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# Overexpression of human SOD1 in VDAC1-less yeast restores mitochondrial functionality modulating beta-barrel outer membrane protein genes

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ABSTRACT

Cu/Zn Superoxide Dismutase (SOD1), the most important antioxidant defense against ROS in eukaryotic cells, localizes in cytosol and intermembrane space of mitochondria (IMS). Several evidences show a SOD1 intersection with both fermentative and respiratory metabolism. The Voltage Dependent Anion Channel (VDAC) is the main pore-forming protein in the mitochondrial outer membrane (MOM), and is considered the gatekeeper of mitochondrial metabolism. *Saccharomyces cerevisiae* lacking VDAC1 (Δ*por1*) is a very convenient model system, since it shows an impaired growth rate on non-fermentable carbon source. Transformation of Δ*por1* yeast with human SOD1 completely restores the cell growth deficit in non-fermentative conditions and re-establishes the physiological levels of ROS, as well as the mitochondrial membrane potential. No similar result was found upon yeast SOD1 overexpression. A previous report highlighted the action of SOD1 as a transcription factor. Quantitative Real-Time PCR showed that β-barrel outer-membrane encoding-genes *por2*, *tom40*, *sam50* are induced by hSOD1, but the same effect was not obtained in Δ*por1*Δ*por2* yeast, indicating a crucial function for yVDAC2. Since the lack of VDAC1 in yeast can be considered a stress factor for the cell, hSOD1 could relieve it stimulating the expression of genes bringing to the recovery of the MOM function. Our results suggest a direct influence of SOD1 on VDAC.

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## 1. Introduction

Cu/Zn Superoxide Dismutase (SOD1) represents the most important antioxidant defense against ROS in eukaryotic cells, since it catalyzes the disproportionation of superoxide anions to oxygen and hydrogen peroxide [1]. SOD1 is an abundant cytosolic enzyme well conserved throughout evolution; however, SOD1 fractions were found also in nucleus [2], lysosomes [3], peroxisome [4] and intermembrane space (IMS) of mitochondria [5,6]. A Mn SOD (SOD2) isoform is expressed in most eukaryotes and it is exclusively located into the mitochondrial matrix [7]. Notably, mutations in SOD1 gene have been associated to the familial form of Amyotrophic Lateral Sclerosis (ALS) [8,9], even though also wild type SOD1 is implicated in sporadic ALS by a mechanism not yet understood [10]. Although structure and dismutase activity of SOD1 have been well characterized, many recent evidences indicate a SOD1 participation in metabolism regulation.

The yeast *Saccharomyces cerevisiae* grows in an exponential way in the presence of glucose, using alcoholic fermentation and repressing respiration, as long as the sugar is available. When glucose level drops, yeast undergoes the "diauxic shift": the mitochondrial respiration is re-activated, allowing the ethanol utilization for energy production [11,12]. A series of metabolic alterations has been found in yeast cells devoid of SOD1 (Δ*sod1*). Yeast lacking endogenous SOD1 does not completely repress respiration upon fermentative conditions and shows impaired growth on glucose, while it better grows on non-fermentable carbon sources, such as lactate and pyruvate [13]. In addition, Δ*sod1* cells show a significant increase in mitochondrial mass and oxygen consumption, and when diauxic shift occurs, they are no longer able to grow using ethanol [14]. The repression of mitochondrial respiration requires the activation of yeast homologous casein kinases I γ, YCK1 and its paralog YCK2, which relieve the transcriptional repression from the glucose transport transcription regulator RGT1, resulting in the constitutive expression of glucose transporter HXT genes [15]. SOD1 stabilizes

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YCK1 and 2, thus participates in the integration of signals from glucose and oxygen concentration to mitochondrial metabolism [16]. Nevertheless, the relationship between SOD1 and mitochondrial metabolism looks much more extensive.

Metabolic flux within mitochondria and cytosol occurs mainly through the pore-forming proteins Voltage Dependent Anion Channel (VDAC) on the mitochondrial outer membrane (MOM). VDAC pores are permeable to ions and small molecules, such as nucleotides, playing a key role in mitochondrial metabolism [17,18]. In mammals, three different VDAC isoforms are expressed and VDAC1 represents the most abundant and ubiquitously expressed [19,20]. According to the determined structure, human VDAC1 is composed of a β-barrel, made of 19 anti-parallel β-strands, and a N-terminal domain, containing a predicted α-helix and located inside the pore's lumen [21,22]. A main VDAC feature is its voltage dependence [17,23]. The yeast *S. cerevisiae* VDAC1 homologous, encoded by the *por1* gene and called yVDAC1 or POR1, shares with the human protein the voltage dependence and the other functional features; moreover, human or mouse VDAC isoforms 1 and 2 are able to complement the absence of endogenous porin in a defective yeast strain while VDAC3 is not [19,24,25]. The yVDAC1 is important but not essential for yeast viability. Indeed, yeast cells lacking endogenous VDAC1 (Δ*por1*) show a reduced growth on non-fermentable carbon source (i.e. glycerol) at 30 °C, condition exacerbated at higher temperature of 37 °C [26]. The analysis of yeast genome resulted in the discovery of a *por1* gene paralog, called *por2*. The encoded protein, yVDAC2 or POR2 is definitely less characterized, even if it has often been considered responsible of the survival of the yeast in these conditions [24].

Reports suggest that expression levels and activity of SOD1 and VDACs are interconnected [27]. Indeed, both cytosolic and IMS-located SOD1 are required in order to protect MOM proteins, and especially VDACs, from carbonylation, the most common damage induced by superoxide anion [28]. Interestingly, the expression level of yVDAC1 and other MOM proteins, such as the outer membrane translocase components Tom40 and Tob55, is lower in Δ*sod1* yeast compared to wt. On the other hand, in Δ*por1* cells (exponential phase) the activity of IMS-located SOD1 is significantly reduced [29].

All together, the literature indicates an involvement of SOD1 in the regulation of both fermentative and respiratory metabolism, and strengthens the SOD1-VDAC relationship. Here we attempted to get more insight in this issue by overexpressing the human SOD1 in *S. cerevisiae* devoid of endogenous VDAC1 (Δ*por1*). Human and yeast SOD1 indeed share most of the sequence, the tridimensional structure and the mechanism of action, all features that make hSOD1 perfectly active in yeast cells. Despite this, the alignment of human and yeast SOD1 reveals several significative differences (see Suppl. Fig. 1). Our overall results strongly support the concerted role of SOD1 and VDAC in regulating mitochondrial metabolism.

## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

The *por1*-depleted mutant (Δ*por1*) *S. cerevisiae* yeast strain was derived from wild type (wt) strain BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) by replacement of *por1* open reading frame with a kanamicine cassette (EUROSCARF (Frankfurt, Germany)). The double mutant M22.2.1 Δ*por1*Δ*por2* strain was derived from the wt strain M3 (MATa, lys2, his4, trp1, ade2, leu2, ura3) by replacement of *por1* and *por2* genes with respectively LEU2 and TRP1. Yeast strains were grown on the rich medium YP (1% yeast extract, 2% peptone (Bacto)) supplemented with 2% glucose (YPD), 2% or 3% glycerol (YPY) according to the strain request, or on the minimal synthetic defined medium (0.67% yeast nitrogen base w/o amino acids (Sigma)) supplemented with 2% glucose (SD) or glycerol (SY) and with 10 μg/ml of the appropriate nutritional requirements according to the genotype of the strains. Agar (2%) was added for solid plates. Growth was performed at 28 °C or 37 °C.

