1 The nuclear RNA binding protein RBP33 influences mRNA and spliced leader RNA
2 abundance in *Trypanosomabrucei*
3
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17 RNA recognition motif (RRM) containing proteins are important regulators of gene
18 expression in trypanosomes. Here we expand our current knowledge on the exclusively
19 nuclear localized RRM domain containing protein RBP33 of *Trypanosomabrucei*.
20 Overexpression of RBP33 leads to a quick growth arrest in G2/M in bloodstream form cells
21 likely due to an overall mRNA- and spliced leader abundance decrease while the ribosomal
22 RNAs remain unaffected. The recombinant RBP33 binds to poly(A) and random sequence
23 RNA *in vitro* confirming its role as a RNA binding protein. Finally super-resolution
24 microscopy detects RBP33 in small punctaethroughout the nucleus and surrounding the
25 nucleolus, however the signal is depleted inside the nucleolus.
26

27 Introduction
28

1 *Trypanosomabrucei* is the causative agent of human African trypanosomiasis and nagana in
2 cattle. The protozoan parasite employs a digenetic life cycle alternating between the insect
3 vector, a fly of the genus *Glossina* (the tsetse fly) and a mammalian host. In preparation for,
4 and as a response to these very different environments the parasite undergoes dramatic
5 changes in morphology and metabolism, which are controlled at the transcriptome and
6 proteome level (for review of the transcriptome studies see [1]; [2-4]). The rather unusual
7 genome organization in trypanosomes into large polycistronic transcription units requires the
8 proper processing of the individual transcript units by trans-splicing/polyadenylation and the
9 extensive control of the mRNA levels at the post-transcriptional level to compensate for the
10 lack of transcriptional control [5]. Both processes require RNA binding proteins (RBPs) such
11 as the RNA recognition motif (RRM) containing RBPs, which belong to the most abundant
12 RNA binding motives in biology. The basic RRM topology forms a four-stranded β -sheet
13 packed against two α -helices, where the β -sheets form the primary RNA binding surface [6].
14 There are about 70 different RRM containing proteins in trypanosomes but only few of these
15 have been characterized (reviewed in [7]). Currently two RRM containing RBPs are
16 implicated in the differentiation of the parasite: (i) overexpression of RBP6, which
17 contains two RRMs, has the remarkable ability to trigger differentiation of insect specific
18 procyclic trypomastigotes all the way to the mammalian infective metacyclic form. However
19 at this point neither the localization of the protein nor its targets are known [8], while (ii)
20 RBP10, a protein with one RRM, localizes to the cytoplasm and seems to promote the
21 expression of bloodstream form (BSF) specific transcripts [9]. Additionally a number of
22 RBPs including several Pumilo domain proteins (PUF7, PUF10, BOP1, NRG1) and zinc
23 finger proteins (ZFP1, ZC3H20, ZC3H11) are either life stage specifically regulated and/or
24 bind to stage specifically regulated transcripts (reviewed in [7]). RBP7, also a zinc finger
25 domain containing RBP was recently shown to be required for proper quorum sensing and
26 differentiation competence of the BSF parasite [10]. Of the remaining RRM containing RBPs
27 that have been characterized, DRBD3, alias PTB1, and hnRNP/H have been implicated in
28 splicing as well as mRNA stability. hnRNP/H is a mostly nuclear localized protein with

