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RSM22, mtYsxC and PNKD-like proteins are required for mitochondrial translation in *Trypanosoma brucei*

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ABSTRACT

Mitochondrial ribosomes evolved from prokaryotic ribosomes, with which they therefore share more common features than with their counterparts in the cytosol. Yet, mitochondrial ribosomes are highly diverse in structure and composition, having undergone considerable changes, including reduction of their RNA component and varying degree of acquisition of novel proteins in various phylogenetic lineages. Here, we present functional analysis of three putative mitochondrial ribosome-associated proteins (RSM22, mtYsxC and PNKD-like) in *Trypanosoma brucei*, originally identified by database mining. While in other systems the homologs of RSM22 are known as components of mitochondrial ribosomes, YsxC was linked with ribosomes only in bacteria. The PNKD-like protein shows similarity to a human protein, the defects of which cause PNKD (paroxysmal non-kinesigenic dyskinesia). Here we show that all three proteins are important for the growth of *T. brucei*. They play an important function in mitochondrial translation, as their ablation by RNAi rapidly and severely affected the *de novo* synthesis of mitochondrial proteins. Moreover, following the RNAi-mediated depletion of RSM22, structure of the small subunit of mitochondrial ribosome becomes severely compromised, suggesting a role of RSM22 in ribosomal assembly and/or stability.

1. Introduction

Mitochondrion is derived from an ancestral symbiotic α-proteobacterium, which evolved into a double membrane-bound cellular compartment housing several key metabolic processes (Gray, 2012). Even though most of the original genes of the endosymbiont have been translocated into the nucleus, a typical aerobe retains at least several genes in its mitochondrial (mt) genome. The most gene-rich mt genomes are those of the jacobid protists (Burger et al., 2013), while the most reduced one, retaining just two protein-coding genes, have recently been described in a free-living alveolate (Flegontov et al., 2015). Apart from these extremes, most mt genomes encode two ribosomal RNAs, a set of tRNAs and several highly hydrophobic proteins involved in the electron transport chain (Chacińska and Boguta, 2000). Consequently, the mitochondrion has retained the replication, transcription and translation machineries, which depend on the import of nuclear-encoded components. Importantly, mutations that disrupt the expression of mt-encoded proteins have been associated with several human diseases (Sylvester et al., 2004; Scharfe et al., 2009; Calvo and Mootha, 2010).

Mitochondrial ribosomes are derived from ribosomes of the ancestral endosymbiont and hence share more features with prokaryotic ribosomes than with ribosomes in the cytosol (O'Brien, 2002). The sedimentation coefficient (S) of a typical prokaryotic ribosome is around 70S, with its large (LSU) and small subunits (SSU) sedimenting at 50S and 30S, respectively, while those parameters for the 80S eukaryotic cytosolic ribosomes are 60S and 40S (Schmeing and Ramakrishnan, 2009). When compared to their cytosolic counterparts, mt ribosomes are considerably more diverse (Desmond et al., 2011), showing various degrees of reduction of rRNA and acquisition of novel proteins in different phylogenetic lineages (Smits et al., 2007). Due to a different protein/RNA ratio, most mt ribosomes have smaller S values; for example, mammalian mt ribosomes are ~55S (O'Brien, 2003), those from the parasitic protist *Leishmania* are 50S (Maslov et al., 2006), yet there are examples of more bacteria-like mt ribosomes, such as the 78S ribosomes found in some plants (Leaver and Harmey, 1972). Since a typical mt ribosome contains smaller size rRNAs compared to its prokaryotic predecessor, it was proposed that the increased number of proteins has a compensatory role (O'Brien, 2002). However, this view was only partly supported

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by a detailed structural analysis of the bovine mt ribosome (Sharma et al., 2003). Some of the newly recruited proteins appear to be bifunctional, with a second role outside of the mt translation (O'Brien, 2003).

Trypanosomatids are a group of parasitic flagellates, including those responsible for devastating human diseases, such as Human African Trypanosomiasis, Chagas disease and several types of leishmaniasis. These organisms carry mt ribosomes which are among the smallest known, with LSU and SSU sedimenting at just 40S and 30S, respectively (Maslov et al., 2006; Sharma et al., 2009). Indeed, they contain very short 9S SSU and 12S LSU rRNAs, which lost some stem-loop regions and underwent a significant reduction in size of others, and yet retained the typical prokaryote-like secondary structure (Eperon et al., 1983; de la Cruz et al., 1985). Remarkably, although the highly protein-rich mt ribosomes of trypanosomatids contain numerous unique features (Maslov et al., 2007; Zíková et al., 2008; Sharma et al., 2009; Aphasizheva et al., 2011; Aphasizheva et al., 2013; Aphasizheva et al., 2016), they still appear strikingly similar to bacterial ribosomes in morphology (Sharma et al., 2009).

Protein composition of mt ribosomes has been investigated in detail in yeast (Saveanu et al., 2001; Graack and Wittmann-Liebold, 1998), mammals (Koc et al., 2001a; Koc et al., 2001b) and trypanosomatids (Maslov et al., 2007; Zíková et al., 2008; Aphasizheva et al., 2011). Moreover, several comparative analyses have been performed (Smits et al., 2007, Desmond et al., 2011,). Due to difficulties with purification of such large (and often loosely bound) protein complexes, the exact composition of mt ribosomes still remains elusive in most cases. This problem may to some extent explain the large compositional diversity described for mt ribosomes, especially when their peripheral proteins are concerned (Zíková et al., 2008; Desmond et al., 2011; O'Brien, 2003; Aphasizheva et al., 2011).

