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RNA Granules Living a Post-transcriptional Life: the Trypanosomes' Case

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Abstract

Trypanosomes are protozoan parasites responsible for recalcitrant infectious diseases such as Sleeping sickness and Chagas disease in Africa and America, respectively. Their complex life-cycles are accompanied by alternation of forms specific of the insect vectors and vertebrate hosts, each with different metabolic and structural requirements. Unlike most other eukaryotes, these single-cell microorganisms seem to control the expression of protein-coding genes mostly by mRNA degradation, silencing and translation efficiency. Recent evidence showed that genuine cytoplasmic Stress Granules are formed as a response to heat stress in *Trypanosoma brucei*, basically formed by stalled translation initiation complexes on mRNA. On the other hand, Processing bodies (P bodies) are constitutive components of cytoplasmic mRNA metabolism in trypanosomes, which could have an important role in translational repression. During physiological starvation conditions in trypanosomes, components from P bodies fuse with other ribonucleoprotein complexes to form mRNA granules, where transcripts are stored and protected from degradation in a quiescent state. Other novel types of *foci* with unknown function that are related to RNA metabolism can be found in these parasites, namely heat-induced granules containing the 5' to 3' exoribonuclease XRNA, and starvation-induced granules containing transfer RNA halves. Thus, trypanosomes make use of non-membranous structures as a strategy to compartmentalize ribonucleoprotein complexes in the cytoplasm, aiding to cope with stressful situations avoiding mRNA translation or degradation. The relevance of stress-induced *foci* in trypanosomes has yet to be scored, although recent evidence suggests that these cytoplasmic organelles are required for survival under adverse growing conditions.

Keywords

gene expression; P bodies; post-transcriptional; RNA granules; RNA-binding protein; Stress Granules; *Trypanosoma*

1. INTRODUCTION

The information in protein-coding genes has to overcome many checkpoints until it is decoded by ribosomes. Several mechanisms regulate the amount of protein that is synthesized in time and space to avoid the appearance of toxic polypeptides due to misfolding or truncation. Gene expression is leaded by DNA transcription, during which several transcription factors are recruited to a promoter and mediate RNA polymerase (RNA

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pol) II binding, thus initiating RNA synthesis. The complexity of transcription initiation varies from unicellular organisms to metazoans, and trypanosomes are a particular case. These protozoa are the etiological agents of neglected diseases such as Sleeping sickness in sub-Saharan Africa and Chagas disease in the Americas, caused by *Trypanosoma brucei* and *T. cruzi*, respectively. The life cycles of these parasites are complex, involving a hematophagous insect vector and a vertebrate host, each one hosting different life-forms with unique characteristics [1]. Both *T. brucei* and *T. cruzi* have a coat of surface proteins that protect them against proteolysis in the insect gut [2, 3], while in the vertebrate host a different surface repertoire permits immune evasion [4, 5]. Also, energy metabolism inside the vertebrate host is completely dependent on substrate-level glucose phosphorylation, while in the insect vector glucose availability is sporadic and depends on blood feeding by the vector [6]. During vector starvation periods trypanosomes rely on the catabolism of amino acids that are abundant in the insect fluids. Treatments with classic drugs have proven to have low effectiveness and high toxicity. Meanwhile, pharmaceutical companies have disregarded a cure against these parasites. This scenario created an interest in the scientific community to seek for differences between the parasite and the human host with potential therapeutic application. These efforts were rewarded by the identification of novel biological processes such as RNA editing, RNA *trans*-splicing and antigenic variation amongst others [7].

DNA transcription in trypanosomes has particular features. Protein coding genes are not interrupted by introns, and have no canonical promoters for RNA pol II [8, 9]. Transcription of protein-coding genes is initiated at few transcription start sites enriched in histone variants with specific epigenetic modifications [10, 11]. Thus, immature messenger RNAs (mRNAs) arise as large polycistronic units that are further processed by the addition of a hypermethylated-capped Splice Leader (SL) molecule at the 5' end of each mature monocistron during RNA *trans*-splicing [12]. Most protein-coding genes in each transcription unit are non-related in function, and usually belong to different metabolic pathways. Therefore transcription initiation does not seem to be a rate-limiting step in maintaining the adequate mRNA levels required for cellular functions. Instead, post-transcriptional mechanisms such as mRNA degradation, silencing and translation efficiency are the core events regulating protein synthesis [8]. Notwithstanding this, relative high expression levels are obtained from genes with high copy number, or by RNA pol I-dependent transcription of surface-protein coding genes [13]. Here, I will briefly review current knowledge on cytoplasmic mRNA metabolism in trypanosomes. Then, I will describe in detail the relevance of RNA granules in these organisms, which manage gene expression essentially at the post-transcriptional level.

