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MINIREVIEWS

Regulation of RNA binding proteins in trypanosomatid protozoan parasites

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Abstract

Posttranscriptional mechanisms have a critical role in the overall outcome of gene expression. These mechanisms are especially relevant in protozoa from the genus *Trypanosoma*, which is composed by death threatening parasites affecting people in Sub-saharan Africa or in the Americas. In these parasites the classic view of regulation of transcription initiation to modulate the products of a given gene cannot be applied. This is due to the presence of transcription start sites that give rise to long polycistronic units that need to be processed cotranscriptionally by trans-splicing and polyadenylation to give mature monocistronic mRNAs. Posttranscriptional mechanisms such as mRNA degradation and translational repression are responsible for the final synthesis of the required protein products. In this context, RNA-binding proteins (RBPs) in trypanosomes have a relevant role as modulators of mRNA abundance and translational repression by associating to the 3′ untranslated regions in mRNA. Many different RBPs have been proposed to modulate cohorts of mRNAs in trypanosomes. However, the current understanding of their functions lacks a dynamic view on the different steps at which these RBPs are regulated. Here, we discuss different evidences to propose regulatory events for different RBPs in these parasites. These events vary from regulated developmental expression, to biogenesis of cytoplasmic ribonucleoprotein complexes in the nucleus, and condensation of RBPs and mRNA into large cytoplasmic granules. Finally, we discuss how newly identified posttranslational modifications of RBPs and mRNA metabolism-related proteins could have an enormous impact on the modulation of mRNA abundance. To understand these modifications is especially relevant in these parasites due to the fact that the enzymes involved could be interesting targets for drug therapy.

Key words: Trypanosoma; Posttranscriptional gene expression; Ribonucleoprotein complexes; RNA-binding protein; Developmental regulation; Sleeping sickness; Posttranslational modification; Phosphorylation; Chagas disease

Core tip: We discuss several ways to regulate the function of RNA-binding proteins in trypanosomes. We highlight the propensity of these proteins to engage in interactions with other proteins and RNA, resulting in the formation of large reversible aggregates induced by environmental stress. Finally, the possible role of posttranslational modifications on the function of these proteins is discussed in the context of recent high-throughput proteomic evidences.

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INTRODUCTION

The synthesis of the required protein products, and their levels, demands coordinated mechanisms to regulate gene expression in all organisms. These mechanisms operate in eukaryotic cells at the level of transcription initiation and/or mRNA processing, localization, stability or translational efficiency. As an exception to the rule, a group of protozoan parasites belonging to the genus *Trypanosoma* have evolved towards regulating gene expression without classical transcription initiation control. Within these parasites are *T. brucei*, the causative agent of Sleeping Sickness in Sub-Saharan Africa[1], and *T. cruzi*, causing Chagas Disease in the Americas[2]. These parasites have complex life cycles, which involve an hematophagous insect vector and a vertebrate host, each one hosting different life forms with unique characteristics and metabolisms[3]. Unfortunately, there are no effective vaccines against these parasites, and current drug treatments are highly toxic, present low tolerance and require long patient compliance[4]. Although the current drug treatments can be effective during the acute stage of the infection, there is a need for newer, safer and more effective treatments against these neglected diseases.

One peculiarity of these parasites is that their genomes have protein-coding genes organized into large directional gene clusters[5,6], which lack canonical RNA polymerase Ⅱ promoters. Instead, histone variants and epigenetic regulation seem to mark the boundaries of yet undefined transcription start sites for these long transcriptional units[7,8]. The resultant polycistronic immature mRNAs are then processed by coordinated trans-splicing and polyadenylation events, giving rise to mature monocistronic mRNAs[9]. However, protein-coding sequences in each trypanosomatid transcriptional unit are not functionally related as they are in bacterial operons[5]. From this point onwards, posttranscriptional regulation of gene expression heavily contributes to the levels of the protein products that are synthesized, depending on the cell's requirements. The expression of functionally related mRNAs seems to be controlled in a coordinated fashion as posttranscriptional regulons[10]. Within this view, structural motifs enriched in 3' untranslated regions seem to govern the fate of the mRNA molecule inside the cell. Different transcripts carrying the same signature motifs are likely to be regulated in a similar way[11]. These structural motifs serve as ligands for different RNA-binding proteins (RBPs) that associate with the mRNA, forming a ribonucleoprotein (RNP) complex, which is also composed by many other proteins that interact with different parts of the transcript. Consequently, the presence or absence of different RBPs, which in turn can recruit other factors to the RNP complex, likely dictates the fate of the mRNA inside the cell. RBPs interact with mRNA motifs using dedicated functional domains such as the RNA-Recognition Motif (RRM), the Zinc Finger domains, the Pumilio domain and the ALBA domain, to name the most relevant domains found in trypanosomatid RBPs[12].