In trypanosomatids, mitochondrial protein synthesis has several unique features: i) most of its mRNAs undergo extensive post-transcriptional remodeling *via* RNA editing of the uridine insertion/deletion type (Read et al., 2016); ii) all tRNAs for mitochindrial translation are imported from the cytosol (Alfonzo and Söll, 2009); iii) its protein synthesis is resistant to several typical inhibitors of prokaryotic translation (Horváth et al., 2002); iv) in addition to individual ribosomal subunits and monosomes, a ribosome-resembling 45S particle (45S SSU*) that contains only the 9S rRNA, is also present in the mitochondrion (Maslov et al., 2007; Sharma et al., 2009; Ridlon et al., 2013; Aphasizheva et al., 2016). Among the proteins uniquely associated with 45S SSU*, the proteins containing the pentatricopeptide or tetratricopeptide repeat domains, usually associated with RNA processing, stand out (Aphasizheva et al., 2011; Aphasizheva et al., 2016). The exact function of the 45S SSU* complex remains unknown, although it is indispensable for translation and possibly acts by facilitating the discrimination between mature mRNAs and their pre-edited versions (Ridlon et al., 2013; Aphasizheva et al., 2016). *T. brucei* is a suitable model organism for studying mt ribosomes, as it is amenable to an array of forward and reverse genetics methods. Moreover, protein synthesis in its single mitochondrion has been already extensively studied and the assays addressing mt translation and ribosome stability have been developed (Horváth et al., 2002; Maslov et al., 2006; Maslov et al., 2007; Zíková et al., 2008; Týč et al., 2010; Ridlon et al., 2013; Verner et al., 2015).

In our survey aimed to identify functions of mt proteins conserved between human and *T. brucei*, we detected three proteins (RSM22; mtYsxC and PNKD-like protein) that prior to this work were co-purified with the mt ribosomes. The first two proteins were identified as possible ribosomal components in an affinity pull-down analysis by Zíková et al. (2008), and all three were present in the list assembled by Aphasizheva et al. (2011), in which RSM22 and PNKD-like proteins were associated with the SSU, while mtYsxC was identified as a putative part of the LSU, also based on the affinity pull-down survey.

From these proteins only the homolog of RSM22 was previously shown to be a genuine and essential part of mt ribosomes in *S. cerevisiae* (Saveanu et al., 2001). This protein is absent from prokaryotes and therefore represents a secondary acquisition by the mt ribosome (Saveanu et al., 2001; Desmond et al., 2011). The second protein, YsxC, is a member of the GTPase family, and GTPases were shown to be important for assembly and maturation of ribosomal subunits (Im et al., 2011; Matsuo et al., 2014; Gulati et al., 2013; Bassler et al., 2006). Proteins containing the same domain as the *T. brucei* mtYsxC, such as YsxC and YihA of *Bacillus subtilis* and *E. coli*, respectively, were attributed a role in the ribosomal LSU assembly (Schaefer et al., 2006; Wicker-Planquart et al., 2008; Cooper et al., 2009; Wicker-Planquart and Jault, 2015). Finally, the PNKD-like protein carries the lactamase B domain, shared with glyoxalase hydroxyacylglutathione hydrolases known to detoxify methylglyoxal, a by-product of glycolysis. Mutations in this gene have been associated with the movement disorder known as paroxysmal non-kinesigenic dyskinesia (PNKD) in humans (Charlesworth et al., 2013). So far, there is no link to mitochondrial translation for this protein. Here we show that all three proteins participate in mt translation of *T. brucei*, and at least one of them, RSM22, is structurally associated with the mt ribosome.

2. Materials and methods

2.1. Generation of RNAi cell lines and cultivation

Procyclic *T. brucei* 29-13 cells were grown in SDM-79 medium containing 10% fetal bovine serum (FBS), 15 μg ml^{-1} geneticin and 50 μg ml^{-1} hygromycin. These conditions were used in all experiments except for growth rate analysis in glucose-free medium where SDM-80 (supplemented with 10% dialyzed FBS) was used. For RNAi, gene fragments of RSM22 (Tb927.11.5060), mtYsxC (Tb927.8.2760) and PNKD-like (Tb927.8.2650) were PCR-amplified from the *T. brucei* genomic DNA using primers listed in Table S1. The PCR amplicons were gel-purified, digested with the indicated restriction enzymes (Table S1), and ligated into the p2T7-177 vector pre-digested with the same enzymes. The plasmids were linearized using *Not*I, electroporated and the transfectants were selected as described previously (Vondrušková et al., 2005). The cell line with RNAi-targeted RNA polymerase, described elsewhere (Grams et al., 2002), was kindly provided by Paul T. Englund (John Hopkins University, Baltimore). RNAi was induced by the addition of tetracycline (1 μg ml^{-1}) to the medium and cell densities were measured using a Beckman Coulter Z2 counter every 24 h over a period of 7 to 11 days after RNAi induction.

