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Defects in Mitochondrial Fatty Acid Synthesis Result in Failure of Multiple Aspects of Mitochondrial Biogenesis in *Saccharomyces cerevisiae*

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Summary

Mitochondrial fatty acid synthesis (mtFAS) shares acetyl-CoA with the Krebs cycle as a common substrate and is required for the production of octanoic acid (C8) precursors of lipoic acid (LA) in mitochondria. MtFAS is a conserved pathway essential for respiration. In a genetic screen in *Saccharomyces cerevisiae* designed to further elucidate the physiological role of mtFAS, we isolated mutants with defects in mitochondrial post-translational gene expression processes, indicating a novel link to mitochondrial gene expression and respiratory chain biogenesis. In our ensuing analysis, we show that mtFAS, but not lipoylation *per se*, is required for respiratory competence. We demonstrate that mtFAS is required for mRNA splicing, mitochondrial translation and respiratory complex assembly, and provide evidence that not LA *per se*, but fatty acids longer than C8 play a role in these processes. We also show that mtFAS- and LA-deficient strains suffer from a mild heme deficiency that may contribute to the respiratory complex assembly defect. Based on our data and previously published information, we propose a model implicating mtFAS as a sensor for mitochondrial acetyl-CoA availability and a coordinator of nuclear and mitochondrial gene expression by adapting the mitochondrial compartment to changes in the metabolic status of the cell.

Keywords

mitochondrial-nuclear coordination; mitochondrial gene expression; heme; lipoic acid; mitochondrial fatty acid synthesis

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Introduction

The presence of the mitochondrial compartment is one of the hallmark features of eukaryotic cells, and there are grounds to argue that the acquisition of an ancestor of α-proteobacteria (Sagan, 1967) was a defining moment of the genesis of eukaryotes (van der Giezen, 2009). Many of the proteins and processes residing in mitochondria still bear witness to the prokaryotic ancestry of these organelles.

As a consequence of the endosymbiotic relationship between the prokaryotic resident and its host, a large fraction of the bacterial genome was ultimately lost or transferred to the nucleus. However, there appears to be a necessity to maintain a core group of genes encoding proteins and several RNA species within the mitochondrial genome (mtDNA) (Gray *et al.*, 1999; Wallace, 2007). Mitochondria have retained the ability to express these genes, devoting a large fraction of the mitochondrial proteome to this task. In the yeast *Saccharomyces cerevisiae*, eight proteins as well as tRNAs, rRNAs and the RNA subunit of mitochondrial RNase P are still synthesized within mitochondria. With the exception of Var1, a mitoribosomal subunit, all of the proteins encoded by the yeast mitochondrial genome are integral membrane proteins that are core subunits of the oxidative phosphorylation system (OXPHOS) complexes. They assemble with nuclear-encoded subunits to form functional respiratory chain complexes III (cytochrome bc_1 complex) and IV (cytochrome *c* oxidase; COX) and the F_1F_O ATP synthase or complex V. Regulation of proper subunit stoichiometry is necessary to prevent accumulation of unassembled surplus protein subunits. In mitochondrial enzyme complex assembly, this process requires coordinate expression of components from two different genomes. In no small part, gene expression in mitochondria is governed by the nucleus, which encodes the vast majority of factors involved in this process. How the coordination of gene expression of both genomes is achieved is still not well understood.

Transcription of numerous nuclear genes encoding mitochondrial proteins is regulated by the availability of heme and oxygen and can be downregulated by glucose through repressor-mediated histone deacetylation (Schuller, 2003; Turcotte *et al.*, 2010). There is little regulation of mitochondrial transcription, although its rate is known to increase a few fold during a shift to respiration (Amiott *et al.*, 2006a; Amiott *et al.*, 2006b). In contrast to the elaborate transcriptional control of the nuclear genes, production of mtDNA-encoded proteins is regulated predominantly post-transcriptionally. Regulation involves factors required for RNA processing, stability, translation, and complex assembly. The regulation of mitochondrial protein synthesis by the action of mRNA-specific translational activators in yeast has been a subject of intense study during the past two decades (Herrmann *et al.*, 2013).

For example, it has been postulated that *COX1* mRNA is bound by Mss51 and Pet309, which activate translation from this template. Subsequently, Mss51 binds to the protein product and forms a stable Cox1 subassembly complex with other complex IV chaperones such as Cox14, Cox25/Coa3 and the Hsp70 chaperone Ssc1 (Perez-Martinez *et al.*, 2003; Barrientos *et al.*, 2004; Fontanesi *et al.*, 2010; Mick *et al.*, 2010; Fontanesi *et al.*, 2011). The translational activator Mss51 is only released from this complex and then recycled when

Cox1 interacts with its assembly partners, thus setting up a feedback translational regulatory loop that prevents gross overproduction of the subunit (Barrientos *et al.*, 2004; Fontanesi *et al.*, 2010).

If the regulation of mitochondrial gene expression was solely dependent on a linear pathway of heme and oxygen activation of nuclear genes encoding mitochondrial post-transcriptional effectors, the process would probably suffer from a delay in the synthesis of mtDNA-encoded subunits of the respiratory complexes compared to the production of nuclear-encoded subunits.

Mitochondrial fatty acid synthesis (mtFAS) is a conserved pathway essential for respiration in yeast. Deletion of mtFAS components results in small, rudimentary mitochondria, while overexpression of enzyme components of the pathway results in a dramatic enlargement of the mitochondrial compartment (Torkko *et al.*, 2001; Kastaniotis *et al.*, 2004). MtFAS has been implicated as the sole mitochondrial source of octanoic acid in eukaryotes. This product is required as a precursor for the synthesis of the lipoic acid (LA) cofactor that is indispensable for the function of several mitochondrial enzyme complexes including: pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and the glycine cleavage system (GCS) (Fig. 1A) (Schonauer *et al.*, 2009; Hiltunen *et al.*, 2010). A deletion of any gene in the mtFAS and LA attachment pathways results in respiratory deficiency.

A central metabolite for mitochondrial function is acetyl-CoA, the substrate of the Krebs cycle. Acetyl-CoA is also the substrate for ketogenesis in mammals and for mtFAS in all eukaryotes. In this work, we show that a fatty acid product of the mtFAS pathway that is longer than octanoic acid (C8) is required for mitochondrial biogenesis on multiple levels, and we present a model implicating mtFAS as a hub for mitochondrial sensing of cellular metabolic status and control of important mitochondrial post-transcriptional gene expression events.

Results

Synthetic petite interactions

To investigate possible roles of mtFAS in mitochondrial function other than supplying octanoic acid for LA synthesis (Fig. 1A), we screened for mutations causing a synthetic respiratory-deficient “petite” phenotype in combination with a compromised step in mtFAS. We have previously shown that a plasmid-borne copy of the *Escherichia coli fabI* gene, encoding a bacterial FAS type II enoyl reductase, fused to a yeast mitochondrial targeting sequence and expressed from a heterologous promoter (*CTA1*), is capable of rescuing respiratory growth of an *etr1*Δ strain (Torkko *et al.*, 2001). For the work presented here, the yeast *ETR1* ORF was replaced in the yeast genome by *fabI* under transcriptional control of *ETR1* regulatory sequences. The strains (isogenic **a** and α) carrying this construct (“the *FabI* strains”) are respiratory competent but display diminished growth on glycerol and a 50 % reduction in lipoic acid content (277 ± 78 ng lipoic acid g^{-1} wet weight cells compared to 536 ± 101 ng lipoic acid g^{-1} in wild-type and 19 ± 7 ng lipoic acid g^{-1} in the *etr1*Δ negative control strain). Unlike the wild-type W1536 5B/8B strains, the *FabI* strains do not shift quickly from fermentation to respiration when transferred from glucose to glycerol media

(not shown) and are sensitive to the *fabI*-specific drug triclosan on non-fermentable media (see Supporting Information Fig. S1A and S1B). The latter observation is consistent with our previous report on triclosan inhibition of the mtFabI-plasmid complemented *etr1*Δ strain (Torkko *et al.*, 2003).

We employed the *FabI* strains in a colony-color based assay to find ethyl methane sulfonate (EMS) generated mutants unable to lose a plasmid harboring wild-type *ETR1* on respiration-requiring glycerol medium, but able to lose the plasmid on medium containing fermentable glucose as the carbon source (Kastaniotis *et al.*, 2004). 18 synthetic petite mutations were identified in 12 different genes (Fig. 1B, Supporting Information Fig. S2, Tables S1 and S2).

The interacting genes can be divided into five different categories: (I) *CEM1*, *HFA1* and *HTD2* are members of the mtFAS pathway, (II) *MEF1*, *MRPL16*, *MRPS5*, *MSS116* and *RSM22* are essential for mitochondrial translation and post-transcriptional gene expression processes, (III) *LIP3* and *KGD1* are linked to LA metabolism and heme synthesis, (IV) *ALD4* is a mitochondrial pyruvate bypass pathway enzyme (Boubekeur *et al.*, 1999), and (V) *ASK10* encodes a transcription factor in the oxidative stress response (Cohen *et al.*, 2003). Isolation of mutants of category I, III and IV confirmed the specificity of our screen. The synthetic mutation in *ASK10*, the sole member of category V, may imply a role of this factor in expression of mtFAS or LA metabolism related genes. Future analysis should shed more light on the role of the Ask10 protein in the regulation of mtFAS.

Because the synthetic mutations were obtained in a strain compromised for enoyl reductase function, initial analysis of effects of mtFAS lesions on mitochondrial gene expression was carried out in the *etr1*Δ strain background. To rule out gene-specific effects, we later expanded our experiments to include the *htd2*Δ strain also. As it became clear that these strains displayed virtually identical phenotypes, later analyses were sometimes done with only one of the deletion strains. Triclosan inhibition experiments were only carried out with an *etr1*Δ control, as triclosan inhibits bacterial enoyl reductase.

Respiratory chain defects in mtFAS deficient strains

Mutants of category II were the most informative in furthering our understanding of the role of mtFAS in mitochondrial respiration. We isolated several mutations in genes involved in mitochondrial post-transcriptional gene expression processes, implicating mtFAS in the production of functional respiratory complexes. While mtFAS mutants had been shown previously to lack mitochondrial cytochromes, they had been reported to have no mitochondrial translation defects (Harington *et al.*, 1994; Yamazoe *et al.*, 1994). In light of isolating category II mutations affecting mitochondrial gene expression in our screen, we decided to reinvestigate the levels of cytochromes and respiratory complexes in strains with lesions in mtFAS and LA attachment processes.

