8620–8631 *Nucleic Acids Research, 2019, Vol. 47, No. 16* *Published online 8 August 2019*
doi: 10.1093/nar/gkz671

The general mRNA exporters Mex67 and Mtr2 play distinct roles in nuclear export of tRNAs in *Trypanosoma brucei*

Eva Hegedűsová[1], Sneha Kulkarni[1,2], Brandon Burgman[1,3], Juan D. Alfonzo[4] and Zdeněk Paris[1,2,*]

[1]Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic, [2]Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic, [3]The University of Arizona, Tucson, AZ, USA and [4]Department of Microbiology, Ohio State Biochemistry Program and The Center for RNA Biology, The Ohio State University, Columbus, OH 43210, USA

Received July 03, 2019; Revised July 18, 2019; Editorial Decision July 20, 2019; Accepted July 29, 2019

ABSTRACT

Transfer RNAs (tRNAs) are central players in protein synthesis, which in Eukarya need to be delivered from the nucleus to the cytoplasm by specific transport receptors, most of which belong to the evolutionarily conserved beta-importin family. Based on the available literature, we identified two candidates, Xpo-t and Xpo-5 for tRNA export in *Trypanosoma brucei*. However, down-regulation of expression of these genes did not disrupt the export of tRNAs to the cytoplasm. In search of alternative pathways, we tested the mRNA export complex Mex67-Mtr2, for a role in tRNA nuclear export, as described previously in yeast. Down-regulation of either exporter affected the subcellular distribution of tRNAs. However, contrary to yeast, TbMex67 and TbMtr2 accumulated different subsets of tRNAs in the nucleus. While TbMtr2 perturbed the export of all the tRNAs tested, silencing of TbMex67, led to the nuclear accumulation of tRNAs that are typically modified with queuosine. In turn, inhibition of tRNA nuclear export also affected the levels of queuosine modification in tRNAs. Taken together, the results presented demonstrate the dynamic nature of tRNA trafficking in *T. brucei* and its potential impact not only on the availability of tRNAs for protein synthesis but also on their modification status.

INTRODUCTION

In eukaryotes, the shuttling of macromolecules between the nuclear and cytoplasmic compartments is a crucial process, mediated by specific factors called exportins, predominantly belonging to the karyopherin-β (KAP-β) protein family. Primary substrates for this process are newly transcribed RNAs. Each type of RNA is transported by one or more dedicated exportins. For example, in yeast, mRNAs are predominantly exported by the Mex67-Mtr2 complex. Similar roles have been attributed to their homologs in Metazoa (Nxf1-Nxt1), humans (TAP-p15), as well as in trypanosomatid parasites (TbMex67-TbMtr2) (1–4). However, a subset of mRNAs can be exported by Crm1, which is also the chief exporter for rRNAs (5,6). Principal export factors for tRNAs were identified as Los1 and Msn5 in yeast; and exportin-t (Xpo-t) in vertebrates (7–12). These proteins recognize common structural features in all tRNAs, and export them in an energy-dependent manner, mediated by Ran-GTP; machinery that is evolutionarily conserved across all eukaryotic supergroups (13). This trimeric tRNA/Xpo-t/Ran-GTP complex is subsequently exported to the cytoplasm where it dissociates. The cargo-free Xpo-t is then recycled to the nucleus (6).

Prior to their export from the nucleus, tRNAs undergo extensive processing including end maturation, 3′ CCA tail addition, and in some systems, intron removal and post-transcriptional modifications. In vertebrates, tRNA splicing is a nuclear event and precedes end processing. Consequently, Xpo-t does not discriminate between intron-containing or intron-less tRNA, instead, it has a clear preference for tRNAs with mature 5′ and 3′ ends that contain a 3′ CCA. Indeed, this serves as a key quality control mechanism to deliver spliced, end-matured and correctly structured tRNAs into the cytoplasm (14). In yeast, key players in nuclear tRNA export are exportins Los1 and Msn5 (9,15–17), which serve partially overlapping roles (12,18). Los1 preferentially interacts with mature 5′ and 3′ termini and does not distinguish between intron-containing, intron-less or spliced tRNAs, or their aminoacylation status. Msn5, however, preferentially binds spliced aminoacylated tRNAs and exhibits very low affinity for intron-

*To whom correspondence should be addressed. Tel: +420 38 777 5427; Email: parda@paru.cas.cz

containing tRNAs (18). In contrast to yeast, the vertebrate homolog of Msn5, called exportin-5 (Xpo-5) exports miRNAs to the cytoplasm, and its role in tRNA export is assumed to be minor (12,19,20). Neither Los1 nor Msn5 is essential for yeast cell viability (21), suggesting their redundancy in tRNA trafficking. Recently, new putative tRNA export pathways were revealed by a genome-wide screen in yeast (22). The candidate proteins included among others, those known to be involved in rRNA, mRNA or protein export. For instance, inactivation of the major mRNA export complex, Mex67-Mtr2, resulted in nuclear accumulation of end processed intron-containing tRNAs. Interestingly, only four out of 10 intron-containing tRNAs were affected in this manner, indicating a possibility of tRNA substrate preferences (23).

tRNA trafficking is not strictly unidirectional; some tRNAs may indeed traverse back to the nucleus via the tRNA retrograde transport pathway and, in turn, be re-exported to the cytosol. Retrograde transport has been documented in several organisms including humans but its biological significance remains poorly understood. In yeast, it was proposed as a mechanism of tRNA quality control, that monitors both end processing and modification state of tRNAs (14). Intron-containing pre-tRNAs travel to the outer mitochondrial surface where the splicing endonuclease complex is localized. Cytoplasmic spliced tRNAs can travel back to the nucleus in order to be further modified (24) or, as a response to certain changes in environmental conditions, such as nutrient deprivation (25–28). Finally, these tRNAs are re-exported to the cytosol where they participate in protein synthesis.

Trypanosoma brucei (order Kinetoplastida) is a unicellular protozoan parasite that causes severe health problems in humans (African sleeping sickness) and livestock (29,30). During their complex life cycle, as these parasites transition between insect vectors and mammalian hosts, they face various environments significantly differing in nutrient availability. Consequently, during this process, trypanosomes undergo major metabolic remodeling, which includes (among others) switching the means of energy production from oxidative phosphorylation to glycolysis and *vice versa*. Such dynamic changes in cellular metabolism call for efficient gene expression regulation. Curiously, trypanosomes have, compared to many other eukaryotes, an unusual genome organization leading to gene expression control occurring primarily at the post-transcriptional level (31). Given that some environmental cues such as nutrient deprivation may influence nucleo-cytoplasmic tRNA distribution, regulation of the primary and secondary export might represent an additional mechanism of gene expression regulation.

Using bioinformatics, only a limited set of eukaryotic nuclear RNA export factors can be easily identified in trypanosomes. Whereas the mechanism of mRNA and rRNA nucleo-cytoplasmic transport in these parasites has been recently described (4,32,33), little is known about tRNA nuclear export. The genome of *T. brucei* encodes only one intron-containing tRNA i.e. $tRNA^{Tyr}$. In this system, tRNA intron splicing takes place in the cytosol and precedes certain modifications (34), hence $tRNA^{Tyr}$ is first exported from the nucleus to enable intron removal. The mature tRNA is then imported into the nucleus, where it acquires certain post-transcriptional modifications. One such modification is the hypermodified analogue of guanosine called queuosine, present at position 34 in the anticodon of tRNAs (Tyr, His, Asp, Asn). The enzyme responsible for this modification, tRNA guanine transglycosylase (TGT) is a nuclear enzyme in *T. brucei* (35). Tracking these compartment-specific events of tRNA processing, we recently reported the existence of the retrograde import pathway in *T. brucei* (35). Utilizing this approach, in the present study, we describe the involvement of different factors in nuclear tRNA export. Our results indicate that similar to other eukaryotes, TbXpo-t is not important for cell viability. Yet, contrary to its yeast homolog, down-regulation of TbXpo-t did not result in nuclear accumulation of mature tRNAs, nor did it abrogate the export of intron-containing tRNA. However, we found that the levels of nucleus-localized tRNAs are significantly increased after the down-regulation of subunits of the mRNA export factor TbMex67-TbMtr2. Moreover, we observed that the absence of either of the subunits affected different sets of tRNAs: Down-regulation of TbMtr2 resulted in nuclear accumulation of all the tRNAs that were part of this study; absence of TbMex67 led to a specific accumulation of tRNAs that are modified with queuosine.

MATERIALS AND METHODS

Cell culture and plasmid construction

Procyclic-form of *T. brucei* strain 29-13 was grown at 27°C in SDM-79 media supplemented with 10% fetal bovine-serum containing hygromycin (50 μg/ml) and geneticin (15 μg/ml). RNA interference (RNAi) constructs were generated by cloning a portion of the coding sequence of TbXpo-t (size of the insert 873 bp), TbXpo-5 (639 bp), TbMex67 (338 bp) and TbMtr2 (408 bp) into plasmid vector p2T7-177 (36). All inserts for the plasmid were generated by PCR from the genomic DNA of *T. brucei* 29-13 using oligonucleotide primers listed elsewhere (Supplementary Table S1). To generate a double knockdown cell line of TbXpo-t/TbXpo-5, RNAi plasmid of TbXpo-t linearized by HindIII was ligated with PCR amplicon of TbXpo-5 amplified by primers EXP5_F and EXP5-dKD_R (Supplementary Table S1). Final plasmids were linearized by NotI and transfected into *T. brucei* for genomic integration. Transfectants were selected with phleomycin (2,5 μg/ml). RNAi was induced with 1 μg/ml of tetracycline. Cell density was measured every 24 hours using Beckman Coulter Z2 counter. For protein tagging, the protein-coding region of TbXpo-t was amplified using primers ExTag_F and ExTag_R (Supplementary Table S1) and cloned into the expression vector pLEW79-MHTAP. In case of TbXpo-5, the ORF was amplified using primers EXP5-Tag_F and EXP5-Tag_R (Supplementary Table S1) and cloned into the pT7-V5c vector. The expression of tagged TbXpo-t and TbXpo-5 was induced for 1 day with 1 μg/ml of tetracycline.

