Aep3p-dependent translation of yeast mitochondrial *ATP8*

Mario H. Barros[a] and Alexander Tzagoloff[b,*]
[a]Departamento de Microbiologia, Universidade de Sao Paulo, Sao Paulo 05508-900, Brazil; [b]Department of Biological Sciences, Columbia University, New York, NY 10027

ABSTRACT **Translation of mitochondrial gene products in *Saccharomyces cerevisiae* depends on mRNA-specific activators that bind to the 5′ untranslated regions and promote translation on mitochondrial ribosomes. Here we find that Aep3p, previously shown to stabilize the bicistronic *ATP8-ATP6* mRNA and facilitate initiation of translation from unformylated methionine, also activates specifically translation of *ATP8*. This is supported by several lines of evidence. Temperature-sensitive *aep3* mutants are selectively blocked in incorporating [^{35}S] methionine into Atp8p at nonpermissive but not at the permissive temperature. This phenotype is not a consequence of defective transcription or processing of the pre-mRNA. Neither is it explained by turnover of Aep3p, as evidenced by the failure of *aep3* mutants to express a recoded *ARG8*^{m} when this normally nuclear gene is substituted for *ATP8* in mitochondrial DNA. Finally, translational of *ATP8* mRNA in *aep3* mutants is partially rescued by recoded allotopic *ATP8* (*nATP8*) in a high-expression plasmid or in a CEN plasmid in the presence of recessive mutations in genes involved in stability and polyadenylation of RNA.**

Monitoring Editor
Thomas D. Fox
Cornell University

Received: Nov 14, 2016
Revised: Mar 29, 2017
Accepted: Apr 4, 2017

INTRODUCTION

Biogenesis of mitochondrial ATP synthase (F_1-Fo complex) in *Saccharomyces cerevisiae* is assisted by a dozen nuclear gene products that intercede at all stages of the assembly pathway (Ackerman and Tzagoloff, 2005). With a few exceptions (Lefebvre-Legendre *et al.*, 2001; Ackerman, 2002), these factors target the mitochondrial *ATP6*, *ATP8*, and *ATP9* mRNAs and their products, which function in the proton-translocating activity of the ATP synthase. *ATP8* and *ATP6* are cotranscribed together with *COX1*. In some strains, the polycistronic transcript includes *ENS2*, which codes for a mitochondrial endonuclease (Simon and Faye, 1984).

Several nuclear genes were previously shown to affect expression of Atp6p and Atp8p by either stabilizing or activating translation of the bicistronic *ATP8-ATP6* mRNAs (Camougrand *et al.*, 1995; Pelissier *et al.*, 1995; Ellis *et al.*, 2004; Zeng *et al.*, 2007). Translational activation by mRNA-specific factors is by no means unique to the *ATP8-ATP6* mRNA but is a general feature of the mitochondrial genetic system in *S. cerevisiae* (Costanzo and Fox, 1990; Fox, 2012; Herrmann *et al.*, 2013). Translational activators may have more than one function. Mss51p, a translational activator of the *COX1* mRNA, has been shown to bind to the Cox1p product as part of a mechanism for regulating expression of this subunit of cytochrome oxidase (Perez-Martinez *et al.*, 2003; Barrientos *et al.*, 2004; Fontanesi *et al.*, 2011). Translational activators have been identified for every mitochondrial gene product except Atp8p and Var1p (Fox, 2012). Even though *ATP8* and *ATP6* are present in the same transcript(s), their translation is probably regulated by different factors. This is supported by the finding that mutations in the *ATP6* mRNA activator Atp22p prevent translation of Atp6p but not Atp8p (Zeng *et al.*, 2007).

In the present study, we obtained temperature-sensitive (ts) *aep3* mutants that are specifically defective in expression of the mitochondrial *ATP8* gene at the nonpermissive temperature. Our evidence indicates that this phenotype stems from the failure of such mutants to translate the *ATP8-ATP6* mRNA. In an attempt to search for additional factors that affect *ATP8*, we took advantage of the ability of allotopically expressed *ATP8* (*nATP8*) to complement and restore respiratory growth of *atp8* mutants (Gearing *et al.*, 1985; Gearing and Nagley, 1986; Barros *et al.*, 2011). (Note: *nATP8* is *ATP8* recoded for expression on cytoplasmic ribosomes. *nATP8*^{GPD}

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E16-11-0775) on April 12, 2017.

*Address correspondence to: Alexander Tzagoloff (spud@columbia.edu).

Abbreviations used: CEN plasmid, plasmid with a centromeric origin of replication; CPF, cleavage and polyadenyation factor; mtDNA, mitochondrial DNA; ρ^-, respiratory deficient yeast mutant with large deletion in mtDNA; ρ^0, respiratory deficient yeast mutant lacking mtDNA.

is *nATP8* downstream of the *GPD* promoter. $nATP8^{GPD\text{-}CYC1}$ is *nATP8* downstream of the *GPD* promoter and followed by the *CYC1* terminator. $nATP8^{ADH1}$ is *nATP8* downstream of the *ADH1* promoter followed by the *ADH1* terminator.)

Using this screen, we obtained *aep3* mutants that were rescued by a combination of *nATP8* and mutations in *pta1* or *nrd1*. The results of the two approaches provide strong evidence that Aep3p is required specifically for translation of *ATP8*. Of interest, Aep3p was first shown to stabilize the *ATP8-ATP6* bicistronic messenger (Ellis *et al.*, 2004). In a subsequent study, Aep3p was reported to be an essential cofactor for mIF2-dependent initiation of translation of mitochondrial gene products with unformylated initiator methionyl-tRNA (Lee *et al.*, 2009). The present study further enlarges the functional repertoire of this interesting protein and highlights the role of Aep3p as a new translational regulator of *ATP8-ATP6* mRNA.

