Mitochondrial membrane potential acts as a retrograde signal to regulate cell cycle progression

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Mitochondria are central to numerous metabolic pathways whereby mitochondrial dysfunction has a profound impact and can manifest in disease. The consequences of mitochondrial dysfunction can be ameliorated by adaptive responses that rely on crosstalk from the mitochondria to the rest of the cell. Such mito-cellular signalling slows cell cycle progression in mitochondrial DNA-deficient (ρ0) Saccharomyces cerevisiae cells, but the initial trigger of the response has not been thoroughly studied. Here, we show that decreased mitochondrial membrane potential (ΔΨm) acts as the initial signal of mitochondrial stress that delays G1-to-S phase transition in both ρ0 and control cells containing mtDNA. Accordingly, experimentally increasing ΔΨm was sufficient to restore timely cell cycle progression in ρ0 cells. In contrast, cellular levels of oxidative stress did not correlate with the G1-to-S delay. Restored G1-to-S transition in ρ0 cells with a recovered ΔΨm is likely attributable to larger cell size, whereas the timing of G1/S transcription remained delayed. The identification of ΔΨm as a regulator of cell cycle progression may have implications for disease states involving mitochondrial dysfunction.

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Introduction

Mitochondria use the electron transport chain (ETC) complexes to convert energy gained from the oxidation of nutrients into an electrochemical gradient across the inner mitochondrial membrane that is then used to drive ATP synthesis through oxidative phosphorylation (OXPHOS). In addition to producing most of the cell’s ATP, however, mitochondria carry out a diverse array of vital cellular functions including synthesis of Fe-S clusters, amino acid and nucleotide biosynthesis, the production of reactive oxygen species (ROS), and apoptosis. It follows that mitochondrial function is required for cell survival even in a facultative anaerobe like the budding yeast Saccharomyces cerevisiae that can survive without mitochondrial ATP production. In line with the manifold functions of the organelle, mitochondrial dysfunction is associated with numerous diseases including neurodegenerative disorders, metabolic syndrome, cancer, and ageing (1, 2). Rather than manifesting merely as an energy defect, mitochondrial dysfunction can contribute to cellular dysfunction and disease aetiology through diverse mechanisms involving, for example, increased levels of ROS that can damage cellular constituents, changes in nuclear epigenetic marks or gene expression patterns, and even instability of the nuclear genome (1, 3, 4, 5).

To avoid or ameliorate the dire consequences of mitochondrial dysfunction, a complex communication network mediates signals of mitochondrial status to other parts of the cell including the nucleus, lysosomes, peroxisomes, and the endoplasmic reticulum (1, 2). This mito-cellular signalling can involve signals ranging from key metabolites to misfolded proteins or ROS, and acts to restore cellular homeostasis, facilitate adaptation to the altered mitochondrial status, or eliminate dysfunctional mitochondria via mitophagy (6).

Mitochondria contain their own genome, mtDNA, that encodes key subunits of the ETC complexes, as well as the F0 component of the ATP synthase, making this small genome essential for cellular respiration and OXPHOS. It follows that cells lacking mtDNA (termed ρ0 cells) cannot maintain the mitochondrial membrane potential (ΔΨm) by proton pumping; instead, they maintain a limited ΔΨm by a mechanism involving the hydrolysis of ATP by the “reverse” action of the F1 component of the ATP synthase in consort with the electrogenic exchange of ATP3+ for ADP3- by the adenine nucleotide translocator (7, 8, 9). Accordingly, certain hypermorphic mutations in subunits of the F1-ATPase result in a higher membrane potential in ρ0 cells (10).

In addition to preventing cellular respiration and decreasing ΔΨm, the acute depletion of mtDNA after a brief ethidium bromide treatment promotes nuclear DNA instability and defective cell cycle progression that manifests as an accumulation of cells in the G1 phase (5). Moreover, although the nuclear DNA instability in mtDNA-depleted cells is driven by defective Fe-S cluster protein assembly, the cell cycle defect was found to be independent of
Fe-S metabolism (5). Subsequent work elaborated that a cell cycle phenotype of mtDNA-depleted cells is seen even in cells that are adapted to their \( \rho^0 \) status, and is attributable to delayed transition from the G1 to the S phase of the cell cycle through a mechanism proposed to involve the Rad53 checkpoint kinase (11). However, despite the undisputed involvement of mitochondrial dysfunction in human disease, the molecular-level events that link mtDNA loss to the cell cycle machinery, and particularly the mitochondria-proximal signal that initiates this form of mitochondrial communication, have not been thoroughly addressed. Here, we sought to identify the mechanism that triggers the delayed G1-to-S progression in \( \rho^0 \) cells.

A combination of genetic and pharmaceutical interventions uncovered decreased mitochondrial membrane potential as a regulator of cell cycle progression not only in \( \rho^0 \) cells, but also in \( \rho^+ \) cells. Accordingly, the cell cycle defect in \( \rho^0 \) cells was rescued by increasing \( \Delta \Psi_m \), confirming that adequate \( \Delta \Psi_m \) rather than full mitochondrial function per se is required for normal cell cycle progression. In contrast, neither inhibition of mitochondrial ATP synthesis nor altered levels of oxidative stress could directly account for the G1-to-S transition delay of \( \rho^0 \) cells. These findings corroborate that mitochondrial function, via mitochondrial membrane potential, acts as a regulator of cell cycle progression, and call for a better understanding of the mechanisms through which dysfunctional mitochondria impact the cell cycle machinery.

