Current Biology 21, 1738–1743, October 25, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.08.060

Report

Mitochondrial Preprotein Translocase of Trypanosomatids Has a Bacterial Origin

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Summary

Mitochondria are found in all eukaryotic cells and derive from a bacterial endosymbiont [1, 2]. The evolution of a protein import system was a prerequisite for the conversion of the endosymbiont into a true organelle. Tom40, the essential component of the protein translocase of the outer membrane, is conserved in mitochondria of almost all eukaryotes but lacks bacterial orthologs [3–6]. It serves as the gateway through which all mitochondrial proteins are imported. The parasitic protozoa *Trypanosoma brucei* and its relatives do not have a Tom40-like protein, which raises the question of how proteins are imported by their mitochondria [7, 8]. Using a combination of bioinformatics and in vivo and in vitro studies, we have discovered that *T. brucei* likely employs a different import channel, termed ATOM (*a*rchaic *t*ranslocase of the *o*uter *m*itochondrial membrane). ATOM mediates the import of nuclear-encoded proteins into mitochondria and is essential for viability of trypanosomes. It is not related to Tom40 but is instead an ortholog of a subgroup of the Omp85 protein superfamily that is involved in membrane translocation and insertion of bacterial outer membrane proteins [9]. This suggests that the protein import channel in trypanosomes is a relic of an archaic protein transport system that was operational in the ancestor of all eukaryotes.

Results and Discussion

Trypanosomatid Genomes Encode an Ortholog of the Bacterial Omp85-Like Protein YtfM

The Omp85 protein superfamily is defined by having a conserved bacterial surface antigen domain and one or more *po*lypeptide *t*ransport-*a*ssociated "POTRA" domains [9, 10]. The genomes of most α-proteobacteria, which are the closest living relatives of mitochondria, encode two main types of Omp85-like proteins. One type is the Sam50 ortholog BamA, which functions in the insertion of β barrel proteins into the bacterial outer membrane [11]. The other type, represented by YtfM in *Escherichia coli*, is an outer membrane protein that is essential for normal growth [12] and has recently been shown to be required for protein translocation in the outer membranes of bacteria (T.L., unpublished data). Our analysis of the genome of *T. brucei* showed that it encodes not only a Sam50 [8, 13] but also a second Omp85-like protein unrelated to Sam50. This novel protein is highly conserved within trypanosomatids (see Figure S1 available online) but absent from all other eukaryotes including excavate species such as *Giardia* and *Trichomonas* whose genomes do encode a Tom40. For reasons outlined below, this protein was termed ATOM for *a*rchaic *t*ranslocase of the *o*uter *m*itochondrial membrane. Multiple sequence alignment shows sequence conservation in the trypanosomatid ATOMs and the central part (amino acids 170–553) of the bacterial surface antigen domain of bacterial Omp85 proteins, which forms the β barrel pore (Figure S1). To more precisely determine the relationship of the trypanosomatid proteins to bacterial Omp85, we used position-specific iterated BLAST (PSI-BLAST). After four iterations, a large number of bacterial sequences were collected, many of which were annotated as Omp85 bacterial surface antigen. Using CLANS (CLuster ANalysis of Sequences) [14], we found that all of the sequences discovered in the PSI-BLAST search belong to the cluster of YtfM-like proteins, and the lines representing significant similarities link these, but not BamA, sequences with those of the ATOMs of trypanosomatids (Figure 1). This indicates that the trypanosomatid ATOMs are most closely related to the YtfM subgroup of bacterial Omp85 proteins.

ATOM Is a β Barrel Protein of the Mitochondrial Outer Membrane

Finding a trypanosome-specific member of the Omp85 protein family was entirely unexpected. We therefore wondered whether it could be the functional analog of the missing Tom40 [7, 8]. Digitonin fractionation of whole cells and carbonate extraction of isolated mitochondria from cells expressing a carboxy-terminally hemagglutinin (HA)-tagged version of ATOM showed that it is located in mitochondria and that it is an integral membrane protein (Figures 2A and 2B). Unlike the inner membrane protein cytochrome *c*1 (Cyt *c*1), which is exposed to the intermembrane space, the epitope-tagged ATOM is sensitive to added protease in isolated mitochondria, indicating that it is localized in the outer membrane (Figure 2C). As an Omp85-like protein, ATOM is expected to be a β barrel protein, and mitochondrial β barrel proteins require Sam50 for assembly into the outer membrane [13, 15]. Figure S2 shows that the levels of voltage-dependent anion channel (VDAC) and ATOM, but not of the cytosolic translation elongation factor 1a (EF-1a), decline in an induced Sam50 RNA interference (RNAi) cell line. This indicates that Sam50 is required for the correct assembly of VDAC and ATOM and provides experimental evidence that ATOM is a mitochondrial β barrel protein. Blue native gel electrophoresis (BN-PAGE) finally showed that the tagged ATOM is associated with a large protein complex of approximately 700 kDa (Figure 2D).

