International Journal for Parasitology 46 (2016) 641–651

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Roles of the Nfu Fe–S targeting factors in the trypanosome mitochondrion

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ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 6 April 2016
Accepted 11 April 2016
Available online 12 May 2016

Keywords:
Trypanosoma brucei
Nfu1
Iron–sulphur cluster
Fe–S
Mitochondrion

ABSTRACT

Iron–sulphur clusters (ISCs) are protein co-factors essential for a wide range of cellular functions. The core iron–sulphur cluster assembly machinery resides in the mitochondrion, yet due to export of an essential precursor from the organelle, it is also needed for cytosolic and nuclear iron–sulphur cluster assembly. In mitochondria all [4Fe–4S] iron–sulphur clusters are synthesised and transferred to specific apoproteins by so-called iron–sulphur cluster targeting factors. One of these factors is the universally present mitochondrial Nfu1, which in humans is required for the proper assembly of a subset of mitochondrial [4Fe–4S] proteins. Although most eukaryotes harbour a single Nfu1, the genomes of *Trypanosoma brucei* and related flagellates encode three Nfu genes. All three Nfu proteins localise to the mitochondrion in the procyclic form of *T. brucei*, and *Tb*Nfu2 and *Tb*Nfu3 are both individually essential for growth in bloodstream and procyclic forms, suggesting highly specific functions for each of these proteins in the trypanosome cell. Moreover, these two proteins are functional in the iron–sulphur cluster assembly in a heterologous system and rescue the growth defect of a yeast deletion mutant.

1. Introduction

Trypanosoma brucei and related flagellates are unicellular parasites that cause devastating diseases of humans and livestock and thus have a major impact on health and economy, mostly in sub-Saharan Africa but also in other tropical regions. The trypanosome life cycle is rather complex with different stages in mammalian and insect hosts that differ dramatically in their morphology and metabolic requirements (Matthews, 2005). These differences are generally necessitated by the different environments the parasites find themselves in. In the glucose-rich blood of their mammalian host, the bloodstream form (BSF) relies mainly on glycolysis for its energy generation, while the insect-dwelling procyclic form (PCF) needs a fully functional mitochondrion with active oxidative phosphorylation to meet its energetic demands (Tielens and van Hellemond, 2009). As a consequence, the BSF mitochondrion is much more reduced while its PCF counterpart is highly elaborate, extensively branched and metabolically active (Verner et al., 2015).

Despite these obvious differences, both BSF and PCF mitochondria harbour a similar cohort of proteins important for iron–sulphur cluster (ISC) biogenesis, although their abundance in the BSF is generally much lower (Lukeš and Basu, 2015). ISC biogenesis is the most fundamental process that defines a mitochondrion, and in fact the only known common denominator of all mitochondria and mitochondrion-derived organelles, since it is also found in the most reduced mitosomes of several anaerobic protists (Maguire and Richards, 2014; Makiuchi and Nozaki, 2014).

These evolutionarily ancient and highly important ISCs are cofactors of proteins involved in a variety of cellular functions such as metabolic catalysis, DNA replication and repair, translation and iron regulation to name the most prominent ones (Brzóska et al., 2006). With almost 20 well conserved proteins participating in the mitochondrial stage of ISC biogenesis, the process is rather complex and still not fully understood. We will now briefly describe what is known about the mitochondrial steps of ISC biogenesis in yeast and mammalian cells (using the yeast

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http://dx.doi.org/10.1016/j.ijpara.2016.04.006
0020-7519/

nomenclature for this well conserved process) and compare this with the situation in trypanosomes.

Mitochondrial ISC assembly takes place on the Isu1/Isu2 scaffold (Isu2 having arisen from a gene duplication event specific to yeast) with sulphur being provided by the reduction of cysteine to alanine which is catalysed by the desulfurase complex Nfs1-Isd11 (Mühlenhoff et al., 2003) (Fig. 1). The sulphur is reduced by a dedicated electron transport chain constituted of ferredoxin and ferredoxin reductase, with the iron possibly provided by a putative donor frataxin (Lill et al., 2012). The *T. brucei* scaffold protein Isu and the desulfurase Nfs are both indispensable for the PCF and their depletion negatively impacts on aconitase activity (used as a readout for the synthesis of [4Fe–4S] clusters) (Smíd et al., 2006). Moreover, Isu is also essential in the BSF, and both Isu and Nfs localise to the mitochondrion as well as to the nucleolus in both life stages, although the role these proteins might play there remains a matter of speculation (Kovářová et al., 2014). The desulfurase complex of *T. brucei* was also shown to be required for tRNA thiolation in the PCF, however it remains to be resolved whether this is due to direct involvement of this complex in the process or indirectly by the provision of an ISC for another component (Paris et al., 2010; Kovářová et al., 2014).

With the help of the heat shock protein 70 (Hsp70) chaperones Ssq1 and Jac1, the nascent [2Fe–2S] cluster is temporarily transferred to the monothiol glutaredoxin Grx5 from which it can be directly handed over to target [2Fe–2S] apoproteins (Uzarska et al., 2013). While the trypanosome mitochondrial Hsp70 is mostly involved in mitochondrial DNA maintenance, its presence is also required for ISC synthesis (Týč et al., 2015). The mitochondrial glutaredoxin Grx1 can bind a [2Fe–2S] cluster in vitro and plays an important role in iron metabolism of the parasite (Comini et al., 2008). The Isa1, Isa2 and Iba57 proteins participate in the conversion of [2Fe–2S] to [4Fe–4S] clusters, which are eventually transferred to distinct apoproteins with the help of ISC targeting factors such as Nfu1, BolA3 or Ind1 (Lill et al., 2012). Putative homologues of all these proteins are present in *T. brucei* but, with the exception of the Isa1 and Isa2 proteins (Long et al., 2011), experimental studies have not been published (Lukeš and Basu, 2015). The mitochondrial ISC biogenesis machinery is also essential for the synthesis of cytosolic and nuclear Fe–S proteins, since it depends on the export of a still unknown sulphur-containing compound to the cytosol. The so-called CIA (for cytosolic ISC assembly) pathway is outside the scope of this research and will hence not be discussed here.

There is still a certain lack of knowledge about how discrete subsets of Fe–S cluster apoproteins are recognised by specific targeting factors such as Nfu1. This protein shows homology to the C-terminal domain of NifU, which is a scaffold in ISC biogenesis in nitrogen-fixing bacteria (Fig. 2A) (Smith et al., 2005). In humans and yeast, Nfu1 is responsible for the transfer of [4Fe–4S] clusters to a small subset of mitochondrial proteins which include components of respiratory complexes I and II and lipoic acid synthase (LipA) (Cameron et al., 2011; Navarro-Sastre et al., 2011). What makes Nfu1 particularly compelling to study is its involvement in human disease. Point mutations in, or deficiencies of, the protein cause a fatal mitochondrial disease called Multiple mitochondrial dysfunction syndrome with functional Nfu1 deficiency (MMDS1), which is characterised by symptoms such as lactic acidosis, hyperglycinemia and reduced activity of respiratory chain complexes I and II (Cameron et al., 2011; Navarro-Sastre et al., 2011). Somewhat surprisingly, given the severity of the human phenotype, depletion or deletion of Nfu1 from HeLa cells (Navarro-Sastre et al., 2011) and yeast *Saccharomyces cerevisiae* (Schilke et al., 1999), respectively, causes only a very mild growth phenotype in culture. However, a specific impact on several enzymatic activities has been detected. The levels of lipoic acid-bound enzymes (E2

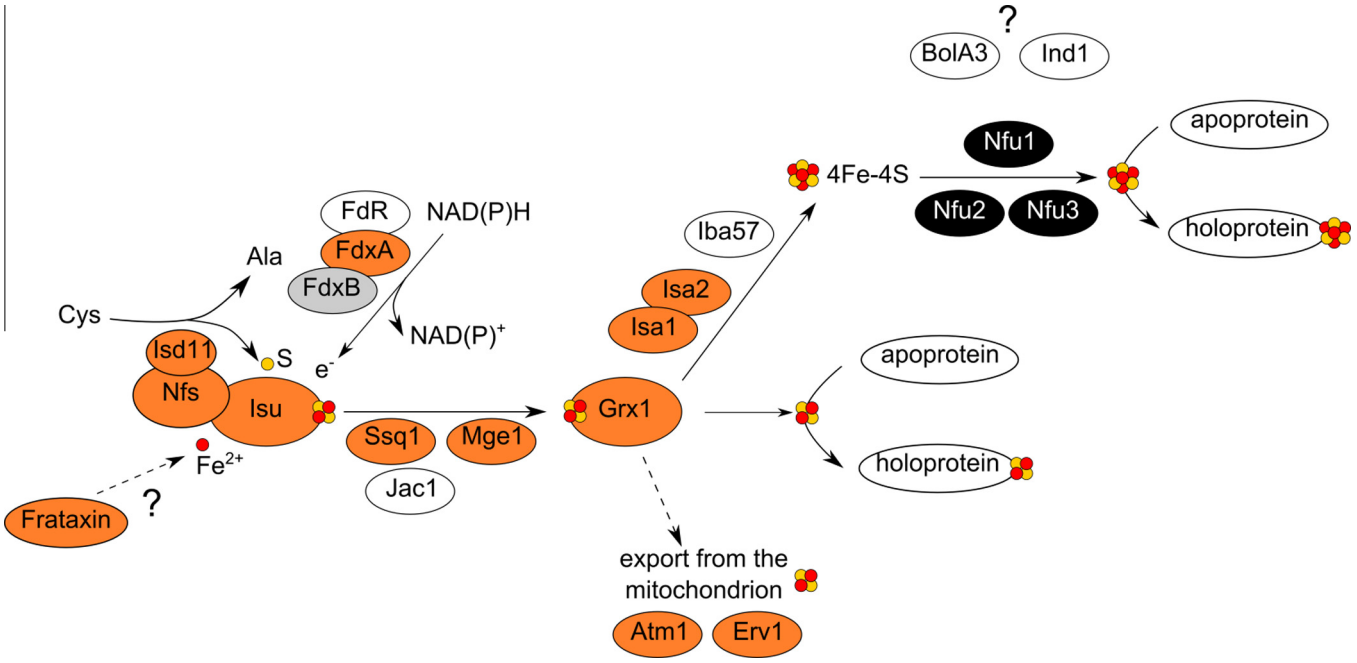

Fig. 1. Scheme of the mitochondrial iron–sulphur clusters assembly pathway in *Trypanosoma brucei*. Components essential in procyclic form are indicated in orange (dark grey), dispensable ones are in light grey, Nfu proteins are in black, and proteins present in the genome but not yet assayed are in white. *Tb*Grx1 is homologous to yeast Grx5. The Nfs-Isd11 desulfurase complex (Paris et al., 2010) provides sulphur on the Isu scaffold (Smíd et al., 2006), while ferredoxins A and B facilitate its reduction (Changmai et al., 2013), and frataxin probably provides iron (Long et al., 2008). Heat shock proteins (Týč et al., 2015) facilitate transfer of newly created [2Fe–2S] on the Grx1 glutaredoxin (Comini et al., 2008). Isa1/2 and Iba 57 proteins enable formation of [4Fe–4S] clusters (Long et al., 2011). A still unknown S-containing component is exported into the cytosol via the inner membrane transporter Atm1 (Horáková et al., 2015), the sulfhydryl oxidase Erv1 of the intermembrane space and glutathione (Basu et al., 2013), and is utilised in the cytosolic iron–sulphur cluster assembly pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

