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Supplementary Information for
Determinism and contingencies shaped the evolution of mitochondrial protein import

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Supplementary text
Figures S1 to S5
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Other supplementary materials for this manuscript include the following:

Dataset S1. SILAC proteomics: ATOM46 RNAi
(Excel files separately submitted)

Dataset S2. SILAC proteomics: ATOM69 RNAi.
(Excel files separately submitted)

Dataset S3. SILAC proteomics: ATOM69 replaced by *T.vaginalis* Tom36.
(Excel files separately submitted)

Dataset S4. SILAC proteomics: ATOM46 replaced by yeast Tom20.
(Excel files separately submitted)

Supplementary Information Text

Material and Methods

Transgenic cell lines

Transgenic procyclic *T. brucei* cell lines were generated using strain 29-13 (1). All cell lines were grown in SDM-79 medium (2) supplemented with 10% (vol/vol) foetal calf serum (FCS) (Sigma Aldrich) at 27 °C, or at 33 °C where indicated. ATOM46 (Tb927.11.7780) and ATOM69 (Tb927.11.11460) RNAi cell lines (Fig. 1 A,B) were generated using the a pLew100-derived stem-loop plasmid in which the phleomycine resistance had been replaced by the blasticidin resistance gene (1, 3). This plasmid facilitates the ligation (using BamHI/XhoI and HindIII/XbaI) of the same RNAi insert in opposite directions. The presence of a 460 bp spacer fragment between the two RNAi inserts generates a stem-loop construct. The RNAi targets the nucleotides in the 3' untranslated region (UTR) of ATOM46 (nt 1 to 254) and in the open reading frame (ORF) of ATOM69 (nt 687 to 1113), respectively. Additionally, for complementation experiments (Fig. 4), another ATOM69 RNAi cell line was generated. Here the RNAi targets the 3' UTR of ATOM69 (nt 194 to 668).

For complementation experiment shown in Fig. S1 and S2, DNA fragments corresponding to ATOM69 and its truncated ORF variants (ΔN103, nt 310-1872; ΔN187, nt 562-1872) were PCR-amplified using *T. brucei* 427 genomic DNA. Subsequently the fragments were cloned into modified pLew100 vectors, carrying puromycine resistance genes, that allows the addition of a segment encoding a C-terminal triple c-Myc cassette. The resulting constructs were transfected into the ATOM69 RNAi cell line targeting the 3'-UTR described above.

For the complementation experiment shown in Fig.S4 and S5, fusion PCR produced a DNA fragment, termed TbTMD-Tom20, encoding the ATOM46 membrane anchor (nt 1 to 276) followed by the yeast Tom20 soluble domain (nt 124-425) using *T. brucei* 427 and *S. cerevisiae* genomic DNA as templates. For complementation experiment shown in Fig. 4 and 5, fusion PCR produced a DNA fragment, termed Tom36-TbTMD, encoding the soluble domain of *T. vaginalis* Tom36 (nt 1-876) followed by the C-terminal membrane anchor of ATOM69 (nt 1689-1869) using *T. brucei* 427 genomic DNA and *T. vaginalis* cDNA as templates. Subsequently both fusion constructs were cloned into a modified pLew100 vector carrying the puromycine resistance gene that allows the addition of a cassette encoding a C- or N-terminal c-Myc epitope, respectively (3). TbTMD-Tom20 plasmid was transfected in the ATOM46 RNAi cell line whereas Tom36-TbTMD plasmid was transfected in the ATOM69 RNAi cell line, both targeting the 3'-UTRs of their corresponding genes as described above.

