SUMMARY

This thesis summarises data included in three publications and one manuscript that describe results of projects I participated in during my PhD study. The results, presented in a context of current knowledge, are briefly outlined in the following paragraphs.

***Giardia intestinalis* incorporates heme into cytosolic cytochrome b_5**

Heme is a prosthetic group of hemoproteins, which are important for transporting electrons in the respiration chain, oxidative stress defense, carrying diatomic gases and other vital functions (Shimizu *et al.*, 2015). Anaerobic pathogens, such as *Giardia*, do not possess the pathway for heme synthesis. They are also devoid of typical hemoproteins involved in stress defense and this function is overtaken by different heme-independent proteins of bacterial origin (Coombs *et al.*, 2004;Nixon *et al.*, 2002). However, these organisms usually retained several genes for hemoproteins from cytochrome b_5 ($cytb_5$) family (Koreny *et al.*, 2013). They typically possess C-terminal transmembrane domain which anchores them in the membrane of ER or mitochondria with N-terminal heme-binding domain facing cytosol. Nevertheless, whether these organisms indeed harbor heme cofactor remained unknown. The only organism which was proven to be able to survive without heme is *Phytomonas serpens*, a protist encoding for 19 cytochrome b5 genes (Koreny *et al.*, 2012).

Giardia contains only few heme-binding proteins, namely flavohemoglobin functioning as a protection against oxygen and nitric oxide (Rafferty *et al.*, 2010) and three $cytb_5$ with yet unknown function (Alam *et al.*, 2012). By bioinformatic searches we identified three known $cytb_5$– gCYTb5-I, gCYTb5-2 and gCYTb5-3 (Alam *et al.*, 2012) and two $cytb_5$-like proteins named gCYTb5-4 and GiTax. Surprisingly, all gCYTb lack the C-terminal anchoring domain typical for previously described cytochromes b5. All genes were subcloned with HA-tag and their localization was shown to be cytosolic.

Furthermore, we tested the ability of four recombinant gCYTb5 proteins to bind heme using UV/VIS-spectroscopy and HPLC analysis. We confirmed that all gCYTb5 proteins are able to bind heme *in vitro*. Moreover, we inserted a mutation into gCYTb5-4 to replace the heme-binding histidine. Consequently, the mutated protein showed significant decrease in the heme-binding ability.

Giardia does not have typical proteins for $cytb_5$ reduction such as P450 reductase. Hence, we tested the ability of protein GiOR-1 to reduce cytochromes as it shows sequence similarity to P450 reductase (Jedelsky *et al.*, 2011; Koreny *et al.*, 2013). Our results clearly indicate that

GiOR-1 is capable of $cytb_5$ reduction *in vitro*. However, given that GiOR-1 was shown to be associated with mitosomes (Jedelsky *et al.*, 2011), it is unknown whether this redox system is functionally relevant for the cells.

Furthermore, we also tested whether the heme cofactor is indeed present in *Giardia* cells. We compared the amount of endogenous heme in wild type cells to the cell line overexpressing gCYTb5-4 and also to gCYTb5-4 with mutated heme-binding site. The heme added to the culture media was incorporated to gCYTb5 as its level was 2-fold larger in gCYTb5-4 overexpressing strain.

In this study, we provided evidence, that *Giardia* is able to utilize heme from the environment and incorporate it into its hemoproteins. Nevertheless the necessity of heme for *Giardia* survival is yet to be discovered due to the lack of the defined growth media.

Minimal cytosolic iron-sulfur cluster assembly machinery of *Giardia intestinalis* is partially associated with mitosomes

The only mitosomal function known in *Giardia* is Fe-S cluster assembly via ISC (iron-sulfur cluster) pathway. The Fe-S clusters are essential for the cells as they enable proteins to transport electrons or sense signals. In eukaryotes, Fe-S proteins are involved in respiration, photosynthesis, DNA metabolism or cell growth. Besides the ISC pathway, there is a cytosolic pathway called CIA machinery (cytosolic iron-sulphur cluster assembly). The CIA machinery is typically formed of at least 8 proteins (Tah18, Dre2, Nbp35, Cfd1, Nar1, Cia1, Cia2 and MMS19) and is dependent on ISC pathway (Netz *et al.*, 2014). The yet-unknown sulfur-containing compound is likely transported from mitochondria to cytosol via Atm1 and Erv1 proteins (Kispal *et al.*, 1999; Lange *et al.*, 2001).

We searched for CIA homologues in metamonads which resulted in the identification of four components present in all of them (Nbp35, Nar1, Cia1 and Cia2). Cfd1 was present in the ancestor of all metamonads and was likely lost secondarily as it is present in less-derived members of the genera. Dre2 and MMS19 are absent in all metamonada. Interestingly, Erv1 and Atm1, two key components which link CIA and ISC miachineries are missing in metamonads as well.