2.2. Quantitative RT-PCR

From all RNAi cell lines, RNA was isolated and processed for subsequent reverse transcription into cDNA as described previously (Hashimi et al., 2008). The resulting random hexamer-primed cDNA was used as a template for quantitative real-time (q) PCR as described in the aforementioned reference using previously designed primers that anneal to the maxicircle mRNA sequences (Carnes et al., 2005; Etheridge et al., 2008); the primers including those designed specifically for this study are listed in Suppl. Table S1. The results were normalized using cytosolic 18S rRNA and β-tubulin. Both standards produced similar results, therefore only data obtained with 18S rRNA are shown in Figs. 1 and 3. The relative abundance of RNAs was determined by the Pfaffl method (Pfaffl, 2001).

2.3. Mitochondrial translation

Analysis of *de novo* mt translation followed procedures described previously (Horváth et al., 2002; Horváth et al., 2000; Ridlon et al., 2013). Approximately 5×10^7 exponentially growing cells were harvested by centrifugation, washed twice and resuspended in 100 μl of the SoTE buffer (0.6 M sorbitol, 20 mM, Tris–HCl, pH 7.5, 2 mM EDTA). Cytosolic translation was inhibited with 100 μg ml^{-1} cycloheximide

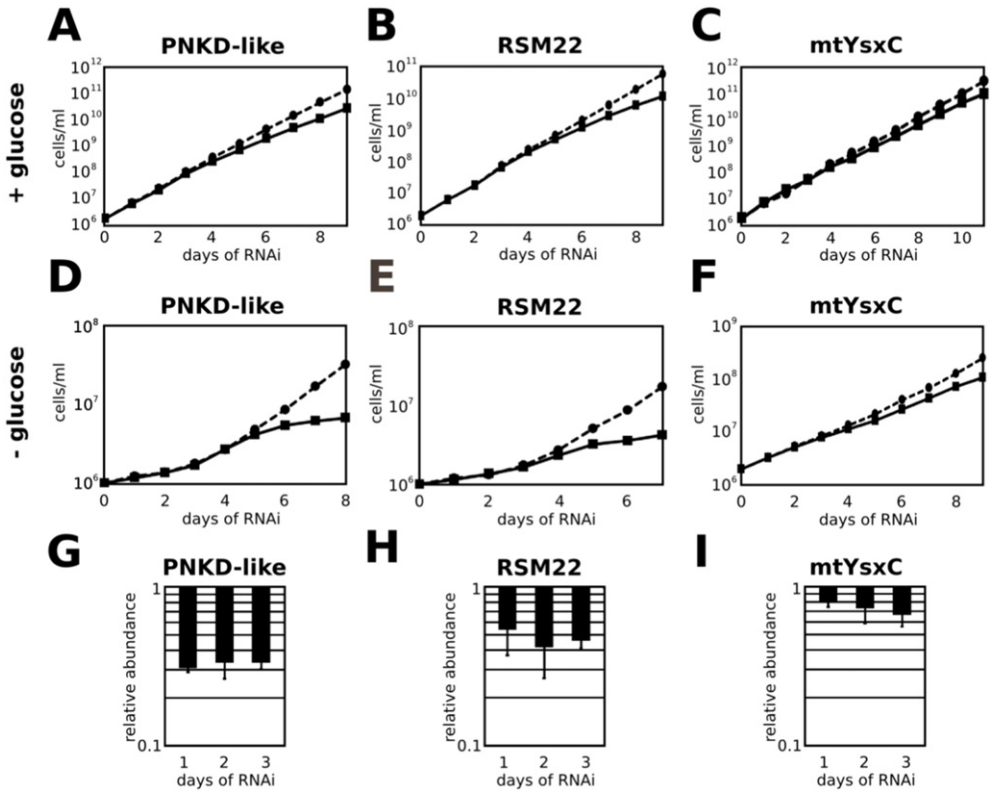

Fig. 1. PNKD-like and RSM22 are important for growth of procyclic *T. brucei* under low-glucose conditions, while the depletion of mtYsxC has only a limited effect. Growth curves of cell lines induced for RNAi in glucose containing SDM-79 medium (A, B and C) and glucose-free SDM-80 medium (D, E and F). Un-induced cells are visualized by circles and dashed line, cells in which RNAi against respective gene was induced are shown by squares with full line. The plots show the calculated cumulative cell numbers, all growth curves were repeated at least three times. Results of qPCR analysis confirming down-regulation of corresponding mRNA (G, H and I). The data show relative amounts of the targeted mRNAs in clonal cell lines upon induction of RNAi. Error bars represent the standard deviation. Experiments for all time points were repeated at least twice. The cytosolic 18S rRNA was used as an internal reference.

and mt translation products were labeled by incubating whole cells for 2 h at 27 °C in the presence of EasyTag™ EXPRESS ^{35}S protein labelling mix (PerkinElmer Life Sciences). Labeled cells were extracted with Triton X-100 to remove soluble proteins and the remaining hydrophobic material was resolved in denaturing 2D (9% *versus* 14%) polyacrylamide Tris–glycine SDS gels; the radioactive products were revealed by fluorography.

