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Tvorba FeS center v cytoplasme Trichomonas vaginalis

FeS cluster assembly in cytosol of Trichomonas vaginalis

Diplomová práce

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Prohlášení:

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Abstract

Iron-sulfur (FeS) cluster assembly is extensively studied in model organisms, e.g. *Saccharomyces cerevisiae*, *Homo sapiens*, and more recently in *Trypanosoma brucei*. However, little is known about FeS assembly in divergent anaerobic organisms such as *Trichomonas vaginalis*, which parasites in the human urogenital tract. This parasitic protist possesses anaerobic form of mitochondria, the hydrogenosome, in which some component of FeS cluster assembly machinery (ISC) has been identified, whereas the cytosolic CIA pathway has not been studied so far.

Our work deals mainly with TvIscU, a component of ISC pathway, and *T. vaginalis* CIA pathway. We suggest that both hydrogenosomal and cytosolic FeS cluster assembly pathways of this parasite differ from typical models. We examined possible ISC-CIA relationship. Next, we found homologues for several key components involved CIA machinery, namely Nbp35, Cfd1, Nar1, Cia1 and Cia2. However, we did not identify any homologous proteins to Tah18, Dre2 and Mms19. We expressed identified proteins with HA-tag and localized them by cell fractionation and immunofluorescence microscopy in *T. vaginalis*. Finally, we immunoprecipitated two Cfd1 paralogues, TvCfd1A and TvCfd1B to search for their interacting partners. The results suggest that these two paralogues interact with each other, however, we did not observe expected interaction with Nbp35.

Abstrakt

Železo-sirné klastry jsou kofaktory celé řady významných proteinů. Pro studium syntézy těchto klastrů se využívají především modelové organismy typu *Saccharomyces cerevisiae, Homo sapiens* a v poslední době také *Trypanosoma brucei.* U *Trichomonas vaginalis,* anaerobního prvoka, který parazituje v urogenitálním traktu lidí, však nebylo toto téma studováno více do hloubky. *T. vaginalis* patří mezi anaeroby, jejichž mitochondrie byla redukována na hydrogenosom. V této organele již byly identifikované některé komponenty ISC dráhy pro syntézu klastrů, zatímco proteiny, které se účastní CIA dráhy v cytosolu, nebyly u *T. vaginalis* doposud charakterizovány.

Tato práce se zabývá především TvIscU, jedním z hlavních proteinů ISC dráhy, a dále také komponenty CIA dráhy. Oba dva typy syntézy klastrů, jak v hydrogenosomech tak i v cytosolu, se zásadně liší od typického modelu založeném na studiu S. cerevisce. V této práci jsme studovali hypotetické funkční propojení ISC-CIA systému. Dále jsme identifikovali homology k charakterizovaným proteinům účastnících se CIA dráhy. Konkrétně jsme identifikovali Nbp35, Cfd1, Nar1, Cia1 Cia2, avšak nenalezli jsme proteiny homologní pro Tah18, Dre2 a Mms19. Nalezené proteiny jsme *v T. vaginalis* exprimovali značené Ha-tagem a lokalizovali je v buněčných frakcích a pomocí imunofluorescenční mikroskopie. Dále jsme imunoprecipitovali dva paralogy Cfd1 proteinu a analyzovali s nimi interagující proteiny. Předběžné výsledky naznačují, že tyto proteiny interagují spolu, namísto jejich obecně známého interakčního partnera proteinu Nbp35.

1 INTRODUCTION:

Trichomonas vaginalis is an anaerobic protist, which parasites on the epithelial tissue in the urogenital tract of human. The trichomoniasis is sexually transmitted disease, which affects about 250 mils. people. *T. vaginalis* contains reduced mitochondria called hydrogenosome. This organelle produces ATP via substrate phosphorylation and more importantly it is involved in a production prosthetic groups containing iron and sulfur.

Iron-sulfur (FeS) clusters are cofactors in various proteins that are truly indispensable for both eukaryotic and prokaryotic cells. So far only bacteria from Lactobacillus genus are proved to live without any iron in the cell. Eukaryotes employ several pathways for FeS cluster synthesis. In general, ISC pathway is situated in mitochondria, SUF machinery is in plastids, and rarely also the NIF pathway localizes to the cytosol or to mitochondria related organelles, called hydrogenosomes. All three pathways were inherited from bacterial endosymbionts (ISC, SUF) or gained by lateral gene transfer (NIF). In recent years, we learned that the distribution and function of various components of these pathways vary considerably. Moreover, additional iron-sulfur assembly (CIA) pathway evolved uniquely in eukaryotes that mediate FeS cluster formation in the cytosol. The CIA pathway appeared to be functionally dependent on the activity of ISC pathway, although the exact mechanism is unknown.

Our work is focused on FeS cluster assembly in *T. vaginalis*. This parasite is known to possess ISC machinery in hydrogenosomes, whereas putative cytosolic CIA pathway has not been experimentally investigated so far. The aims of the thesis were to characterize components of CIA pathways and ISC-CIA relationship. The results revealed significant differences regarding composition and cell localization of CIA pathway in *T. vaginalis* in comparison with generally accepted model based on *Saccharomyces cerevisiae* and mammalian cells.

2 REVIEW OF LITERATURE

1.2. Character and formation of Fe-S clusters

Iron-sulfur (FeS) clusters, an inorganic prosthetic group, are indispensable cofactors of numerous proteins acting e.g. in electron transfer, gene regulation, environmental iron sensing, etc. All these cellular processes are essential and maintain viability of every organism, with exception of *Lactobacillus* species and recently also *B. burgdorferi* that can live entirely without iron(Archibald 1983; Bruyneel, vande Woestyne, and Verstraete 1989; Aguirre et al. 2013) Moreover, recently it has been demonstrated that *Escherichia coli* can survive without any FeS cluster synthesis (Tanaka et al. 2016). The bacteria are able to grow when the 2-C-methyl-D-erythritol-4-phoshate (MEP) pathway for the synthesis of isoprenoids, which is dependent on two proteins containing FeS clusters, was substitute by recombinant mevalonate (MVA) pathway without any link to FeS proteins. Although it is possible to tolerate the loss of FeS proteins in bacteria, it seems rather inconceivable for eukaryotes with complex cell structure and complicated genome organization.

In the nature there are both, the more common homometalic FeS clusters, and heterometalic Fe-S clusters, which are rare and found mainly in enzymes with more specific functions such as nitrogenases (Georgiadis et al. 1992). The core FeS site and the simplest cluster is a single iron atom (Fe) in $^{2+}$ or $^{3+}$ oxidation state, which is tetrahedrally bound by 4 sulfur atoms with oxidation state $^{-2}$. The most common clusters in the nature consist of 2 to 4 irons and the same numbers of inorganic sulfur as well as 4 sulfides originated from cysteinyl residues of the protein backbone ([Fe2S2(SCys4)]n, and [Fe4S4(SCys4)]n) . Unique redox capacity and also enzymatic capability of clusters are given by both, sulfur and iron properties. Metal–ligand covalent bond, spin coupling and spin resonance delocalization are three main characters enable cluster to be enzymatically active and transfer electrons as redox sites of proteins (Beinert 2000). Clusters can achieve potentials ranging from over 400 mV to below -600 mV, and thus serve as excellent redox cofactor (Capozzi, Ciurli, and Luchinat 1998).

Although iron and sulfur can assemble into the cluster spontaneously *in vitro* (Malkin and Rabinowitz 1966), sophisticated biosynthetic machineries are required for the assembly of Fe-S clusters within cells. One of the reasons is that iron and sulfur elements required for the FeS

synthesis are highly toxic in soluble form within the cytoplasm (Fontecave et al. 2005).

In general, sulfur is produced by the activity of cystein desulfurase (e.g. NifS, IscS, SufS) converting L-cystein to alanin. The iron atoms are supplied by an iron donor. Finally 2electrons are provided acquired from ferrous iron and 2 more electrons come from an extra source e.g. protein reductases via electron carrier such as ferredoxin. Further arrangement of these components into cluster is coordinated via cystein residues on scaffold protein. These components are further assembled into various FeS prosthetic groups. [4Fe-4S] cluster can be easily formed from as well as cleaved into two [2Fe-2S] core sites. $[Fe_3S_4(SCys_3)]n$ cluster arises from cubane $[Fe_4S_4(SCys_4)]n$ where one iron and cysteinyl sulfur ligand were remove (Beinert 2000). Heterometalic and more complex clusters are specific cofactors of some enzymes e.g. nitrogenases, CO dehydrogenases etc. Another metals that can potentially form clusters as well are e.g. (e.g. Mo, Ni, Co, Cu).

1.3. The main pathways of Fe-S cluster assembly emerged from bacteria.

There are three types of Fe-S cluster assembly machineries originated from bacteria. Although they share various properties, they differ in distribution across bacteria clades, frequency of their use and their specific functions.

The first pathway named NIF (**ni**trogen **f**ixation) was discovered in *Azobacter vineelandii* (Jacobson et al. 1989). It was demonstrated to synthesize very complex clusters of nitrogenases - nitrogen fixation proteins. These clusters can be very complex (P-cluster) or they contain besides iron an additional metal atom e.g. Mo (Dean, Bolin, and Zheng 1993).Importantly, the deletion of the genes in *nif* operon did not affect the viability of the cells completely (Zheng et al. 1998). As it was expected an additional system for FeS cluster assembly was identified in this bacterium that was named ISC (**i**ron **s**ulfur **c**luster). This pathway produces clusters for wide range of Fe-S proteins occurring throughout the cell cycle, therefore acts primarily as a housekeeping system maintaining the cellular homeostasis and viability (Takahashi and Nakamura 1999; Tokumoto and Takahashi 2001). This type of clusters synthesis was comprehensively studied in *Escherichia coli*, where the third type of Fe-S cluster assembly was discovered afterward (Patzer and Hantke 1999; Takahashi and Tokumoto 2002). The so-called SUF (**su**l**f**ur mobilization) pathway is widely distributed across bacteria and archaebacteria domains. It was designated to substitute ISC pathway during inconvenient conditions such as Fe starvation, oxidative stress or in specific

environment (Nachin et al. 2001; Zheng et al. 2001; Fontecave et al. 2005). Further studies disclosed the relative impact of the SUF machinery in a different species of bacteria, thus cyanobacteria and some of archeabacteria use SUF pathway entirely or at least preferentially compared to ISC (Bandyopadhyay, Chandramouli, and Johnson 2008).

Each of these systems originated in bacteria has a possible equivalent in eukaryotic cell.

1) The ISC is present in every mitochondria or mitochondria like organelle of eukaryotes that evolved from from α-proteobacteria (Tachezy, Sanchez, and Muller 2001; Lill and Mühlenhoff 2005; Richards and Van Der Giezen 2006). This system is indispensible indeed as it forms iron-sulfur clusters utilized in mitochondrial Fe-S proteins. In addition, it produces a component of unclear character, most likely sulfur that is exported to cytosol and then it served for the assembly of FeS clusters in cytoplasm. (Kispal et al. 1999). FeS assembly in the cytosol is catalyzed via CIA (cytosolic iron-sulfur assembly) pathway that is unique to eukaryotes. It supplies the cell with the FeS prosthetic groups for the essential cytosolic and nuclear proteins (Lill and Mühlenhoff 2005). This link between mitochondrial, cytosolic and nuclear Fe-S systems makes the ISC machinery essential in most eukaryotic cells.

2) In eukaryotes, the SUF machinery of Fe-S cluster assembly is present in plastids, the descendents of cyanobacteria (Takahashi and Tokumoto 2002). In addition, recent studies of oxymonades revealed that *Monocercomonoides* is living entirely without mitochondria and ISC pathway in this anaerobic protist is replaced by SUF machinery that is localized in the cytosol (Karnkowska et al. 2016). SUF machinery was previously observed also in the cytosol *Blastocystis hominis*. In this anaerobic protist, SUF machinery serves as an additional system to ISC and CIA pathways and it is upregulated during oxidative-stress condition (Tsaousis et al. 2012). As another example of diversity in distribution and utilization of FeS assembly pathway can be mentioned *Pygsua biforma*, where SUF machinery is situated to MRO (mitochondria related organelle) to replace the ISC, which is apparently absent in this protist (Stairs et al. 2014).

3) We can find components of this NIF machinery in eukaryotes rarely. In such organism, the ISC pathway was replaced by the NIF system that was most probably acquired from ε-proteobacteria by lateral gene transfer (LGT) (Ali et al. 2004; Gill et al. 2007). For instance, the human intestinal parasite *Entamoeba histolytica* and its free-living relative species *Mastigamoeba balamuthii* appear to lose the ISC system completely. NIF pathway is localized presumably to the cytoplasma in *Entamoeba* (Dolezal et al. 2010). In *Mastigamoeba*, the NIF system is duplicated with distinct localization in cytoplasma and organelles (Nývltová et al. 2013). Both *E. histolytica* and *M. balamuthi* are anaerobic protists

with reduced forms of mitochondria called mitosomes, and hydrogenosomes, respectively (Tovar, Fischer, and Clark 1999; Leon-Avila and Tovar 2004; Gill et al. 2007). Whereas hydrogenosome can produce ATP molecules via substrate phosphorylation, the mitosome does not produce ATP at all. On the other side both organelles are nearly universally involved in FeS cluster production (Tachezy, Sanchez, and Muller 2001). In Entamoeba, without functional FeS assembly pathway within mitosome, this organelle accommodates the sulfate activation pathway (Mi-ichi et al. 2009),

1.4. Iron-sulfur cluster (ISC) assembly pathway

ISC assembly machinery is the main system involved in generation Fe-S proteins in both bacteria and eukaryotes. Genes for proteins involved in bacterial ISC machinery are situated by *ISC* operon (Zheng et al. 1998; Tokumoto and Takahashi 2001). These core ISC participants, namely *iscU*, *iscS*, *iscA*, *hscA*, *hscB* and *fdx*, are controlled by specific *isc* repressor. In eukaryotic cell, the ISC pathway is localized exclusively in mitochondria; nevertheless, its components are encoded in nucleus and targeted to organelle later. Majority of the proteins involved in ISC pathway in eukaryotes are homologous to its counterparts in bacteria. However, there were evolved additional members that are essential in eukaryotes for FeS assembly in mitochondria or cytosol, and that are not necessary in original bacterial machinery. The proteins that are involved in eukaryotic iron-sulfur cluster assembly are depicted in more detail farther.

Cystein desulfurase IscS (Nfs1) is the key protein providing the inorganic sulfur atom for *de novo* Fe-S cluster synthesis (Zheng et al. 1993; Kispal et al. 1999). Its substrate - L-cystein is converted to alanin and released sulfur atom is bound to catalytic cystein residue (C328 in *E. coli*) as a persulfide (Zheng et al. 1994; Kato et al. 2002).

Isd11 protein, which is unique for eukaryotes, is required to stabilize the cystein desulfurase Nfs1 (Adam et al. 2006; Wiedemann et al. 2006). The sulfur atom in S^0 oxidative state is further transfer to IscU scaffold domain. Next, S^0 is reduced to S^{2-}. The required electrons are supplied by ferredoxin (Nakamura, Saeki, and Takahashi 1999). Iron in the Fe^{2+} state is imported to mitochondria through Mrs3 and Mrs4 channels (Foury and Roganti 2002; Mühlenhoff et al. 2015). Frataxin is a potential iron donor, however its involvement and its particular function has to be further elucidated (Layer et al. 2006). Moreover, frataxin might be involved also in iron storage (Gerber, Mühlenhoff, and Lill 2003; He et al. 2004). Which component, iron or sulfur, is bound first to the site of the cluster formation is still not clear.

IscU (Isu) interacts directly with IscS and the subsequent conformational change enables the formation of the [2Fe-2S] cluster *de-novo* (Urbina et al. 2001). The IscU scaffold domain acting as a homodimer assembles two [2Fe-2S] cluster per molecule (Fu et al. 1994). Explicitly, IscU binds [2Fe-2S] cluster per monomer while interacts with IscS. However, after detachment of the desulfurase, IscU is able to dimerize and subsequently form more complex [4Fe-4S] cluster (Adrover et al. 2015). Next, the cluster is transferred to apoprotein. IscA protein family and Nfu are known to assist during the formation or transfer of [4Fe-4S] clusters (Pelzer et al. 2000; Tong et al. 2003; Mühlenhoff et al. 2011; Sheftel et al. 2012). Moreover, they were believed to function in bacteria as alternative scaffold domains previously (Krebs et al. 2001; Tong et al. 2003; Angelini et al. 2008). Nevertheless, the ability of the proteins to bind [1Fe-2S] and [4Fe-4S] cluster might be rather a consequence of their role in Fe-S cluster transfer (Gupta et al. 2009; Navarro-Sastre et al. 2011). The occurrence of IscA and Nfu is not universal and indispensable and they are supposed to have various roles in different organisms. IscA and its yeast homologues (Isa, Iba57) are able to bind both, Fe-S cluster or single iron atom (Ding and Clark 2004; Mühlenhoff et al. 2011). The recent view on the role of A-type proteins suggest their involvement during formation 4Fe-4S cluster of various apoproteins in mitochondria (Mühlenhoff et al. 2011).

Finally, the proteins of the chaperon system - mainly Hsp70, Grx5, Jac1, Mge1, and recently identified novel FeS factor Grx5 facilitate the transfer of the cluster to targeted apoprotein (Uzarska et al. 2013).

Interestingly, some of the ISC components mentioned above could also have dual localization and thus fulfill additional tasks. They appear to have play different roles depending in which cellular compartment are localized (Mühlenhoff et al. 2004; Nakai et al. 2004). Apart from FeS cluster biogenesis, IscS was proven to have different functions such as thiomodification of tRNA (Nakai et al. 2001; Mihara and Esaki 2003). In higher eukaryotes, the Isu proteins can also localize to cytoplasm (Tong and Rouault 2000). However, it does not play any role in FeS metabolism, since it differs in N-terminus (Tong and Rouault 2000). Therefore, possibility that mitochondrial ISC pathway can function as well in cytosol or nucleus was excluded (Gerber et al. 2004). On the other side, these study is inconsistent with further research which presents the human cytosolic ISCS and ISCU proteins to be involved in Fe-S assembly outside the mitochondria (Li et al. 2006).

