Bioenergetic consequences of $F_oF_1$–ATP synthase/ATPase deficiency in two life cycle stages of Trypanosoma brucei

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Mitochondrial ATP synthase is a reversible nanomotor synthesizing or hydrolyzing ATP depending on the potential across the membrane in which it is embedded. In the unicellular parasite Trypanosoma brucei, the direction of the complex depends on the life cycle stage of this digenetic parasite: in the midgut of the tsetse fly vector (procyclic form), the $F_oF_1$–ATP synthase generates ATP by oxidative phosphorylation, whereas in the mammalian bloodstream form, this complex hydrolizes ATP and maintains mitochondrial membrane potential ($\Delta\Psi_m$). The trypanosome $F_oF_1$–ATP synthase contains numerous lineage-specific subunits whose roles remain unknown. Here, we seek to elucidate the function of the lineage-specific protein Tb1, the largest membrane-bound subunit. In procyclic form cells, Tb1 silencing resulted in a decrease of $F_oF_1$–ATP synthase monomers and dimers, rerouting of mitochondrial electron transfer to the alternative oxidase, reduced growth rate and cellular ATP levels, and elevated $\Delta\Psi_m$ and total cellular reactive oxygen species levels. In bloodstream form parasites, RNAi silencing of Tb1 by $\sim$90% resulted in decreased $F_oF_1$–ATPase monomers and dimers, but it had no apparent effect on growth. The same findings were obtained by silencing of the oligomycin sensitivity-conferring protein, a conserved subunit in T. brucei $F_oF_1$–ATP synthase. However, as expected, nearly complete Tb1 or oligomycin sensitivity-conferring protein suppression was lethal because of the inability to sustain $\Delta\Psi_m$. The diminishment of $F_oF_1$–ATPase complexes was further accompanied by a decreased ADP/ATP ratio and reduced oxygen consumption via the alternative oxidase. Our data illuminate the often diametrically opposed bioenergetic consequences of $F_oF_1$–ATP synthase loss in insect versus mammalian forms of the parasite.

The $F_oF_1$–ATP synthase is a multisubunit protein complex capable of coupling ATP synthesis/hydrolysis with transmembrane proton translocation. In eukaryotes, this nanomotor is embedded in the inner mitochondrial membrane and consists of two parts, the matrix-facing $F_1$ and the membrane-embedded $F_o$. The $F_1$ domain, known as $F_1$-ATPase, is responsible for the phosphorylation of ADP to ATP, and it consists of a heterohexamer of $\alpha$ and $\beta$ subunits and a central stalk (subunits $\gamma$, $\delta$, and $\epsilon$) that connects the ($\alpha\beta_2$)$_3$-headpiece to the $F_o$ section. The core of the $F_o$ section consists of a ring of $c$ subunits that tightly interacts with subunit $a$, a highly hydrophobic subunit encoded by the mitochondrial genome in most eukaryotes, including trypanosomatids (1, 2). Aside from the central stalk, the interaction between the $F_o$ and $F_1$ domains is mediated by the peripheral stalk, an elongated structure that immobilizes the ($\alpha\beta_2$)$_3$-headpiece during the rotation of the central rotor shaft (central stalk plus c-ring) by directly binding to subunits $\alpha$ and $\beta$ (1). Despite the long period of evolutionary divergence of more than 2 billion years, the structure of prokaryotic and eukaryotic $F_oF_1$–ATP synthases is notably conserved, mainly at the level of tertiary and quaternary structures (2).

Nevertheless, in recent years, purifications and high-resolution structures of $F_oF_1$–ATP synthases from nonclassical model organisms revealed a wider variety in complex composition and structural organization than initially recognized (3–7). This includes the Trypanosoma brucei brucei $F_oF_1$–ATP synthase, an enzyme composed of 23 subunits, of which 14 are either lineage specific or highly divergent (8). For example, the lineage-specific subunits p18 and ATPaseTb2 (Tb2 in short) elaborate the otherwise conserved $F_1$ domain (9, 10) and represent one of the largest peripheral stalk subunits found in $F_oF_1$–ATP synthase to date (11), respectively.

The peculiarities of T. brucei $F_oF_1$–ATP synthase are not restricted only to complex composition. A remarkable feature of this complex is that its activity depends on the parasite’s life cycle. The procyclic form (PCF), also known as insect midgut stage, harbors a conventional mitochondrion where the $F_oF_1$–ATP synthase produces ATP (forward mode) using the electrochemical gradient across the inner mitochondrial membrane generated by the proton-pumping activity of respiratory complexes III and IV (8, 12, 13). In contrast, the infectious stage of the mammalian host, termed long slender bloodstream form (BSF), lacks a cytochrome-mediated electron transport chain and respires exclusively via the alternative
expressing C-terminally v5-tagged Tb1 (Fig. 1). It represents a unique eukaryotic system that allows to study the indispensability of the FoF1-ATPase complex at the expense of ATP (15, 16). Hence, T. brucei represents a unique eukaryotic system that allows to study both modes of the FoF1-ATP synthase in physiological settings and the distinct bioenergetic consequences upon the loss of either of the activities.

The reverse mode of the FoF1-ATP synthase complex is used by some prokaryotes (17), but it is unusual in eukaryotes, where it occurs under rare nonphysiological and stress conditions, such as hypoxia or anoxia. In these cases, the respiratory arrest and subsequent collapse of the ΔΨm causes a reversal of the FoF1-ATP synthase to generate a modest ΔΨm (18, 19). The reversal of FoF1-ATP synthase also takes place in cells lacking mitochondrial DNA, which maintain ΔΨm by an electrogenic exchange of ATP4− for ADP3− by the ADP/ATP carrier (AAC) coupled to ATP hydrolysis by an incomplete FoF1-ATPase (20–22). The depletion of ATP due to the hydrolytic activity of the FoF1-ATP synthase during ischemic conditions is mitigated by a unidirectional inhibitor, the inhibitory factor 1 (23). Noteworthy, in T. brucei, the expression of inhibitory factor 1 is tightly regulated throughout the parasite’s life cycle, and its experimental expression in BSF trypanosomes results in cell death (24, 25), highlighting the indispensability of the FoF1-ATPase complex for BSF T. brucei.

ATPaseTb1 (Tb1 in short; systematic TriTrypDB.org ID Tb927.10.520) (47 kDa) is one of the 14 lineage-specific subunits and the largest Fo subunit of the T. brucei FoF1-ATP synthase complex (8) (named Tb7760 in that study, after its previous systematic TriTrypDB ID TB10.70.7760). Downregulation of Tb1 in PCF trypanosomes inhibits cell growth, destabilizes FoF1-ATP synthase, and affects both the ATP synthetic and hydrolytic activities of the complex (8). Here, we studied in more detail the mitochondrial phenotypes associated with the downregulation of Tb1 in PCF cells and further explore the role of this subunit, as well as that of the peripheral subunit oligomycin sensitivity-conferring protein (OSCP), in the BSF stage.

Results

Tb1 is a membrane-bound subunit of the Fo moiety

Tb1, the largest membrane-associated subunit of the T. brucei FoF1-ATP synthase, has homologs in representatives of the Euglenozoa group but appears to be absent from other eukaryotic lineages (8). In agreement with the reduced size and activity of the mitochondrial in the BSFs of T. brucei (26), Tb1 is less abundant in BSF cells than in PCF cells and barely detectable in the mitochondrial DNA-lacking (aka kinetoplast) strains T. b. brucei Dk 164 and Trypanosoma brucei evansi AnTat 3/3 (Fig. 1A). To confirm the submitochondrial localization of Tb1 and determine how it is associated with the inner mitochondrial membrane, we performed carbonate extraction of mitochondria purified from T. brucei cells expressing C-terminally v5-tagged Tb1 (Fig. 1B). Tb1 is found exclusively in the membrane fraction, and the marker proteins, enolase, mitochondrial RNA-binding protein 1, and AAC, are detected in their expected compartments: cytosol, mitochondrial matrix, and mitochondrial membrane, respectively. This result suggests that Tb1 is an integral membrane protein. In PCF and BSF cells, Tb1 is present in fully assembled FoF1-ATP synthase monomers and dimers, as documented by high-resolution clear native electrophoresis (Fig. 1C) and sedimentation in glycerol gradient (Fig. 1D) followed by Western blot analyses with a specific anti-Tb1 antibody. In glycerol gradients, the Tb1 antibody detected, in addition to Tb1 migrating with the FoF1-ATP synthase, nonspecific bands of ~40 kDa and 42 kDa in PCF and of 55 kDa in BSF, which are identified by the asterisk (Fig. 1D).