Trypanosomes have been the muse for the identification of novel biological mechanisms such as RNA trans-splicing, mitochondrial RNA editing, and antigenic variation[13], allowing us to understand their similarities and differences with higher eukaryotic organisms. Identification and characterization of RBPs in trypanosomes started almost 20 years ago. However, it was the concerted sequencing and annotation of their genomes 10 years ago that handled the field with the tools to characterize factors and deeply describe these mechanisms in trypanosomes[14,15]. While *T. cruzi* lacks some the components required for a functional RNA interference (RNAi) machinery, in *T. brucei* mRNA levels can be easily downregulated by this mechanism[16], making this organism the workhorse in the field. The usefulness of omics-approaches in trypanosomes has also allowed the identification of the mRNA molecules affected when a certain RBP is down or upregulated[17]. This, added to the current ability to identify the associated mRNA molecules of a given RBP by RNA-crosslinking and sequencing technologies[18], can give a deeper understanding of the role of the RBP under study. However, our current understanding of RBP function in trypanosomes is rather static, and is far from reflecting the dramatic changes in posttranscriptional gene expression these protozoa suffer during their life cycles. In their mammalian and insect hosts, these parasites adopt different life forms that are adapted to environmental changing conditions. Thus, any single factor modulating gene expression in trypanosomes needs to be regulated in order to cope with the changing developmental and environmental conditions they face.

Here, we will expose the yet poorly explored regulation of the function of trypanosomatid RBPs, emphasizing on those involved in the modulation of cytoplasmic mRNA levels and translation. Regulation is probably exerted at different levels: RBP expression for a given developmental stage, localization-mediated

Table 1 Developmentally expressed RNA-binding proteins in trypanosomes confirmed by experimental data

RBP	Stage	Ref.
T. brucei		
P34	Procyclics	[23]
P37	Bloodstream	[23]
ZFP1	Procyclics	[26]
ZC3H20	Procyclics	[78]
Alba 3	Tsetse fly except transition stages in proventriculus	[79]
Alba 4	Tsetse fly except transition stages in proventriculus	[79]
RBP6	Metacyclics, epimastigotes	[29]
RBP10	Bloodstream	[28]
hnRNPF/H	Mainly bloodstream	[80]
T. cruzi		
UBP2	Mostly epimastigotes, low in amastigotes	[25]
RBP3	Epimastigotes	[81]
RBP4	Epimastigotes	[81]
RBP19	Amastigotes	[82]
ZFP2	Downregulated in metacyclic trypomastigotes	[83]

RBP: RNA-binding protein.

function including nucleocytoplasmic shuttling and nuclear RNA-binding, condensation and sequestering into RNP complexes, and posttranslational modifications (PTMs). We aim to analyze the possible additional layers of complexity that RBPs bring to the gene expression game in trypanosomes.

Developmental regulation of RBP expression

The simplest way to regulate the function of a protein in a time manner is to prevent or promote its synthesis. This is especially relevant for trypanosomes, which alternate between different life forms in their insect and mammalian hosts during which gene expression seems to be regulated essentially at the posttranscriptional level. In these parasites, certain developmental programs were confirmed to be orchestrated by RBPs[12]. Originally, the developmentally regulated expression of each RBP was detected using specific antibodies (Table 1), although current high-throughput approaches rely on the analysis of the transcriptome and the proteome[19-22]. While the latter approaches can give an extensive amount of information on the regulated abundance of mRNAs and proteins, including RBPs, biochemical or genetic validation for each RBP is required to confirm developmental regulation. The first reported case of developmentally regulated RBPs in *T. brucei* was from P34/P37[23]. These are highly similar RRM-containing RBPs that are required for the assembly of the 60S ribosomal subunit[24], being P34 expressed in procyclics and P37 expressed in bloodstream forms[23]. In *T. cruzi*, the RRM-containing RBP UBP2 was the first to show developmental regulation, being expressed in replicative epimastigotes and amastigotes, while it is almost undetectable in cell-derived trypomastigotes[25]. It remains to be determined if overexpression or the lack of expression of P34/P37, UBP2 or other proteins listed in Table 1, can compromise the developmental program of a trypanosome.

Hendriks *et al*[26] demonstrated that genetic ablation of ZFP1 in *T. brucei* prevented the correct repositioning of the kinetoplast, a specific configuration for the mitochondrial DNA present in trypanosomes and leishmanias[27]. This phenotype was associated with the incomplete morphological differentiation from bloodstream to procyclic form *T. brucei* parasites[26], suggesting a role for ZFP1 in *T. brucei* bloodstream form differentiation. RBP10, which is another RRM-containing protein, showed to be enriched in bloodstream-form *T. brucei* cells[28]. Knock-down of RBP10 by RNAi in bloodstream forms resulted in the downregulation of transcripts with high abundance in this stage, while overexpression of RBP10 in procyclics revealed an increased abundance of bloodstream specific transcripts[28]. Hence, manipulation of RBP10 protein levels seems to modulate the abundance of specific developmentally regulated transcripts, although there was no commitment to a differentiation event. RBP6-mediated developmental differentiation in *T. brucei* is probably the most relevant finding regarding developmental expression[29] (Figure 1A). A genome wide high-throughput RNA-seq analysis revealed RBP6 transcripts in *T. brucei* parasites from the proventriculous were enriched 13-times relatively to procyclics from the midgut. When Kolev *et al*[29] overexpressed RBP6 in cultured procyclic cells, they observed developmental stages that are normally observed in the insect vector and not in culture, namely long and short epimastigotes and metacyclic trypomastigotes (Figure 1A). RBP6-overexpressing parasites in culture showed the hallmarks of undergoing metacyclogenesis, and could be used to infect mice, showing the full potential of an infective form. All these examples reveal the role of developmentally regulated RBPs in the modulation of the abundance of life form-specific transcripts, pointing to these and other RBPs as master factors regulating developmental programs.

