Article

Long lasting reversibility of the mitochondrial permeability transition in *Saccharomyces cerevisiae*.

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Abstract: The *Saccharomyces cerevisiae* mitochondrial unspecific pore ($_{Sc}$MUC) is an uncoupling unspecific pore that shares some similarities with the mammalian permeability transition pore (mPTP). When open, both channels deplete ion and proton gradients across the inner mitochondrial membrane. However, the role of mPTP is to reversibly open to protect cells against stress. If mPTP remains stuck in the open position the cell dies. In contrast, $_{Sc}$MUC is probably dedicated to deplete oxygen from the medium in order to kill competing organisms. Such O_2 depletion would be better achieved if oxidative phosphorylation is at least mildly uncoupled. Still, when oxidative phosphorylation is needed $_{Sc}$MUC should be able to close. To test this, the reversible opening and closing of $_{Sc}$MUC in the presence of different effectors was tested in isolated mitochondria from S. cerevisiae. Evaluations were conducted at different incubation times, monitoring the rate of O_2 consumption, mitochondrial swelling and the transmembrane potential. It was observed that $_{Sc}$MUC did remain reversibly open for minutes. A low energy charge (ATP/ADP) closed the channel. In addition, high Ca^{2+} promoted closing and it was a highly powerful effector.

Keywords: Physiological uncoupling, Yeast mitochondria, Mitochondrial Permeability transition, $_{Sc}$MUC, Reversibility.

1. Introduction

Mitochondrial oxidative phosphorylation (OxPhos) involves two coupled processes. First, the exergonic transit of electrons through the redox complexes in the respiratory chain (RC) drives an active transport of protons across the inner mitochondrial membrane to generate a ΔpH [1] . Then the proton gradient is dissipated by the F_1F_0-ATP synthase to produce ATP [1,2]. In the absence of ATP synthesis, ΔpH remains high, decreasing the rate of electron flow [3]. Stalled electrons form free radicals in the different redox centers located in RC complexes that may react spontaneously with O_2 to produce highly motile Reactive Oxygen Species (ROS) [4]. While a small amount of ROS is always produced, an excess of ROS may damage proteins and lipids, leading to aging and even cell death [5,6]

Increasing the rate of electron flow through the respiratory chain decreases ROS production [7]. In the absence of ATP synthesis, the rate of electron flow may be increased by physiological uncoupling [8]. Physiological uncoupling mechanisms include proton sinks, which may be either non-specific or specific for protons [9] and branched respiratory chains, which express alternative redox enzyme that are not sensitive to the

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ΔpH [8,10]. Uncoupling must be tightly controlled through differential expression and/or activity regulation in order to avoid depleting cellular ATP [11].

Saccharomyces cerevisiae is a unicellular eukaryote that displays two strategies to manipulate its environment, which are meant to eliminate other organisms that compete for nutrients: A P-type H^+-ATPase located in the plasma membrane lowers the external pH to values around 3.0 [12] and in addition its mitochondrial respiratory chain (RC) depletes O_2 from the medium[13]. In these conditions, *S. cerevisiae* cells survives using an anaerobic fermentative metabolism. Thus, mitochondria from *S. cerevisiae* must consume O_2 and oxidize NADH + H^+ at high rates, independently of whether they phosphorylate ATP or not [14,15]. To do this, these mitochondria produce less ATP/O_2 and in addition, a physiological oxidative phosphorylation (OxPhos) uncoupling system is used. Low ATP/O_2 results from the fact that *S. cerevisiae* mitochondria do not have Complex I, instead they have three type two NADH dehydrogenases, which are not proton pumps [16]. This already leads to a high rate of O_2 consumption. In addition, the *S. cerevisiae* Mitochondrial Unspecific Channel ($_{sc}$MUC) may open to uncouple OxPhos. This channel is a proton sink, as it allows the passage of molecules up to 1.1 kDa and depletes all ion gradients, ΔpH included [17–19].

