The depletion of F1 subunit ε in yeast leads to an uncoupled respiratory phenotype that is rescued by mutations in the proton-translocating subunits of F0

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ABSTRACT The central stalk of the ATP synthase is an elongated hetero-oligomeric structure providing a physical connection between the catalytic sites in F1 and the proton translocation channel in F0 for energy transduction between the two subdomains. The shape of the central stalk and relevance to energy coupling are essentially the same in ATP synthases from all forms of life, yet the protein composition of this domain changed during evolution of the mitochondrial enzyme from a two- to a three-subunit structure (γ, δ, ε). Whereas the mitochondrial γ- and δ-subunits are homologues of the bacterial central stalk proteins, the deliberation of subunit ε is poorly understood. Here we report that down-regulation of the gene (ATP15) encoding the ε-subunit rapidly leads to lethal F0-mediated proton leaks through the membrane because of the loss of stability of the ATP synthase. The ε-subunit is thus essential for oxidative phosphorylation. Moreover, mutations in F0 subunits a and c, which slow the proton translocation rate, are identified that prevent ε-deficient ATP synthases from dissipating the electrochemical potential. Cumulatively our data lead us to propose that the ε-subunit evolved to permit operation of the central stalk under the torque imposed at the normal speed of proton movement through mitochondrial F0.

INTRODUCTION All members of the ATP synthase family of proteins are very similar at the structural level, regardless of evolutionary origin. Historically, the enzyme has been described in terms of an integral membrane domain (F0) and a peripheral domain (F1). The wealth of high-definition structural data now available (Devenish et al., 2008; Walker, 2013) reveals that F0 and F1 are divided further into distinct oligomeric substructures. The most hydrophobic F0 subunits associate in a proton-translocating complex, which in its simplest form is composed of one a-subunit adjacent to a ring of 8–15 c-subunits. The remaining F0 subunits assemble a stalk anchored at one end to the stationary a-subunit in the membrane and attached at the other end to the outer periphery of the soluble F1 domain. F1 is largely defined by a globular structure ([αβ]3 hexamer), which contains the catalytic sites, and two or three other proteins that constitute a central stalk, which makes contact with these sites at one end and the c-ring at the other. Together the c-ring and the central stalk comprise a functional “rotor” that turns relative to nonmoving parts to permit energy-coupling reactions catalyzed by ATP synthases. For example, in the direction of ATP synthesis, energy released by proton translocation through F0 activates the rotor, forcing conformational changes in the catalytic sites that effect release of ATP from the enzyme.

One of the least-understood aspects of the ATP synthase coupling mechanism relates to differences in the central stalk
composition between bacterial and mitochondrial enzymes. In the mitochondrial enzyme, it is made of three subunits (γ, δ, and ε), whereas only two (γ and ε) are present in bacterial ATP synthase (Devenish et al., 2008; Walker, 2013). The γ-subunits of F₁ are homologous, and mitochondrial subunit δ is homologous to bacterial subunit ε. However, mitochondrial subunit ε has no counterpart in the bacterial enzyme, identifying it as one of many so-called “supernumerary” ATP synthase subunits that most likely appeared with the establishment of mitochondria as internal organelles during evolution of the eukaryotic cell. (Unless otherwise stated, ε in the text and figures denotes the supernumerary subunit of mitochondrial F₁.) The work reported here defines more clearly the importance of ε in energy coupling.