We analyzed the endogenous cell respiration rate (Fig. 2A) and determined the total mitochondrial cytochrome spectra of the knockout mutants (Fig. 2B) and found them to be diminished, which is consistent with the previously observed respiratory-deficient growth phenotype. Accordingly, respiratory chain enzyme activities also were found to be decreased. Cytochrome *c* oxidase (COX) (Fig. 2C, see also Supporting Information Table S3

for specific activities), NADH cytochrome *c* reductase (NCCR) (Fig. 2D) and succinate cytochrome *c* reductase (SCCR) activities (Fig. 2E) were lowered in the LA attachment deficient *lip2*Δ, *lip3*Δ and *gcv3*Δ strains as well as in the mtFAS deficient *etr1*Δ strain, suggesting a pleiotropic effect in several segments of the mitochondrial respiratory chain. However, COX activity was decreased most in the *etr1*Δ strain, which had a markedly more severe phenotype than the LA deficient strains (Fig. 2C). The ATP hydrolysis activity of the F_1F_o ATPase was lowered only in the *etr1*Δ strain where it was ~70% of wild-type (Fig. 2F). Notably, it has been suggested recently that the *etr1*Δ strain experiences decreased mitochondrial membrane potential due to a defect in the F_1F_o ATPase (Ytting *et al.*, 2012). We examined the effect of triclosan inhibition on the mitochondrial membrane potential of wild-type W1536 8B, *FabI* and *etr1*Δ strains using FACS (fluorescence-activated cell sorting) to monitor the fluorescence signal of the membrane potential-sensitive dye JC-1 (Smiley *et al.*, 1991). Mitochondria from wild-type cells had a high membrane potential as indicated by a high FL2 emission (Fig. 2G, for a more detailed description see Experimental Procedures). In contrast, when the proton translocator FCCP (carbonyl cyanide 4-trifluoromethoxy phenylhydrazone) was added in concert with the JC-1 dye, the FL2 emission was decreased, indicating that membrane potential-dependent mitochondrial uptake of JC-1 was inhibited. The *etr1* Δ strain displayed a low FL2 emission, similar to the FCCP-treated wild-type cells, indicating a strong decrease of mitochondrial membrane potential in mtFAS deficient cells. Addition of FCCP to the *etr1*Δ strain, however, further decreased the FL2 emission, indicating that the membrane potential is not completely abolished in mtFAS deficient yeast strains. The *FabI* strain was indistinguishable from wild-type when grown in the absence of triclosan. Addition of the drug to the *FabI* culture gave a result similar to the *etr1*Δ strain, or to wild-type treated with FCCP, a phenotype suggesting a decrease in mitochondrial membrane potential. The membrane potential drops even further upon FCCP treatment, recapitulating the effect observed in the *etr1*Δ strain. When triclosan was added to the wild-type strain at the same concentration, it did not affect membrane potential, confirming the specificity of the triclosan effect.

Disturbed respiratory complex assembly and translation in mtFAS and LA attachment deficient mutants

To examine if the reductions in respiration and enzyme activities were due to lowered levels of mitochondrial proteins, mitochondrial extracts (Fig. 3A) were analyzed by western blotting. Steady state levels of COX subunits were indeed specifically reduced in the *etr1*Δ mutant, but a much less severe phenotype was detected in the LA attachment deficient strains (*gcv3*Δ, *lip2*Δ and *lip3*Δ). In contrast, all the mutants showed similar levels of Cox5, a nuclear-encoded subunit known to be more stable than the mtDNA-encoded subunits when COX assembly is compromised. In addition, the steady-state levels of other components of the mitochondrial respiratory chain, such as Cyt *b* (a complex III or bc_1 complex subunit), were lowered in some of the mutants (Fig. 3A), while the levels of SDHa (Sdh1 subunit of succinate dehydrogenase/complex II) were decreased in all mutants. The F_1 subunits of ATPase accumulated at normal levels.

To distinguish if decreased steady state levels of mitochondrial proteins were due to decreased synthesis or increased turnover, nascent mtDNA-encoded proteins were labeled

with ^{35}S-methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. MtFAS and lipoylation-defective strains were able to synthesize all mitochondrial translation products. However, all strains had attenuated levels of newly synthesized Cox1, and only the *etr1*Δ strain displayed a mild, more generalized, protein synthesis attenuation phenotype (Fig. 3B).

Usually, accumulation of unassembled respiratory chain enzyme subunits is limited by degradation *via* dedicated proteases (Langer *et al.*, 2001). Additionally, in the case of COX, the accumulation of Cox1 is regulated translationally by a feedback mechanism involving the translational activator Mss51, which plays dual roles in *COX1* mRNA translation and Cox1 chaperoning (Perez-Martinez *et al.*, 2003; Barrientos *et al.*, 2004; Fontanesi *et al.*, 2010). Deletion of the *COX14* gene relieves this feedback inhibition by allowing Mss51 to dissociate from unassembled Cox1 (Barrientos *et al.*, 2004). We aimed to test whether the Cox1 synthesis attenuation observed in mtFAS mutants is due to a translational defect or is merely a result of downregulation due to defective COX assembly. For this purpose, we generated double mutants by introducing the *cox14* deletion into mtFAS and LA deficient mutants. As in *bona fide* COX assembly mutants, deletion of the *COX14* gene in these mutants resulted in a wild-type level of *de novo* Cox1 synthesis (Fig. 3B). These data indicate that a large part of the observed decrease in Cox1 translation is the consequence of a COX assembly defect.

Mitochondrial mRNA splicing inhibition in mtFAS and lipoylation deficient mutants

Often, even minor defects in translation of Cox1 and Cyt *b* result in an inhibition of splicing of the multiple introns present in their pre-mRNAs. This is due to the need to translate intron-encoded maturases for intron excision (Mittelmeier *et al.*, 1995). To investigate whether the decrease in translation efficiency affected splicing, RNAs isolated from mtFAS and lipoylation deficient strains were probed for the *COX1* and *CYTB* transcripts to assess transcript abundance. Our results clearly showed mRNA splicing defects of these transcripts (Fig. 3C). While the overall severity of the defect varied with the growth conditions, the splicing deficiency was consistently more pronounced in the *etr1*Δ and *htd2*Δ strains, which displayed identical phenotypes. Splicing was less severely affected in the lipoylation deficient strains. In addition to these observations, there is a noticeable decrease in *COX2* transcript in the mtFAS deficient strains and the *cbp2*Δ control strain, but not in strains with specific lipoylation defects. Cbp2 is required for the removal of the *CYTB* group I intron bI5 (McGraw *et al.*, 1983) and the *cbp2*Δ deletion mutant, like *etr1*Δ and *htd2*Δ strains, suffers from a severe respiratory chain defect.

To test if the lack of mature transcripts contributed to the lack of mtDNA-encoded proteins, we generated mtFAS deletion strains harboring mitochondria with an intronless genome in the W1536 5B background (*ietr1*Δ and *ihtd2*Δ) (Fig. 4A). We indeed detected reproducible improvement of Cox1 steady-state levels in the intronless mtFAS deficient strains (Fig. 4B), as well as a mild improvement of *de novo* Cox1 synthesis (Fig. 4C). A slightly larger variant of Var1 was present in all strains carrying the intronless mitochondrial genome in comparison to the W303 derivatives otherwise used in this work. This is due to the presence of a different *VAR1* allele in the mitochondrial genome of *kar1* MCC109I (α) strain (Ellis *et*

al., 2004), which was used as the intronless mtDNA donor in the generation of the intronless strains.

To evaluate the impact of the *COX1* mRNA splicing defect on the COX deficient phenotype in *etr1* mutant yeast, we examined *de novo* translation and COX activities in intron-containing and intron-less strains. We detected a mild increase in both Cox1 synthesis (Fig. 4C) and COX activity (from ~ 4 to 13% of wild-type (Fig. 4D, see also Supporting Information Table S4 for specific activities) in *ietr1*Δ. To further assess the contribution of *COX1* mRNA translational regulation to the COX deficient phenotype in *etr1* mutant yeast, *ietr1*Δ was transformed with a plasmid expressing either wild-type or a functional mutant variant of the *COX1* mRNA translational activator Mss51 (mss51^{F199I}). We previously reported that a single copy of the mutant allele *mss51*^{F199I} or additional copies of wild-type *MSS51* partially suppress the respiratory defect caused by mutations in *SHY1*, a COX assembly factor, by increasing Cox1 synthesis (Barrientos *et al.*, 2002). In most COX-deficient strains, mutant or additional copies of *MSS51* do not suppress the COX assembly defect, but significantly increase Cox1 synthesis *via* bypassing the negative feedback regulation (Barrientos *et al.*, 2004; Fontanesi *et al.*, 2010). The restoration of Cox1 synthesis by excess Mss51 or by mss51^{F199I} is due to its increased intrinsic capacity to avoid being trapped in a complex containing newly synthesized Cox1 when COX assembly is defective (Fontanesi *et al.*, 2010). More recently, we have reported that Mss51 is a heme binding protein able to sense heme B levels to promote Cox1 synthesis and assembly (Soto *et al.*, 2012). Notably, mss51^{F199I} can perform its functions in the absence of heme (Soto *et al.*, 2012) and thus it is ideal to test whether Cox1 translation is down-regulated in the absence of Etr1. Our results show that the additional heme-dependent wild-type Mss51 significantly restores Cox1 synthesis and increases COX activity (to 46% of wild type) and respiration (to 20%) in *ietr1*Δ. However, this level was insufficient to enhance respiratory growth on rich glycerol plates (Fig. 4E). In contrast, the heme-independent *mss51*^{F199I} allele fully restored Cox1 synthesis and COX activity in *ietr1*Δ resulting in an increase of respiration to 50% of the wild-type value and a partial rescue of respiratory growth on plates (Fig. 4E).

Lipoylation defects and PDH bypass

Yeast mitochondria and probably also the mitochondria of higher eukaryotes exclusively utilize internally synthesized LA (Hiltunen *et al.*, 2010). Since the level of LA in the *FabI* strain was only ~50% of that in wild-type, we expected that the synthetic petite screen would identify factors involved in LA metabolism. Lip2, Lip3, Lip5 and Gcv3 are all required for the synthesis and attachment of LA to subunits of PDH, α-KGDH, and GCS (see Fig. 1A). Thus, isolation of the *lip3* and *kgd1* mutants of category III as well as the *ald4-1* mutation, which disables the PDH bypass, provides further evidence for the specificity of our screen.