The mammalian permeability transition pore (mPTP) is an uncoupling system similar to $_{sc}$MUC[20]. Both $_{sc}$MUC and mPTP are highly effective to equilibrate proton and ion concentrations between the mitochondrial matrix and the cytoplasm [21]. An open $_{sc}$MUC allows the passage of molecules up to 1.1 kDa, while mPTP has a cutoff limit of 1.5 kDa [17,22]. In spite of their functional similarities, the physiological role of each of these channels seems to be different as mPTP opens to protect the cell against ischemia [23], while $_{sc}$MUC is probably designed to deplete O_2 [24]. Also, these channels express different sensitivity to some effectors [25]. Notably, Ca^{2+} closes $_{sc}$MUC while it opens mPTP. In addition, high Pi closes $_{sc}$MUC but opens mPTP [18,26]. Also, ADP closes both channels, but ATP opens $_{sc}$MUC while it closes mPTP [27–30]

An important feature of physiological uncoupling systems is that they have to be tightly controlled to avoid depleting cellular ATP [31]. Unicellular organisms and some tissues such as cardiac muscle, where mitochondria are often exposed to large variations in metabolic demand or O_2 and solute concentrations, seem to undergo frequent reversible opening and closing of PTP. However, after long incubation times, the pore may be stuck in the open position, leading to cell death [32–34]. Thus, it was decided to evaluate for how long $_{sc}$MUC remains reversible and to determine which important effectors, such as adenine nucleotides of Ca^{2+} continue to control opening and closure of this channel after long incubation times.

2. Results

2.1 *Effects of ADP/ATP on $_{sc}$MUC.*

Both $_{sc}$MUC and mPTP are considered equivalent, except for some differences in effectors, e.g., in contrast to PTP, both Ca^{2+} and Pi close $_{sc}$MUC. Adenine nucleotides are among known effectors of both mPTP and $_{sc}$MUC: in mPTP Pi opens the channel while ATP and ADP close it. in yeast ATP opens $_{sc}$MUC, while ADP and Pi close them[34–37]Thus, to test $_{sc}$MUC reversibility we alternatively added ATP and ADP in the presence of different Pi concentrations and tested the ability of these molecules to counteract the effects of each other (Fig. 1). In agreement with data in the literature, a slow rate of oxygen consumption was observed at 2.0 mM Pi (Fig. 1-A, trace a), suggesting that $_{sc}$MUC was closed[18,19]. Under these conditions, the addition of different concentrations of ATP increased the rate of oxygen consumption (Fig. 1-A, traces b to e). In contrast, at 0.1 mM Pi the rate of oxygen consumption was high (Fig. 1-B trace a), suggesting that $_{sc}$MUC was open[19,38]. Here, addition of increasing concentrations of ADP inhibited the rate of

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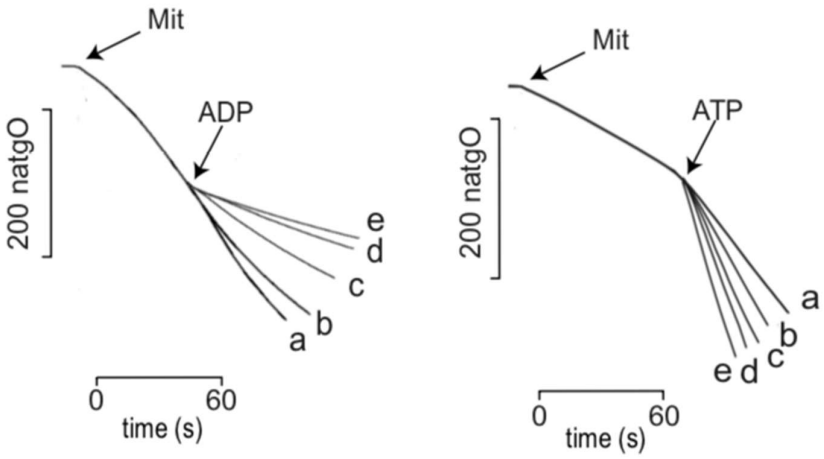
oxygen consumption, suggesting that ADP closed scMUC, i.e., reverted the open state promoted by low Pi (Fig. 1B traces b to e).