### 2.2. Cloning and expression of SOD1 proteins

The encoding sequences of human SOD1 (hSOD1) and yeast SOD1 (ySOD1) were amplified from in stock plasmid by PCR using specific primer couples (see Suppl. Table 1 for primer sequences) and cloned into yeast expression vector pYX142 or pYX212 (Novagen) by EcoRI/SalI digestion. The corresponding SOD1 sequences carrying the eGFP at the C-terminal domain, were produced and cloned as previously indicated. The constructs were confirmed by DNA sequencing.

Each construct was introduced into BY4742 Δ*por1*, M22.2.1 Δ*por1*Δ*por2* and the corresponding wt strains by lithium-acetate transformation; same strains were transformed with the corresponding empty vector as a control. Selection of recombinant clones was performed on SD plates containing all nutritional requirements except leucine (pYX142) or uracil (pYX212). The presence of expressed hSOD1 in the yeast was verified by immunoblotting, using the anti-SOD1 antibody (C17, Santa Cruz Biotechnologies) (1:1000), and the anti-GFP antibody (Roche) (1:500).

### 2.3. Characterization of yeast growth

The ability of yeast transformant cells to grow in the presence of different carbon sources was assayed by plating drop-serial dilution starting from $10^6$ cells, on YPD or YPY. Plates were incubated at 28 °C or 37 °C from 1 to 6 days. Kinetics of yeast transformant growth were obtained incubating yeast in liquid minimal medium SD or SY at the starting $OD_{600}$ of 0.01. The yeast growth was monitored by periodically measuring $OD_{600}$, until the stationary phase was reached. At least, three independent experiments were performed.

### 2.4. Mitochondrial membrane potential measurement

The mitochondrial membrane potential ($\Delta\Psi_m$) was indirectly monitored using the fluorescent probe 2-[4(dimethylamino) styryl]-1-methylpyridinium iodide (DASPMI) (Molecular Probes). Yeast samples grown in SD medium up to the exponential phase (0.3–0.6 $OD_{600}$) were treated with 5 μM DASPMI for 30 min under constant shaking at 30 °C. Cells were analyzed by flow cytometry.

### 2.5. Detection of ROS

Intracellular ROS content was measured using the fluorescent probe dihydrorhodamine 123 (*DHR 123*) (Sigma). Yeast samples were grown in SD medium up to the exponential phase, then treated for 2 h with DHR 123 (5 μg/mL of culture), under constant shaking at 30 °C, as reported in [30]. ROS content was also measured after treatment with the oxidizing agents hydrogen peroxide ($H_2O_2$) or 1,1′-dimethyl-4,4′-bipyridinium dichloride (Paraquat) (Sigma). Yeast samples grown in SD medium up to exponential phase were exposed to 3 mM of $H_2O_2$ or alternatively 1 mM of Paraquat for 2 h under constant shaking at 30 °C, prior the DHR 123 staining. Cells were analyzed by flow cytometry.

### 2.6. Flow cytometry

Fluorescence of yeast cells were analyzed by flow cytometry analysis using a CyFlow® ML flow cytometer (Partec). 50,000 cells per sample were analyzed; data obtained were acquired, gated, compensated, and analyzed using the FlowMax software (Partec), using each time the wt transformed with empty vector (pYX142) as reference sample for relative quantification. Each experiment was repeated at least twice in triplicate. Data were statistically analyzed by one-way ANOVA with Tukey's post hoc test. A value of $P < 0.001$ was taken as significant.

### 2.7. Yeast RNA extraction and RT-PCR

Yeast samples grown in SD medium up to the exponential phase were suspended in spheroplast buffer (100 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl) and lysed with glass beads 425–600 μm (Sigma) by vortexing. Total RNA was extracted and purified using Trizol Plus RNA Purification Kit (Life Technologies) according to manufacturer's protocol. Residual DNA was removed by DNase digestion using RNA-free DNase I (Life Technologies). Total RNA was directly reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. cDNA were used for semi-quantitative PCR using primers described ahead. The PCR products were loaded onto Ethidium Bromide-stained 2% agarose gel in TBE.

### 2.8. Quantitative PCR

The cDNA concentration of specific targets was analyzed by Quantitative Real-Time PCR. Three independent experiments were performed in triplicate by using the QuantiFast SYBR Green PCR Kit (QIAGEN). Analysis was performed by using the *iCycler IQ* (Biorad) in 96-well plates. Thermocycling program consisted in a first activation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, a combined annealing/extension step at 54 °C for 30 s, and a final step at 72 °C for 10 min. Specific couples of primer for *por2*, *tom40* and *sam50* genes were designed and analyzed using Primer 3 Bio-tool software (http://bioinfo.ut.ee/primer3-0.4.0/primer3); in addition, *por1* and *cit1* genes were used as internal control, while the housekeeping *act1* gene was used for normalization (see Suppl. Table 2 for primers sequences). Analysis of relative expression level was performed as described in [31]. Data were statistically analyzed by one-way ANOVA with Tukey's post hoc test. A value of $P < 0.05$ was taken as significant.

## 3. Results

### 3.1. The overexpression of hSOD1, but not ySOD1, completely restores the ability of Δpor1 yeast to grow on glycerol

It is well known that Δ*por1* yeast hardly grow on glycerol at temperature restrictive conditions, due to its defects in the mitochondrial metabolism [26]. To analyze the effect of SOD1 supplementation on yeast growth, yeast strains were transformed with the pYX142 construct carrying the human or the yeast SOD1 sequence, or with the empty vector as a control. In order to verify the expression of heterologous proteins and compare it to the endogenous SOD1, immunoblotting analysis was performed using the corresponding constructs encoding for the C-terminal fused eGFP SOD1 proteins. Immunoblot analysis revealed that both human or yeast SOD1, introduced with the plasmidic expression, were correctly expressed but at significantly lower level than the endogenous protein (see Suppl. Fig. 2).

The effect of SOD1 overexpression on yeast growth phenotype was then analyzed by drop-serial dilution on complete media containing glucose (YPD) or glycerol (YPY), and incubated at 28 °C or 37 °C respectively for 1–2 or 3–4 days, as showed in Fig. 1A. Glucose is the favorite fermentable carbon source, and the yeast commonly metabolizes it via glycolysis. For this reason, Δ*por1* yeast, transformed with empty vector (pYX142), did not exhibit any difference compared to wt strain transformed likewise when cultured on glucose, either at 28 °C or at 37 °C. Conversely, glycerol utilization provides a two-step process: the phosphorylation of glycerol, occurring in cytosol, and the conversion of glycerol-3-phosphate in dihydroxyacetone, in mitochondria [32]. According to the literature, Δ*por1* transformed with pYX142 exhibited an impaired growth on glycerol at 28 °C, which was completely abolished at 37 °C [19,26]. Surprisingly, the overexpression of human SOD1 in Δ*por1* yeast significantly improved the growth rate at 28 °C

