Deletion of conserved protein phosphatases reverses defects associated with mitochondrial DNA damage in *Saccharomyces cerevisiae*

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Mitochondrial biogenesis is regulated by signaling pathways sensitive to extracellular conditions and to the internal environment of the cell. Therefore, treatments for disease caused by mutation of mtDNA may emerge from studies of how signal transduction pathways command mitochondrial function. We have examined the role of phosphatases under the control of the conserved α4/Tap42 protein in cells lacking a mitochondrial genome. We found that deletion of protein phosphatase 2A (PP2A) or of protein phosphatase 6 (PP6) protects cells from the reduced proliferation, mitochondrial protein import defects, lower mitochondrial electrochemical potential, and nuclear transcriptional response associated with mtDNA damage. Moreover, PP2A or PP6 deletion allows viability of a sensitized yeast strain after mtDNA loss. Interestingly, the *Saccharomyces cerevisiae* ortholog of the mammalian AMP-activated protein kinase was required for the full benefits of PP6 deletion and also for proliferation of otherwise wild-type cells lacking mtDNA. Our work highlights the important role that nutrient-responsive signaling pathways can play in determining the response to mitochondrial dysfunction.

mitochondria | TOR | petite-negative | bioenergetics | nutrient signaling

At least one in 10,000 people is afflicted by a mtDNA-associated disease (1). Mitochondrial dysfunction caused by mutation of the mitochondrial genome often manifests as a neuromuscular disorder, but mtDNA damage also may play a role in other ailments, such as diabetes and cancer (2). In many species, alterations to mtDNA mount during the lifespan of the organism (3) and may play a causal role in the aging process.

Conserved signaling pathways closely tie mitochondrial biogenesis to the extracellular environment and to the internal state of the cell. For example, activation of the nutrient-sensitive target of rapamycin complex 1 (TORC1) can promote mitochondrial biogenesis in mice (4), and activation of AMP-activated protein kinase (AMPK) drives mitochondrial biogenesis in muscle upon energy depletion (5). As in human cells, changing the nutritional status of *Saccharomyces cerevisiae* affects mitochondrial biogenesis. For instance, switching from glucose, the preferred carbon source, to other carbon sources leads to large-scale remodeling of cellular metabolism, including activation of many genes required for respiration. This change is controlled, in part, by the yeast ortholog of AMPK, SNF1 kinase (6). Currently, there is no effective treatment for mitochondrial disease (7). However, pharmacological control of signaling complexes with authority over mitochondrial biogenesis, such as TORC1, is likely to be a promising avenue toward the treatment of mitochondrial disorders (8).

Experiments using *S. cerevisiae* offer an opportunity to study many aspects of mitochondrial dysfunction in a genetically and biochemically tractable organism. Although mitochondria are essential organelles, neither respiration nor mtDNA is required for the viability of budding yeast on fermentable medium, because sufficient ATP can be made by glycolysis (9). However, certain nuclear mutations cause an inability to proliferate after loss of a functional mitochondrial genome (conversion from the ρ^+ to the ρ^- state). The term "petite-negative" characterizes those mutations that sensitize cells to mtDNA loss. Studies of the petite-negative phenotype have demonstrated that cellular functions required after mtDNA loss can be conserved between yeast and human cells (10).

Importantly, mtDNA mutation can impair mitochondrial protein import, an indispensable process, by lowering the electrochemical potential ($\Delta\Psi^{mito}$) across the mitochondrial inner membrane (11, 12). Maintenance of the essential $\Delta\Psi^{mito}$ in cells lacking mtDNA depends on electrogenic exchange of ATP^{4-} generated by glycolysis for ADP^{3-} produced by hydrolytic activity of the ATP synthase F_1 sector in the mitochondrial matrix (9). Consequently, when F_1 activity is blocked, ρ^- cells are not able to divide. Previously, we found that a high-copy plasmid harboring Tip41p suppresses the petite-negative phenotype of a mutant deficient in F_1 sector activity and, furthermore, increases the division rate of otherwise WT ρ^- cells (13). Tip41p interacts physically with Tap42p (14), the *S. cerevisiae* ortholog of the mammalian α4 protein, which controls the stability and activity of type 2A and type 2A-related phosphatases downstream of the TOR kinase (15). In this work, we find that deletion of two conserved, related, Tap42p-associated phosphatases can provide substantial advantages to cells deleted of the mitochondrial genome. Furthermore, we demonstrate that the SNF1 kinase plays

Significance

The mitochondrion harbors a genome inherited from its prokaryotic ancestor. Damage to mtDNA causes severe consequences for the cell. Disease-causing mtDNA mutations can be inherited, or harmful changes in the mitochondrial genome can accumulate during treatment with anticancer or antiviral agents. Furthermore, mutation of mtDNA correlates with and may have a causal role in aging. We have used a single-celled organism in which many studies of mitochondrial function have been performed to investigate how pathways that sense the nutritional status of the cell determine the consequences of mtDNA damage. We found that the deletion of two protein phosphatases conserved across many eukaryotes has greatly beneficial effects on cells lacking a mitochondrial genome, suggesting a potential therapeutic approach for mitochondrial disease.

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The authors declare no conflict of interest.

