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“Labile” heme plays a critical role in the regulation of mitochondrial biogenesis -through Hap4p- in  
*Saccharomyces cerevisiae*

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Running title: *Labile heme regulates mitochondrial biogenesis*

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## ABSTRACT

Heme (iron protoporphyrin IX) is well-known as prosthetic group for enzymes involved in metabolic pathways such as oxygen transport and electron transfer through mitochondrial respiratory chain. However, heme has also been shown to be an important regulatory molecule (“labile” heme) for diverse processes such as translation, kinase activity and transcription in mammals, yeast and bacteria. Taking advantage of a yeast strain deficient for heme production, we investigated the role of “labile” heme in the regulation of mitochondrial biogenesis. This process is regulated by the HAP complex in yeast. We show for the first

time that cellular “labile” heme is critical for the post-translational regulation of HAP complex activity most likely through the stability of the co-activator Hap4p. Consequently, “labile” heme regulates mitochondrial biogenesis and cell growth. This work adds another mechanism in the field of mitochondrial biogenesis regulation by cell metabolites.

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Mitochondria are organelles that have a critical role in the energy and intermediary metabolism in eukaryotic cells. They are well-known for their involvement in the synthesis of adenosine

triphosphate (ATP), the currency in charge of fulfilling cell energy demand. Mitochondrial ATP synthesis mostly relies on the oxidative phosphorylation system (OXPHOS) which involves enzymatic complexes that form the mitochondrial respiratory chain and the phosphorylation system. It is well-established that mitochondrial enzymatic content within cells vary in order to match ATP synthesis to ATP demand. This phenomenon has been observed in different species and experimental models, ranging from microorganisms -such as yeast- to mammals and is associated to diverse pathologies (1–6).

Although mitochondria possess their own genome (mtDNA), they are genetically semi-autonomous. Indeed, the mtDNA encodes a few (8 in yeast and 13 in mammals) of the numerous proteins constituting the OXPHOS. All the remaining OXPHOS components and mitochondrial proteins (protein machineries involved in mtDNA replication, transcription and translation, assembly factors, mitochondrial proteins import, intermediary metabolism) are encoded by the nuclear genome. Thus, regulation of the expression of both mitochondrial and nuclear genes encoding mitochondrial proteins is controlled by a defined network of nuclear transcription factors.

In the yeast *Saccharomyces cerevisiae*, the transcription factors Hap2p/3p/4p/5p -HAP complex- are the master regulators of mitochondrial biogenesis (7, 8). This heteromeric complex relies on the binding to the promoter of target genes by the DNA binding subcomplex

Hap2p/Hap3p/Hap5p. These three subunits are constitutively expressed. The activation of transcription is mediated by Hap4p, the co-activator subunit (9–13) and the only subunit whose expression is known to be regulated by the carbon source. Deletion of any of the subunits of the HAP complex impairs yeast growth on non-fermentable substrate such as lactate -on which mitochondria are the only source of ATP production-(11). In mammals, due to multicellular organization, more transcriptional regulators are involved, although the general mechanism is the same, with the well-known co-activator PGC-1 $\alpha$  acting like a functional counterpart of the yeast Hap4p (14, 15). Extensive work is still being carried out in order to identify the molecular regulators of the activity of PGC-1 $\alpha$  and Hap4p. On this matter, it is interesting to point out recent results which highlight that both the yeast and mammalian co-activators share common regulatory signals. For example, it has been recently demonstrated that PGC-1 $\alpha$  is regulated by the glutathione redox state, as we previously showed for Hap4p (16, 17). Previous studies also established the involvement of reactive oxygen species (ROS) and cAMP-dependent signaling in negative and positive regulation of the transcriptional regulators of mitochondrial biogenesis in both yeast and mammals (5, 18–21).

Heme (iron protoporphyrin IX) is well-known as a prosthetic group for enzymes involved in metabolic pathway such as oxygen transport and electron transfer through mitochondrial respiratory chain. However, heme has also been shown to be an important regulatory molecule (“labile” heme), for

diverse processes such as translation, kinase activity and transcription in mammals, yeast and bacteria (22–24). Moreover, the hypothesis that heme can be a regulatory molecule for the HAP complex has been previously formulated (11, 25–27). However, no study has been able to establish molecular details surrounding heme regulation of the HAP complex. Even though it has been recently described a heme dependent regulation of Hap4p transcription (28–32). Thus, while the name HAP historically stands for heme activator protein, the HAP complex is sometimes designated like a heme-independent transcription factor (33). Taking advantage of a yeast strain deficient for heme production, we tackled this question. Our results are the first to show that cellular “labile” heme is critical for the HAP complex activity most likely through an enhancement of the stability of the co-activator Hap4p. Consequently, “labile” heme regulates mitochondrial biogenesis and cell growth. This work adds a new contributor in the field of mitochondrial biogenesis regulation by cell metabolites.

## **Results**

### ***Modulation of cellular “labile” heme pool***

Our cell growth experiments were carried out on a non-fermentable substrate: lactate. In this condition, the mitochondrial compartment is well differentiated and mitochondrial oxidative phosphorylation processes and the HAP complex are mandatory for cell growth and proliferation.

The heme biosynthesis pathway is localized within two distinct compartments: the mitochondria and the cytosol (scheme1). In order to modulate cellular “labile” heme pool, we made use of a strain deleted for the first enzyme of this pathway: hem1. This enzyme, which is localized in the mitochondrial matrix, catalyzes the condensation of glycine with succinylCoA to generate delta-aminolevulinic acid (ALA). A strain deleted for this enzyme is auxotrophic for ALA (35). In an attempt to modulate cellular “labile” heme, the  $\Delta hem1$  strain was supplemented with increasing concentrations of ALA and cellular “labile” heme pool was assessed thanks to the reliable HMG2-lacZ reporter gene (35, 36). In this system, expression of the  $\beta$ -galactosidase is under the regulation of the HMG2 promoter which is strongly repressed by the heme level. Figure 1 shows that whereas this promoter’s activity is low in wild type cells, this activity is strongly increased in  $\Delta hem1$  cells grown in the presence of low (5 and 10 $\mu$ g/mL) ALA concentrations. Increasing extracellular ALA concentrations lead to a decrease in this promoter’s activity. This shows that modulation of extracellular ALA in a  $\Delta hem1$  strain allows modulation of the cellular “labile” heme pool. Extracellular ALA concentration above 100 $\mu$ g/mL in both wild type and  $\Delta hem1$  cells did not increase the repression of HMG2-lacZ reporter gene (data not shown).

### ***Modulation of mitochondrial compartment***

As stipulated above, cells were grown on non-fermentable substrate where growth is strictly

dependent on mitochondrial activity i.e. energy conversion processes (ATP synthesis) take place at the mitochondrial level. Further, mitochondrial oxidative phosphorylation requires heme biosynthesis to generate mitochondrial cytochromes. We thus investigated mitochondrial activities in  $\Delta hemI$  cells supplemented with various concentrations of ALA. Figure 2A shows that there is a 50% decrease in  $\Delta hemI$ - $ALA5$ - cellular respiratory rate compared to the wild type cells. Increasing extracellular ALA up to 100 $\mu$ g/mL allowed a full restoration of cellular respiratory rate. Figure 2B shows that this modulation of cellular respiratory rate affected cellular growth rate and the relationship between these two parameters is linear (figure 2B, inset), as previously shown (3). In addition, figure 2C shows that cytochrome c oxidase (one of the most controlling enzyme of the oxidative phosphorylation system (37–39)) activity exhibits a 50% decrease in  $\Delta hemI$ - $ALA5$ - compared to the wild type cells. Increasing extracellular ALA up to 100 $\mu$ g/mL allows a full restoration of this activity. Furthermore, cytochrome c oxidase activity perfectly correlates with respiratory rate (figure 2C, inset).

The above-mentioned results relate to the oxidative phosphorylation amount/activity. In order to determine whether we observed an overall quantitative regulation of the mitochondrial compartment, we assessed citrate synthase activity, a well-accepted marker of mitochondrial amount within cells (40–42). Figure 2D shows that there is a 60% decrease in  $\Delta hemI$ - $ALA5$ - citrate synthase

activity compared to the wild type cells. Increasing extracellular ALA up to 100 $\mu$ g/mL allows a full restoration of citrate synthase activity. Inset in figure 2D shows a very good correlation between respiratory rate and citrate synthase activity, reinforcing the hypothesis of an overall regulation of the mitochondrial compartment upon ALA addition in  $\Delta hemI$  cells.

Within the cell, mitochondria are highly dynamic organelles that form a network. They undergo fusion and fission events continuously, leading to a diverse range of mitochondrial morphologies, from fragmented states to continuous networks (43–45). It remains unclear how the diverse morphologies interact with bioenergetic properties. To determine whether mitochondrial structure/network was altered when mitochondrial oxidative phosphorylation activities decreased, we performed both fluorescence and electronic microscopy under our experimental conditions. Figure 3A shows that when visualized in fluorescence microscopy, mitochondria exhibit a well-differentiated network, comparable to that of the wild type for any of the extracellular ALA concentration tested. Figure 3B shows that when visualized in electron microscopy, mitochondria exhibit a coherent ultrastructure, comparable to that of the wild type for any of the extracellular ALA concentration tested. However, for the lowest ( $\Delta hemI$ - $ALA5$ - &  $\Delta hemI$ - $ALA10$ -) ALA concentrations, a decrease in mitochondrial diameter was observed. For higher ALA concentrations, the mitochondrial diameter was comparable to that of the wild type.

***Cellular “labile” heme pool regulates mitochondrial biogenesis***

The amount of mitochondria within a cell is controlled by its turnover i.e. the respective rates of mitochondrial biogenesis and mitochondrial degradation. The HAP complex has been shown to be involved in the specific induction of genes involved in gluconeogenesis, metabolism of alternate carbon sources, respiration, and mitochondrial development. The disruption of any subunit of this complex renders the cells unable to grow on non-fermentable carbon sources (10, 12, 46, 47). Moreover, many genes involved in energy metabolism have been shown to be regulated by this complex (8, 48). We thus assessed the activity of the HAP complex with a widely used reporter gene, pCYC1-lacZ (49). Figure 4A shows that under low cellular “labile” heme condition ( $\Delta hem1$ - $ALA5$ -) the activity of the HAP complex is highly decreased and this activity increases proportionally with the amount of cellular “labile” heme, being slightly higher than in the wild type for the  $\Delta hem1$ - $ALA100$  condition. The master regulator of the activity of the HAP multicomplex is the subunit Hap4p (11). Cellular amounts of Hap4p were thus assessed under our experimental conditions. Figure 4B shows that the amount of Hap4p is highly increases proportionally with the amount of cellular “labile” heme, being higher than in the wild type for the  $\Delta hem1$ - $ALA100$  condition.

