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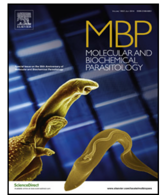

The interaction of a *Trypanosoma brucei* KH-domain protein with a ribonuclease is implicated in ribosome processing

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ABSTRACT

Ribosomal RNA maturation is best understood in yeast. While substantial efforts have been made to explore parts of these essential pathways in animals, the similarities and uniquenesses of rRNA maturation factors in non-Opisthokonts remain largely unexplored. Eukaryotic ribosome synthesis requires the coordinated activities of hundreds of Assembly Factors (AFs) that transiently associate with pre-ribosomes, many of which are essential. Pno1 and Nob1 are two of six AFs that are required for the cytoplasmic maturation of the 20S pre-rRNA to 18S rRNA in yeast where it has been almost exclusively analyzed. Specifically, Nob1 ribonucleolytic activity generates the mature 3′-end of 18S rRNA. We identified putative Pno1 and Nob1 homologues in the protist *Trypanosoma brucei*, named *Tb*PNO1 and *Tb*NOB1, and set out to explore their rRNA maturation role further as they are both essential for normal growth. *Tb*PNO1 is a nuclear protein with limited cytosolic localization relative to its yeast homologue. Like in yeast, it interacts directly with *Tb*NOB1, with indications of associations with a larger AF-containing complex. Interestingly, in the absence of *Tb*PNO1, *Tb*NOB1 exhibits non-specific degradation activity on RNA substrates, and its cleavage activity becomes specific only in the presence of *Tb*PNO1, suggesting that *Tb*PNO1-*Tb*NOB1 interaction is essential for regulation and site-specificity of *Tb*NOB1 activity. These results highlight a conserved role of the *Tb*PNO1-*Tb*NOB1 complex in 18S rRNA maturation across eukaryotes; yet reveal a novel role of their interaction in regulation of *Tb*NOB1 enzymatic activity.
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1. Introduction

Eukaryotic ribosomal biogenesis is an essential and highly conserved process, studied most extensively in yeast and less so in human, mouse and Xenopus [1–4]. In eukaryotic cells, three of the mature rRNA species, 18S, 5.8S and 25–28S rRNA, are co-transcribed as a polycistronic transcript, which matures through a series of endo- and exonucleolytic processing steps [5]. Large ribonucleoprotein processing complexes assemble while rRNA sequences are modified, external and internal transcribed spacer sequences (ETS and ITS, respectively) are removed, and mature rRNAs are assembled into ribosomal subunits [3,6]. The mature rRNA sequences undergo extensive covalent nucleotide modification during this process, guided by small nucleolar RNAs [7]. Concurrently, non-ribosomal proteins called Assembly Factors (AFs) associate transiently with assembling ribosomes to facilitate the processing and folding of rRNA to ensure the sequential recruitment of ribosomal proteins for accurate ribosome assembly [6,8,9]. The 60S and 40S pre-ribosomal particles assemble in the nucleolus, and are exported to the cytoplasm where they mature. Both mature subunits are comprised of many ribosomal proteins, with the large 60S subunit (LSU) containing the 25/28S, 5.8S and 5S rRNA, and the small 40S subunit (SSU) containing our focus, the 18S rRNA [6].

The protozoan parasite *Trypanosoma brucei*, responsible for African sleeping sickness in humans and Nagana in cattle, is positioned, in eukaryotic molecular phylogenetic maps, far from organisms from which our core knowledge of biological processes derives [10,11]. Its RNA metabolism involves exclusive features such as extensive mitochondrial transcript editing, and coupled trans-splicing and polyadenylation of cytosolic mRNAs [12,13].