Antibodies

The following primary antibodies were used for immunoblots at the indicated dilutions: rabbit anti-ATOM46 (1:50); rabbit anti-ATOM69 (1:50) (4); rabbit anti-ATOM40 (dilution 1:1000); rabbit anti-CoxIV (dilution 1:1000); rabbit anti-cytochrome C (Cyt C) (dilution 1:1000) (5); mouse anti-elongation factor 1a (EF1a) (dilution 1:10.000, Product No. 05–235, Merck Millipore); mouse anti-c-Myc (1:2000; Product No. 132500, Invitrogen). Secondary antibodies used in this study were: goat anti-mouse IRDye 680LT, goat anti-rabbit IRDye 800CW (dilution 1:20.000, LI-COR

Biosciences) and horse radish peroxidase-coupled goat anti-mouse/rabbit (dilution 1:5000, Sigma-Aldrich).

Digitonin extraction

Digitonin extractions were done as described (3). In short, expression of the epitope tagged proteins was induced by treating the respective cell lines with 1 µg/ml of tetracycline (Sigma) for 24 hours prior to harvesting. Subsequently 1 × 108 cells were harvested, washed in PBS, resuspended in 1 ml of 1x SoTE buffer (20mM Tris-HCl pH 7.5, 0.6 M sorbitol and 2 mM EDTA) containing 0.015 % (w/v) digitonin and were incubated on ice for 10 min. This mild digitonin treatment ensures effective lysis of the plasma membrane, whereas the mitochondrial membranes remain intact. Differential centrifugation (6.800 g, 5 min, 4 °C) yields an organelle-mitochondria enriched pellet fraction and a cytosolic protein enriched soluble fraction. Equal cell equivalents of both these fractions alongside whole cells were mixed with SDS sample buffer and were analysed by SDS-PAGE and immunoblotting. Furthermore, the above generated pellet fractions were subjected to carbonate extraction.

Carbonate extraction

To distinguish between peripheral/loosely associated membrane proteins from integral membrane proteins, the above generated pellet fractions were resuspended in 160 µl of 100 mM Na2CO3 pH 11.5. 80 µl were removed which served as ‘total’ fraction. The remaining 80 µl were incubated on ice for 10 min and centrifuged (100,000 g, 10 min, 4 °C) to yield a soluble fraction, corresponding to peripheral membrane protein, and a pellet fraction, corresponding to integral membrane proteins. Equal cell equivalents of all 3 fractions were analysed by SDS-PAGE and immunoblotting.

Blue native PAGE

Blue native was done as described (6). In short, the crude mitochondrial fractions generated by digitonin extraction were solubilized in mitochondria lysis buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 10% glycerol, 0.1 mM EDTA containing 1% (w/v) digitonin) for 15 min at 4 °C. Solubilized extracts obtained after centrifugation (20.817 g, 15 min, 4 °C) were analysed by electrophoresis in a gradient gel (4-13% acrylamide). Furthermore, the gels were soaked in SDS-PAGE running buffer (25 mM Tris, 1 mM EDTA, 190 mM glycine, 0.05% (w/v) SDS) prior to western blotting. This ensures efficient transfer of proteins onto membrane during blotting.

Purification of cytosolic receptor domains by Ni-NTA affinity chromatography

The soluble cytosolic domain of ATOM46 (Tb927.11.7780 amino acids: 37–419) and ATOM69 (Tb927.11.11460 amino acids: 1–569) were fused with a hexahistidine tag in the N- or C-terminus, respectively, and expressed in E.coli. The tagged proteins were purified using Ni-NTA affinity chromatography and the purity was assessed by SDS-PAGE. In short, bacterial cultures were grown in LB medium (containing 100 µg/ml kanamycin or ampicillin for ATOM46 or ATOM69 respectively) at 37 °C to an OD600 of 0.5-1. Subsequently, expression was induced by addition of isopropyl- β-D-thiogalactopyranosid (IPTG) to 1 mM for 2 hours at 37 °C. Aliquots of 50ml bacterial cultures were centrifuged (5000 g, 15 min, RT). Following removal of the