**Results**

**Decreased mitochondrial membrane potential delays G1-to-S phase progression**

To study the effects of mtDNA loss, an mtDNA-devoid (\( \rho^0 \)) variant of the mtDNA-containing (\( \rho^+ \)) WT strain was made by standard treatment with ethidium bromide (12), and confirmed by quantitative real-time PCR. In accordance with previous reports, \( \rho^0 \) cells grew slower than their \( \rho^+ \) counterparts in a dextrose-containing rich medium, and exhibited an altered cell cycle profile (Figs 1A and S1A) (11, 13). Specifically, the percentage of G1 cells in an early logarithmic phase–unsynchronized culture of \( \rho^0 \) cells was twice as high as...
as in $\rho^-$ cells grown under identical conditions; this difference remained constant for at least 2 h during exponential growth and was also observed in cells that had lost their mtDNA because of deficiency of known mtDNA maintenance factors such as Rim1, Mip1, and Mgm101 (Figs 1A and B and S1B and C). The increased frequency of G1 cells in the unsynchronized $\rho^0$ culture was attributable to a slower G1-to-S transition rather than a faster progression through other cell cycle stages because $\alpha$-factor–treated $\rho^0$ cells released from G1 synchrony transitioned into the S phase later than their $\rho^+$ counterparts (Fig 1C and D; compare timepoints when % of G1 cells starts to decrease or % of S cells peaks in panel D), corroborating previous findings (11). A G1-to-S transition delay of the $\rho^0$ variant was observed even after release from G2 synchrony achieved by nocodazole treatment; although $\rho^-$ and $\rho^0$ cells showed comparable timing of G2 exit (Fig S1D and E), $\rho^0$ cells accumulated in G1 and displayed delayed entry into the S phase when compared to $\rho^+$ cells (Fig 1E). Using our mtDNA-deficient strain that exhibited the previously reported delay in G1/S transition (5, 11, 14), we proceeded to seek the identity of the proximal signal that triggers the cell cycle phenotype in mtDNA-deficient cells.

Yeast mtDNA encodes proteins that are essential for the ETC and OXPHOS. The impairment of these processes in $\rho^0$ cells results in compromised mitochondrial ATP production, decreased $\Delta\Psi_m$, and increased generation of ROS (15, 16, 17), all of which are reasonable candidates for the initial signal that triggers the G1-to-S phase delay in $\rho^0$ cells. In line with a previous report (11), treatment of $\rho^+$ cells with the ATP synthase inhibitor oligomycin did not cause accumulation of cells in the G1 phase (Figs 2A and S2A); the potency of the oligomycin was confirmed by its effect on mitochondrial membrane potential measured using the fluorescent cationic dye tetramethylrhodamine methyl ester perchlorate (TMRE) (Fig S2B, lower panel). As expected based on the lack of a functional Fo subunit, $\rho^0$ cells were not impacted by oligomycin treatment (Fig S2B, upper panel). Decreased mitochondrial ATP synthesis was therefore excluded as a cause of the G1-to-S transition delay observed in $\rho^0$ cells.

We next tested the importance of the $\Delta\Psi_m$ for normal cell cycle progression. To this end, we treated exponentially growing cells with BAM15, an uncoupler that efficiently dissipates the $\Delta\Psi_m$ without depolarizing the plasma membrane (18). Interestingly, BAM15 treatment of $\rho^+$ cells caused a transient accumulation of cells in the G1 phase (Figs 2B and S2C). The observed accumulation of cells in the G1 phase was accompanied by a corresponding decrease in the percentage of cells in the S phase. Treatment with another uncoupler, CCCP, caused a similar transient increase in G1 phase cells as BAM15 (Fig S2D and E).

To further corroborate the connection between $\Delta\Psi_m$ and G1-to-S progression, we synchronized $\rho^+$ cells in G1 with $\alpha$-factor and released them into a medium containing BAM15. In analogy to the G1-to-S transition delay in $\rho^0$ cells, BAM15 treatment delayed the

![Figure 2. Loss of $\Delta\Psi_m$, but not the inhibition of mitochondrial ATP synthesis, delays G1-to-S progression in WT $\rho^+$ cells.](image)

(A) Percentage of G1 phase (solid lines) and S phase (dashed lines) cells in WT $\rho^+$ in an early logarithmic phase left untreated or treated with 20 $\mu$M of oligomycin to inhibit mitochondrial ATP synthesis. Aliquots were harvested upon the addition of the drug (0 min) and every 30 min thereafter. Values represent the average of two independent experiments, and error bars indicate SD. Representative DNA histograms are shown in Fig S2A.

(B) Percentage of G1 phase (solid lines) and S phase (dashed lines) cells in early logarithmic phase cultures of WT $\rho^+$ cells left untreated or treated with 30 $\mu$M of BAM15. Aliquots were harvested upon the addition of the drug (0 min) and every 15 min thereafter. Values represent the average of three independent experiments, and error bars indicate SD. Representative DNA histograms are shown in Fig S2C.

(C) DNA histogram of WT $\rho^+$ cells in early logarithmic phase cultures of WT $\rho^+$ cells synchronized in G1 with 10 $\mu$g/ml $\alpha$-factor and released into media with or without 30 $\mu$M BAM15. Cells were sampled upon release (time 0) and every 5 min thereafter.

(D) Quantification of G1 phase (solid lines) and S phase (dashed lines) cells in the experiment presented in panel (C). Values represent data from a single experiment. See also Fig S2.
progression of α-factor–synchronized ρ° cells from the G1 into the S phase: although the percentage of G1 cells began to decline 20 min after release of ρ° cells into a normal medium, indicating transition from the G1 into the S phase, ρ° cells released into the BAM15-containing medium did not show signs of G1 exit until 70 min after release (Fig 2C and D). BAM15 treatment also delayed G1-to-S transition in ρ° cells released from G2 synchrony: although the presence of BAM15 did not appreciably affect the transition from the G2 to the G1 phase, ρ° cells released from G2 into the BAM15-containing medium showed an increased percentage of G1 cells and delayed S phase entry, consistent with a specific G1-to-S transition delay (Fig S2G–J).