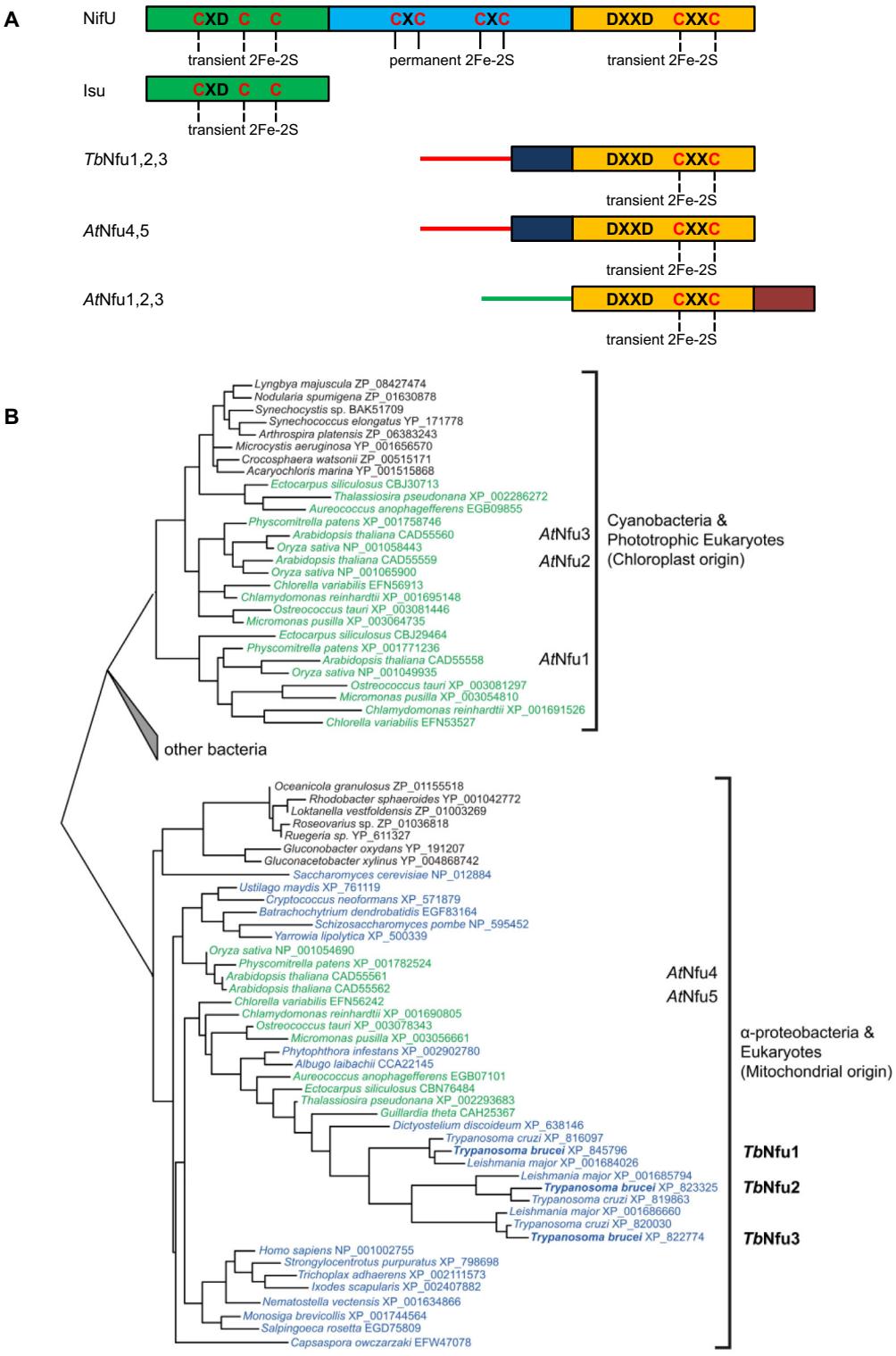

Fig. 2. Bioinformatic analysis of Nfu proteins in prokaryotes and eukaryotes, and the position of the *Trypanosoma brucei* (*Tb*)Nfu proteins among related prokaryotic and eukaryotic proteins. (A) The bacterial NifU protein consists of three domains. The N-terminal part contains three highly conserved cysteine residues which are involved in formation of a new iron–sulphur cluster, and is highly similar to the Isu proteins. The central part of NifU is called ferredoxin-like domain, contains a permanent iron–sulphur cluster and shows similarity to nitrate reductases. The C-terminal part accommodates a conserved motif CXXC, which is presumed to be involved in formation of a new iron–sulphur cluster and is found in the Nfu proteins as well. The conserved aspartate residues were proposed to mediate the transfer of newly formed iron–sulphur clusters. The N-terminal bars represent mitochondrial targeting sequences (the sequence is not well predicted in *Tb*Nfu3), and a plastid targeting sequence from *Arabidopsis thaliana*, respectively. The N-terminal boxes mark Nfu1-like domains in the *T. brucei* proteins and *At*Nfu4 and 5, while the C-terminal box shows a B domain specific for *At*Nfu1-3. (B) Scheme of a maximum likelihood phylogenetic tree of Nfu homologues. The prokaryotic taxa are in black, while eukaryotes are highlighted in colors (shades of grey) (green (light grey) for chloroplast-containing photoautotrophs and blue (dark grey) for heterotrophs). The bootstrap/SH-like aLRT branch supports are shown for the cyanobacteria/chloroplasts clade and the alpha-proteobacteria/mitochondria clade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subunits of pyruvate dehydrogenase (PDH), alpha-ketoglutarate dehydrogenase (α-KGDH]) and the H protein of the glycine cleavage system (GCS)), as well as the amount and activity of complex II (succinate dehydrogenase (SDH)) were decreased in HeLa cells (Navarro-Sastre et al., 2011), while a significant depletion of SDH activity was also described in yeast (Schilke et al., 1999).

Unlike the situation in the benchmark eukaryotes yeast and human, the genomes of plants including *Arabidopsis thaliana* contain five genes with similarity to the C-terminus of NifU. Two of these Nfu homologues (*At*Nfu4 and *At*Nfu5) have such a NifU domain at the C-terminus, as well as a N-terminal Nfu1-like region, thus closely matching the domain organisation of typical mitochondrial Nfu1 proteins from other eukaryotes, while the other three proteins (*At*Nfu1, 2 and 3) contain well-defined predicted plastid targeting sequences (Fig. 2A) (Léon et al., 2003). Most of these plant Nfu proteins where able to complement a yeast deletion mutant when targeted to the mitochondrion. Localisation studies showed that *At*Nfu1, 2 and 3 are localised in the plastid, while *At*Nfu4 was shown to be confined to the mitochondrion, and *At*Nfu5 was predicted to have the same localisation (Léon et al., 2003).

Interestingly, cytosolic and nuclear localisation of some of the mitochondrial ISC pathway members has been documented, suggesting a partial redundancy of the CIA and ISC pathways (reviewed in Rouault, 2012). HeLa cells are reported to contain two Nfu1 isoforms, which are created by differential splicing of a common precursor mRNA, and which localise to the mitochondrion and cytosol, respectively (Tong et al., 2003). Hitherto, no specific targets for the non-mitochondrial isoform have been identified.

Trypanosomatid flagellates, represented in this study by the genetically tractable *T. brucei*, belong to the eukaryotic supergroup Excavata and hence the emergence of multiple copies of Nfu in their genomes must have occurred independently of plants. Here we show that at least two out of three *Tb*Nfu proteins localise to the mitochondrion in both life stages of *T. brucei*. These two *Tb*Nfu proteins (*Tb*Nfu2 and 3) can functionally replace yeast Nfu1 when targeted to its mitochondrion, suggesting a conserved function. Moreover, the same two *Tb*Nfu proteins are essential for the BSF and PCF life stages, suggesting that they are non-redundant and target specific apoproteins for ISC transfer. This is especially intriguing for the BSF trypanosome, where only a small subset of Fe–S containing mitochondrial proteins is expected to be essential (Lukeš and Basu, 2015; Basu et al., 2016).

