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Short communication

The import and function of diatom and plant frataxins in the mitochondrion of *Trypanosoma brucei*

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ABSTRACT

Frataxin is a conserved mitochondrial protein, almost universally present in prokaryotes and eukaryotes, where it is implicated in Fe–S cluster assembly and several other processes. Here we show that frataxins from the diatom *Thalassiosira pseudonana* and the plant *Arabidopsis thaliana* are efficiently targeted and processed in the mitochondrion of the evolutionary distant excavate kinetoplastid flagellate *Trypanosoma brucei*. Moreover, both heterologous frataxins are able to rescue a lethal deficiency for *T. brucei* frataxin.

Frataxin is a small highly conserved protein found in virtually all prokaryotes and eukaryotes. It lacks well-defined motifs and despite its omnipresence and the fact that in humans its deficiency causes serious Friedreich's ataxia, the function of frataxin is far from being understood. Among the roles assigned to this protein are Fe–S cluster and heme biosynthesis, control of reactive oxygen species (ROS), participation in the respiratory complexes, repair of damaged Fe–S clusters and iron storage [1,2]. These and other putative functions of frataxin have been extensively studied in the yeast, mouse and human cells [3–9], and lately also in *Trypanosoma brucei* [10] and *Arabidopsis thaliana* [11,12]. We have shown recently that both hydrogenosomal frataxin proteins of *Trichomonas vaginalis* are not only efficiently imported into the *T. brucei* mitochondrion via their genuine hydrogenosomal import signals, but are also able to rescue the phenotype caused by the downregulation of trypanosome frataxin [10].

The import into the *T. brucei* mitochondrion of proteins equipped with a hydrogenosomal import signal [13] and the lack of obvious homologues in the kinetoplastid genomes of several key components of the mitochondrial import machinery [14] were interpreted as features of a simplified pathway for mitochondrial protein import in trypanosomes [15]. Same as trypanosomes, trichomonads belong to the supergroup Excavata [16], their hydrogenosomes being in fact only highly modified mitochondria [17]. We wondered whether the mitochondrial import system of *T. brucei* will also recognize import signals of mitochondria from very distantly related organisms. For that purpose we have chosen the model representative of land plants *A. thaliana* and the marine diatom protist *Thalassiosira pseudonana*. Marine diatoms are ecologically highly significant protists with an impact on global carbon cycling similar to that of all rain forests combined [18]. A recent view holds that they belong to the supergroup Chromalveolata, while *A. thaliana* is ranked into the supergroup Plantae [16].

In this work we show that the genuine import signals of frataxins from *A. thaliana* and *T. pseudonana* were sufficient to import the respective proteins into the mitochondrion of *T. brucei*, in which both frataxins exerted a rescue effect. Our results indicate that this organelle behaves as a universal importer and represents a useful system for functional analysis of heterologous genes.

1. Frataxins of *T. pseudonana* (Tp-frataxin) and *A. thaliana* (At-frataxin)

The sequence of Tp- and At-frataxins and *T. brucei* (Tb)-frataxin were aligned with CLUSTAL W2 and the mitochondrial signal peptides were predicted with MitoprotII (Fig. 1). At the amino acid level the similarity between Tb- and Tp-frataxins, and Tb- and At-frataxins is 34.8% and 57.7%, respectively. Two α-helices and