The results of Fig 2 demonstrate that a ΔΨm collapse induced by uncoupler treatment causes cells to accumulate in the G1 phase by triggering a G1-to-S phase transition delay similar to the one observed in ρ° cells. These findings implicate ΔΨm in modulating cell cycle progression in ρ° cells.

The cell cycle defect in ρ° cells is exacerbated by a further decrease in membrane potential

In contrast to uncoupler-treated cells where the ΔΨm is largely dissipated, ρ° cells have the ability to maintain a membrane potential that, although lower than in ρ° cells, is still sufficient to support various ΔΨm-dependent processes such as the import of nuclear-encoded mitochondrial proteins (9, 19). Next, we examined whether the decreased ΔΨm could underlie the cell cycle phenotype of ρ° cells and act as the initial signal to trigger the G1-to-S transition delay. We measured the ΔΨm of WT ρ° and ρ° cells using TMRE, and corrected the signal for potential changes in strain mitochondrial mass as detected by nonyl acridine orange (NAO), a green-fluorescent dye that localizes to the mitochondria in a ΔΨm-independent manner (20). Microscopy analysis confirmed the uptake of NAO, and thus its suitability as a readout of mitochondrial mass, into both ρ° and ρ° cells (Fig S3A). Uncoupler-treated samples analysed in parallel provided a measure of the background fluorescence. As expected, the ΔΨm of WT ρ° cells was significantly lower than that of WT ρ° cells, but clearly higher than the baseline value of uncoupler-treated cells (Figs 3A and S3B and C).

In additional support of a role for ΔΨm in regulating cell cycle progression, the accumulation of ρ° cells in the G1 phase was further exacerbated when ΔΨm was fully dissipated by BAM15 or CCCP treatment (Figs 3B and S2C, D, and F). The effect of BAM15 was more sustained in ρ° than in ρ° cells and was evident even 2 h after the addition of the compound (compare Figs 2B and 3B). Expectedly, time-course experiments after release from α-factor– or nocodazole-induced synchrony revealed a more severe G1-to-S phase progression delay in ρ° cells in the presence of BAM15 (G1 exit starting 40 min and 80 min after release from α-factor synchrony into the medium lacking and containing BAM15, respectively) (Figs 3C and D and S2G–J). These observations establish a quantitative correlation between ΔΨm and cell cycle progression, where the extent of the G1-to-S delay is governed by the severity of ΔΨm loss.

Increasing ΔΨm rescues the cell cycle phenotype of ρ° cells

The metabolism of ρ° cells differs significantly from that of ρ° cells given that respiratory-deficient ρ° cells must compensate for the loss of a subset of the citric acid cycle reactions, the products of which are central to many anabolic pathways (21). If the G1-to-S phase progression delay is indeed driven by decreased ΔΨm and not by other functional or metabolic differences between ρ° and ρ° cells, we hypothesized that it should be rescued by increasing ΔΨm in ρ° cells. In the absence of ETC activity, mtDNA-deficient cells maintain ΔΨm by an alternative mechanism that involves the hydrolysis of glycolytically produced ATP by the “reverse” action of the F1 subunit of the mitochondrial ATP synthase and the electrogenic exchange of ATP3+ for ADP3 over the inner mitochondrial membrane (7, 9).

The ΔΨm of ATP1-111 ρ° cells was comparable to that of WT ρ° cells, confirming the ATP1-111 ρ° strain as a suitable model to test our hypothesis in Figs 4A and S3B and C.

We first analysed the cell cycle profile of unsynchronized WT and ATP1-111 ρ° and ρ° cells. As expected based on the relatively high ΔΨm in ATP1-111 ρ° cells, the strain showed a cell cycle profile that was indistinguishable from WT ρ° cells (Fig 4B and C). Moreover, analysis of α-factor–synchronized cells revealed that the ATP1-111 mutation was largely able to rescue the delay in G1-to-S transition observed in ρ° cells: although the percentage of G1 cells in cultures of WT and cox4Δ ρ° cells was still above 90% 30 min after release from synchrony, only 67% of ATP1-111 ρ° cells remained in the G1 phase (Figs 4D and S3D). Finally, the growth of ATP1-111 ρ°, as judged by colony size or cell density measurements in liquid media, was partially restored relative to WT ρ° cells (Figs 4E and S3E), as previously reported by others (5, 22).

The earlier experiments carried out on WT cells revealed a more sustained effect of BAM15 on the cell cycle profile of ρ° than ρ° cells (Figs 2B and 3B). We attributed this to the slower recovery of ΔΨm in ρ° cells that lack a functional ETC. To explore this aspect further, we monitored cell cycle progression in ρ° cells lacking Cox4, a nuclear-encoded subunit of complex IV. Unperturbed cox4Δ ρ° cells were able to maintain a ΔΨm that did not significantly differ from that of WT ρ° cells (Figs 4A and S3B and C). Accordingly, deletion of the COX4 gene did not have a pronounced effect on the cell cycle profile of unsynchronized ρ° cells (Fig 4B and C). However, cox4Δ ρ° cells exhibited a somewhat delayed transition from G1 to S in synchrony experiments (Figs 4D and S3D) and a stronger, more sustained cell cycle response to BAM15 than WT or ATP1-111 ρ° cells, indicating that a functional ETC aids in timely recovery from uncoupling (Figs 4F and S3F). In line with this notion, all three ρ° strains, including the ATP1-111 ρ° with the restored ΔΨm, failed to recover from the BAM15-induced accumulation of G1 cells within the 120-min follow-up period, although the percentage of G1 cells was lower in ATP1-111 ρ° than in WT or cox4Δ ρ° cells (Figs 4G and S3F).