Figure 1. Rates of O_2 consumption of isolated yeast mitochondria



(A) (B)

Figure 1. O_2 consumption rates of isolated yeast mitochondria. Effects of phosphate (Pi) ATP and ADP. A: Reaction mixture: 0.6 M mannitol, 5 mM MES , pH 6.8 , 0.1 mM KCl , 0.5 mM $MgCl_2$, 2µL/mL ethanol, 0.1 mM phosphate. ADP additions were: a) 2 mM; b)1.5 mM; c) 1 mM; d) 0.5 mM and e) none ADP. B: Reaction mixture: as in A, except 2 mM phosphate. ATP additions were: a) none ATP; b) 0.5 mM; c) 1 mM; d) 1.5 mM and e) 2 mM

Oxygen consumption results confirmed that scMUC was regulated by Pi, ADP and ATP. To further explore this phenomenon, it was decided to determine whether changes on ΔΨ were consistent with alternating PT states in response to sequential additions of ADP and ATP (Fig. 2). At low Pi, scMUC was open (Fig. 2A trace a) and ΔΨ was low; here, addition of 2 mM ADP closed scMUC increasing ΔΨ (Fig. 2-A trace b). Then the ADP-mediated increase in ΔΨ was reversed by different [ATP], leading to a second depletion of ΔΨ (Fig. 2A, traces c,d,e). The opposite experiment was also performed: when in the presence of high Pi, scMUC was closed as evidenced by a high ΔΨ (Fig. 2B). Here ATP addition led to opening of scMUC decreasing ΔΨ; (Fig. 2B, trace b). Then the addition of different [ADP] promoted a new rise in ΔΨ, *suggesting scMUC* closed again (Fig. 2B traces c,d,e). Thus, the alternative addition of ADP and ATP resulted in a reversible sequence of opening/closing of scMUC *(Fig. 2).*

Figure 2. Effect on the mitochondrial transmembrane potential.

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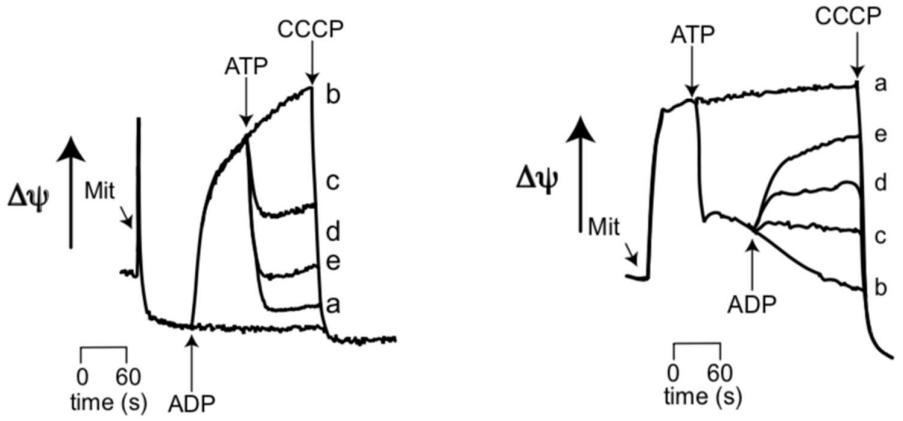
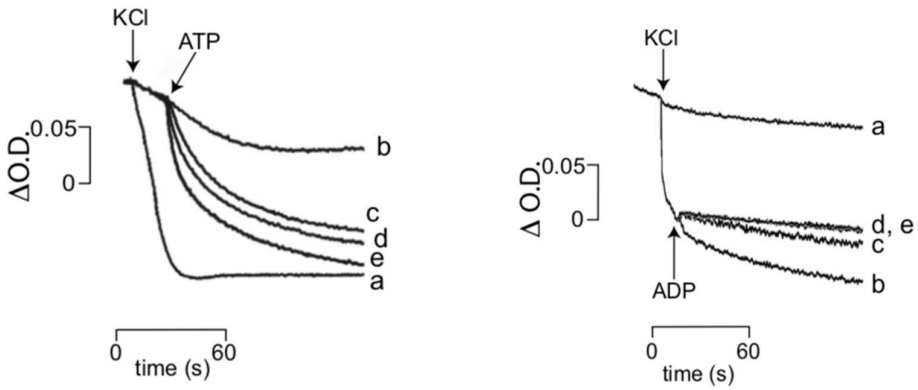