Regulation of RBP localization

A potential way to regulate the function of an RBP is to limit where in the cell it can associate to its RNA target. Although we are beginning to understand the complexity of RBP-mediated posttranscriptional regulation of gene expression, current models often describe static events, where the RBP is functional once it is associated with the RNA. For many RBPs directly involved in nuclear RNA metabolism, or for those showing high sequence identity to characterized factors involved in conserved nuclear mechanisms, a nuclear localization would make sense for the expected function. This has been the case for the LA protein[30], the exoribonuclease XRNE involved in pre-rRNA processing[31], the nuclear cap binding protein CBP20[32], the Splice-Leader RNP complex associated Sm proteins[33], or Lsm proteins[34], to name a few. Notwithstanding this, nuclear localization for other functionally uncharacterized RBPs like ZFP8 in *T. cruzi* requires further understanding of the function of the protein to assign it to a nuclear function[35]. This

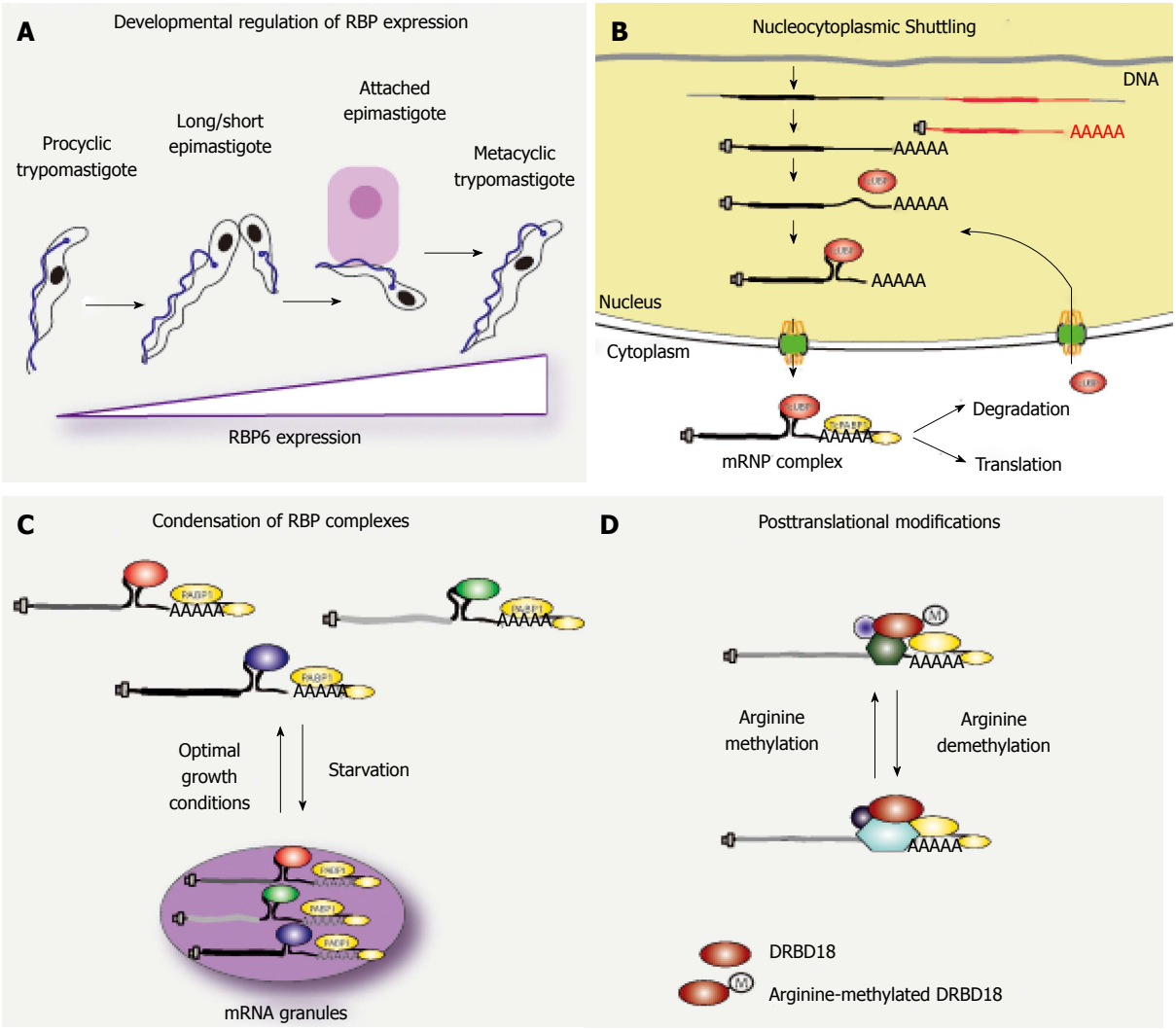

Figure 1 Regulation of RNA-binding proteins function in trypanosomes. Each panel summarizes the proposed mode of regulation of the function of RNA-binding proteins in trypanosomes.

was the case of the nuclear protein RBP33 in *T. Brucei*, which was suggested to be involved in the regulation of nuclear gene silencing[36]. Nuclear localization signals in trypanosomes are difficult to detect because they are not usually predicted by the algorithms developed for mammalian proteins[30,37], adding another layer of complexity to the characterization of RBPs targeted to the nucleus.