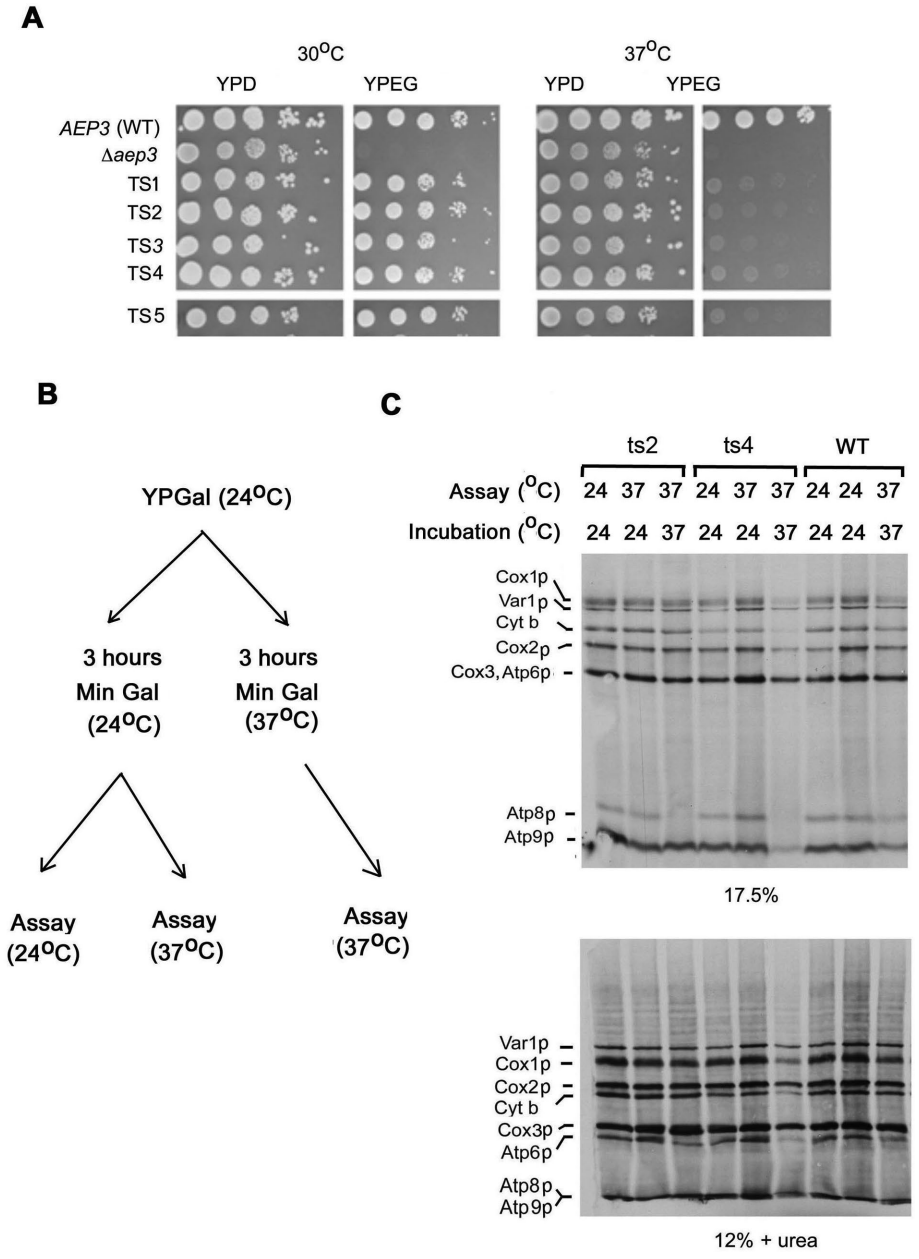


FIGURE 1: Growth and mitochondrial translation activity of ts *aep3* mutants. (A) The wild-type strain W303-1A (*AEP3*), the *aep3*-null mutant (Δ*aep3*), and the null mutant transformed with plasmid bearing five independent ts *aep3* mutant alleles were serially diluted and spotted on rich glucose (YPD) and two rich ethanol/glycerol (YEPG) plates, which were incubated at 30 or 37°C. (B) Three conditions used to measure the effect of temperature on mitochondrial translation in ts mutants. (C) In vivo labeling of mitochondrial gene products by the W303-1A (WT) and two *aep3*-null mutants transformed with two different ts *aep3* alleles (ts2 and ts4). Cells were grown to early stationary phase in YPGal at 24°C, followed by 3 h of incubation at 24°C, and were then assayed at 24°C (left lane) or 37°C (middle lane). In the third assay, cells grown at 24°C were incubated for 3 h at 37°C and assayed at 37°C (right lane). Equivalent number of cells were labeled with [^{35}S]methionine as described in *Materials and Methods*, and total mitochondrial proteins were separated on a 17.5% polyacrylamide gel and separately on a 12% polyacrylamide gel containing 6 M urea (to resolve Cox3p and Atp6p). Proteins were transferred to nitrocellulose and exposed to x-ray film. The mitochondrial translation products are identified on the left side of each gel.

RESULTS

Aep3p is required for translation of *ATP8* mRNA

ATP synthase mutants, including *aep3* mutants, are prone to undergo partial or complete deletions in mitochondrial DNA (Ellis *et al.*, 2004; Zeng *et al.*, 2007). As a result, they display a pleiotropic phenotype, with large reductions in cytochrome oxidase (COX) and the bc1 complex. This property makes it difficult to gather a detailed understanding of the underlying biochemical defect. This experimental complication has been overcome by obtaining ts alleles of the gene in question (Zeng *et al.*, 2007). The ts mutants tend to have a more stable mitochondrial genome, thereby permitting biochemical analysis unencumbered by secondary effects stemming from loss of mtDNA.

A plasmid library with mutations in *aep3* was obtained by PCR mutagenesis of *AEP3* under low-stringency conditions. This library was used to transform a heterozygous *aep3*-null mutant. Diploid transformants were sporulated and haploid progeny tested for ts growth on nonfermentable carbon sources. This protocol was used to collect seven ts mutants capable of growing on rich glycerol plus ethanol medium at 30 but not 37°C (Figure 1A).

Mitochondrial translation in the parental strain and two different ts mutants (ts2 and ts4) was assayed under different conditions (Figure 1B). Cells were grown to log phase at 24°C, transferred to minimal galactose medium, and further incubated for 3 h in minimal galactose medium at 24°C. They were then assayed at 24 or 37°C. Under both conditions, [^{35}S]methionine incorporation into all of the mitochondrial translation products, including Atp8p, was approximately the same in the ts mutants as in wild-type cells (Figure 1C). Translation of Atp8p but not Atp6p or Atp9p, however, was markedly decreased in mutant cells grown

in minimal galactose at 37°C and assayed at 37°C. The selective reduced synthesis of Atp8p in cell preincubated and assayed at the restrictive temperature could be a consequence of defective transcription/processing of the mRNA precursor, stability of the mRNA or of the translation product, or a requirement of Aep3p specifically for translation of *ATP8* mRNA.

ATP8 is part of a polycistronic transcript that includes *COX1* and *ATP6*. This primary transcript is processed by endonucleolytic cleavages that produce *COX1* mRNA and 5.2- and 4.6-kb bicistronic mRNAs with *ATP8* plus *ATP6* (Camougrand *et al.*, 1995; Pelissier *et al.*, 1995; Figure 2A). It is unlikely that *aep3* mutants are defective in transcription or processing of the mRNA, as they have normal amounts of *COX1* mRNA and Cox1p (Ellis *et al.*, 2004). Cox1p and Atp6p levels in the ts mutants at the nonpermissive temperature are comparable to the wild type (Figure 1C). These results suggest that the reduced 35% *ATP8-ATP6* mRNAs detected in the *aep3*-null mutant (Ellis *et al.*, 2004) probably stems from RNA turnover.