Denaturing gel electrophoresis and Northern hybridization

Total RNA was isolated using guanidinium isothiocyanate/phenol/chloroform extraction method as described previously (37). 5 μg of RNA was resolved by

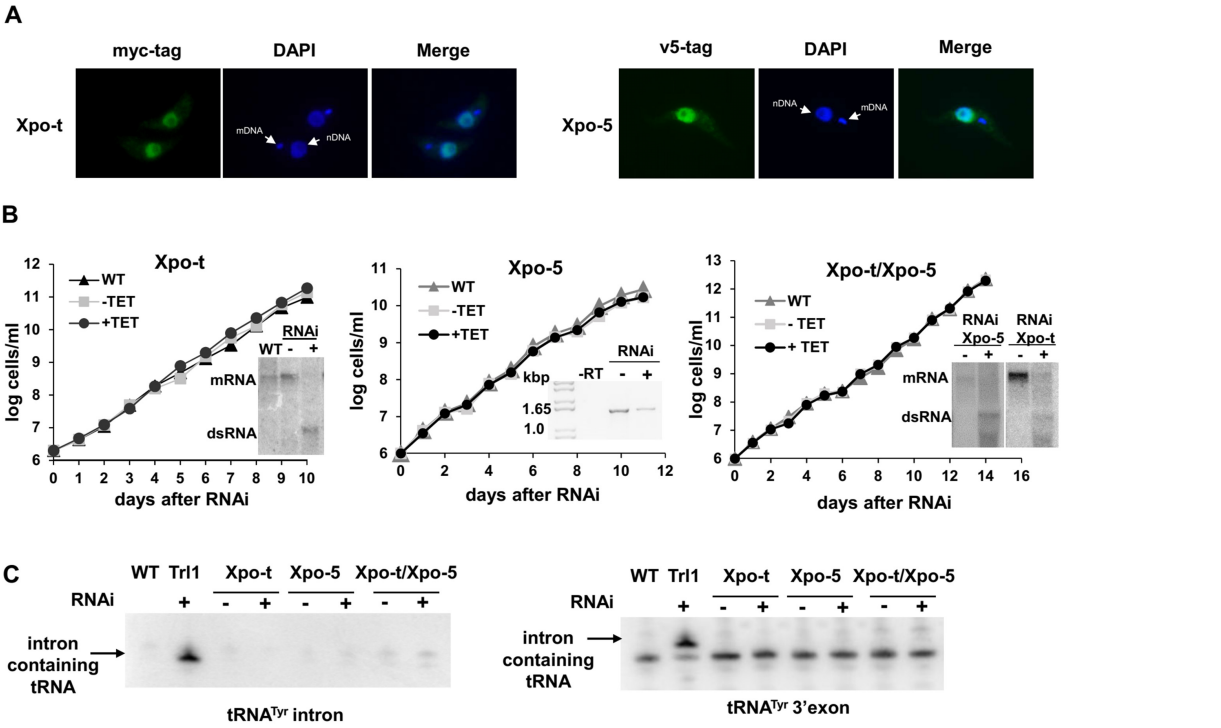

Figure 1. TbXpo-t and TbXpo-5 are not important for nuclear tRNA export. (**A**) Tagged TbXpo-t and TbXpo-5 were visualized by fluorescence microscopy using monoclonal anti-c-Myc or anti-V5 antibodies respectively, coupled with Alexa Fluor 488 conjugated secondary antibody. Co-localization was performed with DAPI staining of the nuclear (nDNA) and mitochondrial DNA (mDNA). (**B**) Growth curves of wild-type (WT; triangle), non-induced (–TET; square) and RNAi-induced (+TET; circle) cell lines of TbXpo-t, TbXpo-5 and double knockdown of TbXpo-t/TbXpo-5. Inset: RT-PCR analysis or Northern blots showing the down-regulation of the mRNA levels. (**C**) The silencing of exportins does not lead to the accumulation of intron-containing $tRNA^{Tyr}$. Total RNA was isolated from wild type (WT), non-induced (–TET) and RNAi induced (+TET) cells (TbXpo-t, TbXpo-5, and TbXpo-t/TbXpo-5). Trl1 RNAi induced cells were used as a positive control. $tRNA^{Tyr}$ 3′ exon and intron probe were used to detect intron accumulation, where the 3′ exon probe detects both intron-containing and spliced $tRNA^{Tyr}$.

denaturing gel electrophoresis (8% acrylamide, 7 M urea), electroblotted to Zeta probe® (Bio-Rad) membranes, and UV cross-linked (1200 μJ ×100). The membranes were probed with oligonucleotides radiolabeled with γ^{32}P-dATP (Supplementary Table S2). Northern hybridization was performed according to the manufacturer´s instructions (Bio-Rad). Subsequently, the membranes were exposed overnight to a Phosphorimager screen and analyzed using Typhoon™ 9410 scanner and ImageQuant TL software (GE Healthcare). Boronate affinity electrophoresis was performed as described previously (35). To verify the down-regulation of mRNA levels of studied genes, total RNA was resolved on denaturing formaldehyde agarose gel and blotted to Zeta probe® membranes. The membranes were probed with α^{32}P-dATP labeled PCR amplicons (RNAi constructs) as described by the manufacturer (DecaLabel DNA labeling kit, Thermo Scientific™).

Fluorescence in situ hybridization (FISH)

1×10^7 cells were harvested and washed with PBS. Cells were resuspended in 4% paraformaldehyde/PBS solution and fixed to poly-L-lysine coated microscope slides for 30 min. Non-adherent cells were removed by washing with PBS and remaining cells were dehydrated by a series of increasing ethanol concentrations (50, 80 and 100%, for 3 min each). Subsequently, the permeabilized cells were pre-hybridized with hybridization solution (2% BSA, 5× Denhardt's solution, 4× SSC, 5% dextran sulphate, 35% deionized formamide, 10 U/ml RNase inhibitor), for 2 h. The slides were then incubated overnight at room temperature in a humid chamber in the presence of 10 ng/μl Cy3, AlexaFluor 488 (AF) or Fluorescein (Flc)-labeled oligonucleotide probes (Supplementary Table S2), in the aforementioned hybridization solution. Afterward, slides were washed for 10 min, once with 4× SSC with 35% deionized formamide, followed by one wash each with 2× SSC and 1× SSC. Finally, the slides were mounted with mounting medium supplemented with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI). Images were taken with confocal microscope Olympus FluoView™ FV1000 and analyzed using Fluoview and ImageJ (NIH) software. FISH data were quantified as described previously (23). In brief, six cells per micrograph were selected randomly. Fluorescence intensities were measured using ImageJ with a plot profile analysis along a 3.5 μm line drawn across the nucleus with overhangs covering the cytoplasm. The results are expressed as the average value of relative fluorescence intensity ±SD.