***Hap1p is not involved in “labile” heme induced regulation of mitochondrial biogenesis through Hap4p***

A number of studies have established that Hap1p is the heme sensor that controls the expression of a number of genes encoding heme and oxygen dependent processes (34). In addition, Hap1p controls the expression of Hap4p (32), which is the activation domain of the Hap2/3/4/5 transcription complex (HAP complex). In order to assess whether Hap1p could play a role in heme induced regulation of mitochondrial biogenesis through Hap4p, we generated a  $\Delta hap1$  and a  $\Delta hap1\Delta hem1$  mutant in which most of the experiments mentioned above were repeated. As shown in figure 5, Hap1p deletion did not modify (A) labile heme titration by ALA in the absence of Hem1p (B) the increase in respiratory rate induced by an increase in labile heme (C) the relationship between growth rate and respiratory rate (D) the increase in cytochrome oxidase activity induced by an increase in labile heme (E) the increase in HAP complex activity induced by an increase in labile heme (F) the increase in Hap4p induced by an increase in labile heme. These results clearly show that Hap1p is not involved in the “labile” heme induced regulation of mitochondrial biogenesis through Hap4p.

***Hemin interacts with Hap4p***

As shown above, intracellular Hap4p amount is strongly correlated to the cellular “labile” heme amount. This led us to formulate the hypothesis of an eventual interaction between this protein and cellular “labile” heme. GST-Hap4<sub>(330-554)</sub> was thus purified and *in vitro* interaction between this Hap4p fragment and hemin was tested through spectrophotometric analysis. Indeed it has

been shown that hemin spectra is strongly modified upon its interaction with a protein (50). Figure 6A shows that GST-Hap4<sub>(330-554)</sub> does not exhibit any absorbance spectra whereas hemin exhibits a strong absorbance signal around 390nm, characteristic of the Soret band. Adding GST-Hap4<sub>(330-554)</sub> (4μM) to hemin (4μM) at a stoichiometric concentration induces a shift in the hemin maximal absorbance signal. Further increasing the GST-Hap4<sub>(330-554)</sub> concentration (up to 8μM) did not modify the GST-Hap4<sub>(330-554)</sub>-hemin absorbance spectra. In the inset is shown the differential spectra between hemin and GST-Hap4<sub>(330-554)</sub>-hemin. Furthermore, figure 6B shows that GST alone does not modify the hemin absorbance spectra. In the inset is shown the differential spectra between hemin and hemin in the presence of GST spectra. These results strongly indicate an interaction between hemin and GST-Hap4<sub>(330-554)</sub>.

In order to determine whether the full-length Hap4p is also able to interact with hemin, we performed co-precipitation of Hap4p on whole-cell lysates using hemin-agarose beads. Figure 6C clearly shows that even though in different amounts, hemin and Hap4p (from both wild type and  $\Delta hemI$ - $ALA100$ -) co-precipitate, strongly reinforcing the possibility of an interaction between hemin and Hap4p. The specificity of this interaction was further confirmed by performing a competition experiment between hemin and hemin-agarose. When cell extracts were preincubated in the presence of hemin, there was no detectable interaction between Hap4p and hemin-agarose

(Figure 6D). To further characterize this interaction *in vitro*, a difference spectroscopy titration of hemin binding to Hap4p was performed and an apparent K<sub>d</sub> of about 1.4μM was measured (Figure 6E).

### ***Cellular “labile” heme pool regulates Hap4p stability***

Our results show that not only cellular “labile” heme amount correlates with the amount of Hap4p but also that Hap4p and hemin can interact *in vitro*. Based on these results, we hypothesized that cellular “labile” heme could play a role in regulating the amount of Hap4p through a stabilization of this protein. Consequently, Hap4p turn-over was assessed through cycloheximide addition to cells under conditions where cellular “labile” heme amount is modulated (see figure 2). Figure 7 clearly shows that increasing cellular “labile” heme increases Hap4p half-life. Indeed, with the lowest concentrations of ALA ( $\Delta hemI$ - $ALA5$ - and  $\Delta hemI$ - $ALA10$ -), Hap4p underwent a decrease of 60% in 3 minutes and 70% in 7 minutes while with high concentrations of ALA ( $\Delta hemI$ - $ALA25$ - and  $\Delta hemI$ - $ALA100$ -) in 3 minutes Hap4p is only reduced by 30% and 50% after 7 minutes similarly to the WT with a low variability between the experiments (Table I).

### ***A non-metabolizable analogue of heme up-regulates mitochondrial biogenesis***

The experiments stated above point to a regulation of mitochondrial biogenesis by cellular “labile” heme. However, in these experiments, “labile” heme levels were manipulated through

ALA addition to a  $\Delta hemI$  strain. ALA is the second metabolite of the heme biosynthesis pathway and can be incorporated in hemoproteins. To strengthen our observations, we decided to reproduce our experiments with the Deuteroporphyrin IX (DP IX), a so-called gratuitous inducer (51). DP IX is a more soluble analog of protoporphyrin IX, which is the penultimate product of the heme biosynthesis pathway (scheme 1) and can be matured by the ferrochelatase. Hence, DP IX is another way to increase intracellular heme level and to emphasize the involvement of the heme molecule in regulatory processes. However, once matured by ferrochelatase, this molecule cannot be incorporated into hemoproteins (51). Consequently, a  $\Delta hemI$  strain cannot grow with DP IX alone (data not shown) and experiments performed with DP IX were done in the presence of the lowest concentration of ALA used all throughout our experiments.

Figure 8A shows that DP IX supplementation to  $\Delta hemI-ALA5-$  cells restores cellular “labile” heme to the wild type level. Figure 8B shows that DP IX supplementation to  $\Delta hemI-ALA5-$  cells strongly increases respiratory rate in such a way that it reaches 90% of the wild type respiratory rate. Growth and cytochrome c oxidase activity are partially restored by DP IX supplementation to  $\Delta hemI-ALA5-$  cells (figure 8C&D). Figure 8E shows that DP IX supplementation to  $\Delta hemI-ALA5-$  cells restores citrate synthase activity to the wild type level.

We then assessed whether DP IX was able to regulate HAP complex activity. Figure 9A shows

that DP IX supplementation to  $\Delta hemI-ALA5-$  cells significantly increases HAP complex activity. Further, in these conditions, Hap4p levels are similar to the ones assessed in the wild type cells (Figure 9B). Last, DP IX supplementation to  $\Delta hemI-ALA5-$  cells strongly decreases Hap4p turnover (Figure 9C).

Taken together, the results obtained by DP IX supplementation to  $\Delta hemI-ALA5-$  cells clearly show that cellular “labile” heme regulates mitochondrial biogenesis.

## **Discussion**

In this paper, making use of a strain deleted for the first enzyme of the heme biosynthesis pathway, we were able to modulate the intracellular “labile” heme concentration. On non-fermentable growth substrate, this “labile” heme concentration modulation positively regulates cellular mitochondrial content. We investigated the origin of this regulation and were able to show that intracellular “labile” heme regulates mitochondrial biogenesis. Indeed, the activity of the master regulator of this process, the HAP complex positively correlates with the cellular “labile” heme concentration. Moreover, both the amount and the stability of the co-activator of this complex, Hap4p positively correlates with the cellular “labile” heme concentration. Further, we were able to show that hemin and Hap4p interact directly *in vitro* as well as by coprecipitation. In vitro experiments allowed us to assess an apparent Kd of about 1.4 $\mu$ M. While that Kd seems quite weak, a/ it is in the same range than the one measured for Mss51, a COX1 mRNA-



specific translational activator and COX chaperone(52) and b/ one should keep in mind that this is *in vitro* measurements that might not completely reflects *in vivo* affinities that can be highly modified for example in the case of channeling processes. It is tempting to speculate that the HAP4-“labile” heme interaction allows an increase in Hap4p stability which itself will increase mitochondrial biogenesis.

It should be pointed out here that despite an important modulation of mitochondrial activities in our experimental conditions, no alteration of mitochondrial network was assessed. Which shows that there is no simple correlation between cellular mitochondrial activities and mitochondrial network.

It has previously been shown that in wild type cells, heme is a limiting factor for Hap4p transcription (32). However, this is only the case in glucose-grown cells, where heme intracellular concentration is increased by supplying the cells with either ALA or hemin. In galactose-grown cells, a situation closer to our study in non-fermentable medium (no glucose repression of oxidative metabolism) neither heme or ALA (at a concentration three times higher than the one used in our study) regulated Hap4p transcription (32). Such a regulation is thus unlikely under our experimental conditions. Further, our assessment of Hap4p half-life clearly shows that this parameter is positively regulated by “labile” heme. To our knowledge, our results are the first ones evidencing a post-translational regulation of the HAP complex.

A crucial point in our study is the link between heme and mitochondrial oxidative phosphorylation. Indeed, heme is mandatory for mitochondrial biogenesis and the generation of mitochondrial cytochromes. Consequently, to evidence the regulation of mitochondrial biogenesis by cellular labile heme implies a distinction between heme that will lead to the generation of cytochromes and labile heme that is involved in cell signaling. To do so we made use of deuteroporphyrin IX, a gratuitous inducer analogous to labile heme (51). This allowed us to show that (i) even at low ALA concentration, an increase in deuteroheme -thanks to DP IX- allows an increase in mitochondrial biogenesis. This indicates that low ALA concentration is not controlling mitochondrial biogenesis through cytochromes generation. (ii) An increase in deuteroheme increases mitochondrial biogenesis through an increase in HAP activity and Hap4p stability.

In conclusion, we show that in the yeast *Saccharomyces cerevisiae*, mitochondrial biogenesis is positively regulated by “labile” heme, via an increase of the HAP4 protein’s stability most likely through its binding to “labile” heme (scheme 2).

