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Short communication

# An RNAi screen of the RRM-domain proteins of *Trypanosoma brucei*

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ABSTRACT

In eukaryotes, proteins containing RNA Recognition Motifs (RRMs) are involved in many different RNA processing reactions, RNA transport, and mRNA decay. Kinetoplastids rely extensively on post-transcriptional mechanisms to control gene expression, so RRM domain proteins are expected to play a prominent role. We here describe the results of an RNA interference screen targeting 37 of the 72 RRM-domain proteins of *Trypanosoma brucei*. RNAi targeting 8 of the genes caused clear growth inhibition in bloodstream trypanosomes, and milder effects were seen for 9 more genes. The small, single-RRM protein *Tb*RBP3 specifically associated with 10 mRNAs in trypanosome lysates, but RBP3 depletion did not affect the transcriptome.

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## 1. Introduction, results and discussion

The genomes of Kinetoplastids are made up of polycistronic transcription units [1]; individual mRNAs are generated by *trans* splicing and polyadenylation. As a consequence of this genomic organisation, trypanosomes and Leishmanias are highly dependent on post-transcriptional mechanisms to regulate gene expression [2]. In addition, the mitochondrial mRNAs are extensively edited [3] and, as in other eukaryotes, the stable catalytic and structural RNAs are subject to processing and modification. All of these processes require the participation of RNA binding proteins. Prominent among these are proteins containing an RNA Recognition Motif (RRM). In a previous survey we described 72 *Trypanosoma brucei* genes encoding RRM-containing proteins. We predicted possible functions for some of them based on sequence homologies or published experimental data [4]. We describe here the results of an RNA interference screen that was designed to find out which of the remaining proteins were important in trypanosome survival.

For RNAi we used a vector in which dsRNA is synthesised from opposing T7 promoters [5,6] (Supplementary Table S1). We transfected 35 plasmids into either bloodstream-form trypanosomes, procyclic-form trypanosomes or both, with a bias towards bloodstream forms. The day after transfection, selecting drug was added and the cultures were cloned by limiting dilution [5]. If no parasites survived the selection, we attempted the transfection once more; failure to get live cells occurred more often for bloodstream forms than for procyclic forms. Overall we obtained clones for 32 genes (Table 1). Failure to get clones is uninformative: although it could be caused by leakage of a lethal RNAi, other technical problems cannot be excluded.

To test the effects of RNAi, we added tetracycline (0.1–0.5 μg/ml) and cultivated the parasites for up to 7 days, diluting as required, and assessed mRNA levels by Northern blotting. The results are summarised in Tables 1 and 2, illustrated in Fig. 1, and presented more fully in Supplementary Table S1. If Northern blotting results demonstrated that tetracycline addition did not cause a decrease in the amount of the target mRNA, clones were not investigated further. Eight of the genes tested were required for normal growth of bloodstream forms (Table 2); in several more cases, transient or mild effects were seen, including decreased growth whether or not tetracycline was added (Table 2 and Fig. 1). The doubling time of the bloodstream trypanosomes used in our experiments, in the absence of deleterious mRNA depletion, is about 7 h [7] (see panels for *DRBD9* and *TRRM3* in Fig. 1) and for procyclics, about 10 h. Most of the bloodstream RNAi lines illustrated in Fig. 1 grew slower than the wild type even in the absence of tetracycline, with even slower growth upon tetracycline addition. Similar observations were made for some of the procyclic lines (see Table 2). These cell lines may have some dsRNA effect on either translation or mRNA levels even in the absence of tetracycline. It is important to note that the less dramatic differences were apparent only if growth was monitored

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**Table 1**
RNAi screen statistics. (1) The plasmid was transfected into the relevant form at least once. (2) Cells resistant to the selecting drug obtained. (3) No cells survived the selection. (4) A decrease of at least 50% in the target mRNA was seen by Northern blot after tetracycline induction. (5) The Northern blot revealed no difference between cells with and without RNAi induction. (6) The mRNA for the RRM protein was not detected in cells without RNAi. (7) "Mild" growth effects were either transient or resulted in an increase in division time of two-fold or less relative to cells without RNAi induction. (8) "Clear growth effect" in bloodstream forms indicates a division time of 12 h or more in the presence of tetracycline. No conclusions can be drawn from any of the negative results.

