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Nuclear mRNA maturation and mRNA export control: from trypanosomes to opisthokonts

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Review

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Abstract

The passage of mRNAs through the nuclear pores into the cytoplasm is essential in all eukaryotes. For regulation, mRNA export is tightly connected to the full machinery of nuclear mRNA processing, starting at transcription. Export competence of pre-mRNAs gradually increases by both transient and permanent interactions with multiple RNA processing and export factors. mRNA export is best understood in opisthokonts, with limited knowledge in plants and protozoa. Here, I review and compare nuclear mRNA processing and export between opisthokonts and *Trypanosoma brucei*. The parasite has many unusual features in nuclear mRNA processing, such as polycistronic transcription and trans-splicing. It lacks several nuclear complexes and nuclear-pore-associated proteins that in opisthokonts play major roles in mRNA export. As a consequence, trypanosome mRNA export control is not tight and export can even start co-transcriptionally. Whether trypanosomes regulate mRNA export at all, or whether leakage of immature mRNA to the cytoplasm is kept to a low level by a fast kinetics of mRNA processing remains to be investigated. mRNA export had to be present in the last common ancestor of eukaryotes. Trypanosomes are evolutionary very distant from opisthokonts and a comparison helps understanding the evolution of mRNA export.

Introduction

All eukaryotic cells rely on the transport of molecules between the nucleus and the cytoplasm, in an efficient and regulated manner. With few exceptions, transport occurs through nuclear pores that pinch the double-layered membrane of the nucleus. Each nucleus possesses hundreds (yeast and trypanosomes) to thousands (human) of such channels. Nuclear pores are immensely complex multiprotein structures built from 500 to 1000 copies of ~30 different proteins, the nucleoporins (NUPs). Each nuclear pore complex (NPC) is an eight-fold symmetric cylindrical structure, consisting of a symmetric core of one inner and two outer concentric rings (the nucleoplasmic and the cytoplasmic rings) that connect the inner and outer nuclear membrane and form the pore (Alber *et al.*, 2007*b*). The nucleoplasmic outer ring is connected to the nuclear basket at the nuclear site of the pore and the cytoplasmic outer ring to eight cytoplasmic filaments at the cytoplasmic site (Alber *et al.*, 2007*b*). About one third of all NUPs possess highly unstructured regions enriched in phenylalanine–glycine (FG) motifs (FG NUPs). These FG motifs can phase-separate (Zilman, 2018) and in this way create a passive diffusion barrier for all molecules larger than ~40 kDa (Stanley *et al.*, 2017). Thus, while molecules up to about 30 kDa can freely enter and exit the nucleus by diffusion, larger proteins and almost all RNA molecules require more complex transport systems and can only pass because they bind to nuclear transporters that specifically interact with the FG-repeat sequences of the central channel (Stanley *et al.*, 2017).

Efficient and regulated mRNA export is of utmost importance to all eukaryotic cells, as all mRNAs must cross the nuclear envelope to reach their final destination in the cytoplasm. Most importantly, mRNA export is no isolated process restricted to the pore, but is tightly coordinated with the entire nuclear mRNA maturation machinery, starting at transcription (Björk and Wieslander, 2017; Wende *et al.*, 2019). An elaborate and collaborative control system ensures, that only mature, fully processed mRNAs can exit to the cytoplasm. mRNAs are transcribed by RNA polymerase II (RNAPII), usually as monogenetic transcripts. The C-terminal domain of the polymerase recruits factors to the transcription site that are needed for the downstream events of RNA processing, including capping, polyadenylation and splicing (Wende *et al.*, 2019). Consequently, many mRNA maturation steps occur co-transcriptionally. During maturation, the pre-mRNA interacts with many proteins and protein complexes and some of these complexes mark the completion of certain mRNA processing steps and recruit the mRNA export factor Mex67-Mtr2 (NXF1-NXT1 or TAP-p15 in human) to the mRNP. The most famous among these complexes is the TREX complex (couples *tr*anscription and *ex*port) (Sträßer *et al.*, 2002; Wende *et al.*, 2019; Ashkenazy-Titelman *et al.*, 2020). Mex67 is the major mRNA export factor (a 'mobile nucleoporin' according to some newer studies (Derrer *et al.*, 2019)) and interacts with the FG Nups of the inner pore channel, this way transporting the large mRNP out of the nucleus. At the cytoplasmic site of the pores, the mRNP is remodelled to replace export factors by proteins required for the mRNAs cytoplasmic functions. This process requires the ATP-dependent RNA helicase Dbp5. Faulty RNAs are degraded by the nuclear exosome, aided by the TRAMP complex. In Metazoa, several

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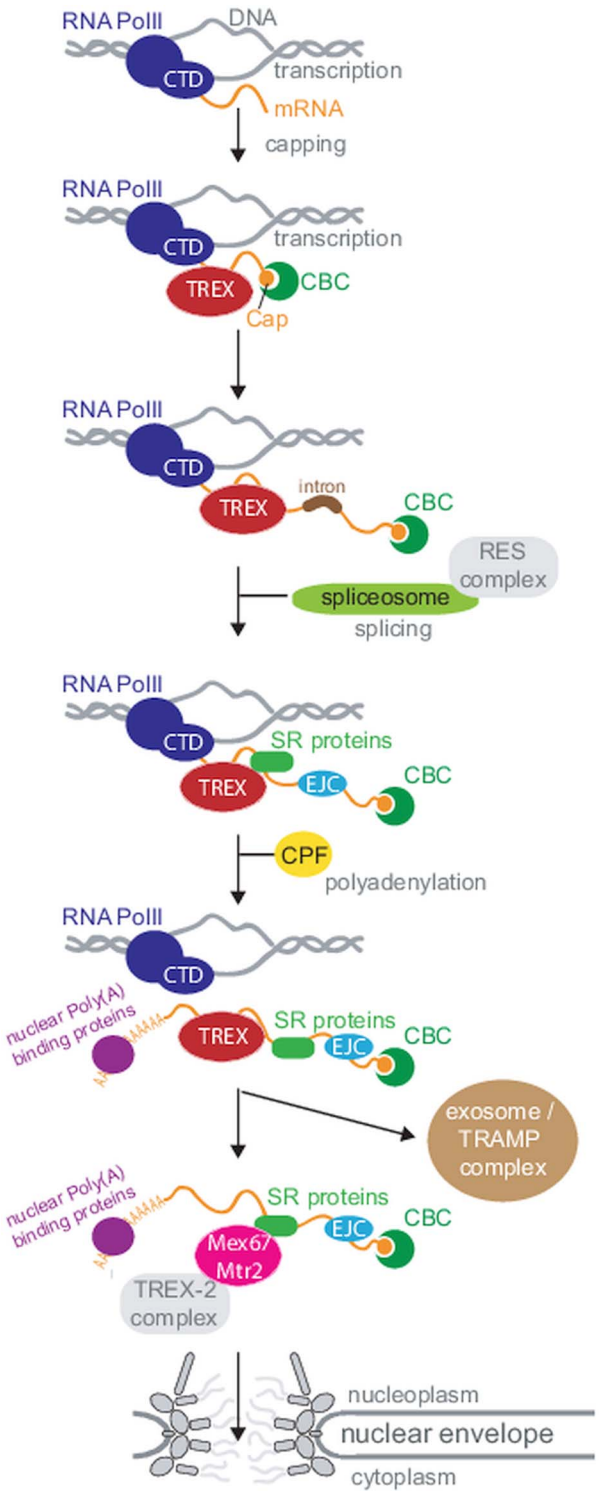


Fig. 1. Overview of nuclear mRNA metabolism. This diagram summarizes the basic steps of nuclear mRNA processing in a simplified version; all steps are discussed in the review in greater detail. Note that not all components are present in all organisms. For clarity, I have sorted processing steps in a pathway, but the order of events is not fully understood and processing steps can also occur in different orders or simultaneously. Abbreviations: CTD: C-terminal domain; CBC: cap binding complex; SR: serine–arginine rich; RNA PolII: RNA polymerase II; CFP: cleavage and polyadenylation factor; TREX: couples transcription and export; TRAMP: Trf4-Air2-Mtr4 polyadenylation.

alternative mRNA export factors and routes exist, and this redundancy may explain why phenotypes after depletion of orthologues to essential yeast export factors are often mild. These factors will mostly not be included here, but are covered well in Scott *et al.* (2019). Figure 1 provides a simplified overview about nuclear mRNA metabolism.

In this review, I will compare the steps of nuclear mRNA maturation and export and its regulation between opisthokonts (mostly yeast and human), where these processes have been mostly studied, and the African trypanosome *Trypanosoma brucei*, the causative agent of African sleeping sickness and related cattle diseases. From an evolutionary point of view, these organisms are highly divergent: opisthokonts belong to one of the two major eukaryotic kingdoms, the Amorphea, while trypanosomes belong to the Discoba, which is now considered an extra clade outside of the two major kingdoms (He *et al.*, 2014; Adl *et al.*, 2019). A comparison offers the unique opportunity to distinguish features of mRNA export that were present in the last common ancestor of eukaryotes from features that have evolved later.

Genome organization and mRNA metabolism of *T. brucei* has several highly unusual and often unique features. The parasite possess a very gene-dense genome with only two introns (Mair *et al.*, 2000; Kolev *et al.*, 2010; Siegel *et al.*, 2010) and almost no regulatory regions (Berriman *et al.*, 2005). Uniquely, genes are arranged head to tail to form about 167 polycistronic transcription units, each consisting out of tens to hundred of transcripts (Berriman *et al.*, 2005). These polycistrons are transcribed by RNA polymerase II from transcription start sites that are epigenetically marked by histone modifications (Siegel *et al.*, 2009). A small number of mRNAs, mostly encoding highly abundant cell surface proteins, are transcribed by RNA polymerase I (Zomerdijk *et al.*, 1991; Chung *et al.*, 1992; Lee and Van der Ploeg, 1997; Gunzl *et al.*, 2003). Polycistronic transcription means that neither 5′ nor 3′ end of the mRNA are accessible for direct capping and polyadenylation. Instead, the 5′ m^7G cap is added by trans-splicing the capped 39-nucleotide exon from the spliced leader RNA to each mRNA's 5′ end, coupled with polyadenylation of the downstream transcript (Campbell *et al.*, 1984; LeBowitz *et al.*, 1993; Ullu *et al.*, 1993; Matthews *et al.*, 1994). Note that after processing, trypanosome mRNAs do not significantly differ from mRNAs of any other organism: they have a 5′ cap and a poly(A) tail of about 100 nucleotides and the open reading frame is flanked by 5′ and 3′ UTR regions. The only trypanosome-unique mRNA features are specific methylations at the cap structure (Perry *et al.*, 1987; Freistadt *et al.*, 1988; Bangs *et al.*, 1992) and the fact that, as a result of the trans-splicing reaction, every mRNA has exactly the same 39 nucleotides (the miniexon sequence) at its 5′ end. While the trypanosome NPC core structure is conserved, it has features indicating fundamental differences in the mRNA export pathway between trypanosomes and opisthokonts (Obado *et al.*, 2016, 2017; Rout *et al.*, 2017) (Fig. 2). First, trypanosome nuclear pores lack the asymmetric distribution of some NUPs that is required for unidirectionality of transport. Second, apparent orthologues to many NUPs with specific functions in mRNA export and export control are missing and this includes the entire ATP-dependent remodelling complex at the cytoplasmic site. Instead, mRNA export in trypanosomes is likely RanGTP dependent (Obado *et al.*, 2016). Third, trypanosomes can initiate mRNA export co-transcriptionally, indicating that the completion of major processing steps such as polyadenylation and splicing is not required (Goos *et al.*, 2019). These fundamental differences raise the question, how and whether trypanosomes do control mRNA export.