Our observations that Tb1 is present in fully assembled FoF1-ATP synthase are in agreement with a recent cryogenic electron microscopy structure of the FoF1-ATP synthase dimer from Euglena gracilis, a closely related free-living species within the Euglenozoa group, in which Tb1 is found on the matrix side of the membrane part of the complex at the peripheral stalk base (6). We predicted the structure of T. brucei Tb1 using E. gracilis Tb1 as a template (Fig. 1E). The modeling showed that both Tb1 orthologs share a fold similar to the mitochondrial distribution and morphology protein 38 (Mdm38) from Saccharomyces cerevisiae, a protein suggested to be involved in the assembly of the mitochondrial-encoded subunit a into the Fo moiety (27). Although several algorithms predicted one transmembrane helix in Tb1, the protein does not span the inner mitochondrial membrane. Instead, the presumptive transmembrane region occurs as a HTH motif intruding into the membrane (Fig. 1E), and therefore, Tb1 is a monotopic membrane protein.

Silencing of Tb1 in PCF cells leads to a transient increase of ΔΨm followed by redirection of respiration toward AOX

Previously, we showed that Tb1 silencing in PCF cells leads to decreased steady-state levels of fully assembled FoF1-ATP synthase complexes, and, therefore, to less ATP produced by substrate-stimulated oxidative phosphorylation in digitonin-permeabilized cells (8). Here, we explored in more detail the effect of Tb1 silencing on mitochondrial physiology and bioenergetics of PCF cells. As expected, Tb1 silencing caused a progressive growth phenotype detected first at day 4 of RNAi induction (Fig. 2A). Western blot analysis using a specific anti-Tb1 antibody showed that Tb1 protein expression was reduced to less than 10% at day 2 of RNAi induction (Fig. 2B). The downregulation of Tb1 was accompanied by a decrease in the steady-state level of the peripheral stalk subunit Tb2, whereas the abundance of the F1 moiety subunit β was not as strongly affected (Fig. 2B). The structural integrity and the activity of the FoF1-ATP synthase complex were assessed by blue native electrophoresis (BNE) followed by Western blotting and in-gel activity staining of the complex, respectively (Fig. 2C). Western blot analyses of native complexes showed a significant reduction of FoF1-ATP synthase monomers and dimers at days 2 and 4 of RNAi induction. In agreement with the steady-state...
levels of subunit β, the free F₁-ATPase moiety was assembled and active, and it accumulated in Tb1-silenced cells. The decreased levels of fully assembled FₐF₁–ATP synthase complexes affected ATP production by oxidative phosphorylation (8), which was reflected by a ~25% reduction in the total cellular ATP levels (Fig. 2D) and by a 50% increase in the ADP/ATP ratio (Fig. 2E) by day 4 of RNAi induction. Suppression of Tb1 expression also caused a mild, but statistically significant, increase in ΔΨm at day 2 of RNAi induction (Fig. 2F), but notably, this increase was only transient. In PCF cells, the ΔΨm is maintained by the activity of respiratory complexes III and IV, passing electrons from ubiquinol to molecular oxygen. However, electrons can be rerouted to another electron acceptor, a plant-like AOX, which reduces molecular oxygen

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To test if the cells responded to the hyperpolarization detected at day 2 by rewiring the electrons toward AOX, we measured the oxygen consumption rate in the presence of potassium cyanide (KCN) and salicylhydroxamic acid (SHAM), inhibitors of complex IV and AOX, respectively. Indeed, we detected that the respiration of Tb1-silenced cells is more sensitive to SHAM compared with KCN, confirming the higher proportion of AOX-mediated respiration (Fig. 2G). This rerouting of electrons was accompanied by an increase in AOX expression as determined by Western blot (Fig. 2B). Because complex III is one of the major producers of harmful superoxide (O₂⁻) molecules, an important subclass of reactive oxygen species (ROS) (29), we measured the mitochondrial concentration of O₂⁻ before and after Tb1 RNAi in PCF cells (Fig. 2H). We found that O₂⁻ decreased over time after Tb1 ablation, supporting the proposed rerouting of electrons from complexes III and IV toward AOX, perhaps as a protective mechanism against oxidative stress (30–32). Despite the lower levels of mitochondrial O₂⁻, the disruption of fully assembled FₐF₁–ATP synthase induced changes in cellular physiology that ultimately led to higher levels of various cytosolic ROS molecules (e.g., peroxyl, hydroxyl) as measured by...
Figure 2. Loss of Tb1 in PCF cells affects the structural integrity of the FoF1–ATP synthase complex and induces changes in mitochondrial physiology. A, growth of noninduced (−tet) and Tb1 RNAi–induced (+tet) PCF cells measured for 8 days. Cumulative cell density was calculated from the cell counts adjusted by the dilution factor needed to seed the cultures at 2 × 10⁶ cells/ml each day. B, Western blot analysis of PCF Tb1 RNAi trypanosomes grown in the absence (−tet) or presence (+tet) of tetracycline for 2 and 4 days. Whole-cell lysates were subjected to SDS-PAGE followed by immunostaining with antibodies against Tb1 and Tb2 (Fo moiety), subunit β (F1 moiety), and AOX. The numbers beneath each blot represent the abundance of immunodetected protein expressed as a percentage of the noninduced sample after normalizing to the signal intensity of the mitochondrial HSP70 probing (loading control). C, BNE of 4 μg of DDM-lysed mitochondria from PCF Tb1 RNAi–noninduced (−tet) and PCF Tb1 RNAi–induced (+tet, 2 and 4 days) cells followed by Western blot analysis using antibodies against subunit β and Tb2 to detect free F1 and monomeric (M) and dimeric (D) ATP synthase complexes (first two panels). BNE of 60 μg of DDM-lysed mitochondria from PCF Tb1 RNAi–noninduced (−tet) and PCF Tb1 RNAi–induced (+tet, 2 and 4 days) cells followed by in-gel staining of ATPase activity (rightmost panel). D, total cellular ATP levels of PCF Tb1 RNAi–noninduced cells (−tet) and cells induced for 2 and 4 days (+tet, 2 days and 4 days) (means ± SD, n = 4, Student’s unpaired t-test). E, relative ADP/ATP ratio of PCF Tb1 RNAi–noninduced cells (−tet) and cells induced for 2 and 4 days (+tet, 2 days and 4 days) (means ± SD, n = 4, Student’s unpaired t-test). F, flow cytometry analysis of TMRE-stained PCF Tb1 RNAi–noninduced cells (−tet) and cells induced for 2, 4, and 6 days (+tet, 2 days, 4 days, and 6 days) to measure ΔΨm. The addition of FCCP served as a control for mitochondrial membrane depolarization (+FCCP). G, the oxygen consumption rate of PCF Tb1 RNAi live cells in the presence of glycerol-3-phosphate. After the addition of the substrate, cells were consuming oxygen at the steady rate. Injection of SHAM inhibited AOX-mediated respiration. The difference between the original values and the values after addition of SHAM is graphed as AOX-mediated respiration. Additional injection of KCN inhibited complex IV–mediated respiration and ceased the oxygen consumption of the cells. The difference between the values after SHAM addition and after KCN addition is graphed as complex IV–mediated respiration (means ± SD, n = 6, Student’s unpaired t-test). H and I, flow cytometry analysis of MittoSOX-treated (H) and H2DCFHDA-treated (I) PCF Tb1 RNAi–noninduced cells (−tet) and cells induced for 2, 4, and 6 days (+tet, 2 days, 4 days, and 6 days) to measure mitochondrial O₂⁻⁻ and total cellular ROS levels, respectively (means ± SD, n = 6, Student’s unpaired t-test). ΔΨm, mitochondrial membrane potential; AOX, alternative oxidase; BNE, blue native electrophoresis; DDM, dodecylmaltoside; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; H2DCFHDA, dichlorodihydrofluorescein; KCN, potassium cyanide; O₂⁻⁻, superoxide; PCF, procyclic form; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; Tb1, ATPaseTb1; Tb2, ATPaseTb2; TMRE, tetramethylrhodamine ethyl ester.
dichlorodihydrofluorescein (H₂DCFHDA), a fluorescent ROS-sensitive dye (Fig. 2f). This gradual increment of oxidative stress might have contributed to the growth phenotype observed in parasites with suppressed Tb1 expression. 