2. Materials and methods

2.1. Cells and plasmids

Trypanosoma brucei BSF 427 cells, BSF single-marker cells and PCF 29:13 cells (Wirtz et al., 1999) were cultured as described elsewhere (Changmai et al., 2013). For RNA interference (RNAi) against *Tb*Nfu1 (Tb927.7.1720) a 573 bp region (nt 1–573) was PCR amplified using primers Nfu1_F and Nfu1_R (see Supplementary Table S1 for all oligonucleotide sequences used and plasmids generated in this study). For *Tb*Nfu2 (Tb927.10.11160), a 480 bp region (nucleotide (nt) 1–480) was amplified using primers Nfu2_F and Nfu2_R, and for *Tb*Nfu3 (Tb927.10.5290), a 480 bp region (nt 1–480) was amplified using primers Nfu3_F and Nfu3_R. All amplicons were cloned into p2T7-177 vector (Wickstead et al., 2002) to create the *Tb*Nfu1-3 RNAi plasmids. An additional plasmid for RNAi of *Tb*Nfu1 was generated by amplifying nt 45–395 of the gene's 3′untranslated region (UTR), followed by ligation to p2T7-177 (generating plasmid pCR49). Restriction enzyme *Not*I was used to linearise all RNAi plasmids prior to electroporation.

To create plasmids for endogenous tagging of *Tb*Nfu1, *Tb*Nfu2 and *Tb*Nfu3 at the N-terminus with PTP, a region of the 5′end of the open reading frames (ORFs) was inserted into the plasmid p2678 (Kelly et al., 2007). Plasmids generated were pCR34 (*Tb*Nfu1), pCR37 (*Tb*Nfu2) and pCR39 (*Tb*Nfu3), and enzymes used for linearisation were *Blp*I (*Tb*Nfu1), *Xcm*I (*Tb*Nfu2) and *Sph*I (*Tb*Nfu3). For C-terminal endogenous tagging with the PTP tag, a fragment of the 3′end of the *Tb*Nfu1-3 ORFs lacking the stop codon was amplified from trypanosome genomic DNA and cloned into a derivative of pC-PTP-Neo with the antibiotic resistance changed to puromycin (Schimanski et al., 2005). Plasmids generated were pCR35 (*Tb*Nfu1), pCR38 (*Tb*Nfu2) and pCR36 (*Tb*Nfu3), while enzymes used for their linearisation were *Sal*I (*Tb*Nfu1), *Bsg*I (*Tb*Nfu2) and *Nsi*I (*Tb*Nfu3), respectively. To create plasmids for tagged overexpression of *Tb*Nfu1 and *Tb*Nfu3, both full-length ORFs were amplified from *T. brucei* genomic DNA and cloned into pJH54 (C-terminal HA_3 tagging vector kindly provided by Christine Clayton) and pT7V5 (adding a C-terminal V5 tag), respectively (Surve et al., 2012). All plasmids were linearised with *Not*I prior to transfection.

Linearised plasmids were electroporated into PCF cells using the standard procedure (Vondrušková et al., 2005). *Tb*Nfu1-3 RNAi plasmids stably integrated in 29:13 cells were selected by the addition of 5 μg/ml of phleomycin. In all cases, RNAi was induced by the addition of 1 μg/ml of tetracycline to the medium and growth curves performed in triplicate (a representative experiment is shown for each construct and life cycle stage). PCF 427/29:13 cells transfected with pCRs 34, 37, 39 (PTP-*Tb*Nfu1-3) and pCRs 35, 38, 36 (*Tb*Nfu1-3-PTP) were all treated with 0.5 μg/ml of puromycin. PCF 29:13 cells transfected with constructs overexpressing *Tb*Nfu1 and *Tb*Nfu3 were selected with 5 μg/ml of phleomycin (pJH54-based construct) and 0.5 μg/ml of puromycin (pT7V5-based construct). BSF 427 and BSF single-marker cells were electroporated using the Amaxa Nucleofector II electroporator and program X-001. BSF 427 cells were transfected with pCR38 and BSF single-marker cells with the *Tb*Nfu1-3 RNAi plasmids and pCRs 36–39. Selection was with 0.2 μg/ml of phleomycin (*Tb*Nfu1-3 RNAi) and 0.2 μg/ml of puromycin (pCRs 36–39). BSF single-marker cells were also transfected with an overexpression construct for *Tb*Nfu1 (*Tb*Nfu1-V5, pCR43) and selected with 0.2 μg/ml of puromycin.

2.2. Bioinformatics

The Nfu homologues were identified by a BLAST search of the National Center for Biotechnology Information (NCBI), USA non-redundant protein database and aligned using MAFFT (Katoh et al., 2005). The alignment was manually edited in BioEdit (Hall, 2011). The phylogenetic tree was constructed in PhyML 3.1 (Guindon et al., 2010) using the default settings and the robustness of individual branches was evaluated by SH-like approximated likelihood ratio test and bootstrap after 100 iterations.

2.3. Antibody production

Specific polyclonal antibodies were commercially produced for two of the *Tb*Nfu proteins. For *Tb*Nfu1, two synthetic oligopeptides were used (CSGKSSQRSIVVEKNE and RRKLKKDEVSASQS) corresponding to amino acids 52–67 and 266–279, respectively, of the *T. brucei* Nfu1 protein. The polyclonal antibodies were raised in a rabbit over 87 days, and subsequently affinity purified by Eurogentec (Belgium). Similarly, oligopeptide SSTYDNFIPDGQTC, corresponding to amino acids 30–43, was used for production of the anti-*Tb*Nfu3 antibody by GenScript (China).

2.4. Immunofluorescence

Following staining with mitotracker red (Sigma–Aldrich, USA), BSF 427, BSF single-marker cells and PCF 29:13 cells were fixed for 30 min in either 2.3% (w/v) paraformaldehyde at room temperature or in methanol at −20 °C. Following permeabilisation (paraformaldehyde fixation only) in PBS with 0.1% (v/v) Triton-X100 or overnight in methanol at −20 °C, slides were incubated with the primary antibody, either anti-protein A (1:5,000, Sigma–Aldrich) or anti-V5 (1:500, Invitrogen, USA) in PBS for 1 h. Following two washes with PBS, the slides were incubated with the secondary antibody, either AlexaFluor488 goat anti-rabbit (1:200, Molecular Probes, USA) or AlexaFluor488 goat anti-mouse (1:200, Molecular Probes) in PBS for 1 h. The slides were washed twice in PBS, then 10 μg/ml of DAPI (Fisher Scientific, USA) was added for 5 min. After two more PBS washes, the cells were examined under an Axioscope II fluorescent microscope.

2.5. Western blot analysis

Cell lysates corresponding to 2×10^6 cells were loaded into each lane and separated by SDS–PAGE. The proteins were transferred to a nitrocellulose or a PVDF membrane (previously activated for 5 min in methanol) by wet transfer. The membrane was blocked for 1 h at room temperature with blocking buffer (5% (w/v) milk powder in PBS). The primary antibody (anti-protein A 1:20,000, anti-*Tb*Nfu1, or anti-*Tb*Nfu3 1:1,000) was diluted to the appropriate concentration and added to the membrane. Incubation was overnight at 4 °C. The membrane was washed 2×5 min with PBS and then incubated with the secondary antibody (anti-rabbit HRP conjugate 1:2,000, Sigma–Aldrich) in blocking buffer for 1 h at room temperature. Finally, the membrane was washed 2×5 min in PBS and antibodies were detected by enhanced chemiluminescence (Clarity Western ECL Substrate, Bio-Rad, USA).

2.6. Digitonin fractionation

For digitonin fractionation, 10^7 cells per sample were collected, incubated in STE-NaCl buffer (250 mM sucrose; 25 mM Tris–HCl, pH 7.4; 1 mM EDTA; 150 mM NaCl) with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 1.5 mM digitonin (Sigma–Aldrich) for 4 min at room temperature. Subsequently, samples were centrifuged (11,337*g* for 2 min), and the obtained supernatants were used for western blot analysis. For detection of tagged proteins, anti-protein A antibody (Sigma–Aldrich) was used at a 1:20,000 dilution. As controls, antibodies against mitochondrial Hsp70 (1:1,000) (mitochondrial marker, (Panigrahi et al., 2008)), and cytosolic enolase (1:10,000) (cytosolic marker) were used.

With the aim to separate their cytosolic and mitochondrial fractions, digitonin fractionation of Nfu1-HA$_3$ and Nfu3-V5 expressing flagellates was performed as follows: cells were harvested, washed twice with SHE buffer (250 mM sucrose, 25 mM HEPES, 1 mM EDTA) and an equivalent of 1 mg of cellular protein was resuspended in 200 μl of HBSS buffer (136.9 mM NaCl; 5.37 mM KCl; 0.81 mM $MgSO_4$; 1.26 mM $CaCl_2$; 0.44 mM KH_2PO_4; 0.33 mM Na_2HPO_4; 4.17 mM $NaHCO_3$; 5.55 mM glucose, pH 7.3) with the addition of 80 μg of digitonin (Sigma–Aldrich). Samples were briefly vortexed and, following incubation for 5 min at room temperature, centrifuged (11,337*g*, 2 min). Supernatant was collected as the cytosolic fraction and pellet was washed with HBSS buffer and then resuspended in 200 μl of the same buffer supplemented with 0.1% Triton X-100. Following incubation on ice for 5 min, samples were centrifuged (11,337*g*, 2 min) and the obtained supernatant was collected as the mitochondrial fraction. Anti-HA and anti-V5 antibodies (Invitrogen, USA) and anti-MRP2 (Vondrušková et al., 2005) were used at 1:2,000 dilution.

2.7. Yeast complementation assay

A ΔIsu1 yeast W303 strain described elsewhere (Gerber et al., 2004) was further modified by a second deletion of Nfu1. This was introduced by PCR amplification of the nourseothricin resistance gene using flanking primers which contained fragments corresponding to the 50 nucleotides down- and upstream of the *S. cerevisiae NFU1* gene, and which was transformed into the ΔIsu1 strain as described elsewhere (Janke et al., 2004). Successful homologous recombinants in which the *NFU1* gene was replaced by the nourseothricin cassette were selected by growth with 100 μg/ml of nourseothricin and subsequent PCR analysis of chromosomal DNA of restreaked clones. Full-length ORFs (*Tb*Nfu2 and3) and the ORF lacking the predicted mitochondrial targeting sequence (*Tb*Nfu1) were amplified from *T. brucei* genomic DNA and cloned into the so-called yeast mitocyto expression vector (p426-TDH-F1β-HIPIP-Myc) (Mühlenhoff et al., 2011) from which the HiPIP-encoding gene was replaced with the *Tb*Nfus. All *Tb*Nfu containing vectors as well as a positive control vector (encoding yeast Nfu1, (Navarro-Sastre et al., 2011)) were transformed into the ΔIsu1ΔNfu1 strain (for description, see Section 2.1) and the expression of C-terminally myc-tagged *Tb*Nfu proteins was verified by western blot analysis. A similar sized colony was picked from each clone as well as from the parental strain and re-suspended in 100 μl of distiled water. Serial 1:10 dilutions were made and approximately 10 μl of each dilution was spotted onto two identical SC-Galactose plates. One set of plates was incubated for 3–4 days at the permissive temperature of 30 °C and the other one at the restrictive temperature of 34 °C.