The F₁ ε-subunit of Saccharomyces cerevisiae is encoded by the nuclear gene ATP15. Previous work (Guelin et al., 1993; Lai-Zhang et al., 1999) using different atp15-deletion (Δε) mutants reported that the yeast grew at poor to modest rates on nonfermentable substrates, which meant that cells had a variable level of respiratory competence. This finding was unexpected because the ε-subunit of mitochondrial F₁ is not a vestigial element inherited from the prokaryotic progenitor but instead represents a new protein function acquired during evolution of the eukaryotic enzyme, and it was assumed to be indispensable for energy-coupled oxidative phosphorylation. However, it is important to note that all of the work thus far investigating the functional relevance of the ε-subunit in mitochondrial F₁ used Δε strains, which are pleiotropic mutants. When cultured on fermentable carbons, upward of 70% of Δε cells fail to maintain mitochondrial DNA (mtDNA) and instead convert to respiratory-deficient ρ⁻/ρ⁻ derivatives. Prompted by the notion that the functional relevance of mitochondrial F₁ subunit ε would remain ambiguous until conditions were established under which the effect on respiration caused by the primary nuclear mutation (atp15-deletion allele) could be assessed in an otherwise respiratory wild-type (ρ⁺) background, we transformed Δε yeast with a plasmid for doxycycline-regulated expression of wild-type ATP15. Our results show that yeast cultures down-regulated for production of the ε-subunit retain partial respiratory activity as a direct function of suppressor mutations in a mitochondrial-encoded F₀ protein (a- or ε-subunit) that rescues the uncoupling defect imposed by the elimination of subunit ε. The effect of the ε-subunit on energy coupling and structure/function relationships in mitochondrial ATP synthase is discussed with respect to the more primitive (ε-less) bacterial enzyme.

### RESULTS

A 25% depletion in subunit ε leads to a total uncoupling of mitochondria

We constructed yeast strain YE1 (henceforth referred to by its acronym Tet-ε), which has a null allele in place of the ε-subunit gene (ATP15) in the chromosome and carries a doxycycline-repressible form of the gene on a low-copy plasmid (see Materials and Methods and genotypes in Table 1). Without the drug, Tet-ε grew in nonfermentable glycerol/ethanol media like its parental strain (SDC22) and displayed normal subunit ε levels. Down-regulation of ATP15 expression was achieved using doxycycline at a concentration (10 μM) known to not affect the respiratory capacity of wild-type yeast (Duvezin-Caubet et al., 2003). The respiratory growth of Tet-ε remained normal for 6 h after the addition of doxycycline to the culture medium and then stopped abruptly (Figure 1A, arrowhead). Samples were removed at this time point from both the –Dox and +Dox Tet-ε cultures, and mitochondria were isolated. Western blots revealed that the amount of the ε-subunit decreased by ~25% at the time of doxycycline-induced growth arrest compared with control mitochondria from Tet-ε cells grown in the absence of doxycycline (Figure 1B). The amount of the ε-subunit was reduced by ~50% in mitochondria from growth-arrested +Dox cells, whereas the levels of other nuclear gene products evaluated (subunits α, γ, Atp4, d, porin, Aac) were quite similar for the two samples. The three mitochondrial-encoded proteins a-Atp6, c-Atp9, and cytochrome b were reduced in samples from +Dox versus –Dox cells, but the effect was less severe than with the Δε-subunit.

Respiratory responses to metabolites and drugs measured for mitochondria from –Dox cells were within normal range (Figure 1C and Table 2). Compared to the maximal respiratory activity, which was measured in the presence of an uncoupling agent (carbonyl cyanide m-chlorophenyl hydrazone [CCCP]), the rate of mitochondrial oxygen consumption was 17% with NADH alone (state 4 respiration) and increased to 53% after addition of ADP (state 3 respiration). Instead, with NADH alone, the rate of oxygen consumption in mitochondria from +Dox cells was essentially equal to the CCCP-stimulated rate. Oligomycin, which blocks proton translocation through the F₀, restored mitochondria to the slower state 4 respiration rate expected under conditions in which the respiratory substrate (NADH) is available but not the phosphorylation substrate (ADP). Hence the partial depletion of subunit ε, which was induced by doxycycline, correlated with 100% mitochondrial uncoupling due to rest of the text.

| Strain (acronym) | Nuclear genotype | mtDNA | Source |
|------------------|------------------|-------|--------|
| SDC22 (WT)       | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 | ρ⁺Arg8⁰ | Duvezin-Caubet et al. (2003) |
| SDC6 (Tet-ε)     | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 atp16::KanMX + pCM189-ATP16 | ρ⁺Arg8⁰ | Duvezin-Caubet et al. (2003) |
| YG1 (Tet-γ)      | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 atp3::KanMX + pCM189-ATP3 | ρ⁺Arg8⁰ | This study |
| YE1 (Tet-ε)      | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 atp15::KanMX + pCM189-ATP15 | ρ⁺Arg8⁰ | This study |
| S1 (Δε+c-L57F)   | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 atp15::KanMX | ρ⁺Arg8⁰ + c-L57F | This study |
| S1+ε (ε+c-L57F)  | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 atp15::KanMX + pCM189-ATP15 | ρ⁺Arg8⁰ + c-L57F | This study |
| S2 (Δε+a-A120V)  | Matγ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 atp15::KanMX | ρ⁺Arg8⁰ + a-A120V | This study |