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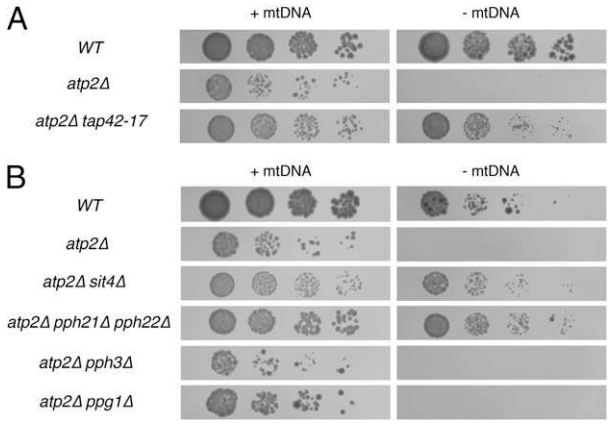


Fig. 1. Mutation of Tap42p or its client phosphatases can suppress the petite-negative phenotype. (*A*) A temperature-sensitive allele of *TAP42* suppresses the inviability of ρ^- cells lacking the F_1 sector of the ATP synthase. Strains CDD205 (WT), CDD298 (*atp2*Δ), or CDD300 (*atp2*Δ *tap42-17*) were tested for the petite-negative phenotype (cells containing mtDNA were incubated for 2 d at 30 °C). (*B*) Deletion of PP6 or PP2A allows viability of *atp2*Δ ρ^- cells. Strains BY4741 (WT), CDD275 (*atp2*Δ), CDD296 (*atp2*Δ *sit4*Δ), CDD332 (*atp2*Δ *pph21*Δ *pph22*Δ), CDD273 (*atp2*Δ *pph3*Δ), and CDD277 (*atp2*Δ *ppg1*Δ) were assayed for a petite-negative phenotype.

a vital role in the proliferation of cells lacking mtDNA and also is partially responsible for the beneficial effects of protein phosphatase 6 (PP6) deletion.

Results

The mtDNA Dependence of a Petite-Negative Mutant Is Suppressed by Reducing the Activity of Type 2A and 2A-Like Phosphatases. To test whether reducing the function of the essential Tap42 protein would, like Tip41p overexpression, allow the proliferation of cells lacking the F_1 sector subunit Atp2 after mtDNA loss, we took advantage of a temperature-sensitive allele of *TAP42* (Fig. S1*A*). When cultured at the semipermissive temperature of 30 °C, *atp2*Δ ρ^- cells, as expected, failed to divide after mtDNA deletion by ethidium bromide (EtBr) treatment (Fig. 1*A*). However, *atp2*Δ *tap42-17* ρ^- cells remained proliferative, suggesting that overexpression of Tip41p suppresses the petite-negative phenotype by inhibiting Tap42p function.

Tap42p is required for the folding and assembly of protein phosphatase 2A (PP2A) and the related phosphatases, protein phosphatase 4 (PP4) and PP6 (15). In *S. cerevisiae*, PP2A activity is provided redundantly by the catalytic subunits Pph21p and Pph22p, PP4 activity is supplied by Pph3p, and PP6 activity is furnished by Sit4p. The Ppg1 protein also is thought to encode a PP2A-like polypeptide. Concomitant deletion of Pph21p and Pph22p successfully suppressed the petite-negative phenotype of *atp2*Δ yeast (Fig. 1*B*). Moreover, *atp2*Δ *sit4*Δ cells proliferated after mtDNA loss. However, unlike strains lacking either Sit4p or both Pph21p and Pph22p, *atp2*Δ cells lacking the related phosphatases Pph3p and Ppg1p were not able to divide after loss of mtDNA, and these phosphatases were not studied further. We confirmed that neither loss of Pph21p/Pph22p (Fig. S2*A*) nor removal of Sit4p (Fig. S2*B*) prevented mtDNA destruction by EtBr. Rescue of the petite-negative phenotype by phosphatase deletion was not limited to mutants lacking the F_1 sector of ATP synthase; deletion of Sit4p also allowed viability of ρ^- cells lacking Tom70p (Fig. S3*A*), an outer membrane receptor for proteins entering the mitochondria, or of ρ^- cells lacking the mitochondrial i-AAA protease (Fig. S3*B*). However, Sit4p deletion was not able to suppress the need for Aac2p, the major mitochondrial ATP/ADP antiporter, after mtDNA deletion (Fig. S3*C*). Synthetic lethality resulting from combined loss of PP2A and PP6 (16) prevented us from analyzing *atp2*Δ *pph21*Δ *pph22*Δ *sit4*Δ ρ^- cells.

Sit4p is directed toward specific substrates by four Sit4p-associated proteins (SAPs): Sap4p, Sap155p, Sap185p, and Sap190p (17). Deletion of no individual SAP rescued the petite-negative phenotype of *atp2*Δ ρ^- cells (Fig. S4*A*). However, double deletion of specific pairs of SAPs allowed *atp2*Δ proliferation after mtDNA loss: *atp2*Δ *sap155*Δ *sap190*Δ ρ^- cells divided nearly as quickly as *atp2*Δ *sit4*Δ ρ^- cells (Fig. S4*B*). *atp2*Δ *sap185*Δ *sap190*Δ ρ^- and *atp2*Δ *sap155*Δ *sap185*Δ ρ^- cells also exhibited some proliferation, although at a reduced rate compared with *atp2*Δ *sap155*Δ *sap190*Δ ρ^- cells. Therefore, Sit4p is directed by multiple SAPs toward substrates that govern mitochondrial function.

Like Sit4p, the Pph21 and Pph22 proteins are directed toward substrates by regulatory subunits (18). Individual deletion of regulatory proteins Rts1 or Cdc55 did not allow suppression of the *atp2*Δ ρ^- petite-negative phenotype (Fig. S4*C*). However, combined deletion of both Cdc55p and Rts1p allowed proliferation of *atp2*Δ ρ^- cells as efficiently as deletion of the catalytic subunits, suggesting that either regulatory subunit is able to direct PP2A toward targets that determine the outcome of mtDNA damage.

