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Fe/S protein biogenesis in trypanosomes — A review☆

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ABSTRACT

Trypanosoma brucei, the causative agent of the African sleeping sickness of humans, and other kinetoplastid flagellates belong to the eukarytotic supergroup Excavata. This early-branching model protist is known for a broad range of unique features. As it is amenable to most techniques of forward and reverse genetics, *T. brucei* was subject to several studies of its iron-sulfur (Fe/S) protein biogenesis and thus represents the best studied excavate eukaryote. Here we review what is known about the Fe/S protein biogenesis of *T. brucei*, and focus especially on the comparative and evolutionary interesting aspects. We also explore the connections between the well-known and quite conserved ISC and CIA machineries and the tRNA thiolation pathway. Moreover, the Fe/S cluster protein biogenesis is dissected in the procyclic stage of *T. brucei* which has an active mitochondrion, as well as in its pathogenic bloodstream stage with a metabolically repressed organelle. This article is part of a Special Issue entitled: Fe/S proteins: Analysis, structure, function, biogenesis and diseases.

1. Introduction

Trypanosomes and related parasites belong to the well-studied protist group Kinetoplastida, which is ranked into the eukaryotic supergroup Excavata. Kinetoplastids are comprised of free-living (e.g. *Bodo* spp.), commensalic (e.g. *Cryptobia* spp.) and parasitic flagellates (e.g. *Trypanosoma* spp., *Leishmania* spp.), which are equipped with one or two flagella, which can rarely be lost. Their unifying feature is a huge and complex mitochondrial (also termed kinetoplast) DNA, composed of thousands of circular molecules that gave the name to the whole group. For the purpose of this review we will deal only with flagellates belonging to the family Trypanosomatidae, an extremely speciose assembly of obligatory parasitic protists. Trypanosomatids belong to the most successful and widespread parasites, being able to infect virtually any insect and vertebrate on all the continents. The flag species are *Trypanosoma brucei* (Fig. 1), the causative agents of the African sleeping sickness of humans and nagana in cattle, *Trypanosoma cruzi*, which is responsible for the still widespread Chagas disease in South and Central America [1], and *Leishmania* spp. present (often endemic) in most tropical countries including southern Europe, where they causes leishmaniases, a wide range of clinically variable diseases [2]. Numerous other members of the genus *Trypanosoma* are known to cause diseases of veterinary importance, while the genus *Phytomonas* is responsible for several serious diseases of commercial plants [3].

While we will occasionally resort to the above species, by far main attention will be given to *T. brucei*. This is caused by one principal fact – this is the only trypanosomatid in which RNA interference (RNAi) is not only fully functional (with some exceptions, such as the restricted RNAi in *Leishmania braziliensis*), but also technically straightforward. Moreover, *T. brucei* is amenable to homologous recombination, protein tagging and overexpression, its genome has been fully sequenced already in 2005, and a range of selectable markers is available for its studies [4,5]. Thanks to these qualities, this trypanosome qualifies not only as a model kinetoplastid, but also as the best-studied member of the supergroup Excavata. In an already quite stable taxonomic system, all known eukaryotes fall into six to seven supergroups, out of which Excavata represent a highly supported and thus undisputed supergroup. Bringing together mostly parasitic protists, the Excavata are known to harbor a wide range of mitochondria and mitochondrion-derived organelles, which reflects the spectrum of environments they occupy, ranging from strictly anaerobic to fully aerobic ones [6,7]. Whether the earliest extant eukaryotes can be found within the Excavata is a hotly debated and unresolved question, but it is certain that they generally constitute long branches that branched off early from the eukaryotic tree. It is this long independent evolutionary history which is most likely responsible for a number of deviations from a prototypical eukaryotic cell that have been described in these protists.

Trypanosomes are therefore a particularly useful model species for comparative studies of key eukaryotic pathways and evaluations of the age and extent of a given evolutionary invention. Among main oddities of *T. brucei* belongs the baroquely complex RNA editing of mitochondrial transcripts, in which small guide RNA molecules along with dozens of specialized proteins specify the pattern of post-transcriptional uridine insertions and deletions [8], massive *trans-*

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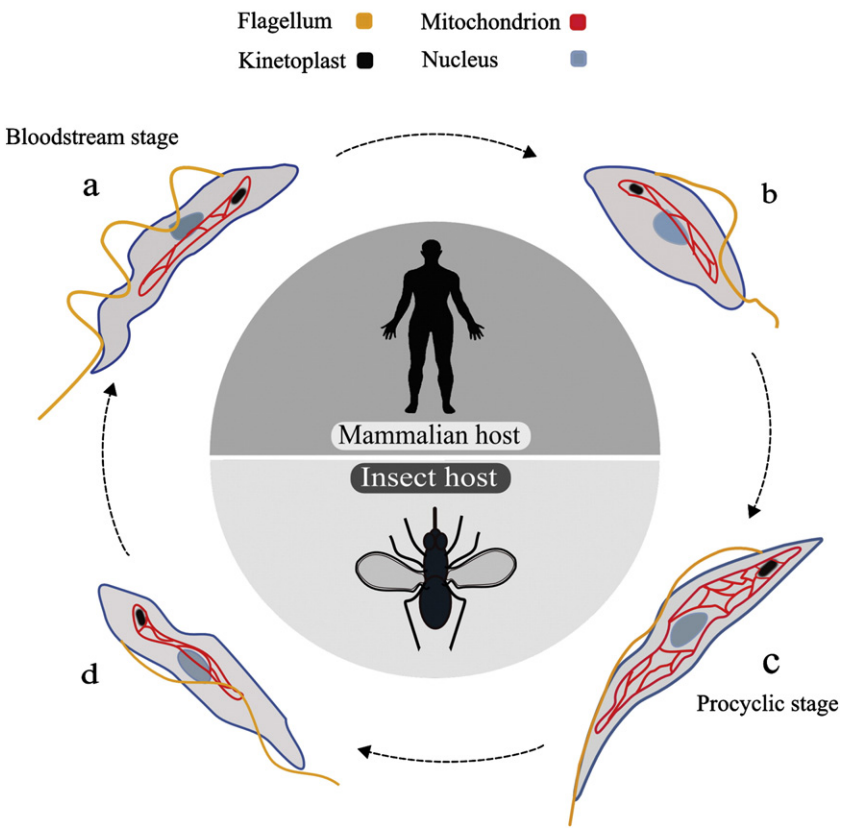

Fig. 1. Life cycle of *Trypanosoma brucei*. *T. brucei* undergoes a digenetic life cycle between a mammalian host (human, cattle etc.) and an insect vector (tse-tse fly *Glossina* spp.). This simplified life cycle depicts the proliferative bloodstream stage (BS) (a), which resides in the mammalian bloodstream. The subsequent stage is the non-dividing bloodstream short stumpy form (b), which is eventually transmitted to the vector during the bloodmeal. Successively, inside the tse-tse fly gut it transforms into the proliferative insect procyclic stage (PS) (c). Next, it is converted into the non-dividing metacyclic form in the salivary glands of the vector (d). While taking a successive bloodmeal, the vector transmits the metacyclic trypanosomes to another mammalian host, maintaining the cycle. The PS cells (c) possess a typical active mitochondrion, while the organelle becomes highly reduced in BS (a).

splicing of nuclear transcripts, which are transcribed non-canonically by RNA polymerase I as huge polycistrons covering hundreds of genes [9], as well as the capacity of these parasites to orchestrate, by a unique mechanism, switching its proteinaceous surface coat, allowing them to comfortably escape the immune response of the host [10]. Moreover, as highly pathogenic agents, trypanosomes are studied with the aim of designing novel drugs against them, with the currently used drugs being old, toxic and prone to trigger resistance [11].