The compound containing sulfur (X-S), which is produced by ISC and required for further maturation of Fe-S clusters in the cytosol, is exported via Atm1 to the cytosol (Kispal et al. 1999). Most likely Erv1 and glutathione molecule assist during the X-S transport as well

(Sipos et al. 2002; Mesecke et al. 2005).

1.6. Cytosolic Iron-sulfur cluster assembly (CIA)

The CIA machinery is a unique and ubiquitous system for biogeneses of Fe-S proteins in the cytosol of eukaryotic cells (Lill and Mühlenhoff 2005). The CIA members identified thus far are Nbp35, Cfd1, Nar1, Cia1, Cia2, MMS19, Dre2 and Tah18 (Netz et al. 2014)

Moreover, Grx3 and Grx4 of the glutaredoxin protein family are proposed to be involved and essential for function of CIA, regarding their role in iron trafficking and iron homeostasis ((Li et al. 2009; Mühlenhoff et al. 2010).

The main experimental work was performed and most of information about CIA was revealed using *Saccharomyces cerevisiae* as a model organism. Hence, the majority of following overview is based on this yeast model.

The *de-novo* formation of the cluster in cytoplasm takes place on a scaffold domain comprising Cfd1 and Nbp35 (Vitale, Fabre, and Hurt 1996; Roy et al. 2003; Hausmann et al. 2005). The proteins are assembled as a heterotetramer (Netz et al. 2007). Both proteins belong to the large protein family of P-loop NTPases performing various cellular functions (Leipe et al. 2002; Roy et al. 2003). N-terminus of Nbp35 contains four cystein residues that binds stable [4Fe-4S] cluster. Thus, Nbp35 belong among the cytosolic Fe-S proteins, and simultaneously it is member of Fe-S cluster production machinery (Hausmann et al. 2005).

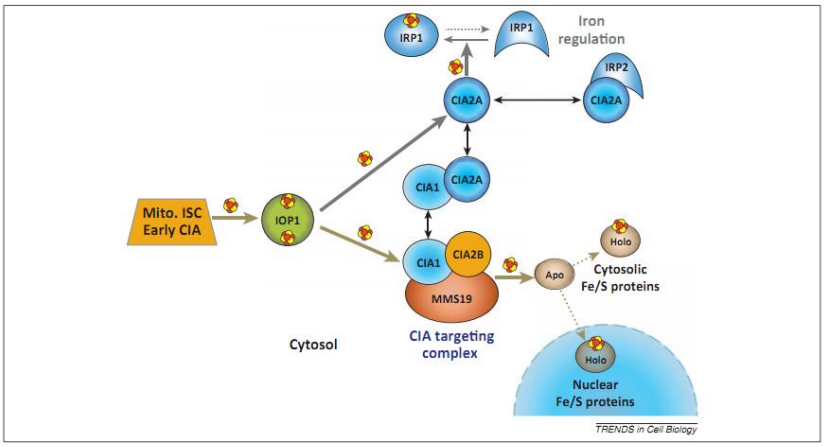

Figure1. CIA pathway by Netz et al. 2014

The function of the protein and so the entire scaffold complex is dependent on the presence of NADH-Dre2-Tah18 electron transfer chain that provides electrons for assembly of the [4Fe-4S] cluster on Nbp35 (Netz et al. 2010, 2012). In addition to this intrinsic cluster, Nbp35 and Cfd1 complex bind two labile [4Fe-4S] clusters via cysteins residues in CX2C conserved motif at the C-termini of both proteins (Netz et al. 2012). Additionally, the NTP binding motif is necessary for the loading of the cluster, although any ATPase or GTPase activity was observed neither for Nbp35 nor Cfd1 (Netz et al. 2012). The Cfd1 was first identified cytosolic FeS factor however it is absent in several protist lineages and in plants (Tsaousis et al. 2014). In these organisms, Nbp35 is acting as homodimer (Bych et al. 2008).

Nar1 shows sequence homology with iron-only hydrogenases (Nicolet, Cavazza, and Fontecilla-Camps 2002). Nevertheless, it completely lacks the hydrogenase activity. Nar 1 retains two 4Fe-4S clusters on C- and N- terminus of the protein (Balk et al. 2004). It serves as a link between early and late steps of CIA pathway. Nar1 directly interacts with Nbp35 and also with CIA targeting complex (Cia1, Cia2, Mms19) *in vivo* (Balk et al. 2005; Hausmann et al. 2005; Seki et al. 2013; Stehling et al. 2013). However, its precise role needs to be elucidated. Interestingly, two homologues were observed in human cells named IOP1, and IOP2 (Barton and Worman 1999; Song and Lee 2008). While IOP1 is essential for cytosolic Fe-S proteins, IOP2 may have different cellular role and it is localized in nucleus (Barton and Worman 1999; Song and Lee 2011).

Nar directly interacts with the CIA targeting complex, which deliver FeS cluster to targeted protein. In the yeast, the complex comprises of Cia1, Cia2 and Mms19. Cia1, as a member of WD40-repeat protein family, most probably recognizes its partner proteins through the surfaces and acts as a docking site for transfer of the Fe-S cluster to targeted protein (Balk et al. 2005; Xu and Min 2011). Cia2, a small protein with reactive cystein residue, and Mms19assembly together with Cia1 and mediate the transfer of the cluster to targeted proteins (Weerapana et al. 2010; Gari et al. 2012; Stehling et al. 2012, 2013). Moreover, the proteins involved in the targeting complex are able to form different subcomplexes for specific targeting of the cluster to apoproteins (Paul and Lill 2015).

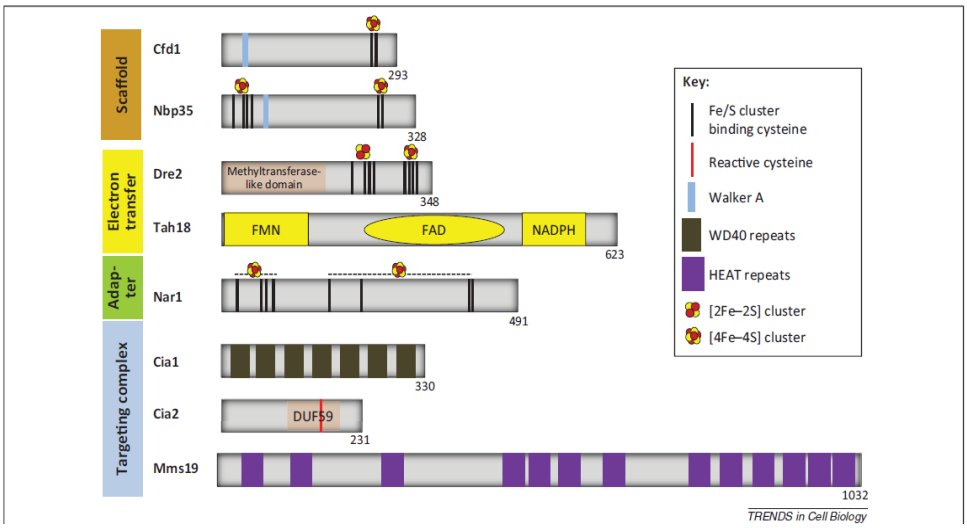

Figure 2: Function domains of the CIA proteins by Netz et al. 2014

Interestingly, some CIA proteins, e.g. Nbp35 and Cia1, have a dual localization in the cell. In addition to cytosol, they were observed in the nucleus too (Vitale, Fabre, and Hurt 1996; Balk et al. 2005). Therefore, it was proposed that CIA machinery could be present in both, cytosol and nucleus, depending on which compartment the targeted Fe-S proteins occur in (Hausmann et al. 2005). Recent surveys suggest the localization of the CIA machinery more likely to cytosol as it was shown for CIA targeting complex acting with XPD (xenoderma pigmentosum group) helicase which is an intrinsic nuclear protein (Vashisht et al. 2015).

1.7. The Cytosolic and Nuclear Fe-S proteins

The importance of various Fe-S proteins in the cytosol and nucleus can be explained by their involvement in various aspects of DNA replication, genome stability, ribosome or tRNA biosynthesis and modification. Cells with defect in cluster assembly were observed to have DNA defects, as they are more sensitive to mutagens and DNA damage agents (Stehling et al. 2012). As examples of essential proteins containing Fe-S cluster we could mention polymerases, helicases, DNA glycosylases, translation initiation factor Rli1 etc. (Oliver Stehling and Roland Lill 2013; Netz et al. 2014; Paul and Lill 2015). Rli1 was the first identified cytosolic Fe-S protein and its function appeared to be both, ISC and CIA, dependent (Kispal et al. 2005). In the cells depleted for *rli1* was impaired formation of

ribosome (Yarunin et al. 2005). Moreover, Rli1 directly interacts with at least one translation initiation factor eIF3 (Dong et al. 2004; Yarunin et al. 2005). These particular outcomes farther depict and give more evidence about the versatility and importance of FeS proteins.

Moreover, several components of CIA machinery themselves play additional roles within the nucleus. The proteins of CIA targeting complex – Cia2, Cia1 and Mms19 were observed to interact with enzymes participating in DNA metabolism. As these proteins interact and form various complexes, comprising of selected two or all three components, they can operate in different location and processes. For instance, human CIA2 is present in two paralogues – CIA2A, CIA2B, which both play different roles. CIA2B is a member of targeting complex, while CIA2A is required for the maturation of integral Fe-S cluster of the iron regulatory protein (IRP1) and thus control iron homeostasis in the cell (Stehling et al. 2013).

1.8. Iron-sulfur cluster assembly in *Trichomonas vaginalis*

Trichomonas vaginalis, is sexually transmitted pathogen of humans, which parasites onthe host epithelial tissue in urogenital tract. Members of the Trichomonadidae family contain hydrogenosomes, the anaerobic form of mitochondria (Müller and Lindmark 1973). The energy metabolism of hydrogenosomes is dependent on substrate-level phosphorylation. Pyruvate or malate is converted to CO_2, acetate and hydrogen with concomitant ATP synthesis (Hrdy et al. 2008). The function of this pathways is dependent on several FeS proteins, including pyruvate:ferredoxin oxidoreductase, ferredoxin, hydrogenase, NADH dehydrogenase. The hydrogenosomes contain the complete set of components of ISC pathway. In view of the fact that the genom of *Trichomonas* is highly repetitive, most of proteins ivolved in ISC machinery are encoded by multiple gene copies (Carlton). Cystein desulfurase, IscS, is present in two paralogues copies, but only one copy is expressed (Tachezy et al. 2001, Sutak et al. 2004). *iscu* is the only one single-copy gene in *T. vaginalis*. However, IscA and Nfu are encoded by multiple gene copies, four and three respectively, that are all upregulated in iron depleted conditions (Carlton et al. 2007, Beltran et al. 2013). Hence, it is not known whether they assist during the assembly and transfer of the cluster or whether they even substitute IscU protein as alternative scaffold domains. Ferredoxin (Fdx), the donor of electrons for the cluster assembly is reduced by pyruvate-ferredoxin oxidoreductases (PFO). Fdx is present in 7 copies, which are supposed to replace each other as the knockout of the most abundant Fdx1 has not significant effect for the cells (Carlton et al. 2007, Land et al. 2004). Frataxin (Fra) and Isd1, are present in 2 copy-genes, which are

both expressed (Beltran et al. 2013). The chaperon system comprises of Jac1, Mge1 and Hsp70 (Beltran et al. 2013). These proteins are universally required to dissociate Fe-S cluster from IscU and further to stabilize and transfer it into apoproteins (Uzarska et al. 2013, Lill et al 1999, Voisine et al. 2001, Deloche and Georgopoulos 1999, Dutkiewicz et al. 2003). Ind1, P-loop Ntpase, is relative of Nbp35 and Cfd1 acting in the cytosol. It is present in 4 copies and most likely it assists in maturation of complex I in hydrogenosomes as shown previously for mitochondria (Sheftel et al. 2009). Additionally, hydrogenosomes possess hydrogenase maturases (HydE, HydG, HydF) that are necessary for formation of H-cluster in hydrogenases (Putz et al. 2006).

The specific counterparts of transport machinery, which facilitate the connection between ISC and CIA machineries, e.g. Erv1, Atm1 and glutathion were not identified in *T. vaginalis* (Rada et al. 2011).

The CIA components for which corresponding genes were found in *T. vaginalis* genome include - Cfd1, Nbp35, Nar1, Cia1 and Cia2 (Carlton et al. 2007). Interestingly, some core components of CIA pathway in *Trichomonas* are missing, particularly Tah18, Dre2, Grx3 and Grx4. However, in contradiction to indispensability of these proteins in aerobic eukaryotes, it seems to be a general trend in anaerobes (Tsaousis et al. 2014).

3 THE AIMS OF THE THESIS

This study deals with an iron-sulfur cluster assembly in *Trichomonas vaginalis.*

The main goals of the thesis were:

1) To establish and use the gene knockout technique for TvIscU and observe its consequence for FeS cluster assembly in *T. vaginalis*
2) To identify the proteins that are involved in putative CIA pathway of *T. vaginalis*
3) To express the recombinant CIA proteins in *T. vaginalis* and localize them using subcellular fractionation and immunofluorescence microscopy.
4) To immunoprecipitate TvCfd1A-HA and TvCfd1B-HA to find their putative partner proteins.

4 MATERIALS AND METHODS

4.1 Cultivation of the organisms

4.1.1 Cultivation of T. vaginalis

An axenic culture of T. vaginalis T1 (TVT1) strain (J.H.Tai, Taiwan) was grown in Diamond´s medium (TYM) pH 6,2 in semianaerobic conditions at 37 °C (Diamond, L. S. (1957).

4.1.2 Cultivation of E. coli

For cloning was used *E. coli* strain TOP10 or XL-1 Blue. The bacteria were cultivated in LB medium at 37 °C on the shaker (220 rpm). Transformed cells were grown on LB plates with X-gal (30 µl of the storage solution), Ampicilin (100 µg/ml) or Kanamycin (50 µg/ml) depending which vector was used. For long period E. coli cells were stored in LB medium with 20 % glycerol in -80 °C.

4.1.2.1 Cultivation media

Medium TYM pH 6,2:

K_2HPO_4 (Sigma)	0,8 g
KH_2PO_4 (Sigma)	0,8 g
ascorbic acid (Sigma)	0,2 g
L-cystein (Sigma)	1 g
maltose (Sigma)	5 g
yeast extract (Oxoid)	10 g
trypton (Oxoid)	20 g
ammonium ferric citrate (2,28/100 ml)	1 ml
(agar	0,5 g)
H_2O	fill to 900 ml
inactivated horse serum (Gibco)	100 ml (added after sterilization)

LB medium:

LB medium (Sigma)	20 g
H_2O	500 ml

LB plates:

LB agar	17 g
H_2O	500 ml

SOC medium (pH 7):

trypton	2 g
yeast extract	0,5 g
NaCl	0,058 g
250mM KCl	1 ml
H_2O	fill to 100 ml
20% glucose (sterile)	1,8 ml (added after sterilization)
2M $MgCl_2$ (sterile)	0,5 ml (added after sterilization)

Store in -20 °C.

X-gal:

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galaktosid)	100 mg
N,N'-dimethyl-formamid	2 ml

Store in -20 °C and protect it from light.

Antibiotics:

Ampicilin (Sigma)	100 mg/ml
Geneticin (G418) (Sigma)	200 µl/ml
Kanamycin (Sigma)	50 mg/ml
Penicillin-Streptomycin (Sigma)	100 000 IU/ml

4.2 Buffers and solutions

10x PBS:

NaCl	8 g
KCl	0,2 g
$NaH_2PO4 \cdot 12H_2O$	1,53 g
KH_2PO_4	0,2 g
H_2O	fill to 1000 ml

8M Urea buffer

$NaH_2PO_4 \cdot H_2O$	13,8 g
Tris base	1.2 g
urea	480,5 g
H_2O	fill to 1000 ml

Adjust the pH of the buffers.

25mM DSP solution

1 mg of DSP (dithiobis[succinimidylpropionate]) per 100 µl of DMSO

4.2.1 SDS and Western blot analysis

Coomassie Brilliant Blue solution

Coomassie Brilliant Blue	200 mg
ethanol	225 ml
H_2O	225 ml
acetic acid	50 ml

Blotting buffer:

10x concentrated SDS buffer	100 ml
methanol	200 ml
H_2O	700 ml

Blocking buffer:

dry milk (Laktino)	10 g
Tween 20 (Sigma)	200 µl
PBS	200 ml

Ponceau S:

Ponceau S	0,5%
glacial acetic acid	1%

4.2.2 Immunofluorescence

2x PEM buffer, pH 6,9:

PIPES	30,2 g
0,5M EGTA	2 ml
1M $MgSO_4$	100 µl

Mix the chemicals and add NaOH until the solution is transparent. Set the pH 6,9.

H_2O	fill to 500 ml

Sterilize using autoclave.

PEMBALG:

1x PEM pufr	100 ml
BSA	1 g
lysin	1,8 g
cold water fish skin gelatin (Sigma)	0,5 g

Dissolve the gelatin in approximately 30 ml sterile H_2O. Heat to 45°C. Cool to room temperature and add BSA and lysin. Fill up to 50 ml using sterile H_2O and add 50 ml of 2x PEM. Store in -20 °C.

4.2.3 Antibodies

Primary antibodies:

anti-HA monoclonal antibody (mouse IgG), (Exbio) (1:400 western blot, 1:1000 immunofluorescence)

anti-ME (Malic Enzyme) polyclonal antibody (rabbit), (Eurogentec) (1:500)

anti-Fdx1 polyclonal antibody (rat), (provided by Patricia Johnson) (1:1000)

Secondary antibodies:

A. Western blot analysis:

antibody against mouse conjugated with alkaline phosphatase (ICN/CAPPEL) (1:2000)

antibody against rat conjugated with alkaline phosphatase (ICN/CAPPEL) (1:2000)

antibody against mouse IgG conjugated with horse radish peroxidase (Novex ECL) (1:2000)

B. Immunofluorescence

antibody against mouse IgG labeled with ALEXA FLUOR 594 (Molecular Probes) (1:1000)

antibody against rabbit IgG labeled withALEXA FLUOR 488 (Molecular Probes) (1:1000)

antibody against rat IgG labeled with ALEXA FLUOR 594 (Molecular Probes) (1:1000)

4.2.4 Fractionation of T. vaginalis cells

ST buffer, pH 7,2:

sacharose	42,85 g
Tris base (Sigma)	0,6 g
KCl	18,5 mg
H_2O	fill to 500 ml

Adjust the pH 7,2. Store in -20 °C.