1 three RRM motifs that differentially affect the transcriptome of BSF and procyclic form (PCF)
2 cells [11]. PTB1 (two RRM motifs), on the other hand, is mostly localized to the cytoplasm and
3 influences the stability of some transport/metabolism related transcripts through binding to
4 the 3' UTR[12,13]. The poly(A) binding proteins (PABP1/2) both contain 4 RRM motifs and
5 associate with polysomes. Under stress conditions PABP2 but not PABP1 re-localizes to the
6 nucleus[14]. The small RBPs UBP1/2 both harbor only one RRM motif and are localized to the
7 cytoplasm. Overexpression but not RNAi knockdown of UBP1/2 cause changes in the
8 transcriptome[15]. RBP42 is essential in PCF and localizes to the cytoplasm where it
9 associates with polysomes. RNA sequencing studies using CLIPseq have demonstrated
10 specific binding of RBP42 to transcripts involved in energy metabolism. Recently a new RRM
11 domain containing protein (RBP33) was described to be nuclear localized in BSF and PCF
12 parasites[16]. Immunoprecipitation experiments coupled to RNA sequencing demonstrated that
13 RBP33 associates with more than 400 different transcripts including its own mRNA. RNAi
14 knockdown of RBP33 showed a strong growth phenotype and led to increased expression of
15 RNAs from regions that are otherwise silenced including retroposon and repeat sequences.
16 Based on these results Fernández-Moya and coworkers suggest RBP33 to be involved in
17 controlling expression of these non-coding transcripts. RBP33 (Tb927.8.990; 33.9 kDa)
18 contains one predicted RRM motif in the N-terminal region [16] and resolves as a 37 kDa
19 protein on SDS PAGE (Figure 1A). Recombinantly expressed RBP33 is detected as a
20 monomer and homodimer on native gel electrophoresis (Figure 1B). The native RBP33 is also
21 detected as a higher molecular weight complex in PCF cells (Figure 1B) consistent with the
22 multimerization seen in the recombinant protein and its potential to bind RNA (sample not
23 RNase treated). If other proteins are present in this complex is currently unknown. It is
24 interesting to note that the related human RNA binding protein hnRNP A1 and all proteins of
25 the RRM1 family contain two RNA binding domains, while RBP33 only contains one and
26 thus might require dimerization for function. RBP33 is equally expressed in BSF (NYSm) and
27 PCF (Antat1.1) trypanosomes as detected by western blotting and shown previously (Figure
28 1A and [16]). Immunofluorescence using a polyclonal RBP33 antibody (1:1000) and super-

1 resolution microscopy (STED) localized the protein in about 60 (median; n=20) distinct foci
 2 with a diameter of 50-150 nm in BSF and PCF parasites irrespective of the cell cycle stage
 3 (Figure 1C). Absence of the RBP signal in the nucleolus could be observed. To quantify this,
 4 the microscopic image slice with the most pronounced lack of DAPI staining indicating the
 5 nucleolus[17] was selected and a Gaussian blur filter was applied in order to emphasize the
 6 RBP33 signal. From the DAPI signal, a binary mask was created using an intensity threshold.
 7 The enclosed negative space was used to define the nucleolus. The nucleolus was subdivided
 8 into an inner and outer region; each containing 50% of the area (Figure 1D). Within each
 9 defined region, the mean intensity of the RBP33 signal was measured (Figure 1E) showing a
 10 significant decrease of the RBP33 signal in the inner nucleolus region (p-value <
 11 0.0001). Currently, RBP33 and RRM1 are the only known RRM domain containing protein to
 12 exclusively localize to the nucleus under normal growth conditions [18]. Recently published
 13 results demonstrated that RNAi against RBP33 mRNA is lethal and leads to an increase in Pol
 14 II transcripts from strand-switch regions, retroposons and repeat sequences [16].
 15 Overexpression of RBP33 in bloodstream form parasites (Figure 2A, B) led to growth arrest
 16 eight hours post induction, but not to cell death (Figure S2A, B). The nuclear localisation of
 17 the protein did not change during overexpression (Figure S1). Based on flow cytometry
 18 (FACS-Calibur, Becton Dickinson) using propidium iodide stained cells a large fraction of the
 19 population accumulated in G2/M (12-24h) suggesting a block prior to cytokinesis (Figure
 20 S2C). This was also supported by fluorescence microscopy analysis of the karyotype using
 21 DAPI to stain the nucleus and kinetoplast (Figure S2D). Twelve hours post overexpression of
 22 RBP33 the number of cells in G1 that contain one kDNA (k) and one nucleus (n; 1k1n),
 23 decreased from 80% to 55%, while 2k2n cells, which are in the post mitotic phase of the cell
 24 cycle, increased from 4% to 23%. The overall phenotype rapidly reversed leading to a
 25 normally growing population 36 hours post induction of RBP33 overexpression, at which
 26 point RBP33 mRNA and protein abundance were back to normal levels, despite the
 27 continuous induction with tetracyclin. Based on the previous observation that RBP33 binds to
 28 its own mRNA by Fernández-Moya and coworkers we speculate that the protein is able to