Succinyl-CoA generated by α-KGDH is required in mitochondria along with glycine in the first step of heme synthesis, which is the formation of δ-aminolevulenic acid (δ-ALA). A heme deficiency may be one of the causes of the observed cytochrome assembly defect. To assess heme levels, we measured β-galactosidase activity in yeast strains transformed with a plasmid harboring a fusion of the heme-repressed *ANB1* promoter to *lacZ* (Deckert *et al.*,

1998). Levels of β-galactosidase were increased about threefold in the fatty acid- or LA-deficient strains (Fig. 5A), a change so small that it could not be clearly detected by northern blotting (supplementary Fig. S3). This de-repression was suppressed by the addition of δ-ALA to the growth media (Fig. 5A). Addition of δ-ALA to the deficient strains, however, did not result in improvement of the steady state levels of respiratory complex proteins (Fig. 5B). Full de-repression increases β-galactosidase expression from this reporter by 100 to 200-fold or more (Deckert *et al.*, 1998). Hence our observation is consistent with a mild heme deficiency in strains devoid of LA.

The *ANB1-lacZ* construct assesses "subjective" cellular heme levels, i.e. the levels that the cell senses as sufficient or deficient in terms of its needs. In order to better understand the role of heme in the mtFAS-deficient context, we tested absolute heme content in mtFAS mutants and the effect of δ-ALA supplementation. We treated *etr1* and *lip2* mutant strains with 100 μg/ml δ-ALA. This concentration was chosen because it was enough to complement the growth defect of a *hem1* mutant strain (Haslam *et al.*, 1979) and restored *ANB1-lacZ* repression in our reporter construct assay (Fig. 5A). As shown in the Figure 6, heme B content is reduced in *etr1* and *lip2* mutant mitochondria and whole cells. δ-ALA supplementation induced a mild but consistent increase in heme B content in whole cell extracts from *etr1* and *lip2* mutant strains, and a considerable increase in mitochondrial heme B only in *etr1*Δ. Intriguingly, the suppressor *FAM1-1* (see next paragraph) completely restored heme levels to wild-type values in the *etr1* mutant strain.

Suppression of respiratory deficiency of mtFAS deficient yeast strains by FAM1-1 does not improve lipoylation status

The *FAM1-1* suppressor mutation (Harington *et al.*, 1994) partially rescues the respiratory-deficient phenotype of all mutants carrying deletions of genes encoding mtFAS enzymes (Kastaniotis *et al.*, 2004). The mitochondrially mislocalized peroxisomal Faa2 acetyl-CoA ligase encoded by *FAM1-1* has been proposed to activate free fatty acids in mitochondria, making them available to the organelle to substitute for the lack of endogenously synthesized fatty acids. Faa2 accepts substrates ranging from C7:0 – C17:0 (Knoll *et al.*, 1994). Hence, we investigated if the suppressor allowed lipoylation of LA-dependent mitochondrial enzyme complexes. As presented in Fig. 5C, no lipoylation was detectable in these yeast strains. In contrast, there was a marked increase in the steady state levels of respiratory complex proteins Cox1, Cyt *b* and Sdh2, suggesting that a fatty acid longer than eight carbons is responsible for the improvement in respiratory ability in mtFAS-deficient strains. We also found by northern analysis that *FAM1-1* exerted a mild suppression effect on the processing defect of *RPM1*, the mitochondrial RNA subunit of RNase P (Fig. 5D), which had been previously reported to be severely impaired in mtFAS-deficient strains (Schonauer *et al.*, 2008).

Discussion

In response to a shortage of glucose in conjunction with the availability of non-fermentable carbon sources, yeast mitochondrial biogenesis is ramped up to allow the cells to cope with

the changed environmental conditions. The results we present here indicate that mtFAS is required for biogenesis of respiratory-competent mitochondria at multiple levels.

Our screen for mutations causing a synthetic respiratory-deficient “petite” phenotype in combination with a compromised step in mtFAS allowed us to identified 18 mutants, most of which would not have been found in a “genome-wide screen” using the deletion strain collection. Our ensuing analyses clearly indicate a connection of mtFAS products to mitochondrial gene expression processes. Specifically, several post-transcriptional events are disturbed in mtFAS mutants. (i) The steady-state levels of *COX1* and *CYTB* mRNA and the encoded proteins are markedly decreased in intron-containing strains because splicing of the corresponding mRNA precursors is severely retarded. Poor translation of intron-encoded maturases needed for intron excision is likely to be at least in part due to lower levels of mature tRNAs, as we have previously reported that mtFAS deletion strains are deficient in processing of the *RPM1* RNA subunit of mitochondrial RNase P (Schonauer *et al.*, 2008). (ii) Lack of accumulation of Cox1 in an intronless strain is due to a specific COX assembly defect. We have dissected the respective contributions of *COX1* mRNA splicing, translation and assembly on the COX-deficient phenotype of *etr1*Δ yeast. When these processes were taken out of the equation by using intronless strains plus expressing an additional *mss51*^{F199I} allele in the intronless *ietr1*Δ mutant strain, Cox1 synthesis and COX activity were fully recovered. Remarkably, the COX-sufficient strain only partially recovered respiratory growth competence, suggesting that mtFAS is required for mitochondrial functions other than COX biogenesis.

To unveil the primary cause of the respiratory-deficient phenotype in strains with defects in mtFAS and LA attachment, we pursued the characterization of suppressor strains. In general, LA attachment deficient yeast strains suffer from a milder phenotype than mtFAS mutant strains. Strikingly, our results indicate that mtFAS deficient strains suppressed by *FAM1-1* are completely devoid of lipoylated Lat1, Kgd2 and Gcv3 but show an increase in the abundance of respiratory complex proteins and, as previously demonstrated (Kastaniotis *et al.*, 2004), regain the ability to grow on medium requiring respiration. In addition, it has been reported previously that *FAM1-1* restores the cytochrome spectrum of a *cem1* mutant strain lacking a functional condensing enzyme (Harington *et al.*, 1994). The absence of lipoylation in the respiratory-competent *FAM1-1*-suppressed mutants, in conjunction with the reported substrate preference of Fam1/Faa2, suggests that the physiologically active mtFAS product is longer than eight carbons. Hence, our data demonstrates that a mtFAS product other than octanoic acid or LA is required for respiration.

Strains with defects in mtFAS and LA attachment apparently suffer from mild heme deficiency, a factor that could explain at least part of their respiratory-deficient phenotype. Our β-galactosidase reporter results indicate that mtFAS- or lipoylation-deficient strains are sensing a mild but reproducible shortage of heme, an important cofactor for several mitochondrial respiratory complexes, which is consistent with an earlier observation of a heme deficiency in α-KGDH mutants (McCammon *et al.*, 2003). Northern blotting for the *ANB1* message confirms that the heme deficiency sensed by the cell is minuscule, at least under the growth conditions we used. However, analyses of steady-state heme levels in these strains tell a different story. Heme B is reduced by approximately 50% in both the *etr1*Δ and

*lip2*Δ strains. The considerable increase of mitochondrial heme B in *etr1*Δ mitochondria upon addition of δ-ALA to the growth media may indicate that mitochondria in this strain background suffer more from the heme deficiency than does the rest of the cell. The full restoration of heme levels in the *FAM1-1* suppressed strain is striking. However, since *FAM1-1* does not restore lipoylation of α-KGDH to any detectable level, and since the heme levels in the α-KGDH-deficient *lip2*Δ strain do not respond much to δ-ALA supplementation, it is unlikely that the increase in steady-state cellular heme levels is due to restored α-KGDH activity. In part, this observation may result from the restoration of the respiratory chain, which ensures the retention of more heme in the cell.

Our data indicate that at least in the case of the COX deficiency observed in *etr1*Δ mutant yeast, *COX1* mRNA splicing and Cox1 translational regulation play a role in attenuating the levels of Cox1 synthesis, and that when Cox1 biogenesis is enhanced in intronless *ietr1*Δ by an additional *MSS51* or *mss51*^{F199I} allele, COX assembly and function are restored. Importantly, the restoration of COX by *MSS51* was only to ~50% of wild-type and had a minor impact on cell respiration and respiratory growth, whereas the *mss51*^{F199I} allele fully restored COX activity and cellular respiration to ~50% of wild-type. We have recently shown that Mss51 function is modulated by heme B binding (Soto *et al.*, 2012). Furthermore, restored Cox1 synthesis is also known to promote synthesis of heme A, the prosthetic group of COX (Barros *et al.*, 2002). The possibility that mtFAS could either directly or indirectly affect the ability of wild-type Mss51 to sense heme, even when the cofactor is in abundance, warrants future investigations.

Superficially, *FAM1-1* -and *mss51*^{F199I} -mediated suppression are similar in their effect on respiration and growth of mtFAS deficient strains on a non-fermentable carbon source. Our data indicate however, that *FAM1-1* suppression improves steady state levels of several different respiratory complex proteins, while *MSS51* is a *COX1*- specific translation activator. Although we have not yet rigorously tested the possibility of *mss51*^{F199I} effects on the expression of components of other complexes, we find such an activity not very likely. Instead, we suggest that a major contributing factor responsible for the respiratory deficiency of mtFAS mutants is a COX defect, and repair of this defect allows for improved respiration. Unless a role of *mss51*^{F199I} in restoration of lipoylation is invoked, suppression of mtFAS defects by this allele is also further evidence that lipoylation of mitochondrial proteins is not required for respiration *per se*. Further analyses will clarify these hypotheses.

What is the connection between mtFAS and energy metabolism? The Krebs cycle and mtFAS share acetyl-CoA as their substrate. Thus, the mtFAS pathway is ideally suited to act as the master regulator of respiratory growth *via* synthesis of LA precursors and fatty acids required for post-transcriptional gene expression. As such, it would act as the sensor for the nutritional status of the cell by connecting acetyl-CoA availability to PDH and α-KGDH activities as well as to gene expression in mitochondria. Rather than adapting to change in nutritional status by waiting for increases in nuclear-encoded gene products, mitochondria would receive a direct metabolic signal. This would render the organelles poised to receive incoming derepressed nuclear-encoded respiratory chain gene products that will assemble with proteins synthesized in mitochondria. Prior activation of PDH and α-KGDH by lipoylation would allow for faster adaptation to a changing environment.

We propose a model wherein mtFAS is part of an intricate regulatory circuit, which acts as a hub and coordinator of mitochondrial biogenesis in yeast (Fig. 7). A number of these connections are conserved in higher eukaryotes (Feng *et al.*, 2009; Mayr *et al.*, 2011; Smith *et al.*, 2012).

