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Stage-specific requirement for Isa1 and Isa2 proteins in the mitochondrion of *Trypanosoma brucei* and heterologous rescue by human and *Blastocystis* orthologues

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Summary

IscA/Isa proteins function as alternative scaffolds for the assembly of Fe-S clusters and/or provide iron for their assembly in prokaryotes and eukaryotes. Isa are usually non-essential and in most organisms are confined to the mitochondrion. We have studied the function of TbIsa1 and TbIsa2 in *Trypanosoma brucei*, where the requirement for both of them to sustain cell growth depends on the life cycle stage. The TbIsa proteins are abundant in the procyclic form, which contains an active organelle. Both proteins are indispensable for growth, as they are required for the assembly of Fe-S clusters in mitochondrial aconitase, fumarase and succinate dehydrogenase. Reactive oxygen species but not iron accumulate in the procyclic mitochondrion upon ablation of the TbIsa proteins, but their depletion does not influence the assembly of Fe-S clusters in cytosolic proteins. In the bloodstream form, which has a downregulated mitochondrion, the TbIsa proteins are non-essential. The Isa2 orthologue of the anaerobic protist *Blastocystis* partially rescued the growth and enzymatic activities of TbIsa1/2 knock-down. Rescues of single knock-downs as well as heterologous rescues with human Isa orthologues partially recovered the activities of aconitase and fumarase. These results show that the Isa1 and Isa2 proteins of diverse eukaryotes have overlapping functions.

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Introduction

Iron–sulphur (Fe-S) proteins are present in all domains of life. Most of them are essential and it is estimated that in a typical eukaryotic cell over 100 proteins containing Fe-S clusters are involved in electron transport, catalysis, sensing and DNA/RNA metabolism. However, this is likely an underestimate because numerous other Fe-S cluster-dependent functions are continuously being elucidated and more proteins than previously appreciated have been shown to possess the evolutionarily ancient Fe-S cofactors (Johnson *et al.*, 2005; Lill and Mühlenhoff, 2005; Fontecave, 2006; Lill, 2009).

The current paradigm holds that most, if not all, Fe-S clusters in eukaryotes are synthesized within their mitochondria by the highly conserved iron–sulphur cluster (ISC) assembly machinery that originated from this organelle's prokaryotic endosymbiont ancestor. While a substantial fraction of the clusters formed *de novo* are integrated into organellar proteins, some of them are exported into the cytosol and the nucleus, where they are incorporated into the target proteins via a eukaryote-specific cytosolic Fe-S cluster pathway (= CIA) (Katoh *et al.*, 2002; Lill and Mühlenhoff, 2008). Key components of the ISC system are: (i) cysteine desulphurase (Nfs/IscS) that catalyses desulphurization of L-cysteine into alanine providing sulphur (Zheng *et al.*, 1993); (ii) frataxin (Yfh), which is proposed to deliver iron, although its exact function remains to be established (Lill and Mühlenhoff, 2008; Long *et al.*, 2008a); (iii) ferredoxin (Yah1) that provides electrons for the reduction of sulphur to sulphide in the clusters (Nakamura *et al.*, 1999); and (iv) a metallochaperone (IscU/Isu1), on which the Fe-S cofactors are transiently assembled (Nishio and Nakai, 2000; Mühlenhoff *et al.*, 2003). However, the list of conserved and essential components of the Fe-S cluster assembly pathway is steadily growing and currently includes approximately two dozen proteins (Lill, 2009).

With the exception of the CIA pathway, all dedicated machineries for Fe-S cluster assembly contain the A-type scaffold component (Lill, 2009). In eukaryotic genomes, the homologue(s) of prokaryotic IscA are the Isa proteins (Zheng *et al*., 1998; Rouault and Tong, 2005). In the yeast *Saccharomyces cerevisiae*, as well as in numerous other eukaryotes, two Isa homologues labelled Isa1 and Isa2 were recently shown to have an additional binding partner Iba57, also involved in Fe-S cluster assembly (Gelling *et al*., 2008). Isa1 is found in all multicellular model organisms, while it may be poorly conserved or even missing in protists with reduced mitochondria or mitosomes, such as *Encephalitozoon cuniculi*, *Cryptosporidium parvum*, *Entamoeba histolytica* and *Trichomonas vaginalis* (Lill and Mühlenhoff, 2005; Vinella *et al*., 2009).

It was proposed that the rather small IscA and SufA (member of the bacterial SUF operon) proteins serve in prokaryotic Fe-S cluster biosynthesis as alternative scaffold proteins (Krebs *et al*., 2001; Ollagnier-de-Choudens *et al*., 2001; Wollenberg *et al*., 2003; Lu *et al*., 2008) and a similar function was also suggested for their eukaryotic homologues (Wu *et al*., 2002). Furthermore, IscA may provide iron for the Fe-S cluster assembly in *Escherichia coli*, especially under limited iron conditions (Ding *et al*., 2004), a role consistent with its very strong iron-binding affinity (Ding and Clark, 2004). These features were recently demonstrated also for the human Isa1 (hIsa1) (Lu *et al*., 2010). Deletion of IscA or SufA causes only a minor growth phenotype in *E. coli* (Djaman *et al*., 2004), while the simultaneous elimination of both proteins is lethal (Tan *et al*., 2009) and can be partially rescued by hIsa1 (Lu *et al*., 2010).

Two independent crystallographic studies of the *E. coli* IscA revealed a monodimeric or monotetrameric form with a novel fold and a pocket that can accommodate an iron atom or a Fe-S cluster (Bilder *et al*., 2004; Cupp-Vickery *et al*., 2004). Moreover, Mossbauer spectroscopical analysis was instrumental in showing that IscA can bind both [2Fe-2S] and [4Fe-4S] clusters, although it remains to be established whether the former cluster type is an intermediate step in the construction of [4Fe-4S] cluster, or a derivative thereof (Ollagnier-de-Choudens *et al*., 2004).

Although the IscA/Isa proteins are widely distributed across the tree of Life, they do not seem to be essential, since their elimination in *E. coli* leads to only a mild phenotype (Tokumoto and Takahashi, 2001; Djaman *et al*., 2004). Two different explanations for this result have been put forward: (i) IscA/Isa proteins from different Fe-S cluster machineries can, to some extent, complement each other, or (ii) they are essential only under specific growth conditions (Fontecave and Ollagnier-de-Choudens, 2008). The latter possibility gained some support from the observation that IscA in *E. coli* is indispensable in the presence of high concentrations of oxygen (Johnson *et al*., 2006) but is non-essential under anaerobic conditions (Wang *et al*., 2010). The Isa proteins are also dispensable in *S. cerevisiae* (Jensen and Culotta, 2000; Pelzer *et al*., 2000), where they function as substrate-specific assembly factors required for proper function of biotin and lipoic acid synthases, as well as in the maturation of mitochondrial aconitase (Mühlenhoff *et al*., 2007; Gelling *et al*., 2008). The hIsa1 protein is expressed in multiple tissues (Córaz-Castellano *et al*., 2004) and was recently shown to have dual localization in human cells (Song *et al*., 2009). Even though the majority of the protein in HeLa cells was localized in the mitochondrial matrix, a fraction was also consistently found in the cytosol and it was proposed that hIsa1 might serve as a scaffold for the delivery of Fe-S clusters to aconitase in both cellular compartments (Song *et al*., 2009). In any case, the Isa1 deficiency in yeast was rescued by its human orthologue (Córaz-Castellano *et al*., 2004), while hIsa1 can rescue the IscA and SufA mutant in *E. coli* (Lu *et al*., 2010). Furthermore, a large-scale computational analysis uncovered a strong co-expression between the Isa1 and Isa2 proteins and the synthesis of haem (Nilsson *et al*., 2009). Still, too little is currently known about the potential cross talk between Fe-S cluster and haem biosynthetic processes (Wingert *et al*., 2005) to draw further conclusions.

In order to further clarify the function of the Isa proteins, we undertook their functional analysis in *Trypanosoma brucei*, a parasitic protist responsible for the highly pathogenic African sleeping sickness and other diseases. Trypanosomatids are evolutionarily distant from other eukaryotes that have been well studied, and represent the only excavate protists amenable to functional analyses. Moreover, trypanosomes are very interesting model organisms, in which the fully active mitochondrion of the procyclic stage transforms into a downregulated organelle that retains only basic functions in the bloodstream stage (Lukeš *et al*., 2005). We reasoned that functional analyses in these two different forms of mitochondria in trypanosomes may shed additional light on the still rather enigmatic function of the Isa proteins in the eukaryotic cell.

Results

Identification and phylogenetic analyses of Isa1 and Isa2

The available kinetoplastid genomes were searched for homologues of the yeast Isa1 and Isa2 proteins. A BLAST search identified two homologues in the *T. brucei* genome, here labelled TbIsa1 (Tb927.8.5540) and TbIsa2 (Tb927.5.1030). We identified similar Isa orthologues in the *Trypanosoma cruzi*, *Leishmania major, Leishmania*

braziliensis and *Leishmania infantum* genomes (Fig. S1A and B). Alignment of their deduced amino acid sequences with numerous prokaryotic and eukaryotic homologues revealed high conservation of both proteins among kinetoplastid flagellates, including the three cysteine residues predicted to be involved in the ligation of Fe or Fe-S clusters. From the sequence alignment it is apparent that the characteristic architecture of the *E. coli* IscA protein, composed of two α-helices and seven β-sheets (Bilder *et al*., 2004), is likely conserved in its *T. brucei* homologues, and this prediction was confirmed by structural modelling (data not shown). The TbIsa1 and TbIsa2 genes code for 272 and 173-amino-acid-long proteins with calculated molecular weights of 29.5 kDa and 19.0 kDa, respectively, and their overall identity is 23% at the amino acid level. Both proteins are predicted to contain a cleavable mitochondrial import signal, with probability 0.3733 and 0.9748 for TbIsa1 and TbIsa2, respectively (MitoProtII), and similarly high probabilities predicted by pSORT.