ATOM Is Required for Mitochondrial Protein Import In Vivo and In Vitro

In order to study the function of ATOM, we prepared a tetracycline (tet)-inducible RNAi cell line. Induction of RNAi causes

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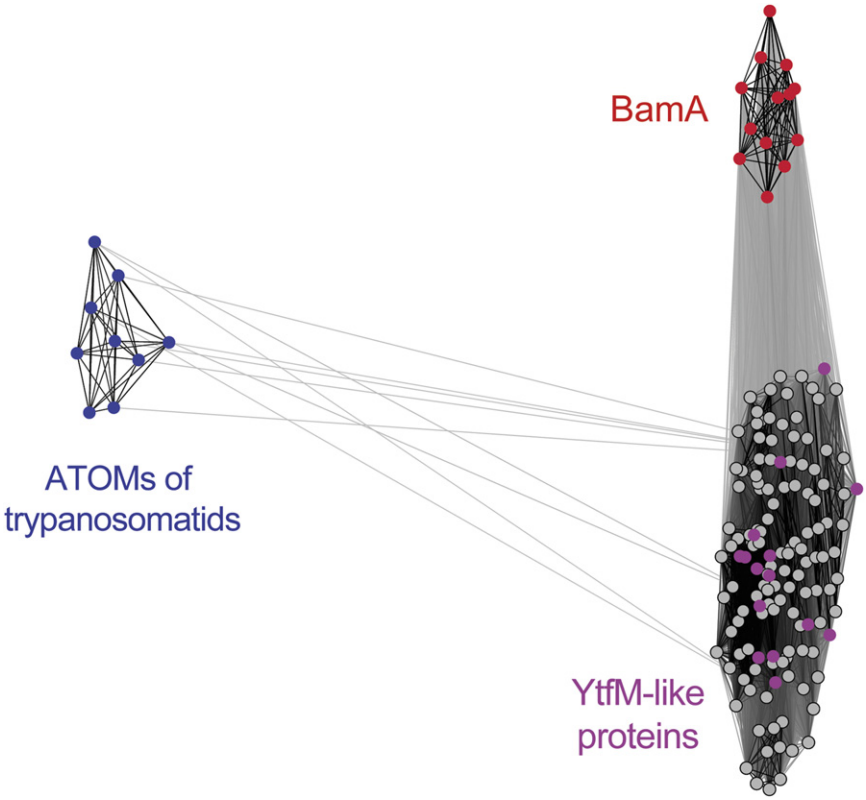

Figure 1. Trypanosomatid Genomes Encode an Ortholog of the Bacterial Omp85-like Protein YtfM

CLANS (CLuster ANalysis of Sequences) for ATOM-like sequences of trypanosomatids (blue), PSI-BLAST hits using ATOM as a query (gray), a set of representative bacterial YtfM-like sequences (purple), and a set of BamA sequences (red). Sequences with greater pairwise similarity are clustered closer together, and lines are drawn between sequences with pairwise BLAST $p < 10^{-3}$. (PSI-BLAST searching used the NCBI nr database, accessed April 5, 2011, with four iterations using default parameters. The set of the top 500 hits was redundancy reduced to a 70% identity threshold before clustering.) See also Figure S1.