## **Experimental procedures**

### Yeast strains, plasmids, and culture medium

The following yeast strains were used in this study: BY4742 (*MAT $\alpha$  ura3 $\Delta$ 0 lys2 $\Delta$ 0 leu2 $\Delta$ 0 his3 $\Delta$ 0*) (Euroscarf);  *$\Delta$ hem1* (*MAT $\alpha$  ura3 $\Delta$ 0 lys2 $\Delta$ 0 leu2 $\Delta$ 0*)

*his3Δ0 ydr232W::kanMX4*) supplied by Dr T. Ferreira (Poitiers, France). Where indicated, yeast strains were transformed with either of the following plasmids: the reporter gene HMG2-LacZ (35, 36); pCYC1-lacZ (pLG669Z) (49); or pVT100U-mtGFP (53). The GST-HAP4(330-554) plasmid (54), a kind gift of Dr. Berger, was expressed in bacteria for protein purification.  $\Delta hap1$  and  $\Delta hap1\Delta hem1$  strains were obtained by homologous recombination of the *hap1::HIS3MX6* deletion cassette in BY4742 and  $\Delta hem1$  strains, respectively. The 1.4kb deletion cassette was PCR-amplified from the plasmid pFA6a-HIS3MX6 (55) using the forward F1-HAP1 (5'GGGAACAATAGGTTAGATGTCTGAATACC CCTTATAATTCACGGATCCCCGGGTAAATTA A) and reverse R1-HAP1 (5'CCATCAATTTCAACATTATCAACGGGTAG AAAATCAACCGGAATTCGAGCTCGTTTAAA C) primers (underlined nucleotides refer to the sequence homology to HIS3MX6 cassette and the remaining of the sequence to HAP1 locus) according to (56). Clones were screened by PCR for integration by homologous recombination using HAP1-Fwd(5'GAACCTCATTCCATCAACTTCC) as a forward primer and either HAP1-Rev (5'TGTTCCATGTAGTGGCAGAG) located in the original HAP1 coding sequence or TEFpro-Rev (5'GATGTGAGAAGTGTATCCTAGC) located in the deletion cassette, as reverse primers.

Cells were grown aerobically at 28°C in the following medium: 0.175% yeast nitrogen base

(Difco), 0.2% casein hydrolysate (Merck), 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.2% lactate (w/v) (Prolabo), pH 5.5, 20mg.L<sup>-1</sup> L-tryptophan (Sigma), 40mg.L<sup>-1</sup> adenine hydrochloride (Sigma) and 20mg.L<sup>-1</sup> uracil (Sigma). When cells carried a plasmid, uracil was omitted in the growth medium. Where indicated, 5/10/25 or 100μg.mL<sup>-1</sup> 5-aminolevulinic acid (Frontier Scientific) and/or 2.5μg.mL<sup>-1</sup> Deuteroporphyrin IX (Frontier Scientific) were added to the medium. Growth was measured at 600nm in a Safas spectrophotometer (Monaco). Dry weight determinations were performed on samples of cells harvested throughout the growth period, washed twice in distilled water and weighed after extensive drying.

#### Oxygen consumption assays:

The oxygen consumption was measured polarographically at 28°C using a Clark oxygen electrode in a 1mL thermostatically controlled chamber. 1mL of culture was transferred to the chamber and respiratory rates (JO<sub>2</sub>) were determined from the slope of a plot of O<sub>2</sub> concentration versus time. The measured activities are normalized per mg dry weight.

#### β-galactosidase activity measurement:

Cells were harvested from cultures at OD<sub>600nm</sub> 0.5-1 and permeabilized using a standard procedure as described in (57). After a 30 minutes preincubation period, 0.4mg/mL ONPG (O-nitrophenyl-b-D-galactopyranoside) were added and the tube was

briefly vortexed. The reaction was stopped after a yellow color had developed by addition of 0.5M Na<sub>2</sub>CO<sub>3</sub>. The samples were centrifuged for 30 seconds at 14000g and the absorbance of the supernatant read at 420nm.  $\beta$ -galactosidase activity is expressed in arbitrary units per OD<sub>600nm</sub> and per minute (57).

#### Proteins Extraction, Electrophoresis, and Western-Blot

Cells were lysed using a mixture of 7.5%  $\beta$ -mercaptoethanol in 1.85M NaOH. After 10 minutes incubation on ice, proteins were precipitated by the addition of an equal volume of 3M trichloroacetic acid for 10minutes on ice. After a rapid centrifugation at 4°C, the protein pellet was suspended in a mixture of 10% SDS and sample buffer (0.06M Tris, 2% SDS, 2%  $\beta$ -mercaptoethanol, 5% glycerol, 0.02% bromophenol blue). Protein amounts corresponding to 0.5 OD units of cells were separated by 10% SDS-PAGE performed according to the method of Laemmli. After electro-transfer onto nitrocellulose membranes (Amersham Biosciences), proteins were probed with the desired primary antibodies:  $\alpha$ -Hap4p and  $\alpha$ -PGK-1 (monoclonal antibody, Invitrogen) and detected using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) ECL Prime reagent (Amersham Biosciences), according to the manufacturer instructions. Signal quantifications were done using the ImageJ software.

#### Protein degradation assay

To quantify Hap4p life span, 1mL of each cell culture was centrifuged at 4°C, corresponding for T0. Then, 0.4mg/mL cycloheximide were added to the cultures. After 3 and 7 minutes, 1mL of the cultures was centrifuged as previously. The dry pellets were conserved at -20°C until proteins extraction and western blot, as described above.

#### Citrate synthase activity

Cells were lysed in 50mM Tris-HCl (pH 7.5) buffer and glass beads with 5 cycles (20 sec at 4m/s) of FastPrep™. Citrate synthase (EC 4.1.3.7) activity was determined spectrophotometrically (Safas Monaco) by monitoring at 412 nm the oxidation of coenzyme A (produced by citrate synthase activity) by 5,5'-dithiobis-2nitrobenzoic acid (DTNB) over time in the following buffer: 50mM Tris-HCl (pH 7.5), 0.1mM acetyl-CoA, 0.2mM DTNB, and 0.5mM oxaloacetate. The enzyme activity was calculated using an extinction coefficient of 13 600M<sup>-1</sup>.cm<sup>-1</sup> at 412nm for DTNB. One citrate synthase unit was equal to 1 $\mu$ mole of DTNB reduced per minute per mg dry weight.

#### Cytochrome c oxidase activity

Cells were lysed in 50mM potassium phosphate (pH 7.5) buffer and glass beads with 5 cycles (20 sec at 4m/s) of FastPrep™. Cytochrome c oxidase (COX) activity (1mM KCN sensitive) was determined by monitoring spectrophotometrically (550nm) the rate of disappearance of reduced

cytochrome c at 28°C in the following buffer: 50mM PiK, 100µM reduced cytochrome c. The enzyme activity was calculated using an extinction coefficient of 18 500M<sup>-1</sup>.cm<sup>-1</sup> at 550nm for reduced cytochrome c (58, 59).

#### Hemin absorption spectra

Recombinant Glutathione-S-transferase (GST) and GST fused to the C-terminal region of Hap4p (330-554, containing the transcriptional activation domain) were purified from bacterial lysate after 3 hours induction with 1mM IPTG. Bacterial cells were washed once in the following buffer: HEPES 25mM, KCl 50mM, glycerol 10%, EDTA 0.5mM, 10mM β-mercaptoethanol, 1mM PMSF, pH 7.7. After three freeze-thaw cycles, cells were sonicated on ice and centrifuged 15 minutes at 16000g. Purification was performed on the supernatant using GST-sepharose 4B resin (GE healthcare) using manufacturer recommendations. The spectra of hemin (Sigma Aldrich) was recorded in absence and presence of different concentrations of the recombinant proteins in potassium phosphate buffer (0.1M; pH 7) using a spectrophotometer (Safas).

Heme titrations were performed by difference spectroscopy at room temperature and aerobically using the Safas spectrophotometer. A concentration of 100nM of the recombinant GST-Hap4p was used in potassium phosphate buffer (0.1M; pH 7). After the baseline was set (Hap4p 100nM against buffer), equal concentrations of hemin were added to the reference and sample cuvette (0.025 to 15µM) to

reflect the binding of recombinant protein and hemin and not the absorbance of the free ligand. To determine the binding parameters, the data obtained from the heme titrations were plotted and fit to an equation describing a single binding site using the GraphPad Prism Software.

#### Hemin co-precipitation assay

Cells were lysed in the following buffer: 50mM Tris, 100mM NaCl, 5mM EDTA, 5% glycerol, 1mM PMSF, pH 7,5 (IP buffer) and an anti-protease cocktail (cOmplete; Roche), with 4 cycles (20 sec at 4m/s) of FastPrep™. For the co-precipitation, 50µL of 50% v/v hemin-agarose or agarose beads (Sigma) were added to 500µL of supernatant corresponding to 80 OD units of cells and incubated for 2 hours at 4°C. Beads were then washed with the IP buffer and proteins were eluted with a mixture of 10% SDS and sample buffer. Inputs were samples without incubation with hemin-agarose beads and precipitated in 35% TCA. Protein pellets were resuspended in a mixture of 10% SDS and sample buffer. After quantification (BioRad), proteins were separated by SDS-PAGE using precast Nupage 4-12% Bis-Tris Plus Gels (Invitrogen).

#### Antibodies

Polyclonal anti-HAP4 antibodies were generated by Eurogentec using the HAP4 fragment 330-554 as an antigen. Phospho-glycerate kinase antibody was a commercial antibody (PGK1; Invitrogen)

#### Epifluorescence Microscopy

For each condition, 900 $\mu$ L of cell cultures were added to 100 $\mu$ L 37% paraformaldehyde. After 8 minutes of RT incubation, samples were pelleted, washed with 1mL of PBS and pellets were resuspended in PBS. Cells were observed in a fully automated inverted microscope Olympus using a 100 $\times$  1.4 NA Plan-Apochromat objective. Stacks of fluorescence images were collected automatically at 0.2 $\mu$ m Z-intervals. The deconvolution of each Z-series was automatically computed with two plugins of ImageJ: PSF generator and DeconvolutionLab. To obtain a 3D image, the deconvolutions were treated with the software Chimera (UCSF).

#### EM Microscopy

The yeast pellets were placed on the surface of a copper EM grid (400 mesh) that had been coated with formvar. Each loop was very quickly submersed in liquid propane precooled and held at -180°C by liquid nitrogen. The loops were then

transferred in a pre-cooled solution of 4% osmium tetroxide in dry acetone in a 1.8 mL polypropylene vial at -82°C for 72 hours (substitution), warmed gradually to room temperature, followed by three washes in dry acetone. Specimens were stained for 1 hour in 1% uranyl acetate in acetone at 4°C, blackroom. After another rinse in dry acetone, the loops were infiltrated progressively with araldite (epoxy resin Fluka). Ultrathin sections were contrasted with lead citrate and observed with a Hitachi 7650 electron microscope (Bordeaux Imaging Center - Electron Microscopy Pole of the University of Bordeaux Segalen).

#### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Statistical analysis was carried out using ANOVA test for all results. Prism software (GraphPad, San Diego, CA) was used for all tests. A p-value of less than 0.05 was considered significant.

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article

**Abbreviations:** HAP : heme activator protein; ATP : adenosine triphosphate; OXPHOS : oxidative phosphorylation; DNA : deoxyribonucleic acid; mt DNA : mitochondrial DNA; PGC-1: PPAR $\gamma$  coactivator-1; ROS: reactive oxygen species; GFP : green fluorescent protein; DP IX : deuteroporphyrin IX; JO<sub>2</sub> :

respiratory rate; ONPG : O-nitrophenyl-b-D-galactopyranoside; PGK-1 : phosphoglycerate kinase 1; DTNB : 5,5'-dithiobis-(2-nitrobenzoic acid); COX : cytochrome c oxidase; KCN : potassium cyanide; GST : Glutathione-S-transferase; TCA : Trichloroacetic acid; ALA :  $\delta$ -aminolevulinate; HEM1 :  $\delta$ -aminolevulinate synthase

**Author contributions:** CB, EDY, LERV, and BS designed and performed the experiments CB, EDY, BS, MR, SDC, and AD analyzed the corresponding results. CB and EDY wrote the paper with SDC, MR and AD.

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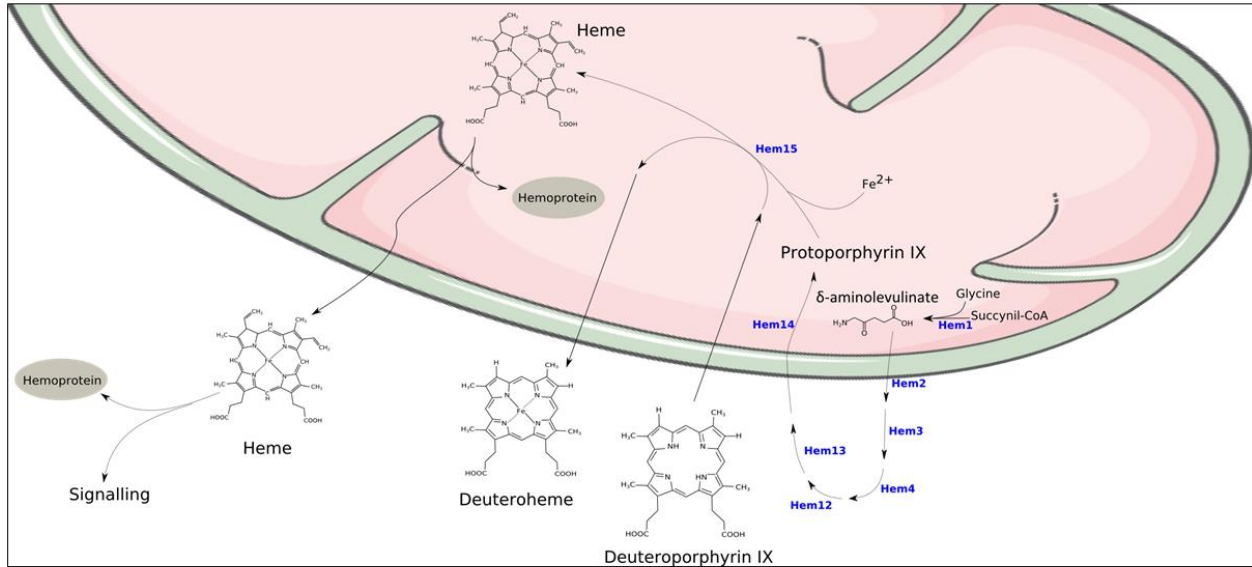
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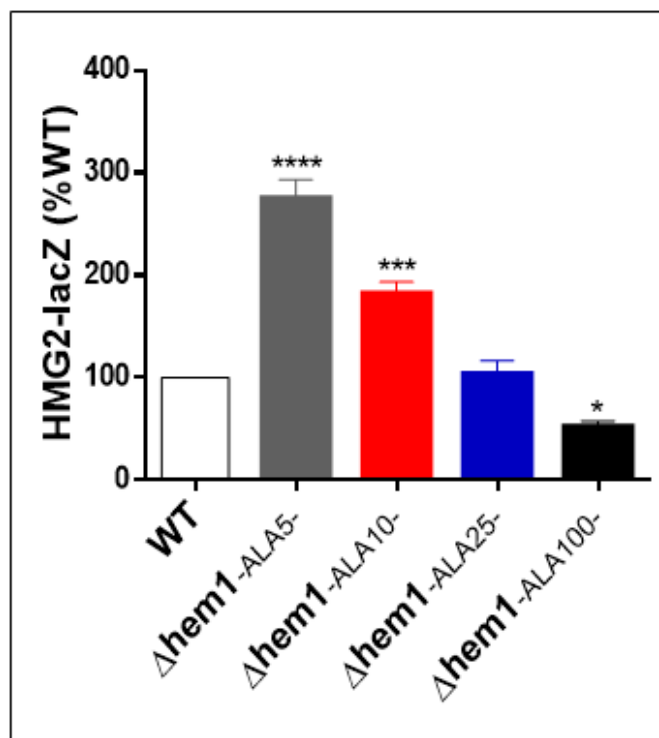
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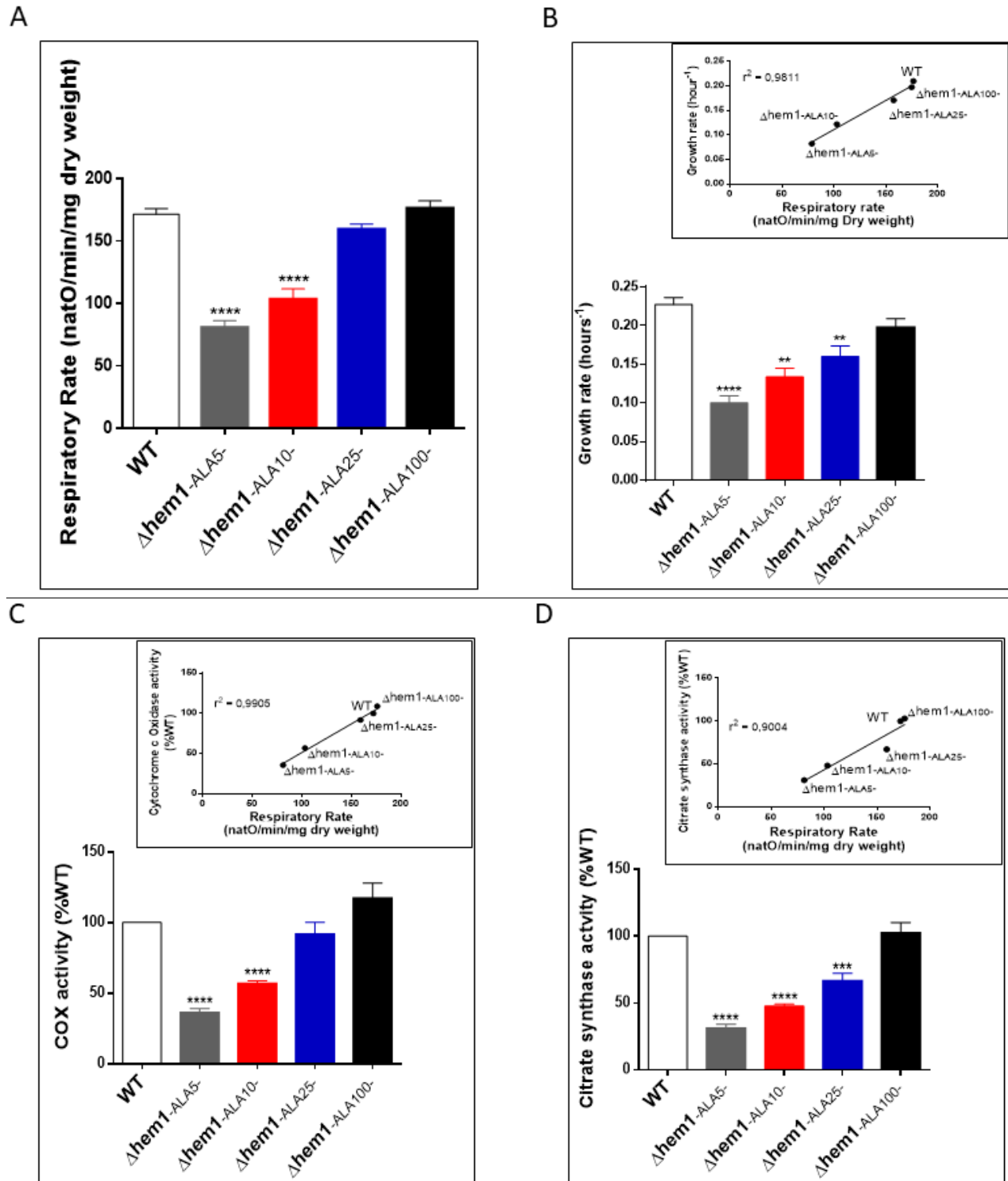
FIGURE LEGENDS



**Scheme 1: Overview of the heme synthesis pathway in yeast.** Heme is synthesized from Glycine and Succinyl-CoA in a pathway initiated (Hem1, 5-aminolevulinic acid synthase) and terminated inside mitochondria (Hem14, protoporphyrinogen oxidase; Hem15, ferrochelatase) with a number of intermediary reactions taking place in the cytosol (Hem2, ALA dehydratase; Hem3, porphobilinogen deaminase; Hem4, uroporphyrinogen III synthase; Hem12, uroporphyrinogen III decarboxylase; Hem13, coproporphyrinogen III oxidase). Heme can then be complexed in mitochondrial or cytosolic Hemoproteins, or act in signaling pathways. Deuteroporphyrin IX is an analogue of protoporphyrin IX that is much more soluble and stable and can be converted to deuteroheme (an analogue of heme) by the ferrochelatase enzyme, but cannot be incorporated into Hemoproteins.