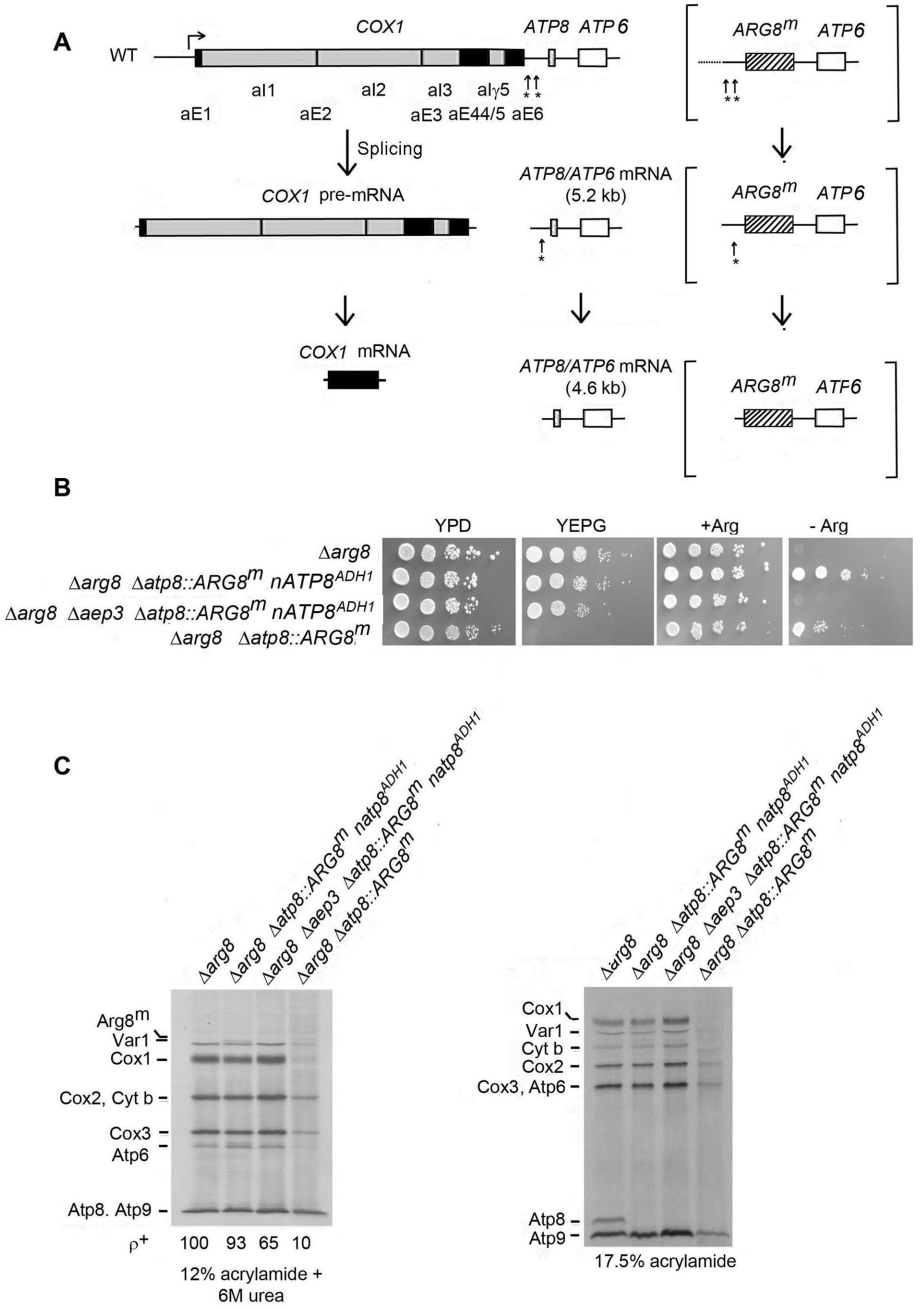


FIGURE 2: Expression of *ARG8*^{m} from the *ATP8* locus. (A) Processing of the *COX1-ATP8-ATP6* and *COX1-ARG8-ATP6* pre-mRNAs (brackets). The introns of *COX1* are in gray and the exons in black. Arrows with asterisks indicate the cleavage sites during maturation of the primary transcript. (B) Inhibition by an *aep3*-null mutation on growth of an atp8::*ARG8*^{m} strain in the absence of arginine. The relocated *nATP8*^{ADH1} was in a plasmid with the 5′ and 3′ sequences of *ADH1*. Strains with the indicated genotypes were serially diluted and spotted on rich glucose (YPD), rich ethanol plus glycerol (YPEG), and minimal glucose with arginine (+ Arg) and without arginine (–Arg). The photograph was taken after 2 d of incubation at 30°C. (C) Mutants used in B were labeled in vivo in the presence of cycloheximide with [^{35}S]methionine for 10 min. Total cellular protein were separated on 12% plus urea and 17.5% polyacrylamide gels and transferred to nitrocellulose, and radiolabeled proteins were detected as in Figure 1C.

Aep3p is required for expression of *ARG8*^{m} at the *ATP8* locus of mtDNA

Synthesis by *aep3* ts mutants of Cox1p and Atp6p but not Atp8p does not exclude the possibility that Aep3p protects newly synthesized Atp8p against proteolysis. To address this question, we tested whether Aep3p is required for expression of *ARG8*^{m} when this gene is substituted for *ATP8* in mtDNA. *ARG8*^{m} differs from nuclear *ARG8* in its sequence to accommodate for differences in the genetic code of yeast mitochondria (Steele *et al.*, 1996). The rationale of this test is that the stability of a nonmitochondrial protein like the *ARG8*^{m} product should not be affected by Aep3p. Failure of mitochondrial *ARG8*^{m} to be expressed in an *aep3* mutant would constitute compelling evidence that Aep3p is required for translation rather than stability of Atp8p.

In agreement with earlier studies (Rak and Tzagoloff, 2009; Barros *et al.*, 2011), substitution of mitochondrial *ATP8* with *ARG8*^{m} restored growth of an *arg8* mutant in medium lacking arginine (Figure 2B). This confirms the expression of a functional *ARG8*^{m} when it is flanked by the 5′ and 3′ flanking sequences of *ATP8*. As expected, absence of *ATP8* in the arginine-independent clones precluded growth on respiratory carbon sources, which was rescued by *nATP8*^{ADH1}, a nuclear version of *ATP8* (Gearing and Nagley, 1986; Barros *et al.*, 2011) flanked by the *ADH1* promoter and terminator in pMGL3 (Barros *et al.*, 2011). The combination of allotopic *ARG8*^{m} and *nATP8*^{ADH1} conferred both arginine prototrophy and respiratory competence on the *arg8* null mutant (Figure 2B). The arginine prototrophy, however, was lost in the double *arg8* and *aep3*–null mutant, which remained respiratory competent because of Atp8p expressed from allotopic *nATP8*. This indicates that translation of *ARG8*^{m} and, by inference, that of *ATP8* depend on Aep3p. Radiolabeled Atp8p is not seen

in the respiratory-competent strain with *nATP8* (Figure 2C, third lane from left), as this gene product is translated on cytoplasmic ribosomes.

