REVIEW ARTICLE

High-throughput Methods for Dissection of Trypanosome Gene Regulatory Networks

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Abstract: From synthesis to decay, mRNA associates with RNA-binding proteins (RBPs) establishing dynamic ribonucleoprotein particles (RNPs). Understanding the composition and function of RNPs is fundamental to understanding how eukaryotic mRNAs are controlled. This is especially relevant for trypanosomes and related kinetoplastid parasites, which mostly rely on post-transcriptional mechanisms to control gene expression. Crucial for trypanosome differentiation, development, or even response to heat shock, RBPs are known to be essential modulators of diverse molecular processes. The recent application of large-scale quantitative methods, such as Next-Generation Sequencing (NGS) and quantitative mass spectrometry, has revealed new exciting features about the parasite RNA-related metabolism. Novel proteins carrying RNA-binding activity, including many proteins without RNA-related ontology were discovered setting a necessary groundwork to get in insights into RNA biology.

Conclusion: This review aims to give the reader an understanding of current trypanosome RNP research, highlighting the progress made using high-throughput approaches.

1. INTRODUCTION

The Kinetoplastid parasites branched very early in eukaryote evolution and several of their members constitute a major public health threat across the world. Because of their medical and veterinary importance, these parasites have been intensively investigated, revealing that regulation of gene expression displays some unique features. Remarkably, there is no evidence for control of expression *via* regulated transcription initiation, which has made Kinetoplastids an attractive model for the study of post-transcriptional control of gene expression [1]. Since then, RNA-binding proteins (RBPs) have emerged to gain a central role in the biology of the parasite [2, 3]. The findings of several papers published recently [4-10] offered important insights into RNA biology renewing questions about which are the main contributors to functional RNP diversity.

1.1. The Diversity of RNA-binding Proteins in Trypanosomes

How many different proteins are associated with mRNAs in trypanosomes? About 150 RBPs are annotated in *T. brucei* genome on the basis of the presence of known RNA-binding domains (RBDs), but the functions and *in vivo* binding specificities of most RBPs are not known. These RBPs have been inferred from sequence homology where the most common RBDs were observed to be RRM (74 proteins, [11]), CCCH (48 proteins [12]) and Pumilio (11 proteins, [13]).

Other classical RBDs commonly found in more complex eukaryotes are far less frequent in trypanosomes (*e.g.* KH, S1 and CSD domains). Few of them were identified by biochemical methods [14-16], and only a minor fraction had been validated for RNA binding *in vivo* (reviewed in [17, 18]). A crucial step towards a comprehensive genome-wide catalogue of proteins with *in vivo* mRNA binding activity has been established in human cells [19, 20]. In these studies, protein-mRNA interactions were covalently crosslinked by UV irradiation and pulled down with oligo (dT). Mass spectrometry analysis revealed the near-complete repertoire of RBPs, including hundreds of novel players. Among these proteins, it was further shown that several metabolic enzymes, such as peptidyl-prolyl isomerases and kinases, also bind RNA *in vivo* [19]. While searching for common motifs among the newly identified RBPs, several non-canonical RBDs were discovered. For instance, proteins carrying single amino acid patches (*e.g.* Lys) can form exposed interfaces with potential to mediate molecular interactions [19].

Using a similar approach, we have recently identified 155 high-confidence RBPs in cultured bloodstream cells [4]. Many of these proteins lack any recognizable domain suggesting the existence of novel RNA-binding features. As expected, significant enrichment was observed for canonical

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RBDs (RRM, PUF and Zinc finger), as well as for less common domains, including PSP1, C/DEAD helicase, ALBA and translation elongation factors (GTP_EFTU_D2 domain). These numbers constitute the most up-to-date estimate of the total number of RBPs that are expressed in *T. brucei*, but presumably many more RBPs await discovery. Indeed, many proteins known to or predicted to bind RNA were not included in the high-confidence group. Selecting a less stringent cut off (FDR 5%), the list expands to 293 hits and would include 16 extra predicted RBPs [4].