| | | Procyclic | Bloodstream |
|---|---|---|---|
| 1 | Plasmid transfected | 24 | 30 |
| 2 | Clones obtained | 19 | 23 |
| 3 | No live cells obtained | 5 | 7 |
| 4 | mRNA decrease confirmed | 6 | 11 |
| 5 | No RNAi effect on mRNA | 1 | 5 |
| 6 | RNA not detected | 6 | 8 |
| 7 | Mild growth effect | 7 | 9 |
| 8 | Clear growth effect | 0 | 8 |

**Table 3**
Protein location studies. A sequence encoding a V5 tag was integrated in frame with the open reading frame, at the 5′-end; alternatively, the protein with a C-terminal myc tag was over-expressed from a procyclin promoter. For the immunofluorescence (IFA), proteins in parentheses gave low signals, not very different from background, so the assignments are tentative. Data for RBPs 20, 25 and 38 are shown in Supplementary Figure 2. Data for the locations of DRBD7, DRBD9 and TRRM3 (Table 2) were obtained by cell fractionation.

| | V5 *in situ* | Myc inducible expression |
|---|---|---|
| Not detected on Western blot | RBP10 | RBP8, RBP9 |
| Detected on Western blot | RBP3, RBP6, RBP20, RBP21, RBP28, RBP38, RBP28, DRBD7, DRBD9, TRRM3 | RBP3, RBP6, RBP10, RBP25 |
| IFA: cytoplasm | RBP3, RBP38 (RBP28, DRBD7) | (RBP6) |
| IFA: nucleus | RBP20 (DRBD9, TRRM3) | |
| IFA: nucleus and cytoplasm | | RBP25 |
| IFA: negative | RBP21 | RBP10 |

over at least 5 days and cumulative growth curves were plotted. Two cell lines with *RBP23* RNAi behaved strangely: they grew extremely slowly in the absence of tetracycline but recovered somewhat in the presence of tetracycline (Fig. 1); the basis for this has not been investigated. Inducible over-expression of RBP3 increased the division time from 8.2 to 12.6 h (Supplementary Figure S1A,B).

Two of the RRM-protein mRNAs were known, from microarray analyses, to be more abundant in bloodstream forms than procyclic forms (R. Queiroz and C. Clayton, in preparation). For both of these – *RBP9* and *RBP10* – RNAi in procyclic trypanosomes did not affect growth. A bloodstream cell line with *RBP10* RNAi showed severe growth inhibition; we have not yet succeeded in generating a bloodstream line with *RBP9* RNAi. Bloodstream trypanosomes with a T7-driven RNAi against *TRRM3* showed no growth effect, but a stem-loop construct revealed growth inhibition (Fig. 1).

Our RNAi methods have two major limitations: the system may be leaky in the absence of tetracycline, and it does not completely remove a gene product. In the "Trypanofan" RNAi screen 197 open reading frames were targeted [8]. Effects on growth were detected for 38% of all genes tested, and 29% of those with a Pfam annotation. These are clearly likely to be under-estimates: on top of the incomplete penetrance of RNAi, the cells were not cloned, so the methodology selected strongly against parasites with leaky RNAi. Our results for RNAi targeting RRM protein mRNAs, in contrast, suggested that 60–70% of the individual gene products were required for normal growth. The difference between these numbers and those seen in Trypanofan could be due to technical differences, but might also reflect the essential role of RNA metabolism.