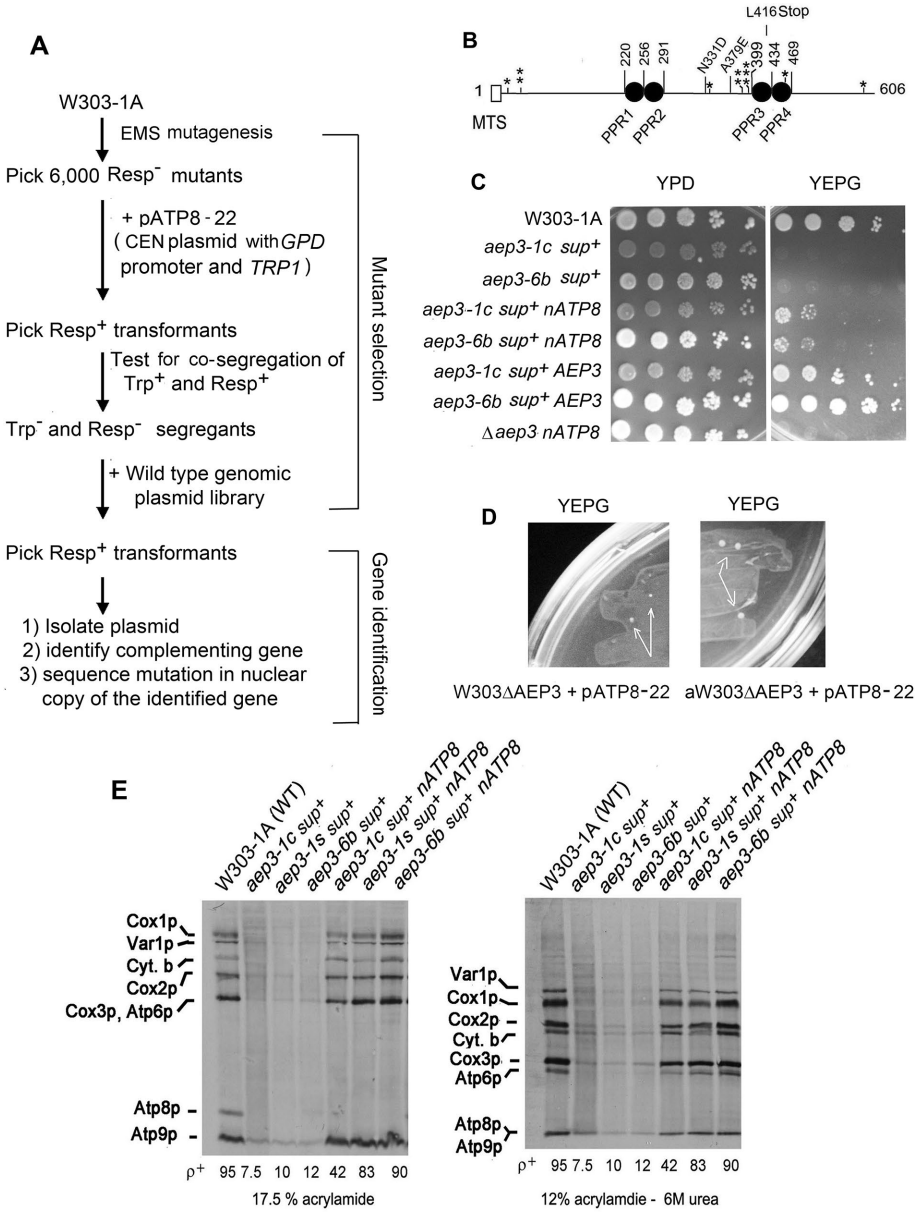


FIGURE 3: Isolation of mutants with *nATP8*-dependent respiration. (A) Genetic screen used to isolate and identify *aep3* mutants with *nATP8*-dependent respiration. (B) Location of the *aep3-1c*, *aep3-6b*, *ts1* (single asterisk), *ts2* (double asterisks), and *ts3* alleles (triple asterisk) relative to the PPR motifs in Aep3p. Open bar, putative mitochondrial targeting sequence (MTS). (C) Growth property of respiratory-competent strains with *nATP8* (pATP8-22) obtained by the genetic screen depicted in A and of the corresponding respiratory-deficient segregants lacking *nATP8*. The respiratory deficient segregants were also transformed with a plasmid containing *AEP3* (bottom two rows). Mutants with the indicated phenotypes were serially diluted and spotted on rich glucose medium (YPD) and rich ethanol/glycerol medium (YPEG).(D) Respiratory-deficient *aep3*-null mutants with *nATP8* (aW303ΔAEP3/nATP8 and W303ΔAEP3/nATP8) were plated on YEPD. The photograph was taken after 4 d of incubation at 30°C. The arrows show respiratory-competent revertant colonies. (E) The parental wild-type strain W303-1A, the two respiratory-competent *aep3* point mutants, and segregants described in B were labeled in vivo and the translation products visualized as in Figure 1C. The mutant *aep3-1s* has the premature termination codon but not the other point mutation of *aep3-1c*. A plasmid (pAEP3-1S) containing this allele was integrated into the nuclear DNA of the null mutant aW303ΔAEP3/S⁺/ST22, which has both a suppressor and *nATP8*.

Screen for mutants defective in expression of *ATP8*

The finding that Aep3p functions in translation of *ATP8* raises the question of whether there may exist additional factors necessary for *ATP8* expression. The genetic screen used in this study to isolate mutants in nuclear gene products required specifically for expression of mitochondrial *ATP8* was based on the ability of *nATP8* to complement *atp8* mutants (Gearing and Nagley, 1986; Barros *et al.*, 2011). A library consisting of 6000 respiratory-defective mutants obtained by ethyl-methanesulfonate (EMS) mutagenesis was transformed with pATP8-22, a tryptophan-selectable CEN plasmid with the *GDP* promoter carrying recoded *nATP8*. In this screen, restoration of respiration by *nATP8* is expected to identify mutations in genes that target mitochondrial *ATP8*. As proof of principle, the mutant library was transformed with a recoded *COX2* (n*COX2*) that has a W56R mutation in the first transmembrane domain of Cox2p (Supekova *et al.*, 2010). This allotopic *nCOX2* partially complemented a *cox2* mutant (Supekova *et al.*, 2010), as well as a *pet111* mutant defective in translation of mitochondrial *COX2* mRNA (Supplemental Figure S1A). The screen of mutants transformed with *nCOX2* yielded 16 clones partially complemented for growth on rich ethanol/glycerol (yeast extract/peptone/glycerol [YEPG]). Further tests indicated that they all had mutations in *PET111* (Supplemental Figure S1B).

A similar screen was used to transform the mutant library with *nATP8* in plasmid pATP8-22 (Figure 3A). The screen yielded two respiratory-competent strains that were ascertained to have mutations in *AEP3* (alleles *aep3-1c* and *aep3-6b*). The aep3-*6b* mutant had a single A379E missense mutation, and *aep3-1c* had the same mutation plus an N331D substitution and a premature L416Ochre termination codon in the third pentatricopeptide repeat of Aep3p (Figure 3B). The mutants with the different alleles grew at similar rates on YEPG medium in the presence of *nATP8* (Figure 3C).

Of interest, *nATP8* by itself did not rescue respiration in mutants with the *aep3-1c*-, *aep3-6b*-, or *aep3*-null allele. Transformation of respiratory-deficient *aep3-1c* or *aep3-6b* segregants with pATP8-22 failed to confer growth on YEPG medium (unpublished data). Similarly, transformation of the *aep3* null mutant with this plasmid also failed to restore growth on YEPG medium. After 4–5 d, however, respiratory-competent revertant colonies began to appear on this medium (Figure 3D). Plasmid segregation tests on the revertants confirmed the requirement of *nATP8* for respiratory

competency (unpublished data). This was also true of the original respiratory-competent mutants with the *aep-1c* and *aep3-6b* alleles (Figure 3B). The *nATP8* requirement correlated with the mitochondrial translational activity. Respiratory-deficient mutants with the *aep3-1c*, *aep3-6b*, and *aep3-1s* (a mutant construct with just the L416Ochre mutation) alleles had unstable mtDNA and very low mitochondrial translation activity compared with the respiratory-competent revertant clones (Figure 3E). No radiolabeled Atp8p was detected in any of the respiratory-deficient or competent -clones, indicating that the restoration of respiratory activity was unrelated to expression of mitochondrial *ATP8*. Together these results indicate that respiration in the different *aep3* mutants depends on *nATP8* and an extragenic suppressor.

The suppressors conferring respiratory growth on *aep3* mutants in the presence of *nATP8* were determined to be in nuclear DNA. A respiratory-competent *aep3* revertant containing *nATP8* (aW303ΔAEP3/nATP8) was depleted of mtDNA (ρ^0). Respiration in this ρ^0 derivative was restored by reintroduction of wild-type mtDNA from W303 (Supplemental Figure S2A). In addition, the sequences of mtDNA from the respiratory-deficient mutant W303ΔAEP3/nATP8 and the isogenic revertant W303ΔAEP3/nATP8/S^+ were identical.

The nuclear suppressors were also determined to be recessive. Diploid cells (Δ*aep3/*Δ*aep3 sup*$^+$*/sup*$^-$ *nATP8*) issued from a cross of the respiratory-competent revertant aW303ΔAEP3/S^+/ST22 (Δ*aep3 sup*$^+$ *nATP8*) to the respiratory-deficient parental strain W303ΔAEP3 (Δ*aep3 sup*$^-$) were respiratory deficient (Supplemental Figure S2B). Growth on respiratory carbon sources, however, was restored by a recombinant plasmid containing *AEP3* (Supplemental Figure S2B). Similarly, cross of the respiratory-competent point mutant *aep3-1c* (*s*$^+$) or *aep3-1s* (*s*$^+$) to W303ΔAEP3 (Δ*aep3 sup*$^-$) produced respiratory-deficient diploid cells. The respiratory deficiency of the different heterozygous *sup*$^+$*/sup*$^-$ genotype is indicative of a recessive suppressor.

Suppression of *aep3* mutants containing *nATP8* is not affected by an *fmt1* mutation

FMT1 codes for the mitochondrial methionyl-tRNA transformylase. Respiration and mitochondrial protein synthesis are not affected in *fmt1* mutants, as they are able to initiate translation with unformylated initiator methionyl-tRNA (Li *et al.*, 2000). A double *fmt1* and *aep3* mutant with a Y305N substitution in Aep3p, however, has been shown to lack general mitochondrial translational activity, indicating a requirement of Aep3p for translation in the absence of formylated methionyl-tRNA fMet (Lee *et al.*, 2009). To ascertain whether respiratory competence and mitochondrial translation of the *aep3*-null mutant with the suppressor and *nATP8* depend on Fmt1p, we crossed the respiratory-competent *aep3*-null mutant expressing *nATP8* to an *fmt1*-null strain. Diploid cells issued from the cross were sporulated, and meiotic progeny with the double *aep3*, *fmt1*–null alleles and *nATP8* indicated that the rescue of respiratory growth (Supplemental Figure S3A) and of mitochondrial translation (Supplemental Figure S3B) by the suppressor does not require methionyl transformylase. The extragenic suppressor in combination with *nATP8*, therefore, rescues translation of mitochondrial gene products in *aep3* mutants even when formylation of the initiator methionyl tRNA is blocked.

