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Review

The role of mitochondrial biogenesis and ROS in the control of energy supply in proliferating cells ☆

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ABSTRACT

In yeast, there is a constant growth yield during proliferation on non-fermentable substrate where the ATP generated originates from oxidative phosphorylation. This constant growth yield is due to a tight adjustment between the growth rate and the cellular mitochondrial amount. We showed that this cellular mitochondrial amount is strictly controlled by mitochondrial biogenesis. Moreover, the Ras/cAMP pathway is the cellular signaling pathway involved in the regulation of mitochondrial biogenesis, with a direct relationship between the activity of this pathway and the cellular amount of mitochondria. The cAMP protein kinase Tpk3p is the catalytic subunit specifically involved in the regulation of mitochondrial biogenesis through regulation of the mitochondrial ROS production. An overflow of mitochondrial ROS decreases mitochondrial biogenesis through a decrease in the transcriptional co-activator Hap4p, which can be assimilated to mitochondria quality control. Moreover, the glutathione redox state is shown as being an intermediate in the regulation of mitochondrial biogenesis. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

In living cells, growth is the result of coupling between substrate catabolism and multiple metabolic processes that can be assumed to take place during net biomass formation and maintenance processes (i.e. maintenance of ionic gradients, protein, lipid, and nucleic acid turnover) [1,2]. If one considers cells for which most of the ATP flux is generated through oxidative phosphorylation, when cell energy demand varies, the activity of oxidative phosphorylation should be modulated. Two mechanisms, which are not exclusive, can account for such a regulation. Short-term adaptation relies on a flux modulation through every functional unit of the mitochondrial oxidative phosphorylation, whereas long-term adaptation to various rates of ATP utilization can be achieved by modifying the number of these functional units, i.e. mitochondrial amount — which refers to the number of respiratory chain units as assessed by mitochondrial cytochromes measurement—. If one considers large variations in ATP turnover, it is highly likely that the amount of enzymes involved in the oxidative phosphorylation pathway plays a significant role in this process in order to allow a constant yield of ATP synthesis —.see below— [3–6]. Moreover, the trade-off between rate and yield of ATP synthesis in heterotrophic organisms has been highlighted as a possible major mechanism of cooperation and competition involved in the evolutionary aspects of energy metabolism [7]. Consequently, the molecular mechanisms involved in the adjustment of energy production to energy demand are of particular interest.

In mammalian cells, both the above-mentioned short-term and long-term regulations have been described. An example that illustrates a short-term kinetic regulation is the cAMP-induced phosphorylation degree of complex I that is a key regulating step modulating the oxidative phosphorylation capacity when glioma cell proliferation varies [8,9]. The long-term modulation of mitochondrial amount is well known in muscle. Indeed, experiments from the 1960s have shown an increase in mitochondrial marker enzymes associated with an increase in the number and shape of mitochondria in response to exercise [10,11]. Muscle mitochondrial content can be increased by 50–100% within 6 weeks of endurance training. Chronic contractile activity produced by electrical stimulation of the motor nerve can mimic this mitochondrial biogenesis. Williams et al. [12] were the first to show that chronic contractile activity led to increases in mRNA levels encoding nuclear and mitochondrial gene products.

As highlighted in [7], there is a trade-off between rate and yield of ATP synthesis in heterotrophic organisms. Indeed, short-term and long-term regulation mechanisms are not equivalent in terms of energy transduction efficiency. This was well studied in the yeast *Saccharomyces cerevisiae* [5,13,14]. A short-term kinetic regulation of the respiratory chain will induce a modulation of the flux through the respiratory chain units. Under steady-state conditions, where ATP synthesis matches ATP consumption, the growth yield may depend on two variables: 1) the

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fraction of ATP utilized for cell maintenance vs. that used for biomass synthesis per se and 2) the amount of ATP synthesized per oxygen consumed during oxidative phosphorylation [i.e., ATP-to-O ratio (ATP/O)]. The actual ATP/O has been shown, in vitro in isolated mitochondria, to vary according to the proton leak, the degree to which the redox proton pump slips [13,14], and the functional steady state of mitochondria. Indeed, ATP/O varies from zero under non phosphorylating conditions (state 4) to the maximal value that can be sustained in the presence of saturating amounts of P_i, ADP, and respiratory substrate (state 3). Thus, by extrapolating these results to the in vivo situation, the growth yield should be decreased when the ATP turnover decreases, because the amount of respiratory substrate consumption required to compensate for the proton leak and proton slippage increases. Furthermore, if one considers the case of adjusting ATP synthesis by adjusting the amount of mitochondria (long-term regulation), the flux through the respiratory chain units can be constant and thus the energy transduction efficiency will be constant.

