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Down regulation of Tim50 in *Trypanosoma brucei* increases tolerance to oxidative stress

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Abstract

Trypanosoma brucei, the causative agent for African trypanosomiasis, possesses a single mitochondrion that imports hundreds of proteins from the cytosol. However, the parasite only possesses a few homologs of the canonical protein translocases found in fungi and animals. We recently characterized a homolog of the translocase of the mitochondrial inner membrane, Tim50, in *T. brucei*. TbTim50 knockdown (KD) moderately reduced cell growth, decreased the mitochondrial membrane potential, and inhibited import of proteins into mitochondria. In contrast to Tim50 KD, we show here that TbTim50 overexpression (OE) increased the mitochondrial membrane potential as well as increased the production of cellular reactive oxygen species (ROS). Therefore, TbTim50 OE also inhibits cell growth. In addition, TbTim50 OE and KD cells showed different responses upon treatment with H₂O₂. Surprisingly, TbTim50 KD cells showed a greater tolerance to oxidative stress. Further analysis revealed that TbTim50 KD inhibits transition of cells from an early to late apoptotic stage upon exposure to increasing concentrations of H₂O₂. On the other hand TbTim50 OE caused cells to be in a pro-apoptotic stage and thus they underwent increased cell death upon H₂O₂ treatment. However, externally added H₂O₂ similarly increased the levels of cellular ROS and decreased the mitochondrial membrane potential in both cell types, indicating that tolerance to ROS is mediated through induction of the stress-response pathway due to TbTim50 KD. Together, these results suggest that TbTim50 acts as a stress sensor and that down regulation of Tim50 could be a survival mechanism for *T. brucei* exposed to oxidative stress.

Keywords

TbTim50; Mitochondria; Membrane potential; *T. brucei*; Oxidative stress; Apoptosis

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1. Introduction

Trypanosoma brucei, a group of hemoflagellated parasitic protozoa, is the causative agent of a deadly disease known as African trypanosomiasis [1, 2]. The disease is spread among humans and domestic animals by an insect vector, the tsetse fly. During its digenetic life cycle within the fly and the mammalian host the parasite faces dramatic changes in its environment such as available nutrients, temperature, pH and oxygen concentration. To adapt to these changes *T. brucei* undergoes a series of developmental modulations involving its morphology, ultrastructure, metabolic patterns, and in particular its mitochondrial activities [3–6]. In eukaryotes, mitochondria play a central role in cellular homeostasis and cell death when exposed to various stresses by regulating different cellular functions such as, energy metabolism, redox balance, autophagy and apoptosis [7–10]. However, the role of mitochondria in a cellular stress response is less understood in parasitic protozoa like *T. brucei*.

Similar to higher eukaryotes, the majority of mitochondrial proteins in *T. brucei* are nuclear-encoded. Therefore these proteins are imported into mitochondria in order to perform their functions. In fungi and higher eukaryotes, there are three major protein translocases in mitochondria, the translocase of the mitochondrial outer membrane (TOM), and two translocases of the mitochondrial inner membrane, TIM23 and TIM22 [11, 12]. TOM and TIMs are multi-subunit protein complexes that have been extensively characterized in fungi and later in animals and plants. Interestingly, trypanosomatids do not have any homologs of the TOM subunits. Instead, an archaic β -barrel protein, ATOM, serves the function of Tom40, the major component of the fungal TOM complex [13]. Similarly, homologs of the TIM22 components could not be detected in this parasite, but it possesses a few homologs of the fungal TIM23 subunits, such as Tim17 and Tim50 [14, 15].

We have shown previously that *T. brucei* Tim17 (TbTim17) is essential for cell survival and is critical for mitochondrial protein import [14]. TbTim17 is present in a larger molecular mass protein complex and it is associated with several novel trypanosome-specific proteins [16]. We also have characterized Tim50 in *T. brucei* (TbTim50), which interacts with TbTim17 *in vivo* [15]. TbTim50 possesses a mitochondrial-targeting signal (MTS) at its N-terminus and a characteristic phosphatase motif at its C-terminus, which shows similarity to the transcription factor II (TFII)-stimulated RNA polymerase II C-terminal domain (CTD)-phosphatase. Knockdown (KD) of TbTim50 inhibits import of proteins into mitochondria that contain an N-terminal MTS, while the recombinant TbTim50 possesses a dual-specificity phosphatase activity [15].