2. CYTOPLASMIC mRNA METABOLISM

The evidence obtained from model organisms indicates that mRNP complexes are remodeled as soon as they reach the cytoplasmic side of the nuclear pore complex [14]. As important as nuclear export is for defining mRNA fate, there is no information on how this process takes place in trypanosomes. Recently, a putative ortholog of the yeast mRNA nuclear export factor Mex67 was identified in *T. brucei*, which unexpectedly contained a CCCH motif [15]. It remains to be determined where and when cytoplasmic messenger ribonucleoprotein (mRNP) complexes are formed or remodeled in trypanosomes. Recent evidence in *T. cruzi* and the related kinetoplastid *Leishmania major* suggests that core proteins from mRNP complexes could be binding to the mRNA in the nucleus prior to export [16, 17], where the burden of factors acquired in this compartment could determine mRNA destiny in the cytoplasm [18]. In trypanosomes, transcripts have signature *cis*-acting motifs mainly at the 3' untranslated region (UTR) that can be recognized in *trans* by RNA-

binding proteins (RBPs) thus forming mRNP complexes. The combination of RBPs in the mRNP complex will play a significant role in the cytoplasmic fate of the mRNA [19, 20].

Once in the cytoplasm, the mRNA can be efficiently translated, silenced or degraded. This strategy might look quite expensive for the trypanosomatid cell, as it involves the synthesis of mRNAs that won't achieve their final goal. However, it allows bulk transcription of multi-copy surface protein genes such as procyclins in *T. brucei* and mucin genes in *T. cruzi* [4, 21]. Recent evidence on the co-regulation of cohorts of functionally related transcripts in trypanosomes has placed post-transcriptional operons as a general mechanism to regulate the stability of related mRNAs independently of transcription initiation of individual transcriptional units [22–24]. In this scenario, a genome loaded with RBPs as *trans*-acting factors involved in mRNA regulation may be predicted. Intriguingly, trypanosomes do not possess an increased number of RBPs compared to other organisms [15, 25]. This suggests that the mechanisms of post-transcriptional gene regulation in trypanosomes are conserved with higher eukaryotes, but does not necessarily underscore the complexity or efficiency these mechanisms have acquired through evolution in these protozoa.

3. TRANSLATION INITIATION

The SL molecule that mature trypanosomatid mRNAs acquire during *trans*-splicing contain a complex 5'-end 7-methyl guanosine cap structure that is modified in its first four nucleotides (cap4 structure) [12]. Translation initiation mechanisms have not been studied in detail in trypanosomes. However, the groups of Shapira and de Melo Neto have revealed the composition of the core translation initiation machinery in the related kinetoplastid parasite *Leishmania spp.*, a topic recently reviewed by Clayton and Shapira [26]. Four different cytoplasmic cap binding proteins (eIF4E) have been identified in *Leishmania spp.* [27, 28]. None of these orthologs was able to complement their yeast counterpart [29], presumably due to structural divergence in order to recognize the cap4 structure. The RNA helicase eIF4A-1 and the large scaffolding protein eIF4G-3 have also been identified as part of the eIF4F complex, which is required for ribosome recruitment and translation initiation [30, 31].

Translation initiation is the most regulated step in eukaryotic protein synthesis and is highly influenced by cellular stress. An early step in eukaryotic translation initiation begins with the formation of a ternary complex between GTP, the initiator Methionyl-transfer RNA_i (Met-tRNA_i) and the translation initiation factor eIF2 [32], which is composed by the α , β and γ subunits. While the γ subunit binds GTP and Met-tRNA_i, the α subunit interacts with the guanine nucleotide exchange factor eIF2B [33]. In mammalian cells, distinct types of stress trigger the phosphorylation of eIF2 α at the conserved Ser51 residue by specific eIF2-kinases [34]. This provokes a significant increase in the affinity of eIF2-GDP for eIF2B, therefore sequestering the guanine nucleotide exchange factor in unproductive complexes and preventing new translation initiation events [35].