2.4. Analysis of mitochondrial ribosomal complexes

The procedure was carried out as described previously (Maslov et al., 2007; Ridlon et al., 2013). Approximately 10^8 procyclic cells were recovered by brief centrifugation, washed with SoTE and lysed in DM buffer (1% dodecyl maltoside, 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 0.1 mM EDTA) supplemented with 400 units of RNasin® Plus RNase inhibitor (Promega). Lysate was cleared by centrifugation at 17,000 ×*g* for 15 min and subsequently loaded on top of 7–30% sucrose gradients made with SGB buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 0.1 mM EDTA, 0.05% dodecyl maltoside). Gradients were centrifuged in an SW41 rotor at 50,000 ×*g* for 16 h and separated into 32 fractions. Phenol-chloroform method was used to extract RNA from these fractions, and RNA transferred onto BrightStar®-Plus hybridization membrane (Ambion) was immobilized by UV cross-linking. Membranes were hybridized with probes against 9S and 12S rRNA and the results were quantified by a Typhoon Phosphoimager. The signals obtained for 9S and 12S rRNA were normalized by hybridization to the *in vitro*-transcribed tandem 12S-9S rRNA immobilized on each hybridization membrane.

3. Results

3.1. Identification of the proteins of interest

Three proteins analyzed in this study (Tb927.8.2650, Tb927.11.5060 and Tb927.8.2760) were initially studied using BlastP, Psi-BLAST and pHMMER algorithms to identify conserved domains within their sequences and to search for potential homologs in other organisms. Conserved domain database, Pfam and Interpro databases were then used to further analyze the conserved domains.

The first candidate, Tb927.8.2650, contains a lactamase B superfamily domain that resembles the domain of glyoxalase hydroxyacylglutathione hydrolases. These proteins are ubiquitously present in bacteria, archaea and eukaryotes. In trypanosomatid flagellates, some homologs are annotated as metallo-β-lactamase-like proteins. The lactamase B superfamily domain of Tb927.8.2650 gene is interrupted by an unrelated sequence. The closest human counterparts include hypothetical metallo-β-lactamase domain-containing proteins, some of which are predicted to be mitochondrial due to the presence of the mt targeting sequence. Isoforms of the human PNKD gene appeared among the highest scoring hits. The PNKD protein is connected with paroxysmal nonkinesigenic dyskinesia, but its exact role in this disease remains elusive (Charlesworth et al., 2013). Therefore, we termed the *T. brucei* protein as PNKD-like protein.

The second target protein, Tb927.11.5060, was identified as a homolog of the *S. cerevisiae* RSM22 (Ribosomal Small subunit Mitochondrial) protein. The yeast RSM22 appeared in affinity pull-downs of other ribosomal proteins and is essential for yeast growth (Saveanu et al., 2001). It has no homologs in prokaryotes. In this study its *T. brucei* counterpart is referred to as RSM22 protein.

Homologs of the third target, Tb927.8.2760, are in databases annotated as hypothetical proteins. They contain the YsxC GTPase domain, which is shared with homologous proteins YsxC and YihA from *B. subtilis* and *E. coli*, respectively. While also present in eukaryotes (Leipe et al., 2002), these proteins were so far functionally associated only with LSU of bacterial ribosome (Wicker-Planquart et al., 2008), where they are required for its assembly (Wicker-Planquart and Jault, 2015; Cooper et al., 2009). We termed the *T. brucei* protein mtYsxC (= mitochondrial YsxC).

3.2. Cell growth inhibition upon RNAi of the targets

Functional analysis of all three proteins was initiated by their RNAi-mediated depletion. Each culture displayed a growth retardation observable at days 4 or 5 post-induction (p.i.) (Figs. 1A, B and C). As the growth phenotype was rather mild, we next measured the growth of uninduced and RNAi-induced cells in a glucose-depleted medium, where they cannot rely on glycolysis and need a fully functional mitochondrion to survive. In the absence of glucose, RNAi-mediated targeting of PNKD-like and RSM22 proteins resulted in slower or limited growth after day 5 p.i. (Figs. 1D and E). Under these conditions, the mtYsxC RNAi knock-down phenotype was also more pronounced as compared to the glucose-containing medium, but no growth arrest was observed (Fig. 1F). Subsequently, the following experiments were performed in the presence of glucose.

The RNAi-mediated efficiency of mRNA ablation was determined by qPCR. The analysis showed a decline in the amount of each targeted mRNA (Figs. 1G, H and I). The most efficient knock-down was achieved in case of PNKD-like mRNA, with its level decreasing by almost 70% as compared to the uninduced cells. RSM22 mRNA was depleted by 50%, while mtYsxC responded relatively poorly to RNAi, with corresponding mRNA being down-regulated by only 30%. This fact can explain the absence of growth arrest in the glucose-free medium.

3.3. Inhibition of de novo synthesis of mt proteins

We then asked whether the ablation of the PNKD-like, RSM22 and mtYsxC proteins affects the *de novo* mt protein synthesis in *T. brucei*. Two out of the 18 maxicircle-encoded proteins, cytochrome *c* oxidase subunit I (Co1) and cytochrome *c* reductase (cytochrome bc_1 complex) subunit *b* (CyB), are the most suitable for direct visualization by incorporation of ^{35}S *in vivo* (Horváth et al., 2000; Horváth et al., 2002). After inhibition of the cytosolic translation by cycloheximide and separation of the labeled polypeptides in a 2D gel, the Co1 and CyB products become visible as two predominantly labeled spots, with nearly equal intensities, positioned off the main diagonal on the 2D gel (Fig. 2). The faint spots, also present off the diagonal (indicated by asterisks), likely represent aggregated forms of Co1 and CyB and some other mt-encoded proteins (Škodová-Sveráková et al., 2015). The characteristic off-diagonal positions of Co1 and CyB in the gel are due to extreme hydrophobicity of these proteins (Horváth et al., 2000; Horváth et al., 2002).

