# TbLOK1/ATOM19 is a novel subunit of the non-canonical mitochondrial outer membrane protein translocase of *Trypanosoma brucei*

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*Summary*

**TbLOK1 has previously been characterized as a trypanosomatid-specific mitochondrial outer membrane protein whose ablation caused a collapse of the mitochondrial network, disruption of the membrane potential and loss of mitochondrial DNA. Here we show that ablation of TbLOK1 primarily abolishes mitochondrial protein import, both *in vivo* and *in vitro*. Co-immunprecipitations together with blue native gel analysis demonstrate that TbLOK1 is a stable and stoichiometric component of the archaic protein translocase of the outer membrane (ATOM), the highly diverged functional analogue of the TOM complex in other organisms. Furthermore, we show that TbLOK1 together with the other ATOM subunits forms a complex functional network where ablation of individual subunits either causes degradation of a specific set of other subunits or their exclusion from the ATOM complex. In summary these results establish that TbLOK1 is an essential novel subunit of the ATOM complex and thus that its primary molecular function is linked to mitochondrial protein import across the outer membrane. The previously described phenotypes can all be explained as consequences of the lack of mitochondrial protein import. We therefore suggest that, in line with the nomenclature of the ATOM complex subunits, TbLOK1 should be renamed to ATOM19.**

***Introduction***

Mitochondria perform a number of important functions and are essential for all eukaryotic life (Friedman & Nunnari, 2014). The origin of mitochondria can be traced back to the engulfment of α-proteobacterium by an archeal host cell (Williams *et al.*, 2013). During evolution the endosymbiotic ancestor of mitochondria was converted into an organelle that largely is under the control of the nucleus. Crucial for this conversion was the acquirement of a protein import system that allowed the endosymbiont to make use of genes that previously had been transferred to the nucleus (Lithgow & Schneider, 2010, Hewitt *et al.*, 2011, Gray, 2012). Today more than 95% of the more than 1000 mitochondrial proteins are synthesized in the cytosol and subsequently imported across one or both mitochondrial membranes.

Mitochondrial protein import and the membrane complexes that mediate it have been studied in great detail (Chacinska *et al.*, 2009, Schmidt *et al.*, 2010, Schulz *et al.*, 2015). The protein translocase of the mitochondrial outer membrane (TOM), is of special interest as it is the first translocase with which imported proteins have to engage. In yeast and humans the TOM complex consists of 7 subunits. The β-barrel membrane protein Tom40, a member of the VDAC-like protein family (Pusnik *et al.*, 2009), forms the translocation pore and is tightly associated with the single membrane spanning protein Tom22 that functions as TOM complex organizer and as a secondary receptor. Tom7, Tom6 and Tom5 are important for assembly of the TOM complex and regulate its dynamics. Tom20 and Tom70, finally, are in part redundant protein import receptors with partially overlapping substrate specificities (Perry *et al.*, 2008, Mani *et al.* 2015).

Most of our knowledge on mitochondrial protein import systems stems from studies done in yeast, *Neurospora* and humans, which on a global scale are closely related and only represent a very small segment of the eukaryotic diversity (Burki, 2014, Adl *et al.*, 2005). Bioinformatic analysis and experimental studies in plants and more recently in

the parasitic protozoa *Trypanosoma brucei* have shown that surprisingly of all TOM subunits only Tom40 and Tom22 are conserved in all eukaryotes (Mani *et al.* 2015). The most highly diverged TOM complex found to date is the archaic translocase of the outer membrane (ATOM) of trypanosomatids (Pusnik *et al.*, 2011), which are one of the most early diverging eukaryotes that have mitochondria capable of oxidative phosphorylation (Cavalier-Smith, 2010, He *et al.*, 2014). A recent study has shown that the ATOM complex consists of six subunits (Pusnik *et al.*, 2011, Mani *et al.* 2015). ATOM40 forms the highly diverged protein import channel and shows weak sequence similarity to both the VDAC-like and the Omp85-like protein family (Pusnik *et al.*, 2011, Zarsky *et al.*, 2012). Besides Tom40 of yeast, *Neurospora* and humans, all of which belong to the same eukaryotic supergroup of the Opisthokonts, trypanosomal ATOM40 is the only TOM complex import pore that has been analyzed by electrophysiological methods. These experiments showed that recombinant ATOM40 behaved more similar to Omp85-like proteins rather than to yeast Tom40 in regard of its oligomeric state and its fast flickering conductance states (Harsman *et al.*, 2012). The second conserved subunit is ATOM14. It is a remote orthologue of Tom22 which, similar to the plant Tom22 orthologue Tom9, has a very short cytosolic domain that lacks a cluster of acidic residues (Mani *et al.* 2016). The remaining four subunits are specific for Kinetoplastids. They include: ATOM11 and ATOM12 which regulate ATOM complex assembly; ATOM69 which contains a CS/Hsp20-like domain and tetratricopeptide repeat (TPR) motifs; and ATOM46 which has an armadillo repeat domain. ATOM69 and ATOM46 are protein import receptors that recognize mitochondrial targeting signals. Interestingly, despite the functional conservation of the import signals between yeast and Kinetoplastids the receptors that recognize them, Tom20/Tom70 in yeast and ATOM69/ATOM46 in trypanosomes, evolved independently of each other (Mani *et al.* 2015). The same appears to be the case for the protein import receptors that have been characterized in plant mitochondria (Perry *et al.*, 2006).

In the present study we show that TbLOK1, a recently characterized mitochondrial morphology factor (Povelones *et al.*, 2013), in fact is an additional core subunit of the ATOM complex. Interestingly, unlike any other TOM complex subunits in yeast, plants or trypanosomes, it likely has two transmembrane domains.