As seen in other organisms, the list also includes proteins involved in vesicular transport and the glycolytic enzyme pyruvate kinase. This enzyme was found to interact with mRNA not only in trypanosomes but also in several human cell lines and budding yeast [4, 19-22]. Even though in trypanosomes most glycolytic enzymes are localized within glycosomes, the last step catalyzed by pyruvate kinase occurs at the cytosol. Whether such enzyme/RNA interactions are biologically relevant warrants further investigation. Another interesting protein displaying RNA-binding activity was a putative phosphatase (Tb927.6.640) linked to the RNP remodeling as it was also shown to localize at Stress Granules (SG) upon starvation [6]. As seen in diverse organisms [11, 19, 23, 24], the *T. brucei* mRNA interactome was enriched in intrinsically disordered regions. These disordered segments were overrepresented in low complexity and repetitive amino acid sequences (our unpublished data). The unstructured segments consist of repetitive stretches of glycine (G), asparagine (N), glutamine (Q) and histidine (H) residues. This highlights a conserved trypanosome to man function of short repetitive amino acid motifs exhibiting potential RNA-binding activity.

Comparative genomic analyses of *Leishmania major*, *Trypanosoma cruzi* and *T. brucei* revealed a remarkable degree of conservation [25]. Consequently, most proteins (151 out of 155) that were identified in the mRNA-bound proteome are conserved in these parasites. Four potential RBPs are *T. brucei*-restricted, so are likely to control unique features of the parasite biology. As expected, it includes the AGO1 protein as RNAi is known to occur in *T. brucei* but not in *T. cruzi* and *L. major*. A second protein (Tb927.11.2250) was also identified in a genome-wide screen for genes involved in driving stumpy formation [26], a stage exclusive to *T. brucei*. Finally, ZC3H13 [27] and a 'Cold-shock' DNA-binding domain containing protein (Tb927.4.4520) were restricted to *T. brucei*. The ZC3H13 was found to repress reporter expression [4] but its targets and function are not known.

It is important to note that this study would not have detected proteins that may act during other stages of the parasite's life cycle, may have low expression levels or may be only transiently expressed. Moreover, transcripts are captured *via* the poly(A) tail, so proteins binding pre-mRNA and non-polyadenylated RNA are not purified. Nevertheless, the number of potential RBPs has a reasonable relative complexity compared with similar studies in metazoans [19, 20] and protozoa [28].

1.2. From Protein Discovery to Function Assignment

A complete functional understanding of any RBP requires the identification of its mRNA targets. Initial genome-wide attempts to identify associated transcripts in trypanosomes employed microarrays (RIP-ChIP: RNA-Binding Protein Immunoprecipitation-Microarray (Chip) Profiling) ([15, 29-35]) and once next-generation sequencing was established, RNA sequencing (RIP-Seq: RNA-Binding Protein Immunoprecipitation followed by deep sequencing) [36-41]. RIP-Seq often involves cross-linking of proteins to RNA, followed by immunoprecipitation of the protein-RNA complex. Protein-bound transcripts are then used to make a cDNA library that is subjected to RNA sequencing (RNA-Seq). Analysis of the RBP33-bound transcripts by this technique [38] revealed that a large proportion of targets derived from the strand switch regions and also from the end of transcriptional units. Since these transcripts are not normally detected, a role in nuclear gene silencing was suggested. RIP-seq analysis after heat shock revealed that many mRNAs containing AUU repeats, mostly encoding chaperones, are specifically stabilized by ZC3H11 [37]. A survey of the ZC3H39-associated transcripts in *T. cruzi* epimastigotes suggested that, under stress conditions, the protein sequesters into SG to mRNAs normally expressed at high levels [39]. A putative recognition motif, AAACAA, was identified. Similarly, in *T. brucei,* ZC3H39 was shown to bind poly(A) mRNA and to decrease reporter expression [4]. In *T. cruzi*, NRBD1 binds specifically to some mRNAs encoding mainly ribosomal proteins [40]. Also in epimastigotes, a specific subset of mRNAs associates with the elongation factor TEF1 (EF-1α) [41].