Deletion of PP2A or PP6 Increases the Fitness of Otherwise WT Cells Lacking mtDNA. Prompted by initial results suggesting that mutation of *TAP42* could increase the proliferation rate of ρ^- cells Fig. S1*B*), we wondered whether deletion of Sit4p or of both

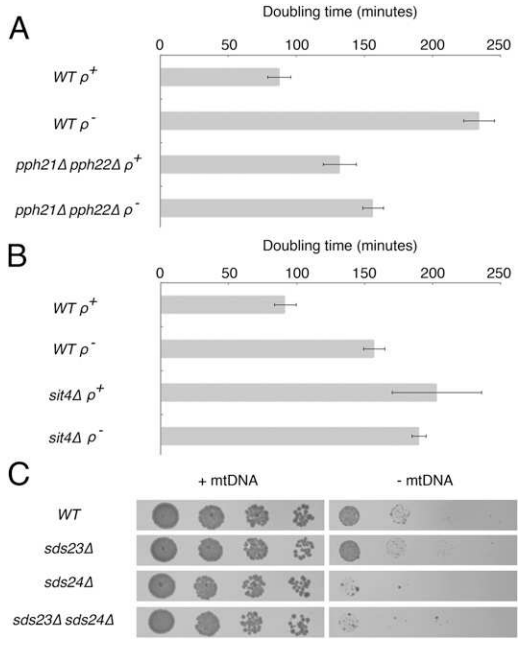


Fig. 2. Proliferation of petite-positive strains lacking mtDNA depends upon the magnitude of PP6 and PP2A signaling. (*A*) Combined removal of Pph21p and Pph22p raises the proliferation rate of ρ^- cells. Strains CDD290 (WT) and CDD453 (*pph21*Δ *pph22*Δ) either lacking or containing mtDNA were measured during proliferation in logarithmic phase. Average division times are plotted ($n = 9$ in all cases, except $n = 8$ for CDD290 cells containing mtDNA); error bars indicate SD. Proliferation rates were determined as in ref. 23. (*B*) Deletion of Sit4p protects cells from the reduced relative proliferation rate associated with mtDNA loss. The doubling time of strains CDD463 (WT) and CDD250 (*sit4*Δ) either lacking or harboring mtDNA were assayed as in *A* ($n = 9$). (*C*) Elimination of the protein phosphatase regulator Sds24p causes defective proliferation after mtDNA loss. Strains CDD289 (WT), CDD337 (*sds23*Δ), CDD335 (*sds24*Δ), and CDD336 (*sds23*Δ *sds24*Δ) were assayed for a petite-negative phenotype, with ρ^+ and ρ^- cells incubated for 2 d.

Pph21p and Pph22p would provide any advantage to cells lacking mtDNA, even in the absence of a petite-negative mutation. Therefore, we compared the proliferation rate of cells of a given nuclear genotype either containing or deleted of mtDNA. WT cells typically slow their proliferation by 40–60% upon mtDNA loss (Fig. 2 *A* and *B*). However, the effect of mtDNA loss on proliferation is not as severe for isogenic cells lacking PP2A activity: The division rate of *pph21*Δ *pph22*Δ cells forced to lose mtDNA was diminished by only 16% compared with *pph21*Δ *pph22*Δ cells harboring a mitochondrial genome (Fig. 2*A*). Furthermore, *pph21*Δ *pph22*Δ ρ^- cells proliferated more rapidly than isogenic WT ρ^- cells ($P < 0.0005$ by two-tailed Student's paired *t* test, two-tailed distribution).

Similarly, Sit4p deletion benefited cells whose mtDNA was destroyed: The proliferation rate of cells lacking Sit4p was completely unaffected by mtDNA loss (Fig. 2*B*). We note that although *sit4*Δ mutants proliferate slowly on glucose-containing medium and are unable to proliferate on nonfermentable medium, cells lacking Sit4p do contain mtDNA (Fig. S2*B*). Therefore, protection from a decline in proliferation rate after mtDNA destruction is not caused by a defect in mtDNA maintenance in *sit4*Δ mutants. Our results demonstrate that deletion of PP2A or of PP6 can protect cells from the detrimental effects of mtDNA damage, even in the absence of a mutation causing a petite-negative phenotype.

Sit4p and Pph21p/Pph22p are thought to be inhibited by the Sds23 family of proteins (19). We asked whether deletion of the two paralogous members of this family in *S. cerevisiae*, Sds23p and Sds24p, would reduce the proliferation of otherwise WT cells after mtDNA loss. Indeed, Sds24p deletion diminished the fitness of ρ^- cells (Fig. 2*C*). Deletion of Sds23p alone had no effect on the proliferation of ρ^- cells and did not enhance the damaging effects of Sds24p deletion on cells lacking mtDNA. These results indicate that the outcome of mtDNA loss is closely tied to the level of PP2A and PP6 function: Reduced Pph21p/Pph22p or Sit4p activity is advantageous for cells lacking mtDNA, whereas mutations likely to augment the activity of these phosphatases impair the proliferation of ρ^- cells.

