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Abstract: Iron-sulfur clusters (ISCs) are protein co-factors essential for a wide range of cellular functions. The core ISC 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 ISC assembly. In mitochondria all [4Fe-4S] ISCs are synthesized and transferred to specific apoproteins by so-called ISC 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 harbor a single Nfu1, the genomes of Trypanosoma brucei and related flagellates encode three Nfu genes. All three Nfu proteins localize to the mitochondrion in the procyclic form of T. brucei, and TbNfu2 and TbNfu3 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 ISC assembly in a heterologous system and rescue the growth defect of a yeast deletion mutant.

***Cover Letter**

Dear Professor Loukas,

We thank the reviewers for their comments, which have helped improve the manuscript.

We have dealt with every comment and provided our response in blue text in the separate file entitled ‘response to reviews’. Since there was substantial support from both reviewers we hope that this improved manuscript is now acceptable for publication.

Yours sincerely,

Julius Lukeš

***Response to Reviewers**

Reviewers' comments:

Reviewer #1: This submission presents expression, localization, and functional data on the three phylogenetically related TbNfuI proteins of Trypanosoma brucei. These TbNfuI proteins function in the assembly of mitochondrial iron-sulfur clusters that have been shown to participate in multiple vital cellular roles, but this is the first such study in protozoan parasites. Fundamentally, the authors demonstrate that: 1) all three TbNfu1 proteins are clustered in a phylogenetic tree (Figure 1); 2) all three C-tagged TbNfu proteins localize to the mitochondrion in procyclic forms (Figure 2); 3) at least TbNfu2 and TbNfu3 localize to the mitochondrion in blood stream forms (Figure 3); 4) TbNfu2 and TbNfu3 complement a <DELTA>TbNfu1 lesion in Saccharmomyces cerevisiae; and 5) RNAi knockdowns of TbNfus have growth inhibitory effects in both procyclic and bloodstream forms, although to differing degrees. Overall, although the impact of this study is not enormous, the study is well-designed, sufficiently novel, and the manuscript adequately clear to warrant publication in the journal. There are some issues, however, that should be addressed.

Major point:

The Introduction and Discussion sections are much too long and should be reduced by approximately two thirds. The Introduction is six pages long and much of it is review-like material that is not directly pertinent to TbNfu function. The Discussion is basically a rehash of the Results section and the remainder is highly speculative.

We have reduced the introduction to 4.5 pages and the discussion to 4 pages. We feel that any further reductions would remove essential information and also not be compatible with suggestions from reviewer 2 (addition of the ISC assembly scheme and speculation about the diversification of trypanosome Nfus).

Minor points:

1) The title should be changes. This reviewer did not understand why the three NbNfus had "non-redundant" roles.

Since the Nfu proteins are apparently all required for normal trypanosome growth, we felt justified in saying that they play non-redundant roles in the organism. We however agree that the situation with *Tb*Nfu1 is not entirely conclusive and have hence changed the title to ‘Roles of the Nfu Fe-S targeting factors in the trypanosome mitochondrion’.

2) Line 197 - It is not clear why the authors conclude that there is a differential distribution of TbNfu2 and TbNfu3 based on a photograph of a single parasite transfected with either tagged gene.

We have removed this conclusion about the differential distribution of *Tb*Nfu2 and 3 from the manuscript.

3) Lines 338-340 - Are the authors differentiating between "similarity" and identity?

We now include both similarity and identity values in the text for clarity.

4) The data in Figure 6A, per the authors' statement on lines 455-456, are not reproducible. Therefore, the Figure should be removed.

We feel that the figure should stay in the manuscript for the sake of completeness. We have reworded the text to make it more obvious that the obtained phenotype - while transient and weak - is consistently observed immediately after transfection of *Tb*Nfu1 RNAi constructs and only lost upon continued cultivation of cells or freezing.

5) Table 1 could go in a supplement.

We have put table 1 into the supplementary material.

Reviewer #2: The authors have presented experimental evaluation of T. brucei Nfu1-3, and supported their role in ISC pathway. They appear to be mitochondrial, and have distinct targets. At least 2 are essential, while results for Nfu1 are somewhat inconclusive in that regard. Overall the paper is clearly written, and reasonably straightforward.

Points to address:

1) While not absolutely necessary, it would be helpful to include a schematic overview of the ISC assembly pathway, to orient the background information in the introduction and indicate the position that the TbNfus occupy within this pathway.

We now include this scheme as Fig. 1 to improve clarity of the background information.

2) In Figure 2B, right panel, there appear to be 2 bands in the region of Nfu3 in the whole cell lysate. Upon fractionation, the upper band stays primarily in the cytoplasm, and the lower is in the mitochondrion. Would the authors speculate that the upper band is a pre-import intermediate, prior to N-terminal cleavage, or do they believe it is merely a cross-reacting protein?

This fractionation experiment uses a cell line overexpressing a tagged version of *Tb*Nfu3. Although the explanation suggested by the reviewer is possible, we did not observe this band in other Western blots (of this cell line or cell lines expressing *Tb*Nfu3-PTP from the endogenous locus) and hence suspect that it is merely a cross-reacting band.

3) In both Figures 5 & 6, the y-axis scales for growth curves vary from panel to panel, for no apparent reason. I would recommend using a uniform scale on all of these graphs to facilitate comparisons between cell lines.

We have unified the y-axes of these graphs.

4) The sentence starting on line 437, "We suspect that suppression of the phenotype occurs..." is not clear. I think that the authors are circuitously referring to the common RNAi phenomenon in which repression is eventually lost over time; however, the way it is written implies to me that something is actively suppressing the effect of Nfu1 loss. Also, the following note that cryopreserved lines no longer display RNAi phenotype upon tet induction is a very common occurrence for this technique, and it doesn't reflect anything particular to Nfu1 - this point should be made clear to the reader.

The text has been rephrased to emphasise that the RNAi effect is simply lost over time and a sentence added to mention that this is a common occurrence.

5) Line 498: replace the word "only" with either "lone" or "single". I think this fits the intended meaning more clearly.

The word ‘only’ has been replaced by ‘single’.

6) Line 505: the supposition that Nfu1 was not locatable in BSF "probably due to very low expression" is addressable in a couple immediate ways. First, ribosome profiling work comparing BSF and PCF have been published (e.g. Jensen, et al. 2014) that the authors should check to determine if their hypothesis is substantiated. Second, the authors possess antibodies to Nfu1 that should be sufficient to test the relative amount of protein per cell equivalent in each cell type. Either or both of these analyses should be included to address this hypothesis.

Ribosomal profiling suggests that all *Tb*Nfus are similarly expressed in both life stages, so we have removed the ‘probably due to low expression’. Unfortunately the remainder of the antibody does not produce convincing signals on Western blots anymore (see supplementary figure 2B) which might be

due to repeated freeze-thaw cycles (it was produced many years ago) and we do not have an old blot comparing levels in BSF and PCF carefully.