The major part of this review will systematically describe and compare nuclear mRNA processing steps of opisthokonts and trypanosomes, with a particular focus on factors and mechanisms that are relevant for mRNA export (see section 'Nuclear mRNA processing steps in opisthokonts and trypanosomes'). The review will not discuss the spatial organization of part of the RNA processing machinery into nuclear bodies, as this is well covered by another review in this issue (Faria, 2021). The review closes

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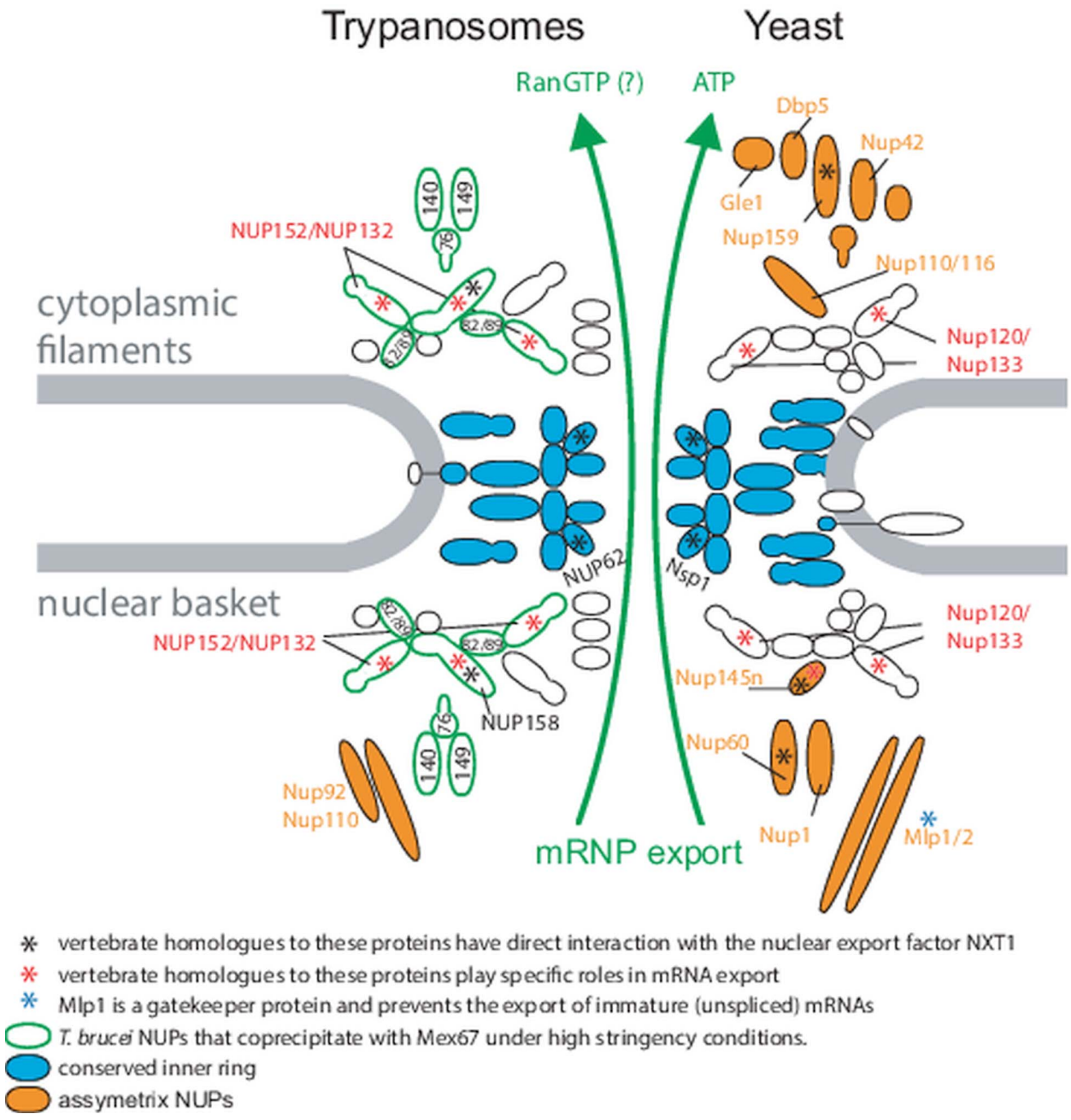


Fig. 2. Nuclear pores in trypanosomes and yeast. Schematics of trypanosome (left) and yeast (right) nuclear pores. The conserved inner ring is shown in blue, and all asymmetric nuclear pore proteins are indicated in orange. Homologues of vertebrate NUPs that were shown to engage in direct interaction with the C-terminal domain of the nuclear export factor NXT1 (Bachi *et al*., 2000; Forler *et al*., 2004) are marked with a black asterisks. Homologues to vertebrate NUPs with suspected roles in intermediate mRNA export are marked with red asterisks (Powers *et al*., 1997; Vasu *et al*., 2001; Blevins *et al*., 2003). *T. brucei* NUPs that coprecipitate with the mRNA export factor Mex67 under high stringency conditions are encircled in green (Obado *et al*., 2016). The figure is modified from Obado *et al*. (2016).

with a discussion on mRNA export control in trypanosomes (see section 'Co-transcriptional initiation of RNA export indicates the lack of major mRNA export checkpoints in trypanosomes') and a small outlook.

Nuclear mRNA processing steps in opisthokonts and trypanosomes

The C-terminal domain of RNAPII

mRNAs are transcribed by RNA polymerase II (RNAPII). The C-terminal domain of RNAPII (CTD) contains heptapeptide repeats with the consensus sequence YSPTSPS that get differentially phosphorylated during the different steps of transcription, this way successively recruiting mRNA processing and export factors to the transcription site (Jeronimo *et al*., 2013). At the promoter region, the CTD is hypophosphorylated, during initiation it gets phosphorylated at S5 and S7 and elongation causes dephosphorylation at S5 and increases phosphorylation at Y1, S2 and T4. At the end of the transcription unit, Y1 gets dephosphorylated (upstream of the polyadenylation site) and finally S2 and T4 are dephosphorylated once the polyadenylation site has been passed (Heidemann *et al*., 2013). RNA processing proteins and their complexes that are recruited specifically *via* CTD phosphorylation patterns include for example the capping enzyme (Cho *et al*., 1997; McCracken *et al*., 1997; Ghosh *et al*., 2011), the TREX complex (Meinel *et al*., 2013) or, in yeast, the SR protein Npl3 (Dermody *et al*., 2008; Meinel *et al*., 2013).

Like many protozoans, trypanosomes have an RNA polymerase II with a non-canonical CTD lacking the repetitive motifs.

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Still, the CTD is essential for parasite survival and serine-rich with at least 17 phosphorylation sites (Das and Bellofatto, 2009; Urbaniak *et al.*, 2013; Das *et al.*, 2017). These 17 phosphorylation sites are all in the only stretch of the CTD that is indispensable for RNAPII function, evidence for their importance (Das and Bellofatto, 2009). The function of CTD phosphorylation in trypanosomes remains unclear. Trypanosome mRNAs can be processed in the absence of CTD phosphorylation, for example when mRNA is transcribed by a different RNA polymerase, such as RNA polymerase I or by phage RNA polymerase T7 (albeit processing efficiency has never been studied). Moreover, CTD phosphorylation appears not required for transcription *per se* and also not for co-transcriptional m^7G capping (Badjatia *et al.*, 2013; Gosavi *et al.*, 2020). The trypanosome kinase CRK9 was suspected to act as a CTD kinase, because its depletion causes CTD hypophosphorylation (Badjatia *et al.*, 2013), but a recent study using analogue-sensitive CRK9 resulted in inhibition of splicing within 5 min, while the loss in CTD phosphorylation took 12–24 h (Gosavi *et al.*, 2020). These data indicate that there is cross-talk between the mRNA processing machinery and RNAPII, but argue against CRK9 being directly involved in CTD phosphorylation.

Despite of not being essential for transcription, the trypanosome CTD mediates correct positioning of RNAPII at transcriptional start sites within the chromatin (Das *et al.*, 2017). In trypanosomes, these transcription start sites can stretch over several kilobases (Siegel *et al.*, 2009; Thomas *et al.*, 2009; Aslett *et al.*, 2010; Kolev *et al.*, 2010), as the parasites lack conventional RNAPII promoters, with the exception of the atypical promoter of the spliced leader RNAs (Gilinger and Bellofatto, 2001). Interestingly, transcription starts predominantly in the correct direction (Wedel *et al.*, 2017); and it remains to be investigated whether the CTD is also involved in defining transcription directionality. Another potential function of trypanosome CTD phosphorylations could be to mediate the pausing of RNAPII that was observed downstream of SL addition sites of each gene and may facilitate trans-splicing (Wedel *et al.*, 2017). Such a function (currently purely speculative) would be analogous to the CTD function in higher eukaryotes in connecting mRNA transcription with downstream processing steps.

The TREX complex

The TREX complex, so named because it couples *tr*anscription to mRNA *ex*port, is one of the first complexes that associates with the newly transcribed transcripts (Sträßer *et al.*, 2002; Wende *et al.*, 2019; Ashkenazy-Titelman *et al.*, 2020). It consists of the THO complex, and, in its minimal version, of the RNA helicase Sub2 (UAP56/DDX39B in Metazoa) and the adaptor protein Yra1 (ALYREF/THOC4 in Metazoa) (Table 1). Several further subunits specific to either yeast or human have been described (reviewed in Wende *et al.*, 2019).

The THO complex is the core of the TREX complex and creates a binding platform for the other TREX proteins on the chromatin during transcription (Aguilera and Klein, 1990; Piruat and Aguilera, 1998; Gewartowski *et al.*, 2012). In yeast, the THO complex is heteropentameric and consists of Tho2, Hpr1, Mft1, Thp2 and Tex1.

The DEAD box RNA helicase Sub2 (UAP56/DDX39B) plays multiple roles in the TREX complex and has conserved functions in mRNA export (Luo *et al.*, 2001; Reed and Hurt, 2002; Taniguchi and Ohno, 2008; Dufu *et al.*, 2010; Kammel *et al.*, 2013; Serpeloni *et al.*, 2016). It binds progressively to the newly transcribed mRNA (Kiesler *et al.*, 2002), promotes spliceosome assembly together with U2AF65 and is essential in the pre-mRNA splicing process (Fleckner *et al.*, 1997; Shen *et al.*, 2007). One important function of Sub2 is the recruitment of the adaptor protein Yra1 (discussed below) to the mRNP (Strässer and Hurt, 2001). Because Sub2 and Mex67 bind to the same domain of Yra1, Sub2 is released from the transcript as soon as Mex67 binds: Sub2 hands over the transcript to the downstream key-players of export (Strässer and Hurt, 2001) and this mechanism appears conserved in metazoans (Hautbergue *et al.*, 2008). The function of the helicase in mRNA export is ATP-dependent (Kota *et al.*, 2008; Taniguchi and Ohno, 2008) and thus one of only two energy-dependent steps in mRNA export (the other is the RNP remodelling by RNA helicase Dbp5 at the cytoplasmic site of the pore).

Table 1. Proteins of the TREX core complex

Sub complex	Yeast	Human	*T. brucei*
	Sub2	UAP56/DDX39B	Sub2 (Tb927.10.540)
	Yra1	ALYREF/THOC4	–
THO	Hpr1	THOC1	–
THO	Tho2	THOC2	–
THO	Tex1	THOC3	–
THO	Mft1	–	–
THO	Thp2	–	–

Note that only *S. cerevisiae* TREX complex components are listed with the respective human homologues; TREX subunits unique to human are not listed. The SR proteins Gbp2 and Hrb1 are specific components of the yeast TREX complex and are also not included in this table but discussed in section 'SR proteins'.

The export adaptor Yra1 (ALYREF/THOC4) is recruited to the mRNP in multiple ways. With the exception of Sub2 (discussed above), these ways differ between yeast and higher eukaryotes. In yeast, a protein of the cleavage and polyadenylation complex (Pcf11 (Johnson *et al.*, 2009)), the DEAD-box RNA helicase Dbp2 (Ma *et al.*, 2013), RNA itself (Meinel *et al.*, 2013) and ubiquitylation of several proteins, e.g. Histone 2B (Vitaliano-Prunier *et al.*, 2012) can recruit Yra1 or contribute to the recruitment. In higher eukaryotes, ALYREF can be recruited to the RNP by the spliceosome *via* interaction with the exon junction complex (Masuda *et al.*, 2005; Gromadzka *et al.*, 2016) or by the cap-binding complex (CBC) (Cheng *et al.*, 2006; Nojima *et al.*, 2007; Sen *et al.*, 2019) (see section 'Cap-binding complex'). ALYREF is also present at the mRNAs 3′ ends, dependent on the cap binding protein CBP80 and the nuclear poly(A) binding protein PABPN1 (Shi *et al.*, 2017). These differences in the loading mechanism of the TREX complex between yeast (mostly transcription dependent) and metazoans (mostly splicing dependent) likely reflect the higher number of introns in metazoans. Independent on how Yra1/ALYREF is recruited to the mRNP, once bound it interacts directly with the export receptor Mex67-Mtr2 (NXF1-NXT1, TAP-p15) and recruits it to the mRNP and thus plays an essential role in mRNA export (Strässer and Hurt, 2000; Zenklusen *et al.*, 2001; Hautbergue *et al.*, 2008). The binding of NXF1 to ALYREF causes a conformational change in ALYREF that decreases its affinity for the RNP: ALYREF hands over the RNP to Mex67 at the nuclear basket and does not accompany the RNP through the pore (Kim *et al.*, 2001; Kiesler *et al.*, 2002; Lund and Guthrie, 2005). While Yra1 is the major Mex67 adaptor protein in yeast and essential for mRNA export (Portman and Gull, 2012; Segref *et al.*, 1997; Santos-Rosa *et al.*, 1998; Zenklusen *et al.*, 2002), depletion of the metazoan orthologue has only a mild effect on mRNA export (Gatfield and Izaurralde, 2002; Longman *et al.*, 2003; Katahira *et al.*, 2009). The likely reason is that Metazoa have many alternative NXF1 adaptors, including for example organism-specific

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Table 2. Enzymes involved in nuclear mRNA capping and cap methylation

Activity	*S. cerevisiae*	Metazoa	*T. brucei*
RNA triphosphatase	Cet1	Mce1	CET1 (Tb927.3.2190)
RNA guanylyltransferase	Ceg1		CGM1 (Tb927.7.2080)
Guanine-N7-methyltransferase	Abd1	Hcm1	
2′-*O*-ribose MTases (cap1)	–	Mtr1	MTr1 (Tb927.10.7940)
2′-*O*-ribose MTases (cap2)	–	Mtr2	MTr2 = MT48 = MT417 = Com1 (Tb927.11.4890)[a,b]
2′-*O*-ribose MTases (cap3)	–	–	MTr3 = MT57 = MT511 (Tb927.9.12040)
2′-*O*-ribose MTases (cap4)	–	–	(?) MTr3 = MT57 = MT511 (Tb927.9.12040)[c]
Enzymes involved in base methylation	–	–	Not yet identified

[a]Misleading nomenclature: note that MTr2 is not the MEX67 interacting protein Mtr2.
[b]The trypanosome enzyme is homologous to the vaccinia virus (VP39) methyltransferase and not to the metazoan Mtr2.
cIt is likely but not proven that MTr3 methylates ribose at both the third and fourth nucleotide, as MTr3 -/- cells lack 2′-O ribose methylations on both positions (Arhin *et al.*, 2006*b*).