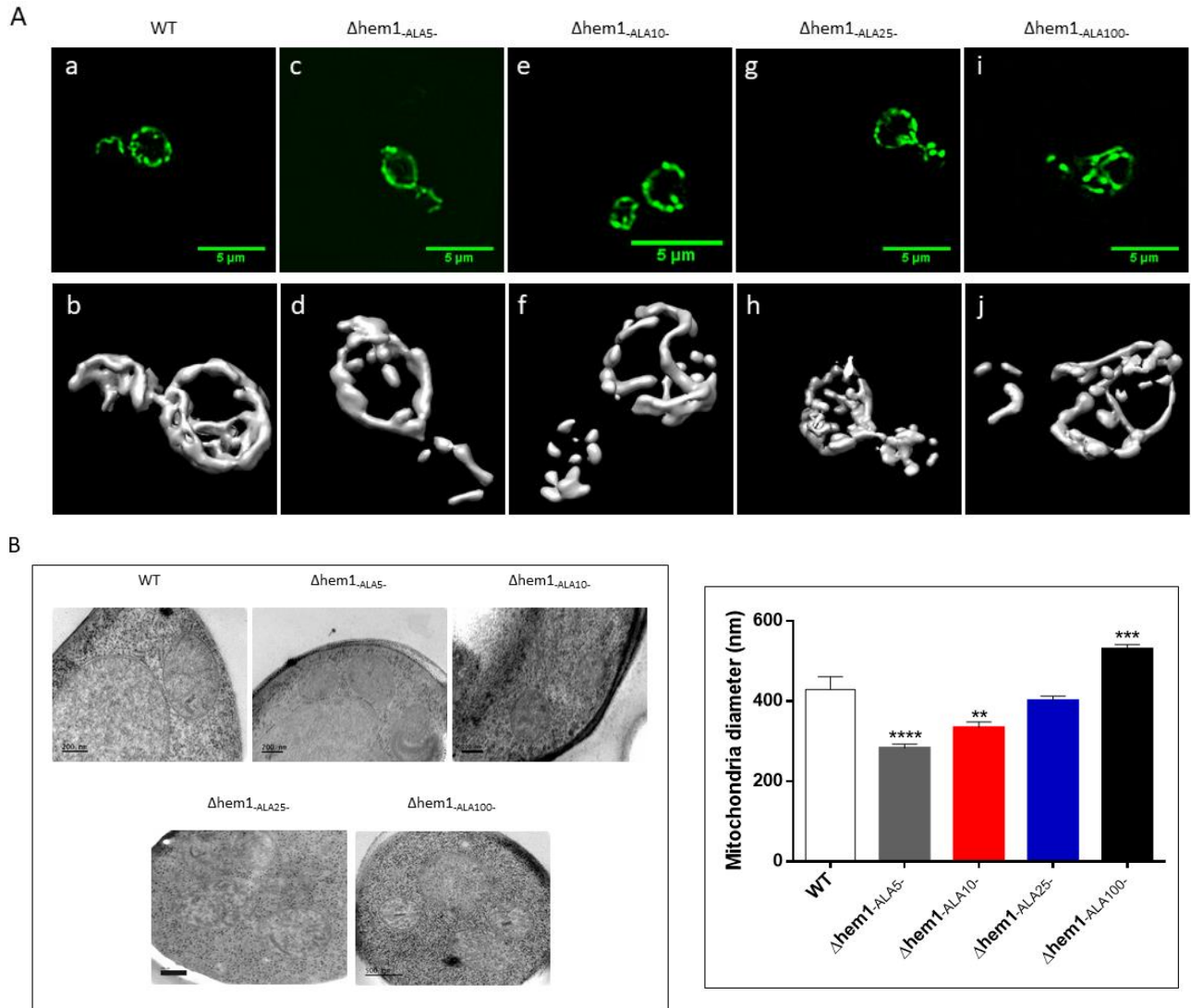


**Figure 1: Assessment of the “labile” heme level through the activity of HMG2 promoter-lacZ gene at different concentrations of ALA.** Wild type (WT) and  $\Delta hem1$  cells were transformed with a plasmid encoding the reporter gene HMG2-LacZ that is negatively regulated by “labile” heme. The growth medium of  $\Delta hem1$  cells was supplemented with  $5\mu\text{g.mL}^{-1}$  ( $\Delta hem1$ -ALA5-),  $10\mu\text{g.mL}^{-1}$  ( $\Delta hem1$ -ALA10-),  $25\mu\text{g.mL}^{-1}$  ( $\Delta hem1$ -ALA25-) or  $100\mu\text{g.mL}^{-1}$  ( $\Delta hem1$ -ALA100-) of ALA ( $\delta$ -aminolevulinic acid). Cells were harvested and  $\beta$ -galactosidase activities were measured and expressed as percentage of WT. Results shown represent means of at least three separate experiments  $\pm$  SEM.



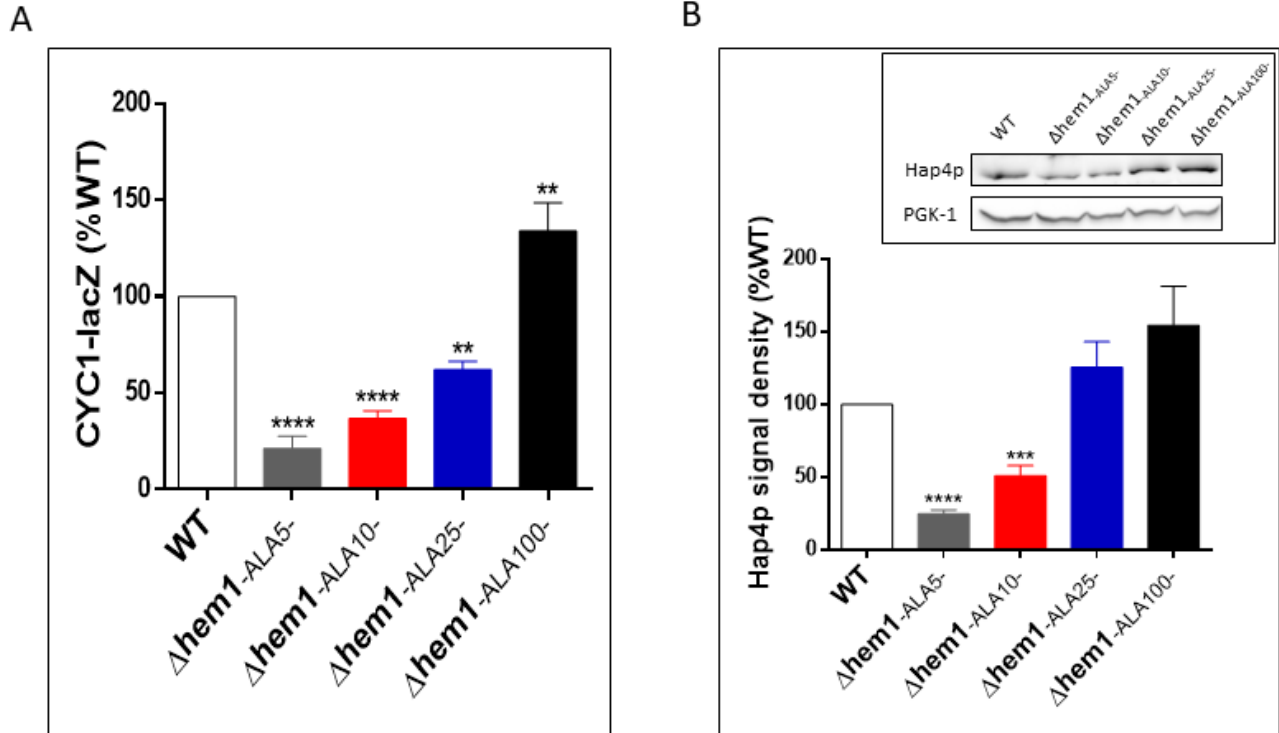
**Figure 2: Effect of ALA addition on respiratory rates, growth, cytochrome c oxidase and citrate synthase activities in wild type and  $\Delta hem1$  cells.** Wild type (WT) and  $\Delta hem1$  cells were grown aerobically in the presence of increasing concentrations of ALA for the latter as described in Figure 1. Each culture was

subjected to several analyses during exponential phase all repeated 5 times over a 4 hours period. The experiments were reproduced at least three times with independent cultures. (A) Spontaneous respiratory rates measured on cultures in the different conditions are shown. Given values for each condition are means of all data points  $\pm$  SEM. (B) Growth rate measured on cultures in the different conditions are shown. Given values for each condition are means of all data points  $\pm$  SEM. Inset: A linear regression line between respiratory rate and growth rate is presented for which the determination coefficient is indicated ( $r^2$ ). Growth rates for each condition were calculated in each experiment from measured OD<sub>600nm</sub> of cultures over a 4 hours period. (C) Cytochrome c oxidase activities measured on cultures in the different conditions are shown. Given values for each condition are means of all data points  $\pm$  SEM. Inset: A linear regression line between respiratory rate and cytochrome c oxidase activity is presented for which the determination coefficient is indicated ( $r^2$ ). (D) Citrate synthase activities measured on cultures in the different conditions are shown. Inset: A linear regression line between respiratory rate and citrate synthase activity is presented for which the determination coefficient is indicated ( $r^2$ ). Given values for each condition are means of all data points  $\pm$  SEM