Protease inhibitors:

Tosyl-lysin-chlormetylketon (TLCK)	25 mg/ml
Leupeptin	5 mg/ml

4.2.5 Immunoprecipitation

Boiling buffer, pH 7,4:

50mM Tris base

1mM EDTA

1% SDS

Incubation buffer pH7,4:

50mM Tris base

150mM NaCl

5mM EDTA

1% Triton (TX100)

4.3 General protocols used in the thesis

4.3.1 Transformation of T. vaginalis using the electroporation time-constant protocol

For transformation of T. vaginalis by circular or linear DNA we used following protocol:

1. all steps are done sterile
2. start with cell culture 500 ml for approximately 6 shots
3. spin the cells at 1250 x g, 10 min., 4 °C and weigh the pellet
4. use 500 ml of fresh ice cold TYM 6,2 medium to 1 g of the pellet and gently resuspend the cells
5. cool the 4 mm cuvettes (Bio-Rad) and G 23 needle to 4°C
6. pass the cells once through G 23 needle
7. pipette 300 µl of cell suspension to cuvette and add 50 µg of circular or 100 µg of linear DNA
8. electroporate using the time-constant protocol: 175 ms, 350 V (GenePulser Xcell, Bio-Rad)
9. transfer the cells immediately to 50 ml of fresh tempered TYM 6,2 medium with added Penicillin-Streptomycin (1000 IU/ml)
10. to cells transformed by circular plasmid add G418 (200 µg/ml) after 4 hours / to cells transformed by linear DNA add G418 (110 µg/ml) after 6 hours
11. let undergo the selection for max 7 days
12. passage the cells to 15 ml culture tube with fresh TYM 6,2 medium (G418 added)

Optionally (for T. vaginalis cells transformed by linear DNA to get clonal population)

11. 17 hours after transfection take 25 ml of transformed cell culture (50 ml) and harvest by centrifugation at 1250 x g, 10 min, 4°C, simultaneously, 12,5 ml from the transformed culture keep in 15 ml plastic tube.
12. removed the supernatant and gently resuspend the pellet in 50 ml of fresh tempered TYM 6,2 medium with agar (Pen-Strep and G418 added)
13. invert the solution twice and plated it on 96-well plate
14. incubate anaerobically for 5-8 days
    pick up grown colonies and transfer it to culture tube

4.3.2 Transformation of E. coli

To transform E. coli we used following protocol:

1. thaw the cells on the ice
2. thaw the SOC medium
3. pipette ligation reaction to bacteria and incubate for 30 min
4. incubate in 42°C water bath for 30 sec
5. incubate 2 min on the ice
6. add 250 µl of SOC medium
7. incubate at 37 °C , 220 RPM, 1 hour
8. distribute the selection antibiotics and X-gal on the LB agar plate
9. distribute 200-250 µl of the transformed cells on the plate
10. incubate at 37 °C overnight
11. store the plate at 4°C

4.3.3 SDS page

For further analysis by western blot, the protein samples were dissolved in the SDS sample buffer and denaturated at 100 °C for 5 min. Then the proteins were separated under denaturating conditions using 12% or 13,5% polyacrylamide gel with SDS. As standard to determine the molecular weights we used PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific).

4.3.4 Western blot analysis

To analyze proteins samples we used following protocol:

1. soak the SDS page gel together with 6 filter papers and nitrocellulose membrane of the same size
2. stack the 3 filter papers, the nitrocellulose membrane, the gel and 3 filter papers on blotting machine (semi-dry blot Biometra)
3. blot at 1,5 mA per square cm (1 h and 10 min)
4. visualize the blotted proteins on the membrane by Ponceau S (0,5%) (1 min and wash shortly in H_2O)
5. incubate the membrane in the blocking buffer at the room temperature (2 h) or at 4 °C (overnight)

6. incubate the membrane with primary antibody in the blocking buffer at the room temperature (2 h) or at 4 °C (overnight)
7. wash the membrane 3 times (15 min) in the blocking buffer
8. incubate the membrane with secondary antibody in the blocking buffer at room temperature (1-2 h)
9. wash the membrane 2 times (15 min) in the blocking buffer and once in PBS (15 min)

incubate the membrane with substrate for alkaline phosphatase AP Fast BCIP/NBT (Sigma) or horse radish peroxidase (HRP) Novex ECL Substrate Enhancer Kit (Invitrogen life technologies

4.3.5 Immunofluorescence microscopy

To prepare immunofluorescence slides we used following protocol:

1. start with 10 ml of the culture
2. fix the cells using 1% formaldehyde at 37 °C, 30 min
3. spin the cells at 900 x g, 5 min, 4 °C
4. wash the pellet in 5 ml of PEM buffer and repeat the step 3.
5. discard the supernatant by aspiration and gently resuspend the pellet in 500 ml of PEM buffer by 1ml plastic pasteur pipette
6. distribute the cells on the silanized microscope slide (approximately 150 µl per slide) andlet it dry
7. all following steps will be held in the box with wet tissues (do not let it dry)
8. permeabilize the cells using 0,1% triton TX100 in PEM buffer, 15 min
9. remove the excess PEM buffer with 0,1% triton TX-100 and wash in PEM buffer 3 times for 5 min
10. block the slides in 0,5 ml of PEMBALG buffer, 1 hour
11. remove excess buffer and incubate in PEMBALG with primary antibody, 1,5 hours
12. remove excess buffer and wash in PEM buffer 3 times for 5, 10 and 15 min
13. remove excess buffer and incubate in PEMBALG with secondary antibody in the dark, 1 h
14. wash in PEM buffer 3 times for 5, 10 and 15 min
15. remove excess liquid

16. use Vectashield with DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) to stain the nuclei
17. cover the slides with a cover slip
18. after 1 min fix the cover slip with nail polish
19. keep the slides in a dark and use for immunofluorescence microscopy

4.4 Sources of the genomic data

BLASTP program and its extension PSI-BLAST (position-specific iterative BLAST) from the NCBI web server (blast.ncbi.nlm.nih.gov) were used to search for *T. vaginalis* homologues of proteins that were previously identified in *Saccharomyces cerevisiae*. The genomic data of *T. vaginalis* are available on trichdb.org (Carlton et al. 2007).

4.5 Genes and proteins used in the thesis

The data are available on TrichDB.

Ferredoxin 1	TVAG_003900
TvIscU	TVAG_432650
TvCfd1A	TVAG_390510
TvCfd1B	TVAG_447450
TvNbp35A	TVAG_157490
TvNbp35B	TVAG_178350
TvCia1A	TVAG_350560
TvCia1B	TVAG_441510
TvCia2	TVAG_198590
TvNar1	TVAG_129510

4.6 Isolation of genomic DNA

Genomic DNA of *T. vaginalis* T1 and its transformed strains were isolated using High Pure PCR Template Preparation Kit (Roche).

4.7 Preparation of polyclonal rat anti-TvIscU antibody

4.7.1 PCR amplification of tviscu

The gene for TvIscU was amplified by PCR (polymerase chain reaction) using specific primers and reaction conditions. As a template, we used genomic DNA of *T. vaginalis*.

Primers for PCR amplification of *tviscu* are shown in the table below. Primers contain restriction sites (RS) for NdeI and BamHI restriction enzymes (RE) for cloning to pET42b vector. The gene is 459 bp long.

Name		RE	RS	Sequence (5´-3´)
tviscu	forward	NdeI	CATATG	TGACCATATGCTCGCTGCAGTTTCCCGC
	reverse	BahmHI	GGATCC	TGACGGATCCTTTAGCAGCAGCCTTCTTGGC

PCR protocol:

DNA template	1 µl
primer forward(10mM)	1 µl
primer reverse (10mM)	1 µl
dNTP (10mM)	0,5 µl
Mg_2SO_4 (20 mM)	2,5 µl
10x PFU buffer	2,5 µl
H_2O	16,5 µl
PFU polymerase	0,5 µl

Cycle:

3:00 95°C, 30 x [0:30 95°C, 0:30 55°C, 2:00 72°C], 7:00 72°C

PCR product was analyzed by horizontal electrophoresis using 1% agarose gel. The DNA was visualized by SYBRSafe dye (Invitrogen). GeneRuler™ DNA Ladder Mix (Fermentas) was used as a standard.

4.7.2 Cloning and expression of tviscu

PCR product was purificated by high Pure PCR Product Purification Kit (Roche) and ligated to the pGEM-T Easy vector (Invitrogen) using manufacturer protocol. Then the ligation product was transformed to *E. coli* XL1-Blue competent cells that were subsequently incubated on LB plates with ampicillin (100µg/ml) and X-Gal over night at 37°C. White colonies were picked up and screened for the insert using PCR method with specific primers. Positive colonies were incubated in 5 ml of LB medium with ampicillin (100µg/ml) over

night at 37°C. After that the cell culture was pelleted (6000 x g, 10 min) and plasmid was isolated using the QIAGEN Miniprep Kit.

Plasmid was further digested by the NdeI and BamHI restriction enzymes. Products of digestion were separated in 1% agarose gel. The band corresponding to *tviscu* was excised and isolated using QIAGEN Gel Extraction Kit. This insert was ligated into the pET42b plasmid (Novagen), adding a poly-His tag to the 3' end of the open reading frame of the *tviscu*. The ligation product was transformed to the XL1-Blue competent cells. The cells were incubated on LB plates with kanamycin (50μg/ml) over night at 37 °C. Colonies were screened for the insert using PCR with insert-specific primers. Positive colony was picked and incubated in 5 ml LB with kanamycin (50 μg/ml) over night at 37 °C. The cell culture was pelleted and the plasmid was isolated using the QIAGEN Miniprep Kit. The correct insertion of *tviscu* into the pET42b plasmid was verified by sequencing. DNA was further used for transformation of *E.coli* and production of the recombinant protein.

4.7.3 Production of recombinant protein

The *E. coli* culture expressing TvIscU incubated in 2 l of LB medium on shaker at 37 °C. When the optical density at 600 nm reached 0.6 we added IPTG to final concentration of 250 μM and incubated the culture for 4 hours.

4.7.4 Purification of recombinant proteins

The protein purification was done under denaturing conditions in 8M urea buffers with different pH. To purify the recombinant His-tagged protein, we used following protocol:

1. spin the cells and resuspend the pellet in 15 ml of 8M urea buffer pH 8
2. sonicate the cells at amplitude 60, 15 sec. pulses, 5 times (sonicater Vibra Cell)
3. spin the lysate at 17 000 x g for 20 min
4. incubate the supernatant with 1 ml of PerfectPro NiNTA Agarose (5 Prime) for 15min and then transfer the mixture to a column
5. collect the flow-through
6. wash the column two times with 4ml of 8M urea buffer pH 6.4 and collect flow-through

7. wash the column four times with 500 µl of 8M urea buffer pH 5.9 and collect each flow-through (D1-4)
8. wash the column four times with 500 µl of 8M urea buffer pH 4,5 and collect each flow-through (E1-4)

Next, we analysed the fractions using SDS-PAGE and western-blot with an anti-His antibody. Fractions (E3, E4) that contained the purified protein were separated using a preparative electrophoresis. We stained the gel in Coomasie Brilliant Blue and then washed in destain solution. Finally, we excised band corresponding to our protein and confirm it by mass spectrometry. Gel was washed in PBS buffer and homogenized. The rat was immunized by four doses of acrylamide gel each containing 250 µg of the protein. We separate the serum and used as the anti-TvIscU antibody.

4.8 The knockout of *fdx1* and *tviscu*

For gene knockout by homologous recombination we used plasmids pFdKO and pTvIscUKO. We obtained the pFdKO by Dr. Sven Gould. It contains *neomycin phosphotransferase* cassette and *ampicilin* cassette. The *neomycin phosphotransferase* is flanked by 5´UTR and 3´UTR of *fdx1*. The 5´UTR fragment is cloned through NotI/KpnI and 3´UTR fragment is cloned through BamHI/HindIII. We modified pFdKO by replacement of both *fdx1* UTRs with *tviscu* 5´and 3´UTR fragments.

The primers that were used for amplification of *tviscu* UTRs are shown.

Name		RE	RS	Sequence (5´-3´)
***tviscu* 5´UTR**	forward	NotI	CGGCCG	AGTGCGGCCGCATCATAATCTTTTTATCAG
	reverse	KpnI	GGTACC	AGTGGTACCTCTGTTGAAATGAAATTAGGGAA
***tviscu* 3´UTR**	forward	BamHI	GGATCC	AGTGGATCCGCATTTCATGCATTGTTCTC
	reverse	HindIII	AAGCTT	AGTAAGCTTAAAGAGGCCCAATCCATTTT

We transformed E.coli TOP10 by plasmid pFdKO/pTvIscUKO and selected the bacteria cells using Ampicilin (100µg/ml). Positive colony was picked up and incubated in 100 ml of LB medium. We isolated pFdKO using Wizard Plus Midipreps (Promega). The concentration of DNA in midipreps was measure by NanoDrop ND – 1000 Spectrophotometer.

4.8.1 Digestion of pFdKO and pTvIscUKO

For gene knockout we needed high amount of linear DNA. We digested the plasmid using ScaI, NotI and HindIII restriction enzymes (RE) and then we used shrimp alkaline phospshatase (SAP) to dephosphorylate the 5´and 3´ends of DNA (all chemicals by Thermo Scientific). Due to loses during digestion and DNA precipitation we started with higher amount of DNA that was aproximatelly 130 µl of plasmid DNA (1100 µg/ml). To digest the pFdKO/pTvIscUKO we used following protocol:

plasmid DNA (1100 µg/ml)	130 µl
10x FastDigest buffer	50 µl
dH_2O	310 µl
ScaI	10 µl
2 hour, 37°C	

Precipitate the DNA (25 EtOH : 1 NaOAc pH 5,4) to 50 µl and proceed with further digestion.

DNA	50 µl
10x Red buffer	15 µl
SAP	6,25 µl
dH_2O	78,75 µl
1 hour 45 min, 37°C	

Inactivate the SAP at 65°C, 15 min. Use the sample for further digestion.

sample with DNA	150 µl
10x Red buffer	50 µl
H_2O	280 µl

NotI	10 µl
HindIII	10 µl

overnight, 37°C

Precipitate the DNA to 50 µl (2000 µg/ml) . Proceed with *T. vaginalis* transfection.

T. vaginalis cells were transfected by the linear DNA using electroporation time-constant protocol (chap. 2.3.1). The transformed cells were analyzed after seventh passage using western blot (chap. 2.3.4) with the polyclonal rat anti-Fdx1 antibody.

PCR reaction for *fdx1* was used to test the knockout cells. Primers hybridize with complementary DNA at ORF (forward) and 3´UTR (reverse) of the gene. The fragment is 609 bp long.

Name		Sequence
***fdx1* ORF**	forward	CAGTGGTGAAAACGATGGTG
***fdx1* 3´UTR**	reverse	CGTGCAGAATGGTTGCAT

PCR protocol:

Template DNA	1 µl
primer forward (10 mM)	1 µl
primer reverse (10 mM)	1 µl
PCR Master Mix (Promega)	10 µl
H_2O	7 µl

Cycle:

3:00 95°C, 30 x [0:30 95°C, 1:0 57°C, 1:00 72°C], 5:00 72°C

We separated the PCR products as previously for *tviscu.*

4.9 PCR amplification of genes for CIA proteins

Genes were amplified by PCR (polymerase chain reaction) with specific primers and reaction conditions. Genomic DNA of *T. vaginalis* was used as a template. Primers that were used for PCR amplification of the genes for CIA proteins are shown in table below. Primers contain restriction sites (RS) for NdeI and BamHI restriction enzymes (RE) for cloning to TagVag2 vector. Forwad primers of *tvnbp35a* and *tvcfd1b* contain modified nucleotides to mutate restriction sites of BamHI and NdeI, respectively.

Name		RE	RS	Sequence (5´-3´)
tvnbp35a	forward	NdeI	CATATG	CTAGCATATGAGCTGCTCAGGAAATTGTGGCT CCTGTTCACATGCTGGCACA
	reverse	BahmHI	GGATCC	GACTGGATCCTGCAGAGAGTTTCTGCTGGATCTTTTC
tvnbp35b	forward	NdeI	CATATG	CTAGCATATGTCTTGTAACGGAGATTGC
	reverse	BahmHI	GGATCC	GACTGGATCCTTTTGATACAGCATCCATGAT
tvcfd1a	forward	NdeI	CATATG	CTAGCATATGAGCACACAGAATTTCATC
	reverse	BahmHI	GGATCC	GACTGGATCCTTGAGCTGCTTTTTGTTGTGG
tvcfd1b	forward	NdeI	CATATG	ATACATATGAGCACGCAAAATTTCATCCTTGTCATGT CTGGCAAGGGAGGAGTTGGAAAGTCCACAACAGCA GCTAATATAGCACGTGCTTATGCTGCCAAATATGGAAAA
	reverse	BahmHI	GGATCC	GACTGGATCCTTGTGCTACAGGTTGGGCTGG
tvnar1	forward	NdeI	CATATG	CTAGCATATGAGCGCAGACCCTGCTGCT
	reverse	BahmHI	GGATCC	GACTGGATCCCCAAGCGAATTGATCTTTTTC
tvcia1a	forward	NdeI	CATATG	CTAGCATATGAAAGTCTCGACCATCGAT
	reverse	BahmHI	GGATCC	GACTGGATCCTTTTGATTTTTCTATATCGAA
tvcia1b	forward	NdeI	CATATG	CTAGCATATGAAAACTGTTTTTGAGTGC
	reverse	BahmHI	GGATCC	GACTGGATCCGTATATTGAACTGAGTTCATG
tvcia2	forward	NdeI	CATATG	CTAGCATATGGCAGCAAATCCAAATCCA
	reverse	BahmHI	GGATCC	GACTGGATCCTTCCTCTTCATCATCACATGC

UTR´s of *tvnbp35b* (572 bp) and *tvcfd1a* (616 bp) were amplified by PCR (polymerase chain reaction) with specific primers. Primers contain restriction sites (RS) for SacII and NdeI restriction enzymes (RE) for cloning to TagVag2 vector.