TREX components or splicing factors of the SR (serine −arginine-rich) protein family (Scott *et al.*, 2019; Wende *et al.*, 2019; Ashkenazy-Titelman *et al.*, 2020).

Trypanosomes have an orthologue to the TREX-complex protein Sub2 (Table 1). This helicase has nuclear localization in *Trypanosoma cruzi* and *T. brucei* (Serpeloni *et al.*, 2011*a*; Dean *et al.*, 2017; Goos *et al.*, 2017), binds mRNA (Lueong *et al.*, 2016) and, importantly, its depletion by RNAi in *T. brucei* caused growth arrest and accumulation of polyadenylated mRNA in the nucleus, all indicative of an essential function in mRNA export (Serpeloni *et al.*, 2011*a*). Still, trypanosomes are unlikely to possess a conventional TREX complex, as obvious orthologues to all other TREX subunits (Yra1, Tho2, Hpr1, Mft1, Thp2 and Tex1) are absent, TbSub2 fails to complement yeast Sub2 (Serpeloni *et al.*, 2011*a*) and TbSub2 was not detected in a Mex67 affinity purification approach (Obado *et al.*, 2016). Moreover, TbSub2 differs from its yeast orthologue by a faster ATP hydrolysis rate and an activity less dependent on RNA binding (de Bittencourt *et al.*, 2017). The exact function of TbSub2 in mRNA export remains to be discovered. Interestingly, the Sub2 orthologue of *Toxoplasma gondii*, a parasite with distant phylogenetic relationship to trypanosomes, also is the only TREX component that can be identified by homology, and its involvement in mRNA export was shown (Serpeloni *et al.*, 2016); one protein with no similarity to Yra1 apart from the presence of an RRM domain could be a functional *T. gondii* Yra1 orthologue based on its mRNA export phenotype (Serpeloni *et al.*, 2016).

Recently, the retrotransposon hot spot proteins (RHS proteins) were suggested to be the trypanosomes alternative to the TREX complex. This multigene family has 118 members (classified into six groups) that are characterized by a retrotransposon insertion site in the 5′ region of the coding sequence, resulting in ~60% pseudogenes (Bringaud *et al.*, 2002). Proteins of five of the six subfamilies (RHS1,3,4,5,6) show nuclear localization, while RHS2 is in the cytoplasm (Bringaud *et al.*, 2002; Florini *et al.*, 2019). An extensive analysis of retrotransposon hot spot proteins RHS2, RHS4 and RHS6 showed association with active PolII transcription sites (Chip Seq), even for the cytoplasmic RHS2, which can shuttle to the nucleus (Florini *et al.*, 2019). RNAi depletion of all proteins caused growth arrest and a global reduction in transcription as well as a block in mRNA export. RHS4 is part of the PolII complex (Das *et al.*, 2006; Devaux *et al.*, 2006), while RHS2 mostly co-precipitated with ribosomal proteins and translation factors and RHS6 mostly with nuclear proteins involved in transcription and mRNA processing (Florini *et al.*, 2019). In conclusion, retrotransposon hot spot proteins have essential functions in transcription and further nuclear and cytoplasmic mRNA processing steps. Whether they act in connecting transcription with export and thus can be considered functional orthologues to the TREX complex remains to be investigated. In particular, direct binding to RNA has not been shown (albeit chromatin interaction appears partially mediated by RNA for RHS2 and RHS4 (Florini *et al.*, 2019) and RHS4 co-precipitates with oligo(dT) beads (Lueong *et al.*, 2016)). Moreover, it remains unclear, whether the multiple phenotypes observed after RNAi depletion of an RHS protein represent functions in different RNA processing steps (e.g. transcription and export), or, whether block of one RNA processing step (e.g. transcription) subsequently affects multiple downstream pathways without the direct involvement of RHS proteins.

Adding the cap

The first modification added to every newly transcribed mRNA is the 5′ m^7G cap (Table 2). mRNA capping is done co-transcriptionally as soon as the first 25–30 nucleotides are transcribed, by capping enzymes that are recruited to the transcription start site; serine 5 phosphorylation of the CTD serves as one recruitment signal (Ramanathan *et al.*, 2016). mRNA capping involves three enzymatic activities. First, RNA triphosphatase (TPase) removes the γ-phosphate from the triphosphorylated mRNA's 5′ end, creating a 5′ diphosphate mRNA. Second, RNA guanylyltransferase (GTase) transfers a GMP group from GTP to the 5′ diphosphate mRNA, creating the 5′-5′ triphosphate linkage between the cap and the first base of the mRNA. Third, the guanine-N7-methyltransferase (MTase) methylates the N7 amine of the guanine cap using S-adenosylmethionine (SAM) as methyl-donor, to form the cap 0 structure. In *Saccharomyces cerevisiae*, the three capping activities are on three separate proteins (Cet1, Ceg1 and Abd1) (Martinez-Rucobo *et al.*, 2015). In Metazoa, TPase and GTase activities reside on the same protein (Mce1 in mouse) (Yue *et al.*, 1997; Chu *et al.*, 2011), while the guanine-N7-methyltransferase activity (Hcm1) is on a separate protein (Saha *et al.*, 1999). In higher eukaryotes, the 2′O of the ribose of the first base or first and second base is methylated, creating the predominant cap 1 and cap 2 structures, respectively (Furuichi *et al.*, 1975; Bélanger *et al.*, 2010; Werner *et al.*, 2011; Furuichi, 2015); the responsible human methylases are hMtr1 and hMtr2 (Bélanger *et al.*, 2010; Werner *et al.*, 2011). Ribose methylations are absent in yeast (Mager *et al.*, 1976; Sripati *et al.*, 1976). In metazoans, the 5′ m^7G cap is important for the

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Table 3. The cap binding complex in *S. cerevisiae*, metazoans and trypanosomes

S. cerevisiae	Metazoa	*T. brucei*
Cbp20	CBP20 = NCBP1	CBP20 (Tb927.6.1970)
Cbp80	CBP20 = NCBP2	–
–	–	CBP110 (Tb927.10.2990)
–	–	CBP30 (Tb927.10.15210)
–	–	CBP66 (Tb927.3.1340)

export of spliced mRNAs but not of intron-less mRNAs, probably because it is involved in recruiting the TREX complex upstream to the first exon–exon junction (Cheng *et al.*, 2006).

The process of mRNA capping is unusual in trypanosomes (Table 2). Because of the polycistronic transcription, the 5′ ends of mRNAs are not directly accessible for capping enzymes. Therefore, the cap is added by trans-splicing the capped 39 nucleotide long miniexon of the spliced leader RNA to the 5′ end of each transcript (reviewed in Michaeli, 2011; Preusser *et al.*, 2012). The spliced leader RNA itself is separately transcribed from a tandem array of about 100 SL RNA genes, each copy from its own promoter (these are the only PolII promoters present in trypanosomes) (Günzl *et al.*, 1997; Gilinger and Bellofatto, 2001; Srivastava *et al.*, 2017). Capping of the spliced leader RNA is different from both the yeast and metazoan system, in that the RNA triphosphatase activity is on a separate protein (TbCet1, (Ho and Shuman, 2001) while the RNA guanylyltransferase and the guanine-N7-methyltransferase activity reside on the bifunctional enzyme TbCgm1 (Hall and Ho, 2006; Ruan *et al.*, 2007; Takagi *et al.*, 2007). Note that trypanosomes also have the RNA guanylyltransferase and guanine-N7-methyltransferase activity on two individual enzymes (Ce1 and Cmt1, respectively) (Silva *et al.*, 1998; Hall and Ho, 2006) but these enzymes are cytoplasmic (Dean *et al.*, 2017) and not involved in SL RNA capping (Ruan *et al.*, 2007; Takagi *et al.*, 2007; Ignatochkina *et al.*, 2015; Silva *et al.*, 1998). One further unique feature of trypanosomes is that the mRNA cap is of the heavily methylated type 4: the first four transcribed nucleotides (AACU) have ribose 2′-O methylations and there are additional base methylations on the first (m_2^6A) and fourth (m^3U) position (Perry *et al.*, 1987; Freistadt *et al.*, 1988; Bangs *et al.*, 1992). Cap methylation is essential for trans-splicing (Ullu and Tschudi, 1991) and ribose methylation is required for efficient translation (Zeiner *et al.*, 2003*b*; Zamudio *et al.*, 2009). Three 2′-O-ribose methyltransferases have been described in trypanosomes, MTr1 (Zamudio *et al.*, 2007; Mittra *et al.*, 2008), MTr2 (Hall and Ho, 2006; Zamudio *et al.*, 2006; Arhin *et al.*, 2006*b*) and MTr3 (Zamudio *et al.*, 2006; Arhin *et al.*, 2006*a*); MTr2 and MTr3 are related to the vaccinia virus VP39 methyltransferase. The deletion of some but not all three 2′-O-ribose methyltransferases is viable, with some effects on growth and translation (Zamudio *et al.*, 2006, 2009; Arhin *et al.*, 2006*b*). The function of the unusual base methylations is unknown, and the responsible enzymes have not yet been identified. The SL RNA is also pseudouridinylated at position −12 relative to the 5′ splice site, but this modification is not essential for growth in culture (Hury *et al.*, 2009). It is not known yet, whether the trypanosome cap (and its methylations) is required for mRNA export.

Cap-binding complex

As soon as the m^7 G cap is synthesized, it is bound by the nuclear cap-binding complex (CBC), a heterodimer out of Cbp20 and Cbp80 (CBP20/CBP80 or NCBP1/NCBP2 in metazoans) that protects the new transcript from degradation (Table 3). Only Cbp20 binds the m^7G cap directly; the Cbp80/CBP80 subunit serves as a binding platform for many regulatory factors with key function in multiple diverse pathways, including transcription, splicing, export and translation (Gonatopoulos-Pournatzis and Cowling, 2013; Müller-McNicoll and Neugebauer, 2014; Rambout and Maquat, 2020). For example, the CBC binds to the Yra1/ALYREF subunit of the TREX complex and recruits it co-transcriptionally to the mRNA's 5′ end, likely facilitating nuclear export (Cheng *et al.*, 2006; Nojima *et al.*, 2007; Sen *et al.*, 2019); in yeast, Npl3, an SR protein which contributes to the formation of an export-competent RNP, is also recruited by the CBC (Sen *et al.*, 2019). The CBC indirectly promotes nuclear export in many ways by promoting various steps in mRNP maturation and it accompanies its RNP target through the pore. However, there is no genetic evidence for a direct involvement of the CBC complex in mRNA export and whether it is strictly required for export is still debated.

The nuclear cap binding complex of trypanosomes has only one conserved subunit (CBP20) and at least three trypanosome-specific subunits (CBP30, CBP66, CBP110) (Li and Tschudi, 2005) (Table 3). It is essential and required for trans-splicing and has 15-fold higher affinity to the hypermethylated trypanosome type 4 cap than to a type 0 cap (Li and Tschudi, 2005). It is not known whether and how the CBP is remodelled during trans-splicing, whether the CBP plays a role in mRNA export and when and where it is replaced by the cytoplasmic cap binding complex.