T. brucei undergoes a complex digenetic life cycle, comprising a mammalian host (human, cattle) and an insect vector (tse-tse fly *Glossina* spp.) (Fig. 1). It is a particularly useful model for studying the mitochondrion, since there is just a single organelle per cell, which undergoes dramatic remodeling of its morphology and metabolism in the course of the life cycle [12]. Due to the drastic change of the host environment, the parasite devised notable morphological and metabolic variations. The key stages are the mammalian bloodstream stage (BS) and the insect-specific procyclic stage (PS) (Fig. 1), which can both be cultured *in vitro*. PS possesses a large reticulated and fully functional mitochondrion, equipped with a standard respiratory chain, somewhat unusual Krebs cycle and two terminal oxidases [13]. Inside the insect gut the PS flagellates dwell in carbohydrate-poor conditions. The tsetse fly-metabolized bloodmeal is a rich source of amino acids (particularly proline and glutamate) supporting the PS energy metabolism [14]. On the other hand, by metabolizing the plentiful glucose present in the mammalian bloodstream, the BS trypanosomes rely solely on glycolysis, which is carried out in specialized organelles called glycosomes [15]. As a substitute to the standard respiratory chain of PS, glycerol-3-phosphate dehydrogenase supplies electrons to the terminal alternative oxidase (TAO) as the electron acceptor in BS. Moreover, cytochrome *c*-dependent respiratory complexes III and IV are entirely lost from the BS cells, with the function of its complexes I and II remaining enigmatic [16,17]. The BS complex V (ATP synthase) functions in an unusual fashion, as its F1 part rotates in an opposite direction compared to that in the PS flagellates. It hydrolyzes ATP and pumps H^+ out of the mitochondrion to generate membrane potential required for protein import [18].

For all the above reasons, we have decided to study the Fe/S protein biogenesis pathways in *T. brucei*. Fe/S clusters are simple primordial protein cofactors, involved in a wide range of essential biological processes of each domain of life. The biogenesis of Fe/S cluster is a remarkably complex procedure engaging ~30 proteins. Hitherto, four Fe/S protein biosynthetic systems have been identified, specifically the nitrogen fixing (NIF), sulfur mobilization (SUF), iron sulfur cluster (ISC) and cytosolic iron sulfur protein assembly (CIA) machineries [19]. *T. brucei* retains the conserved ISC and CIA machineries (Fig. 2 and Table 1). We will first briefly discuss the processes concerning iron acquisition, then we will describe the mitochondrion-located ISC machinery, and conclude with what is known about the CIA machinery. Further, we will address the communication between Fe/S protein biogenesis and tRNA thiolation. We will also pay particular attention to the instances when the *T. brucei* proteins were used in rescue experiments, and when the data from this protist seem to have a wider significance.

2. Iron acquisition

Fe/S cluster assembly commences in the mitochondrion, to which free iron is imported in the reduced form, Fe^{2+}. Membrane potential and the inner mitochondrial membrane proteins Mrs3 and Mrs4 are