***Results***

**TbLOK1 is essential under all conditions**

A recent study in *T. brucei* characterized a kinetoplastid-specific protein of 19 kDa that was termed TbLOK1 for loss of kinetoplast DNA (kDNA), the mitochondrial genome of trypanosomes (Povelones *et al.*, 2013). Ablation of TbLOK1 interfered with normal mitochondrial morphology and with the maintenance of the single unit kDNA. Moreover, the protein was shown to be essential for an intact mitochondrial membrane potential and transcription of the kDNA. Biochemical experiments indicated that TbLOK1 is an integral protein of the mitochondrial outer membrane.

In order to study the function of TbLOK1 in more detail we produced an inducible RNAi cell line for the bloodstream form of *T. brucei*. Fig. 1A shows that TbLOK1 is also essential in this life cycle stage. Moreover, as observed in procyclic forms, ablation of the protein affects the morphology of the mitochondrion (Fig. S1). Mitochondrial translation and thus the kDNA are essential for the survival of *T. brucei* throughout its life cycle (Cristodero *et al.*, 2010). To find out whether the growth arrest observed in induced TbLOK1-RNAi cells is linked to kDNA maintenance or another as yet unknown function, we used an engineered cell line that as a bloodstream form can grow in the absence of mitochondrial DNA (Dean *et al.*, 2013). This is possible because it carries a single point mutation in the nuclear-encoded γ-subunit of the mitochondrial ATPase that allows to compensate for the lack of the mitochondrially encoded subunit 6 of the ATPase. In this cell line ablation of genes involved in mitochondrial gene expression will therefore not cause a growth arrest. However, TbLOK1 remains essential also in this cell line (Fig. 1B), which strongly suggests that the primary function of TbLOK1 is not linked to mitochondrial gene expression.

**Ablation of TbLOK1 interferes with mitochondrial protein import**

The only known essential mitochondrial outer membrane proteins in *T. brucei* that are not directly linked to mitochondrial gene expression are subunits of the ATOM complex (Mani *et al.*, 2015a), Sam50 (Sharma *et al.*, 2010, Schnarwiler *et al.*, 2014) and pATOM36 (Pusnik *et al.*, 2012), all of which are involved in mitochondrial protein import. We therefore wondered whether TbLOK1 might also be involved in mitochondrial protein import. To test this we prepared protein extracts from a procyclic tetracycline-inducible TbLOK1-RNAi cell line at different times after induction and analyzed them on immunoblots using a panel of antibodies recognizing imported mitochondrial proteins (Fig. 2A). It has previously been shown that for many substrates *in vivo* ablation of protein import factors causes cytosolic accumulation of unprocessed precursor proteins and/or a decrease of the mitochondrially localized mature forms (Pusnik *et al.*, 2011). It should be noted though that accumulation of precursors is not seen for all substrates because mislocalized cytosolic precursor proteins are often rapidly degraded.
The results in Fig. 2A show that ablation of TbLOK1, simultaneously with the growth arrest, results in a decrease of the imported mitochondrial proteins RNA editing associated protein 1 (REAP1), initiation factor 2 (IF2), cytochrome oxidase subunit IV (CoxIV) and voltage dependent anion channel (VDAC). Moreover, for REAP1 and CoxIV accumulation of unprocessed precursor forms is observed. Cytosolic elongation factor 1a (Ef1a) however is not affected.

In order to exclude that the observed growth arrest and the concomitant accumulation of the unprocessed CoxIV precursor are due to off target effects, we complemented the TbLOK1-RNAi cell line with a recoded version of the TbLOK1 gene expected to be resistant to the RNAi Fig. S2 shows that in this cell line addition of tetracycline still causes ablation of the endogenous TbLOK1 mRNA. However, due to expression of the recoded TbLOK1 the cells grow normally and accumulation of CoxIV precursor is not observed anymore.

Mitochondrial protein import was also assayed *in vitro* using mitochondria isolated from the uninduced and induced TbLOK1-RNAi cell line. To prevent pleiotropic effects, organelles from induced cells were isolated only 1.5 days after induction of RNAi prior to the onset of the growth arrest (Fig. S3). Fig. 2B shows that import of the *in vitro* translated chimeric protein consisting of the presequence-containing 150 N-terminal amino acids of lipoamide dehydrogenase (LDH) fused to mouse dihydrofolate reductase (DHFR) (Hauser *et al.*, 1996) as well as of the trypanosomal inner membrane protein alternative oxidase (TAO) (Hamilton *et al.*, 2014) was essentially abolished in mitochondria isolated from the induced cell line.

In summary, these results show that TbLOK1 plays a prominent role in mitochondrial protein import.

**TbLOK1 is a subunit of the ATOM complex**

The mitochondrial OM of *T. brucei* contains two protein translocases: the ATOM complex (Mani *et al.*, 2015), which is the general entry gate for essentially all mitochondrial proteins, and the more specialized SAM complex, which mediates biogenesis of β-barrel proteins (Sharma *et al.*, 2010). Moreover, a protein termed pATOM36 that is involved in import of a subset of proteins has also been identified (Pusnik *et al.*, 2012). The results in Fig. 2 show that ablation of TbLOK1 affects import of matrix, inner membrane as well as β-barrel proteins very early after induction of RNAi. This could be explained if TbLOK1 is implicated in the function of the ATOM complex.