Increasing evidence indicates that besides mitochondrial protein import, these Tom and Tim proteins are also involved in other functions, such as maintaining mitochondrial morphology, regulation of fission and fusion of the organelle and recruitment of anti-apoptotic and autophagy proteins [17–19]. Tom proteins are phosphorylated by cytosolic kinases to control mitochondrial protein biogenesis as a means of regulating mitochondrial activities [20–22]. In addition Tim50 is also known to be involved in developmental regulation and apoptosis in zebra fish, drosophila, and human [23, 24], although the mechanisms responsible for these actions are not well understood.

Here we discovered that TbTim50 plays a role in the stress response pathway in *T. brucei*. We found that maintenance of a particular level of TbTim50 is required in order to maintain cell growth and mitochondrial membrane potential at normal levels. Down regulation of TbTim50 inhibited the transition of cells from an early to a late apoptotic stage and thus increased the tolerance of *T. brucei* to peroxide treatment. Whereas, TbTim50 overexpression hyperpolarized the mitochondrial inner membrane, increased ROS production and promoted cell apoptosis. These results further indicate the important role of the *T. brucei* mitochondrion and its protein translocator in the maintenance of cellular homeostasis.

2. Materials and methods

2.1. Strains, media, cell growth and isolation of mitochondria

The procyclic form of *T. brucei* 427 cells was grown in SDM-79 medium containing 10% heat inactivated fetal bovine serum [15, 16]. *T. brucei* cells expressing TbTim50 RNAi (TbTim50 KD) and TbTim50 with a C-terminally 3x hemagglutinine (HA) tag (TbTim50 OE) were developed as previously reported [15]. These cell lines were maintained in the same medium supplemented with hygromycin (50 µg/ml), G418 (15 µg/ml) and phleomycin (2.5 µg/ml). Cell growth was assessed by inoculating the procyclic form at a cell density of 2×10^6 /ml in fresh medium containing antibiotics in the presence or absence of doxycycline (1 µg/ml). Cells were counted at different growth time points with a Neubauer hemocytometer. The log of the cumulative cell number was plotted against time of incubation in culture.

Mitochondria were isolated from the parasite after lysis by nitrogen cavitation in isotonic buffer [14, 15]. The isolated mitochondria were stored at a protein concentration of 10 mg/ml in SME buffer (250 mM sucrose, 20 mM MOPS/KOH, 2 mM EDTA, pH 7.4) containing 50% glycerol at -70°C . Before use, mitochondria were washed twice with 9 volumes of SME buffer to remove glycerol.

2.2. Isolation of RNA and northern analyses

Total RNA was isolated from the procyclic form of *T. brucei* using Trizol (Invitrogen) according to the manufacturer's protocol. Northern blot analysis was performed as described [14, 15]. Specific probes were made using a random primer labeling protocol (Invitrogen) from cDNA fragments, which were generated by PCR using sequence-specific primers designed from the coding regions of TbTim50 and actin.

2.3. SDS-PAGE and immunoblot

Proteins from isolated mitochondria were resolved on a 10% SDS/PAGE gel and then transferred to nitrocellulose membranes (Bio-Rad) [14, 15]. The antigens were visualized by using an enhanced chemiluminescence kit (ECL; Amersham). Antibodies against TbTim50 [15], HA-epitope (Clone 12CA5, Roche Applied Science), and *T. brucei* β -tubulin [25] were used as probes. The secondary antibodies used were either anti-rabbit or anti-mouse immunoglobulins linked to horseradish peroxidase (Amersham Life Science).

2.3. Hydrogen peroxide treatment

TbTim50 RNAi and TbTim50-3HA *T. brucei* were induced for four days with doxycycline (1 µg/ml). Parental *T. brucei* 427 cells were also grown in parallel for 4 days. Cells were then exposed to different concentration of hydrogen peroxide (0–800 µM) for 2 h. Treated cells were immediately used for further analysis.

2.4. Live/dead assay

T. brucei parental 427, TbTim50 RNAi and TbTim50-3HA cells (2×10^7) were harvested, washed once with 1x PBS and resuspended in 200 µl of 1 x PBS. C12-resazurin and SYTOX Green dyes (Molecular Probes®) were then added to each 100 µl cell suspension at a final concentration of 500 nM. Cells were incubated at 27 °C for 15 min. Next, 400 µl of the 1x PBS was added to the cells and the mixtures kept on ice. The samples were analyzed immediately by flow cytometry, with excitation at 488 nm and measurement of the fluorescence emission at 530 nm and 575 nm.