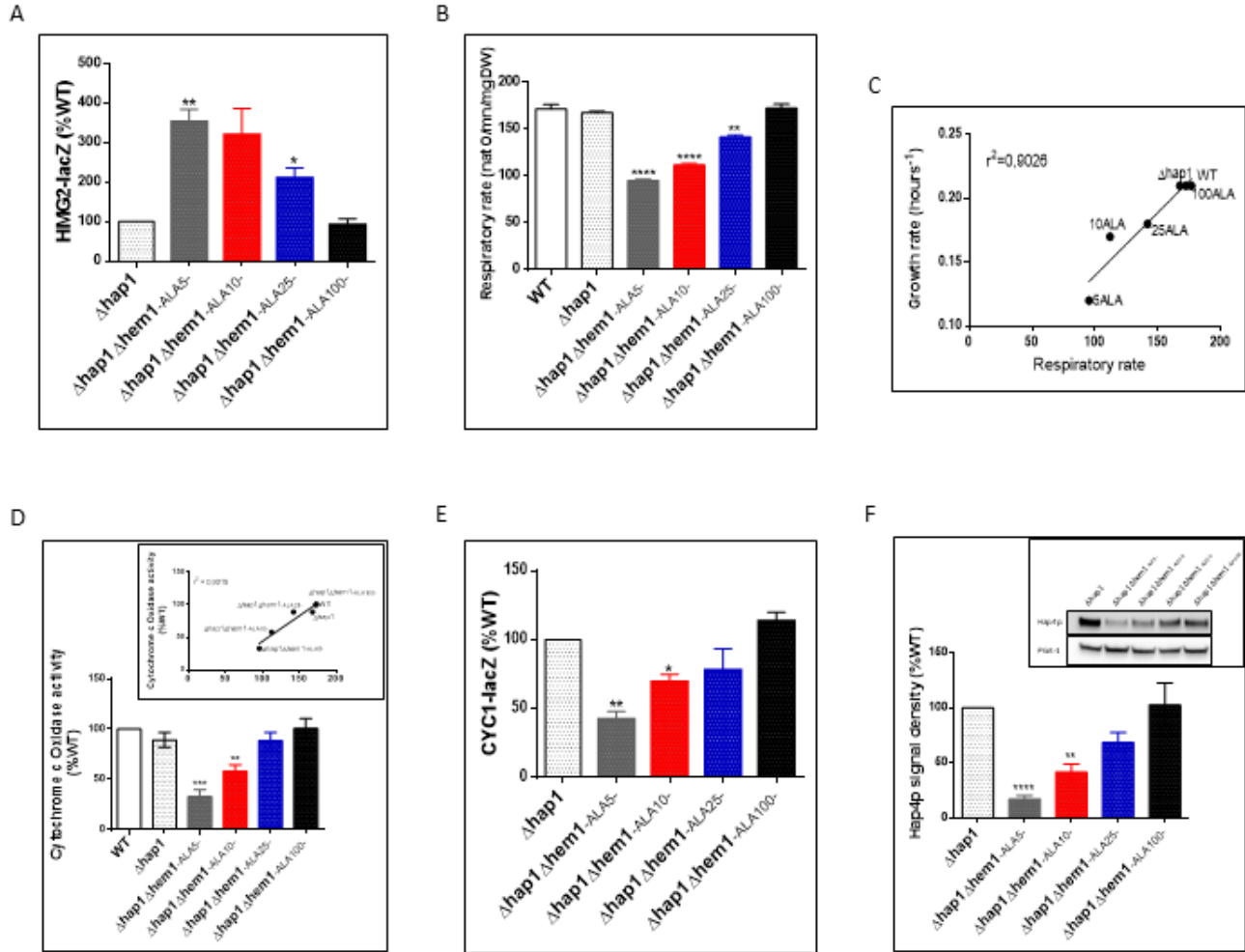


**Figure 3: Effect of ALA addition on mitochondrial network and ultrastructure in WT and mutant cells cultured in the different conditions.** (A) Mitochondrial network morphology observed by epifluorescence microscopy. WT or mutant cells were transformed with pVT100u-mtGFP, and observed by fluorescence microscopy. Representative images are shown for each condition. Deconvolved fluorescence images (higher panel) and 3D reconstructed images are shown (lower panel). (B) Electron micrographs of cells with increasing concentrations of ALA. Ultrastructural studies were performed as described under Experimental procedures. Images of representative cells shown observed in the considered conditions are shown. Average diameter of mitochondrial sections observed as described in B in the different conditions. More than 2 sections from 7 different cells in total were analyzed in each condition. The bar-graph shows mean  $\pm$  SEM for all experiments.



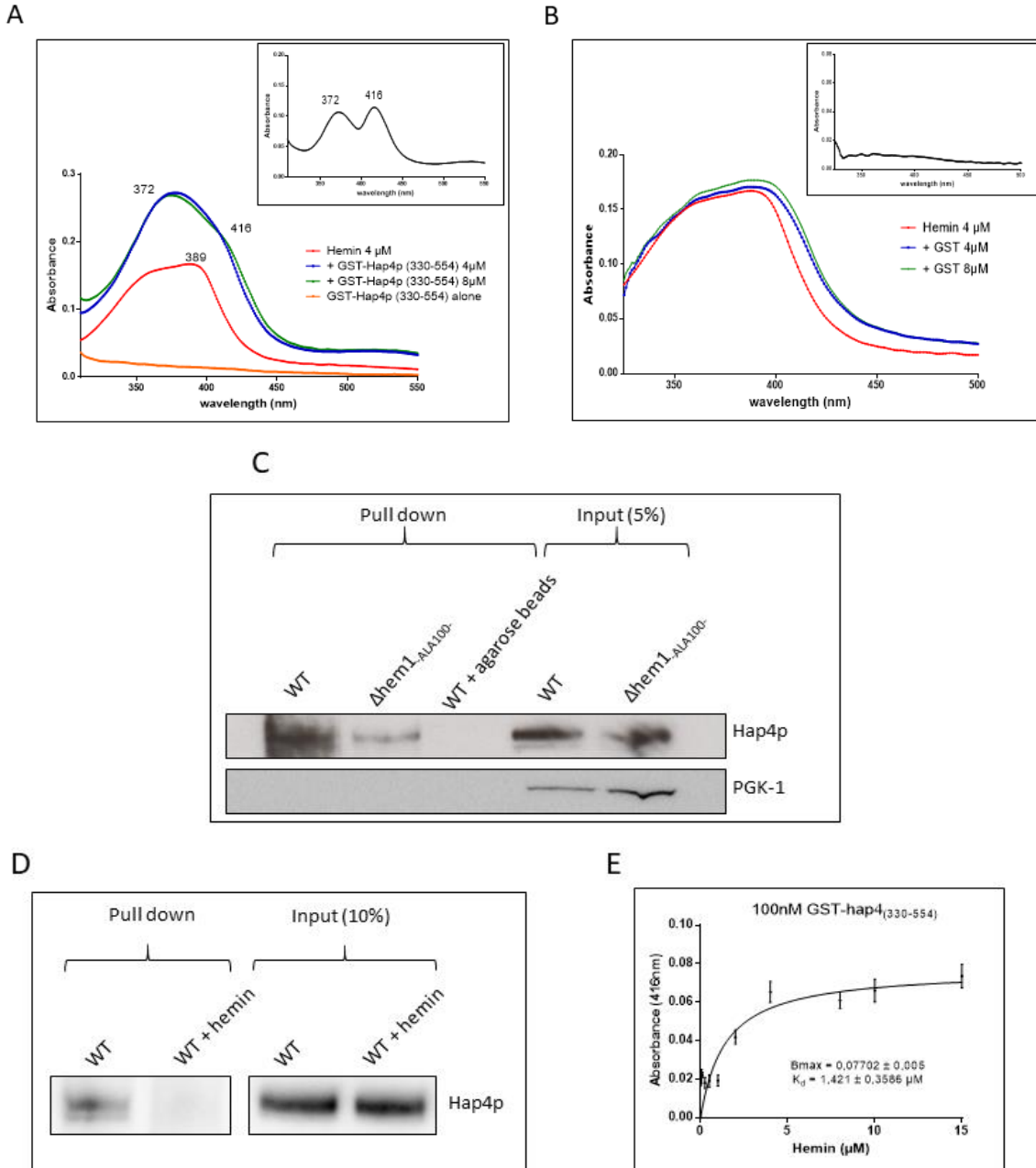


**Figure 4: Effect of ALA addition on the HAP complex activity in wild type and  $\Delta hem1$  cells.** (A) WT and  $\Delta hem1$  cells were transformed with the plasmid p-CYC1-LacZ bearing the lacZ reporter gene under the control of the CYC1 promoter regulated by the HAP transcription factor. The cells were grown in the conditions described in Figure 1 and  $\beta$ -galactosidase activities were measured. Results are mean % of WT  $\pm$  SEM of at least three measurements performed on independent cell cultures. (B) The relative levels of the co-activator hap4 were determined in WT and  $\Delta hem1$  cells in the presence or the absence of the different concentrations of ALA (see legend to figure 1 for ALA concentrations). Total protein extracts were analyzed by SDS-PAGE and western blot using antibodies directed against Hap4p and phospho-glycerate kinase (PGK1) as a loading control. Results representative of at least four experiments are shown in the inset. Hap4p signal was quantified. Signal intensity was normalized to PGK1 signal and expressed in percentage of WT for each experiment. The bar-graph shows mean % of WT  $\pm$  SEM for all experiments.