Suppression by *SMT1*

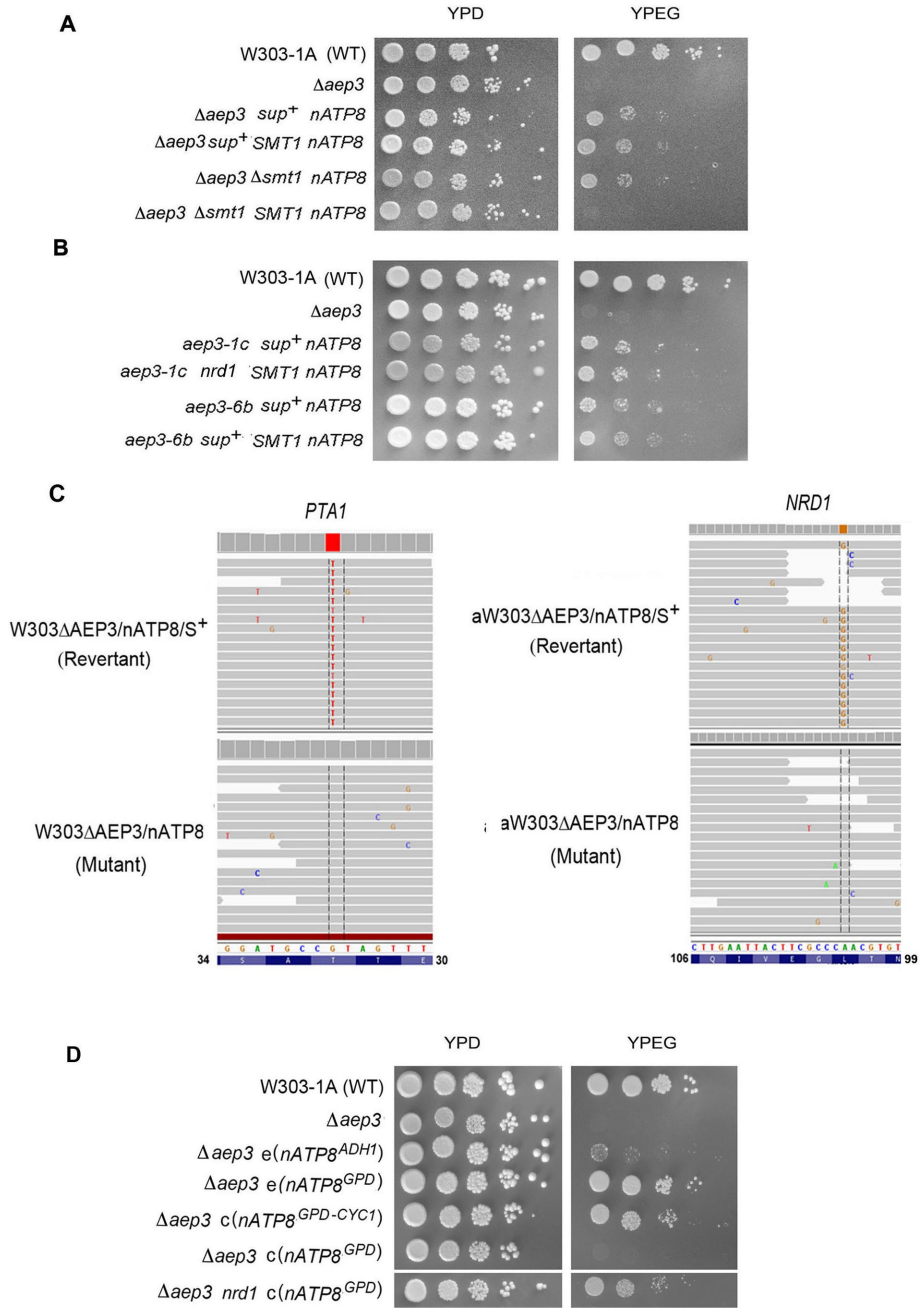
The product of the nuclear *SMT1* gene was recently proposed to be a translational repressor of the mitochondrial *ATP8-ATP6* mRNA (Rak *et al.*, 2016). The translational defect of A*TP6* and *ATP8* in F_1 mutants is suppressed by recessive mutations in *SMT1* (Rak *et al.*, 2016). Deletion of *SMT1* in the presence of *nATP8* partially restored respiration in the *aep3*-null mutant (Figure 4A). The rescue also improved mtDNA maintenance. In contrast to the *smt1* deletion, overexpression of *ATP22*, an *ATP6*-specific translational activator, did not rescue the respiratory growth of the *aep3*-null or point mutants (unpublished data). Suppression of growth of *aep3* mutants by the *smt1*-null mutation is recessive, as is the restoration of *ATP8-ATP6* mRNA translation (Figure 4A).

The rescue by the *smt1*-null mutation of respiration in the *aep3* point mutants, however, was countered by an extra copy of *SMT1*, indicating that the *smt1* was recessive in this context as well and therefore distinct from the suppressor(s) in the *aep3*-null mutants (Figure 4A).

Suppressors of *aep3* mutations

The suppressor mutations in the *aep3*-null and point mutants transformed with *nATP8* were identified by comparing whole-genome sequences of respiratory-competent revertants and the parental respiratory-deficient *aep3* mutants containing *nATP8*. A total of 22,944,148 reads were obtained for the respiratory-deficient mutant W303ΔAEP3/nATP8 and 27,045,134 for the corresponding respiratory-competent revertant W303ΔAEP3/nATP8/S^+. The reads of the mutant aligned 83% and of the revertant 96% with the *S. cerevisiae* reference genome. Only two consistent differences were found in the nuclear genome of the revertant. One was a C-to-A mutation in *PTA1*, resulting in a nonconservative T32K change in a subunit of the holo-CPF complex and a conservative L11V change in the product of *BMH2*, respectively. Similarly, 48,641,033 reads were obtained for the independent revertant aW303ΔAEP3/nATP8/S^+ and 14,641,033 for the corresponding respiratory-deficient parental strain aW303ΔAEP3/nATP8. The reads of the mutant aligned 93% and of the revertant 95% with the *S. cerevisiae* reference genome. The following mutations were detected in this strain: a G-to-A and a C-to-A mutation in *LEU3*, resulting in the amino acid changes G490E and S492R, and an A-to-G mutation in *NRD1*, resulting in a L101R substitution. Sanger sequencing confirmed the *PTA1* and *NRD1* mutations. These two genes were also sequenced in the mutants with *aep3-1c* and *aep3-6b* alleles. The *pta1* mutation was not found in either strain. The *aep3-1c* mutant, however, had A-to-G and G-to-A mutations in *NRD1*, resulting in Q206R and P526Q changes in the protein product, respectively. The *aep3-6b* mutant had only the G-to-A mutation in *NRD1*, leading to the P526Q substitution in the protein.

The genome sequencing data pointed to mutations in *PTA1* and *NRD1* as the best candidates for suppressing the respiration defect of *aep3* mutants with *nATP8*. Common to both genes is their function in RNA metabolism and more specifically the reported role of *PTA1* in cleavage and polyadenylation of nuclear RNAs (Zhao *et al.*, 1999). This suggested that the lack of complementation of *aep3* mutants by *nATP8* might be related to turnover of the RNA as a result of inefficient posttranscriptional modification of mRNA lacking proper cleavage and polyadenylation signals. This is supported by the observation that *nATP8* restores respiration of an *aep3*-null mutant when cloned in a centromeric plasmid with the strong *GPD* promoter (Mumberg *et al.*, 1995) and *CYC1* terminator (Figure 4D). The ability of *nATP8* to rescue *aep3* mutants when expressed from a high-copy plasmid with the *GPD* promoter indicates that the requirement of a terminator can be circumvented by overexpression of the *nATP8* (Figure 4D). The inefficiency of allotopic *nATP8* to bypass the Aep3p requirement is also evident in the very poor rescue by a plasmid (pATP8/ST4) that contains the weaker *ADH1* promoter



even though it also has the *ADH1* terminator (Figure 4D).