2.5. Mitotracker staining

Cells were harvested and suspended in fresh culture medium at a density of $4-5 \times 10^6$ /ml. Cells were stained with MitoTracker® Red, fixed and permeabilized as previously described [14, 15]. Briefly, MitoTracker® Red CMXRos (Molecular Probe®) was dissolved in DMSO at a concentration of 1 mM and added to the cell suspension at a final concentration of 0.5 µM. The mixtures were incubated at 27°C for 10 min. Cells were then washed and incubated in fresh culture medium for an additional 30 min. Cells were washed twice with 1x PBS and fixed in 0.37% paraformaldehyde at 4°C for 5 min. Next, cells were centrifuged, washed and resuspended in cold PBS and stored at 4 °C until FACS analysis. Fluorescence intensity (Geom. Mean) was measured with a FACSCalibur (Becton Dickinson) analytical flow cytometer using absorption at 578 nm and emission at 599 nm.

2.6. Measurement of ROS

T. brucei cells (1×10^7 /ml) were incubated with 10 µM 2',-7' dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 27 °C. After incubation, cells were washed once with 1x PBS and analyzed by flow cytometry, by measuring the fluorescence emission at 520 nm using the excitation wavelength at 492 nm.

2.7. Annexin staining

T. brucei cells were stained with Alexa FluorR 488 conjugated annexin V and propidium iodide (PI) using an apoptosis assay kit (Molecular Probe®) according to the manufacturer's protocol. Briefly, cells (2×10^7) were harvested and washed in cold PBS. The washed cells were resuspended in 1x annexin-binding buffer (200 µl). Alexa FluorR 488 annexin V and propidium iodide were added to the cell suspensions as directed in the protocol and cells were incubated at room temperature for 15 min. After the incubation period, 400 µl 1x annexin-binding buffer was added, mixed gently and kept on ice. The stained and unstained cells were analyzed by flow cytometry, by measuring the fluorescence emission at 530 nm and 575 nm using the excitation wavelength at 488 nm.

2.8. TUNEL assay

T. brucei cells (1×10^7) were harvested, washed with 1x PBS and resuspended in 500 μ l of the same buffer. Cells were fixed in para-formaldehyde (0.37%) and permeabilized using Triton X-100 (0.1%). Cells were stained with fluorescein-12-dUTP using the DeadEnd™ fluorometric TUNEL system (Promega) according to manufacturer's protocol. Cells were then analyzed by flow cytometry by measuring the green fluorescence of fluorescein-12-dUTP at 520 nm using the excitation wavelength at 488 nm.

2.8. Flow cytometry

Samples were analyzed on a Beckman Coulter Epics XL-MCL and results were analyzed using FlowJo software (Treestar software). Compensation of spectral overlap was performed using single-stained control cells. Compensation was manually corrected as needed to ensure that single-stained cells did not fall below the axis. All flow cytometry experiments were performed at least three times for each condition.

3. Results

3.1. TbTim50 OE reduces *T. brucei* cell growth

A stable cell line of *T. brucei* containing an inducible construct for TbTim50 with 3xHA epitope tag at the C-terminal was developed [15]. After induction with doxycycline, TbTim50 OE cells produced an ectopic copy of the TbTim50 transcript, which was smaller in size than the endogenous copy because of having different 3' and 5' untranslated regions. The endogenous TbTim50 transcript was also expressed at similar levels in the presence and absence of doxycycline (Fig. 1A). Immunoblot analysis using anti-HA antibody detected TbTim50-3HA (~54 kDa) in the mitochondria samples isolated from TbTim50 OE cells but not from those in the parental wild type control (Fig. 1B). A pair of smaller molecular size proteins (~52 kDa and ~50 kDa) was also detected by anti-HA antibody from TbTim50 OE mitochondrial proteins, which could represent the degradation products of TbTim50-3xHA. Reprobing the same blot with anti-TbTim50 antibody recognized both the endogenous and ectopically expressed TbTim50 in TbTim50 OE samples and only the endogenous protein in the mitochondrial samples isolated from the parental *T. brucei*. There was about a 2-fold increase of the TbTim50 protein levels in TbTim50 OE compared to parental controls.

Interestingly, we found that overexpression of TbTim50 did not favor cell growth of *T. brucei* (Fig. 1C). Induced cells grew at a moderately lower rate than uninduced controls. However, when compared to the parental controls both uninduced and induced cells grew at a slower rate. The cellular doubling times calculated at the exponential phases of growth (2–6 days) were 10 ± 0.5 , 13.6 ± 1.6 , and 19.4 ± 0.15 for parental, uninduced, and induced *T. brucei*, respectively. Previously, we reported that TbTim50 KD caused a moderate reduction in cell growth [15]. Together, these results suggest that an optimal level of TbTim50 is required for normal cell growth. We did not observe any growth inhibition due to overexpression of TbTim17, another mitochondrial protein translocator in *T. brucei* [16], indicating that TbTim50 may play an additional role in cell function other than translocation of proteins into mitochondria.