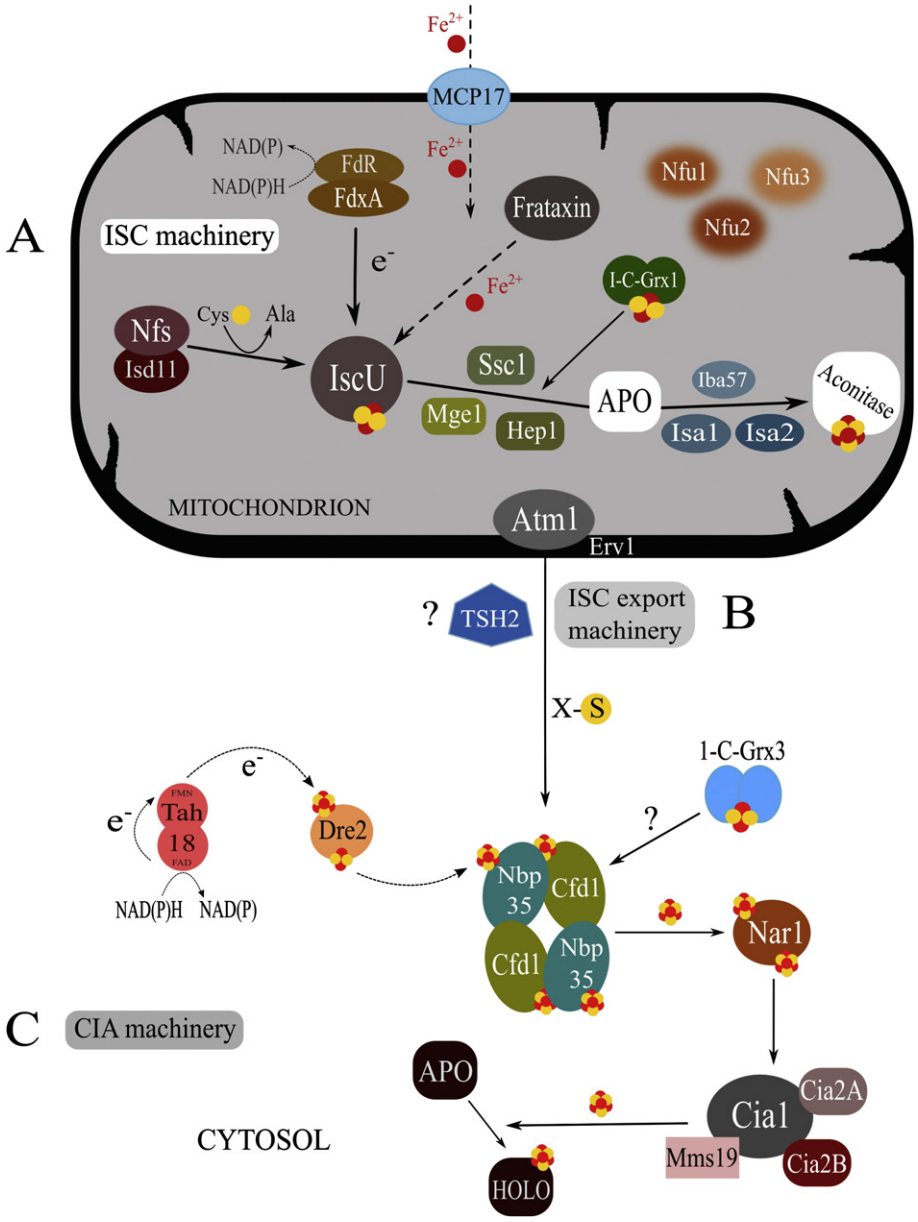

Fig. 2. Fe/S protein biosynthetic machineries of *T. brucei*. **A. ISC machinery:** The *T. brucei* genome contains all main ISC components. For the mitochondrial and cytosolic Fe/S cluster protein biogenesis, the Nfs-Isd11-IscU (a [2Fe-2S] cluster is assembled on IscU) module and subsequently FdxA are required. Frataxin is another essential ISC component. Ferrous iron (Fe^{2+}, red sphere) is imported into the mitochondrion by the putative MCP17 (putative function; broken arrow). RNAi against frataxin (broken arrow) did not alter the mitochondrial iron content, hence it may not be involved in iron storage. The final step engages Ssc1, Mge1 and Hep1, ensuring the incorporation of Fe/S clusters into apo-proteins. The function of three Nfu homologues (blurred blobs) remains unknown. The Fe/S cluster-coordinating trypanosome-specific monothiol glutaredoxins are functionally capable to participate in the ISC machinery. The mitochondrial 1-C-Grx1 was shown to coordinate [2Fe-2S] cluster *in vitro*. Aconitase, possessing a [4Fe-4S] cluster, is shown as a typical holoprotein, which is specifically matured by Isa1, Isa2 and Iba57. **B. ISC export machinery:** In trypanosomes, this machinery is comprised of the ABC transporter Atm1 of the mitochondrial inner membrane and the sulfhydryl oxidase Erv1 of the mitochondrial intermembrane space. The unique trypanosome-specific dithiol trypanothione (TSH2) most likely replaced glutathione. X-S is the unknown sulfur-containing substrate exported by mitochondrial Atm1. **C. CIA machinery:** The overall CIA pathway, as it is known in yeast and human, is depicted. *T. brucei* contains a functionally conserved CIA system. A bridging [4Fe-4S] cluster is assembled on the Cfd1/Nbp35 scaffold protein complex, with the contribution of the electron transport chain Tah18/Dre2. Dre2 itself is a Fe/S protein coordinating one [4Fe-4S] and one [2Fe-2S] cluster. The bridging [4Fe-4S] cluster is then transferred to the apo-proteins via Nar1 (which contains two [4Fe-4S] clusters) and the targeting complex composed of Cia1, Cia2A, Cia2B and Mms19. 1-C-Grx3 might play a role as an iron donor. All of the above-mentioned CIA factors are present and essential throughout the life cycle of *T. brucei*.

essential for this step in *Saccharomyces cerevisiae* [19]. In *T. brucei* the putative mitochondrial carrier protein TbMCP17, situated at the inner mitochondrial membrane, is possibly functioning as the iron importer (Fig. 2A) [20]. The BS trypanosomes acquire iron via the host transferrin, which is taken up by a receptor-mediated endocytosis at the flagellar pocket [21]. The transferin-receptor complex endocytosis is followed by acidification in endosomes, which releases the iron [22]. The precise iron acquisition mechanism of PS in the tse-tse fly remains unknown, although it was shown recently that this life stage can internalize iron from ferric complexes mediated by a reductive mechanism [23]. This process involves reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) and the subsequent transportation, enabling the PS flagellates to uptake iron from various ferric complexes in the transferrin-deficient insect gut [23].

Cytosolic iron is never allowed to remain free due to its capacity to produce reactive oxygen species. The chelatable and redox-active iron is assembled as a transitory cellular labile iron pool (LIP) comprised of

Table 1
T. brucei ISC and CIA components.

Fe/S protein biogenesis components	Full name/Function	TriTrypDB accession number	Yeast homologue	Human homologue
TbNfs	Cysteine desulfurase	Tb927.11.1670	Nfs1	NFS1
TbIsd11	ISC biogenesis desulfurase-interacting protein of 11 kDa	Tb10.389.1785	Isd11	ISD11
TbIscU	Scaffold protein	Tb927.9.11720	Isu1	ISCU
TbIsa1	A-type ISC assembly proteins	Tb927.8.5540	Isa1	ISCA1
TbIsa2		Tb927.5.1030	Isa2	ISCA2
TbIba57	Isa-interacting protein	Tb927.8.6480	Iba57	IBA57
TbFrataxin	Frataxin	Tb927.3.1000	Yfh1	FXN
TbFdxA	Ferredoxin	Tb927.7.890	Yah1	FDX2
TbFdxB		Tb927.4.4980		——
TbFdR	Ferredoxin reductase	Tb10.70.5510	Arh1	FDXR
TbNfu1	Scaffold proteins	Tb927.7.1720	Nfu1	NFU1
TbNfu2		Tb10.26.0860		
TbNfu3		Tb10.70.1780		
Tb1-C-Grx1	Monothiol glutaredoxin 1	Tb09.160.2210	Grx5	GLRX5
TbSsc1	Mitochondrial Hsp70 chaperone	Tb927.6.3740	Ssc1	GRP75
TbMge1	Nucleotide exchange factor	Tb927.6.2170	Mge1	GRPE-L 1/2
TbHep1	Heat shock protein with a zinc-finger motif	Tb927.3.2300	Hep1/Zim17	Hep
TbErv1	Sulfhydryl oxidase, ISC export system	Tb09.160.4440	Erv1	ALR
TbAtm1	ABC transporter, ISC export system	Tb11.01.8700	Atm1	ABCB7
TbCfd1	P-loop NTPase, scaffold proteins	Tb927.7.1500	Cfd1	CFD1
TbNbp35		Tb927.10.1690	Nbp35	NBP35
TbDre2	Electron acceptor from NAD(P)H-Tah18	Tb927.8.1750	Dre2	CIAPIN1
TbTah18	Electron transfer from NAD(P)H to Dre2	Tb927.4.1950	Tah18	NDOR1
TbCia1	CIA targeting complex docking protein	Tb927.8.3860	Cia1	CIAO1
TbNar1	CIA adaptor protein	Tb927.10.10650	Nar1	IOP1
TbCia2A	CIA targeting complex	Tb927.9.10360	Cia2	CIA2A
TbCia2B		Tb927.8.720		CIA2B
TbMms19		Tb927.8.3920	Met18/	MMS19
		Tb927.8.3500	Mms19	

CIA components are highlighted in bold face.

Fe^{2+} plus Fe^{3+} and accompanied by diverse type of ligands, organic anions (phosphates and carboxylates), polypeptides (glutathione) and components of membranes such as phospholipids [24]. The exact chemical composition of LIP in trypanosomes is unknown, although trypanothione, their most abundant intracellular low molecular mass thiol, is thought to be the key physiological ligand [25] (see Section 3.7).

3. Mitochondrial ISC machinery

The ISC machinery involves three central biogenesis steps [26]. First, synthesis of a [2Fe-2S] cluster takes place on the scaffold protein Isu1, working as an assembly platform in a process involving the cysteine desulfurase module Nfs1-Isd11 as the sulfur donor [27]. The accurate mechanism in which iron binds to the scaffold Isu1 remains unclear, although the association of the iron-binding protein frataxin is evident [28,29]. An electron transfer chain is necessary for the reduction of the Nfs1-held persulfide to the Fe/S cluster-specific sulfide form. Ferredoxin, Fdx (Yah1 in yeast), is responsible for this phenomenon, delivering electrons with the assistance of ferredoxin reductase (FdR) and NAD(P)H [30]. In the penultimate step, the Isu1-attached Fe/S cluster is released, and resolves the temporary association with the transfer proteins. This stage is assisted by a Hsp70 chaperone system involving ATPase Ssq1, the co-chaperone Jac1 and the nucleotide exchange factor Mge1 [29]. The released Fe/S cluster can be transported to the apo-proteins directly, or by means of the mitochondrial monothiol glutaredoxin, Grx5 [29]. A unique interaction of Grx5 and Ssq1 has been described recently, showing their crucial participation in all cellular Fe/S protein maturations [31].