In *Giardia*, Nar1, Cia1 and Cia2 are encoded as a single-copy genes, Nbp35 was found in three copies (Nbp35-1, Nbp35-2, Nbp35-3). Two putative Tah18 homologues – GiOR-1 and GiOR-2 were previously characterized (Jedelsky *et al.*, 2011). We fused all the genes with the

C-terminal HA-tag and addressed their localization using fluorescent microscopy as well as cell fractionation. Cytosolic localization was observed in case of Nar1, Cia1 and Nbp35-3. Interestingly, Cia2 was found to be localized both in cytosol as well as inside mitosomes. Nbp35-1 and Nbp35-2 also localize in cytosol and are also peripherally associated with the outer mitosomal membrane. The GiOR-1 protein was shown to be inner mitosomal protein.

The subsequent *in vivo* biotinylation and coprecipitation of GiOR-1 protein revealed, that it mainly interacts with matrix proteins. However, Cia2 coprecipitation revealed no matrix proteins. Given that the protease protection assay showed its clear distribution inside mitosomes, we propose that Cia2 is likely to be localized in the intermembrane space.

As GiOR-1 and GiOR-2 proteins show sequence similarity to Tah18, we further addressed their possible involvement in CIA pathway. Their ability to rescue the function of Tah18 in *T. brucei* was tested. We used the strain with double RNAi knock-down for Tah18 and Dre2. Both *Giardia* proteins were able to partially rescue the growth of RNAi cell line. Moreover, both proteins were able to rescue Fe-S cluster dependent activity of aconitase. Nevertheless, despite the fact that the proteins partially rescue the RNAi phenotype, they are likely not functioning in CIA pathway in *Giardia*. The phylogenetic analysis revealed that Tah18-like proteins including GiOR-1 and GiOR-2 in metamonads are of different evolutionary origin than canonical Tah18 proteins. Moreover, GiOR-1 is mitosomal matrix protein and GiOR-2 was identified to be associated with unknown peripheral vesicules in *Giardia* (Jedelsky *et al.*, 2011). Therefore they likely act in different cellular compartments then CIA machinery.

Taken together, we propose, that GiOR proteins in *Giardia* are not a part of CIA machinery, although their function remains to be characterized. It thus makes *Giardia* one of few eukaryotes lacking Tah18/Dre2 complex. Furthermore, we furher hypothesize, that the function of proteins Atm1 and Erv1 linking the ISC and CIA pathways was probably overtaken by Nbp35-1 and Nbp35-2 associated with outer membrane as well as Cia2 in intermembrane space.

Probing the biology of *Giardia intestinalis* mitosomes using *in vivo* enzymatic tagging

Current knowledge of mitosomal proteome in *Giardia* is limited due to the difficulty of their purification and very divergent sequences, which restrain the search for homologues of typical mitochondrial proteins. Hence, we developed a new method for studying protein-protein interactions in *Giardia*. We made use of bacterial enzyme Biotin ligase (BirA). This enzyme

is able to add biotin to the short specific sequence called biotin acceptor peptide (BAP) (Howarth and Ting, 2008). Biotinylated protein can be used for protein purification with streptavidin-coupled beads, since streptavidin-biotin bond is very strong. Therefore, we made a chimeric construct containing *E. coli* BirA fused with N-terminal mitosomal targeting signal and C-terminal HA-tag. Another vector was carrying a bait mitosomal protein with C-terminal BAP-tag. *Giardia* cells were cotransfected with both vectors and the successful transformation was observed as specific biotinylation of given protein on Western blot and fluorescent microscopy.

As a bait we used the only known inner membrane protein Pam18 (Jedelsky *et al.*, 2011). Cells expressing biotinylated Pam18 were used for isolation of mitosome enriched fraction. This fraction was then chemically crosslinked, solubilized and the lysate was purified on magnetic streptavidin-coupled beads. The obtained eluate was measured using mass spectrometry. Amongst the identified proteins we found one, which was after profile-sequence comparisons conducted with HHpred assigned as putative homologue of Tim44, a key component of the TIM complex. This protein was shown to localize in mitosomes when episomally expressed. However, bioinformatic analysis showed high divergence of the sequence as well as truncation of N-terminal part of the protein, which is responsible for TIM binding and interaction with Hsp70 (Ting *et al.*, 2014).

Nevertheless, using Tim44 as a biotinylation bait, we obtained another dataset of proteins, which revealed its proximity to Hsp70 and other mitosomal proteins so it might function in mitosomal TIM complex. However, the exact role of the truncated Tim44 in mitosomes remains to be investigated. Interestingly, no possible candidate for inner membrane import channel from Tim17 famiy was identified. This might be due to the limitations of mass spectrometry analysis, which uses trypsin cleavage of the proteins and which may thus be affected by inefficiency of trypsin to cleave hydrophobic proteins. Using outer membrane channel Tom40 as a biotinylation bait, we identified new mitosomal protein MOMP35. It is an outer membrane protein, which can trigger mitosomal aggregation when overexpressed.