**Stringent Tb1 silencing leads to a virtually complete loss of F₉F₁-ATPase complex in BSF parasites and mitochondrial membrane depolarization, followed by cell death**

In contrast to the canonical role of the F₉F₁-ATP synthase in PCF cells, BSF cells use the reverse activity of this complex, which generates ΔΨm at the expense of ATP (15, 16). We silenced Tb1 in BSF cells by using an RNAi construct targeting the 3’ region of the Tb1 ORF. This caused a decrease of Tb1 to 11% at day 2 of RNAi induction, reaching undetectable levels of the protein at day 3 (Fig. 3A). As expected, the BSF Tb1 3’ RNAi cell line exhibited a strong growth phenotype and ceased growing entirely by day 4 of induction (Fig. 3B). The steady-state levels of F₁-ATPase subunits β and p18 were decreased by ~40% at day 2, whereas the steady-state levels of the F₉-associated subunits Tb2 and OSCP dropped to ~10% (Fig. 3C). Moreover, the levels of AAC were decreased by 60% (Fig. 3C), suggesting that disruption of the F₉F₁-ATPase might affect the stability of this transporter. As in PCF cells, Tb1 downregulation caused a strong decrease of F₉F₁-ATPase monomers and dimers while there was a simultaneous increase in free F₁ subcomplex (Fig. 3D, Fig. S1A).

Similarly, a conditional double knock-out (cDKO) of OSCP resulted in undetectable levels of OSCP at day 2 (Fig. 3E), which was accompanied by a strong growth phenotype (Fig. 3F). In this cell line, termed BSF OSCP cDKO, both OSCP alleles were replaced by drug resistance cassettes, and an ectopic copy of the OSCP gene, whose expression depends on the presence of tetracycline in the culture medium, was introduced (Fig. S2). The removal of tetracycline led to ablation of OSCP expression, reduced levels of F₁ subunits to ~80%, and F₉ subunits to ~10% (Fig. 3G), as well as virtually complete loss of the monomeric and dimeric forms of the complex (Fig. 3H, Fig. S1B), by day 2 of tetracycline removal.

To investigate the effect of the F₉F₁-ATPase loss on ΔΨm, we used flow cytometry analysis of intact, live cells stained with tetramethylrhodamine ethyl ester (TMRM). In both BSF Tb1 3’ RNAi and OSCP cDKO cell lines, the ΔΨm was strongly compromised at day 2 of RNAi induction and tetracycline removal, respectively (Fig. 4, A and B), preceding the observed growth defect. To corroborate these results, we determined the ability of the mitochondrion to generate ΔΨm in digitonin-permeabilized cells using safranin O dye. Control BSF Tb1 3’ RNAi and OSCP cDKO cells were able to build up and retain a ΔΨm, as addition of ATP caused a decrease in safranin O fluorescence, indicating stacking of the dye within the energized organelle. This decrease was completely reversed by adding either oligomycin (Fig. 4, C and D, black lines) or carboxyatractyloside (Fig. S3, A and D, black lines), inhibitors of the F₉F₁-ATP synthase and the AAC, respectively. Subsequent addition of the uncoupler SF 6847 had no further effect on depolarization. In contrast, cells silenced for Tb1 or ablated for OSCP were unable to generate ΔΨm in situ (Fig. 4, C and D, red lines). No changes in fluorescence were detected when the addition of oligomycin or carboxyatractyloside preceded that of ATP (Fig. S3, B, C, E and F), confirming that the decrease in safranin O fluorescence observed in control cells indeed depends on the ATP-hydrolyzing activity of the F₉F₁-ATPase and on the ADP/ATP exchanging activity of the AAC. In summary, these measurements indicate that, in the absence of either Tb1 or OSCP, BSF cells cannot generate ΔΨm, presumably because the proton-pumping F₉F₁-ATPase is completely disrupted in these cells.

Because the activity of the F₁-ATPase is tightly connected to the cellular ADP/ATP pool (33), we measured the cellular ADP/ATP ratio in control BSF Tb1 3’ RNAi and OSCP cDKO cells and in cells with diminished Tb1 and OSCP expression. The ADP/ATP ratio was already significantly decreased at day 1 (Fig. 4, E and F), the opposite of the effect observed in PCF cells (compare Fig. 2E), likely because of the dysfunctional F₉F₁-ATPase. Higher levels of ATP than ADP may also affect the glycolytic flux, which is directly linked to respiration via the mitochondrial glycerol-3-phosphate dehydrogenase (34) and/or activity of AOX (35). Indeed, decreased levels of F₉F₁-ATPase caused lower glycerol-3-phosphate-stimulated oxygen consumption in digitonin-permeabilized BSF cells depleted of Tb1 or OSCP (Fig. 4, G and H). As expected, in both samples, the measured respiration was fully inhibited by addition of SHAM.

**Suppression of F₉ subunits expression to ~10% is compatible with BSF cell viability in vitro**

The virtually complete ablation of either Tb1 or OSCP (Fig. 3, A and E) is lethal for BSF trypanosomes, and this is in agreement with previous studies examining F₉F₁-ATPase subunits (9, 11, 16). Surprisingly, these parasites can withstand a 90 to 95% loss of the same proteins as revealed by analysis of two different cell lines: a BSF Tb1 5’ RNAi cell line, in which the dsRNA targets the 5’ region of the Tb1 ORF, and a BSF OSCP RNAi cell line. In these cell lines, Tb1 and OSCP expressions were stably downregulated to 7 to 9% and 3 to 4%, respectively, over the period of 7 days of RNAi induction (Fig. 5, A and B), with no obvious effect on their growth rate (Fig. 5, C and D). The different outcomes in the viability of the BSF Tb1 RNAi cell lines might be attributed to different efficiencies of the 3’ and 5’ targeted RNAi probes or to their distinctive genetic backgrounds (Tb1 3’ RNAi: EATRO 1125 AnTat 1.1 versus Tb1 5’ RNAi: BSF Lister 427). However, as the same difference in viability was observed for BSF OSCP cDKO and OSCP RNAi cells, which share the same genetic background, the distinct phenotypes were most likely due to the differences in stringency of suppression. Thus, we decided to further investigate the basis for the observed differences.

Western blot analyses revealed a correlative decrease in F₉ subunits in both BSF Tb1 5’ and OSCP RNAi cell lines, whereas F₇ subunits stayed largely unaffected. Similar to BSF Tb1 3’ RNAi cells, the expression of AOX was slightly
upregulated, whereas the levels of AAC were halved (Fig. 5, E and F). Next, we analyzed the assembly of $F_o F_1$–ATPase complexes in BSF Tb1 5’ and OSCP RNAi cells by BNE. Silencing of Tb1 and OSCP induced monomer and dimer instability as $F_1$–ATPase subcomplexes accumulated in RNAi-induced cells, and less monomeric and dimeric forms were detected using antibodies against F1 and Fo moieties (Fig. 5, G and H, Fig. S1, C and D), but compared with BSF Tb1 3’
RNAi–induced cells and OSCP cDKO cells (Fig. 3, D and H), the decrease was not as profound.