All knock-down cell lines showed a decreased *de novo* synthesis of mt proteins. The effect of the RNAi on Co1 and CyB synthesis was already visible on day 1 p.i. for RSM22 and mtYsxC, and on day 2 in case of the PNKD-like protein (Fig. 2). The impact was even more pronounced at day 5 p.i., when all labeled products became noticeably reduced in each induced cell culture. Despite the translational products being only incompletely depleted after day 5 p.i., the effect on the organellar translation appears to be specific, as it occurs as early as day 1 and 2 p.i., clearly predating the onset of the growth retardation phenotype.

3.4. Transcription and RNA editing appear to be unaffected

To further investigate whether the synthesis of Co1 and CyB is specifically affected upon the depletion of PNKD-like, RSM22 and mtYsxC proteins, we turned our attention to transcription and RNA editing, which precede mt translation. The analysis was done using a qPCR-based assay (Carnes et al., 2005). The *T. brucei* Co1 and CyB genes encode never-edited and minimally-edited transcripts. RPS12 mRNA was included in the analysis as a representative of extensively pan-edited mRNAs. RNAi cell line of mt RNA polymerase (mtRNAP) (Grams et al., 2002) was used as a positive control. In case of PNKD-like RNAi cell line, there was no significant change of mRNA levels of the followed transcripts, except a 20–30% decrease in edited RPS12 mRNA (Figs. 3A, E, I, M, Q). In case of the RSM22 and mtYsxC RNAi knock-downs, almost no change was detected in pan-edited RPS12 (Figs. 3N, O, R, S) and Co1 mRNA abundances (Figs. 3B, C), a 20–30% decrease was observed for both edited and unedited species of CyB mRNAs (Figs. 3F, G, J, K). Following the mtRNAP ablation, the pre-edited transcripts remained unchanged, while the levels of edited and unedited mRNAs were significantly decreased over the time of RNAi induction (Figs. 3D, H, L, P, T). It is rather unexpected that following the ablation of mtRNAP no change was detected for pre-edited transcripts. Although this could be due to inefficient ablation of mtRNAP, we cannot rule out that the qPCR assay used here was not sensitive enough to detect changes in transcript level.

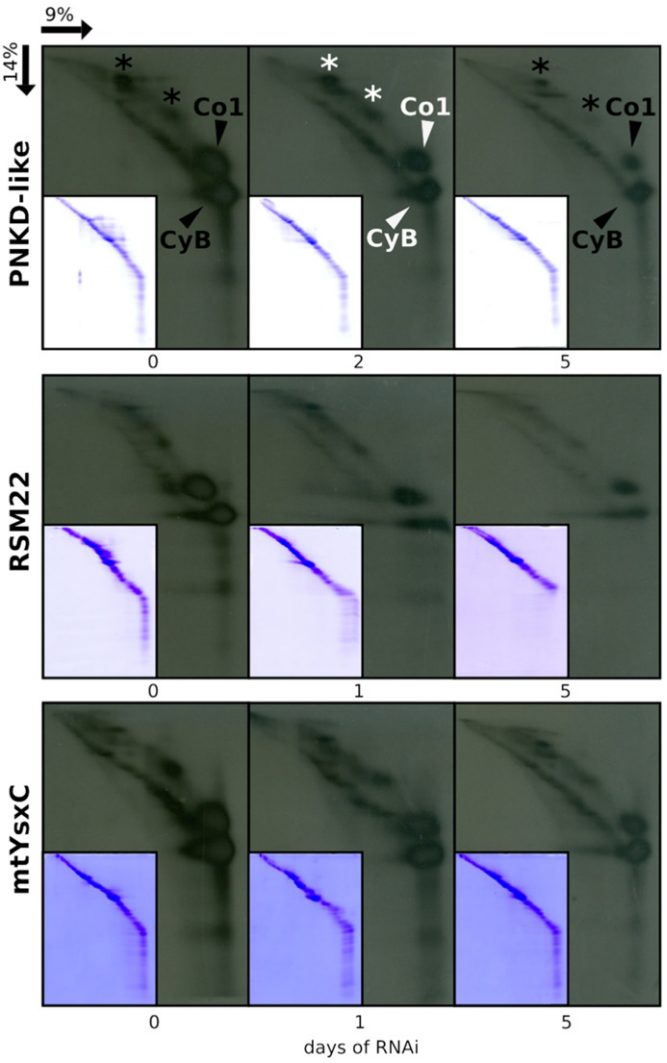

Fig. 2. Mitochondrial protein synthesis is affected upon RNAi-mediated depletion of PNKD-like, RSM22 or mtYsxC proteins. Radioactively labeled *de novo* synthesized proteins were separated in a 9% *vs* 14% denaturing polyacrylamide gels and detected by fluorography. Positions of the mitochondrial-encoded cytochrome *c* oxidase subunit I (Co1) and cytochrome *c* reductase subunit *b* (CyB) are indicated. Asterisks mark other mitochondrial translation products. Insets show the respective Coomassie-stained gels.