In *T. brucei*, the eIF2 α ortholog contains an unusually large N-ter extension, and the position of the conserved Ser51 residue is replaced by a Threonine at position 169 [36]. Out of three potential eIF2 α -kinases found in *T. brucei*, only eIF2K2 was able to phosphorylate yeast and mammalian eIF2 α at Ser51, or *T. brucei* eIF2 α at Thr169. On the contrary, yeast GCN2 or mammalian PKR kinases failed to phosphorylate *T. brucei* eIF2 α at Thr169. Whether *T. brucei* eIF2 α has other phosphorylatable residues than Thr169 that could be involved in regulating translation initiation remains to be investigated. Intriguingly, eIF2K2 is a membrane associated protein that localizes to the flagellar pocket of both insect dwelling and bloodstream forms of *T. brucei* [36]. This may be linked to nutrient sensing, since the flagellar pocket is involved in nutrient endocytosis [37]. Even so, eIF2 α is cytoplasmic in *T.*

brucei [38], which opens up the possibility of the existence of undocumented, yet functional cytoplasmic eIF2 α kinases that might have low sequence identity to the orthologs identified in other species.

4. STRESS GRANULES

In mammalian cells, eIF2 α phosphorylation by stress-activated kinases prevents new translation initiation events, resulting in the formation of unproductive and abortive preinitiation complexes on the mRNA. This leads to the formation of microscopically visible cytoplasmic structures called Stress Granules (SGs). SGs only appear under stress conditions and their presence correlates with translational silencing. The protein composition of these structures depends on the type of cell and kind of stress stimuli, but has a core composition of translation initiation factors such as eIF4E, eIF4A, eIF4G, eIF3, eIF2, the poly(A)-binding protein PABP1, and proteins from the small but not the large ribosomal subunit [39, 40]. Other mammalian SGs components are aggregation-prone proteins such as TIA-1, TIAR, G3BP and Lsm4, which nucleate SGs [35]. Nevertheless, the SGs in a single cell may be different in composition. Many excellent reviews on this subject have been published in the recent years, where the composition of SGs and other RNA granules is exhaustively described [34, 41, 42].

The information gathered from SG physiology indicates that these *foci* serve as mRNA triage centers, from where re-initiation, mRNA decay or storage is decided [43], but their function is still an open matter of debate [41, 44]. Sodium arsenite is the stressor of preference to induce the formation of SGs in mammalian cell cultures [45]. The physiologically important targets during arsenite exposure are unknown. Many cellular responses to the exposure of this drug could be the consequence of protein misfolding due to cysteine oxidation by arsenite [46]. SGs can also be induced in mammalian cells by blocking translation initiation downstream of the pre-initiation complex [47].

In *Saccharomyces cerevisiae*, SGs were first identified as novel cytoplasmic structures that appeared under glucose deprivation-stress, and due to the presence of eIF4E, eIF4G and PABP1 were named EGP bodies [48]. These foci were subsequently characterized as yeast SGs based on two characteristics: the recruitment of proteins orthologous to those found in mammalian SGs, and enhanced formation by eIF2 α phosphorylation [49]. Heat shock is also a potent inducer of SGs in *S. cerevisiae* [50], *Drosophila melanogaster* [51], and *T. brucei* [38], which acts independently of eIF2 α phosphorylation. Accordingly, yeast SGs induced by heat-shock have a slightly different protein composition compared to those induced by glucose-deprivation [42, 50].