3.2. TbTim50 OE increases the mitochondrial membrane potential

In fungi, Tim50 maintains the permeability barrier across the MIM by closing the TIM23 channel in the absence of precursor proteins [26]. In order to investigate the effect of TbTim50 OE on the mitochondrial membrane potential, cells were stained with Mitotracker red which accumulates in mitochondria in a membrane potential-dependent manner. The parental and TbTim50 KD cells were also used in parallel to compare. We found that compared to the parental controls, TbTim50 OE increased the mitochondrial membrane potential ~20% and ~50% on day 2 and day 4, respectively (Fig. 2). This is in contrast to TbTim50 KD, which reduced the mitochondrial membrane potential by ~30% on day 2 and ~40% on day 4 post-induction compared to parental controls (Fig. 2) [15]. The change in the membrane potential due to alteration of TbTim50 levels progressed with the term of the induction period. As expected, pretreatment of cells with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) at a final concentration of 50 μ M reduced the intensity of staining more than 80%. Therefore, these results indicate that similar to fungi [26] and *Drosophila* [24], TbTim50 plays a role in maintaining the mitochondrial inner membrane permeability barrier in *T. brucei*.

3.3. TbTim50 OE increases cellular ROS

Mitochondria are the primary site for the production of cellular ROS. To investigate the effect of an alteration of TbTim50 levels on cellular ROS production, we treated cells with the fluorogenic probe, DCFH-DA, which diffuses into cells and is deacetylated by cellular esterases to non-fluorescent DCFH. Inside cell, DCFH is rapidly oxidized to highly fluorescent DCF by ROS. After measurement of the fluorescent intensity of the DCFH-DA treated cells revealed that the levels of cellular ROS was increased about 4 fold on day 2 and 12 fold on day 4 post-induction in TbTim50 OE cells in comparison to parental controls (Fig. 3). Whereas, the ROS level in TbTim50 KD cells was about the same level at day 2, and was slightly (~2 fold) increased on day 4 in comparison to that in parental *T. brucei* controls. Therefore, TbTim50 OE caused more distress in cells than TbTim50 KD. We did not see a similar change in cellular ROS due to over-production of Tim17 (data not shown), suggesting that the effect was specific for TbTim50.

3.4. TbTim50 KD increases *T. brucei* tolerance to oxidative stress

In order to investigate the effect of externally added oxidative stress on TbTim50 KD and OE *T. brucei*, we treated cells with various concentrations of H₂O₂ and assayed cell viability on the basis of incorporation of the two dyes sytox and C12-resazurin, followed by flow-cytometry analyses (Fig. 4). Sytox is cell impermeable, thus it only stains dead or necrotic cells, whereas C12-resazurin is cell permeable, thus it is capable of penetrating living cells [27, 28]. Within metabolically active cells, the nonfluorescence C12-resazurin is converted to fluorescent C12-resorufin, thus fluorescence intensity is directly proportional to the number of live cells in population. Cells that were unstained or stained with resorufin alone and those that were heat-killed and treated with sytox were used as controls. Flow-cytometry results revealed that with increasing concentrations of H₂O₂, the parental cells became more and more injured as indicated by the number of cells present in the upper right corner of each plot (Fig. 4A). A similar situation was observed for Tim50 OE cells. However, Tim50

KD cells were less injured as seen with the increasing concentrations of H₂O₂. Even after treatment with 800 µM H₂O₂ about 80–90 % of the cells were alive and healthy as shown by the number present in the lower right corner of the plot. Comparing the live versus injured ratio of each cell type at different concentrations of H₂O₂ (from three independent experiments) clearly confirmed that Tim50 KD cells were relatively more resistant to exposure to an oxidative stress than either parental or Tim50 OE cells (Fig. 4B).