Phenotypes Associated with the Reduced $\Delta\Psi^{mito}$ Characteristic of Cells Lacking mtDNA Are Reversed in the Absence of PP2A or PP6. Cells lacking mtDNA exhibit a characteristic transcriptional signature. Most prominently, genes activated upon iron starvation are highly up-regulated after loss of the mitochondrial genome (12). Moreover, the retrograde (RTG) signaling pathway is activated by mtDNA loss, driving expression of enzymes

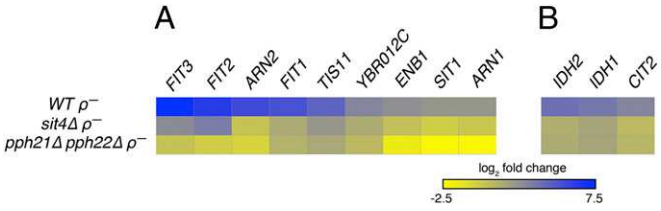


Fig. 3. PP2A or PP6 elimination attenuates the transcriptional response to mtDNA removal. (*A*) Activation of iron-responsive genes in ρ^- cells is diminished by Sit4p deletion or by combined removal of Pph21p and Pph22p. Genes selected for analysis were activated more than fourfold in ρ^- strain CDD410 in comparison with ρ^+ strain CDD463 and were annotated as increased under iron depletion conditions (40). Gene-expression levels of strains CDD410 (*mrpl16*Δ ρ^-), CDD415 (*mrpl16*Δ *sit4*Δ ρ^-), and CDD454 (*mrpl16*Δ *pph21*Δ *pph22*Δ ρ^-) were normalized to that of cells from WT strain CDD463, $\log_2$ transformed, and plotted. (*B*) Activation of RTG signaling pathway targets in ρ^- cells is abated by Sit4p removal or by Pph21p/Pph22p deletion. Transcript abundance is represented as in *A*. The ρ^- status of all *mrpl16*Δ strains was confirmed by mating assays.

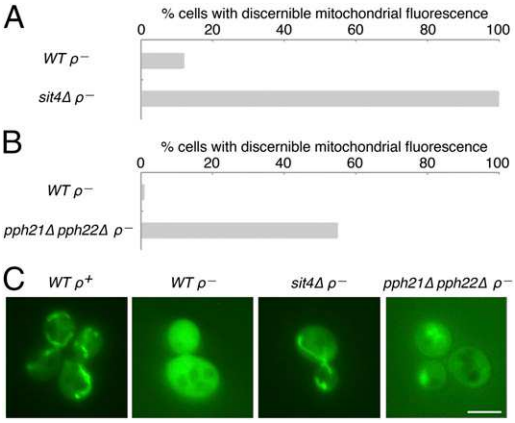


Fig. 4. Deletion of PP6 or PP2A increases protein import by mitochondria lacking mtDNA. (*A*) Sit4p removal increases protein import into ρ^- mitochondria. Strains CDD463 (WT) and CDD250 (*sit4*Δ) carrying pHS12 (41) and deleted of mtDNA were visualized and scored for perceivable mitochondria ($n > 200$ cells). (*B*) Coincident Pph21p and Pph22p deletion accelerates the import of proteins into mitochondria in ρ^- cells. Strains CDD290 (WT) and CDD453 (*pph21*Δ *pph22*Δ) were assayed as in *A* ($n > 200$ cells). (*C*) Representative images from *A* and *B*. Mitochondria are discernable in >99% of ρ^+ cells. Microscopy was performed as in ref. 23. (Scale bar, 5 μm.)

catalyzing anaplerotic reactions that are important in the absence of a respiratory chain (20). Activation of the iron regulon and of the RTG pathway is tightly linked to the $\Delta\Psi^{mito}$ of ρ^- cells (12, 21). We tested by quantitative mRNA sequencing whether the transcriptional response to mtDNA damage is attenuated in cells lacking both Pph21p and Pph22p or deleted of Sit4p. For these experiments, rather than destroying mtDNA using EtBr, we forced mtDNA loss by blocking mitochondrial translation through removal of the mitochondrial ribosomal protein Mrpl16p (22). As previously reported (12, 21), several genes responsive to iron deprivation or to RTG pathway activation were up-regulated at least fourfold in ρ^- cells. We found that deletion of Sit4p or Pph21p/Pph22p considerably weakened the expression of iron regulon (Fig. 3*A*) and RTG pathway (Fig. 3*B*) targets induced by mtDNA loss. Most strikingly, rather than the ~150-fold overexpression of *FIT3* manifested upon mtDNA deletion, *sit4*Δ ρ^- cells overexpressed *FIT3* less than sevenfold as compared with WT ρ^+ cells, and *pph21*Δ *pph22*Δ ρ^- cells did not overexpress *FIT3* (Fig. 3*A*). Because the transcriptional response to mtDNA loss is associated with reduced $\Delta\Psi^{mito}$ (12, 21), our results suggest that the $\Delta\Psi^{mito}$ of *sit4*Δ ρ^- or *pph21*Δ *pph22*Δ ρ^- cells might be higher than that of otherwise WT ρ^- cells.

Reduced $\Delta\Psi^{mito}$ can be visualized by the localization of a fluorescent protein targeted by a Cox4 presequence (12). This substrate is imported efficiently by ρ^+ mitochondria, but ρ^- mitochondria with reduced $\Delta\Psi^{mito}$ cannot import this polypeptide efficiently. Consequently, fluorescent signal accumulates in the cytosol, and mitochondria become difficult to visualize. Importantly, mutations that boost $\Delta\Psi^{mito}$ relocalize mitochondria-targeted fluorescent proteins to the organelle (12, 23).