In *T. brucei*, mammalian-like SGs appear in response to severe heat-shock, which is concomitant with polysome disruption and an overall increase in the decay rate of bulk mRNA [38]. Still, stress induced by arsenite at several concentrations does not seem to engage SGs in *T. cruzi* as revealed by distinct protein markers [16]. SGs in *T. brucei* contain both poly(A)-binding proteins PABP1 and PABP2, all four isoforms of eIF4E (1 to 4), eIF2 α , and eIF3B [38]. Ribosomal proteins from the 40S subunit or eIF4G orthologs have not been tested for their presence in *T. brucei* SGs, but it seems very likely that they might contain these two core constituents (Fig. 1), resembling the composition of mammalian SGs. Heat-shock SGs in *T. brucei* seem to be in equilibrium with polysomes, since cycloheximide prevented their formation, suggesting that polysome disruption is required before SGs are formed. The release of mRNAs from polysomes by treating *T. brucei* parasites with puromycin did not induce the formation of SGs in the absence of a stress insult, suggesting that an unbalance of the equilibrium towards free transcripts is not enough to induce their formation [38]. When the normal growing temperature is restored to the culture of parasites,

SGs protein components restore their normal cytoplasmic localization, suggesting that the stress response is reversible. It could be hypothesized that the transcripts silenced in SGs could return to the translation pool once growth is resumed by the parasites (Fig. 1). Although the precise function of SGs in the physiology of *T. brucei* is not completely understood, their formation could be relevant in a context of environmental heat-stress, avoiding commitment to grow through the silencing/degradation of bulk mRNA (Fig. 1). This condition would allow the translation of Heat-Shock Proteins required to maintain homeostasis until stress is over, or until cells adapt to the new non-lethal growing conditions.

5. PROCESSING BODIES (P BODIES)

In mammalian cells, SGs can interact, share proteins or sometimes completely fuse with P bodies. P bodies were initially identified as cytoplasmic *foci* containing the 5' to 3' exoribonuclease XRN1 [52], the decapping complex Dcp1/Dcp2 and the Lsm1-7 protein complex in mammalian cells [53]. Later on, Sheth and Parker [54] named these structures accordingly, due to the concentration of decapping enzymes and activators found in yeast. Unlike SGs, P bodies are constitutive components of cells, although their size and number can increase with stress [41]. In almost all studied organisms P bodies concentrate proteins involved in decapping and 5' to 3' mRNA degradation (DCP1, DCP2, and XRN1) but exclude exosome components [55, 56]. Ribosomal proteins are also absent from P bodies in all studied species [57], although these *foci* contain a few translation initiation factors, such as eIF4E [57, 58], eIF4G, and PABP1 [48, 59]. In mammalian cells, P bodies contain components of the RNA-induced silencing complex (RISC), namely Argonaute, micro RNAs (miRNAs), and GW182 [60–63]; proteins involved in Nonsense Mediated Decay (NMD) [64], and the translational repressor RCK/p54 (DHH1) [65]. For an exhaustive list of components see the reviews by Buchan and Parker [42], and Thomas *et al.* [34]. Even so, P bodies in a single cell frequently show different composition, [34] likely reflecting that different functions occur in individual P bodies.

P bodies contain mRNA in equilibrium with polysomes, as evidenced by the cycling of transcripts between P bodies and the translating pool in both yeasts [57] and mammalian cells [66]. This dynamics argues for a function in transcript storage, in addition to their role as mRNA degradation factories as initially thought. However, not all untranslated transcript is stored in P bodies [44]. In spite of being ubiquitous, microscopically visible P bodies are not required for decapping, translational repression, mRNA stabilization during stress, miRNA mediated repression, AU-rich element mediated mRNA degradation, or degradation of mRNAs with premature termination codons by NMD in mammals [44]. It seems likely that these functions take place in submicroscopic mRNP complexes, and that exogenously altering macroscopic P body formation does not affect normal mRNA metabolism. In a more general view, the formation of granules could be the consequence of the rise in the concentration of these factors/complexes, removing them from the bulk of the cytoplasm.

The decapping proteins DCP1 and DCP2 have no clear orthologs that can be easily identifiable by sequence identity in the genomes of kinetoplastid parasites, although the decapping enzymatic activity could be detected in trypanosomes extracts [67]. The identification and localization of these enzymes in trypanosomes would represent an important contribution to the field of P bodies, and would provide a marker for these structures in these parasites. Nevertheless, P bodies have been described both in *T. cruzi* and *T. brucei*. The initial characterization of the DEXD/H-box RNA helicase DHH1, and the ortholog of XRN1 (XRNA) in *T. cruzi* showed that these proteins were localized in cytoplasmic *foci* that resembled P body-like structures [68, 69]. P bodies from *T. cruzi* also contain the Pumilio-like protein PUF6 (Fig. 1), which has been shown to colocalize with