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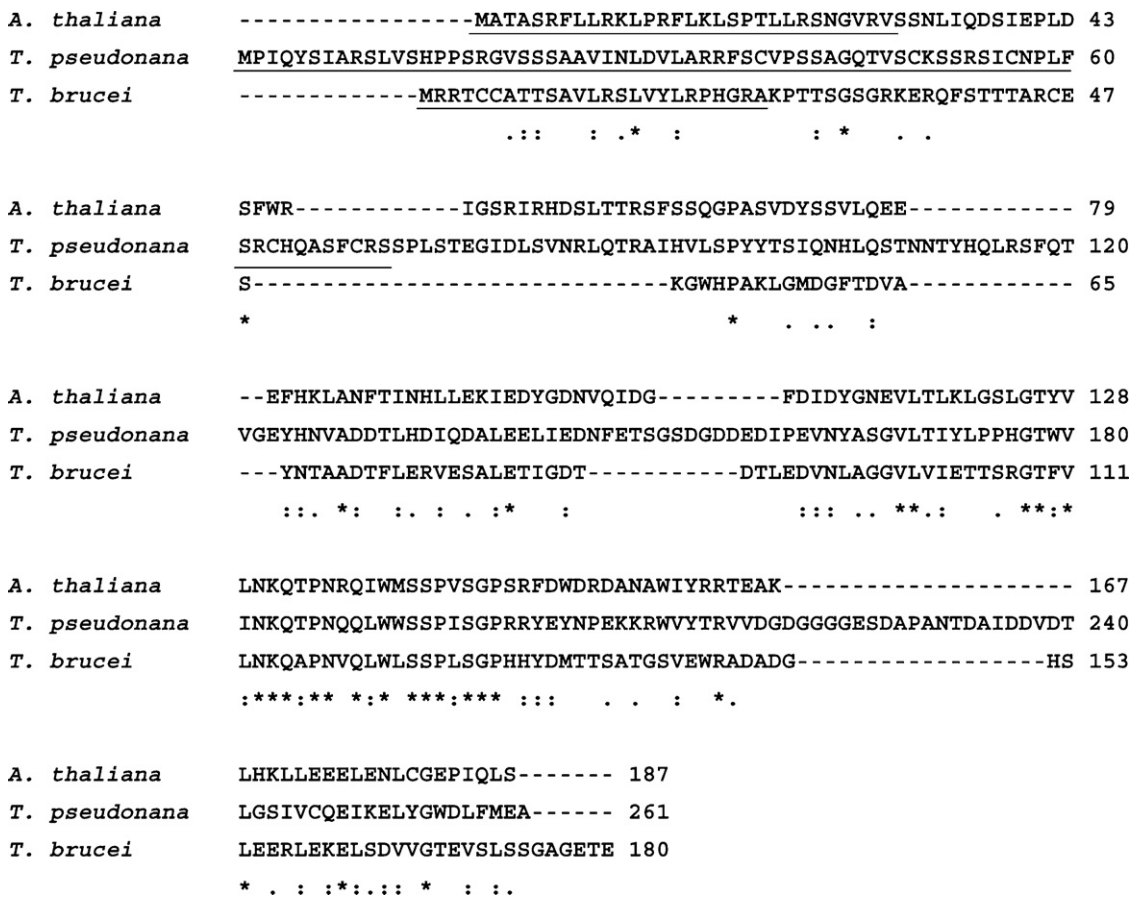

Fig. 1. Multiple alignment of predicted of mitochondrial signal peptides and protein sequences of frataxin from *Arabidopsis thaliana* (NP192233), *Thalassiosira pseudonana* (JGI euk. genomics; fgenesh1_pg.C_chr_3000375) and *Trypanosoma brucei*. (GeneDB Tb927.3.1000). Amino acids are numbered to the right of the respective sequences. Stars denote identical residues, dots denote similar residues. Gaps were introduced to optimize the alignment. The mitochondrial signal peptides predicted with MitoprotII are underlined.

six β-sheets seem to be conserved in all analyzed frataxins, with sequence conservation confined to the C-terminal part (Fig. 1). The predicted signal peptides for At-frataxin and Tp-frataxin are 30 and 71 amino acids long (probabilities 0.9981 and 0.8304), respectively (Fig. 1).

2. Tp-frataxin and At-frataxin rescue the growth of *T. brucei* with downregulated Tb-frataxin

Since antibodies are not available against either frataxin, we have tagged both genes as follows. The HA_3 tag was amplified from the pJH54 vector and cloned into the pABPURO vector [19]. The full-length 261 amino acids-long Tp-frataxin gene was amplified from total DNA of the protist diatom *T. pseudonana* using primers GAAGCTTATGCCAATACAGTACAGCA and GGATCCTGCCTCCATAAACAAGTC (added HindIII and BamHI restriction sites are underlined, respectively). Amplification of the known 187 amino acids-long At-frataxin gene from cDNA of the multicellular bikont *A. thaliana* was performed using primers GCTAGCATGGCTACAGCTTCAAGGTT and GGATCCTGAGAGTTGGATTGGTTC (added NheI and BamHI restriction sites are underlined, respectively). Next, both full-size frataxin genes lacking stop codons but containing predicted mitochondrial import signals were separately inserted 5′ to the HA_3 tag. The constructs were upon linearization with BstXI introduced into the 29–13 procyclic *T. brucei* inducible for RNA interference (RNAi) against the *T. brucei* frataxin [10]. Clonal cell lines have been obtained as described elsewhere [20]. The empty pABPURO vector containing HA_3 tag and Tb-frataxin RNAi knockdown were used as controls.