Spliceosome

All pre-mRNAs that contain introns are processed by the spliceosome in a complex series of reactions that serve to remove the introns and join the 3′ end of each exon with the 5′ end of the next exon (Wahl *et al.*, 2009; Fica and Nagai, 2017). Splicing is guided by sequence elements within the intronic region, such as the branch point sequence (BPS) that attacks the phosphodiester bond of the 5′ splice site to create a free 5′ exon and the intron-lariat-3′ exon intermediate (Wahl *et al.*, 2009). Another highly conserved intronic sequence element is the polypyrimidine tract (a sequence rich in polypyrimidine) that is located between the branch point site and the 3′ splice site and is required for early steps of spliceosome assembly (Wahl *et al.*, 2009). The spliceosome consists of five small nuclear RNAs (snRNAs) U1, U2, U4, U5 and U6 that associate with both snRNA-specific proteins as well as with Sm proteins (LSm proteins in the case of U6) (Wahl *et al.*, 2009). The Sm/LSm proteins form a ring-like structure around a conserved motif of the respective snRNA, the Sm site (Kambach *et al.*, 1999). About 75% of splicing events of budding yeast and human occur co-transcriptionally (Neugebauer, 2019).

The core spliceosome machinery is highly conserved across all eukaryotes (Will and Lührmann, 2011), albeit trypanosomes have some variations in the Sm core proteins and Sm sites (Preusser *et al.*, 2012). Similar to opisthokonts, splicing in trypanosomes occurs mainly co-transcriptionally (Ullu *et al.*, 1993), albeit at least one exception has been reported (Jäger *et al.*, 2007). The trans-splicing reaction is performed by a variant of the spliceosome that has its U1 snRNP replaced by the SL RNP: the SL RNA serves as both snRNA and trans-splicing substrate (Preusser *et al.*, 2012)). Like *cis*-splicing sites in other organisms, trans-splicing sites are preceded by polypyrimidine tracts (Smith *et al.*, 2008; Kolev *et al.*, 2010). The splicing reaction is analogous to *cis* splicing, except that a branched Y-structure intermediate instead of a lariat structure is formed, when the SL RNA intron is joined 2′-5′ to an A residue upstream of the polypyrimidine tract. The conventional U1 snRNP is still present in trypanosomes

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Table 4. EJC components in yeast, metazoans and trypanosomes

S. cerevisiae	Metazoan	*T. brucei*
eIF4AIII	eIF4AIII	FAL1 = HEL54 = eIF4AIII (Tb927.11.8770)[a]
Y14	Y14	Y14 (Tb927.7.1170)
Magoh	Magoh	Magoh (Tb927.6.4950)
–	MLN51	–

[a]interaction with Y14 and Magoh could not be shown (Bercovich *et al.*, 2009*b*). Whether this protein is the functional orthologue of eIF4AIII is still debated.

and needed for the *cis*-splicing of the two intronic RNAs, encoding the poly(A) polymerase PAP1 and the DEAD box RNA helicase DBP2B. Interestingly, these two intronic mRNAs are conserved across Trypanosomatidae (Mair *et al.*, 2000; Camacho *et al.*, 2019). Moreover, both intronic mRNAs encode for proteins with (potential) functions in nuclear RNA processing: PAP1 presumably adenylates pre-snoRNAs (Chikne *et al.*, 2017) and DBP2B is one of two trypanosome homologues of Dbp2 that in yeast recruit Yra1 to the mRNP (Ma *et al.*, 2013). One, purely speculative, model is that the trypanosome *cis*-splicing is not a random left over of a general loss of introns but could have autoregulatory functions. Furthermore, interaction of the U1 snRNP protein U1A with the polyadenylation factor CPSF73 (Tkacz *et al.*, 2010) and the finding that the U1 snRNP proteins U1C and U1-70K also interact with the SL RNA and the U6 snRNA (Preusser *et al.*, 2014) indicate additional functions of the U1 RNP in connecting *trans*-splicing with *cis*-splicing and polyadenylation. This could explain the relatively high abundance of the U1 snRNP despite the presence of only two introns. An alternative explanation for the high abundance of the U1 snRNP could be yet undiscovered functions of this snRNP unrelated to mRNA processing: the mammalian U1 snRNP for example has been recently shown to regulate chromatin retention of non-coding RNAs (Yin *et al.*, 2020).

Several further trypanosome proteins with a function in splicing have been described; next to the SR proteins (see section 'SR proteins') these are DRBD3 (=PTB1), DRBD4 (=PTB2) and HNRNPH/F (De Gaudenzi *et al.*, 2005; Stern *et al.*, 2009; Gupta *et al.*, 2013; Das *et al.*, 2015; Clayton, 2019). In addition, the cyclin-dependent kinase CRK9 is essential for the first step in splicing, presumably by phosphorylating proteins of the pre-mRNA processing machinery (Badjatia *et al.*, 2013; Gosavi *et al.*, 2020).

Exon Junction Complex

All splice sites are imprinted to the mRNA by the exon junction complex, EJC, that binds 20−24 nucleotides upstream of exon −exon junctions and has many important cytoplasmic functions (e.g. the recognition of premature stop codons during the pioneer round of translation leading to NMD) (Woodward *et al.*, 2017). The EJC consists of the subunits eIF4AIII, Y14 and Magoh; animals have one additional subunit (MLN51) (Table 4). In mammals, the spliceosomal protein CWC22 is involved in recruiting the EJC to the splice site *via* its interaction with eIF4AIII (Alexandrov *et al.*, 2012; Barbosa *et al.*, 2012; Steckelberg *et al.*, 2012). At least in metazoans, splicing appears to be a significant activator of mRNA export: microinjected intronic pre-mRNAs are far more efficiently exported than the same transcripts microinjected without the intron (Luo and Reed, 1999). The likely reason is that the EJC plays a major role in recruiting the TREX complex to the mRNA by directly interacting with several of its components, including UAP56 and ALYREF (Le Hir *et al.*, 2001; Masuda *et al.*, 2005; Gromadzka *et al.*, 2016; Gerbracht and Gehring, 2018; Viphakone *et al.*, 2019). Still, in Drosophila, the EJC appears dispensable for bulk mRNA export (Gatfield and Izaurralde, 2002). Moreover, in *S. cerevisiae*, most mRNAs have no introns and TREX recruitment occurs predominantly *via* transcription.

Trypanosomes and Leishmania have putative orthologues to the three major components of the exon junction complex, Magoh, eIF4AIII and Y14 (Bannerman *et al.*, 2018) (Table 4). Whether eIF4AIII (also called FAL1 and HEL54) is a true EJC component is still debated. *T. brucei* eIF4AIII is a low abundance protein that can be depleted with only minor effect on cell growth and massively overexpressed as wild type or ATPase inactive mutant with no effect on growth (Dhalia *et al.*, 2006). There are contradicting reports on eIF4EIII localization: eYFP fusions localize to the nucleus and nucleolus (Dhalia *et al.*, 2006; Dean *et al.*, 2017) and the same localization was found with immunofluorescence using antiserum raised to the *T. brucei* protein (Dhalia *et al.*, 2006). In contrast, antiserum raised to the *T. cruzi* homologue (called HEL54) indicates cytoplasmic localization of both the *T. cruzi* and *T. brucei* proteins, mainly to dots close to the nucleus, in addition to nuclear localization; immuno gold electron microscopy confirmed localization to the outside of the nuclear pore as well as to the nucleus (Inoue *et al.*, 2014). Interestingly, nuclear localization of *T. cruzi* HEL54 could by enforced by (i) deleting the putative NES, indicating that the protein shuttles between the nucleus and the cytoplasm (ii) inhibition of transcription by Actinomycin D, indicating that export requires the presence of RNA and (iii) RNAi of Mex67, indicating that HEL54 export is Mex67 dependent (RNAi was done in *T. brucei* for technical reasons) (Inoue *et al.*, 2014). The localization of a shuttling protein to either nucleus or cytoplasm is sensitive to how cells were treated prior to fixation or imaging, a possible explanation for the discrepancy between these datasets. eIF4AIII failed to be co-precipitated by Y14-TAP, possibly because several residues essential for eIF4AIII interaction are mutated in Y14 and Magoh (Bercovich *et al.*, 2009*b*). In contrast, the interaction between Y14 and Magoh could be detected both by Y2H and by co-immunoprecipitation (Bercovich *et al.*, 2009*b*) and both Y14 and Magoh have nuclear localizations (Bercovich *et al.*, 2009*b*; Dean *et al.*, 2017). RNAi depletion of either Y14 or Magoh only caused minor or no growth effects, respectively, but the reduction in protein levels is not known (Bercovich *et al.*, 2009*b*) and only knock-out experiments can answer the question, whether either protein is essential. In addition, the NTF2 domain protein Tb927.10.2240 was co-precipitated with Y14 (Bercovich *et al.*, 2009*b*) but the genome-wide localization database TrypTag found cytoplasmic localization with tags at either C- or N- terminus (Dean *et al.*, 2017), questioning its presence in the EJC. The available data provide evidence for the presence of an EJC in trypanosomes (note that trypanosomes also have a CWC22 homologue) but its composition and function remain unclear. With every mRNA trans-spliced in trypanosomes, the EJC has the potential to mark successful completion of mRNA 5′ end processing and promote nuclear export, however, this model is not supported by any data yet.

RES complex

The RES complex (pre-mRNA REtention and Splicing) was identified in yeast as a trimeric complex consisting of Pml1p, Snu17p and Bud13p, that associate with the spliceosome before the first catalytic step (Dziembowski *et al.*, 2004). It has multiple functions in splicing that are only partially understood, but in particular Pml1p deletion caused leakage of pre-mRNAs to the cytoplasm while splicing was hardly affected, indicating that at least this subunit may have a direct role in regulating mRNA export (Dziembowski *et al.*, 2004). The RES complex subunits and

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their spliceosome association are conserved in human (Deckert *et al.*, 2006; Bessonov *et al.*, 2008). Trypanosomes have no readily identifiable orthologues of the RES complex components Pml1p, Snu17p and Bud13p.

SR proteins

SR proteins are multifunctional RNA binding proteins that bind mRNAs throughout their journey from transcription to translation (Änkö, 2014; Wegener and Müller-McNicoll, 2019) and are conserved across eukaryotes (Busch and Hertel, 2012). They are best known for their essential roles in splicing and as regulators of alternative splicing but have many functions beyond, including an important role in selective mRNA export and retention (Müller-McNicoll *et al.*, 2016; Hautbergue *et al.*, 2017; Zhou *et al.*, 2017). Classical SR proteins consist of an N-terminal RRM domain, a glycine−arginine-rich spacer region of variable length and a C-terminal RS domain with at least 40% RS dipeptide content (Manley and Krainer, 2010). SR protein activity is tightly regulated by posttranslational modifications, in particular by reversible phosphorylations of the serine residues within the RS domain through a range of kinases and phosphatases (Zhou and Fu, 2013). Most SR proteins are adaptors for NXF1 and are required for selective nuclear export of specific mRNA isoforms (Müller-McNicoll *et al.*, 2016). The binding to NXF1 is mediated by two pairs of arginine residues flanking a glycine-rich region in the SR protein linker region (Lai and Tarn, 2004; Huang and Steitz, 2005; Hargous *et al.*, 2006; Tintaru *et al.*, 2007; Botti *et al.*, 2017). Importantly, SR proteins bind NXF1 only in their hypophosphorylated stage (Zhou and Fu, 2013), and, given that SR protein dephosphorylation is required for the release of the splicing machinery this suggests a possible mechanism for the selective export of spliced mRNAs (Huang and Steitz, 2005). Just like ALYREF of the TREX complex, the SR proteins SRSF3 and SRSF7 increase the RNA binding ability of NXF1 upon binding, possibly by inducing a structural change (Hautbergue *et al.*, 2008; Viphakone *et al.*, 2012; Müller-McNicoll *et al.*, 2016). However, while ALYREF hands the mRNA over to NXF1, SR proteins bind close to NXF1 and remain in the complex during export (Müller-McNicoll *et al.*, 2016; Botti *et al.*, 2017). SR proteins add another level of complexity to the regulation of mRNA export: in mammalian cells, more than 1000 endogenous mRNAs require specific SR proteins for export (Müller-McNicoll *et al.*, 2016). The function of SR proteins in nuclear export is not restricted to mammals: even though *S. cerevisiae* lacks classical SR proteins, it has three SR-like proteins that share the basic SR protein domain structure: Npl3, Gbp2 and Hrb1. All these shuttle between the cytoplasm and the nucleus, when bound to newly transcribed mRNA (Lee *et al.*, 1996; Windgassen and Krebber, 2003; Häcker and Krebber, 2004). Gbp2 and Hrb1 are yeast-specific subunits of the TREX complex and important mRNA surveillance factors: they bind their mRNA targets *via* the THO complex (Hurt *et al.*, 2004) and recruit either Mex67 or the TRAMP complex (discussed in section 'The nuclear exosome, the TRAMP complex and the NNS complex'), targeting the mRNA for export or decay, respectively (Hackmann *et al.*, 2014). Npl3 acts as an adaptor protein for Mex67 and mediates mRNA export, regulated by a very similar phosphorylation and dephosphorylation cycle of Npl3 as described for mammalian SR proteins (Gilbert and Guthrie, 2004).

