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Short communication

# The intermembrane space protein Erv1 of *Trypanosoma brucei* is essential for mitochondrial Fe-S cluster assembly and operates alone

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ABSTRACT

Sulfhydryl oxidase Erv1 is a ubiquitous and conserved protein of the mitochondrial intermembrane space that plays a role in the transport of small sulfur-containing proteins. In higher eukaryotes, Erv1 interacts with the mitochondrial import protein Mia40. However, *Trypanosoma brucei* lacks an obvious Mia40 homologue in its genome. Here we show by tandem affinity purification and mass spectrometry that in this excavate protist, Erv1 functions without a Mia40 homologue and most likely any other interaction partner. Down-regulation of TbErv1 caused a reduction of the mitochondrial membrane potential already within 24 h to less than 50% when compared with control cells. The depletion of TbErv1 was accompanied by accumulation of trCOIV precursor, with a concomitant reduction of aconitase activity both in the cytosol and mitochondrion. Overall, TbErv1 seems to have a role in the mitochondrial translocation and Fe-S cluster assembly in the organelle.



The mitochondrion or organelles derived from it are omnipresent organelles of eukaryotic cells. A notable event in the mitochondrion becoming an integrated organelle was the development of an efficient protein import system to facilitate the targeting nuclear-encoded mitochondrial proteins into one of the four sub-compartments: the mitochondrial outer membrane, intermembrane space (IMS), inner membrane and matrix [1]. As a part of this highly conserved and complex system, the import and folding of cysteine-rich proteins smaller than 20 kDa is facilitated by the mitochondrial IMS assembly (MIA) machinery. In higher plants and opisthokonts, the MIA machinery is comprised of two proteins, Erv1 and Mia40, with the latter partner likely emerging later in evolution, as it is absent in several protist lineages [2]. In contrast, Erv1 has been found in nearly all eukaryotes, being lost only in a small group of organisms lacking substrates of the MIA pathway [2]. In addition to its function in mitochondrial import, Erv1 has been associated with Atm1, a key component of the mitochondrial iron-sulfur (Fe-S) cluster export machinery [3], and is therefore considered essential for the cytosolic Fe-S cluster assembly (CIA) pathway [4]. This notion is based on the observations that the depletion of either Erv1 or Atm1 in yeast led to a less efficient insertion of Fe-S clusters into the cytosolic proteins, together with an accumulation of iron in the mitochondria [3]. Interestingly, eukaryotes which in the course of evolution lost Erv1 from their genome seem to have lost Atm1 in parallel, which is another indication for the interplay between these two proteins [2]. However, a recent study challenged the essentiality of Erv1 and the MIA machinery for the CIA pathway [5].

In this paper we further investigated the role of Erv1 in the parasitic flagellate *Trypanosoma brucei*, the causative agent of African sleeping sickness and member of the eukaryotic supergroup Excavata [6]. Regardless of the presence of an essential Erv1, no homologues of Mia40 were so far identified in the genome of *T. brucei* and related trypanosomatids [2,7]. Moreover, Erv1 in *T. brucei* (labeled TbErv1) was recently shown to be able to utilize both oxygen and cytochrome *c* (cyt *c*) as electron acceptors *in vitro* [7]. Consequently, Erv1 was predicted to operate alone and co-localize in the IMS with cyt *c*.

To address these questions, we initiated our study with an immunofluorescence assay using previously generated rabbit polyclonal antibodies raised against TbErv1 [7]. Indeed, in the procyclic