The concluding step engages several ISC targeting factors that transfer and insert the Fe/S cluster to specific apo-proteins. The dedicated insertion of [4Fe-4S] clusters is performed by alternative scaffold proteins Isa1 and Isa2 [32,33], supported by Iba57 [34]. Various maturation factors presenting target protein specificity are associated with this final step, as is the case of Ind1, which is essential for the maturation of complex I [35], and Nfu1, which matures a broad range of proteins like respiratory complexes I, II and lipoic acid synthase [36,37]. The core ISC members involved in the first two steps are also crucial for the maturation of extra-mitochondrial Fe/S cluster proteins.

3.1. Nfs and IscU

Upon the release of the Tritryp (*T. brucei*, *T.cruzi* and *L. major*) genomes in 2005 [38–40] it was straightforward to find genes encoding the key components of the ISC pathway (Fig. 2A and Table 1). The level of conservation of all of them was such that most were already properly annotated in the TriTrypDB [41]. Due to their critical role in the ISC pathway, cysteine desulfurase TbNfs (in this review, the *T. brucei* proteins will be labeled with the prefix Tb) and the scaffold TbIscU were selected for initial analysis in the procyclic stage (Fig. 2A). Functional analysis was not attempted in the mammalian-infective bloodstream stage because its transfection was initially much more challenging than that of the PS. Moreover, the importance of TbNfs and TbIscU was questionable in the BS organelle, as it lacks respiratory complexes, and with the sole exception TAO, all other proteins seem to be either down-regulated or absent from the mitochondrion, when compared with the PS organelle [42]. Since TAO contains a non-Fe/S cluster bi-iron center [43], and all Fe/S rich respiratory complexes are not expressed at this life stage (ATPase [= complex V] is an exception but does not contain Fe/S clusters), any cluster-containing mitochondrial proteins were unknown at that time and hence, essentiality of the ISC pathway in the BS *T. brucei* was doubtful [44].

By means of the tetracyclin-inducible RNAi vector p2T7-177, equipped with opposing T7 promoters (the linearized vector containing fragment of the target gene becomes integrated into the *T. brucei* genome by homologous recombination at the unique minichromosomal 177 bp repeat site and allows an inducible mRNA depletion) [45], either TbNfs or TbIscU mRNA was efficiently down-regulated. That resulted in a substantial growth phenotype, which was somewhat stronger in the

case of TbNfs. Next, the impact of these ablations was assayed on the activity of the [4Fe-4S]-containing aconitase. In *T. brucei*, aconitase is encoded by a single gene, but the protein is present in both the mitochondrion and cytosol of the PS, yet it is absent from the BS cells [46]. Its dual localization makes aconitase particularly suitable for the parallel measurement of the Fe/S cluster-dependent enzymatic activities in these two cellular compartments. Indeed, its activity dropped in both RNAi-induced knock-downs, proving that TbNfs and TbIscU are required for the maturation of the mitochondrial as well as cytosolic Fe/S clusters [44]. It was also shown that in their absence, the *T. brucei* aconitase is inactive but not destabilized. The perturbation of the ISC pathway was followed by the EPR spectroscopy, and the impact on overall mitochondrial metabolism was assessed by measuring the metabolic end-products, revealing dramatic increase of pyruvate. This is highly reminiscent of the mitochondrial suppression in the BS, which switches to glycolysis, producing pyruvate as a major metabolic end product [44]. This initial study showed that two key ISC components have a highly conserved function in trypanosomes as compared to their yeast homologues [47,48].

Using digitonin fractionation and transmission cryoelectron microscopy, both TbNfs and TbIscU were recently shown to have a dual localization in the mitochondrion and nucleolus of the PS and BS flagellates. Although the bulk of these proteins is present in the organelle, the amount in the nucleolus must be physiologically relevant [49]. It should be mentioned here that in yeast *Saccharomyces cerevisiae* Nfs1 is known to contain a nuclear localization signal [50] and a putative signal for this compartment was also detected in the *T. brucei* protein. The BS mitochondrion is highly reduced when compared with the PS organelle [51], and it was reasonable to assume that in it a demand for the Fe/S clusters may be very low, if any. Due to the absence of a measurable Fe/S cluster-dependent activity, we overexpressed the mitochondrial-targeted aconitase and its activity proved that the Fe/S cluster assembly occurs also in this BS cell compartment. Depletion of TbIscU in the BS was lethal, providing further evidence of a functionality of this pathway in the silenced BS mitochondrion [49].

3.2. Selenocysteine lyase and Isd11

Besides TbNfs, the genome of *T. brucei* and other trypanosomatids contains a Nfs-like gene. Extensive phylogenetic analysis confirmed that TbNfs falls into a single clade with other eukaryotic cysteine desulfurases, whereas a Nfs-like protein branches off within a clade composed of selenocysteine lyases (SCL), less frequent enzymes than the omnipresent cysteine desulfurases [52]. Targeting of the TbSCL transcript by RNAi in the PS did not result in a growth phenotype, whereas TbNfs was previously shown to be essential [44].

It has been described that the Nfs and Nfs-like proteins have both cysteine desulfurase and selenocysteine lyase activities, although a typical Nfs has a higher preference for cysteine, whereas the activity of a typical SCL is much higher towards selenocysteine cleaving it to alanine and selenium [53]. SCL activity is crucial for organisms that demand selenium, as first reported in bacteria and later in mammals, both of which require selenoproteins [54]. Indeed, RNAi-induced knock-downs of TbNfs or TbSCL had both their cysteine desulfurase and selenocysteine lyase activities strongly reduced, supporting the view that in this protist their activities are transposable [52]. HA_3-and TAP-tagged TbSCL was localized to the nucleus and cytosol in PS, while TbNfs is known to be present in the mitochondrion [44] and the nucleolus [49]. This dual localization is strongly supported by the fact that upon ablation of TbNfs, both activities significantly drop in the mitochondrion and cytosol [52]. However, thiolation of the cytosolic and mitochondrial tRNAs was reduced in cells ablated for TbNfs, but not in those with lower level of TbSCL [55]. The lack of growth phenotype following the loss of TbSCL has been interpreted by the capacity of TbNfs to functionally complement TbSCL, whereas the same does not apply *vice versa* [52]. Moreover, the depletion of TbNfs is associated with concurrent decrease of its binding partner TbIscU, while the same effect was not observed in the TbSCL RNAi knock-downs (P. Changmai and J. L., unpubl. results).

The *T. brucei* genome also contains TbIsd11, the binding partner forming a functional complex with TbNfs, expressed in both life stages (Fig. 2A). As anticipated, TbIsd11 is an essential component for the mitochondrial and cytosolic Fe/S cluster assemblies, and involved in the tRNA thiolation too [56] (see also Section 6 below).

3.3. Frataxin

Although perhaps the largest body of literature of all participants of the Fe/S cluster assembly is available on frataxin [57], discussion about its function is far from settled [58]. It is a small highly conserved multifunctional protein which, besides its role in the pathogenesis of Friedreich's ataxia, has been implicated with functions in iron storage, control of radical oxygen species and heme biosynthesis [59–62].