supernatant the bacterial pellets were stored at -20 °C. 4 bacterial pellets were resuspended in 10 ml of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8) containing 2 mM phenylmethylsulfonyl fluoride (PMSF). ATOM46 and ATOM69 bacterial suspensions were lysed by sonication on ice (3 x 10 pulses of 20 seconds) and two rounds of French press, respectively. Following cell disruption, the corresponding suspensions were centrifuged (26.000 g, 4 °C, 30 min) and the supernatants were incubated for 2 hours at 4 °C with Ni-NTA magnetic beads (Catalog number: 78606, Thermo Scientific) that had been pre-equilibrated with lysis buffer. Beads were recovered using a magnetic stand and were washed once with 10 ml wash buffer I (50 mM NaH2PO4, 300 mM NaCl, 20 mM Imidazole, pH 8) and twice with 10 ml wash buffer II (50 mM NaH2PO4, 300 mM NaCl, 50 mM imidazole, pH 8). The corresponding protein loaded beads were stored in storage buffer (wash buffer I containing 2 mM DTT and 1% (w/v) of bovine serum albumin (BSA)) at 4 °C for 24 hours prior to use. The purity of proteins bound to the beads were analysed on an SDS-PAGE after elution with imidazole buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM Imidazole, pH 8). BSA standard was used to determine the quantity of proteins. Equilibrated Ni-NTA beads that were not exposed to His-tag proteins (control beads) were also resuspended in storage buffer and kept at 4 °C for 24 hours prior to use to serve as bed volume control to access the background of the binding assays.

Receptor binding assays

Radioactive ([35S]-Met-labelled) precursor proteins LDH (aa 1-14)-DHFR (mouse), substrates Tb927.11.14730, Tb927.11.9560, Tb927.10.13600 and Tb927.5.3640 were synthesized in vitro using the TNT T7 Quick coupled transcription/translation systems (Promega) as previously described (4). The quantity and quality of the radioactive precursor proteins was determined via SDS-PAGE and autoradiography. For binding assays, bed volume beads loaded with 800 pmol of the soluble receptor domains and equal volume of control beads were used. After washing the beads three times with 500 µl of assay buffer (20 mM imidazole, 10 mM MOPS-KOH pH 7.2, 1% (w/v) BSA, 0.5% (w/v) digitonin), containing either 50 mM or 400 mM KCl, the beads were resuspended in one bed volume of assay buffer. 3 µl [35S]-Met-labelled precursor proteins were added to the assay volume. The beads were incubated for an hour at 27 °C on a thermomixer at 600 rpm. Subsequently, they were washed three times with 500 µl assay wash buffer without BSA (20 mM imidazole, 10 mM MOPS-KOH pH 7.2, 0.5% (w/v) digitonin) containing either 50 mM or 400 mM KCl. Finally, bound proteins were eluted with 200 µl of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, pH 8). The eluted proteins were precipitated with trichloroacetic acid (TCA), washed with acetone, resuspended in 1x SDS sample buffer and analysed on a 13% SDS-PAGE followed by digital autoradiography along with the input (13%).

Immunoprecipitations

Digitonin-extracted crude mitochondrial fraction of 1x108 cells expressing the c-Myc tag protein of interest were washed in PBS and subsequently solubilized in 200 µl of lysis buffer (20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 50 mM KCl) containing 1% (w/v) digitonin and 1x protease inhibitor mix (Roche, EDTA-free Product No. 11873580001) for 15 mins on ice. After centrifugation (20.817 g, 15 min, 4 °C), the lysate (input: IN) was incubated with 40 µl of anti-c-Myc beads (EZview red anti-c-Myc affinity gel, Sigma) that had been pre-equilibrated with 1.5 ml of wash buffer (20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 50 mM KCl) containing 0.1% (w/v) digitonin). After incubation at 4 °C for 2 hours, the supernatant with the unbound proteins (flow through: FT) was discarded and the beads were washed thrice with 500 µl of wash buffer (20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 50 mM KCl) containing 1% (w/v) digitonin and 1x protease inhibitor mix. Bound proteins were eluted by boiling the beads for 3 mins in 40 µl of SDS-PAGE sample buffer without β-mercaptoethanol. The input (IN), flow

through (FT) (5% each) and eluate (IP) (100%) were subjected to SDS- PAGE and immunoblotting.