7) Line 564: how is it "known" that Tb927.8.6190 and Tb927.11.11730 are mitochondrial ISC targeting factors? My understanding is that they have not been characterized experimentally, and are putative predictions based on homology.

We have added a 'putative' to the sentence.

8) The conclusions drawn regarding Nfu1 in the final paragraph of the Discussion (Lines 581-2) are not justified. While the Nfu1 RNAi results do not indicate it is essential, they also do not indicate it is not essential. When you have a significant but intermediate growth defect, you cannot discern whether cells can biologically compensate for the loss of the target, or whether the RNAi knockdown is incomplete. The authors should refrain from claiming that Nfu1 is not essential, and either state its essentiality is inconclusive, or note that it is required for normal growth. Also, the conclusion from the Nfu1 yeast complementation experiment is underdeveloped. The authors chose to try complementation with an N-terminal truncation of Nfu1, for reasons not made clear to me, which could very well be the root cause of its inactivity. Comparison of truncated Nfu1 to complete Nfu2 and Nfu3 is therefore tricky, and the negative result for Nfu1 cannot be a reliable proxy for claiming that Nfu1 doesn't have similar ISC targeting activity to Nfu2 or Nfu3. These points need to be clarified in the revised manuscript

We have reworded our conclusions regarding *Tb*Nfu1 accordingly and also added a sentence regarding the removal of the mitochondrial targeting sequence from this protein only in the results section.

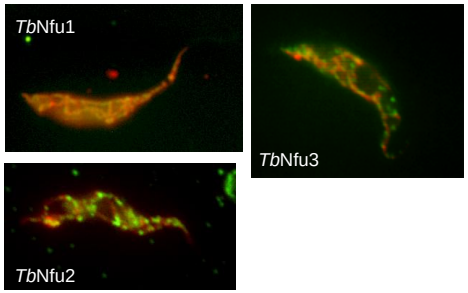
8) The authors should present at least one hypothesis in the Discussion to explain the unprecedented diversity of ISC targeting proteins in trypanosomes.Why have tryps done this? While it is clear that the answer is unknown at present, the question deserves emphasis and at least an attempt to explain it.

We fully agree that this question deserves attention, especially since the presence of multiple Nfus excited us from the beginning. We have now come up with a hypothesis, which is presented in the Discussion section.

9) As experimental localization of Nfu1 in BSF was not successful, perhaps the words "All three" should be deleted from line 51 of the abstract.

We have removed this from the abstract and elsewhere in the text.

***Graphical Abstract (for review)**



***Highlights (for review)**

- *Trypanosoma brucei* expresses three Nfu proteins whereas other eukaryotes generally have only one
- Two of these three proteins are essential in both the mammalian-infective and the insect stage of the parasite
- These two Nfu proteins are also functional in a heterologous context in *S. cerevisiae*

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

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Abstract

Iron-sulfur clusters (ISCs) are protein co-factors essential for a wide range of cellular functions. The core ISC 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 ISC assembly. In mitochondria all [4Fe-4S] ISCs are synthesized and transferred to specific apoproteins by so-called ISC 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 harbor a single Nfu1, the genomes of *Trypanosoma brucei* and related flagellates encode three Nfu genes. All three Nfu proteins localize 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 ISC assembly in a heterologous system and rescue the growth defect of a yeast deletion mutant.

Keywords: *Trypanosoma brucei*, Nfu1, iron-sulfur cluster; Fe-S; mitochondrion

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 harbor a similar cohort of proteins important for iron-sulfur 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 evolutionary 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 just 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 nomenclature for this well conserved process) and compare this to 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 sulfur 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 sulfur 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 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 BSF, and both Isu and Nfs localize 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 PCF, however, it remains to be resolved if this is due to direct involvement of this complex in the process or indirectly by the provision of an ISC for another component (Kovářová et al., 2014; Paris et al., 2010).

With the help of the 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 mtHsp70 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 homologs of all these proteins are present in *T. brucei* but, with the exception of the Isa1 and Isa2 proteins (Long et al., 2011), have thus far not been experimentally studied (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 sulfur-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 characterized by symptoms such as lactic acidosis, hyperglycinemia, and reduced activities 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 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 man, the genomes of plants including *Arabidopsis thaliana* contain five genes with similarity to the C-terminus of NifU. Two of these Nfu homologs (*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. Localization studies showed that *At*Nfu1, 2 and 3 are localized in the plastid, while *At*Nfu4 was shown to be confined to the mitochondrion, and *At*Nfu5 was predicted to have the same localization (Léon et al., 2003).

Interestingly, cytosolic and nuclear localization 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 localize 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

T. brucei BSF 427 cells, BSF single-marker cells and PCF 29:13 (Wirtz et al., 1999) cells were cultured as described elsewhere (Changmai et al., 2013). For 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 1 for all oligonucleotide sequences used and plasmids generated in this study). For *Tb*Nfu2 (Tb927.10.11160), a 480 bp region (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'UTR, followed by ligation to p2T7-

177 (generating plasmid pCR49). *Not*I was used to linearize 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 linearization 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 linearization 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 linearized with *Not*I prior to transfection.

Linearized 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 phleomycin. In all cases, RNAi was induced by the addition of 1 µg/ml 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 puromycin. PCF 29:13 cells transfected with constructs overexpressing *Tb*Nfu1 and *Tb*Nfu3 were selected with 5

µg/ml phleomycin (pJH54-based construct) and 0.5 µg/ml 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 phleomycin (*Tb*Nfu1-3 RNAi) and 0.2 µg/ml 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 puromycin.

2.2 Bioinformatics

The Nfu homologs were identified by BLAST search of the NCBI 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. Similarly, oligopeptide SSTYDNFIPDGQTC, corresponding to amino acids 30 – 43, was used for production of the anti-*Tb*Nfu3 antibody by GenScript.

2.4 Immunofluorescence

Following staining with mitotracker red (Sigma-Aldrich), 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 permeabilization (paraformaldehyde fixation only) in phosphate buffered saline (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) in PBS for 1 hr. Following two washes with PBS, the slides were incubated with the secondary antibody, either AlexaFluor488 goat anti-rabbit (1:200, Molecular Probes) or AlexaFluor488 goat anti-mouse (1:200, Molecular Probes) in PBS for 1 hr. The slides were washed twice in PBS, then 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Fisher Scientific) 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 x 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 hr 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 x 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 hr at room temperature. Finally, the membrane was washed 2 x 5 min in PBS and antibodies were detected by enhanced chemiluminescence (Clarity Western ECL Substrate, BioRad).

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 (13,000 rpm 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 digitonin (Sigma-Aldrich). Samples were briefly vortexed and following incubation for 5 min at room temperature centrifuged (13,000 rpm, 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 (13,000 rpm, 2 min) and the obtained supernatant was collected as the mitochondrial fraction. Anti-HA and anti-V5 antibodies (Invitrogen) were used in dilution 1:2,000 and anti-MRP2 (Vondrušková et al., 2005) at 1:2,000.