We determined the location of Cox4p (1–21)-GFP in otherwise WT ρ^- cells and in ρ^- cells lacking either Sit4p or both Pph21p and Pph22p. Fluorescent mitochondria are not discernable in the great majority of ρ^- cells expressing the Cox4p (1–21)-GFP reporter (Fig. 4 *A* and *C*). In contrast, all ρ^- cells also lacking Sit4p exhibited a radical relocalization of fluorescent substrate to mitochondria, signaling a raised $\Delta\Psi^{mito}$ in *sit4*Δ ρ^- cells. Similarly, *pph21*Δ *pph22*Δ ρ^- cells manifested a significant relocalization of Cox4p (1–21)-GFP to mitochondria (Fig. 4 *B* and *C*). Methylene blue staining confirmed that cultures of ρ^- cells expressing Cox4p (1–21)-GFP consisted almost exclusively

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of viable cells. Notably, we found that mitochondria in cells lacking both Pph21p and Pph22p form an amorphous mass (Fig. 4*C*) rather than the serpentine structures found in WT cells, warranting future studies on the role of PP2A in maintaining mitochondrial morphology.

To investigate further whether $\Delta\Psi^{mito}$ might be raised in ρ^- cells lacking Sit4p or Pph21p and Pph22p, we quantified by flow cytometry a fluorescent dye, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), whose concentration by mitochondria is dependent on $\Delta\Psi^{mito}$ (24), with the caveat that such dyes first must pass through the plasma membrane. As a population, ρ^+ cells exhibited significantly higher $DiOC_6$ fluorescence than ρ^- cells, confirming the utility of this assay (Fig. 5*A*). Quantification of cell fluorescence indicated that the median fluorescence intensity (MFI) of ρ^+ cells was approximately threefold higher than that of ρ^- cells (Fig. S5*A*). Consistent with an increase in $\Delta\Psi^{mito}$ provoked by Sit4p deletion in cells lacking mtDNA, *sit4*Δ ρ^- yeast exhibited higher fluorescence intensity than WT ρ^- cells (Fig. 5*B*), with *sit4*Δ ρ^- cells exhibiting more than twice the MFI of ρ^- cells (Fig. S5*A*). Coincident deletion of Pph21p and Pph22p similarly increased dye uptake by ρ^- cells (Fig. S6); however, we note that measurements of fluorescence intensity in cells lacking PP2A should be interpreted with caution because of the aberrant mitochondrial morphology of *pph21*Δ *pph22*Δ cells.

SNF1 Kinase Activity Determines the Consequences of mtDNA Loss. We used suppression of the petite-negative phenotype by Sit4p deletion to consider further the downstream effects of Tap42-controlled phosphatase removal in ρ^- cells. Sit4p inhibits Snf1p, the catalytic subunit of the *S. cerevisiae* ortholog of AMPK, through dephosphorylation of its conserved activation loop (25). Consequently, we tested whether Snf1p might facilitate rescue of the petite-negative phenotype. Indeed, *atp2*Δ *sit4*Δ *snf1*Δ ρ^- cells proliferated much more slowly than *atp2*Δ *sit4*Δ ρ^- cells (Fig. 6*A*), indicating that Snf1p plays an important role in the rescue of *atp2*Δ ρ^- lethality by Sit4p removal. A well-characterized target of Snf1p is the transcriptional repressor Mig1p, which Snf1p phosphorylates and inactivates to promote the expression of genes involved in metabolism of carbon sources other than glucose (26). Accordingly, we asked whether deletion of Mig1p would reverse the harmful effects of Snf1p deletion on *atp2*Δ *sit4*Δ ρ^- cells; indeed, the effect of Snf1p removal on *atp2*Δ *sit4*Δ ρ^- proliferation was countermanded by the *mig1*Δ mutation. Therefore, for ρ^- cells to receive the full benefits of Sit4p deletion, Snf1p likely allows the activation of one or more genes repressed by the Mig1 transcription factor. Snf1p is directed toward downstream targets by associated β-subunits Sip1p, Sip2p, and Gal83p (6). However, deletion of no individual β-subunit affected the ability of *sit4*Δ to suppress *atp2*Δ ρ^- lethality (Fig. S7*A*). Likewise, deletion of neither Sak1p nor Tos3p, upstream kinases known to activate Snf1p (6), was able to reduce the proliferation of *atp2*Δ *sit4*Δ ρ^- cells (Fig. S7*B*). Deletion of a third upstream Snf1p-activating kinase, Elm1p, caused synthetic lethality in combination with the *sit4*Δ allele, precluding our analysis of Elm1p in this context.

Because Snf1p is important for the full effect of Sit4p removal on proliferation of *atp2*Δ cells lacking mtDNA, we asked whether mutants solely lacking Snf1p are petite-negative. In fact, cells lacking Snf1p do exhibit extremely slow proliferation after mtDNA loss (Fig. 6*B*), but the petite-negative phenotype of *snf1*Δ cells is not rescued by Mig1p deletion. However, the proliferation of *snf1*Δ cells lacking mtDNA was rescued by expression of the *ATP3-5* allele (Fig. S8*A*), which raises the $\Delta\Psi^{mito}$ of ρ^- mitochondria (Figs. S5*B* and S8*B*), suggesting that the petite-negative phenotype of cells lacking Snf1p might be caused by reduced $\Delta\Psi^{mito}$ after mtDNA loss. Sit4p deletion also rescued the slow proliferation of *snf1*Δ ρ^- cells (Fig. 6*B*), raising the possibility that loss of Sit4p can elevate $\Delta\Psi^{mito}$ even in the absence of the SNF1 kinase.