To investigate the phylogenetic distribution of the Isa1 and Isa2 proteins (ISA1/2) we used a previously published alignment from a genome wide search on these proteins (Vinella *et al*., 2009). To this alignment, we added the sequences of Isa1 and Isa2 from all available identified homologues but also some additional eukaryotic sequences recently available from genomic and transcriptomic projects. Maximum likelihood and Bayesian phylogenetic analyses demonstrated that Isa1 and Isa2 sequences from two clades (Fig. S1A). Interestingly, while most eukaryotes have both isoforms, all homologues from parasites such as *T. vaginalis* and *Giardia lamblia* cluster within the Isa2 clade, while the diatom *Thalassiosira pseudonana* homologues group within the Isa1 cluster. The genome of another parasite, *E. histolytica*, appears to be completely devoid of either Isa homologue (Fig. S1A).

Expression, subcellular localization and interaction of TbIsa

In order to establish the subcellular localization, and to follow the sedimentation properties and depletion of the TbIsa proteins, we have overexpressed the full-size Isa1 protein in *E. coli*, from which the abundantly expressed insoluble protein has been purified (data not shown). Specific polyclonal antibodies generated against TbIsa1 in a rat were used for further experiments. The polyclonal antibodies against TbIsa2 were generated against a synthetic oligopeptide derived from the *T. brucei* protein (see *Experimental procedures*). In order to verify the predicted mitochondrial localization of both proteins, we have obtained cytosolic and mitochondrial fractions from the procyclic form (PF) (strain 29–13) cells of *T. brucei*. The purity of both fractions, obtained by digitonin fractionation was confirmed by compartment-specific cytosolic and mitochondrial markers enolase and mitochondrial RNA binding protein (MRP) 2 respectively. As shown in Fig. 1A, all detectable TbIsa1 and TbIsa2 proteins are indeed confined to the organelle.

Based on the crystal structure of IscA it was predicted that in *E. coli* this protein forms one of the two alternative tetrameric oligomers (Bilder *et al*., 2004). We wondered whether the two trypanosome Isa proteins form a complex or bind the Iba57 protein, their interacting partner in yeast (Gelling *et al*., 2008). First, we tested possible interaction by sedimentation in glycerol gradients. Western analysis on the fractions showed that both proteins co-sediment in fractions 4–6 (Fig. S2), suggesting that Isa proteins may be in a complex. Sedimentation of mitochondrial proteins TbRGG1 and KREL1, used as controls, was in agreement with previously reported data (Hashimi *et al*., 2008). However, mass spectrometry analysis of proteins pulled down via TAP-tagged TbIsa1 and TbIsa2 did not identify TbIba57 (data not shown). Next, co-immunoprecipitations in the cell lysates with anti-TbIsa1 or anti-TbIsa2 antibodies, under physiological or high salt conditions, did not reveal mutual interactions between these two proteins (data not shown). It remains plausible though that, like the *E. coli* IscA, the TbIsa proteins form homo-dimers.

Expression of both TbIsa genes is essential in procyclic trypanosomes

In order to assess the function of individual Isa proteins in the PF of *T. brucei*, we used RNAi to selectively down-regulate the mRNA levels of TbIsa1 and/or TbIsa2. A fragment of the TbIsa1 gene or the entire TbIsa2 gene was cloned into the p2T7-177 vector containing opposing tetracycline-regulatable promoters. Moreover, another cell line was transfected with a NotI-linearized p2T7-177 vector containing fragments of the TbIsa1 and TbIsa2 genes cloned in tandem, which allowed their parallel ablation. For each construct, several clonal cell lines have been obtained by limiting dilution using phleomycin as a selectable marker. In representative clones RNAi was induced by the addition of 1 μg ml^{-1} tetracycline to the medium and the growth was monitored using cell counter. Upon RNAi induction, the growth of the TbIsa knockdowns was identical with the non-induced cells until day 3. Thereafter, the growth of the RNAi-induced TbIsa1 and TbIsa2 cells slowed significantly down (Fig. 1B and C). A cumulative effect occurred in the induced double knockdown cells, which virtually stopped dividing on day 4 post induction (Fig. 1D), and did not recover even after 12 days. The depletion of the target proteins was monitored by Western blot analysis using specific antibodies. In cells in which the TbIsa1 mRNA was targeted, a very strong

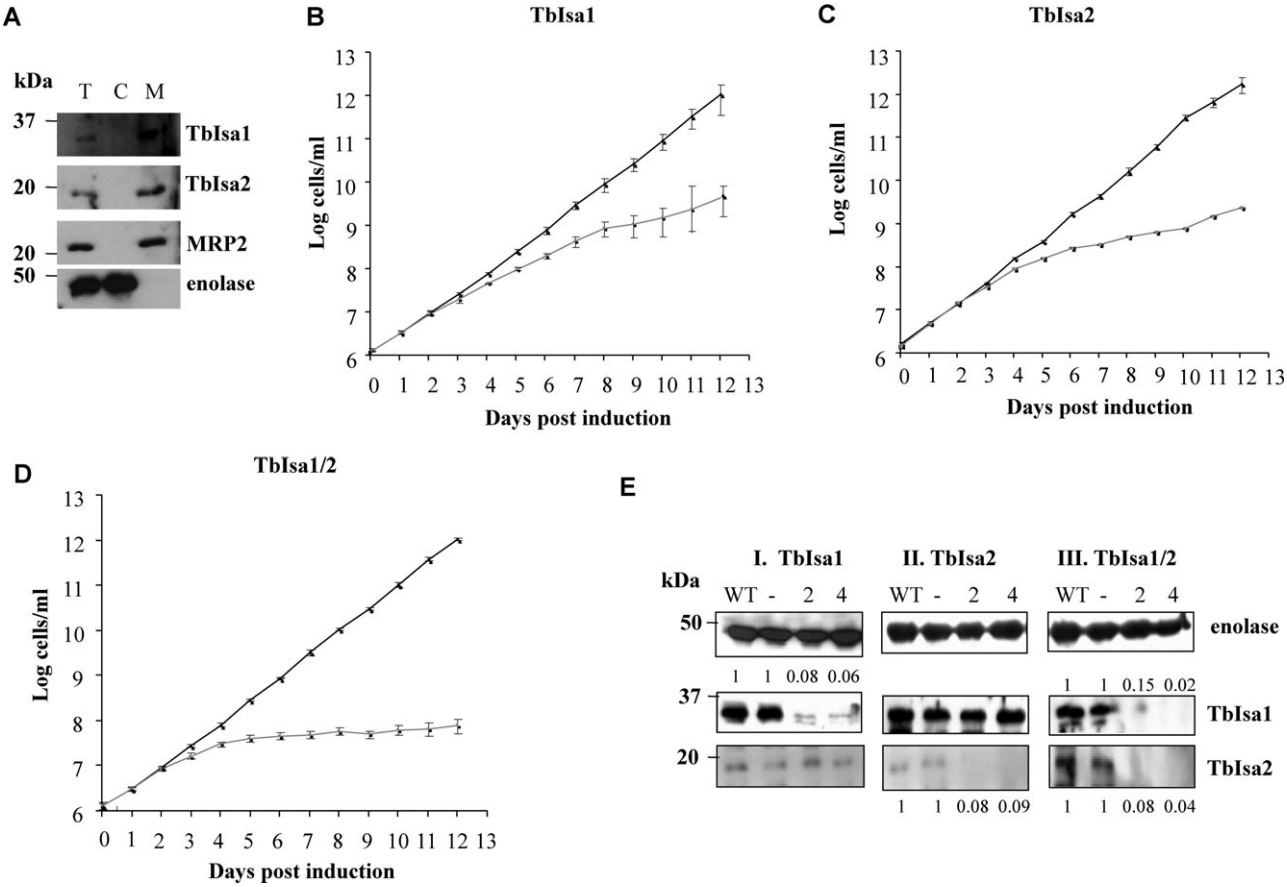

Fig. 1. TbIsa proteins are essential for growth of PF *T. brucei* and RNAi is specific for target TbIsa.
A. Western blot analysis of TbIsa1 and TbIsa2 in total lysates (T) and purified cytosolic fractions (C) and mitochondrial (M). Mitochondrial RNA binding protein 2 (MRP2) and enolase were used as mitochondrial and cytosolic markers respectively.
B–D. Growth inhibition following RNAi induction against TbIsa1 (B), TbIsa2 (C) and TbIsa1/2 (D). Cell numbers were measured using cell counter. The numbers of non-induced cells (squares; black line) and those after RNAi induction (circles; grey line) are indicated. The *y*-axis is labelled by a log scale and represents the product of cell densities measured and total dilution. The mean and the SD values represent the average of three independent RNAi experiments after selecting of *T. brucei* RNAi cell lines by Northern and Western blots.
E. Protein levels were analysed by Western blot analysis with α-TbIsa1 and α-TbIsa2 antibodies in whole-cell extracts from wild-type (WT) PF cells, non-induced (–) and RNAi-induced single (I. TbIsa1; II, TbIsa2) and double (III, TbIsa1/2) knock-downs after 2 and 4 days of induction. The band densities of TbIsa1 and TbIsa2 in the RNAi-induced cells are indicated above the bands, as compared with the bands in the non-induced cells. α-Enolase antibody was used as a loading control. Protein size marker positions are indicated.

decrease of the TbIsa1 protein occurred already by day 2 of RNAi induction (Fig. 1E). While more than 90% of the protein was eliminated at this early time point, residual TbIsa1 protein remained present at a stable level even by day 4.