Figure 2. Effect of the sequential addition of ATP and ADP on the mitochondrial transmembrane potential (ΔΨ). Reaction mixture as in Fig. 1, except 15 μM safranine-O and Pi. Mit: 0.5mg/mL mitochondria; 0.1 mM Pi (a-e) and 1 mM ADP (b-e) . ATP additions were: a) 0 mM; b) 0.5 mM; c) 1 mM d) 1.5 mM; e) 2mM. B 2 mM Pi (a-e) and 1 mM ATP (b-e). ADP additions were: a) 0 mM; b) 0.5 mM; c) 1 mM d) 1.5 mM; e) 2mM. Measurements were conducted in a Ollis/Aminco spectrophotometer in Dual mode at 511-533nm.

Another parameter commonly used to follow PT is mitochondrial swelling. At high Pi or ADP $_{sc}$MUC was closed and the rate of swelling was slow (Fig. 3, traces a) At low Pi or high ATP, $_{sc}$MUC was open and added K^+ ion entered the mitochondrial matrix causing a rapid rate of swelling (Fig. 3, traces b) [39]. Alternation between opening and closing was followed. In the presence of 2.0 mM Pi and 1 mM ADP, swelling was slow (Fig. 3A, trace a) and then, addition of different [ATP] caused increasing rates of swelling, suggesting opening of $_{sc}$MUC (Fig. 3A traces c, d and e). In the opposite sense, at 0.1 mM Pi plus 1 mM ATP a rapid rate of swelling was observed (Fig. 3B, trace b), then, addition of different [ADP] inhibited the rate of swelling (Fig. 3-B traces c, d, and e) suggesting that ADP closed $_{sc}$MUC. Therefore, the effect of Pi, ADP or ATP was known in isolation, with these results we observed that the phenomenon of these three effectors; in the same experiment, can open and close making the $_{sc}$MUC dynamic.

Figure 3. . Effect on mitochondrial swelling.



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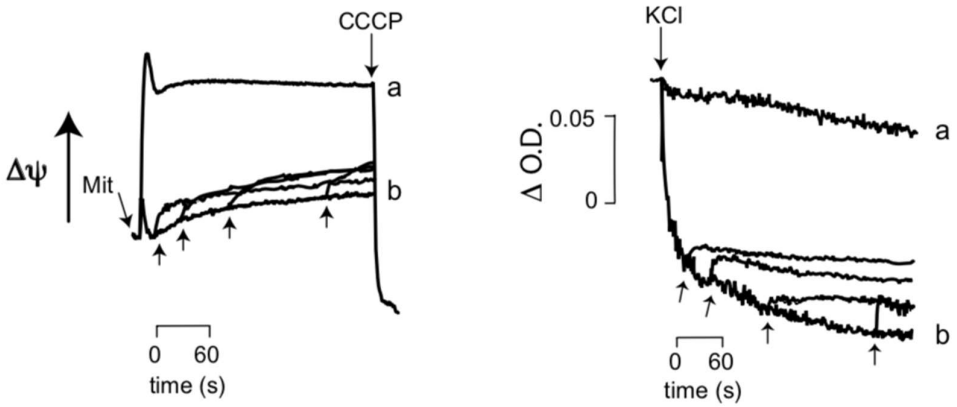
(A) (B)

Figure 3. Effect of the sequential addition of ATP and ADP on mitochondrial swelling. A: Reaction mixture, as in Fig. 1, except 0.3 M mannitol. The arrow indicates the addition of 0.1 M KCl . Swelling was measured in an Ollis/Aminco spectrophotometer in Split mode at 560 nm. 0.1 mM Pi (a-e); 1 mM ADP(b-e) . ATP : a) 0 mM; b) 0.5 mM; c) 1 mM ; d) 1.5 mM; e) 2mM B: as in Fig. 2 except 2 mM Pi. Additions were (a-e); 1 mM ATP (b-e). ADP: a) 0 mM; b) 0.5 mM; c) 1 mM ; d) 1.5 mM; e) 2mM

