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Title: 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 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.

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

Reviewers' comments:

Reviewer #1: This submission presents expression, localization, and functional data on the three phylogenetically related TbNfu proteins of *Trypanosoma brucei*. These TbNfu 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 TbNfu 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 Δ TbNfu1 lesion in *Saccharomyces 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 changed. This reviewer did not understand why the three Nfus 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 *TbNfu1* 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 *TbNfu2* 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 *TbNfu1* 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 *TbNfu3*. 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 *TbNfu3*-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 *TbNfus* 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 *TbNfu1* accordingly and also added a sentence regarding the removal of the mitochondrial targeting sequence from this protein only in the results section.

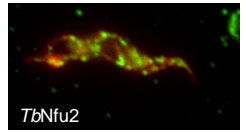
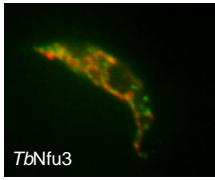
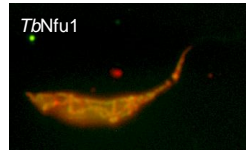
- 8) The authors should present at least one hypothesis in the Discussion to explain the unprecedented diversity of ISC targeting proteins in trypanosomes. Why have trypanosomes done this? While it is clear that the answer is unknown at present, the question deserves emphasis and at least an attempt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

1 **Roles of the Nfu Fe-S targeting factors in the trypanosome**

2 **mitochondrion**

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43 **Abstract**

44 Iron-sulfur clusters (ISCs) are protein co-factors essential for a wide range of cellular
45 functions. The core ISC assembly machinery resides in the mitochondrion, yet due to
46 export of an essential precursor from the organelle, it is also needed for cytosolic and
47 nuclear ISC assembly. In mitochondria all [4Fe-4S] ISCs are synthesized and
48 transferred to specific apoproteins by so-called ISC targeting factors. One of these
49 factors is the universally present mitochondrial Nfu1, which in humans is required for
50 the proper assembly of a subset of mitochondrial [4Fe-4S] proteins. Although most
51 eukaryotes harbor a single Nfu1, the genomes of *Trypanosoma brucei* and related
52 flagellates encode three Nfu genes. All three Nfu proteins localize to the mitochondrion
53 in the procyclic form of *T. brucei*, and *TbNfu2* and *TbNfu3* are both individually essential
54 for growth in bloodstream and procyclic forms, suggesting highly specific functions for
55 each of these proteins in the trypanosome cell. Moreover, these two proteins are
56 functional in the ISC assembly in a heterologous system and rescue the growth defect
57 of a yeast deletion mutant.

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

65 **1. Introduction**

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

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

86 These evolutionary ancient and highly important ISCs are cofactors of proteins involved
87 in a variety of cellular functions such as metabolic catalysis, DNA replication and repair,
88 translation and iron regulation, to name just the most prominent ones (Brzóška et al.,

89 2006). With almost 20 well conserved proteins participating in the mitochondrial stage
90 of ISC biogenesis, the process is rather complex and still not fully understood. We will
91 now briefly describe what is known about the mitochondrial steps of ISC biogenesis in
92 yeast and mammalian cells (using the yeast nomenclature for this well conserved
93 process) and compare this to the situation in trypanosomes.

94 Mitochondrial ISC assembly takes place on the Isu1/Isu2 scaffold (Isu2 having arisen
95 from a gene duplication event specific to yeast) with sulfur being provided by the
96 reduction of cysteine to alanine which is catalysed by the desulfurase complex Nfs1-
97 Isd11 (Mühlenhoff et al., 2003) (Fig. 1). The sulfur is reduced by a dedicated electron
98 transport chain constituted of ferredoxin and ferredoxin reductase, with the iron possibly
99 provided by a putative donor frataxin (Lill et al., 2012). The *T. brucei* scaffold protein Isu
100 and the desulfurase Nfs are both indispensable for PCF and their depletion negatively
101 impacts on aconitase activity (used as a readout for the synthesis of [4Fe-4S] clusters)
102 (Smíd et al., 2006). Moreover, Isu is also essential in BSF, and both Isu and Nfs
103 localize to the mitochondrion as well as to the nucleolus in both life stages, although the
104 role these proteins might play there remains a matter of speculation (Kovářová et al.,
105 2014). The desulfurase complex of *T. brucei* was also shown to be required for tRNA
106 thiolation in PCF, however, it remains to be resolved if this is due to direct involvement
107 of this complex in the process or indirectly by the provision of an ISC for another
108 component (Kovářová et al., 2014; Paris et al., 2010).

109 With the help of the Hsp70 chaperones Ssq1 and Jac1, the nascent [2Fe-2S] cluster is
110 temporarily transferred to the monothiol glutaredoxin Grx5, from which it can be directly
111 handed over to target [2Fe-2S] apoproteins (Uzarska et al., 2013). While the
112 trypanosome mtHsp70 is mostly involved in mitochondrial DNA maintenance, its
113 presence is also required for ISC synthesis (Týč et al., 2015). The mitochondrial

114 glutaredoxin Grx1 can bind a [2Fe-2S] cluster *in vitro* and plays an important role in iron
115 metabolism of the parasite (Comini et al., 2008). The Isa1, Isa2 and Iba57 proteins
116 participate in the conversion of [2Fe-2S] to [4Fe-4S] clusters, which are eventually
117 transferred to distinct apoproteins with the help of ISC targeting factors such as Nfu1,
118 BoIA3 or Ind1 (Lill et al., 2012). Putative homologs of all these proteins are present in *T.*
119 *brucei* but, with the exception of the Isa1 and Isa2 proteins (Long et al., 2011), have
120 thus far not been experimentally studied (Lukeš and Basu, 2015). The mitochondrial
121 ISC biogenesis machinery is also essential for the synthesis of cytosolic and nuclear
122 Fe-S proteins, since it depends on the export of a still unknown sulfur-containing
123 compound to the cytosol. The so-called CIA (for cytosolic ISC assembly) pathway is
124 outside the scope of this research and will hence not be discussed here.

125 There is still a certain lack of knowledge about how discrete subsets of Fe-S cluster
126 apoproteins are recognised by specific targeting factors such as Nfu1. This protein
127 shows homology to the C-terminal domain of NifU, which is a scaffold in ISC biogenesis
128 in nitrogen-fixing bacteria (Fig. 2A) (Smith et al., 2005). In humans and yeast, Nfu1 is
129 responsible for the transfer of [4Fe-4S] clusters to a small subset of mitochondrial
130 proteins, which include components of respiratory complexes I and II and lipoic acid
131 synthase (LipA) (Cameron et al., 2011; Navarro-Sastre et al., 2011). What makes Nfu1
132 particularly compelling to study is its involvement in human disease. Point mutations in
133 or deficiencies of the protein cause a fatal mitochondrial disease called Multiple
134 mitochondrial dysfunction sndrome with functional Nfu1 deficiency (MMDS1), which is
135 characterized by symptoms such as lactic acidosis, hyperglycinemia, and reduced
136 activities of respiratory chain complexes I and II (Cameron et al., 2011; Navarro-Sastre
137 et al., 2011). Somewhat surprisingly, given the severity of the human phenotype,
138 depletion or deletion of Nfu1 from HeLa cells (Navarro-Sastre et al., 2011) and yeast

139 *Saccharomyces cerevisiae* (Schilke et al., 1999) respectively, causes only a very mild
140 growth phenotype in culture. However, a specific impact on several enzymatic activities
141 has been detected. The levels of lipoic acid-bound enzymes (E2 subunits of pyruvate
142 dehydrogenase [PDH], alpha-ketoglutarate dehydrogenase [α -KGDH] and the H protein
143 of the glycine cleavage system [GCS]), as well as the amount and activity of complex II
144 (succinate dehydrogenase [SDH]) were decreased in HeLa cells (Navarro-Sastre et al.,
145 2011), while a significant depletion of SDH activity was also described in yeast (Schilke
146 et al., 1999).