Based on the findings in Fig 4, we conclude that the delayed G1-to-S phase progression in ρ° cells can be attributed to a decreased ΔΨm. Moreover, the cell cycle defect and the consequent petite phenotype and slow growth of ρ° cells can be rescued by increasing ΔΨm through the ATP1-111 mutation, implicating membrane potential—rather than other metabolic adaptations in ρ° cells—as the main determinant of
the cell cycle delay. Accordingly, recovery from transient uncoupler-induced cell cycle delay is expedited by a functional ETC.

**Altered ROS is not the signal for the G1-to-S phase progression delay in \( \rho^0 \) cells**

Mitochondria are a major source of ROS, and mitochondrial dysfunction is often associated with increased levels of intracellular ROS (3). Furthermore, ROS are documented to influence cell proliferation (1). Therefore, we wanted to determine whether ROS levels play a role in the \( \rho^0 \) G1-to-S phase progression delay. Rather than increasing the percentage of cells in G1, however, \( \text{H}_2\text{O}_2 \) treatment primarily caused the accumulation of WT \( \rho^+ \) cells in the early S phase, as previously reported by others (Figs 5A and Band S4A) (23). In contrast, decreasing ROS by treatment of WT \( \rho^+ \) cells with the antioxidants N-acetylcysteine (NAC) or reduced glutathione (GSH) caused a transient accumulation of cells in the G1 phase along with a decrease in the percentage of cells in the S phase (Fig 5C, left panel; Fig S4B). A similar but more sustained effect of treatment with either NAC or GSH was observed in G1 with 10 \( \mu \text{g/ml} \) \( \alpha \)-factor and released into media with or without 30 \( \mu \text{M} \) BAM15. Aliquots were sampled upon release (time 0) and every 10 min thereafter. Values represent the average of five independent experiments, and error bars indicate SD. Representative DNA histograms are shown in Fig S2C. This analysis revealed comparable levels of lipid peroxidation in all six strains, while accurately detecting an increase in lipid peroxidation in control samples treated with \( \text{H}_2\text{O}_2 \) (Figs 5E and S4E). Also, levels of mitochondrial superoxide as indicated by MitoSOX Red fluorescence were comparable across all strains (Fig S4F for controls). Based on these results, we conclude that the cell cycle phenotype observed in \( \rho^0 \) cells does not correlate with the cellular levels of oxidative stress and that the rescued cell cycle phenotype in \( \text{ATP1-111} \rho^0 \) cells cannot be attributed to normalized ROS levels per se.

To further explore the relationship between ROS, \( \Delta\text{Ψ}_\text{m} \), and cell cycle progression, we gauged the levels of oxidative stress in our strains of interest by measuring lipid peroxidation, a reliable indicator of cellular oxidative damage (26, 27). This analysis revealed comparable levels of lipid peroxidation in all six strains.
Figure 4. Cell cycle delay can be rescued by increasing the ΔΨm of ρ⁰ cells.

(A) ΔΨm normalized to mitochondrial mass was measured in WT (AC403), ATP1-111, and cox4Δ ρ⁺ and ρ⁰ cells as described in the Materials and Methods section. Histograms of TMRE and NAO fluorescence are presented in Fig S3B and C. The average of seven independent experiments is shown; error bars represent SD. The groups were compared by one-way ANOVA. ****P < 0.0001, **P < 0.01, ns P > 0.05 compared with respective ρ⁺; ####P < 0.0001, #P < 0.05, ns P > 0.05 compared with the indicated controls.

(B) G1 synchronized cells

(C) Unsynchronized ρ⁰ cells

Mt membrane potential controls the cell cycle  Gorospe et al.  https://doi.org/10.26508/lsa.202302091  vol 6 | no 12 | e202302091  6 of 14
ΔΨm modulates G1/S transition through cell size

The G1/S transition is controlled by a late-G1 checkpoint called Start, passage through which requires attainment of a critical cell size and commits cells to cell division (28, 29). In brief, progression through G1 is driven by the G1 cyclin Cln3 in complex with the cyclin-dependent kinase Cdk1 that phosphorylates and partly inactivates the transcriptional inhibitor Whi5, leading to the induction of a transcriptional program encompassing over 200 genes that promotes G1/S transition (30). This transcriptional wave is controlled by two transcription factors, SBF and MBF. SBF regulates the expression of genes involved in cell morphogenesis and the timing of cell cycle commitment such as those encoding the G1 cyclins Cln1 and Cln2. MBF targets, including the S phase cyclins Clb5 and Clb6, drive DNA replication and repair (31). Once expressed, the association of Cln1/2 with Cdk1 promotes features such as bud emergence and pheromone resistance, and Clb5/6-Cdk1 complexes induce DNA replication. A prominent model for how Start transition is coupled to attainment of a critical cell size involves the growth-dependent dilution of the Whi5 repressor (32, 33), although other mechanisms have also been proposed (e.g., Refs. (34, 35)). Moreover, cell size, G1/S transcription, and S phase entry are influenced by additional factors and pathways including the cAMP/protein kinase A, TOR, and Snf1/AMPK pathways, the full extent and interaction of which are currently not understood (36). In fact, passage through Start is regulated by the integration of multiple internal and external signals including, but not limited to, cell size, nutrient availability, and stress (37).

To gain insight into the mechanisms connecting mitochondrial metabolism and ΔΨm to Start passage, we first explored the cell size of WT versus ATP1-111 ρ0 and ρ1 cells. Given that Start passage is coupled to cell size, we asked whether the rescued G1/S transition in ATP1-111 ρ0 cells is merely a consequence of larger cell size and thus a shorter “pre-Start” G1 period compared with WT ρ0 cells, or whether it is explained by changes to some other aspect regulating Start passage, as is observed upon switching to non-fermentable carbon sources when the critical cell size required for G1/S transition decreases (38). Microscopy-based measurement of the area of G1 phase (=unbudded) cells revealed that although WT and cox4Δ Δρ0 cells were smaller than their ρ0 counterparts, the size of ATP1-111 ρ0 cells was comparable to that of ATP1-111 ρ+ and WT ρ0 cells (Figs 6A and S5A). Thus, the rescued G1/S transition in ATP1-111 ρ0 cells with a partially recovered ΔΨm may be explained by their larger size and the subsequently shorter pre-Start G1 period.