Knockdown or overexpression of the RBP of interest, followed by global transcriptome analyses also provides clues about the function of the RBP. This is especially useful when combined with RIP-seq analysis as it helps to identify the direct targets of the protein under investigation. Combining these datasets, it was shown in procyclic form that DRBD13 would act as a negative regulator of transcripts encoding ribosomal, cell membrane and proteins involved in transport [36]. One of the most enriched transcripts in this analysis encoded RBP6, a protein involved in the regulation of the developmental stages in the insect vector [42] (see below). Consequently, downregulation of DRBD13 by RNAi increased RBP6 mRNA levels [36]. How the crosstalk between these two proteins leads through the developmental stages of the parasite is not yet clear.

Although RIP-seq technique has proved to be useful for mRNA target discovery, it provides limited information about the specific binding site. Since in many cases these motifs are relatively short (4-5 nucleotides) the use of simple bioinformatics tools to predict *in vivo* protein binding sites is precluded. To overcome this issue, a CLIP (ultraviolet cross-linking immunoprecipitation) protocol was initially developed to identify the neuron-specific RBP Nova targets in the brain [43]. The technique exploits the irreversible protein-nucleic acid cross-linking to stringently purify a specific protein-RNA complex using immunoprecipitation (IP) followed by SDS-PAGE. To facilitate identification of binding sites, the RNAs are intentionally reduced in size with RNases before IP. In HITS-CLIP (High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation), the isolated RNAs are then converted into cDNA libraries for sequencing on NGS platforms [44]. Since developed, a plethora of RNA-protein interactions have been mapped in

diverse organisms [45]; however, this technique has been barely used in trypanosomes. Analysis of the RBP42-bound transcripts by HITS-CLIP showed that the protein binds to the coding regions of about 2000 transcripts [46]. However, a more stringent cut off identified as targets to genes involved in energy metabolism (*e.g.* glycolysis and Krebs cycle enzymes). In another study, the analysis of DRBD3/PTB1-bound mRNAs concluded with 265-mRNA targets encoding mainly proteins involved in translation [47]. Motif analysis of the CLIP data identified polypyrimidine-rich binding motifs mainly located within the 3' untranslated region (UTR).

Since the mRNA-bound proteome and CLIP-seq use identical cross-linking conditions, it is likely that most of the RBPs recently uncovered by Lueong *et al.* [4] are easily amenable to CLIP-seq protocols. An alternative strategy, called photo-activatable cross linking (PAR-CLIP), is to supply 4-thiouridine (4sU) to cells, which is then incorporated into nascent RNA and can be crosslinked to protein by 365 nm UV light irradiation [48]. For *T. brucei*, this strategy possibly requires the use of transgenic parasites able of salvaging pyrimidines from its environment. Although trypanosomes express a highly efficient uracil transporter [49], it appears to work poorly for the ribonucleotide analog. Attempts to label nascent transcripts with 4sU in wild-type cells showed low incorporation levels [50]. Besides, although PAR-CLIP was initially reported to be more efficient than conventional UV 254 nm crosslinking [48], it may hold true only for specific RBPs [19, 51].