**TABLE 1: Genotypes of yeast strains.**
specifically to proton leakage through $F_0$. This finding was corroborated in experiments that used rhodamine 123 as a reporter of mitochondrial membrane potential ($\Delta \Psi$; Figure 1D). In mitochondria from untreated Tet-$\varepsilon$ cells, supplying the respiratory chain with electrons from ethanol produced a large fluorescent quenching of the dye that collapsed after subsequent addition of potassium cyanide. In contrast, the mitochondria partially depleted of subunit $\varepsilon$ could not be energized with ethanol until the proton leak in $F_0$ was blocked by the addition of oligomycin to the reaction sample. The severity of the uncoupling defect observed for doxycycline-treated Tet-$\varepsilon$ cells was not unexpected because the steady-state level of the $\delta$-subunit was reduced by half in these cells (Figure 1B; see earlier discussion), and we knew from previous work (Duvezin-Caubet et al., 2003) that decreasing the amount of $F_1$ $\delta$ by 50% was sufficient to uncouple yeast mitochondria completely. Assays of oligomycin-sensitive mitochondrial ATPase activity in the two samples showed that although comparable rates were obtained in the absence of the inhibitor, the sample from +Dox cells was less sensitive than normal to oligomycin (77 vs. 89% inhibition; Table 3). The latter finding suggests that there is a higher percentage of $F_1$ in the free, oligomycin-insensitive state in mitochondria isolated from +Dox yeast, which was predicted given the fact that these cells are partially depleted for two proteins (subunits $\delta$ and $\varepsilon$) that are known to be critical factors in mitochondria for physically coupling $F_1$ to $F_0$.

**Differential effects on mtDNA stability in $\Delta \varepsilon$ versus $\Delta \gamma$ or $\Delta \delta$ null mutants**

The oligomycin-sensitive uncoupling defect we observed in response to partial depletion of the $\varepsilon$-subunit is similar to the phenotype others reported for yeast in which the cellular level of $\gamma$- or $\delta$-subunit
proteins was effectively reduced, by either dilution through the creation of heterozygous diploids (Δγ/γ or Δδ/δ; Xiao et al., 2000) or the use of a repressible promoter (Duvezin-Caubet et al., 2003). Hence a partial deficit in any one of the three rotor subunits can interfere with the mitochondrial ΔΨ by allowing protons to leak through F0. The circumstances are exacerbated in yeast null mutants that are completely deleted for one or another rotor subunit protein. Pertinent to our work, previous characterization of such strains revealed a dichotomy in mtDNA phenotypes that could not be explained. On one hand, Δγ and Δδ cells rapidly lost mtDNA and converted 100% to the p−/p0 state (Giraud and Velours, 1997; Lai-Zhang et al., 1999). Instead, cultures of Δε mutants harboring one (Guelin et al., 1993) or another (Lai-Zhang et al., 1999) null allele in place of the F0-subunit genes enable yeast to maintain mtDNA in the absence of the F1-ε-subunit. Samples of the p+ Tet-ε cells that had accumulated in galactose media after 40 generations in the presence of doxycycline (Figure 2) formed either medium-sized (S1) or small (S2) colonies on rich glycerol/ethanol medium (Figure 3A), and both types of clones failed to grow on glucose plates lacking uracil (Figure 3B). The latter condition indicated that strains S1 and S2 had lost the URA3-plasmid (pCM189-ATP15) bearing the doxycycline-repressible gene for wild-type subunit ε and were, in fact, ε-subunit null (Δε) strains. Missense mutations were identified in F0 subunits c (c-L57F) and a (a-A120V) of strains S1 and S2, respectively (see Materials and Methods). S1

![Molecular Biology of the Cell](image)

**FIGURE 2:** Kinetics of p+/p0 cell production. The Tet-γ, Tet-δ, and Tet-ε strains and their parental strain SDC22 (WT) were grown in rich galactose in the presence of 10 μM doxycycline. The cultures were refreshed several times with the same medium to produce a total of 40 generations, which was estimated by measuring the turbidity at 650 nm. The contents in p+/p0 cells were determined at the indicated number of generations.