SILAC RNAi experiments

SILAC RNAi experiments were primarily done as described in (7). *T. brucei* cell lines allowing inducible RNAi and/or simultaneous expression of c-Myc-tagged proteins were washed in filter sterile PBS and were transferred to SDM-80 medium (8) supplemented with 5.55mM glucose, either with light (unlabelled: 12C6/14Nχ) or heavy (labelled: 13C6/15Nχ) isotopes of lysine (0.4mM) and arginine (1.1mM) (Euroisotop) and 10-15% dialyzed FCS (BioConcept, Switzerland). For efficient labelling of proteins with either light or heavy amino acids *T. brucei* cells were cultured for at least 8-10 doubling times in SILAC medium.

T. brucei RNAi cell lines for ATOM46 (Fig. 1A) and ATOM69 (Fig. 1B), the ATOM69 RNAi cell line complemented with Tom36-TbTMD (Fig. S3A) and the ATOM46 RNAi cell line complemented with TbTMD-Tom20 (Fig. S5A), were induced with tetracycline for 6, 5, 4 and 9 days respectively. Subsequently the induced (1x108 cells) and the non-induced (1x108 cells) cultures were mixed in a 1:1 ratio prior to harvest. A crude mitochondrial pellet of the mixed cells (2x108) was generated by digitonin treatment and later analysed by MS. All SILAC RNAi experiments were done in three biological replicates including a label-switch.

Quantitative proteomics

Proteins of mitochondria-enriched fractions were either separated by SDS-PAGE and subsequently digested in-gel (ATOM69 RNAi experiments) or directly digested in solution (ATOM46 RNAi, Tom36-TbTMD and TbTMD-Tom20 complementation experiments) using trypsin and processed for liquid chromatography-mass spectrometry analysis as described before (9). Peptide mixtures were analysed using an Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) coupled to an UltiMate 3000 RSLCnano HPLC system (Thermo Fisher Scientific, Dreieich, Germany) applying a 60-min solvent gradient ranging from 7.5% methanol/4.5% acetonitrile to 49.5% methanol/29.7% acetonitrile in 0.1% formic acid (samples from in-gel digests) or a 310-min solvent gradient ranging from

0.5% methanol/0.3% acetonitrile to 45.6% methanol/28.5% acetonitrile in 4% dimethyl sulfoxide/0.1% formic acid (samples from in-solution digests) at flow rates of 250 nl/min. Mass spectrometric raw data, acquired in data-dependent mode, were processed using MaxQuant/Andromeda (version 1.6.3.4 for the ATOM46 RNAi dataset and version 1.6.5.0 for the other datasets; (10, 11)) and all entries for *T. brucei* TREU927 downloaded from the TriTryp database (version 8.1; https://tritrypdb.org/tritrypdb/) plus the sequences for the Tom36-TbTMD and TbTMD-Tom20 hybrid proteins for protein identification and SILAC-based relative protein quantification. MaxQuant default settings were used except that protein identification and relative quantification were based on ≥ 1 unique peptide and SILAC peptide pair, respectively. See supplementary datasets S1- S4 for lists of proteins identified in the individual datasets.

In silico tools for receptor preference analysis

Receptor preference analysis using SILAC data (Fig. 3 and Fig. 5) was done using three online tools. Presence of mitochondrial presequence was determined using MitoFates (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi), hydrophobicity of substrates was calculated using

the GRAVY score (http://www.gravy-calculator.de/index.php) and the transmembrane prediction was done using HMMTOP (http://www.enzim.hu/hmmtop/). Statistical significance was calculated using Analysis of covariance (ANCOVA) in R Archive Network (http://cran.r-project.org/). $p < 0.05$ was considered significant.