For most models of cytoplasmic RBP function there is no information on where and when the RNP complex is formed. This is especially relevant in posttranscriptional regulation, which is believed to happen mostly in the cytoplasm, but has a main actor (the mRNA) with a nuclear history. In *T. brucei* bloodstream forms, most mRNA half-lives are under 20 min, with few long lived mRNAs with half lives of more than 2 h[17]. In *Saccharomyces cerevisiae*, expression dynamics of RBPs showed an increased protein abundance and higher half-life as compared to non-RBP proteins[38]. If this is also the case for trypanosomes, it could suggest that a single RBP molecule might complete more than one cycle of RNP complex biogenesis and degradation. In mammalian cells, RNP complex remodeling and recycling of RBPs has been studied from a nuclear perspective, with many factors associating to mRNA in the nucleus, travelling together through the nuclear pore complex and returning to the nucleus for another event of RNP biogenesis[39]. It is clear that analyzing a snapshot for the localization of an RBP will tell where the molecule spends most of its time, but will fail to determine whether it spends time in other cellular locations albeit transiently. The evidence of RBP nucleocytoplasmic shuttling in trypanosomes points to a possible nuclear step for proper cytoplasmic function[40]. This hypothesis arose from the behavior of six single RRM-containing RBPs (UBP1-2, RBP3-6) in *T. cruzi*[37]. In this case, these cytoplasmic proteins reversibly accumulated in the nucleus when cells were stressed with sodium arsenite. Although this is not a physiological stressor, its use allowed the nuclear accumulation of these RBPs, events that would have been undetected under normal conditions[40]. Active transcription and an intact RRM were required for

Table 2 List of components identified in starvation mRNA granules in trypanosomes

Component	Organism
poly(A)	*T. brucei, T. cruzi*
UBP1	*T. brucei, T. cruzi*
UBP2	*T. cruzi*
RBP3	*T. cruzi*
RBP4	*T. cruzi*
RBP5	*T. cruzi*
RBP6b	*T. cruzi*
PABP1	*T. brucei, T. cruzi*
PABP2	*T. brucei, T. cruzi*
DRBD3	*T. brucei*
DRBD4	*T. brucei*
ALBA1	*T. brucei*
ALBA2	*T. brucei*
ALBA3	*T. brucei*
ALBA4	*T. brucei*
ZFP3	*T. brucei*
DHH1	*T. brucei, T. cruzi*
XRNA	*T. brucei, T. cruzi*
SCD6	*T. brucei*
PBP1	*T. brucei*
MKT1	*T. brucei*
CAF1	*T. brucei*
NOT1	*T. brucei*
NOT5	*T. brucei*
UPF1	*T. brucei*
DBP1	*T. brucei*
AGO1	*T. brucei*
eIF4G1	*T. brucei*
eIF4G4	*T. brucei*
Serine-threonine kinase receptor-associated protein	*T. brucei*
mRNA cap guanine-N7 methyltransferase	*T. brucei*
Phosphatase	*T. brucei*
Methyltransferase	*T. brucei*

UBP1 nuclear accumulation, suggesting dependence on nuclear availability of newly synthesized mRNAs for this to happen[37]. Colocalization of UBP1 with target transcripts and poly (A) mRNA in the nucleus suggests that arsenite could be inhibiting nuclear export of mRNA. In the same line of evidence, the cytoplasmic DRBD3 in *T. brucei* showed a similar response to arsenite stress[41], showing that similar events of nucleocytoplasmic shuttling could be affected by arsenite in both species. Also in *T. brucei*, PABP2 could accumulate in the nucleus under a combined stress of heat shock and sinefungin addition[42], a drug that prevents trans-splicing of mRNA by inhibiting Cap4 formation of Splice leader molecules[43]. All these evidences point towards a nuclear mRNA-binding event taking place during mRNP biogenesis (Figure 1B). It remains to be determined if this nuclear event is required for the function of these RBPs in the cytoplasm.

Regulation of RBP interactions and condensation in RNP complexes

In the cytoplasm of trypanosomatid cells mRNA molecules are associated with general and specific RBPs. It is believed that these specific RBPs will dictate the fate of the mRNA[44]. Thus, mRNA molecules and RBPs are distributed in RNP complexes throughout the cytoplasm. However, these RNP complexes can be very different in protein composition from each other, with proteins colocalizing in some foci but not in every one of them[45].

Under starvation conditions in *T. cruzi* and *T. brucei*, large cytoplasmic granules are formed as a result of the condensation of different RNP complexes[46]. Several evidences suggest that these are not genuine Stress Granules, and consequently were named mRNA granules, since these were characterized based on the dynamics of the mRNA[46]. The list of proteins colocalizing with mRNA granules is in expansion (Table 2), since these structures have been a valuable tool to involve a putative RBP in mRNA metabolism in trypanosomes. No ribosomal proteins from the 40S or 60S subunits were detected in mRNA granules in *T. cruzi*[46], suggesting that the transcripts accumulating in these structures have already exited translation. In *Saccharomyces cerevisiae*, metabolic enzymes also reorganize from a homogenous distribution in the cytoplasm to reversible granule-like assemblies under starvation[47]. In this work it was proposed that the recruitment of enzymes to cytoplasmic foci during the nutrient deprivation period could function as a reservoir of critical cellular components for cell-cycle reentry once nutrients are readily available. This would empty the cytoplasm of these proteins, and would prevent their function due to condensation in granules. This hypothesis is especially relevant in trypanosomes for proteins with enzymatic activity like the exoribonuclease XRNA, which is involved in 5′ to 3′ enzymatic digestion of highly unstable and developmentally regulated mRNA[48] (Figure 1C). XRNA is in fact a component of starvation mRNA granules, where it does not seem to be promoting mRNA degradation. In fact, the transcripts stored in mRNA granules in trypanosomes are intact, since these do possess a poly(A) tail and the characteristic mini-exon at the 5′ end[46,49]. This, added to the fact that transcripts in granules can reenter translation if a new source of nutrients is available[50], suggests that these structures can play a role as reservoirs of transcripts during a physiological stress like starvation. This setup would provide fast templates for synthesis of cellular components that would allow differentiation or cell-cycle re-entry.