Name		RE	RS	Sequence (5´-3´)
***tvcfd1a* 5´UTR**	forward	SacII	CCGCGG	ATACCGCGGATTGAAATCATAAATCTT
tvcfd1a	reverse	NdeI	CATATG	ACCCATATGTCACAAAATCTTAGTAAA
***tvnbp35b* 5´UTR**	forward	SacII	CCGCGG	ATACCGCGGTTCTGTCTAAAGCTTTAA
tvnbp35b	reverse	NdeI	CATATG	ACCCATATGTATGATTTTTAAGTTGTTCAG

PCR protocol (chemicals New England Biolabs):

Template DNA	1 µl
primer forvard (10mM)	1,25 µl
primer reverse (10mM)	1,25 µl
5x Q5 reaction buffer	5 µl
5x Q5 High GC enhancer	5 µl
10 mM dNTPs	0,5 µl
Q5 High-fidelity DNA polymerase	0,25 µl
dH_2O (PCR sterile)	10,75 µl

PCR Cycle:

0:30 98°C, 30 x [0:10 98°C, 0:45 65°C, 1:25 72°C], 2:00 72°C

4.10 Cloning and expression of the genes for CIA proteins

We digested the PCR products and TagVag2 vector by NdeI and BamHI or SacII and NdeI restriction enzymes in FastDigest Buffer (chemicals by Thermo Scientific) at 37°C for 45 min. We precipitated the PCR products using High Pure PCR Template Purification Kit (Roche), separated the digested plasmid using 1% agarose gel and excised the corresponding band. To isolate the plasmid from the gel, we used Gel Extraction Kit (GENEAID).

We cloned the PCR products to TagVag2 vector using following protocol.

10x T4 DNA ligation buffer	2 µl
TagVag2 vector	4 µl
insert	13 µl
T4 DNA ligase	1 µl
at 16 °C, overnight	

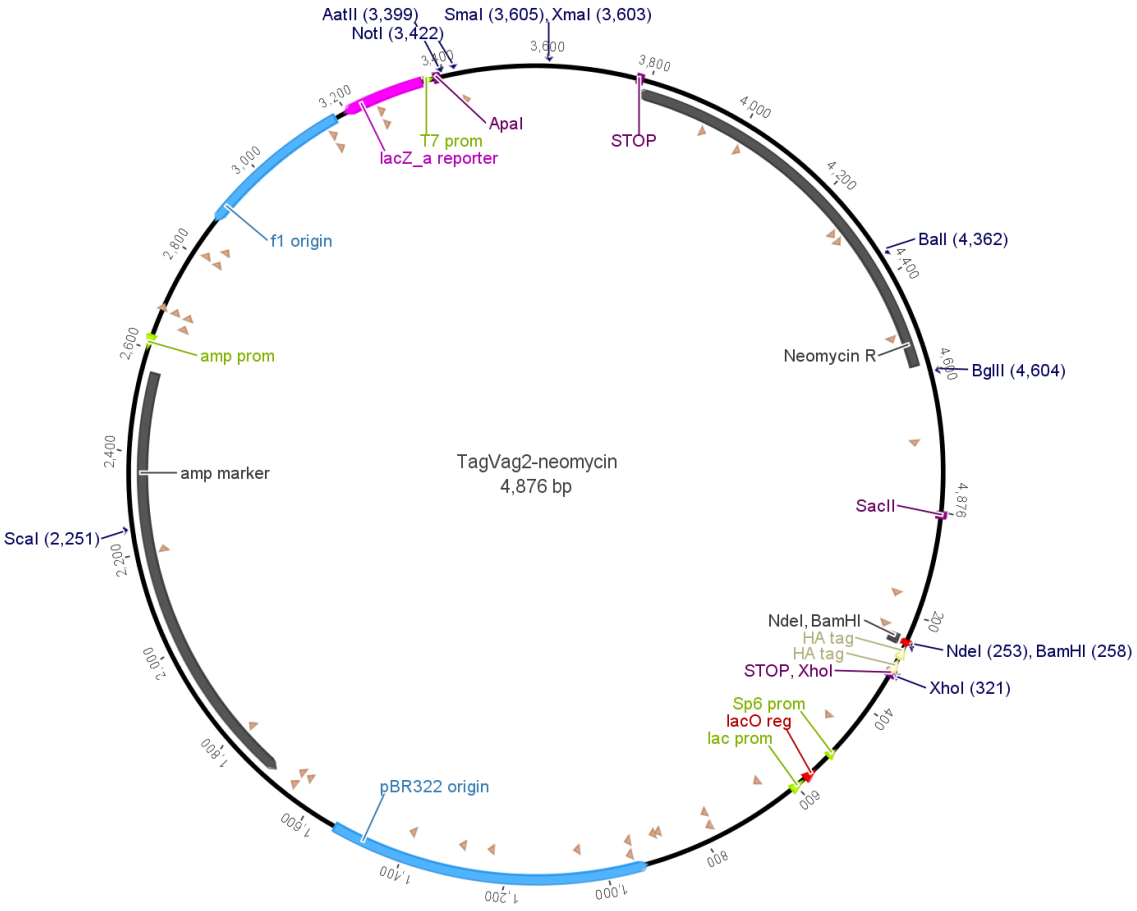

Figure 3: The sequence of TagVag2 expression plasmid (Hrdý et al. 2004).

E. coli TOP10 competent cells were transformed by the ligation. Plasmid isolated using Midiprep Kit (GENEAID) was used for T. vaginalis transfection (chap.4.3.1.).

After approximately sixth passage, the transformed cells were further analyzed by western blot analysis and immunofluorescence microscopy.

4.11 Cell fractionation by differential centrifugation

1. all steps are done at 4°C
2. start with cell culture about 250 ml in amount
3. spin down the cells at 2500 x g, 15 min, 4°C
4. discard the supernatant
5. resuspend the pellet in 45 ml of PBS (non-sterile) and transfer it to 50 ml plastic tube.
6. spin down at 1000 x g, 10 min, 4°C

7. resuspend the cells in 15 ml of ice cold ST buffer and spin down as in previous step
8. resuspend the cells in approximately 10 – 15 ml of ice cold ST buffer per 250 ml of starting culture
9. per each ml add 10 μg of Leupeptin and 50 μg TLCK
10. sonicate on ice at amplitude 40, 1 sec. pulses, 1 minute intervals until only few cells remain unbroken (sonicater Vibra Cell)
11. after each interval check the cells using microscope
12. spin the homogenized cells at 800 x g, 10 min, 4°C
13. transfer carefully the supernatant (do not swirl the pellet containing living cells, nuclei and membranes) to new plastic tube and spin down at 8900 x g, 20 min, 4°C
14. transfer 1 ml of supernatant (crude cytosolic fraction) to 1,5 ml tube
15. discard rest of the supernatant by aspiration and get a pellet (large granular fraction enriched with hydrogenosomes)
16. discard the contamination of lysosomes (white hem of the pellet) by aspiration
17. resuspend the pellet in 1 ml of ST, transfer it to 1,5 ml tube and spin down at 20 000 x g, 20 min, 4°C
18. repeat the step 16. and get the pellet of hydrogenosomes (proceed with further experiment or store at -80°C)
19. Spin down the crude cytosolic fraction at 20 000 x g, 30 min, 4°C

4.11.1 Protein protection assay

To reveal whether the proteins in the hydrogenosomal fraction are localized on the surface or whether they are inside the organelle we used the trypsin treatment. Trypsin digests proteins at 37°C. It cannot cross the hydrogenosomal membrane and therefore the proteins within the organelle remain untouched.

For trypsin treatment, we used the following protocol:

1. weigh the pellet of purified hydrogenosomes and make 4 aliquots containing 2,5 mg of pellet, each

2. resuspend them in 250 ml of ST buffer (TLCK (50 µg/ml), leupeptin (10 µg/ml) added)
3. add trypsin (200 µg /ml)to one of the aliquots, trypsin (200 µg /ml) and 1% Triton TX-100 to another aliquot and 2 aliquots keep without any treatment
4. one of the aliquots without treatment keep on ice and the three rest incubate at 37°C (30min)
5. spin the samples 20 000 x g, 5 min., 4°C
6. resuspend the pellets with SDS loading buffer

4.12 Immunoprecipitation of the Cfd1A proteins

4.12.1 Preparation of anti-HA Dynabeads using Dynabeads Antibody Coupling Kit (Life Technologies)

Day 1:

1. weigh 10 mg of Dynabeads M-270 Epoxy to 1,5 ml tube
2. add 1 ml of C1 buffer , pipette up and down 3 times
3. place it on the magnet for 1 min and remove the supernatant
4. Add 24 µl of anti-HA antibody (anti-HA Mouse antibody, Exbio) to get final concentration 15 µg of antibody per 1 mg of dynabeads
5. add 476 µl of C1 buffer and pipette 3 times
6. add 500 µl of C2 and mix by pipette
7. incubate on a roller/rotator over night at 37°C

Day 2:

1. place on the magnet for 1min and remove the supernatant
2. wash in 800 µl of HB buffer
3. wash in 800 µl of LB buffer
4. wash in 800 µl of SB buffer
5. add 1000 µl of SB buffer and incubate on the roller at room temperature for 15min, then remove the supernatant
6. resuspend in 980 µl of SB buffer and add 20 µl of 1% sodium azid
7. Store in 4°C until further use

4.12.2 Immunopreciptation protocol

all steps are done at 4°C

1. start with cell culture about 250 ml
2. spin down the cells at 1200 x g, 10 min, 4°C (Hettich 32 R centrifuge)
3. discard the supernatant and wash in PBS, repeat the step 2.
4. discard the supernatant and resuspend the pellet into dense suspension of PBS approximately 1 – 1,5 ml, transfer it to 1,5 ml tube
5. use the Biorad Assay to measure the proteins of whole cells
6. dilute the suspension to final concentration of 1,5 µg/ml
7. ad DSP to final 1mM concentration
8. incubate on ice, 30 min
9. spin down the sample at 1200 x g, 10 min, 4°C
10. resuspend in 1 ml boiling buffer
11. incubate at 80°C, 10 min
12. spin down at 1000 x g, 10 min, at room temperature
13. take the supernatant and dilute it 1:20 in incubation buffer supplemented with protease inhibitors (cOmplete mini EDTA-free, Roche Life Science)
14. add 100 µl of dynabeads
15. incubate over night at 4°C on rotator, speed 2-3
16. next day – magnet it 1 min and remove the supernatant
17. wash it with 1 ml of incubation buffer , three times
18. ad 50 µl of SDS sample buffer (without dye) and incubate at 70°C, 10 min
19. proceed for Mass Spectrometry analysis

Mass spectrometry analyses were performed in the Mass Spectrometry Laboratory of BIOCEV using label free quantificance, NanoLC/MS – Orbitrap fusion.

5 RESULTS

5.1 IscU in hydrogenosomes of *T. vaginalis*

To test whether hydrogenosomal ISC machinery is required for assembly of FeS clusters in the cytosol via CIA machinery, we selected TvIscU, an important component of ISC, as a target for a gene knockout. TvIscU is the only ISC component, which is coded by a single gene in *T. vaginalis* genome and thus suitable candidate for a gene deletion.

5.1.1 Cellular localization of TvIscU

First, we decided to verify expected hydrogenosomal localization of TvIscU. The putative TvIscU is encoded by a single gene (TVAG_432650) of 563 bp. Calculated size of the protein is 16,1 kDa with predicted N-terminal targeting sequence of 8 amino acids (MLAAVSRS/SA) (Smíd et al. 2008) For the protein localization within *T. vaginalis* cell, we prepared specific polyclonal rat anti TvIscU antibody.

The *tviscu* gene was amplified by PCR and cloned to the pet42b expression vector which allows production of TvIscU with C-terminal poly-His tag. The protein was expressed in *E. coli* BL21 cells and then purified using a nickel column. The recombinant protein was used for immunization of rat.

Subsequently, we used the antibody to detect the protein in *T. vaginalis* using immunofluorescence microscopy. We observed that TvIscU labels spherical structures that are typical for hydrogenosomes. Moreover, it co-localizes with malic enzyme, which is used as the hydrogenosomal marker protein. These results confirmed that TvIscU is present in hydrogenosomes.

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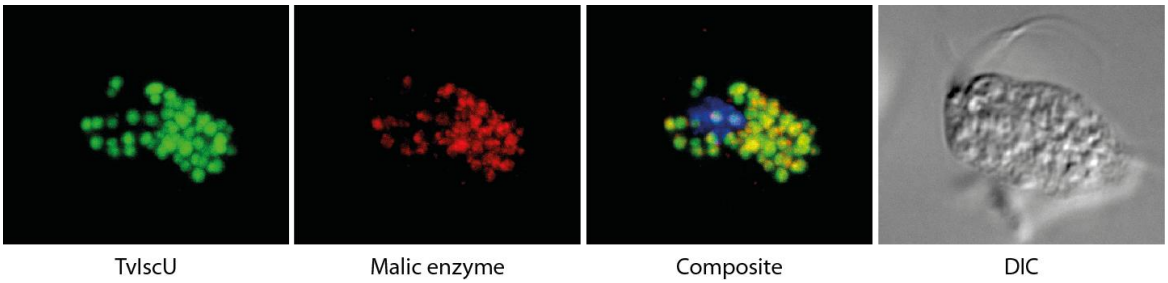


Figure 4: Localization of TvIscU in hydrogenosomes

TvIscU was detected by rat anti-TvIscU antibody (green). Malic enzyme was used as a hydrogenosomal marker that was detected by rabbit anti-malic enzyme antibody (red). The nuclei were stained with DAPI (blue). DIC - differential interference contrast.

5.1.2 Knockout of TvIscU coding gene

The most efficient methods for functional studies of components of FeS assembly machinery are methods of reverse genetics. However, these methods are not amenable for *T. vaginalis*. The most promising approach is based on homologous recombination that was successfully used for knockout of limited number of *T. vaginalis* genes (Land et al. 2004, Bras et al. 2013). Therefore, we decided to establish this method in our laboratory and initially we attempted to reproduce the gene knockout for hydrogenosomal [2Fe2S] Ferredoxin (Fdx1). The plasmid pFdKO for replacement of *ferredoxin 1* (*fdx1*) (TVAG_003900) was kindly provided by Dr. Sven Gould (University of Düsseldorf). The vector contains the gene for Neomycin phosphotranferase as a selectable marker, which is flanked by of 5´ and 3´ untranslated regions (UTR) of *fdx1*. The 5´ and 3´ UTR sequences are each approximately 1000 bp in length. Once the sequence of the plasmid is linearized, the homologous recombination occurs via the untranslated regions which are identical to targeted genomic DNA (Figure X).

For this reason, we digested the plasmid pFdKO by NotI and HindIII and the linear sequence was used for transformation of *T. vaginalis* via electroporation. The high amount (100 μg/μl) of digested pKOFdx was used per 600 mg cells in 300 μl of TYM medium. After electroporation, the cells were transferred to 50 ml of TYM medium and selected using geneticin (110 μg/ml). However, these initial experiments did not lead to selection of cells with *fdx1* gene knockout. western blot analysis using anti-Fdx1antibody revealed expression of Fdx1 in all selected lines. The *fdx1*gene was also detected by PCR using primers hybridizing to ORF and 3´ untranslated region (Figure X).

Therefore, we tried to improve the protocol for the preparation of linear DNA from the pFdKO plasmid. We used the additional ScaI restriction enzyme to digest the backbone of the circular plasmid and shrimp alkaline phosphatase (SAP) to prevent re-joining of cleaved DNA (Figure X). Next we electroporated the cells in 5 parallels by DNA (100 μg/μl) and transferred each of them to 50 ml TYM media. According to protocol, we added geneticin (G418) (110 μg /ml) 6 hours after electroporation. Seventeen hours after electroporation, we span down the cells and resuspended them in 50 ml newly prepared TYM 6,2 medium with agar and G418. Next, we took 12,5 ml of each cell culture and cultivated them in 15 ml plastic tubes. The rest of the cells were distributed on 96-well plates and selected as clonal populations. The cells electroporated by circular pFdKO were used as a control for electroporation and cloning protocol. After 5 days of selection we obtained viable

transformants of the control cells in 96-well plate and in the tube. However, we observed living cells only in one population of cells transformed by linear pFdKO in 15 ml tube, labeled TvR41 and we did not observe any living cells on the 96-well plates. To test expression of *fdx1*, we analyzed the lysate of TvR41 cells via western blot using anti-Fdx1 antibody (Figure X). No visible signal for the protein was detect, whereas Fdx1was observed in the control cells. Next, TvR41 strain was used for cloning on 96-well plate. We obtained 8 viable clones of *T. vaginalis* with no expression of Fdx1.

To conclude, we reproduced gene knockout for fdx1,performed previously in the laboratory of Patricia Johnson (Land et al. 2004) and Sven Gould (personal communication). Once we established the knockout technique by homologous recombination and deleted the gene for Fdx1, we focused on deletion of gene for TvIscU. In the pFdKO plasmid, we replaced the UTRs of fdx1 by 5' and 3'UTRs of *tviscu*, of 1083 and 1057 bp, respectively. *T. vaginalis* cells were submitted to electroporation with linearized pIscUKO in three independent experiments, each in triplicates. In parallel experiments, cells were transformed with circular plasmid as positive controls for electroporation. However, no viable cells were selected under these conditions. These results suggest that knockout of *tviscu* is most probably lethal for *T. vaginalis* cells. Consequently, we were unable to investigate, whether the deletion of *tviscu* affect function of the CIA machinery.