In the TbIsa2 knock-downs, the anti-TbIsa2 antibody allowed observing the virtual disappearance of the target protein by day 2 (Fig. 1E). In both cell lines, Western analysis was instrumental to show that both antibodies and RNAi knock-downs are specific for their targets, as the levels of the non-interfered TbIsa protein remained unaltered even after 4 days of RNAi induction. Western analysis with the anti-TbIsa1/Isa2 antibodies confirmed a very efficient elimination of both proteins in the double knock-downs (Fig. 1E) and the lack of their mutual interaction.

TbIsa are required for the assembly of mitochondrial Fe-S clusters only

While early studies implicated the yeast Isa proteins with the assembly of both cytosolic and mitochondrial Fe-S clusters (Jensen and Culotta, 2000; Pelzer *et al.*, 2000), according to more recent data their function is associated only with the mitochondrion (Mühlenhoff *et al.*, 2007). We have tested the effect of individual and parallel depletion of the TbIsa proteins on selected enzymes in both cellular compartments of the PF trypanosomes. The Fe-S cluster-containing aconitase (encoded by one gene) and fumarase (encoded by two different genes) have dual localization in trypanosome cells, with 30 and 50% of aconitase and fumarase activities in the mitochondrion respectively (Saas *et al.*, 2000; Coustou *et al.*, 2006),

allowing us to measure their activities separately in the mitochondrion and cytosol (Fig. 2A and B). The purity of each compartment fraction was assessed by Western blot analysis using antibodies against mitochondrial MRP2 and cytosolic enolase (Fig. 2E). As shown in Fig. 2A, in all three RNAi knock-downs, 2 days upon the addition of tetracycline into the medium the mitochondrial aconitase activity dropped by 60%. Following additional 48 h, only about 25% of the activity persisted (Fig. 2A). The depletion of any of the TbIsa proteins had an even more dramatic effect on mitochondrial fumarase, which on day 2 decreased by between 60 to 75%, while on day 4 only 10–25% residual activity remained (Fig. 2B). A parallel measurement of the activities of aconitase and fumarase in pure cytosolic fractions obtained from the non-induced and RNAi-induced TbIsa knock-downs revealed that neither of these activities was decreased (Fig. 2A and B). The activity of succinate dehydrogenase (complex II), an Fe-S cluster containing enzyme that is exclusively mitochondrial, followed a pattern very similar to both above-mentioned enzymes. Its activity dropped most in the double knock-downs, with only 15% of its activity remaining after 4 days of RNAi induction (Fig. 2C). The activity of threonine dehydrogenase, an enzyme lacking Fe-S clusters, was used as a control; its activity remained unaffected in both the non-induced and RNAi-induced cells (Fig. 2D).

In order to evaluate possible associations of the TbIsa proteins with other components of the mitochondrial Fe-S cluster assembly machinery, we have followed the levels

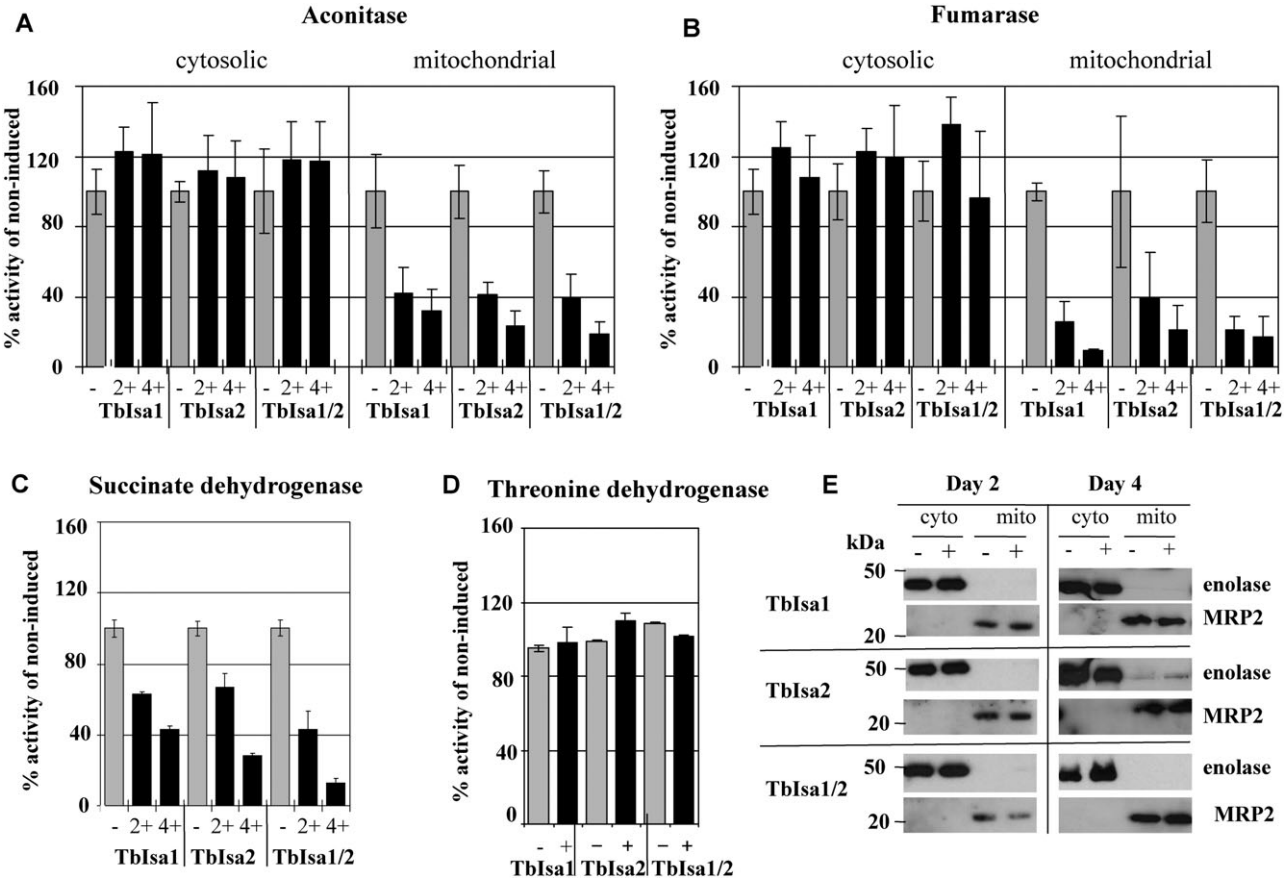

Fig. 2. Biochemical consequences of RNAi-mediated downregulation of TbIsa proteins in PF *T. brucei*.
A. The effect of RNAi against TbIsa1, TbIsa2 and TbIsa1/2 on the activity of the Fe-S cluster-containing cytosolic (left panel) and mitochondrial (right panel) aconitase. Enzymatic activities were measured in non-induced cells (grey columns) (–) and cells 2 and 4 days of RNAi induction (black columns) (+). Specific activities are shown as percentage of activities in non-induced cells; the mean and the SD values represent the average of three independent RNAi experiments.
B. Enzymatic activities of Fe-S cluster-containing fumarase are shown same as in (A).
C. Enzymatic activities of Fe-S cluster-containing mitochondrial succinate dehydrogenase (= complex II) are shown same as in (A).
D. Enzymatic activities of Fe-S cluster lacking threonine dehydrogenase are shown same as in (A).
E. The purity of the cellular fractions (cyto – cytosolic; mito – mitochondrial) obtained for the above enzymatic measurements from non-induced (–) cells, as well as from TbIsa1, TbIsa2 and TbIsa1/2 knock-downs after 2 and 4 days upon RNAi induction (+) was verified by Western analysis using α-enolase and α-MRP2 antibodies, which served as cytosolic and mitochondrial markers respectively. Protein size marker positions are indicated.

of the cysteine desulphurase Nfs, metallochaperone IscU and frataxin in the interfered cells 2, 4 and 6 days of RNAi induction. However, in both the single and double knock-downs the abundance of all of these proteins remained unaltered (Fig. S3).

Reactive oxygen species is elevated but iron content remains unaltered in the mitochondrion

We were interested to determine if the disturbance of mitochondrial Fe-S cluster-containing enzymes had a more general impact on functions of the organelle. Using dihydroethidium we have shown that all TbIsa RNAi-induced cells accumulate ROS (Fig. 3A–C). In yeast, disruptions of the ISC pathway, such as the depletion of the Isa proteins, frequently lead to iron accumulation in the mitochondrion. We wondered whether the same effect will also occur in the trypanosome cells. The concentration of iron was followed in mitochondrial fractions by the ferene method, which detects all intracellular iron except the one bound by haem (Pieroni *et al*., 2001). The measurement revealed an unaltered iron level in the organelle of RNAi-induced double knock-downs when compared with the non-induced cells (Fig. 3D).