Several modules of this proposed circuit may constitute positive feedback loops. The PDH complex is a source for the acetyl-CoA substrate of mtFAS and hence LA synthesis. The essential requirement of LA in the function of the PDH complex constitutes the clearest positive feedback loop (Schonauer *et al.*, 2009). The proposed regulatory circuit suggests that lower levels of the acetyl-CoA substrate for mtFAS cause the respiratory chain defects detected in LA-deficient mutants.

A second positive feedback module may be represented by the RNase P maturation process (Schonauer *et al.*, 2009), as RNase P requires mature *RPM1* RNA for its activity, and one of the precursor *RPM1* processing steps requires RNase P activity itself. This would feed forward into translation, as the 5´ cleavage step in tRNA maturation is carried out by RNase P.

Thirdly, impaired COX complex assembly, which blocks Cox1 translation initiation, contributes to a backup in the splicing of the *COX1* mRNA precursor. Our model implies a dual role for heme in this regulatory circuit, as a redox cofactor in respiratory enzymes such as COX, and as a signaling molecule. In response to oxygen availability, heme levels are sensed in the nucleus and in mitochondria. Heme acts as a ligand to activate the major aerobic transcriptional activators Hap1 and the Hap2/3/4 complex in the nucleus (Zitomer *et al.*, 1992) as well as the translational activation function of Mss51 in mitochondria (Soto *et al.*, 2012). In our model, heme serves to integrate information of both oxygen and acetyl-CoA substrate availability.

It has been shown that the mtFAS pathway is not required for the expression, assembly or function of respiratory complexes *per se* (Merz *et al.*, 2009). In the absence of the mitochondrial pyruvate importer or in strains lacking functional PDH, the level of mitochondrial protein complex lipoylation corresponds to the availability of ketogenic amino acids in the media (Herzig *et al.*, 2012), demonstrating a possible acetyl-CoA dosage response of mtFAS. Also, our isolation of synthetic petite mutants with the *FabI* strain, which is not detectably translation deficient, indicates a response of mitochondrial gene expression to mtFAS output. In addition, the suppression of the *COX1* translation defect and full restoration of COX activity by the heme-independent mss51^{F199I} in the *ietr1*Δ mutant is consistent with a bypass of a defect in a regulatory step of COX expression by this mutant allele in the mtFAS-deficient strain. A role for mtFAS as a mitochondrial acetyl-CoA sensor is also consistent with the strict conservation of mtFAS and LA synthesis and the metabolic isolation of these processes in the organelle.

There is some evidence that this regulatory circuit may be conserved in higher eukaryotes. In a mouse mtFAS conditional knockout model, the animals suffer from a premature aging phenotype characteristic for mice with mitochondrial dysfunction (Smith *et al.*, 2012). Remarkably, apart from LA deficiency, the authors observed lower levels of all respiratory

complexes, particularly complexes II and IV. Additionally, a patient with a defect in LA synthase was reported to display various neuromuscular symptoms, defects in mitochondrial energy metabolism and acute respiratory chain deficiency, including a marked decrease in complex II activity (Mayr *et al.*, 2011). We attribute this defect to a feedback effect of low lipoylation on mtFAS. MtFAS patients, yet to be identified, would be expected to display a rather similar phenotype.

None of our results points to a primary involvement of membrane phospholipids in the phenotypes caused by mtFAS deficiency. Ultimately, the precise identity and destination of mtFAS products and how they exert their effects on mitochondrial biogenesis will have to be determined by metabolic labeling studies. In conclusion, our model provides a framework to understand how mitochondria in single cell and more complex eukaryotes obtain information about the cellular metabolic status independent of nuclear signals.

Experimental Procedures

Strains, media and growth conditions

For strains used in this study see Table 1. Yeast cells were grown on either rich YP (1% yeast extract, 2% peptone) with 2% glucose YPD, 2% galactose YPGal, 2% ethanol, 3% glycerol YPEG, YPD supplemented with 300 μg ml^{-1} of geneticin, synthetic complete (SIGMA-Aldrich, Helsinki, Finland) with 2% glucose (SCD), 3% glycerol (SCG) or SCD and SCG media lacking one or more nutrients, WO-Gal (2% galactose, 0.67% yeast nitrogen base), WO-EG (2% ethanol, 3% glycerol, 0.67% yeast nitrogen base). 100–400 μg ml^{-1} δ-aminolevulinic acid (δ-ALA) was supplemented in media buffered at pH 5.5. In some cases media was supplemented with triclosan (Dermatologica Widmer, Helsinki, Finland). Glycerol sectoring medium has been described previously (Kastaniotis *et al.*, 2004). Solid media contained 2% agar. Yeast was grown at 30°C with vigorous shaking for liquid cultures.

Plasmids

Plasmids YCp*lac*22, YCp*lac*33 and YCp*lac*111 (Gietz *et al.*, 1988), pYE352-*CTA1* (Filppula *et al.*, 1995), pYE352::*YBR026c* and pYE352::*mtFabI* (Torkko *et al.*, 2001), the HeAl library, pTSV30A, pYE352-*YHR067w* and pYE*FAM1-1* (Kastaniotis *et al.*, 2004), YCp(33)AZ*SU -lacZ* (Deckert *et al.*, 1998) and the Lacroute library (Harington *et al.*, 1993) and plasmids for genomic integration of *MSS51* and $mss51^{F199I}$ (Barrientos *et al.*, 2002) have been described. Plasmid YEp352 was generated by digesting plasmid pYE352-*CTA1* with EcoRI and then religating the gapped plasmid to remove the *CTA1* insert. For clarity, pYE352-*CTA1*, pYE352::*YBR026c*, pYE352-*YHR067w* and pYE*FAM1-1* will be called YEp*CTA1*, YEp*ETR1*, YEp*HTD2* and YEp*FAM1-1*, respectively.

Manipulations of DNA and plasmid constructions were carried out using standard techniques (Ausubel *et al.*, 1989). All new constructs were verified by sequencing. The *ETR1* gene was amplified from yeast genomic DNA and cloned into vectors pTSV30A and YCp*lac*22 to create plasmids pTSV30*ETR1* and YCp22*ETR1* expressing the *ETR1* gene from its native regulatory sequences. The construct containing the fusion of the sequence

encoding the *COQ3* mitochondrial targeting sequence fused to the *E. coli fabI* gene was amplified from pYE352::*mtFabI* and the regulatory region of the *ETR1* gene was amplified from the pTSV30*ETR1* plasmid. These PCR products were cloned into vector YCp*lac*22 to create plasmid YCp22*mtFabI* harboring the chimeric *mtFabI* gene under control of the *ETR1* promoter and the *CTA1* terminator. The *ALD4*, *ald4-1*, *HTD2*, *MRPS5* and *mrps5-1* genes were cloned into vectors YCp*lac*33 or YCp*lac*111 to create plasmids YCp33*ALD4*, YCp33*ald4-1*, YCp33*HTD2*, YCp33*MRPS5*, YCp111*MRPS5* and YCp111*mrps5-1* expressing all of the genes from their native regulatory sequences.

Disruption of open reading frames

Each open reading frame was deleted by homologous recombination with the *kanMX4* cassettes amplified from the deletion strains of the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) using primers specific to the 5′ upstream and 3′ downstream regions of the genes. The PCR products were purified and transformed into the wild-type strains using the lithium acetate method (Gietz *et al.*, 2002). Proper integration of the constructs was verified by PCR and complementation. Double mutants were obtained from crosses of the respective single mutants and sporulation of the heterozygous diploids.

Generation of the etr1::mtFabI (FabI) strains

The *mtFabI* knock-in DNA was amplified from the YCp22*mtFabI* plasmid with 435 base pairs of the *ETR1* promoter region on the 5’ end and a 3’ tail with 40 base pairs homologous to *ETR1* terminator sequence. After selection of the respiratory competent transformants on YPG plates integration of the *mtFabI* construct into the *ETR1* locus of W1536 5B (**a**) and 8B (α) *etr1*Δ strains was tested by sensitivity to geneticin and triclosan. Both of the strains were verified by PCR.

Generation of rho^0 strains

W1536 5B (**a**) *etr1*Δ and *htd2*Δ strains were treated with ethidium bromide to induce loss of mitochondrial DNA (Ellis *et al.*, 1999). 1 ml of an overnight YPD culture grown to approximately optical density (OD) at 600 nanometers of 4 were collected, washed with 5 ml Sorenson’s solution (60 mM NaPi, 140 mM KPi) and resuspended in 2 ml Sorenson’s solution with 10 μg ml^{-1} or 20 μg ml^{-1} ethidium bromide and shaken for 2 or 4 hours. After washing with Sorenson’s solution, the cells were suspended in YPD and plated on YPD plates for single colonies. The ρ^0 genotype was verified by staining candidates in buffered 250 ng ml^{-1} DAPI (Thermo Scientific) (0.01 M $MgCl_2$, 0.01 M Tris pH 8.0) solution and inspection by fluorescence microscopy (Olympus BX51).

Generation of the intronless strains

The intronless mitochondrial genome was transferred from the *kar1* MCC109I (α) cytosolic donor strain (Ellis *et al.*, 2004), to W1536 5B (**a**) *etr1*Δ and *htd2*Δ ρ^0 strains harboring plasmids YEp*ETR1* and YCp33*HTD2*, respectively, to allow for selection on SCG. Saturated SCD-ura cultures of both strains were mixed with an YPD culture of MCC109I and incubated 3 hours without shaking and an additional 3 hours with mild shaking after adding fresh SCD-ura. Cultures were diluted and plated on SCD-ura. Candidates unable to

grow on SCD-trp and growing on SCG were chosen for further tests. The intronless genotype was confirmed by PCR. Intronless strains are designated by the cytosolic receptor strain, preceded by lower case “i”, as in (Ellis *et al.*, 2004).

Generation of W1536 5B ietr1Δmss51^{F199I} and ietr1ΔMSS51

W1536 5B *ietr1*Δ *mss51*^{F199I} and *ietr1*Δ *MSS51* were constructed as previously described (Soto *et al.*, 2012).

Growth curve, FACS analysis and spotting assay

For the growth curves and FACS analysis, cells were inoculated at an OD of 0.2 and grown on SCD medium for 72 hours. Triclosan was added to the samples at the 5 hour time point at 1 μg ml^{-1} concentration (growth curve) or 0.5 μg ml^{-1} (FACS) as indicated. Samples for FACS were harvested after the 72 hour growth period.

For spotting assays, strains were grown to logarithmic growth phase, harvested and adjusted to an OD of 0.5. A dilution series of undiluted, 1:10, 1:100 and 1:1000 was made and 2 μl of cells of each dilution were spotted on SCD and SCG plates and grown at 30°C for 2 and 5 days, respectively.