147 Unlike the situation in the benchmark eukaryotes yeast and man, the genomes of plants
148 including *Arabidopsis thaliana* contain five genes with similarity to the C-terminus of
149 NifU. Two of these Nfu homologs (*AtNfu4* and *AtNfu5*) have such a NifU domain at the
150 C-terminus, as well as a N-terminal Nfu1-like region, thus closely matching the domain
151 organisation of typical mitochondrial Nfu1 proteins from other eukaryotes, while the
152 other three proteins (*AtNfu1*, 2 and 3) contain well-defined predicted plastid targeting
153 sequences (Fig. 2A) (Léon et al., 2003). Most of these plant Nfu proteins were able to
154 complement a yeast deletion mutant when targeted to the mitochondrion. Localization
155 studies showed that *AtNfu1*, 2 and 3 are localized in the plastid, while *AtNfu4* was
156 shown to be confined to the mitochondrion, and *AtNfu5* was predicted to have the same
157 localization (Léon et al., 2003).

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

164 Trypanosomatid flagellates, represented in this study by the genetically tractable *T.*
165 *brucei*, belong to the eukaryotic supergroup Excavata and hence, the emergence of
166 multiple copies of Nfu in their genomes must have occurred independently of plants.
167 Here we show that at least two out of three *TbNfu* proteins localize to the mitochondrion
168 in both life stages of *T. brucei*. These two *TbNfu* proteins (*TbNfu2* and 3) can
169 functionally replace yeast Nfu1 when targeted to its mitochondrion suggesting a
170 conserved function. Moreover, the same two *TbNfu* proteins are essential for the BSF
171 and PCF life stages, suggesting that they are non-redundant and target specific
172 apoproteins for ISC transfer. This is especially intriguing for the BSF trypanosome,
173 where only a small subset of Fe-S containing mitochondrial proteins is expected to be
174 essential (Lukeš and Basu, 2015; Basu et al., 2016).

175

176 **2. Materials and methods**

177 *2.1 Cells and plasmids*

178 *T. brucei* BSF 427 cells, BSF single-marker cells and PCF 29:13 (Wirtz et al., 1999)
179 cells were cultured as described elsewhere (Changmai et al., 2013). For RNAi against
180 *TbNfu1* (Tb927.7.1720) a 573 bp region (nt 1 – 573) was PCR amplified using primers
181 Nfu1_F and Nfu1_R (see Supplementary Table 1 for all oligonucleotide sequences
182 used and plasmids generated in this study). For *TbNfu2* (Tb927.10.11160), a 480 bp
183 region (nt 1 – 480) was amplified using primers Nfu2_F and Nfu2_R, and for *TbNfu3*
184 (Tb927.10.5290), a 480 bp region (nt 1 – 480) was amplified using primers Nfu3_F and
185 Nfu3_R. All amplicons were cloned into p2T7-177 vector (Wickstead et al., 2002) to
186 create the *TbNfu1-3* RNAi plasmids. An additional plasmid for RNAi of *TbNfu1* was
187 generated by amplifying nt 45-395 of the gene's 3'UTR, followed by ligation to p2T7-

188 177 (generating plasmid pCR49). *NotI* was used to linearize all RNAi plasmids prior to
189 electroporation.

190 To create plasmids for endogenous tagging of *TbNfu1*, *TbNfu2* and *TbNfu3* at the N-
191 terminus with PTP, a region of the 5' end of the open reading frames (ORFs) was
192 inserted into the plasmid p2678 (Kelly et al., 2007). Plasmids generated were pCR34
193 (*TbNfu1*), pCR37 (*TbNfu2*) and pCR39 (*TbNfu3*) and enzymes used for linearization
194 were *BlnI* (*TbNfu1*), *XcmI* (*TbNfu2*) and *SphI* (*TbNfu3*). For C-terminal endogenous
195 tagging with the PTP tag, a fragment of the 3' end of the *TbNfu1-3* ORFs lacking the
196 stop codon was amplified from trypanosome genomic DNA and cloned into a derivative
197 of pC-PTP-Neo with the antibiotic resistance changed to puromycin (Schimanski et al.,
198 2005). Plasmids generated were pCR35 (*TbNfu1*), pCR38 (*TbNfu2*) and pCR36
199 (*TbNfu3*), while enzymes used for their linearization were *SalI* (*TbNfu1*), *BsgI* (*TbNfu2*)
200 and *NsiI* (*TbNfu3*), respectively. To create plasmids for tagged overexpression of
201 *TbNfu1* and *TbNfu3*, both full length ORFs were amplified from *T. brucei* genomic DNA
202 and cloned into pJH54 (C-terminal HA₃ tagging vector kindly provided by Christine
203 Clayton) and pT7V5 (adding a C-terminal V5 tag), respectively (Surve et al., 2012). All
204 plasmids were linearized with *NotI* prior to transfection.

205 Linearized plasmids were electroporated into PCF cells using the standard procedure
206 (Vondrušková et al., 2005). *TbNfu1-3* RNAi plasmids stably integrated in 29:13 cells
207 were selected by the addition of 5 µg/ml phleomycin. In all cases, RNAi was induced by
208 the addition of 1 µg/ml tetracycline to the medium and growth curves performed in
209 triplicate (a representative experiment is shown for each construct and life cycle stage).
210 PCF 427/29:13 cells transfected with pCRs 34, 37, 39 (PTP-*TbNfu1-3*) and pCRs 35,
211 38, 36 (*TbNfu1-3*-PTP) were all treated with 0.5 µg/ml puromycin. PCF 29:13 cells
212 transfected with constructs overexpressing *TbNfu1* and *TbNfu3* were selected with 5

213 $\mu\text{g/ml}$ phleomycin (pJH54-based construct) and $0.5 \mu\text{g/ml}$ puromycin (pT7V5-based
214 construct). BSF 427 and BSF single-marker cells were electroporated using the Amaxa
215 Nucleofector II electroporator and program X-001. BSF 427 cells were transfected with
216 pCR38 and BSF single-marker cells with the *TbNfu1-3* RNAi plasmids and pCRs 36-39.
217 Selection was with $0.2 \mu\text{g/ml}$ phleomycin (*TbNfu1-3* RNAi) and $0.2 \mu\text{g/ml}$ puromycin
218 (pCRs 36-39). BSF single-marker cells were also transfected with an overexpression
219 construct for *TbNfu1* (*TbNfu1-V5*, pCR43) and selected with $0.2 \mu\text{g/ml}$ puromycin.

220 *2.2 Bioinformatics*

221 The Nfu homologs were identified by BLAST search of the NCBI non-redundant protein
222 database and aligned using MAFFT (Kato et al., 2005). The alignment was manually
223 edited in BioEdit (Hall, 2011). The phylogenetic tree was constructed in PhyML 3.1
224 (Guindon et al., 2010) using the default settings and the robustness of individual
225 branches was evaluated by SH-like approximated likelihood ratio test and bootstrap
226 after 100 iterations.

227 *2.3 Antibody production*

228 Specific polyclonal antibodies were commercially produced for two of the *TbNfu*
229 proteins. For *TbNfu1*, two synthetic oligopeptides were used (CSGKSSQRSIVVEKNE
230 and RRKLKKDEVSASQS) corresponding to amino acids 52 – 67 and 266 - 279,
231 respectively, of the *T. brucei* Nfu1 protein. The polyclonal antibodies were raised in a
232 rabbit over 87 days, and subsequently affinity purified by Eurogentec. Similarly,
233 oligopeptide SSTYDNFIPDGQTC, corresponding to amino acids 30 – 43, was used for
234 production of the anti-*TbNfu3* antibody by GenScript.

235 *2.4 Immunofluorescence*

236 Following staining with mitotracker red (Sigma-Aldrich), BSF 427, BSF single-marker
237 cells and PCF 29:13 cells were fixed for 30 min in either 2.3% (w/v) paraformaldehyde
238 at room temperature or in methanol at -20°C. Following permeabilization
239 (paraformaldehyde fixation only) in phosphate buffered saline (PBS) with 0.1% (v/v)
240 Triton-X100 or overnight in methanol at -20°C, slides were incubated with the primary
241 antibody, either anti-protein A (1:5,000, Sigma-Aldrich) or anti-V5 (1:500, Invitrogen) in
242 PBS for 1 hr. Following two washes with PBS, the slides were incubated with the
243 secondary antibody, either AlexaFluor488 goat anti-rabbit (1:200, Molecular Probes) or
244 AlexaFluor488 goat anti-mouse (1:200, Molecular Probes) in PBS for 1 hr. The slides
245 were washed twice in PBS, then 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Fisher
246 Scientific) was added for 5 min. After two more PBS washes, the cells were examined
247 under an Axioscope II fluorescent microscope.