Not counting the auxiliary splicing factor U2AF65 (=RBSR4), trypanosomes have at least five SR proteins: RBSR1 (Tb927.9.6870), RBSR2 (Tb927.9.6870), RBSR3 (Tb927.3.5460), TRRM1 (=RRM1, Tb927.2.4710) and TSR1 (Tb927.8.900) (Clayton, 2019) and all localize primarily to the nucleus (Manger and Boothroyd, 1998; Ismaïli *et al.*, 1999; Dean *et al.*, 2017; Wippel *et al.*, 2019*b*). Studies indicate essential functions of trypanosome SR proteins in *cis*- and *trans* splicing, mRNA stability, processing of snoRNA, rRNA, and snRNAs, and modelling of chromatin structure (Manger and Boothroyd, 1998; Ismaïli *et al.*, 1999; Gupta *et al.*, 2014; Levy *et al.*, 2015; Naguleswaran *et al.*, 2015; Wippel *et al.*, 2019*b*). Even though none of the SR proteins was co-purified with *T. brucei* MEX76 in cryomill-affinity purification (Obado *et al.*, 2016), at least RRM1 has a potential function in mRNA processing and export: RRM1 co-precipitates with the nuclear non-canonical poly(A) polymerase NPAPL/ncPAP1, a putative subunit of the trypanosome TRAMP-complex ((Cristodero and Clayton, 2007; Etheridge *et al.*, 2009), see section 'The nuclear exosome, the TRAMP complex and the NNS complex'), and also with retrotransposon hot spot proteins (Naguleswaran *et al.*, 2015), which may in trypanosomes connect transcription with mRNA export (Florini *et al.*, 2019). *T. brucei* DRBD2 was suggested to be the orthologue of the yeast SR-like protein Gbp2, but it has cytoplasmic localization and no RS domain (Wippel *et al.*, 2019*a*) and is unlikely a functional orthologue.

Adding the poly(A) tail

The 3′ end processing of an mRNA also occurs co-transcriptionally (Kumar *et al.*, 2019; Stewart, 2019*a*). In yeast, the 3′ end processing machinery is composed of the cleavage and polyadenylation factor (CPF) and the two accompanying cleavage factors CF1A and CF1B. The yeast CPF is a large multiprotein complex with three enzymatic activities. The first two activities are required for the addition of the poly(A) tail: the endonuclease (Ysh1/Brr5) cleaves the new transcript and the poly(A) polymerase (Pap1) successively adds AMP to the resulting free hydroxyl group at the 3′ end (Kumar *et al.*, 2019). The third activity links mRNA 3′ end processing to regulation of transcription elongation and termination and consists of two phosphatases that dephosphorylate serine 5 and tyrosine 1 of the CTD (Krishnamurthy *et al.*, 2004; Schreieck *et al.*, 2014) (compare section 'The C-terminal domain of RNAPII'). CF1A and CF1B contribute to RNA recognition and nuclease activation and bind specific RNA sequences (Yang and Doublié, 2011; Xiang *et al.*, 2014). The human homologue of CPF is the cleavage and polyadenylation specificity factor (CPSF), which shares many orthologues with the yeast machinery (Kumar *et al.*, 2019). In human, the highly conserved AAUAAA motif of the polyadenylation signal (PAS) directs the cleavage of the pre-mRNA 10−30 nucleotides downstream (Hu *et al.*, 2005; Derti *et al.*, 2012; Chan *et al.*, 2014; Schönemann *et al.*, 2014; Gruber *et al.*, 2016) and this motif is conserved in fission yeast (Mata, 2013; Schlackow *et al.*, 2013), albeit less well in budding yeast (Zhao *et al.*, 1999). Further *cis*-acting sequences contribute to poly(A) site recognition, but these are less well conserved between species. Whether and how poly(A) addition is connected to nuclear export is still largely unknown; an attractive model is that the release of the CPF from the RNP signals the completion of the export competent RNP (Stewart, 2019*a*).

The cleavage and polyadenylation complex is mostly conventional in trypanosomes (Hendriks *et al.*, 2003; Bercovich *et al.*, 2009*a*; Tkacz *et al.*, 2010; Koch *et al.*, 2016), except that it contains at least two trypanosome-specific subunits (Tb927.11.13860 and Tb927.8.4480) and no potential CTD phosphatase is among the CPF components (Koch *et al.*, 2016). However, the recognition of the poly(A) site is non-conventional: instead of recognizing specific *cis*-elements on the mRNA, the cleavage takes place at a conserved distance to the polypyrimidine tract used for the *trans*-splicing of the upstream gene. This distance varies between different species of Trypanosomatida and is about 100 nucleotides in *T. brucei* (Campos *et al.*, 2008; Kolev *et al.*, 2010; Clayton and Michaeli, 2011; Dillon *et al.*, 2015). The likely reason for this unusual poly(A) site recognition is the strict coupling of

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Table 5. Poly(A) binding proteins in yeast, human and trypanosomes

Poly(A) binding protein	*S. cerevisiae*	*S. pombe*	Human	*T. brucei*
Nuclear, CCCH	Nab2	Nab2	ZC3H14	–
Nuclear, 1 RRM	–	Pab2	PABPN1	–
Cytoplasmic, 4 RRM	Pab1	Pab1	PABP1 (major)[a]	PABP1, PABP2

[a]Mammals have several further cytoplasmic PABP isoforms: tPABP (testis-specific), ePABP (embryonic) and PABP4 (Gray *et al.*, 2015).

trans-splicing with polyadenylation of the upstream transcript (LeBowitz *et al.*, 1993; Ullu *et al.*, 1993; Matthews *et al.*, 1994), which is also reflected by the finding that RNAi knock-down experiments of most CPF proteins inhibit both polyadenylation and *trans*-splicing (Hendriks *et al.*, 2003; Koch *et al.*, 2016). Whether the presence of a poly(A) tail supports mRNA export is, like in other systems, not known. However, the fact that trypanosomes can export mRNAs co-transcriptionally ((Goos *et al.*, 2019) discussed below), indicate that polyadenylation is at least not essential for export.

Poly(A) binding proteins

Once the poly(A) tail is synthesized, it is covered by nuclear poly (A) binding proteins, that come in two domain variants for RNA binding, either with zinc-finger domains (Nab2 in *S. pombe* and *S. cerevisiae* and ZC3H14 in human) or with a single RRM domain (Pab2 in *S. pombe* and PABPN1 in human) (Table 5). The *S. cerevisiae* zinc-finger protein Nab2 (which belongs to the SR protein family) plays a major role in mRNA export, as it shuttles between the nucleus and the cytoplasm, recruits Mex67-Mtr2 to the mRNA and interacts with the nuclear pore-associated protein Mlp1 (Fasken *et al.*, 2019); less mechanistic information is available for the orthologous human zinc-finger protein ZC3H14 or the single RRM domain protein PABPN. Once the mRNA reaches the cytoplasm, its nuclear poly(A) binding proteins are replaced by cytoplasmic poly(A) binding proteins, that all have four RRM domains (Table 5). Most of these proteins can shuttle to the nucleus and also function in mRNA export; this is best established for Pab1 of *S. cerevisiae* (Brune *et al.*, 2005; Dunn *et al.*, 2005; Brambilla *et al.*, 2019).

Trypanosomatidae have no obvious homologues to yeast or human nuclear poly(A) binding proteins with CCCH zinc-finger domains or single RRMs (Table 5). Instead, they have two (*T. brucei*) or three (*T. cruzi* and *Leishmania*) essential poly(A) binding proteins with four RRM domains that have a dominant cytoplasmic localization and co-purify with translating polysomes (Bates *et al.*, 2000; da Costa Lima *et al.*, 2010; Kramer *et al.*, 2013). In *T. brucei*, PABP2 appears to be the major poly(A) binding protein for bulk mRNAs, as it co-purifies a diverse range of RNA binding proteins and localizes across both small and large polysomal fractions; while PABP1 is found in small polysomes only and binds to only few proteins (Zoltner *et al.*, 2018). Interestingly, *T. brucei* PABP2 and Leishmania PABP2 and PABP3 (but not PABP1 of either organism) can be trapped inside the nucleus under certain conditions (da Costa Lima *et al.*, 2010; Kramer *et al.*, 2013). This indicates that these isoforms are shuttling, fulfilling both the function of a nuclear and a cytoplasmic PABP, analogous to yeast Pab1. Whether PABP2 is involved in nuclear export is not known, but given that nuclear export can occur co-transcriptionally prior to poly(A) tail synthesis ((Goos *et al.*, 2019), see section 'Co-transcriptional initiation of RNA export indicates the lack of major mRNA export checkpoints in trypanosomes'), its binding to the poly(A) is at least not essential for export.

The TREX-2 or THSC complex

Just like TREX-1, the TREX-2 or THSC complex has multiple roles in nuclear mRNA metabolism, ranging from regulation of transcription to mRNA export (García-Oliver *et al.*, 2012; Stewart, 2019*b*). It consists of the Sac3 (GANP) scaffold, bound to Thp1 (PCID2), Cdc31 (centrin-2/CENP), Sem1 (DSS1) and two copies of Sus1 (ENY1) (Stewart, 2019*b*). In resting cells, TREX-2 is mainly found at the nucleoplasmic site of the NPC, where it interacts (via Sac3) with the export factor Mex67-Mtr2 (NXF1-NXT1) as well as the NUPs Nup1 (NUP153) and, at least in vertebrates, TPR (the metazoan homologue to Mlp1, Fig. 2) (Ullman *et al.*, 1999; Fischer *et al.*, 2002; Soop *et al.*, 2005; Wickramasinghe *et al.*, 2010; Rajanala and Nandicoori, 2012; Umlauf *et al.*, 2013; Jani *et al.*, 2014; Aksenova *et al.*, 2020). The TREX-2 complex is essential for mRNA export (Fischer *et al.*, 2002; Wickramasinghe *et al.*, 2010; Umlauf *et al.*, 2013; Jani *et al.*, 2014). In yeast, TREX-2 also interacts with complexes involved in transcription (Rodríguez-Navarro *et al.*, 2004; García-Oliver *et al.*, 2012; Schneider *et al.*, 2015; García-Molinero *et al.*, 2018) and it has been suggested to play a role in repositioning transcribed genes to the NPC (Jani *et al.*, 2009), a phenomenon called 'gene gating' (Ben-Yishay *et al.*, 2016). TREX-2 may therefore play a major role in connecting mRNA transcription with export, but the detailed function of the complex remains to be explored. Trypanosomes have no obvious orthologues to the core components of the yeast TREX-2/THSC complex, Sac2, Thp1, Sem1 and Sus1.

Mex67-Mtr2 (NXF1-NXT1 or TAP-p15): the major mRNA export factor

The Mex67-Mtr2 heterodimer (NXF1-NXT1 or TAP-p15 in metazoans) is the major mRNA export complex, conserved across most eukaryotes (Segref *et al.*, 1997; Katahira *et al.*, 1999). It binds its mRNA targets directly, or more often indirectly (for example *via* the Yra1/ALYREF/THOC4 subunit of the TREX complex) and then mediates the export of its cargo by interacting with FG NUPs of the NPC. Mex67/NXF1/TAP has five domains: (i) the N-terminal arginine-rich RNA binding domain binds RNA and this activity is essential for RNA export (Zolotukhin *et al.*, 2002; Hautbergue *et al.*, 2008). This domain becomes accessible to RNA by a conformational change of the protein induced by its binding to the TREX complex (Viphakone *et al.*, 2012). (ii and iii) the pseudo RRM (RNA recognition motif) domain and the LRR (leucine-rich repeat) domain of NXF1/TAP are both involved in export mostly by binding splicing factors of the SR (serine–arginine rich) protein family (Huang *et al.*, 2003; Müller-McNicoll *et al.*, 2016). (iv and v) the NTF2L and UBA (ubiquitin associated) domains mediate the interactions between NSF1/TAP and the FG Nups of the NPC, allowing transport (Fribourg *et al.*, 2001). The smaller partner of the complex, Mtr2/NXT1, also has an NTF2-like fold, which binds to the NTF2L domain of Mex67/NXF1. Recent data indicate that the interaction of Mex67/NXF1 with the nuclear pores is independent on mRNA (Ben-Yishay *et al.*, 2019; Derrer *et al.*, 2019) and, at least in yeast, is not even interrupted by the Dbp5 remodelling in the cytoplasm (Derrer *et al.*, 2019). Strikingly, a fusion of Mex67 and Nup116 is sufficient to compensate for Mex67 deletion, indicating that at least the essential function of Mex67 is fully restricted to its nuclear pore localization (Derrer *et al.*, 2019).