A previous blue native polyacrylamide electrophoresis (BN-PAGE) analysis has shown that ATOM40, the pore forming subunit of the ATOM complex, migrates in a high molecular weight complex of approximately 700 kDa (Pusnik *et al.*, 2011). In order to test whether TbLOK1 is a physical component of the ATOM complex we performed BN-PAGE and subsequent immunobloting using antisera against TbLOK1 and the different ATOM subunits. The result in Fig. 3A shows that TbLOK1 co-migrates with all ATOM

complex subunits except the peripheral receptor ATOM69. As previously shown ATOM69 is present in higher molecular weight complex that only contains little of the ATOM40 protein (Mani *et al.*, 2015). Similar experiments were performed in the bloodstream form of *T. brucei.* They showed that also in this life cycle stage the TbLOK1-containing complexes co-migrate with the ATOM40 complex when analyzed by BN-PAGE (Fig. S4). Furthermore, reciprocal co-immunoprecipitations (co-IPs) using a procyclic cell line expressing Myc-tagged TbLOK1 in combination with C-terminally hemagglutinine (HA)-tagged ATOM40 demonstrate that pulling on either the Myc- or the HA-tag results in the co-precipitation of the other tagged protein, irrespectively of whether TbLOK1 is N- or C-terminally tagged (Fig. 3B). A co-IP was also performed using a cell line which expresses N-terminally Myc-tagged TbLOK1 in a wild-type background and the resulting fractions were analyzed using antibodies against the different ATOM subunits. As shown in Fig. 3C all ATOM subunits co-immunoprecipitate with the tagged TbLOK1, whereas VDAC, which serves a control, remained in the supernatant. The results in Fig. 3C cannot be explained by an artefactual association of tagged TbLOK1 with the ATOM complex due to its overexpression, since a pull down using tagged ATOM12 recovers native untagged TbLOK1 and ATOM40, respectively (Fig. 3D).

In summary, these results show that TbLOK1 is a previously unidentified component of the ATOM complex.

**Ablation of TbLOK1 affects ATOM subunits and the ATOM complex**

To reveal the functional connections between TbLOK1 and the other ATOM subunits, we measured how its ablation affects the abundance of the other subunits as detected by sodium dodecyl sulfate (SDS)–PAGE and subsequent immunoblotting (Fig. 4, top panels). In addition we analyzed the same samples by BN–PAGE to investigate how TbLOK1 affects the steady state levels of the ATOM complexes (Fig. 4, bottom panels).

To minimize indirect effects in these experiments, the TbLOK1-RNAi cell line was analyzed very early, 1.5 days after induction, which is 1.5 days prior to the onset of the growth arrest (Fig. S3). The results show that ablation of TbLOK1 leads to ablation of ATOM12, whereas all other ATOM subunits remain stable. Thus, TbLOK1 is required for the stability or for import of ATOM12 (Fig. 4, top panels). However, whereas ATOM14, ATOM11 and ATOM46 are stable in the absence of TbLOK1 their assembly into high molecular weight complexes is much reduced (Fig. 4, bottom panels). The assembly of the β-barrel pore ATOM40 and the receptor subunit ATOM69 is also affected but to a much lesser extent.

Interestingly, the ATOM40-containing complexes that are still present in induced cells are more heterogenous due to the accumulation of various lower molecular weight forms (Fig. 4 second panel, Fig. S5, control). This could either be due to less efficient assembly or to a decreased stability of TbLOK1-lacking complexes. Indeed the BN-PAGE in Fig S5 shows that while ATOM40-containing complexes that lack TbLOK1 remain stable in the presence of increasing concentrations of lauryl-β-D-maltoside (DDM) they were destabilized at elevated temperature. These results suggest that under these conditions TbLOK1 exerts a stabilizing function on the ATOM complex.

As expected if TbLOK1 is indeed an essential subunit of the ATOM complex we should see similar effects in the bloodstream form since mitochondrial protein import is essential throughout the life cycle of the parasite. Fig. S6 shows that this is the case as ablation of TbLOK1 in the bloodstream form causes a decrease in the level of ATOM complex subunits as well as of other mitochondrial proteins.

**Ablation of ATOM subunits affects TbLOK1**

We also did the complementary experiments. Thus, the levels of TbLOK1 as determined by SDS-PAGE and immunoblots as well as its assembly state as revealed by BN-PAGE were analyzed in cell lines in which each of the ATOM subunits was individually ablated

(Fig. 5). The results show that ablation of ATOM40 and ATOM14 results in the degradation of TbLOK1 (Fig. 5, top panels). Alternatively the reduction in the steady state levels of TbLOK1 might be due to fact that import of TbLOK1 directly depends on ATOM40 and ATOM14. However, as shown above, the dependence of TbLOK1 on ATOM40 and ATOM14 is not reciprocal since both proteins remain stable even in the absence of TbLOK1 (Fig. 4, top panels). In all other cell lines ablated for the different ATOM subunits TbLOK1 remains stable. We have previously performed an extensive analysis of the effects that ablation of the different ATOM subunits has on the ATOM complexes as analyzed by BN-PAGE (see Fig. S4 in (Mani *et al.*, 2015)). Looking at the presence of TbLOK1 in the same type of experiments confirms the picture. In the ATOM11 and ATOM46 RNAi cell lines TbLOK1 accumulates in a lower molecular weight complex of 440 kDa (Fig. 5, bottom panels). It is worth mentioning that for unknown reasons this low molecular weight complex is sometimes (Fig. 5, bottom panels) but not always (Fig. 3A and Fig. 4, bottom panels) also visible in the uninduced RNAi cell lines. As shown previously (Mani *et al.*, 2015) ablation of ATOM12 results in a shift of the ATOM complex to a higher molecular weight that contains the major fraction of ATOM69 (Fig. 3A). TbLOK1 gets shifted in the same way. Ablation of ATOM69 on the other hand does not appear to affect TbLOK1-containing complexes. In the ATOM46/ATOM69 double knock down cell line, finally, the result looks like in the ATOM46 RNAi cell line alone.

In summary, these results (Fig. 5) together with the analysis of the TbLOK1-RNAi cell line (Fig. 4) suggest that TbLOK1 is a core subunit of the ATOM complex.