3. Results

3.1. Bioinformatics

All three trypanosome Nfu proteins are small (approximately 30 kDa) and have the typical domain architecture of this family with a conserved cysteine motif (CxxC) at their C-termini, thus theoretically enabling them to bind a Fe–S cluster (Fig. 2A). *Tb*Nfu1 and *Tb*Nfu2 sequences share 24.5% identity (38.7% similarity), *Tb*Nfu1 and *Tb*Nfu3 share 24.5% identity (41.7% similarity), with *Tb*Nfu2 and *Tb*Nfu3 being least homologous (19.5% identical, 31.2% similar) according to the EMBOSS stretcher algorithm (Rice et al., 2000). Phylogenetic analysis of the Nfu proteins sampled across all eukaryotic super-domains shows that there are two eukaryotic lineages of Nfu. The first lineage is present across all eukaryotes and branches together with alpha-proteobacteria, indicative of its mitochondrial origin. Phototrophic eukaryotes also acquired a Nfu gene with a different lineage, which groups with cyanobacteria and very likely has a plastidial origin (Fig. 2B). In all trypanosomatids sequenced to date, the mitochondrial Nfu protein underwent a triplication (Fig. 2B), suggesting that this multiplication event occurred already in the common ancestor. Indeed, the trypanosomatid Nfu group clusters with mitochondrial Nfu proteins from other eukaryotes including *A. thaliana* Nfu4 and 5, while the plastid Nfu proteins from this plant species are more distantly related (Fig. 2B).

*Tb*Nfu1 (Tb927.7.1720) encodes a protein with a molecular mass of 31.1 kDa including the predicted mitochondrial targeting sequence. Cleavage at amino acid position 23 (probability 0.9880, MitoProt (Claros and Vincens, 1996)) would result in a mature protein mass of 28.25 kDa. *Tb*Nfu2 (Tb927.10.11160) has a predicted molecular mass of 26.7 kDa following the original annotation; however, this short version of the protein lacks the N-terminal Nfu1-like domain. Analysis of the *trans*-splice sites suggests an upstream start codon (Parsons et al., 2015) which would result in

a larger protein of 37.6 kDa. This longer version of *Tb*Nfu2 is predicted to be mitochondrial with a cleaved size of 31.78 kDa (probability 0.869, TargetP 1.1 (Emanuelsson et al., 2000)). C-terminal, endogenous PTP tagging of the protein suggests that it is in fact the longer version that is produced, since the observed size including the tag is approximately 52 kDa, which is close to the expected size of 50.8 kDa. Tagging with PTP at the N-terminus produces a slightly bigger protein of approximately 55 kDa, consistent with a non-cleaved species (Supplementary Fig. S1). Finally, *Tb*Nfu3 (Tb927.10.5290) has a predicted molecular mass of 29.5 kDa and is predicted to be mitochondrial with rather low confidence scores (probability: 0.374, MitoProt; probability: 0.438, TargetP1.1 (Claros and Vincens, 1996; Emanuelsson et al., 2000)).

3.2. Localisation in PCF cells

Cell lines carrying *Tb*Nfu proteins either tagged at their endogenous locus with the PTP tag or overexpressed and containing either the HA_3- or V5-epitope tag were used for localisation studies. Both immunofluorescence analysis and digitonin fractionation techniques were employed to corroborate results in the PCF. All proteins were tagged at their C-termini since two *Tb*Nfu proteins are predicted to have an N-terminal mitochondrial targeting signal and all three belong to the mitochondrial lineage. Importantly, PTP-tagging at the N-terminus at the endogenous locus apparently masked the critical endogenous N-terminal mitochondrial targeting sequences since diffuse cytoplasmic staining due to mistargeting was observed (data not shown).

*Tb*Nfu1-PTP detected with an antibody against protein A clearly localised to the elaborate PCF mitochondrion where it was evenly distributed and completely overlapped with mitotracker red staining (Fig. 3A). This result was confirmed by digitonin fractionation, where an overexpressed, HA-tagged *Tb*Nfu1 protein was clearly mitochondrial (Fig. 3B). Finally, the same HA-tagged protein also localised to the mitochondrion by immunofluorescence analysis (data not shown).

*Tb*Nfu2-PTP, detected with an antibody against protein A, also localised to the mitochondrion, although the distribution of the tagged protein was more punctate than that of its tagged *Tb*Nfu1 counterpart (Fig. 3A). Digitonin fractionation of the same cell line using increasing concentrations of digitonin showed a higher amount of the protein in fractions with increased concentration of the detergent, corresponding to the mitochondrial compartment, while some of it was also present in other fractions (Fig. 3B). This suggests that an - albeit small - amount of the *Tb*Nfu2 protein may also be cytosolic. Another possible explanation of this observation would be the presence of *Tb*Nfu2 processing intermediates in the cytosol.

The same approach was used to localise the third *Tb*Nfu homologue, which was endogenously PTP-tagged and detected with an antibody against protein A. Indeed, all *Tb*Nfu3 clearly localised to the reticulated mitochondrion where it showed a staining pattern reminiscent of *Tb*Nfu2, with the obtained signal having a more focal distribution in the organellar lumen (Fig. 3A). Mitochondrial localisation was further confirmed by digitonin fractionation where an overexpressed, V5-tagged protein was also found

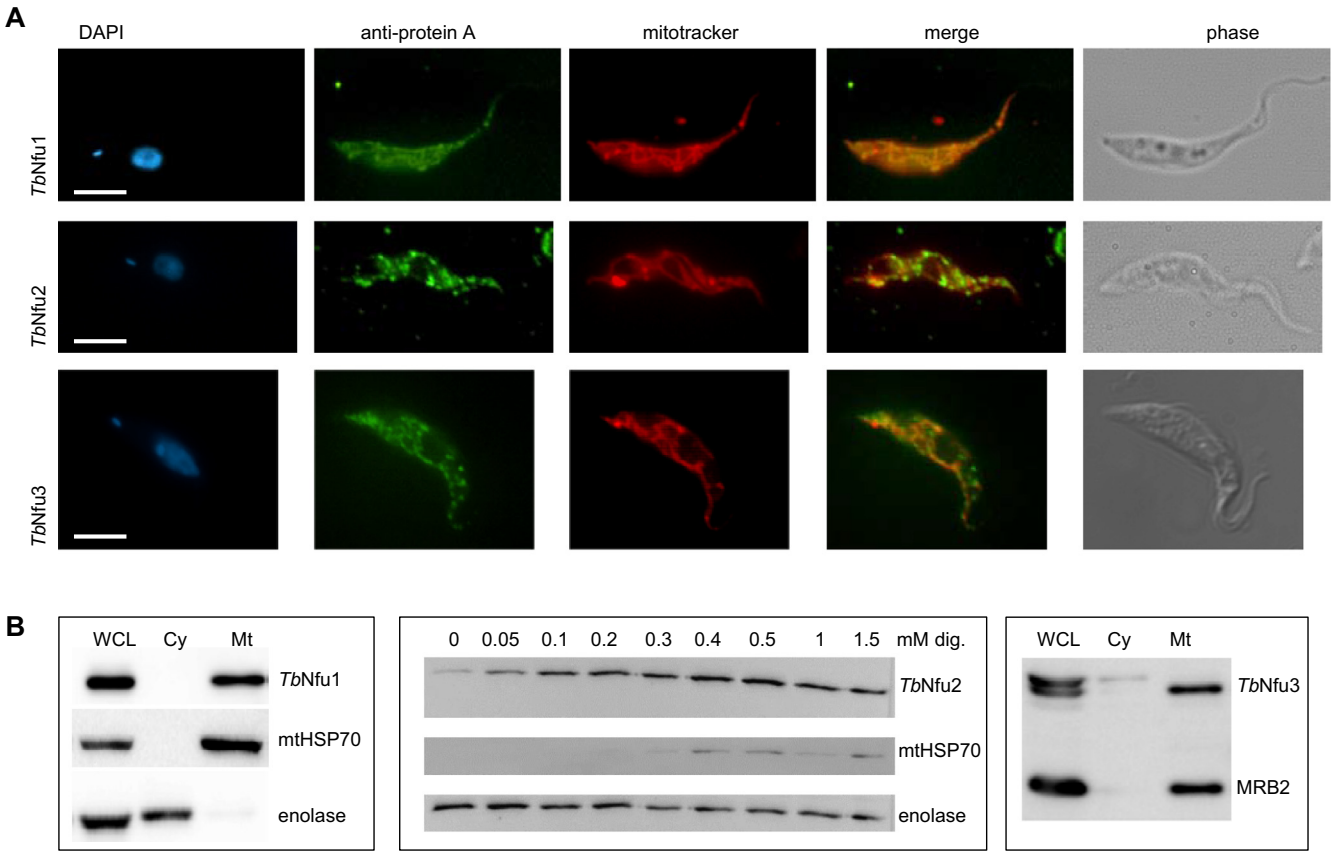

Fig. 3. Localisation of *Trypanosoma brucei* (*Tb*)Nfus in procyclic form cells. (A) Immunofluorescence analysis of endogenously PTP-tagged *Tb*Nfu proteins. DAPI, anti-protein A, mitotracker, a merge of protein A and mitotracker, and a phase contrast image of a representative cell are shown. Scale bar = 5 μm. (B) Subcellular fractionation of cell lines expressing tagged *Tb*Nfu proteins. Fractionation into cytosolic (Cy) and mitochondrial (Mt) fractions as well as the whole cell lysate (WCL) (*Tb*Nfu1-HA and *Tb*Nfu3-V5), and fractionation with increasing concentrations of digitonin (dig.) (*Tb*Nfu2-PTP) are shown. Mitochondrial heat shock protein 70 (mitochondrial Hsp70) and MRB2 were used as mitochondrial controls and enolase as a cytosolic control.

exclusively in the organelle (Fig. 3B). This result was corroborated when *Tb*Nfu3-V5 was localised to the mitochondrion by immunofluorescence analysis in the same cell line (data not shown).