We next wished to investigate the subcellular distributions of some of the essential proteins. After checking the protein sequences for targeting signals (to rule out the presence of a mitochondrial pre-sequence) we either tagged genes *in situ*, to give proteins with an N-terminal V5 tag [9], or inducibly expressed proteins with a myc tag at the N- or C-terminus (see e.g. [10]). Four proteins were found in the cytoplasm, three were restricted to the nucleus and one was in both compartments. Some tagged proteins that were detectable by Western blotting gave no signal by immunofluorescence; a few were not detected at all. The results are summarised in Table 3 and some data shown in Supplementary Figure S2.

**Table 2**
Effects of RNAi on trypanosome growth. Data are shown only for genes whose growth of RNAi lines was affected by tetracycline addition, or the lines had defective growth even without tetracycline. Numbers are the division time in hours. Where several numbers are given for one gene, they represent the results for independent cloned cell lines, and the numbers are given in order: the first number is for clone 1, the second number is for clone 2, etc. "Transient" means that an effect was seen in the 24-well plates but not after transfer to 5 ml flasks; this must be regarded with extreme caution. Bloodstream *DRBD5* lines (stem-loop construct, not included in the table) showed poor growth independent of tetracycline addition; quantitation was difficult because of clumping.

| | PC −tet | PC +tet | BS −tet | BS +tet | Location |
|---|---|---|---|---|---|
| *RBP3* | 8.4 | 8.7 | 8.2 | 12.6 | Cytoplasm |
| *RBP10* | [a] | No effect[a] | 11 | Dead[b] | Cytoplasm |
| *RBP14A* | ND | ND | 7.4, 7.5 | 8.0, 7.9 | ND |
| *RBP20* | 13.0 | 13.5 | [a] | No effect[a] | Nucleus (spots) |
| *RBP21* | [a] | Transient[a] | 9.5, 9.6 | 10.0, 10.2 | ND |
| *RBP23* | ND | ND | 19, 17 | 13, 10 | ND |
| *RBP25*[c] | ND | ND | 11.5 | 23 | Nucleus and cytoplasm |
| *RBP26* | ND | ND | 12 | 14 | ND |
| *RBP28* | 11.7, 10.8 | 13.1, 12.2 | ND | ND | Unclear |
| *RBP30* | [a] | Transient[a] | 9.2 | 9.8 | ND |
| *RBP31* | ND | ND | 9.8 | 17 | ND |
| *RBP38* | 11.8, 12.5, 12.6 | 12.7, 12.6, 12.8 | 9.1, 9.5, 8.9 | 10.0, 9.8, 9.6 | Cytoplasm |
| *DRBD6 A/B, 11* | NC | | 10.2, 9.5 | 12.0, 12.7 | ND |
| *DRBD7* | ND | ND | 9.8, 9.5, 10.1 | 13.1, 13.9, 16.8 | Cytoplasm |
| *DRBD9* | ND | ND | 7.4 | 8.4 | Nucleus |
| *DRBD12* | NC | | 10.0, 12.6, 11.7 | 11.5, 13.7, 13.4 | ND |
| *DRBD14* | [a] | No effect[a] | [a] | Transient[a] | ND |
| *TRRM3* | 10.5 | 13.5[b] | 7.0 | Dead[b] | Nucleus |

[a] Not measured in detail. ND: not done; NC: no clones obtained.
[b] Using p2T7 derivatives, no effect or clones not obtained; results are for a stem-loop.
[c] Results for *RBP25* were very variable, ranging from no effect to complete growth inhibition.