Both TbIsa are non-essential in bloodstream trypanosomes

Since the depletion of TbIsa's exhibited an effect on the mitochondrial Fe-S cluster-containing proteins in the PF cells, we also generated RNAi knock-downs in the bloodstream form (BF), which parasitizes mammalian blood. These cells are known to have a largely downregulated organelle, as compared with the PF cells (Schneider, 2001; Lukeš *et al*., 2005; Tielens and van Hellemond, 1998). First, using the anti-TbIsa1 and anti-TbIsa2 antibodies, the levels of the TbIsa proteins in total lysates of the BF cells purified from the blood of a rat were shown to

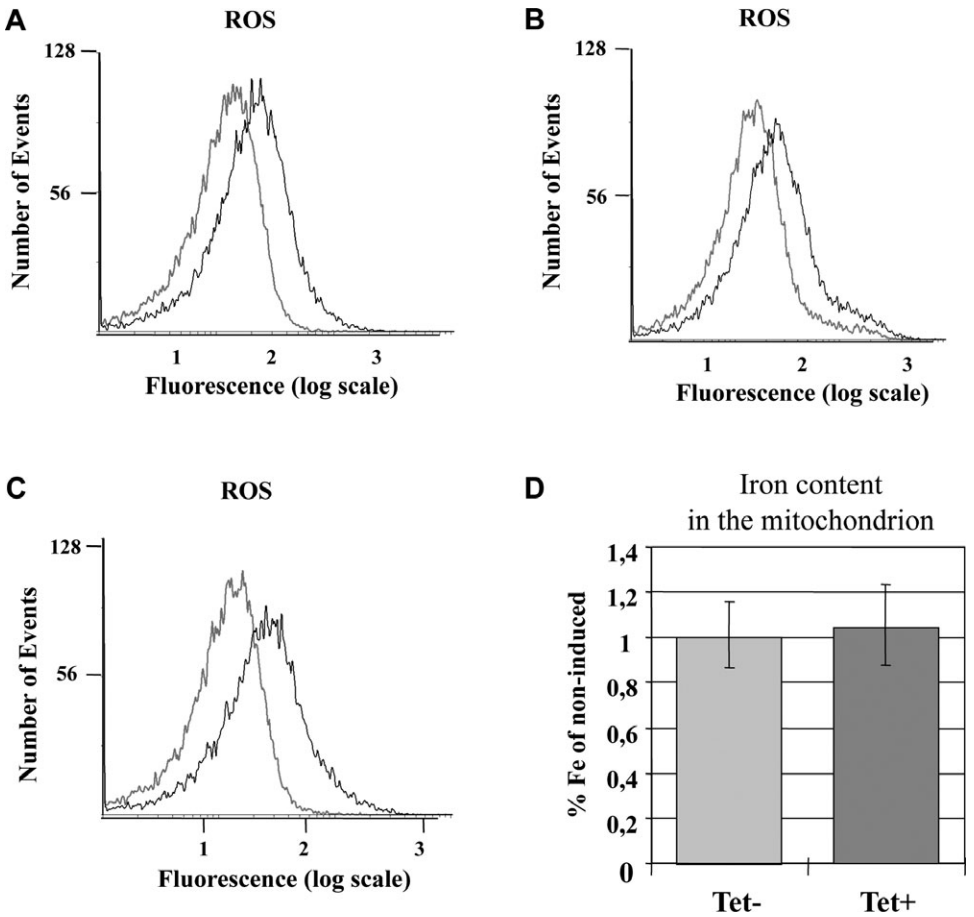

Fig. 3. Measurement of ROS and iron content in mitochondrion of PF *T. brucei* RNAi cell lines.
A–C. ROS were measured using dihydroethidium in TbIsa1 RNAi cells (A), TbIsa2 RNAi cells (B) and TbIsa1/2 RNAi cells (C) after 4 days of RNAi induction (black line), as compared with the respective non-induced cells (grey line). Representative data from three independent experiments are shown.
D. The concentration of iron was quantified by the ferene method for mitochondrial fraction obtained from the non-induced cells (–) and cells, in which TbIsa1/2 were ablated (4 days of RNAi induction) (+). Experiments were performed for three independent RNAi inductions.

be substantially lower than in the PF cells (Fig. 4A). Signal quantification revealed that there is about 4× and 6.2× less of TbIsa1 and TbIsa2, respectively, in the BF than in the PF cells. Next, the TbIsa1, TbIsa2 and TbIsa1/2 RNAi constructs used to deplete the target proteins in the PF cells were electroporated into the *T. brucei* 427 BF cells grown in HMI-9 medium. The growth of phleomycin-resistant non-induced and RNAi-induced clones was then followed for one week, with cells diluted on a daily basis. Unexpectedly, no or only a very weak growth phenotype was observed in either of the transfectants (Fig. 4B–D). The target TbIsa transcripts were undetectable in the BF