***Discussion***

TbLOK1 was discovered by a global RNAi screen for proteins whose ablation causes loss of kDNA. Further work showed that TbLOK1 is an integral mitochondrial outer membrane protein specific for Kinetoplastids (Povelones *et al.*, 2013). Its ablation caused a growth arrest in the procyclic form of *T. brucei*. The first detected phenotypes in the induced RNAi cells were a collapse of the mitochondrial network that was followed by a loss of the mitochondrial membrane potential and a decline in the respiration rate (Povelones *et al.*, 2013). Later effects included: asymmetric division of the kDNA network resulting in smaller kDNAs in daughter cells; a reduction of transcription of the maxicircle DNA and a subsequent loss of the kDNA. Based on these results it was argued that TbLOK1 might be a morphology factor that regulates mitochondrial fission and/or fusion and by doing so would influence mitochondrial function. Alternatively it was suggested that the morphology changes might be caused by an indirect effect of TbLOK1 such as modulating the lipid composition of the mitochondrial membranes (Povelones *et al.*, 2013).

Here we show that TbLOK1 likely is a novel and essential component of the trypanosomal ATOM complex that mediates import of proteins across the mitochondrial outer membrane (Mani *et al.*, 2015). This conclusion is based on the following evidences: i) Ablation of TbLOK1 interferes with mitochondrial protein import both *in vivo* and *in vitro*; ii) Co-IP analysis shows that TbLOK1 is found in the same complex as all other ATOM subunits, a result that is supported by the observation that TbLOK1 co-migrates with ATOM40, ATOM46, ATOM14, ATOM12 and ATOM11 on BN-gels; iii) TbLOK1 is functionally connected to the other ATOM subunits in a complex network of interdependencies and iv) TbLOK1 is essential in a bloodstream form cell line that can grow in the absence of the kDNA. All outer membrane proteins that behave like that in other systems are involved in mitochondrial protein import.

As in other eukaryotes more than 1000 proteins are imported into the mitochondrion of *T. brucei* (Mani *et al.*, 2016). Depletion of TbLOK1 reduces the abundance of the ATOM complex, which abolishes protein import and leads to a depletion of nuclear-encoded mitochondrial proteins. These likely include factors involved in the maintenance of mitochondrial morphology, in oxidative phosphorylation as well as in the replication and segregation of the kDNA which fully explains the previously reported phenotypes. The subunits of the ATOM complex were originally defined by the overlap between the *T. brucei* outer membrane proteome and the proteins identified in co-IPs from cells expressing HA-tagged ATOM40 (Mani *et al.*, 2015). For the co-IPs the elution was either done under denaturing conditions or under native conditions with subsequent size selection by BN–PAGE. TbLOK1 was present in two of these data sets, but for unknown reasons it was absent in the co-IP sample that was eluted under native conditions, which explains why it was missed in the previous study (Mani *et al.*, 2015).

The present study now reveals that TbLOK1 is an essential subunit of the ATOM complex, the general protein translocase of the outer membrane. We therefore suggest, in line with the nomenclature of the other ATOM subunits (Mani *et al.*, 2015), to rename TbLOK1 to ATOM19, the number reflecting its predicted molecular weight.

ATOM subunits can be divided into three groups: i) The core subunits ATOM40 and ATOM14, which are remote orthologues of the yeast Tom40 and Tom22; ii) The small subunits ATOM12 and ATOM11 which inhibit or promote the assembly of the complex and iii) The peripheral import receptors ATOM46 and ATOM69, whose ablation does not affect the stability of any of the other subunits (Mani *et al.*, 2015).

ATOM19/TbLOK1 best fits into the second group as it does not show homology to any known protein import factor and appears to influence the assembly and/or the stability of the ATOM complex. It appears to have two transmembrane domains according to various prediction algorithms. This is highly unusual for a subunit of the mitochondrial

outer membrane protein translocase, since except for the β-barrel pore Tom40, all TOM subunits in all eukaryotes that have been analyzed to date have only a single membrane-spanning domain each.

Modern eukaryotic phylogeny resolves five to six supergroups that diverged very early in evolution (Burki, 2014, Adl *et al.*, 2005). However, biochemical and functional data is only available on the TOM complexes of the yeast, *N. crassa*, higher plants and most recently from *T. brucei*. These organisms represent the three supergroups: Opisthokonts, Archeaplastidae and Excavates.

A comparative analysis shows that the only subunits, that are shared between all three TOM complexes, are a β-barrel membrane protein of the VDAC-like protein family (Tom40 in yeast and plants, ATOM40 in trypanosomatids) that forms the import channel and a closely associated conserved protein (Tom22 in yeast, Tom9 in plant, ATOM14 in trypanosomatids) that acts as a secondary import receptor and as an organizer of the whole complex (Mani *et al.*, 2015). Orthologues of Tom5, Tom6 and Tom7 are found in both the TOM complexes of yeast and plants but are absent from the trypanosomal ATOM complex (Maćasev *et al.*, 2004, Mani *et al.*, 2016). Finally, TOM complexes of all three systems have two receptor subunits each: Tom20/Tom70 in yeast; Tom20/OM64 in plants and ATOM46/ATOM69 in trypanosomatids. They do not share sequence similarity and in some cases not even the same topology indicating that the three protein receptor pairs arose independently of each other by convergent evolution.

The discovery of TbLOK1/ATOM19 as a novel subunit of the ATOM complex that is specific for Kinetoplastids and that has two transmembrane domain therefore sets the trypansomal outer membrane protein translocase even further apart from its counterparts in yeast and plants. The fact that 5 out of 7 ATOM subunits are specific for

Kinetoplastids suggests that they are a deep-branching clade within the supergroup of the Excavates or that the latter have polyphyletic evolutionary origin.