efficient ablation of the ATOM mRNA and shows that the protein is essential for growth and survival of insect-stage *T. brucei* (Figure 3A). The RNAi led to a time-dependent accumulation of the uncleaved precursor form of mitochondrial heat shock protein 70 (mHsp70) (Figure 3B) in the cytosol and to a concomitant decrease of its mitochondrially localized mature form (Figure 3C). A decrease is also seen for the inner membrane protein cytochrome oxidase subunit IV (CoxIV), and to a lesser extent for the outer membrane-localized VDAC (Figure 3B). In the case of CoxIV, as for most of the tested proteins, no cytosolic accumulation of the precursor form was observed. Thus, the decrease of the steady-state levels of imported proteins can serve as a proxy for the inhibition of mitochondrial protein import because mislocalized proteins seem to get rapidly degraded. Cell lines in which inducible RNAi has been combined with inducible expression of various tagged proteins allow tracking import of newly synthesized proteins, which improves the sensitivity of the assay. Figure 3D shows that ablation of ATOM leads to a rapid reduction of the levels of two newly synthesized matrix proteins, pentatricopeptide repeat protein 2 (PPR2) [16] and tryptophanyl-tRNA synthetase 2 (TrpRS2) [17], as well as of newly synthesized tagged VDAC (Figure 3E) [7]. The reduction of the protein levels is specific for cell lines undergoing RNAi (Figure 3F) and precedes the growth arrest, indicating that it is a direct effect of the lack of ATOM. Moreover, Figure S3 shows that induction of RNAi for 48 hr after which inhibition of protein import is observed affects neither the mitochondrial membrane potential nor organellar ATP production [18]. The requirement of ATOM for mitochondrial protein import was confirmed in vitro by comparing protein import into mitochondria isolated from uninduced and induced ATOM RNAi cell lines. Figure 3G shows that import of an in vitro-translated fusion protein consisting of the N-terminal 150 amino acids of mitochondrial dihydrolipoamide dehydrogenase of *T. brucei* [19] and mouse dihydrofolate reductase (LDH-DHFR) was greatly reduced in mitochondria isolated from ATOM-ablated cells. The same was the case for assembly of in vitro-translated VDAC as visualized by BN-PAGE (Figure 3H). In summary, these results show that ATOM is required for import of matrix and inner and outer membrane proteins both in vivo and in vitro.

Methotrexate Induces the Formation of a Stable Translocation Intermediate

To directly test for an interaction between ATOM and an imported matrix protein in the process of translocation, we developed import experiments that generate an import intermediate that is arrested in the import channel. Mitochondrial protein import requires unfolding of the transported substrate [20]. The folate agonist methotrexate (MTX) binds to DHFR with very high affinity, stabilizing the protein in its folded form [20]. Thus, when using LDH-DHFR as a substrate in import experiments, addition of MTX should not affect membrane translocation of the N-terminal part of the fusion protein, but it is expected to block import of the irreversibly folded DHFR moiety. The blue native gel in Figure 4A shows that addition of MTX to an import reaction induces the formation of a large complex that contains radioactively labeled LDH-DHFR. The complex is only formed in the presence of MTX and depends on ATOM. Moreover, as expected due to the fact that inner membrane translocation drives protein import across the outer membrane [21], the LDH-DHFR-containing complex does not form in the absence of the membrane potential or in the absence of Tim17, the core component of the trypanosomal inner membrane protein translocase [22, 23]. In summary, these results show that addition of MTX induces the formation of a stable import intermediate that is strictly dependent on the presence of ATOM.

ATOM Is a Component of the Active Outer Membrane Translocase

In order to test whether ATOM is a component of the import intermediate complex, we sought to purify the imported