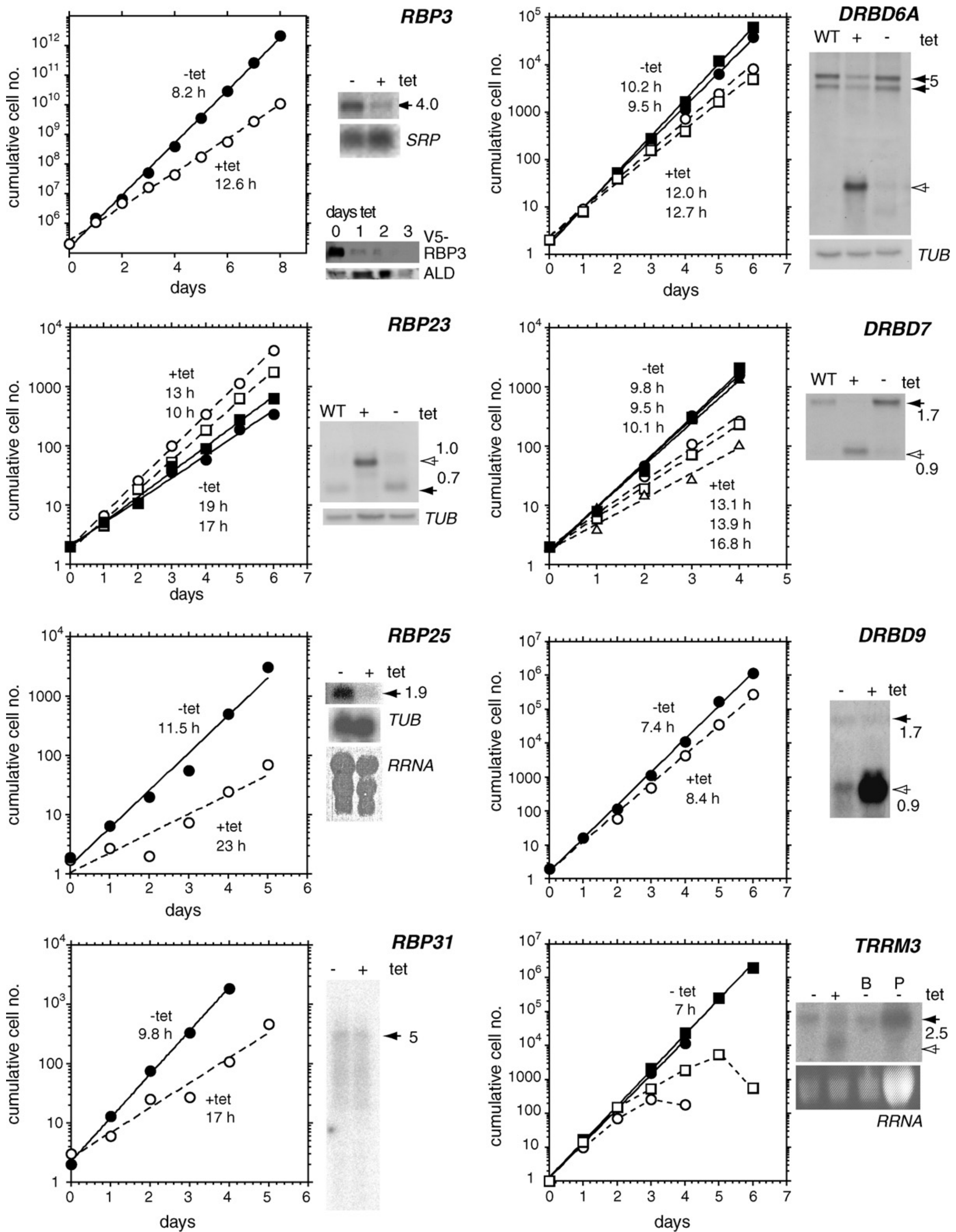

**Fig. 1.** Growth data for bloodstream trypanosomes with RNAi. The targeted protein is indicated. Cells were grown with (+) or without (−) tetracycline and diluted as required to maintain exponential growth; cumulative growth curves are shown along with division times calculated using Kaleidograph. RNA was prepared on day 3 after tetracycline addition, except for *DRBD9* (day 2). Sizes of mRNAs in kb are indicated next to the blots (solid arrow); the open arrow indicates the dsRNA. *TUB*: tubulin control probe. The Northern for *TRRM3* is from the clone with the less severe effect. All RNAi plasmids were based on p2T7 except those for *RBP3* and *TRRM3*, which were stem-loops (Supplementary Table S1). For RBP3 we also induced RNAi in cells expressing V5-tagged RBP3; the lower panel shows depletion of V5-RBP3 1, 2 and 3 days after RNAi induction, with aldolase (ALD) as a control.