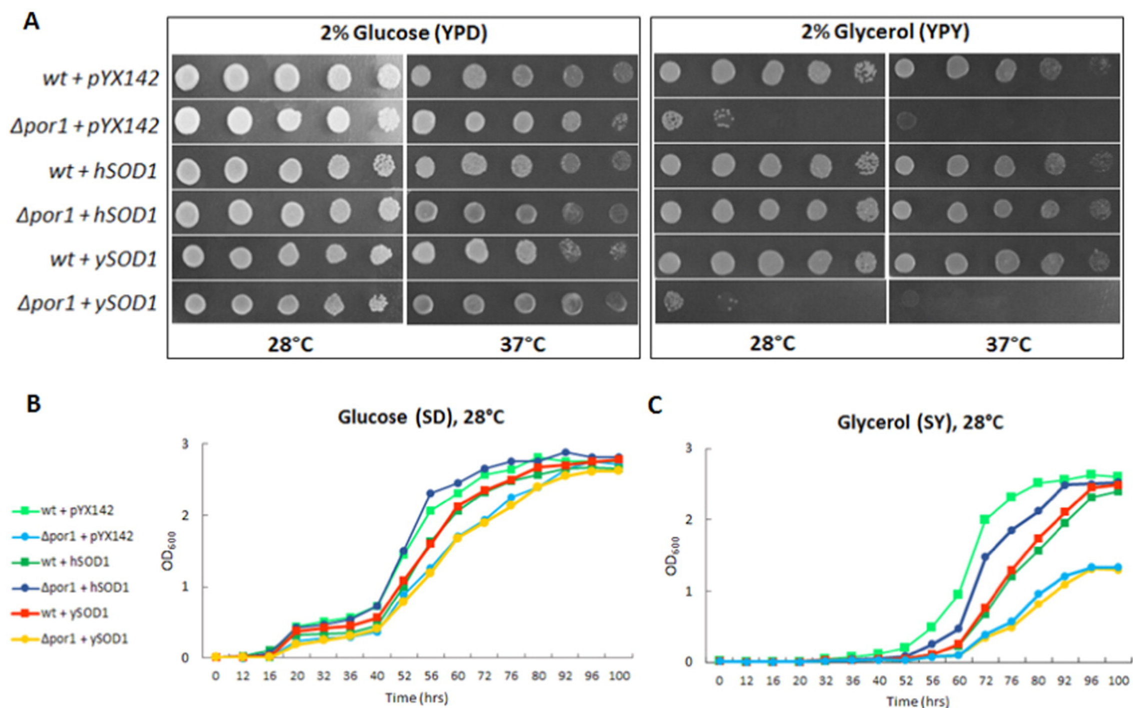


**Fig. 1.** Yeast growth phenotype analysis of Δpor1 cells overexpressing SOD1 proteins. (A) A representative panel of drop-serial dilutions assay of wt or Δ*por1* yeast, transformed as described. Yeast samples were plated on YPD or YPY and incubated at 28 °C or 37 °C, respectively for 1–2 or 3–4 days. As shown, no major difference among the strains and the delivered constructs was detected in the presence of glucose as substrate. On glycerol, while Δ*por1* transformed with pYX142 strain shows a significative impairment of the growth rate, the addition of the plasmid expressing hSOD1, but not ySOD1, fully restores the yeast growth defect. (B–C) Representative curves of yeast cell concentration against time, obtained for the strains indicated in the panel upon growth in minimal medium containing glucose (B) or glycerol (C) as carbon source. While no significant difference in the kinetic of growth was found on glucose, the growth rate of Δ*por1* transformed with pYX142 on glycerol resulted dramatically impaired. The overexpression of hSOD1, but not ySOD1, in Δ*por1* strain significantly improves the growth of this defective strain.

and completely restored the temperature-sensitive growth defect at 37 °C, making the Δ*por1* capable of growing on glycerol in a comparable way than the wt yeast transformed with pYX142. On the contrary, the overexpression of yeast SOD1 in Δ*por1* cells did not change the growth phenotype of Δ*por1* cells, since its growth was equivalent to that of Δ*por1* transformed with pYX142.

To quantitatively analyze the impact of SOD1 on the yeast growth rate, growth curves were produced by monitoring the optical density against time in liquid minimal media at 28 °C. Fig. 1B shows the yeast concentration curves at $OD_{600}$ in glucose (SD). Although Δ*por1* transformed with pYX142 is able to use glucose as well as the wt yeast, the curve obtained highlights that its growth is slightly slower than the other strains, but also that it is able to reach at the end a very similar saturation (maximum $OD_{600}$ value at plateau phase). The hSOD1 supplementation in Δ*por1* strain improved, although minimally, the yeast growth rate. Fig. 1C shows the yeast growth curves in glycerol (SY). Although all yeast strains were previously made adapted to glycerol, they showed a longer lag phase compared to the growth in glucose, 36–40 h for wt yeast, transformed or not with SOD1 proteins, and of ~60 h for the Δ*por1* yeast transformed with empty pYX142 or expressing ySOD1. In addition, the Δ*por1* transformed with pYX142 grew slower using glycerol as carbon source and the maximal concentration ($OD_{600}$) after 100 h was significantly lower than in the other cells. Thus, the growth delay of Δ*por1* strain previously measured in glucose became much more pronounced in glycerol. Again, the overexpression of hSOD1 in Δ*por1* cells significantly improved both yeast growth rate and maximum saturation, up to values similar to wt strain, while no similar effect was detected upon ySOD1 overexpression. For unknown reasons, the overexpression of hSOD1 or ySOD1 in the wt yeast resulted in a minimum delay in the growth. Overall, these results indicate that human, but not yeast, SOD1 overexpression in the mitochondrial-defective strain Δ*por1* allows a greater and more efficient mitochondrial utilization of glycerol, suggesting the ability of hSOD1 to recover the cellular bioenergetic deficit due the absence of yVDAC1.

### *3.2. The overexpression of hSOD1 restores physiological level of mitochondrial membrane potential and ROS in Δpor1 cells*

Once verified the peculiar and exclusive effect of human SOD1 supplementation on Δ*por1* growth phenotype, we investigated whether it affects the mitochondrial membrane potential ($\Delta\Psi_m$) and ROS production. $\Delta\Psi_m$ is strictly related to the ATP production via oxidative phosphorylation [33], thus represents a direct measurement of mitochondrial health. Yeast transformants were treated with the fluorescent probe DASPMI and then analyzed by flow cytometry. Results reported in Fig. 2A clearly show that the fluorescence emission peak of Δ*por1* cells, transformed with the empty vector, is shifted towards lower fluorescence level compared to the peak obtained by wt yeast containing the same empty vector (used as reference control). Since DASPMI uptake is $\Delta\Psi_m$-dependent [34], this result suggests that lack of yVDAC1 affects the mitochondrial energization. A relative quantification of DASPMI positive cells was performed and it is showed in Fig. 2B: according to previous results (Fig. 2A), Δ*por1* transformed with pYX142 shows a reduction of around 35% in DASPMI staining compared to the control. A similar result was reported in [35]. However, the overexpression of hSOD1 in Δ*por1* cells promotes a strong increase of DASPMI uptake, as clearly indicated by the emission peak that almost overlaps the wt yeast peak (Fig. 2A). In addition, the percentage of DASPMI positive cells is significantly increased, up to values similar to the control (Fig. 2B), suggesting that hSOD1 supplementation promotes a recovery of the compromised membrane potential of Δ*por1* mitochondria.