The functions of the mitochondrial protein import system, to import more than 1000 different proteins and to sort them to the correct intramitochondrial localization are conserved between yeast, plant and trypanosomatids. Revealing the detailed function of TbLOK1/ATOM19 and the other ATOM subunits in the context of protein import will help us to define the irreducible features of the import machinery that are conserved among all eukaryotes and thus will provide us with a deeper insight into the physical and biochemical constraints that shape mitochondrial protein import.

***Experimental procedures***

**Transgenic cell lines**

Procyclic RNAi cell lines of *Trypanosoma brucei* were based on the 29-13 strain (Wirtz *et al.*, 1998, Wirtz *et al.*, 1999) and grown at 27°C in SDM-79 supplemented with either 5 or 10% of fetal calf serum (FCS). Bloodstream form RNAi cell lines were based on the *T. brucei* New York single marker strain or a derivative thereof that can grow in the absence of kDNA, termed $F_1\gamma$L262P (Dean *et al.*, 2013). All bloodstream form cells were grown at 37°C in HMI-9 supplemented with 10% FCS.

RNAi cell lines directed against ATOM subunits have been described before (Mani *et al.*, 2015). RNAi of TbLOK1 (Tb927.9.10560) was done by using a pLEW100-derived stem-loop based plasmid (Wirtz *et al.*, 1999, Bochud-Allemann & Schneider, 2002). As insert, we used a 396bp long fragment covering nucleotides 48-443 of the ORF. The complementation of the TbLOK1-RNAi cell line was done using a synthetically synthesized TbLOK1 gene (GeneScript) predicted to be resistant to RNAi (Fig. S2) that was cloned into pLEW100. TbLOK1 was epitope tagged using pLEW100 derived plasmids designed to allow for addition of a triple Myc-tag at either the N- or C-terminus of the protein (Mani *et al.*, 2015). The complementation of the TbLOK1 RNAi cell lines was done using a synthetically synthesized gene (GeneScript) predicted to be resistant to RNAi that was cloned into pLEW100. The same plasmids were used to transfect a cell line expressing an in situ C-terminally HA-tagged ATOM40 (Mani *et al.*, 2015) which yielded cell lines expressing both HA-tagged ATOM40 and the corresponding Myc-tagged TbLOK1. Transformation and selection of clones were done as described in (McCulloch *et al.*, 2004). To obtain growth curves the cells were counted daily using a Neubauer hematocytometer and subsequently diluted to $4x10^6$ for the procyclic form and $10^5$ cells/ml for the bloodstream form.

**Isolation of Mitochondria**

Mitochondria of the cell lines ablated for the ATOM complex subunits were isolated at the time of the onset of the growth phenotype. Mitochondria from the induced TbLOK1-RNAi cell line were isolated after 1.5 days of tetracycline induction (the onset of the growth arrest was observed at 3.5 days of induction), in order to avoid secondary effects.

Mitochondria used for Co-IP experiments contained Myc-tagged TbLOK1. Expression of the tagged protein was induced for 3 days. Mitochondria from all cell lines mentioned above were isolated using the isotonic procedure as previously described (Schneider *et al.*, 2007b, Hauser *et al.*, 1996) and stored in 20 mM Tris pH 7.5, 0.6 M sorbitol, 2 mM EDTA containing 10 mg/ml fatty acid free bovine serum albumin.

***In vitro* protein import**

$^{35}$S-Met labeled substrate proteins were synthesized using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Product No L1170). For the LDH-DHFR precursor, the same plasmid as described before (Pusnik *et al.*, 2011) encoding for a chimeric protein consisting of the first 150 amino acids of the *T. brucei* LDH fused to mouse DHFR was used. To produce the TAO precursor a PCR product consisting of the complete ORF of the protein (Tb927.10.7090) that was flanked by a T7-RNA polymerase promoter and 3' DNA stretch encoding a poly A-tail was used as a template.

*In vitro* protein import was basically done as described (Hauser *et al.*, 1996). 25μg of isotonically isolated mitochondria were incubated in 20 mM HEPES-KOH pH 7.4, 0.6 M sorbitol, 25 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM $KH_2PO_4$, 5 mg/ml fatty acid free BSA containing 4 mM ATP pH 7. 0.5 μg creatine kinase and 20mM phosphocreatine. The radiolabeled precursor proteins were added for the indicated times. The total reaction volume was 25 μl. The reactions were stopped by adding 0.5 μl of 0.2 mM valinomycine and 5 mM of carbonyl cyanide m-chlorophenyl hydrazone. All samples were digested

with 80 μg/ml of proteinase K for 10 min on ice. Proteinase K was inactivated by the addition of 3.6 mM of phenylmethylsulfonyl fluoride. Mitochondria were reisolated by centrifugation and the resulting pellets were processed for SDS-PAGE analysis. Autoradiographs of the SDS-PAGE were analysed using a PhosphoImager (FujiFilm FLA3000).

**Blue native PAGE**

Isotonically isolated mitochondria (Schneider *et al.*, 2007b, Hauser *et al.*, 1996) or pellets from digitonin fractionations corresponding to crude mitochondria (Fig. S4) were solubilized in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% glycerol and 0.1mM EDTA containing 1 to 1.5% (w/v) digitonin. The samples were centrifuged with 20'800g at 4°C and the resulting supernatants were separated on 4-13% gradient BN gels. Before blotting the gels on polyvinylidene fluoride membranes (Merck Millipore, Product No IPFL00010) the gels were soaked in SDS running buffer (25mM Tris, 190mM glycine, 1mM EDTA, 0.05% (w/v) SDS). Proteins were detected using horseradish peroxidase-coupled secondary antibody with the Super Signal West Pico Chemiluminescence Substrate or Femto Maximum Sensitivity Substrate Kits (Thermo Scientific, Product No 34080 or 35095).