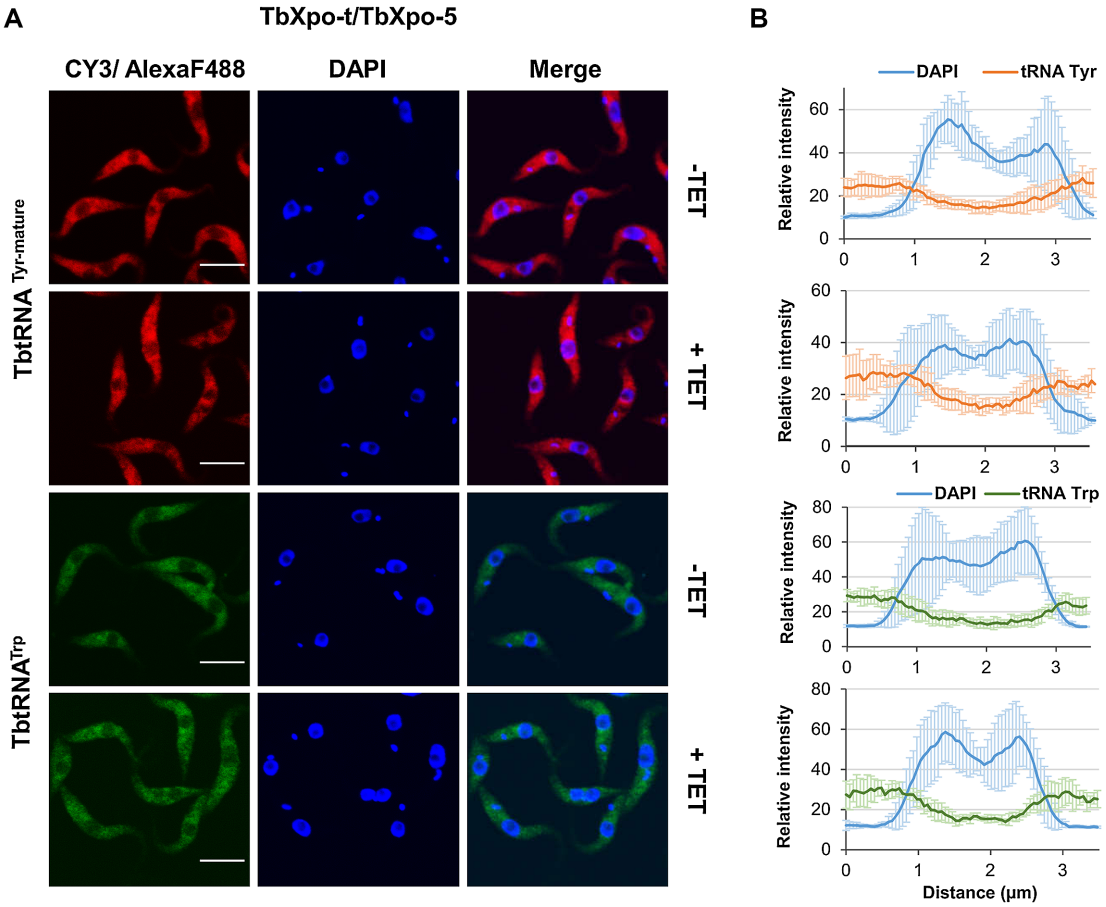

Figure 2. Simultaneous down-regulation of TbXpo-t and TbXpo-5 does not cause nuclear accumulation of tRNAs. (**A**) To determine the subcellular localization of spliced $tRNA^{Tyr}$ and $tRNA^{Trp}$, non-induced (–TET) and RNAi-induced (+TET) cells of double knockdown were examined by FISH. Micrographs show the subcellular localization for spliced $tRNA^{Tyr}$ (red-Cy3), $tRNA^{Trp}$ (green-AF488) and the nuclear and mitochondrial DNA (blue-DAPI). Bars, 5 µm. (**B**) Quantification of the fluorescence intensity of tRNAs and DAPI. Each graph shows the intensity profile of individual fluorophores of 6 randomly selected cells, where the values represent the relative intensity average ± SD.

Immunofluorescence assay

1×10^7 cells were harvested and fixed to glass slides with 4% paraformaldehyde, for 10 min. Cells were then permeabilized with 100% ice-cold methanol for 20 min and washed once with PBS. All subsequent incubation steps were performed in a humid chamber. To block non-specific binding sites, the slides were incubated with 5% milk in PBS supplemented with 0.05% Tween 20, for 45 min. The slides were then probed with primary mouse anti-V5 or mouse anti-myc antibodies (1:500 dilution), followed by extensive washes with PBS, and finally with secondary donkey anti-mouse antibody coupled with AlexaFluor488. Slides were air-dried and mounted with mounting media with DAPI (Invitrogen). Images were taken with fluorescent microscope Zeiss Axioplan 2.

RT-PCR analysis

Reverse transcription was carried out with total RNA, as described by the manufacturer (QuantiTect Reverse Transcription Kit, Qiagen). The resulting cDNA was PCR amplified with corresponding oligonucleotide primers (Supplementary Table S1).

RESULTS

Neither TbXpo-t nor TbXpo-5 plays a primary role in nuclear tRNA export in *T. brucei*

In yeast, Los1 was described as the main exporter involved in (re-)export of tRNAs (9,11,17). Hence, using its sequence, we searched for potential orthologs in *T. brucei,* in the trypanosomatid genome database (TriTrypDB) (38). We found an orthologous gene we termed TbXpo-t (Tb927.2.2240). To verify its subcellular localization, a strain expressing a C-terminally-tagged version of the TbXpo-t was generated. Using immunofluorescence microscopy, we observed that TbXpo-t localized to the nucleus (Figure 1A). We then established a tetracycline-inducible TbXpo-t RNAi cell line. Down-regulation of TbXpo-t expression by RNA interference was confirmed by Northern blot (Figure 1B, inset), but led to no significant differences in the growth rate between RNAi-induced cells and wild type, or a non-induced control (Figure 1B), leading to the conclusion that TbXpo-t is not important for cell viability. Recently, we demonstrated that in *T. brucei,* tRNA splicing occurs in the cytoplasm (34). Thus, the impairment of nuclear export would be expected to result in the ac-

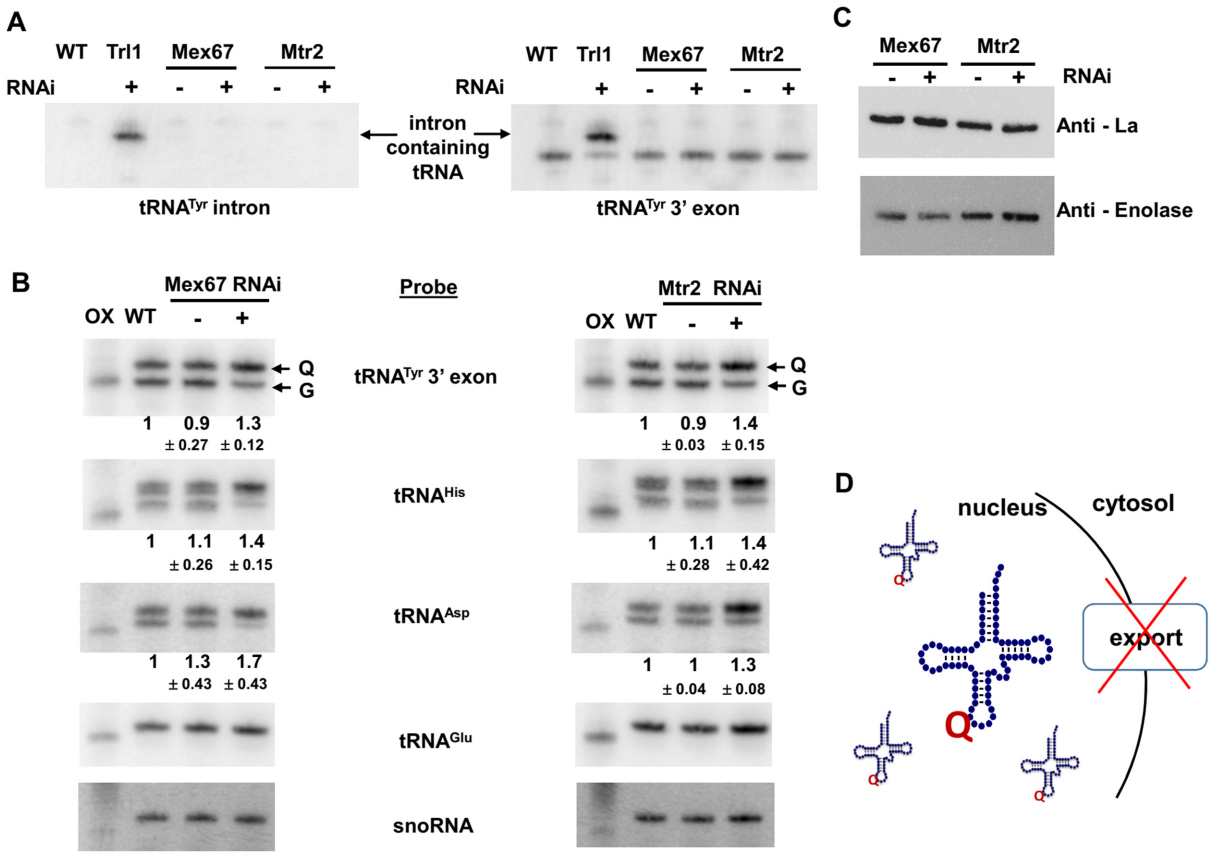

Figure 3. Down-regulation of TbMex67 or TbMtr2 does not disrupt the export of intron-containing tRNA but leads to an increased level of Q-modified tRNAs. (**A**) Total RNA extracted from TbMex67 and TbMtr2 RNAi-induced (+TET) and non-induced (–TET) cells was separated on urea gel, followed by Northern blotting. TbTrl1 RNAi was used as a positive control for the detection of intron-containing $tRNA^{Tyr}$. Probes for $tRNA^{Tyr}$ 3′ exon and intron were used to detect the accumulation of intron-containing tRNA. (**B**) Total RNA was collected at different time points. Boronate affinity electrophoresis-Northern blotting was carried out for RNAi induced (+TET) and non-induced (–TET) cells. Probes for $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asp}$ were used to determine Q levels, based on the electrophoretic shift caused by the presence of Q as described elsewhere (46). $tRNA^{Glu}$ and snoRNA probes were used as loading controls. Representative Northerns from three biological replicates. The relative levels of Q-modified tRNA are shown below each panel. WT values of Q modification were set to 1 and the mean ±SD of three independent experiments is shown. (**C**) Western blot analysis with protein samples collected from non-induced (–TET) and tetracycline-induced (+TET) TbMex67 and TbMtr2 cells after 36 h, with antibodies for the compartment-specific protein markers: La protein (nuclear marker) and enolase (cytoplasmic marker). (**D**) Model showing tRNAs modified with Q as a result of longer retention in the nucleus.

cumulation of the intron-containing $tRNA^{Tyr}$. To test this possibility, we performed Northern hybridization with an intron-specific probe for $tRNA^{Tyr}$. Additionally, the tRNA ligase (TbTrl1) RNAi cell line was used as a positive control for intron-containing tRNA (34). This experiment did not reveal any accumulation of intron-containing $tRNA^{Tyr}$ in TbXpo-t RNAi (Figure 1C). Next, we investigated the effect of TbXpo-t knockdown on the subcellular distribution of mature $tRNA^{Tyr}$ and $tRNA^{Trp}$, by fluorescence *in situ* hybridization (FISH). Still, no significant changes in the subcellular localization of these tRNAs were observed, as compared to the control (Supplementary Figure S1). Taken together, these results indicate, that TbXpo-t is not important for the primary export of intron-containing tRNAs, nor is it necessary for the export of mature tRNAs.