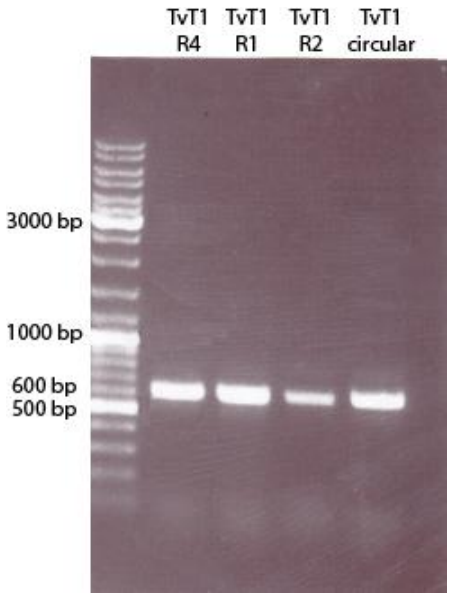


Figure 5 PCR analysis of *T. vaginalis* cell lines electroporated with digested pFdKO

DNA electrophoresis detects 609 bp long DNA fragment in all *T. vaginalis* cell lines electroporated by pFdKO that was digested by NotI and HindIII (TvT1 R4, TvT1 R1, TvT1 R2), and circular pFdKO as a control (TvT1 circular). The DNA fragment was amplified by PCR using specific primers complementary to ORF of *fdx1* and 3´UTR of *fdx1* gene using genomic DNA of transformed cell lines as a template. The results indicate that the fdx1 was not deleted. Therefore, we suggest that the plasmid, which was used to knockout the gene, was digested incompletely and remained partially circular.

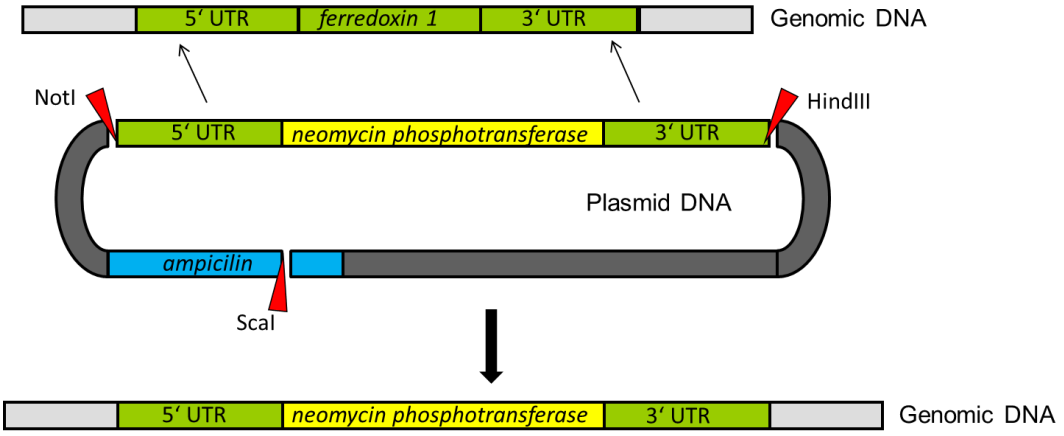


Figure 6: The model of pFdKO plasmid and scheme of homologous recombination

The plasmid possesses an ampicilin and neomycin resistance cassete. We digest the plasmid by NotI, HindIII, ScaI. The homologous recombination occurs via untranslated regions (UTR), which are identical to genomic DNA.

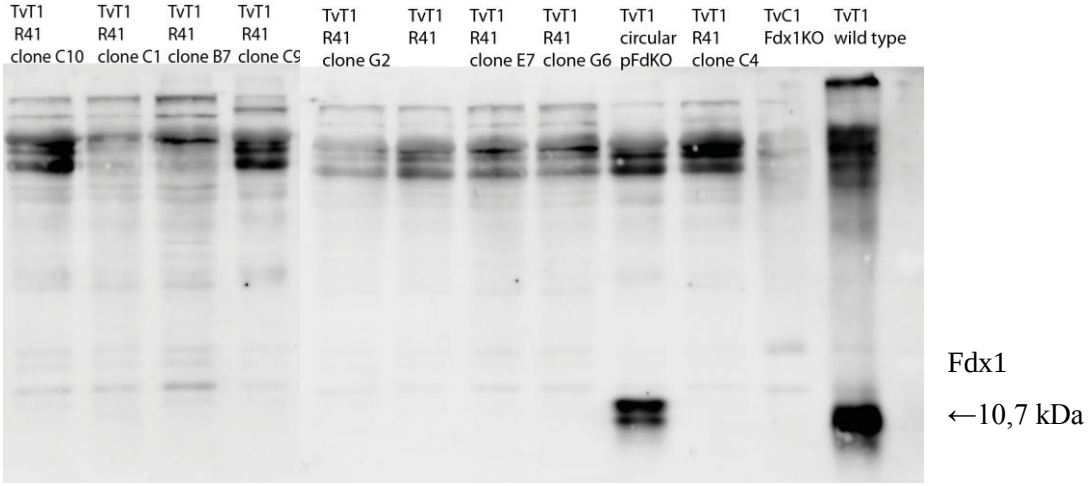


Figure 7: Western blot analysis of TvT1 knockout lineages

T. vaginalis R41 strain (TvT1 R41) and R41 clones (C10, C1, B7, C9, G2, E7, G6 and C4) have no signal for Fdx1. *T. vaginalis* Fdx1 knockout cells (TvC1 Fdx1KO) obtained previously by Land et al. (2004) was used as a negative control for Fdx1. *T. vaginalis* wild type cells (TvT1 wild type) and cells transformed by circular plasmid (TvT1 circular pFdKO) were used as positive controls for Fdx1.

5.3 Characterization of CIA pathway components

Cytosolic iron-sulfur assembly pathway (CIA) comprises of 8 core components in *Saccharomyces cerevisiae*. Nbp35, Cfd1, Dre2, Tah18, Nar1, Cia1, Cia2 and Mms19 are proposed to be cytosolic FeS assembly factors. However, their distribution is not consistent among other organisms. We focused on CIA pathway in *T. vaginalis* and characterized its CIA proteins.

5.3.1 Identification of genes coding CIA proteins in the genome of T. vaginalis

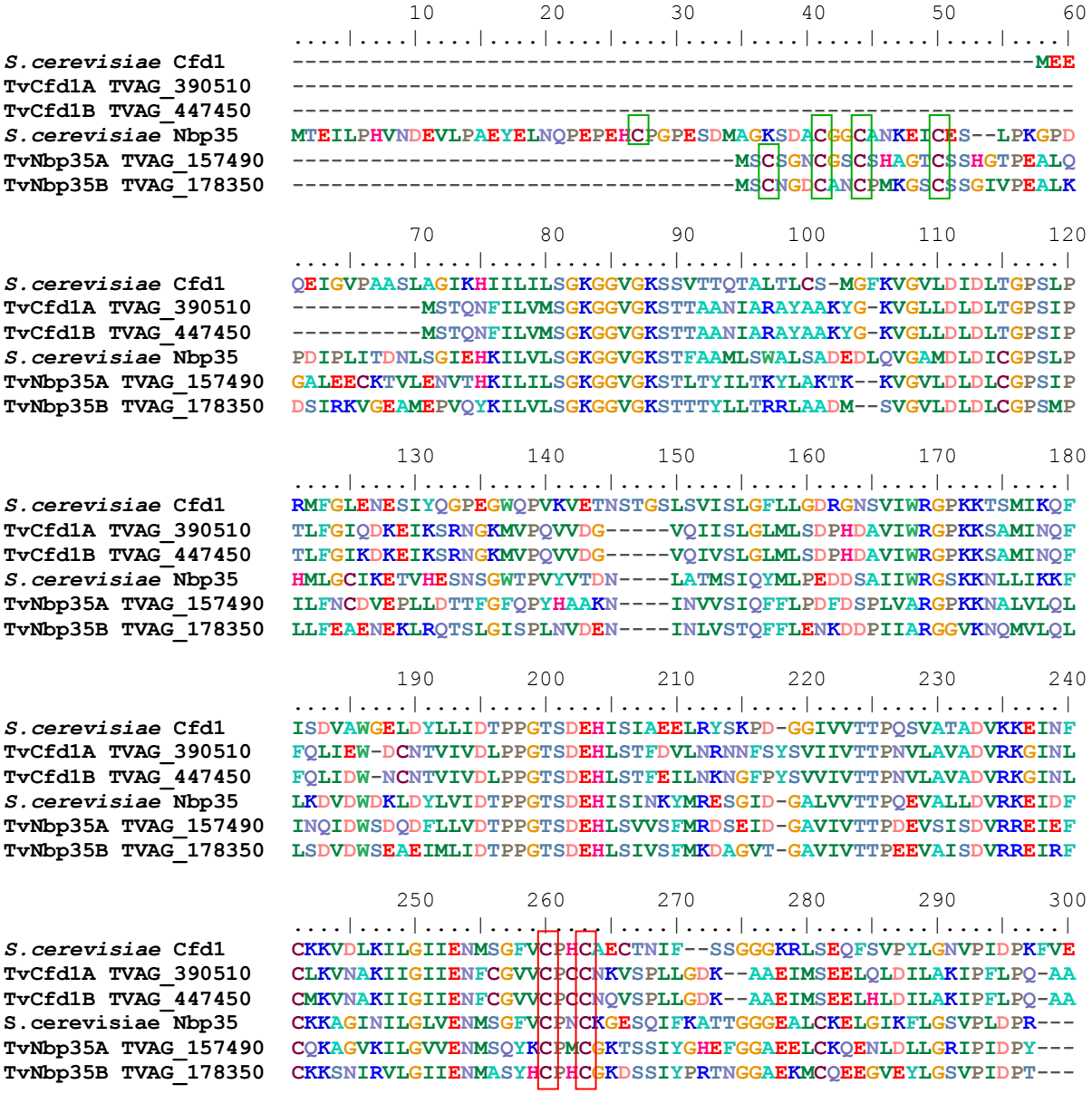
We searched for components of CIA proteins in the genomic data of *T. vaginalis* available at TrichDB. We used BLAST as a basic tool to search for protein homologues using proteins of CIA machinery previously described in *S. cerevisiae* as queries. We found 5 putative components of CIA pathway: Nbp35, Cfd1, Cia1, Cia2, and Nar1 (Table. X). We did not find any homologues to Tah18, Dre2 and Mms19 as reported previously (Tsaousis et al. 2014).

Protein	Accession number	Predicted molecular weight
TvCfd1A	TVAG_390510	27108 Da
TvCfd1B	TVAG_447450	27034 Da
TvNbp35A	TVAG_157490	31283 Da
TvNbp35B	TVAG_178350	31034 Da
TvNar1	TVAG_129510	44548 Da
TvCia1A	TVAG_350560	34053 Da
TvCia1B	TVAG_441510	35473 Da
TvCia2	TVAG_198590	18143 Da

Figure 8: Table of CIA components identified in *T. vaginalis* genome

Nbp35 and Cfd1 are both coded by two paralogous genes named Nbp35A and Nbp35B, and Cfd1A and Cfd1B. Analysis of predicted protein sequences revealed presence of the CPXC conserved motif, which facilitates the binding of transient labile FeS cluster at the C-termini of the Nbp35/Cfd1 proteins. In addition, both Nbp35 paralogues possess 4 Cys residues at the N-termini coordinating additional [4Fe4S] cluster (Figure 9). Protein sequence identity of TvNbp35A/B and TvCfd1A/B to yeast orthologues is 39,865/39,865 and

34,831/35,338, respectively. Similarly, we identified two paralogues of Cia1, containing WD40 repeat domain and named *Tv*Cia1A and *Tv*Cia1B (Figure 10), whereas a single genes for putative Cia2 was detected (Figure 11). Protein sequence identity of TvCia1A/B, and TvCia2 to yeast orthologue is 21,515/25,228, and 31,073 respectively. Finally, we were able to identified highly divergent Nar1 that displayed only 16,735 sequence similarity/identity to yeast Nar1. This protein is similar to hydrogenases of *T. vaginalis* however, differs in modified H-cluster that is required for hydrogen synthesis in hydrogenases. (Figure X).



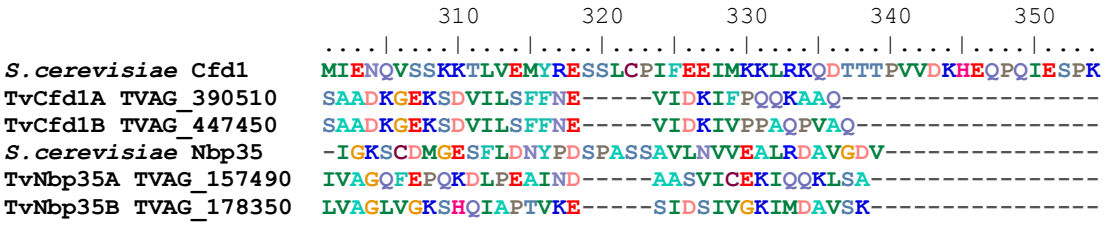


Figure 9: Alignment of Cfd1 and Nbp35 proteins

Putative Cfd1A and Nbp35 proteins were found in *Trichomonas vaginalis* using *Saccharomyces cerevisiae* orthologs as queries. The conserved motif CPXC at C-terminus of the proteins is highlighted (red boxes). Nbp35 proteins possess additional 4 conserved cysteins at N-terminus (green boxes).

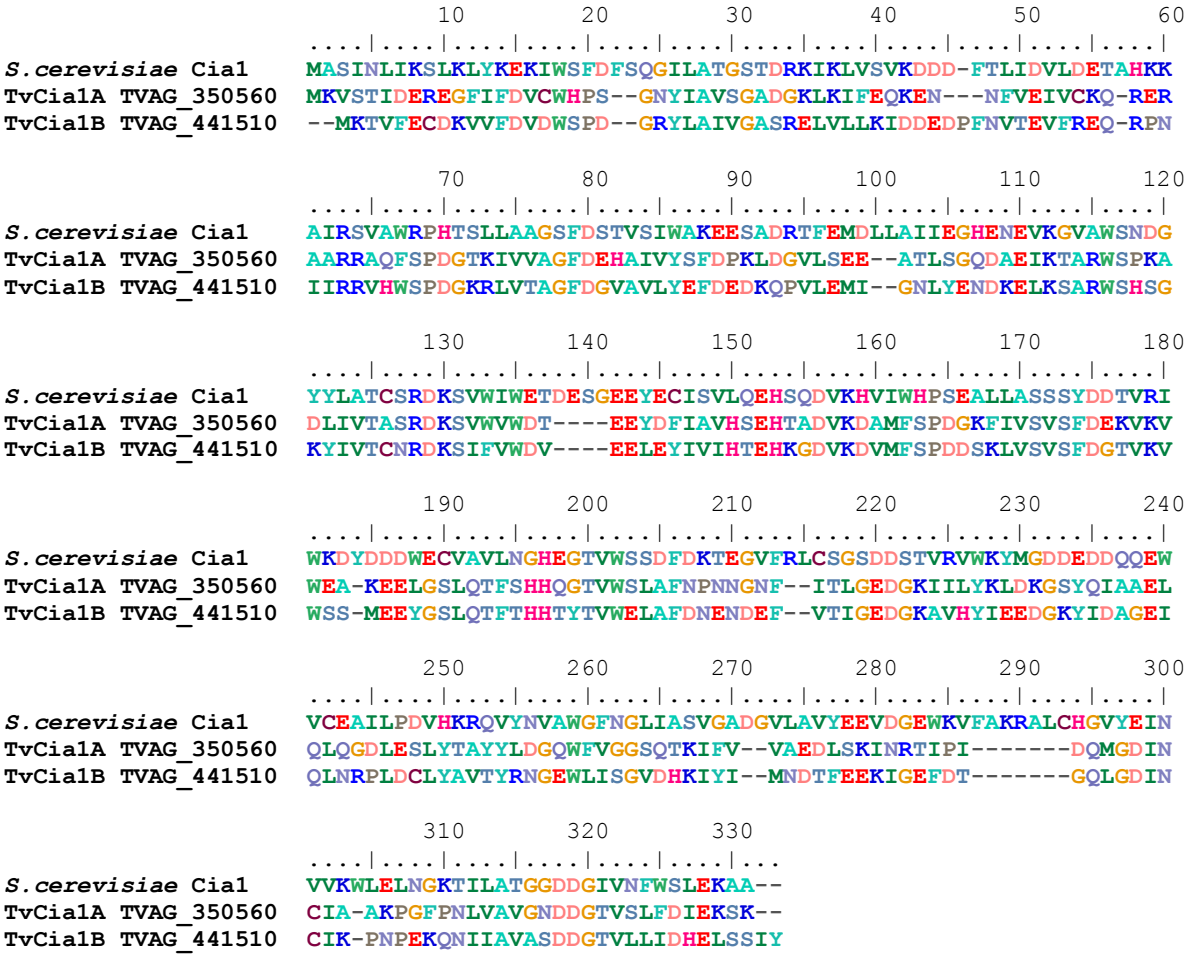


Figure 10: Alignment of Cia1 proteins

Putative Cia1 paralogues were found in *Trichomonas vaginalis* genome using *Saccharomyces cerevisiae* amino acid sequence of Cia1 as a query.