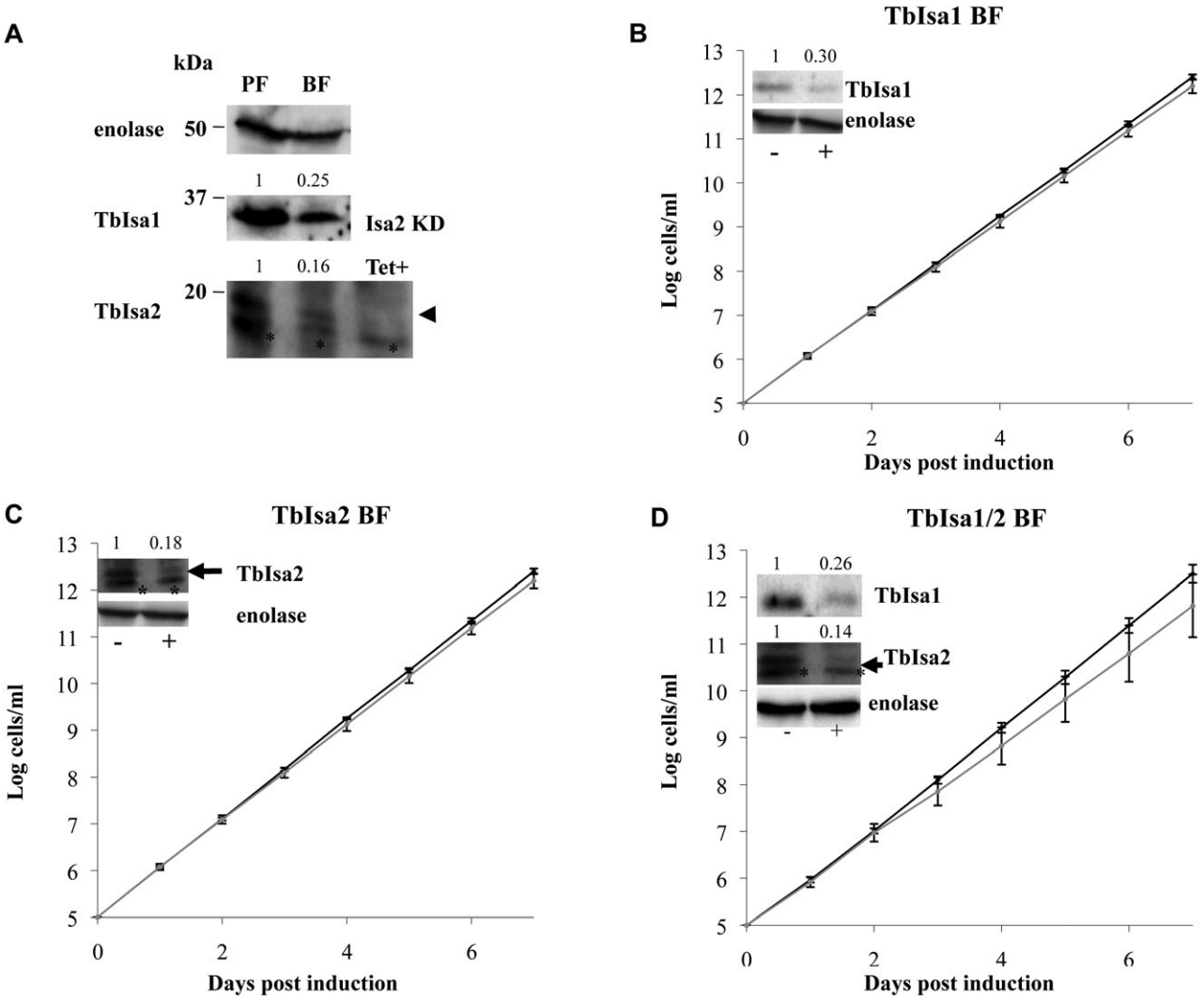

Fig. 4. TbIsa proteins are non-essential for BF *T. brucei*.
A. TbIsa1 and TbIsa2 are much less abundant in the BF cells (strain 920) than in the PF cells (strain 29–13), as shown by Western blot analysis with the α-TbIsa1 and α-TbIsa2 antibodies. The specific and non-specific bands recognized by the α-TbIsa2 antibodies are labelled with an arrow and asterisk respectively. Induced PF Isa2 RNAi cells were used as a control for the TbIsa2-specific band. Numbers above the bands indicate their relative density in the RNAi-induced cells as compared with the non-induced ones. Cytosolic enolase was used as a loading control. Protein size marker positions are indicated.
B–D. Growth curves of TbIsa1 (B), TbIsa2 (C) and TbIsa1/2 double knock-down (D) cells over 7 days showed that RNAi silencing altered only very weakly the growth of the BF cells. Cells were diluted every 24 h to a density of 10^5 cells ml^{-1} and their numbers were measured using cell counter. The numbers of non-induced cells (black line) and those after RNAi induction by the addition of 1 μg ml^{-1} tetracycline (grey line) are indicated. The *y*-axis is labelled by a log scale and represents the product of cell densities measured and total dilution. The mean and the SD values represent the average of three independent RNAi experiments after selecting RNAi cell lines by Northern and Western blots. The arrow indicates a sampling point for Western blot analysis. Western blot analysis of total protein extracted from the non-induced (–) cells, and knock-down cells 4 days of RNAi induction (+) are shown as insets. Numbers above the bands indicate their relative density in the RNAi-induced cells as compared with the non-induced ones. The α-TbIsa1 and α-TbIsa2 antibodies followed the target proteins, the α-enolase antibody was used as a loading control. The specific and non-specific bands recognized by α-TbIsa2 antibodies are indicated by arrow and asterisk respectively.

cells assayed by Northern blot analysis (data not shown), yet Western blot analysis with anti-TbIsa1/Isa2 antibodies confirmed a decrease by 70% and 85% of the targeted protein upon RNAi induction in the respective single knock-down (Fig. 4B and C). Equally efficient downregulation of both proteins was documented in the double knock-down cells (Fig. 4D).

Functional rescues with human Isa homologues

While human Isa1 has been intensely studied and can rescue *E. coli* (Lu *et al*., 2010) and yeast cells depleted for its homologues (Córaz-Castellano *et al*., 2004), nothing is known about the function of human Isa2. Here we have attempted to use the trypanosome model to study the human Isa proteins using a complementation assay. Based on our observation that TbIsa1 can to some extent substitute for TbIsa2, and vice versa, since both proteins have similar biochemical properties, we prepared various rescue combinations with the human Isa genes. To this end, the following rescue cell lines were generated: (i) TbIsa1 + hIsa1; (ii) TbIsa2 + hIsa2; (iii) TbIsa1 + hIsa2; (iv) TbIsa2 + hIsa1; (v) TbIsa1/2 + hIsa1; (vi) TbIsa1/2 + hIsa1/2; and (vii) TbIsa1/2 + hIsa2.

In both the human and *T. brucei* cells, most mitochondrion-targeted proteins are synthesized as precursors that are, upon import, proteolytically matured by mitochondrial processing peptidase. We have shown recently that the signal peptide on the human frataxin will efficiently target the downstream protein into the trypanosome organelle (Long *et al*., 2008b). Therefore, the coding sequence for the human frataxin signal peptide (1–54 residues) was introduced to replace the predicted signal peptide (1–13 residues) of hIsa1 gene obtained from the human cDNA library, after an attempt with full-length hIsa1 failed due to the lack of its import into the *T. brucei* mitochondrion (data not shown). The full-length hIsa2 with its genuine import signal inserted in the pFC4 vector was used in all rescue experiments with this protein. Import of these heterologous proteins was followed using antibody against the HA_3-tag attached to the C-termini of hIsa1 protein and with a specific antibody against hIsa2. As shown in Fig. 5A, all tagged hIsa1 was imported into the single mitochondrion of PF cells, while the situation was more complex in the case of hIsa2, which was equipped with its genuine import signal. Western blot analysis detected two forms, apparently representing the pre-processed (arrow) and processed (arrowhead) hIsa2, while no signal was detected in cell lines lacking the hIsa2 construct (Fig. 5C). Therefore, we resorted to Western blot analysis of cellular fractions from the TbIsa2 + hIsa2 cells. As expected, the resulting mitochondria contained only the shorter processed form of hIsa2, whereas the most abundant species in the cytosol was the pre-processed protein. In the total cell lysate, the processed hIsa2 protein predominated, although the processing of this alien protein was apparently less efficient relative to the genuine trypanosome mitochondrial proteins (Fig. 5B).

Next, we measured the activities of aconitase and fumarase in the mitochondrion, as activities of these proteins in the cytosol were not influenced by the status of the TbIsa proteins (Fig. 2). Comparative measurement of these activities in the above-described PF cell lines, as well as in cells with ablated TbIsa2 and TbIsa1/2 showed that both activities are, to some extent, rescued in all cases (Fig. 6). Particularly efficient is the rescue of TbIsa2 by hIsa2, while the other combinations resulted in less efficient rescues, especially when aconitase is considered (Fig. 6A). The activity of fumarase was restored to almost wild-type levels even in both cross-rescues (TbIsa1 + hIsa2 and TbIsa2 + hIsa1) (Fig. 6B), strongly indicating that the functions of the human Isa proteins are overlapping in *T. brucei*. However, although the cell growth was not recovered by the human Isa proteins (data not shown), high level of enzymatic activities were measured in the rescue cells.

Rescue with Blastocystis *Isa2*

So far, no information is available on the function of the Fe-S cluster assembly proteins identified in the anaerobic mitochondrion-related organelles (MROs) of *Blastocystis hominis*. Since MROs display a mixture of mitochondrial and hydrogenosomal features, they are functionally and evolutionarily distinct from the canonical aerobic mitochondria of yeast and mammals (Stechmann *et al*., 2008). Being the only Isa homologue identified in *B. hominis* (Fig. S1), BhIsa2 was used to rescue the TbIsa deficiency in *T. brucei*. The full-length BhIsa2 gene amplified from a cDNA molecule was cloned into the pABPURO vector and introduced into the PF *T. brucei* inducible for RNAi against the *T. brucei* Isa1/2 double knock-down. The empty pABPURO vector and TbIsa1/2 RNAi knock-down were used as controls. The growth phenotype triggered by the depletion of TbIsa1/2 was partially rescued by the expression of BhIsa2 (data not shown). Furthermore, the activities of mitochondrial fumarase (Fig. 7A), aconitase (Fig. 7B), as well as succinate dehydrogenase (Fig. 7C), almost reached the wild-type levels.

Discussion

Trypanosoma brucei and related flagellates are responsible for devastating diseases of humans and other vertebrates in most tropical regions. These parasites are also the most genetically tractable excavate protists, and as a consequence, they are the only representatives of this