248 *2.5 Western blot analysis*

249 Cell lysates corresponding to 2×10^6 cells were loaded into each lane and separated by
250 SDS-PAGE. The proteins were transferred to a nitrocellulose or a PVDF membrane
251 (previously activated for 5 min in methanol) by wet transfer. The membrane was
252 blocked for 1 hr at room temperature with blocking buffer (5% (w/v) milk powder in
253 PBS). The primary antibody (anti-protein A 1:20,000, anti-*TbNfu1*, or anti-*TbNfu3*
254 1:1,000) was diluted to the appropriate concentration and added to the membrane.
255 Incubation was overnight at 4°C. The membrane was washed 2 x 5 min with PBS and
256 then incubated with the secondary antibody (anti-rabbit HRP conjugate 1:2,000, Sigma-
257 Aldrich) in blocking buffer for 1 hr at room temperature. Finally, the membrane was
258 washed 2 x 5 min in PBS and antibodies were detected by enhanced
259 chemiluminescence (Clarity Western ECL Substrate, BioRad).

260 2.6 Digitonin fractionation

261 For digitonin fractionation, 10^7 cells per sample were collected, incubated in STE-NaCl
262 buffer (250 mM sucrose; 25 mM Tris-HCl, pH 7.4; 1 mM EDTA; 150 mM NaCl) with
263 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
264 temperature. Subsequently, samples were centrifuged (13,000 rpm for 2 min), and the
265 obtained supernatants were used for Western blot analysis. For detection of tagged
266 proteins, anti-protein A antibody (Sigma-Aldrich) was used at a 1:20,000 dilution. As
267 controls, antibodies against mitochondrial Hsp70 (1:1,000) (mitochondrial marker,
268 (Panigrahi et al., 2008)), and cytosolic enolase (1:10,000) (cytosolic marker) were used.

269 With the aim to separate their cytosolic and mitochondrial fractions, digitonin
270 fractionation of Nfu1-HA₃ and Nfu3-V5 expressing flagellates was performed as follows:
271 cells were harvested, washed twice with SHE buffer (250 mM sucrose, 25 mM HEPES,
272 1 mM EDTA) and an equivalent of 1 mg of cellular protein was resuspended in 200 μ l of
273 HBSS buffer (136.9 mM NaCl; 5.37 mM KCl; 0.81 mM MgSO₄; 1.26 mM CaCl₂; 0.44
274 mM KH₂PO₄; 0.33 mM Na₂HPO₄; 4.17 mM NaHCO₃; 5.55 mM glucose, pH 7.3) with the
275 addition of 80 μ g digitonin (Sigma-Aldrich). Samples were briefly vortexed and following
276 incubation for 5 min at room temperature centrifuged (13,000 rpm, 2 min). Supernatant
277 was collected as the cytosolic fraction and pellet was washed with HBSS buffer and
278 then resuspended in 200 μ l of the same buffer supplemented with 0.1 % Triton X-100.
279 Following incubation on ice for 5 min, samples were centrifuged (13,000 rpm, 2 min)
280 and the obtained supernatant was collected as the mitochondrial fraction. Anti-HA and
281 anti-V5 antibodies (Invitrogen) were used in dilution 1:2,000 and anti-MRP2
282 (Vondrušková et al., 2005) at 1:2,000.

283 2.7 Yeast complementation assay

284 A Δ I_{su1} yeast W303 strain described elsewhere (Gerber et al., 2004) was further
285 modified by a second deletion of Nfu1. This was introduced by PCR amplification of the
286 nourseothricin resistance gene using flanking primers, which contained fragments
287 corresponding to the 50 nucleotides down- and upstream of the *S. cerevisiae NFU1*
288 gene, and which was transformed into the Δ I_{su1} strain as described elsewhere (Janke
289 et al., 2004). Successful homologous recombinants in which the *NFU1* gene was
290 replaced by the nourseothricin cassette were selected by growth with 100 μ g/ml
291 nourseothricin and subsequent PCR-analysis of chromosomal DNA of restreaked
292 clones. Full length ORFs (*TbNfu2* and *3*) and the ORF lacking the predicted
293 mitochondrial targeting sequence (*TbNfu1*) were amplified from *T. brucei* genomic DNA
294 and cloned into the so-called yeast mitocyto expression vector (p426-TDH-F1 β -HiPIP-
295 Myc) (Mühlenhoff et al., 2011) from which the HiPIP-encoding gene was replaced with
296 the *TbNfus*. All *TbNfu* containing vectors as well as a positive control vector (encoding
297 yeast Nfu1, (Navarro-Sastre et al., 2011)) were transformed into the Δ I_{su1} Δ Nfu1 strain
298 (for description, see above) and the expression of C-terminally myc-tagged *TbNfu*
299 proteins was verified by Western blot analysis. A similar sized colony was picked from
300 each clone as well as from the parental strain and re-suspended in 100 μ l of distilled
301 water. Serial 1:10 dilutions were made and about 10 μ l of each dilution was spotted
302 onto two identical SC-Galactose plates. One set of plates was incubated for 3-4 days at
303 the permissive temperature of 30 °C and the other one at the restrictive temperature of
304 34 °C.

305

306 **3. Results**

307 *3.1 Bioinformatics*

308 All three trypanosome Nfu proteins are small (around 30 kDa) and have the typical
309 domain architecture of this family with a conserved cysteine motif (CxxC) at their C-
310 termini, thus theoretically enabling them to bind a Fe-S cluster (Fig. 2A). *TbNfu1* and
311 *TbNfu2* sequences share 24.5% identity (38.7% similarity), *TbNfu1* and *TbNfu3* share
312 24.5% identity (41.7% similarity), with *TbNfu2* and *TbNfu3* being least homologous
313 (19.5% identical, 31.2% similar) according to the EMBOSS stretcher algorithm (Rice et
314 al., 2000). Phylogenetic analysis of the Nfu proteins sampled from across all eukaryotic
315 super-domains shows that there are two eukaryotic lineages of Nfu. The first lineage is
316 present across all eukaryotes and branches along with alpha-proteobacteria, indicative
317 of its mitochondrial origin. Phototrophic eukaryotes also acquired a Nfu gene with a
318 different lineage, which groups with cyanobacteria and has very likely a plastidial origin
319 (Fig. 2B). In all trypanosomatids sequenced so far, the mitochondrial Nfu protein
320 underwent a triplication (Fig. 2B), suggesting that this multiplication event occurred
321 already in the common ancestor. Indeed, the trypanosomatid Nfu group clusters with
322 mitochondrial Nfu proteins from other eukaryotes including *A. thaliana* Nfu4 and 5, while
323 the plastid Nfu proteins from this plant species are more distantly related (Fig. 2B).

324 *TbNfu1* (Tb927.7.1720) encodes a protein with a molecular mass of 31.1 kDa including
325 the predicted mitochondrial targeting sequence. Cleavage at amino acid position 23
326 (probability 0.9880, MitoProt (Claros and Vincens, 1996)) would result in a mature
327 protein mass of 28.25 kDa. *TbNfu2* (Tb927.10.11160) has a predicted molecular mass
328 of 26.7 kDa following the original annotation; however, this short version of the protein
329 lacks the N-terminal Nfu1-like domain. Analysis of the *trans*-splice sites suggests an
330 upstream start codon (Parsons et al., 2015) which would result in a larger protein of
331 37.6 kDa. This longer version of *TbNfu2* is predicted to be mitochondrial with a cleaved
332 size of 31.78 kDa (probability 0.869, TargetP 1.1 (Emanuelsson et al., 2000)). C-

333 terminal, endogenous PTP tagging of the protein suggests that it is in fact the longer
334 version that is produced, since the observed size including the tag is around 52 kDa,
335 which is close to the expected size of 50.8 kDa. Tagging with PTP at the N-terminus
336 produces a slightly bigger protein of about 55 kDa consistent with a non-cleaved
337 species (Suppl. Fig. 1). Finally, *TbNfu3* (Tb927.10.5290) has a predicted molecular
338 mass of 29.5 kDa and is predicted to be mitochondrial with rather low confidence
339 scores (probability: 0.374, MitoProt; probability: 0.438, TargetP1.1 (Claros and Vincens,
340 1996; Emanuelsson et al., 2000)).