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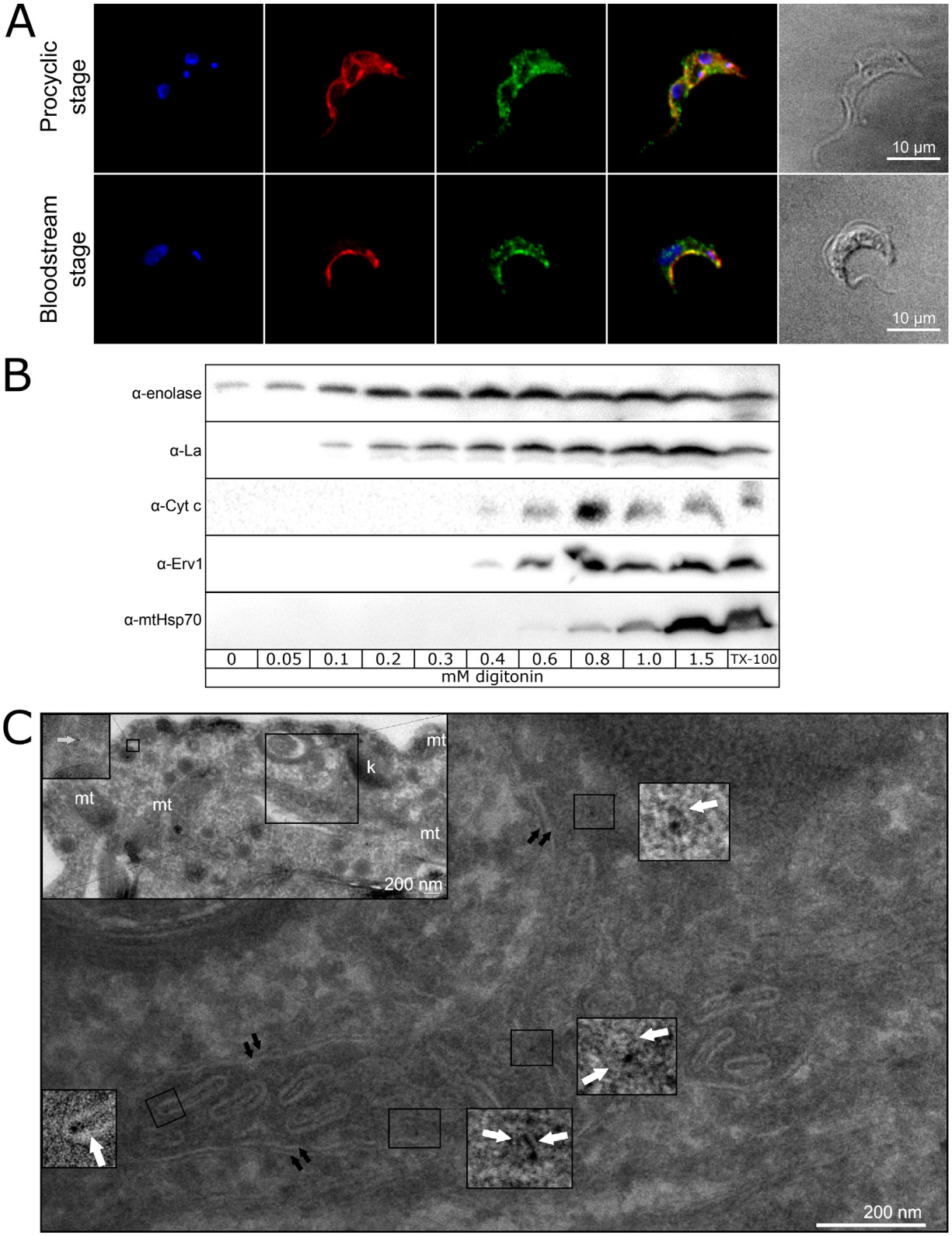


**Fig. 1.** TbErv1 is a mitochondrial intermembrane space protein. (A) Immunolocalization of TbErv1 in the procyclic and bloodstream stages of *T. brucei* using α-TbErv1 antibody (green) shows its co-localization with the mitochondrion. The organelle was stained with MitoTracker Red (red) (final concentration 100 nM; Thermo Fisher), and DNA was stained with DAPI (blue) (final concentration 1 μg/ml; Sigma-Aldrich). Scale bar is 10 μm. (B) Procyclic cells were lysed with increasing concentrations (0–1.5 mM) of digitonin as described previously [7], the whole cell lysate was produced by treatment with 0.1% (v/v) Triton X-100. The TbErv1 protein is released after the cytosolic (enolase) and nuclear (La) marker proteins, but before the mitochondrial matrix component (mtHsp70), and in parallel with the intermembrane space protein cyt *c*, pinpointing the localization of TbErv1 to the mitochondrial intermembrane space. (C) Immuno-electron microscopy of TbErv1 were performed on cryosections of procyclic *T. brucei* fixed in 4% formaldehyde with 0.1% glutaraldehyde and processed as described elsewhere [19]. For better accuracy of the immuno-detection, we used the protein specific antibody conjugated to 6 nm gold nanoparticles and images (TEM JEOL 1010) were captured at range of ±20° tilt angles (insets). Nanoparticles were localized in the cytosol (grey arrow) and within the inner space of the mitochondrial cristae (insets). Black arrows show both outer and inner mitochondrial membranes; the cristae membranes are indicated by white arrows; mitochondrion (mt); kinetoplast DNA (k). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