Recent phylogenetic studies in trypanosomes, revealed possible candidates belonging to the KAP-β protein family, including a putative ortholog of the yeast Msn5, which we define here, as TbXpo-5 (Tb927.10.7580) (13). As Msn5, has been shown to play a role in nuclear export of mature tRNAs in yeast (14), we tested TbXpo-5 for its role in tRNA nuclear export in *T. brucei*. Nuclear localization of TbXpo-5 was confirmed by expression of the C-terminally tagged version of the protein, which was subsequently visualized by immunofluorescence microscopy (Figure 1A). To test the possible role of TbXpo-5 in tRNA export, an RNAi cell line was generated and confirmed by RT-PCR (Figure 1B, inset). Even after several days of RNAi silencing of TbXpo-5, cells did not show any growth defect as compared to the non-induced control (Figure 1B). We also did not observe any accumulation of intron-containing tRNA in these cells (Figure 1C). Moreover, the down-regulation of TbXpo-5 expression did not result in nuclear accumulation of mature $tRNA^{Tyr}$ or $tRNA^{Trp}$, as assessed by FISH (Supplementary Figure S2). In order to test mutual functional redundancy of TbXpo-t and TbXpo-5, we then performed a simultaneous double knockdown, and the down-regulation of expression of both genes was confirmed (Figure 1B, inset). Surprisingly, neither growth defects nor nuclear accumulation of intron-containing $tRNA^{Tyr}$ was observed in this cell line

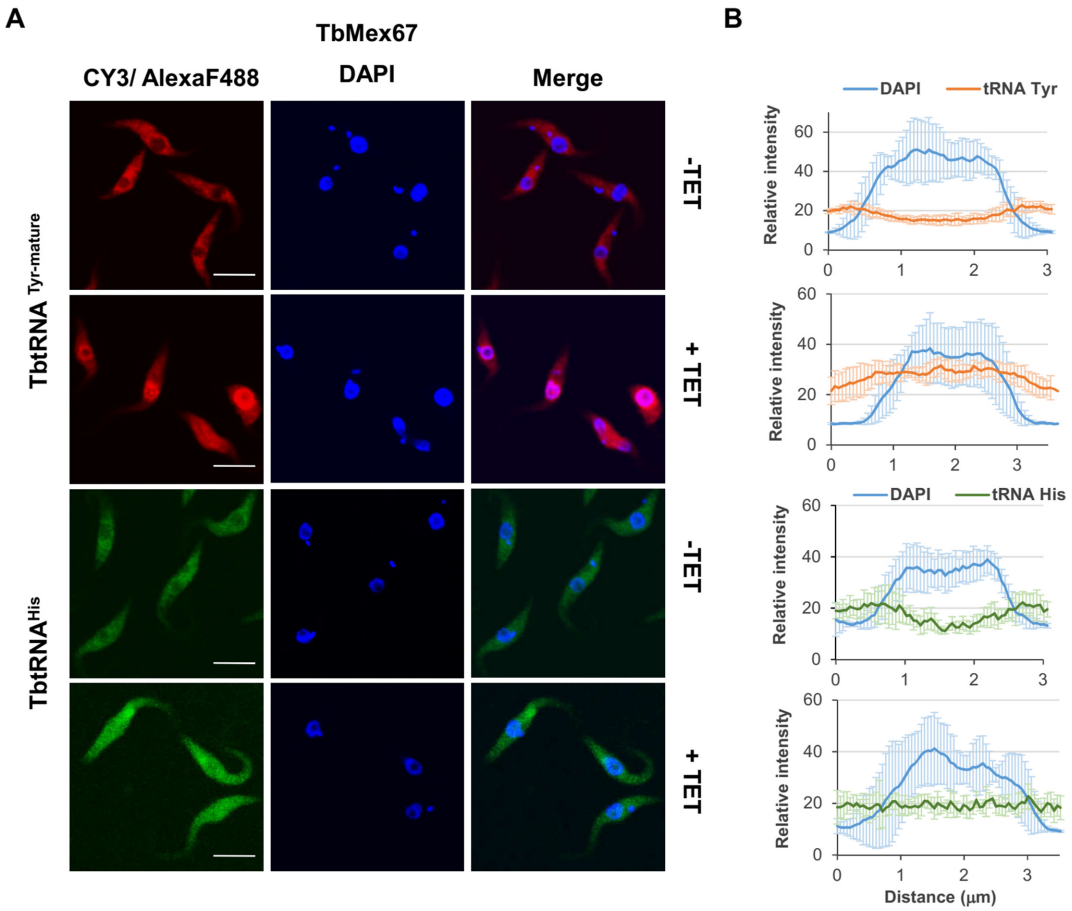

Figure 4. TbMex67 exports tRNAs which are substrates for Q modification. (**A**) Subcellular localization of spliced $tRNA^{Tyr}$ and $tRNA^{His}$ in non-induced (–TET) and RNAi-induced (+TET) cells of TbMex67 was shown by FISH using fluorescent probes: spliced $tRNA^{Tyr}$ (red-Cy3), $tRNA^{His}$ (green-AF448). Co-localization was performed with DAPI staining of the nuclear and mitochondrial DNA. Bars, 5 μm. (**B**) Quantification of the fluorescence intensity of tRNAs and DAPI. Each graph shows the intensity profile of the individual fluorophores of 6 randomly selected cells, where the values represent the relative intensity average ± SD.

(Figure 1B, C). FISH analysis of the double knockdown cell line also did not show any significant nuclear accumulation of mature $tRNA^{Tyr}$ or $tRNA^{Trp}$ (Figure 2). These results suggest that alternative, yet to be characterized pathways for tRNA nuclear export are present in trypanosomes.

Identification of an alternative export pathway for translocation of tRNAs through the NPC

Previously, the complex consisting of Mex67 and Mtr2 (or their homologs) has been described as a major mRNA exporter (1–4). Interestingly, yeast *mex67-5* and *mtr2* temperature-sensitive mutants rapidly accumulate end processed intron-containing tRNAs at the non-permissive temperature (23), showing for the first time an additional function of this complex in the export of intron-containing tRNAs. Additionally, spliced $tRNA^{Ile}$ was co-purified with Mex67, suggesting a role for this protein in nuclear re-export of tRNAs, in yeast (23). To investigate the potential function of TbMex67 and TbMtr2 in tRNA export in *T. brucei*, we constructed RNAi-inducible cell lines of these two genes. The down-regulation of expression of each gene was confirmed by either RT-PCR or Northern blotting (Supplementary Figure S3B). In agreement with recently published data (4), both RNAi cell lines exhibited strong growth defects (Supplementary Figure S3A), evidence of the importance of Mex67 and Mtr2 in *T. brucei*. To determine if the knockdown of the expression of either of these genes results in the disruption of primary export of intron-containing $tRNA^{Tyr}$, total RNA from RNAi induced and non-induced cell lines was analyzed by Northern hybridization, using an intron-specific probe. However, contrary to yeast, we did not observe any significant accumulation of intron-containing $tRNA^{Tyr}$ in these knockdown cell lines when compared to the positive control (TbTrl1 RNAi) (Figure 3A). To exclude the possibility that Mex67-Mtr2 knockdowns may indirectly affect tRNA export as a secondary consequence of defective mRNA transport, we also performed western blot analysis to assess the levels of the La protein (39) and enolase as nuclear and cytoplasmic markers, respectively. At the early time-point of 36 hours, chosen for this study, the levels of these proteins remained unchanged (Figure 3C).

We have previously shown that the spliced $tRNA^{Tyr}$ is imported into the nucleus of *T. brucei*, by the tRNA retrograde import pathway (35). One of the consequences of

