counterselect the *petite* cells that could be present in the population and then inoculated in YNB supplemented with 2% glucose and incubated for 4 h at 28°C. Then 2% ethanol was added and the cultures were shifted at 37°C. After either 7 or 15 generations of growth at 37°C, cells were plated on YNB agar plates supplemented with 2% ethanol and 0.25% glucose and 1 g/l of drop-out powder (9) at a dilution giving approximately 200 cells/plate. *Petite* frequencies were defined as the percentage of colonies showing the *petite* phenotype after a 5-day incubation at 28°C.

RNA preparation and northern analysis

Total RNA was prepared by extraction with hot acidic phenol (33), from cells grown in YP supplemented with the appropriate carbon source. Northern analysis was carried out as previously described (34). The *SYM1* probe was a 512 bp fragment obtained by PCR amplification with pFL38-*SYM1* plasmid as template (1). The primers used for the amplification were: Fw: 5′-cagctagggctgtcatctatg-3′; Rv 5′-ccccaaggaacaaggtctgataaat-3′. The amount of RNA loaded on the gel was estimated by hybridization with an actin gene probe (*ACT1*). All the probes were labeled with [α- ^{32}P]dCTP using the rediprime DNA labelling system (Amersham). The signals were quantified with a PhosphorImager (BioRad).

Isolation of mitochondria and enzyme assay

Cells, pregrown at 28°C in YPE medium, were exponentially grown in YPD at 28°C and transferred to YP medium supplemented with 0.6% glucose plus 2% ethanol (medium B) at 37°C for 13 h. Preparation of mitochondria was performed as previously described (35). Protein concentration was determined by the method of Bradford (36), using the Bio-Rad Protein Assay following the manufacturer's instructions. Enzymatic activities were measured spectrofotometrically at a standard temperature of 30°C. The Succinate Dehydrogenase DCPIP assay was conducted as described (37) with minor modifications. The initial reaction rate at 600 nm was recorded in the cuvette containing 0.15 mM dichlorophenolindophenol, 2.5 mM phenazine methosulfate, 100 mM sodium azide, 0.1 M phosphate buffer, pH 7.8 and 10 mM succinate (sodium salt). NAD(H)-specific isocitrate dehydrogenase activity was measured spectrophotometrically at 340 nm as previously described (38). Citrate synthase was assayed spectrophotometrically at 412 nm, according to Srere (39). MDH activity was performed at 340 nm as described (40) with the following modifications: 60 mM KPO_4 (pH 7), 0.28 mM NADH and 0.67 mM oxaloacetate. Cytochrome c oxidase (complex IV) was measured spectrophotometrically as described by Warthon and Tzagoloff (41). Enzyme activities of ubiquinone cytochrome c reductase (complex III) and ATPase (complex V) were measured spectrophotometrically as described (42,43). The exogenous substrates were added to the reaction mixture after exhaustion of endogenous substrates.

Gel electrophoresis and western blot analysis

The detection of complexes II, IV and V in *SYM1*, *sym1Δ* and/or *sym1Δ* transformed with HA-tagged *SYM1* strains was performed by using electrophoresis and western blot (WB) analysis. A total of 50 μg mitochondrial protein/lane were loaded on 12% SDS–polyacrylamide gel. For two-dimensional BNGE, 100 μg of protein from isolated mitochondria was treated as described by Mijtmans *et al*. (44); then the samples were loaded and run into a 5–13% gradient non-denaturating 1D-BNGE. For denaturing 2D-BNGE electrophoresis, the 1D-BNGE lane was excised, treated for 1 h at room temperature with 1% SDS and 1% β-mercaptoethanol and then run through a 16.5% tricine-SDS–polyacrylamide gel, using a 10% spacer gel (45). For WB analysis, the gels were electroblotted onto nitrocellulose filters and sequentially immunostained with specific antibodies against Sdh2 (a kind gift from prof. B. Lemire), COIII (cytochrome c oxidase, subunit III) (Molecular Probes, Invitrogen), HA (Roche Applied Science), Atp4 (ATP synthase, subunit 4) (a kind gift from prof. J. Velours) and Odc1 (a kind gift from prof. L. Palmieri).

Electron microscopy

Exponentially phase cultures in medium A at 37°C or cells exponentially grown in YPD at 28°C, shifted in YPE medium at 37°C and harvested after 13 h, of the yeast strains BY4741 and *sym1Δ* were fixed with 2% glutaraldehyde in distilled water for 1 h at room temperature. Cells were washed with water and postfixed with freshly prepared 4% $KMnO_4$ in H_2O on a nutator for 2 h at 4°C. After five washes in water, cells were incubated with 2% uranyl acetate on a nutator for 2 h at 4°C, washed and dehydrated in increasing (30–100%) concentrations of ethanol. The samples were infiltrated overnight at 4°C in a mixture of ethanol with Spurr-low viscosity embedding

medium (Electron Microscopy Sciences, Ft Washington, PA, USA). The mixture was replaced with pure embedding medium, and the samples were allowed to polymerize at 60°C for 48 h. For further contrast, ultrathin sections were stained with uranyl acetate and lead citrate before examination at a CM 10 Philips electron microscope at 80 kV (Eindhoven, The Netherlands).

Determination of glycogen content

Qualitative glycogen determinations were made by adding 10 ml of a 0.4% KI-0.2% iodine solution onto plates. Cells that accumulate glycogen stain brown, whereas those that do not remain yellow.

Miscellaneous yeast methods

Transformation of yeast strain was obtained by the lithium-acetate method (46). Restriction-enzyme digestions, *Escherichia coli* transformation and plasmid extractions were performed using standard methods (47). Yeast DNA was prepared as previously described (48).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank Antonietta Cirasolo and Roberto Silva for their skilful technical assistance; Luigi Palmieri for the generous gift of antiOdc1 antibody, Bernard D. Lemire for the generous gift of antiSdh2 antibody and Jean Velours for the generous gift of antiAtp4 antibody.

Conflict of Interest statement. None declared.

FUNDING

This work was funded by grants from Fondazione Telethon, Italy no. GGP030039, Fondazione Pierfranco and Luisa Mariani, and Research Grant of the Italian Ministry of Health no. RF-INN-2007-634163.

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