The study of the *T. brucei* frataxin has focused mainly on establishing its function and testing the extent of its conservation across eukaryotic supergroups. As anticipated, frataxin is essential for the PS, and its ablation results in a spectrum of phenotypes. Activities of three proteins, the function of which relies on the Fe/S clusters (aconitase, fumarase and succinate dehydrogenase), were shown to be largely decreased in the RNAi-interfered cells [63]. Moreover, the ablated trypanosomes suffered from increased concentration of reactive oxygen species (ROS), lower mitochondrial membrane potential and decrease in oxygen consumption, yet no change in their mitochondrial iron content was observed. This can be explained by the fact that in this ancestral protist, frataxin monomers do not form large aggregates observed in yeast and humans [64,65], and hence this protein may not play any role in iron storage [63] (Fig. 2A). Therefore, the function(s) of frataxin in intracellular iron distribution, documented in the Opisthokont eukaryotes may represent derived, rather than ancestral feature(s). Furthermore, it is unlikely that frataxin has any function in heme metabolism of trypanosomes and related flagellates, as these are heme auxotrophs, lacking either the entire heme synthesis pathway or a substantial part of it [66]. Recently, frataxin was shown to be present also in the highly reduced mitochondrion of the BS trypanosomes and, same as in the PS, conspicuously absent from the cytosol [49].

Frataxin was reported from the hydrogenosome of the strictly anaerobic *Trichomonas vaginalis*, which is a highly modified hydrogen-producing organelle derived from the mitochondrion [67]. It was of interest to assay whether the functions of hydrogenosomal frataxin are at least distantly related to those of its mitochondrial orthologue. Moreover, most genes that are single-copy in virtually all eukaryotes, exist in multiple copies in the *T. vaginalis* genome, which is also the case of frataxin. For technical reasons, functional analysis cannot be performed within the trichomonad cell, so we overexpressed in the PS of *T. brucei* the tagged *T. vaginalis* frataxin equipped with its genuine hydrogenosomal import signal [63]. Interestingly, while the same signal is not recognized by the mitochondrial import complexes of *S. cerevisiae* [68], we were able to show that it is sufficient for the import into the *T. brucei* mitochondrion. Since the import of both slightly different *T. vaginalis* frataxins into trypanosomes depleted for their *T. brucei* orthologue resulted in an almost perfect rescue of all phenotypic alterations, one can conclude that both of them remain fully functional [63].

Thanks to the encouraging rescue by the hydrogenosome-derived frataxin, additional rescues were attempted from even more distantly related eukaryotes, namely those of the model land plant *Arabidopsis thaliana* and the ecologically important marine diatom *Thalassiosira pseudonana*. Mitochondrial protein import complexes of *T. brucei* are either highly derived or reduced [69], which was interpreted by some as a uniquely retained, highly ancestral feature [69–71]. Therefore, we wondered whether the genuine mitochondrial import signals of these two unrelated photosynthesizing eukaryotes would be sufficient for the import of their respective frataxins into the *T. brucei* mitochondrion.

Indeed, in both cases the tagged proteins were efficiently imported into the PS organelle, supporting the argument that the mitochondrial import complexes of trypanosomes possess features of a universal importer [72]. Moreover, while the rescues by the *A. thaliana* or *T. pseudonana* frataxins of the trypanosome Fe/S cluster-containing mitochondrial and cytosolic proteins were not as efficient as in the case of their *T. vaginalis* orthologue, these experiments validate eukaryotic frataxin as a universal functionally conserved protein [72].

Due to the capacity of the trypanosome mitochondrion to recognize and efficiently import alien signal peptides, we have attempted a rescue of frataxin-depleted *T. brucei* with human frataxin. Even when equipped with its genuine signal peptide, in trypanosomes human frataxin gets processed by the processing peptidase in exactly the same manner as in human cells [73]. While human frataxin is by some authors confined to the mitochondrion [74,75], others submit that it is also present in the cytosol and nucleus [76,77]. However, human frataxin can exercise its rescuing function in *T. brucei* only when targeted to the mitochondrion, although it can do so even when its processing has been inhibited by a mutation [73].

3.4. ISA proteins

Two Isa proteins (Isa1 and Isa2) are present in the *T. brucei* genome (Fig. 2A). These rather small proteins were proposed to serve as alternative scaffold proteins in both prokaryotes and eukaryotes [32,33]. This rather limited essentiality in eukaryotes was explained by overlapping function with other scaffold proteins and/or their requirement only under specific conditions [78]. In human cells, Isa1 seems to have a dual localization in the mitochondrion and cytosol, serving as a scaffold for the aconitase-targeted Fe/S clusters [79].

Since the BS and PS flagellates dramatically differ in the overall activity and morphology of their single mitochondrion, we went to test the hypothesis that the Isa proteins may be essential in the latter but dispensable in the former life stage. Indeed, RNAi-based depletion of Isa1 and/or Isa2 resulted in cessation of growth of the insect-dwelling PS, whereas their down-regulation did not alter growth of the mammalian-infective BS trypanosomes [80]. This is a reflection of the fact that the PS mitochondrion contains an array of proteins functionally dependent on the Fe/S clusters (e.g. aconitase, fumarase and succinate dehydrogenase), whereas the highly reduced organelle of the BS cells appears to be devoid of these proteins. While the CIA pathway is essential throughout the entire life cycle of *T. brucei* (see below, Section 5), the lack of growth phenotype, under the standard culture conditions, upon depletion of both Isa proteins strongly indicates that none of them participates in the CIA pathway. Recently, the confinement of the *T. brucei* Isa proteins to the organelle was confirmed by immunoelectron microscopy also in BS [49].

Furthermore, by an exhaustive series of cross-rescues we have shown that both human Isa proteins are able to (partially) rescue, in cells depleted for either single or both of their Isa proteins, the activities of the followed set of the Fe/S cluster-containing enzymes and hence showed for the first time that human Isa2 is likely to have an overlapping function with human Isa1 [80].

In yeast, Isa1 and Isa2 were shown to bind Iba57 in an interaction required for the maturation of a subset of Fe/S proteins, i.e. [4Fe-4S] cluster-containing enzymes including mitochondrial aconitase and radical S-adenosyl methionine enzymes biotin and lipoic acid synthases [81]. We have shown that the well-conserved TbIba57 is dispensable for the BS and essential for the PS, which can be explained by different requirements of these stages for the aconitase activity (T. Skalický and J. L., unpubl. results). Indeed, aconitase was shown to be destabilized in the PS *T. brucei* depleted for TbIba57, and these cells were, same as in *E. coli* manipulated in a similar manner [82], sensitive to oxidative stress, as demonstrated by their dramatic sensitivity to paraquat (T. Skalický and J. L., unpubl. results).

3.5. Ferredoxins

Ferredoxins are highly conserved proteins operating as electron transporters, having crucial role in the biogenesis of the Fe/S proteins. *S. cerevisiae* possesses a single orthologue of ferredoxin (Yah1) [30], whereas in humans two of its counterparts are present, human ferredoxin 1 (HsFdx1) and 2 (HsFdx2) [83]. Both proteins are expressed ubiquitously, although HsFdx1 is upregulated in the adrenal cortex and medulla, while the central nervous system is rich with HsFdx2 [83,84].