DHH1 [70]. PUF6 seems to be involved in the destabilization of mRNA in *T. cruzi*, since target transcripts showed to be down-regulated by overexpression of the protein [70]. Another protein we have identified in P bodies from *T. cruzi* is UBP1, as evidenced by colocalization in parasites expressing XRNA-green fluorescent protein (GFP) (Fig. 2A). UBP1 is one of the most characterized RBPs from trypanosomes, which has been shown to be involved in the selective stabilization or destabilization of target transcripts mainly coding for surface proteins [23, 71, 72]. Strikingly, not all P bodies contain UBP1, and not all microscopically visible UBP1 particles include XRNA (Fig. 2A). This argues for the existence of P bodies with heterogeneous composition in trypanosomes, which could putatively be specific either in function or in mRNA content.

In *T. brucei*, P bodies contain DHH1, XRNA and SCD6, which is the ortholog of the translational silencer RAP55, also known as LSM14 [38]. Apparently, P bodies from both *T. brucei* and *T. cruzi* species contain mRNA in equilibrium with polysomes, as cycloheximide treatment reduces the amount and size of foci by trapping transcripts in the polysomal fraction [38, 69]. Environmental stresses such as heat-shock [38] and carbon source starvation [68, 69] increase the number and size of P bodies in *T. cruzi* and *T. brucei*, respectively (see below). Recently, the expression of a dominant negative DHH1 mutant (E182Q) with abolished ATP hydrolysis activity, was shown to impair *T. brucei* growth [73]. With no over-expression at all, the DHH1 E182Q mutant showed an increase in the size of P bodies and a reduction in polysomes that could not be ascribable to a reduction in total mRNA levels [73]. Given that the ATPase activity of DEAD-box helicases is required for recycling onto mRNP complexes, but not for RNA helicase activity [74], it could be speculated that the DHH1 E182Q mutant from *T. brucei* spends more time on mRNA targets, increasing translational repression by kidnapping translatable mRNAs. This interaction seems to be extremely transient as DHH1, or the E182Q mutant, have not been detected in polysomal fractions from *T. brucei* [73] or *T. cruzi* [69]. Also, the E182Q mutant showed to be absent from P bodies in half of the cells, suggesting that there is not a clear route from polysomes to P bodies for the mRNAs that are targeted by DHH1 [73]. Further work will determine if the effect of the DHH1 mutant on reducing polysomes and enhancing P bodies is the cause or the consequence of growth arrest of the parasites. Recent evidence in *S. cerevisiae* suggests that decapping can occur in polysomes [75], with a concomitant degradation of the mRNA. This leads to ask which functions take place in P bodies from unstressed trypanosomes, and if mRNAs within P bodies can be directly degraded in a 5' to 3' direction after decapping.

In addition to P bodies enhancement, the heat-shock response in *T. brucei* induces the formation of a new type of granule composed by XRNA, and that exclude DHH1 and SCD6, at the posterior end of the cell [38] (Fig. 1). It is relevant to point out that the GFP fusions of XRNA forming these granules are expressed from an endogenous tagged locus, thus discarding overexpression artifacts. These granules could represent to some type of reservoir or confinement for XRNA, removing part of the enzyme out of the cytoplasm or P bodies as a strategy for inhibition by sequestration. Further work will determine the function of XRNA granules, contributing to the nature of cytoplasmic granular structures during stress.

6. mRNA Granules

As mentioned above, starvation stress in *T. cruzi* and *T. brucei* induce the formation of large cytoplasmic granules that, unlike most other mRNA related granules, were characterized based on poly(A) mRNA dynamics [68]. Although these granules appear in a stress condition, several evidences suggest that these are not genuine SGs, and accordingly were named mRNA granules. In starved *T. cruzi* parasites, PABP1, PABP2 and eIF4E, which are components of *T. brucei* heat-SGs (see above), colocalize with the P body markers XRNA