We hypothesized that the observed differences in the viability of the examined cell lines may lay in the varying amounts of remaining active $F_oF_1$–ATPase complexes. Therefore, we quantified and compared the changes in the steady-state levels of $F_1$ (β and p18) and $F_o$ (Tb1, Tb2 and OSCP) subunits for the four BSF cell lines. At day 4 of RNAi induction, when the steady-state levels of the target proteins in BSF Tb1 3' and OSCP RNAi cells were the lowest in their respective cell lines (Fig. 5, A and B), the steady-state levels of the tested $F_o$ subunits remained significantly higher (25.1% ± 12.3%, means ± SD) than those in BSF Tb1 3' RNAi and OSCP cDKO cells by day 2 of RNAi induction or tetracycline removal, respectively (9.2% ± 4.2%, means ± SD) (Fig. 5I). We propose that in the BSF Tb1 3' RNAi and the OSCP cDKO cell lines, the levels of $F_o$ subunits drop under a threshold that does not allow the parasite to assemble a sufficient amount of $F_oF_1$–ATPase complexes to maintain its viability. Assuming a direct relationship between the steady-state levels of individual $F_o$ subunits and the total levels of $F_oF_1$ holocomplex, it would suggest that at least 10% of assembled $F_oF_1$–ATPase complexes is necessary to maintain the viability of BSF $T. brucei$ cells in vitro.

To test if the detected decrease in the levels of $F_oF_1$–ATPase monomers and dimers in BSF Tb1 5' and OSCP RNAi cells affects $\Delta\Psi_m$, we measured TMRE fluorescence in live cells from both cell lines by flow cytometry. We did not detect

**Figure 4.** Virtually complete loss of $F_oF_1$–ATPase leads to a sudden collapse of $\Delta\Psi_m$, lower ADP/ATP ratio, and reduced respiration rate in BSF parasites. A, flow cytometry analysis of TMRE-stained BSF Tb1 3' RNAi–noninduced cells (−tet) and cells induced for 1 day and 2 days (+tet, 1 day and 2 days) to detect changes in the $\Delta\Psi_m$. The addition of FCCP served as a control for mitochondrial membrane depolarization (+FCCP) (means ± SD, n = 4, Student's unpaired t-test). B, flow cytometry analysis of TMRE-stained BSF OSCP cDKO cells grown in the presence (+tet) or absence of tetracycline for 1 day and 2 days (−tet, 1 day and 2 days) to detect changes in the $\Delta\Psi_m$. The addition of FCCP served as a control for mitochondrial membrane depolarization (+FCCP) (means ± SD, n = 4, Student's unpaired t-test). C, mitochondrial membrane polarization detected using safranin O dye in digitonin-permeabilized BSF Tb1 3' RNAi–noninduced cells (−tet, black line) and cells induced for 2 days (+tet, 2 days, red line) in the presence of ATP, oligomycin (OLM), carboxyatractyloside (cATR), and SF 6847, an uncoupler, were added where indicated. cATR was added after OLM to test for any further depolarization of the mitochondrial membrane due to inhibition of the AAC, whose electrogenic activity can potentially contribute in the generation of ATP-stimulated $\Delta\Psi_m$. D, mitochondrial membrane polarization detected using safranin O dye in digitonin-permeabilized BSF OSCP cDKO cells grown in the presence (+tet) or absence of tetracycline for 2 days (−tet, 2 days, red line) after the addition of ATP. ATP, oligomycin (OLM), carboxyatractyloside (cATR), and SF 6847, an uncoupler, were added where indicated. cATR was added after OLM to test for any further depolarization of the mitochondrial membrane due to inhibition of the AAC, whose electrogenic activity can potentially contribute in the generation of ATP-stimulated $\Delta\Psi_m$. E, relative ADP/ATP ratio in BSF OSCP cDKO cells cultured in the presence (−tet) or absence of tetracycline for 1 day and 2 days (−tet, 1 day and 2 days) (means ± SD, n = 4, Student's unpaired t-test). F, relative ADP/ATP ratio in BSF OSCP cDKO cells cultured in the presence (−tet) or absence of tetracycline for 1 day and 2 days (−tet, 1 day and 2 days) (means ± SD, n = 4, Student's unpaired t-test). G, oxygen consumption rates of digitonin-permeabilized BSF Tb1 3' RNAi–noninduced cells (−tet, black line) and cells induced for 2 days (+tet, 2 days, red line) in the presence of glycerol-3-phosphate. Respiration was arrested by the addition of SHAM where indicated. H, oxygen consumption rates of digitonin-permeabilized BSF OSCP cDKO cells grown in the presence (+tet, black line) or absence of tetracycline for 2 days (−tet, 2 days, red line) after addition of glycerol-3-phosphate. Respiration was arrested by addition of SHAM where indicated. $\Delta\Psi_m$, mitochondrial membrane potential; AAC, ADP/ATP carrier; BSF, bloodstream form; cDKO, conditional double knock-out; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; OSCP, oligomycin sensitivity-conferring protein; SHAM, salicylhydroxamic acid; TMRE, tetramethylrhodamine ethyl ester.

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any differences in ΔΨm between the measured time points (Fig. 6A). Furthermore, we assessed the ability of the F$_0$F$_1$–ATPase to polarize the mitochondrial membrane in digitonin-permeabilized BSF Tb1 5' and OSCP RNAi cells using safranin O dye in the presence of ATP. Albeit reduced, compared with control cells (Fig. 6, B and C, Fig. S4, A and D,
black lines), RNAi-induced cells from both cell lines were still able to generate a ΔΨm (Fig. 6, B and C, Fig. S4, A and D, red lines). In all four cases, the inner mitochondrial membrane was fully depolarized by the addition of oligomycin (Fig. 6, B and C) or carboxyatractyloside (Fig. S4, A and D). The results were further validated by measuring safranin O fluorescence when either oligomycin or carboxyatractyloside was present before ATP addition (Fig. S4, B, C, E and F). These observations show that the FoF1–ATPase activity is affected in BSF Tb1 5’ and OSCP RNAi–induced cells but is not fully abolished as observed for BSF Tb1 3’ RNAi and OSCP cDKO cells (Fig. 4, C and D, Fig. S3, A and D, red lines).

The difference between the ΔΨm measured in vivo (by flow cytometry) and in situ (by safranin O) may reflect the different aspects of ΔΨm generation interrogated by these assays: the former measures the actual magnitude of ΔΨm in living cells, and the latter, the capacity of cells to generate ΔΨm. The differences observed imply that BSF cells do not use FoF1–ATPase complexes at full capacity to generate ΔΨm, and therefore, a significant decrease in these assemblies has no effect on their total ΔΨm and the viability of cells grown in culture. The need for the ~10% of remaining FoF1–ATPase complexes (Fig. 5) in the mitochondria is corroborated by increased sensitivity of BSF Tb1 5’ and OSCP...
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RNAi–induced cells to carboxyatractyloside (\(~12\) and \(~73\) times, respectively) and to oligomycin (\(~2.5\) and \(~2.1\) times, respectively) as measured by resazurin cell viability assay (Fig. 6, D and E).

In summary, our data provide insight into the distinct bioenergetic consequences of the loss of \( F_oF_1\)–ATP synthase/ATPase in PCF and BSF parasites. We showed that a decrease in the \( F_oF_1\)–ATP synthase levels in PCF cells led to lowered ATP levels and transiently increased \( \Delta\Psi_m\), which manifested in the form of oxidative stress and slower cell growth. In BSF trypanosomes, as reported before, a full disruption of the \( F_oF_1\)–ATPase caused the loss of \( \Delta\Psi_m\) followed by cell death, but we observed that these parasites can withstand a substantial loss of the complex without an obvious effect on viability in culture.