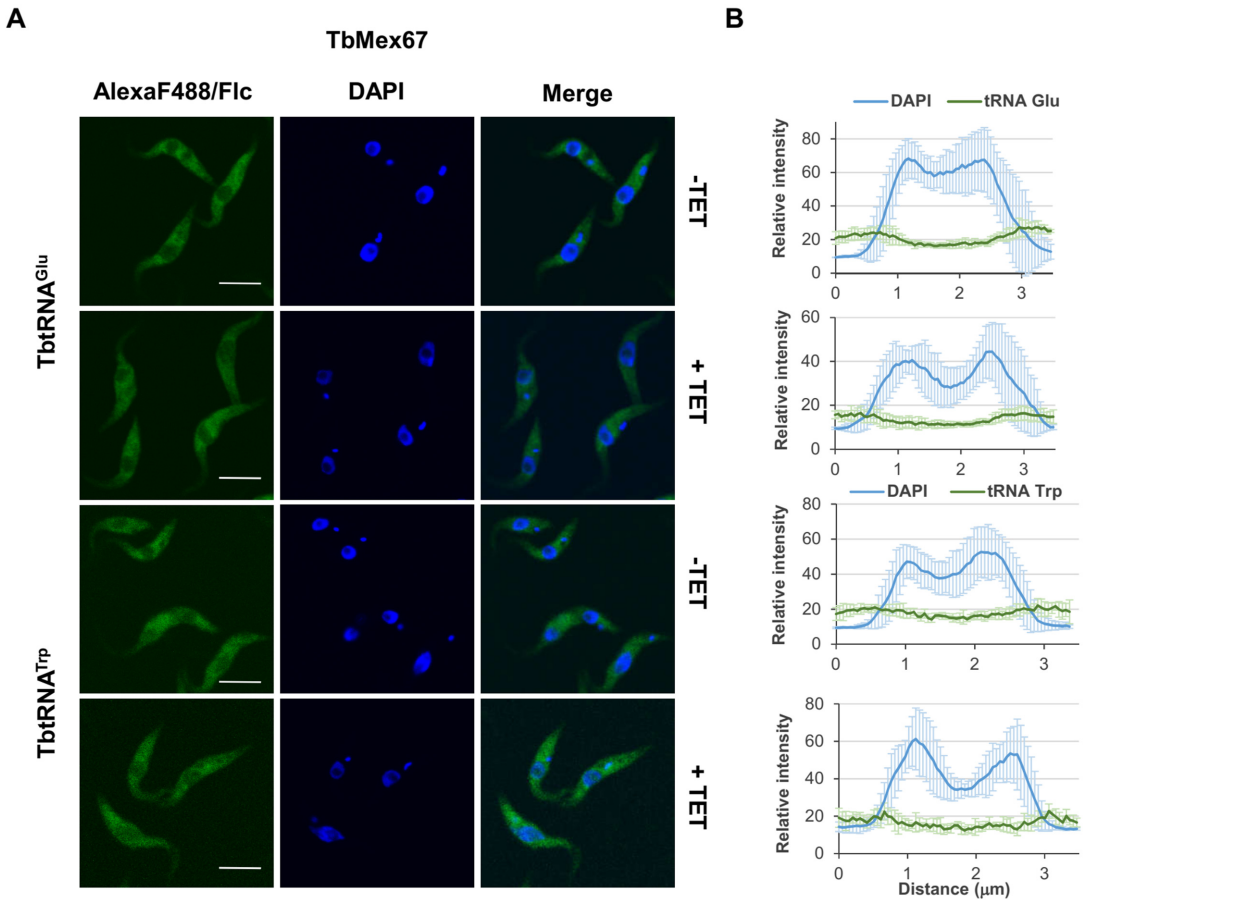

Figure 5. Subcellular localization of $tRNA^{Glu}$ and $tRNA^{Trp}$ are not affected by the knockdown of TbMex67. (**A**) Subcellular localization of $tRNA^{Glu}$ and $tRNA^{Trp}$ was shown by FISH in TbMex67 non-induced (–TET) and RNAi-induced (+TET) cells. Micrographs show the localization of $tRNA^{Glu}$ and $tRNA^{Trp}$ (green-AF488 or Flc) and the nuclear and mitochondrial DNA (blue-DAPI). Bars, 5 μm. (**B**) Quantification of the fluorescence intensity of tRNAs and DAPI. The relative intensities represent average ±SD from six randomly selected cells.

this pathway is the acquisition of nucleotide modifications such as queuosine (Q), that is, in *T. brucei,* catalyzed by a nucleus-localized enzyme, TbTGT (35). In addition to mature $tRNA^{Tyr}$, this modification is also present on $tRNA^{His}$, $tRNA^{Asp}$, and $tRNA^{Asn}$, which do not contain introns. We hypothesized that, if TbMex67 or TbMtr2 were involved in the re-export of mature $tRNA^{Tyr}$ or the export of other Q-containing tRNAs, down-regulation of these genes would lead to changes in tRNA transport dynamics and this could, in turn, affect their modification levels. Therefore, we analyzed queuosine modification levels of the selected tRNA species, at different time points post-RNAi induction, using boronate affinity gel electrophoresis. As described previously, this method is based on the affinity of *cis*-diol groups to 3-(acrylamido) phenylboronic acid (APB), resulting in differential electrophoretic mobility of Q-tRNAs as compared to their unmodified counterparts. Furthermore, the treatment of RNA with an oxidizing agent (sodium periodate) results in the conversion of *cis*-diols into *cis*-dialdehydes that do not exhibit any affinity to APB, therefore, oxidized RNA was employed as a negative control of the technique. Additionally, $tRNA^{Glu}$ and snoRNA, which are not substrates for Q modification, were used as loading controls for normalization. The resulting Northern blots have, indeed, showed a gradual increase in Q-levels following the depletion of either of the putative tRNA nuclear exporters (Figure 3B). Thus, the increasing modification levels are likely consequent to the decreased rate and/or blockage of nuclear export in regard to these tRNA species, possibly increasing the likelihood of tRNA-TGT interaction (Figure 3D).

To verify the role of TbMex67 and TbMtr2 in the export of mature tRNAs, we analyzed the subcellular localization of different tRNAs, using FISH. This experiment revealed that the depletion of TbMex67 results in nuclear accumulation of not only mature $tRNA^{Tyr}$ but also $tRNA^{His}$ (Figure 4) and $tRNA^{Asp}$ (Supplementary Figure S4). Surprisingly, other tRNAs ($tRNA^{Glu}$ and $tRNA^{Trp}$) were not affected in this manner (Figure 5). Unlike TbMex67, RNAi of TbMtr2 led to the nuclear accumulation of all the mature tRNAs tested (Figure 6,7; Supplementary Figure S4). To quantify the tRNA subcellular distribution, we plotted the fluorescence intensities of the FISH and DAPI signals, with the expectation that the signal for a given tRNA approaches that of DAPI, this would be indicative of nuclear retention. The maximum FISH signal for spliced $tRNA^{Tyr}$ co-localized with DAPI signal in the TbMex67 RNAi cell line (Figure 4B), while the FISH signal of $tRNA^{Trp}$ and

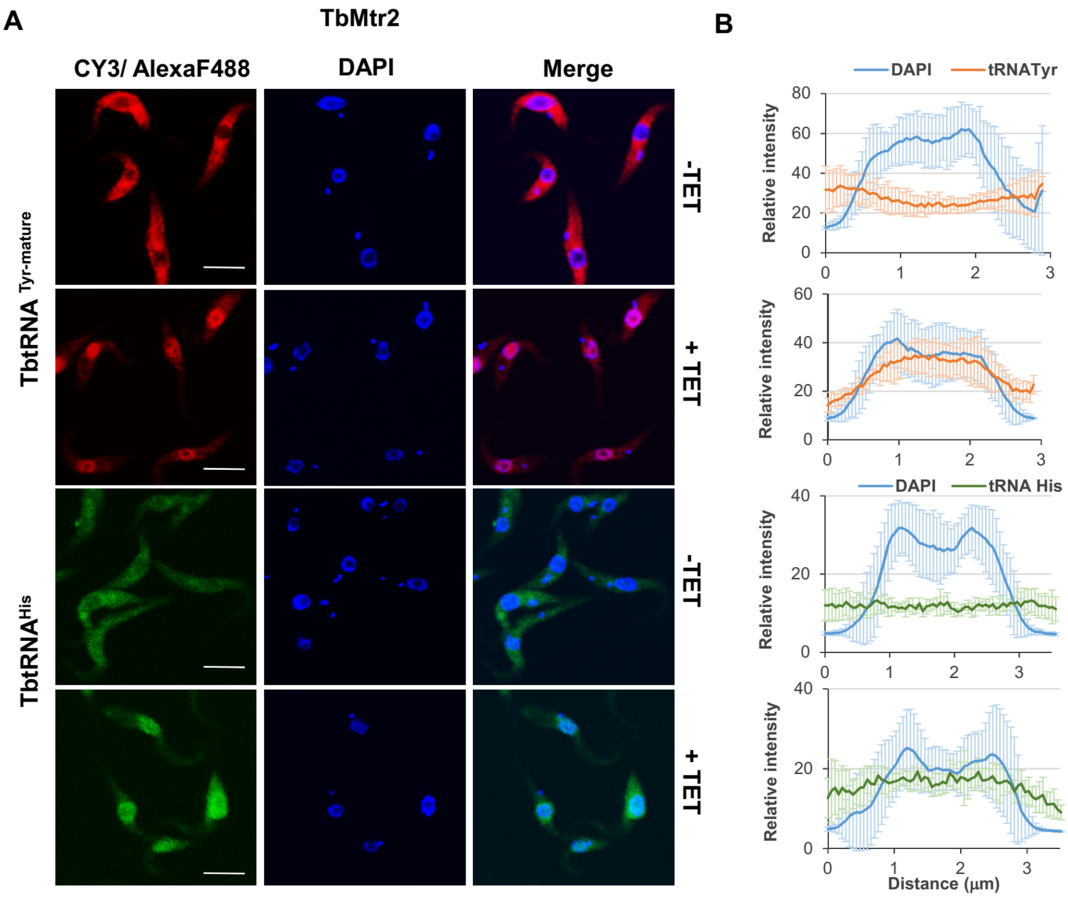

Figure 6. TbMtr2 is involved in the export of $tRNA^{Tyr}$ and $tRNA^{His}$. (**A**) To determine the subcellular localization of spliced $tRNA^{Tyr}$ and $tRNA^{His}$ in non-induced (–TET) and RNAi-induced (+TET) cells of TbMtr2 were examined by FISH assay using fluorescent probes. Micrographs show the subcellular localization for spliced $tRNA^{Tyr}$ (red-Cy3), $tRNA^{His}$ (green-AF488) and the nuclear and mitochondrial DNA (blue-DAPI). Bars, 5 μm. (**B**) Quantification of the fluorescence intensity of tRNAs and DAPI. Each graph shows the intensity profile of the individual fluorophores of 6 randomly selected cells, where the values represent the relative intensity average ± SD.