The complete thermodynamic description of growth processes has been obtained by establishing the balanced chemical reactions for anabolism and catabolism. A crucial parameter for growth evaluation is its yield, i.e. the efficiency of the transformation processes from substrate consumption to biomass formation. The quantification of enthalpy efficiency – the energy converted into biomass divided by the energy input – has been successfully achieved for microorganisms [15–17] as well as for cultured mammalian cells [18]. This approach is based on the continuous measurement of heat production and on the exhaustive determination of substrates and by-products, thus allowing the construction of enthalpy balances (see [1,19] for reviews). Using this approach, we have previously shown [20] that the enthalpic growth yield remains constant during any growth phase in yeast on respiratory substrate (i.e. exponential and transition phases). This constant enthalpic growth yield is owed to a tight adjustment of mitochondrial amount to cellular energy demand [20]. This indicates that molecular mechanisms are involved in the growth yield homeostasis allowing a constant yield all throughout growth for a considered strain. In this review, we will develop the mechanisms involved in the regulation of mitochondrial amount by mitochondrial biogenesis in yeast proliferating on non-fermentable substrate. The signal transduction pathway involved and the cellular consequences of a deregulation of this pathway will be addressed, more particularly in the case of mitochondrial ROS (reactive oxygen species) generation and their cellular consequences.

1.1. Growth yield homeostasis in respiring yeast is due to a strict mitochondrial content adjustment

As stipulated above, we have shown that during yeast cell proliferation on non-fermentable substrate, the growth yield is constant whichever the growth phase. By comparing a number of yeast – *S. cerevisiae* – strains that have very different growth rates, we were able to show that the growth rate is directly proportional to the cellular amount of mitochondrial cytochromes that allow the quantification of the amount of mitochondrial respiratory chains within a cell (Fig. 1). Moreover, we have shown that in all these strains, the same amount of oxygen is necessary to generate 1 g of dry weight [5], which implies that the growth yield is constant. Consequently, in these conditions, the growth rate depends strictly on the cellular amount of mitochondria. In order to go further in the understanding of this process, we investigated the molecular mechanisms involved in the adjustment of mitochondrial amount to cell energy demand.

1.2. Cellular adjustment of mitochondrial content is due to mitochondrial biogenesis

The main process involved in the adjustment of ATP synthesis in response to energy demand is a modulation of cellular mitochondrial amount. Two processes are known to control the cellular steady state of mitochondrial amount: mitochondrial biogenesis and mitochondrial