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 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 above) 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 distilled water. Serial 1:10 dilutions were made and about 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 (around 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 from across all eukaryotic super-domains shows that there are two eukaryotic lineages of Nfu. The first lineage is present across all eukaryotes and branches along with alpha-proteobacteria, indicative of its mitochondrial origin. Phototrophic eukaryotes also acquired a Nfu gene with a different lineage, which groups with cyanobacteria and has very likely a plastidial origin (Fig. 2B). In all trypanosomatids sequenced so far, 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 around 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 about 55 kDa consistent with a non-cleaved species (Suppl. Fig. 1). 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 Localization 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 localization studies. Both immunofluorescence analysis and digitonin fractionation techniques were employed to corroborate results in 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 localized to the elaborate PCF mitochondrion where it was evenly distributed and completely overlapped with mitotracker red staining (Fig. 3A, top row). This result was confirmed by digitonin fractionation, where an overexpressed, HA-tagged *Tb*Nfu1 protein was clearly mitochondrial (Fig. 3B, left panel). Finally, the same HA-tagged protein also localized to the mitochondrion by immunofluorescence analysis (data not shown).

*Tb*Nfu2-PTP detected with an antibody against protein A also localized to the mitochondrion, although the distribution of the tagged protein was more punctate than that of its tagged *Tb*Nfu1 counterpart (Fig. 3A, middle row). 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, middle panel). 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 localize the third *Tb*Nfu homologue, which was endogenously PTP-tagged and detected with an antibody against protein A. Indeed, all *Tb*Nfu3 clearly localized 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, bottom row). Mitochondrial localization was further confirmed by digitonin fractionation, where an overexpressed, V5-tagged protein was also found exclusively in the organelle (Fig. 3B, right panel). This result was corroborated when *Tb*Nfu3-V5 was localized to the mitochondrion by immunofluorescence analysis in the same cell line (data not shown).

3.3 Localization in BSF cells

Localization 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 localize 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 when 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 to 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.

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 Materials and methods), 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 of the *Tb*Nfu1 protein level, the depletion did not trigger a stable phenotype (Fig. 6A; Suppl. Fig. 2A). 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 PCF started to show a growth phenotype between day 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 recognize the PTP-tag. Consistent 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 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 Materials and methods).

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 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 PCF (Suppl. Fig. 2B).

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 procyclic and bloodstream form 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 been dissected to a considerable detail also 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, as compared to 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-localized 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 harbor the same set of three Nfu genes. Such an amplification of ISC components is rare, and when Nfu proteins are concerned, is known only from higher plants (Balk and Schaedler, 2014).

Since the plant Nfu proteins show different localizations, we wondered if this was also the case with the Nfu proteins in *T. brucei*. All three Nfu1 homologs were, however, localized to the mitochondrion in PCF and, at least in the case of the latter two Nfu proteins, also in the BSF flagellates. This is in agreement with the localization 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 localization of *Tb*Nfu1 in BSF were unsuccessful, its mitochondrial localization is highly anticipated due to the presence in mitochondria in PCF, the easily discernible mitochondrial targeting sequence, and the observation that so far all components of the ISC machinery had the same localization in both trypanosome life stages (Changmai et al., 2013; Kovářová et al., 2014; Paris et al., 2010).

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 homolog is expected in 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 lipoic acid synthase (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, just like most Fe-S proteins, are not likely to be essential (Lukeš and Basu, 2015; Surve et al., 2012; Tielens and Van Hellemond, 1998). 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 (Basu et al., 2016; Lukeš and Basu, 2015; Stephens et al., 2007). 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

503 indeed the case in yeast and humans (Navarro-Sastre et al., 2011; Schilke et al., 1999).
504 An additional possible explanation for the indispensability of the *Tb*Nfu proteins –
505 particularly in the BSF stage – is their involvement in hitherto unknown or trypanosome-
506 specific holoenzyme synthesis.

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

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

521 It is quite counterintuitive that a parasitic protist carries in its mitochondrion more
522 dedicated late-acting targeting factors than all multicellular organisms in which the ISC
523 pathway has been examined (Lill, 2009). It indicates that although the core ISC
524 machinery is highly conserved throughout the investigated eukaryotic superkingdoms
525 (Basu et al., 2016; Lukeš and Basu, 2015), it is flexible in its peripheral elements, where
526 gene duplications, expansions or perhaps even novel acquisitions can accommodate

for 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 hypothesize 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.

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Author contributions

Planned experiments: CB, JL, JK, AP; performed experiments: CB, JK, IK-H; analyzed data: CB, JL, JK, AP, contributed reagents: JL, AP; wrote the paper: CB, JL, JK, AP. All authors agreed on the final version of the manuscript.

References

Balk, J., Schaedler, T. A, 2014. Iron cofactor assembly in plants. Annu. Rev. Plant Biol. 65, 125–53. doi: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. doi:10.1128/EC.00304-12

Basu, S., Netz, D.J., Haindrich, A.C., Herlerth, N., Lagny, T.J., Pierik, A.J., Lill, R., Lukeš, J., 2014. Cytosolic iron-sulphur protein assembly is functionally conserved and essential in procyclic and bloodstream *Trypanosoma brucei*. Mol. Microbiol. 93, 897–910. doi:10.1111/mmi.12706

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

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

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

583 Changmai, P., Horáková, E., Long, S., Černotíková-Stříbrná, E., McDonald, L.M., Bontempi,
584 E.J., Lukeš, J., 2013. Both human ferredoxins equally efficiently rescue ferredoxin
585 deficiency in *Trypanosoma brucei*. Mol. Microbiol. 89, 135–51. doi:10.1111/mmi.12264

586 Chen, Y., Hung, C.-H., Burderer, T., Lee, G.-S.M., 2003. Development of RNA interference
587 revertants in *Trypanosoma brucei* cell lines generated with a double stranded RNA
588 expression construct driven by two opposing promoters. Mol. Biochem. Parasitol. 126,
589 275–279. doi:10.1016/S0166-6851(02)00276-1

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

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

596 Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular
597 localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300,
598 1005–1016. doi:10.1006/jmbi.2000.3903

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

602 Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New
603 Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the
604 Performance of PhyML 3.0. Syst. Biol. 59, 307–321. doi:10.1093/sysbio/syq010

605 Horáková, E., Changmai, P., Paris, Z., Salmon, D., Lukeš, J., 2015. Simultaneous depletion of
606 Atm and Mdl rebalances cytosolic Fe-S cluster assembly but not heme import into the
607 mitochondrion of *Trypanosoma brucei*. FEBS J. 282(21), 4157-75. doi:10.1111/febs.13411

608 Hall, T., 2011. BioEdit: An important software for molecular biology. GERF Bull. Biosci. 2, 60–
609 61.

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

614 Katoh, K., Kuma, K., Miyata, T., Toh, H., 2005. Improvement in the Accuracy of Multiple
615 Sequence Alignment Program MAFFT. Genome Inf. 16(1), 22-33.
616 doi.org/10.11234/gi1990.16.22