Discussion

Tb1 is the largest membrane-associated subunit of the \( F_oF_1\)–ATP synthase in *T. brucei*. Inferring from the \( F_oF_1\)–ATP synthase structure of the related organism *E. gracilis*, Tb1 is located on the matrix side of the \( F_o\) periphery (6) and contains an Mdm38-like fold. Mdm38 is a component of the yeast mitochondrial cotranslational membrane insertion machinery, and it has been reported to assist in the assembly of the mitochondrial-encoded proton channel subunit a into the \( F_oF_1\)–ATP synthase (27). Despite the structural similarity between Mdm38 and Tb1, the former was never copurified with either electron transport chain complexes or \( F_oF_1\)–ATP synthase (27, 36, 37), whereas the latter is a *bona fide* component of the euglenozoan \( F_oF_1\)–ATP synthase (6, 8, 38). In addition, the *E. gracilis* Tb1 ortholog contacts nearly exclusively a species-specific extension of subunit a (6), suggesting that it is not involved in the incorporation of subunit a into the complex in the same way as in yeast. Based on the predicted structural conservancy of Tb1 and compositional similarity of the \( F_oF_1\)–ATP synthase in *T. brucei* and *Euglena*, it is reasonable to assume that Tb1 in *T. brucei* is located on the periphery of the dimer. Presumably, it establishes very few, or none at all, contacts with subunits involved directly in the proton translocation or dimerization of the complex. Yet, Tb1 is absolutely crucial for the \( F_oF_1\)–ATP synthase integrity (8), potentially by aiding in the stability or assembly of auxiliary \( F_o\) subunits.

Here, we silenced Tb1 subunit by RNAi with high efficiency in two cultivable forms of *T. brucei*. In PCF cells, the severe loss of the monomeric and dimeric forms of the \( F_oF_1\)–ATP synthase after depletion of Tb1 causes a transient hyperpolarization of the inner mitochondrial membrane. This effect is then reversed by redirecting electrons from the conventional respiratory chain complexes III and IV toward AOX, as documented by the increased SHAM-sensitive respiration. The increased respiration through the AOX pathway is accompanied by a gradual but mild reduction in the \( \Delta\Psi_m\) (\(~25\%\) by day 6 of RNAi induction), consistent with the fact that AOX is incapable of pumping protons across the inner mitochondrial membrane, and therefore, does not contribute to the generation of \( \Delta\Psi_m\) (28). Moreover, the increase in AOX-mediated respiration led to lowered levels of mitochondrial \( O_2^-\), presumably produced to a large extent by complex III. The rerouting of electrons toward AOX is a phenomenon that was already reported in PCF *T. brucei* cells when complexes III and IV were downregulated (39, 40) and may reflect a protective mechanism also used by plants to cope with the increase in ROS generation associated with inhibition of the cytochrome pathway (39). Similarly, artificial expression of AOX in fruit fly and mouse cells was shown to limit mitochondrial ROS formation when respiration was inhibited by antimycin (inhibitor of complex III) or cyanide (inhibitor of complex IV) (31, 32).

Furthermore, we observed that the total cellular ATP levels decreased only by \(~25\%\) at day 4 of RNAi induction, reflecting the ability of PCF cells to rely also on ATP production through both cytosolic and mitochondrial substrate-level phosphorylation via the pyruvate kinase and the succinyl-CoA synthetase, respectively, when grown in glucose-rich conditions (41, 42). Although the ATP levels were reduced just \(~25\%\), there was a 50% increase in the ADP/ATP ratio. This could be explained by the avid ATP consumption by the accumulated \( F_o\) subcomplexes, as reflected by the in-gel staining of ATPase activity. An increased ADP/ATP ratio could also reflect a higher intracellular ATP demand, namely by processes intended to restore homeostasis. Although the increased respiration through AOX contributed to the reduction of intramitochondrial \( O_2^-\) levels, prolonged inhibition of the oxidative phosphorylation pathway due to Tb1 downregulation led to an increase of various cytosolic ROS, causing oxidative stress. Because the synthesis of certain ROS-detoxifying molecules requires ATP (43), we hypothesize that the inability of PCF Tb1 RNAi–induced cells to cope with the increasing concentrations of cytosolic ROS might be due to the lowered levels of intracellular ATP caused by inhibition of oxidative phosphorylation.

In BSF *T. brucei*, the \( F_oF_1\)–ATP synthase complex operates in reverse mode to generate the vital \( \Delta\Psi_m\) (15, 16). Therefore, the cell death that followed the collapse of the \( \Delta\Psi_m\) in parasites fully depleted of Tb1 or OSCP was expected and is consistent with the earlier studies. Nevertheless, while Tb1 and OSCP suppression down to 3 to 10% caused a corresponding loss of \( F_oF_1\)–ATPase complexes, these BSF trypanosomes were able to thrive in culture without any obvious effect on their viability. Similarly, perturbation of mitochondrial translation in BSF cells, manifested by decreased levels of \( F_oF_1\)–ATPase due to reduced production of its mitochondrial encoded subunit a, had no effect on growth in culture (44). Moreover, the \( \Delta\Psi_m\) of BSF Tb1 5’ and OSCP RNAi–induced cells was not affected when measured by flow cytometry of live cells, but there was a significant reduction in the ability of the \( F_oF_1\)–ATPase to generate a proton gradient when estimated in permeabilized cells by safranin O assay. This phenomenon can be explained by a combination of two factors: the higher sensitivity of safranin O to changes in \( \Delta\Psi_m\) compared with TMRE (45) and a possible overcapacity of the \( F_oF_1\)–ATPase...
complex in BSF trypanosomes. The overcapacity of certain enzymes has already been documented in *T. brucei*. For instance, the reduction of the maximum rate ($V_{\text{max}}$) of AOX reaction by 50% was predicted to have no effect on oxygen consumption (46). Taken together, our observations would suggest that although the $F_{\text{f}}F_{\text{i}}$–ATPase is a promising drug target, the potential drug would have to inhibit the majority of the assembled complexes to exert cytotoxic effects, although the proportion of $F_{\text{f}}F_{\text{i}}$–ATPase complexes that is required to sustain the viability of the parasite in the host environment might be different compared with the situation *in vitro*.

Dyskinetoplastic trypanosomes are BSF cells that partially or entirely lack their mitochondrial DNA, termed kinetoplast DNA, and consequently, the mitochondrial-encoded subunit a of the FoF1 ATP synthase. Unlike yeast “petite mutants” or mammalian $\rho^0$ cells, dyskinetoplastic trypanosomes are successful in nature, where they are transmitted mechanically by bloodsucking insects among a vast range of mammals or by coitus in equids (50). It is striking that in African trypanosomes of the trypanozoon group, the kinetoplast DNA loss has occurred repeatedly and does not appear to impair cell viability of the replicative stage in the mammalian bloodstream (48). The loss of kinetoplast DNA in BSF trypanosomes is most likely facilitated by the facts that (i) these cells rely only on a single mitochondrial-encoded protein, the subunit a of the $F_{\text{f}}F_{\text{i}}$–ATPase, (ii) they do not use the $F_{\text{f}}F_{\text{i}}$–ATPase synthase for energy production, as glycolysis fulfills their ATP demands, thanks to the abundant glucose in the mammalian host’s bloodstream, and (iii) these cells acquire a compensatory mutation that affects $F_{\text{f}}$–ATPase function (22). We speculate that the long-term tolerance of reduced levels of $F_{\text{f}}F_{\text{i}}$–ATPase, exemplified by our BSF $\text{Tb1}^*$ and OSCP RNAi cell lines, can provide BSF cells with a time frame to gain nuclear mutations allowing them to compensate for the loss of mitochondrial DNA, an event that is deleterious, or even lethal, for the majority of eukaryotes (57).