The larger size of ATP1-111 ρ0 cells is expected to lead to a faster activation of G1 transcription because of earlier dilution of the transcriptional inhibitor Whi5 (Fig 6A). We analysed the timing of the G1/S transcriptional wave in WT versus ATP1-111 ρ+ and ρ0 cells released from α-factor synchrony, an experimental approach previously used by others (39, 40). As expected (41), no fluctuation of the levels of the non-cycling G1 cyclin CLN3 was observed after release, and transcript levels of CLN3 or WHI5 did not significantly differ across the analysed strains (Figs 6C and S5B and C). In contrast, the induction of the late-G1 cyclins CLN1 and CLN2 was delayed in WT ρ0 cells as compared to WT ρ+ cells. A similar but somewhat weaker tendency was observed for the S phase cyclins CLB5 and CLB6 (Fig S5C). Unexpectedly, and in discordance with the rescued G1/S transition phenotype, the transcriptional pattern of these cyclins was not restored in ATP1-111 ρ0 cells (Fig 6C; compare ATP1-111 ρ0 with WT ρ+). The rescued cell cycle phenotype of ATP1-111 ρ0 cells does therefore not appear to be explained by an earlier accumulation of CLN1/2 or CLB5/6 gene products.

Taken together, the findings in Fig 6 indicate that ATP1-111 ρ0 cells are larger in size than WT ρ0 cells, likely explaining their rescued cell cycle phenotype. However, the rescued timing of their Start passage appears to involve improvements downstream of G1 and S cyclin transcription. Signalling through numerous pathways, including the PKA, TOR, or Snf1/AMPK pathways, is known to impinge on Start timing, and their involvement, and that of any Cdk inhibitors acting on the Cin1/2-Cdk1 complex, should be addressed in future work to gain further insight into cell cycle regulation in cells with mitochondrial dysfunction.

Discussion

The complexes of the ETC and ATP synthase are encoded on both cellular genomes, making retrograde communication from the mitochondria to the nucleus a prerequisite for mitochondrial biogenesis and the correct assembly of these critical complexes. Mitochondrial signalling is also required to trigger any compensatory responses that allow the cell to adjust to mitochondrial dysfunction. Therefore, inter-organellar communication initiated in the mitochondria is crucial under both physiological and pathological conditions.

A number of studies have implicated signals of mitochondrial status in the control of cell cycle progression. Mitochondrial dysfunction brought about by ETC mutations was shown to delay G1-to-S transition in Drosophila melanogaster imaginal disc cells (42, 43). Interestingly, these studies found different mitochondrial cues to be responsible for triggering the G1/S cell cycle checkpoint
Figure 5. Levels of oxidative stress do not correlate with the extent of the G1-to-S delay.

(A) Representative DNA histogram of WT (AC402) ρ⁺ cells grown until an early logarithmic stage and treated with 400 μM H₂O₂ for 60 min. Additional timepoints are shown in Fig S4A.

(B) Quantification of G1 phase (solid lines) and S phase (dashed lines) cells in the experiment presented in Fig 5A and three additional experiments; error bars indicate SD. Representative DNA histograms are shown in Fig S4A.

(C) Quantification of % of G1 phase (solid lines) and S phase (dashed lines) cells in WT ρ⁺ (left panel) and WT ρ⁰ (right panel) strains treated with 30 mM NAC or 20 mM GSH for 2 h. Values represent the average of three experiments, and error bars indicate SD. Representative DNA histograms are shown in Fig S4B.

(D) ΔΨm normalized to mitochondrial mass was measured in WT (AC402) cells left untreated and treated with either 30 mM NAC or 20 mM GSH for 10 min. The average of six independent experiments is shown; error bars represent SD. The control and the treated samples were compared by one-way ANOVA. **** P < 0.0001. Raw TMRE intensity data and related controls are shown in Fig S4C and D.

(E) Quantification of lipid peroxidation in WT (AC402), ATP1-111, and cox4Δ ρ⁺ and ρ⁰ cells grown on YPDA until an early logarithmic phase. Values are expressed as MDA equivalent (nM) per ng total protein. The average of eight independent experiments is shown; error bars represent SD. There is no significant difference between sample groups after comparison by one-way ANOVA. (F) Quantification of MitoSOX Red fluorescence as an indicator of mitochondrial superoxide in WT (AC402), ATP1-111, and cox4Δ ρ⁺ and ρ⁰ cells grown until an early logarithmic stage. The average of four independent experiments is shown; error bars represent SD. There is no significant difference between sample groups after comparison by one-way ANOVA. See also Fig S4.
depending on the underlying ETC subunit mutation: although a complex IV mutation that decreased ATP levels signalled through AMPK and p53 to prevent S phase entry, a complex I mutation induced high ROS and signalled through the Foxo/p27 pathway. These findings highlight the fact that the signals and pathways that modulate the cell cycle in response to mitochondrial dysfunction can vary even within a single type of cell.

In *S. cerevisiae*, the identity of the mitochondrial signal that triggers the accumulation of respiratory-deficient ρ0 cells in G1 has remained unknown. In the current study, we rule out the two cues of mitochondrial dysfunction identified in *Drosophila*—loss of mitochondrial ATP production and high ROS—as triggers of the yeast cell cycle delay. Instead, we show that the G1-to-S transition delay observed in ρ0 cells is caused by decreased ΔΨm. Accordingly, the cell cycle phenotype of ρ0 cells could be recovered by increasing ΔΨm, indicating that low ΔΨm rather than respiratory deficiency per se restricts cell cycle progression in ρ0 cells (Figs 3–5). Dissipation of ΔΨm in ρ+ cells induced a similar but transient G1-to-S delay (Fig 2). These results therefore establish mitochondrial membrane potential as a general modulator of cell cycle progression in *S. cerevisiae* cells.