617 Kelly, S., Reed, J., Kramer, S., Ellis, L., Webb, H., Sunter, J., Salje, J., Marinsek, N., Gull, K.,
618 Wickstead, B., Carrington, M., 2007. Functional genomics in *Trypanosoma brucei*: a
619 collection of vectors for the expression of tagged proteins from endogenous and ectopic
620 gene loci. Mol. Biochem. Parasitol. 154, 103–9. doi:10.1016/j.molbiopara.2007.03.012

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

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

627 Lill, R., 2009. Function and biogenesis of iron-sulphur proteins. Nature 460, 831–838.
628 doi:10.1038/nature08301

629 Lill, R., Hoffmann, B., Molik, S., Pierik, A.J., Rietzschel, N., Stehling, O., Uzarska, M. a.,
630 Webert, H., Wilbrecht, C., Mühlenhoff, U., 2012. The role of mitochondria in cellular iron-
631 sulfur protein biogenesis and iron metabolism. Biochim. Biophys. Acta - Mol. Cell Res.
632 1823, 1491–1508. doi:10.1016/j.bbamcr.2012.05.009

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

637 Lukeš, J., Basu, S., 2015. Fe/S protein biogenesis in trypanosomes — A review. Biochim.
638 Biophys. Acta - Mol. Cell Res. 1853, 1481–1492. doi:10.1016/j.bbamcr.2014.08.015

639 Maguire, F., Richards, T.A., 2014. Organelle evolution: a mosaic of “mitochondrial” functions.
640 Curr. Biol. 24, R518–20. doi:10.1016/j.cub.2014.03.075

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

643 Matthews, K.R., 2005. The developmental cell biology of *Trypanosoma brucei*. J. Cell Sci. 118,
644 283–90. doi:10.1242/jcs.01649

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

648 Mühlenhoff, U., Richter, N., Pines, O., Pierik, A.J., Lill, R., 2011. Specialized function of yeast
649 Isa1 and Isa2 proteins in the maturation of mitochondrial [4Fe-4S] proteins. J. Biol. Chem.
650 286, 41205–41216. doi:10.1074/jbc.M111.296152

651 Navarro-Sastre, A., Tort, F., Stehling, O., Uzarska, M. a., Arranz, J.A., Del Toro, M., Labayru,

652 M.T., Landa, J., Font, A., Garcia-Villoria, J., Merinero, B., Ugarte, M., Gutierrez-Solana,
653 L.G., Campistol, J., Garcia-Cazorla, A., Vaquerizo, J., Riudor, E., Briones, P., Elpeleg, O.,
654 Ribes, A., Lill, R., 2011. A fatal mitochondrial disease is associated with defective NFU1
655 function in the maturation of a subset of mitochondrial Fe-S proteins. Am. J. Hum. Genet.
656 89, 656–667. doi:10.1016/j.ajhg.2011.10.005

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

661 Paris, Z., Changmai, P., Rubio, M.A.T., Zíková, A., Stuart, K.D., Alfonzo, J.D., Lukeš, J., 2010.
662 The Fe/S cluster assembly protein Isd11 is essential for tRNA thiolation in *Trypanosoma*
663 *brucei*. J. Biol. Chem. 285, 22394–22402. doi:10.1074/jbc.M109.083774

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

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

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

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

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

678 Smíd, O., Horáková, E., Vilímová, V., Hrdý, I., Cammack, R., Horváth, A., Lukeš, J., Tachezy,
679 J., 2006. Knock-downs of iron-sulfur cluster assembly proteins IscS and IscU down-
680 regulate the active mitochondrion of procyclic *Trypanosoma brucei*. J. Biol. Chem. 281,
681 28679–28686. doi:10.1074/jbc.M513781200

682 Smith, A.D., Jameson, G.N.L., Dos Santos, P.C., Agar, J.N., Naik, S., Krebs, C., Frazzon, J.,
683 Dean, D.R., Huynh, B.H., Johnson, M.K., 2005. NifS-mediated assembly of [4Fe-4S]
684 clusters in the N- and C-terminal domains of the NifU Scaffold protein. Biochemistry 44,
685 12955–12969. doi:10.1021/bi051257i

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

688 Surve, S., Heestand, M., Panicucci, B., Schnaufer, A., Parsons, M., 2012. Enigmatic presence
689 of mitochondrial complex I in *Trypanosoma brucei* bloodstream forms. Eukaryot. Cell 11,
690 183–193. doi:10.1128/EC.05282-11

691 Tielens, a G.M., Van Hellemond, J.J., 1998. Differences in energy metabolism between
692 Trypanosomatidae. Parasitol. Today 14, 265–271.doiI: 10.1016/S0169-4758(98)01263-0

693 Tielens, A.G.M., van Hellemond, J.J., 2009. Surprising variety in energy metabolism within
694 Trypanosomatidae. Trends Parasitol. 25, 482–90. doi:10.1016/j.pt.2009.07.007

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

699 Týč, J., Klingbeil, M.M., Lukeš, J., 2015. Mitochondrial Heat Shock Protein Machinery Hsp70 /
700 Hsp40 Is Indispensable for Proper Mitochondrial DNA Maintenance and Replication. MBio
701 10, e02425–14. doi:10.1128/mBio.02425-14.

702 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, 1830-41. doi: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. doi: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. doi: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, 211–6. doi: 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. doi: 10.1016/S0166-6851(99)00002-X

Figure legends

Fig. 1: Scheme of the mitochondrial ISC assembly pathway in *T. brucei*. Components essential in PCF are indicated in orange, dispensable ones are in green, Nfu proteins in blue, and proteins present in the genome but not assayed so far in grey. *Tb*Grx1 is homologous to yeast Grx5. The Nfs-Isd11 desulfurase complex (Paris et al., 2010) provides sulfur 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 utilized in the CIA pathway.

Fig. 2: Bioinformatic analysis of Nfu proteins in prokaryotes and eukaryotes/ Position of the *Tb*Nfu proteins among related prokaryotic and eukarytic proteins.

A) The bacterial NifU protein consists of three domains. The N-terminal part (in green) contains three highly conserved cysteine residues, which are involved in formation of a new ISC, and is highly similar to the Isu proteins. The central part of NifU (in blue) is called ferredoxin-like domain, contains a permanent ISC and shows similarity to nitrate reductases. The C-terminal part (in yellow) accommodates a conserved motif CXXC, which is presumed to be involved in formation of a new ISC and is found in the Nfu proteins as well. The conserved aspartate residues were proposed to mediate the transfer of newly formed ISCs. The red bars represent N-terminal mitochondrial

targeting sequences (the sequence is not well predicted in *Tb*Nfu3), and the green bar represents a plastid targeting sequence from *A. thaliana*, respectively. Dark blue boxes mark Nfu1-like domains in the *T. brucei* proteins and *At*Nfu4 and 5, while the maroon box shows a B domain specific for *At*Nfu1-3. B) Scheme of maximum-likelihood phylogenetic tree of Nfu homologs. The prokaryotic taxa are in black, while eukaryotes are highlighted in colors (green for chloroplast-containing photoautotrophs and blue for heterotrophs). The bootstrap/SH-like aLRT branch supports are shown for the cyanobacteria/chloroplasts clade and the alpha-proteobacteria/mitochondria clade.