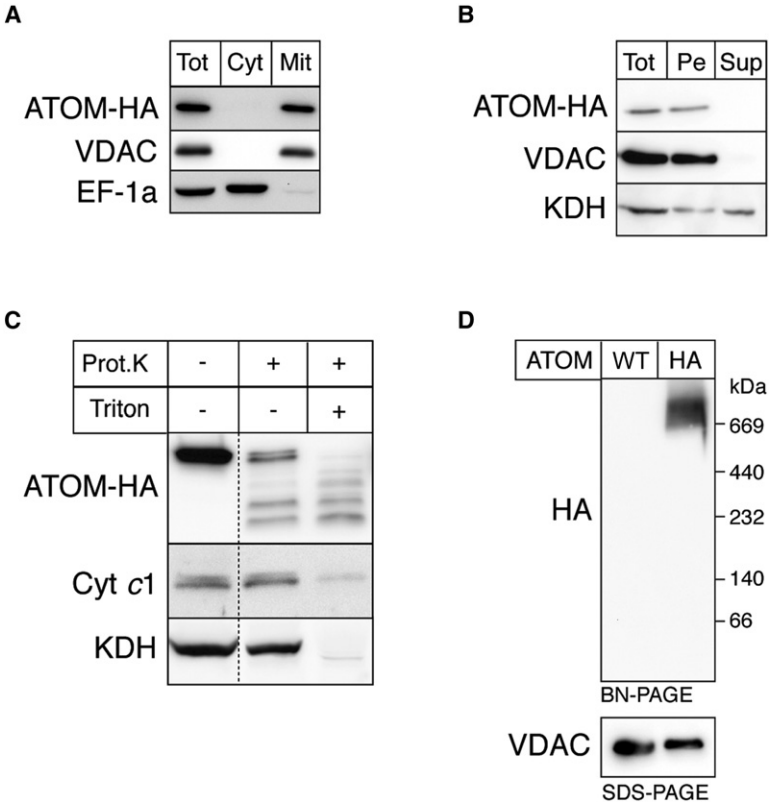

Figure 2. ATOM Is an Integral Protein of the Mitochondrial Outer Membrane

(A) Digitonin fractionation of HA-tagged ATOM-expressing cells. VDAC, voltage-dependent anion channel; EF-1a, eukaryotic elongation factor 1a.

(B) Alkaline carbonate extraction of ATOM-containing mitochondria. KDH, α-ketoglutarate dehydrogenase.

(C) Proteinase K treatment (50 μg/ml) of ATOM-HA-containing mitochondria. Cyt *c*1, cytochrome *c*1.

(D) Immunoblot of the ATOM-HA-containing complex separated by BN-PAGE. VDAC serves as a loading control. See also Figure S2.

LDH-DHFR from mitochondria isolated from a cell line expressing HA-tagged ATOM. After import, mitochondria were solubilized with digitonin and subjected to immunoprecipitation using anti-HA antibodies coupled to agarose beads. Figure 4B shows that the radioactive LDH-DHFR could be immunoprecipitated only from the reaction that received MTX, and only from mitochondria containing the tagged ATOM. Under all other conditions, only background levels of the precursor are seen. Further controls demonstrate the specificity of the immunoprecipitation because only HA-tagged ATOM, but not VDAC, CoxIV, or mHsp70, was recovered in the pellet fraction.

MTX-induced jamming of the DHFR-containing precursor protein in the translocation channel was essential for the initial characterization of Tom40 [24]. Using the same classical approach, we show that ATOM is a component of the active translocase complex in *T. brucei* that is formed by an import-arrested precursor protein (Figure 4).

Taken together, its similarity to Omp85-like protein translocases, its characteristics as a β barrel protein (Figure S2), and the observation that it is essential for cell viability—like Tom40, but unlike other subunits of the classical translocase of the outer membrane [15]—suggest that ATOM is the protein translocation pore of the outer membrane translocase in *T. brucei*.

Conclusions

Mitochondria have a monophyletic evolutionary origin, and the core elements of the protein import pathway are found in all eukaryotes that have been analyzed [4, 5]. The only exception to this evolutionary rule was the translocase of the outer mitochondrial membrane (TOM). The essential component of the TOM, Tom40, was found in all eukaryotic groups, including organisms such as *Giardia* and *Trichomonas* from the supergroup Excavata, to which trypanosomes belong. Only in the trypanosomes is Tom40 absent. The presence of ATOM as the protein import pore of the *T. brucei* outer mitochondrial membrane explains the absence of Tom40.

Currently, there is insufficient evidence to map the evolutionary acquisition of ATOM and Tom40 with certainty. It is possible that Tom40 was present in the ancestor of all eukaryotes and during excavate evolution was replaced by ATOM in the line leading to the trypanosomatids. However, for reasons outlined below, we believe that our results can best be explained by one of three models, each of which is built on a common, novel scenario for the early evolutionary history of eukaryotes in which the last common ancestor lacked Tom40 and imported proteins across the outer mitochondrial membrane using an ATOM-like protein translocase. In the first model, ATOM descended from a YtfM-like bacterial translocase present in the original endosymbiont (Figure S4A). A variation of this model would allow that the YtfM ancestor of ATOM was derived by horizontal gene transfer from other bacterial sources (Figure S4B), as has been suggested in explaining acquisition of other mitochondrial functions [25, 26]. Sequence comparison does not help to distinguish between the two models because, although it revealed a strong association of ATOM with YtfM-like proteins of proteobacteria (Figure 1), it lacks the resolution to trace it to a specific group within the proteobacteria. In both cases, the ATOM function is replaced by Tom40, which serves as the common core in the TOM complex of all eukaryotes bar the trypanosomes [5]. Either this replacement event occurred subsequent to the split between trypanosomes and other eukaryotes or, if it occurred earlier, the TOM complex did not establish itself in the trypanosomatid lineage.