3.5. Treatment with H₂O₂ decreases the mitochondrial membrane potential and increases the levels of ROS in the parental, TbTim50 OE and TbTim50 KD *T. brucei*

As we observed that TbTim50 KD cells were more tolerant to H₂O₂ treatment than TbTim50 OE and parental *T. brucei*, we wanted to investigate the effect of such treatment on the mitochondrial membrane potential and levels of ROS in these cells. Results showed that the mitochondrial membrane potential dropped over 75% in all three types of cells after H₂O₂ treatment (Fig. 5A–D). A reduction in mitochondrial membrane potential was observed even at the lowest concentration of H₂O₂ (200 µM). This indicated that oxidative stress similarly depolarized the mitochondrial membrane in parental, TbTim50 OE and KD cells. These results also explained our observation that longer incubation times (> 4h) killed most of these cells even at 200 µM H₂O₂. Therefore an increased tolerance in Tim50 KD cells is due to an increase in the immediate adaptive response of *T. brucei* upon H₂O₂ treatment.

In addition to mitochondrial membrane potential, we found that the levels of ROS increased gradually with increasing concentrations of H₂O₂ in parental and Tim50 KD cells (Fig. 5E, G, and H). Therefore, these results showed that although the level of ROS increased during peroxide treatment, TbTim50 KD cells were able to partially resist that stress. Peroxide treatment also increased the level of ROS in TbTim50 OE cells however the results were more adverse (Fig. 5F and H). With an increasing concentration of H₂O₂, the number of TbTim50 OE cells with higher DCF-staining was reduced and a sub-population of cells stained at a lower intensity increased (Fig. 5F). This is because those TbTim50 OE cells that were injured/dead due to H₂O₂ treatment were weakly stained with DCF. Some weakly stained cells also accumulated in parental and Tim50 KD *T. brucei* after treatment with higher concentrations (400–800 µM) of H₂O₂, however, the number of cells in these populations was relatively lower in comparison to that found for TbTim50 OE. Therefore, these results further indicate that oxidative stress caused more injury to TbTim50 OE cells in comparison to the parental and Tim50 KD *T. brucei*. The tolerance of TbTim50 KD cells to oxidative stress is not likely due to an increased capacity of these cells to neutralize ROS. Rather it could be due to an increase in a signaling ability of these cells, which mediates cell survival upon oxidative stress. On the other hand, TbTim50 OE cells were more vulnerable to oxidative stress because these cells already have more ROS due to OE of TbTim50.

3.6. TbTim50 KD reduces the transition of cells from early to late apoptotic stages upon treatment with an increasing concentration of H₂O₂

Next we determined if Tim50 KD or OE had any effect on the development of an apoptotic phenotype in cells treated with H₂O₂. For this purpose we simultaneously stained cells with propidium iodide (PI) and annexin V and then analyzed these cells by flow-cytometry (Fig.

6). Cells that were either unstained or treated with annexin V alone, and heat-killed cells treated only with PI were used as positive and negative controls. FACS analysis showed that parental cells not treated with H_2O_2 were sorted in the lower-left quadrant, indicating that these were live cells minimally stained with both dyes (Fig. 6A). With increasing concentration of H_2O_2 , cells became more apoptotic, thus more cells sorted to the upper- and lower-right quadrants. Cells in the upper-right quadrant (Annexin+ /PI+) are at the late-apoptotic stage while those in the lower-right quadrant (Annexin+ /PI-) of the plot are in the early-apoptotic stage. We found that a large proportion (50–60%) of Tim50 OE cells were already in the early apoptotic stage without any H_2O_2 treatment. This could be due to increased ROS levels and hyperpolarization of the mitochondrial membrane in this cell type due to Tim50 OE. After treatment with a low dose (200 μM) of H_2O_2 , these cells were sorted as being dead. With increasing concentrations of H_2O_2 , the numbers of Tim50 OE cells sorted into the late apoptotic stage were relatively greater in comparison to the parental cell type (Fig. 6A). In contrast to OE, most of the Tim50 KD cells were alive to begin with. After treatment with H_2O_2 these cells were primarily in an early apoptotic stage and only 2–3% of cells were in the late apoptotic stage even after treatment with the highest concentration of H_2O_2 (Fig. 6A). Comparing the ratio of the early versus late apoptotic cell populations for different types of *T. brucei* tested at different concentrations of H_2O_2 revealed that the parental and Tim50 OE cells quickly reached the late apoptotic stage as H_2O_2 concentrations were raised (Fig. 6B). However, transition from the early to the late apoptotic stage upon exposure to oxidative stress was somehow blocked in Tim50 KD cells (Fig. 6B).