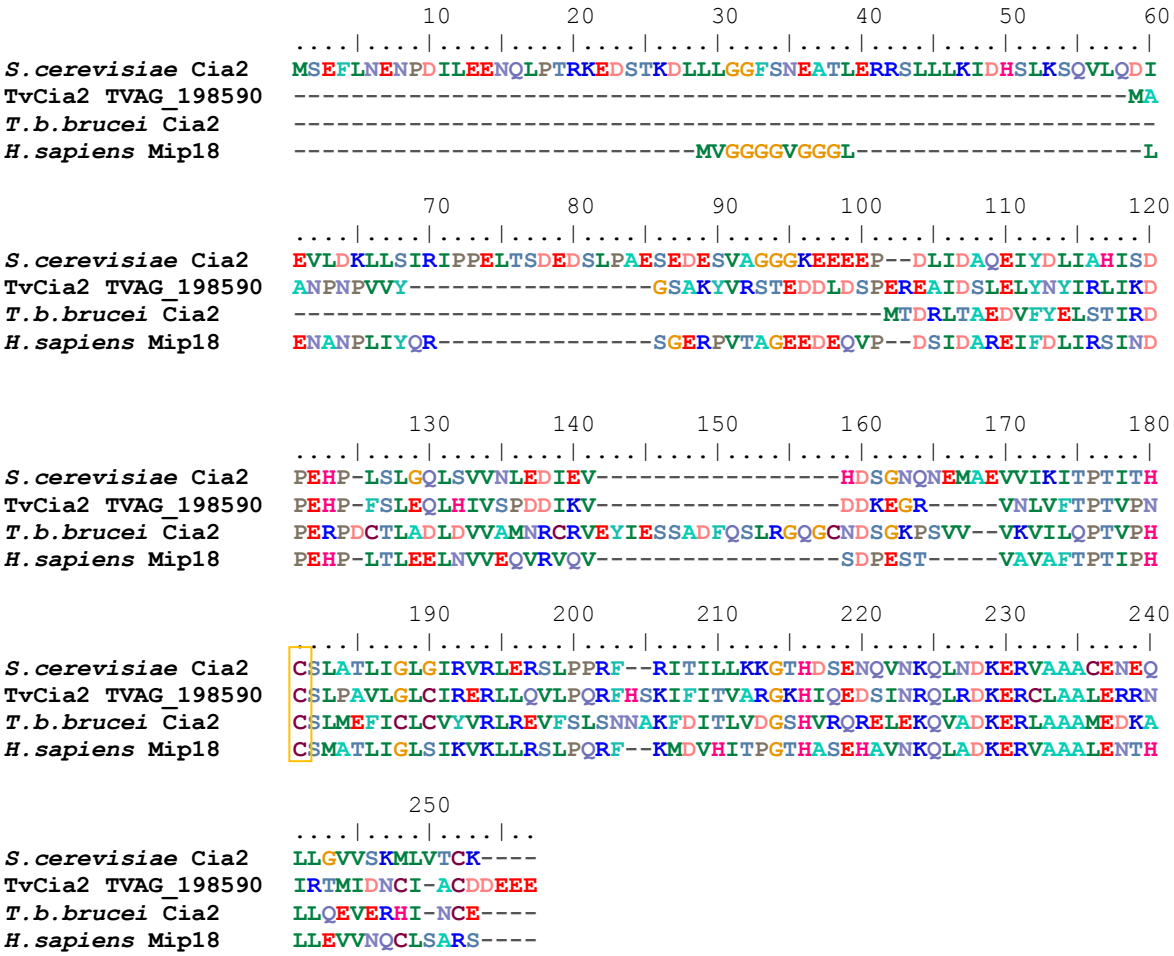
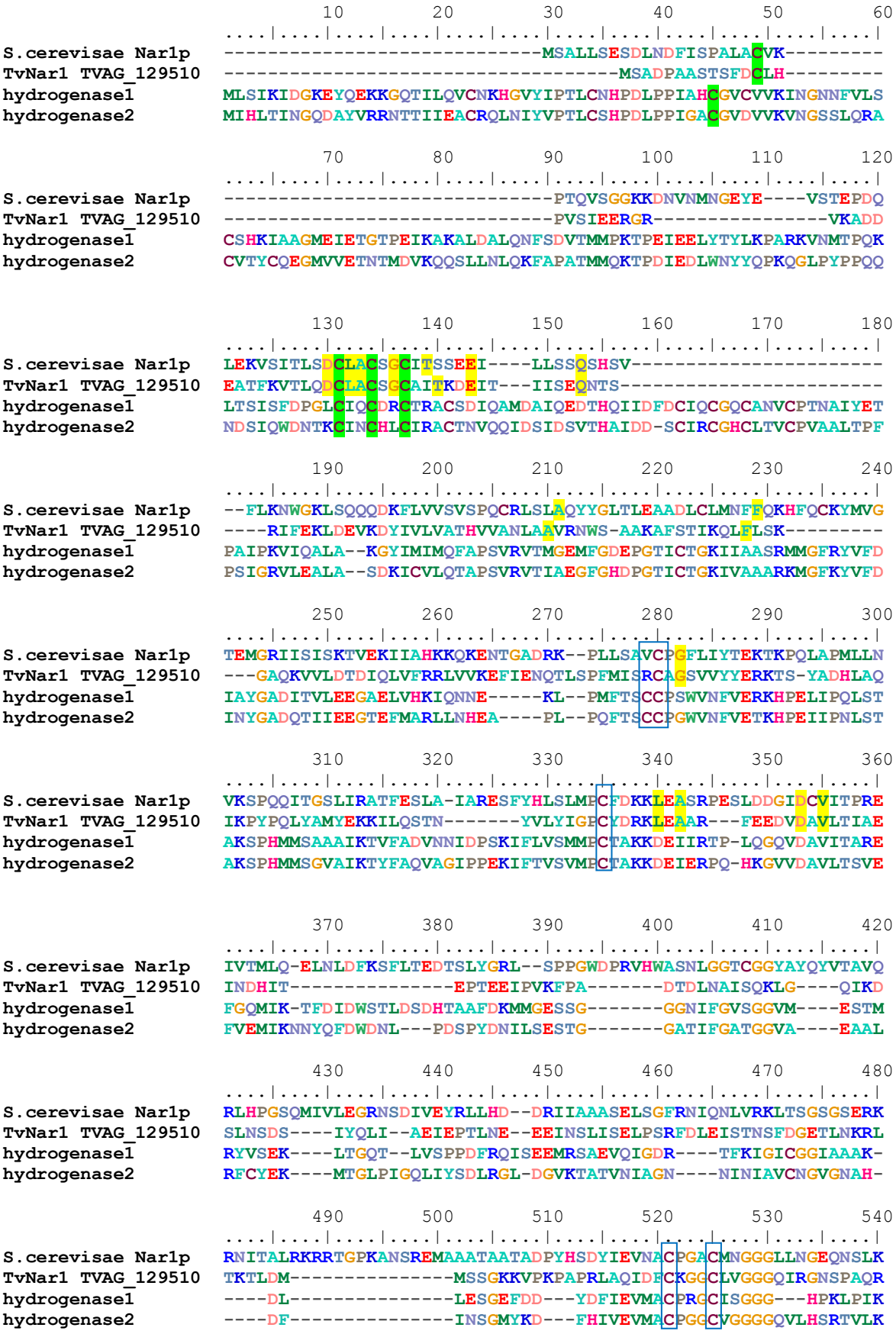


Figure 11: Alignment of Cia2 proteins

Cia2 was identified in *Trichomonas vaginalis* genome using *Saccharomyces cerevisiae* Cia2 (Mip18 in *Homo sapiens*) as a query. Cia2 is conserved in most eukaryotes. The highly conserved reactive cystein is highlighted (yellow box).


S.cerevisae Nar1p
TvNar1 TVAG_129510
hydrogenase1
hydrogenase2

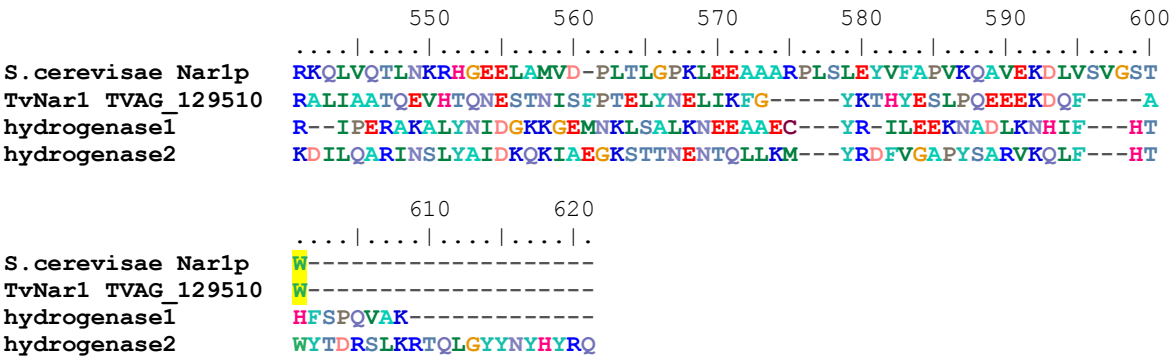
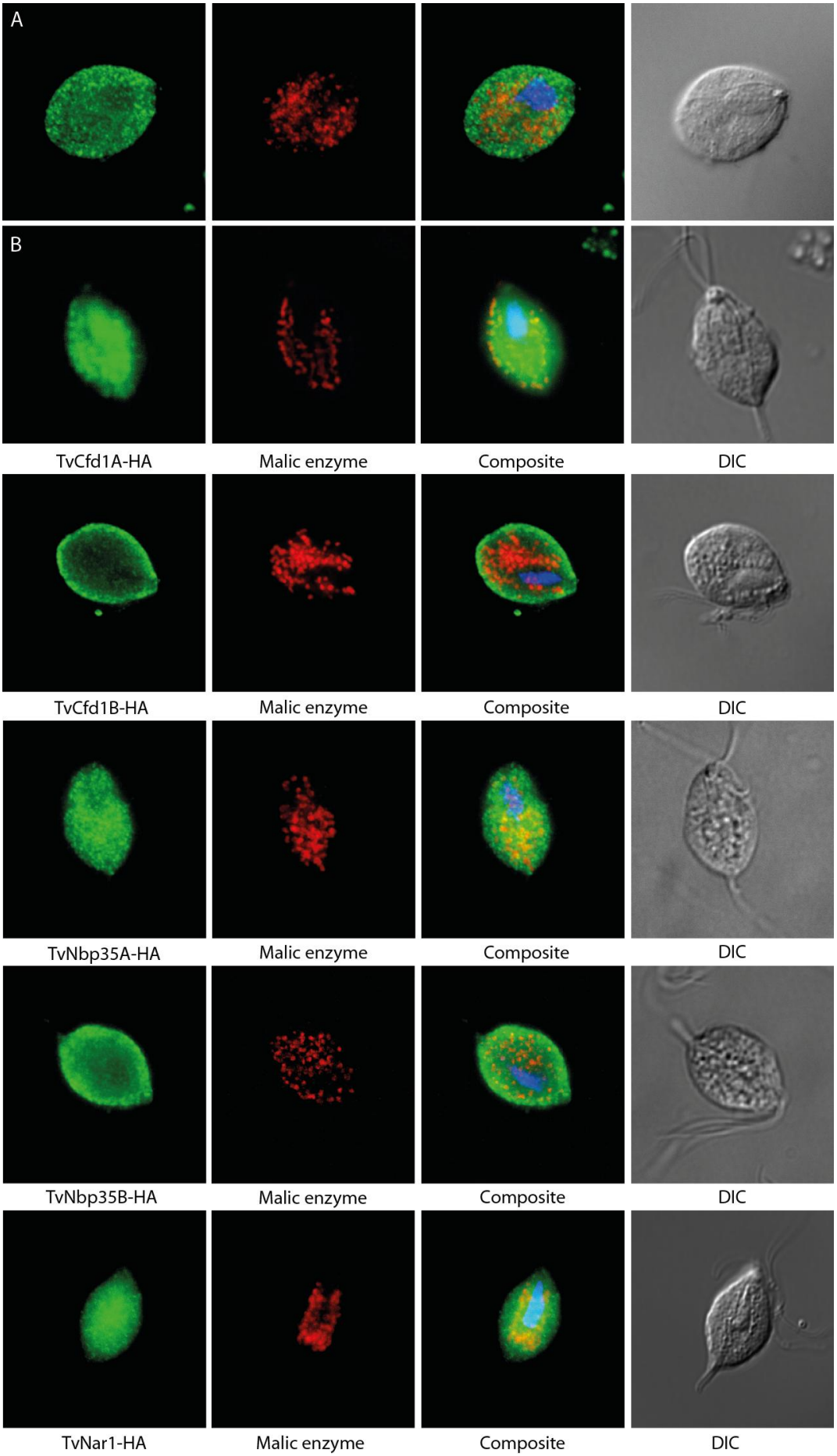


Figure 12: Alignment of Nar1 proteins found in and iron dehydrogenases.

Alignment of putative Nar1 protein of *T. vaginalis* against *S. cerevisiae* Nar1 and *T. vaginalis* hydrogenases. Conserved cysteins are highlighted in green color. The cysteine residues coordinating H- cluster are indicated in blue boxes. Aminoacid residues, which are highly conserved in Nar1 proteins are in yellow color (Nicolet, Cavazza, and Fontecilla-Camps 2002; Balk et al. 2004).

5.3.2 Localization of the members of CIA machinery by immunofluorescence microscopy

The subcellular localization of CIA proteins were investigated using fluorescence microscopy. The genes coding for CIA components were subcloned to expression vector TagVag2 that allows their expression with hemagglutinin tag (HA) at C-terminus in *T. vaginalis*. TvCfd1A-HA, TvCfd1B-HA, TvNbp35A-HA, TvNbp35B-HA, TvNar1-HA, TvCia1A-HA and TvCia2-HA, appeared to be localized exclusively in the cytosol. Interestingly, TvCia1B-HA and TvCfd1A-HA appear to have in some experiments dual localization in the cytosol and hydrogenosomes, however, in other experiments, these protein were observed only in the cytosol (Figure 13).


A
B
TvCfd1A-HA
Malic enzyme
Composite
DIC
TvCfd1B-HA
Malic enzyme
Composite
DIC
TvNbp35A-HA
Malic enzyme
Composite
DIC
TvNbp35B-HA
Malic enzyme
Composite
DIC
TvNar1-HA
Malic enzyme
Composite
DIC

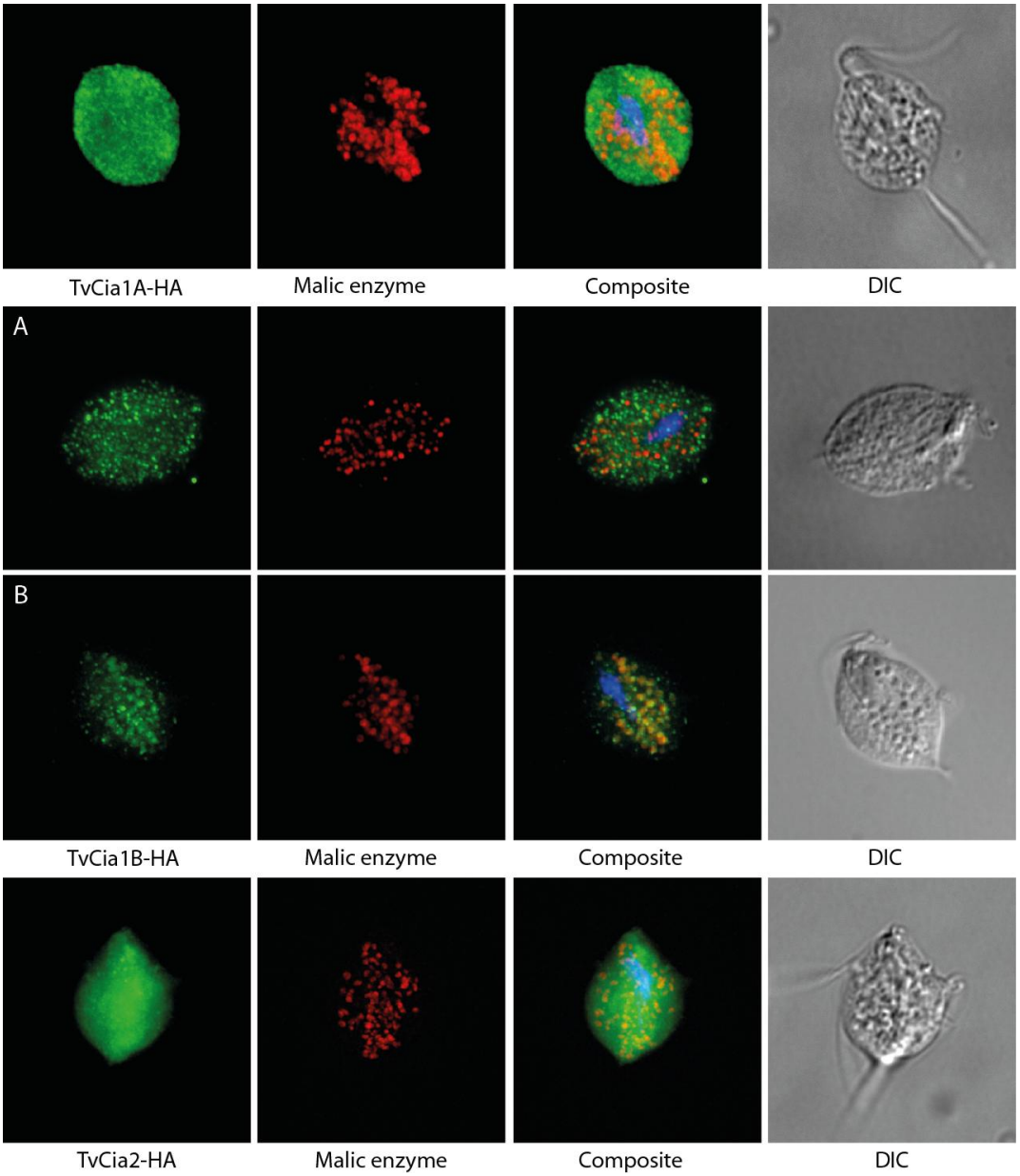


Figure 13: Localization of HA-tagged components of CIA machinery in *T. vaginalis* by fluorescent microscopy

HA-tagged proteins were detected using monoclonal mouse anti-HA antibody (green). Malic enzyme was used as a marker for hydrogenosomes and detected using rabbit polyclonal anti-malic enzyme antibody (red). The nuclei were stained with DAPI (blue). DIC - differential interference contrast. TvCfd1A-HA and TvCia1B-HA display dual localization. Localization exclusively in the cytosol (A), and in the cytosol and hydrogenosomes (B) is shown.