Lipoic acid analysis

Analysis of lipoic acid in yeast has been described before (Brody *et al.*, 1997; Schonauer *et al.*, 2009).

Yeast mitochondrial membrane potential analysis

Cells were harvested after the diauxic shift (OD of ~ 4.0) and subsequently treated with JC-1, a cationic, lipophilic dye used for the visualization of mitochondrial membrane potential (Smiley *et al.*, 1991). The JC-1 lipophilic cation dye dye (5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolocarbocyanine iodide) has been shown to form so-called J-aggregates at high concentrations, resulting in a shift of absorption and fluorescence maxima. JC-1 uptake into mitochondria, where the molecules aggregate due to a concentration increase relative to the cytosol, is membrane potential dependent. Aggregated, mitochondrially-imported JC-1 emits orange fluorescence (FL2, 590 nm) while unaggregated JC-1 shows green fluorescence (FL1, 520 nm). The graphs were divided into quadrants at the x (2) y (2) to facilitate analysis. Fractions of cell populations present in the quadrants are indicated (Q1-Q4). 10 μM FCCP (carbonyl cyanide 3- trifluoromethoxy phenylhydrazone) added at the same time as JC-1 was used as the negative control. The triclosan concentration in the triclosan–supplemented media was 0.5 μg ml^{-1}. Staining was performed following the SIGMA Mitochondria Staining Kit protocol (SIGMA-Aldrich, St. Louis, MO, USA). The fluorescence signal was detected using a Pertec fluorescence spectrophotometer equipped with a 20 mW solid state 488 nm laser (Partek, Münster, Germany), following the MitoProbe™ JC-1 Assay Kit for Flow Cytometry (M34152) protocol. Data analysis was performed by FlowMax software from Partek.

β-Galactosidase assays

Strains W1536 5B and derivatives thereof, transformed with plasmids YCp(33)AZ (Deckert *et al.*, 1998) and YCp*lac*33 as a control, were inoculated in 2 ml SCD-Ura medium and grown over night. The next day, these cultures were used to inoculate fresh cultures to an OD of 0.2, and these fresh cultures grown to OD of ~ 0.5, when the cells were harvested for β-galactosidase assays using the permeabilized cell assay as described (Adams *et al.*, 1997).

Heme content determination

Mitochondria were isolated from cells grown for 16 hours in rich galactose media (YPGal) supplemented, when indicated, with 100 μg/ml δ-aminolevulinic acid (δ-ALA), as previously described (Haslam *et al.*, 1979). Spheroplasts were obtained by zymolyase (5 mg/ml) digestion from cells grown under the same conditions. Hemes were extracted from mitochondria (1 mg) and spheroplasts (2 mg) with 250 mM HCl acetone and 50% acetonitrile and analyzed by HPLC with a C18 column as described (Soto *et al.*, 2012).

Statistical Analysis

All experiments were done at least in triplicate. For the enzymatic and polarographic assays, data are presented as means ± SD. The values obtained for wild-type and mutants strains were compared by the t-Student test. $P < 0.05$ was considered significant. For quantification of western blot and radioactive signals, the images were digitalized and densitometry performed using the histogram function of the Adobe Photoshop program. The values measured in three independent assays did not differ by more than 5%.

Mitochondrial isolation for western blot analysis

Mitochondria were prepared from strains grown in media containing 2% galactose or 2% glucose as described (Meisinger *et al.*, 2000) and (Herrmann *et al.*, 1994).

Synthetic petite screen

The colony sectoring screen has been described (Kastaniotis *et al.*, 2004). W1536 5B and 8B *FabI* strains carrying pTSV30*ETR1* plasmid were subjected to EMS mutagenesis, plated on glycerol sectoring media and grown at 30°C for 10 days and at 22°C for 7 days. Non-sectoring red colonies were isolated and retested to confirm the phenotype. Mutants that depended on the presence of the pTSV30*ETR1* plasmid on SCG but not on SCD were tested with YCp22*ETR1*, YCp22*mtFabI* and YEp*CTA1* (negative control) for ability to grow on SCG to rule out mutations in the *mtFabI* replacement cassette and with YEp*FAM1-1* to identify possible mutations in mtFAS pathway members (Kastaniotis *et al.*, 2004).

We were unable to completely resolve complementation groups in complementation analysis, presumably because of a propensity of the mutants to lose mitochondrial DNA. Mutations were identified by genomic DNA library complementation of the respiratory growth defect. All these candidate genes also complemented the mutants when present on single copy plasmids.

For some of the mutants, we chose to confirm the synthetic petite phenotype in a strain background that had not undergone EMS treatment. The synthetic petite phenotypes of both

mrps5-1 (Fig. S2) and *ald4-1* (data not shown) were reproduced in the respective knockout strains carrying a plasmid harbouring the respective mutant allele in the *FabI* genetic background.

Western blot analysis

Equivalent amounts of total mitochondrial proteins were separated by SDS-PAGE (12% gel) in order to quantify the steady-state levels of mitochondrial respiratory chain enzymes and individual subunits. Proteins were transferred to nitrocellulose membrane and decorated with the indicated antisera. Anti- Porin antibody was used as loading control.

Characterization of yeast mitochondrial respiratory chain

Endogenous cell respiration was assayed in whole cells in the presence of galactose using a Clark type polarographic oxygen electrode from Hansatech Instruments (Norfolk, UK) at 24°C as described (Barrientos *et al.*, 2002). Mitochondria prepared from the different strains were used for spectrophotometric assays carried out at 24°C. KCN-sensitive cytochrome *c* oxidase (COX) activity, antimycin A-sensitive NADH cytochrome *c* reductase (NCCR) activity and antimycin A-sensitive succinate cytochrome *c* reductase (SCCR) were assayed with 50 μg of mitochondria permeabilized with 0.5% sodium deoxycholate, as described (Barrientos *et al.*, 2002). Briefly, COX activity was measured by following the oxidation of 50 μM reduced cytochrome *c* at 550 nm in a buffer containing 20 mM KPi (pH 7.4). The addition of 0.3 mM KCN inhibited the reaction. NCCR and SCCR activities were measured by following the reduction of oxidized 50 μM cytochrome *c* at 550 nm, using respectively 0.4 mM NADH or 10 mM succinate as the electron donor in a buffer containing 20 mM KPi (pH 7.4) and 2 mM EDTA. The addition of 0.4 μM antimycin A inhibited both reactions. ATPase activity was assayed by measuring release of inorganic phosphate from ATP at 37°C in the presence and absence of oligomycin (King, 1932).

Mitochondrial cytochrome spectra

Isolated mitochondria were extracted at a protein concentration of 5 mg/ml in 20 mM Tris-HCl, pH 7.5, 1 M KCl, 1% sodium deoxycholate, conditions that quantitatively solubilize all mitochondrial cytochromes (Tzagoloff *et al.*, 1975). Samples of the extract were either oxidized with ferricyanide or reduced with sodium dithionite and the difference spectra were measured at room temperature using a UV-2401PC Shimadzu spectrophotometer.

In vivo mitochondrial protein synthesis

Mitochondrial gene products were labeled with ^{35}S-methionine (7 mCi mmol^{-1}, PerkinElmer) in whole cells at 30°C for 10 minutes pulse in the presence of 0.2 mg ml^{-1} cycloheximide. Equivalent amount of total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane and exposed to Kodak X-OMAT X-ray film.

Northern blot analysis

Northern blot analysis was done as described previously (Schonauer *et al.*, 2008). For anti-sense oligonucleotide probes see Supporting Information Table S5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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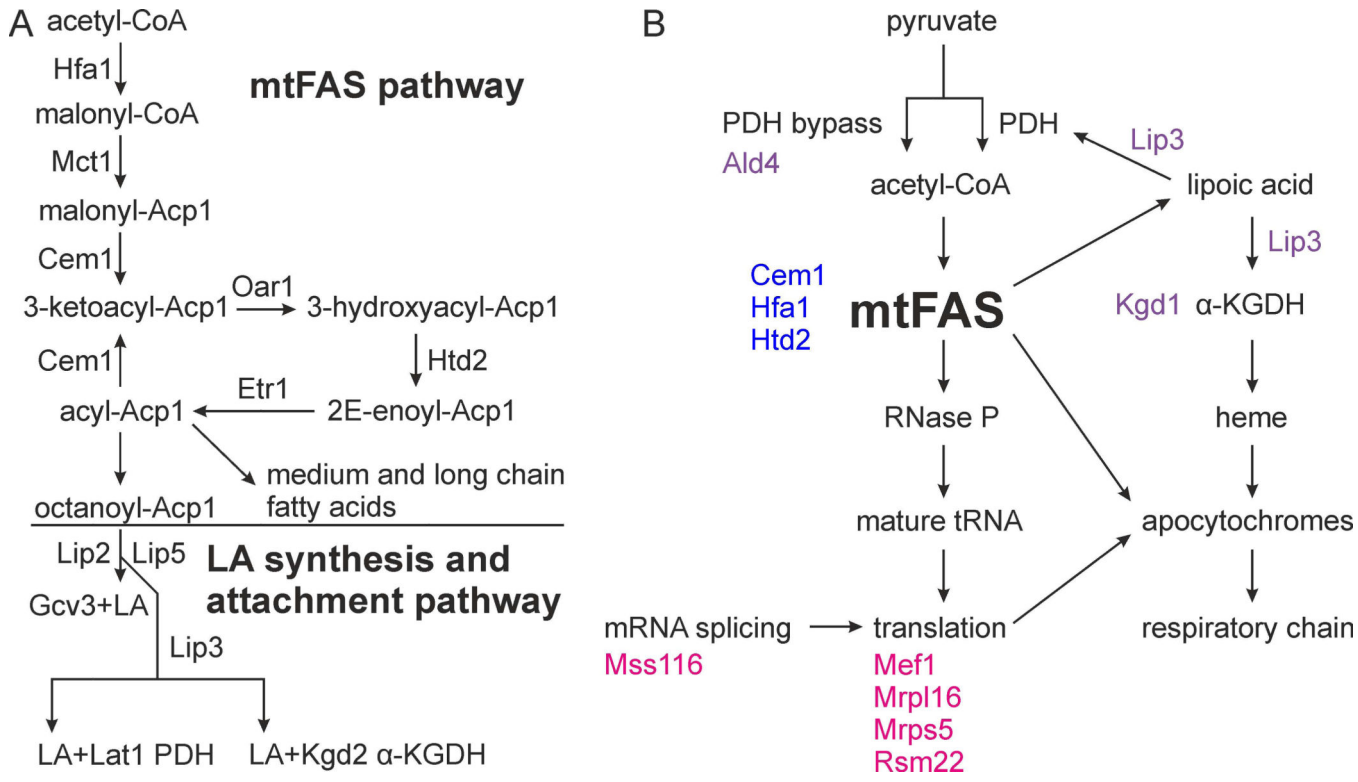

Fig. 1.