Fig. S1.

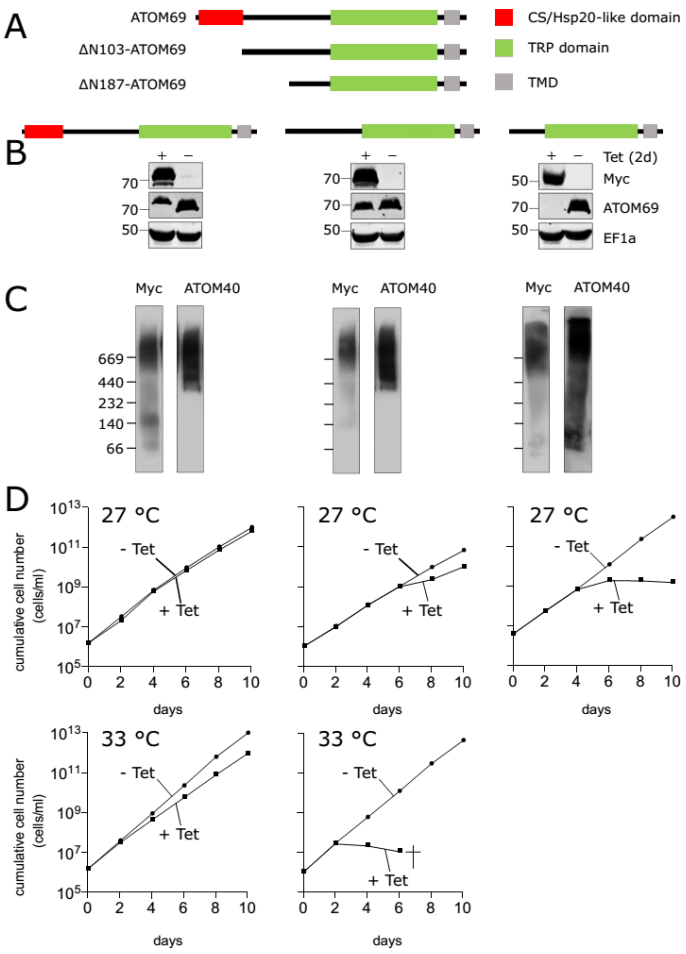

Fig. S1. The CS/Hsp20-like domain of ATOM69 is essential for growth at 33°C

(A) Schematic representation of ATOM69 and its truncated versions used in complementation experiments. All versions are expressed in the background of ATOM69 3' UTR RNAi. **(B)** Immunoblots probed with antisera against Myc and ATOM69 shows the efficient replacement of endogenous ATOM69 by tagged ectopically expressed versions. EF1a serves as a loading control. **(C)** Crude mitochondrial fractions of the indicated complemented cell lines were analysed by BN-PAGE. The corresponding immunoblots were probed using antisera against Myc and ATOM40. **(D)** Growth curves of the indicated uninduced (-Tet) and induced (+ Tet) complemented cell lines at 27 °C (top panel) and at 33 °C (bottom panel).

Fig. S2.