To test the reversibility of $_{sc}$MUC after long incubation times, yeast mitochondria were incubated for several minutes under conditions where the pore was open and then ADP was added. Both the ΔΨ and mitochondrial swelling were monitored. When ADP was added after different incubation times, it was observed that the ΔΨ recovered and swelling was reverted suggesting that the $_{sc}$MUC closed, and thus its reversibility was quite robust. (FIG 4A&B)

Figure 4. Effect of the addition of ADP on the mitochondrial transmembrane potential and mitochondrial swelling



(A) (B)

Figure 4. Effect of the addition of ADP on the mitochondrial transmembrane potential and mitochondrial swelling. Reaction mixture as in Fig. 2 excepts 2 mM ADP were added at different times: 30 seconds, 1, 2 and 4 minutes (upward arrows). B: Reaction mixture, as in Fig. 3. The arrow indicates the addition of 0.1 M KCl . Additions of 2 mM ADP were at different times: 30 seconds, 1, 2 and 4 minutes (arrows upward).

The above results indicate that the opening response of $_{sc}$MUC to the sequential additions of ATP and ADP is reversible for several minutes of incubation. The next question addressed here is whether $_{sc}$MUC reversibility is observed in the presence of other effectors, so we decided to test the effect of Ca^{2+} and EGTA at different incubation times.

2.2 Effects of Ca^{2+}/EGTA additions on $_{sc}$MUC.

Ca^{2+} withdrawal from the medium opens $_{sc}$MUC, while addition of Ca^{2+} closes it. This is an efficient signaling system that controls ATP production during different events such as the cell cycle or mating. At short incubation times, the effects of Ca^{2+} and EGTA are fully reversible. To test the reversibility after several minutes when using Ca^{2+} as an effector, mitochondria were incubated in the presence of EGTA for increasing incubation times and then Ca^{2+} was added to promote closing. Measurements of ΔΨ and mitochondrial swelling were conducted to evaluate PT reversibility. Yeast mitochondria were incubated in the presence of EGTA for 15, 30 seconds and 1, 2 and 4 min. Then Ca^{2+} was added and the effects were evaluated. At low Pi and in the presence of EGTA, the addition of K^+ led to a decrease of ΔΨ. Then, the addition of Ca^{2+} at the indicated times pro-

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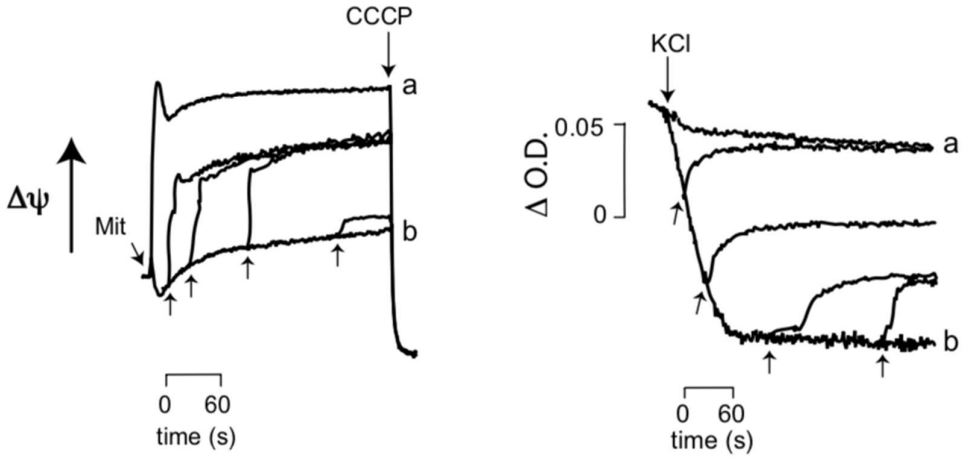
moted a recovery of the ΔΨ. This was observed at all the incubation times tested (FIG 5A&B). When swelling of mitochondria was tested at low Pi and in the presence of EGTA, K^+ promoted swelling, which was reverted by Ca^{2+} addition at all the incubation times tested. Thus, addition of Ca^{2+} led to full recovery of ΔΨ (FIG 5A) and to reversal of swelling (FIG 5B) indicating that scMUC closed after long incubation times in the open state. Reversal was even more pronounced than the one obtained using ATP/ADP additions.