Mitochondria are the most important intracellular source of ROS, producing superoxide anion as by-product of mitochondrial respiration. Although elevated ROS levels are considered deleterious, it is now widely acknowledged that physiological ROS levels are involved in redox signaling [36]. Thus, physiological ROS production is associated to a proper mitochondrial function. The total ROS content in transformed yeast was estimated using flow cytometry and measuring the fluorescence emission of rhodamine, the oxidation product of DHR123.

In Fig. 3A and B the DHR123 fluorescence profile and percentage of positive cells to DHR123 staining, respectively, directly proportional to the total ROS content, are shown. These results clearly indicate that ROS in the Δ*por1* cells transformed with pYX142 were significantly lower than in wt yeast transformed likewise (control). Indeed, the emission peak of Δ*por1* transformed with pYX142 is shifted towards lower fluorescence values and the ROS relative quantification indicates a significant reduction of total ROS, corresponding to approximately a fifth of what measured for reference control. This result can be explained with the reduced mitochondrial function of the Δ*por1* strain. The lack of yVDAC1 could affect the organelle activity and, as a consequence, ROS production would be depressed. Although SOD1 is an antioxidant enzyme, the overexpression of hSOD1 in Δ*por1* yeast increased the ROS content, restoring the condition of the control (see the overlapping peaks of Δ*por1* transformed with hSOD1 with wt yeast transformed with pYX142 in Fig. 3A), indicating a role for this enzyme in mitochondrial reactivation. Moreover, the supplementation of wt yeast with hSOD1 promotes a light decrease of ROS production, as reported in literature [37].

Taken together these results indicate that the overexpression of hSOD1 in Δ*por1* cells promotes a recovery of the mitochondrial functionality by restoring the physiological level of $\Delta\Psi_m$ and of ROS.

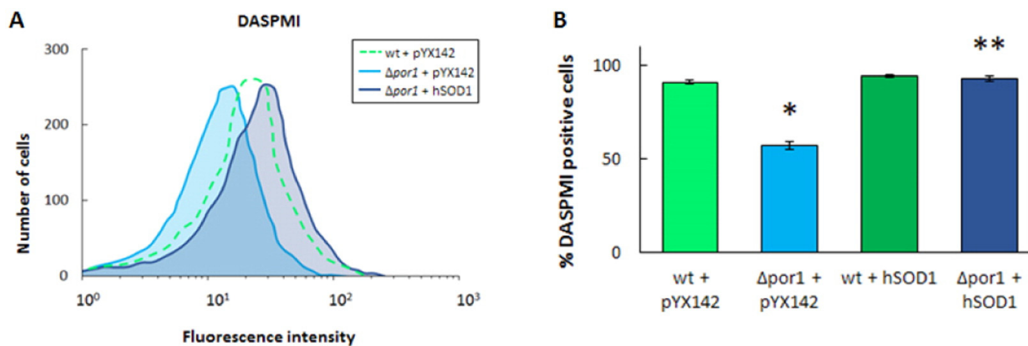


**Fig. 2.** Mitochondrial membrane potential analysis by DASPMI staining of Δpor1 yeast cells after hSOD1 overexpression. (A) A representative image of fluorescence emission profile of yeast cells after incubation with DASPMI. Peak corresponding to Δ*por1* transformed with pYX142 is shifted towards lower level of fluorescence compared to the other samples, indicating a minor sensitivity of Δ*por1* cells to DASPMI uptake. (B) Relative quantification of DASPMI-positive cells in relation to wt transformed with pYX142 (reference control). Δ*por1* transformed with pYX142 showed the lowest amount of positive cells compared to wt yeast expressing or not hSOD1, indicating a significant decrease of the physiological polarization of the mitochondrial membrane. The overexpression of hSOD1 in Δ*por1* cells dramatically increases the percentage of DASPMI positive cells, indicating a re-establishment of the physiological $\Delta\Psi_m$. (*) P < 0,001 related to the wt + pYX142; (**) P < 0,001 related to Δ*por1* + pYX142.

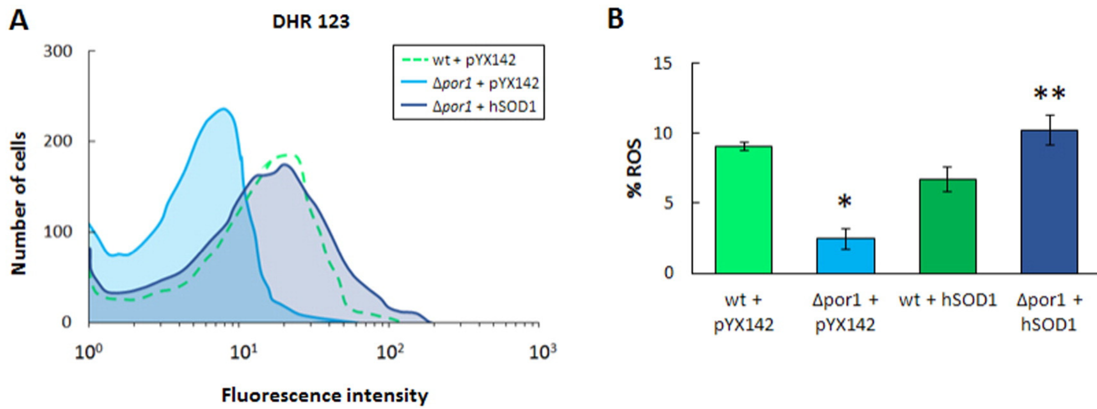


**Fig. 3.** Total ROS measurement in yeast samples in basal condition. (A) A representative image of fluorescence emission profile of rhodamine for Δ*por1* transformed with pYX142 or expressing hSOD1 compared to wt yeast transformed with pYX142 yeast. Peak corresponding to Δ*por1* sample is shifted towards a lower level of fluorescence, indicating a decreased amount of ROS. (B) Relative quantification of total ROS in yeast cells; wt yeast transformed with pYX142 was used as reference control. Δ*por1* transformed with pYX142 shows a smaller ROS content than control, probably due to poorly active mitochondria. However, the overexpression of hSOD1 in Δ*por1* restores the physiological level of ROS. (*) P < 0,001 related to the wt + pYX142; (**) P < 0,001 related to Δ*por1* + pYX142.

### *3.3. hSOD1 overexpression changes sensitivity of Δpor1 yeast to oxidants*

In order to analyze the anti-oxidant properties provided by hSOD1 supplementation, we analyzed the sensitivity of transformed yeast to the oxidant agents hydrogen peroxide ($H_2O_2$) and the ROS-generator Paraquat (PQ).

$H_2O_2$ is commonly produced in the cell as final product of the dismutation reaction catalyzed by SOD1. Notwithstanding $H_2O_2$ is not a SOD1 substrate, it has been recently reported that yeast SOD1 actively participates in oxidative stress response upon incubation with hydrogen peroxide [38]. To analyze the effect of hSOD1 overexpression on ROS production in oxidative stress conditions, yeast samples were treated with appropriate concentration of $H_2O_2$ and the increase in ROS content was analyzed by flow cytometry. Fig. 4A shows the change in ROS after $H_2O_2$ exposure for each sample normalized with the corresponding untreated sample. Although $H_2O_2$ treatment promoted a significant increase of ROS in all samples, Δ*por1* yeast transformed with the empty pYX142 showed the largest increase (about 85%) in comparison with its untreated control, while wt yeast transformed likewise (control) showed an increase in ROS content of about 30%. This result is in clear agreement with the literature, indicating that Δ*por1* is much more sensitive to $H_2O_2$ treatment than the wt yeast [35,39]. Anyway, the presence of hSOD1 in the Δ*por1* yeast improved the cell response to $H_2O_2$, since the increase of ROS content was about 35%, similar to what found for the control.