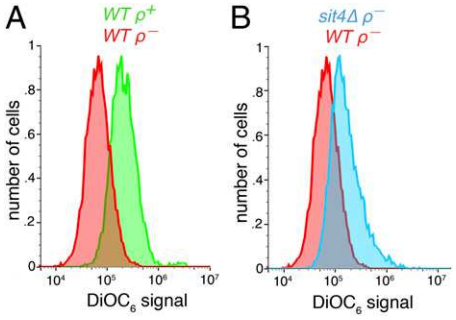


Fig. 5. Deletion of PP6 increases uptake of a $\Delta\Psi^{mito}$-dependent fluorescent dye by cells lacking mtDNA. (*A*) Fluorescence intensity of $DiOC_6$-treated ρ^- cells is reduced in comparison with ρ^+ cells. Strain CDD463 either containing (WT ρ^+) or lacking (WT ρ^-) mtDNA were stained with $DiOC_6$. Fluorescence of >10,000 events was assessed. Relative fluorescence is plotted using a logarithmic scale on the *x*-axis, and the number of cells plotted on the *y*-axis is normalized to the mode. (*B*) Removal of Sit4p increases $DiOC_6$ concentration by ρ^- cells. Fluorescence of $DiOC_6$-stained CDD463 cells lacking mtDNA (WT ρ^-, plotted in *A*) was compared with that of ρ^- cells of strain CDD250 (*sit4*Δ ρ^-) stained with $DiOC_6$. Data are represented as in *A*.

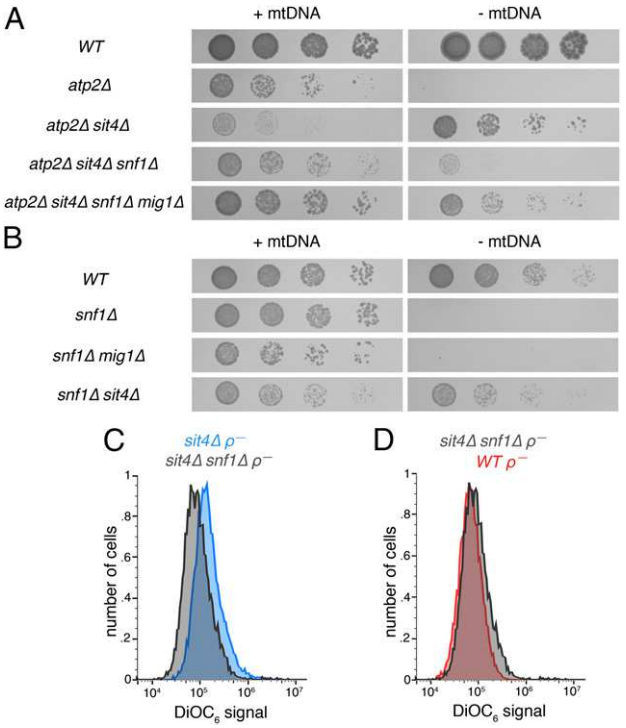


Fig. 6. The SNF1 kinase promotes proliferation of cells lacking mtDNA. (*A*) Snf1p inactivates the Mig1 transcription factor to potentiate the effects of Sit4p deletion on *atp2*Δ ρ^- cells. Strains CDD463 (WT), CDD215 (*atp2*Δ), CDD249 (*atp2*Δ *sit4*Δ), CDD458 (*atp2*Δ *sit4*Δ *snf1*Δ), and CDD614 (*atp2*Δ *sit4*Δ *snf1*Δ *mig1*Δ) were tested for the petite-negative phenotype; ρ^+ cells and ρ^- cells were cultured for 2 d and 5 d, respectively. (*B*) Snf1p is required for normal proliferation of ρ^- cells. Strains CDD463 (WT), CDD604 (*snf1*Δ), CDD653 (*snf1*Δ *mig1*Δ), and CDD602 (*snf1*Δ *sit4*Δ) were assayed for the petite-negative phenotype; both ρ^+ and ρ^- cells were incubated for only 2 d. (*C*) Removal of Snf1p decreases $DiOC_6$ uptake by *sit4*Δ ρ^- cells. CDD602 cells lacking mtDNA (*sit4*Δ *snf1*Δ ρ^-) were compared with ρ^- cells of strain CDD250 (*sit4*Δ ρ^-) as in Fig. 5. (*D*) ρ^- cells lacking both Sit4p and Snf1p still exhibit higher $DiOC_6$ staining than otherwise WT cells. Strains CDD602 (*sit4*Δ *snf1*Δ, plotted in *C*) and CDD463 (WT), both lacking mtDNA, were compared as in Fig. 5.

To investigate further the ability of Sit4p deletion to raise $\Delta\Psi^{mito}$ independently of Snf1p, we assayed the uptake of $\Delta\Psi^{mito}$-sensitive dye in ρ^- cells deleted of Sit4p and either lacking or expressing Snf1p. Consistent with a requirement for Snf1p in obtaining the entire benefit of Sit4p deletion (Fig. 6*A*), the $\Delta\Psi^{mito}$ of *sit4*Δ *snf1*Δ ρ^- cells was reduced in comparison with *sit4*Δ ρ^- cells (Fig. 6*C*), quantified as a 40.1% decrease in $DiOC_6$ MFI (Fig. S5*C*). However, congruous with suppression of the proliferation defect of *snf1*Δ ρ^- cells by Sit4p removal, *sit4*Δ *snf1*Δ ρ^- cells still exhibited a shifted fluorescence profile, in contrast to otherwise WT ρ^- cells (Fig. 6*D*). This shift corresponded to a 25.4% higher MFI for *sit4*Δ *snf1*Δ ρ^- cells than for WT ρ^- cells (Fig. S5*C*). This positive effect on $\Delta\Psi^{mito}$ is sufficient to increase the efficiency of import and thereby drive the relocalization of Cox4p (1–21)-GFP from the cytosol to mitochondria (Fig. S9). Taken together, our results indicate a role for the SNF1 kinase in controlling $\Delta\Psi^{mito}$ in ρ^- cells, and our findings establish that augmentation of $\Delta\Psi^{mito}$ by Sit4p removal in ρ^- cells relies on both Snf1p-dependent and Snf1p-independent mechanisms (Fig. 7).