Current evidence failed to identify a single RBP required for the formation of mRNA granules, although the overexpression of SCD6 is sufficient to induce the formation of aggregates similar to starvation granules, which contain mRNA, DHH1 and eIF4E[49]. However, depletion of SCD6 by RNAi in *T. brucei* did not affect the formation of starvation mRNA granules[51], suggesting that redundant factors might be involved in the formation of these structures. Of the experimentally validated components of these structures, many contain RNA-binding domains (RBD), such as the RRM or ALBA domain, or contain domains involved in interactions with other mRNA metabolism-related proteins (Table 2). Very recently, Fritz *et al*[52] developed a very ingenious approach to purify starvation induced mRNA granules

that is based on the release of these structures from the subpellicular microtubule cage after cytoskeleton depolymerization, followed by mass spectrometry (MS)[52]. By this approach, they could detect many proteins already identified in mRNA granules, and many new components, 17 of which could be efficiently validated by eYFP-fusion protein colocalization to mRNA granules markers. These included RNA metabolism related proteins, as well as one phosphatase, one methyltransferase, and four hypothetical proteins (Table 2).

Other domains accompanying RBDs in RBPs can provide a scaffold for protein interactions and additional regulation[53]. This is the case of low complexity (LC) sequences, defined by low information content due to the repetition of a few amino acids. LC sequences enriched in RBPs and mRNA metabolism related proteins resemble prion-like domains, allowing these proteins to self-assemble. For instance, the yeast Lsm4 protein, a core P-body component, appears to be involved in granule formation by self-aggregation of its Q/N rich domain[54]. Reijns *et al*[55] identified 20 yeast P-body proteins with above average Q/N content, and demonstrated a reduced association to these structures when these sequences were removed from Ccr4p, Pop2p and Dhh1p. In trypanosomes, proteins like UBP1, UBP2, RBP3 and RBP6, which contain Q-rich sequences, are found in starvation mRNA granules[46]. In spite of this, the presence of a functional RRM is necessary and sufficient for the localization of UBP1 to these structures, showing a direct association to transcripts in starvation mRNA granules for this RBP[46]. Q or G-rich sequences were also identified in many other RRM-type RBPs[56], and a poly-Q sequence has been proposed to be a motif involved in the interaction of MKT1 with several mRNA-related proteins[57]. In mammalian cells, the LC sequence of Fused in Sarcoma (FUS) is able to form reversible hydrogel droplets *in vitro*, which can trap other LC sequence-containing molecules[58]. These sequences are predicted to form intrinsically unstructured domains, and were shown to orchestrate the dynamic assembly of RNP granules in yeasts and mammalian cells through homotypic and heterotypic associations[59]. Thus, the polymeric association of different RBPs in RNP complexes seems to control the dynamic assembly of RNA granules. It is therefore the presence of LC sequences, or a combination of these with an RBD, that could be regulating the function of an RBP, and probably of the bound mRNA, through their recruitment to RNP granules.

To add a layer of complexity, Han *et al*[60] demonstrated that phosphorylation of FUS LC sequence inhibits its association to homotypic hydrogel droplets. The hypothesis arising from these results is that regulatory phosphorylations can prevent LC sequence-containing RBPs to transition into the RNA granule, thus regulating RNP formation and composition[61]. This hypothesis guides our review to probably the most important, yet the less explored, way to modulate the function of an RBP in trypanosomes.

Regulation by PTMs

PTM of proteins has shown to increase the functional diversity of the proteome by the covalent addition of functional groups, peptides or proteins[62]. Amongst the many PTM a protein can suffer, we will discuss here those that were shown to modify cytoplasmic or nuclear RBPs in trypanosomes, namely phosphorylation, SUMOylation, and arginine-methylation. To understand the role these PTMs on RBPs, and many other proteins, will have a huge impact in trypanosomes, since these modifications involve one or more enzymes that could be used as targets for pharmacological intervention for these parasitic diseases.