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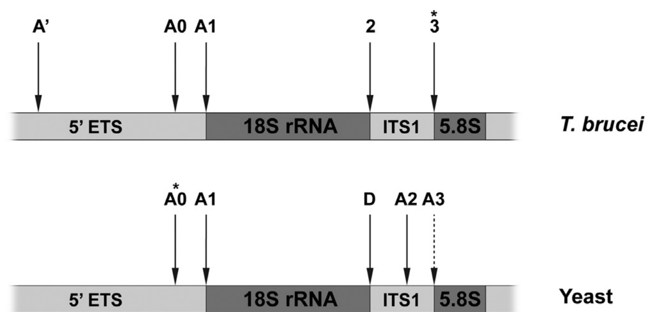

Fig. 1. A schematic representation of partial *T. brucei* and yeast pre-rRNA sequences (not drawn to scale), indicating the cleavages that lead to 18S rRNA production. ETS-External transcribed spacer, ITS-Internal transcribed spacer. * indicates the site where the first cleavage event occurs. (Adapted from [44]).

Likewise, it has distinctive features of rRNA processing, such as fragmentation of the 28S rRNA into six stable fragments [14,15], highly diverged processing enzymes [16], and an 18S rRNA that is the largest known [17,18].

The first cleavage event during the processing of primary rRNA transcripts also exhibits differences. In yeast and metazoans rRNA processing commences by cleavages within the 5′ or 3′-ETS sequences [2,4]. For instance, 18S pre-rRNA processing in yeast is initiated by cleavage at site-A0 within the 5′-ETS sequences, followed by A1 to generate the mature 5′-end of 18S rRNA and then A2 within ITS1 to separate the 18S rRNA from the 5.8S/LSU precursor rRNA (Fig. 1, bottom) [2,5]. Initial 5′ ETS cleavages further upstream have been observed in vertebrates and other organisms [4,14], yet are notably absent from yeast pre-rRNAs [1].

The process is reversed in *T. brucei,* where the earliest cleavage event occurs at site 3 within the first ITS, ITS1, to separate the 18S SSU precursor from the 5.8S/LSU precursor (Fig. 1, top) [18,19]. The *T. brucei* 5′ ETS is then removed from pre-18S rRNA by subsequent cleavages at two 5′ ETS sites, A' and A0 (A0 being the initial cleavage site in yeast, as indicated). As in yeast, cleavage at A1 forms the mature 5′-end of *T. brucei* 18S rRNA. [18,19]. In all species, these steps occur in the nucleolus.

The final step of 18S rRNA maturation takes place after export of the 40S precursor to the cytoplasm, where the cleavage at site D (similar or identical to site 2 in *T. brucei*) [18,19] generates the mature 3′-end of 18S rRNA. In organisms investigated to date, this cleavage step is carried out by the nuclease Nob1, with the overall structure and function of 3′ end maturation of the small subunit rRNA apparently universally conserved [20–25]. Nob1 is one of the seven AFs that remain bound to late cytoplasmic pre-40S ribosomes in yeast, the other six being Pno1, Dim1, Enp1, Tsr1, Rio2 and Ltv1 [26]; other sources suggest that late-stage associations with AFs Ppr43 and Rio1 are a possibility [24,27]. All these AFs, with the exception of Ltv1 are essential, and their deletion stalls cytoplasmic maturation of 18S rRNA [28–33]. Nob1 has been shown to directly interact with the RNA-binding AF Pno1 (also called Dim2). Nob1-Pno1 interaction is required for optimal yeast ribosome biogenesis, playing a role in cleavage at the 3′-end of 18S rRNA (site D) [34].