Paraquat is a widely used herbicide that promotes superoxide anion generation at mitochondrial level [40]. Differently from $H_2O_2$, PQ cytotoxicity is related to its redox cycling: the di-cation ($PQ^{2+}$) crosses mitochondrial membranes through putative carriers and, once in the matrix, undergoes reduction to monocation and high reactive radical $PQ^{\bullet -}$ [41]. Notably, $PQ^{2+}$ uptake into mitochondria is driven by $\Delta\Psi_m$ of energized mitochondria [41]. Results in Fig. 4B show the ROS increase in samples treated with PQ and normalized with the corresponding untreated samples. Similarly to what found after hydrogen peroxide treatment, 20% of ROS increase was induced in wt yeast transformed with pYX142 after PQ exposure; on the contrary, the ROS increase in Δ*por1* transformed with pYX142 was only 4%. This result indicates that Δ*por1* yeast is much less sensitive to PQ than wt yeast. Since PQ uptake follows the $\Delta\Psi_m$ [41], this result is in agreement with previous data showing a drop $\Delta\Psi_m$ in Δ*por1* cells (see Fig. 2). Again, the overexpression of hSOD1 in Δ*por1* restored the sensitivity to PQ as in wt yeast, promoting a ROS increase similar to what found in wt yeast transformed with pYX142 (about 18%).

In conclusion, hSOD1 overexpression significantly reduces ROS production in Δ*por1* after hydrogen peroxide exposure and restores the cell physiological sensitivity to PQ, confirming an involvement of

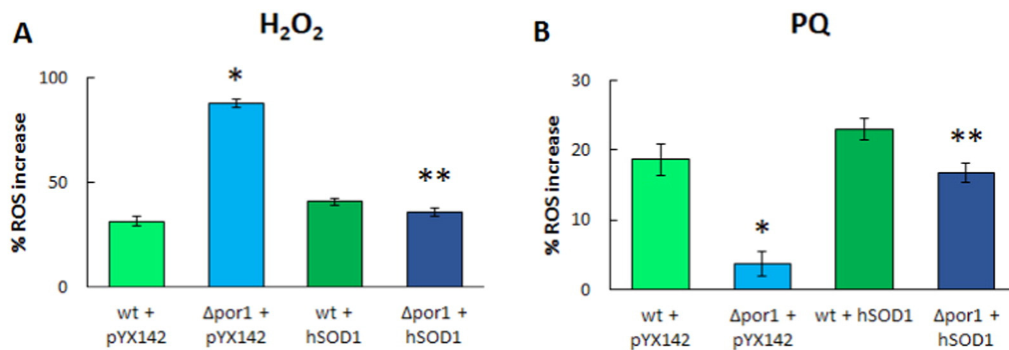


**Fig. 4.** Quantification of ROS in yeast cells after oxidant treatments. (A) Quantification of ROS increment in transformant yeast upon incubation with $H_2O_2$. Each sample was normalized to the corresponding untreated sample. A significative increase of ROS was detected both in wt and in Δ*por1* yeast, transformed or not with hSOD1. However, the effect of $H_2O_2$ was much more prominent in Δ*por1* yeast compared to both untreated Δ*por1* and treated wt yeast. hSOD1 overexpression in Δ*por1* yeast significantly lowers the ROS increment. (B) Quantification of ROS increment in transformant yeast after PQ exposure. Each sample was normalized to the corresponding untreated sample. Differently from hydrogen peroxide treatment, Δ*por1* yeast shows low sensitivity to PQ due to poorly energized mitochondria. hSOD1 supplementation in Δ*por1* restores the yeast sensitivity to PQ up to a similar level of that of wt. (*) P < 0,001 related to the wt transformed with pYX142; (**) P < 0,001 related to Δ*por1* transformed with pYX142.

this enzyme in the improvement of the mitochondrial functionality with respect to oxidative stress control.

### *3.4. hSOD1 regulates gene expression of β-barrel proteins of MOM in Δpor1 yeast*

Based on our results and the literature [38] we speculated that hSOD1 overexpression in Δ*por1* yeast can stimulate mitochondrial metabolism by modulating the expression of specific genes encoding for proteins possibly involved in the process. Quantitative Real Time PCR experiments were undertaken in order to verify our hypothesis. Since VDAC is involved in the permeability of the MOM, other β-barrel mitochondrial proteins were considered, such as the second VDAC isoform (yVDAC2) and the subunits of import complexes TOM and SAM supposedly containing a pore structure [42], i.e. Tom40 and Sam50. As a mitochondrial control the *cit1* gene, encoding for the mitochondrial matrix enzyme citrate synthase, was used. The expression of *cit1*, *por2*, *tom40*, *sam50* (and *por1* as control for wt yeast), were normalized with the actin housekeeping gene *act1*. The relative expression quantification is shown in Fig. 5. In Fig. 5A the gene expression in wt and Δ*por1* yeast transformed with the empty vector, here used as our reference control, is presented. No significant difference was found between the two yeast strains, for all tested genes, indicating that the lack of yVDAC1 per se does not affect the expression of the other genes encoding for MOM protein in our experimental conditions. The impact of hSOD1 overexpression on porin's gene expression in wt yeast was next considered. As reported in Fig. 5B, both *por1* and *por2* expression levels are substantially unchanged. Similar results were found for the others genes analyzed. In Fig. 5C a similar panel reports results in Δ*por1* yeast obtained in two conditions: upon transformation with hSOD1 or without any additional gene. The expression of target genes is shown. While the expression of *cit1* remains substantially unchanged, *por2*, *tom40* and *sam50* are significantly increased in the presence of hSOD1. In particular, in the Δ*por1* yeast expressing hSOD1, there is an increase of the *por2* of about 8 times, as well as an increase of 5,5–6 times for *tom40* and *sam50*, compared to the control Δ*por1* yeast transformed with the empty vector. An overlapping result was found by semi-quantitative PCR, showed in Fig. 5D.

Since *por2* gene expression was strongly stimulated in the Δ*por1* strain upon hSOD1 transformation, we expected that this isoform would actively mediate the mitochondrial recovery. It was indeed reported that when *por2* gene is overexpressed in Δ*por1* cells, yVDAC2 is able to complement the absence of yVDAC1 [24]. To assess this hypothesis, we transformed a yeast strain devoid of both porin genes (Δ*por1*Δ*por2*) with pYX212 carrying the sequence encoding for hSOD1. The Δ*por1*Δ*por2* growth was analyzed by drop-serial dilution on 2% YPD or 3% YPY after incubation at 28 °C or 37 °C respectively for 1–2 or 3–6 days. The results are shown in Fig. 6.