It was more than 20 years ago when the first RBP with PTMs was identified in *T. brucei*. This was the case of Nopp44/46, which is an RBP belonging to a family composed by several members with different number of RGG repeats[63]. Nop44/46 is phosphorylated on serine and tyrosine residues, and has been proposed to be required for formation of the large ribosomal subunit in *T. brucei*[64]. Numerous proteomic approaches have recently been used to identify phosphorylated proteins in *T. brucei* and *T. cruzi* (Table 3), since phosphorylation events are especially relevant due to their impact in a broad spectrum of processes and regulatory functions[65]. In *T. brucei*, the phosphoproteome of the bloodstream form was the first to be explored based on phosphopeptide enrichment coupled to MS[66]. In this study, serine, threonine and tyrosine phosphorylated residues were detected, showing conserved phosphorylation sequence motifs in regulatory kinases, suggesting that phosphorylation-based signaling mechanisms are conserved in trypanosomes[66]. However, a previous genome wide survey for protein kinases in *Leishmania*, *T. brucei* and *T. cruzi* showed these parasites lack receptor-linked tyrosine and tyrosine kinase-like kinases, but instead posses dual specificity kinases[67]. Urbaniak and collaborators[68] reported a quantitative phosphorylation study of the *T. brucei* bloodstream and procyclic forms by SILAC labeling. The results from this work revealed a significant number of phosphorylated ZFPs, RRM-type RBPs, and mRNA metabolism-related proteins (Table 3), some of which showed lifecycle specific regulation of phosphorylation status. In this work it was proposed that the dynamic phosphorylation of RBPs could have the potential to modulate gene expression, as these proteins could be acting as ultimate effector molecules of some of the trypanosomatid signaling cascades[68]. *T. cruzi* has also been explored extensively at the phosphoproteomic level[69-71], revealing many new and already *T. brucei* identified phosphorylated RBP and related proteins (Table 3). In a very interesting study, Queiroz *et al*[71] performed a comparative proteomic and phosphoproteomic analysis during amastigogenesis, the transition from non-replicating and infective trypomastigotes to replicating non-infective amastigotes. From these analyses several

Table 3 RNA-binding proteins and mRNA metabolism-related proteins with identified posttranslational modifications in trypanosomes

RBP	*T. brucei*	*T. cruzi*	Posttranslational modification
PUF domain			
PUF1	Tb927.10.4430	TcCLB.397937.10/TcCLB.508625.160	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[71]
PUF2	Tb927.10.12660	TcCLB.507831.110	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[69]
PUF3	Tb927.10.310	TcCLB.508787.30/TcCLB.468005.9	Phosphorylated in *T. brucei*[66,68]
PUF4	Tb927.6.820	TcCLB.509399.190/TcCLB.510073.30	Phosphorylated in *T. brucei*[66,68]
PUF6	Tb927.10.15870	TcCLB.510125.10	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[71] Sumoylated in *T. cruzi*[73]
PUF7	Tb927.11.14960	TcCLB.508445.99/TcCLB.511715.100	Sumoylated in *T. cruzi*[73]
PUF9	Tb927.1.2600	TcCLB.506563.10/TcCLB.503869.40	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
PUF10	Tb927.11.6740	TcCLB.506773.130/TcCLB.508799.70	Arginine-methylated in *T. brucei*[75]
PUF11	Tb927.11.10810	TcCLB.503757.30/TcCLB.503719.39	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
RRM domain			
PABP1	Tb927.9.9290	TcCLB.506885.70	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70,71] Sumoylated in *T. cruzi*[73]
PABP2	Tb927.9.10770	TcCLB.508461.140	Phosphorylated in *T. brucei*[68]
UBP1	Tb927.11.500	TcCLB.507093.220	Phosphorylated in *T. cruzi*[84]
RBP3	Tb927.11.530	TcCLB.507093.250	Phosphorylated in *T. brucei*[68]
RBP8	Tb927.7.320	TcCLB.504165.20/TcCLB.508981.20	Phosphorylated in *T. brucei*[68]
RBP9	Tb927.11.12120	TcCLB.511127.10/TcCLB.511481.70	Phosphorylated in *T. brucei*[68]
RBP10	Tb927.8.2780	TcCLB.508989.30/TcCLB.509569.120	Phosphorylated in *T. brucei*[66,68]
RBP12	Tb927.10.12740	TcCLB.511277.200	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[71]
U1 snRNP (RBP24)	Tb927.8.4830	TcCLB.508837.140/TcCLB.511383.30	Phosphorylated in *T. brucei*[68]
RBP28	Tb927.3.1030	TcCLB.511871.110/TcCLB.511863.20	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
RBP29	Tb927.10.13720	TcCLB.511277.580	Phosphorylated in *T. cruzi*[69,71]
RBP30	Tb927.5.1750	TcCLB.505229.20/TcCLB.510823.20	Phosphorylated in *T. brucei*[68]
RBP33	Tb927.8.990	TcCLB.503733.50/TcCLB.508569.90	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
RBP35	Tb927.9.12360	TcCLB.510741.40	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70]
RBP38	Tb927.11.5850	TcCLB.508641.180/TcCLB.508515.30	Phosphorylated in *T. brucei*[68]
RBP42	Tb927.6.4440	TcCLB.509167.140	Phosphorylated in *T. cruzi*[71]
RBSR1	Tb927.9.6870	TcCLB.506625.70	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[69]
RBSR4	Tb927.10.3500	TcCLB.510265.40/TcCLB.510311.50	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
DRBD2	Tb927.9.13990	TcCLB.510755.120/TcCLB.508413.50	Phosphorylated in *T. cruzi*[69]
DRBD5	Tb927.6.3480	TcCLB.469785.40/TcCLB.507025.50	Phosphorylated in *T. brucei*[68]
DRBD7	Tb927.4.400	TcCLB.507873.30/TcCLB.510689.60	Phosphorylated in *T. brucei*[68]
DRBD10	Tb927.11.16020	TcCLB.507037.20/TcCLB.508707.80	Phosphorylated in *T. brucei*[68]
DRBD11	Tb927.3.3940	TcCLB.503683.30/TcCLB.509999.120	Phosphorylated in *T. brucei*[68]
DRBD13	Tb927.8.6650	TcCLB.506399.40/TcCLB.509243.20	Phosphorylated in *T. brucei*[68]
DRBD17	Tb927.8.710	TcCLB.507649.100/TcCLB.508567.100	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
DRBD18	Tb927.11.14090	TcCLB.511727.190	Arginine-methylated in *T. brucei*[75]
HNRNPH	Tb927.2.3880	TcCLB.511109.130/TcCLB.504157.10	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70,71] Sumoylated in *T. cruzi*[73]
TRRM1	Tb927.2.4710	TcCLB.509317.60/TcCLB.511621.50	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[69] Sumoylated in *T. cruzi*[73]
TRRM3	Tb927.3.3670	TcCLB.506989.100/TcCLB.510149.140	Arginine-methylated in *T. brucei*[75]
PPCTI	Tb927.5.3750	TcCLB.503619.20/TcCLB.511647.40	Phosphorylated in *T. brucei*[68]
MRD1	Tb927.8.4170	TcCLB.509561.110/TcCLB.503897.90	Phosphorylated in *T. brucei*[68]
TSR1	Tb927.8.900	TcCLB.509607.30/TcCLB.503715.10	Phosphorylated in *T. brucei*[68]
NRBD1/2 (P34/P37)	Tb927.11.14000/Tb927.11.14020	TcCLB.511727.270/TcCLB.511727.290	Arginine methylated in *T. brucei*[75]
Conserved	Tb927.10.7030	TcCLB.506779.100/TcCLB.511153.100	Arginine-methylated in *T. brucei*[75]
Conserved	Tb927.11.7310	TcCLB.506779.100/TcCLB.511153.100	Arginine-methylated in *T. brucei*[75]
Conserved	Tb927.11.14090	TcCLB.511727.190	Arginine-methylated in *T. brucei*[75]
Conserved	Tb927.11.6240	TcCLB.506297.230/TcCLB.510101.80	Arginine-methylated in *T. brucei*[75]
Zinc finger domains			
ZFP1	Tb927.6.3490	TcCLB.511511.6	Phosphorylated in *T. brucei*[66,68]
ZFP3	Tb927.3.720	TcCLB.509719.69/TcCLB.509231.39	Phosphorylated in *T. brucei*[68]
ZC3H5	Tb927.3.740	TcCLB.511867.10/TcCLB.507775.10	Phosphorylated in *T. brucei*[68]
ZC3H7	Tb927.3.1340	TcCLB.509233.210	Phosphorylated in *T. brucei*[68]
ZC3H8	Tb927.3.5250	TcCLB.510143.120/TcCLB.508409.310	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
ZC3H11	Tb927.5.810	TcCLB.507305.40	Phosphorylated in *T. brucei*[68,85]
ZC3H12	Tb927.5.1570	TcCLB.510819.119/TcCLB.506739.99	Phosphorylated in *T. brucei*[86]
ZC3H13	Tb927.5.1580	No homologue	Phosphorylated in *T. brucei*[66,68]