Fig. 3: Localisation of *Tb*Nfus in PCF 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 = 5µm. B) Subcellular fractionation of cell lines expressing tagged *Tb*Nfu proteins. Fractionation into cytosolic and mitochondrial fractions (*Tb*Nfu1-HA and *Tb*Nfu3-V5), and fractionation with increasing concentrations of digitonin (*Tb*Nfu2-PTP) are shown.

Fig. 4: Localisation of *Tb*Nfus in BSF 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 = 5µm.

772 Fig. 5: Complementation of ΔIsu1ΔNfu1 yeast strain.

773 ΔIsu1ΔNfu1 strain transfected or not with the indicated rescue plasmids encoding
774 *Tb*Nfus and *S. cerevisiae* Nfu1 incubated at 30 °C.

775

776 Fig. 6: RNAi against the *Tb*Nfu proteins in PCF cells.

777 A) Cumulative growth of *Tb*Nfu1 RNAi cell line, in the presence (open squares, dashed
778 line) or absence of tetracycline (tet) in the medium (solid squares, unbroken line).
779 Western blot shows depletion of the endogenous *Tb*Nfu1 protein detected with anti-
780 *Tb*Nfu1 antibodies. B) Cumulative growth of *Tb*Nfu2 RNAi cell line, in the presence
781 (open squares, dashed line) or absence of tetracycline (tet) in the medium (solid
782 squares, unbroken line). Western blot shows depletion of the endogenously PTP-
783 tagged *Tb*Nfu2 protein detected with anti-protein A. C) Cumulative growth of *Tb*Nfu3
784 RNAi cell line, in the presence (open squares, dashed line) or absence of tetracycline
785 (tet) in the medium (solid squares, unbroken line). Western blot shows depletion of the
786 endogenous *Tb*Nfu3 protein detected with anti-*Tb*Nfu3 antibodies. Cytosolic enolase
787 was used as a loading control in all three panels.

788

789 Fig. 7: RNAi against the *Tb*Nfu proteins in BSF cells.

790 A) Cumulative growth of *Tb*Nfu1 RNAi cell line, in the presence (open squares, dashed
791 line) or absence (solid squares unbroken line) of tetracycline (tet). Western blot shows
792 depletion of endogenous *Tb*Nfu1 protein detected with anti-*Tb*Nfu1. B) Cumulative
793 growth of *Tb*Nfu2 RNAi cell line, in the presence (open squares, dashed line) or
794 absence (solid squares, unbroken line) of tetracycline (tet). Western blot shows

depletion of endogenously PTP-tagged *Tb*Nfu2 detected with anti-protein A. C) Cumulative growth of *Tb*Nfu3 RNAi cell line, in the presence (open squares, dashed line) or absence (solid squares, unbroken line) of tetracycline (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 panels.

Supp. Table 1:

Oligonucleotides used and plasmids generated in this study

Fig. 1: Apparent molecular weight of *Tb*Nfu2

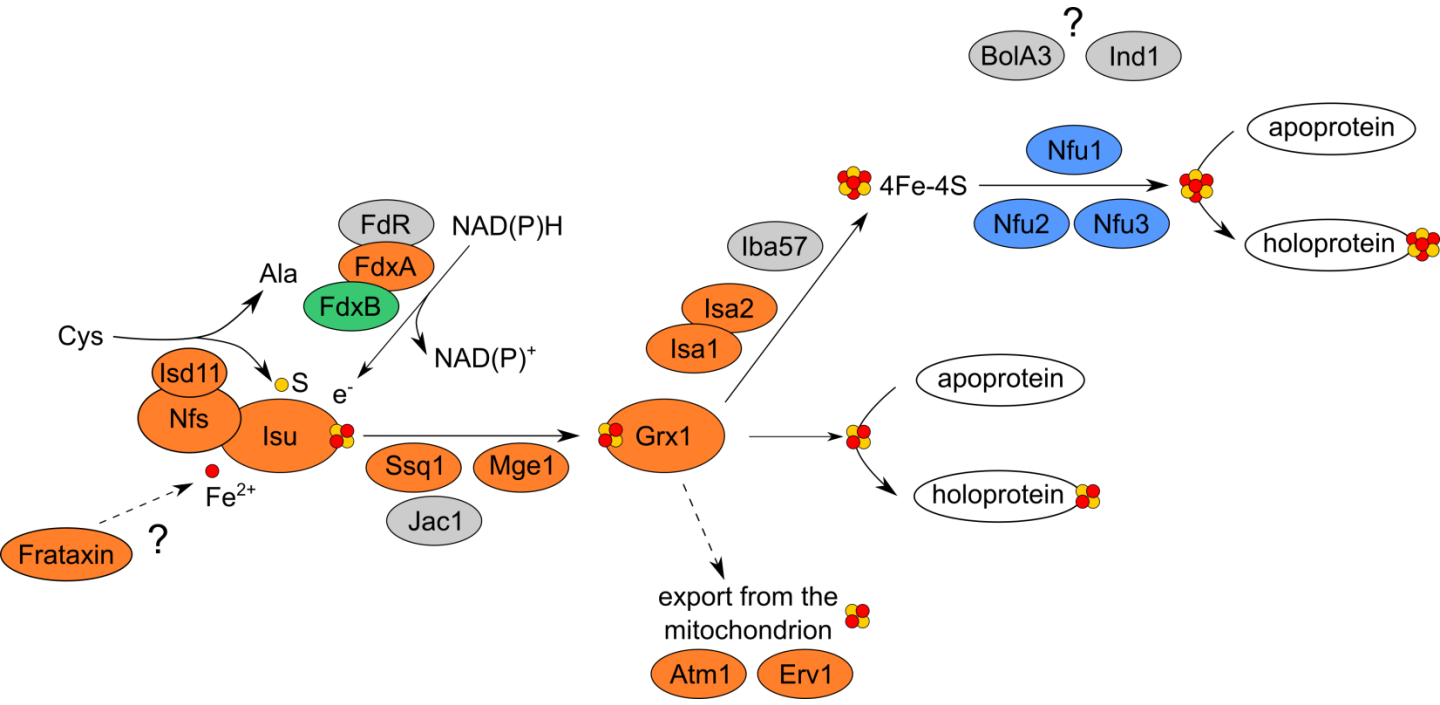
Western blot of N- and C-terminally PTP-tagged *Tb*Nfu2. The protein was visualized with an antibody against the PTP tag (anti-protein A).

Supp. Fig.2: Loss of RNAi response in BSF and PCF *Tb*Nfu1 RNAi lines.