**TABLE 2:** ATP synthesis/hydrolysis activities of mitochondria.

| Strain (acronym) | Dox | ATP synthase (nmol ATP/min/mg) | ATPase (μmol P/min/mg) | Inhibition (%) | p−/p0 (%) |
|-----------------|-----|-------------------------------|------------------------|---------------|-----------|
| YE1 (Tet-ε)    | −   | 697 ± 141                     | 3030 ± 163             | 89 ± 1        | 2 ± 1     |
| YE1 (Tet-ε)    | +   | 3063 ± 216                    | 706 ± 66               | 77 ± 2        | 4 ± 1     |
| S1 (Δε+c-L57F) | −   | 488 ± 96                      | 3102 ± 77              | 90 ± 1        | 3 ± 1     |
| S1+ε (c-L57F)  | +   | 188 ± 22                      | 1133 ± 191             | 11 ± 2        | 12 ± 2    |

The strains were grown in rich glycerol/ethanol medium in the presence/absence of 10 μM doxycycline (Dox). Oxygen consumption was measured in freshly isolated mitochondria. The percentage of inhibition of ATPase activity by oligomycin is indicated.
(Δε+c-L57F) was notably impaired for growth in liquid glycerol/ethanol medium in comparison with the plasmid-bearing Tet-ε cells from which it originated but could be restored to the control level after reintroduction of pCM189-ATP15 (Figure 3C, S1+ε).

The c-L57F mutation slows F₀ activity
Respiration in the S1 (Δε+c-L57F) mitochondria was only partially uncoupled, as evidenced by a significant stimulation by both ADP and CCCP (Figure 4A and Table 2). These mitochondria could sustain a significant ΔΨ with ethanol without adding oligomycin (Figure 4B), and 27% the control level of ATP synthesis activity was observed (Table 3). These data provide evidence that the c-L57F mutation enables partial recovery of the physical and functional coupling of F₁ to F₀ defect imposed by the absence of subunit ε. However, although Western blots revealed the presence of near-normal to modestly reduced amounts of ATP synthase subunits α, γ, δ, and c in S1 mitochondria (Figure 4C), F₁F₀ complexes minus the ε-subunit could not detected by blue native PAGE (BN-PAGE) by in-gel activity assays or Western blotting with antibody against the β subunit of F₁ (Figure 4D). Instead, Western blots showed evidence of high–molecular weight oligomers of the hydrophobic F₀ proteins, subunits a and c. Such findings indicate that the S1 variant produces an ε-minus form of the ATP synthase that is fragile and dissociates in response to detergent extraction and/or gel electrophoresis.

The nuclear and mitochondrial chromosomal genotypes of the strains S1 and S1+ε are identical (Δε+c-L57F), the difference being that the latter strain is transformed with plasmid pCM189-ATP15, in which the wild-type gene for subunit ε is subject to down-regulation by doxycycline. In the absence of drug, S1+ε yeast are replete with the ε-subunit and provide a cell model to study the c-L57F substitution as a stand-alone mutation. Strain S1+ε grew as well as the control Tet-ε cells in rich glycerol/ethanol medium (Figure 3C). State 4 respiration was also normal in S1+ε mitochondria and was stimulated efficiently (4.5-fold) by CCCP (Table 2). However, state 3 respiration (Table 2) and ATP synthesis (Table 3) were both diminished, by ~20–30%, compared with the control, notwithstanding the fact that BN-PAGE (Figure 4D) revealed control amounts of fully assembled ATP synthase in the S1+ε strain. These data suggest that, by itself, the c-L57F mutation reduces the rate of proton translocation through F₀ without any effect on F₁F₀ coupling and assembly.