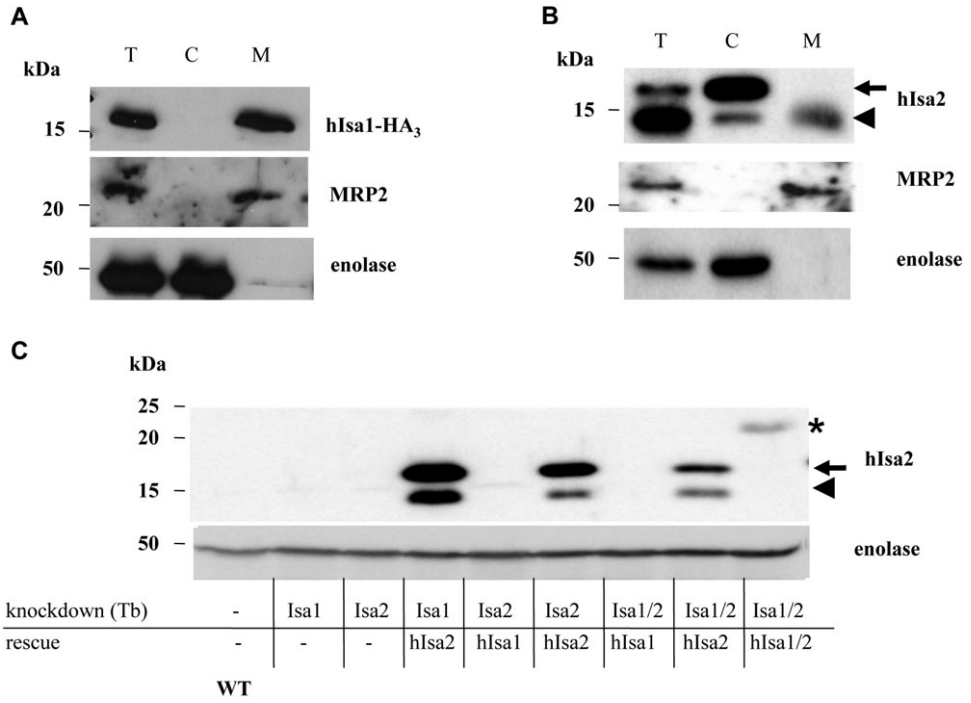

Fig. 5. Human Isa proteins are expressed, processed and efficiently targeted into the mitochondrion of PF *T. brucei*.
A. Western blot analysis of the expression and targeting of the HA_3-tagged human frataxin signal peptide lead hIsa1. Total cell lysate (T), cytosolic (C) and mitochondrial (M) fractions isolated by digitonin fractionation were probed with antibody against the HA_3 tag. α-MRP2 and α-enolase antibodies were used as mitochondrial and cytosol-specific markers respectively.
B. Western blot analysis of the expression, processing and targeting of hIsa2. Total cell lysate (T), cytosolic (C) and mitochondrial (M) fractions isolated by digitonin fractionation were probed with antibody against human Isa2. α-MRP2 and α-enolase antibodies were used as mitochondrial and cytosolic markers respectively. The pre-processed and processed forms of hIsa2 are indicated with an arrow and arrowhead respectively.
C. Western blot analysis of the expression and processing of hIsa2 in various PF cell lines. The table below describes which TbIsa protein was downregulated (knockdown-Tb) by RNAi, and which human (h) Isa protein was used to complement the RNAi knock-down cells (rescue).Total lysate was obtained from the following cells: 29–13 PF cells (WT), TbIsa1 knock-down, TbIsa2 knock-down, TbIsa1 knock-down transfected with pFC4-hIsa2, TbIsa2 knock-down transfected with pABPURO-hIsa1, TbIsa2 knock-down transfected with pFC4-hIsa2, TbIsa1/2 double knock-down transfected with pABPURO-hIsa1, TbIsa1/2 double knock-down transfected with pFC4-hIsa2, TbIsa1/2 double knock-down transfected with both pABPURO-hIsa1 and pFC4-hIsa2-HA. The pre-processed and processed forms of hIsa2 are indicated with an arrow and arrowhead respectively. The HA_3-tagged hIsa2 corresponds to the pre-processed form and is labelled with an asterisk. α-Enolase antibody was used as loading control.

eukaryotic super-group, in which functional studies are routinely made. We are interested in investigating how Fe-S clusters are generated in this deeply divergent and medically highly relevant protist.

So far, we have demonstrated that several key components of the pathway are present in *T. brucei* and their function seems to be conserved with respect to the well-studied eukaryotes, such as *S. cerevisiae*, *Arabidopsis thaliana* and humans. A preliminary search for genes encoding members of the ISC and CIA pathways in the *T. brucei* genome revealed not only the presence of all conserved components but in a few cases even the existence of several homologues of otherwise single-copy genes (S.L and J.L., unpubl. results). Therefore, it is reasonable to assume that the complexity of the Fe-S cluster assembly in this unicellular eukaryote will be similar to that of the sophisticated machinery emerging from the studies of multicellular organisms.

Proper assembly of the Fe-S clusters in trypanosomes clearly requires the function of cysteine desulphurase Nfs, its interaction partner Isd11 and the metallochaperone IscU, the bulk of which is localized in the single reticulated mitochondrion (Smid *et al.*, 2006; Paris *et al.*, 2010). More recently, we have shown that, although undetectable by Western blot analysis, a tiny amount of Nfs is also present in the cytosol/nucleus and the same seems to apply for selenocysteine lyase, which has overlapping functions with the above-mentioned protein (Poliak *et al.*, 2010). Furthermore, the *T. brucei* Nfs is indispensable for the thiolation of tRNAs in both mitochondrial and cytosolic compartments (Wohlgamuth-Benedum *et al.*, 2009). Another core ISC component, frataxin, is essential for Fe-S clusters incorporated not only in the mitochondrial proteins, but also in the cytosolic ones, although for survival of the trypanosome, mitochondrial localization of frataxin is mandatory (Long *et al.*, 2008a,b).

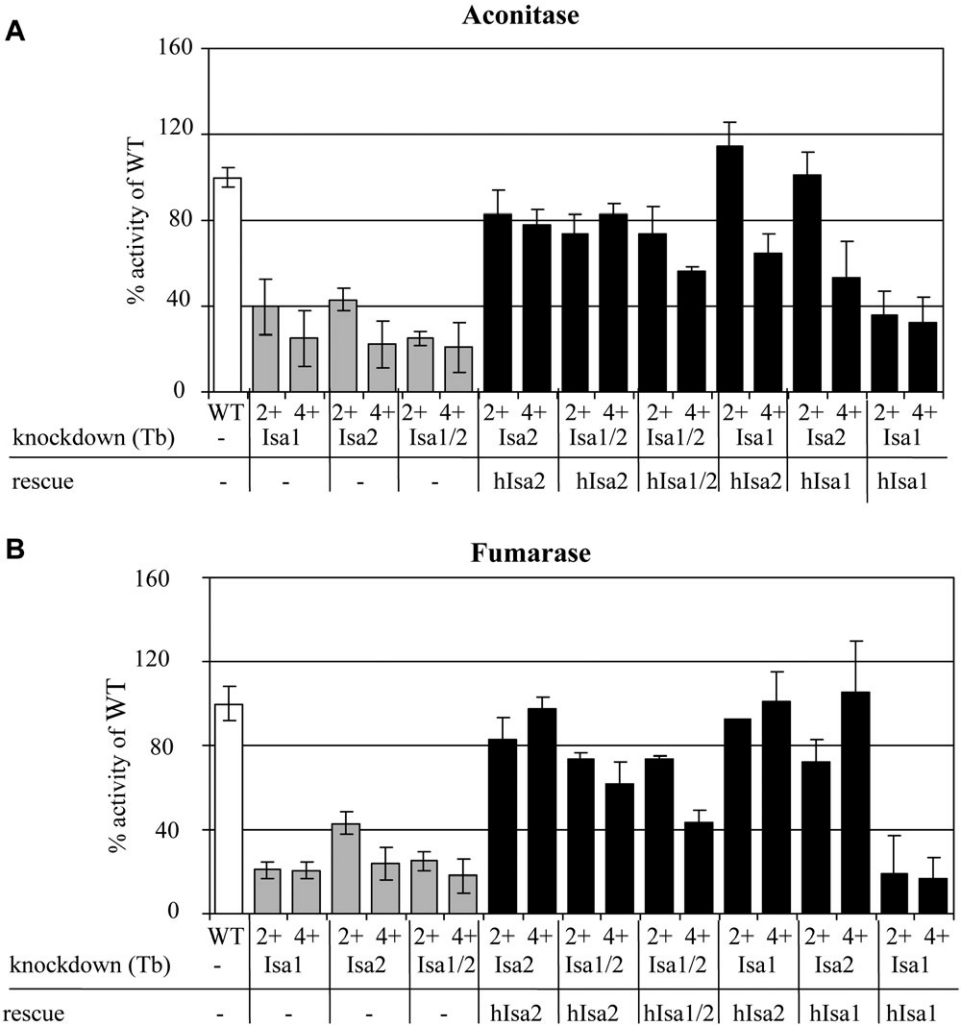

Fig. 6. Biochemical consequences of TbIsa knock-downs (cross) rescued with human Isa proteins in PF *T. brucei*.
A. Enzymatic activity of aconitase was measured for mitochondrial fractions obtained with digitonin fractionation. Its activity in 29–13 PF cells represents 100% (white column), activities in TbIsa1, TbIsa2 and TbIsa1/2 double knock-down 2 and 4 days upon RNAi inductions (+) (grey columns). Activities shown in black columns are from the following cells: TbIsa2 knock-downs transfected with pFC4-hIsa2, TbIsa1/2 double knock-down transfected with pFC4-hIsa2, TbIsa1/2 double knock-down transfected with pABPURO-hIsa1 and pFC4-hIsa2, TbIsa1 knock-down transfected with pFC4-hIsa2, TbIsa2 knock-down transfected with pABPURO-hIsa1, TbIsa1 knock-down transfected with pABPURO-hIsa1+HA_3; the mean and the SD values represent the average of measurements of three independent RNAi inductions. The table below describes which TbIsa protein was downregulated (knockdown-Tb) by RNAi, and which human (h) Isa protein was used to complement the RNAi knock-down cells (rescue).
B. Enzymatic activity of Fe-S cluster-containing mitochondrial fumarase. Cell lines are same as in (A).

Here we have focused on the role of the eukaryotic Isa proteins, functionally studied so far mostly in *S. cerevisiae*. Until recently, they were affiliated with the Fe-S cluster assembly of mitochondrial proteins such as aconitase and biotin synthase (Pelzer *et al.*, 2000; Mühlenhoff *et al.*, 2007). This view was, however, recently challenged, as human Isa1 was proposed to play an important role in both mitochondrial and cytosolic Fe-S cluster assembly in HeLa cells (Song *et al.*, 2009). We have therefore inspected activities of the Fe-S cluster-containing fumarase and aconitase, which have a dual localization in the PF trypanosomes (Saas *et al.*, 2000; Coustou *et al.*, 2006) and are thus particularly suitable for assessing the impact on different cellular compartments. Whereas activities of both enzymes invariably dropped in both the mitochondrion and cytosol of the PF cells upon the depletion of Nfs, IscU, Isd11 or frataxin (Smid *et al.*, 2006; Long *et al.*, 2008a; Paris *et al.*, 2010), the consequence of depletion of the Isa proteins was different, as it impacts only on the organellar Fe-S cluster-containing enzymes. Moreover, the activity of succinate dehydrogenase was strongly depleted in all TbIsa knock-downs, a phenomenon which went unnoticed in other eukaryotes (Jensen and Culotta, 2000;