A striking phenotype exhibited by BSF $\text{Tb1}^*$ RNAi and OSCP cDKO trypanosomes is the reduced respiration rate upon Tb1 and OSCP depletion, respectively, as there is no direct link between the activities of AOX and the $F_{\text{f}}F_{\text{i}}$–ATPase. Rather, the oxygen consumption rate by AOX is a measure of glycolysis, which provides BSF trypanosomes with the majority, if not all, of the cellular ATP (34, 58). We hypothesize that disruption of the $F_{\text{f}}F_{\text{i}}$–ATPase complex leads to a lower consumption rate of intracellular ATP. In support of this argument, we observed a gradual reduction in the cellular ADP/ATP ratio after suppression of Tb1 or OSCP expression. A recent study showed that *T. brucei* AOX can be inhibited by a transient accumulation of intramitochondrial ATP caused by inhibition of the $F_{\text{f}}F_{\text{i}}$–ATPase hydrolytic activity with oligomycin (35), representing the first report proposing that the $F_{\text{f}}F_{\text{i}}$–ATPase activity can affect the glycolytic flux in BSF trypanosomes. Similarly, oligomycin treatment inhibits the rates of oxygen consumption and pyruvate production with a concomitant dissipation of $\Delta\Psi$m when glucose is the substrate (15, 59). This effect that was originally contributed to the inhibition of glycolysis before triose phosphate oxidation (60) can now be linked to the inhibition of the $F_{\text{f}}F_{\text{i}}$–ATPase. According to the metabolic control theory (61), the flux control of a pathway can be exerted not solely by enzymes from the particular pathway but also by processes outside of it, such as the ATP-consuming processes. In *T. brucei*, the glycolytic flux is controlled mainly by enzymes outside of the pathway, specifically, by hexose transporters responsible for glucose uptake into the cell (62). The absence of regulation by components from the pathway is not restricted to this organism, as it was reported that overexpression of glycolytic enzymes does not exert significant control on the pathway in both yeast (63) and bacteria (64). Interestingly, a study in *Escherichia coli* demonstrated that incrementing the cytosolic ATP consumption by expression of free $F_{\text{f}}$–ATPase resulted in a 70% increase in the glycolytic rate, concluding that enzymes consuming ATP can affect the glycolytic flux (65). Taken all together, our results further corroborate the idea that the levels of $F_{\text{f}}F_{\text{i}}$–ATPase activity can affect the respiration rate, playing an unexplored role in controlling the glycolytic rate in the BSF stage of the parasite.

**Trypanosoma brucei $F_{\text{f}}F_{\text{i}}$–ATP synthase**

**Experimental procedures**

**Plasmid construction and generation of cell lines**

The generation of the PCF $\text{Tb1}^*$ RNAi cell line was described in an earlier study (8). To generate the BSF $\text{Tb1}^*$ and $\text{Tb1}^*$ RNAi cell lines, $\text{Tb1}$ (Tb927.10.520) ORF fragments of 460 bp (between nucleotides 30 and 489) and 636 bp (between nucleotides 424 and 1060), respectively, were amplified from the genome of the wild type (WT) BSF *T. brucei* strain Lister 427 using primers 1 and 2 for the $\text{Tb1}^*$ RNAi fragment, and primers 3 and 4 for the $\text{3\'RNAi}$ fragment. The resulting 5′ and 3′ RNAi amplicons were cloned into p2T7-177 vector (66) *via* BamHI and XhoI restrictions sites and transfected into the puromycin-resistant *T. brucei* SmOxB427 cell line (67) and the neomycin- and hygromycin-resistant *T. b. brucei* EATRO 1125 AnTat 1.1 90:13 cell line (68), respectively. For the inducible expression of $\text{Tb1}$ fused with a C-terminal 3x v5 tag, the $\text{Tb1}$ coding sequence was PCR-amplified from *T. brucei* strain Lister 427 genome using primers 5 and 6. Using the HindIII and BamHI restriction sites inherent in the primers, the fragment was cloned into the pT7_v5 vector (69). The construct was linearized with NotI and transfected into the neomycin-resistant transgenic BSF427 single-marker cell line as described previously (11).
Trypanosoma brucei $F_0F_1$–ATP synthase

To generate the BSF OSCP RNAi cell line, an OSCP (Tb927.10.8030) ORF fragment of 446 bp (between nucleotides 13 and 458) was amplified from the genome of the WT BSF *T. brucei* strain Lister 427 using primers 7 and 8. The resulting amplicon was cloned into p2T7-177 vector via BamHI and XhoI restrictions sites, and the construct was linearized with NotI before transfection into the neomycin-resistant transgenic BSF427 single-marker cell line as described previously (11). For the generation of the BSF OSCP cDKO cell line, 5′ and 3′ intergenic region fragments from OSCP gene were amplified using primers 9 and 10 for the 5′ intergenic region, and primers 11 and 12 for the 3′ intergenic region. The resulting 5′ intergenic region amplicon of 452 bp was cloned into NotI and MluI restriction sites within pLEW13 vector to generate pLEW13:5′IR452bp vector. Subsequently, the 3′ intergenic region amplicon of 414 bp was inserted into pLEW13:5′IR452bp vector via XhoI and Stsi restriction sites to obtain the OSCP single KO construct. This construct was linearized using NotI and transfected into the WT BSF *T. brucei* strain Lister 427 to knock out the first OSCP allele. To generate the construct for the KO of the second OSCP allele, the T7 RNA polymerase and neomycin cassette from the OSCP single KO construct was replaced by a cassette containing a 10% activity T7 promoter, the tetracycline repressor, and the hygromycin resistance gene from pLEW90 vector. For the generation of the tetracycline-inducible OSCP ectopic copy construct, the OSCP ORF was amplified from the genome of the WT BSF *T. brucei* strain Lister 427 using primers 13 and 14. The OSCP ORF was cloned into *Bam*HI and *XhoI* restriction sites within pLEW79 vector, replacing the luciferase ORF. It is important to mention that the pLEW79 vector was mutagenized for the purpose of this study to include an *XhoI* restriction site downstream its HindIII site due to the existence of a *HindIII* site within the sequence of the OSCP ORF. BSF *T. brucei* OSCP single KO cells were transfected with the OSCP ectopic copy construct before knocking out the second OSCP allele. The strategy used for the conditional KO of OSCP in BSF cells was adapted from the reference (70). The correct integration of the OSCP single KO construct was verified by PCR using the following primer pairs: primers 15 (annealing upstream the 5′ intergenic region of the OSCP gene used as homologous recombination site) and 17 (binding the T7 RNA polymerase sequence); primers 18 (binding the neomycin resistance gene sequence) and 16 (annealing downstream the 3′ intergenic region of the OSCP gene used as homologous recombination site). The correct integration of the OSCP double KO construct was verified by PCR using the following primer pairs: primers 15 and 19 (which binds the tetracycline repressor sequence); primers 20 (binding the hygromycin resistance gene sequence) and 16. The presence of the OSCP inducible copy was verified by PCR using primers 21 (which anneals to the procytoclastic acidic repetitive protein promoter) and 22 (annealing downstream the aldolase 3′ UTR that follows the OSCP ORF). A schematic representation of the PCR verification is depicted on Fig. S2. The sequences of all the primers used in this study are found in Table S1.

**T. brucei culture conditions**

The PCF Tb1 RNAi cell line was grown at 27 °C in the glucose-rich medium SDM-79 (27) (Invitrogen 07490916N) supplemented with 10% fetal bovine serum (BioSera FB-1090/500) and 7.5 mg/ml hemin (Sigma H9039) and containing 15 μg/ml G418 (Sigma G8168), 25 μg/ml hygromycin B Gold (InvivoGen ant-hg-1), and 2.5 μg/ml phleomycin (InvivoGen ant-ph-2p).

The BSF *T. b. brucei* Lister 427 strain, EATRO 1125 AnTat 1.1 (68), stable acriflavine-induced dyskinetoplastic *T. b. brucei* EATRO 164 (71), and dyskinetoplastic *T. b. evansi* AnTat 3/3 that lost mitochondrial DNA upon culturing (16) were all grown at 37 °C and 5% CO2 in HMI-11 medium (Invitrogen 07490915N) supplemented with 10% fetal bovine serum. The BSF 5′ Tb1 RNAi cell line was grown in the presence of puromycin and phleomycin. The BSF 3′ Tb1 RNAi cell line was grown in the presence G418, hygromycin, puromycin, and phleomycin. The BSF OSCP RNAi cell line was grown in the presence of G418 and phleomycin. The BSF OSCP cDKO cell line was grown in G418, hygromycin, and phleomycin. The final concentrations of antibiotics in the culture medium were the following: 0.1 μg/ml puromycin, 2.5 μg/ml G418, 5 μg/ml hygromycin, and 2.5 μg/ml phleomycin.