3.3. Localisation in BSF cells

Localisation of both endogenously tagged *Tb*Nfu2-PTP and *Tb*Nfu3-PTP detected with an antibody against protein A in BSF cells was highly similar to PCF cells with the proteins distributed unevenly throughout the mitochondrial lumen (Fig. 4). We have thus far been unable to localise either tagged or endogenous versions of *Tb*Nfu1 in the BSF cells by immunofluorescence and currently have no explanation for this.

3.4. Functional complementation in yeast

A *S. cerevisiae* ΔIsu1ΔNfu1 strain was generated by introducing the Nfu1 deletion into an existing ΔIsu1 strain ((Gerber et al., 2004) and data not shown). This strain shows temperature-dependent slow growth, especially on non-fermentable carbon sources as well as minor defects in aconitase and succinate dehydrogenase activities (Schilke et al., 1999). In our hands the ΔIsu1ΔNfu1 strain already showed a severe phenotype at 30 °C compared with the W303 parent strain. The ability of *Tb*Nfu1-3 to rescue this growth phenotype was assessed following transformation with plasmids separately encoding each of these proteins. The N-terminal *Neurospora crassa* F1ß-presequence was employed to guide the *Tb*Nfu proteins efficiently into yeast mitochondria. For *Tb*Nfu1 the predicted trypanosomal mitochondrial targeting sequence was excluded, while full-length *Tb*Nfu2 and *Tb*Nfu3 were cloned into the yeast expression vector since a putative presequence could not be predicted with as much confidence as for *Tb*Nfu1. Different dilutions of the strains were spotted on SC-Gal plates and grown at 30 °C (Fig. 5). Following 3–4 days of incubation, *Tb*Nfu2 and *Tb*Nfu3 protein-expressing transformants as well as a control strain expressing *S. cerevisiae* Nfu1 grew well, while the parental ΔIsu1ΔNfu1 or *Tb*Nfu1 expressing strain showed a growth defect (Fig. 5). This experiment strongly suggests that at least two of the three *Tb*Nfu proteins are functional scaffolds of ISC biosynthesis; moreover, they can operate out of context in a heterologous system and complement the ΔIsu1ΔNfu1 strain.

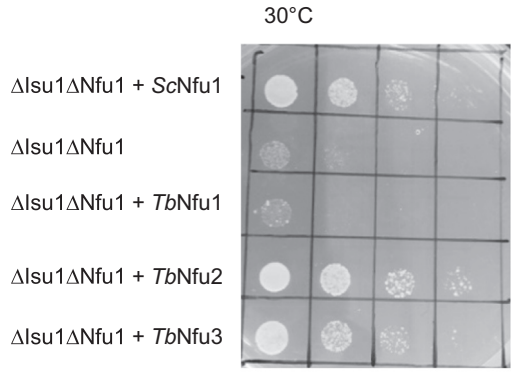

Fig. 5. Complementation of ΔIsu1ΔNfu1 yeast strain. ΔIsu1ΔNfu1 strain transfected or not with the indicated rescue plasmids encoding *Trypanosoma brucei* (*Tb*) Nfus and *Saccharomyces cerevisiae* (*Sc*)Nfu1 incubated at 30 °C. Serial 10-fold dilutions of the different strains are shown (1, 1:10, 1:100 and 1:1,000 from left to right).

3.5. RNAi in PCF cells

We employed the RNAi strategy to analyse the function of *T. brucei* Nfu proteins. Results for *Tb*Nfu1 were somewhat ambiguous with growth being affected very late upon RNAi induction (from day 7 post-induction onwards, Fig. 6A). Moreover, the phenotype was unstable. In order to confirm the efficiency of RNAi, a specific antibody against a *Tb*Nfu1-derived oligopeptide was generated (see Section 2.3), and an RNAi cell line also carrying a C-terminally PTP-tagged endogenous copy of *Tb*Nfu1 was prepared. Both approaches indicate that despite a marked decrease in the *Tb*Nfu1 protein level, the depletion did not trigger a stable phenotype (Fig. 6A; Supplementary Fig. S2A). However, we suspect that our cell lines became refractory to RNAi over time and also note a loss of phenotype after storage in the frozen state, both phenomena that are commonly observed in *T. brucei*.

Depletion of *Tb*Nfu2 in the PCF started to show a growth phenotype between days 3 and 5 of RNAi induction. From day 6 post-induction onwards, cells grew extremely slowly, after which growth almost completely ceased (Fig. 6B). A PTP tag was introduced into one allele of *Tb*Nfu2 in this RNAi cell line and depletion of the tagged protein was monitored by western blot analysis with anti-protein A antibodies which recognise the PTP tag. Consistent

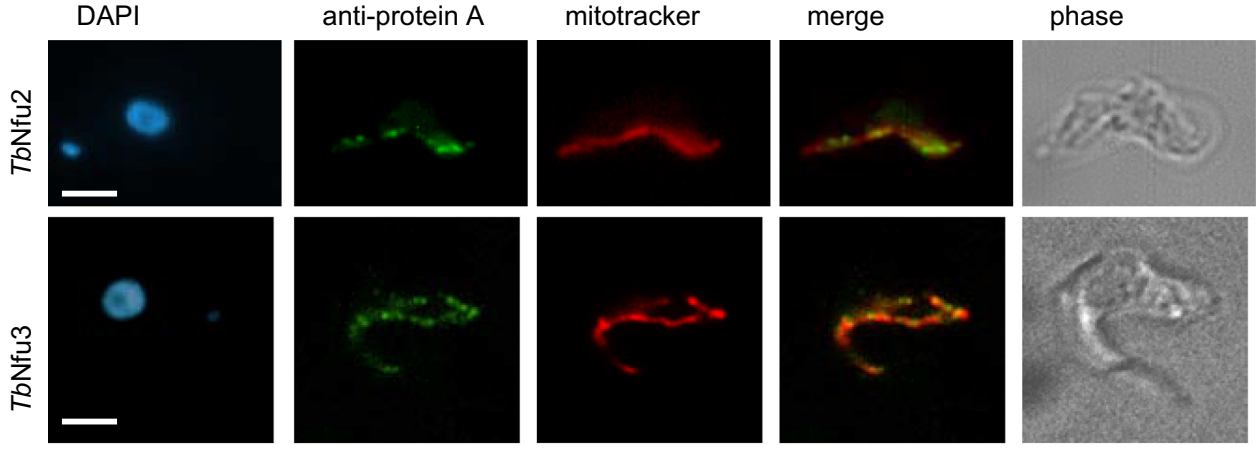

Fig. 4. Localisation of *Trypanosoma brucei* (*Tb*)Nfus in bloodstream form cells. Immunofluorescence analysis of endogenously PTP-tagged *Tb*Nfu proteins. DAPI, anti-protein A, mitotracker, a merge of protein A and mitotracker, and a phase contrast image of a representative cell are shown. Scale bar = 5 μm.

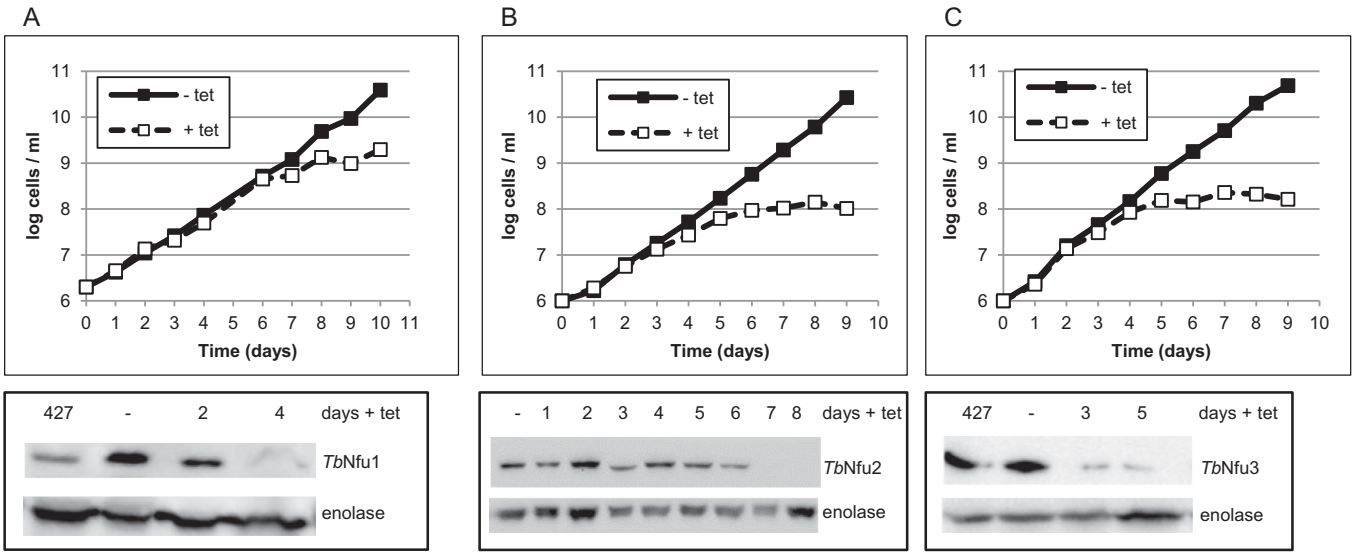

Fig. 6. RNA interference against the *Trypanosoma brucei* (*Tb*)Nfu proteins in procyclic form cells. (A) Cumulative growth of a *Tb*Nfu1 RNAi cell line in the presence (open squares, dashed line) or absence (solid squares, unbroken line) of tetracycline (tet) in the medium. Western blot shows depletion of the endogenous *Tb*Nfu1 protein detected with anti-*Tb*Nfu1 antibodies. (B) Cumulative growth of a *Tb*Nfu2 RNAi cell line in the presence (open squares, dashed line) or absence (solid squares, unbroken line) of tet in the medium. Western blot shows depletion of the endogenously PTP-tagged *Tb*Nfu2 protein detected with anti-protein A. (C) Cumulative growth of a *Tb*Nfu3 RNAi cell line in the presence (open squares, dashed line) or absence (solid squares, unbroken line) of tet in the medium. Western blot shows depletion of the endogenous *Tb*Nfu3 protein detected with anti-*Tb*Nfu3 antibodies. Cytosolic enolase was used as a loading control in all three experiments.

with the severe growth phenotype observed, depletion of the protein became apparent on day 6 and the protein remained undetectable for the rest of the induction time course (Fig. 6B). RNAi against *Tb*Nfu3 in the PCF produced a growth phenotype similar to that of *Tb*Nfu2 with growth slowing down around day 4 post RNAi induction and more or less arresting around day 5 (Fig. 6C). Efficient depletion of the *Tb*Nfu3 protein was confirmed by western blot analysis (Fig. 6C) using a specific polyclonal antibody developed against part of the endogenous *Tb*Nfu3 protein (see Section 2.3).