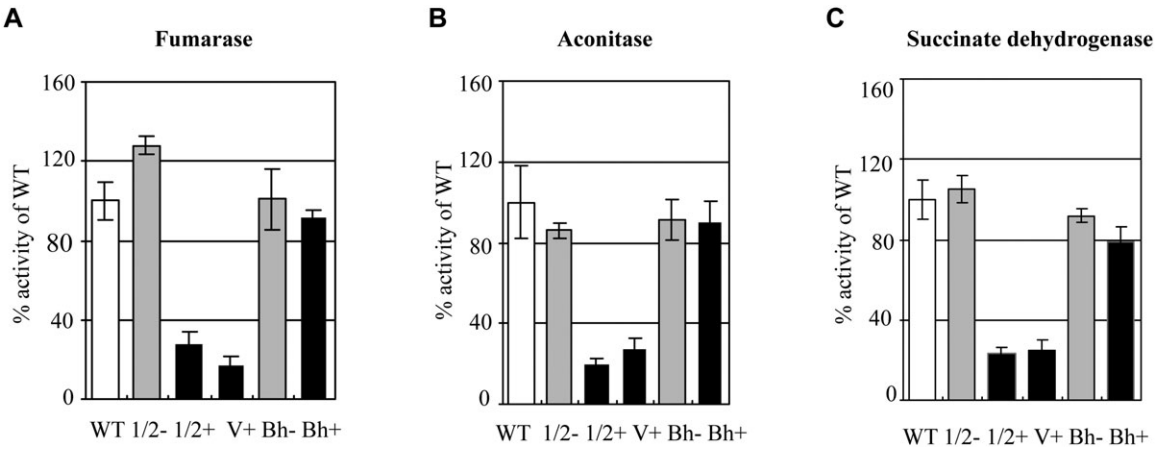

Fig. 7. Biochemical consequences of TbIsa1/2 double knock-down rescued with *B. hominis* Isa2 proteins in PF *T. brucei*. Enzymatic activities of fumarase (A), aconitase (B) and succinate dehydrogenase (C) were measured in the digitonin isolated mitochondria. A given activity in 29–13 PF cells represents 100% (white column). Activity in non-induced TbIsa1/2 cells (1/2–) and the same cell transfected with BhIsa2 (Bh–) is shown in grey columns. Activity in RNAi-induced TbIsa1/2 (1/2+), TbIsa1/2 with an empty pABPURO vector (V+), and those transfected with BhIsa2 (Bh+) after 4 days of RNAi induction is shown in black columns; the mean and the SD values represent the average of measurements from three independent RNAi induction.

Kaut *et al*., 2000; Pelzer *et al*., 2000; Mühlenhoff *et al*., 2007; Gelling *et al*., 2008).

It is likely that this drop in activities of all three inspected mitochondrial Fe-S proteins is responsible for the observed lethal phenotype. Moreover, the ablation of the TbIsa proteins caused a dramatic increase of ROS in all RNAi-induced cell lines, particularly in the double knock-down. We attribute this effect to the disruption of the respiratory chain due to the shortage of Fe-S clusters needed for its components, not to the Fenton reaction, since iron did not accumulate in the TbIsa-depleted organelle. Particularly telling is the apparent lack of importance of TbIsa in the BF cells, in which both proteins can be ablated with no impact on cell growth. It is well known that the BF mitochondrion is considerably reduced and lacks respiratory complexes, yet it still efficiently imports proteins, maintains membrane potential, and replicates and transcribes its mitochondrial DNA, the transcripts of which are properly edited and translated (Schneider, 2001; Hashimi *et al*., 2010). This organelle lacks most if not all Fe-S cluster-containing proteins such as respiratory complexes I thru III (Besteiro *et al*., 2005; Lukeš *et al*., 2005; Tielens and van Hellemond, 1998), fumarase (Coustou *et al*., 2006) and aconitase (P.C and J.L., unpubl. results). The only currently known [2Fe-2S] cluster-containing mitochondrial protein in the BF *T. brucei*–monothiol glutaredoxin 1 is, however, non-essential (Comini *et al*., 2008). Our results provide indirect, but rather strong evidence that except for a very low amount of aconitase, no other proteins containing the [4Fe-4S] clusters are made in this downregulated organelle, rendering the two TbIsa proteins fully dispensable for this life stage. Moreover, non-essentiality of the TbIsa proteins for the BF *T. brucei* further confirmed that they do not participate in Fe-S cluster export and cytosolic Fe-S cluster assembly. It is reasonable to assume that both these pathways are required also at the BF stage, since numerous cytosolic Fe-S proteins, such as Rli1 are essential for yeast (Kispal *et al*., 2005) as well as trypanosomes (Estévez *et al*., 2004). Still, the low amount of TbIsa in BF cells may reflect the existence of as yet unidentified [4Fe-4S] proteins in their mitochondrion.

Recently, Song *et al*. (2009) showed that in HeLa cells the majority of hIsa1 is located in the mitochondrion, while a miniscule but functionally important amount of this protein is also present in the cytosol. Although we cannot entirely rule out a similar situation in the studied flagellates, two lines of evidence presented in this study – the lack of an effect of TbIsa ablation on the cytosolic Fe-S cluster proteins and the non-essentiality of these proteins in the BF cells – indicate that the TbIsa proteins only have a functional role in the mitochondrion of *T. brucei*. In *E. coli* IscA was shown to be required for maturation of the [4Fe-4S] enzymes (Tan *et al*., 2009). The non-essentiality of TbIsa for cytosolic Fe-S proteins indicates that ferredoxin, an [2Fe-2S] protein and electron donor for the ISC machinery (Lill and Mühlenhoff, 2008) indeed remains unaffected by the lack of the TbIsa proteins in the organelle. It thus appears that in *T. brucei* the Isa proteins specifically function for mitochondrial [4Fe-4S] proteins, such as fumarase, aconitase and succinate dehydrogenase. However, the cofactor of IscA/Isa proteins, whether it is iron (Ding and Clark, 2004; Ding *et al*., 2004; Lu *et al*., 2010) or Fe-S cluster (Ollagnier-de-Choudens *et al*., 2004), remains elusive. Our

attempts to identify it in the TbIsa proteins failed due to low expression of the tagged proteins in *T. brucei*.

It is also worth noting that haem is not synthesized in trypanosomes (Kořený *et al*., 2010), so the association of Isa1 with haem synthesis, supported by the data obtained in mouse and zebra fish (Nilsson *et al*., 2009), is certainly not contributing to its essentiality in trypanosomes. Furthermore, the [4Fe-4S] cluster-carrying biotin synthase, which requires the assistance of Isa proteins for the synthesis of biotin in the *S. cerevisiae* mitochondria (Mühlenhoff *et al*., 2007), is not encoded in the *T. brucei* genome and thus cannot contribute to the observed growth phenotype.

We have demonstrated that TbIsa1 and TbIsa2 have similar functions in mitochondrion-confined Fe-S cluster assembly, the essentiality of which is particularly visible in PF RNAi double knock-downs, a situation unlike yeast (Jensen and Culotta, 2000; Pelzer *et al*., 2000). It was shown recently that human Isa1 was able to partially rescue the growth of the *E. coli* IscA and SufA double knock-out (Lu *et al*., 2010), but no characterization of hIsa2 rescues has been provided so far. Here we demonstrate the capacity of hIsa1 and/or hIsa2 to partially cross-rescue the activities of mitochondrial aconitase and fumarase in both single and double TbIsa1 and/or TbIsa2 knock-downs, proving their overlapping function. Ablation of TbIsa2 and complementation of the *T. brucei* cell line with hIsa2 seems to have a stronger effect on mitochondrial Fe-S proteins than that of their counterparts. However, in none of the various rescue combinations cell growth was fully recovered, indicating that hIsa failed to replace an as yet unknown activity of the TbIsa proteins.

The fact that both TbIsa proteins are essential components of the mitoproteome of PF *T. brucei*, their function being conserved as confirmed by rescue with orthologues from evolutionarily very distantly related anaerobic unicellular and aerobic multicellular eukaryotes, testifies to the unique and indispensable role of the Isa proteins in the Fe-S cluster assembly.

Experimental procedures

Phylogenetic analysis

For Isa1/2 sequences, multiple sequence alignments were created using MAFFT (Katoh *et al*., 2002) based on a seed alignment of ATC sequences kindly provided by Celine Brochier-Armanet (Vinella *et al*., 2009). The complete multiple sequence alignment was trimmed using MANUEL (Blouin *et al*., 2009) and phylogenetic trees were estimated from alignments by using RAxML 7.04 (Stamatakis *et al*., 2005) with the LG+F model of amino acid substitution and a γ model of rate heterogeneity. Bayesian phylogenetic analysis was carried out using MrBayes with the same model settings.

RNAi constructs, transfections, cloning and RNAi induction

To downregulate the Isa1 and Isa2 mRNAs by RNAi, 530-nt- and 522-nt-long fragments of the Isa1 and Isa2 genes were amplified using primer pairs Isa1-FP and Isa1-RP, and Isa2-FP and Isa2-RP (Table S1), respectively, from total genomic DNA of the *T. brucei* strain 29–13. Both amplicons were separately cloned into the p2T7-177 vector, which was, upon NotI-mediated linearization, introduced into procyclic (PF) *T. brucei* 29–13 cells using a BTX electroporator and selected as described elsewhere (Vondrušková *et al*., 2005). The double knock-down was prepared by cloning the same fragment of the Isa2 gene, into the p2T7-177 + Isa1 construct, which was stably integrated into the *T. brucei* 29–13 PF cells as described above. All three RNAi constructs were introduced into the 427 BF *T. brucei* cells using the Amaxa Nucleofector II electroporator, with transfectants kept in the HMI-9 medium and selected following a protocol described elsewhere (Hashimi *et al*., 2008).