1.3. Anticipating the Impact of an RBP on its mRNA Target

One way to analyze the functional impact of a protein on mRNA targets is by "tethering" it to a reporter mRNA [52]. In this technique, the protein under study is attached to an mRNA reporter through an artificial RNA-protein interaction. The fusion protein is generally tethered to the 3'UTR, which is the frequent site of action. This tethered function assay was adapted into an approach for a survey of post-transcriptional regulatory activity in *T. brucei* proteins [5]. For the screen, a library made from random genomic fragments was cloned in a plasmid designed for expression of proteins fused to an RNA-binding domain. Transfection into cells expressing mRNAs encoding a positive or negative selectable marker with the cognate RNA recognition sequence allowed identification of more than 300 candidates [5]. Not surprisingly, the screens confirmed the role of RBPs in the regulation of mRNA fate in trypanosomes but also shed light on many proteins unrelated to mRNA metabolism. Indeed, more than 150 hypothetical proteins were seen to affect gene expression. This approach does not discern at which step gene expression is affected by the tethered protein (*e.g.* mRNA stability or translation) and can give artefacts such as the effects of tethering proteases. In any case, tethering results must be taken with caution. In general, negative results are most likely meaningless and results obtained with protein fragments may not hold true for full-length proteins (discussed in [4, 5]). Having this in mind, an Open reading frame collection containing the most interesting candidates was recently used to perform complementary tethering screens [4]. These screens resulted in a functional catalogue of 44 putative up- and 48 down-regulating proteins including canonical RBPs and novel uncharacterized proteins (Fig. **1**). Intriguingly, only a fraction of these regulators were seen to interact with poly(A) mRNA [4]. Thus, a subset of regulators may act as "bridges" between the RBPs and the translation/degradation apparatus. In addition, the screen results supported previous findings showing that proteins carrying an HNPY (His/Asn/Pro/Tyr) sequence linear motif have a positive regulatory effect on the fate of the target mRNA [4, 53]. The motif is required to interact with the master regulator MKT1 which associates with poly(A)-binding protein (PABP1) promoting translation [53]. Interestingly, the mRNA-bound proteome showed that not only the HNPY-containing proteins are canonical or novel RBPs, but also MKT1. Thus, a possible cooperative binding between MKT1 and these RBP partners might regulate mRNA expression. The discovery of the HNPY motif as a signature necessary and sufficient for binding MKT1 was also possible thanks to the application of NGS technologies [53]. The authors applied high-throughput DNA sequencing to identify gene fragments within colonies produced in a traditional yeast two-hybrid format by scraping the resulting colonies together followed by PCR amplification and sequencing. Hence, an additional benefit of using a shotgun library is the ability to delineate functional domains.

1.4. RBPs and Developmental Regulation Control

So far, two RBPs were seen implicated in overall life-cycle-stage-specific gene expression control in *T. brucei*. RBP10, a bloodstream-specific protein, was shown to correlate with a bloodstream expression pattern [54]. While depletion of RBP10 resulted in the downregulation of abundant bloodstream-specific transcripts, its overexpression in procyclic form increased them. Unfortunately, RBP10 target mRNAs are not yet known; however, preliminary results indicate that RBP10 binds to, and promotes degradation of a subset of procyclic-specific mRNAs (Clayton, Mugo personal communication).

An analysis of the *T. brucei* transcriptome in infected tsetse tissues by RNA-Seq revealed that trypanosomes occupying the proventriculus exhibit much higher levels of RBP6 transcripts in comparison to procyclics from the midgut [42]. Remarkable, by overexpressing RBP6 in cultured procyclic trypanosomes, the authors *in vitro* recapitulated the developmental stages observed in the vector, including not only the generation of short and long epimastigote forms but also infective metacyclic trypomastigotes expressing Variant Surface Glycoprotein (VSG). The activated VSG genes were originated from monocistronic expression sites and carried a metacyclic-type VSG Pol I promoter, confirming the identity of the metacyclics generated *in vitro*. Since these stages are transitory and non-proliferative (or intimately attached to the epithelium in the salivary glands), this finding provides an amenable *in vitro* system for understanding the molecular basis of parasite differentiation within the fly. RBP6 target mRNAs are not yet known but *in vitro*, it binds AU-rich elements [35]. Overexpression of RBP6 in procyclic cells was found to downregulate mRNAs containing the identified motif [35]. In *T. cruzi*, the RBP6 ortholog gene has not been characterized; however, RBP6 transcripts abundance increases in the trypomastigote stage, suggesting it may also
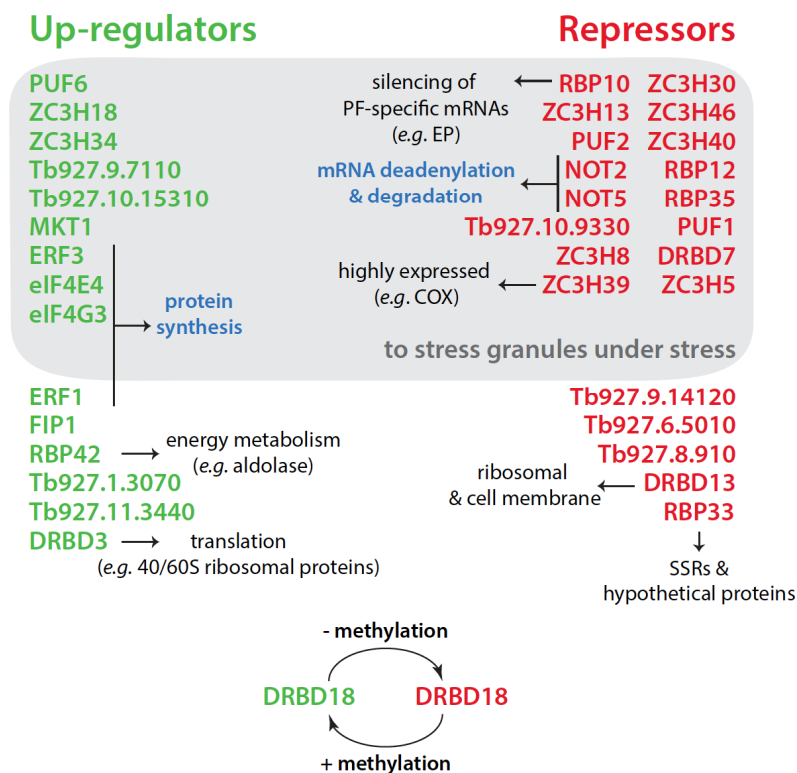
Up-regulators