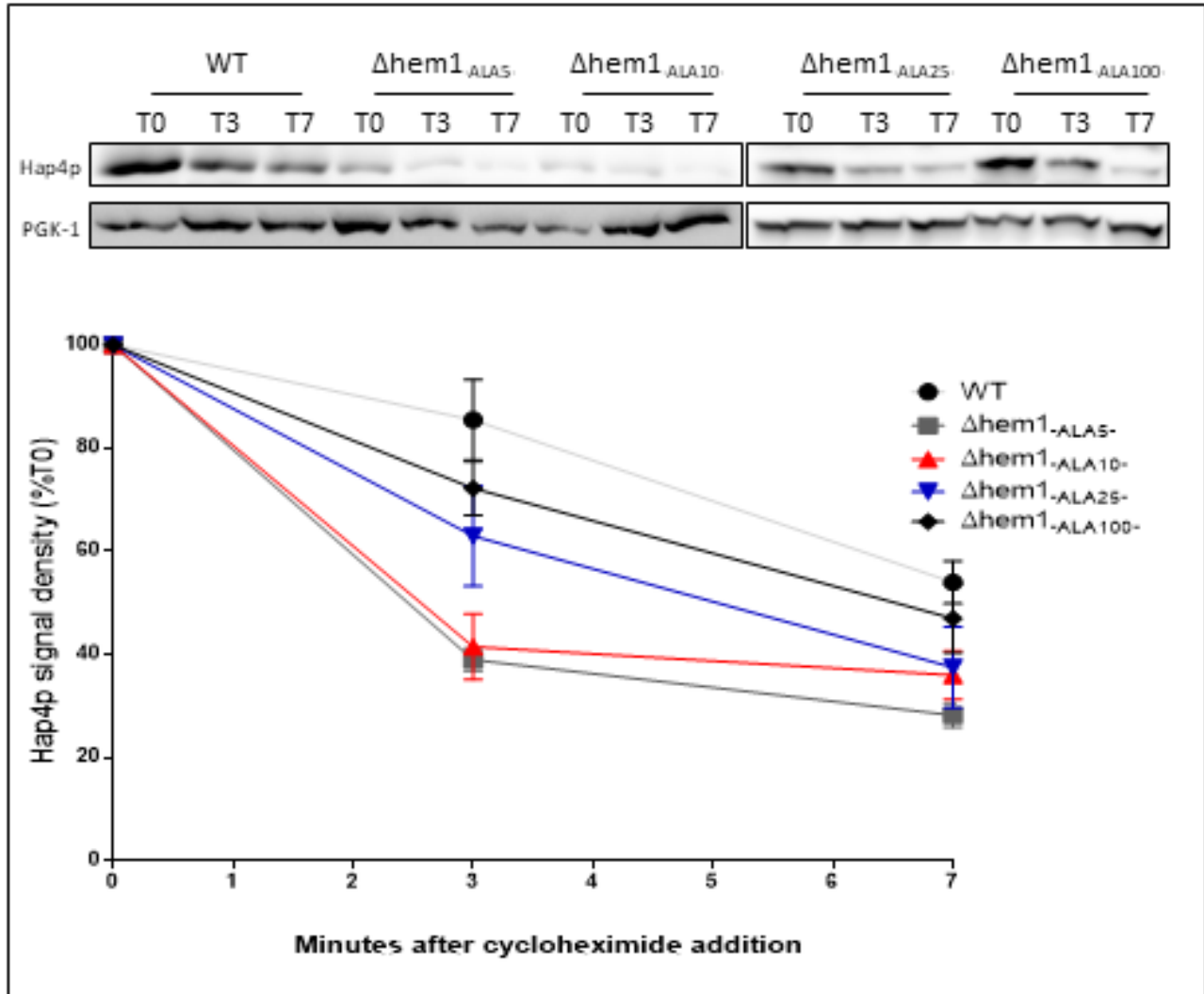
**Figure 5: Study of the role of Hap1p on the regulation of mitochondrial biogenesis by heme.** (A) *Δhap1* and *Δhap1Δhem1* cells were transformed with a plasmid encoding the reporter gene HMG2-LacZ that is negatively regulated by “labile” heme. The growth medium of *Δhap1Δhem1* cells was supplemented with  $5\mu\text{g.mL}^{-1}$  (*Δhap1Δhem1-ALA5-*),  $10\mu\text{g.mL}^{-1}$  (*Δhap1Δhem1-ALA10-*),  $25\mu\text{g.mL}^{-1}$  (*Δhap1Δhem1-ALA25-*) or  $100\mu\text{g.mL}^{-1}$  (*Δhap1Δhem1-ALA100-*). *Δhap1* and *Δhap1Δhem1* cells grown aerobically. Cells were harvested and  $\beta$ -galactosidase activities were measured and expressed as percentage of *Δhap1*. Results shown represent means of at least three separate experiments  $\pm$  SEM. (B) Spontaneous respiratory rates measured on cultures in the different conditions of WT, *Δhap1* and *Δhap1Δhem1* are shown. Given values for each condition are means of all data points  $\pm$  SEM. (C) A linear regression line between respiratory rate and growth rate is presented for which the determination coefficient is indicated ( $r^2$ ). Growth rates for each condition of WT, *Δhap1* and *Δhap1Δhem1* cells were calculated in each experiment from measured  $\text{OD}_{600\text{nm}}$  of cultures over a 4 hours period. (D) Cytochrome c oxidase activities measured on cultures in the different conditions are shown. Given values for each condition of WT, *Δhap1* and *Δhap1Δhem1* cells are means of

all data points and expressed as percentage of  $\Delta hap1 \pm SEM$ . (E)  $\Delta hap1$  and  $\Delta hap1\Delta hem1$  cells were transformed with the plasmid p-CYC1-LacZ bearing the lacZ reporter gene under the control of the CYC1 promoter regulated by the HAP transcription factor. The cells were grown in the conditions described in Figure 1 and  $\beta$ -galactosidase activities were measured. Results are mean % of  $\Delta hap1 \pm SEM$  of at least three measurements performed on independent cell cultures. (F) The relative levels of the co-activator hap4 were determined in  $\Delta hap1$  and  $\Delta hap1\Delta hem1$  cells in the presence or the absence of the different concentrations of ALA. Total protein extracts were analyzed by SDS-PAGE and western blot using antibodies directed against Hap4p and phospho-glycerate kinase (PGK1) as a loading control. Results representative of at least four experiments are shown in the inset. Hap4p signal was quantified. Signal intensity was normalized to PGK1 signal and expressed in percentage of  $\Delta hap1$  for each experiment. The bar-graph shows mean % of  $\Delta hap1 \pm SEM$  for all experiments.



**Figure 6: Physical interaction between Hemin and Hap4p.** (A) Absorption spectra of hemin, GST-hap4<sub>(330-554)</sub> purified recombinant protein, or a mixture of both at the indicated concentrations. In the inset is shown the difference spectrum between hemin with hap4 (+ GST-hap4<sub>(330-554)</sub> 4 $\mu$ M) and hemin alone (Hemin 4 $\mu$ M). The wavelengths of major peaks are indicated above them. (B) Spectra of hemin in the presence or absence of indicated concentrations of purified recombinant GST. In inset, the difference spectrum between hemin with GST (+ GST 4 $\mu$ M) and hemin alone (Hemin 4 $\mu$ M). (C) Coprecipitation of Hap4p in hemin pull-down experiments. WT or  $\Delta$ hem1 cells were cultured aerobically in the absence or in the presence of 100 $\mu$ g.ml<sup>-1</sup> of ALA respectively. Cell extracts were subjected to co-precipitation assay with

Hemin-Agarose beads and analyzed by SDS-PAGE and western blot using antibodies directed against Hap4p or PGK1. A negative control experiment was also performed with WT cell extracts and unconjugated agarose beads (WT + agarose beads). Representative western blot results or at least 3 replicates are shown. (D) Coprecipitation of Hap4p in hemin pull-down experiments. Cell extracts were preincubated with 100 $\mu$ g.ml<sup>-1</sup> of hemin for 2 hours then subjected to co-precipitation assay with Hemin-Agarose beads and analyzed by SDS-PAGE and western blot using antibodies directed against Hap4p. Representative western blot results of at least 3 replicates are shown. (E) Difference absorption spectra and titration of GST-hap4<sub>(330-554)</sub> at 100nM with increasing concentrations of hemin (from 0 to 15 $\mu$ M). The curves were generated from fits to an equation describing a single binding site ( $Y=B_{max}*X/(Kd+X)$ ) with GraphPad Prism

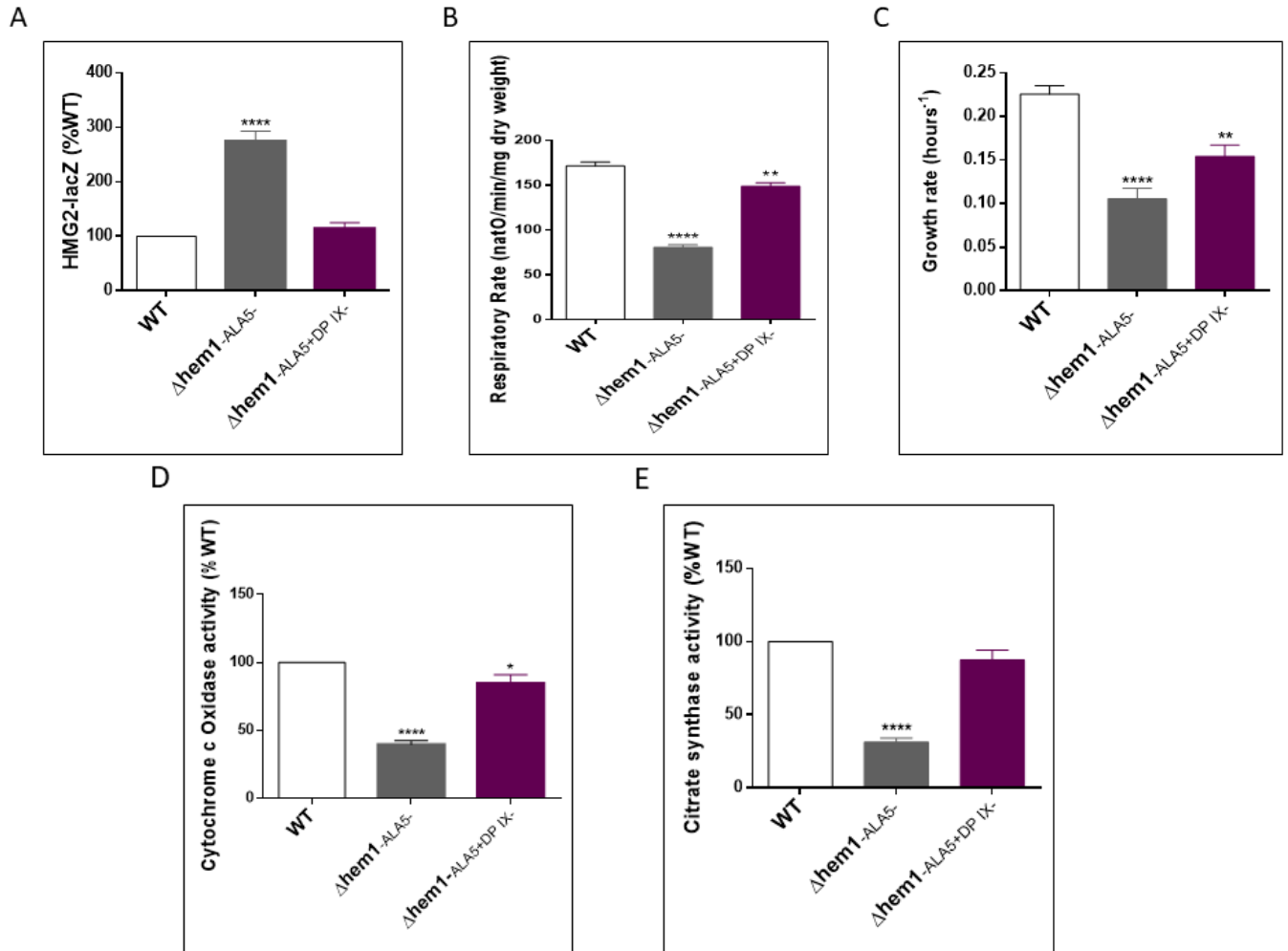


**Figure 7: Assessment of Hap4p protein stability at different concentrations of ALA.** Cycloheximide (0.4 mg.ml<sup>-1</sup>) was added to wild type (WT) and *Δhem1* cells grown aerobically in the absence or the presence of different concentrations of ALA (as described in figure 1). Aliquots of cells were harvested for each condition right before (T0), 3 min (T3), and 7 min (T7) after adding cycloheximide. Total protein extracts were analyzed by SDS-PAGE and western-blot using antibodies directed against Hap4p or PGK1. Top: Representative results of four replicates are shown. Bottom: Hap4p signal was quantified, normalized to PGK1 signal (loading control) and expressed in percentage of T0 for each condition. The data points are mean % of T0 ± SEM over 4 independent experiments.