Parasite cell culture

PF *T. brucei* (cell line 29–13) was cultivated at 27°C in SDM-79 medium containing G418 and hygromycin (Vondrušková *et al*., 2005). BF *T. brucei* (strain 427) was grown *in vitro* in HMI-9 medium supplemented with hygromycin (Hashimi *et al*., 2008). Wild-type BF (strain 920) were harvested from the blood of an infected rat by cardiac exsanguination when the parasitaemia reached 1×10^9 cell ml^{-1}. Blood was placed onto a DE52 DEAE cellulose (Whatman) column, washed with PSG buffer (38 mM Na_2HPO_4, 2 mM KH_2PO_4, 80 mM glucose, 29 mM NaCl, pH 8.0), eluted parasites were collected by centrifugation and the pellet was stored at–80°C until further use.

Following Northern and/or Western blot analyses, one clone out of four of Tb-Isa1, TbIsa2 and TbIsa1/2 RNAi knock-downs were used for further experiments. In the *T. brucei* PF and BF cells RNAi was induced by the addition of 1 μg ml^{-1} tetracycline to the SDM-79 and HMI-9 medium respectively. Cell density was measured every 24 h using the Beckman Z2 Coulter counter over a period of 12 days for PF and 7 days for BS after the induction of double stranded RNA synthesis. Cell morphology was analysed under the light microscope and by staining with DAPI and Giemsa.

Expression of recombinant TbIsa1, glycerol gradient and digitonin fractionation

The full-size TbIsa1 gene was amplified by PCR with primers Isa1-FP/O and Isa1-RP/O (containing the stop codon) (Table S1). The amplicon was gel-purified and cloned into the pET/100D expression vector (Invitrogen). The resulting expression plasmid encoding His_6-tagged TbIsa1 was transformed into the *E. coli* strain BL21 (star DE3) (Novagen). Insoluble protein was obtained from induced bacterial cells (incubation at 37°C for 3 h, and induced with 1 mM IPTG) under denaturing conditions using ProBond Ni-chelating resin (Invitrogen). Digitonin fractionation of the *T. brucei* PF and BF cells was performed following a protocol described

elsewhere (Smid *et al*., 2006). Glycerol gradient of the PF cells was performed as described previously (Hashimi *et al*., 2008).

Preparation of antibodies and Western blot analyses

Polyclonal antibody against the TbIsa1 protein was prepared by immunizing a rat at two-week intervals with the purified recombinant Isa1 protein by Cocalico Biologicals (Reamstown, PA, USA). In the case of TbIsa2, a synthetic oligopeptide (QPKSQELRTVAEGEC) corresponding to amino acids 97–110 of the *T. brucei* protein was used to raise polyclonal antibodies in a rabbit, which were subsequently affinity-purified by GeneScript. Cell lysates corresponding to 5×10^6 cells/lane of the PF cells, or 1.5×10^7 cells of the BF cells were separated on a 15% SDS-polyacrylamide gel, transferred to membranes and probed. The anti-TbIsa1 polyclonal rat antibodies and the anti-TbIsa2 rabbit antibodies were used at 1:1000 and 1:25 dilutions respectively. The polyclonal antibodies against MRP2 (Vondrušková *et al*., 2005), frataxin (Long *et al*., 2008a), IscS and IscU (Smid *et al*., 2006), enolase (provided by P.A.M. Michels), aconitase (provided by M. Boshart), and human Isa2 (provided by H. Puccio) were used at 1:1000, 1: 1500, 1:500, 1:1000, 1:200 000, 1:500 and 1:1000 dilutions respectively. Antibodies against TbRGG1 and KREL1 were used as described elsewhere (Hashimi *et al*., 2008). The HA_3- or TAP-tagged *in vivo* expressed TbIsa proteins were determined by Western blot analysis using corresponding polyclonal antibodies followed by appropriate secondary antibodies conjugated with horse radish peroxidase (Sigma), and visualized using ECL substrates (Pierce) (Hashimi *et al*., 2008; Long *et al*., 2008a). For quantification of signals in Western blots, the program ImageJ 1.44p (NIH) was used.

Measurement of intracellular iron

Cytosolic and mitochondrial fractions were prepared by digitonin fractionation as described above. After protein concentration was estimated with the Bradford reagent (Bio-Rad), the volume of the fractions was decreased using a vacuum centrifuge, but the protein pellet was kept wet. Iron content was quantified by the ferene method (Hennesy and Reid, 1984; Pieroni *et al*., 2001). Briefly, 3–5 mg protein per sample was resuspended in 100 µl of phosphate buffered saline, treated with 50 µl concentrated HCl in 100°C for 15 min, the sample was centrifuged and supernatant was transferred to a new tube. Fifty microlitres of the supernatant was mixed with 0.3 ml 1 M acetic acid-sodium acetate buffer (pH 5.5), then briefly treated with 5 µl thioglycolic acid with subsequent addition of 10 µl 6.25 mM ferene-S (Sigma). The reaction was incubated for 1 h at room temperature, and the absorbance was measured at 593 nm using a Tecan Spectrometer. To calculate the number of moles of iron bound to the protein, the molar extinction coefficient for ferene-S of 33 850 L cm^{-1} mol^{-1} was used. A standard iron solution was used as a control.

Measurement of mitochondrial ROS

After centrifugation, exponentially growing PF *T. brucei* (5×10^6 cells) were resuspended in 1 ml of fresh SDM-79 medium and ROS were measured by oxidation of dihydroethidium (Sigma), added to the final concentration 5 µg ml^{-1}, in the time frame of 30 min at 27°C (Long *et al*., 2008a). After staining, the cells were resuspended in Iso-flow buffer and instantly measured by flow cytometry using an Epics XL flow cytometer (Coulter) with excitation and emission settings of 488 and 620 nm. Dihydroethidium can be oxidized by superoxide to fluorescent ethidium, which interacts with DNA (Carter *et al*., 1994).

Measurement of enzymatic activities

The activities of fumarase, aconitase and threonine dehydrogenase were measured in cytosolic and mitochondrial fractions obtained with digitonin fractionation, the purity of which was controlled by compartment-specific antibodies against MRP2 and enolase using Western blot analysis. The activities of fumarase and aconitase were determined spectrophotometrically at 240 nm as the rate of production of fumarate and *cis*-aconitate respectively. The activity of threonine dehydrogenase was established at 340 nm as the rate of NAD^+ reduction as described elsewhere (Saas *et al*., 2000). The activity of succinate dehydrogenase was measured in crude mitochondrial membrane extract as described elsewhere (Horváth *et al*., 2005).

Rescue with human Isa

A 390-nt-long full-length cDNA of the human Isa1 (hIsa1) gene fragment (AAH02675) with the stop codon (or without the stop codon in case of tagging) were amplified from a commercial human liver cDNA library (Invitrogen) using primers hFxn-hIsa1, hFxn-hIsa1-M and hIsa1-R-RP or hIsa1-HA_3, and cloned into the pABPURO vector with or without HA_3 tag in their 3′ end, respectively, following a strategy described previously (Long *et al*., 2008b). Next, the mitochondrial targeting peptide (1–13 amino acids) of hIsa1 predicted by MitoProt II (0.9807) was replaced with mitochondrial targeting peptide of human frataxin (amino acids 1–55), which is known to efficiently import proteins in the *T. brucei* organelle (Long *et al*., 2008b), using primers hFxn-hIsa1 and hIsa1-R-RP or hIsa1-HA_3 (Table S1). A 465-nt-long full-length cDNA (AAH15771) was amplified from a commercial hIsa2 construct (NITE BioResource Center) using primers hIsa2-R-FP and hIsa2-R-RP, and cloned into the pFC4 vector with blasticidin resistance, producing pFC4-hIsa2. All constructs were verified by sequencing. pABPURO- and pFC4-based constructs were linearized with BstXI and NotI, respectively, and used for transfection of the PF cells, in which RNAi against TbIsa1, TbIsa2 and TbIsa1/2 can be induced. In these cells, the activities of mitochondrial fumarase and aconitase were measured as described above.

Rescue with Blastocystis *Isa*

A partial sequence of the Isa2 gene was identified among the expressed sequence tags of *Blastocystis* sp. (Stechmann *et al*., 2008). The full-length sequence of this gene was obtained using RACE techniques as described previously (Stechmann *et al*., 2008). The 498-nt-long full-length cDNA of

the *Blastocystis* Isa2 (BhIsa2) gene was cloned into the pABPURO vector (using primers BhIsa-F and BhIsa-R; Table S1), following the cloning strategy described above for hIsa. A putative mitochondrial targeting peptide of the *Blastocystis* Isa2 protein, predicted by MitoProt II with high probability (> 0.99), was retained in the construct for the rescue experiments. The linearized construct was introduced into the inducible knock-downs PF cells for TbIsa1 and TbIsa2. In the obtained cell lines, the activities of fumarase, aconitase, succinate dehydrogenase and threonine dehydrogenase were measured in total cell lysates, as well as in subcellular fractions as described above.

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