1 destabilize its own mRNA thus leading to a normal expression level after 36 hours of
2 overexpression. To investigate whether RBP33 has a general effect on mRNA abundance we
3 analyzed total RNA from different time points during the overexpression by northern blotting
4 using the spliced leader as a probe (Figure 2C). Overexpression of RBP33 led to a decrease of
5 total mRNA and the spliced leader RNA when normalized to 18S rRNA (Figure 2C, D). The
6 decrease in mRNA and SL RNA abundance was followed by a recovery towards normal levels
7 of expression 36 hours post induction of RBP33 overexpression. Based on the observation that
8 overexpression of RBP33 changed the overall mRNA abundance we tested the ability of the
9 recombinant RBP33 to bind poly(A) RNA oligonucleotides applying an electrophoretic
10 mobility shift assay (EMSA; Figure 2E-G). Biotin labeled poly(A) RNA was shifted such that
11 three distinct complexes with decreased electrophoretic mobility in the native gel were
12 detected by northern blotting (Figure 2E). The addition of the anti-RBP33 antibody to the
13 RNA/protein mixture led to a supershift of the RBP33/poly(A) RNA complexes (Figure 2E).
14 On the other hand, the addition of excess non-labeled poly(A) RNA led to the decrease of
15 some of the higher molecular weight complexes, however no complete competition of the
16 labeled RNA was observed. The specificity of the RBP33 induced RNA shift could be
17 demonstrated by probing for RBP33 with and without poly(A) RNA (Figure 2F). For this we
18 incubated RBP33 with or without poly(A) RNA, subsequently resolved the mixture on a
19 native gel and probed for RBP33 by western blotting (Figure 2F). Without the addition of
20 poly(A) RNA RBP33 resolved as a monomer and apparent homodimer while the addition of
21 poly(A) RNA to RBP33 led to a shift similar to what was seen on the northern blot (Figure
22 2F). Supershift with the anti-RBP33 antibody added to the RBP33/poly(A) RNA mixture was
23 observed by western blotting as well as by northern blotting (Figure 2E, F). We also tested if
24 RBP33 was able to bind RNA in general. Using an RNA oligonucleotide with a randomized
25 sequence, we repeated the EMSA experiment (Figure 2G) and observed (by northern blotting)
26 effects similar to those described above for the poly(A) RNA oligonucleotide indicating that
27 under our conditions RBP33 has little specificity in binding to RNA substrates.

1 From the study presented here and the previously published work[16], the following picture
 2 of RBP33 emerges; It is exclusive localized in about 60 punctae in the nucleus outside the
 3 nucleolus. RBP33 is present in equal amounts in PCF and BSF cells throughout the cell cycle.
 4 The protein binds mRNAs *in vivo*, including its own mRNA, and short RNA oligonucleotides
 5 *in vitro*. Its overexpression and depletion both suggest that RBP33 is involved in decreasing
 6 the steady state levels of RNAs; the rapid onset of the RNAi and overexpression phenotypes
 7 together with the lack of stage specific regulation in BSF and PCF parasites suggest that
 8 RBP33 is likely involved in a central process of RNA biogenesis.

9 References

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Figure legends

Figure 1: RBP33 in PCF and BSF.