The induction of RNAi and ectopically expressed tagged Tb1 was triggered by the addition of 1 μg/ml of tetracycline into the medium. BSF OSCP cDKO cells were constantly grown in the presence of 1 μg/ml of tetracycline to sustain OSCP expression. The expression of the OSCP ectopic copy was suppressed by cultivating BSF OSCP cDKO cells in the absence of tetracycline, preceded by a two-step washing of the cells with tetracycline-free medium. For all the experiments in this study, cells were maintained in a mid-late exponential growth phase, meaning 0.6 × 10^7 to 1.2 × 10^7 cells/ml in the case of PCF cells, and 0.6 × 10^6 to 1.2 × 10^6 cells/ml in the case of BSF cells.

**SDS-PAGE and Western blotting**

Appropriate volumes of PCF and BSF culture were spun down at 1400g for 10 min at 4 °C, and cell pellets were washed once with 1× PBS (10-mM phosphate buffer, 130-mM NaCl, pH 7.3). To prepare whole-cell lysates at a concentration of 1 × 10^7 cells in 30 μl, cell pellets were resuspended in 1× PBS before the addition of 3× Laemmli buffer (150-mM Tris-HCl, pH 6.8, 300-mM 1,4-dithiobitol, 6% (w/v) SDS, 30% (w/v) glycerol, 0.02% (w/v) bromophenol blue). The mixture was boiled at 97 °C for 10 min and stored at −20 °C. For Western blot analysis, a volume of sample corresponding to 3 × 10^6 cells per well was separated by SDS-PAGE (Bio-Rad 4568094), blotted onto a polyvinylidene difluoride membrane (Pierce 88518), and probed with the appropriate monoclonal (mAb) or polyclonal (pAb) antibody. This was followed by incubation with a secondary HRP-conjugated anti-rabbit (Bio-Rad 1721019) or anti-mouse (Bio-Rad 1721011) antibody (1:2000), which immunoreacts, respectively, with the polyclonal or monoclonal primary antibodies. Proteins were visualized using the Clarity Western ECL substrate (Bio-Rad 1705060EM) on a ChemiDoc instrument (Bio-Rad). The PageRuler prestained protein ladder (Thermo Fisher Scientific 26617) was used to determine the size of the detected bands.
The primary antibodies used in this study were the following: mAb anti-v5 epitope tag (1:2000, Invitrogen), mAb anti-mitochondrial HSP70 (1:5000; 72 kDa) (72), mAb anti-AOX (PCF 1:300, BSF 1:1000, kindly provided by Minu Chaudhuri; 33 kDa), pAb anti-mitochondrial RNA-binding protein 1 (1:1000; 23 kDa), pAb anti-enolase (1:1000; 47 kDa) (73), pAb anti-ACE (1:1000; 34 kDa), pAb anti-mitochondrial RNA-binding protein 1 (PCF 1:300, BSF 1:1000, kindly provided by Minu Chaudhuri; 33 kDa), pAb anti-mitochondrial RNA-binding protein 1 (PCF 1:300, BSF 1:1000, kindly provided by Minu Chaudhuri; 33 kDa), and antibodies against F$_o$F$_1$–ATP synthase subunits β (1:2000; 54 kDa), p18 (1:1000; 18 kDa), Tb1 (1:1000; 47 kDa), Tb2 (1:1000; 43 kDa), and OSCP (1:1000; 27 kDa). The latter antibodies were produced in the Zíková lab and are available upon request. The densitometric analysis of the bands was carried out using the ImageLab software by relating the signal intensity from the bands corresponding to RNAi-induced/tetracycline-depleted cells to that of the lane pertinent to control cells. The percentage of downregulation relative to the control sample was then normalized to the corresponding signal intensity of the bands from the blots probed with anti-enolase or anti-mitochondrial HSP70 (loading controls).

**Isolation of crude mitochondrial vesicles**

Crude mitochondrial vesicles were obtained by hypotonic lysis as described earlier (11, 72). In summary, cell pellets from 3 × 10$^8$ BSF cells were washed once with a buffer I (150-mM NaCl, 20-mM glucose, 20-mM phosphate buffer, pH 7.9), resuspended in a buffer II (1-mM Tris-HCl, pH 8.0, 1-mM EDTA), and homogenized in a Dounce homogenizer. Alternatively, cell pellets from 1 × 10$^9$ PCF cells were washed in a mitochondrial lysis buffer (2-mM $\varepsilon$-aminocaproic acid (ACA), 50-mM imidazole-HCl, 1-mM EDTA, 50-mM NaCl, pH 7.0) and lysed for 1 h on ice with 4 mg digitonin/1 mg protein. Samples were centrifuged at 16,000 g for 30 min at 4 °C, and the protein concentrations of the cleared lysates were determined by bicinchoninic acid assay. Samples were mixed with 5x loading dye (0.1% (w/v) Ponceau-S, 50% (w/v) glycerol) and loaded onto a 3 to 12% native gradient gel. After electrophoresis (3 h, 100 V, 4 °C), the resolved mitochondrial proteins were transferred onto a nitrocellulose membrane (overnight, 20 V, 4 °C) and probed with selected antibodies (p18 1:1000 and Tb1 1:1000).

BNE was performed as described in an earlier study (9) with some modifications. Crude mitochondrial vesicles from 2 × 10$^8$ cells were resuspended in 1 M ACA and solubilized with either 2% (PCF) or 4% (BSF) dodecylmaltoside (DDM) for 1 h on ice. Samples were centrifuged at 16,000g for 30 min at 4 °C, and the protein concentrations of the cleared lysates were estimated using the bicinechonic acid assay (Pierce 23225). Samples were mixed with 1.5 μl of the loading dye (500-mM ACA, 5% (w/v) Coomassie Brilliant Blue G-250) and loaded onto a 3 to 12% native gradient gel. After the electrophoresis (3 h, 140 V, 4 °C), proteins were blotted onto a polyvinylidene difluoride membrane (2 h, 100 V, 4 °C, stirring) and immunodetected using antibodies against different F$_o$F$_1$–ATP synthase subunits (subunit β 1:2000, p18 1:1000, Tb2 1:500, and OSCP 1:100). Alternatively, the gel was transferred into the ATPase reaction buffer (35-mM Tris-HCl, pH 8.0, 270-mM glycine, 19-mM MgSO$_4$, 0.3% (w/v) Pb(NO$_3$)$_2$, 11-mM ATP) for overnight incubation under slow agitation (in-gel staining of F$_o$F$_1$–ATPase activity). Subsequently, the gel was soaked in 30% methanol to stop the reaction. The ATPase activity appears as a white precipitate.

**Sodium carbonate submitochondrial fractionation**

Sodium carbonate extraction of mitochondrial membranes was adapted from an earlier study (76). Mitochondrial vesicles from 3 × 10$^8$ cells were isolated by hypotonic lysis as described previously. The resulting supernatant from a 25G needle homogenization step was kept as a cytosolic fraction. The mitochondrial pellet was further treated with digitonin (80 μg/ml) for 15 min on ice to disrupt the mitochondrial outer membrane. The material was then cleared by centrifugation at 12,000g for 20 min at 4 °C and the pelleted mitoplasts were resuspended in 0.1 M Na$_2$CO$_3$ buffer (pH 11.5) before incubation on ice for 30 min. A final ultracentrifugation step at 100,000g for 1 h at 4 °C carried out in an SW50Ti rotor of a Beckman Instrument yielded a supernatant comprised of proteins from the mitochondrial matrix, including stripped peripheral membrane proteins, and a pellet containing integral proteins isolated from the mitochondrial membrane fraction.