Whether the ATOM was derived from the symbiont or by horizontal gene transfer, there remains a question over the steps in the ancestry of Tom40. There is no evidence that Tom40 is closely related to any protein family represented in extant species of bacteria, yet its outer membrane location and β barrel structure strongly suggest that it did evolve from a bacterial protein and that considerable sequence changes, acquired through evolution, obscure its ancestry [27, 28].

Tom40 and mitochondrial VDAC share a common ancestry [7]. VDAC serves as a metabolite carrier in the outer mitochondrial membrane and is the functional counterpart of bacterial porins, with which it shares some structural similarity [29].

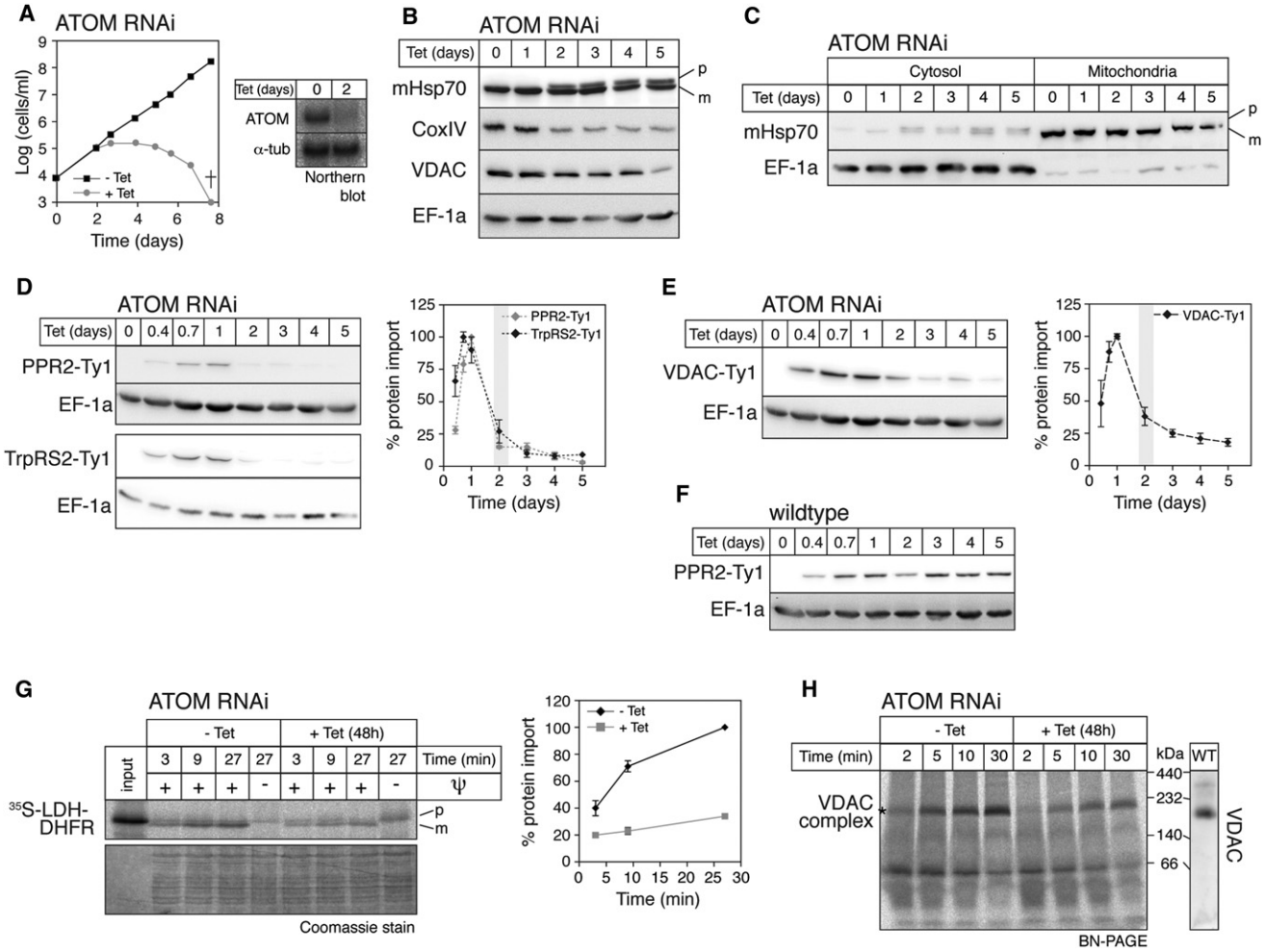

Figure 3. ATOM Is Required for Mitochondrial Protein Import In Vivo and In Vitro

(A) Growth curve of uninduced and tet-induced ATOM RNAi cell line. α-tub, α-tubulin mRNA.

(B) Levels of precursor mHsp70 accumulate whereas levels of mature mHsp70, CoxIV, and VDAC decline in total cellular extracts during tet induction of ATOM RNAi cells.

(C) ATOM RNAi causes cytosolic accumulation of mHsp70 precursor. Levels of mHsp70 and EF-1a were analyzed by immunodecoration. p, precursor; m, mature form.

(D) Levels of tet-inducible Ty1-tagged PPR2 and TrpRS2 decline during tet induction of ATOM RNAi cells. Gray bar in graphs indicate time of onset of growth arrest.

(E) As in (D), but level of tagged VDAC was monitored.

(F) Level of tet-inducible Ty1-tagged PPR2 does not decline in wild-type cells.

(G) Inhibition of in vitro import in isolated mitochondria from induced ATOM RNAi cells. ^{35}S-labeled LDH-DHFR was imported and analyzed by SDS-PAGE. ψ, membrane potential. The Coomassie-stained gel is shown as a loading control.