DISCUSSION

Aep3p is a 70-kDa mitochondrial protein with four pentatricopeptide (PPR) motifs, each consisting of a degenerate 35–amino acid sequence repeated in tandem in two antiparallel α-helices leading to a helix-turn-helix domain. In series, the repeats form an RNA-binding groove (Manna, 2015). PPR proteins have been described in chloroplasts and in plant, human, and fungal mitochondria (Shikanai, 2006; Lipinski *et al.*, 2011; Solotoff *et al.*, 2015). Pet309p, Atp22p, and Msc6p are examples of yeast mitochondrial PPR proteins involved in the stability and/or translation of mitochondrially encoded RNAs (Krause *et al.*, 2004; Zeng *et al.*, 2007; Moda *et al.*, 2016). Like other PPR proteins, Aep3p was recently shown to cosediment with mitochondrial ribosomes in a large complex termed Miorex (Kehrein *et al.*, 2015).

Consistent with the presence in Aep3p of the PPR RNA-binding motif is its previously described function in stabilizing the *ATP8-ATP6* mRNA (Ellis *et al.*, 2004) and the requirement for mIF2-dependent initiation of translation of mitochondrial gene products with unformylated initiator methionyl-tRNA (Lee *et al.*, 2009). Our results indicate that in addition to these activities, Aep3p is also required specifically for translation of *ATP8*. This is supported by three lines of evidence. At the restrictive temperature, *aep3* ts mutants are impaired in expression of *ATP8* but not *ATP6* or other mitochondrial gene products. Second, the Cox2p deficit is not a consequence of either defective transcription or processing of the *COX1-ATP8-ATP6* primary transcript. It is also unlikely that Aep3p is required for stability of Atp8p.

FIGURE 4: Suppressors of *aep3* mutants. (A) The respiratory-competent wild-type W303-1A and *aep3*-null mutants with the indicated genotypes were serially diluted and spotted on rich glucose (YPD) and rich ethanol/glycerol (YPEG) media and photographed after 3 d of incubation at 30°C. Wild-type *SMT1* in an integrative plasmid. (B) Growth of revertants with and without an extra copy of *SMT1*: *aep3-1c nrd1 nATP8* and *aep3-6b nrd1 nATP8* are revertants of two *aep3* point mutants; *aep3-1c nrd1 nATP8* and *aep3-6b nrd1 nATP8* are the same revertants with an extra copy of *SMT1*. The strains were serially diluted and spotted on rich glucose and rich ethanol plus glycerol media as in A. (C) Whole-genome analysis of mutations in the two independent revertants W303ΔAEP3/S+/22 and aW303ΔAEP3/S+/22. Alignments, obtained with the Integrative Genomics Viewer software developed by the Broad Institute (MIT and Harvard), of reads of the revertants (top) with a consistent positional base change differing from that of the parental mutant genome (bottom). Left, alignments of reads from W303ΔAEP3/S+/22 with the G-to-T mutation in the reverse strand of *PTA1*. The T was present in 270 of 271 reads of the revertant. The single exception had the G of the mutant. At the same nucleotide position the mutant had a G in 260 out of 261 reads. The single exception had a C at this position. Right, results obtained with the genome of aW303ΔAEP3/S+/22 and the parental mutant. In this strain, a consistent A-to-G change was found in *NRD1* (reverse strand shown). The revertant had the G in 92 and a C in two reads. (D) Growth of the following strains was measured as in A: W303-1A, the parental wild-type strain; Δ*aep3*, the *aep3*-null mutant; Δ*aep3* *e(nATP8^{ADH1})*, the *aep3* mutant transformed with an episomal plasmid containing *nATP8* flanked by the *ADH1* promoter and terminator; Δ*aep3 e(nATP8^{GPD})*, the *aep3* mutant transformed with an episomal plasmid containing *nATP8* downstream of the *GPD* promoter; Δ*aep3 c(nATP8$^{GPD\text{-}CYC1}$)*, the *aep3* mutant transformed with an centromeric plasmid containing *nATP8* flanked by the *GPD* promoter and *CYC1* terminator; Δ*aep3 c(nATP8^{GPD})*, the *aep3* mutant transformed with a centromeric plasmid containing *nATP8* downstream of the *GPD* promoter; and Δ*aep3 nrd1 nATP8*, the *aep3* mutant with the *nrd1* suppressor mutation transformed with a centromeric plasmid containing *nATP8* downstream of the *GPD* promoter.

This is evidenced by the finding that complementation of an *arg8* mutant by the *atp8::ARG8*^{m} allele requires the presence of wild-type *AEP3*. These results constitute strong evidence that Aep3p function is required for translation rather than stability of Arg8p and, by analogy, of Atp8p. Finally, although it does not exclude other possible mechanisms, rescue of *aep3* mutants by allotopic *nATP8* is also consistent with a role of Aep3p in translation of mitochondrial *ATP8*.

When expressed from a plasmid lacking a proper termination sequence, rescue of *aep3* point and null mutants by *nATP8* depends on recessive mutations in nuclear DNA. This is true even when transcription of *nATP8* is under the control of a strong promoter such as *GDP* (pATP8-22). *NRD1* and *PTA1* have been identified as nuclear suppressors that enable allotopic *nATP8* on a CEN plasmid lacking a transcriptional terminator to rescue *aep3* mutants. *NRD1* and *PTA1* are involved in mRNA termination. Pta1p is an essential 90-kDa protein required for both cleavage and poly(A) addition of mRNA precursor (Zhao *et al.*, 1999). Nrd1p acts both on transcription termination and an RNA processing factor that has been shown to recruit RNA to the nuclear exosome, which uses its 3′ to 5′ exonuclease activity to degrade aberrant transcripts but also mature the 3′ of precursor RNAs (Heo *et al.*, 2013). The overlapping activities of these suppressors suggest that the mechanism of suppression involves an enhancement of 3′-end maturation of the *nATP8* RNA, thereby sparing the RNA from degradation. This explanation gains further support from the finding that a plasmid construct with *nATP8* flanked by the *GPD* promoter and the *CYC1* terminator rescues the *aep3* mutants in the absence of additional suppressor mutations. The recessive nature of the *pta1* and *nrd1* suppressor mutations indicates that they probably spare the *nATP8* transcript from degradation. Normal growth of the suppressor strains on rich glucose indicates that the *pta1* and *nrd1* mutations do not seriously compromise processing or degradation of other cellular RNAs. We have no information on whether the *pta1* and *nrd1* mutations would improve complementation by other relocated mitochondrial genes lacking 3′ processing and polyadenylation sequences. Conceivably, previous attempts to relocate mitochondrial genes to the nucleus (Claros *et al.*, 1995) were unsuccessful for reasons related to incorrect 3′ processing of the primary transcripts.

Complementation of the *aep3* mutants by *nATP8* is also achieved by deletion of *SMT1*. This gene has been proposed to repress translation of the *ATP8-ATP6* mRNA (Rak *et al.*, 2016) and regulate expression of these genes by adjusting the rate of their synthesis to the availability of the F_1 component of ATP synthase with which they interact during assembly of the ATP synthase complex (Rak and Tzagoloff, 2009; Rak *et al.*, 2016). The block in translation of the *ATP8-ATP6* mRNA is suppressed by mutations in *SMT1*. The reduction of *ATP8-ATP6* mRNA in *aep3* mutants (Ellis *et al.*, 2004) would lead to a concomitant decrease of Atp6p synthesis. Mutations in *smt1* may compensate for the Atp6p insufficiency by increasing its synthesis in *aep3* mutants. Alternatively, the absence of Smt1p in *aep3* mutants may influence some other aspect of Atp8p biogenesis from the allotopic *nATP8* gene. Either of these mechanisms could explain the partial rescue by *SMT1* deletion of respiration in *aep3* mutants.