Discussion

Deletion of Conserved Protein Phosphatases Can Raise the $\Delta\Psi^{mito}$ Across the Inner Membrane of Mitochondria Lacking DNA. The reduced $\Delta\Psi^{mito}$ that is characteristic of mitochondria lacking mtDNA is certainly a major contributor to, if not the cause of, the petite-negative phenotype (9, 27). Accordingly, transcriptomic, microscopic, and flow cytometric assays all indicate that $\Delta\Psi^{mito}$ is raised when either Sit4p or both Pph21p and Pph22p are deleted from cells lacking a mitochondrial genome. How might deletion of these phosphatases raise $\Delta\Psi^{mito}$ in ρ^- cells? Because removal of PP2A or PP6 allows cells completely lacking the F_1 sector of ATP synthase to live in the absence of mtDNA, one potential mechanism of $\Delta\Psi^{mito}$ augmentation might be transcriptional up-regulation of a different ATP hydrolase residing in the mitochondrial matrix. However, transcriptomic analyses of strains lacking mtDNA and either Sit4p or both Pph21p and Pph22p provided no evidence for a substantial increase in the mRNA of any mitochondrial ATPase. It remains possible that the increase in the levels or activity of such a hydrolase may occur at the translational or posttranslational level; our present analysis would not detect such an increase.

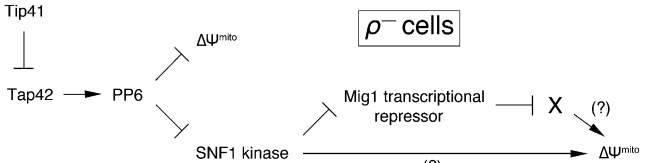


Fig. 7. Removal of PP6 improves the fitness of ρ^- cells through SNF1 kinase-dependent and -independent mechanisms. Tip41p overexpression suppresses the petite-negative phenotype and increases ρ^- cell proliferation through its role as a potential Tap42p inhibitor. Blocking Tap42 function leads to Sit4p instability and inactivity (15), promoting the fitness of ρ^- cells. Tap42p's described role in blocking the activity of fully assembled Sit4p (14) is not shown here, for simplicity and because of the apparently epistatic effects of longer-term Tap42p inhibition on the petite-negative phenotype. The full benefits of Sit4p deletion on a petite-negative mutant are realized only when Snf1p is present and combats repression of transcriptional target(s) (X) of the Mig1 protein. Still, Sit4p deletion raises the apparent $\Delta\Psi^{mito}$ of ρ^- cells even when Snf1p is inactivated. Snf1p also may have an independent role in maintaining $\Delta\Psi^{mito}$ in ρ^- cells, as indicated by the rescue of the *snf1*Δ petite-negative phenotype by F_1-sector hyperactivation and by the reduction of $\Delta\Psi^{mito}$ in *sit4*Δ ρ^- cells by Snf1p deletion. Notably, Mig1p deletion does not rescue the petite-negative phenotype of *snf1*Δ cells, implying that if Mig1p function impinges upon the $\Delta\Psi^{mito}$ of ρ^- cells, there also is likely to be an alternative, Mig1p-independent pathway impacting $\Delta\Psi^{mito}$ downstream of Snf1p. We note that an alternative model of Mig1p control, in which Snf1p and Sit4p independently impinge upon Mig1p function, is possible and has not been excluded.

Another plausible explanation for increased $\Delta\Psi^{mito}$ of *sit4*Δ ρ^- and *pph21*Δ *pph22*Δ ρ^- cells is a change in ion mobility and distribution across the mitochondrial inner membrane. Interestingly, a proteomic analysis of *S. cerevisiae* mitochondria has demonstrated phosphorylation of the major ATP/ADP antiporter Aac2p (28). Moreover, Aac2p is essential for the generation of $\Delta\Psi^{mito}$ by electrogenic exchange of ATP^{4-} for ATP^{3-} across the inner membrane of ρ^- mitochondria (9), and Aac2p still is required for proliferation of ρ^- cells, even when Sit4p is deleted. It is feasible that phosphorylation of Aac2p plays a role in improving the $\Delta\Psi^{mito}$ of cells lacking mtDNA. Alternatively, $\Delta\Psi^{mito}$ might be affected more indirectly through a change in the phosphorylation state and therefore in the substrate preference and/or efficiency of the mitochondrial import machinery (29), thereby altering the abundance of one or more ion transporters. Notably, at least two SAPs have been found to be associated with highly purified mitochondria (30), providing compelling evidence that Sit4p has a direct role in controlling the phosphorylation state of proteins localized to this subcellular location.