ZC3H17	Tb927.7.930	TcCLB.508879.10/TcCLB.508215.10	Phosphorylated in *T. brucei*[68]
ZC3H18	Tb927.7.2140	TcCLB.511807.160	Phosphorylated in *T. brucei*[68]
ZC3H20	Tb927.7.2660	TcCLB.503567.9/TcCLB.506859.204	Phosphorylated in *T. brucei*[68]
ZC3H21	Tb927.7.2670	TcCLB.506859.230/TcCLB.511817.10	Phosphorylated in *T. brucei*[68]
ZC3H22	Tb927.7.2680	TcCLB.506859.240/TcCLB.511817.20	Phosphorylated in *T. brucei*[68]
ZC3H23	Tb927.7.4980	TcCLB.509149.20/TcCLB.508175.350	Phosphorylated in *T. brucei*[68]
ZC3H28	Tb927.9.9450	TcCLB.506885.200/TcCLB.510729.220	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70]
ZC3H29	Tb927.9.9520	TcCLB.510729.210/TcCLB.506885.204	Phosphorylated in *T. brucei*[68]
ZC3H30	Tb927.10.1540	TcCLB.506977.110	Phosphorylated in *T. brucei*[68]
ZC3H31	Tb927.10.5150	TcCLB.506009.10/TcCLB.510295.59	Sumoylated in *T. cruzi*[73]
ZC3H32	Tb927.10.5250	TcCLB.503795.10 /TcCLB.506679.10	Phosphorylated in *T. brucei*[68]
ZC3H34	Tb927.10.12330	TcCLB.507787.140/TcCLB.507625.70	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
ZC3H35	Tb927.10.12740	TcCLB.511263.30/TcCLB.507831.40	Phosphorylated in *T. brucei*[68]
ZC3H37/38	Tb927.10.12780	TcCLB.507831.20/TcCLB.511267.20/ TcCLB.511263.50	Phosphorylated in *T. brucei*[68]
ZC3H39	Tb927.10.14930	TcCLB.508895.50	Phosphorylated in *T. brucei*[68]
ZC3H40	Tb927.10.14950	TcCLB.508895.60/TcCLB.506211.60	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[69,71]
ZC3H41	Tb927.11.1980	TcCLB.508355.330/TcCLB.508357.9	Phosphorylated in *T. brucei*[68]
ZC3H43	Tb927.11.7450	TcCLB.511151.20/TcCLB.508241.90	Phosphorylated in *T. brucei*[68]
ZC3H44	Tb927.11.7890	TcCLB.506933.50	Phosphorylated in *T. brucei*[68]
ZC3H46	Tb927.11.16550	TcCLB.507089.30/TcCLB.504085.70	Phosphorylated in *T. brucei*[68]
ZC3H47	Tb927.6.4960	TcCLB.506945.210	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[69]
Conserved	Tb927.11.3970	TcCLB.509229.90/TcCLB.506733.140	Phosphorylated in *T. brucei*[68]
Conserved	Tb927.9.14120	TcCLB.510759.100/TcCLB.506999.120	Arginine-methylated in *T. brucei*[75]
Other			
NOP44/46	Tb927.8.760	TcCLB.510859.17/TcCLB.510859.10	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
SMD2	Tb927.2.5850	TcCLB.508667.49/TcCLB.511189.80	Phosphorylated in *T. brucei*[68]
DCL2	Tb927.3.1230	No homologue	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
CAF1	Tb927.6.600	TcCLB.511827.60/TcCLB.510535.60	Phosphorylated in *T. brucei*[68]
Not1	Tb927.10.1510	TcCLB.509247.30	Phosphorylated in *T. cruzi*[70]
XRNA	Tb927.7.4900	TcCLB.507817.80	Phosphorylated in *T. cruzi*[69]
ALBA3	Tb927.4.2040	TcCLB.510877.40	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
ALBA4	Tb927.4.2030	TcCLB.510877.30	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
eIF4E	Tb927.11.11770	TcCLB.508827.30	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70]
	Tb927.6.1870	TcCLB.509037.40/TcCLB.421959.10	Phosphorylated in*T. brucei*[68]
eIF4G2	Tb927.9.5460	TcCLB.508277.340/TcCLB.506445.20	Phosphorylated in *T. brucei*[68]
	Tb927.11.10560	TcCLB.510285.100/TcCLB.504827.130	Phosphorylated in *T. brucei*[68]
eIF4G5	Tb927.8.4500	TcCLB.508989.90	Phosphorylated in *T. brucei*[68]