In mammalian cells, the ΔΨm has been reported to fluctuate over the course of the cell cycle, with the highest ΔΨm, mitochondrial O2 consumption, and ATP synthesis measured just before G1-to-S transition (44, 45). Furthermore, (45) elegantly demonstrated that the boost in ΔΨm in late-G1 cells was required for S phase entry (45). The ΔΨm therefore appears to regulate cell cycle progression even in higher eukaryotes. In addition, the mitochondrial membrane potential is known to govern, for example, mitochondrial dynamics and quality control through mitophagy (46, 47), highlighting its role as a central readout of mitochondrial status.
Mt membrane potential controls the cell cycle

We further found that the improved \(\Delta \Psi_m\) of \(ATP1-111\) \(p^0\) cells resulted in a larger cell size, which likely explains the faster G1/S transition as cells need less time to reach the critical cell size required for Start. Based on the Whi5 dilution model, a larger cell size is predicted to lead to an earlier activation of G1/S transcription, but this was not observed in \(ATP1-111\) \(p^0\) cells (Fig 6).

Another factor connecting cell size to the timing of Start is Cln3 concentration (e.g., Ref. (34)). Although not studied here, the position of Cln3 upstream of CLN1/2 and CLB5/6 expression, which were not rescued, makes changes to Cln3 activity an unlikely explanation for the faster G1/S transition in \(ATP1-111\) \(p^0\). However, multiple additional pathways, for example, some involved in nutrient sensing and stress, are known to modulate Start (36, 37, 38). Future investigations should seek to identify the mechanisms that connect \(\Delta \Psi_m\) changes to the cell cycle machinery. This and many related aspects of the mito-cellular communication can in the long term help advance our understanding of mitochondrial disease.

Mitochondrial dysfunction underlies a diverse group of diseases that is caused by defects in mitochondrially localized proteins encoded on either the nuclear or the mitochondrial genome. The common feature of mitochondrial diseases is a defective function of the respiratory chain and/or ATP synthase that leads to insufficient mitochondrial ATP synthesis. In most cases, mitochondrial disorders also involve decreased \(\Delta \Psi_m\) (48). However, not all symptoms of mitochondrial disease can be directly attributed to changes in energy metabolism, as some stem from (mal)adaptive responses to the mitochondrial dysfunction. For example, dysregulated immune signalling triggered by mtDNA instability was shown to aggravate the metabolic dysfunction in a mouse model with an increased mtDNA mutation load (49). Similarly, patient fibroblasts carrying the common m.3242 A>G mtDNA point mutation exhibited constitutive activation of the PI3K-Akt-mTORC1 signalling axis, the inhibition of which partly improved mitochondrial function (50). These examples illustrate the importance of understanding the various forms of mito-cellular crosstalk and the possible therapeutic potential of inhibiting signalling that in some cases may be more detrimental than beneficial. It remains to be seen whether inhibition of normal cell cycle progression in response to

Table 1. Yeast strains used in this study.

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<th>Strain</th>
<th>Genotype</th>
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<tr>
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<td>PW66-1C</td>
<td>MATa cox4Δ::NatMX (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW66-1C (p^0)</td>
<td>MATa cox4Δ::NatMX (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW66-1A</td>
<td>MATa cox4Δ::NatMX (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW66-1A (p^0)</td>
<td>MATa cox4Δ::NatMX (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW72</td>
<td>MATa ATP1-111 (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW72 (p^0)</td>
<td>MATa ATP1-111 (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW130-1A</td>
<td>MATa ATP1-111 (p^0)</td>
<td>This study</td>
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<td>PW130-1A (p^0)</td>
<td>MATa ATP1-111 (p^0)</td>
<td>This study</td>
</tr>
<tr>
<td>PW104-A</td>
<td>MATa rim1Δ::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>PW111</td>
<td>MATa mip1Δ::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>PW114</td>
<td>MATa mgm101Δ::KanMX4</td>
<td>This study</td>
</tr>
</tbody>
</table>

All strains are isogenic to W303 and derivatives of W4069-4c (AC402); only genotypes differing from AC402 are listed.

Table 2. Oligonucleotides used in the study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin forward</td>
<td>5’-CAT GAT ACC TTG GTG TCT TGG-3’</td>
</tr>
<tr>
<td>Actin reverse</td>
<td>5’-CTA CAG ATA CAG TTG GCA AA-3’</td>
</tr>
<tr>
<td>Cox1 forward</td>
<td>5’-GAAG GGA TGA TGA GGT GAA-3’</td>
</tr>
<tr>
<td>Cox1 reverse</td>
<td>5’-GTG CCT GAA TAG ATG ATA ATG GT-3’</td>
</tr>
<tr>
<td>CLN1 forward</td>
<td>5’-CTA CAG ATT GAT CAT CGC CG-3’</td>
</tr>
<tr>
<td>CLN1 reverse</td>
<td>5’-CCA CCC TTT GCT TCT ACT CC-3’</td>
</tr>
<tr>
<td>CLN2 forward</td>
<td>5’-ATG TAG TGG ACT TGG CC-3’</td>
</tr>
<tr>
<td>CLN2 reverse</td>
<td>5’-AGA GAG AAC CAC GAC CAT GA-3’</td>
</tr>
<tr>
<td>CLN3 forward</td>
<td>5’-TTA CCA CTC TGG GTG GCT AA-3’</td>
</tr>
<tr>
<td>CLN3 reverse</td>
<td>5’-TTA CCA CTC TGG GTG GCT AA-3’</td>
</tr>
<tr>
<td>CLB5 forward</td>
<td>5’-TAGG CAC GAC CAT CGC TCT CTA CTA CTG-3’</td>
</tr>
<tr>
<td>CLB5 reverse</td>
<td>5’-TTC GAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG-3’</td>
</tr>
<tr>
<td>WHI5 forward</td>
<td>5’-CCG TGT CTG GTG TCT CTG GTG-3’</td>
</tr>
<tr>
<td>WHI5 reverse</td>
<td>5’-CCG TGT CTG GTG TCT CTG GTG-3’</td>
</tr>
<tr>
<td>ACT1 forward</td>
<td>5’-CAAT GAG AAT TGG TGA TGA TGA TGA TGA TGA TGA-3’</td>
</tr>
<tr>
<td>ACT1 reverse</td>
<td>5’-CAAT GAG AAT TGG TGA TGA TGA TGA TGA TGA TGA-3’</td>
</tr>
</tbody>
</table>