Upon the addition of tetracycline, which triggers the synthesis of double stranded RNA, Tb-frataxin was eliminated within five days (Fig. 2A). In the absence of this protein cells stop dividing and eventually die [10]. However, procyclic cells, in which Tb-frataxin has been eliminated by RNAi, and which express Tp-frataxin, retain almost the same growth as their non-induced counterparts (Fig. 2B). The growth rate is also rescued by the expression of At-frataxin, although not to the same level (Fig. 2B). However, no rescue was observed in the RNAi cell line containing the empty HA_3-tagged pABPURO vector (Fig. 2B).

3. Localization of Tp- and At-frataxins in procyclic *T. brucei*

Since antibodies are available only against the Tb-frataxin [10], we have used the tagging strategy to follow the intracellular localization of the Tp- and At-frataxins. The HA_3 tag at the C-terminus should not interfere with the mitochondrial import signal located at the N-termini of the proteins. Subcellular fractions obtained with digitonin treatment performed as described previously [21] were immunoprobed with antibodies against the HA_3 tag. The tagged protein was present in the mitochondrion, whereas no signal was observed in the cytosol (Fig. 3A). The antibody detects two bands in the mitochondrial and total cell lysates. Since the size difference among them is in correlation with the size of the mitochondrial import signals of both heterologous frataxins (shorter and longer in At- and Tp-frataxins, respectively), we interpret the upper band as