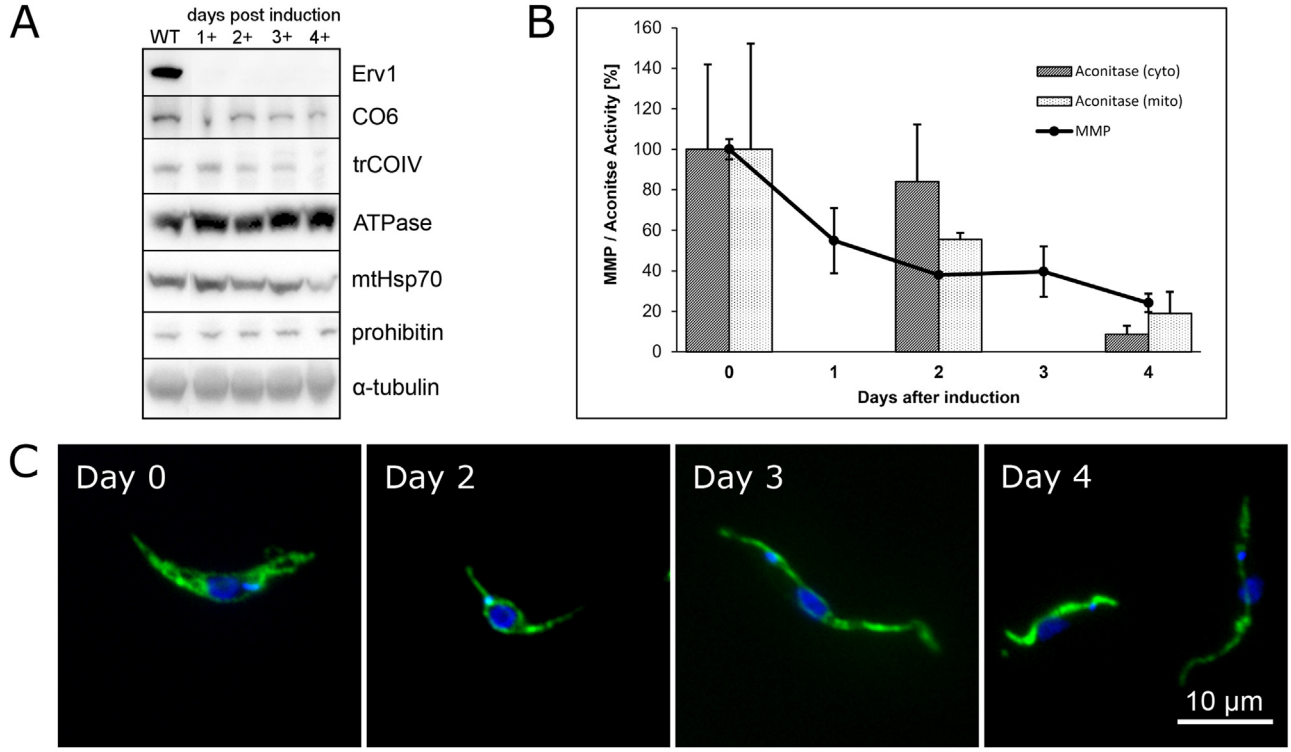


**Fig. 2.** Down-regulation of TbErv1 disrupts Fe-S cluster assembly. (A) Western blot analysis of mitochondrial proteins after RNAi of TbErv1. Cytochrome *c* oxidase subunit 6 (CO6), trypanosome-specific subunit 4 of cytochrome *c* oxidase (trCOIV), prohibitin and the F1 moiety of ATP synthase (ATPase) are membrane-bound proteins; mtHsp70 is targeted to the mitochondrial matrix; α-tubulin was used as loading control. (B) Aconitase activity and mitochondrial membrane potential in procyclic TbErv1 RNAi cells. The aconitase activity was measured in the cytosolic (cyto) and mitochondrial fractions (mito) as described elsewhere [20] and is shown as a bar graph. Error bars show the 95% confidence interval calculated from three measurements of independently induced cultures. The mitochondrial membrane potential was determined by the uptake of tetramethylrhodamine measured by flow cytometry and is shown as line graph. Error bars show the 95% confidence interval calculated from two technical replicates. (C) Mitochondrial morphology of procyclic TbErv1 RNAi cells. Mitochondria were immuno-stained with α-mtHsp70 (green), DNA was stained with DAPI (blue) (used at 1 µg/ml; Sigma-Aldrich). Scale bar is 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stage of *T. brucei*, TbErv1 shows a mitochondrial localization pattern that co-localizes with MitoTracker Red (Fig. 1A). However, a very weak signal is present also in the cytosol, and a similar pattern was observed in the bloodstream stage (Fig. 1A). To investigate this mitochondrial localization in more detail, we performed selective permeabilization of procyclic stage cells with digitonin (Fig. 1B). This experiment confirms that TbErv1, like the IMS protein cyt *c*, was released later than cytosolic enolase but earlier than the heat shock protein 70 (mtHsp70), a mitochondrial matrix marker. This finding indicates that TbErv1 is most likely a constituent of the IMS. We further validated the localization of TbErv1 by electron microscopy using immunogold labelling of cryosectioned procyclic *T. brucei*. This approach confirmed the localization within the IMS of the cristae-rich mitochondrion (Fig. 1C; insets). This result is in agreement with those from yeast, human and plants, where the Erv1 protein also localizes into the IMS [8–10].