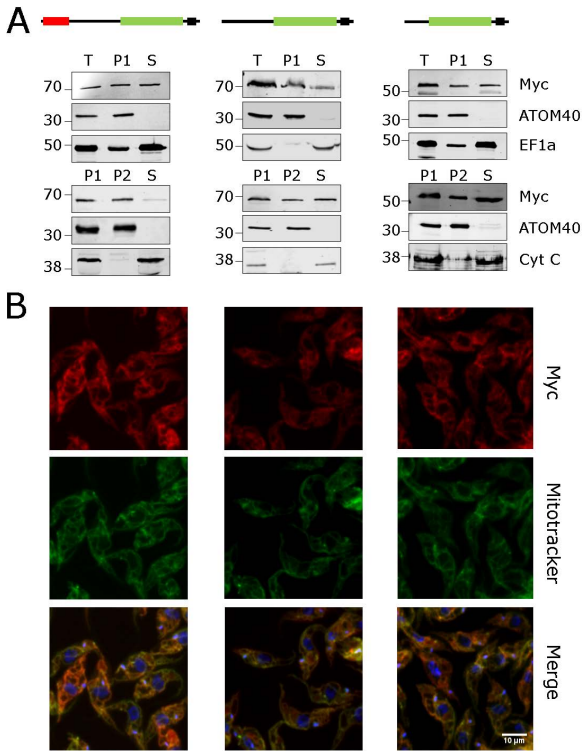

Fig. S2. ATOM69 and its truncated versions are integral mitochondrial membrane proteins.

(A) Immunoblot analysis of total-cell (T), digitonin-extracted crude mitochondrial pellet (P1) and soluble (S) fractions of cells expressing c-Myc-tagged ATOM69 variants (top panel). ATOM40 and EF1a serves as mitochondrial and cytosolic markers, respectively. The digitonin-extracted crude mitochondrial pellet (P1) was subjected to carbonate extraction. Bottom panel shows immunoblot analyses of the starting P1 pellet, the P2 pellet and the soluble fraction (S) after carbonate extraction (bottom panel). ATOM40 and cytochrome C (Cyt C) serves as integral and peripheral membrane markers, respectively. **(B)** IF analysis of cells expressing the c-Myc-tagged ATOM69 variants. Mitotracker serves as a mitochondrial marker. Scale bar, 10 µm.

Fig. S3.

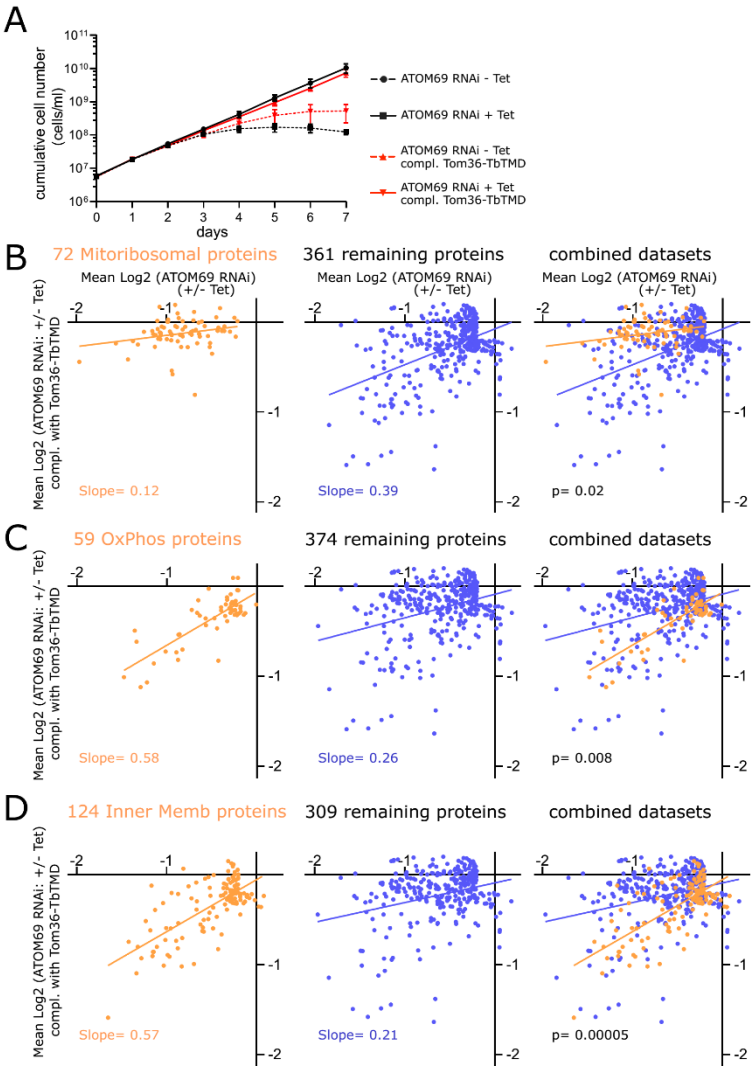

Fig. S3. Chimeric Trichomonas Tom36 does not complement loss of function of ATOM69 however restores a subset of *T. brucei* mitochondrial proteins.