DISCUSSION
Energy coupling in the ATP synthase occurs by means of an elongated hetero-oligomeric structure called the central stalk, which makes contact at one end with the catalytic sites in the soluble F₁ domain and at the other end with the proton-translocating unit in the membrane (Davenport et al., 2008; Walker, 2013). In the mitochondrial enzyme, the central stalk is made of three subunits (γ, δ, and ε) instead of two as found in bacterial ATP synthase (γ and ε). Bacterial subunit ε is homologous to mitochondrial subunit δ, whereas the mitochondrial ε-subunit has no counterpart in bacteria. Our investigation into the functional relevance of mitochondrial ε provides information that helps explain the appearance of this novel protein with the evolution of mitochondrial ATP synthase. The results also offer valuable insight into previous publications in which the phenotype of yeast strains lacking the chromosomal gene encoding the ε-subunit (Δε) was described but could not be explained fully.

Deleting the yeast gene for one of the two conserved, central stalk proteins (γ or δ) destroys oxidative phosphorylation in the cell (Paul et al., 1994; Giraud and Velours, 1997; Lai-Zhang et al., 1999).

FIGURE 3: Mutations in F₀ a- and c-subunits can bypass the need for subunit ε. (A) Samples of the Tet-ε galactose cultures after 40-generation growth in the presence of doxycycline (Figure 2) produce colonies that grow on rich glycerol/ethanol medium. Arrowheads indicate medium-size (S1) and small (S2) clones that carry mutations in subunit c (c-L57F) and subunit a (a-A128V), respectively. (B, C) Growth of Tet-ε, S1, and S1 retransformed with the Tet-ε gene on glucose plates lacking uracil (B) and in liquid glycerol/ethanol medium (C).
Instead, poor to modest growth on nonfermentable substrates has been reported for yeast Δε strains (Guelin et al., 1993; Lai-Zhang et al., 1999). Another complicating feature is that primary mutations in any of the three central stalk proteins promote the loss of mtDNA. The mtDNA instability is most severe in Δγ and Δδ yeast, which convert 100% to ρ−/ρ0 cells (Giraud and Velours, 1997; Lai-Zhang et al., 1999; Duvezin-Caubet et al., 2003, 2006; this study). This phenomenon is best described as a survival tactic based on the fact that the proton-translocating subunits of F0 are mitochondrial gene products, and the seal provided by the central stalk can be compromised if the structure of this element is incomplete (Lai-Zhang et al., 1999; Duvezin-Caubet et al., 2006; Godard et al., 2011). By eliminating mtDNA, the ρ−/ρ0 derivatives of Δγ and Δδ yeast lose the capacity to synthesize an integral membrane F0 domain that can collapse the ΔΨ by leaking protons.

In contrast, Δε yeast convert only partially to cytoplasmic petite derivatives such that the constitution of stable cultures is a heterogeneous mixture of ρ+ and ρ−/ρ0 cells (∼30:70 split). The degree of
mtDNA instability makes it impossible to distinguish the effects on respiration attributed solely to the missing ε-subunit. For this reason we used a transformed Δε yeast strain (Tet-ε) for our experiments that carried a doxycycline-repressible form of the ε-subunit gene, ATP15, on a plasmid. In the absence of drug, Tet-ε is ρ+ and behaves just like the parental control strain (SDC22), in which ATP15 is expressed from its native promoter in the chromosome. Instead, a doxycycline-induced block in subunit ε production was rapidly followed by a growth arrest in nonfermentable media (Figure 1A). At the point of growth arrest, the mitochondria were only partially depleted of subunit ε (25% depletion; Figure 1B), had a normal content of mtDNA, but were totally uncoupled due to F0-mediated proton leaks (Figure 1, C and D, and Table 1).

The correlation of 25% reduction in the ε-subunit with 100% mitochondrial uncoupling in growth-arrested Tet-ε cells supports the idea that a full complement of ε protein is required to support energy coupling in the organelle. However, this conclusion is difficult to reconcile with the phenotype of simple Δε strains, which lack completely the ε-subunit but retain partial respiratory competence (Guelin et al., 1993; Lai-Zhang et al., 1999). A solution to the puzzle was revealed from experiments in which Tet-ε cells were cultured for 40 generations with a fermentable carbon source (galactose) in a rich medium that also contained doxycycline (Figure 2). Because rich galactose media can support the growth of respiratory-deficient yeast that are auxotrophic for uracil, it was no surprise to find that many cells from the original culture had lost the URA3-linked plasmid, which rendered them equivalent to untransformed Δε yeast that were incapable of producing any ε-subunit (Figure 3B). Remarkably, whereas after two or three generations ATP15 plasmid-replete Tet-ε cells stopped growing on respiratory substrates in media that contained 10 μM doxycycline (Figure 1A), the cured cells that were recovered after extensive culturing in a rich fermentable medium supplemented with the drug formed two populations (S1 and S2) of respiring colonies on glycerol/ethanol plates (Figure 3A).