This localization is consistent with the canonical role of Erv1 in the mitochondrial IMS assembly [11–13]. In *Saccharomyces cerevisiae*, Mia40 and Erv1 are involved in the stabilization of small TIMs (translocases of the inner mitochondrial membrane) in the IMS, which are required for the assembly of the TIM22 complex [12]. To determine whether the down-regulation of TbErv1 has an effect on protein import, we assessed levels of several mitochondrial proteins by Western blot (Fig. 2A). Accumulation of the cytochrome *c* oxidase subunit IV (trCOIV) precursor after 2 days of RNAi-induction indicates that the mitochondrial translocation machinery may be impaired.

In *Saccharomyces cerevisiae*, Erv1 was experimentally shown to be essential for maturation of the cytosolic Fe-S proteins Leu1p and Rli1p, while its absence had no influence on the incorporation of iron into the mitochondrial Fe-S protein Bio2p [3]. Whether this is also the case in *T. brucei* was assessed by activity measurements of the Fe-S cluster-containing aconitase [14], in cells in which TbErv1 was inducibly depleted by RNAi [7]. Due to the dual localization of aconitase in the studied flagellate [14], the activity was measured separately in cytosolic and mitochondrial fractions. Previous studies have shown that in *T. brucei* the apo-aconitase undergoes maturation by specific Fe-S assembly machineries residing in either the cytosol or mitochondrion [15,16].

A substantial drop in mitochondrial aconitase activity was noted already 2 days after RNAi induction, and nearly complete ablation of the activity in both the mitochondrion and the cytosol occurred on day 4 post-induction (Fig. 2B). The disruption of the CIA pathway may be a secondary effect following the impairment of the mitochondrial Fe-S cluster machinery [4], rather than a consequence of the involvement of Erv1 in the export of Fe-S clusters from the mitochondrion [5]. The phenotype triggered by the ablation of TbErv1 can thus be explained by the disrupted mitochondrial import of either small TIM proteins or the source of sulfur for the organellar Fe-S cluster machinery.

In *S. cerevisiae*, the activity of Erv1 was also shown to influence mitochondrial morphology [16]. To test whether a similar effect occurs in trypanosomes, TbErv1-depleted procyclic cells were immunodecorated with α-mtHsp70 antibody [17], which allowed monitoring of the mitochondrial morphology by fluorescent microscopy. The non-induced TbErv1 RNAi cells have their mitochondrial morphologically unaltered (Fig. 2C), regardless of the already significantly lower level of TbErv1 when compared with the wild type trypanosomes [7]. However, the RNAi-induced cells, in which TbErv1 is almost completely eliminated [7], experienced a