$tRNA^{Glu}$, showed minimum overlap with the DAPI signal (Figure 5B). Despite the fact that the signal of $tRNA^{His}$ and $tRNA^{Asp}$ was increased in the nucleus in the TbMex67 RNAi-induced cell line (Figure 4B; Supplementary Figure S4B) the fluorescent intensities of these tRNAs did not exhibit the similar pattern of exclusive nuclear accumulation as that observed in case of $tRNA^{Tyr}$. A possible explanation for this can be linked with a potential role of TbMex67 in nuclear re-export, exclusively. As mentioned before, the only intron-containing tRNA in trypanosomes, $tRNA^{Tyr}$, undergoes splicing in the cytosol and is imported retrogradely to the nucleus to be modified. On the other hand, since other Q-containing tRNAs do not need splicing and can be immediately modified in the nucleus after end-processing, it is difficult to differentiate between their primary and secondary export. If TbMex67 is specifically involved in the re-export of these tRNAs, we would still detect their signal in the cytosol in TbMex67 RNAi cell line, owing to the tRNAs that were exported by the primary export machinery. Therefore, the increased level of fluorescence of these tRNAs in the nucleus is not as pronounced as in the case of $tRNA^{Tyr}$, where we are exclusively detecting the defect in nuclear re-export. In the case of RNAi-induction of TbMtr2 cells, the FISH signal for all the tested tRNAs overlapped with DAPI (Figures 6B and 7B; Supplementary Figure S4B).

The results above support the potential bifurcation of the export pathway by the TbMex67-TbMtr2 complex, whereby TbMtr2 works as a more general tRNA exporter, TbMex67 may have a more specialized function, including, but not limited to, the re-export of mature $tRNA^{Tyr}$. The role of TbMex67 in tRNA re-export is reported for the first time here and, is provisionally unique to *T. brucei*. Furthermore, its role in the export of only a subset of tRNA species, which are typically modified with queuosine may indicate a certain substrate specificity. It remains to be seen whether the modification status of the tRNAs, is a recognition determinant for the TbMex67 export pathway, with possible implications for the regulation of their function in translation.

DISCUSSION

Subcellular trafficking of tRNAs between the nucleus and cytoplasm is a dynamic process, which provides fully functional tRNAs to the eukaryotic cell. This nucleocytoplasmic shuttling of tRNAs is governed by the nuclear export factors (exportins), which can also act as the sen-

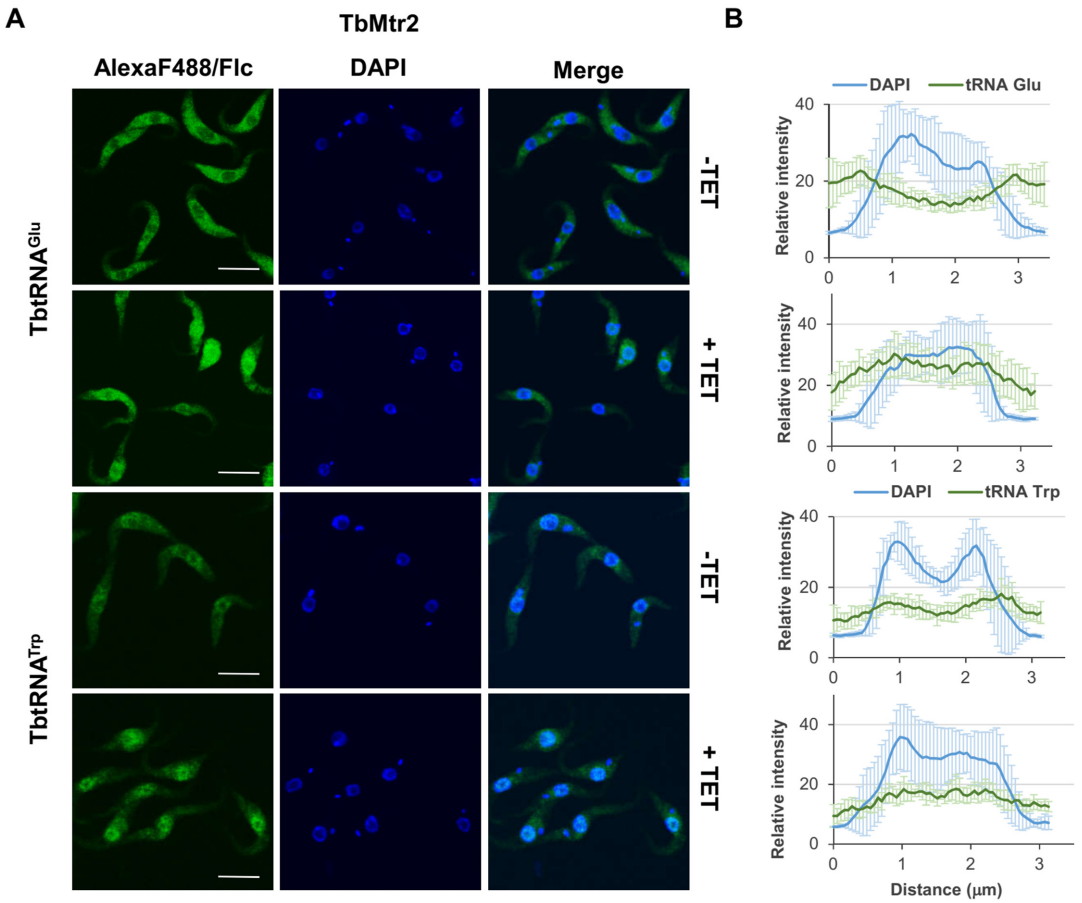

Figure 7. Silencing of TbMtr2 leads to nuclear accumulation of tRNAs which are not substrate for Q modification. (**A**) Subcellular localization of $tRNA^{Glu}$ and $tRNA^{Trp}$ was shown by FISH in non-induced (–TET) and RNAi-induced (+TET) cells of TbMtr2. Micrographs show the localization of $tRNA^{Glu}$ and $tRNA^{Trp}$ (green-AF488 or Flc) and the nuclear and mitochondrial DNA (blue-DAPI). Bars, 5 μm. (**B**) Quantification of the fluorescence intensity of tRNAs and DAPI. The relative intensities represent average ± SD from the six cells.

tinels of tRNA quality control. It is well known, that tRNAs can localize to different subcellular compartments depending on their maturation status, and also in response to changing environmental conditions (25,28). Nuclear export of tRNAs has been extensively studied for decades in several model systems, yet, there are key factors missing and this pathway is not fully understood. Hence, our present study of the factors of tRNA nuclear export in the early diverging protozoan parasite, *Trypanosoma brucei*, may contribute towards a better understanding of the mechanisms and evolution of these pathways in the metazoan lineages.

Metazoan exportin-t, and its yeast ortholog, Los1, were considered the main tRNA exporters, however, they were later proven to be redundant (9,40–43). Moreover, in *Drosophila melanogaster,* the absence of any apparent homolog of exportin-t (44), corroborated possible involvement of some additional factors needed for tRNA nuclear export. In yeast, Msn5 (exportin-5) was suggested as an alternative. While Los1 was shown to bind intron-containing tRNAs, Msn5 preferentially bound spliced aminoacylated tRNAs (16,18). The low affinity of Msn5 for intron-containing tRNAs suggests its role in the re-export of mature tRNAs. Though *los1*Δ *msn5*Δ double mutant cells had a larger nuclear pool of tRNAs than either mutant alone, the double mutant cells were viable indicating that there are additional Los1- and Msn5-independent tRNA exporters.

Here, we report that similar to yeast, TbXpo-t and TbXpo-5 are not important for the growth of trypanosomes. Since the tRNA splicing machinery is localized to the cytosol in *T. brucei*, we attempted to detect intron-containing $tRNA^{Tyr}$ as a hallmark of defective primary tRNA export (34). However, the depletion of either TbXpo-t or TbXpo-5 was not accompanied by the accumulation of this pre-tRNA, thus excluding their role in the primary export of intron-containing tRNAs. Simultaneous down-regulation of these two proteins did not affect the nucleo-cytoplasmic trafficking of spliced mature $tRNA^{Tyr}$, ruling out their role in the secondary export of this tRNA. Taken together, these results pointed towards yet to be identified alternative pathways for nuclear export of tRNAs in trypanosomes.