**Glycerol gradient sedimentation**

Hypotonically purified mitochondrial vesicles from ~2.5 × 10$^8$ cells were resuspended in glycerol gradient lysis buffer (10-mM Tris-HCl, pH 7.2, 10-mM MgCl$_2$, 200-mM KCl, 1-mM 1,4-dithiothreitol) and solubilized with 1% Triton X-100 for 30 min on ice. The lysates were cleared by a centrifugation step (2× 16,000g, 30 min, 4 °C), and the protein concentration was determined by the Bradford assay. Cleared mitochondrial lysates were resolved by ultracentrifugation (Beckman Instrument, SW40 rotor) at 38,000g for 5 h on an 11-ml 10 to 30%
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glycerol gradient, which was poured using the Gradient Station (Bio-comp) according to the manufacturer’s protocol. The glycerol gradients were then fractionated with the Gradient Station, and 500 µl fractions were stored at −80 °C.

$\Delta \Psi m$ measurement

The $\Delta \Psi m$ of live cells was estimated using the cell-permeant red-fluorescent dye TMRE (Thermo Fisher Scientific T669), whose fluorescence intensity is proportionally dependent on the $\Delta \Psi m$ values. Equal number of cells ($3 \times 10^{6}$) were harvested for each time point and resuspended in the culture medium containing 60-nM TMRE. The staining of the cells was carried out for 30 min at the appropriate temperature for each life stage. Subsequently, cells were spun down at 1400g for 10 min at room temperature, resuspended in 1 ml of 1× PBS (see composition above) and immediately analyzed by flow cytometry using BD FACSCanto II instrument and its blue laser (488 nm) with the band pass PE filter (585/15). For each sample, 10,000 events were collected. Treatment with 20-μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (Sigma C2920) was used as a control for mitochondrial membrane depolarization. Data were evaluated using BD FACSDiva (BD Company) software. The TMRE signal corresponding to RNAi-induced/tetracycline-depleted cells was normalized to that of control cells and expressed in percent.

In situ $\Delta \Psi m$ of permeabilized cells was determined fluorometrically using safranin O dye (Sigma S2255) (77). For each sample, 2 × $10^{7}$ cells were harvested and washed once with ANT buffer (8-mM KCl, 110-mM K-gluconate, 10-mM NaCl, 10-mM free-acid Heps, 10-mM K$_2$HPO$_4$, 0.015-mM EGTA potassium salt, 10-mM mannitol, 0.5 mg/ml fatty acid–free bovine serum albumin, 1.5-mM MgCl$_2$, pH 7.25) (78). The cell pellet was resuspended in 2 ml of ANT buffer containing 5-μM safranin O and 4-μM digitonin. Fluorescence was recorded in a Hitachi F-7100 spectrofluorometer (Hitachi High-Technologies) at a 5-Hz acquisition rate, using 495 nm and 585 nm excitation and emission wavelengths, respectively. Substrates (1-mM ATP, PanReac AppliChem A13480025) and inhibitors (10 µg/ml oligomycin or 1-µM carboxyatractyloside, Sigma O4876 and Biorbyt orb259156-10, respectively) were added where indicated. Final addition of the uncoupler SF 6847 (250 nM; Enzo Life Sciences BML-EI215-0050) served as a control for maximal depolarization. All the experiments were performed at room temperature and constant stirring.

Mitochondrial $O_2^{-•}$ and cellular ROS measurements

For the measurement of mitochondrial and cellular ROS molecules, the red mitochondrial $O_2^{-•}$ indicator MitoSOX (Thermo Fisher Scientific M36008) and H$_2$DCFHDA dye (Thermo Fisher Scientific) were used, respectively. The staining procedure followed was essentially the same as for the determination of $\Delta \Psi m$ in vivo except that TMRE was replaced by 5-μM MitoSOX or 10-μM H$_2$DCFHDA in the corresponding assay. MitoSOX and H$_2$DCFHDA fluorescence signals were recorded using BD FACSCanto II instrument and its blue laser (488 nm) with the band pass phycoerythrin (585/15 nm) and fluorescein isothiocyanate (530/30 nm) filters, respectively. The fluorescence signal of RNAi-induced/tetracycline-depleted cells was normalized to that of control cells and expressed in percentage. The values were plotted and analyzed statistically using GraphPad Prism 8.0 software.

High-resolution respirometry

The oxygen consumption rate was determined using the Orobos Oxygraph-2K (Oroboros Instruments Corp). For each sample, 2 × $10^{7}$ cells were harvested and washed once with Mir05 mitochondrial respiration medium (0.5-mM EGTA, 3-mM MgCl$_2$, 60-mM lactobionic acid, 20-mM taurine, 10-mM KH$_2$PO$_4$, 20-mM Heps, 110-mM sucrose, 1 mg/ml fatty acid–free bovine serum albumin, pH 7.1). The cell pellet was resuspended in 2.1 ml of Mir05 and transferred into the respiration chamber at the appropriate growth temperature for each life stage and under constant stirring. In the experiments carried out with intact PCF cells, 10-mM glycerol-3-phosphate (Sigma, 17766) was added, and complex IV- and AOX-mediated respirations were inhibited by injection of 1-mM KCN and 250-µM SHAM (Sigma S607), respectively. For the experiments performed with permeabilized BSF cells, the addition of 4-µM digitonin (Sigma D141) preceded the injection of 20-mM glycerol-3-phosphate into the chamber, and respiratory inhibition was achieved by addition of 250-µM SHAM. The most stable portion of either the oxygen consumption rate slope (PCF experiments) or the oxygen concentration in the chamber slope (BSF experiments) was determined for each biological replicate after the addition of substrates and inhibitors. The values were plotted and analyzed statistically using GraphPad Prism 8.0 software.

ADP/ATP ratio and total cellular ATP levels

Both the ADP/ATP ratio and the total cellular ATP were estimated using the bioluminescence-based ADP/ATP assay kit (Sigma MAK135) following the manufacturer’s protocol. In brief, 1 × $10^{6}$ cells per sample were harvested and washed once with PBS-G (1× PBS plus 6-mM glucose). Cells were resuspended in 10 µl of 1× PBS-G and transferred into a white flat-bottom 96-well microtiter plate. Luminescence was recorded in an Orion II microplate luminometer (Titerterk-Berthold). The ATP levels and calculated ADP/ATP ratios of RNAi-induced/tetracycline-depleted cells were normalized to those of control cells and expressed in percentage. The values were plotted and analyzed statistically using GraphPad Prism 8.0 software.

Resazurin cell-viability assay

BSF cells were inoculated into a transparent flat-bottom 96-well microtiter plate at a density of 500 trypanosomes in a final volume of 200 µl of the culture medium per well. The cells were incubated in the presence of various drug concentrations (0.98- to 4000-µM carboxyatractyloside and 9.78–5000 ng/ml oligomycin) for 72 h at 37 °C. Wells without the drug served as
the control for cell viability. Subsequently, 20 μl of a 125 μg/mL resazarin (Sigma R7017) stock solution was added and fluorescence was measured 24 h later (total drug incubation time of 96 h) in a Tecan plate reader using 544-nm and 590-nm excitation and emission wavelengths, respectively. Data were analyzed with GraphPad Prism 8.0 software using a nonlinear regression and a sigmoidal dose–response analysis. All the experiments were performed in triplicate.

**Modeling of Tb1 structure**

The structure of Tb1 was predicted with I-TASSER (34) using the structure of *E. gracilis* Tb1 as a template (PDB ID: 6TDU (6)).

**Data availability**

All data discussed are presented in the article.

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**Abbreviations**—The abbreviations used are: ΔΨm, mitochondrial membrane potential; AAC, ADP/ATP carrier; ACA, ε-aminocaproic acid; AOX, alternative oxidase; BNE, blue native electrophoresis; BSF, bloodstream form; cDKO, conditional double knockout; DDM, dodecylmaltoside; H2DCFHDA, dichlorodihydrorhodamine; KCN, potassium cyanide; Mdm38, mitochondrial ATP synthase subunit 1); Tb1, ATPaseTb1 (T. brucei F0F1,–ATP synthase subunit 1); Tb2, ATPaseTb2 (T. brucei F0F1,–ATP synthase subunit 2); TMRE, tetramethylrhodamine ethyl ester; WT, wild type.

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