Suppressor mutations were identified in the mitochondrial genes coding for subunits of the proton translocation machinery in strains S1 and S2. For S1, a missense mutation was found in ATP9 that resulted in phenylalanine substitution for Leu-57 in the subunit ε structure (S1+Δε strain). Partial structures of the enzyme show that most of the contacts between the different subunits of the enzyme are within 3.5 Å of the ε-subunit (Figure 4D). S2 was a genetic lesion located to the ATP9 subunit a. Because the respiratory function of S2 was much worse relative to S1, the latter strain was investigated in detail (Figure 4 and Tables 2 and 3). Experiments with S1 that queried the characteristics of respiration and energy coupling in the ATP synthase revealed significant levels of coupled enzyme activities that indicated the assembly in mitochondria of an ε-deficient F1F0 complex that did not leak protons. The fact such complex was not detected by BN-PAGE analysis of digitonin-extracted mitochondria (Figure 4D) suggests that the physical association of F1 with F0 is compromised significantly in S1 yeast. Under conditions in which the c-l57F mutation could be investigated in the context of an otherwise normal F1F0 structure (S1+Δε strain), the results showed that in this configuration the ATP synthase is fully coupled but reduced in capacity to translocate protons across the membrane (Tables 2 and 3).

Having shown that combining either the c-l57F or a-A120V mutation with the null allele for subunit ε enables yeast to retain mtDNA, we suggest that similar mutations may have occurred in the genetic background of the Δε strains that have been described (Guelin et al., 1993; Lai-Zhang et al., 1999), which permitted a subpopulation of ρ+ cells to persist. In contrast, it has not been possible to recover ρ+ derivatives of Δγ and Δδ that are respiratory competent. Previously we showed that the mtDNA instability in Δδ yeast can be suppressed by mutations in the mitochondrial genes for subunits c and a, but whereas these maintain the mitochondrial inner membrane in a proton-impermeable state, such mutant ATP synthases fail to ensure proton-linked energy-transducing activities (Godard et al., 2011).

Information on the atomic structure of ATP synthases supports an explanation for phenotypic differences between Δε and Δδ yeast strains. Partial structures of the enzyme show that most of the contacts between the central stalk and the proton translocation apparatus in the membrane involve the δ-subunit (Stock et al., 1999; Daunt et al., 2010). Hence transduction of the proton motive force to the F1 catalytic sites largely depends on subunit δ. However, within the central stalk, the ε-subunit provides more than half of the atomic contacts for the δ-subunit (Figure 5, blue vs. red spheres). It follows that the stability of δ in the protein structure is likely compromised under conditions in which the ε-subunit is missing (e.g., Δε). Accelerated degradation of unbound subunit δ in doxycycline-treated Tet-ε is supported by Western analysis, which shows a reduction in the levels of this protein in comparison with mitochondria from control cells (Figure 1B).

We propose that the ε-subunit evolved as a means of securing the contact between γ and δ, which otherwise might be compromised by the torque imposed on subunit δ when the ε-ring rotates. Consistent with this idea is that the c-l57F mutation acquired by Δε yeast to sustain coupled oxidative phosphorylation did so by slowing the rate of proton-linked activities in the enzyme, as this may have been necessary to reduce the amount of strain on the structure.