3.6. RNAi in BSF cells

A relatively mild growth defect was observed with BSF clones induced for *Tb*Nfu1 RNAi immediately after transfection (Fig. 7A); this could, however, not be reproduced in later experiments with frozen cell lines. A western blot produced at the same time as the initial growth curve and probed for endogenous *Tb*Nfu1 showed temporary and mild depletion of the protein at day 3 post-induction (Fig. 7A). New cell lines were generated targeting the *Tb*Nfu1 3′UTR to also enable rescue of the potential phenotype

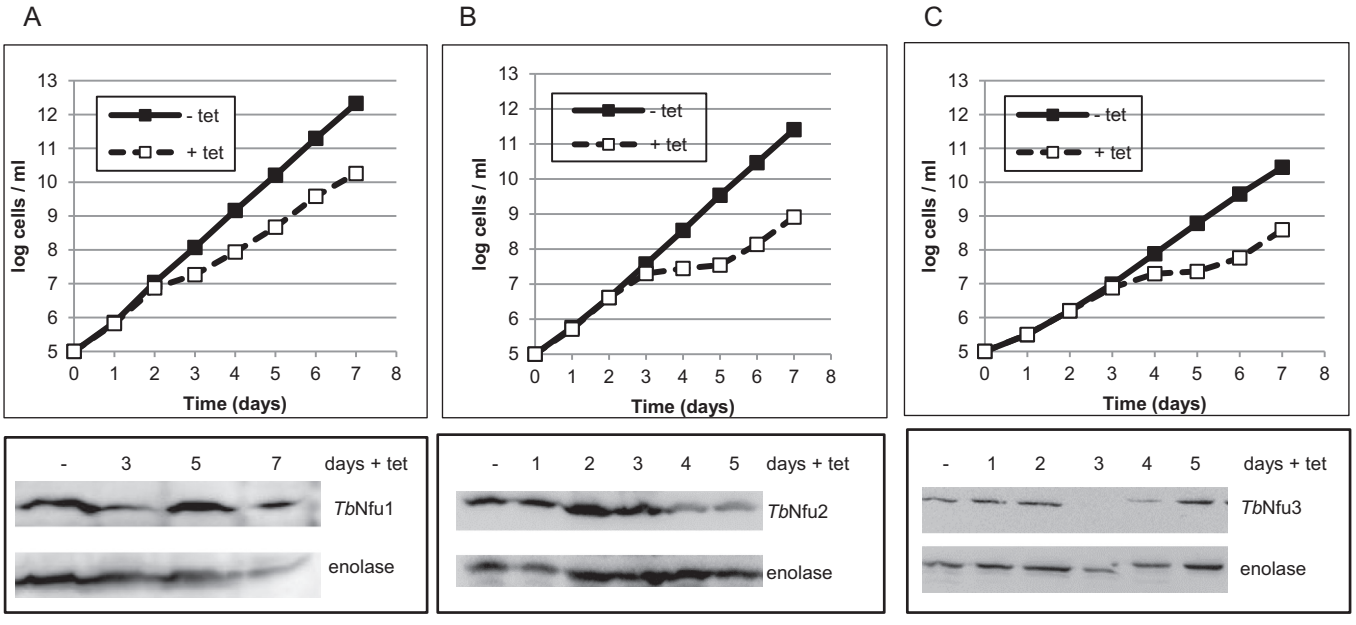

Fig. 7. RNA interference against the *Trypanosoma brucei* (*Tb*)Nfu proteins in bloodstream form cells. (A) Cumulative growth of a *Tb*Nfu1 RNAi cell line in the presence (open squares, dashed line) or absence (solid squares unbroken line) of tetracycline (tet). Western blot shows depletion of endogenous *Tb*Nfu1 protein detected with anti-*Tb*Nfu1. (B) Cumulative growth of a *Tb*Nfu2 RNAi cell line in the presence (open squares, dashed line) or absence (solid squares, unbroken line) of tet. Western blot shows depletion of endogenously PTP-tagged *Tb*Nfu2 detected with anti-protein A. (C) Cumulative growth of a*Tb*Nfu3 RNAi cell line in the presence (open squares, dashed line) or absence (solid squares, unbroken line) of tet. Western blot shows depletion of endogenously PTP-tagged *Tb*Nfu3 with anti-protein A. Cytosolic enolase was used as a loading control in all three experiments.

by overexpression of the *Tb*Nfu1 ORF from mRNA with a non-endogenous 3′UTR. RNAi of cell lines with or without *Tb*Nfu1-V5 rescue gave a very similar result with no persistent growth defect observed in either case (data not shown). Prolonged cultivation as required for subsequent transfection and freezing might have abolished the growth phenotype which was transient and not very stable. Western blots using anti-*Tb*Nfu1 antibody showed inefficient depletion of the protein in cell lines recovered from liquid nitrogen, similar to the situation in the PCF (Supplementary Fig. S2B).

Down-regulation of *Tb*Nfu2 in the BSF cells caused a slower growth around day 3, with cells escaping from RNAi at day 6. Western blot analysis using a cell line also carrying an endogenously C-terminal PTP-tagged allele showed depletion of the protein after 4 and 5 days of induction (Fig. 7B). Targeted depletion of *Tb*Nfu3 gave similar results with BSF trypanosomes showing a growth phenotype at day 3 post RNAi-induction and eventual escape from the RNAi response between days 5 and 6 (Fig. 7C). Western blot analysis with specific antibodies showed efficient depletion of the endogenously tagged protein at days 3 and 4 with the cells re-expressing *Tb*Nfu3-PTP upon escape from RNAi (Fig. 7C). These RNAi revertants are frequently observed in *T. brucei*, especially in the BSF cells, and are no cause for concern (Chen et al., 2003). Regardless of these findings, our experiments clearly show that *Tb*Nfu2 and *Tb*Nfu3 are critical for fitness in PCF and BSF parasites.

4. Discussion

Although the increasingly complex synthesis of Fe–S clusters in eukaryotes has been studied primarily in model organisms such as yeast, *A. thaliana*, rats and humans, the ISC pathway has also been dissected in considerable detail in *T. brucei*, which is in this respect the best studied representative of the eukaryotic supergroup Excavata (Lukeš and Basu, 2015). Despite frequent and substantial departures from even the most basic mechanisms and processes in this diverged parasitic protist, compared with a typical eukaryotic cell, this does not seem to be the case when ISC synthesis is concerned. Indeed, trypanosomes contain all components of the mitochondria-localised Fe–S synthesis pathway. However, the Nfu proteins are an exception, since three homologues have apparently emerged in the ancestor of the kinetoplastid flagellates, as both trypanosomes and leishmanias harbour the same set of three Nfu genes. Such an amplification of ISC components is rare, and where Nfu proteins are concerned, is known only from higher plants (Balk and Schaedler, 2014).

Since the plant Nfu proteins show different localisations, we wondered if this was also the case with the Nfu proteins in *T. brucei*. All three Nfu1 homologues were, however, localised to the mitochondrion in the PCF and, at least in the case of the latter two Nfu proteins, also in the BSF flagellates. This is in agreement with the localisation of the single homologue in other non-plant eukaryotes (Schilke et al., 1999; Tong et al., 2003). Even though repeated attempts to determine the subcellular localisation of *Tb*Nfu1 in the BSF were unsuccessful, its mitochondrial localisation is highly anticipated due to the presence in mitochondria in the PCF, the easily discernible mitochondrial targeting sequence, and the observation that so far all components of the ISC machinery had the same localisation in both trypanosome life stages (Paris et al., 2010; Changmai et al., 2013; Kovářová et al., 2014).

Our results suggest that at least two (*Tb*Nfu2 and *Tb*Nfu3) out of the three trypanosome proteins are capable of transferring ISCs to target proteins in the yeast mitochondrion, even though these might be very different from their endogenous targets in *T. brucei*. This is in good agreement with rescue assays performed with the *A. thaliana* Nfu proteins (Léon et al., 2003), suggesting that even though a divergence in function might result in the creation of different isoforms of a protein in a given organism, these different isoforms are still capable of fulfilling at least some of their evolutionary ancestral functions.