Although both porins are absent, the Δ*por1*Δ*por2* transformed with the empty vector is still able to grow on glucose at both 28 and 37 °C. It is known that the Δ*por1*Δ*por2* double mutant on glycerol shows a more severe growth impairment than the single Δ*por1* mutant at 28 °C, while, as for the single gene mutant, the yeast growth is completely abolished at 37 °C [26]. According to literature, the growth of Δ*por1*Δ*por2* yeast transformed with pYX212 on glycerol is extremely reduced at 28 °C, and is completely arrested at 37 °C. The double mutant Δ*por1*Δ*por2* indeed needs at least 1 additional day of incubation to reach a growth phenotype comparable to that of Δ*por1*. On the opposite to Δ*por1* yeast, the hSOD1 overexpression in the Δ*por1*Δ*por2* strain did not improve the yeast growth on non-fermentable substrate: as shown in Fig. 6, the growth of this strain is really similar to the Δ*por1*Δ*por2* yeast transformed with the empty vector (control), resulting in a reduced growth at 28 °C and a complete inhibition at 37 °C.

Overall, these results support the hypothesis that hSOD1 specifically affects the expression of several target genes in Δ*por1*, a defective strain in which the lack of endogenous yVDAC1 is a strong stress factor for the cell, and clearly indicates that the *por2* gene is essential to allow the

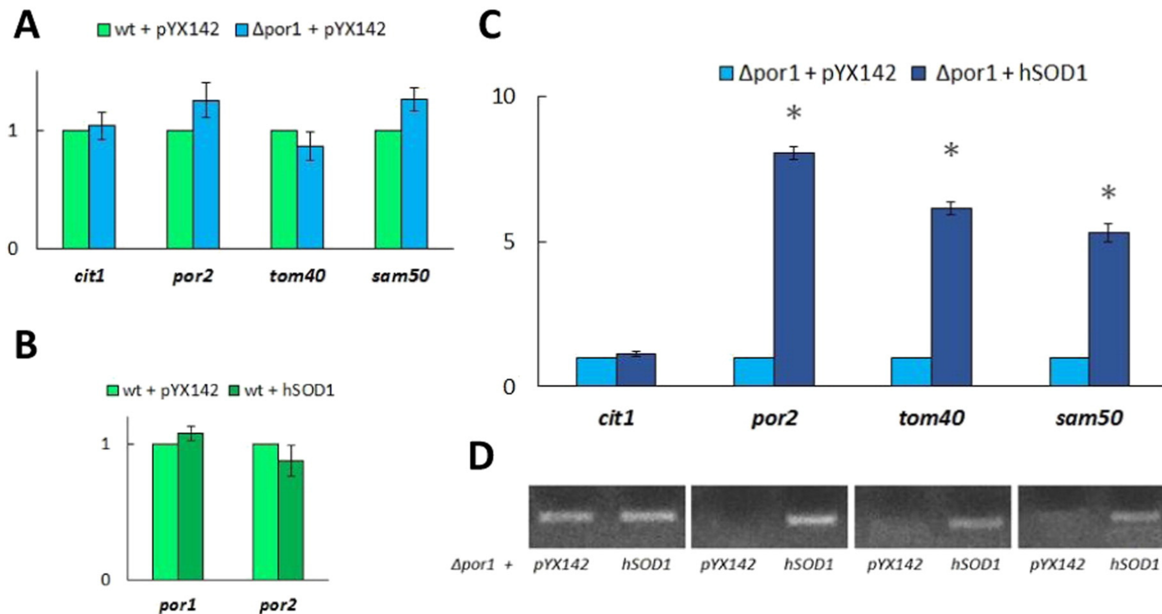


**Fig. 5.** Relative quantification of the expression level of MOM β-barrel protein genes in transformed and control yeast performed by quantitative Real-Time PCR. Data were normalized by *act1* gene; the *cit1* gene was used as internal control. (A) Relative variations of target genes in Δ*por1* yeast compared to the wt yeast. No significative difference was found in target genes between wt and Δ*por1* both transformed with empty vector. (B) Relative variations of *por1* and *por2* genes in wt yeast expressing hSOD1 compared to wt. The overexpression of hSOD1 in wt strain does not change the expression level of porin genes. (C) Relative variations of target genes in Δ*por1* yeast expressing hSOD1 compared to control. hSOD1 supplementation in Δpor1 strain significantly increases the expression level of *por2*, *tom40* and *sam50* genes, while the internal control *cit1* remains unchanged. (*) $P < 0{,}001$ is related to Δ*por1* + pYX142. (D) Analysis of target gene expression after semi-quantitative PCR in agarose gel. Similar amount of amplification reaction was loaded on agarose gel. Differences between expression of target genes are evident.

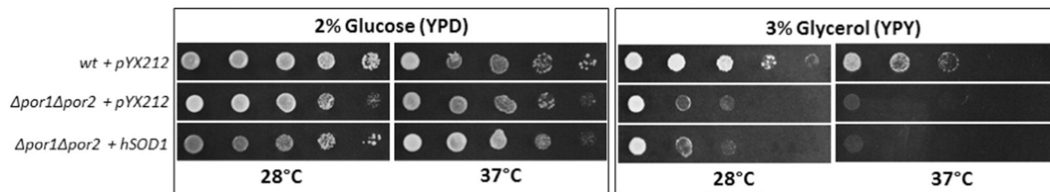


**Fig. 6.** Drop-serial dilution of Δ*por1*Δ*por2* yeast expressing hSOD1. A representative panel of drop-serial dilution of Δ*por1*Δ*por2* yeast transformants on YPD or YPY and incubated at 28 °C or 37 °C for respectively 1–2 and 3–4 days. Both Δ*por1*Δ*por2* cell transformed or not with hSOD1 grow hard on glycerol, suggesting a key role for *por2* gene in mitochondrial reactivation.

mitochondrial recovery mediated by hSOD1, suggesting a recovery function for yVDAC2.

## 4. Discussion

The *por1* gene was first inactivated in *S. cerevisiae* in 1987 [26]. Surprisingly the strain was viable, but the haploid *por*$^{-}$ mutant grew very slowly on glycerol at 37 °C. In addition the strain showed greatly lowered levels of cytochrome c and cytochrome oxidase subunit IV, suggesting that the mitochondrial impairment in the utilization of not fermentable substrate was due to a reduced biogenesis of the organelle. Later, the same group attempted to identify other pore-forming activities responsible for the viability of the defective yeast cell. The addition of a detergent solubilized mitochondrial membrane extract to a planar lipid bilayer set-up showed a pore-forming activity with a single channel conductance of 2.0 nS in 1 M KC1 and the characteristics of general diffusion pores with an estimated effective diameter of 1.2 nm [43,44].

Another group produced more *por1*-less *S. cerevisiae* strains and performed bioenergetic experiments aimed at studying the mitochondrial activity in vivo (oxygen consumption of yeast cells) and in vitro (isolated mitochondria). Their results suggested a tight coupling and a similar flux of ADP in the parent strain and in the Δ*por1* mutant and consequently the accessibility of hydrophilic molecules through the membrane [45]. In 1997 it was discovered a second porin isoform gene in *S. cerevisiae*. The second yeast VDAC gene, *por2*, encodes a protein (POR2 or yVDAC2) with 49% amino acid sequence identity to yVDAC1. It was reported that it can functionally complement the defects present in Δ*por1* strains only when it is overexpressed, while the deletion of the *por2* gene alone has no detectable phenotype. Intriguingly, no evident yVDAC2 channel was detected in electrophysiological experiments or in reconstituted systems [24]. Moreover, mitochondria isolated from yeast Δ*por1* had profoundly reduced outer membrane permeability to exogenous NADH, up to 20-fold, even when yVDAC2 was expressed in a comparable manner to yVDAC1 [46], suggesting that the two proteins are not interchangeable.