3.7. TbTim50 OE increases nuclear DNA fragmentation upon treatment with an increasing concentration of H_2O_2

Nuclear DNA fragmentation is a hallmark for the late stage of apoptosis. To assess nuclear DNA fragmentation, TUNEL assays were performed after treating the parental, Tim50 OE, and Tim50 KD cells with different concentrations of H_2O_2 . Unstained cells were used as the negative controls for FACS analysis. The TUNEL system measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the recombinant enzyme terminal deoxynucleotidyl transferase (rTDT) [29]. Results showed that TUNEL-positive cell populations increased with increasing concentration of peroxide due to Tim50 OE (Fig. 7A and B). The shift in fluorescence intensity was maximum when Tim50 OE cells were treated with 800 μM H_2O_2 (Fig. 7B). In contrast, the fluorescence intensities were comparable in Tim50 KD cells when treated with 200–800 μM concentration of H_2O_2 . At 800 μM H_2O_2 , parental cells were stained relatively more than Tim50 KD *T. brucei*. Together these results support the concept that Tim50 is required for transition of *T. brucei* cells to late apoptotic stages in the presence of oxidative stress.

4. Discussion

Although the major function of mitochondria is cellular energy production, this organelle is intimately involved in various other cellular processes, such as cell death, aging, and stress [30–32]. Therefore, communication between mitochondria and the rest of the cell is critical to maintaining cell homeostasis during normal stages and during various challenges due to

environmental stimuli [33–35]. This communication is critical in the case of *T. brucei*, which faces various environmental challenges during its digenetic life cycle and it is supported by the fact that the parasite extensively modulates its mitochondrial activities to cope with such assaults. Here we show that the mitochondrial protein translocator Tim50 in *T. brucei* is associated with the cellular stress-response pathway. TbTim50 works as a sensor for mediating stress signals during oxidative assault. Therefore, both up and down regulation of this protein hampered cellular homeostasis. TbTim50 OE increased the level of cellular ROS and hyperpolarized the mitochondrial membrane leading to cellular apoptosis upon externally added ROS. In contrast, TbTim50 KD induced an unknown signaling event that protected *T. brucei* during oxidative stress.

In fungi, it has been reported that Tim50 controls the mitochondrial membrane potential by closing the import channel formed by Tim23 in the absence of any presequence-containing protein to avoid proton leakage [26]. The N-terminal targeting signal or presequence of mitochondrial proteins interact with the Tim50 C-terminal domain, which is topologically positioned in the inter-membrane space of mitochondria [36, 37]. This interaction then activates the TIM23 channel for protein translocation [26]. Therefore it can be assumed that TbTim50 has a similar function by closing the protein import channel that is possibly formed by TbTim17 (*T. brucei* lacks an equivalent of Tim23). We have shown that TbTim50 interacts *in vivo* with TbTim17 [15]. Although the membrane topology of TbTim50 has not been determined, the predicted primary and the 3D-structure analyses revealed that TbTim50 does possess a unique region within amino acid residues 259–274, which could be responsible for its interaction with TbTim17 [15]. Therefore this unique region of TbTim50 may play a role in keeping the channel closed in the absence of incoming precursor proteins. However, further investigation is required to verify these assumptions. In any case it is most likely that TbTim50 OE hyperpolarized the mitochondrial inner membrane by tightly closing the import channel. It is known in other systems that hyperpolarization increases swelling of mitochondria, which in turn induce mitochondrial permeability transition pores formed by the mitochondrial ADP/ATP carrier and VDAC, thus inducing apoptosis [38, 39]. A similar mechanism may exist in *T. brucei*. In a unicellular parasitic protozoan like *T. brucei*, the path to apoptotic cell death has not been well defined [40, 41]. However, typical hallmarks of apoptosis such as mitochondrial membrane depolarization, cytochrome C release, phosphatidyl serine exposure and fragmentation of nuclear DNA are induced in this parasite upon induction of oxidative stress or treatment with staurosporin, concanavalin A, and prostaglandin A2 [40–43]. We found that a large proportion of the TbTim50 OE cells (~60%) were annexin-positive even before addition of H₂O₂, which shows that TbTim50 OE is apoptotic in *T. brucei*. In addition, we found that after H₂O₂ treatment, more cells became TUNEL positive due to TbTim50 OE than TbTim50 KD, indicating that Tim50 OE enhances apoptotic events under such conditions in *T. brucei*.