The essentiality of a Nfu1 homologue is expected in the PCF, especially bearing in mind that the *Tb*Nfu proteins likely serve as specific targeting factors for the same enzymes as in other organisms, namely complexes I and II and LipA. The Fe–S co-factors are crucial for proper function of the respiratory chain, thus rendering their transfer factors essential in any organism that depends on oxidative phosphorylation for energy generation, such as the PCF of *T. brucei*. However, this fails to explain the indispensability of the Nfu scaffold proteins in the BSF stage, where the known Nfu1 targets, similar to most Fe–S proteins, are not likely to be essential (Tielens and Van Hellemond, 1998; Surve et al., 2012; Lukeš and Basu, 2015). Moreover, the amounts of ferredoxin, glutaredoxin and lipoic acid, which might be affected by depletion of a component of the [4Fe–4S] ISC biosynthesis machinery, are rather low in the BSF stage and potentially not required for cell survival (Stephens et al., 2007; Lukeš and Basu, 2015; Basu et al., 2016). Preliminary studies to assay aconitase activity and tRNA thiolation in the *Tb*Nfu RNAi cells did not show any departure from the wild type situation (data not shown). Measuring aconitase activity is a generic test, used for assaying the functionality of the core ISC machinery. However, it is unlikely to be affected by the depletion of specific targeting factors such as the Nfu proteins, which is indeed the case in yeast and humans (Schilke et al., 1999; Navarro-Sastre et al., 2011). An additional possible explanation for the indispensability of the *Tb*Nfu proteins – particularly in the BSF stage – is their involvement in hitherto unknown or trypanosome-specific holoenzyme synthesis.

One of the biggest gaps in our understanding of the ISC pathway is what causes the specificity of Fe–S cluster transfer, i.e. why a given dedicated ISC factor such as Nfu1 transfers clusters to distinct target protein(s) and not to others. These cluster transfer reactions might be too transient for their identification by protein pull-downs, and hence phenotypical analyses of depletion/deletion mutants may provide the only clues towards identifying affected molecules and pathways.

None of the other putative mitochondrial ISC targeting factors (BolA3-Tb927.8.6190 and Ind1-Tb927.11.11730) has thus far been investigated in *T. brucei*, despite their presence in the parasite genome and their conservation in trypanosomatid flagellates. Bearing in mind the extra-mitochondrial localisation of the core ISC biosynthesis components Isu and Nfs, which are also found in the nucleolus of *T. brucei* (Kovářová et al., 2014), it is tempting to speculate that the parasite has diversified its repertoire of ISC targeting factors even further to meet the needs for specific ISC transfer in different pathways.

It is quite counterintuitive that a parasitic protist carries in its mitochondrion more dedicated late-acting targeting factors than all multicellular organisms in which the ISC pathway has been examined (Lill, 2009). It indicates that although the core ISC machinery is highly conserved throughout the investigated eukaryotic superkingdoms (Lukeš and Basu, 2015; Basu et al., 2016), it is flexible in its peripheral elements where gene duplications, expansions or perhaps even novel acquisitions can accommodate special requirements of lineage-specific Fe–S cluster proteins. Since there are only two intron-containing genes in *T. brucei* (Siegel et al., 2010), alternative splicing to generate protein diversity as has been observed for human Nfu1 (Tong et al., 2003) is not likely to occur in the parasite. We therefore hypothesise that contrary to higher eukaryotes, *T. brucei* has instead evolved to produce multiple Nfu gene copies dedicated to different target proteins.

Altogether, the *T. brucei* Nfu2 and Nfu3 proteins are bona fide ISC targeting factors, but each of them has a specific essential function, possibly distinct from the Nfu functions known from other organisms. *Tb*Nfu1 might be different, since it does not appear to be essential in either trypanosome life stage or rescue the yeast ΔIsu1ΔNfu1 mutant. However, we cannot exclude that the depletion of *Tb*Nfu1 by RNAi was simply insufficient to cause a phenotype, and the protein might still be required for normal trypanosome growth. As for the yeast assay, removal of the putative mitochondrial targeting sequence might have caused inactivity of the protein and could explain the failed rescue. Overall, our results concerning *Tb*Nfu1 are rather inconclusive. Future work to establish the exact roles played by the *Tb*Nfu proteins in the mitochondrion will require further analysis of the mitochondrial Fe–S metalloproteome of *T. brucei*, which is currently in progress.

Acknowledgements

We thank Zuzana Vávrová-Bláhová (Institute of Parasitology, University of South Bohemia, Czech Republic) for her valuable contributions at the beginning of the project, Luděk Kořený (University of Cambridge, UK) for help with phylogenetic analysis, and Paul A. M. Michels (University of Edinburgh, UK) for kindly providing anti-enolase antibody. The ΔIsu1 yeast strain and the *S. cerevisiae* Nfu1 rescue plasmid were kindly provided by Roland Lill (Philipps University Marburg, Germany). This work was supported by the Czech Grant Agency 16-18699S, and a Bioglobe (European Union) grant CZ.1.07/2.3.00/30.0006 to CB and JL. We acknowledge the use of research infrastructure that has received funding from the European Union 7th Framework Program (FP7/2007–2013) under agreement no. 316304.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2016.04.006.

References

Balk, J., Schaedler, T.A., 2014. Iron cofactor assembly in plants. Annu. Rev. Plant Biol. 65, 125–153. http://dx.doi.org/10.1146/annurev-arplant-050213-035759.

Basu, S., Leonard, J.C., Desai, N., Mavridou, D.A.I., Tang, K.H., Goddard, A.D., Ginger, M.L., Lukeš, J., Allen, J.W.A., 2013. Divergence of Erv1-associated mitochondrial import and export pathways in trypanosomes and anaerobic protists. Eukaryot. Cell 12, 343–355. http://dx.doi.org/10.1128/EC.00304-12.

Basu, S., Horáková, E., Lukeš, J., 2016. Iron-associated biology of *Trypanosoma brucei*. Biochim. Biophys. Acta 1860, 363–370. http://dx.doi.org/10.1016/j.bbagen.2015.10.027.

Brzóska, K., Męczyńska, S., Kruszewski, M., 2006. Iron–sulfur cluster proteins: electron transfer and beyond. Acta Biochim. Pol. 53, 685–691, doi:20061382 [pii].

Cameron, J.M., Janer, A., Levandovskiy, V., MacKay, N., Rouault, T.A., Tong, W.H., Ogilvie, I., Shoubridge, E.A., Robinson, B.H., 2011. Mutations in iron–sulfur cluster scaffold genes NFU1 and BOLA3 cause a fatal deficiency of multiple respiratory chain and 2-oxoacid dehydrogenase enzymes. Am. J. Hum. Genet. 89, 486–495. http://dx.doi.org/10.1016/j.ajhg.2011.08.011.

Changmai, P., Horáková, E., Long, S., Černotíková-Stříbrná, E., McDonald, L.M., Bontempi, E.J., Lukeš, J., 2013. Both human ferredoxins equally efficiently rescue ferredoxin deficiency in *Trypanosoma brucei*. Mol. Microbiol. 89, 135–151. http://dx.doi.org/10.1111/mmi.12264.

Chen, Y., Hung, C.-H., Burderer, T., Lee, G.-S.M., 2003. Development of RNA interference revertants in *Trypanosoma brucei* cell lines generated with a double stranded RNA expression construct driven by two opposing promoters. Mol. Biochem. Parasitol. 126, 275–279. http://dx.doi.org/10.1016/S0166-6851(02)00276-1.

Claros, M.G., Vincens, P., 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur. J. Biochem. 241, 779–786. http://dx.doi.org/10.1111/j.1432-1033.1996.00779.x.

Comini, M.A., Rettig, J., Dirdjaja, N., Hanschmann, E.M., Berndt, C., Krauth-Siegel, R. L., 2008. Monothiol glutaredoxin-1 is an essential iron–sulfur protein in the mitochondrion of African trypanosomes. J. Biol. Chem. 283, 27785–27798. http://dx.doi.org/10.1074/jbc.M802010200.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005–1016. http://dx.doi.org/10.1006/jmbi.2000.3903.

Gerber, J., Neumann, K., Prohl, C., Mühlenhoff, U., Lill, R., 2004. The yeast scaffold proteins Isu1p and Isu2p are required inside mitochondria for maturation of cytosolic Fe/S proteins. Mol. Cell. Biol. 24, 4848–4857. http://dx.doi.org/10.1128/MCB.24.11.4848-4857.2004.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321. http://dx.doi.org/10.1093/sysbio/syq010.

Hall, T., 2011. BioEdit: an important software for molecular biology. GERF Bull. Biosci. 2, 60–61.

Horáková, E., Changmai, P., Paris, Z., Salmon, D., Lukeš, J., 2015. Simultaneous depletion of Atm and Mdl rebalances cytosolic Fe–S cluster assembly but not heme import into the mitochondrion of *Trypanosoma brucei*. FEBS J. 282, 4157–4175. http://dx.doi.org/10.1111/febs.13411.

Janke, C., Magiera, M.M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., Knop, M., 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21, 947–962. http://dx.doi.org/10.1002/yea.1142.

Katoh, K., Kuma, K., Miyata, T., Toh, H., 2005. Improvement in the Accuracy of Multiple Sequence Alignment Program MAFFT. Genome Inf. 16, 22–33. http://dx.doi.org/10.11234/gi1990.16.22.

Kelly, S., Reed, J., Kramer, S., Ellis, L., Webb, H., Sunter, J., Salje, J., Marinsek, N., Gull, K., Wickstead, B., Carrington, M., 2007. Functional genomics in *Trypanosoma brucei*: a collection of vectors for the expression of tagged proteins from endogenous and ectopic gene loci. Mol. Biochem. Parasitol. 154, 103–109. http://dx.doi.org/10.1016/j.molbiopara.2007.03.012.

Kovářová, J., Horáková, E., Changmai, P., Vancová, M., Lukeš, J., 2014. Mitochondrial and nucleolar localization of cysteine desulfurase Nfs and the scaffold protein Isu in *Trypanosoma brucei*. Eukaryot. Cell 13, 353–362. http://dx.doi.org/10.1128/EC.00235-13.

Léon, S., Touraine, B., Ribot, C., Briat, J.-F., Lobréaux, S., 2003. Iron–sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from *Arabidopsis thaliana*. Biochem. J. 371, 823–830. http://dx.doi.org/10.1042/BJ20021946.

Lill, R., 2009. Function and biogenesis of iron–sulphur proteins. Nature 460, 831–838. http://dx.doi.org/10.1038/nature08301.