(A) Western blot of recombinant RBP33 tagged to the maltose binding protein (MBP-RBP33) and total cell extracts from bloodstream form (BSF) and procyclic form (PCF) trypanosomes separated on SDS PAGE. Blots were decorated with polyclonal rabbit anti-RBP33 antibodies produced against recombinant RBP33 in this study. Equal cell numbers (5×10^6) of BSF and PCF were loaded. (B) Western blot from purified recombinant MBP-RBP33 (100 ng of MBP-RBP33) and PCF total cell extract (1×10^6 cells, not RNase treated) resolved on clear native gel (4%). Blot was decorated with antibodies against RBP33 and a core component of the mitochondrial import machinery ATOM. (C) Immunofluorescence localization of RBP33 (red). RBP33 is located in the nucleus (cyan) during all cell cycle stages in PCF and BSF (maximum z-projection are shown). The nucleus and kDNA is stained with DAPI. RBP33 builds a granular structure in the nucleus, whereas the nucleolus is less stained. The upper panel shows the enlarged nucleus of the second panel (white box). The images of the last panel were acquired by super-resolution microscopy. For super-resolution microscopy goat anti-rabbit IgG Oregon green antibody (1:100, Invitrogen) was used. The nuclei were visualized with Draq5 (1:2000, Abcam). The images were acquired with Leica SP8 Confocal Microscope System with STED. Deconvoluted was performed using Huygens professional software. (D) RBP33 does not localize to the nucleolus. The nucleus is stained with DAPI (cyan) and acquired with confocal microscopy in PCF (n=26). For the RBP33 signal (red) a polyclonal antibody (1:1000) and as a secondary goat anti-rabbit Alexa Fluor 594 (1:1000, Invitrogen) was used and acquired by super-resolution microscopy. For each nucleus, the slice with the most pronounced nucleolus was selected and by creating a binary mask, the DAPI signal was subdivided into nucleus (black), outer nucleolus (grey) and inner nucleolus (light grey). For the RBP33 signal a Gaussian blur filter (GB) was applied and the mean intensity for each region was measured. (E) Measurement of the RBP33 signal in the

1 inner and outer nucleolus in comparison to the nucleus. Scale bar 1 μ m

2
3 Figure 2: Overexpression and RNA binding of RBP33.

4 (A) Western blot using anti-RBP33 antibody and total cell protein extracts during the time
5 course of RBP33 overexpression; normalization to a cytoplasmic protein (Alba3). (B)
6 Quantification of RBP33 overexpression from three independent biological replicates as seen
7 in (A). (C) Northern blot of total mRNA abundance from different timepoints post
8 overexpression of RBP33 using a spliced leader probe that detects all mRNA transcripts
9 (mRNA) and the spliced leader RNA itself (SL). The blot was normalized to the 18S rRNA.
10 (D) Quantification of three biological replicates of (C) where the non-induced (0h) level was
11 set as 100%. (E): RNA protein complex formation detected by northern blotting with probing
12 for biotinylated poly(A) RNA. Binding reactions were performed with purified recombinant
13 MBP-RBP33 fusion protein and synthetic oligoribonucleotides (biotinylated at the 3'-end or
14 unmodified). Biotinylated oligonucleotides were used at the concentration of 25 nM,
15 unlabelled oligonucleotides – at 200 $\times$ or 500 $\times$ excess, and 300 ng RBP33 was used. Reactions
16 were resolved in 4% clear native PAGE. When purified recombinant RBP33 is added, three
17 distinct complexes appear. When the antibody against RBP33 (AB) is added, a supershift is
18 observed. Presence of a competitor (comp) reduces complex formation. (F): Similar effects are
19 observed when probing with antibodies for RBP33 by western blot. Arrowheads point to
20 RBP33 signal. Addition of poly(A) RNA leads to shifted complexes; presence of a competitor
21 (comp) does not change complex formation; addition of the antibody (AB) causes a supershift
22 (star). (G): Northern blot, same as in (A), EMSA done with random sequence RNA
23 oligonucleotides.

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Figure1

Figure2

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