5.3.3 Localization of the members of CIA machinery in subcellular fractions

The transformed cells that expressed the recombinant protein of interest were subjected to cellular fractionation and the protein localization was tested by western blotting. To test the protein topology within hydrogenosomes, the hydrogenosomal fractions were treated with trypsin to discriminate between proteins that are attached to the organellar surface and internal proteins. In this so-called protein protection assay, the hydrogenosomes were incubated for 30 min at 37°C with trypsin to remove organellar surface proteins. In controls, hydrogenosomes were incubated under same conditions without trypsin at 4°C, and 37°C.

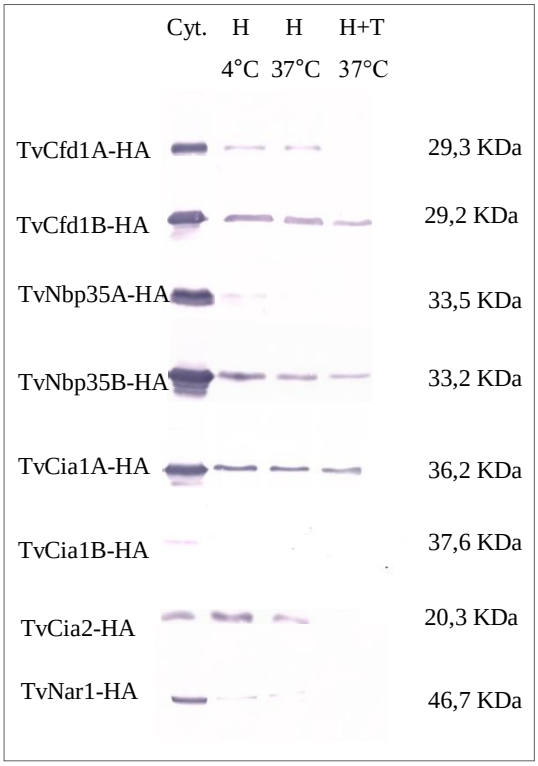


Figure 14: Identification of HA tagged CIA proteins that were expressed in *T. vaginalis* under control of STK promotor in subcellular fractions.

Cyt, cytosol; H, hydrogenosomal fraction; H+T, hydrogenosomal fraction treated with trypsin. For protein protection assay, the hydrogenosomal fractions were incubated for 30 min. at 4°C or 37°C. TvCfd1B, TvCia1A-HA and TvNbp35B-HA is present also in inner hydrogenosomal fraction (H+T). The mouse anti-HA antibody was used against Ha-tagged proteins together with secondary alkaline phosphatase antibody.

As expected, the majority of CIA components is localized in the cytosol. However, significant signal within the hydrogenosomal fractions was observed also for TvCfd1A-HA, TvCfd1B-HA, TvNbp35B-HA, TvCia1A-HA, and TvCia2-HA. Weak signals in the hydrogenosomal fractions were noticed also for TvNar1-HA and TvNbp35A-HA.TvCia1B-HA displays very low expression and was detected only in the cell lysate and cytosol. The trypsin treatment removed signals for TvCfd1A-HA, Nbp35A-HA, TvCia2-HA, and TvNar1-HA, which indicates that these proteins are partially attached to the outer hydrogenosomal membrane but they are not inside of hydrogenosomes. Interestingly, the protein protection assay revealed that part of Cfd1B-HA, Nbp35B-HA, and Cia1A-HA is present inside of the organelles.

5.3.4 Localization of TvCfd1A-HA and TvNbp35-HA expressed under native promoter

The STK promoter is known to drive strong expression of associated genes. Together with unexpected localization of some CIA components observed above, we suspected that gene expression under control of STK promoter may cause miss-localization of tagged proteins. Therefore we decided to verify cell localization of selected proteins under the control of their native promoters.

Initially, we chose TvCfd1A, TvCfd1B, TvNbp35B and TvCia1A. For TvCfd1A and TvNbp35B genes, we were able to amplified approximately 500 bp of the sequence prior to ORF and cloned it to TagVag2 instead of STK promoter. Unfortunately, we failed to clone upstream regions of TvCfd1B and TvCia1A. Therefore, we proceeded only with TvNbp35B and TvCfd1A in further experiments. The *T. vaginalis* cells transformed with TvCfd1A-HA and TvNbp35B-HA under the control of native promoters (TvCfd1-HA-N, TvNbp35A-HA-N) were analyzed by immunofluorescence microscopy and western blotting of cellular fractions. The fluorescence microscopy results of Nbp35B-HA-N were similar to Nbp35B-HA. TvCfd1A-HA-N did not display dual localization, as we observed previously for TvCfd1A-HA. Although, the protein, labeled by anti-HA tag antibody, seems to have granular pattern, it did not co-localize with organellar marker .

Significant changes were observed using western blot analysis of subcellular fractions (Figure X). In the case of Cfd1A-HA-N, we noticed the presence of this protein exclusively in the cytosolic fraction. Nbp35B-HA-N was associated with the hydrogenosomal fraction; however, the signal disappeared under protein protection assay. This observation indicates that Nbp35B-HA is attached to the surface of hydrogenosomes yet not imported into the organelles when the protein is expressed under control of the native promoter.

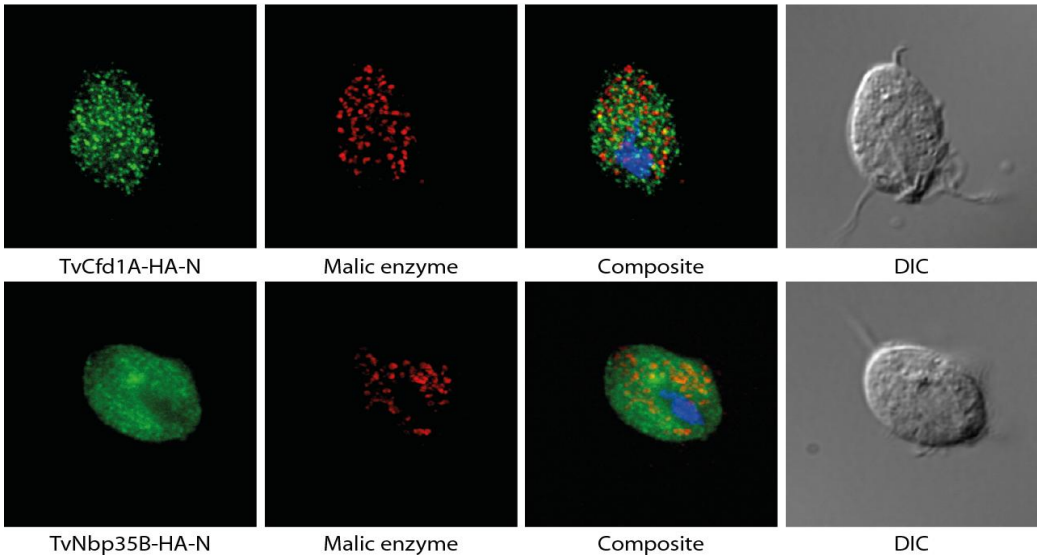


Figure 15: Localization of HA-tagged components of CIA machinery in *T. vaginalis* by fluorescence microscopy

HA-tagged proteins were detected using monoclonal mouse anti-HA antibody (green). Malic enzyme was used as a marker for hydrogenosomes and detected using rabbit polyclonal anti-malic enzyme antibody (red). The nuclei were stained with DAPI (blue). DIC - differential interference contrast.

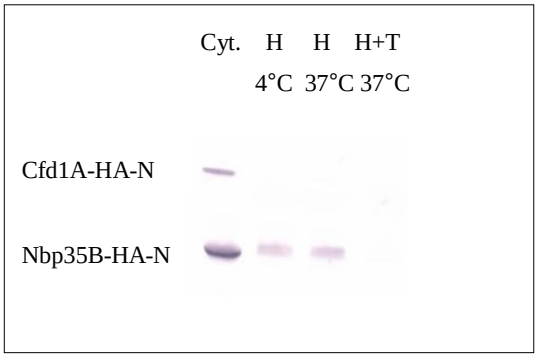


Figure 16 : Localization of TvCfd1A-HA-N and Nbp35-HA-N that were expressed in *T. vaginalis* under native promotors.

Subcellular fractions were isolated and analyzed by western blot. Cyt, cytosolic fraction; H, hydrogenosomal fraction; H+T, hydrogenosomal fraction treated with trypsine. For protein protection assay, the hydrogenosomal fractions were incubated for 20 min. at 4°C or 37°C. The mouse monoclonal anti-HA antibody was used to visualized HA-tagged proteins together with secondary anti mouse antibody conjugated with alkaline phosphatase.

5.4 Immunoprecipitation of TvCfd1A-HA and TvCfd1B-HA

Cfd1 and Nbp35 are known to form heterotetrameric complex in yeast. As we detected two paralogues for each protein in *T. vaginalis*, we were interested whether all paralogues participate in formation of such a complex in this protist.

Thus, we employed strains expressing TvCfd1A-HA and TvCfd1B-HA for immunoprecipitation of interacting partners. We followed the co-IP protocol using dithibis[succinimidylpropionatel] (DSP) to crosslink the interacting proteins or proteins in the proximity. DSP is water-insoluble homobifunctional N-hydroxysuccimide ester which reacts with primary amines forming covalent amide bonds. The spacer arm length is 12 Å.

The sample prepared by the same protocol employing parent T1 strain was used as a control in each experiment. Both HA-tagged strains were immunoprecipitated in duplicates and analyzed by mass spectrometry (The Laboratory of Mass Spectrometry in BIOCEV).

Each sample was analyzed in three technical parallels. Complete raw data are presented in Supplementary Tables. Only proteins for which 2 unique peptides were identified were approved for further analysis. As significant partners immunoprecipitated with TvCfd1A-HA and TvCfd1b-HA were considered only the proteins that were either unique or enriched with fold change over 4 when compared with immunoprecipitated proteins in control samples (Table 2). Unique proteins are these that were present only in HA-tagged immunoprecipitated

samples (log2 of intensity is shown in the Table X) and were not identified in the control. All identified proteins were automatically annotated based on TrichDB database and manually edited.

Altogether, we identified 144 proteins enriched in the samples TvCfd1A-HA_1 and 2, relative to the control. Of these proteins, 30 proteins (counted with Cfd1A-HA) meet our criteria for interacting partner i.e. 15 protein with fold change >4, and 15 unique proteins. Interestingly, 22 interacting proteins are most likely cytosolic proteins, and 8 proteins were previously identified in hydrogenosomes. Particularly, C-tail anchored protein-8 and Hmp36 and Tom40-2 are outer membrane proteins.

Proteomic analysis of the TvCfd1B-HA samples revealed considerably less proteins. Altogether we identified 82, of these x 11 (counted with both TvCfd1B-HA and TvCfd1A) we considered as interacting partners.

Importantly, as the main interaction partner we found Cfd1A. Although, TvCfd1A and B displayed high sequence identity that results in large number of common peptides, we identified unique peptides of TvCfd1A in the samples of immunoprecipitated TvCfd1B-HA. However regarding to similarity of these paralogues and number of identical razor peptides, we cannot discern the TVCfd1 proteins quantitatively. Further, we indentified hydrogenosomal C-tail anchored protein-8, and ATP dependent phosphofructokinase that were also present in TvCfd1A-HA sample. The other proteins are most likely cytosolic.

We did not find any of the Nbp35 paralogues that were expected to be interaction partner proteins. To disclose whether TvCfd1A interacts with TvCfd1B without any Nbp35 protein in *T. vaginalis*, these experiments have to be thoroughly repeated and supplemented with further analysis.

	TvCfd1A-HA_1						TvCfd1A-HA_2		
protein with fold change 4 over TvT1control	Fold change TvCfd1A/ TvT1 ctrl	Accession numbers	Characterization of the proteins	Fold change TvCfd1A/ TvT1 ctrl	Accession numbers	Characterization of the proteins	Fold change TvCfd1A/ TvT1 control	Accession numbers	Characterization of the proteins
	39,77	TVAG_277930*	conserved hypothetical protein	6,02	TVAG_318870	Cell division control protein	7,80	**TVAG_390510**	**Cfd1A**
	30,91	**TVAG_390510**	**Cfd1A**	4,41	TVAG_073860	Pyruvate phosphate dikinase	4,45	TVAG_332970*	Putative Tom40-2
	13,30	TVAG_430830/ TVAG_079260	PPi-dependent phosphofructokinase (cytosolic)	4,24	TVAG_230580*	Pyruvate-ferredoxin oxidoreductase	2,50	TVAG_283610	Conserved hypothetical protein
	10,65	TVAG_1143108*/ TVAG_484570	Thioredoxin peroxidase	4,12	TVAG_137820	Chaperonin 60 kDa	2,29	TVAG_203620*	Hydrogenosomal Cpn60
	7,97	TVAG_031860*	Hmp36	4,10	TVAG_153560/ TVAG_155010	Hsp90 cytosolic			
	6,90	TVAG_044510/ TVAG_163000	Heat shock proteins Hsp70 (cytosolic)	4,03	TVAG_363390/ TVAG_345360	Fructose-bisphosphate aldolases			
unique proteins in the sample	log2 intensity	Accession numbers	Characterization of the proteins	log2 intensity	Accession numbers	Characterization of the proteins	log2 intensity	Accession numbers	Characterization of the proteins
	21,97	TVAG_422780	Alcohol dehydrogenase	20,64	TVAG_075580	onserved hypothetical protein	21,86	TVAG_486930/ TVAG_462810	40S ribosomal protein S6
	21,72	TVAG_178890/ TVAG_177930	Chaperonins 60kDa	20,53	TVAG_220990/ TVAG_069560	TPR repeat containning protein	19,96	TVAG_151920/ TVAG_145570	WD repeat domain/receptor for activated protein kinase C
	21,55	TVAG_466720/ TVAG_466660	Chaperonins 60 kDa	20,39	TVAG_462530/ TVAG_006250	30S ribosomal protein S8	19,61	TVAG_009720	Adaptin
	21,24	TVAG_473510	Conserved hypothetical protein	19,96	TVAG_491670/ TVAG_320780	Cytosolic malic enzymes			
	21,11	TVAG_496160*	ATP-dependent phosphofructokinase	19,70	TVAG_532880	Clathrin heavy chain			
	20,72	TVAG_336320*	Hybrid cluster protein	19,47	TVAG_454490	Purine nucleoside phosphorylase			

TvCfd1B-HA_1			TvCfd1B-HA_2		
Fold change TvCfd1B/ TvT1 control	Accession numbers	Characterization of the proteins	Fold change TvCfd1B/ TvT1 ctrl	Accession numbers	Characterization of the proteins
96,15	TVAG_390510/**TVAG_447550**	Cfd1A/**Cfd1B**	8,70	**TVAG_447450**/TVAG_390510	Cfd1B/Cfd1A
9,82	TVAG_277930*	Conserved hypothetical protein			
4,68	**TVAG_447450**	**Cfd1B**			
log2 intensity	Accession numbers	Characterization of the proteins	log2 intensity	Accession numbers	Characterization of the proteins
21,06	TVAG_473510	Conserved hypothetical protein	22,46	TVAG_486930 TVAG_462810	40S ribosomal protein S6
20,44	TVAG_178890/ TVAG_177930	Chaperonins-60kDa	20,66	TVAG_151920 TVAG_145570	WD repeat domain/receptor for activated protein kinase C
19,02	TVAG_496160*	ATP-dependent phosphofructokinase			
18,88	TVAG_220990 /TVAG_069560	chaperone binding protein			

Table 2: Immunoprecipitation of TvCfd1A-HA and TvCfd1B-HA

The table shows the data from two experiments performed in parallels. TvCfd1A-HA_1 and TvCfd1A_2 samples contain 30 putative partner proteins for TvCfd1A. TvCdf1B-HA_1 and TvCfd1B-HA_2 samples contain 11 putative partner proteins for TvCfd1B. In these samples, we found unique peptides for TvCfd1A. Immunoprecipitated Ha-tagged proteins are highlighted in bold. Proteins that are differentiated are stated both (/). Predicted hydrogenosomal proteins are marked (*).

6 DISCUSSION

6.1 ISC-CIA relationship

In model organisms such as *S. cerevisiae* and *A. thaliana*, the ISC pathway was repeatedly shown to be indispensable for maturation of FeS clusters in mitochondria as well as in the cytosol (Balk and Pilon 2011; Lill et al. 2012). Moreover, the components of ISC pathway has to be localized within mitochondria to fulfill their proper roles (Gerber et al. 2004). The link between mitochondrial ISC machinery and the cytosolic CIA pathway is facilitated by the proteins Atm1 and Erv1 of the inner mitochondrial membrane and intermembrane space respectively, along with glutathione (GSH) (Kispal et al. 1999; Mesecke et al. 2005; Schaedler et al. 2014). These components export sulfur containing compound of unclear character (X-S) to the cytosol. Two hypothetical structures of X-S compound have been proposed including tetra-GSH-coordinated [2Fe2S] cluster, and GSSSG (the oxidized form of GSSH).

Intriguingly, *Trichomonas* and other anaerobic parasites possess no protein homologous to Atm1 or Erv1 (Loftus et al. 2005; Rada et al. 2011). Moreover, *T. vaginalis* does not synthesize GSH. The question is, whether these components could be replaced by alternative export machinery or whether the ISC pathway is required for CIA machinery equally in anaerobic and aerobic organisms that differ in oxygen-metabolism significantly. Therefore, we attempt to investigate this phenomenon using *T. vaginalis* as a model anaerobic organism. In our study we attempted to establish a method of reverse genetics based on homologous recombination in *T. vaginalis* to investigate the role of hydrogenosomal ISC machinery for function of CIA machinery, and we partially characterized components of CIA machinery. We primarily searched for single copy genes coding CIA components as suitable candidates for gene knockout. In *T. vaginalis*, most of genes are present in multiple copies and only one component of ISC pathway, particularly IscU was found to be a single copy (Carlton et al. 2007). On the other side the IscU protein was shown to be essential in other organisms and therefore the complete knockout might be lethal. However, Nfu and IscA were previously suggested to be potential alternatives for IscU scaffold domain in *T. vaginalis* (Beltrán et al. 2013). There were identified 4 paralogues for Nfu and 3 for IscA proteins, which are all expressed in iron dependent manner (Carlton et al. 2007; Beltrán et al. 2013). To test whether *T. vaginalis* cells would be able to overcome the complete loss of TvIscU, we attempted to delete TvIscU by methods of reverse genetics. Moreover, deletion of IscU might provide information about the character of hydrogenosomal ISC - CIA relationship.