Lill, R., Hoffmann, B., Molik, S., Pierik, A.J., Rietzschel, N., Stehling, O., Uzarska, M.A., Webert, H., Wilbrecht, C., Mühlenhoff, U., 2012. The role of mitochondria in cellular iron–sulfur protein biogenesis and iron metabolism. Biochim. Biophys. Acta 1823, 1491–1508. http://dx.doi.org/10.1016/j.bbamcr.2012.05.009.

Long, S., Jirků, M., Mach, J., Ginger, M.L., Sutak, R., Richardson, D., Tachezy, J., Lukeš, J., 2008. Ancestral roles of eukaryotic frataxin: Mitochondrial frataxin function and heterologous expression of hydrogenosomal *Trichomonas* homologues in trypanosomes. Mol. Microbiol. 69 (2008), 94–109. http://dx.doi.org/10.1111/j.1365-2958.2008.06260.x.

Long, S., Changmai, P., Tsaousis, A.D., Skalický, T., Verner, Z., Wen, Y.Z., Roger, A.J., Lukeš, J., 2011. Stage-specific requirement for Isa1 and Isa2 proteins in the mitochondrion of *Trypanosoma brucei* and heterologous rescue by human and *Blastocystis* orthologues. Mol. Microbiol. 81, 1403–1418. http://dx.doi.org/10.1111/j.1365-2958.2011.07769.x.

Lukeš, J., Basu, S., 2015. Fe/S protein biogenesis in trypanosomes – a review. Biochim. Biophys. Acta 1853, 1481–1492. http://dx.doi.org/10.1016/j.bbamcr.2014.08.015.

Maguire, F., Richards, T.A., 2014. Organelle evolution: a mosaic of "mitochondrial" functions. Curr. Biol. 24, R518–R520. http://dx.doi.org/10.1016/j.cub.2014.03.075.

Makiuchi, T., Nozaki, T., 2014. Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa. Biochimie 100, 3–17. http://dx.doi.org/10.1016/j.biochi.2013.11.018.

Matthews, K.R., 2005. The developmental cell biology of *Trypanosoma brucei*. J. Cell Sci. 118, 283–290. http://dx.doi.org/10.1242/jcs.01649.

Mühlenhoff, U., Gerber, J., Richhardt, N., Lill, R., 2003. Components involved in assembly and dislocation of iron–sulfur clusters on the scaffold protein Isu1p. EMBO J. 22, 4815–4825. http://dx.doi.org/10.1093/emboj/cdg446.

Mühlenhoff, U., Richter, N., Pines, O., Pierik, A.J., Lill, R., 2011. Specialized function of yeast Isa1 and Isa2 proteins in the maturation of mitochondrial [4Fe–4S] proteins. J. Biol. Chem. 286, 41205–41216. http://dx.doi.org/10.1074/jbc.M111.296152.

Navarro-Sastre, A., Tort, F., Stehling, O., Uzarska, M.A., Arranz, J.A., Del Toro, M., Labayru, M.T., Landa, J., Font, A., Garcia-Villoria, J., Merinero, B., Ugarte, M., Gutierrez-Solana, L.G., Campistol, J., Garcia-Cazorla, A., Vaquerizo, J., Riudor, E., Briones, P., Elpeleg, O., Ribes, A., Lill, R., 2011. A fatal mitochondrial disease is associated with defective NFU1 function in the maturation of a subset of mitochondrial Fe–S proteins. Am. J. Hum. Genet. 89, 656–667. http://dx.doi.org/10.1016/j.ajhg.2011.10.005.

Panigrahi, A.K., Zíková, A., Dalley, R.A., Acestor, N., Ogata, Y., Anupama, A., Myler, P.J., Stuart, K.D., 2008. Mitochondrial complexes in *Trypanosoma brucei*: a novel complex and a unique oxidoreductase complex. Mol. Cell. Proteomics 7, 534–545. http://dx.doi.org/10.1074/mcp.M700430-MCP200.

Paris, Z., Changmai, P., Rubio, M.A.T., Zíková, A., Stuart, K.D., Alfonzo, J.D., Lukeš, J., 2010. The Fe/S cluster assembly protein Isd11 is essential for tRNA thiolation in

Trypanosoma brucei. J. Biol. Chem. 285, 22394–22402. http://dx.doi.org/10.1074/jbc.M109.083774.

Parsons, M., Ramasamy, G., Vasconcelos, E.J.R., Jensen, B.C., Myler, P.J., 2015. Advancing *Trypanosoma brucei* genome annotation through ribosome profiling and spliced leader mapping. Mol. Biochem. Parasitol. 202, 1–10. http://dx.doi.org/10.1016/j.molbiopara.2015.09.002.

Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet. 16, 276–277. http://dx.doi.org/10.1016/j.cocis.2008.07.002.

Rouault, T.A., 2012. Biogenesis of iron–sulfur clusters in mammalian cells: new insights and relevance to human disease. Dis. Model. Mech. 5, 155–164. http://dx.doi.org/10.1242/dmm.009019.

Schilke, B., Voisine, C., Beinert, H., Craig, E., 1999. Evidence for a conserved system for iron metabolism in the mitochondria of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. 96, 10206–10211. http://dx.doi.org/10.1073/pnas.96.18.10206.

Schimanski, B., Nguyen, T.N., Günzl, A., 2005. Highly efficient tandem affinity purification of trypanosome protein complexes based on a novel epitope combination. Eukaryot. Cell 4, 1942–1950. http://dx.doi.org/10.1128/EC.4.11.1942-1950.2005.

Siegel, T.N., Hekstra, D.R., Wang, X., Dewell, S., Cross, G.A.M., 2010. Genome-wide analysis of mRNA abundance in two life-cycle stages of *Trypanosoma brucei* and identification of splicing and polyadenylation sites. Nucleic Acids Res. 38, 4946–4957. http://dx.doi.org/10.1093/nar/gkq237.

Smíd, O., Horáková, E., Vilímová, V., Hrdý, I., Cammack, R., Horváth, A., Lukeš, J., Tachezy, J., 2006. Knock-downs of iron–sulfur cluster assembly proteins IscS and IscU down-regulate the active mitochondrion of procyclic *Trypanosoma brucei*. J. Biol. Chem. 281, 28679–28686. http://dx.doi.org/10.1074/jbc.M513781200.

Smith, A.D., Jameson, G.N.L., Dos Santos, P.C., Agar, J.N., Naik, S., Krebs, C., Frazzon, J., Dean, D.R., Huynh, B.H., Johnson, M.K., 2005. NifS-mediated assembly of [4Fe–4S] clusters in the N- and C-terminal domains of the NifU Scaffold protein. Biochemistry 44, 12955–12969. http://dx.doi.org/10.1021/bi051257i.

Stephens, J.L., Lee, S.H., Paul, K.S., Englund, P.T., 2007. Mitochondrial fatty acid synthesis in *Trypanosoma brucei*. J. Biol. Chem. 282, 4427–4436. http://dx.doi.org/10.1074/jbc.M609037200.

Surve, S., Heestand, M., Panicucci, B., Schnaufer, A., Parsons, M., 2012. Enigmatic presence of mitochondrial complex I in *Trypanosoma brucei* bloodstream forms. Eukaryot. Cell 11, 183–193. http://dx.doi.org/10.1128/EC.05282-11.

Tielens, A.G.M., Van Hellemond, J.J., 1998. Differences in energy metabolism between Trypanosomatidae. Parasitol. Today 14, 265–271. http://dx.doi.org/10.1016/S0169-4758(98)01263-0.

Tielens, A.G.M., van Hellemond, J.J., 2009. Surprising variety in energy metabolism within Trypanosomatidae. Trends Parasitol. 25, 482–490. http://dx.doi.org/10.1016/j.pt.2009.07.007.

Tong, W.-H., Jameson, G.N.L., Huynh, B.H., Rouault, T.A., 2003. Subcellular compartmentalization of human Nfu, an iron–sulfur cluster scaffold protein, and its ability to assemble a [4Fe–4S] cluster. Proc. Natl. Acad. Sci. U.S.A. 100, 9762–9767. http://dx.doi.org/10.1073/pnas.1732541100.

Týč, J., Klingbeil, M.M., Lukeš, J., 2015. Mitochondrial heat shock protein machinery Hsp70/Hsp40 Is indispensable for proper mitochondrial DNA maintenance and replication. MBio 10, e02425–e02514. http://dx.doi.org/10.1128/mBio.02425-14.

Uzarska, M.A., Dutkiewicz, R., Freibert, S., Lill, R., Fox, T.D., 2013. The mitochondrial Hsp70 chaperone Ssq1 facilitates Fe/S cluster transfer from Isu1 to Grx5 by complex formation. Mol. Biol. Cell 24, 1830–1841. http://dx.doi.org/10.1091/mbc.E12-09-0644.

Verner, Z., Basu, S., Benz, C., Dixit, S., Dobáková, E., Faktorová, D., Hashimi, H., Horáková, E., Huang, Z., Paris, Z., Peña-Diaz, P., Ridlon, L., Týč, J., Wildridge, D., Zíková, A., Lukeš, J., 2015. Malleable mitochondrion of *Trypanosoma brucei*. Int. Rev. Cell Mol. Biol. 315, 73–151. http://dx.doi.org/10.1016/bs.ircmb.2014.11.001.

Vondrušková, E., Van Den Burg, J., Zíková, A., Ernst, N.L., Stuart, K., Benne, R., Lukeš, J., 2005. RNA interference analyses suggest a transcript-specific regulatory role for mitochondrial RNA-binding proteins MRP1 and MRP2 in RNA editing and other RNA processing in *Trypanosoma brucei*. J. Biol. Chem. 280, 2429–2438. http://dx.doi.org/10.1074/jbc.M405933200.

Wickstead, B., Ersfeld, K., Gull, K., 2002. Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 125, 211–216. http://dx.doi.org/10.1016/S0166-6851(02)00238-4.

Wirtz, E., Leal, S., Ochatt, C., Cross, G.A., 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 99, 89–101. http://dx.doi.org/10.1016/S0166-6851(99)00002-X.