Mitochondrial fatty acid synthesis (mtFAS)/ lipoic acid (LA) synthesis and attachment pathways and synthetic mutants identified in the screen. (A) Schematic depiction of the mtFAS and LA synthesis and attachment pathways. (B) Graphic presentation of the results of the synthetic petite screen. Mutated factors are color-coded to indicate functional groups. Blue: mtFAS enzymes, magenta: post-transcriptional gene expression related proteins, purple: mutations related to LA attachment (Lip3), LA-dependent complexes (Kgd1) or the pyruvate dehydrogenase complex (PDH) bypass (Ald4).

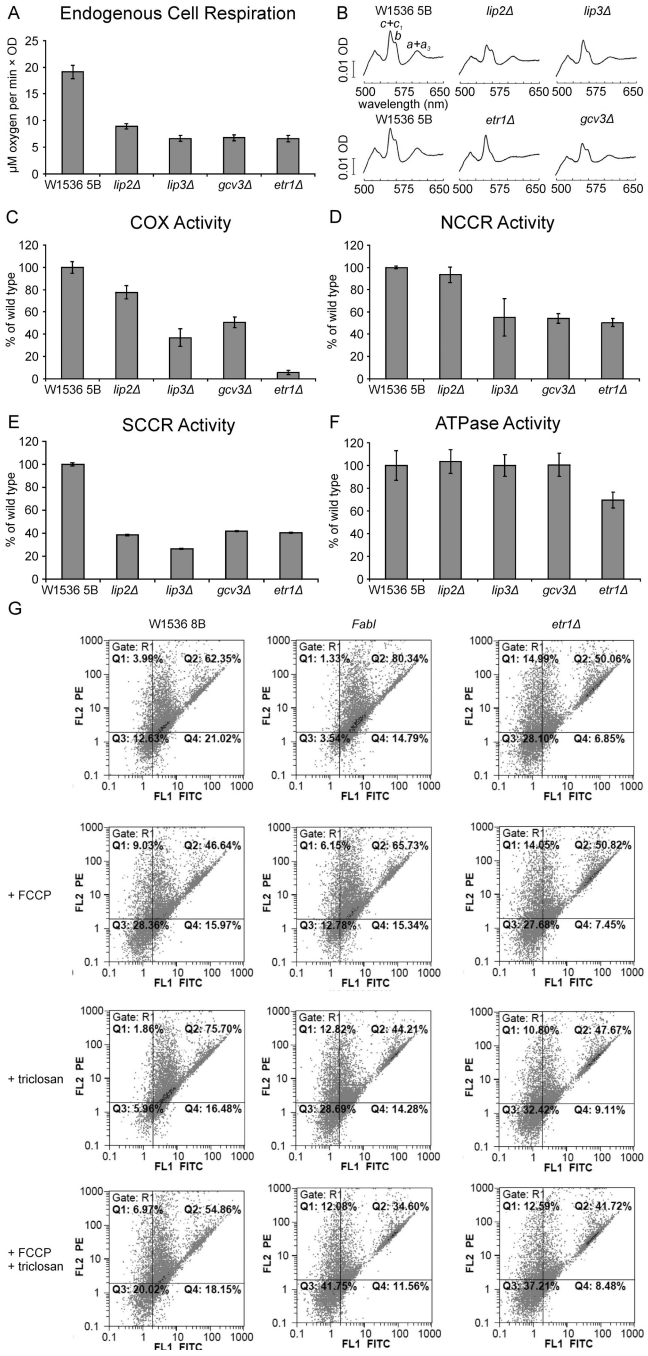

Fig. 2.

Characterization of yeast strains carrying deletion mutations in genes required for lipoic acid synthesis and attachment and mitochondrial fatty acid synthesis deletion. (A) Endogenous cellular respiration, (B) cytochrome spectra, (C) cytochrome *c* oxidase complex (COX) activity, (D) NADH cytochrome *c* reductase activity (complex I + III) (NCCR) activity, (E) succinate cytochrome *c* reductase (complex II + III) (SCCR) activity, and (F) ATPase activity. Data are shown as the mean of three independent repetitions ± SD. (G) Mitochondrial membrane potential in *etr1*Δ and *FabI* strains inhibited by triclosan, assessed

by measuring JC-1 fluorescence with FACS (fluorescence-activated cell sorting). Orange fluorescence (FL2) indicates high membrane potential, while green fluorescence (FL-1) is observed when the mitochondrial membrane potential is strongly decreased (see Experimental Procedures). FCCP (carbonyl cyanide 3- trifluoromethoxy phenylhydrazone) is an uncoupler that causes dissipation of membrane potential.

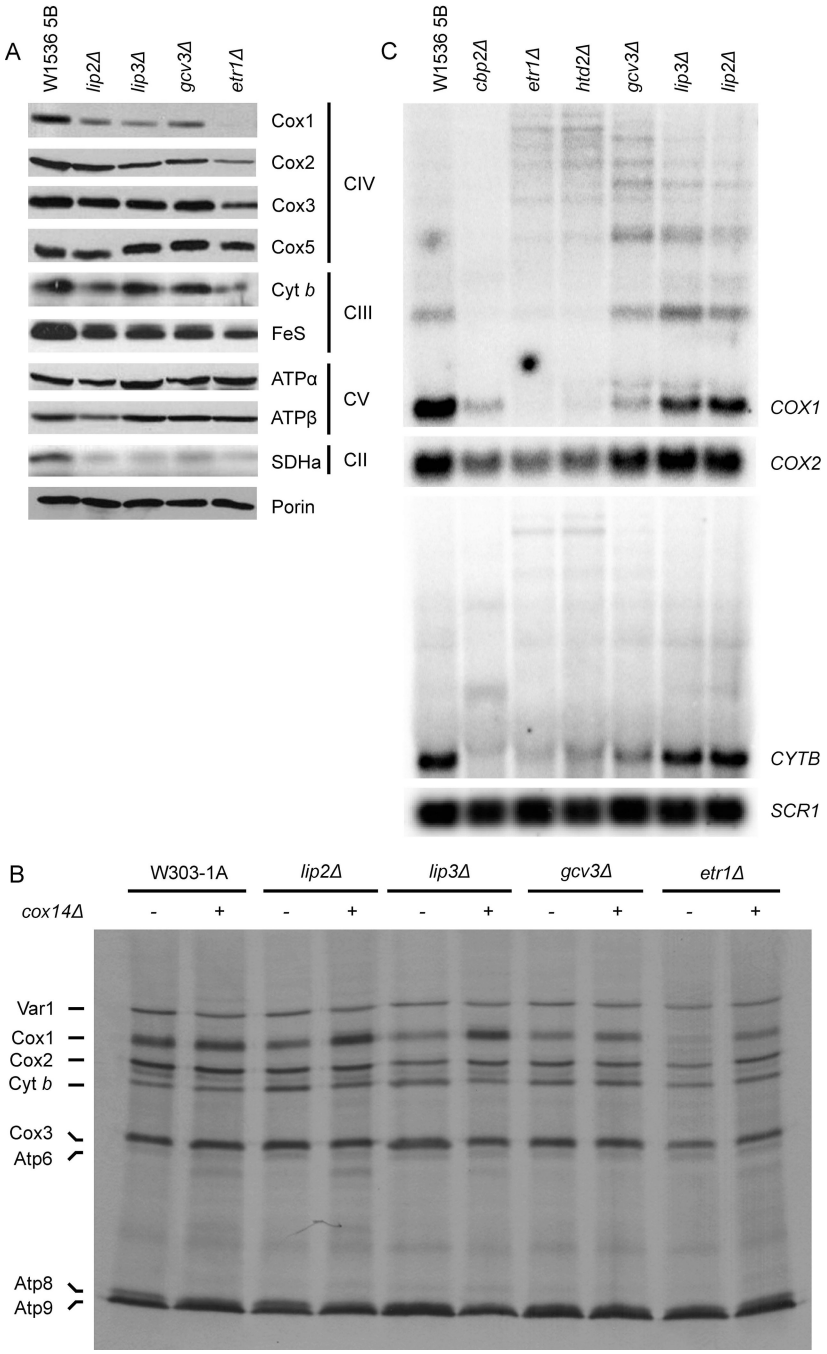

Fig. 3.

Mitochondrial DNA gene expression and OXPHOS enzyme subunit levels in intron-containing mitochondrial fatty acid synthesis mutant strains. (A) Steady state levels of indicated proteins by western blot analysis of isolated mitochondria. Loading control: Porin. (B) *De novo* translation of mtDNA-encoded proteins. (C) Processing of mitochondrial transcripts. A *cpb2*Δ strain was used as a non-lipoylation/non-mtFAS-defective respiratory deficient control. Total RNA extracted from the strains was hybridized with a probe

complementary to *COX1*, *COX2* and *CYTB*. Loading control: *SCR1*, the RNA subunit of the Signal Recognition Particle (SRP), a housekeeping gene.