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Table 6. Summary of Mex67 interactions partners in trypanosomes, identified by two independent studies

Protein	GeneID	Dostalova *et al.* (2013)	Obado *et al.* (2016)
Mex67 (bait)	Tb927.11.2370	x	x
Mtr2	Tb927.7.5760	x	x
Importin 1 (IMP1)	Tb927.9.13520	x	x
TbNUP149 (NUP76 complex)	Tb927.11.11080		x
TbNUP140 (NUP76 complex)	Tb927.11.11090		x
TbNUP76 (NUP76 complex)	Tb927.8.6250		x
TbNUP158	Tb927.11.980		x
TbNUP152	Tb927.10.9650		x
TbNUP132	Tb927.7.2300		x
TbNUP89	Tb927.11.2950		x
Ran (RTB2)	Tb927.3.1120		x
RanBP1	Tb927.11.3380		x
GAP TBC-RootA	Tb927.10.7680		x

The trypanosome Mex67 protein was identified by homology searches in the trypanosome genome (Schwede *et al.*, 2009). Affinity purification of Mex67 resulted in the identification of the trypanosome Mtr2 homologue, a 15.2 kDa protein with an NTF2 domain that shares higher similarity to the human p15 than to yeast Mtr2 (Dostalova *et al.*, 2013). Trypanosome Mex67/Mtr2 fulfils all characteristics expected of a functional mRNA export complex: (i) Mex67 localizes to the nuclear pores (Kramer *et al.*, 2010; Dean *et al.*, 2017) and Mtr2 to the nucleus (Dostalova *et al.*, 2013; Dean *et al.*, 2017) and possibly to the nuclear pores (Dean *et al.*, 2017). (ii) Depletion of either Mex67 or Mtr2 causes a growth effect and accumulation of polyadenylated mRNAs in the nucleus (Schwede *et al.*, 2009; Dostalova *et al.*, 2013). (iii) In affinity capture experiments Mex67 co-isolates the Nup76 complex (Nup76, Nup140, Nup149) and the NUPs Nup152, Nup158 and Nup89; and, under low stringency conditions, many further NUPs, indicating interactions with the nuclear pore (Obado *et al.*, 2016) (Fig. 2). Uniquely, TbMex67 possess a CCCH type zinc finger at its N-terminus (Kramer *et al.*, 2010; Dostalova *et al.*, 2013) that is essential for its function (Dostalova *et al.*, 2013). It is tempting to speculate, that the CCCH finger mediates mRNA binding of Mex67, perhaps even specific to the miniexon sequence that is present on every mRNA (Dostalova *et al.*, 2013). Consistent with this hypothesis is the absence of a TREX-1 and TREX-2 complex in trypanosomes (see sections 'The TREX complex' and 'The TREX-2 or THSC complex') and the fact that no putative Mex67 adaptor protein co-purified with Mex67 (Dostalova *et al.*, 2013; Obado *et al.*, 2016). Two independent studies have analysed Mex67 interacting proteins (Table 6): The first study was a classical immunoprecipitation using TbMex67 with a C-terminal PTP tag as a bait (Dostalova *et al.*, 2013). This resulted in two equally strong bands on a Coomassie gel that were identified by mass spectrometry as Mtr2 (as expected) and, surprisingly, importin 1 (IMP1): importins transport proteins from the cytoplasm to the nucleus. TbIMP1 has nuclear pore localization (Dean *et al.*, 2017) and its depletion by RNAi is lethal and causes poly(A) accumulation in the nucleus, indicating an important role in mRNA export, perhaps as a transporter of Mex67 (Dostalova *et al.*, 2013). The closest homologue to IMP1 in human is the karyopherin TRN2 (transportin 2). TNR2 has been shown to interact with NXF1 in two independent studies; however, the studies contradict each other: in one study this interaction was RanGTP dependent (Shamsher *et al.*, 2002), in the other it was RanGTP sensitive (Güttinger *et al.*, 2004), indicating a function in protein export or protein import, respectively. To date, a function in protein import is considered more likely (Twyffels *et al.*, 2014). It remains unclear, whether *T. brucei* IMP1 is an importin or exportin and whether it has other targets than Mex67 (TNR2 has many additional cargoes (Güttinger *et al.*, 2004)). Interestingly, upon depletion of IMP1 in trypanosomes, cell fractionation experiments detected a shift of Mex67 from the cytoplasmic to the nuclear fractions (Dostalova *et al.*, 2013), indicative of IMP1 functioning in Mex67 export. In this context, the results from the second Mex67 interaction study (based on cryomilled trypanosomes and mass spectrometry detection of all Mex67 co-purified proteins) are highly interesting: next to Mtr2 and IMP1 and many nuclear pore proteins (Fig. 2) Mex67 also co-purified stoichiometric amounts of the small GTPase Ran and Ran binding proteins (RanBP1 and GAP TBC-RootA) (Obado *et al.*, 2016). It is unlikely that Mex67 binds Ran directly: binding to Ran would probably be *via* the NTF2-like domain of Mex67 (as NTF2 binds to and imports Ran-GDP into the nucleus (Nehrbass and Blobel, 1996; Ribbeck *et al.*, 1998; Smith *et al.*, 1998; Stewart *et al.*, 1998) but the structure of the mammalian TAP/p15 complex shows that the NTF2-like domain is not accessible to Ran (Fribourg *et al.*, 2001) and a high confidence model of the trypanosome complex based on this structure shows the same (Obado *et al.*, 2016). One model consistent with all data would be (i) Inside the nucleus, Mex67-Mtr2 binds its mRNA cargo *via* the zinc-finger domain of Mex67 and it also binds to IMP1-RanGTP. (ii) This complex passes the nuclear pore. (iii) At the cytoplasmic site, Ran hydrolyses the bound GTP aided by GTPase activating protein (GAP) and RanBP1, resulting in disassembly of the complex and release of the mRNA cargo to the cytoplasm. The last step would be analogous to the ATP-dependent remodelling of the RNP export complex by the DEAD box RNA helicase Dbp5 in opisthokonts, potentially compensating for the absence of Dbp5 in trypanosomes (see section 'Nuclear pores and NUPs'). Note that this model is purely speculative and more experimental work is required to determine the exact functions of all Mex67 interacting proteins.

T. brucei Mex67/Mtr2 has additional functions in tRNA export ((Hegedűsová *et al.*, 2019), review in this issue from Zdenek Paris)

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and in ribosome biogenesis (Rink and Williams, 2019; Rink *et al.*, 2019).

mRNA export by the rRNA transporters NMD3 and XPO1?

The proteins Crm1/Xpo1 (exportin 1) and Nmd3 mediate transport of the large ribosomal subunit subunits through the pore: the nuclear export signal containing protein Nmd3 acts as an adaptor to recruit the Crm1/Xpo1 export receptor to the pre-60S subunit, facilitating its export (Johnson *et al.*, 2002; Baßler and Hurt, 2019). Trypanosomes have orthologues for both Nmd3 and Xpo1 (Zeiner *et al.*, 2003*a*; Prohaska and Williams, 2009) and the function in nuclear export of the large ribosomal subunit appears conserved: TbXPO1 depletion causes nuclear accumulation of ribosomal RNAs (Biton *et al.*, 2006), TbNMD3 depletion inhibited processing of the large ribosomal subunit (Droll *et al.*, 2010; Rink *et al.*, 2019) and both XPO1 and NMD3 associate with *T. brucei* 60S ribosomal subunits (Prohaska and Williams, 2009).

Surprisingly, unlike in other systems, trypanosome NMD3 and XPO1 appear also involved in mRNA export (Bühlmann *et al.*, 2015): RNAi depletion of NMD3 caused the poly(A) FISH (fluorescence *in situ* hybridization) signal to shift from being mainly cytoplasmic to being almost entirely nuclear (Bühlmann *et al.*, 2015), exactly like RNAi depletion of MEX67 (Schwede *et al.*, 2009; Dostalova *et al.*, 2013). Moreover, RNAi depletion of either NMD3, XPO1 or MEX67 have identical effects on mRNA levels: there is a minor stabilization of most mRNAs, and a pronounced stabilization of mRNAs encoded by the so-called PAG genes (procyclin-associated genes), short-lived transcripts that are co-transcribed with the very stable and abundant mRNA encoding the cell surface proteins of the procyclic life cycle stage (the stage that resides in the tsetse fly midgut) (Bühlmann *et al.*, 2015). The reason for this massive stabilization of this group of mRNAs upon block in RNA export is not fully understood, but stabilization depends on the mRNAs conserved 5′ UTR and is independent on transcription or translation (Bühlmann *et al.*, 2015). The likeliest explanation is that the block in mRNA export prevents the mRNAs to reach their cytoplasmic destiny of degradation.

Do trypanosomes have two alternative pathways to export mRNAs? MEX67 RNAi is lethal, indicating that the XPO1/NMD3 system cannot compensate for the absence of MEX67. It is therefore more likely that MEX67-Mtr2 and XPO1/NDM3 export pathways interact and depend on each other, in a way that needs to be established.

Nuclear pores and NUPs

Many NUPs of the NPC play active roles in regulating or mediating mRNA export (Ashkenazy-Titelman *et al.*, 2020). In vertebrates, five FG Nups have direct interactions with the C-terminal region of NXT1, namely Nup62 (cytoplasmic), Nup98 (outer ring), Nup153 (nucleoplasmic), Nup214 (cytoplasmic) and Nup358 (cytoplasmic outer ring) (Bachi *et al.*, 2000; Forler *et al.*, 2004); if present, the respective homologues in yeast and trypanosomes are indicated by black asterisks in Fig. 2. At the nuclear basket Nup1 (NUP153 in vertebrates) binds the export competent RNP *via* the TREX-2 complex; in vertebrates TPR (the homologue to yeast Mlp1) contributes to this interaction (Ullman *et al.*, 1999; Soop *et al.*, 2005; Rajanala and Nandicoori, 2012; Umlauf *et al.*, 2013; Jani *et al.*, 2014; Aksenova *et al.*, 2020). In yeast, the basket NUP Mlp1 acts as a gatekeeper to prevent the export of immature mRNAs, in particular of unspliced mRNAs (Green *et al.*, 2003; Galy *et al.*, 2004; Vinciguerra *et al.*, 2005) and this function appears conserved in Metazoans (Coyle *et al.*, 2011; Rajanala and Nandicoori, 2012). In yeast, the Mlp1/Mlp2 interacting protein Pml39 is equally essential for retention of unspliced transcripts and may work as an upstream regulator of Mlp1 (Palancade *et al.*, 2005). These interactions of the RNP with proteins of the nuclear basket dock the export-competent RNP to the pore, in preparation for export. The human β-actin mRNA resides on average 80 ms at the basket (Grünwald and Singer, 2010). Translocation through the export channel is fast (5–20 ms for β-actin (Grünwald and Singer, 2010)) and the contributing mRNA-specific Nups are less well-known, possibly because central channel Nups are structurally too essential to test specific roles. In vertebrates, Nup98, Nup133 and Nup160 have suspected roles in intermediate mRNA export (Powers *et al.*, 1997; Vasu *et al.*, 2001; Blevins *et al.*, 2003) (red asterisks in Fig. 2). The final steps of RNA export at the cytoplasmic filaments (80 ms for β-actin (Grünwald and Singer, 2010)) are better understood. Central is the DEAD-box RNA helicase Dbp5 (DDX19 in vertebrates) that remodels the RNP complex by separating double-stranded RNA regions and RNA−protein interactions, to release export factors, including Mex67 (Lund and Guthrie, 2005; von Moeller *et al.*, 2009; Lin *et al.*, 2018). The ATP dependency of this process ensures directionality of mRNA export. The second key-player in this cytoplasmic remodelling process is the NUP **Gle1** (Murphy and Wente, 1996; Watkins *et al.*, 1998), which is required to activate Dbp5 (Alcázar-Román *et al.*, 2006; Weirich *et al.*, 2006). In yeast, inositol hexakisphosphate as an essential co-activator of Dbp5 (Alcázar-Román *et al.*, 2006; Weirich *et al.*, 2006); whether this small molecule is also needed in vertebrates is still debated (Adams *et al.*, 2017; Lin *et al.*, 2018). Both Dbp5 and Gle1 have direct interactions with NUPs of the cytoplasmic filaments: Dbp5 binds Nup159 (Nup214 in vertebrates) and Gle1 binds Nup42 (hCG1 in human) (Murphy and Wente, 1996; Strahm *et al.*, 1999; Kendirgi *et al.*, 2005; Alcázar-Román *et al.*, 2010).