Mitochondrial respiration has been shown to be defective in the *aep3* Y305 mutant when formylation of methionine is blocked by an *fmt1*-null mutation (Lee *et al.*, 2009). Our results indicate that the respiratory deficiency and mitochondrial translation of the *aep3 fmt1* double-null mutant is rescued by *nATP8* in the presence of the nuclear suppressor (s^+). The rescue of mitochondrial translation in a mutant lacking both Fmt1p and Aep3p (Supplemental Table S1) differs from the phenotype of the *aep3* (Y305N) *fmt1* double mutant, which is not only respiratory deficient, but also exhibits severely depressed mitochondrial translation (Lee *et al.*, 2009; Supplemental Table S1). The discrepancy in the two phenotypes may be explained by the different genetic backgrounds of the strains used in the two studies, although we cannot exclude that it may also be related to the Y305N mutation itself.

In summary, our results show that in addition to its previously described activities, Aep3p is also required for translation of *ATP8*. The finding that mutations in *pta1* and *nrd1* are able to activate complementation of *aep3* mutants by *nATP8* suggests that they increase cellular concentration of the protein by stabilizing the mRNA through 3′-end processing and modification.

MATERIALS AND METHODS

Yeast strains and growth media

The genotypes and sources of the strains of yeast used in this study are listed in Supplemental Table S2. The compositions of YPD, YEPG, and minimal glucose medium (supplemented with the appropriate auxotrophic requirements) have been described elsewhere (Ellis *et al.*, 2004).

Construction of *aep3* ts mutants

Temperature-sensitive *aep3* alleles were obtained by PCR amplification of the gene under conditions favoring misincorporation of deoxynucleotides. Primers 5′-ggaattgtgagcggataac and 5′-gggtaacgccagggttttccc used for the synthesis started from the cloning site in YEp351-AEP3 (Ellis *et al.*, 2004). The gene was amplified in four separate reactions containing 0.25 mM $MnCl_2$, 1.5 mM $MgCl_2$, and 0.2 mM dITP and with the concentration of one of the four deoxynucleotides reduced from 0.2 to 0.04 mM. The four 2004–base pair products were pooled, digested with *Hin*dIII, and cloned in the centromeric plasmid YCplac22 containing *TRP1* (Gietz and Sugino, 1988). The plasmid library obtained from the ligation was used to transform the heterozygous diploid strain a/αW303ΔAEP3 (Ellis *et al.*, 2004). The pooled tryptophan prototrophic transformants were sporulated, and histidine- and tryptophan-independent meiotic progeny were checked for growth at 30 but not 37°C on YEPG. The screen yielded mutants with a clear ts phenotype. The *aep3* allele in pAEP3/TS1 had five nucleotide changes that lead to amino acid changes–K20R, K154R, V336E, I460V, and I559V. The five other acids changes detected in pAEP3/TS2 were V40A, N172S, L216P, K213R, and H389R. Plasmids pAEP3/TS3, TS4, TS5, TS6, and TS7 had the single–amino acid changes F392S C347R, D310V, A480V, and E281G, respectively (Figure 3B).

Mutagenesis and plasmid isolation

The haploid strains W303-1A and W303-1B were mutagenized with EMS (Lindegren *et al.*, 1965), and respiratory-deficient mutants were selected based on growth on rich glucose (YPD) but not on rich ethanol/glycerol (YPEG) medium. Approximately 6000 respiratory-deficient mutants were pooled and transformed with pATP8-22, a centromeric *TRP1*-based plasmid containing *nATP8* (recoded for allotopic expression) under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter (GDP; Zampol *et al.*, 2010). This plasmid is expected to complement mutants that cannot express mitochondrial *ATP8*. To identify the gene responsible for the respiratory deficiency, the *nATP8*-dependent, respiratory-competent clones were spread for single colonies on YPD to isolate respiratory-deficient segregants auxotrophic for tryptophan. Such segregants are expected to have lost the plasmid with *nATP8*. To identify the mutant gene responsible for the respiratory deficiency, we transformed the respiratory-deficient segregants with a yeast

genomic library consisting of partial Sau3A fragments of wild-type yeast nuclear DNA ligated to the *Bam*HI site of the episomal plasmid YEp24 (Botstein and Davis, 1982). This plasmid library was a gift of Marian Carlson (Department of Genetics and Development, Columbia University). Respiratory-competent transforms were expected to have a recombinant plasmid of the library with the complementing gene. The screen yielded two respiratory-deficient clones that were ascertained to have mutations in *aep3*. The mutant alleles were named *aep3-1c* and *aep3-6b*.

The *aep3-1c* and *aep3-6b* mutations were identified by PCR amplification of nuclear DNA from each mutant with primers 5′-ggggagctcctgtccaaatgccaggg and 5′-ggcctgcagggacgattccatcgaag. The 2213–base pair product was digested with *Sac*I and *Pst*I and cloned into YIp351 (Hill *et al.*, 1986), yielding plasmids pAEP3-1C and pAEP3-6b. Sequencing of the plasmids revealed that *aep3-1c* had three nucleotide changes in *aep3*, resulting in amino acids changes N331D, A379E, and the nonsense mutation, L416Stop (Figure 1). The gene of *aep3-6b* had a single-nucleotide change, which resulted in the A379E substitution. An *aep3* gene expressing the truncated product of *aep3-1* without the additional mutations was reverse transcribed with the oligonucleotides 5′-ggcctgcagggacgattccatcgaag and 5′-ggcaagcttaaatcacttcttccc. The 1608–base pair product was digested with *Pst*I plus *Hin*dIII and cloned into YIp351 to obtain pAEP3-1S.

Whole-genome sequencing

The Wizard Genomic Purification Kit (Promega) was used to extract total DNA from the parental respiratory-deficient mutant W303ΔAEP3/nATP8 and from the revertant W303ΔAEP3/nATP8/sup. The DNAs were quantified using the QUBIT DNA high-sensitivity assay, and 1 ng of the normalized DNA was tagged by the Nextera XT (Illumina) protocol. The libraries were amplified and cleaned up, and 1 µl of the undiluted libraries was analyzed on an Agilent Technology 2100 Bioanalyzer using a high-sensitivity DNA chip. The libraries were pooled and adjusted to 2 nmol in 15 µl. The pooled libraries were subjected for sequencing with the NextSeq (Illumina) equipment in the Genome Investigation and Analysis Laboratory of the Institute of Biomedical Sciences at the University of Sao Paulo. The BWA Aligner tool, version 1.1.4 (Base Space Labs Illumina), was used to align the 71,585,181 reads obtained from the four strains with the reference genomes of *S. cerevisiae*. The alignments were compared using the Integrative Genomics Viewer (Base Space Labs Illumina).

In vivo labeling of mitochondrial gene products in wild-type and *aep3* mutants

Yeast grown to early stationary phase in YPGal were harvested, washed, and inoculated at a cell density of $A_{600} = 2$ into 2% galactose in nitrogen base without amino acids supplemented with auxotrophic requirements. After 2 h growth, or 3 h growth in the ts experiment, cells were harvested, washed twice, treated with cycloheximide, and labeled at room temperature for 30 min with [^{35}S]methionine (1000 Ci/mmol; MP Biochemicals). Total cellular proteins were separated by SDS–PAGE on 17.5 and 12% polyacrylamide gels (Laemmli, 1970) without and with 6 M urea, respectively. Proteins were transferred to a nitrocellulose membrane and the radiolabeled mitochondrial gene products visualized by autoradiography.

Miscellaneous procedures

Standard methods were used for plasmid manipulations (Sambrook *et al.*, 1989). The procedure of Schiestl and Gietz (1989) was used for yeast transformations. Protein concentrations were determined by the method of Lowry *et al.* (1951).

ACKNOWLEDGMENTS

This research was supported by grants from the Fundação de Amparo a Pesquisa de São Paulo (2013/09482-8 and 2013/07937-8) and the Conselho Nacional Pesquisa (302935/2014-2) to M.B. and National Institutes of Health Grant HL02274 to A.T.

REFERENCES

Ackerman SH (2002). Atp11p and Atp12p are chaperones for F(1)-ATPase biogenesis in mitochondria. Biochim Biophys Acta 1555, 101–105.