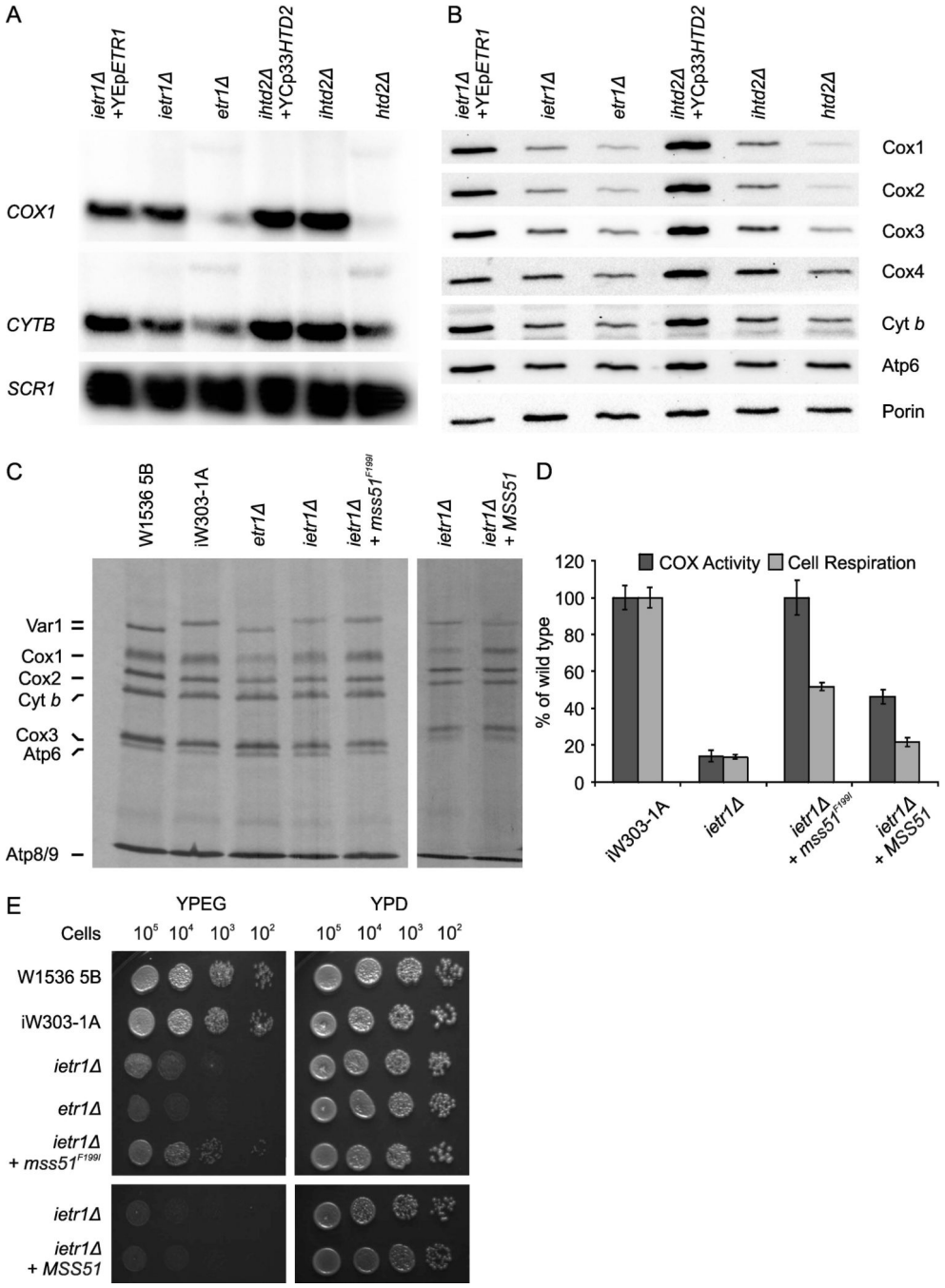

Fig. 4.

Mitochondrial gene expression, OXPHOS enzyme subunit levels, COX activity, respiration and growth of intronless mtFAS mutant strains. (A) Processing of mitochondrial transcripts in mitochondrial fatty acid synthesis deletion strains with or without mitochondrial introns. Total RNA extracted from the strains was hybridized with a probe complementary to *COX1*, *COX2* or *CYTB*. Loading control: *SCR1*. (B) Analysis of the steady state levels of indicated proteins from mitochondrial extracts by western blot. Loading control: Porin. (C) *De novo* protein synthesis in intronless and control strains. (D) Cytochrome oxidase activity

measurements and cell respiration in intron-containing and intronless W1536 5B *etr1*Δ strains and the *ietr1*Δ strain expressing one extra copy of either *mss51*^{F199I} or wild-type *MSS51*. Endogenous cell respiration was measured polarographically. Data are shown as the mean of three independent repetitions ± SD. (E) Dilution series testing for growth of strains analyzed in panels C and D on non-fermentable and fermentable carbon sources.

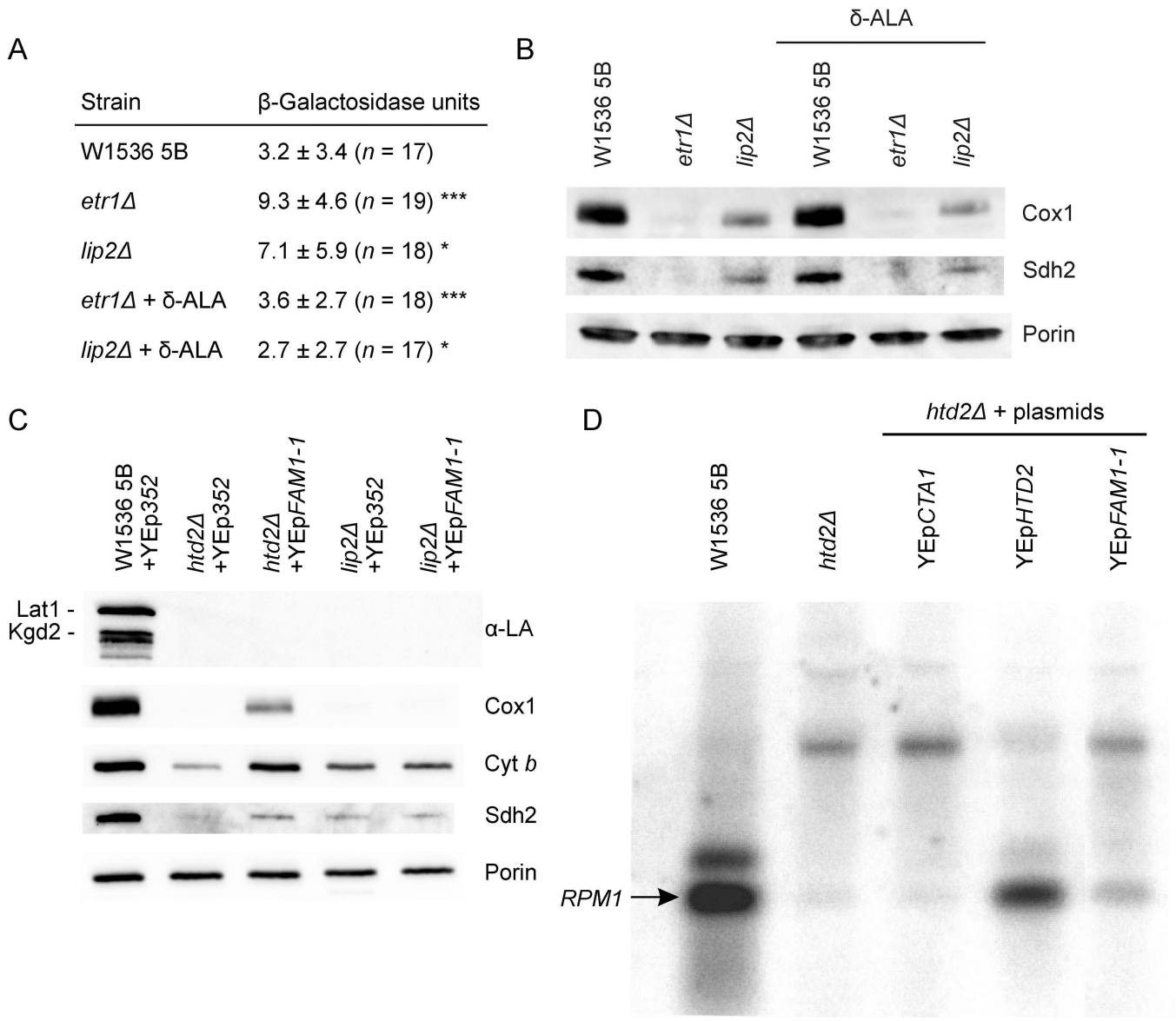

Fig. 5.

Effect of δ-aminolevulenic acid (δ-ALA) supplementation and *FAM1-1* overexpression on the OXPHOS phenotypes of mtFAS and LA attachment mutant strains. (A) β-Galactosidase assay. Medium was supplemented with 100 μg ml^{-1} δ-ALA. Asterisk indicates statistical significance determined by Student's t test (***$p < 0.0001$, *$p < 0.05$). Data are represented as the mean of at least 17 measurements ± SD. (B) Supplementation with δ-ALA does not improve accumulation of respiratory complex subunits. Western blot analysis of Cox1 and Sdh2 steady state levels in mitochondria from wild-type, mtFAS and LA attachment-deficient cells grown in the presence or absence of 400 μg ml^{-1} δ-ALA. Loading control: Porin. (C) Western blot analyses of *the* steady state levels of the indicated proteins in control and mtFAS or LA attachment deficient strains overexpressing *FAM1-1*. Lat1 and Kgd2 are the lipoylated E2 subunits of pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex, respectively. Loading control: Porin. (D) Processing of the *RPM1*

RNA subunit of RNase P is improved in mtFAS deficient strains overexpressing *FAM1-1*. Total RNA extracted from the indicated strains was hybridized with a probe complementary to *RPM1*. The arrow indicates mature *RPM1*.