We have previously shown that over-expression of the abundant small RRM proteins UBP1 and UBP2 affects gene expression in *T. brucei* [11]. RBP3 is related to UBP1 and UBP2 [4] and was reported to colocalise with the helicase DHH1 and polyA + RNA in granules in stressed procyclic trypanosomes [12]. In bloodstream forms, V5-*in situ* tagged RBP3 was all over the cytoplasm but also showed some concentration in regions containing DHH1 (Fig. 2A). In other respects V5-RBP3 behaved similarly to UBP1 and UBP2 [11]:

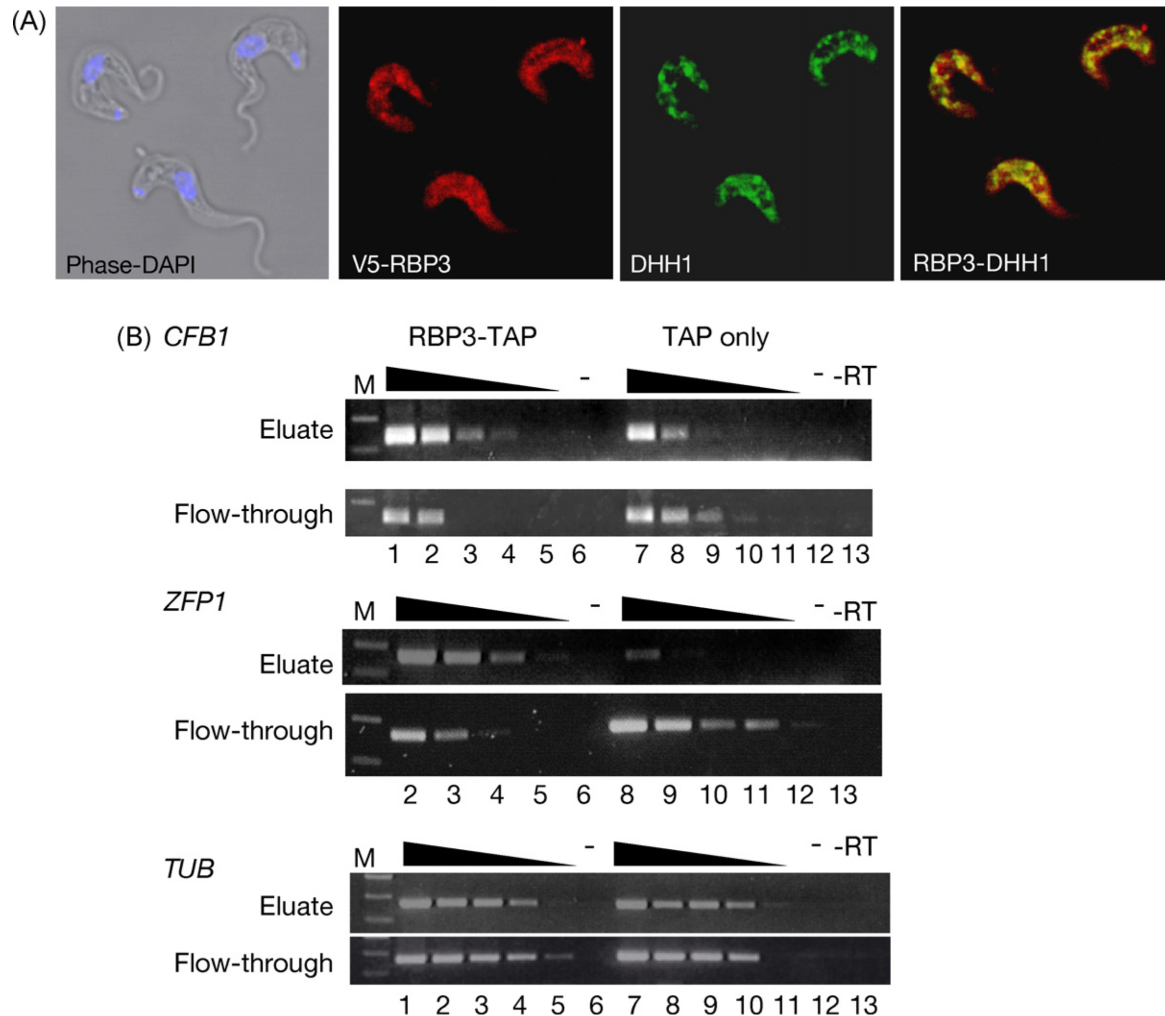


**Fig. 2.** Localisation and RNA binding assays for RBP3. (A) Immunofluorescence of bloodstream-form trypanosomes with *in situ* V5-tagged *Tb*UBP3 (red) counterstained for *Tb*DHH1 (green) and for DNA (DAPI). (B) Cells expressing *Tb*RBP3-TAP and the TAP tag alone were bound to IgG sepharose, and the bound complexes were released by TEV protease [14]. RNA was prepared from the entire eluate, and from 0.3 ml out of the 1 ml flow-through fraction [14], and reverse transcribed using an oligo-dT primer. 1:10 serial dilutions (until 1:10,000) of the cDNA generated were used as a template for the PCR, using specific primers for each transcript. After 30 amplification cycles, 10 μl of each sample were run into an agarose gel. The photograph shows the ethidium bromide stain. Lanes 1 and 7 represent cDNA from $3 \times 10^6$ cells for the eluate, and $9 \times 10^5$ cells for the flow-through; lanes 2 and 8, 10 times less, and so on. Thus the flow-through lanes represent four times less cDNA than the eluate lanes immediately above them.

it showed a similar abundance by Western blotting, was predominantly cytoplasmic after cell fractionation, and was not associated with polysomes on sucrose gradients (not shown).