and DHH1, and poly(A) mRNA [68] (Fig. 1). These granules also contain UBP1 and several RBPs from the same family of proteins (UBP2, RBP3, RBP4, RBP5a, RBP6b) [68]. It is most probable that PUF6 also colocalizes with mRNA granules in trypanosomes given its localization to P bodies [70], although this has not been tested. Neither ribosomal proteins from the 40S or 60S subunits [68], nor the eIF4G scaffold protein (our unpublished results) showed accumulation in mRNA granules in *T. cruzi*, suggesting that the transcripts destined for accumulation in these structures are not primed by translation initiation complexes. These evidences show that there is only one type of granule present in the cell under starvation conditions. In mammalian, yeast or *T. brucei* cells, P bodies can always be identified independently of SGs in response to stress. Since P bodies are constitutive, it is most probable that P bodies, different mRNP complexes, and translating mRNAs converge in mRNA granules in trypanosomes (Fig. 1) [68]. As mentioned above, in unstressed *T. cruzi* cells, not all XRNA *foci* (P bodies) contain UBP1 (Fig. 2A), while in starved parasites XRNA completely colocalizes with UBP1 (Fig. 2B), indicating the fusion of the two *foci* in of the mentioned mRNA granules. In an independent study, Holetz *et al.* [69] characterized and studied the localization of DHH1 in *T. cruzi*. Similarly, they found DHH1 in cytoplasmic *foci* that increased in size during starvation of the parasites, and named these granules P body-like structures due to their similarities to yeast P bodies [69]. Our results indicate that DHH1 localizes to mRNA granules under starvation stress in *T. cruzi*, although there is a certain amount of DHH1 that remains free in the cytoplasm. However, there are no granules composed by DHH1 that do not colocalize with mRNA granules [68]. Thus, it can be suggested that mRNA granules and DHH1 P body-like *foci* are the same structure.

There is no information on the phosphorylation status of eIF2 α under starvation conditions in trypanosomes. Certainly, under nutritional stress there is a marked reduction in polysomes in *T. cruzi*, indicating translational silencing of mRNAs [69]. In fact, these transcripts are recruited to mRNA granules under starvation stress, where they are kept quiescent and protected from degradation [68]. Most notably, transcripts in mRNA granules from *T. brucei* can return to polysomes for translation once starvation stress is over (J. De Gaudenzi, G. Noé, V. Campo, A. C. Frasch and A. Cassola, unpublished results), indicating that these structures indeed function as reservoirs of mRNA during stress. Unexpectedly, mRNA granules contain transcripts that are not normally translated in the epimastigote stage, like amastin (A. Cassola and A. C. Frasch, unpublished results). This observation argues that there is no selectivity in the transcripts that are recruited to mRNA granules, but rather contain bulk poly(A) mRNA.

In *Plasmodium berghei*, translational repression plays an essential role in the zygote to ookinete transformation through the silencing of specific maternal mRNAs in P granules [76]. These are P body-related cytoplasmic *foci* that contain PABP, eIF4E and DOZI, which is the ortholog of DHH1, but apparently exclude eIF4G and the RNA degradation enzymes such as XRN1 or DCP1 and 2 orthologs [77]. This protein composition is similar, yet not identical, to mRNA granules from trypanosomes [68]. The precedent of *P. berghei* P granules allows hypothesizing whether translational silencing in *T. cruzi* or *T. brucei* mRNA granules is required for survival or development. The identification of protein mutants with impaired mRNA granules formation will answer this question, and will also aid to define if mRNA granules formation is a cause or a consequence of translational repression.

In insect-dwelling parasites, mRNA granules appear as soon as 15 minutes in *T. brucei* (Fig. 3A), while it takes almost 24 hours to obtain a homogenous population of *T. cruzi* parasites with this phenotype (Fig. 3B). This apparently late response in *T. cruzi* could be just a matter of experimental conditions, since faster responses have been obtained with other strains [78], although never in the same time range as *T. brucei*. These differences in the response-time between the two species could be ascribable to their basic physiology and metabolism,

since *T. brucei* belongs to the salivaria group, while *T. cruzi* is a stercoraria parasite that transmits through feces [79]. The fact that *T. cruzi* parasites with mRNA granules can be obtained from the intestine of the insect vector [68], suggests an involvement of these structures in the physiology of the parasite during the life-cycle, where starvation periods of the insect vector are common [80]. Nonetheless, mRNA granules are not formed during differentiation of non-infective epimastigotes to infective metacyclic trypomastigotes [68], but rather might form during the initial starvation stress that consequently leads to differentiation. Recently, the global reorganization of metabolic enzymes into reversible assemblies during *S. cerevisiae* nutrient starvation was described [81]. In this work the authors propose 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 re-entry once nutrients are readily available. In the same line of evidence, the transcripts stored in mRNA granules in trypanosomes could provide fast templates for synthesis of cellular components that would allow differentiation or cell-cycle re-entry.