The genome of *T. brucei* contains two homologues of ferredoxin, namely TbFdxA (Fig. 2A) and TbFdxB [85]. Their functional analysis revealed that TbFdxA, but not TbFdxB, is essential in the PS, taking part in the Fe/S cluster biogenesis. Depletion of TbFdxA resulted in a drop of the Fe/S cluster-containing enzymatic activities in both the mitochondrial and cytosolic compartments. By disrupting both alleles of TbFdxA via a knockout strategy using homologous recombination and introducing a regulatable copy of TbFdxA, we have verified its indispensability in the disease-causing BS [85]. TbFdxA and TbFdxB appear in distantly related branches, with TbFdxA more closely related to its human and yeast counterparts than to TbFdxB, which is affiliated with the most basal eukaryotic ferredoxin branch [85].

Interestingly, both human ferredoxins equipped with their endogenous mammalian mitochondrial import signals, complemented the TbFdxA-ablated PS cells, rescuing both their growth and the aconitase activity. This successful complementation supports the notion of the human ferredoxins being functional homologues produced by gene duplication [85]. Ferredoxin reductase (TbFdR) (Fig. 2A) is present in the *T. brucei* genome and anticipated to transfer the reducing equivalents between NAD(P)H and ferredoxin.

3.6. Ssc1, Mge1 and Hep1

An efficient incorporation of the Fe/S clusters into apoproteins requires involvement of a dedicated chaperone system. Ssc1, a member of the chaperone family Hsp70, has a versatile role in several biological functions in all spheres of life [86–88]. In *S. cerevisiae*, Ssc1 helps to transfer the Fe/S clusters from the scaffold IscU to the target proteins in a cooperation with the co-chaperone Jac1 and the nucleotide release factor Mge1 [86]. Furthermore, to maintain the structural and functional properties of Ssc1, participation of the mitochondrial protein Hep1 is required, in the absence of which Ssc1 tends to undergo self-aggregation [89].

We have unambiguously identified homologues of Ssc1, Mge1 and Hep1 in the *T. brucei* genome (J. Týč et al., unpubl. results) (Fig. 2A). Preliminary experiments showed that depletion of TbSsc1 leads to a growth effect in both life stages, also affecting the mitochondrial and cytosolic aconitase activity in the PS. In contrast to the rat Hsp70 that is up-regulated under stress conditions [90], the PS and BS flagellates with down-regulated TbSsc1 do not show any increased sensitivity to stress (J. Týč et al., unpubl. results).

3.7. Glutaredoxins, trypanothione and Fe/S protein metabolism

Glutaredoxins are small thioredoxin-fold architecture exhibiting proteins present in most living organisms. Initially identified as thiol-disulfide oxidoreductases involved in redox reactions, they were later found to be involved in heme biosynthesis and Fe/S cluster biogenesis. Indeed, the deletion of Grx5 in yeast resulted in an anomalous assembly of Fe/S clusters on aconitase and succinate dehydrogenase [91]. Glutaredoxins are typically classified based on the number of the cysteine residues in the active site as monothiolic (CXXS, 1-C-Grx) or dithiolic (CXXC, 2-C-Grx) [92]. 1-C-Grxs were first discovered in *S. cerevisiae* [93] and subsequently found to be universally distributed.

Trypanosomatids encode three monothiol glutaredoxins; Tb1-C-Grx1 occurs solely in the mitochondrion (Fig. 2A), Tb1-C-Grx2 and

Tb1-C-Grx3 are anticipated to be of mitochondrial and cytosolic localization, respectively. All of them are expressed in both the BS and PS of *T. brucei* [94,95]. Tb1-C-Grx3, the orthologue of yeast Grx3-Grx4 [94,96], might play a role in cytosolic iron regulation (Fig. 2A). It has been reported that Tb1-C-Grx2 and Tb1-C-Grx3 failed to complement the yeast Grx5 mutants [96]. Recently, Tb1-C-Grx1, which is an abundant mitochondrial protein, was shown *in vitro* to bind a Fe/S cluster using glutathione as a cofactor [95]. Furthermore, a N-terminal extension, which is unique for the trypanosomatid-specific 1-C-Grx1, grants the conformational flexibility of the protein ensuring a more stable ISC assembly. The Cys104 in the active-site of Tb1-C-Grx1 is essential for binding the Fe/S cluster [95]. Strikingly, overexpression of the Cys104 mutant of Tb1-C-Grx1 *in vitro* (under iron deprivation or oxidative stress) and *in vivo* in mice significantly lowered the parasite's fitness, hinting to the involvement of iron and Fe/S cluster biogenesis in its infectivity [95]. A recent NMR study of the residues 42–184 of Tb1-C-Grx1 interpreted this N-terminal extension to be an unstructured element [97]. Nevertheless, it is thought to be involved in structural rearrangements of the protein [95].

The trypanosome redox system tackling antioxidant and xenobiotic stress-involved cues has always been an intriguing area of research. The occurrence of glutathione in trypanosomatids has been first shown in 1980 [98] and later the enigmatic redox system of trypanosomatids was shown to be composed of the unfamiliar N^1, N^8-bis-glutathionylspermidine (trypanothione) [99], NAD(P)H-dependent trypanothione reductase and the thioredoxin-like oxidoreductase tryparedoxin. Trypanothione (TSH2) (Fig. 2B) biosynthetic pathway has been identified in *T. brucei, L. major* and to some extent also in *T. cruzi.* [100]. The trypanothione metabolism proved to be crucial for cell viability and virulence [101], as it is involved in many processes, Fe/S protein metabolism being one of them [100]. All three monothiolic Tb1-C-Grxs and Tb2-C-Grx1 coordinate [2Fe-2S] clusters engaging the trypanosomal low molecular weight thiol, mono-glutathionylspermidine or trypanothione [95]. Fe/S cluster binding could lead to the dimerization of Tb2-C-Grx1 and Tb1-C-Grx2 [94]. Fe/S cluster can be incorporated into Tb1-C-Grx1, in the absence of the low molecular weight thiol, resulting in a conformational change [95]. Interestingly, trypanothione can form a protein-free iron-containing complex that is further incorporated into the reduced Tb2-C-Grx1 [102,95]. The available data featuring the ability of trypanothione to perform as an efficient thiol ligand of iron and Fe/S clusters *in vitro*, suggest the probability for unusual roles of this unique dithiol in the iron metabolism and redox sensing in the kinetoplastid protists, which are yet to be elucidated.

4. ISC export machinery

This dedicated system connects the mitochondrial ISC and cytosolic CIA machineries by exporting the mitochondrion-produced glutathione-sulfur-moiety [103], necessary for the maturation of the cytosolic and nuclear Fe/S proteins (Fig. 2B). It is comprised of the ABC transporter Atm1 of the inner mitochondrial membrane [104], the sulfhydryl oxidase Erv1 of the mitochondrial intermembrane space (IMS) [105] and the tripeptide glutathione [106]. Depleting each of the members leads to severe defects of the cytosolic and nuclear Fe/S proteins but not of the mitochondrial ones [107].