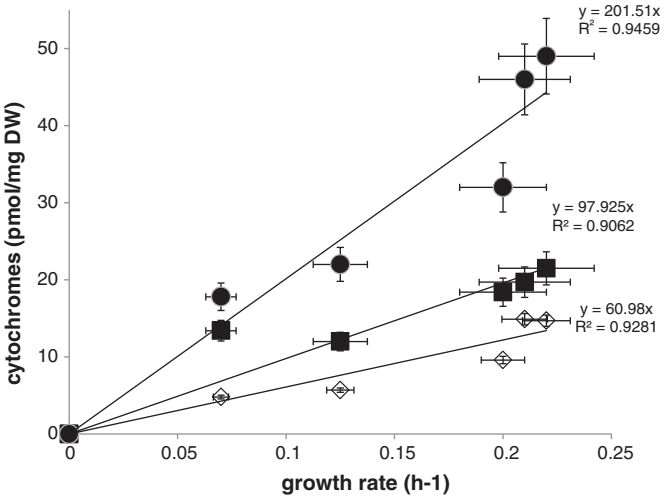


Fig. 1. Relationship between growth rate and the amount of mitochondrial cytochromes. The cellular content in c c1 (●), b (■), and a a3 (◊) hemes was calculated as described in Dejean et al. 2000, taking into account the respective molar extinction coefficient values and the reduced minus oxidized spectra recorded using a dual beam spectrophotometer (Aminco DW2000). Growth was measured spectrophotometrically by assessing the turbidity at 600 nm. Dry weight determinations were performed on samples of cells harvested throughout the growth period and washed twice in distilled water. For each strain, the relationship between dry weight and optical density was determined. The cells were grown aerobically at 28 °C in a synthetic minimal medium (0.175% yeast nitrogen base (Difco), 0.2% casein hydrolysate (Merck), 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 (w/v), pH 5.5, 20 mg·L^{-1} L-tryptophan (Sigma), 20 mg·L^{-1} L-uracil (Sigma), 40 mg·L^{-1} adenine hydrochloride (Sigma) with 2% lactate (w/v) as carbon source.

degradation. These two processes govern the turnover of the mitochondrial compartment. In order to determine whether one of these or both were involved in the cellular adjustment of mitochondrial amount, we assessed both mitochondrial autophagy (mitophagy) and mitochondrial biogenesis in yeast proliferating on non-fermentable substrate (Fig. 2). Both autophagy – that is required for the induction of mitophagy – and mitophagy itself were evaluated in growing yeast thanks to the stability of the GFP protein [21,22]. Autophagy was assessed using ATG8-GFP [22]. Indeed, autophagy-related protein 8 (Atg8) is an ubiquitin-like protein required for the formation of autophagosomal membranes. The transient conjugation of ATG8 to the autophagosomal membrane through an ubiquitin-like conjugation system is essential for autophagy in eukaryotes. Once the autophagosome is ready for fusion with the lysosome, Atg8p gets degraded in the autolysosome. This can be detected by following the GFP protein itself thanks to its stability (Fig. 2A). Mitophagy was assessed in a similar way, as described previously [21] using IDP-GFP, a fusion between mitochondrial isocitrate dehydrogenase and the GFP — Fig. 2B. Fig. 2A&B shows that there is neither an autophagic nor a mitophagic process in growing yeast. Moreover, in yeast, the Hap complex (Hap2/3/4/5 — Scheme 1) has been shown to be involved in the specific induction of genes involved in gluconeogenesis, metabolism of alternate carbon sources, respiration, and mitochondrial development. Indeed, the disruption of any subunits of this complex renders the cells unable to grow on non-fermentable carbon sources [23–26]. Many genes involved in energy metabolism have been shown to be regulated by this complex [27–29]. Assessment of the activity of this complex as a whole – thanks to a previously described lacZ reporter gene – [30] showed no significant variation during yeast growth on non-fermentable substrate (data not shown). Moreover, previous work from our laboratory has shown that the activity of this complex is proportional to the amount of the HAP4 subunit [31]. We thus assessed Hap4p amount in growing yeast, and found that there is a constant amount of Hap4p all throughout growth (Fig. 2C). This data and the fact that the activity of the Hap complex is constant throughout growth indicate a constant mitochondrial