First, we confirmed the predicted hydrogenosomal localization of TvIscU. For this purpose, we developed specific polyclonal rat anti TvIscU antibody and using immunofluorescence microscopy, we demonstrated its localization within the organelles. This result is in agreement with previous proteomic studies, in which TvIscU has been detected in hydrogenosomal cellular fractions (Rada et al. 2011).

The methods of reverse genetics are effective tools to investigate the function and phenotype of genes in various model organisms (Hardy et al. 2010). However, applications of these methods for the non-model organisms, albeit being often important human pathogens, are still limited. Particularly, no satisfying technique for gene manipulation has been established for *T. vaginalis*. Utilization of the method of RNA silencing by antisense RNA was reported a decade ago (Mundodi, Kucknoor, and Alderete 2007). However, this technique was not successfully reproduced by other research groups. Neither antisense oligonucleotides (Muñoz et al. 2012) seem to be a suitable technique for gene knockdown for this parasite, as it failed in further experiments in our and other laboratories (data not published). The most effective method appears to be a gene replacement (knockout) by homologous recombination (Land et al. 2004; Brás et al. 2013)This approach is based on a spontaneous homologous recombination of identical sequences of DNA. In the cells, the homologous recombination generally serves as DNA repair mechanism. Under selection pressure of suitable antibiotic, the cells are force to undergo recombination process in a specific position within the genome (Müller 1999). In our experiments, the quality of a linearized DNA that it delivered by electroporation to the cells appears to be crucial for this process, while contamination of circular plasmid might significantly decrease the chance to achieve the homologous recombination. The gene knockout was performed in two laboratories until these days. The first knockout of *fdx1* did not show any phenotype (Land et al. 2004). The observation was explained later by presence of 7 paralogues for Ferredoxin that can substitute each other (Carlton et al. 2007). The report about knockout of the second gene coding for hydrogenosomal protein HMP23 (Brás et al. 2013), was published almost ten years later. The deletion of this gene led to slight change in the size of hydrogenosomes, since the deleted gene encodes the hydrogenosomal membrane protein. Unfortunately, the genome of *T. vaginalis* appeared to be highly repetitive with multiple gene copies for most genes, which significantly limits utilization of homologous recombination for functional genomics.

In our laboratory, we established the knockout technique using the protocol by Land et al. (2004). The crucial condition for successful gene replacement is complete linearization of DNA that is used for electroporation. Initially, we observed frequent contamination by the circular plasmid that led to the selection of cell without accomplished recombination of the gene within the genome. When we improved the protocol to get highly purified linear DNA for targeted gene replacement, we successfully selected *T. vaginalis* population with deleted gene for *fdx1*. Moreover, we cloned these populations and obtained 8 *fdx1* knockout clones.

The same protocol was used for gene deletion of *tviscu*. However, our repeated attempts to select *T. vaginalis* with deleted gene for TvIscU were not successful. These results suggest that TvIscU is essential for *T. vaginalis* and cannot be substitute by alternative scaffold proteins such as IscA, and Nfu. The paralogues for IscA and Nfu would more probably perform additional roles e.g. transfer of the cluster to specific apoproteins as proposed for *S. cerevisiae* (Mühlenhoff et al. 2011). We also cannot exclude the possibility that our linearized construct failed to replace homologous region in *T. vaginalis* genome upon electroporation. However, to limit this possibility, we repeated this experiment three times each in triplicates using pFDKO as positive control.

Therefore, further experiments are desired in future with genes for IscS, Isd11 or Frataxin that are encoded by two genes, thus the knockout of one of the paralogues might display some phenotype related to FeS cluster assembly in viable cells, as was shown previously for Isa1 paralogues in *S. cerevisiae* (Pelzer et al. 2000).

6.2 Components of CIA machinery in *T. vaginalis*

CIA pathway that provides the Fe-S cluster assembly in cytosol is unique for eukaryotic cells (Lill and Mühlenhoff 2005). The members of CIA are highly conserved among eukaryotes. Moreover, they are believed to be present already in the last eukaryotic common ancestor (LECA) (Tsaousis et al. 2014). We focused on CIA pathway of *T. vaginalis* and found 8 candidates for CIA components. Namely we detected two putative paralogues of Cfd1 (Cfd1A, Cfd1B), Nbp35 (Nbp35A, Nbp35 B), Cia1 (Cia1A, Cia1B), and single copy genes for Nar1 and Cia2.

Paralogues for Cfd1 and Nbp35 are P-loop NTPases proteins that possess similar sequence motif CPXC for binding transient cluster (Netz et al. 2007). Moreover, Nbp35 proteins contain also additional conserved cysteins to coordinate a stable [4Fe4S] cluster at N-termini (Hausmann et al. 2005). It was point out that Nbp35 is ubiquitous protein, while Cfd1

is absent in some lineages of eukaryotes including *Giardia intestinalis*, a close relative of trichomonads (Tsaousis et al. 2014). The absence of Cfd1 could be explained by the observation that Nbp35 can form functional homodimer as observed in Viridiplantae (Bych et al. 2008).

Two putative Cia1 proteins with WD40 repeat domain were named TvCia1A and TvCia1B, respectively. Further, we identified TvCia2 which contains a reactive cysteins residue common for other Cia2 proteins (Weerapana et al. 2010). Also, we found TvNar1 which is a homologous to FeS protein Nar1 (Balk et al. 2004)

6.2.1 Expression under control of strong promotor caused protein miss-localization

We tested the cellular localization for all identified CIA components using fluorescent microscopy and subcellular fractionation. The fluorescent microscopy clearly displayed the localization of all CIA proteins in the cytosol apart from TvCfd1A-HA and TvCia1B-HA that were observed to have dual localization in some experiments.

TvCfd1A, TvNbp35A, TvNar1 and TvCia2 were observed to be associated with surface of the organelles after subcellular fractionation. TvCfd1B-HA, Nbp35B-HA and Cia1A-HA were detected within the hydrogenosomes. It is known that expression of genes driven by strong STK promoter may result in unnatural amount of proteins and consequently leads to the protein miss-localization within the cells (Rada et al. 2015). Therefore, we suspected that the unusual localization of TvCfd1B, TvNbp35B and TvCia1A within the organellar fractions might be caused by the over-expression of the proteins.

Indeed, when we analyzed localization of the Nbp35B-HA-N that is expressed under native promoter, the signal decreased and protein appeared to be associated only with hydrogenosomal surface. TvCfd1-HA-N completely lost organellar localization and was present exclusively in the cytosol. These results indicate that high protein expression that is under control of STK promotor leads to the protein miss-localization within the cells. Similar observation was reported for expression of Fdx1 by Rada et al. (2015). The authors investigated the role of N-terminal presequence of Fdx1 for its targeting to hydrogenosomes. When the targeting presequence was deleted and ΔFdx was expressed under STK promotor, significant part of the protein was delivered into the organelles. However, when the same protein was expressed under control of native promotor, ΔFdx remains exclusively in the cytosol (Rada et al. 2015). Regarding these results, we recommend to use the native promoters for expression of recombinant proteins in *Trichomonas vaginalis* if possible.

6.2.2 Organellar localization of CIA components

Observed association of Nbp35B-HA-N with hydrogenosomal surface is noteworthy. Presence of some CIA components in crude mitochondrial fractions was previously observed in other eukaryotes. Namely, Nar1 and Cfd1 in *S. cerevisiae* appeared to be present in crude mitochondria fraction and membrane-enriched fractions, respectively (Roy et al. 2003; Balk et al. 2004). Although this phenomenon appears to be rather an artifact, two cytosolic FeS factors, Tah 18 and Dre2, were shown to have dual localization in both, cytosol and mitochondria (Vernis et al. 2009; Banci et al. 2011). According to recent studies, Dre2 is suggested to associate with the surface of mitochondria (Peleh et al. 2014).

However, Nbp35 was found only in the cytosol and nucleus of *S. cerevisiae* (Hausmann et al. 2005). In contrary, in *G. intestinalis* two Nbp35 paralogues and Cia2 revealed dual localization in the cytosol and mitosomes, reduce forms of mitochondria (Pyrih et al., under review). Nbp35 proteins are most likely associated with the surface of mitosomes whereas Cia2 is present in the intermembrane space. In this work, we proposed that Nbp35 and Cia2 may substitute the absence of Atm1 and Erv1 in *G. intestinalis*. Similarly, we observed association of Nbp35 with surface of *T. vaginalis* hydrogenosomes. In the case of Cia2, its attachment to the surface of hydrogenosomes was observed when the gene was expressed under control of STK; however, we did not succeed to express this protein under native promoter yet. Therefore, further experiments are needed to resolve Cia2 topology in *T. vaginalis*. Collectively, our results suggest that there is significant variation in cellular localization and possibly functions of CIA components in eukaryotes.

6.2.3 Missing CIA components in T. vaginalis

We did not find any homologues for MMS19, Dre2 and Tah18. The patchy distribution of Mms19 which seems to be not essential was reported previously (Tsaousis et al. 2014). The absence of Dre2 and Tah18 seems to be a general trend in anaerobic protists (Pyrih et al. under review, Basu et al. 2013; Tsaousis et al. 2014). We suggest that the Mms19 might be substituted by different protein, which would be able to bind and interact with nuclear proteins in *Trichomonas vaginalis*. Otherwise, its function can be substitute by other members

of CIA targeting complex, particularly TvCia1A/B and TvCia2 themselves, as the CIA targeting complex appeared to vary in its member´s composition (Paul and Lill 2015)

We cannot explain the loss of Dre2 and Tah18 satisfactory, yet. These proteins form a short electron chain in the cytosol (Zhang et al. 2008; Netz et al. 2010). Dre2 directly interacts with Nbp35 and donates electrons to reduce stable N-terminal [4Fe-4S] cluster (Netz et al. 2010, 2014). It remains the matter of debate whether there exists a different electron donor to supply the electrons for this cluster in T. vaginalis, or whether it does not need any electron transfer. Interestingly, Tah18 and Dre2 are also involved in unrelated reduction of ribonucleotide reductase metallocofactor, so it is unclear how this cofactor is assembled in *T. vaginalis* and other anaerobes (Zhang et al. 2014). This revelation therefore leads to doubts about its main function as essential FeS factors.

6.2.4 Cfd1 interacting partners

Finally, we investigated interactions between Cfd1 and Nbp35 proteins that are known to form a scaffold domain for cluster assembly during initial steps of the CIA machinery (Netz et al. 2007; Stehling et al. 2008). In the species containing both, Cfd1 and Nbp35, these components form functional heterotetramer (Netz et al. 2012). However, *T. vaginalis* possess two gene paralogues for each protein that are all transcribed (data provided by Dr. Sven Gould). To investigate composition of expected Cfd1/Nbp35 complex in *T. vaginalis*, HA-tagged TvCfd1A and TvCfd1B were immunoprecipitated using anti-HA dynabeads and analyzed by mass spectrometry.

When TvCfd1B-HA protein was immunoprecipitated, TvCfd1A was repeatedly detected among interacting partners. However, in the immunoprecipitated TvCfd1A-HA samples, we were unable to detect unique peptides for TvCfd1B. This non-reciprocal data could result from high sequence identity between TvCfd1A and TvCfd1B that cause difficulties in identification of unique peptides for each protein when large number of common peptide is present. However, we failed to detect any TvNbp35 paralogue as expected interaction partner for TvCfd1. Cfd1 is often missing in various eukaryotes including members of SAR, Archaeplastida, and *G. intestinalis* (Tsaousis et al. 2014). In *A. thaliana*, Nbp35 protein was shown to form functional homomeric complex without contribution of Cfd1 (Bych et al. 2008). However, the formation of a complex that consists of only Cfd1 subunits has not been observed so far. Moreover, Cfd1 subunits cannot form N-terminal [4Fe4S] cluster, thus function of such a complex in FeS cluster formation is doubtful. On the other side the Cfd1

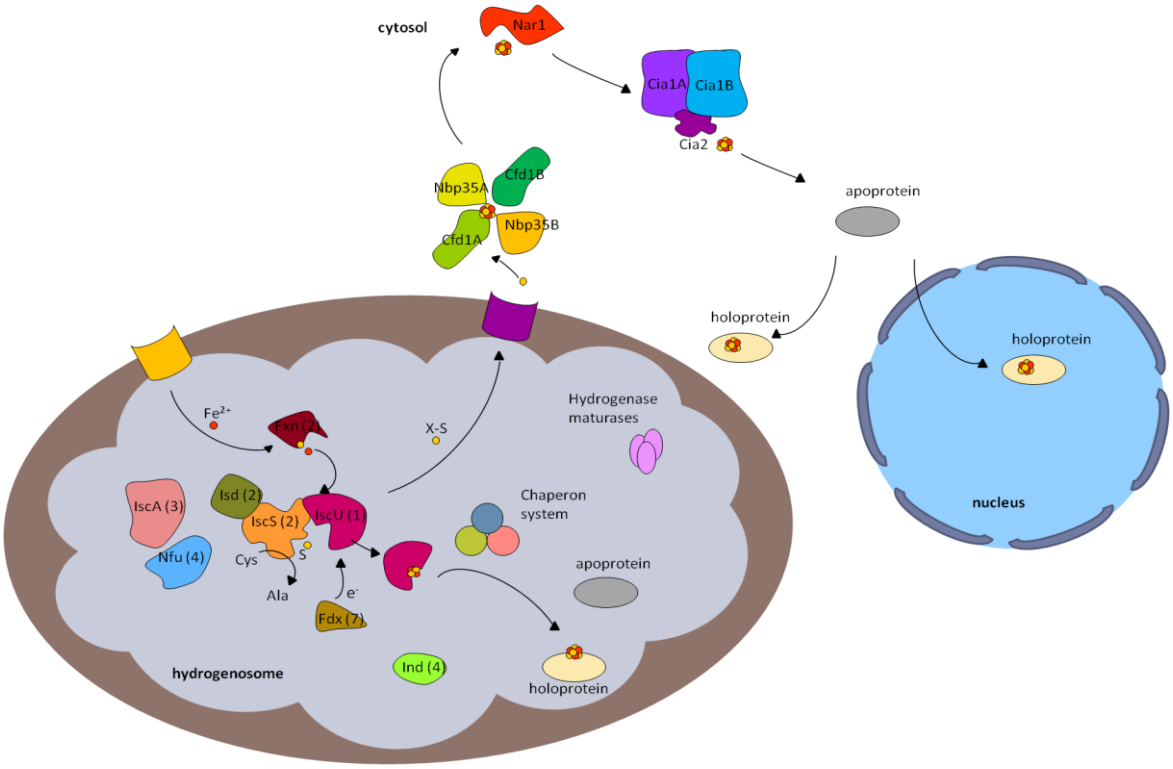
homodimer is able to bind one [4Fe4S] via its C-terminus (Netz et al. 2012). Another possible explanation is that high amount of recombinant Cfd1 that was expressed under control of STK promotor, does not allow natural interaction with Nbp35 proteins that are present in innate concentrations. Therefore, other strategies need to be employed to resolve Cfd1-Nbp35 interactions in *T. vaginalis*: (i) immunopreciptation of Nbp35 paralogues that are expressed under native promotor, (ii) simultaneous co-expression of two proteins of CIA pathway (e.g. Cfd1A and Nbp35B) with different tags. We expect to detect the interaction partners more likely once they have a similar expression level. As a control, we plan to use cells, which co-express other CIA protein and CIA non-related protein.

Interestingly, the proteomic analysis of proteins co-precipitated with TvCfd1A-HA and TvCfd1B-HA revealed that both proteins interact with cytosolic as well as hydrogenosomal proteins. For example, among interacting partners of TvCfd1A-HA, we identified typical cytosolic enzymes, such as PPi-dependent phosphodructokinase, Alcohohol dehydrogenase, or malic enzymes, proteins that are present at outer hydrogenosmal membrane (C-tail anchored protein-8, putative Tom-40) as well as protein that reside in hydrogenosomal matrix (Pyruvate-ferredoxin oxidoreductase and ATP-dependent phosphofructokinase). These findings indicate that both recombinant proteins have dual localization in the cytosol and hydrogenosomes when expressed under the strong STK promoter. This is in agreement with observed dual localization of TvCfd1A and B using western blot analysis of subcellular fractions as discussed above. It is unlikely that all identified proteins are natural functional interacting partners. Probably they are randomly crosslinked in the close proximity with abundant recombinant proteins. Therefore, this method seems to be an excellent tool for investigation of cellular localization of the tagged proteins. Indeed, we used similar approach for investigations of Cia2 and Nbp35 topology in *G. intestinalis*, previously (Pyrih et al. under review).

7 CONCLUSIONS

In conclusion, in this work we focused on selected components of ISC pathway and CIA machinery in *Trichomonas vaginalis*. We prepared specific antibody against TvIscU, and demonstrated localization of TvIscU in the hydrogenosomes. We established gene knock out system for *T. vaginalis* and attempt to delete gene for TvIscU. The results suggest that TvIscU is essential for the parasite. Next, we identified 8 components of CIA pathway and demonstrated their localization in the cytosol. Moreover, we showed that TvNbp35A is also associated with the hydrogenosomal surface. We established a protocol for protein immunoprecipitation and analysis by mass spectrometry. We propose a tentative scheme for FeS cluster biogenesis via ISC and CIA machineries in *T. vaginalis* (FigX), which need further investigations to clarify several points:

(i) Which membrane transporters and proteins are involved in the transport of X-S compound from hydrogenosomes to the cytosol and which proteins are involved in the transport of iron from the cytosol to hydrogenomes?, (ii) Are there other hypothetical proteins participating in ISC or CIA machinery in *T. vaginalis*?, (iii) Do Cfd1 and Nbp35 proteins form proposed complex?, (iv) Do the paralogous proteins have similar or different function?, (v) How does the X-S arise and what it is composed of? (vi) How is the loss of Dre2 and Tah18 related to anaerobiosis and how do the cells deal with electron transfer to stable cluster of Nbp35?



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