341 *3.2 Localization in PCF cells*

342 Cell lines carrying *TbNfu* proteins either tagged at their endogenous locus with the PTP
343 tag or overexpressed and containing either the HA₃- or V5-epitope tag were used for
344 localization studies. Both immunofluorescence analysis and digitonin fractionation
345 techniques were employed to corroborate results in PCF. All proteins were tagged at
346 their C-termini since two *TbNfu* proteins are predicted to have an N-terminal
347 mitochondrial targeting signal and all three belong to the mitochondrial lineage.
348 Importantly, PTP-tagging at the N-terminus at the endogenous locus apparently
349 masked the critical endogenous N-terminal mitochondrial targeting sequences since
350 diffuse cytoplasmic staining due to mistargeting was observed (data not shown).

351 *TbNfu1*-PTP detected with an antibody against protein A clearly localized to the
352 elaborate PCF mitochondrion where it was evenly distributed and completely
353 overlapped with mitotracker red staining (Fig. 3A, top row). This result was confirmed by
354 digitonin fractionation, where an overexpressed, HA-tagged *TbNfu1* protein was clearly
355 mitochondrial (Fig. 3B, left panel). Finally, the same HA-tagged protein also localized to
356 the mitochondrion by immunofluorescence analysis (data not shown).

357 *TbNfu2*-PTP detected with an antibody against protein A also localized to the
358 mitochondrion, although the distribution of the tagged protein was more punctate than
359 that of its tagged *TbNfu1* counterpart (Fig. 3A, middle row). Digitonin fractionation of the
360 same cell line using increasing concentrations of digitonin showed a higher amount of
361 the protein in fractions with increased concentration of the detergent, corresponding to
362 the mitochondrial compartment, while some of it was also present in other fractions
363 (Fig. 3B, middle panel). This suggests that an - albeit small - amount of the *TbNfu2*
364 protein may also be cytosolic. Another possible explanation of this observation would
365 be the presence of *TbNfu2* processing intermediates in the cytosol.

366 The same approach was used to localize the third *TbNfu* homologue, which was
367 endogenously PTP-tagged and detected with an antibody against protein A. Indeed, all
368 *TbNfu3* clearly localized to the reticulated mitochondrion where it showed a staining
369 pattern reminiscent of *TbNfu2*, with the obtained signal having a more focal distribution
370 in the organellar lumen (Fig. 3A, bottom row). Mitochondrial localization was further
371 confirmed by digitonin fractionation, where an overexpressed, V5-tagged protein was
372 also found exclusively in the organelle (Fig. 3B, right panel). This result was
373 corroborated when *TbNfu3*-V5 was localized to the mitochondrion by
374 immunofluorescence analysis in the same cell line (data not shown).

375 *3.3 Localization in BSF cells*

376 Localization of both endogenously tagged *TbNfu2*-PTP and *TbNfu3*-PTP detected with
377 an antibody against protein A in BSF cells was highly similar to PCF cells with the
378 proteins distributed unevenly throughout the mitochondrial lumen (Fig. 4). We have thus
379 far been unable to localize either tagged or endogenous versions of *TbNfu1* in the BSF
380 cells by immunofluorescence and currently have no explanation for this.

381 3.4 Functional complementation in yeast

382 A *S. cerevisiae* Δ Isu1 Δ Nfu1 strain was generated by introducing the Nfu1 deletion into
383 an existing Δ Isu1 strain ((Gerber et al., 2004) and data not shown). This strain shows
384 temperature-dependent slow growth, especially on non-fermentable carbon sources as
385 well as minor defects in aconitase and succinate dehydrogenase activities (Schilke et
386 al., 1999). In our hands the Δ Isu1 Δ Nfu1 strain already showed a severe phenotype at
387 30 °C when compared with the W303 parent strain. The ability of *TbNfu1-3* to rescue
388 this growth phenotype was assessed following transformation with plasmids separately
389 encoding each of these proteins. The N-terminal *Neurospora crassa* F1 β -presequence
390 was employed to guide the *TbNfu* proteins efficiently into yeast mitochondria. For
391 *TbNfu1* the predicted trypanosomal mitochondrial targeting sequence was excluded,
392 while full-length *TbNfu2* and *TbNfu3* were cloned into the yeast expression vector since
393 a putative presequence could not be predicted with as much confidence as for *TbNfu1*.
394 Different dilutions of the strains were spotted on SC-Gal plates and grown at 30 °C (Fig.
395 5). Following 3 to 4 days of incubation, *TbNfu2* and *TbNfu3* protein-expressing
396 transformants as well as a control strain expressing *S. cerevisiae* Nfu1 grew well, while
397 the parental Δ Isu1 Δ Nfu1 or *TbNfu1* expressing strain showed a growth defect (Fig. 5).
398 This experiment strongly suggests that at least two of the three *TbNfu* proteins are
399 functional scaffolds of ISC biosynthesis; moreover, they can operate out of context in a
400 heterologous system and complement the Δ Isu1 Δ Nfu1 strain.

401 3.5 RNAi in PCF cells

402 We employed the RNAi strategy to analyse the function of *T. brucei* Nfu proteins.
403 Results for *TbNfu1* were somewhat ambiguous with growth being affected very late
404 upon RNAi induction (from day 7 post-induction onwards, Fig. 6A). Moreover, the

405 phenotype was unstable. In order to confirm the efficiency of RNAi, a specific antibody
406 against a *TbNfu1*-derived oligopeptide was generated (see Materials and methods),
407 and an RNAi cell line also carrying a C-terminally PTP-tagged endogenous copy of
408 *TbNfu1* was prepared. Both approaches indicate that despite a marked decrease of the
409 *TbNfu1* protein level, the depletion did not trigger a stable phenotype (Fig. 6A; Suppl.
410 Fig. 2A). However, we suspect that our cell lines became refractory to RNAi over time
411 and also note a loss of phenotype after storage in the frozen state, both phenomena
412 that are commonly observed in *T. brucei*.

413 Depletion of *TbNfu2* in PCF started to show a growth phenotype between day 3 and 5
414 of RNAi induction. From day 6 post-induction onwards, cells grew extremely slowly after
415 which growth almost completely ceased (Fig. 6B). A PTP-tag was introduced into one
416 allele of *TbNfu2* in this RNAi cell line and depletion of the tagged protein was monitored
417 by Western blot analysis with anti-protein A antibodies which recognize the PTP-tag.
418 Consistent with the severe growth phenotype observed, depletion of the protein
419 became apparent on day 6 and the protein remained undetectable for the rest of the
420 induction time course (Fig. 6B). RNAi against *TbNfu3* in PCF produced a growth
421 phenotype similar to that of *TbNfu2* with growth slowing down around day 4 post RNAi-
422 induction and more or less arresting around day 5 (Fig. 6C). Efficient depletion of the
423 *TbNfu3* protein was confirmed by Western blot analysis (Fig. 6C) using a specific
424 polyclonal antibody developed against part of the endogenous *TbNfu3* protein (see
425 Materials and methods).

426 3.6 RNAi in BSF cells

427 A relatively mild growth defect was observed with BSF clones induced for *TbNfu1* RNAi
428 immediately after transfection (Fig. 7A); this could, however, not be reproduced in later

429 experiments with frozen cell lines. A Western blot produced at the same time as the
430 initial growth curve and probed for endogenous *TbNfu1* showed temporary and mild
431 depletion of the protein at day 3 post-induction (Fig. 7A). New cell lines were generated
432 targeting the *TbNfu1* 3'UTR to also enable rescue of the potential phenotype by
433 overexpression of the *TbNfu1* ORF from mRNA with a non-endogenous 3'UTR. RNAi of
434 cell lines with or without *TbNfu1*-V5 rescue gave a very similar result with no persistent
435 growth defect observed in either case (data not shown). Prolonged cultivation as
436 required for subsequent transfection and freezing might have abolished the growth
437 phenotype which was transient and not very stable. Western blots using anti-*TbNfu1*
438 antibody showed inefficient depletion of the protein in cell lines recovered from liquid
439 nitrogen, similar to the situation in PCF (Suppl. Fig. 2B).