(A) Growth curves of uninduced (-Tet) and induced (+ Tet) ATOM69 3' UTR RNAi cell line (black) and ATOM69 3' UTR RNAi cell line complemented with N-terminal c-Myc-tagged Tom36-TbTMD (red) in SDM-79 medium. **(B)** 72 detected mitoribosomal proteins (orange dots) were significantly (p= 0.02) restored when compared with 361 remaining proteins (blue dots). Linear regressions of both groups are indicated by the orange and blue lines, respectively. **(C)** 59 detected proteins of the oxidative phosphorylation complex (orange dots) are significantly down-regulated (p= 0.008) even after expression of Tom36-TbTMD when compared with the 374 remaining proteins (blue dots) indicating that these proteins are not restored. Linear regressions of both groups are indicated by the orange and blue lines, respectively. **(D)** Like in (C), 124 detected inner membrane proteins (orange dots) are not restored (p= 0.00005) when compared with 309 remaining proteins (blue dots). Linear regressions of both groups are indicated by the orange and blue lines, respectively.

Fig. S4.

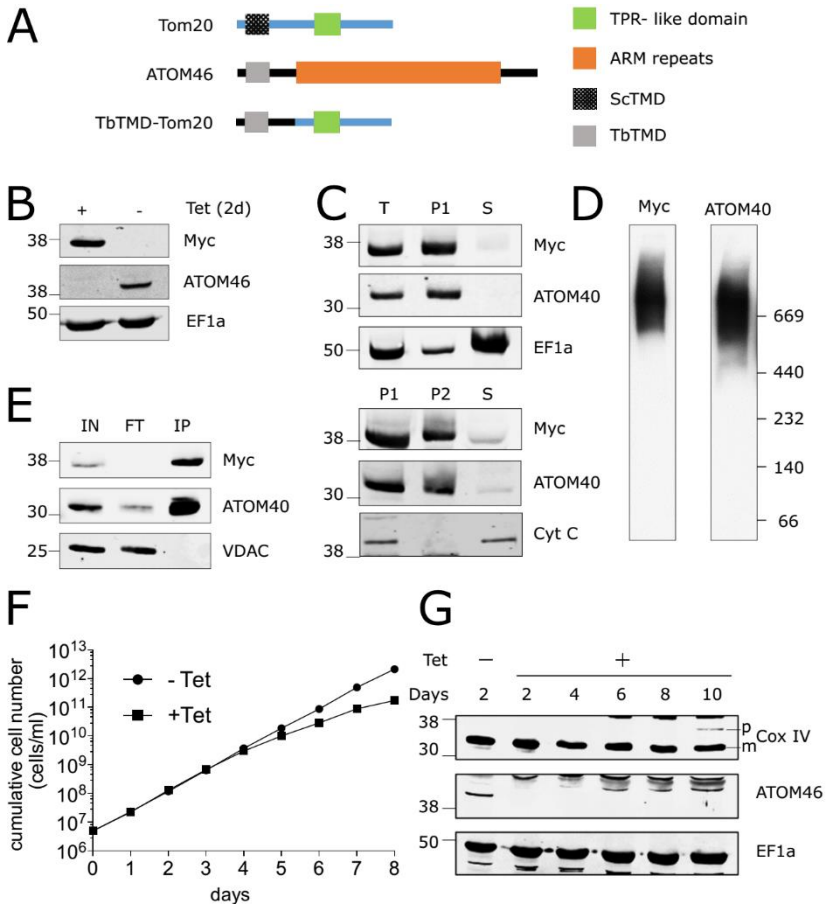

Fig. S4. Chimeric yeast Tom20 integrates into the ATOM complex

(A) Domain structure of S. cerevisiae Tom20, ATOM46 and its chimera (TbTMD-Tom20). TbTMD-Tom20 is expressed in the background of ATOM46 3' UTR RNAi. **(B)** Immunoblots probed with antisera against Myc and ATOM46 shows the efficient replacement of endogenous ATOM46 by the chimeric TbTMD-Tom20. Elongation factor 1a (EF1a) serves as a loading control. **(C)** Immunoblot analysis of total-cell (T), digitonin-extracted mitochondria-enriched pellet (P1) and soluble (S) fractions of cells expressing c-Myc tagged TbTMD-Tom20 (top panel). ATOM40 and EF1a serves as mitochondrial and cytosolic markers, respectively. The digitonin-extracted mitochondria-enriched pellet (P1) was subjected to carbonate extraction. Immunoblot analysis of total (P1), pellet (P2) and soluble fraction (S) after carbonate extraction (bottom panel). ATOM40 and cytochrome C (Cyt C) serves as integral and peripheral membrane markers, respectively. **(D)** Crude mitochondrial fractions of the tagged TbTMD-Tom20 expressing line were analysed by BN-PAGE. The corresponding immunoblots were probed using antisera against c-Myc (Myc) and ATOM40. **(E)** Co-immunoprecipitation using tagged c-Myc-tagged TbTMD-Tom20 (Myc) as the bait. VDAC serves as a negative control. **(F)** Growth curves of uninduced (-Tet) and induced (+ Tet) ATOM46 3' UTR RNAi cell line ectopically expressing the tagged TbTMD-Tom20. **(G)** Immunoblots showing steady state levels of CoxIV, ATOM46 in whole cell extracts of the same cell line as in (F) collected at the indicated time points after Tet induction. EF1a serves as loading control.

Fig. S5.

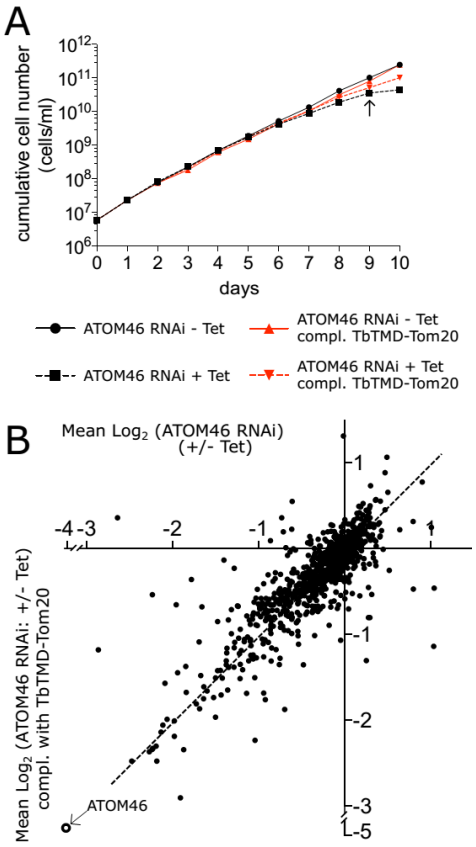

Fig. S5. Tom20 cannot restore import of *T. brucei* proteins

(A) Growth curves of uninduced (-Tet) and induced (+ Tet) ATOM46 3' UTR RNAi cell line (black) and ATOM46 3' UTR RNAi cell line complemented with c-terminal c-Myc-tagged TbTMD-Tom20 (red) in SILAC medium. Arrow indicates the day when the cells were harvested for SILAC proteomics. **(B)** Scatter plot depicting fold change in abundance of 950 mitochondrial proteins upon ablation of ATOM46 (X-axis) and after ATOM46 has been replaced by TbTMD-Tom20 (Y-axis).

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