**Co-Immunoprecipitations**

The reciprocal co-IPs shown in Fig. 3B were done as previously described for the other ATOM complex subunits (Mani *et al.*, 2015). In short, digitonin-purified crude mitochondrial fractions were solubilized in lysis buffer (20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 25 mM KCl) containing 1% (w/v) digitonin and 1 x protease inhibitor mix (Roche, Product No. 11873580001). After 15 min incubation on ice, the lysate was cleared by a 10 min centrifugation step at 20'800g and incubated with either anti-Myc beads (Clontech Laboratories, Inc., Product No. 631208) or anti-HA beads

(Roche, Product No. 11815016001) that had previously been equilibrated with lysis buffer containing 0.1% (w/v) digitonin. The resulting mixtures were incubated for 2 h at 4°C under constant mixing, washed three times with lysis buffer, containing 0.1% (w/v) digitonin and 1 x protease inhibitor mix, and eluted by boiling in SDS-PAGE sample buffer.

The procedure for the Co-IPs shown in Fig. 3C was essentially identical as described above, except that instead of digitonin-purified crude mitochondrial fractions 520 μg of isotonically isolated mitochondria purified from the Myc-TbLOK1-expressing cells were used. The mitochondrial pellet was solubilized in 600 μl lysis buffer as described above. Incubation was with 100 μl of 1:1 slurry of anti-Myc beads (Sigma, Product No E6654). Washes and elution were done as above.

**Miscellaneous**

To prepare a crude mitochondrial fraction cells were extracted with low concentrations of digitonin (Schneider *et al.*, 2007a). In short, $10^8$ cells were harvested, washed in phosphate buffered saline and resuspended in 0.5 ml 1 x SoTE, prewarmed to ambient temperature. Next 0.5 ml of ice-cold 1 x SoTE containing 0.03% (w/v) of digitonin was added. The mixture was incubated on ice for 5 min and centrifuged at 4°C for 5 min with 6'800 g. The resulting pellets correspond to the crude mitochondrial fractions used in some of the experiments mentioned above.

The immunofluorescence analysis shown in Fig. S1 was done as described (Mani *et al.*, 2015). To stain the mitochondrion polyclonal rabbit anti-ATOM40 (dilution 1:1'000) and polyclonal mouse anti-mtHsp60 (dilution 1:200) antisera were used. The secondary antibodies used were goat anti-rabbit FITC (dilution 1:100, Sigma Product No. F0382) and goat anti-mouse Alexa Fluor 633 (dilution 1:1'000, Thermo Scientific Product No. A-21052).

Images were acquired with a DVC360 FX monochrome camera (Leica Microsystems) mounted on a DMI6000B microscope (Leica Microsystems). Downstream analysis and deconvolution was done using the Leica Application Suite X software (Leica Microsystems).

The polyclonal TbLOK1 rabbit antisera used for immunoblots was produced commercially by Eurogentec. The antigen, a his-tagged version of TbLOK1, was expressed in *E. coli*. Subsequently TbLOK1-containing inclusion bodies were isolated and the protein was further purified by SDS-PAGE. The band corresponding to TbLOK1 was cut out and used for immunization. For immunoblots derived from BN gels blotting, a previously described TbLOK1 antibody was used (Povelones *et al.*, 2013).

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**Author contributions**

S.D. and J.M. carried out most of the experiments and analyzed the data. S.K. discovered that TbLOK1 migrates in a high molecular weight complex on BN-gels. A.H. performed the complementation experiments. A.S. analyzed the data and wrote the paper.

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**Figure legends**

**Fig. 1. TbLOK1 is essential in a cell line that does not depend on mitochondrial DNA.** Growth curves of uninduced (-Tet) and induced (+Tet) bloodstream form TbLOK1-RNAi cell lines. Top panel: bloodstream form TbLOK1-RNAi cell line. Solid and broken lines depict two independent experiments. Bottom panel: TbLOK1-RNAi cell line based on the $F_1\gamma$L262P bloodstream strain that can grow in the absence of the kDNA. Mean and standard deviation of triplicate experiments are indicated. Inset Western blots show the successful downregulation of TbLOK1 after two days of induction. Ef1a is used as loading control. The cross indicates that after three days of induction no living cells could be detected.

**Fig. 2. Ablation of TbLOK1 interferes with mitochondrial protein import. A.** Upper panel: *In vivo* analysis of mitochondrial protein import during ablation of TbLOK1. Immunoblots of whole cell extracts showing the steady state levels of the mitochondrial proteins REAP1, IF2, CoxIV, VDAC and TbLOK1 in the procyclic TbLOK1-RNAi cell line. Cytosolic Ef1a serves as a loading control. Time of induction in days (days + Tet) is indicated at the top. Black triangle indicates the onset of the growth arrest. OM, outer membrane; IM, inner membrane; Cyt, cytosol. Precursor (p) and imported mature proteins (m) are indicated. Lower panel: quantification of immunoblots of four independent experiments demonstrating the TbLOK1-dependent reduction of the steady state levels of the indicated mitochondrial proteins, relative to Ef1a. Standard errors are indicated. **B.** *In vitro* import of the $^{35}$S-methionine-labeled precursor proteins LDH-DHFR and TAO into mitochondria isolated from the procyclic TbLOK1-RNAi cell line grown in the absence or the presence (1.5 days) of tetracycline. IN, input. Precursor

(p) and imported mature proteins (m) are indicated. The samples where the membrane potential (ψ) was inactivated served as negative controls. Segments of the Coomassie-stained gels that do not coincide with the gels containing the labeled proteins are shown as loading controls.