440 Down-regulation of *TbNfu2* in the BSF cells caused a slower growth around day 3, with
441 cells escaping from RNAi at day 6. Western blot analysis using a cell line also carrying
442 an endogenously C-terminal PTP-tagged allele showed depletion of the protein after 4
443 and 5 days of induction (Fig. 7B). Targeted depletion of *TbNfu3* gave similar results with
444 BSF trypanosomes showing a growth phenotype at day 3 post RNAi-induction and
445 eventual escape from the RNAi response between days 5 and 6 (Fig. 7C). Western blot
446 analysis with specific antibodies showed efficient depletion of the endogenously tagged
447 protein at days 3 and 4 with the cells re-expressing *TbNfu3*-PTP upon escape from
448 RNAi (Fig. 7C). These RNAi revertants are frequently observed in *T. brucei*, especially
449 in the BSF cells, and are no cause for concern (Chen et al., 2003). Regardless of these
450 findings, our experiments clearly show that *TbNfu2* and *TbNfu3* are critical for fitness in
451 procyclic and bloodstream form parasites.

452

453 **4. Discussion**

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

467 Since the plant Nfu proteins show different localizations, we wondered if this was also
468 the case with the Nfu proteins in *T. brucei*. All three Nfu1 homologs were, however,
469 localized to the mitochondrion in PCF and, at least in the case of the latter two Nfu
470 proteins, also in the BSF flagellates. This is in agreement with the localization of the
471 single homologue in other non-plant eukaryotes (Schilke et al., 1999; Tong et al., 2003).
472 Even though repeated attempts to determine the subcellular localization of *TbNfu1* in
473 BSF were unsuccessful, its mitochondrial localization is highly anticipated due to the
474 presence in mitochondria in PCF, the easily discernible mitochondrial targeting
475 sequence, and the observation that so far all components of the ISC machinery had the
476 same localization in both trypanosome life stages (Changmai et al., 2013; Kovářová et
477 al., 2014; Paris et al., 2010).

478 Our results suggest that at least two (*TbNfu2* and *TbNfu3*) out of the three trypanosome
479 proteins are capable of transferring ISCs to target proteins in the yeast mitochondrion,
480 even though these might be very different from their endogenous targets in *T. brucei*.
481 This is in good agreement with rescue assays performed with the *A. thaliana* Nfu
482 proteins (Léon et al., 2003), suggesting that even though a divergence in function might
483 result in the creation of different isoforms of a protein in a given organism, these
484 different isoforms are still capable of fulfilling at least some of their evolutionary
485 ancestral functions.

486 The essentiality of a Nfu1 homolog is expected in PCF, especially bearing in mind that
487 the *TbNfu* proteins likely serve as specific targeting factors for the same enzymes as in
488 other organisms, namely complexes I and II and lipoic acid synthase (LipA). The Fe-S
489 co-factors are crucial for proper function of the respiratory chain, thus rendering their
490 transfer factors essential in any organism that depends on oxidative phosphorylation for
491 energy generation, such as the PCF of *T. brucei*. However, this fails to explain the
492 indispensability of the Nfu scaffold proteins in the BSF stage, where the known Nfu1
493 targets, just like most Fe-S proteins, are not likely to be essential (Lukeš and Basu,
494 2015; Surve et al., 2012; Tielens and Van Hellemond, 1998). Moreover, the amounts of
495 ferredoxin, glutaredoxin and lipoic acid, which might be affected by depletion of a
496 component of the [4Fe-4S] ISC biosynthesis machinery, are rather low in the BSF stage
497 and potentially not required for cell survival (Basu et al., 2016; Lukeš and Basu, 2015;
498 Stephens et al., 2007). Preliminary studies to assay aconitase activity and tRNA
499 thiolation in the *TbNfu* RNAi cells did not show any departure from the wild type
500 situation (data not shown). Measuring aconitase activity is a generic test, used for
501 assaying the functionality of the core ISC machinery. However, it is unlikely to be
502 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 *TbNfu* 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 (BoIA3-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

527 for special requirements of lineage-specific Fe-S cluster proteins. Since there are only
528 two intron-containing genes in *T. brucei* (Siegel et al., 2010), alternative splicing to
529 generate protein diversity as has been observed for human Nfu1 (Tong et al., 2003) is
530 not likely to occur in the parasite. We therefore hypothesize that contrary to higher
531 eukaryotes, *T. brucei* has instead evolved to produce multiple Nfu gene copies
532 dedicated to different target proteins.

533 Altogether, the *T. brucei* Nfu2 and Nfu3 proteins are *bona fide* ISC targeting factors, but
534 each of them has a specific essential function, possibly distinct from the Nfu functions
535 known from other organisms. *TbNfu1* might be different, since it does not appear to be
536 essential in either trypanosome life stage or rescue the yeast Δ Isu1 Δ Nfu1 mutant.
537 However, we cannot exclude that the depletion of *TbNfu1* by RNAi was simply
538 insufficient to cause a phenotype, and the protein might still be required for normal
539 trypanosome growth. As for the yeast assay, removal of the putative mitochondrial
540 targeting sequence might have caused inactivity of the protein and could explain the
541 failed rescue. Overall, our results concerning *TbNfu1* are rather inconclusive. Future
542 work to establish the exact roles played by the *TbNfu* proteins in the mitochondrion will
543 require further analysis of the mitochondrial Fe-S metalloproteome of *T. brucei*, which is
544 currently in progress.

545

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556

557 **Author contributions**

558

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

562

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726 **Figure legends**

727 Fig. 1: Scheme of the mitochondrial ISC assembly pathway in *T. brucei*. Components
728 essential in PCF are indicated in orange, dispensable ones are in green, Nfu proteins in
729 blue, and proteins present in the genome but not assayed so far in grey. *TbGrx1* is
730 homologous to yeast Grx5. The Nfs-Isd11 desulfurase complex (Paris et al., 2010)
731 provides sulfur on the Isu scaffold (Smíd et al., 2006), while ferredoxins A and B
732 facilitate its reduction (Changmai et al., 2013), and frataxin probably provides iron (Long
733 et al., 2008). Heat shock proteins (Týč et al., 2015) facilitate transfer of newly created
734 [2Fe-2S] on the Grx1 glutaredoxin (Comini et al., 2008). Isa1/2 and Iba 57 proteins
735 enable formation of [4Fe-4S] clusters (Long et al., 2011). A still unknown S-containing
736 component is exported into the cytosol via the inner membrane transporter Atm1
737 (Horáková et al., 2015), the sulfhydryl oxidase Erv1 of the intermembrane space and
738 glutathione (Basu et al., 2013), and utilized in the CIA pathway.

739

740 Fig. 2: Bioinformatic analysis of Nfu proteins in prokaryotes and eukaryotes/ Position of
741 the *TbNfu* proteins among related prokaryotic and eukaryotic proteins.

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

750 targeting sequences (the sequence is not well predicted in *TbNfu3*), and the green bar
751 represents a plastid targeting sequence from *A. thaliana*, respectively. Dark blue boxes
752 mark Nfu1-like domains in the *T. brucei* proteins and *AtNfu4* and 5, while the maroon
753 box shows a B domain specific for *AtNfu1-3*. B) Scheme of maximum-likelihood
754 phylogenetic tree of Nfu homologs. The prokaryotic taxa are in black, while eukaryotes
755 are highlighted in colors (green for chloroplast-containing photoautotrophs and blue for
756 heterotrophs). The bootstrap/SH-like aLRT branch supports are shown for the
757 cyanobacteria/chloroplasts clade and the alpha-proteobacteria/mitochondria clade.

758

759 Fig. 3: Localisation of *TbNfus* in PCF cells.

760 A) Immunofluorescence analysis of endogenously PTP-tagged *TbNfu* proteins. DAPI,
761 anti-protein A, mitotracker, a merge of protein A and mitotracker and a phase contrast
762 image of a representative cell are shown. Scale bar = 5µm. B) Subcellular fractionation
763 of cell lines expressing tagged *TbNfu* proteins. Fractionation into cytosolic and
764 mitochondrial fractions (*TbNfu1*-HA and *TbNfu3*-V5), and fractionation with increasing
765 concentrations of digitonin (*TbNfu2*-PTP) are shown.

766

767 Fig. 4: Localisation of *TbNfus* in BSF cells.

768 Immunofluorescence analysis of endogenously PTP-tagged *TbNfu* proteins. DAPI, anti-
769 protein A, mitotracker, a merge of protein A and mitotracker and a phase contrast
770 image of a representative cell are shown. Scale bar = 5µm.