Ackerman SH, Tzagoloff A (2005). Function, structure, and biogenesis of mitochondrial ATP synthase. Prog Nucleic Acid Res Mol Biol 80, 95–133.

Barrientos A, Zambrano A, Tzagoloff A (2004). Mss51p and Cox14p jointly regulate mitochondrial translation of Cox1p in *Saccharomyces cerevisiae*. EMBO J 23, 3274–3282.

Barros MH, Rak M, Paulela JA, Tzagoloff A (2011). Characterization of Gtf1p, the connector subunit of yeast mitochondrial tRNA-dependent amidotransferase. J Biol Chem 286, 32937–32947.

Botstein D, Davis RW (1982). Principles and practice of recombinant DNA research with yeast. In: The Molecular Biology of the Yeast *Saccharomyces Cerevisiae*: Metabolism and Gene Expression, ed. JN Strathern, EW Jones, and JR Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 607–636.

Camougrand N, Pelissier P, Velours G, Guerin M (1995). *NCA2*, a second nuclear gene required for the control of mitochondrial synthesis of subunits 6 and 8 of ATP synthase in *Saccharomyces cerevisiae*. J Mol Biol 247, 588–596.

Claros MG, Perea J, Shu Y, Samatey FA, Popot JL, Jacq C (1995). Limitations to in vivo import of hydrophobic proteins into yeast mitochondria. The case of a cytoplasmically synthesized apocytochrome b. Eur J Biochem 228, 762–771.

Costanzo MC, Fox TD (1990). Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. Annu Rev Genet 24, 91–113.

Ellis TP, Helfenbein KG, Tzagoloff A, Dieckmann CL (2004). Aep3p stabilizes the mitochondrial bicistronic mRNA encoding subunits 6 and 8 of the H+-translocating ATP synthase of *Saccharomyces cerevisiae*. J Biol Chem 279, 15728–15733.

Fontanesi F, Clemente P, Barrientos A (2011). Cox25 teams up with Mss51, Ssc1, and Cox14 to regulate mitochondrial cytochrome c oxidase subunit 1 expression and assembly in *Saccharomyces cerevisiae*. J Biol Chem 286, 555–566.

Fox TD (2012). Mitochondrial protein synthesis, import and assembly. Genetics 192, 1203–1234.

Gearing DP, McMullen GL, Nagley P (1985). Chemical synthesis of a mitochondrial gene designed for expression in the yeast nucleus. Biochem Int 10, 907–915.

Gearing DP, Nagley P (1986). Yeast mitochondrial ATPase subunit 8, normally a mitochondrial gene product, expressed in vitro and imported back into the organelle. EMBO J 5, 3651–3655.

Gietz RD, Sugino A (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74, 527–534.

Heo DH, Yoo I, Kong J, Lidschreiber M, Mayer A, Choi BY, Hahn Y, Cramer P, Buratowski S, Kim M (2013). The RNA polymerase II C-terminal domain-interacting domain of yeast Nrd1 contributes to the choice of termination pathway and couples to RNA processing by the nuclear exosome. J Biol Chem 288, 36676–36690.

Herrmann JM, Woellhaf MW, Bonnefoy N (2013). Control of protein synthesis in yeast mitochondria: the concept of translational activators. Biochim Biophys Acta 1833, 286–294.

Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986). Yeast/*E.coli* shuttle vectors with multiple unique restriction sites. Yeast 2, 163–167.

Kehrein K, Möller-Hergt BV, Ott M (2015). The MIOREX complex: lean management of mitochondrial gene expression. Oncotarget 6, 16806–16807.

Krause K, Lopes de Souza R, Roberts DG, Dieckmann CL (2004). The mitochondrial message-specific mRNA protectors Cbp1 and Pet309

are associated in a high-molecular weight complex. Mol Biol Cell 15, 2674–2683.
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lee C, Tibbetts AS, Kramer G, Appling DR (2009). Yeast *AEP3p* is an accessory factor in initiation of mitochondrial translation. J Biol Chem 284, 34116–34125.
Lefebvre-Legendre L, Vaillier J, Benabdelhak H, Velours J, Slonimski PP, di Rago JP (2001). Identification of a nuclear gene (*FMC1*) required for the assembly/stability of yeast mitochondrial F(1)-ATPase in heat stress conditions. J Biol Chem 276, 6789–6796.
Li Y, Holmes WB, Appling DR, RajBhandary UL (2000). Initiation of protein synthesis in *Saccharomyces cerevisiae* mitochondria without formylation of the initiator tRNA. J Bacteriol 182, 2886–2892.
Lindegren G, Hwang YL, Oshima Y, Lindegren CC (1965). Genetical mutants induced by ethyl methanesulfonate in *Saccharomyces*. Can J Genet Cytol 7, 491–499.
Lipinski KA, Puchta O, Surendranath V, Kudla M, Golik P (2011). Revisiting the yeast PPR proteins–application of an Iterative Hidden Markov Model algorithm reveals new members of the rapidly evolving family. Mol Biol Evol 28, 2935–2948.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J Biol Chem 193, 265–275.
Manna S (2015). An overview of pentatricopeptide repeat proteins and their applications. Biochimie 113, 93–99.
Moda BS, Ferreira-Júnior JR, Barros MH (2016). Partial suppression of the respiratory defect of *qrs1/her2* glutamyl-tRNA amidotransferase mutants by overexpression of the mitochondrial pentatricopeptide Msc6p. Curr Genet 62, 607–617.
Mumberg D, Muller R, Funk M (1995). Yeast vectors for the controlled expression of heterologous protein in different genetic backgrounds. Gene 156, 119–122.
Pelissier P., Camougrand N, Velours G, Guerin M (1995). *NCA3*, a nuclear gene involved in the mitochondrial expression of subunits 6 and 8 of the F_o–F_1 ATP synthase of *S. cerevisiae*. Curr Genet 27, 409–416.
Perez-Martinez X, Broadley SA, Fox TD (2003). Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. EMBO J 22, 5951–5961.
Rak M, Su CH, Xu JT, Azpiroz R, Singh A, Tzagoloff A (2016). Regulation of mitochondrial translation of the *ATP8-ATP6* mRNA by Smt1p. Mol Biol Cell 27, 919–929.
Rak M, Tzagoloff A (2009). F1-dependent translation of mitochondrially encoded Atp6p and Atp8p subunits of yeast ATP synthase. Proc Natl Acad Sci USA 106, 18509–18514.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Schiestl RH, Gietz RD (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acid as a carrier. Curr Genet 16, 339–346.
Shikanai T (2006). RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol Life Sci 63, 698–708.
Simon M., Faye G (1984). Organization and processing of the mitochondrial *oxi3/oli2* multigenic transcript in yeast. Mol Gen Genet 196, 266–274.
Solotoff V, Moseler R, Schulte U (2015). Two pentatricopeptide repeat domain proteins are required for the synthesis of respiratory complex I. Curr Genet 61, 19–29.
Steele DF, Butler CA, Fox TD (1996). Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc Natl Acad Sci USA 93, 5253–5257.
Supekova L, Supek F, Greer JE, Schultz PG (2010). A single mutation in the first transmembrane domain of yeast COX2 enables its allotopic expression. Proc Natl Acad Sci USA 107, 5047–5052.
Zampol MA, Busso C, Gomes F, Ferreira-Junior JR, Tzagoloff A, Barros MH (2010). Over-expression of *COQ10* in *Saccharomyces cerevisiae* inhibits mitochondrial respiration. Biochem Biophys Res Commun 402, 82–87.
Zeng X, Hourset A, Tzagoloff A (2007). The *Saccharomyces cerevisiae ATP22* gene codes for the mitochondrial ATPase subunit 6-specific translation factor. Genetics 175, 55–63.
Zhao J, Kessler M, Helmling S, O'Connor JP, Moore C (1999). Pta1, a component of yeast CF II, is required for both cleavage and poly(A) addition of mRNA precursor. Mol Cell Biol 19, 7733–7740.