Recently, several putative Los1- and Msn5-independent tRNA nuclear export pathways were identified in a genome-wide screen, in yeast (22). It was discovered that disruption of Mex67/Mtr2, the canonical heterodimeric mRNA export machinery, caused accumulation of end-processed intron-containing pre-$tRNA^{Ile}$ and $tRNA^{Tyr}$ in the nucleus (23). Concurrently, overexpression of Mex67-Mtr2 rescued

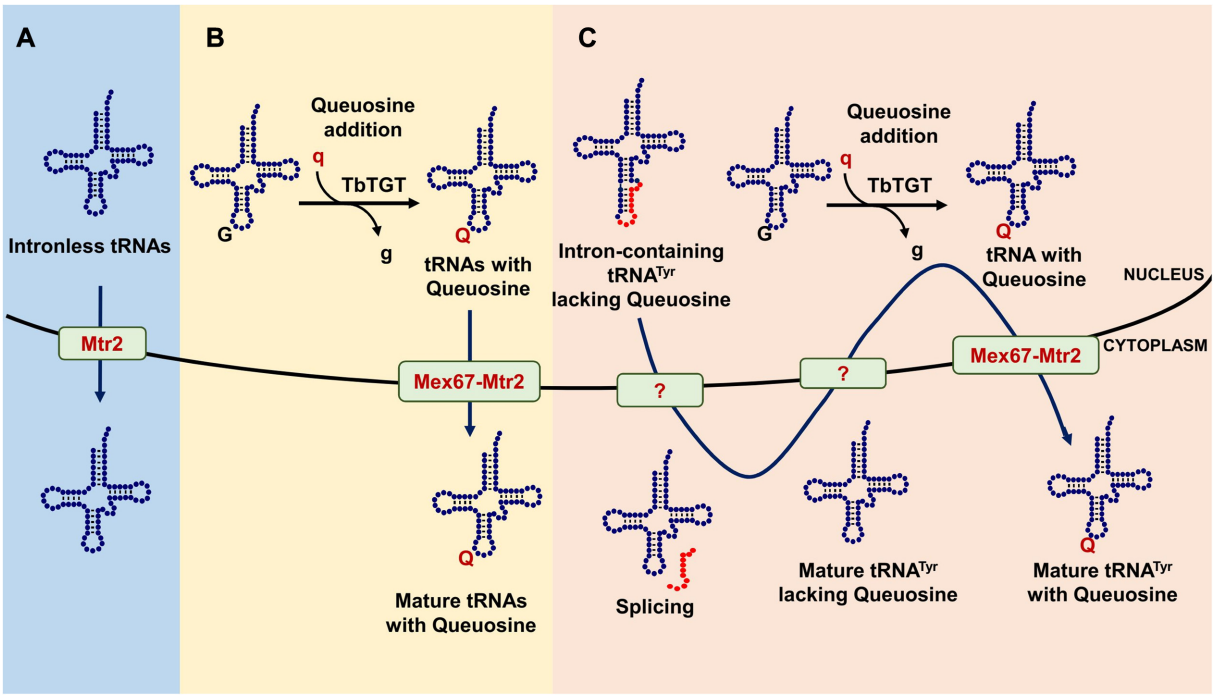

Figure 8. Model for tRNA nuclear export in *T. brucei*. Three possible pathways for primary and re-export of tRNAs in *T. brucei*, where general mRNA exporters TbMex67 and TbMtr2 serve distinct roles. (**A**) TbMtr2 has a more general role in tRNA nuclear export exporting intronless, mature, modified or unmodified tRNAs. (**B**) TbMex67 has a more specialized role in the export of queuosine-containing tRNAs. (**C**) Intron-containing $tRNA^{Tyr}$ is exported by an unknown exporter (question mark). After intron cleavage, the spliced $tRNA^{Tyr}$ undergoes retrograde transport to the nucleus to get modified with queuosine by the nuclear enzyme TGT (35). Finally, queuosine-containing $tRNA^{Tyr}$ is re-exported by TbMex67 and TbMtr2 to the cytoplasm to be used in cytoplasmic translation.

*los1*Δ mutants, attesting to the role of this complex in primary tRNA export (23).

Hence, we generated TbMex67 and TbMtr2 knockdown strains in *T. brucei*, to ascertain whether these may represent the missing piece of the puzzle. TbMex67 and TbMtr2 are important for cell growth due to their canonical role in mRNA export, which has been elucidated previously (4). However, here we provide evidence that Mex67 and Mtr2 have a role in nuclear tRNA export in *T. brucei.* Interestingly, the depletion of either of the exporters respectively impacts different subsets of mature tRNA species (with some overlaps). Whereas the ablation of TbMtr2 affected the subcellular trafficking of all the tRNAs tested, the down-regulation TbMex67 appeared to impair the export of Q-tRNAs exclusively. We took advantage of the fact that queuosine addition to tRNAs is a nuclear event in *T. brucei*, which uniquely provides an easily traceable marker for nuclear retention (35). The observed increased levels of Q in tRNAs in TbMex67 and TbMtr2 RNAi cell lines suggest that these factors play a role in the efficient export of these tRNAs from the nucleus. Longer nuclear retention may then increase the likelihood of being modified by the TbTGT enzyme, which in turn also suggests a competition between the efficiency of Q formation and the rate of tRNA export. In this realm, the levels of Q are not solely dependent on the catalytic activity of TGT; modification levels may be influenced by re-export efficiency.

It is possible that TbMex67 specifically recognizes some unique features within the substrate, such as the Q modification. Hence, while TbMtr2 may serve as a general scaffold, its binding with various interaction partners possibly provides the specificity for given tRNA species. This idea is additionally supported by the detection of increased Q modification levels (in the nucleus-localized tRNA species) following the depletion of either TbMtr2 or TbMex67.

In yeast, Mex67 functions in conjunction with Mtr2, consequently, in the absence of either of the subunits of the complex, identical tRNA species are affected (23). Our data, however, strongly suggests a distinct function for TbMex67 and TbMtr2 leading to the accumulation of different sets of tRNAs in *T. brucei*. This implies that beyond their possible heterodimeric nature suggested by immunoprecipitation experiments (4), the two proteins may act independently of each other. Along these lines, it was reported that human TAP (homolog of Mex67) can act independently of p15 (homolog of Mtr2) (45). In addition, yeast Mex67 requires Mtr2 for association with the nuclear pores (2), while Mtr2 alone is capable of interaction with NPC. Thus, there is circumstantial evidence that in addition to the role of the Mex67/Mtr2 complex, each of the components may also perform distinct functions independently of each other, which we believe may also be true for their orthologs in trypanosomes.

Despite our efforts, we have not identified the factor(s) responsible for the export of the intron-containing tRNA from the nucleus of *T. brucei*. We provide two possible explanations for this: First, there may be a specific nuclear export factor for intron-containing $tRNA^{Tyr}$ yet to be discovered;

alternatively, there is a high degree of redundancy in tRNA export pathways, which coupled with technical limitations may prevent us from completely blocking nuclear export of intron-containing tRNA. In conclusion, we show that the canonical mRNA export complex TbMex67/TbMtr2 also functions in the nuclear export of tRNAs. Whereas TbMtr2 serves a general role, TbMex67 appears to selectively export only a subset of tRNA species (Figure 8). Although these tRNAs are characterized by the presence of Q modification, the exact nature of the recognition element critical for TbMex67 binding remains to be elucidated. Finally, we view the nuclear export of tRNAs as a dynamic process, that controls not only the availability of tRNAs for protein synthesis but also their modification status, both of which can, in turn, significantly impact the cellular proteome. Such a scenario may be especially relevant in *T. brucei*, where the regulation of gene expression occurs mostly at the post-transcriptional level.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We would like to thank Anita Hopper for insightful discussions and guidance concerning tRNA retrograde transport and to all members of the Alfonzo (The Ohio State University, Columbus, USA) and Paris (Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic) laboratories for their comments and useful suggestions. We would also like to thank Paul A.M. Michels for the anti-enolase antibody.

FUNDING

National Institutes of Health [GM084065-10 and AI131248 to J.D.A.]; Czech Science Foundation [15-21450Y to Z.P.]; ERDF/ESF project Centre for Research of Pathogenicity and Virulence of Parasites [CZ.02.1.01/0.0/0.0/16_019/0000759 to Z.P.]; University of South Bohemia [036/2017/P to S.K.]; University of Arizona [NIH MHIRT T37 MD001427 to B.B.]. Funding for open access charge: Biology Centre CAS, Institute of Parasitology.
Conflict of interest statement. None declared.