**Fig. 3. TbLOK1 is a subunit of the ATOM complex. A.** BN-PAGE immunoblots of mitochondrial membrane extracts from procyclic wildtype cells were probed with antisera against all six ATOM subunits and TbLOK1. Molecular weight markers (kDa) are indicated. **B.** Reciprocal Co-IPs of C-terminally tagged ATOM40 and N- as well as C-terminally tagged TbLOK1. Mitochondrial membrane extracts of procyclic cell lines co-expressing C-terminally HA-tagged ATOM40 and N- or C-terminally Myc-tagged TbLOK1, respectively, were subjected to Co-IPs using anti-HA or anti-Myc antisera, respectively. Immunoblots containing 5% input (IN), 100% eluate (IP) and 5% flow through fractions (FT) were probed for the presence of HA-tagged ATOM40 and the Myc-tagged TbLOK1, respectively. As a control the panel stained for Myc-tagged TbLOK1 was reprobed for the abundant mitochondrial OM protein VDAC. **C.** Mitochondrial membrane extracts of a procyclic cell line expressing N-terminally Myc-tagged TbLOK1 was subjected to IP using an anti-Myc antiserum. The resulting fractions were probed for tagged TbLOK1, for all ATOM subunits and for VDAC which serves as a control. **D.** Mitochondrial membrane extracts of a procyclic cell line expressing C-terminally Myc-tagged ATOM12 was subjected to IP using an anti-Myc antiserum. The resulting fractions were probed for native untagged TbLOK1 and ATOM40. VDAC served as a control.

**Fig. 4. Ablation of TbLOK1 affects ATOM subunits and the ATOM complex.** Top panels: mitochondrial membrane extracts of the uninduced (-Tet) and induced (+Tet) procyclic TbLOK1-RNAi cell line were separated by SDS-PAGE and the resulting immunoblots were probed using antisera against TbLOK1 and the indicated ATOM

subunits. Bottom panels: the same extracts were separated by BN-PAGE and the resulting immunoblots were analyzed as in the top panel. The precentage of the remaining complex after RNAi is indicated for each cell line.

**Fig. 5. Ablation of ATOM subunits affects TbLOK1.** Top panels: mitochondrial membrane extracts of the indicated uninduced (-Tet) and induced (+Tet) procyclic RNAi cell lines were separated by SDS-PAGE and the resulting immunoblots were probed using the TbLOK1 antiserum. Bottom panels: the same extracts were separated by BN-PAGE and the resulting immunoblots were analyzed as in the corresponding panel on the top. A detailed characterization of the cell lines allowing inducible ablation of each individual ATOM subunit including growth curves and immunoblots has been published before (Mani *et al.*, 2015).

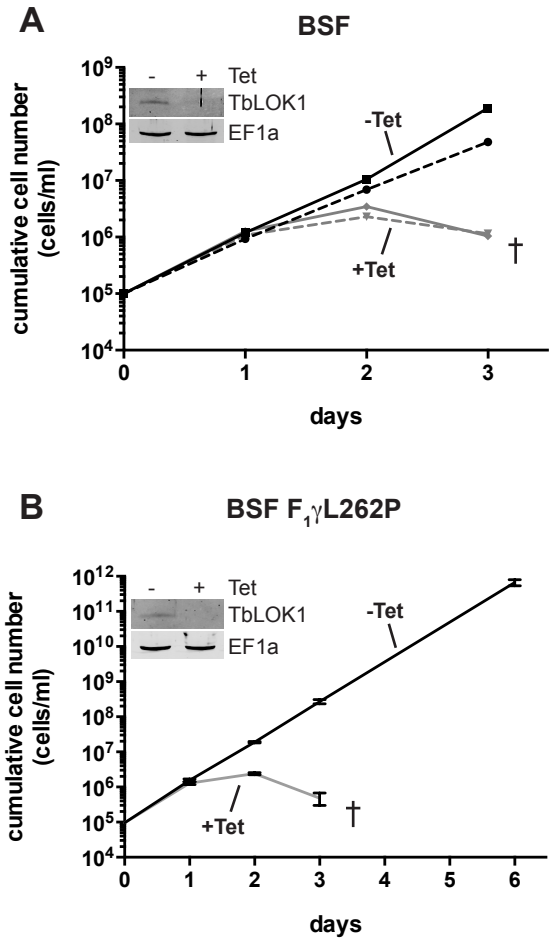
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Figure 1

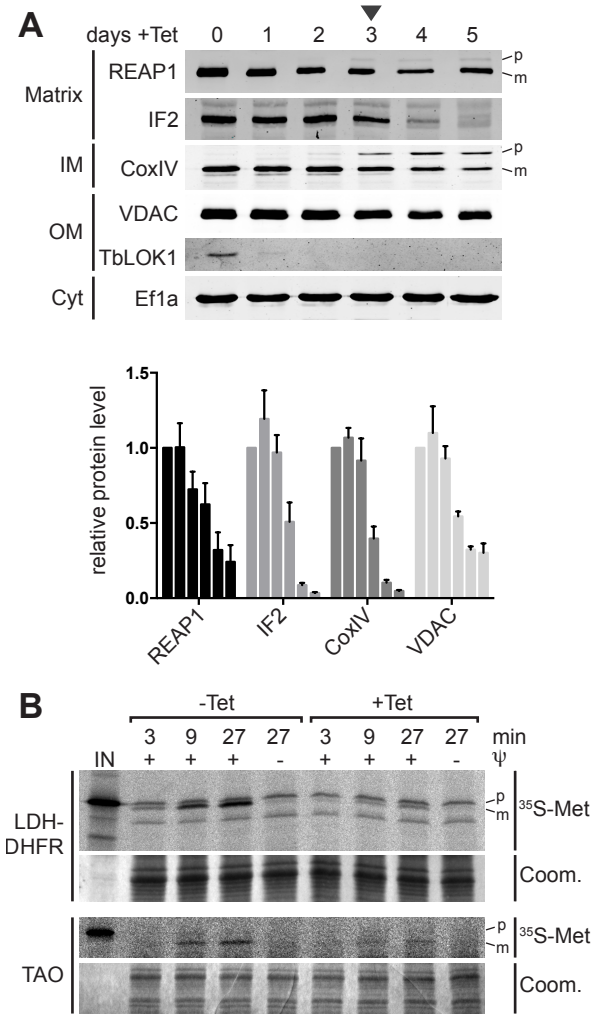
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Figure 2