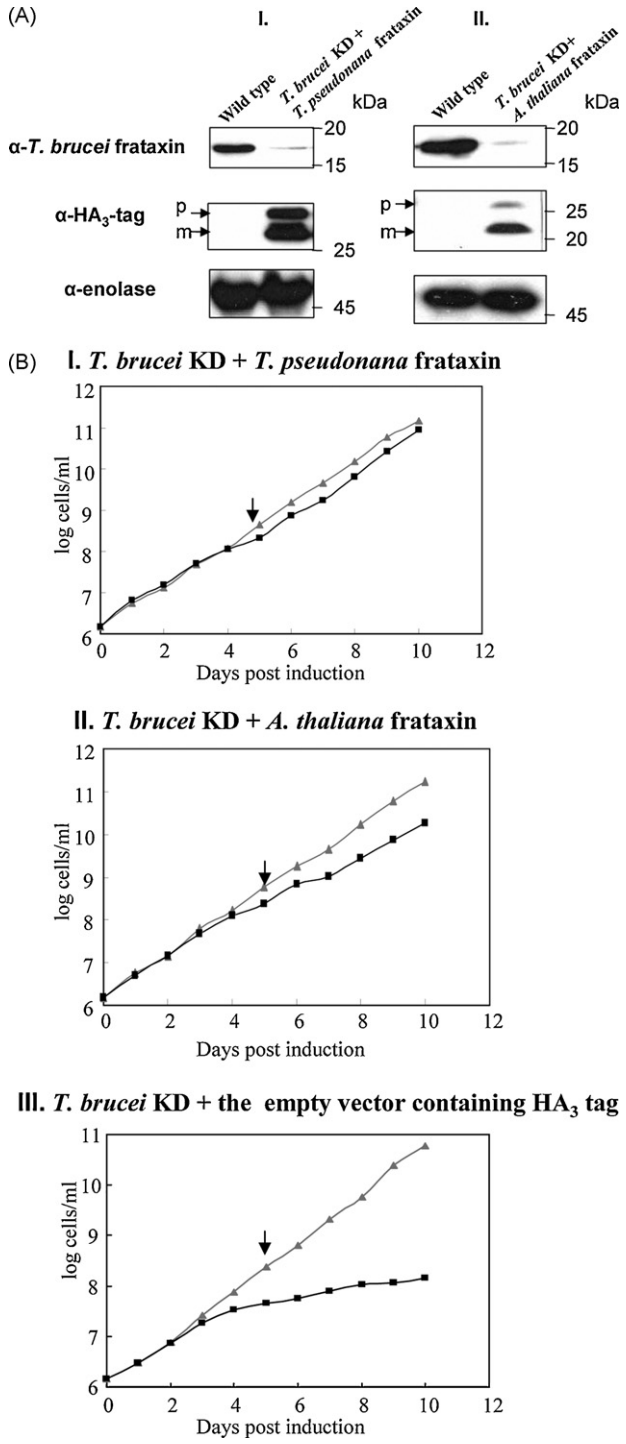

Fig. 2. Immunoblot and growth analysis of 29–13 cells and RNAi knockdowns for *T. brucei* frataxin containing HA_3-tagged heterologous frataxins. (A) Immunoblot analysis of the *T. brucei* frataxin and the HA_3-tagged frataxins of *Thalassiosira pseudonana* (panel I) and *Arabidopsis thaliana* (panel II) in 29–13 cells, and in RNAi cells with eliminated *T. brucei* frataxin (5 days of RNAi induction), containing constitutively expressed heterologous frataxins. 5×10^6 cells were analyzed by SDS-PAGE and immunoprobed with α-Tb-frataxin and α-HA_3-tag antibodies. The α-enolase antibody was used as a loading control; p = precursor; m = mature form. (B) Effect of constructs on cell growth. Numbers of non-induced (gray triangles) and RNAi-induced cells (black squares) are shown. Tb-frataxin RNAi knockdown cells transfected with either *T. pseudonana* frataxin (panel I) or *A. thaliana* frataxin (panel II) or the empty pABPURO vector containing only the HA_3 tag. Cell densities were measured daily to day 10 after RNAi induction using a Beckman Z2 Coulter. The arrow represents sampling time point for the measurement of enzyme activities.

the precursor (p) and the lower band as the mature form (m) of the protein (Figs. 2A and 3A). From these Western blot analyses we conclude that the *T. brucei* mitochondrial processing peptidase is able to properly process, albeit perhaps less efficiently, both heterologous proteins.

To corroborate this observation, we resorted to fluorescent microscopy. The treatment of cells for immunocytochemistry and DAPI staining were performed as described elsewhere [10]. Using the α-HA_3 tag antibody we show in both cell lines that either of the heterologous epitope-tagged frataxins is evenly distributed throughout the reticulated mitochondrion of procyclic cells, as also confirmed by DAPI staining of the kinetoplast and by co-localization with the mitochondrial RNA binding protein 2 (MRP2) (Fig. 3B). Again, no tagged protein was detected in the cytosol.

4. Tp- and At-frataxins restore activities of Fe–S proteins

The rescued growth already indicated that both heterologous proteins are able, at least partially, to complement for the deficiency of Tb-frataxin. It was shown previously that downregulation of Tb-frataxin causes a disruption of Fe–S cluster assembly [10]. Therefore, we decided to measure the activities of several Fe–S cluster-containing proteins with mitochondrial and/or cytosolic localization following protocols described elsewhere [10,20]. Activity of the marker Fe–S cluster enzyme, aconitase, dropped in induced RNAi cells and in the same cells containing an empty pABPURO vector to less than 20% of its wild type activity (Fig. 4A). However, in the presence of Tp- or At-frataxins its activity increased to about 50% of the wild type level (Fig. 4A). Similarly, activities of fumarase and succinate dehydrogenase (=mitochondrial respiratory complex II) in the induced RNAi cell lines rescued with either of the used heterologous frataxins are three to four times higher than in their absence (Figs. 4B and C). However, the diatom frataxin rescued activities of both Fe–S cluster-containing proteins more efficiently. As expected, the activities of a control enzyme that does not require Fe–S clusters for its activity (threonine dehydrogenase) did not differ in the studied cell lines (Fig. 4D).

While hundreds of proteins of *A. thaliana* have been subjected to functional analyses, to our knowledge this is the first functional analysis of a protein from *T. pseudonana*, the only diatom with known genome [18]. While having enormous impact on global ecosystem, diatoms are still inaccessible to current methods of functional genomics. Therefore, probing functions of their genes in other organisms remains a method of choice. Here we show that *T. brucei* represents a suitable host organism for this purpose, as its mitochondrion readily imports proteins equipped with mitochondrial import signal of organelles from very distantly related eukaryotes. Moreover, the solid rescues of the Tb-frataxin knockdown testify of proper folding of the imported protein.