(H) Inhibition of VDAC assembly (asterisk) in isolated mitochondria from induced ATOM RNAi cells analyzed by BN-PAGE. The immunoblot shows the VDAC complex in wild-type cells.

For the graphs in (D), (E), and (G), standard errors are indicated. See also Figure S3.

Evolution of VDAC from bacterial porins therefore cannot be excluded [29]. The scenario that Tom40 might have evolved from the ATOM also cannot be rejected (Figure S4C). However, it is worth noting that Tom40 has fewer predicted β strands in the β barrel [30] than the 16-stranded β barrel characteristic of Omp85-family proteins.

Whichever model best describes the evolution of the outer membrane protein translocase, ATOM likely provides a missing link in mitochondrial evolution that bridges the gap between bacterial protein export systems and the modern Tom40-based mitochondrial protein import systems. Based on an a analysis of a whole range of trypanosomatid-specific characters, including (1) the absence of Tom40 [7], (2) a unique cytochrome *c* with only one cysteine for heme binding and a mechanism for its biogenesis that is unique in nature [31], and (3) a much simplified origin of replication complex [32], it has been suggested to place the root of the eukaryotic phylogenetic tree between the trypanosomatids and all other eukaryotes [33]. The discovery of the bacterial-type mitochondrial protein translocase ATOM supports this proposal.

Experimental Procedures

Cells and RNAi

RNAi of ATOM was performed by using a pLew-100-derived stem-loop construct and the procyclic *T. brucei* strain 29-13 [34]. As an insert, we