Figure 5. Effect of the addition of Ca^{2+} on the mitochondrial transmembrane potential and mitochondrial swelling



(A) (B)

Figure 5. Effect of the addition of Ca^{2+} on the mitochondrial transmembrane potential and mitochondrial swelling. A: Reaction mixture as in Fig. 2. Ca^{2+} 600 µM additions (upward arrows) were at different times: 30 sec, 1, 2 and 4 min. B: Reaction mixture as in Fig. 3. Where 0.1 M KCl . Additions of 600 µM Ca^{2+} were at: 30 sec, 1, 2 and 4 min (upward arrows).

3. Discussion

S. cerevisiae exhibits a high rate of electron flow through the RC that is further accelerated in the presence of glucose [40]. This is reminiscent of the Crabtree effect originally reported by Otto Warburg in cancer cells [40]. Both in cancer cells and in yeast, energy for growth is provided by anaerobic glycolysis [41]. It has been proposed that in tumor cells, the uncoupled, fast rate of O_2 consumption is needed to inhibit production of ROS, helping the cells to survive [42,43]. In contrast, in *S. cerevisiae* this does not seem to be the case as these cells deplete O_2 thriving in anoxic environments.

The high rate of O_2 consumption by *S. cerevisiae* is slightly uncoupled due to different factors. First, yeast mitochondria do not have a proton-pumping respiratory Complex I, as it is substituted by three alternative NADH dehydrogenases (ND2) [15]. ND2s catalyze a much faster electron transfer than Complex I, which has a slow, complicated catalytic cycle. ND2s do not contribute to the protonmotive force so the ADP/O is lower than in other (mammalian) mitochondria. In addition, they express scMUC, a

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channel regulated by the energy charge [19]. Indeed in our hands, ADP closed the $_{sc}$MUC while ATP opened it. So a high energy charge uncouples OxPhos releasing O_2 consumption, while a low energy charge couples OxPhos to optimize the synthesis of ATP [8].

A system to deplete O_2 would confer yeast with an advantage over other organisms as these cells thrive in hypoxic/anoxic conditions. The reversibility of the permeability transition by the alternate addition and withdrawal of calcium has been documented [18]. Mitochondria from yeast do not have a calcium transporter and the slow uptake of this ion may have been a factor in our results [44]. In contrast mammalian cells move Ca^{2+} through a mitochondrial uniporter (MCU) controlling respiration, mitophagy/ autophagy, and mitochondrial apoptosis [45].

Unlike mammals, in *S. cerevisiae* cytoplasmic Ca^{2+} transients may last up to 60 minutes, signaling for processes such as mating or during the cell cycle. Perhaps these are the times when the highest mitochondrial ATP production is needed. In addition, external events such as, alteration in osmolarity or the recovery response from starvation once a substrate becomes available [46]. When a haploid type a cell detects alpha-pheromone, it needs to form a large projection designed to reach for an alpha cell nearby. Then, both cells mate and become a diploid, a process that probably requires a large amount on energy. In these circumstances no apoptotic mechanisms are triggered [47]. Instead, Ca^{2+} probably enhances OxPhos. Indeed, Ca^{2+} efficiently closed $_{sc}$MUC increasing $\Delta\Psi$ and reversing mitochondrial swelling. Thus, in *S. cerevisiae*, PT was alternatively evoked by ATP and reversed by ADP plus Pi. i.e. a decrease in energy charge optimizes OxPhos. In each case, OxPhos is stimulated by either low energy charge (an decrease in ATP/ADP) or an increase in Ca^{2+}, which indicates that a large quantity of ATP will be needed.

In mammals, mPTP is related to a stress response. It is interesting to note that training confers heart mPTPs with both, the ability to open and thus avoid overproducing ROS and to close after an ischemic event, re-coupling OxPhos and helping the cell to survive an ischemic event [24,48]. In this regard, it has been observed that during cardiac stress situations, intermittent episodes of reperfusion allow the cell to recover ATP pools and avoid ROS overproduction, greatly improving survival probabilities. This procedure is termed conditioning [49,50].