So far, the organelle of *T. brucei* was shown to import proteins preceded by the trypanosomatid mitochondrial or trichomonad hydrogenosomal import signals [10,13]. In both cases, the import signals for these excavate organelles are short. Their shared simplicity was correlated with the apparent absence of most translocases of the outer and inner membranes that are highly conserved throughout eukaryotes, but appear to be missing in the genomes of *T. brucei*, *T. cruzi* and *Leishmania major* [15]. In fact, only the Tim17 translocase has been unambiguously identified in the kinetoplastid genomes, and its elimination by RNAi lead to the predicted disruption of mitochondrial protein import [14,22]. It is therefore quite interesting that the very long import signal [22] is able to import the Tp-frataxin so efficiently into the *T. brucei* mitochondrion, where it rescues the knock-down for the genuine trypanosome frataxin.

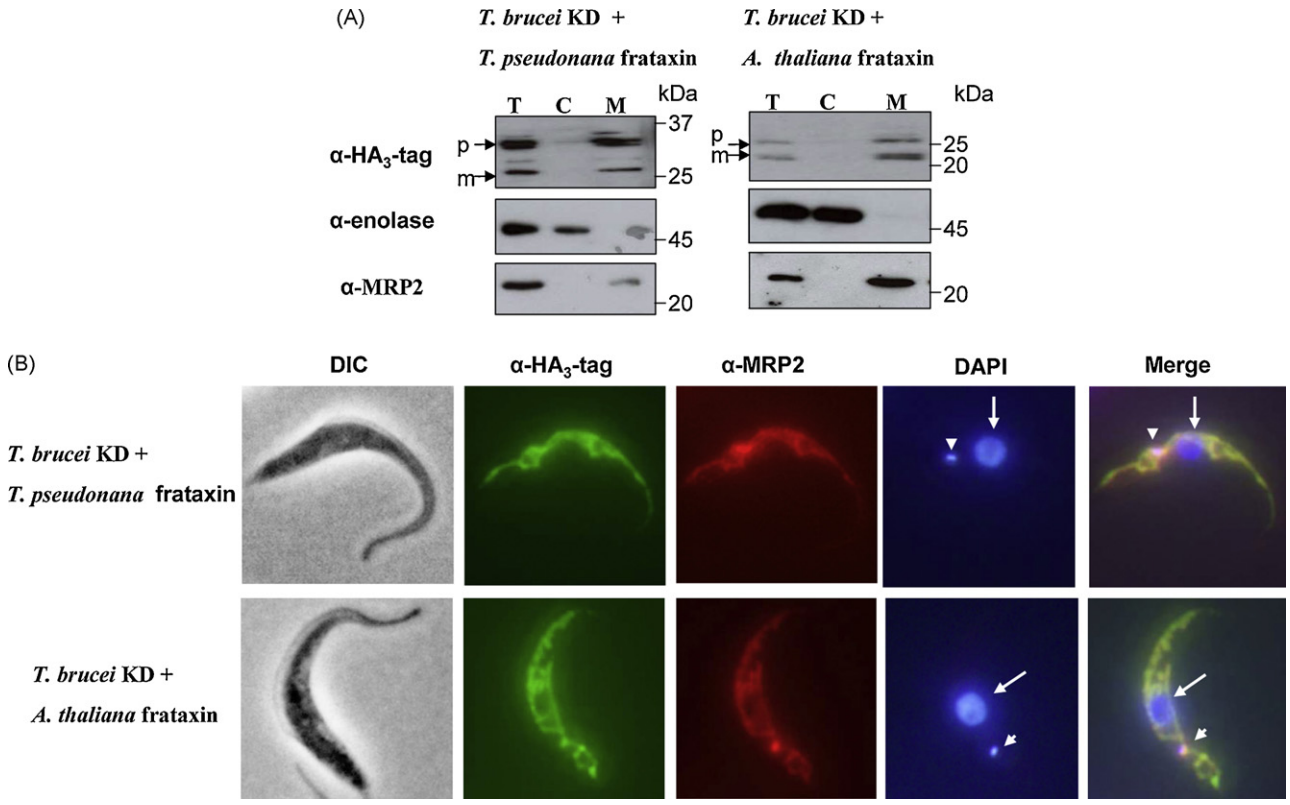

Fig. 3. Mitochondrial localization of constitutively expressed HA_3-tagged *T. pseudonana* and *A. thaliana* frataxins in the rescued *T. brucei* knockdowns. (A) Immunoblot analysis of the HA_3-tagged protein in total cell lysates (T), cytosolic (C) and mitochondrial fractions (M). The α-MRP2 (mitochondrial RNA binding protein 2) and α-enolase antibodies were used as mitochondrial and cytosolic markers, respectively. (B) Immunolocalization of the HA_3-tagged *T. pseudonana* and *A. thaliana* frataxins. Cells stably expressing the heterologous frataxins were fixed and treated with α-HA_3-tag mouse monoclonal antibody and α-MRP2 rabbit polyclonal antibody, followed by Alexa Fluor-488 (green) donkey α-mouse antibody and Alexa Fluor-594 (red) donkey α-rabbit antibody. Finally, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI). The merged images (merge) show colocalization of the HA_3-tagged *T. pseudonana* or *A. thaliana* frataxin with the mitochondrial protein MRP2 and DAPI. Arrowhead and arrow indicate the kinetoplast and nuclear DNA, respectively. DIC, differential interference contrast.