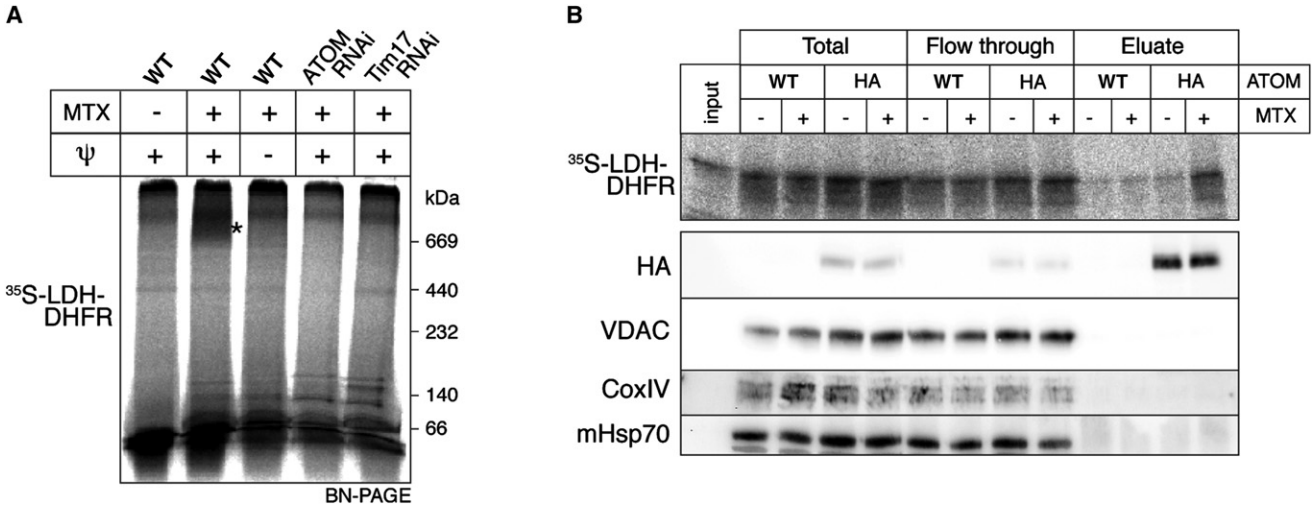

Figure 4. ATOM Is a Component of the Active Outer Membrane Translocase

(A) Radiograph of a BN-PAGE showing that ^{35}S-labeled LDH-DHFR forms a methotrexate (MTX)-dependent complex (asterisk) in an vitro import reaction in wild-type mitochondria, but not in mitochondria from induced ATOM or Tim17 RNAi cell lines. ψ, membrane potential.

(B) Import reactions using ^{35}S-labeled LDH-DHFR in the absence and presence of MTX in wild-type and ATOM-HA-containing mitochondria, respectively. Digitonin-solubilized mitochondrial extracts were immunoprecipitated using anti-HA antiserum. Labeled substrate protein is recovered in the pellet only in ATOM-HA-containing mitochondria in the presence of MTX. Outer membrane, inner membrane, and matrix markers are not precipitated.

used a 490 bp DNA fragment (nucleotides 22–511) of the ATOM open reading frame (Tb09.211.1240).

In Vitro Import and Assembly of Precursor Proteins

Import of ^{35}S-labeled precursor proteins (rabbit reticulocyte lysate) into isolated mitochondria of *T. brucei* was performed as described [35], except that the import buffer contained 5 mg/ml fatty acid-free bovine serum albumin. In vitro assembly of ^{35}S-labeled VDAC into complexes and subsequent analysis on BN-PAGE was performed as described for yeast, except that 1.5% (w/v) of digitonin was used for solubilization [36].

Immunoprecipitations

For the immunoprecipitations, the import intermediate was produced in a 160 μl import reaction containing 160 μg of mitochondria, 40 μl of ^{35}S-labeled LDH-DFHR, and 10 μM of methotrexate (MTX). All subsequent steps were performed in the presence of 10 μM of MTX. Mitochondria were reisolated and resuspended in 160 μl lysis buffer (20 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, 100 mM NaCl, 25 mM KCl, 10% [w/v] glycerol) containing 1.5% (w/v) digitonin and a protease inhibitor cocktail (Roche Applied Science). After a clearing step, 80 μl of lysis buffer containing 1.5% (w/v) digitonin was added, and the mixture (240 μl) was incubated for 2 hr at 4°C with 25 μl bed volume of anti-HA agarose (Roche Applied Science). The beads were extensively washed in lysis buffer containing 0.2% of digitonin (w/v) prior to elution with SDS sample buffer. SDS polyacrylamide gels were blotted onto polyvinylidene fluoride membranes (Millipore). Proteins were detected by digital autoradiography or immunodecoration and enhanced chemiluminescence detection (Pierce).

Supplemental Information

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.cub.2011.08.060.

Acknowledgments

We thank E. Horn and B. Schönfisch for technical assistance and N. Wiedemann for helpful discussions. This study was funded by grants from the Swiss National Foundation (31003A_121937 to A.S.), the Peter und Traudl Engelhorn Stiftung (to M.N.), the Bundesministerium für Bildung und Forschung (Dynamo) (to C.M.), the Deutsche Forschungsgemeinschaft (to C.M.), and the Excellence Initiative of the German Federal & State Governments (EXC 294 BIOSS) (to B.W. and C.M.). T.L. is a Federation Fellow of the Australian Research Council.

Received: June 27, 2011
Revised: August 22, 2011
Accepted: August 30, 2011
Published online: October 13, 2011

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