The architecture of the yeast NPC is well known (Alber *et al.*, 2007*a*, 2007*b*), in subnanometer resolution (Kim *et al.*, 2018) and the structure of the trypanosome NPC was modelled based on homology studies and affinity capture/mass spectrometry interactomic studies (DeGrasse *et al.*, 2008, 2009; Obado *et al.*, 2016, 2017) (Fig. 2). A comparison shows that structure and composition of the NPCs are in principle conserved between yeast and trypanosomes, in particular within the inner ring of the pore (Fig. 2). However, there are some striking differences: (a) Trypanosome nuclear pores are highly symmetrical, with the only exception of the trypanosome-specific proteins NUP110 and NUP92, which are exclusively found at the nuclear basket. In contrast, yeast (and also metazoan) NPCs contain several nuclear pore proteins that specifically localize to either the nuclear basket or the cytoplasmic site of the pore. This asymmetry is crucial for the directionality of mRNP export in opisthokonts (Hurwitz *et al.*, 1998; Schmitt *et al.*, 1999; Folkmann *et al.*, 2011) and it remains unknown, how directionality of transport is achieved in trypanosomes. (b) Among the proteins that are asymmetrically distributed in yeast and absent in trypanosomes are many proteins with important and well-characterized functions in mRNP export, namely Gle1, Dbp5 and Nup159 at the cytoplasmic filaments. Moreover, whether the only trypanosome proteins with asymmetric distribution, NUP92 and NUP110, are orthologues of the Opisthokont Mlp proteins is not certain, as evidence indicates independent ancestry (Holden *et al.*, 2014). Function of NUP92 in chromosome segregation appears conserved, albeit a knock-out is viable and can adapt to normal growth over time (Holden *et al.*, 2014). (c) Of the five FG NUPs that in vertebrates have direct interactions with NXF1, trypanosomes only have two (NUP62 and NUP158) while yeast only lack the metazoan specific protein NUP358 (Fig. 2). (d) In

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Table 7. Exosome and TRAMP complex subunits in trypanosomes and yeast

	Domains	*S. cerevisiae*	Human	*T. brucei*
Exo13	S1 subunit	Csl14	EXOSC1	TbCSL14 (Tb927.5.1200)
	S1/KH	Rrp4	EXOSC2	RRP4 (Tb927.7.4670)
	S1/KH	Rrp40	EXOSC3	RRP40 (Tb927.9.7070)
	RNAse PH	Rrp41	EXOSC4	RRP41A (Tb927.10.7450) RRP41B (Tb927.2.2180) RRP45 (Tb927.6.670) EAP1 (Tb927.1.2580) EAP2 (Tb927.11.16600) EAP4 (Tb927.11.11030) [a]
	RNAse PH	Rrp46	EXOSC5	
	RNAse PH	Mtr3	EXOSC6	
	RNAse PH	Rrp42	EXOSC7	
	RNAse PH	Rrp43	EXOSC8	
	RNAse PH	Rrp45	EXOSC9	
	3′–5′ exonuclease (RNase D)	Rrp6	EXOSC10	RRP6 (Tb927.4.1630)
	3′–5′ exonuclease (RNase II), PIN endonuclease domain	Dis3 (Rrp44)	DIS3, DIS3L	RRP44 (Tb11.02.5380)
	C1D	Lrp1 (Rrp47)	C1D (LRP1)	EAP3 (Tb927.7.5460)
		Mpp6	MPP6	absent ?
TRAMP	RNA helicase	Mtr4	MTREX (MTR4, SKIV2L2)	MTR4 (Tb927.10.7440)
	Poly(A) polymerase	Trf1, Trf5	PAPD5 (TRF4-2)	NPAPL (ncPAP1) (Tb927.8.1090)
	Zn-knuckle	Air1, Air2	ZCCHC7 (AIR1)	? NOP47 (Tb927.11.6620)[b]? RRM1 (Tb927.2.4710)[b]

[a]The six trypanosome RNAse PH subunits cannot be clearly assigned to the yeast orthologues.
[b]It is not yet established, which of these proteins (if any) is the functional orthologue to Air1.

opisthokonts, mRNP export is thriven by ATP hydrolysis that is used by the RNA helicase Dbp5 for remodelling the mRNP complex at the cytoplasmic site of the pore. Trypanosomes have no Dbp5 and the interaction of Mex67 with importin1, Ran, RanBP1 and the corresponding GAP (see section 'Mex67-Mtr2 (NXF1-NXT1 or TAP-p15): the major mRNA export factor') indicates that mRNA export is GTP dependent instead: another fundamental difference between trypanosomes and yeast mRNA export.

The nuclear exosome, the TRAMP complex and the NNS complex

Faulty RNAs and all processing by-products that accumulate in the nucleus are degraded by the nuclear exosome. The core of the eukaryotic RNA exosome is a barrel-shaped structure out of six RNAse PH-like proteins (that are enzymatically inactive) with three S1/KH RNA-binding-domain containing proteins positioned at the top of the barrel (Schmid and Jensen, 2019). RNA degradation activity is provided by the processive 3′–5′ exonuclease and endonuclease Dis3 (also called Rrp44) at the bottom of the barrel and the distributive 3′–5′ exonuclease Rrp6 (EXOSC10 in humans) localized at the top (Schmid and Jensen, 2019). Two further proteins are found at the top of the barrel: Lrp1 (also Rrp47, C1D in human) and Mpp6 (MPP6 in human). This 13-subunit nuclear exosome (also called Exo13, Table 7) is already active, but requires further subunits for efficient and target-specific RNA degradation.

One is the TRAMP complex (Trf4-Air2-Mtr4 polyadenylation), that consists of the RNA helicase Mtr4 (MTR4 (SKIV2L2) in human), the poly(A) polymerase Trf4 (PAPD5 (TRF4-2) in human) and the RNA binding protein Air1 (ZCCHC7 (AIR1) in human) (Schmid and Jensen, 2019) (Table 7). Trf4 is thought to add short A-tails to 3′ ends of exosome targeted RNAs; these tails are believed to facilitate loading of the RNA substrate to Mtr4, which resides at the top of the exosome barrel and probably unwinds the RNA substrate prior to presenting it either to Rrp6 or injecting it into the barrel for degradation by Dis3 (Schmid and Jensen, 2019). The Zn-finger containing Air1 protein likely provides RNA binding activity to the TRAMP complex. The TRAMP complex is engaged in multiple functions, including the decay of highly structured RNAs that would be insensitive to exosomal digestions without the Mtr4 helicase.

Substrate recognition of the TRAMP complex can occur *via* its RNA binding protein Air1, however, RNA polymerase II products are often recognized by the NNS complex (Schmid and Jensen, 2019). In yeast, this complex consists of the RNA binding proteins Nrd1 and Nab3 and the RNA helicase Sen1. Nrd1 and Nab3 have sequence-specific RNA binding domains involved in exosome substrate recognition. Next to its interaction with the exosome, Nrd1 also interacts with serine phosphorylated CTD of RNA polymerase II, linking early transcription with decay (Schmid and Jensen, 2019). One outstanding question is how the exosome distinguishes faulty RNAs from correctly processed RNAs destined for export. The model that currently fits best to the available data is that nuclear RNA degradation is not very selective but rather the default pathway (Schmid and Jensen, 2018; Tudek, 2019). The turn-over rate of nuclear RNAs is in general high ((Wyers *et al.*, 2005; Preker *et al.*, 2008) and RNAs prevented from nuclear export are therefore more likely to be degraded than RNAs that exit fast. For example, in yeast, the spliceosome and the exosome compete for intron-containing RNAs and more than half are degraded instead of spliced (Gudipati *et al.*, 2012). After splicing, cap and poly(A) tail appear to provide a certain protection, but if nuclear export is inhibited these mature transcripts are doomed to degradation too (Tudek *et al.*, 2018). Consistently, long-lived nuclear RNAs (such as snRNAs or

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snoRNAs) require specific protective measures to escape the default RNA decay pathway in the nucleus (Schmid and Jensen, 2018).

Trypanosomes have orthologues to all RNA exosome subunits and most co-precipitate with each other (Estevez *et al.*, 2001, 2003) (Table 7). The lack of co-precipitation of the Rrp44 orthologue questioned whether this subunit is part of the complex (Estevez *et al.*, 2001; Clayton and Estevez, 2010), but given that Rrp44 and Rrp6 have identical, characteristic localization patterns to the nucleoplasm and to the periphery of the nucleolus (Kramer *et al.*, 2016) and both are involved in 5.8S rRNA processing (Estevez *et al.*, 2001) the lack of co-precipitation is likely to reflect a weak interaction rather than none. All evidence points towards trypanosomes having a conserved RNA exosome with mostly or entirely nuclear localization and with mostly conserved and essential function in rRNA processing (Estevez *et al.*, 2001), snoRNA processing (Fadda *et al.*, 2013) and removal of unspliced mRNAs (Kramer *et al.*, 2016). Importantly, all these exosomal functions were concluded from accumulation of the respective RNA species upon depletion of exosome components; thus, whether the exosome specifically targets these RNAs, or, whether these RNAs are degraded because they have an extended exposure time to the exosome is not known. The later model, which is in agreement to the current model in opisthokonts (Schmid and Jensen, 2018; Tudek, 2019), is supported by a simulation of trypanosome mRNA decay pathways that predicts co-transcriptional degradation of mRNA precursors by the exosome: accordingly, mRNA processing and degradation compete and longer mRNAs are more likely degraded than short mRNAs simply because processing time and thus exosomal exposure is longer (Fadda *et al.*, 2014). These data explain the negative correlation between mRNA abundance and mRNA size (Fadda *et al.*, 2014). The model of a rather unspecific exosome is supported by the findings that several short-lived RNA species are stabilized, when nuclear export is inhibited in various ways ((Bühlmann *et al.*, 2015) and see section 'mRNA export by the rRNA transporters NMD3 and XPO1?') and that developmentally regulated mRNAs are enriched in nuclear fractions in the related parasite *T. cruzi* (Pastro *et al.*, 2017): in both cases, mRNA levels appear controlled by transcript-specific cytoplasmic RNA degradation systems rather than by the exosome.

Trypanosomes have nuclear-localized orthologues to at least two of the three subunits of the TRAMP complex, MTR4 and NPAPL (also called ncPAP1) and both are essential for growth (Cristodero and Clayton, 2007; Etheridge *et al.*, 2009) (Table 7). MTR4 and ncPAP1 can be co-isolated together from trypanosome extracts using either protein as a bait, and both respective purified complexes exhibit PAP activity (Etheridge *et al.*, 2009). MTR4 is involved in 5.8S rRNA processing and controls RNA quality by a process that involves polyadenylation (Cristodero and Clayton, 2007). Three further proteins with nuclear localization were co-purified with ncPAP1 (Etheridge *et al.*, 2009). Two have zinc-knuckles and could theoretically be Air1 orthologues: the nucleolar protein NOP47 and the SR protein RRM1 (Table 7). However, neither is the closest homologue to yeast Air1, NOP47 associates with the spindle during mitosis (Zhou *et al.*, 2018) and HA-RRM1 does not co-purify MTR4 or ncPAP1, at least not in amounts resulting in detectable bands on a Coomassie gel (Naguleswaran *et al.*, 2015); whether either is the functional orthologue to Air1 remains to be investigated. Interestingly, the third protein co-purified with ncPAP1 is PUF10, a Pumilio domain protein with a function in 5.8S rRNA processing (Schumann Burkard *et al.*, 2013) and perhaps a trypanosome-specific TRAMP complex subunit. No exosome subunits were co-purified with ncPAP1 (Etheridge *et al.*, 2009). Thus, trypanosomes are likely to have a TRAMP-like complex that awaits further characterization. Trypanosomes have no homologues to the proteins of the NNS complex.

Co-transcriptional initiation of RNA export indicates the lack of major mRNA export checkpoints in trypanosomes

The major differences in nuclear mRNA metabolism between trypanosomes and opisthokonts detailed above, in particular the absence of many factors involved in mRNA export control raise the question, whether and how trypanosomes regulate mRNA export. With only two introns present in trypanosomes, the main question is how and whether trypanosomes prevent the export of polycistronic mRNA precursors that have not undergone trans-splicing and polyadenylation. It is established that mRNA export control is at least not tight in trypanosomes: Polycistronic mRNAs were detected in the cytoplasm by fractionations (Jäger *et al.*, 2007; Kramer *et al.*, 2012) and also by single molecule RNA FISH: ¼ of all tubulin dicistronic RNAs were in the cytoplasm (Goos *et al.*, 2019).