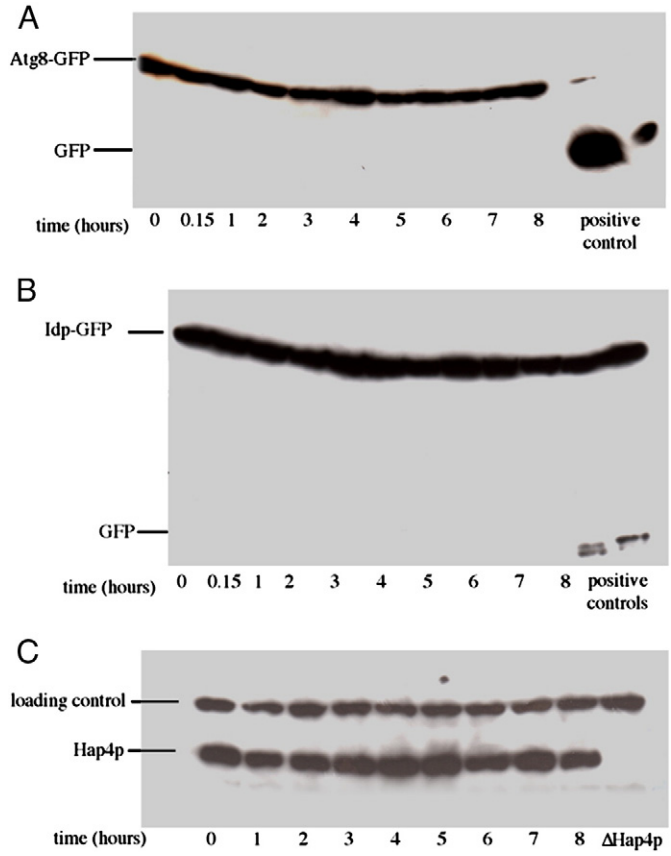


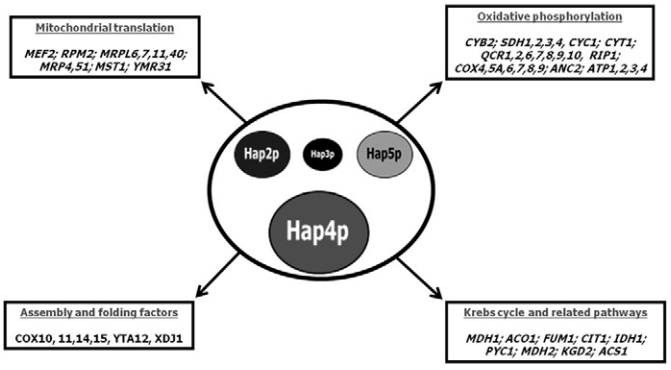
Fig. 2. Mitophagy is not induced during cell proliferation whereas mitochondrial biogenesis is constant. Yeast cells of the strain BY4742 (Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) were first transformed with a plasmid expressing either a fusion protein between the Atg8p and the GFP (Atg8–GFP — A) or a fusion protein between the isocitrate dehydrogenase and the GFP (Idp–GFP — B). Idp–GFP and Atg8–GFP are reporters of the autophagy and mitophagy processes, respectively (see text for details). Positive controls were induced by the addition of rapamycin (0.2 μg/ml) to the culture medium. The cells were grown aerobically at 28 °C in a synthetic minimal medium (0.175% yeast nitrogen base (Difco), 0.2% casein hydrolysate (Merck), 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 (w/v), pH 5.5, 20 mg·L^{-1} L-tryptophan (Sigma), 40 mg·L^{-1} adenine hydrochloride (Sigma) with 2% lactate (w/v) as carbon source. At the indicated time points during growth, cells were collected and protein extracts were resolved by SDS-PAGE in reducing conditions and analyzed by Western-blot with a GFP-antibody (Roche) or Hap4 antibody following the manufacturer's instructions.

biogenesis rate. This shows that in exponentially proliferating yeast, cellular mitochondrial content is tightly controlled by mitochondrial biogenesis.

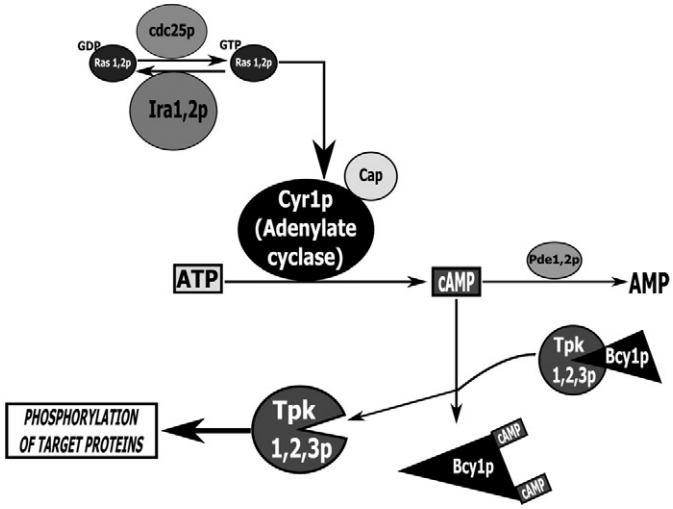
These results raise the question of the role of this tight regulation of mitochondrial biogenesis on cell proliferation. Indeed, the decision to divide is executed either through mitochondrial proliferation, with DNA synthesis, membrane synthesis, mitosis released from energy-limitation and allowed to proceed, or it is made elsewhere to drive DNA replication, membrane synthesis, and the signal to the mitochondria is permissive only, to provide the required fuel for a decision made elsewhere. Results from our laboratory [31] and others show that there is a tight link between cellular phosphate potential and mitochondrial biogenesis. This has been shown in two different ways: either upon uncoupling of the respiratory chain by deletion of the ATPsynthase epsilon subunit – which induces a decrease in cellular phosphate potential and an increase in mitochondrial biogenesis – (Dr E. Tetaud personal communication) or by manipulating the activity of the Ras/cAMP pathway in yeast [31]. These results tend to show that the signal to the mitochondria is permissive only, to provide the fuel required for a decision made elsewhere.