In *S. cerevisiae*, the opening of the $_{sc}$MUC probably does not trigger apoptosis [10] but instead seems to help deplete O_2. Most likely, the widely different roles that mPTP and ScMUC have is a classical example of exaptation, where two similar structures with similar mechanisms of action serve different functions in their respective organisms.[51–54] This is further complicated by the fact that the molecules constituting each channel are still undefined.

4. Materials and Methods

Reagents: All chemicals were analytic grade. Mannitol, MES hydrate, D- Galactose, TEA (triethanolamine), ADP, ATP, safranine-O, oligomycin were from Sigma Chem Co. Bovine serum albumin from Probulmin TM. Bacto-peptone and yeast extract from MCD LAB. H_3PO_4, KCl and $MgCl_2$ were from J.T. Baker.

Yeast strain. Experiments were conducted using either a commercial strain of baker's yeast *Saccharomyces cerevisiae* ("La Azteca, S. A." Mexico City) was used [26] or a laboratory strain W303 (*MATα; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100*). Results were similar foe each strain. Each strain was maintained in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% bacto-agar) plate cultures. Cells were grown as follows: pre-cultures were prepared immersing a loophole of yeast into 100 mL YPD and incubated for 24 hours under continuous agitation in an orbital shaker (New Brunswick Scientific) at 250 rpm in a constant-temperature room (30 ºC). Then each 100

mL flask was used to inoculate 900 mL YPGal of (1% yeast extract, 2% bacto-peptone and 2% galactose). Incubation was continued for 48 h under the same conditions.

Isolation of yeast mitochondria. After incubation, yeast was centrifuged (5000 xg for 5 min, twice) and resuspended in 0.6 M mannitol, 5 mM MES, 0.1% bovine serum albumin, pH 6.8 (TEA). The cell suspension was mixed with 50% (v/v) of 0.5mm diameter glass beads and disrupted in a Bead Beater and mitochondria were isolated from the homogenate by differential centrifugation as previously described [55]. The concentration of mitochondrial protein was determined by Biuret [56].In all assays 0.5 mg mitochondrial protein/mL. In all experiments the mitochondrial sample was pre-incubated for 5 min with oligomycin (4 µg per mg protein).

Oxygen consumption. Experiments were conducted using a Clark electrode (Oximeter model 782, Warner/Strathkelvin Instruments) in a water-jacketed chamber. Temperature was kept at 30°C using a water bath (PolyScience 7 L). Total volume 1.0 mL. The reaction mixture for yeast mitochondria was 0.6 M mannitol, 5 mM MES (TEA), pH 6.8 plus 0.1 M KCl, 0.5 mM $MgCl_2$ and 2 µL/mL ethanol. Where indicated, oligomycin 4 µg/mg prot. preincubating for 5 min.

Transmembrane potential. ΔY was determined as described by Akerman and Wikström [57], following the changes in absorbance of safranine-O at 511-533 in a double beam Aminco-Ollis spectrophotometer in dual mode. The concentrations of ATP, ADP, Ca^{2+} and EGTA used are indicated under each figure. At the end of each trace, the collapse in ΔΨ was induced by addition of 6 µM CCCP**.**

Mitochondrial swelling. Experiments were performed by following the decrease in absorbance of a mitochondrial suspension in a DW2000 Ollis/Aminco spectrophotometer in split mode at a wavelength of 540 nm [37].

Author Contributions: Conceptualization, S Uribe-Carvajal and N Chiquete-Félix.; methodology ; software; formal analysis, .; investigation, P Castañeda Tamez, N Chiquete-Félix, L Morales-García and C Ricardez-García; writing—original draft preparation,. L Morales-García and C Ricardez-García ; writing—review and editing, L Morales-García, C Ricardez-García and S Uribe-Carvajal .; supervision, S Uribe-Carvajal. All authors have read and agreed to the published version of the manuscript.". All have contributed substantially to the work reported.

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