Repressors

PUF6
ZC3H18
ZC3H34
Tb927.9.7110
Tb927.10.15310
MKT1
ERF3
eIF4E4
eIF4G3

silencing of ← RBP10   ZC3H30
PF-specific mRNAs   ZC3H13   ZC3H46
(*e.g.* EP)   PUF2   ZC3H40

mRNA deadenylation   NOT2   RBP12
& degradation ←   NOT5   RBP35
Tb927.10.9330   PUF1

highly expressed   ZC3H8   DRBD7
(*e.g.* COX) ←   ZC3H39   ZC3H5

protein synthesis

to stress granules under stress

ERF1
FIP1
RBP42 → energy metabolism
Tb927.1.3070   (*e.g.* aldolase)
Tb927.11.3440
DRBD3 → translation
(*e.g.* 40/60S ribosomal proteins)

Tb927.9.14120
Tb927.6.5010
Tb927.8.910
ribosomal ← DRBD13
& cell membrane   RBP33
↓
SSRs &
hypothetical proteins

- methylation

DRBD18   DRBD18

+ methylation

Fig. (1). Proteins found to bind poly(A) mRNA in *T. brucei* [4, 36-38, 47, 104] affecting reporter expression upon tethering [4] are colored in green for up-regulators and red for repressors. Others RBPs that not fulfill these criteria are not shown. Under starvation, several of these proteins migrate to stress granules (grey box) [6]. RBP42 and DRBD3 are associated with abundant mRNAs, indicating a stabilizing function [46, 47]. RBP33 interacts with region usually not detected which would be consistent with a destabilizing function [38]. Arginine methylation status regulates DRBD18 functions [8]. In *T. cruzi*, ZC3H39 sequesters highly expressed transcripts to stress granules [39]. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

exert important stage-specific functions in the non-dividing, cell-invasive forms of this parasite [55]. These and other examples [56-59] demonstrate the role of developmentally regulated RBPs in the modulation of the abundance of life form-specific transcripts.

1.5. RBPs Regulating Quorum Sensing

Stumpy differentiation is thought to occur *via* a quorum-sensing mechanism in response to an uncharacterized extracellular factor known as Stumpy Induction Factor (SIF) [60]. Genome-wide RNAi screens for cells that are resistant to SIF mimetics (cell-permeable analogues of cAMP or AMP), have proven to be effective for identifying genes required for differentiation in the slender to stumpy forms [26]. Among others hits, RBP7 was the only canonical RBP found to drive this step. While overexpression of RBP7 prematurely drove parasites to stumpy forms, its knock-down resulted in a delayed response to SIF mimetics. Global transcriptome analyses of cells with decreased RBP7 levels *via* RNAi revealed the upregulation of genes likely required for continued cell proliferation as slender forms (*e.g.* histones and glucose transporters). However, attempts to determine the RBP7 mRNA targets have not been made. The study also identified as regulators of stumpy formation to the hypothetical proteins Tb927.11.6600, Tb927.9.4080 and Tb927.11.2250 [26]. All three proteins were initially identified as putative post-transcriptional regulators [5] and exhib-