The question we address in this work is whether in *T. brucei* divergent processing within ITS1 in the nucleus is mirrored by differences in the essential cleavage to generate the mature 18S 3′ end in the cytosol. Or instead, is this final cytosolic cleavage event, including its enzymology, consistent with what is observed in other eukaryotes and even archaea? We have identified *T. brucei* homologues of yeast proteins crucial for cytosolic 3′ processing of 18S rRNA. Here we report the characterization of Nob1 and Pno1 homologues in *T. brucei*, *Tb*NOB1 and *Tb*PNO1, which function in many ways remarkably similarly to their yeast counterparts. Silencing of either protein in the *T. brucei* insect life stage (procyclic form; PF) results in slowed growth, and reduced cleavage activity at sites 2, 3 and A1 of the 18S rRNA precursor. *Tb*PNO1 associates with four other putative pre-40S ribosome AFs. The majority of *Tb*PNO1 is nuclear with limited cytosolic localization; and its expression patterns exhibit differences from that of yeast. We show that *Tb*NOB1 and *Tb*PNO1 directly interact, and most interestingly, demonstrate likely *in vitro* site 2-specific endonuclease activity of *Tb*NOB1 only in the presence of *Tb*PNO1. Thus we predict that at least in trypanosomes, *Tb*NOB1 requires its partnering AF *Tb*PNO1 in order to execute specific 18S rRNA 3′ end cleavage.

2. Materials and methods

2.1. Plasmid constructs

2.1.1. RNAi silencing vectors

The plasmid expressing tetracycline (tet)-inducible RNAi for *Tb*PNO1 was constructed by cloning a 460-bp fragment of Tb927.9.11840 from *T. brucei* PF 29-13 genomic DNA using the primers 5′-ATGACTCGAGAGCAACGGTAATGACGAACC-3′ and 5′-GTCGAAGCTTTGTGCGTATCTGCAATGACA-3′. Product was digested with XhoI and HindIII (sites underlined), and inserted into similarly digested pZJM (Wang et al., 2000) to create pZJM-*Tb*PNO1. pZJM-*Tb*NOB1 was constructed in a similar manner, except that the 599-bp fragment of Tb927.11.10860 was amplified using primers 5′-AGATCTCGAGCAACGCACCTCCATGTATTG-3′ and 5′-CACGAAGCTTATTACCGCTACCGCATTCAC-3′, digested with HindIII only, and inserted into pZJM by one sticky end-one blunt end ligation.

2.1.2. Expression constructs

To generate C-terminally TAP tagged *Tb*PNO1 for exogenous expression in *T. brucei* (expression from extra copy of gene introduced in rRNA locus), full-length *Tb*PNO1 was amplified with primers 5′- AGTCCAAGCTTATGCTCTCAGCAGCT-3′ and 5′- GAGCTGGATCCAAAGGTATCGTTAAC-3′. After digestion with HindIII and BamHI, the insert was cloned into similarly digested pLEW79-TAP vector (Panigrahi 2003).

To generate N-terminal 6X His-tagged *Tb*PNO1 construct for both cell free and recombinant expression in *E. coli*, full-length *Tb*PNO1 was amplified with primers 5′- TCAGAAGATCTGATGCTCTCAGCAG-3′ and 5′-ACAGCCTCGAGTTAAAAGGTATCG-3′, digested with BglII and XhoI, and ligated into similarly digested pET30a (Novagen). To generate N-terminal 6X His + Xpress-tagged *Tb*NOB1, full length *Tb*NOB1 was amplified with primers 5′-ATGTAGGATCCATATGTCGTGGGCTGCA-3′ and 5′-ATCTAGAATTCTATCACTTCCGTCGCGC-3′, digested with BamHI and EcoRI, and ligation into similarly digested pRSET-C (Invitrogen).

2.2. Cell culture and manipulations

The *T. brucei* PF 29-13 cell line [35] was transfected with 10 μg of NotI-linearized plasmid. Stable cell lines were selected by growth in SDM-79 medium containing 10% fetal bovine serum, 15 μg/ml of G418, and 25 μg/ml of hygromycin, with 2.5 μg/ml of phleomycin as the selective agent for both RNAi and tagged protein expression cell lines. RNAi silencing was induced with 1 μg/ml tet, and the uninduced and induced cells were counted daily to obtain growth curves. The cells were maintained between 1.0×10^6 and 2.5×10^7 cells/ml throughout the course of RNAi induction.

2.3. Purification of TAP-tagged *Tb*PNO1 and mass spectrometry

Expression of the TAP-tagged *Tb*PNO1 protein was induced by adding 100 ng/ml of tet to the culture at a density of 1.2×10^6

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