We further found that the improved \(\Delta \Psi_m\) of \(ATP1-111\) \(p^0\) cells resulted in a larger cell size, which likely explains the faster G1/S transition as cells need less time to reach the critical cell size required for Start. Based on the Whi5 dilution model, a larger cell size is predicted to lead to an earlier activation of G1/S transcription, but this was not observed in \(ATP1-111\) \(p^0\) cells (Fig 6).
decreased Δψm contributes to any of the symptoms of mitochondrial disease. If so, targeting the factors that mediate this signalling may provide a potential avenue for treating that subset of disease symptoms.

Materials and Methods

Yeast strains and growth conditions

Unless otherwise indicated, all S. cerevisiae strains used in this study are congenic to W4069-4c, which is in a W303 background (51), and are listed in Table 1. To ensure fitness before each experiment, all strains were streaked out from glycerol stocks onto a YPDA solid medium (1% yeast extract, 2% peptone, 50 mg/l adenine, 2% glucose, and 2% agar). Unless otherwise indicated, yeast cells were grown in YPDA at 30°C with shaking at 180 rpm.

P0 strains lacking mtDNA were generated by growth in the YPDA medium supplemented with 25 μM etidium bromide for 4–15 d depending on the strain (12). Loss of mtDNA was confirmed by lack of growth on the YPDA medium (1% yeast extract, 2% peptone, 50 mg/l adenine, and 3% glycerol) in combination with the absence of amplification of a region of the mitochondrially encoded COX1 gene by quantitative real-time PCR in samples where a region of the nuclear-encoded ACT1 gene did amplify. Real-time PCRs contained 0.2 μM forward and reverse primers targeting COX1 or ACT1 (Table 2) and 10 μl 2X SyGreen Mix (PCR Biosystems), and were run on a LightCycler 96 instrument (Roche) using the following program: 95°C for 180 s, 45 cycles of (95°C for 10 s, 56°C for 10 s, and 72°C for 1 s with signal acquisition), and melting curve (95°C for 5 s, 65°C for 60 s, and heating to 92°C at 1 s with continuous signal acquisition).

The strain bearing the ATP1-T11l mutation was generated by introduction of the ATP1 variant carrying a T>G substitution at position 331 from a prS316 integration vector as previously described (19, 22). Briefly, the ATP1-T11l insert in prS306 was excised with BamHI and XbaI and re-cloned into the prS316 integration vector. Yeast cells were transformed using a standard lithium acetate procedure (53), and the presence of the mutation in cells that grew in a medium supplemented with 5-FOA was confirmed by sequencing.

Deletion strains were constructed by replacing the entire ORF with the kanMX4 or natMX cassette (54). Gene deletion was confirmed by growth in geneticin or nourseothricin, respectively, and by PCR using both a primer pair that flanks a ORF and a pair internal to it.

Colony size and growth curves

Yeast strains grown in a YPDA liquid medium for at least 24 h were counted using Neubauer Improved Hemocytometer and adjusted to 100 cells per 100 μl dH2O. 100 μl of the cell suspension was spread on a single-partitioned YPDA plate and incubated at 30°C for 48 h before imaging on a ChemiDoc imaging system (Bio-Rad). The area of all colonies, except for ones with another colony attached to them, was analysed using Fiji (55), and individual values were normalized to the average of all WT ρ− cell colonies.

For analysis of growth in liquid media, exponentially growing cultures were re-inoculated into fresh YPDA liquid media with a starting OD600 = 0.1. OD600 readings were recorded every hour by spectrophotometry.

Cell cycle analysis by flow cytometry

Cells grown overnight in YPDA were diluted to a fresh medium to a starting OD600 of 0.1. Cells were harvested at OD600 0.35–0.5, fixed with cold 70% ethanol, and incubated at 4°C overnight. The fixed cells were washed with distilled water once, resuspended in 50 mM Tris–HCl, pH 7.5, with 15 mM NaCl, and treated with 2 mg/ml RNase A at 37°C overnight. The next day, samples were treated with 1.8 mg/ml proteinase K at 50°C for 1 h, spun down, and resuspended in 50 mM Tris, pH 7.5. An aliquot was added to 50 mM Tris, pH 7.5, containing a 1:10,000 dilution of SYBR Green nucleic acid stain (Invitrogen). The cell suspension was sonicated for 10 s with an amplitude of 20% before analysis (Q500; QSonica). DNA content was detected in the FL1 channel using the Cytomics FC500 (Beckman Coulter). Data analysis and quantification of cells in G1, S, and G2 phases was performed using FCS Express 7 Flow (De Novo Software). The y-axis scale of the cell cycle histograms was adjusted to fit the highest cell count.