7. Granules of transfer RNA (tRNA)

Several efforts have proven to be unsuccessful in identifying a functional RNA interference (RNAi) pathway in *T. cruzi*, which is completely functional in *T. brucei* [82]. Silencing of retroposons and the maintenance of genome stability are two endogenous events where the RNAi machinery has been implicated in this parasite. Up to now, miRNAs have been elusive to identification in trypanosomes. With the aim to identify novel small RNAs that could have a role in mRNA metabolism, Garcia-Silva and coworkers [78] performed a library of small RNAs up to 40 nucleotides. Although there was no evidence of the existence of miRNAs, they identified populations of truncated tRNAs just before the anticodon position, suggesting regulated cleavage. The amount of cleaved tRNAs increases upon starvation stress, but cleaved tRNAs are already detectable in unstressed trypanosomes [78]. Nevertheless, the amount of intact tRNAs is not affected enough to compromise translation, suggesting that the tRNA cleavage does not involve translation inhibition by depletion of the tRNA pool in *T. cruzi*.

The phenomenon of tRNA cleavage at the anticodon loop has been described as well in the bacterium *Streptomyces coelicolor* [83], the protozoan *Tetrahymena thermophila* [84], the filamentous fungus *Aspergillus fumigatus* [85], *S. cerevisiae*, *Arabidopsis thaliana* and mammalian cells [86]. In all cases, the increase in cleaved tRNA is a response to some type of stress, like differentiation, amino acid deprivation or oxidative stress, although not all stress insult induces tRNA cleavage [87]. In vitro experiments showed that artificially cleaved tRNAs, presumably fully loaded tRNAs with a nick in the anticodon loop, can inhibit translation [88]. In mammalian cells, 5' derived tRNAs, but not 3' halves, induce translational arrest independently of eIF2 α phosphorylation [89] and stimulate formation of SGs [90]. The mechanisms by which translation is inhibited by cleaved tRNA or tRNA halves remains to be identified [91].

Albeit tRNA cleavage seems to be ubiquitous, the most interesting thing about this response in *T. cruzi* is that 5' and 3' derived tRNA halves accumulate in independent cytoplasmic *foci* that do not colocalize with the P body-like mRNA granules [78] (Fig. 1). This type of confinement for these small RNAs has not been described in other organisms, and its significance has yet to be determined. Whether *T. cruzi* tRNA halves have a function in, or are a consequence of the effect of starvation on mRNA metabolism is an enigma. Further pursuing of this and other responses to environmental changing conditions will help to move from the actual static model of mRNA metabolism to a more dynamic one.

CONCLUSION

Post-transcriptional regulation is widespread in all living organisms and is of particular importance in trypanosomes, where life is post-transcriptional. This independence from the mainstream promoter-dependent regulation of gene expression is probably one of the advantages that made these organisms so successful in their adaptation to different hosts during their life-cycle. These early-divergent eukaryotes seem to have a plethora of non-membranous cytoplasmic structures, some of which are shared with mammals and yeasts. The next step in the characterization of these structures would be to determine if their formation is a cause or a consequence of the global translational silencing. Unlike yeast P bodies and mammalian SGs, where an important number of proteins that mediate nucleation and aggregation have been identified, there is scarce information about this issue in trypanosomes. The culmination of the genome projects in trypanosomes has pushed forward the identification of proteins involved in mRNA metabolism. The identification of additional core components of these cytoplasmic *foci* in trypanosomes seems now possible to achieve by immunoprecipitation of native or tagged components, coupled to mass spectrometry. In the near future, these strategies will allow the identification of novel components. Their importance will be easily assessed through the use of deletion or knockdown mutants, negative dominants, or overexpressed proteins interfering with the correct formation of these granules. Moreover, testing these tools directly in parasitic environmental conditions as similar as possible to the natural ones is a plus towards stepping in the natural transmission cycle of trypanosomes. This direction will allow to manipulate the normal gene-expression program of these life-threatening parasites, making them to induce or repress the expression of selected antigens in a deregulated-fashion that would convert them in possible sources for vaccines.