The *T. brucei* mitochondrion has several advantages for the mitochondrial research (single mitochondrion per cell, extensive up- and down-regulation of the organelle during life cycle, easy purification, substantial body of already available knowledge, etc.). Therefore, it is important to know that its either simplified or highly divergent translocases are able to efficiently import into the organelle proteins equipped with very short as well as very long import signals. Moreover, our study showed that even highly

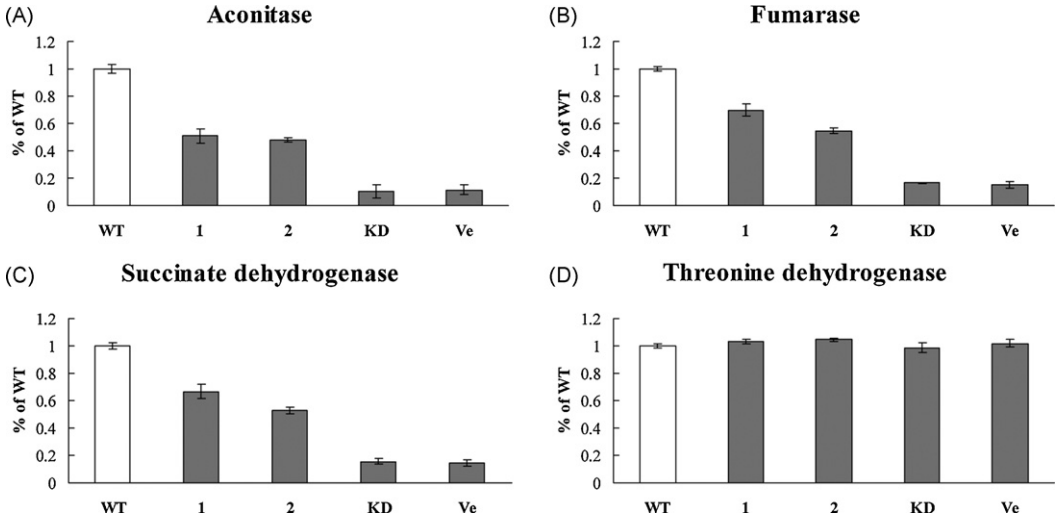

Fig. 4. Rescue effects on the activity of the Fe–S cluster-containing aconitase, fumarase and succinate dehydrogenase, and the Fe–S cluster-lacking threonine dehydrogenase, used as a control. The percentage of specific activity of a given enzyme in 29–13 cells (empty column), the induced (gray columns) Tb-frataxin RNAi knockdowns (KD), and the same cells transfected with either the HA_3-tagged *T. pseudonana* frataxin (1), the HA_3-tagged *A. thaliana* frataxin (2), or an empty pABPURO vector containing the HA_3-tag (Ve). The mean and the SD values of the activities of aconitase (A), fumarase (B), succinate dehydrogenase (C) and threonine dehydrogenase (D) in three independent RNAi inductions are shown.

diverged frataxins that retained only less than 40% sequence similarity with their *T. brucei* homologue are still able to mutually rescue its functions at least in part, in particular function related to the Fe–S cluster assembly. We propose that this information may be valuable for the identification of conserved residues with critical role in the function(s) of eukaryotic frataxins.