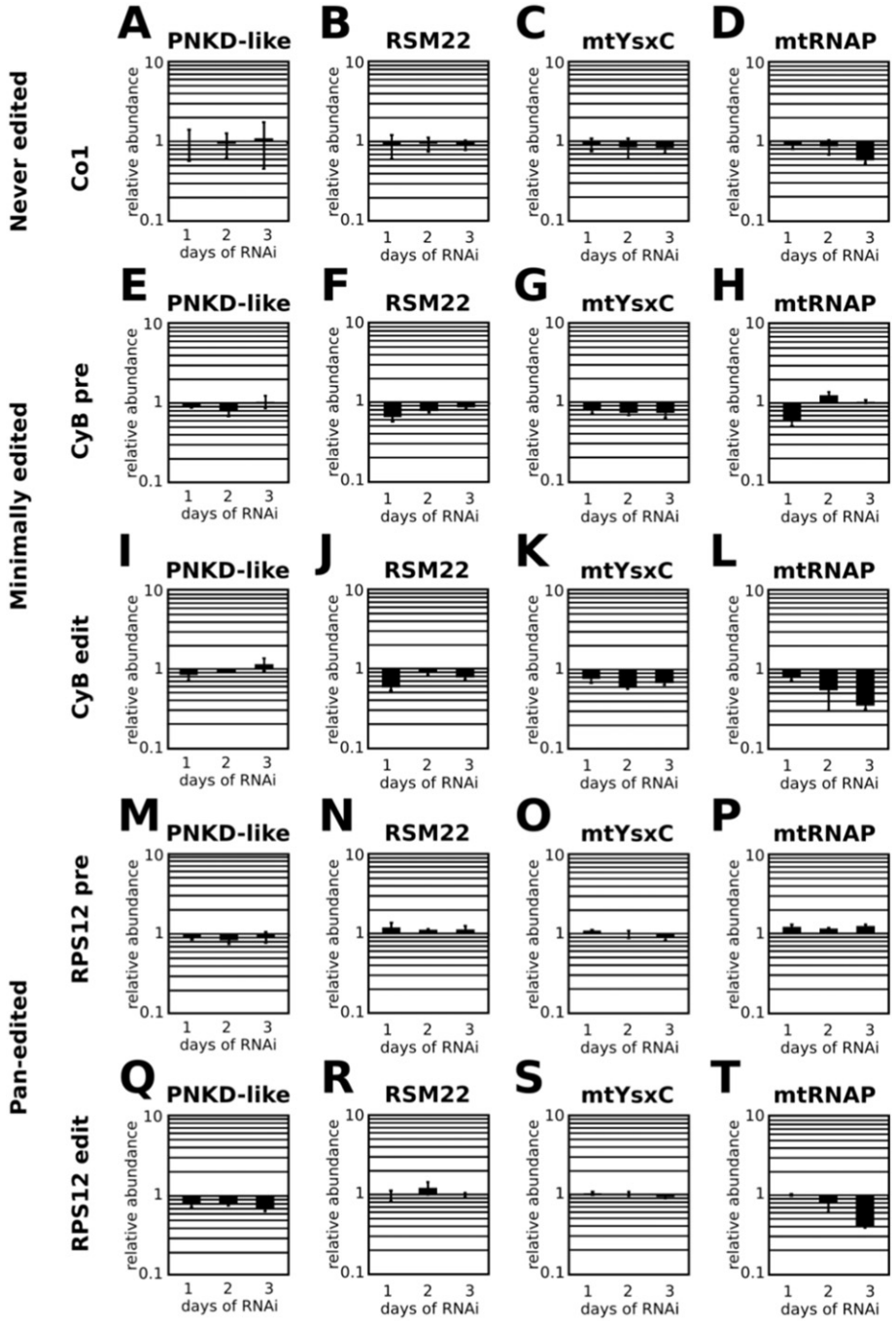

Fig. 3. Relative amounts of the mitochondrial-encoded mRNAs in clonal cell lines upon induction of RNAi, as determined by qPCR. The mean relative abundance of each amplicon is plotted as a column graph on a logarithmic scale on the *y*-axis, in which levels above and below the middle axis signify up- or down-regulation, respectively, while the absence of a column indicates that there was no change in steady-state levels of the transcript upon RNAi silencing. The following mRNAs were assayed: cytochrome *c* oxidase subunit (Co1), cytochrome *c* reductase subunit *b* (CyB), ribosomal protein S12 (RPS12). Pre-edited (pre) and fully edited (edit) mRNAs were followed. RNAi-knock-down cell line of mtRNA polymerase was used as a positive control. Error bars represent the standard deviation. Experiments for all time points were repeated at least twice. The cytosolic 18S rRNA was used as an internal reference.

3.5. Impact on structural integrity of mt ribosomes

Ablation of ribosomal components important for ribosomal structure or assembly affects the integrity of the ribosomal complexes in *T. brucei* (Ridlon et al., 2013). To determine whether the studied proteins are important for structural integrity of the ribosome, we investigated how their depletion affected the population of ribosomal complexes. Only RNAi cell lines of PNKD-like and RSM22 were investigated; the mtYsxC RNAi cell line was not included into this technically rather demanding analysis, as the RNAi efficiency was relatively low (Fig. 1H) and the impact on growth of the cell line was more limited (Figs. 1C, D). Under these conditions it is unlikely that ribosomal integrity would be affected, as that would definitely lead to a growth defect.