1.3. ROS-induced down-regulation of mitochondrial biogenesis

We then investigated molecular signals that govern the regulation of mitochondrial biogenesis. We were able to show that the Ras/cAMP pathway is the key signal transduction pathway involved in this process (Scheme 2). Indeed, the *ccs1-1* yeast strain, mutated in the *ira2* gene encoding an activating protein of the Ras-GTPase activity, exhibiting an overactivation of the Ras/cAMP pathway, was characterized in the late exponential phase by a higher cellular respiration associated with a higher cytochrome content [32–34]. Moreover, yeast mutants with over-activated Ras/cAMP pathway (i.e., Ras2(val19), Δira1-Δira2) or with a constitutive downstream activation of protein kinases A (i.e., Δbcy1) showed an increase in the cellular mitochondrial amount [35,36]. In contrast, loss of Ras activity (i.e., Ras2 mutant) resulted in a slight decrease in this amount [35]. The yeast harbors three A kinase catalytic subunits, which have greater than 75% identity and are encoded by the *TPK* (*TPK1*, *TPK2* and *TPK3*) genes [37]. Although they are redundant for viability and functions such as glycogen storage regulation, the three A kinases are not redundant for other functions such as pseudohyphal growth, regulation of genes involved in trehalose degradation and water homeostasis as well as iron uptake, which are all regulated by Tpk2p [38–40]. Tpk1p is required for the de-repression of branched chain amino acid biosynthesis genes that seem to have a second role in the maintenance of iron levels and DNA stability within mitochondria [41]. These data provide evidence for a specificity of signaling through the three PKA catalytic subunits. In order to elucidate a potential role of one or more of these subunits in the regulation of mitochondrial biogenesis in response to energy demand during growth, we investigated the role of each one of the TPKs in this process. We have shown that the yeast protein kinase Tpk3p is the catalytic subunit involved in the regulation of mitochondrial amount [42]. In the absence of the yeast protein kinase Tpk3p, a significant decrease in cellular mitochondrial amount occurs, when cells are grown in non-fermentable medium [42]. This generates a drastic decrease in cell growth in the Δ*tpk3* cells versus the wild type cells, since when yeast cells are grown on respiratory substrate, energy transformation processes involve oxidative phosphorylation [5]. Briefly, in Δ*tpk3* cells (i) respiratory rates are decreased in these cells when compared to the wild type cells, (ii) cellular mitochondrial content that was assessed quantitatively by measuring the amount of mitochondrial cytochromes namely aa_3, b and cc_1 is decreased, and (iii) growth rate is decreased. We further investigated the mechanisms involved in the regulation of mitochondrial biogenesis via the yeast protein kinase Tpk3p. We showed that the decrease in mitochondrial content in the Δ*tpk3* cells originates in a decrease in mitochondrial biogenesis. Indeed, the activity of the transcription factors (HAP complex) involved in this process is decreased in the Δ*tpk3* cells



Scheme 1. The HAP complex — master regulator of the mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*. The four subunits constituting the complex are represented here with size in line with the predicted molecular weights of each subunit. Hap2p, Hap3p and Hap5p are the DNA-binding subunits and Hap4p is the activating subunit. The mitochondrial proteins encoding genes regulated by the complex are also indicated. See text for references.