To find out whether RBP3 is able to bind to specific RNAs, we expressed TAP-tagged RBP3 in bloodstream trypanosomes, pulled down RBP3-TAP from cell lysates, and identified the bound mRNAs using microarrays. The method ([13,14], see Supplementary Methods) did not distinguish between RNAs bound *in vivo*, and RNAs bound subsequent to cell lysis, but could nevertheless reveal whether RBP3 binds preferentially to specific RNA sequences. The microarrays used contain random genomic fragments, so many genes are represented more than once. Only 10 sequences reproducibly showed more than three-fold enrichment in the RBP3-bound sample (Table S2); of these, five were represented twice or more. They encode the cyclin F-box protein CFB1 [7]; two CCCH zinc finger proteins, ZFP1 [15] and ZC3H11 (Tb927.5.810); and two proteins with no annotation (loci Tb927.4.1000 and Tb927.8.7820). To find out what proportions of the *CFB1* and *ZFP1* mRNAs co-purified with RBP3-TAP, we compared their abundances in the bound and flow-through fractions by reverse transcription and PCR. The highly abundant tubulin (*TUB*) mRNA, and a purification using TAP tag alone, served as controls: these suggested non-specific binding of 10–15% of *TUB* RNA to either the tag, or the resin used for purification. In contrast, at least 25% of *CFB* and *ZFP1* RNAs were reproducibly specifically selected by RBP3-TAP; moreover, these RNAs were depleted in the RBP3-TAP flow-through fraction (Fig. 2B). A full tandem affinity purification of RBP3-TAP, in contrast, revealed no stably-associated protein binding partners (not shown).

To find out whether RBP3 depletion affects mRNA abundances, we compared the transcriptomes of RBP3-depleted cells with those of normal cells by microarray. No differences were seen, and Northern blots for some of the RBP3-selected mRNAs also showed little effect, apart from a slight increase in *ZC3H11* mRNA upon RBP3 over-expression (Figure S1C). Another possible role of RBP3 might be in translation. Antibodies are available for CFB1 [7] or ZFP1 [16] but we were unable to detect either in our cells, whether or not RBP3 levels were altered (not shown). Thus the biological role of RBP3 remains unknown.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.09.001.

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