771

772 Fig. 5: Complementation of Δ Isu1 Δ Nfu1 yeast strain.

773 Δ Isu1 Δ Nfu1 strain transfected or not with the indicated rescue plasmids encoding

774 *TbNfus* and *S. cerevisiae* Nfu1 incubated at 30 °C.

775

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

777 A) Cumulative growth of *TbNfu1* 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 *TbNfu1* protein detected with anti-

780 *TbNfu1* antibodies. B) Cumulative growth of *TbNfu2* 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 *TbNfu2* protein detected with anti-protein A. C) Cumulative growth of *TbNfu3*

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 *TbNfu3* protein detected with anti-*TbNfu3* antibodies. Cytosolic enolase

787 was used as a loading control in all three panels.

788

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

790 A) Cumulative growth of *TbNfu1* 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 *TbNfu1* protein detected with anti-*TbNfu1*. B) Cumulative

793 growth of *TbNfu2* RNAi cell line, in the presence (open squares, dashed line) or

794 absence (solid squares, unbroken line) of tetracycline (tet). Western blot shows

795 depletion of endogenously PTP-tagged *TbNfu2* detected with anti-protein A. C)
796 Cumulative growth of *TbNfu3* RNAi cell line, in the presence (open squares, dashed
797 line) or absence (solid squares, unbroken line) of tetracycline (tet). Western blot shows
798 depletion of endogenously PTP-tagged *TbNfu3* with anti-protein A. Cytosolic enolase
799 was used as a loading control in all three panels.

800

801 Supp. Table 1:

802 Oligonucleotides used and plasmids generated in this study

803 Fig. 1: Apparent molecular weight of *TbNfu2*

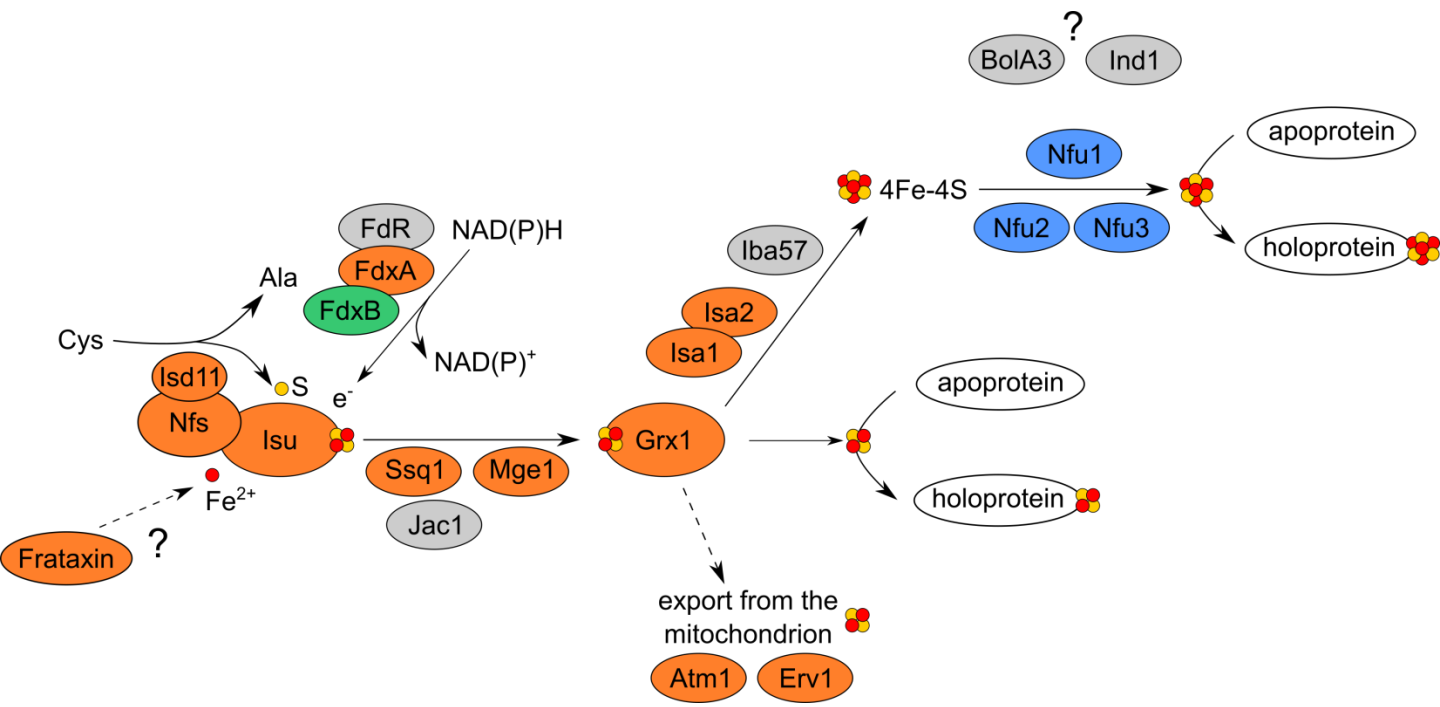
804 Western blot of N- and C-terminally PTP-tagged *TbNfu2*. The protein was visualized
805 with an antibody against the PTP tag (anti-protein A).

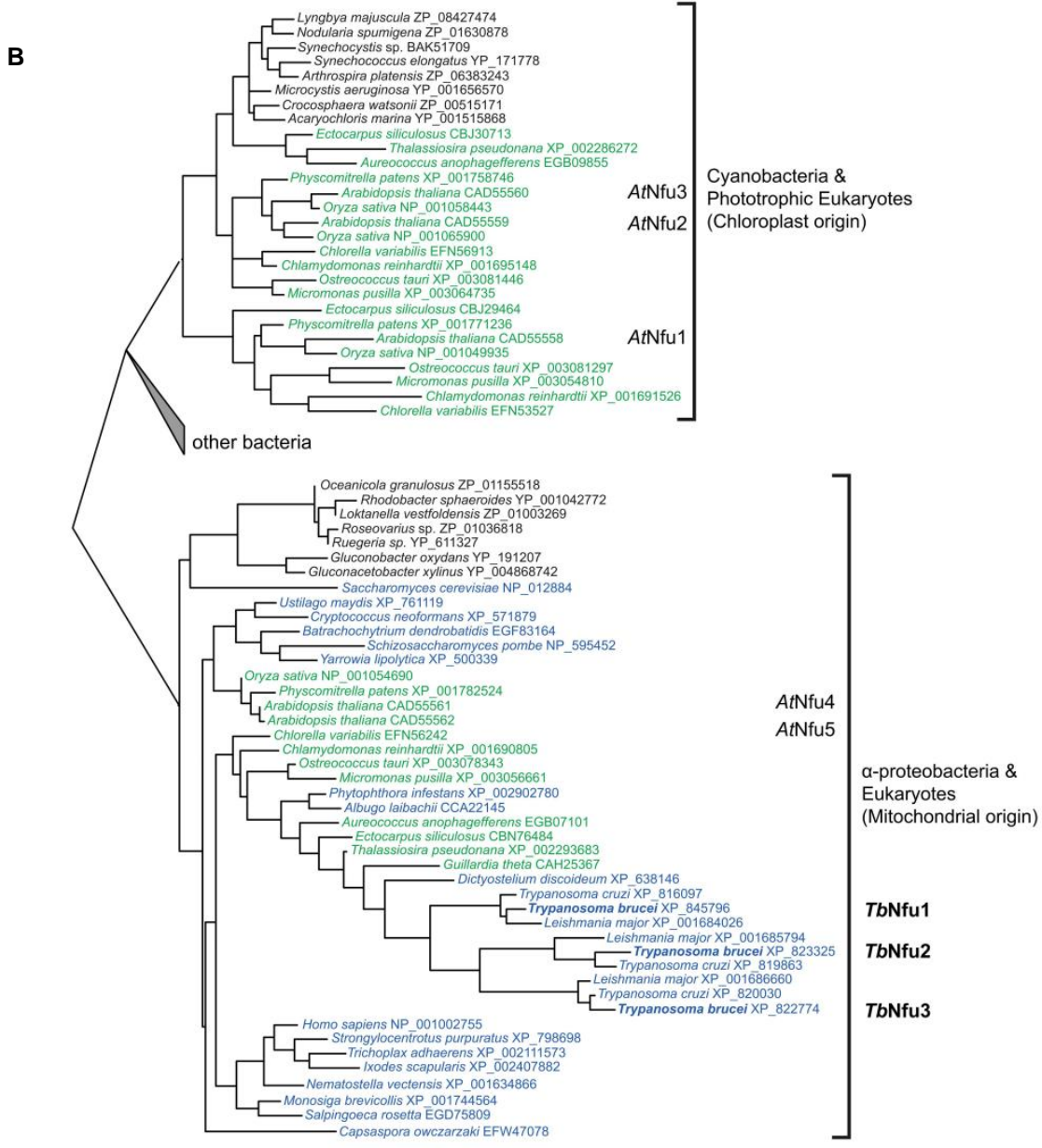
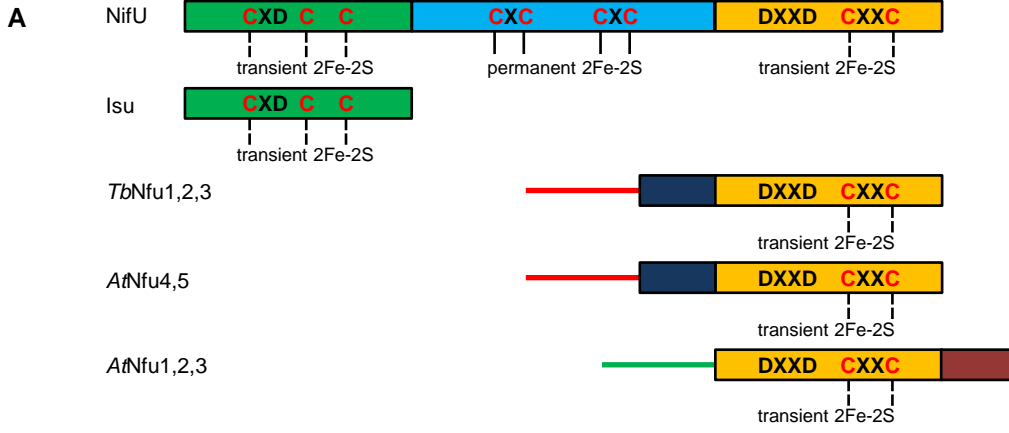
806 Supp. Fig.2: Loss of RNAi response in BSF and PCF *TbNfu1* RNAi lines.