The SNF1 Kinase Plays an Important Role in Cells Lacking mtDNA. Because Snf1p is inhibited by Sit4p (25), we asked whether Snf1p might be required for the rescue of the petite-negative phenotype of *atp2*Δ ρ^- cells by Sit4p deletion. Indeed, upon deletion of Snf1p, *atp2*Δ *sit4*Δ ρ^- cells divided more slowly. The proliferation defects caused by Snf1p removal in *atp2*Δ *sit4*Δ ρ^- cells were reversed by the additional deletion of Mig1p, a transcriptional repressor inhibited by the SNF1 kinase (26). Therefore, we favor a model in which Mig1p release activates one or more genes in cells lacking PP6, allowing maximal suppression of the petite-negative phenotype. We note that lack of Sit4p does not reverse defects associated with mtDNA deletion solely through Snf1p activation: Despite their slow proliferation, *atp2*Δ *sit4*Δ *snf1*Δ ρ^- cells still proliferate, and *sit4*Δ *snf1*Δ ρ^- cells still exhibit a perceptibly higher $\Delta\Psi^{mito}$ than otherwise WT ρ^- cells, as measured by $DiOC_6$ uptake and by an in vivo import assay. So far, we have no evidence that hyperactivation of Snf1p alone is sufficient to increase the fitness of ρ^- cells: Deletion of Mig1p does not rescue the petite-negative phenotype of *atp2*Δ mutants (Fig. S10*A*), nor does deletion of Reg1p, a phosphatase that inhibits Snf1p activation (Fig. S10*B*) (31, 32).

Interestingly, Snf1p not only was necessary for the full benefits of Sit4p removal but also was required for normal proliferation of otherwise WT ρ^- cells. The petite-negative phenotype of *snf1*Δ cells was not suppressed by deletion of Mig1p but instead was suppressed by elevating $\Delta\Psi^{mito}$ through increased ATP hydrolysis in the mitochondrial matrix or by PP6 deletion. Therefore, the aspects of Snf1p activation that are important for the proliferation of ρ^- cells expressing Sit4p may differ from or may be larger in scope than those that are consequential for *atp2*Δ ρ^- yeast lacking Sit4p. Importantly, a portion of Snf1p associates with purified mitochondria (30), indicating a potentially direct role for the SNF1 kinase in modulating mitochondrial function.

Large-scale, mass spectroscopy-based analyses are beginning to elucidate the phosphoproteome changes resulting from the removal of specific kinases and phosphatases (33). Using this approach, we now are searching for the substrates of PP6, PP2A, and the SNF1 kinase that determine the cellular consequences of mtDNA loss. We expect that such studies will provide a valuable foundation for future inquiry into how the extracellular and intracellular environments, through conserved signaling pathways, control mitochondrial biogenesis and function in eukaryotic cells.

Materials and Methods

Yeast Strains and Culture Conditions. Yeast media recipes were as described previously (34). Gene disruptions were carried out as in ref. 35. Strain

genotypes and the details of their construction details are listed in Table S1*A*. Oligonucleotides used for strain construction are listed in Table S1*B*. All strains are congenic with the S288C-based strains described in ref. 36, except for strains CDD619, CDD631, and CDD632. EtBr (Thermo Fisher Scientific) was used at a concentration of 25 μg/mL to destroy mtDNA rapidly (37). Serial dilution assays were accomplished by diluting cultures to an OD_{600} of 0.1 and then performing three additional fivefold dilutions. Four microliters of each dilution were spotted to solid medium. Strains were tested for a petite-negative phenotype as in ref. 23; cells were incubated for 3 d (ρ^+) or 4 d (ρ^-) at 30 °C, except where the temperature or incubation time is otherwise noted.

Plasmids. Plasmids M532 encoding WT *TAP42* and M533 expressing *tap42-17* were generated in the former laboratory of Kim Arndt (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and were kindly provided by Maria Cardenas-Corona (Duke University, Durham, NC). The *tap42-17* allele in plasmid M533 was sequenced, revealing the following changes to the WT Tap42 protein: T61N, L73Q, S100P, E192T, K241E, and E353K.

Transcriptome Analysis. Strains CDD410, CDD415, CDD450, CDD452, CDD454, and CDD463 were diluted from overnight cultures into yeast extract peptone dextrose (YEPD) medium and were allowed to divide at least three times before harvesting. Total cellular RNA was extracted from 0.5 OD_{600} units of cells, using the Nucleospin RNA II kit (Macherey-Nagel) per the manufacturer's instructions. cDNA and Illumina library preparation were performed by the Otogenetics Corporation, followed by sequencing to 2×10^7 paired-end 100-nucleotide reads using the HiSeq2000 platform. Sequencing data from one of the paired ends were mapped against the S288C genome (assembly sacCer3, April 2011) on the Galaxy web server (38) using Tophat version 2.0.6, followed by determination of gene-expression levels using Cuffdiff version 2.1.1 (39) with geometric library normalization, a blind dispersal estimation method, multiread correction, and bias correction based on the sacCer3 genome. Transcript assignments were made using the Ensembl EF4 GTF file for *S. cerevisiae*. Gene expression of all tested strains, provided in fragments per kilobase exon per million fragments mapped on all exons, can be found under Gene Expression Omnibus record GSE52242.

Flow Cytometry. Strains were prepared for flow cytometry as if for an assay of cell proliferation (23). Cells were stained for 150 s in YEPD medium with 350 nM $DiOC_6$ (Anaspec), before immediate analysis using a Becton Dickinson Accuri C6 Flow Cytometer running BD CSampler software. Cells were excited at 488 nM. Fluorescence was collected at 533 nm and plotted using FlowJo 10.0 (TreeStar). The MFI for experimental samples was calculated after subtracting the median innate fluorescence of unstained cells from those stained by $DiOC_6$.

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