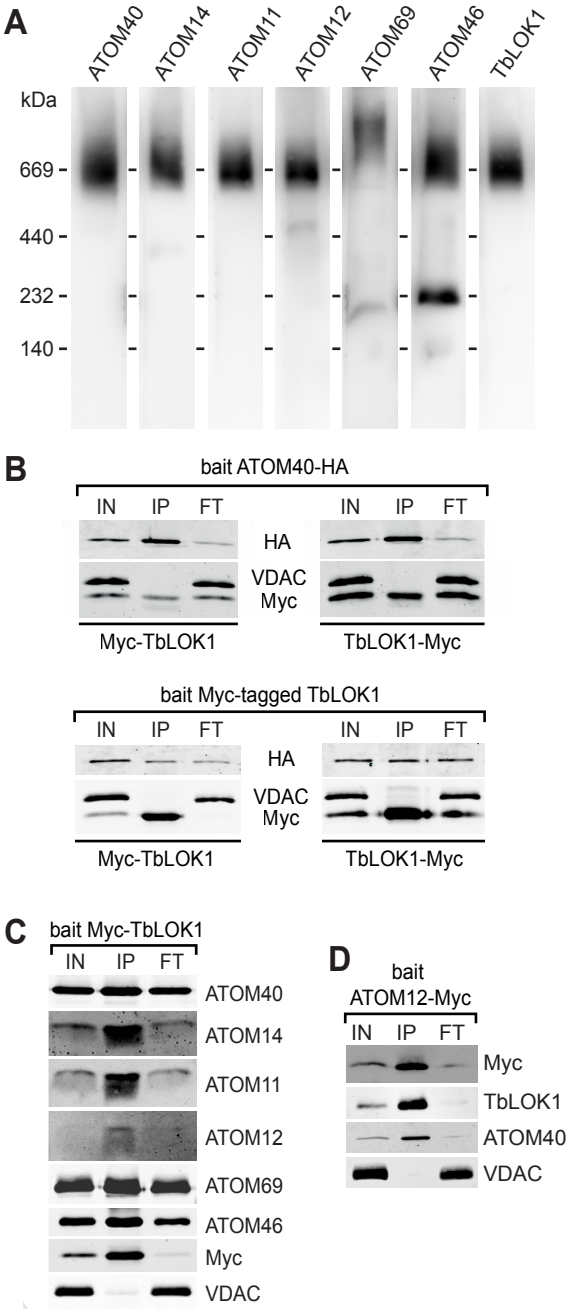
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Figure 3

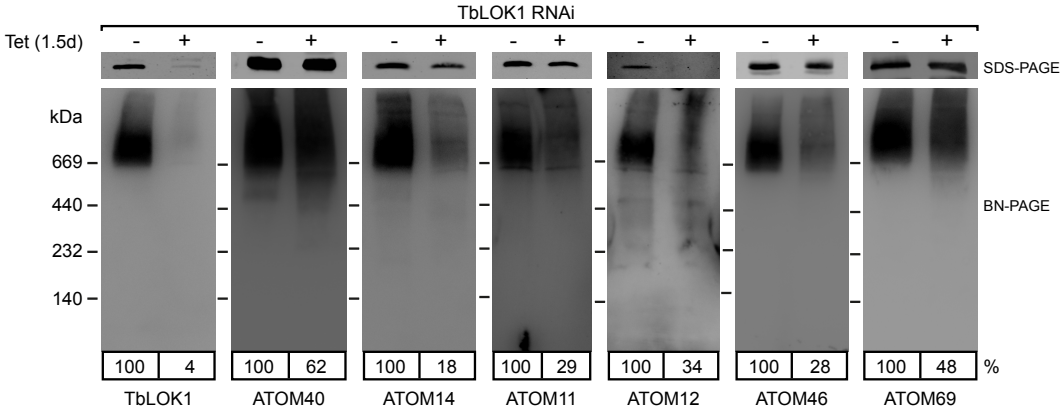


Figure 4

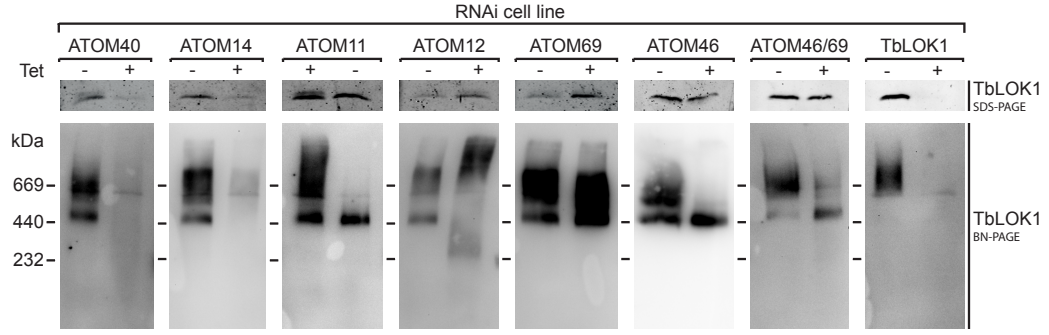


Figure 5