Ribosomal complexes in total cell lysates of uninduced and RNAi-induced cells were fractionated in sucrose gradients and relative levels of the ribosomal RNAs were determined by hybridization with specific probes (Ridlon et al., 2013). No change was observed in rRNA levels in gradient fractions after depletion of PNKD-like mRNA (Figs. 4A, B), while in case of RSM22 a strong destabilization effect on the 45S SSU* and 50S monosome fractions was observed at day 4 p.i., as illustrated by the significantly decreased levels of the 9S rRNA in the gradients (Figs. 4C, D). The destabilization of monosomes appears to have been

accompanied by accumulation of the LSU apparently still in association with other proteins as illustrated by the second peak in the 12S profile (Fig. 4D) that was not resolved from the remaining monosomes. The decline of the 45S SSU* and 50S monosome complexes was not accompanied by corresponding accumulation of the free 9S rRNA in the gradients, indicating that the unassembled RNA was degraded. These results show that PNKD-like protein, despite being important for the function of the mt ribosome, likely plays no structural role. On the other hand, RSM22 is crucial for stability of the SSU subunit shared by the 45S SSU* and monosome complexes.

4. Discussion

Our goal was to investigate whether PNKD-like, RSM22 and mtYsxC proteins play a functional role in translation and a structural role the mt ribosome in *T. brucei*. Previously all three proteins appeared to be at least transiently associated with the *T. brucei* mt ribosome by affinity pull-down assay: PNKD-like and RSM22 proteins by Zíková et al. (2008), and all three by Aphasizheva et al. (2011).

Trypanosomatids have recruited numerous unique proteins to their mt ribosomes (Zíková et al., 2008; Aphasizheva et al., 2011). At least in part that could be due to the need to compensate for the extremely small rRNAs (Sharma et al., 2009), but is more likely related to other aspects of these ribosomes function, such as close interaction with the system of RNA editing (Aphasizheva et al., 2013; Aphasizheva et al., 2016). Our studied proteins, however, contain highly conserved domains, which suggest that their function in mt translation is also conserved. The reason why they were not yet identified in other mt ribosomes (with the exception of RSM22) may be explained by their loose association with the core ribosomal particle and the subsequent loss during purification. Alternatively, these proteins might be present but remain unrecognizable. Mitochondrial ribosomal proteins are evolving quite rapidly making their identification in the genome databases challenging (O'Brien, 2003; Desmond et al., 2011; Smits et al., 2007). As the rates of evolution of mt proteins and rRNAs are comparable (Pietromonaco et al., 1986), trypanosomatids with highly diverged rRNAs (de la Cruz et al., 1985) are also likely to have a set of very divergent proteins in comparison to other eukaryotes. This may be especially true for the PNKD-like protein, with the lowest level of sequence conservation among all three analyzed proteins.

The alternative explanation is that these proteins are widespread, but only in trypanosomatids they gained a new function in the mt ribosome. This scenario corresponds to the view that the reduction of rRNA length and proteins acquisition, presumably compensating for this loss, appeared independently in several eukaryotic lineages. Such changes would be therefore specific for individual phylogenetic groups (Smits et al., 2007; Zíková et al., 2008). There is also a possibility that the unusual proteins associated with SSU in *T. brucei*, such as PNKD-like, may in fact be part of the trypanosomatid-specific 45S SSU* complex (Maslov et al., 2006; Maslov et al., 2007; Ridlon et al., 2013).

All three examined proteins are important for the growth of *T. brucei*, with higher impact of their ablation observed in the absence of glucose, where the energy produced by mitochondrial oxidative phosphorylation becomes essential. Indeed, similar results have been reported previously for other ribosome-associated proteins (Pusnik et al., 2007). Ablation of each protein leads to a severe defect in mt *de novo* protein synthesis long before the commencement of the growth phenotype, strongly suggesting their important functions in organellar translation. Although the final effect at day 5 p.i. looked similar in all cases, the relative impact of each protein's ablation was probably different due to variable RNAi efficiencies in the knock-down cell lines generated in frame of this study. The strongest impact would be expected for mtYsxC, where the decrease in protein synthesis occurs just after mere 30% down-regulation of the corresponding mRNA. Nevertheless, the results might be affected by the target mRNA fragments cleaved in the RNAi pathway that may persist longer than the functional full-length mRNA.

While *de novo* protein synthesis was affected, mt transcription and RNA editing seems to remain unaltered. Limited decrease in mRNA levels was detected for the edited form of RPS12 mRNA in the PNKD-like RNAi knock-down, and for both pre-edited and edited form of CyB in cells depleted for RSM22 and mtYsxC. It was shown previously that in *T. brucei* mt transcription, editing and translation are functionally coupled more closely than in other eukaryotes. Indeed, the accumulating data indicate that they actually interact with each other more than anticipated (Aphasizheva et al., 2013; Aphasizheva et al., 2016), although these connections still need to be fully revealed.

In case of RSM22, the ribosomal SSU was noticeably destabilized, with the monosome peak having been greatly reduced in the sedimentation profile. As the same gradient fractions also contain the trypanosomatid-specific 45S SSU* complex, we cannot exclude the possibility that RSM22 is also important for stability of this complex. Nevertheless, considering that the 45S SSU* complex and the monosome both contain the SSU subunit, combined with the fact that RSM22 protein was found in SSUs in other organisms, it is more likely that depletion of this protein primarily affects the SSU itself with the ensuing effect on the 45S SSU* and monosomes. No such destabilization was detected upon ablation of PNKD-like protein suggesting that, despite being important for mt translation, this protein does not play an integral structural role in the ribosome. The mtYsxC RNAi cell line has not been examined so far, due to low efficiency of RNAi.