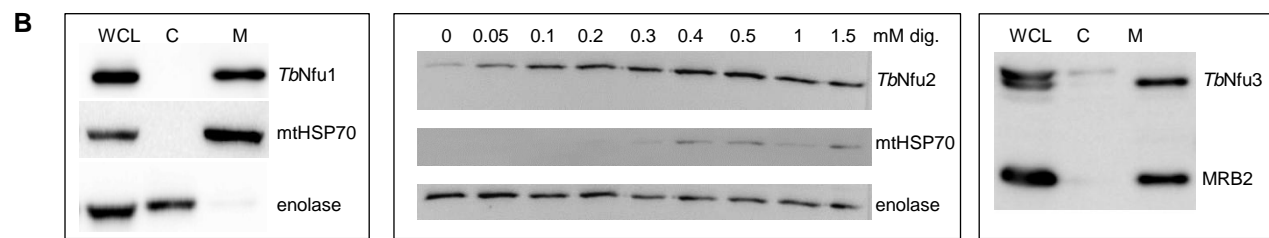
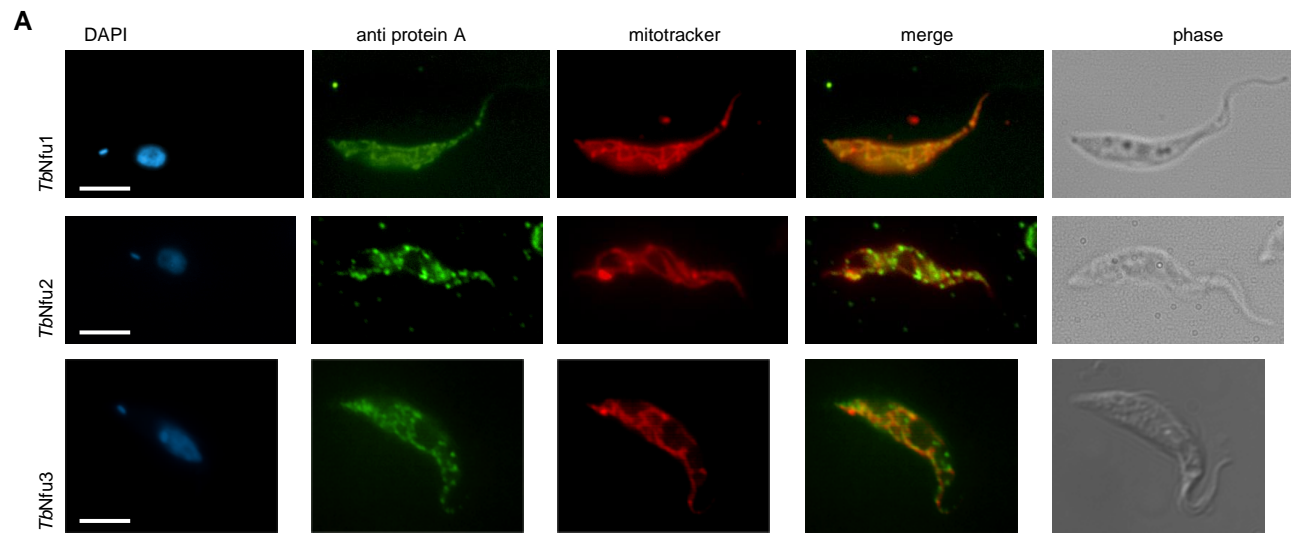
807 A) Western blot of PCF *TbNfu1* RNAi cell line also bearing a PTP-tagged allele. Cells
808 were induced for 10 days with tetracycline and protein samples prepared at the
809 indicated time intervals. *TbNfu1* was visualized with an antibody against the PTP tag
810 (anti-protein A). B) Western blot of BSF *TbNfu1* RNAi cell line. Cells were induced for 7
811 days with tetracycline and protein samples prepared every day. *TbNfu1* was visualized
812 with the *TbNfu1* antibody.