**Table I. Mean percentages of Hap4p protein stability assay**

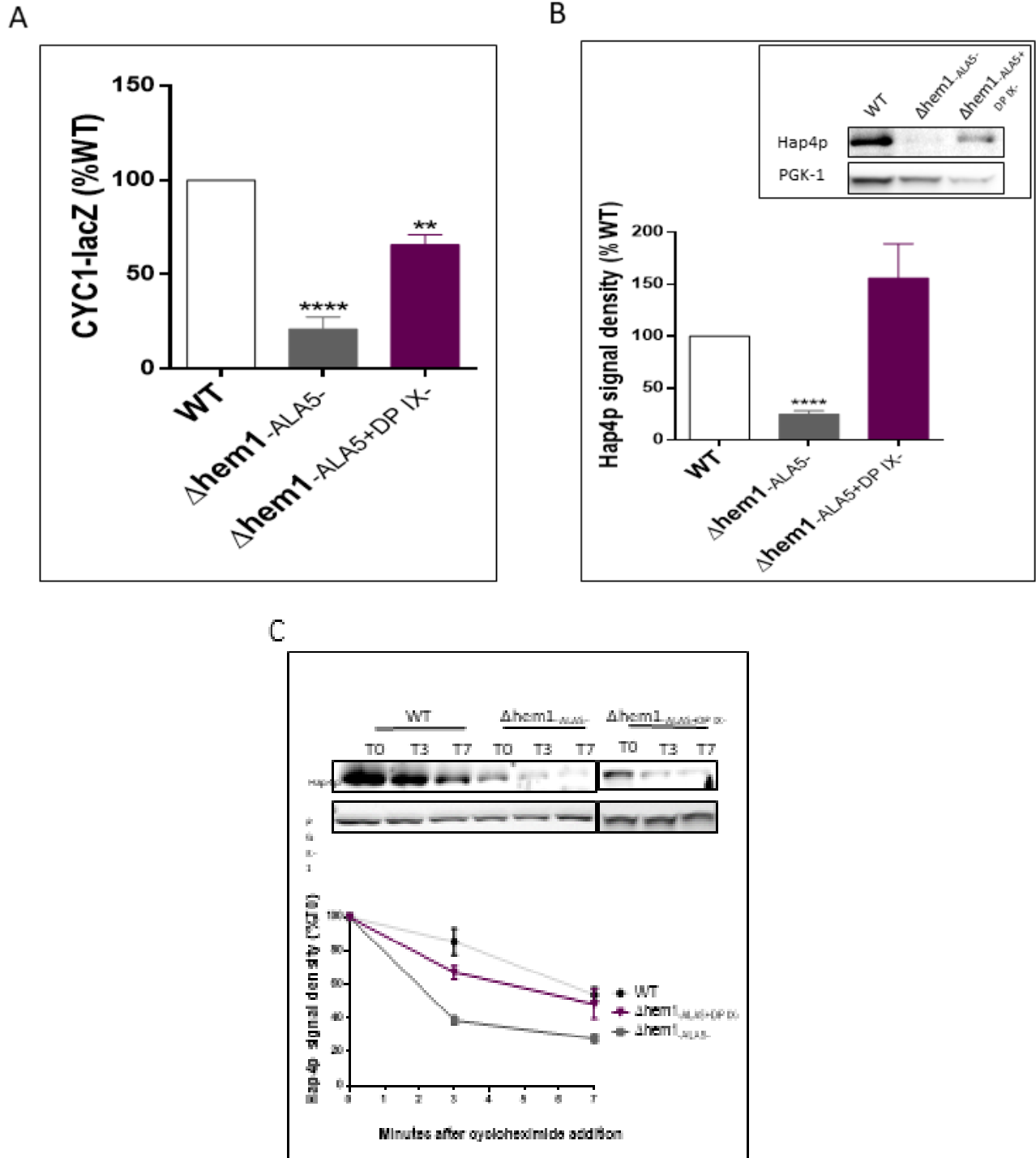
	WT	DHEM1 <sub>-ALA5-</sub>	DHEM1 <sub>-ALA10-</sub>	DHEM1 <sub>-ALA25-</sub>	DHEM1 <sub>-ALA100-</sub>
T0	100	100	100	100	100
T3	85,5 (±7,8)	38,75 (±2,1)	41,5 (±6,4)	63 (±9,7)	72,25 (±5,2)
T7	54 (±4,1)	28 (±2,1)	36 (±4,7)	37,5 (±7,9)	47 (±6,6)

For each condition and each time, mean percentages of T0 (shown on Figure 7) were reported ± (SEM) - data were extracted from figure 7-.



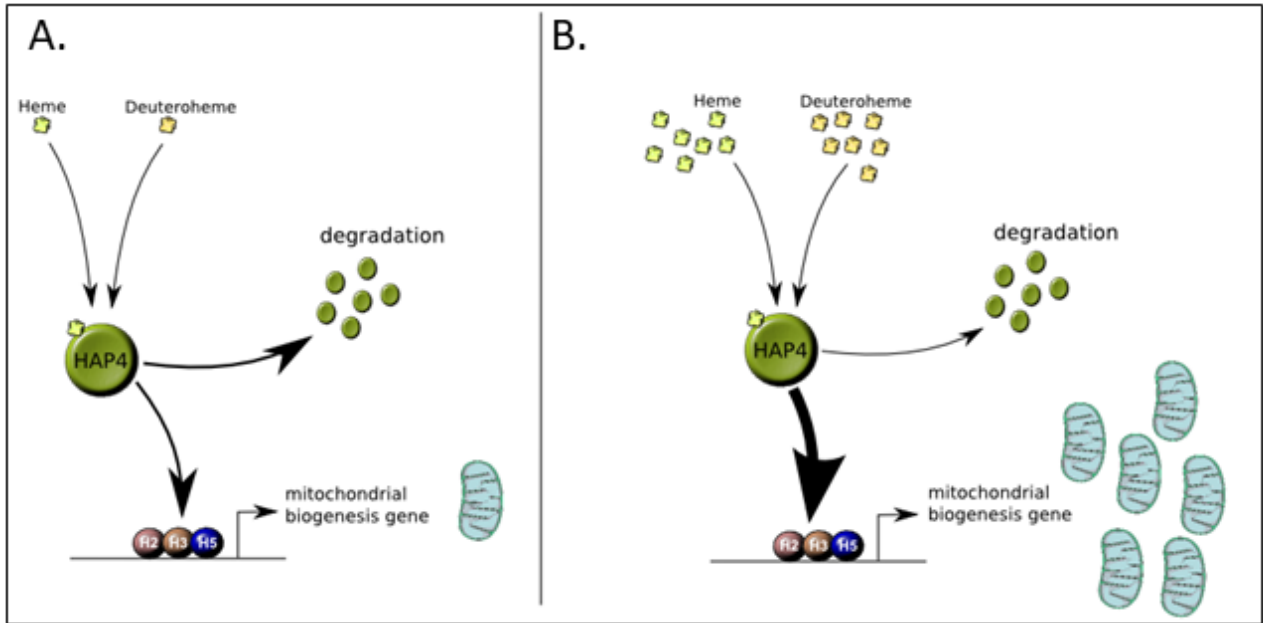
**Figure 8: Effect of the deuteroporphyrin IX analogue on labile heme pool, growth, respiratory rate, cytochrome c oxidase and citrate synthase activities.** Wild type (WT) and  $\Delta hem1$  cells were grown aerobically.  $\Delta hem1$  cultures were supplemented with  $5\mu\text{g}\cdot\text{ml}^{-1}$  of ALA ( $\Delta hem1-ALA5-$ ) or  $5\mu\text{g}\cdot\text{ml}^{-1}$  of ALA and  $2.5\mu\text{g}\cdot\text{ml}^{-1}$  of deuteroporphyrin IX ( $\Delta hem1-ALA5+DP IX-$ ). Results are from at least three independent experiments. (A) WT and  $\Delta hem1$  cells transformed with the plasmid encoding the reporter gene HMG2-LacZ were grown in the conditions described above. Cells were harvested and  $\beta$ -galactosidase activities were measured and expressed as percentage of WT. The bar graph shows mean % of WT of at least three separate experiments  $\pm$  SEM. (B) Spontaneous respiratory rates were measured in the different conditions as described in figure 1. Mean values  $\pm$  SEM are shown. (C) Growth rates in each condition were determined for each experiment from measured OD<sub>600nm</sub> of cultures over a 4 hours period. Mean values  $\pm$  SEM are shown. (D) Cytochrome c oxidase activities were measured and expressed for each experiment as percentage of WT. Mean values  $\pm$  SEM are shown. (E) Citrate synthase activities were measured in the different conditions and expressed as percentage of WT. Mean values  $\pm$  SEM are shown.





**Figure 9: Effect of deuteroporphyrin IX on the HAP complex activity and the Hap4p subunit in wild type and  $\Delta hem1$  cells.** (A) The activity of the transcription factor HAP was assessed with the specific and widely used reporter gene pCYC1-LacZ (49) as described under “Experimental Procedures” from cells grown aerobically in minimal medium containing 0.2% (w/v) DL-lactate + 5 $\mu$ g/ml ALA and in the presence

or the absence of 2.5µg.ml<sup>-1</sup> of DP IX. Results are mean SEM of at least a minimum of three measurements performed on three independent cell cultures. (B) Effect of DP IX on the protein level of the activator subunit Hap4p. The result is representative of at least four blots. Loading control was assessed using a commercial antibody directed against phospho-glycerate kinase (PGK1; Invitrogen). (C) numbers on the quantification graph correspond to the percentage between hap4 levels in the mutant versus the wild type after a normalization at the input. Quantification was assessed using the ImageJ software.



**Scheme 2: Heme-induced regulation of mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*.** (A) In the presence of low concentrations of heme or deuteroheme, HAP4 turnover rate is high and mitochondrial biogenesis is low. (B) An increase in heme or deuteroheme concentration within the cell stabilizes HAP4 and mitochondrial biogenesis rate is higher.