Our findings regarding RSM22 confirmed that it represents a *bona fide* mt ribosomal protein. The only available information about this protein prior to this work was that it is important for survival of *S. cerevisiae* (Saveanu et al., 2001). Since RSM22 was found in most examined eukaryotes and is thus well conserved (Desmond et al., 2011), it is most likely an important component of mt ribosomal SSU. Here we are showing for the first time that this ribosomal protein is indeed necessary for mt translation and also for SSU integrity.

In contrast, the YsxC GTPase protein was so far only known from prokaryotic ribosomes. Proteins containing the same domain as the *T. brucei* mtYsxC, such as YsxC and YihA from *B. subtilis* and *E. coli*, respectively, are associated with the large ribosomal subunit (Wicker-Planquart et al., 2008) and are important for ribosome assembly

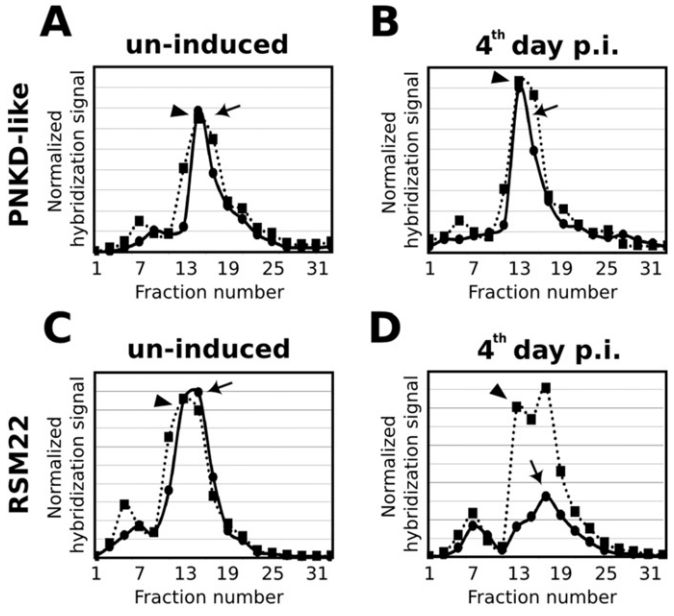

Fig. 4. Sedimentation profiles of rRNAs in sucrose gradient fractions of total cell lysate showing mitochondrial ribosomal complexes in *T. brucei* before and after depletion of the PNKD-like and RSM22 mRNAs. 9S rRNA is visualized by circles and full line, 12S rRNA is shown by squares with dotted line. Arrowhead indicates position of free 45S LSU and arrow points at 45S SSU*-50S monosome complexes. The plots show the normalized hybridization signal in arbitrary units.

(Wicker-Planquart and Jault, 2015; Schaefer et al., 2006; Cooper et al., 2009). Most interacting partners of YsxC identified by TAP-tag analysis in *Staphylococcus aureus* are homologous to proteins that are located on the surface of the *E. coli* ribosome, implying its peripheral localization (Cooper et al., 2009). It was also proposed recently that YsxC binds directly rRNAs (Wicker-Planquart and Jault, 2015).

While eukaryotes were suggested to generally bear organelle-targeted homologs of YsxC (Leipe et al., 2002), almost nothing is known about its function. To our knowledge, YsxC was found associated with mt ribosomes so far only in *T. brucei* (Zíková et al., 2008; Aphasizheva et al., 2011). Here we provide first direct evidence that YsxC is important for translation in the mitochondrion of *T. brucei*. The fact that all known YsxC domain-carrying GTPases, including the *T. brucei* mtYsxC, are associated with the large ribosomal subunit further demonstrates evolutionary conservation of its function. It should be noted that the presence of YsxC in the mitochondrion represents a possible hindrance for its validation as a potential drug target against bacterial pathogens, such as *Staphylococcus aureus* (Cooper et al., 2009).

The PNKD-like protein is likely peripheral and/or loosely associated with the *T. brucei* mt ribosome, as it was identified only in one study (Aphasizheva et al., 2011), and as shown here, its depletion does not lead to structural changes of the ribosome. As protein composition of mt ribosomes is thought to be group-specific to a large degree (O'Brien, 2003), it is possible that this protein was recruited to the ribosome only in the trypanosomatid lineage. In fact it could play a role with the trypanosomatid-specific ribosome-related 45 SSU* particle, which was actually shown to contain the small subunit moiety (Maslov et al., 2006; Maslov et al., 2007; Ridlon et al., 2013).

The *T. brucei* PNKD-like protein shows similarity to the human PNKD protein. Mutations in the corresponding gene have been associated with paroxysmal non-kinesigenic dyskinesia (PNKD), a movement disorder in humans (Charlesworth et al., 2013). All identified mutations alter cleavage of the targeting sequence and subsequently the stability of the whole protein (Ghezzi et al., 2009; Shen et al., 2011). Although suggested to play an important role in maintaining cellular redox status, oxygen consumption and calcium homeostasis the exact function of the protein is still unknown (Ghezzi et al., 2015). As the matter of fact, these observations might be easily explained by its role in the mitochondrial ribosome. The role that we did described here for the PNKD-like protein in *T. brucei*.

In summary, we provide evidence that PNKD-like, RSM22 and mtYsxC are essential for protein translation in the mitochondrion of *T. brucei* and that the depletion of RSM22 leads to the destabilization of small ribosomal subunit.

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