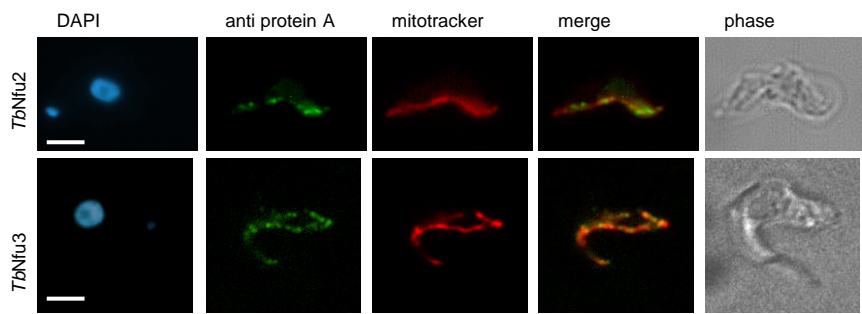
Figure

Figure 1









30°C

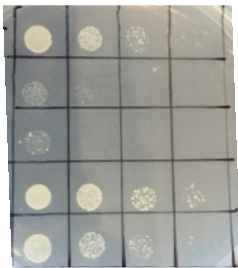
Δ Isu1 Δ Nfu1 + ScNfu1

Δ Isu1 Δ Nfu1

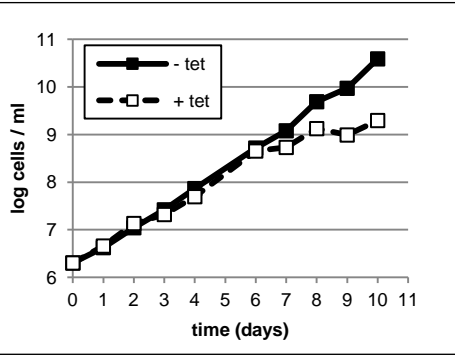
Δ Isu1 Δ Nfu1 + TbNfu1

Δ Isu1 Δ Nfu1 + TbNfu2

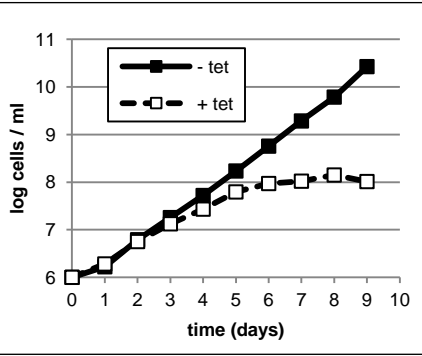
Δ Isu1 Δ Nfu1 + TbNfu3



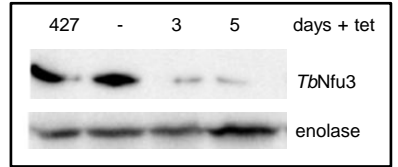
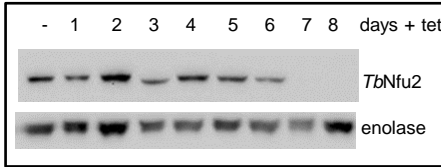
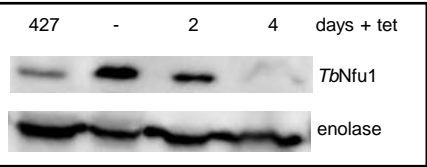
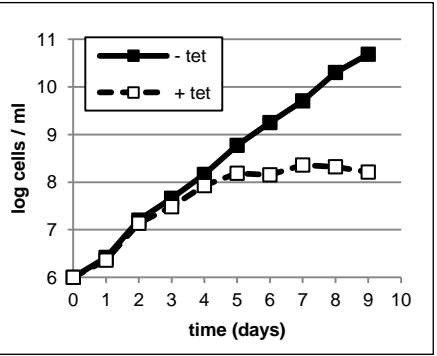
A



B



C



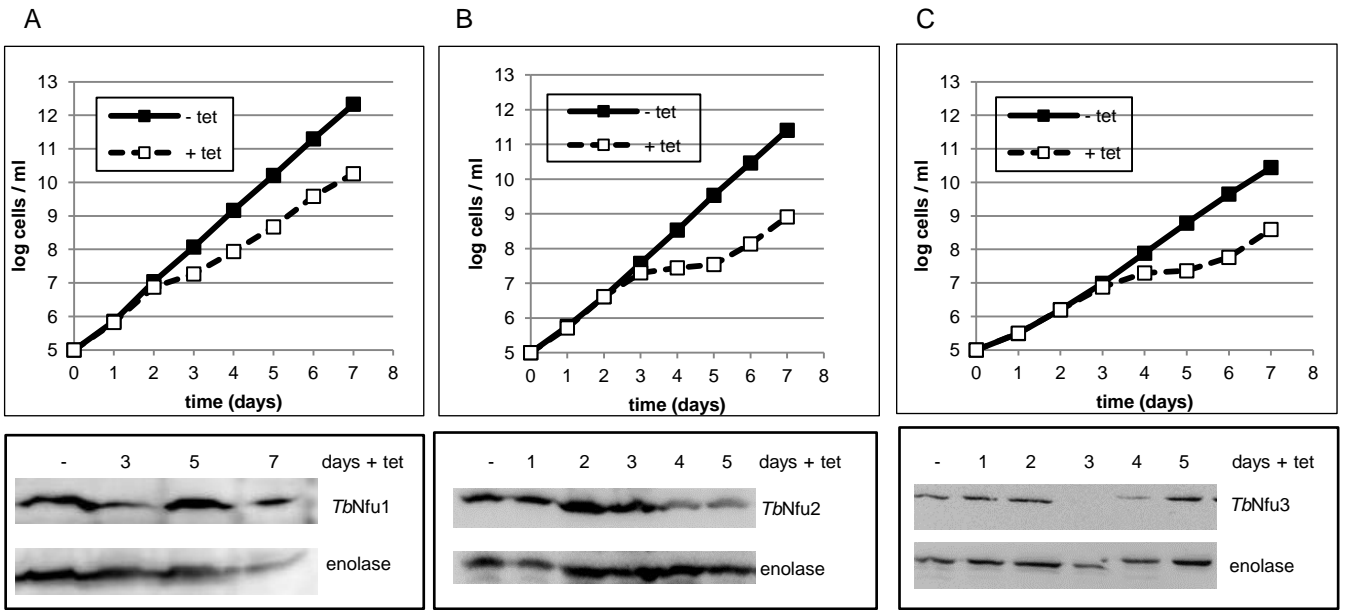
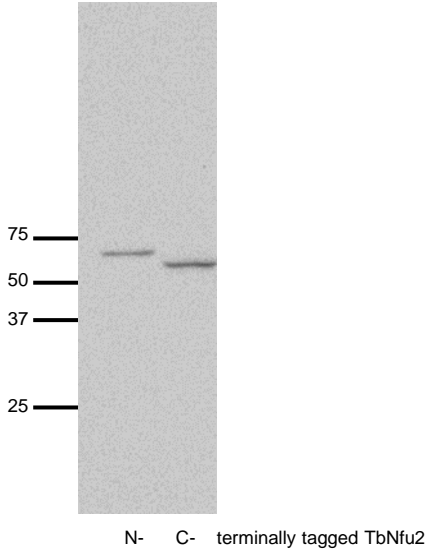


Figure
Supp. Table 1: Oligonucleotides used and plasmids generated in this study

Purpose	Sense oligo	Antisense oligo	Plasmid
RNAi against 3'UTR	CB104: GTCAGGATCCgctgacgacggtgctgtctg	CB105: GTACCTCGAGgttttcccgtaacccgactaac	pCR49 (Nfu1 RNAi)
RNAi against Nfu1	Nfu1_F: CTCGAGATGATAAAGTTCACCTCTGC GGTACTT	Nfu1_R: GGATCCGAGCAACTCCTTCACTG C	Nfu1 RNAi
Endogenous tagging – PTP(N)	CB51: ctaagcttTGATAAAGTTCACCTCTGCGG TAC	CB52: tagatatcCCGTCTGCTCGAAGAAGC GG	pCR34 (PTP- Nfu1)
Endogenous tagging – PTP (C)	CB53: taggtaccGGGTTCCCTCGTACCGCAG	CB77: tcgcgccgcTTTGATTGTGAGGCGC TCAC	pCR35 (Nfu1- PTP)
yeast rescue	CB93: atggtaGGATCCCCTAACCCCTGATTG CCTTCGG	CB94: ATGGTAgaattcGTTTGATTGTGAGG CGCTCAC	Nfu1- rescue
Overexpression – 3xV5 (C)	CB106: GTCAaagcttATGATAAAGTTCACCTCT GCGG	CB107: GTACggatccGTTTGATTGTGAGGC GCTCAC	pCR43 (Nfu1- V5)
RNAi against Nfu2	Nfu2_F: CTCGAGATGGTGGAGGAAGTTAC	Nfu2_R: GGATCCCACCACCACTACCTCA	Nfu2 RNAi
Endogenous tagging – PTP (N)	CB76: gcaagctTgcgggctcgttctcgtgtg	CB56: tagatatcagccgcccgtgctgcatcg	pCR37 (PTP- Nfu2)
Endogenous tagging – PTP (C)	CB79: caGGGCCCGattttgtgacgggtgcgccg	CB80: tagcggccgctccttgccctgcccgtg	pCR38 (Nfu2- PTP)
yeast rescue	CB95: atggtaGGATCCcgggtcgggtcctcgtgtgtt	CB96: ATGGTAgaattcgtccttgccctgcccgtg	Nfu2- rescue
RNAi against Nfu3	Nfu3_F: CTCGAGATGCTACGTGGCACACG	Nfu3_R: GGATCCGAAGTTAAGGAGTTTCA CGTC	Nfu3 RNAi
Endogenous tagging – PTP(N)	CB59: ctaagcttTGCTACGTGGCACACGGC	CB60: tagatatcCCTCTGAGTCATCCTCGT GG	pCR39
Endogenous tagging – PTP (C)	CB61: taggtaccCGGTGGTGACGTGAAACTC C	CB78: atcggccgcTCATCCCCATCGGGCT CA	pCR36
yeast rescue	CB97: ATGGTAagatccCTACGTGGCACACG GCTCATG	CB98: ATGGTAgaatTCCTCATCCCCATCG GGCTC	Nfu3- rescue

Supp. Fig.1



Supp. Fig.2