**MATERIALS AND METHODS**

**Strains and media**

*Escherichia coli* XL1-Blue strain (Stratagene, Santa Clara, CA) was used for the cloning and propagation of plasmids. The...
S. cerevisiae strains used and their genotypes are listed in Table 1. The following rich media were used for the growth of yeast: 1% (wt/vol) yeast extract, 1% (wt/vol) peptone, 40 mg/l adenine, 2% (wt/vol) glucose, 2% (wt/vol) galactose, or 2% (wt/vol) glycerol. The glycerol medium was buffered at pH 6.2 with 50 mM potassium phosphate, and 2% (wt/vol) ethanol was added after sterilization. We also used complete synthetic medium (CSM; 0.17% [wt/vol] yeast nitrogen base without amino acids and ammonium sulfate, 0.5% [wt/vol] ammonium sulfate, 2% [wt/vol] glucose, and 0.8% [wt/vol] of a mixture of amino acids and bases from ForMedium, Norfolk, UK). The solid media contained 2% (wt/vol) agar.

Construction of a yeast strain expressing the subunit \(\gamma\) under the control of a doxycycline-repressible promoter

The coding sequence of the \(\gamma\)-subunit gene (ATP15) was amplified by PCR using DNA from strain W303-1B as a template and the primers ATP15tet1 (5′-cgcggatccATGTCTGCCTGGAGGAAAGCTG-3′) for the sense strand and ATP15tet2 (5′-ataagatggccgcCTATTTTGTTATTGGAGTGCGG-3′) for the antisense strand. The PCR product was digested with BamHI and ligated into the vector pCM189 (Duvezin-Caubet et al., 2003) to produce plasmid p2VT1/13. The cloned gene was verified by DNA sequencing. The SDC22 strain (Duvezin-Caubet et al., 2003) was transformed with p2VT1/13 and selected on synthetic complete medium lacking uracil. SDC22 containing p2VT1/13 was transformed with the deletion cassette of ATP15 obtained by PCR amplification using DNA from strain YPL271w (Δatp15 from the EuropeanSaccharomyces cerevisiae Archive for Functional Analysis, Frankfurt, Germany) as a template and the primers ATP15-300 (5′-AGCATTGACTAGTCTCTC-3′) for the sense strand and ATP15+300 (5′-AGGATGGTGACCAAAGACCG-3′) for the antisense strand according to a previously described procedure (Wach et al., 1994). The transformants were selected on YPGA (yeast extract, peptone, glucose, adenine) supplemented with 200 μg/ml G418 and analyzed by PCR analysis. One clone, called YE1, carrying the expected deletion in the chromosomal ATP15 gene, in a \(\rho^+\) state and containing pTE1, was retained for further analysis.

Construction of a yeast strain expressing the subunit \(\epsilon\) under the control of a doxycycline-repressible promoter

The coding sequence of the \(\epsilon\)-subunit gene (ATP3) was amplified by PCR using DNA from strain W303-1B as a template and primers ATP3tet1 (5′-ataggtcatCGGTCAGAGGACAAATGCGG-3′) for the sense strand and ATP3tet2 (5′-ataaataattgagtttttttaCAGCTTAGTGGATCT-3′) for the antisense strand. The PCR product was digested with PstI and ligated into the vector pCM189 (Duvezin-Caubet et al., 2003) to produce plasmid pP2VT1/13. The PCR product was digested with BamHI and ligated into the vector pCM189 (Duvezin-Caubet et al., 2003) to produce plasmid pTE1. The 3′ untranslated region of ATP3 was amplified by PCR using DNA from strain W303-1B as template and primers GUTR1 (5′-cattcgagTCATAAGATTGCTGAGGCGAAGAA-3′) for the sense strand and GUTR2 (5′-gataagcggCAAACTACTGATGCTGAGGCGAAGAA-3′) for the antisense strand. The PCR product was digested with PstI-HindIII and ligated into the vector pSE1 to produce plasmid pTE1. The cloned gene was verified by DNA sequencing. The SDC22 strain was transformed with pTE1 and selected on synthetic complete medium lacking uracil. SDC22 containing pTE1 was transformed with the deletion cassette of ATP3 obtained by PCR amplification of the pUG6 plasmid containing the KanMX4 module (Wach et al., 1994) as template and primers ATP3/Kan/Ter (5′-CTCG-3′) for the sense strand and ATP3/Kan/Pro (5′-atagcggccgcTCTCG-3′) for the antisense strand. The PCR product was digested with NotI and ligated into the vector pSE1 to produce plasmid pTE1. Molecular Biology of the Cell

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