We have recently studied nuclear export in trypanosomes using three-colour intramolecular single molecule fluorescence *in situ* hybridization (smFISH) (Goos *et al.*, 2019). For this, a large endogenous mRNA (*FUTSCH*, >22 000 nts long) is stained in three colours by hybridization with three nucleotide probe-sets: red at the 5′ end, infrared (pink false-colour) in the middle part, and green at the 3′ end (Fig. 3A). This allows the simultaneous detection and classification of multiple mRNA metabolism intermediates, based on colour combinations (Kramer, 2017; Goos *et al.*, 2019). Using this approach, we observed mRNAs with their 5′ end (red dot) already in the cytoplasm, the middle part (infrared dot) still in the nucleus, and no 3′ end (no green dot) (Goos *et al.*, 2019) (Fig. 3B), suggestive of co-transcriptional nuclear export. Further experiments (using orthogonal methods and different mRNAs) confirmed the presence of co-transcriptional mRNA export in trypanosomes (Goos *et al.*, 2019). Importantly, not all mRNAs are exported co-transcriptionally: only about half of the very long transcripts leave the nucleus while still in transcription. Instead, what the data show is that trypanosomes lack a quality control checkpoint that prevents unprocessed mRNAs from starting export. Given that long mRNAs have longer transcription and processing times than short mRNAs, they are more likely to reach the pore while still in transcription. Average-sized mRNAs, in contrast, have fast processing times and, in addition, are probably too short to reach the pore from their site of transcription. The data do not exclude the presence of a checkpoint that prevents the completion of mRNA export, if these are unprocessed, for example by recognizing the absence of a poly(A) tail and/or associated factors.

To investigate any possible mRNA quality control mechanism further, we have massively increased the amount of polycistronic mRNAs by inhibiting *trans*-splicing. This can be done equally well in two independent ways, either using sinefungin (a drug that inhibits cap methylation (McNally and Agabian, 1992)) or by transfecting a morpholino antisense to the U2 snRNA (Matter and König, 2005; Kramer *et al.*, 2012). When *trans*-splicing is blocked, we observed a large proportion of polycistronic tubulin mRNAs in the cytoplasm (Goos *et al.*, 2019), confirming the absence of a rigid mRNA export control machinery. To our surprise, inhibition of *trans*-splicing also correlated with many RNA binding proteins localizing to granular structures at the outside of the nuclear pores and we named these granules NPGs (nuclear pore granules) (Kramer *et al.*, 2012; Goos *et al.*, 2019) (Fig. 3C). NPG-like structures were not observed after inhibition of splicing in HeLa cells or inhibition of *trans*-splicing in *C. elegans* (Kramer *et al.*, 2012; Goos *et al.*, 2019), indicating that they may be unique to trypanosomes. We determined the proteome of purified NPGs and found that the granules contain

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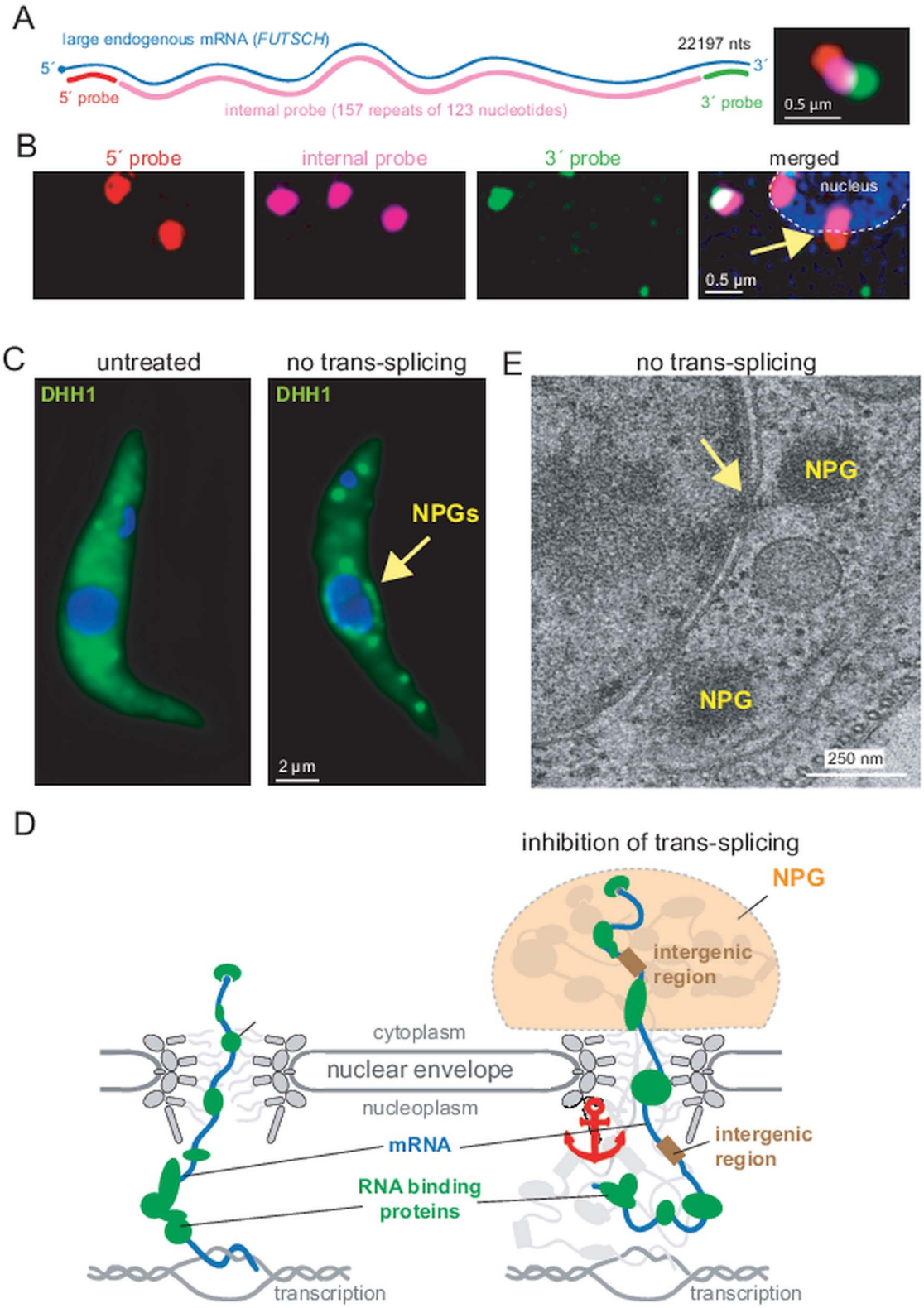


Fig. 3. Trypanosomes can initiate mRNA export co-transcriptionally. (A) Principle of three-colour intramolecular single molecule FISH: a large transcript is simultaneously probed with smFISH probe sets in three different colours, covering the 5′ end, the middle part and the 3′ end. A rare example of an mRNA with all three colours visible as separate dots is shown on the right. (B) Detection of co-transcriptional mRNA export in trypanosomes by three-colour intramolecular single molecule FISH: the mRNA 5′ end (red) is already in the cytoplasm, while the middle part (pink false colour) is still in the nucleus and the 3′ end (green colour) has not yet been transcribed. (C) Trypanosome cells that express DHH1-eYFP as a marker for RNA granules are shown untreated (left) and treated with sinefungin to inhibit trans-splicing (right). DHH1 localizes to nuclear periphery granules (NPGs) at the outside of the nucleus (yellow arrow). (D) Model of co-transcriptional mRNA export in untreated cells (left) and in cells treated with sinefungin to inhibit *trans*-splicing (right). In both conditions, transcripts leave the nucleus while still in transcription. When *trans*-splicing is inhibited, progress of export is slowed or prevented by an unknown mechanism (anchor), resulting in the formation of granules at the cytoplasmic site of the nuclear pore. These granules contain unspliced transcripts stuck in export as well as cytoplasmic RNA binding proteins. (E) NPGs are visible by transmission electron microscopy as dense areas, connected to the nuclear pores *via* electron dense fibre-like structures (yellow arrow). All microscopy images of this figure are taken from Goos *et al.* (2019).

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the full set of cytoplasmic RNA binding proteins (Goos *et al.*, 2019). Proteins involved in nuclear mRNA processing steps, such as splicing (Lsm5, SmE), capping (CGM1) and export (XPO1, MEX67, NUP96, RANBP1) were absent (Kramer *et al.*, 2012). Also, most translation initiation factors were absent, with the exception of some isoforms of the eIF4F complex (eIF4E3, eIF4E1 and possibly eIF4E5, eIF4G1 and eIF4G2) (Goos *et al.*, 2019), consistent with the granules being insensitive to translational inhibitors (Kramer *et al.*, 2012). Moreover, we could detect polycistronic mRNAs in these granules by smFISH (Goos *et al.*, 2019). The easiest explanation consistent with all data is that these granules are newly exported 5′ ends of polycistrons, bound to their natural set of RNA binding proteins that have not yet started translation (Fig. 3D). At least part of the polycistron is still stuck inside the pore and possibly extending into the nucleus: in electron microscopy images an electron-dense string-like structure is often visible that connects the pore with the NPGs (Fig. 3E). The major, still unanswered question is: why is this structure visible? Perhaps, export is somewhat slowed and otherwise transient structures of export accumulate. Export could be slowed because large polycistrons physically block the pores, perhaps because some remain attached to the transcription site and fail to exit completely. Alternatively, a quality control checkpoint could act at the nuclear basket that recognizes an mRNA as not fully processed, slowing or preventing export. Interestingly, while most cytoplasmic RNA binding proteins relocalize to NPGs upon inhibition of *trans*-splicing, we found four cytoplasmic RNA binding proteins that relocalized fully or partially to the nucleus. Most (ZFP1, ZFP2, ZC3H29) are CCCH-type zinc-finger proteins and most (ZFP1, ZFP2, Tb927.11.6600) function in trypanosome life cycle regulation (Hendriks *et al.*, 2001; Hendriks and Matthews, 2005; Paterou *et al.*, 2006; Mony *et al.*, 2014). Importantly, this relocalization was not detected when transcription was blocked by actinomycin D, indicating that it is not the absence of mature mRNA, but rather the presence of polycistronic RNA that causes relocalization. One further protein, XPO-5, moved from the nucleoplasm to the nuclear pores upon inhibition of *trans*-splicing. The function of this putative transporter protein is unknown and it is not essential in procyclic cells (Hegedűsová *et al.*, 2019). Whether either of these five proteins acts in RNA export control remains to be investigated.

Summary and outlook

A fully processed trypanosome mRNA bears no major differences to an mRNA from opisthokonts and mRNA processing appears conserved in its mains features. However, with the exception of Mex67-Mtr2, all complexes and proteins involved in regulating mRNA export in opisthokonts are either absent (TREX, TREX-2, RES, DBP5, probably Mlp1-2) or have no reported functions in mRNA export (non-classical CTD, SR proteins except perhaps RRM1, EJC, TRAMP complex). Moreover, trypanosomes evolved several unique complexes and pathways. For example, mRNA export in trypanosomes is likely thriven by GTP using the RanGTP system instead of ATP and it may also use the XPO1-NMD pathway in addition or together with Mex67-Mtr2. The missing mRNA export control elements in trypanosomes may explain the leakage of unspliced mRNAs into the cytoplasm and the fact that export can start co-transcriptionally, rather than being dependent on the completion of all processing steps. In the near absence of introns, a leakage of unspliced (usually dicistronic) mRNAs may be tolerable to the parasite, with the worst damage being a misregulation in gene expression, but no production of faulty proteins. To keep the leakage of unprocessed mRNAs to a sufficiently low level, it may be sufficient to ensure fast, efficient and mostly co-transcriptional mRNA processing, perhaps supported by preferential cytoplasmic degradation of faulty mRNAs (the latter has not been shown).

A comparison of RNA export pathways throughout the tree of life came to the conclusion that RanGTP-dependent RNA export pathways (exporting rRNA, tRNA and snRNA) are relatively well conserved, while the RanGTP-independent export pathway of mRNA is not (Serpeloni *et al.*, 2011*b*). The Apicomplexa *Toxoplasma gondii*, for example, also lacks the TREX complex with the exception of the Sub2 helicase, has no Mex67 (albeit an unrelated C2H2 zinc-finger protein may act as a functional orthologue) and whether the mRNA export is RanGTP dependent is not certain (albeit a Dbp5 homologue is present in the genome) (Avila *et al.*, 2018). Plants have a TREX complex and a TREX-2 complex with some plant-specific adaptations and also a Dbp5 homologue, but homologues to Mex67 are absent (Ehrnsberger *et al.*, 2019). It is likely that the highly conserved RanGTP-dependent transport system was the export system that has evolved first and was originally used for all RNA and protein transport processes. Later, export systems became more specialized to serve the specific needs of the eukaryotes. Trypanosomes may have experienced little pressure to evolve a sophisticated mRNA export control system and it will be highly interesting to investigate mRNA export in other protozoa with mostly intron-less transcripts.

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