A) Western blot of PCF *Tb*Nfu1 RNAi cell line also bearing a PTP-tagged allele. Cells were induced for 10 days with tetracycline and protein samples prepared at the indicated time intervals. *Tb*Nfu1 was visualized with an antibody against the PTP tag (anti-protein A). B) Western blot of BSF *Tb*Nfu1 RNAi cell line. Cells were induced for 7 days with tetracycline and protein samples prepared every day. *Tb*Nfu1 was visualized with the *Tb*Nfu1 antibody.

Figure

Figure 1



Figure

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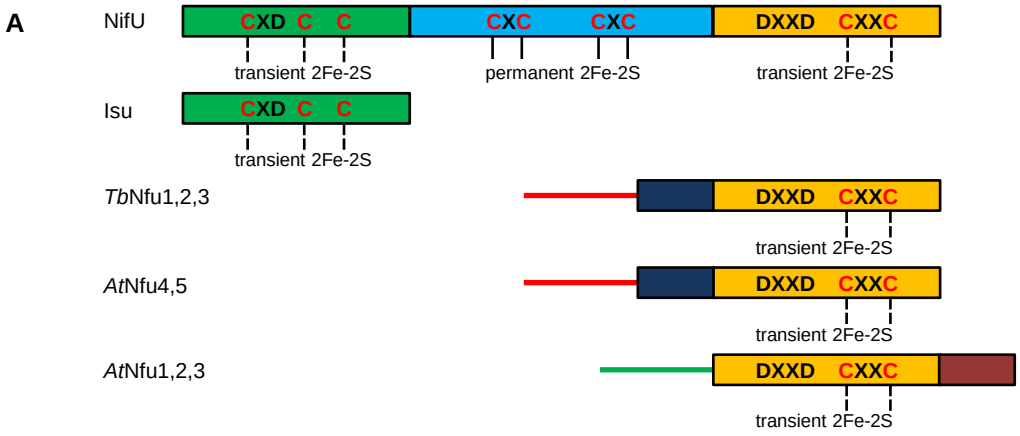