In contrast to Tim50 OE, Tim50 KD cells are somehow resistant to progression to the late stage of apoptosis during oxidative assault. Since TbTim50 is critical for mitochondrial protein import, Tim50 KD alters mitochondrial protein homeostasis (or proteostasis). We speculate that this change induces the mitochondrial unfolded protein response (UPR^{mt})

pathway. It has been reported recently in human cells that chemical stress induces degradation of Tim17A, a membrane integral component of the TIM23 translocase [44]. Degradation of Tim17A by mitochondrial proteases is adequate to activate the UPR^{mt} pathway in these cells. Furthermore, in *Caenorhabditis elegans*, shRNA mediated down regulation of Tim17 increased resistance to oxidative stress [44]. In addition, down regulation of Tim23 in the same organism caused an accumulation of ATFS in the nucleus. ATFS is a bZip transcription factor, which translocates to the mitochondria and degrades quickly via mitochondrial protease in the absence of oxidative stress [45]. Once accumulated in the nucleus under stress, ATFS induces expression of various stress response proteins, which includes mitochondrial chaperones and protein translocators, to restore mitochondrial functions [45]. It is not clear yet how UPR^{mt} works in *T. brucei*, however, it can be assumed that inhibition of mitochondrial protein import by down regulation of TbTim50 induces a stress response pathway that increases the levels of the stress-response proteins post-transcriptionally (since transcriptional regulation is rarely found in trypanosomatids [46]), thus increases tolerance of *T. brucei* to oxidative stress.

We reported previously that TbTim50 up- and down-regulation inversely regulate the steady state levels of the mitochondrial voltage-dependent anion channel (VDAC) [15]. VDAC is a β -barrel protein and is the most abundant protein in the mitochondrial outer membrane (MOM) [47]. VDAC forms an aqueous channel that mediates the major metabolite flux between mitochondria and the cytosol [48, 49]. In *T. brucei* down regulation of VDAC reduces cell growth, mitochondrial ATP production, and alters the respiratory phenotype [47, 50]. In metazoans, VDAC plays a crucial role in programmed cell death (PCD) [51–53]. VDAC interacts with various pro- and anti-apoptotic proteins and regulates the fate for cell death/survival [54–57]. Here, we speculated that an increased level of VDAC in TbTim50 KD cells inhibits apoptosis by recruiting some uncharacterized anti-apoptotic proteins in *T. brucei*. Therefore, these cells appear more tolerant to oxidative stress. However, further investigations are required to prove this hypothesis.

Overall our results showed that there is a well-orchestrated regulatory process in *T. brucei* to help maintain cellular homeostasis upon extrinsic stress signals; mitochondrial proteins like Tim50 play a crucial role in this process. Future investigations will explore the communication between mitochondrial and other cellular functions in *T. brucei*.

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Highlights

- Tim50 acts as a stress-sensor
- Tim50 OE increases ROS and the mitochondrial membrane potential
- Tim50 KD inhibits transition of cells from an early to late apoptotic stage
- Tim50 KD increases *T. brucei*'s tolerance to oxidative stress
- Tim50 KD could be a survival mechanism for *T. brucei* exposed to oxidative stress.

Fig. 1.

TbTim50 overexpression and its effect on cell growth. (A) Northern blot analysis of total RNA isolated from *T. brucei* with a genome-integrated copy of TbTim50-3x hemagglutinin antigen (HA). Cells were grown in the presence (+) or absence (-) of doxycycline for 4 days. Northern blot was probed using radiolabelled TbTim50 and tubulin cDNAs. The ectopic (ecto) and the endogenous (endo) TbTim50 transcripts in TbTim50 OE are indicated. (B) Western blot analysis of mitochondrial proteins from the parental control and TbTim50 OE *T. brucei* after induction for 4 days with doxycycline were performed using antibodies for HA-epitope, TbTim50, and *T. brucei* β -tubulin. One hundred and 50 μ g of mitochondrial proteins was loaded per sample. (C) Growth kinetics of *T. brucei* parental, and TbTim50 OE cells. The TbTim50 OE cells were grown either in the absence (Uninduced) or presence (Induced) of doxycycline. Cell numbers were counted every alternate day post-induction. Parasite cultures were diluted when the cell number reached 1×10^7 /ml. The log of the cumulative cell number was plotted versus time. Results are representatives of three independent experiments. Standard errors were less than 0.1%.

Fig. 2.

Effects of TbTim50 up- and down-regulations on the mitochondrial membrane potential. The TbTim50 KD and OE cells were induced with doxycycline and cells (2×10^7) were harvested at day 2 (A) and day 4 (B). Live cells were stained with Mitotracker Red as described in the materials and methods. Cells were fixed with para-formaldehyde, washed and resuspended in cold PBS for FACS analysis. Fluorescence intensity was measured with a FACSCalibur (Becton Dickinson) analytical flow cytometer using absorption at 578 nm and emission at 599 nm. Flowjo software was used to analyze the results. Parental cells were also pre-treated with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) at a final concentration of 50 $\mu\text{g/ml}$ to disrupt mitochondrial membrane potential. (B) Quantitation of the fluorescence intensity from triplicate samples was performed using the same software. Fluorescence intensity of TbTim50 OE and KD cells were calculated as percent of that in the parental control in respective days. Values shown are mean $\pm$ standard errors (* $p < 0.05$).