ited potential mRNA-binding activity [4]. However, later analysis where full-length proteins were tethered revealed that only Tb927.11.6600 would have an effect on mRNA-fate [4]. It is also possible that the outcome of the tethering approach might be different in pleomorphic cells. Interestingly, Tb927.9.4080 and Tb927.11.2250 proteins were also identified as components of induced mRNA granules strengthening a link with the RNA biology [6].

1.6. Controlling RBP Functions by Post-translational Modifications

In complex eukaryotes, at least five types of post-translational modifications (PTMs) namely phosphorylation, ubiquitination, methylation, acetylation and SUMOylation have been reported for RBPs [61, 62]. These PTMs were shown to modify RNA-binding activities, protein-protein interaction or subcellular localization. In trypanosomatid species they also occur [7, 63-66], but the impact on gene expression has been evaluated for few individual RBPs [8, 67, 68]. Probably the most striking example of PTMs affecting gene expression was that described by Lott *et al.* [8]. By downregulating the endogenous DRBD18 while complementing with hypomethylated, or methylmimic mutant versions, the authors demonstrated that methylation promotes the stabilization of mRNAs. As methylation-deficient and methylation-mimicking DRBD18 mutants showed differential interaction partners, it was suggested that the differential

methylation status shapes the composition of RNP complexes. However, the direct DRBD18 targets have not been yet discovered. As mentioned, the *T. brucei* mRNA-bound proteome is enriched in intrinsically disordered regions. Considering they are frequent sites of PTMs [69, 70], it will be interesting to see whether these modifications occur and how they impact on RNA-binding activities.

1.7. Condensing the Message into Granules

In eukaryotic cells, non-translating mRNAs can accumulate in different types of RNP granules [71]. P-bodies were initially thought to be localized sites for mRNA translational repression, storage or decay as they typically contain mRNA, translation repressors, RBPs, and most enzymes required for mRNA degradation [72]. Since microscopically detectable P-bodies are not essential for mRNA degradation [73] and decay also occurs on actively translating ribosomes [74], it is still unclear whether they function in mRNA degradation [75]. A second type of particle, the stress granule (SG), is thought to be storage sites where mRNAs are temporarily sequestered from translation due to unfavorable conditions [76].

Trypanosomes have a large repertoire of different RNA granules. Although their exact function remains poorly understood, the compartmentalization of mRNA and proteins in the cytoplasm may be crucial for a rapid adaptation to stress and environmental demands (Reviewed in [77, 78]). Recently, an original proteomic-wide screen to identify components of mRNA granules was performed [6]. In this approach, mRNA granules induced by starvation were trapped within the subpellicular microtubule array of the cytoskeleton while soluble proteins were washed away. Then, the microtubules were depolymerised and the released granules sedimented and analyzed by Mass Spectrometry (MS). The authors detected 463 potential candidates including many known but also new components, 17 (out of 49) of which co-localized to mRNA granule markers. More than 100 of these granule-enriched proteins were also seen to bind poly(A) mRNA (FDR 5%; [4]). Consequently, it is not surprising that this subset of proteins is enriched in low complexity regions and short linear motifs, as the assembly mechanism into granules generally involves the self-aggregation of proteins carrying these features [69]. Interestingly, as it was later shown for heat-shock granules [79], ribosomal protein mRNAs were absent from starvation SG [6]. How and why granule exclusion of the ribosomal protein mRNAs occur remains unclear. While in mammals and other organisms, ribosomal protein mRNAs contain a 5′ terminal oligopyrimidine tract (TOP), a conserved sequence motif in the 3′ UTR was found in nematodes [80] and *T. cruzi* [81]. However, initial attempts failed to find conserved linear motifs within the UTR regions of ribosomal mRNAs in *T. brucei*; hence a possible role of the secondary structure was suggested [79].