RBP: RNA-binding protein; RRM: RNA-recognition motif.

RBPs and mRNA-related proteins were identified as being developmentally regulated between the final morphological stages, or during the transformation process[71]. To note was that several RBPs also showed developmental phosphorylation, suggesting that these modifications could be regulating the function of these proteins. It has been suggested that phosphorylation may induce global conformational changes in an RBP, either promoting or inhibiting protein-protein or protein-RNA interactions allosterically[72]. Thus, regulatory phosphorylation events in RBPs could have an enormous impact on gene expression regulation in trypanosomes due to the prevalence of posttranscriptional mechanisms in these parasites.

In another study Bayona *et al*[73] reported the functionality of the small ubiquitin-like modifier (SUMO) pathway in *T. cruzi*. The addition of SUMO is a covalent and reversible modification that can usually affect a protein's normal function. Initially SUMOylation was believed to be only involved in nuclear events such as nucleocytoplasmic transport, DNA replication, gene transcription and DNA damage response, but with the identification of numerous other targets it now seems that SUMOylation can affect numerous processes at both sides of the nuclear envelope[74]. Within the many proteins modified by SUMO addition in *T. cruzi*, some belong to the RBP category (Table 3)[73]. Given that this type of modification is usually very unstable and it is lost during typical experimental manipulations, adequate experimental approaches will be required in the future to address the effect of SUMOylation on RBPs in trypanosomes.

Last but not least, the potential of arginine-methylation was explored by a global proteomic analysis in *T. brucei*[75]. In this work, methylated arginine residues were identified mainly in glycine-rich contexts, as described in other organisms. The functional classification of proteins derived from this work revealed an interesting amount of proteins involved in mRNA metabolism (Table 3)[75], and other related proteins. The relevance of this finding in *T. brucei* is highlighted by the impact that arginine-methylation can have on protein-protein and protein-nucleic acid interactions and subcellular localization of proteins[76]. DRBD18, a double RRM-containing RBP, was initially identified as having three methyl-arginines[75]. In a posterior characterization of DRBD18 in *T. brucei*, methylmimetic or hypomethylated mutants were expressed in parasites

downregulated for endogenous DRBD18. Both mutants exhibited a differential modulation of the *T. brucei* transcriptome, clearly showing opposing effects of due to methylarginine content[77]. Surprisingly, these differences did not seem to be regulated at the level of subcellular localization or affinity for RNA molecules, since both mutants showed similar association to RNA molecules in the cytoplasm. Instead, the methylation state of DRBD18 arginine residues seemed to modulate the interactions of these DRBD18 variants with other protein factors involved in mRNA metabolism, since they showed to associate to different RNP complexes (Figure 1D). Thus, differential arginine-methylation was proposed to shape the composition of DRBD18 RNP complexes, and thus to modulate the fate of the associated mRNA[77].

CONCLUSION

Although the above-described events might seem yet unrelated, future work might provide evidences to connect different mechanisms to regulate the function of an RBP in trypanosomes. This is especially relevant for localization, aggregation and interaction events that might be regulated by posttranslational modifications. Given that RBPs do not have enzymatic activities, and hence cannot be tested as druggable targets, it is tempting to speculate that the enzymes (kinases, phosphatases, SUMO E3 ligases and methyltransferases) responsible for these posttranslational modifications might be indeed good candidates for drug therapy. Approaches targeting any of these protein modifications are likely to have a very high impact for these neglected diseases, either by discovery of new drugs or by drug repurposing.

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