Fig. 3.

Effects of TbTim50 up- and down-regulations on the levels of cellular reactive oxygen species (ROS). The TbTim50 KD and OE cells were induced with doxycycline and cells (2×10^7) were harvested at day 2 (A) and day 4 (B). Cells were washed with 1x PBS and stained with DCFH-DA as described in the materials and methods. Fluorescence intensity was measured with a FACSCalibur (Becton Dickinson) analytical flow cytometer at 520 nm. Flowjo software was used to analyze the results. (C) Fluorescence intensity was calculated from triplicate samples using the same software and plotted as percent of the parental control at the respective time periods. Values shown are mean $\pm$ standard errors (* $p < 0.05$).

Fig. 4.

Effects of TbTim50 up- and down-regulations on cell injury during H₂O₂ treatment. TbTim50 KD and TbTim50 OE cells were grown in the presence of doxycycline for 4 days. Parental control *T. brucei* were also grown in parallel. (A) Cells were treated with different concentration of H₂O₂ (0–800 μM) for 2 h, washed with PBS and stained with sytox green and resorufin as described. FACS analysis was performed immediately and data were analyzed by Flowjo software. FACS analysis of the unstained, heat killed (Dead) and live parasites were performed in parallel. (B) Ratios of the live and injured cell (%) were calculated from three independent experiments and plotted versus the different cell type at different concentrations of H₂O₂. Values shown are mean ± standard errors from triplicate samples.

Fig. 5.

Effects of hydrogen peroxide treatment on mitochondrial membrane potential and cellular ROS. *T. brucei* parental (A and E), TbTim50 OE (B and F), and TbTim50 KD (C and G) cells were treated with different concentrations of H₂O₂ (0, 200, 400, and 800 μM) for 2h. Before treatment, TbTim50 OE and KD cells were induced for 4 days in the presence of doxycycline. Cells were washed once with 1x PBS and immediately used for Mitotracker (A–C) and DCFH-DA (E–G) staining as described. FACS analysis results are shown as histograms. Fluorescence intensity from Mitotracker (D) and DCFH (H) staining were measured using Flowjo software and plotted for different types of cells. Values for intensity were presented in arbitrary unit (A.U.). Standard errors were calculated from three independent experiments.

Fig. 6.

Effects of TbTim50 up- and down-regulations on apoptosis upon oxidative stress. The TbTim50 KD and TbTim50 OE cells were grown in the presence of doxycycline for 4 days. Parental control *T. brucei* were also grown in parallel. (A) Cells were treated with different concentration of H_2O_2 (0–800 μM) for 2 h, washed with PBS and stained with Annexin V and Propidium iodide (PI) as described. FACS analysis was performed immediately and data were analyzed by Flowjo software. FACS analysis of the unstained, heat killed (Dead) and live parasites of each type were performed in parallel. (B) Cell populations in the lower-right and upper-right quadrants were considered to be the late- and early-apoptotic stages,

respectively. The populations of early- and late- apoptotic cells (%) were calculated from three independent experiments and plotted for different cell types [parental (Wt), TbTim50 OE (OE) and TbTim50 KD (KD)] and for different H₂O₂ concentrations.

Fig. 7.

Effect of TbTim50 up- and down-regulations on nuclear DNA degradation during H_2O_2 treatment. TbTim50 KD and TbTim50 OE cells were grown in the presence of doxycycline for 4 days. Parental control *T. brucei* were also grown in parallel. (A) Cells were treated with different concentrations of H_2O_2 (0–800 μM) for 2 h, washed with 1x PBS and prepared for TUNEL assay as described. Incorporation of BrdU into nicked DNA was assessed by FACS analysis. Fluorescence intensity was measured with a FACSCalibur (Becton Dickinson) analytical flow cytometer at 520 nm. Flowjo software was used to analyze the results. (B) Quantitation of the fluorescence intensity from triplicate samples was performed using the same software. Fluorescence intensity in arbitrary units (A.U.) of the parental, TbTim50 OE and KD cells was plotted with respect to H_2O_2 concentrations (0–800 μM). Values shown are mean $\pm$ standard errors (* $p < 0.05$).