1.8. Translational Control

Trypanosomes and related species evolved several eukaryotic initiation factors (eIF4) paralogous that encode distinctly featured proteins [82-86]. In *T. brucei*, EIF4G3 is essential for protein synthesis, a role that depends on its in-

teraction with EIF4E4 and EIF4AI [85]. In the tethering screen, not only EIF4G3 and eIF4E4 increased reporter gene expression but also eIF4G1, eIF4G4 and eIF4E3 [4]. On the other hand, eIF4E1 represses expression when tethered to a reporter, as does its binding partner 4E-IP [4, 5, 87]. Similarly, in metazoans 4E-BP proteins compete with eIF4G for the same eIF4E1 binding motif, thus inhibiting the initiation of protein synthesis [88]. However, eIF4E1 has been proposed as an active translation factor in Leishmania amastigotes [87]. The existence of multiple factors may correspond to the preferential translation of particular mRNAs under specific conditions [89]. In future, it would be interesting to analyze the eIF4E target specificities, and how this preference influences translation. Upon tethering, the putative homologs of the release factors eRF1 and eRF3, and the pre-initiation complex factor SUI1 also increased expression [4].

Historically in trypanosomes, changes in transcript levels have been considered the best predictors of developmentally regulated changes in protein abundance. This can be partially explained by the fact that only few examples documented gene functions controlled at the level of translation or protein turnover [67, 90-93]. However, large-scale studies aim to compare mRNA abundance and protein levels showed a moderate correlation between the transcriptome and the proteome [94]. The contribution of differential translation to regulating gene expression during trypanosomatid life cycles was also examined by ribosome profiling [9, 10, 95]. In this assay, ribosome binding protects mRNA from cleavage by nucleases generating a collection of sequence fragments. These protected fragments are used to generate a library representing regions of mRNAs potentially undergoing translation [96]. Once again, this analysis suggested that changes in protein production are more pronounced than changes in mRNA abundance alone. By comparing translational efficiencies, about twice as many genes were detected as stage-regulated between *in-vivo*-derived slender bloodstream and procyclic compared to those identified based on mRNA abundance [10]. However, since high ribosome density could also indicate pausing or slow elongation the results may not hold true for some transcripts. In addition, protein stability must count for some discrepancies. While in some organisms, ribosome profiling data correlates well with protein abundance [97], it has not been definitely proven in trypanosomes. Nonetheless, for the bulk of genes, a good correspondence between changes in translation and relative protein level comparing bloodstream and procyclic forms was observed [9].

CONCLUSION

A major long-term challenge in the field is to determine the role of each RNP component in a dynamic context. When the interaction occurs and how the combination of RBPs affects the mRNA-fate will help to decode the mechanisms involved. Currently, few RNPs have been studied extensively and the functions and specificities of most RBPs remain unknown. Even though we have expanded the known universe of protein-RNA interactions, we still do not know how they are influenced by the host or vector environments. Since the methods for the discovery and characterization of RNPs are laborious and resource intensive, efficient and ro-

bust techniques are needed to fully characterize gene regulatory networks that occur in the natural habitat. McMahon *et al.* recently developed a new method to identify RBP targets that could circumvent partially these shortcomings [98]. The approach is relatively simple and do not require any biochemistry; if proven successful it could be useful to explore, for instance, RBP targets occurring along the developmental cycles in the tsetse fly.

Although technically challenging, the field of single-cell genomics is advancing rapidly and is providing many new perspectives [99]. As even genetically identical cells (such as a parasite culture) display different expression levels of the same gene [100], it is expected that single cell measurements yield a more detailed understanding of the gene expression program. For instance, single-cell sequencing after RNAi could reveal kinetics that are masked or difficult to see from non-synchronized populations. The importance of capturing single-cell processes may be also particularly critical for differentiation research, where progression between distinct cellular states is fundamental for understanding the biology of the parasite. In the next few years, the implementation of genome editing protocols based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) will certainly transform the molecular parasitology field. Indeed, the application of this technology to organisms traditionally challenging to manipulate genetically such as *T. cruzi* has begun to pay off [101-103]. Finally, since in general both NGS and mass spectrometry rely on already existing databases, only a complete and well-annotated genome will provide the ultimate resource for genomic approaches.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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