In total, we identified 16 new mitosomal proteins and verified their localization. Surprisingly, most of them share no homology to proteins from other organisms. These *Giardia*-specific proteins indicate that mitosomes bare yet unknown functions unique for *Giardia*.

Furthermore, *in vivo* biotinylation tagging allowed us to follow the mode of mitosomal targeting. Using cytosolic BirA, we showed that a newly translated BAP-tagged protein is

biotinylated in cytosol before its transport into mitosome. We also tested weather the proteins are transported in folded or unfolded state. We used mouse dihydrofolate reductase (DHFR), which can be folded upon addition of folate analog (Eilers and Schatz, 1986). Construct comprising mitosomal leader fused to DHFR was unable to be transported into mitosome after addition of folate analog and remained in cytosol.

Single Tim translocase in the mitosomes of *Giardia intestinalis* illustrates the convergence of the protein import machines in anaerobic eukaryotes

Since proteomics have failed to identify possible mitosomal inner membrane translocase, we used the newly published transcriptomic data from related Metamonads (Leger *et al.*, 2017). These sequences were used to enrich Hidden-Markov models and after the HMMsearch, the model was enriched again with newly identified metamonad sequences. This step was repeated three times until no new sequences were identified. We found a candidate sequence which shows weak homology to Tim17 in HHpred analysis. Phylogenetic analysis revealed its affinity to Tim17 homologues in *Giardia* closest relatives – Carpediomonas-like organisms. The protein is thus likely of Tim17 family origin.

However, it lacks some of the conserved features of canonical Tim17, such as multiple GxxxG motifs responsible for Tim23 binding (Demishtein-Zohary *et al.*, 2015) or conserved cysteine residues functioning in voltage-dependent gating (Ramesh *et al.*, 2016). Nevertheless, it has four transmembrane domains, and the arginine residues responsible for Tim44 binding. Although Tim44 is N-terminally truncated, it was recently shown, that the C-terminal part of Tim44 is responsible for direct interaction of Tim44 and Tim17 (Banerjee *et al.*, 2015). Therefore these proteins are likely to interact together in *Giardia*.

Therefore, we addressed the function and localization of the hypothetical Tim17. We revealed, that it is localized in the mitosomal inner membrane and that it is expressed under natural conditions. We also used it as a biotinylation bait for the co-precipitation experiments. The results indicated its close proximity to Tim44 and Hsp70, which points to its function in protein import pathway. It also interacts with some *Giardia*-specific proteins, which could reflect the loss of other components typically involved in other organisms. The similar situation was described for *T. brucei*, where the canonical pathway is reduced and consists of many kinetoplastid-specific proteins (Singha *et al.*, 2012).

Although we were not able to bring direct evidence of Tim17 functioning as a protein translocase, all our results indicated that the protein probably retained its original function. We propose, that the channel could be formed of Tim17 dimer. Using yeast two hybrid system we showed that the protein interacts with itself. Moreover, Tim17 is a part of a protein complex bound by disulfide bond of approximately doubled size of single protein, which was observed on SDS-PAGE using the sample buffer without mercaptoethanol. The ability to dimerize is further supported by the appearance of complex of appropriate size in BN-PAGE upon *in vitro* translation.

Altogether, it seems that the inner membrane transport complex in *Giardia* is a mosaic of ancestral and *Giardia*-specific proteins which has evolved to meet the special needs of this extremely divergent organelle.

CONCLUSIONS

Giardia intestinalis has long been considered as amitochondriate protist. Since the discovery of extremely reduced mitochondria – mitosomes, many publications dedicated to mitosomal proteome were released. Although the only described function so far is the Fe-S cluster synthesis via ISC machinery (Tovar *et al.*, 2003), recent studies suggest that mitosome is much more complex organelle (Martincova *et al.*, 2015; Rout *et al.*, 2016).

The characterization of mitosomal proteome is very difficult, since these extremely small organelles cannot be purified in a clean fraction and also due to very divergent protein sequences. Nevertheless the newly sequenced closely related organisms can be used for identification of more divergent proteins, as was recetly demonstrated on Tim17 homologue (Martincova et al., 2017, manuscript). We can thus expect the identification of more and more extremely divergent proteins.

Mitosomes contain many *Giardia*-specific proteins function of which remains elusive (Martincova *et al.*, 2015; Rout *et al.*, 2016). We propose that these are involved in unique functions without homology to any other organisms. Furthermore, these *Giardia*-specific proteins have probably overtaken the function of canonical components of protein import pathway creating an unique mosaic of ancestral and species-specific proteins.

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