### *4.1. Human SOD1 transformation of Δpor1 yeast changes the mitochondrial metabolism*

In the course of a project aimed at studying the interaction between human VDAC1 and human SOD1 in a cellular system devoid of the endogenous pore-forming protein, we noticed that transformation of Δ*por1* yeast with the vector expressing the human Superoxide Dismutase 1 resulted in the unexpected recovery of a phenotype related to the mitochondrial function. Surprisingly, the Δ*por1* transformation with the yeast SOD1 isoform did not have any similar effect. In our experiments, the overexpression of hSOD1 in Δ*por1* strongly improved the growth rate on glycerol at 28 °C, and fully restored the temperature-dependent growth defect at 37 °C. These experiments provide strong evidences of hSOD1 intersection with the mitochondrial functionality. This is further strengthen by $\Delta\Psi_m$ and cellular ROS analysis, indicating that hSOD1 significantly enhances the amount of polarized mitochondria in Δ*por1* cells and re-establishes the physiological level of ROS. Moreover, from the literature, it is known that Δ*por1* strain is significantly more sensitive to hydrogen peroxide treatment than wild type yeast [35,39]. In our analysis, the hSOD1 overexpression in Δ*por1* drastically reduces the ROS level after $H_2O_2$ treatment, confirming the anti-oxidant properties of human enzyme in yeast cells. Being $H_2O_2$ a product and not a substrate of SOD1, how hSOD1 can protect from oxidative stress induced by hydrogen peroxide?

In a recent work, it was demonstrated that under oxidative stress, the endogenous yeast SOD1 translocates into the nucleus where it acts as transcription factor and regulates the expression of genes involved in oxidative resistance and repair mechanisms [38]. In the same paper the treatment of yeast cell with hydrogen peroxide was sufficient to promote SOD1 nuclear translocation [38]. On these bases, we hypothesize that in Δ*por1* yeast, hSOD1 mediates the expression of genes involved in the response to oxidants and in restoring MOM permeability, as demonstrated by our qPCR experiments.

### *4.2. Hypotheses about the mechanism of mitochondrial metabolism reactivation*

To prove this concept we have studied the modifications in the expression of genes that could be involved in the response to the *por1* deficiency. In particular, we studied the expression level of genes encoding for the other putative pore-forming proteins of the MOM: yVDAC2, the yVDAC1 paralog, and Tom40 and Sam50, containing a transmembrane β-barrel [42] and thus possible substitute of the porin-pore. It has been shown that *por2* overexpression in Δ*por1* yeast is able to partially complement the absence of yVDAC1 [24], and that Tom40 participates in metabolic exchange in Δ*por1* mitochondria [47]. Quantitative Real-Time PCR has revealed that all these three genes overexpress their mRNA in the absence of yVDAC1 and in the presence of hSOD1, but not just in the absence of the yVDAC1 gene.

The Kmita laboratory tried to find a correlation between Δ*por1* mutant and TOM complex proteins. In a first report, they found that TOM complex proteins are upregulated in the Δ*por1* yeast [47]. Then they indicated that the increase in the levels of TOM complex proteins, Tom40 and Tom70, results from changes in the respective gene expression, proposing that, in the absence of the yVDAC1 pore, a signaling pathway leading to their up-regulation has to be triggered. Also mRNA of Tom40 evaluated by Real-Time PCR was found increased in another work from the same group [48]. However, in both cases yeast was grown in the presence of a non-fermentable substrate (glycerol). Transition from fermentation to respiration leads to a series of adaptive mechanisms in yeast cells involving the regulation of genes of mitochondrial utilization of not fermentable carbon source [49]. Thus, it cannot be excluded that the increased amounts of TOM complex components in the defective Δ*por1* found in [48] was a response to glycerol adaptation. In our work, RNA extraction was performed from yeast grown in fermentative condition, excluding any possible mechanism depending from carbon source. Real-Time PCR experiments have shown that no significative differences were detected for *por2*, *tom40* and *sam50* mRNAs in Δ*por1* strain compared to wt. On the contrary, it is the presence of hSOD1 in Δ*por1* yeast to enhance the expression profile of analyzed genes.

### 4.3. Is yVDAC2 a valid substitute of yVDAC1?

The most unexpected result, for us, was the relevant overexpression of *por2*, promoted by hSOD1 addition. *Por2* expression was increased 8 times compared to control. To verify its importance, we used another mutant yeast where both VDAC genes are inactivated. The transformation of this Δ*por1*Δ*por2* yeast with hSOD1 does not show the same effect observed in the Δ*por1* yeast, i.e. there is no visible re-activation of the mitochondrial metabolism on glycerol at 37 °C. This clearly suggests that the lack of the second porin gene has profound effects on the viability of the yeast.

Although any pore-forming activity of yVDAC2 by experimental techniques has not been demonstrated, our results point to a functional substitution of yVDAC1 by yVDAC2 in Δ*por1* upon the hSOD1 stimulus. It is known that the promoter of yeast *por2* gene is a weak promoter [24]. It is possible that only a specific stimulus, like the presence of a different SOD1 (hSOD1), can engage and activate it to replace the lack of yVDAC1.

Since *por2* is likely essential to guide mitochondrial re-activation, we can speculate that the increase of TOM and SAM complex components is a consequence of the yVDAC2 increase: yVDAC2 needs active and abundant specific transport and assembly machinery for incorporation into MOM. However, this does not exclude that Tom40 plays a key role in maintaining the mitochondrial metabolism of Δ*por1* mutants, participating in metabolic exchange.

### 4.4. Hypotheses about reactivation of yeast mitochondrial metabolism mediated by human but not yeast SOD1

Undoubtedly, VDAC1 removal represents a strong stress factor for yeast cells, which could induce hSOD1 to promote the recovery of mitochondrial metabolism through the expression, or the overexpression, of specific genes. It is anyway unexpected and unclear why either the endogenous or the overexpressed yeast SOD1 is not able to trigger by itself the answer obtained with added human SOD1.