T. brucei contains in its genome a complete ISC export machinery (Fig. 2B and Table 1). Ablation of TbAtm1 affects the downstream cytosolic CIA machinery with a disrupted Fe/S cluster incorporation into the cytosolic proteins (P. Changmai et al., unpubl. results). TbErv1 is essential for both life stages. Its RNAi depletion resulted in a massively swollen mitochondrion, a phenomenon derived directly from the specific role of TbErv1 in mitochondrial protein import [108]. A comparative genomics approach represented first *in silico* analysis on the conservation in trypanosomatids of proteins involved in the delivery and/or assembly of the cytosolic Fe/S clusters [108]. The presence of conserved TbDre2 and TbTah18 proteins, as well as other known CIA components in the trypanosomatid genomes allowed to make a confident extrapolation that TbErv1 is a multifunctional protein, with a function in both the mitochondrial IMS protein import [108] and maturation of the cytosolic Fe/S proteins, even though Mia40, the prototype partner of Erv1 in yeast [109] and human [110], is unexpectedly absent from these early-branching protists [108,111].

Mia40 and Erv1 form a disulfide relay system, which ensures the import of the small cysteine-rich proteins into the IMS and folding by transferring disulfide bonds to them [112]. While the respiratory chain of PS flagellates contain cytochrome *c*, the disease-causing BS undergo a cytochrome *c*-independent respiration [113]. For human and yeast Erv1 proteins, cytochrome *c* is the favored electron acceptor [114,115], whereas in *T. brucei* both oxygen and cytochrome *c* are utilized as electron acceptors [108]. The situation is favorable for the cytochrome *c*-deprived BS parasites, where oxygen has to be the dominant electron acceptor. Recently, Mia40 in *S. cerevisiae* has been identified to carry a novel cluster-binding motif, and the dimer of this protein is bridged by a [2Fe-2S] cluster coordinated by its four cysteine residues. Though the exact function of the Fe/S cluster-coordinating Mia40 is unknown, it has been speculated to participate in the ISC export machinery and/or in a novel pathway [116]. The important absence of Mia40 in *T. brucei* proves the TbErv1 protein to be a multifaceted entity. Still, it has to be established whether trypanosomes evolved a Erv1-only streamlined oxidative protein import pathway or a third, so far unidentified player acting like Mia40, is involved [108,111].

5. CIA machinery

Cytosolic and nuclear Fe/S cluster proteins include essential components of protein translation [117], DNA synthesis and DNA repair [118–122]. In yeast and human cells, assembly of their Fe/S clusters is undertaken by the CIA (cytosolic iron-sulfur protein assembly) machinery comprised of some ten proteins [123] (Fig. 2C and Table 1). Functionality of the CIA machinery depends on the ISC assembly (Fig. 2A) and the ISC export machinery (Fig. 2B) [19].

The assembly procedure can be divided into two separate phases. Firstly, a bridging [4Fe-4S] cluster is transiently assembled on the heterotetrameric P-loop NTPases Cfd1 and Nbp35, operating as the scaffolds [124,125]. This step depends on the mitochondrial core ISC components and the ISC export machinery (see Section 3 and 4). Production of the functionally indispensable N-terminal Fe/S cluster of Nbp35 depends on the flavoprotein Tah18 and the Fe/S protein Dre2, which serve as a NAD(P)H-dependent electron transfer chain [126]. Secondly, the bridging [4Fe-4S] cluster coordinated by the Cfd1-Nbp35 complex is released [125], aided by the Fe/S protein Nar1 [127] and the CIA targeting complex Cia1-Cia2-Mms19 [122,121,128,129]. The CIA targeting complex facilitates both Fe/S cluster transfer and target-specific cluster insertion into the apo-forms of several cytosolic and nuclear Fe/S proteins.

The CIA pathway seems to be conserved in a number of single-celled eukaryotes including trypanosomatids, although several departures from the prototypic pathway have been described [108,130]. For example, the anaerobic *Trichomonas vaginalis* and *Giardia intestinalis* lack not only Dre2 [130–132], but also Erv1, a key player in the ISC export machinery, which is otherwise essential for the CIA pathway [105]. This startling flexibility or the lack of conservation of individual components of the CIA pathway among the highly diverse and diverged excavates make *T. brucei* an organism of choice for its functional analyses and dissection.

Individual depletions of the *T. brucei* CIA components by RNAi (strongly) suggested the dispensability of most of them, except the scaffold proteins TbNbp35 and TbCfd1 [133]. Due to this unexpected result, an RNAi double knockdown strategy was implemented, depleting two components in parallel, of the early and intermediate part of the CIA pathway, which invariably affected viability of both life stages. This observation specifies that only a strong depletion of the CIA pathway

triggers phenotypic effects in *T. brucei*. The pathway can be segmented into components principally dealing with 'electron transfer chain', 'scaffolds', and 'targeting complex' and assumed to function as distinct modules [123]. When just a single component of the module is depleted, its binding partner seems to be capable of at least partially complementing for the absence. However, simultaneous depletion of two partners leads to a harsh deficiency of the whole 'module', causing downstream disruption of the CIA pathway [133].

Biochemical characterization by UV–VIS studies revealed that TbTah18, like its yeast, human and plant homologs, is a diflavin reductase. In comparison to its yeast and human counterparts, TbDre2 lacks the entire N-terminal *S*-adenosylmethionine methyltransferase-like domain [134]. However, TbDre2 retains the Fe/S cluster-binding domain, which is indispensable for viability in yeast [135], and, based on UV–VIS and EPR spectroscopy, binds a [2Fe-2S] cluster [133]. This truncated structure is a common character of all kinetoplastid flagellates [133].

To support the notion of a functionally conserved CIA machinery in *T. brucei*, complementation assays have also been performed. Initial analyses using yeast mutants Gal-Dre2 and Gal-Tah18 showed that neither TbDre2, nor TbTah18 were able to complement the growth defect of these cells under depletion conditions. Additionally, the parasite's proteins failed to rescue the growth, not even after their overexpression using strong promoters. Since in the yeast, plant and human cells, Dre2 and Tah18 are known to tightly interact [126,136], the complementation effects were assessed after parallel expression of a pair of plasmid-encoded proteins using the double yeast mutant Gal-Dre2/Gal-Tah18. Coexpression of *T. brucei* orthologues rescued the growth phenotype of the double mutant. Moreover, partially rescued activity of isopropylmalate isomerase, a cytosolic Fe/S cluster-containing protein, further confirmed functionality of the TbTah18-TbDre2 module in the yeast [133].

Equivalent to humans, *T. brucei* also possesses two Cia2 proteins, TbCia2A and TbCia2B (our unpubl. results). Having in mind the differential target specificity of human CIA2A and CIA2B [128] it will be intriguing to see whether or not the same phenomenon evolved in the unrelated *T. brucei*.

6. Fe/S protein assembly and tRNA thiolation

The connection between tRNA thio-modifications and Fe/S protein maturation has been described in bacteria [137], yeast [138] and mammals [74]. Therefore, its presence in *T. brucei* [138] is not surprising. Due to the complete loss of tRNA genes from the mitochondrial DNA of trypanosomes and related flagellates, all tRNAs have to be imported into the mitochondrion from the cytosol [139] and likely dethiolated henceforth [140]. TbNfs was shown to be indispensable for the thiolation of both cytosolic and mitochondrial tRNAs [55]. Apart from TbNfs, its binding partner TbIsd11 is also mandatory for this process, whereas TbMtu1 is essential for mitochondrial tRNA thiolation only [56] (Fig. 3). A structural study of the *E. coli* IscS has revealed that its cysteine-328 loop, required for the transfer of sulfur to several acceptors, allows the enzyme to communicate with different partners by making it accessible to the Fe/S cluster assembly or tRNA thiolation [141].