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References

[1] Bencze KZ, Kondapalli KC, Cook JD, et al. The structure and function of frataxin. Crit Rev Biochem Mol Biol 2006;41:269–91.
[2] Lill R, Mühlenhoff U. Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. Annu Rev Cell Dev Biol 2006;22:457–86.
[3] Cavadini P, Gellera C, Patel PI, Isaya G. Human frataxin maintains mitochondrial iron homeostasis in *Saccharomyces cerevisiae*. Hum Mol Genet 2000;9:2523–30.
[4] Duby G, Foury F, Ramazzotti A, Herrmann J, Lutz J, Lutz T. A non-essential function for yeast frataxin in iron-sulfur cluster assembly. Hum Mol Genet 2002;11:2635–43.
[5] Gakh O, Park S, Liu G, et al. Mitochondrial iron detoxification is a primary function of frataxin that limits oxidative damage and preserves cell longevity. Hum Mol Genet 2006;15:467–79.
[6] González-Cabo P, Vazquez-Manrique RP, García-Gimeno MA, Sanz P, Palau F. Frataxin interacts functionally with mitochondrial electron transport chain proteins. Hum Mol Genet 2005;14:2091–8.
[7] Lesuisse E, Santos R, Matzanke BF, Knight SA, Camadro JM, Dancis A. Iron use for haeme synthesis is under control of the yeast frataxin homologue (Yfh1). Hum Mol Genet 2003;12:879–89.
[8] Martelli A, Wattenhofer-Donze M, Schmucker S, Bouvet S, Reutenauer L, Pucci H. Frataxin is essential for extramitochondrial Fe–S cluster proteins in mammalian tissues. Hum Mol Genet 2007;16:2651–8.
[9] Park S, Gakh O, O'Neill HA, et al. Yeast frataxin sequentially chaperones and stores iron by coupling protein assembly with iron oxidation. J Biol Chem 2003;278:31340–51.
[10] Long S, Jirků M, Mach J, et al. Ancestral roles of eukaryotic frataxin: mitochondrial frataxin function and heterologous expression of hydrogenosomal *Trichomonas* homologs in trypanosomes. Mol Microbiol 2008;69:94–109.
[11] Busi MV, Maliandi MV, valdez H, et al. Deficiency of *Arabidopsis thaliana* frataxin alters activity of mitochondrial Fe–S proteins and induces oxidative stress. Plant J 2006;48:873–82.
[12] Vazzola V, Losa A, Soave C, Murgia I. Knockout of frataxin gene causes embryo lethality in arabidopsis. FEBS Lett 2007;58:667–72.
[13] Hausler T, Stierhof YD, Blattner J, Clayton C. Conservation of mitochondrial targeting sequence function in mitochondrial and hydrogenosomal proteins from the early-branching eukaryotes *Crithidia, Trypanosoma* and *Trichomonas*. Eur J Cell Biol 1997;73:240–51.
[14] Singha UK, Peprah E, Williams S, Walker R, Saha L, Chaudhuri M. Characterization of the mitochondrial inner membrane protein translocator Tim17 from *Trypanosoma brucei*. Mol Biochem Parasitol 2008;159:30–43.
[15] Schneider A, Bursac D, Lithgow T. The direct route: a simplified pathway for protein import into the mitochondrion of trypanosomes. Trends Cell Biol 2008;18:12–8.
[16] Keeling PJ, Burger G, Durnford DG, et al. The tree of eukaryotes. Trends Ecol Evol 2005;20:670–6.
[17] Hrdý I, Hirt RP, Dolezal P, et al. Trichomonas hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. Nature 2004;432:618–22.
[18] Armbrust EV, Berges JA, Bowler C, et al. The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. Science 2004;306:79–86.
[19] Rusconi F, Durand-Dubief M, Bastin P. Functional complementation of RNA interference mutants in trypanosomes. BMC Biotechnol 2005;5 [Art. No. 6].
[20] Horváth A, Horáková E, Dunajčíková P, et al. Down-regulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic *Trypanosoma brucei*. Mol Microbiol 2005;58:116–30.
[21] Smíd O, Horáková E, Vilímová V, et al. Knock-downs of mitochondrial iron-sulfur cluster assembly proteins IscS and IscU down-regulate the active mitochondrion of procyclic *Trypanosoma brucei*. J Biol Chem 2006;281:28679–86.
[22] Gentle IE, Perry AJ, Alcock FH, et al. Conserved motifs reveal details of ancestry and structure in the small TIM chaperones of the mitochondrial intermembrane space. Mol Biol Evol 2007;24:1149–60.