For time-course analysis of unsynchronized cells, 30 ml culture (with a starting OD600 of 0.1) was grown to an OD600 of 0.4–0.6 and split into two portions: the specified drug added to one portion, whereas the other served as a control. Cell aliquots were harvested and immediately fixed every 15 min for 2 h. Time 0 indicates the addition of the drug.

For analysis of synchronized cells, cultures were synchronized in G1 by the addition of 5 μg/ml α-factor pheromone every hour for 2 h. For G2 synchronization, cells were treated with 10–25 μg/ml nocodazole for 2 h. Cells were washed twice with one volume of ice-cold distilled water and released from synchrony by resuspension in the fresh medium. Cells were harvested and immediately fixed every 10 min for 90–120 min after release.

Mitochondrial membrane potential

Cells grown overnight in YPDA were diluted in the fresh medium to a starting OD600 of 0.1 and grown for 24 h; 1 ml of the culture was harvested and washed twice with one volume of PBS buffer (0.14 M NaCl, 0.0027 M KCl, and 0.010 M phosphate buffer, pH 7.4). Cells were reconstituted in 5 ml PBS to an OD600 of 0.05. A 1 ml aliquot was treated with 2 μM tetramethylrhodamine methyl ester perchlorate (TMRE) (Molecular Probes) and incubated at 37°C for 30 min. As a control, another aliquot of the cell suspension was treated with the same concentration of TMRE for 20 min followed by the addition of 20 μM carbonyl cyanide 3-chlorophenyl hydradrazone (CCCP) for 10 min to collapse the membrane potential. When different strains were compared, mitochondrial mass of each strain was estimated by adding 250 nM NAO (Invitrogen) to a third aliquot of the cell suspension and incubated at 37°C for 30 min. Both mitochondrial membrane potential and mitochondrial mass were determined by flow cytometry on a Beckman Coulter Cytomics FC500. Δψm was calculated by subtracting the TMRE fluorescence of the untreated
and uncoupled sample, and, when comparing across strains, normalized for mitochondrial mass (19).

Lipid peroxidation analysis and detection of mitochondrial ROS

Lipid peroxidation was determined by measurement of the amount of thiobarbituric acid reactive substance using QuantiChrom Thiobarbituric Acid Reactive Substance Assay Kit (BioAssay Systems) following the manufacturer’s instructions. The concentration of malondialdehyde (MDA) was measured on a Synergy H4 hybrid microplate reader (BioTek) with a fluorescence intensity of (λem/em = 530 nm/550 nm). Lipid peroxidation was expressed as moles of MDA equivalent equilibrated to the total amount of protein (nM of MDA equivalent/ng protein). Protein concentration was measured using the Quick Start Bradford protein assay (Bio-Rad).

Detection of mtROS was as previously described with some modifications (56). Briefly, overnight cell cultures were diluted to an OD600 of 0.1 in a fresh YPDA medium and grown until an OD600 of 0.5–0.6. Cells were harvested and washed twice with PBS. Cells were resuspended in PBS to an OD600 of 0.5 and incubated with 5 μM MitoSOX Red (Invitrogen) at 30°C for 15 min before analysis by flow cytometry.

Gene expression analysis (RT–qPCR)

Cells released from α-factor–induced G1 synchrony were harvested every 10 min and frozen in liquid nitrogen. Cells were lysed using 0.5 mm Glass Beads (BioSpec) in a FastPrep-24 bead beater (MP Biomedical), and RNA was extracted using RNAeasy Mini Kit (QIAGEN) according to the manufacturer instructions. 1 μg of the harvested RNA was treated with 1U DNase I (Thermo Fisher Scientific), and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Gene expression was analysed in technical duplicates by quantitative real-time PCR using 12.5 ng of cDNA in a 20 μl reaction containing 0.4 μM forward and reverse primers (Table 2) and 10 μl 2x SyGreen Mix (#PB2014–05; qPCRBlO) in a LightCycler instrument (Roche). The 180 s of preincubation at 95°C was followed by 40 cycles of amplification consisting of 95°C for 10 s, 56°C for 10 s, and 72°C for 1 s with single signal acquisition, and melting curve of 95°C for 5 s, 65°C for 60 s, and 97°C at 0.2°C/sec with continuous signal acquisition. Cq values determined by LightCycler 96 software (Roche) were used to calculate the expression of the cyclin genes relative to actin using the Pfaffl method (57). Statistical comparisons between selected timepoints of individual strains were performed by a standard two-tailed t test in GraphPad Prism (GraphPad Software, Inc.).

Cell imaging and size analysis

For mitochondrial staining, yeast cells grown in YPDA until an early stationary phase were harvested and washed twice with PBS. Cells were then resuspended in 1 ml PBS at a total cell density of OD600 = 1.0. The cell suspension was co-stained with 250 nM NAO and 20 μg/ml trypan blue (Invitrogen), and applied to a standard microscopy glass slide and coverslip. A Leica Widefield Thunder microscope equipped with an HC PL FLUOTAR 100X/1.32 OIL PH3 objective, with filters and laser settings for DAPI, FITC, TRITC, and Alexa Fluor 674, was used for all microscopic studies. Images were analysed using Fiji software (55). The area of unbudded (i.e., G1) cells was determined by drawing a region of interest around the stained cell wall and measuring the mean grey value using Fiji.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202302091.

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G Carvalho: formal analysis, investigation, methodology, and writing—original draft, review, and editing.
A Herrera Curbelo: formal analysis, investigation, and writing—original draft, review, and editing.
K Niedźwiecka: formal analysis, investigation, and writing—review and editing.
PH Wanrooij: formal analysis, investigation, and writing—review and editing.
Ake Wiberg: conceptualization, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, project administration, and writing—original draft, review, and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.
Mt membrane potential controls the cell cycle

Gorospe et al.

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