In wild-type yeast the endogenous SOD1 protects the cell from oxidative stress promoted by hydrogen peroxide, activating SOD1 nuclear translocation and the transcription of nuclear factors involved in stress response [38]. However, the *por1* gene deletion per se increases significantly the yeast sensitivity to $H_2O_2$ treatment [39] and, consequently, the apoptosis rate [50]. Thus, the "protective mechanism" described in [38] seems impaired in Δ*por1* yeast. These data correlate with our results, which show the highest increase of ROS in Δ*por1* yeast compared to the other samples after incubation with $H_2O_2$. Overall, these evidences suggest that Δ*por1* yeast shows an intrinsically susceptibility to hydrogen peroxide, probably due to a combination of factors, as well as the changed activity of catalase [51] and reduced activity of IMS-located SOD1 [29]. Thus, we can hypothesize that in Δ*por1* yeast the endogenous SOD1 is possibly less susceptible to activate a rescue answer, even though yeast SOD1 is overexpressed. On the contrary, the overexpression of hSOD1 restores mitochondrial

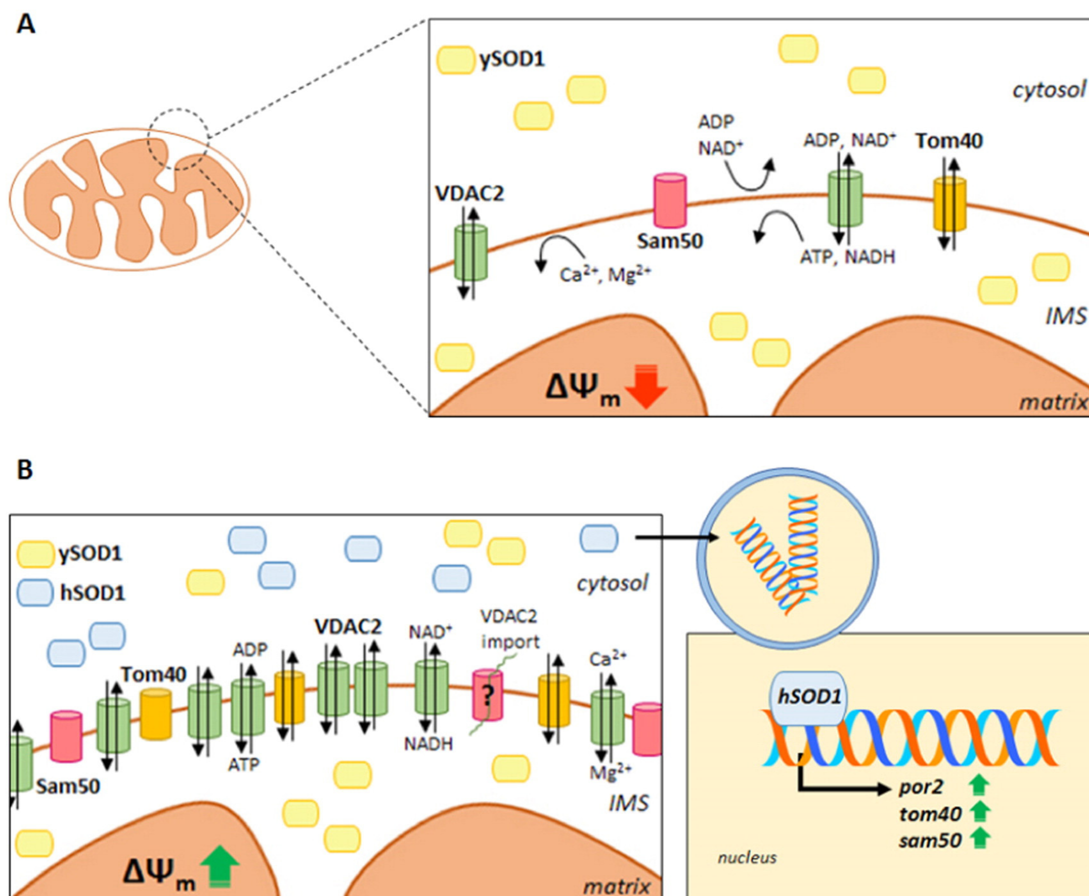


**Fig. 7.** A proposed model of the reactivation of mitochondrial metabolism mediated by hSOD1 transformed in yeast cell. (A) Δ*por1* mitochondria before hSOD1 supplementation have small amounts of yVDAC2 in the outer membrane. In this contest, Tom40 actively participates in metabolite exchanges, thus partially substituting yVDAC1 function. However, lack of yVDAC1 causes a reduced exchange between cytosol and mitochondria and consequently a decrease in the inner membrane potential. Endogenous SOD1 is distributed in cytosol and IMS, but cannot affect the expression of nuclear genes. (B) In Δ*por1* yeast transformed with hSOD1 the pool of SOD1 molecules in the cytosol is deregulated and increases. This triggers the translocation of hSOD1 to the nucleus where it selectively switches on genes involved in the reactivation of the metabolic flux through the outer membranes and consequently in restoring the inner membrane potential and the usual mitochondrial activity. Yellow oval: ySOD1; pale blue oval: hSOD1; green cylinders: yVDAC2; orange cylinders: Tom40; pink cylinders: Sam50.

bioenergetics. In these conditions modifications in the expression profile of specific genes, with functions close to VDAC1, are found.

Human and yeast SOD1 proteins show 70% of sequence similarity and hSOD1 in yeast is perfectly active [37]. Moreover, several crucial amino acids residues are conserved, especially those subjected to modification, which can regulate functional or structural features (see Suppl. Fig. 1). E. g., both human and yeast SOD1 proteins maintain the serine residues 60 and 99, whose phosphorylation promotes SOD1 translocation to the nucleus, with a similar mechanism in yeast or mammalian cells [38]. Also cysteine 57 and 146 are conserved since they form an intramolecular disulfide bonds essential for proper folding.

Oxidation of cysteines is also a key event that can influence the SOD1 subcellular distribution. SOD1 is localized in cytosol and in IMS. Although the alternative targeting mechanism of the same protein to these two compartments remains unsolved, SOD1 apoenzyme crosses the MOM through TOM complex and, once in IMS, it interacts with the Copper Chaperon of SOD1 (CCS), which promotes metal insertion, dimerization and formation of disulfide bond between cysteine 57–146 [52]. Vice versa, the formation of the disulfide bond in cytosol strongly prevents SOD1 import to IMS. The human SOD1 possesses two more cysteine residues, in position 6 and 111, which are not involved in intramolecular disulfide bond. Interestingly, Cys 111 is almost exclusively present in the human SOD1, while in yeast and in most species is replaced by a serine residue. It has been proposed that Cys 111 may bind metals or other ligands, such as glutathione or thioredoxins, through disulfide bonds formation [53], or may be the target of oxidative modification [54] and may modulate aggregation in ALS [55]. Thus, a modification involving Cys111 can influence hSOD1 localization in the cell. It is thus possible hypothesize that hSOD1 in yeast is more susceptible to Cys-modifications that lead to impairment of hSOD1 redistribution and trigger its translocation to the nucleus, thus pushing towards a recovery of the mitochondrial metabolism. Similarly, the absence of Ser111, an amino acid subjected to phosphorylation, could subtract the overexpressed hSOD1 to other regulatory mechanism. Fig. 7 depicts in a cartoon this proposed mechanism of action.

## 5. Conclusions

Our findings indicate that VDAC channels are involved in the communication between mitochondria and nucleus. This function of the VDAC channel could be exerted by means of other proteins associated with the channel, such as TspO as reported recently [56]. Furthermore this is the first clear demonstration that the expression of VDAC isoforms genes maybe coordinated to answer to a cellular stimulus.

In addition these results support the proposal [38] that SOD1 in the yeast cell has additional effects and not only the detoxifying one, acting as a modulator of the expression of target genes. Our data can explain how hSOD1 ectopic expression in yeast acts in promoting mitochondrial stress relief.

In conclusion, although further analyses have to be made, our work shows how much the fate of VDAC is evidently linked to SOD1, and vice versa, with relevant consequence in the study of physiology and pathology of mitochondria.

## Competing interests

The authors declare that no competing interest exists.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2016.03.003.

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