Scheme 2. The Ras/cAMP pathway in the yeast *Saccharomyces cerevisiae*. The synthesis of cAMP by the adenylate cyclase is favored by the active forms (GTP-bound) of the Ras1,2 proteins. In a similar way to their mammalian counterparts, the activation of the cAMP dependent protein kinases (PKA) catalytic subunits (Tpk1,2,3p) is mediated by the release of the regulatory subunit (Bcy1p) after cAMP-binding. The regulation of the cAMP concentration is also dependent on the activity of phosphodiesterases (Pde1,2p) which degrade cAMP in AMP. See text for references.

[43]. Moreover, we showed that the decrease in Hap complex activity is due to an oxidative stress as shown by reversion of the Δtpk3 cell phenotype by an antioxidant as well as by the overexpression of Sod1p — superoxide dismutase. The oxidative stress originates from an increase in mitochondrial ROS production due to the loss of cAMP dependent regulation of mitochondrial ROS production by Tpk3p protein kinase [43]. This was the first report showing that the activity of the HAP complex is sensitive to ROS signaling, clearly involving ROS in mitochondria-to-nucleus signaling. This ROS-induced decrease in the amount of HAP complex is due to a ROS-induced decrease in the amount of Hap4p (functional homolog of PGC1α in mammalian cells) [43]. Moreover, this oxidative-stress sensitivity of Hap4p is a general phenomenon since other oxidative stress inducers also induce a decrease in the amount of this protein (Fig. 3).

Interestingly, in the Δtpk3 cells, although the cells sense the oxidative stress and respond to it by increasing the amount of antioxidant enzymes (i.e. superoxide dismutase and catalase), this increase is not sufficient to suppress the overflow of reactive oxygen species. Such an increase can be deleterious to the cell and is often associated with a mitochondrial malfunction. Through this signaling pathway, the cell protects itself by decreasing mitochondrial biogenesis and thus the amount of dysfunctional mitochondria. Hence, oxidative stress down-regulates mitochondrial biogenesis.

1.4. The glutathione redox state, an intermediate in mitochondrial biogenesis regulation?

In order to further investigate the molecular mechanisms involved in the regulation of Hap4p by oxidative stress, we used a yeast strain in which the activity of the Ras/cAMP pathway can be modulated through exogenous addition of cAMP. In this conditions, various concentrations of cAMP will induce various levels of activation of this pathway and thus of Tpk3p. Cellular cAMP content is under tight control, in particular through its degradation by the type 2 phosphodiesterase gene (*PDE2*) product. The OL556 strain is an engineered *S. cerevisiae* strain that allows manipulation of intracellular cAMP through its addition in the extracellular medium [44]. In this strain, the gene encoding Pde2p is deleted, and Cdc25p activity is attenuated by a point mutation. Extracellular cAMP concentrations of 1–3 mM were used, and led to physiological variations of the intracellular cAMP concentration from 4 to 50 μM [44–46]. As previously shown with Ras/cAMP overactivated strains, cAMP treatment induced an overall increase in mitochondrial amount within the cell proportional to the intracellular cAMP concentration. It should be stressed here that this is associated with a decrease in cellular phosphate potential [31], which again sustains the idea that the signal to the mitochondria is permissive only, to provide the fuel required for a decision made elsewhere. The increase in mitochondrial amount in due to an increased activity of the HAP complex driven by a regulation in the amount of Hap4p, the master regulator of this complex. This increase in Hap4p amount originated in an increase in the protein stability. Moreover, since we had previously shown a crucial role of mitochondrial ROS in the mitochondria-to-nucleus signaling (see above), we investigated whether the cellular redox state was part of the molecular mechanisms governing the regulation of cellular mitochondrial amount and were able to show that an increase in the activity of the Ras/cAMP pathway induced an increase in the intracellular glutathione redox state. Cellular mechanisms that maintain redox homeostasis are crucial, because they provide a buffer against conditions that may perturb the redox environment of cells and/or induce oxidative stress [47–49]. The abundance of glutathione (1–10 mM) in cells and its low redox potential (−240 mV) make the glutathione system a major intracellular redox buffer in most cells [49–51]. Evidence is now accumulating that cell fate and proliferation depend on its redox status [52–55]. We showed that a crucial intermediate in the process of redox control of mitochondrial biogenesis and cell growth is the glutathione redox state. Moreover, the transcriptional complex in charge of mitochondrial biogenesis, the HAP complex, is a key component of this system. It is noteworthy that recent analysis of the cellular consequences of glutathione depletion demonstrated a down-regulation of genes that encode mitochondrial proteins and are regulated by the HAP complex [56]. Using the Δ*glr1* strain, which has a highly oxidized redox status, mitochondrial biogenesis is highly impaired, and this can be reversed by the addition of reduced glutathione to the cells. Altogether, this shows that a crucial intermediate in the regulation of mitochondrial biogenesis is the glutathione redox state, which controls the amount of Hap4p through its stabilization, the master regulator of mitochondrial biogenesis [31]. It is very interesting to note that it has been recently demonstrated that in mammalian cells, the control of PGC1α activity also relies on the glutathione redox state [57].