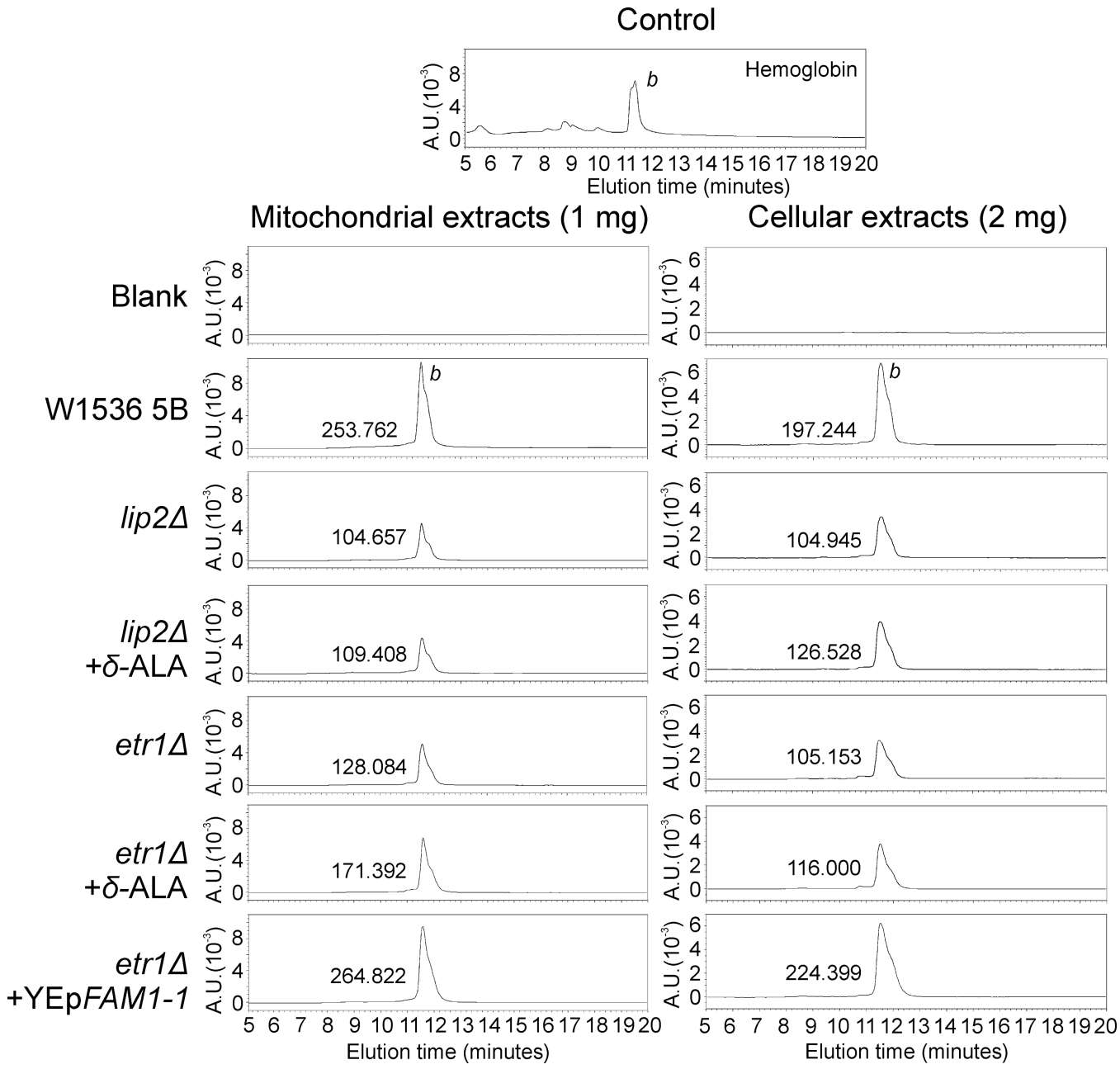

Fig. 6.

Effect of δ-aminolevulenic acid (δ-ALA) supplementation and *FAM1-1* overexpression on heme content in mitochondrial fatty acid synthesis and lipoic acid attachment mutant strains. Hemes were extracted from isolated mitochondria (left panel) or spheroplasts (right panel) and analyzed by HPLC on a reverse phase C18 column. Hemoglobin was used to calibrate the column. The peaks corresponding to heme B were quantified by calculating the areas under the peaks and expressed in μV*sec. The peaks corresponding to heme O and heme A were basically undetectable at the represented scale and have been omitted from the figure.

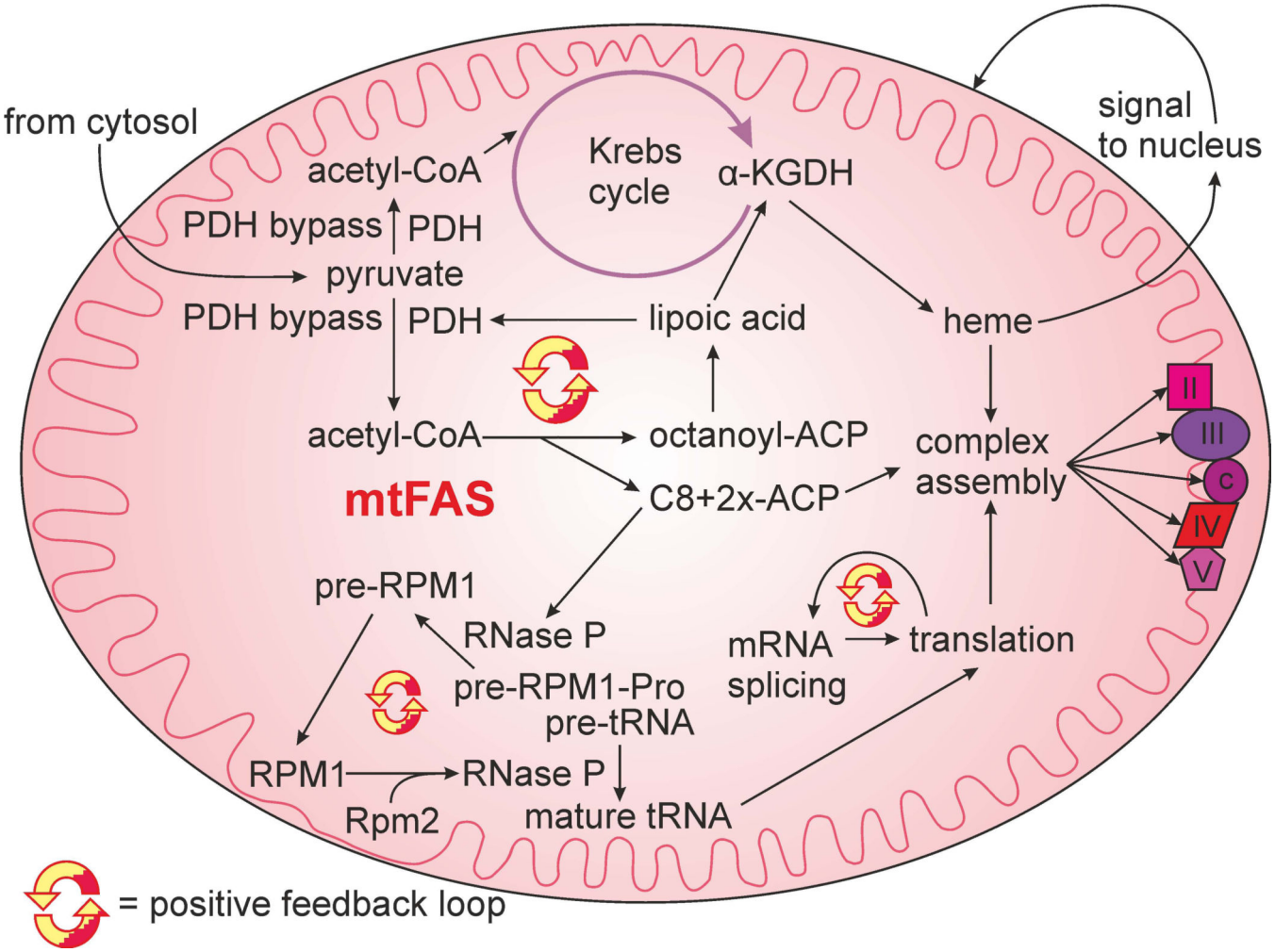

Fig. 7.

Simplified model of mtFAS-dependent regulatory circuits controlling mitochondrial gene expression. MtFAS is needed for octanoyl-ACP production, the sole octanoate source for lipoic acid (LA) synthesis. The pyruvate dehydrogenase complex (PDH) activity is part of a positive feedback loop (red and yellow circular arrow loop) with LA production and acetyl-CoA substrate production. The Krebs cycle and α-ketoglutarate dehydrogenase complex (α-KGDH) provide reducing power, succinate and precursors for heme biosynthesis. The arrows represent the flow of material and the requirement of products of the mtFAS pathway in LA synthesis, *RPM1* RNA processing and respiratory complex assembly. *RPM1* must be processed to assemble fully active RNase P for processing of the tRNA-containing *RPM1* precursor RNA (second proposed positive feedback loop). Translation is needed for mRNA splicing, possibly resulting in mtFAS signal amplification (third proposed feedback loop). Some subunits of the cytochrome *c* reductase complex (III), cytochrome *c* oxidase complex (IV) and F_1F_o ATP synthase (V) are translated in the mitochondria. The succinate dehydrogenase complex (II), as well as complexes III, IV and cytochrome *c* (c), require heme for their assembly and function.

Table 1

Strains used in this study.

Strain	Genotype	Reference
S. cerevisiae		
MCC109I	*MATaade2-101, ura3-52, kar1-1, intronless mtDNA*	(Ellis *et al.*, 2004)
W303-1A	*MATaade2-1, his3-1,15; leu2-2,112; trp1-1, ura3-1*	R. Rothstein (Columbia University, New York, NY)
iW303-1A	*intronless mtDNA*	This study
W303 *cox14*Δ	*MATα, ade2-1, his3-1,15; leu2-2,112; trp1-1, ura3-1, yml129c::TRP1*	(Barrientos *et al.*, 2004)
*cox14*Δ, *lip2*Δ	*yml129c::TRP1, ylr239c, kanMX4*	This study
*cox14*Δ, *lip3*Δ	*yml129c::TRP1, yjl046w, kanMX4*	This study
*cox14*Δ, *gcv3*Δ	*yml129c::TRP1, yal044c, kanMX4*	This study
*cox14*Δ, *etr1*Δ	*yml129c::TRP1, ybr026c, kanMX4*	This study
W1536 5B	*MATaade2*Δ, *ade3*Δ, *can1-100, his3-11,15; leu2-3,112; trp1-1, ura3-1*	(Kastaniotis *et al.*, 2004)
*ald4*Δ	*yor374w, kanMX4*	This study
*cbp2*Δ	*yhl038c, kanMX4*	This study
*cem1*Δ	*yer061c, kanMX4*	This study
*etr1*Δ	*ybr026c, kanMX4*	This study
*etr1*Δ ρ^0	*ybr026c, kanMX4,* ρ^0	This study
*ietr1*Δ	*ybr026c, kanMX4, intronless mtDNA*	This study
*ietr1*Δ *MSS51*	ybr026ckanMX4, intronless mtDNA, integrated MSS51	This study
*ietr1*Δ *mss51*^{F199I}	*ybr026ckanMX4, intronless mtDNA, integrated mss51*^{F199I}	This study
FabI	*ybr026c, kanMX4, mtFabI*	This study
*FabI ald4*Δ	*ybr026c, kanMX4, mtFabI, yor374w, kanMX4*	This study
*FabI mrps5*Δ	*ybr026c, kanMX4, mtFabI, ybr251w, kanMX4*	This study
*gcv3*Δ	*yal044c, kanMX4*	This study
*htd2*Δ	*yhr067w, kanMX4*	This study
*htd2*Δ ρ^0	*yhr067w, kanMX4,* ρ^0	This study
*ihtd2*Δ	*yhr067w, kanMX4, intronless mtDNA*	This study
*lip2*Δ	*ylr239c, kanMX4*	This study
*lip3*Δ	*yjl046w, kanMX4*	This study
*mrps5*Δ	*ybr251w, kanMX4*	This study
W1536 8B	*MATα, ade2*Δ, *ade3*Δ, *can1-100, his3-11,15; leu2-3,112; trp1-1, ura3-1*	(Kastaniotis *et al.*, 2004)
*etr1*Δ	*ybr026c, kanMX4*	This study
FabI	*ybr026c, kanMX4, mtFabI*	This study