It has been shown previously that Nbp35, Cfd1 and Cia1 of *S. cerevisiae* are essential for 2-thio-modification of the cytosolic but not mitochondrial tRNAs, suggesting a steady involvement of the CIA machinery [142]. Indeed, it was demonstrated that the TbNbp35 and TbCfd1 scaffolds of the CIA machinery are along with TbNfs required

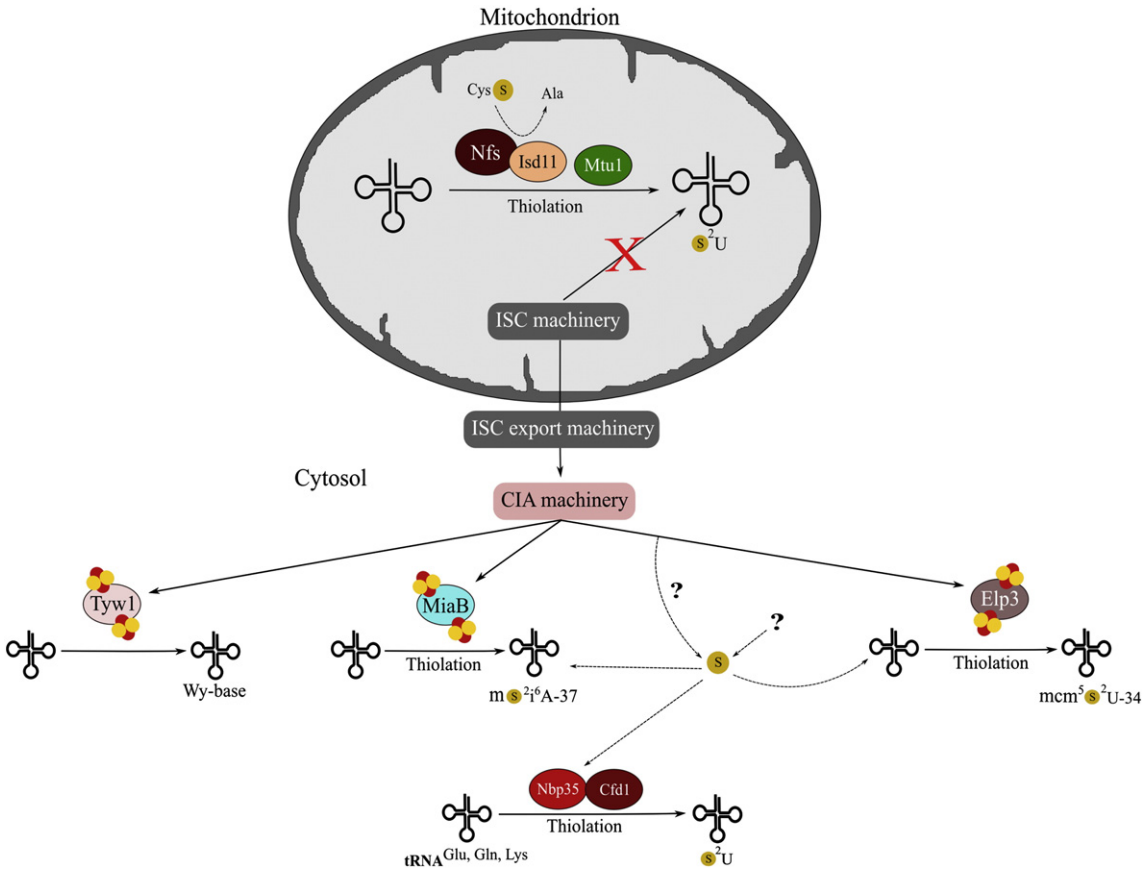

Fig. 3. Fe/S protein assembly and tRNA thiolation. The connection between the tRNA thio-modification and *T. brucei* Fe/S protein biogenesis has been represented here. The mitochondrial tRNA thiolation and ISC machinery share the TbNfs-TbIsd11 module as a source of sulfur, apart from that there is no direct involvement of the ISC machinery. Engagement of CIA machinery is rather direct, via the scaffold proteins TbNbp35/TbCfd1 shown to be associated with the thiolation of cytosolic $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Lys}$, and possible maturation of the cytosolic Fe/S cluster-containing tRNA modifying enzymes Tyw1, MiaB and Elp3. The source of sulfur in the cytosol remains unknown.

for the thiolation of cytosolic $tRNA^{Glu}$ (UUC), $tRNA^{Gln}$ (UUG), and $tRNA^{Lys}$ (UUU) of *T. brucei* (Fig. 3). Moreover, TbNbp35 was shown not to affect the tRNA import machinery in this protist [140]. The presence of Fe/S clusters in several tRNA modification enzymes, namely MiaB, Tyw1 and Elp3, further underlines the mutual dependence of tRNA metabolism and the Fe/S protein assembly machinery [138] (Fig. 3). Several potential homologs of these tRNA-modifying proteins are annotated as such in TriTrypDB [138]. Tyw1 is a cytosolic protein, likely responsible for tRNA recognition and tricyclic base ring formation of wybutosine [143], while Elp3, a component of the elongator complex, is linked to the early-step thiolation of the position 34 wobble uridine of particular cytosolic tRNAs with 5-methoxycarbonylmethyl group (mcm^5) [144]. On the other hand, MiaB catalyses the post-transcriptional modification at position 37 of tRNAs by forming the thiolated nucleoside 2-methylthio-*N*-6-iso-pentenyladenosine (ms^2i^6A-37) [145] (Fig. 3).

Recently, it has been observed that the ablation of TbIscU leads to down-regulation of several cytosolic tRNAs in the PS but not BS [49]. Since tRNA modifications and import are carried out equally in both stages [146], this stage-specific phenotype might be a consequence of the non-functional Fe/S protein biogenesis, but not tRNA thiolation. Combined, the available data are compatible with close association of the tRNA thiolation pathway and the Fe/S cluster biogenesis in *T. brucei*, although a direct involvement of the CIA pathway, apart from the scaffolds, has yet to be scrutinized.

7. Future directions and possible challenges

The latest advancements of our understanding of the Fe/S cluster biogenesis in trypanosomes is particularly important for assessing the evolutionary conservation of this process in eukaryotes, as well as for exploring differences in this essential process between these protist parasites and their mammalian hosts for potential drug design. Outstanding questions worth attention in close future include (functional) studies of: (i) the nucleolus-localized TbNfs-TbIscU complex; (ii) three homologues of Nfu proteins (Fig. 2A); (iii) the 'CIA targeting complex' comprised of TbCia1, TbCia2A, TbCia2B and TbMMS19. Furthermore, it will be interesting to (iv) search by proteomic approaches for targets of the CIA pathway, as well as to (v) fish for novel Fe/S proteins specific to this ancestral protist.

Current limitations include a shortage of the Fe/S cluster-dependent marker activities, not yet established method using radioactively labelled compounds, and difficulties in obtaining sufficient amount of material for mass spectrometric analyses. However, we are firmly convinced that these and other limitations can and will be overcome, allowing trypanosomes to share many of their secrets with us.

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