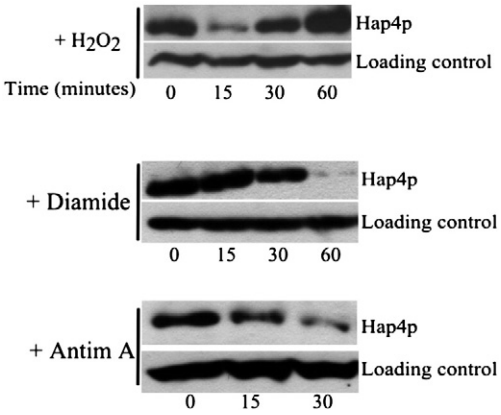


Fig. 3. Oxidative stress inducers decrease the level of Hap4p. Wild type yeast cells were grown aerobically at 28 °C in a synthetic minimal medium (0.175% yeast nitrogen base (Difco), 0.2% casein hydrolysate (Merck), 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 (w/v), 2% lactate (w/v) (Prolabo), pH 5.5, 20 mg·L^{-1} L-tryptophan (Sigma), 40 mg·L^{-1} adenine hydrochloride (Sigma) and 20 mg·L^{-1} L-uracil. H2O2 (400 μM), Diamide (1 mM) or Antimycine A (0.1 μg·mg dry weight^{-1}) was added to the culture and cells were collected at the indicated time points and protein extracts were resolved by SDS-PAGE in reducing conditions. Analyses by Western-blot were performed with a polyclonal antibody against Hap4p. The protein Ade4p was probed as loading control.

2. Conclusion

It is well-established that mitochondrial amount within a cell can vary massively in both unicellular and multicellular organisms. Mitochondria are central platforms in the energy metabolism since they are key sites for ATP production, through the oxidative phosphorylation system (OXPHOS). Thus, one of the main goals of the variation of the mitochondrial amount is to adapt to the variation of the energy demand. As highlighted in this review article, modulating the mitochondrial amount rather than the flux of the OXPHOS machinery allows maintenance of a constant yield of the oxidative phosphorylation machinery and thus allows growth yield homeostasis.

In this adaptation process, the regulation of the synthesis of new mitochondrial proteins (mitochondrial biogenesis) has emerged as a key step. The identification of the transcription factors regulating the expression of the genes encoding for mitochondrial proteins helped to make huge steps forward. Numerous studies are performed to identify the regulatory elements of the activity of these transcription factors. Our previously published studies and the data presented in this article point to the role of oxidative agents in the regulation of the mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae* through the regulation of the co-activator Hap4p. Similar observations have been made about its mammalian counterpart (Pgc1α), notably in severe pathophysiological conditions. Mitochondria being one of the main sites of ROS production, the ROS-mediated downregulation of the mitochondrial biogenesis can be considered like a protective quality-control process. We also showed that the glutathione redox state is an intermediary for the sensing of cell redox stress by the transcription factors involved in the mitochondrial biogenesis regulation. Post-translational consequences of cellular redox perturbation most likely constitute one of the main regulatory processes and the question of the relationship between the cell proliferation and its redox status is a highly discussed research field. Supplementary studies should be performed to address the involvement of other redox players in the fine-tuned regulation of mitochondrial biogenesis.

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