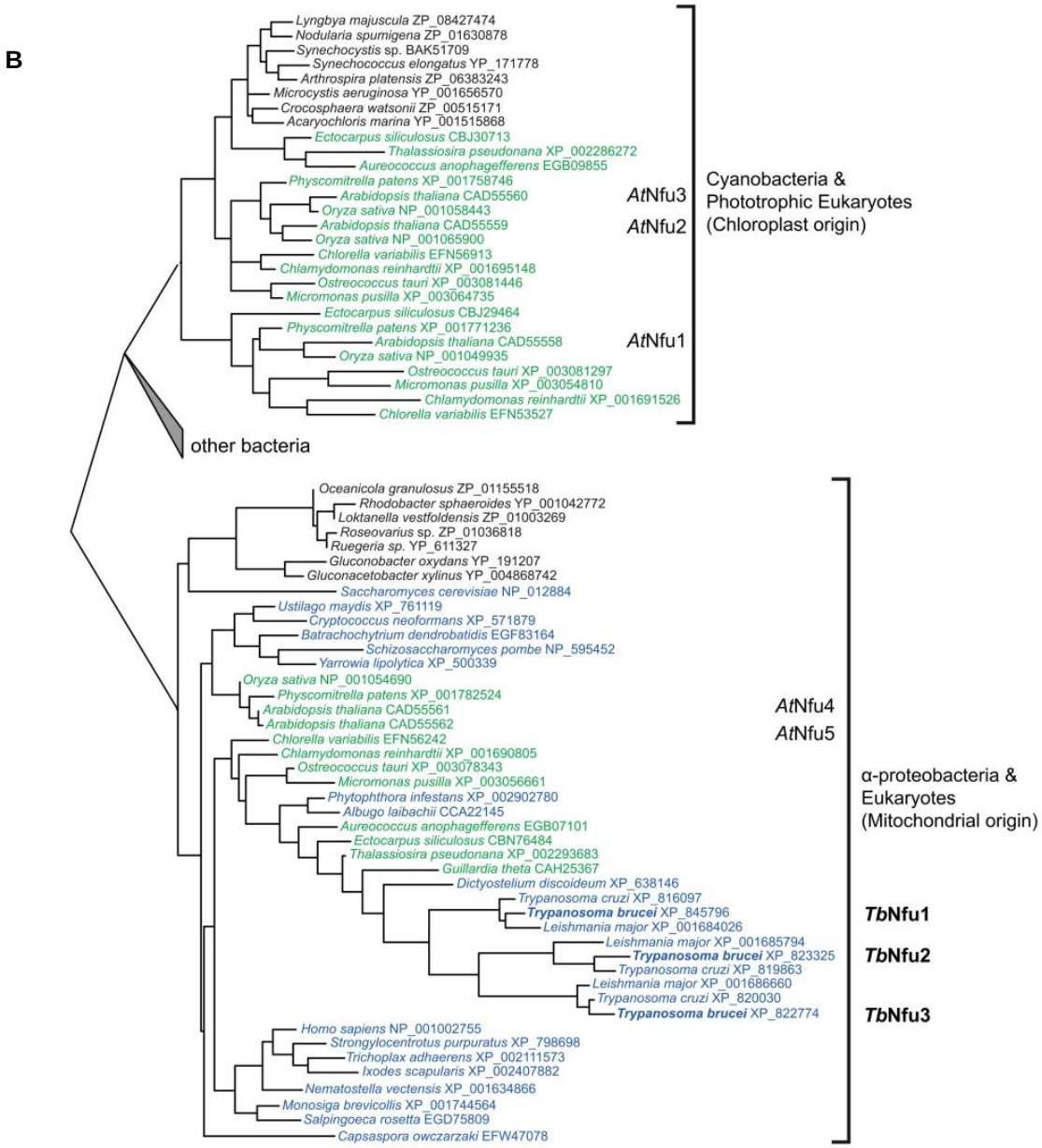

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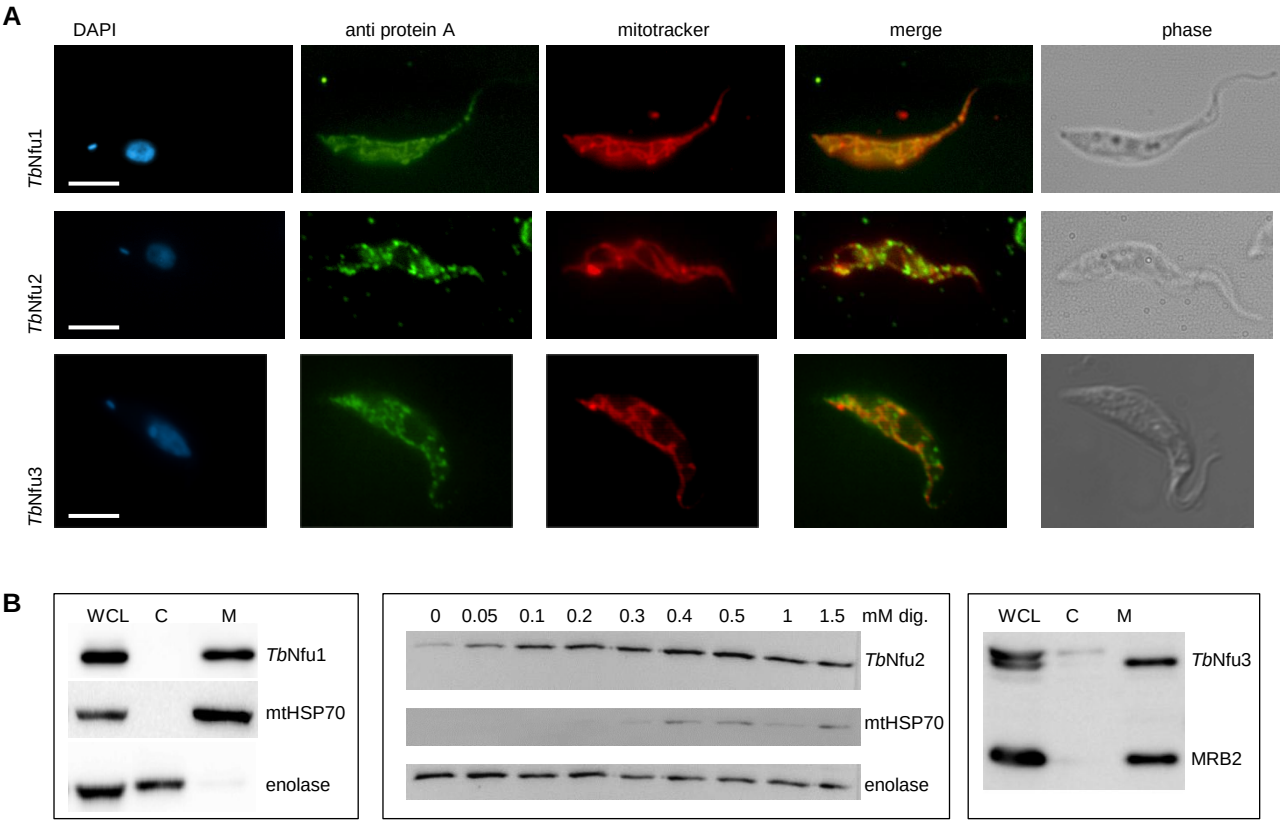

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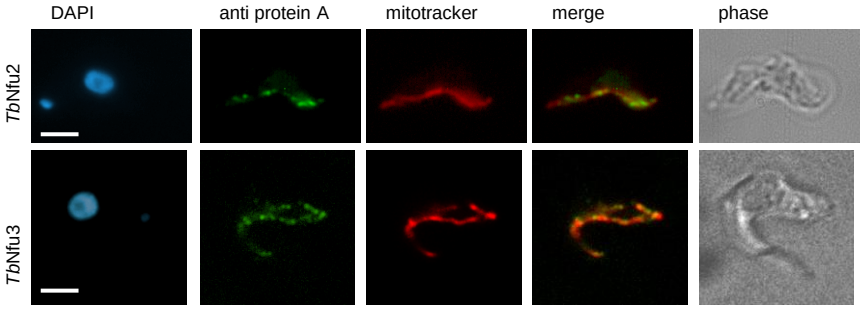

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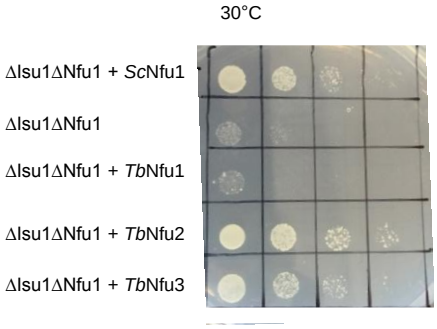

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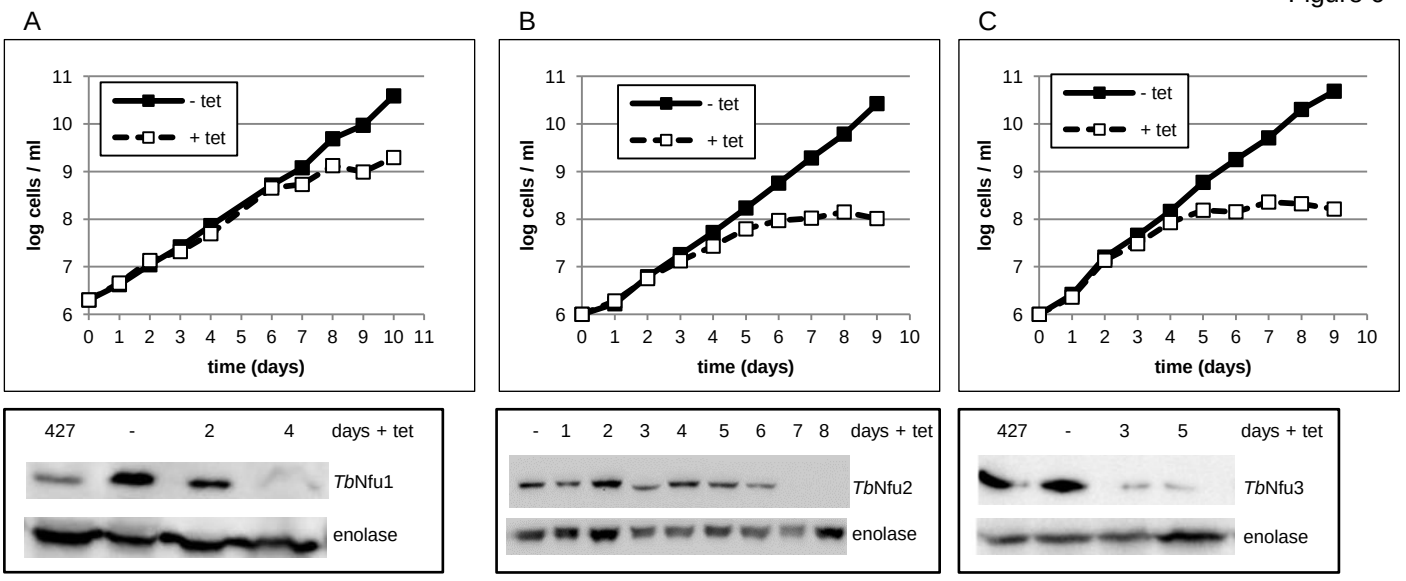