**Table 1**
Mass spectrometry analysis of proteins that co-immunoprecipitated with TbErv1. TbErv1 was purified using Dynabeads linked to α-TbErv1 antibody, and the cells were lysed using 0.1% Triton X-100. The most abundant hits were ordered according to the relative abundance factor subtracted by the abundance in the control sample (RAFC), and the fold change of the proteins over the abundance in the control sample (FCoC). The localization of the co-immunoprecipitated proteins are indicated as follows: CYT – cytoplasm; IMS – inter membrane space; IMM – inner mitochondrial membrane; PM – peroxisomal membrane; GLY – glycosomal matrix; PM – plasma membrane.

| Name | TriTryp accession | Localization | RAFC | FCoC |
|---|---|---|---|---|
| Sulfhydryl oxidase (Erv1) | Tb927.9.6060 | IMS | 1.111E+09 | *57.03* |
| Tryparedoxin peroxidase | Tb927.9.5750 | CYT | 1.134E+07 | *1.70* |
| Putative mitochondrial carrier protein | Tb927.10.14840 | IMM | 7.585E+06 | *3.33* |
| Gim5 B protein | Tb927.9.11600 | PM | 7.429E+06 | *2.39* |
| Cytochrome *c* oxidase subunit IV (trCOIV) | Tb927.1.4100 | IMM | 6.513E+06 | *6.59* |
| Glyceraldehyde-3-phosphate dehydrogenase | Tb927.6.4300 | GLY | 6.437E+06 | *1.38* |
| Proteins associated with differentiation | Tb927.7.5940,Tb927.7.5930 | PM | 4.258E+06 | *3.14* |

gradual reduction of the mitochondrial reticulation. Already on day 2 post RNAi-induction, the majority of flagellates developed a phenotype with the mitochondrion closely surrounding the nucleus (Fig. 2C). On day 4, most trypanosomes had their mitochondria reduced into a small crescent-shaped organelle, which is otherwise characteristic for the bloodstream stage (Fig. 2C). Upon prolonged RNAi-induction the mitochondrion became fragmented, the cells attained an elongated shape and eventually died (data not shown). The collapse of the organelle was accompanied by the drop of the mitochondrial membrane potential, which was followed by staining the cells with tetramethylrhodamine and the measurement of its uptake by flow cytometry (Fig. 2B).

While in opisthokonts and plants, the MIA pathway is composed of Mia40 and Erv1, in other eukaryotes, IMS protein import is mediated solely by Erv1 [2]. Contrary to Erv1, Mia40 was shown to be dispensable for the import of MIA substrates in plants [18], giving rise to the possibility that Erv1 alone may be able to perform the import of proteins to the IMS. In an alternative model, an unknown protein would take over the function of Mia40, although any evidence for this scenario has not been provided. Furthermore, Erv1 was proposed to interact with Atm1 in the context of the Fe-S cluster export machinery [3]. Therefore, to search for possible interaction partners of TbErv1 in procyclic trypanosomes we have performed co-immunoprecipitation using specific polyclonal α-TbErv1 antibody. The precipitated proteins were subjected to mass spectrometry (for methodology see legend of Table 1), and the proteins that co-purified with TbErv1 are listed in Table 1. While we cannot exclude that any of the detected proteins interact with TbErv1, neither seem to be localized to the IMS or to be present in a stoichiometric ratio to TbErv1. Hence, they are unlikely to functionally replace Mia40. Several detected proteins are components of the IMM, which confirms that the conditions used for the pull-down experiment were indeed suitable for detecting putative membrane-bound interacting partners of TbErv1. Since TbAtm1 is lacking among the pulled-down proteins, we found no evidence for any interaction between TbErv1 and TbAtm1.

In summary, we showed that in *T. brucei* TbErv1 is located in the IMS, where it is most likely involved in mitochondrial protein import. Based on mass spectrometry data, we conclude that TbErv1 functions without Mia40 or any related protein and thus performs its role in the mitochondrial import and/or export machineries alone. Importantly, the ablation of TbErv1 in procyclic trypanosomes leads to a nearly complete impairment of both mitochondrial and cytosolic Fe-S cluster assembly machineries. Moreover, morphological changes induced in the mitochondrion by the ablation of TbErv1 triggered a gradual decrease of the mitochondrial membrane potential and a subsequent morphological reduction of the organelle.

## Conflict of interest

Authors declare no conflict of interest.

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