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Abbreviations

GFP	green fluorescent protein
mRNA	messenger RNA
mRNP complex	messenger ribonucleoprotein complex
Met-tRNA_i	Methionyl-transfer RNA _i
miRNAs	micro RNAs
NMD	Nonsense Mediated Decay
P bodies	Processing bodies
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNA pol	RNA polymerase
SL	Splice Leader
SGs	Stress Granules
tRNA	transfer RNA

UTR

untranslated region

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Fig. 1.

A model for RNA granules in trypanosomes. The events depicted here summarize the evidence so far obtained from the study of RNA granules in *T. brucei* and *T. cruzi*. Of note, not all the events depicted here in a single cell have been described in both parasite species (see text for details). (A) Under optimal growth conditions, new translation initiation events (1) continuously take place, leading to mRNA translation in polysomes (2), P bodies (3) are constitutive components of the cytoplasm of trypanosomes [38, 68]. These structures are heterogeneous in protein composition, but protein factors are here depicted together for simplicity. P bodies repress translationally competent transcripts through the function of DHX1, and seem to be able to recruit mRNAs from polysomes (2), which can later return to translation (1). It is not clear yet if mRNAs in P bodies are readily available for 5' to 3' degradation (4), or if targeted 5' to 3' degradation can occur in polysome-associated mRNAs (5), as in yeasts [75]. (B) Under heat stress, stalled translation initiation complexes appear to be the main component of heat-induced SGs (6). Heat stress also induces the formation of XRNA granules (7) at the posterior pole of *T. brucei* parasites [38], which await functional

characterization. The movement of XRNA from P bodies to XRNA granules is shown with a dashed line. Upon return to the optimal growth temperature, mRNAs from SGs could re-engage in translation (1). (C) Under starvation stress, mRNAs exit the translating pool before fusing with P bodies to form P body-like mRNA granules (8), which are composed by a mixture of P body and SGs components. Transcripts in mRNA granules are protected from degradation, but can return to polysomes once optimal nutritional conditions are restored, acting as mRNA reservoirs during stress. Starvation stress also induces the formation of granules composed by 5' (9) or 3' (10) tRNA halves, which are independent structures. The movement of tRNA halves from the tRNA pool, thought to occur in the polysome fraction, is shown with a dotted line. SL, splice leader.

Fig. 2.

Heterogeneous P bodies converge into P body-like mRNA granules evidenced by UB1 co-localization with XRNA-GFP in starved *T. cruzi* parasites. **(A)** The localization of UB1 using specific antibodies is shown in untreated *T. cruzi* cells transfected with XRNA-GFP. UB1 localizes in some XRNA-GFP P bodies (marked with arrowheads), but not all XRNA-GFP P bodies contain UB1 (marked by arrows). **(B)** Parasites were starved by incubation during 24 hours in Phosphate Buffered Saline (PBS). XRNA-GFP and UB1 completely colocalize, indicating fusion of the respective mRNP complexes in P-body-like mRNA granules (marked by arrowheads). **(A, B)** DAPI staining reveals the position of nuclear (N) and kinetoplast (k) DNA. Scale bar: 5 μ m.

Fig. 3. Localization of poly(A) mRNA to P body-like mRNA granules in *T. brucei* and *T. cruzi*. (**A**) Poly(A) was detected by FISH using an Cy3-labelled oligo(dT) probe (see ref [68] for details) in *T. brucei* procyclic trypomastigotes subjected to a fifteen minutes starvation in PBS. The arrow shows a parasite delayed in the formation of mRNA granules that has the typical uniform distribution of poly(A) mRNA of non-starved parasites. (**B**) Poly(A) was detected by FISH, as above, in epimastigotes of *T. cruzi* subjected to a 24 hours-exposure to PBS. The typical granular distribution of poly(A) mRNA upon starvation is depicted. DAPI staining reveals the position of nuclear (N) and kinetoplast (k) DNA. Phase contrast images allows observation of the parasite morphology. Scale bar: 5 μ m.