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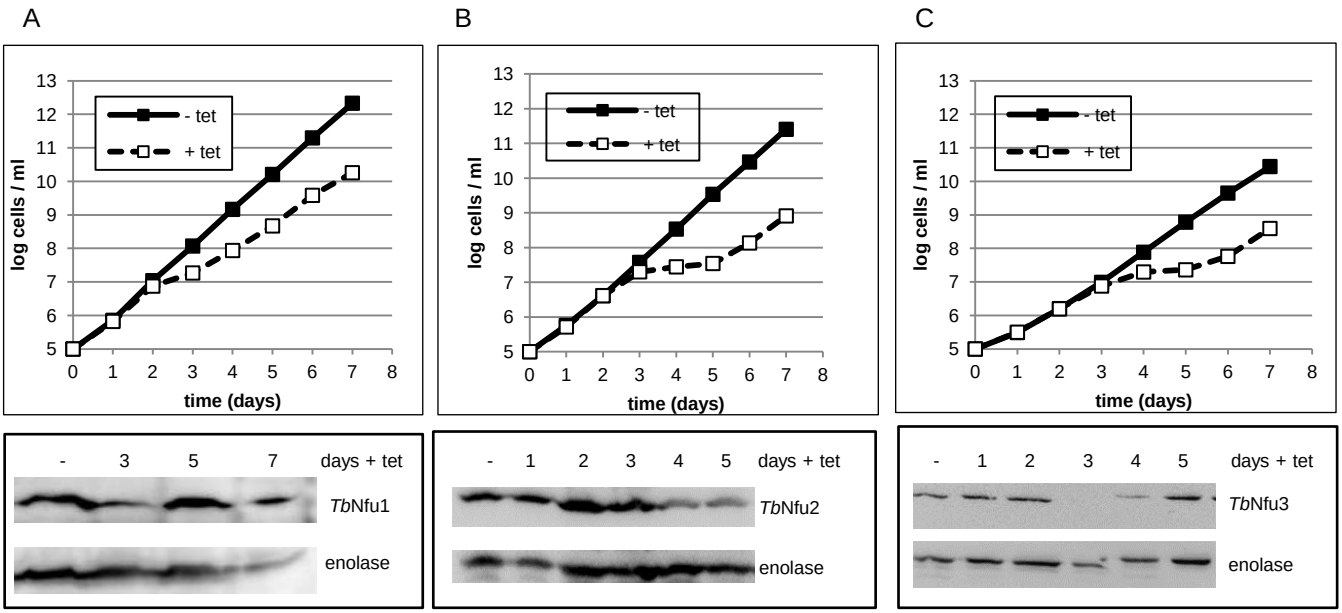
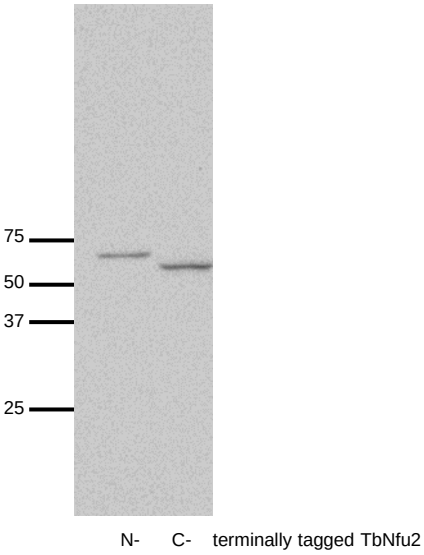


Figure

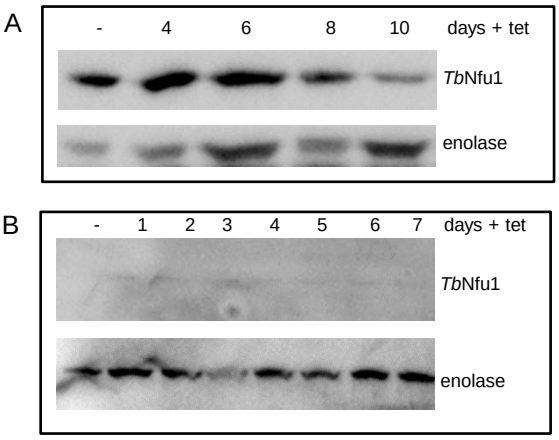
Supp. Table 1: Oligonucleotides used and plasmids generated in this study

Purpose	Sense oligo	Antisense oligo	Plasmid
RNAi against 3'UTR	CB104: GTCAGGATCCgctgacgacggtgcgtgtctg	CB105: GTACCTCGAGgttttcccgtaaccgactaaac	pCR49 (Nfu1 RNAi)
RNAi against Nfu1	Nfu1_F: CTCGAGATGATAAAGTTCACTCTGCGGTACTT	Nfu1_R: GGATCCGAGCAACTCCTTCACTGC	Nfu1 RNAi
Endogenous tagging – PTP(N)	CB51: ctaagcttTGATAAAGTTCACTCTGCGGTAC	CB52: tagatatcCCGTCTGCTCGAAGAAGCGG	pCR34 (PTP-Nfu1)
Endogenous tagging – PTP (C)	CB53: taggtaccGGGTTCCCTCGTACCGCAG	CB77: tcgcggccgcTTTGATTGTGAGGCGCTCAC	pCR35 (Nfu1-PTP)
yeast rescue	CB93: atggtaGGATCCCCTAACCCTGATTGCCTTCGG	CB94: ATGGTAgaattcGTTTGATTGTGAGGCGCTCAC	Nfu1-rescue
Overexpression – 3xV5 (C)	CB106: GTCAaagcttATGATAAAGTTCACTCTGCGG	CB107: GTACggatccGTTTGATTGTGAGGCGCTCAC	pCR43 (Nfu1-V5)
RNAi against Nfu2	Nfu2_F: CTCGAGATGGTGGAGGAAGTTAC	Nfu2_R: GGATCCCACCACCACTACCTCA	Nfu2 RNAi
Endogenous tagging – PTP (N)	CB76: gcaagcttTgcgggtcggttcctggttg	CB56: tagatatcagccgccgtgtcgtcatcg	pCR37 (PTP-Nfu2)
Endogenous tagging – PTP (C)	CB79: caGGGCCCgattttgtgacggtgcgccg	CB80: tagcggccgctccttgccttgccgttgc	pCR38 (Nfu2-PTP)
yeast rescue	CB95: atggtaGGATCCcgggtcggttcctggttgttt	CB96: ATGGTAgaattcgtccttgccttgccgttgc	Nfu2-rescue
RNAi against Nfu3	Nfu3_F: CTCGAGATGCTACGTGGCACACG	Nfu3_R: GGATCCGAAGTTAAGGAGTTTCACGTC	Nfu3 RNAi
Endogenous tagging – PTP(N)	CB59: ctaagcttTGCTACGTGGCACACGGC	CB60: tagatatcCCTCTGAGTCATCCTCGTGG	pCR39
Endogenous tagging – PTP (C)	CB61: taggtaccCGGTGGTGACGTGAAACTCC	CB78: atgcggccgcTCATCCCCATCGGGCTCA	pCR36
yeast rescue	CB97: ATGGTAggatccCTACGTGGCACACGGCTCATG	CB98: ATGGTAgaatTCCTCATCCCCATCGGGCTC	Nfu3-rescue

Supp. Fig.1



Supp. Fig.2


A
- 4 6 8 10 days + tet
TbNfu1
enolase
B
- 1 2 3 4 5 6 7 days + tet
TbNfu1
enolase