REFERENCES

1. Segref,A., Sharma,K., Doye,V., Hellwig,A., Huber,J., Lührmann,R. and Hurt,E. (1997) Mex67p, a novel factor for nuclear mRNA export, binds to both poly (A)+ RNA and nuclear pores. *EMBO J.*, **16**, 3256–3271.
2. Santos-Rosa,H., Moreno,H., Simos,G., Segref,A., Fahrenkrog,B., Panté,N. and Hurt,E. (1998) Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores. *Mol. Cell Biol.*, **18**, 6826–6838.
3. Herold,A., Klymenko,T., Izaurralde,E., Herold,A., Klymenko,T. and Izaurralde,E. (2001) NXF1 / p15 heterodimers are essential for mRNA nuclear export in Drosophila. *RNA*, **7**, 1768–1780.
4. Dostalova,A., Käser,S., Cristodero,M. and Schimanski,B. (2013) The nuclear mRNA export receptor Mex67-Mtr2 of *Trypanosoma brucei* contains a unique and essential zinc finger motif. *Mol. Microbiol.*, **88**, 728–739.
5. Köhler,A. and Hurt,E. (2007) Exporting RNA from the nucleus to the cytoplasm. *Nat. Rev. Mol. Cell Biol.*, **8**, 761–773.
6. Okamura,M., Inose,H. and Masuda,S. (2015) RNA export through the NPC in eukaryotes. *Genes (Basel)*, **6**, 124–149.
7. Arts,G.J., Fornerod,M. and Mattaj,I.W. (1998) Identification of a nuclear export receptor for tRNA. *Curr. Biol.*, **8**, 305–314.
8. Hopper,A.K., Schultz,L.D. and Shapiro,R.A. (1980) Processing of intervening sequences: a new yeast mutant which fails to excise intervening sequences from precursor tRNAs. *Cell*, **19**, 741–751.
9. Hurt,D.J., Wang,S.S., Lin,Y.H. and Hopper,A.K. (1987) Cloning and characterization of LOS1, a *Saccharomyces cerevisiae* gene that affects tRNA splicing. *Mol. Cell Biol.*, **7**, 1208–1216.
10. Görlich,D., Dabrowski,M., Bischoff,F.R., Kutay,U., Bork,P., Hartmann,E., Prehn,S. and Izaurralde,E. (1997) A novel class of RanGTP binding proteins. *J Cell Biol.*, **138**, 65–80.
11. Hellmuth,K., Lau,D.M., Bischoff,F.R., Kunzler,M., Hurt,E.D. and Simos,G. (1998) Yeast Los1p Has Properties of an Exportin-Like Nucleocytoplasmic Transport Factor for tRNA. *Mol. Cell Biol.*, **18**, 6374–6386.
12. Calado,A., Treichel,N., Müller,E.-C., Otto,A. and Kutay,U. (2002) Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. *EMBO J.*, **21**, 6216–6224.
13. O'Reilly,A.J., Dacks,J.B. and Field,M.C. (2011) Evolution of the karyopherin-beta family of nucleocytoplasmic transport factors; ancient origins and continued specialization. *PLoS One*, **6**, e19308.
14. Kramer,E.B. and Hopper,A.K. (2013) Retrograde transfer RNA nuclear import provides a new level of tRNA quality control in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.*, **110**, 21042–21047.
15. Feng,W. and Hopper,A.K. (2002) A Los1p-independent pathway for nuclear export of intronless tRNAs in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 5412–5417.
16. Murthi,A., Shaheen,H.H., Huang,H.-Y., Preston,M.A., Lai,T.-P., Phizicky,E.M. and Hopper,A.K. (2010) Regulation of tRNA bidirectional Nuclear-Cytoplasmic trafficking in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, **21**, 639–649.
17. Sarkar,S. and Hopper,A.K. (1998) tRNA nuclear export in *Saccharomyces cerevisiae*: In situ hybridization analysis. *Mol. Biol. Cell*, **9**, 3041–3055.
18. Huang,H.Y. and Hopper,A.K. (2015) In vivo biochemical analyses reveal distinct roles of Beta-importins and eEF1A in tRNA subcellular traffic. *Genes Dev.*, **29**, 772–783.
19. Lund,E., Guttinger,S., Calado,A., Dahlberg,J.E. and Kutay,U. (2004) Nuclear export of MicroRNA precursors. *Science*, **303**, 95–99.
20. Brownawell,A.M. and Macara,I.G. (2002) Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. *J. Cell Biol.*, **156**, 53–64.
21. Takano,A., Endo,T. and Yoshihisa,T. (2005) tRNA actively shuttles between the nucleus and cytosol in Yeast. *Science*, **309**, 140–142.
22. Wu,J., Bao,A., Chatterjee,K., Wan,Y. and Hopper,A.K. (2015) Genome-wide screen uncovers novel pathways for tRNA processing and nuclear–cytoplasmic dynamics. *Genes Dev.*, **29**, 2633–2644.
23. Chatterjee,K., Majumder,S., Wan,Y., Shah,V., Wu,J., Huang,H.Y. and Hopper,A.K. (2017) Sharing the load: Mex67–Mtr2 cofunctions with Los1 in primary tRNA nuclear export. *Genes Dev.*, **31**, 2186–2198.
24. Ohira,T. and Suzuki,T. (2011) Retrograde nuclear import of tRNA precursors is required for modified base biogenesis in yeast. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 10502–10507.
25. Shaheen,H.H. and Hopper,A.K. (2005) Retrograde movement of tRNAs from the cytoplasm to the nucleus in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 11290–11295.
26. Huang,H.-Y. and Hopper,A. (2016) Multiple layers of Stress-Induced regulation in tRNA Biology. *Life*, **6**, 16.
27. Yoshihisa,T., Yunoki-Esaki,K., Ohshima,C., Tanaka,N. and Endo,T. (2003) Possibility of cytoplasmic pre-tRNA Splicing: the Yeast tRNA slicing endonuclease mainly localizes on the mitochondria. *Mol. Biol. Cell*, **14**, 3266–3279.
28. Whitney,M.L., Hurto,R.L., Shaheen,H.H. and Hopper,A.K. (2007) Rapid and reversible nuclear accumulation of cytoplasmic tRNA in response to nutrient availability. *Mol. Biol. Cell*, **18**, 2678–2688.
29. Barrett,M.P., Burchmore,R.J.S., Stich,A., Lazzari,J.O., Frasch,A.C., Cazzulo,J.J.J. and Krishna,S. (2003) The trypanosomiases. *Lancet*, **362**, 1469–1480.

30. Büscher,P., Cecchi,G., Jamonneau,V. and Priotto,G. (2017) Human African trypanosomiasis. *Lancet.*, **390**, 2397–2409.
31. Daniels,J.-P., Gull,K. and Wickstead,B. (2010) Cell biology of the trypanosome genome. *Microbiol. Mol. Biol. Rev.*, **74**, 552–569.
32. Biton,M., Mandelboim,M., Arvatz,G. and Michaeli,S. (2006) RNAi interference of XPO1 and Sm genes and their effect on the spliced leader RNA in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.*, **150**, 132–143.
33. Bühlmann,M., Walrad,P., Rico,E., Ivens,A., Capewell,P., Naguleswaran,A., Roditi,I. and Matthews,K.R. (2015) NMD3 regulates both mRNA and rRNA nuclear export in African trypanosomes via an XPOI-linked pathway. *Nucleic Acids Res.*, **43**, 4491–4504.
34. Lopes,R.R.S., Silveira,G. de O., Eitler,R., Vidal,R.S., Kessler,A., Hinger,S., Paris,Z., Alfonzo,J.D. and Polycarpo,C. (2016) The essential function of the *Trypanosoma brucei* Trl1 homolog in procyclic cells is maturation of the intron-containing tRNATyr. *RNA*, **22**, 1190–1199.
35. Kessler,A.C., Kulkarni,S.S., Paulines,M.J., Rubio,M.A.T., Limbach,P.A., Paris,Z. and Alfonzo,J.D. (2017) Retrograde nuclear transport from the cytoplasm is required for $tRNA^{Tyr}$ maturation in *T. brucei*. *RNA Biol.*, **15**, 528–536.
36. Wickstead,B., Ersfeld,K. and Gull,K. (2002) Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.*, **125**, 211–216.
37. Chomczynski,P. (1987) Single-Step method of RNA isolation by acid guanidinium extraction. *Anal. Biochem.*, **162**, 156–159.
38. Aslett,M., Aurrecoechea,C., Berriman,M., Brestelli,J., Brunk,B.P., Carrington,M., Depledge,D.P., Fischer,S., Gajria,B., Gao,X. *et al.* (2010) TriTrypDB: a functional genomic resource for the Trypanosomatidae. *Nucleic Acids Res.*, **38**, 457–462.
39. Marchetti,M.A., Tschudi,C., Kwon,H., Wolin,S.L. and Ullu,E. (2000) Import of proteins into the trypanosome nucleus and their distribution at karyokinesis. *J. Cell Sci.*, **906**, 899–906.
40. Hunter,C.A., Aukerman,M.J., Sun,H., Fokina,M. and Poethig,S.R. (2003) PAUSED encodes the Arabidopsis Exportin-t ortholog. *Plant Physiol.*, **132**, 2135–2143.
41. Li,J. and Chen,X. (2003) PAUSED, a Putative Exportin-t, acts pleiotropically in arabidopsis development but is dispensable for viability. *Plant Physiol.*, **132**, 1913–1924.
42. Cherkasova,V., Lopez Maury,L., Bacikova,D., Pridham,K., Bahler,J. and Maraia,R.J. (2012) Altered nuclear tRNA metabolism in La-deleted *Schizosaccharomyces pombe* is accompanied by a nutritional stress response involving Atf1p and Pcr1p that is suppressible by Xpo-t/Los1p. *Mol. Biol. Cell*, **23**, 480–491.
43. Blomen,V.A., Majek,P., Jae,L.T., Bigenzahn,J.W., Nieuwenhuis,J., Staring,J., Sacco,R., van Diemen,F.R., Olk,N., Stukalov,A. *et al.* (2015) Gene essentiality and synthetic lethality in haploid human cells. *Science*, **350**, 1092–1096.
44. Lippai,M., Tirián,L., Boros,I., Mihály,J., Erdélyi,M., Belecz,I., Máthé,E., Pósfai,J., Nagy,A., Udvardy,A. *et al.* (2000) The Ketel gene encodes a Drosophila homologue of importin-beta. *Genetics*, **156**, 1889–900.
45. Braun,I.C., Herold,A., Rode,M. and Izaurralde,E. (2002) Nuclear export of mRNA by TAP/NXF1 requires two nucleoporin-binding sites but not p15. *Mol. Cell Biol.*, **22**, 5405.
46. Igloi,G.L. and Kössel,H. (1985) Affinity electrophoresis for monitoring terminal phosphorylation and the presence of queuosine in RNA. Application of polyacrylamide containing a covalently bound boronic acid. *Nucleic Acids Res.*, **13**, 6881–6898.