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Identification of the interactomes associated with SCD6 and RBP42 proteins in *Leishmania braziliensis*

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ABSTRACT

Leishmania are protozoan parasites responsible for leishmaniasis. These parasites present a precise gene regulation that allows them to survive different environmental conditions during their digenetic life cycle. This adaptation depends on the regulation of the expression of a wide variety of genes, which occurs, mainly at the post-transcriptional level. This differential gene expression is achieved by mechanisms based mainly in RNA binding proteins that regulate the translation and/or stability of mRNA targets by interaction with *cis* elements principally located in the untranslated regions (UTR). In recent studies, our group identified and characterized two proteins, SCD6 and RBP42, as RNA binding proteins in *Leishmania braziliensis*. To find clues about the cellular processes in which these proteins are involved, this work was aimed to determine the SCD6- and RBP42-interacting proteins (interactome) in L. *braziliensis* promastigotes. For this purpose, after an *in vivo* UV cross-linking, cellular extracts were used to immunoprecipitated, by specific antibodies, protein complexes in which SCD6 or RBP42 were present. Protein mass spectrometry analysis of the immunoprecipitated proteins identified 96 proteins presumably associated with SCD6 and 173 proteins associated with RBP42. Notably, a significant proportion of the identified proteins were shared in both interactomes, indicating a possible functional relationship between SCD6 and RBP42. Remarkably, many of the proteins identified in the SCD6 and RBP42 interactomes are related to RNA metabolism and translation processes, and many of them have been described as components of ribonucleoprotein (RNP) granules in *Leishmania* and related trypanosomatids. Thus, these results support a role of SCD6 and RBP42 in the assembly and/or function of mRNA-protein complexes, participating in the fate (decay/accumulation/translation) of L. *braziliensis* transcripts.

Significance: Parasites of the *Leishmania* genus present a particular regulation of gene expression, operating mainly at the post-transcriptional level, surely aimed to modulate quickly both mRNA and protein levels to survive the sudden environmental changes that occur during a parasite's life cycle as it moves from one host to another. This regulation of gene expression processes would be governed by the interaction of mRNA with RNA binding proteins. Nevertheless, the entirety of protein networks involved in these regulatory processes is far from being understood. In this regard, our work is contributing to stablish protein networks in which the *L. braziliensis* SCD6 and RBP42 proteins are involved; these proteins, in previous works, have been described as RNA binding proteins and found to participate in gene regulation in different cells and organisms. Additionally, our data point out a possible functional relationship between SCD6 and RBP42 proteins as constituents of mRNA granules, like processing bodies or stress granules, which are essential structures in the regulation of gene expression. This knowledge could provide a new approach for the development of therapeutic targets to control *Leishmania* infections.

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1. Introduction

Leishmania parasites, causal agent of leishmaniasis, present a complex digenetic life-cycle, alternating between a vector insect and a mammalian host. During their life cycle, these parasites require a precise gene regulation that allows them to achieve the morphological and biochemical changes to survive the different stressful conditions encountered into each one of their hosts [1,2]. Nevertheless, gene expression regulation in these organisms is mostly achieved post-transcriptionally by controlling mRNA stability and translation [3,4]. This peculiar manner of handling gene regulation is dictated by the particular genomic organization found in *Leishmania*: genes are organized in long clusters with the same transcriptional orientation [5–7]. Moreover, canonical promoters for RNA polymerase II are missing, and a constitutive transcription of these gene clusters generates polycistronic RNA precursors that ultimately are processed into individual mRNAs by the processes of trans-splicing and polyadenylation [8,9]. The relative abundance of the mRNAs and their translational activity are mediated by mechanism chiefly based in RNA-binding proteins (RBPs). These proteins orchestrate the processing, transport, stability, translation and decay of mRNAs by interactions with *cis*-elements present in the mRNAs, specially located in their untranslated regions (UTR) [4,8,10,11]. These interactions between RNA and RBPs lead to the formation of discrete foci commonly called RNA granules, which are microscopically visible as intracellular membrane-free structures. Broadly, RNA granules have been classified in two classes: processing bodies (PB), which are constitutively present in the cells and mainly contain mRNA degradation enzymes; and several types of stress granules (SG), which contain mRNAs associated with components of the translation initiation machinery [12]. Thus, these RNA granules would be involved in regulating gene expression by mRNA storing or promoting mRNA decay in the cells.

In the last two decades, the availability of sequences for complete genomes of several trypanosomatids has allowed the uncovering of a large number of putative RBPs, based on the presence of classical structural motifs of mRNA binding such as RNA-Recognition Motifs (RRM), zinc finger (ZF), Pumilio (PUM), and Alba domains [11,13,14]. However, few of these proteins have been characterized in detail and their role in the regulation of gene expression is not fully understood.

Considering the relevance of RBPs in regulating gene expression, our group opted to search for proteins interacting with transcripts coding for proteins involved in survival and virulence of *Leishmania* [15]. Thus, by pull down assays, 52 proteins were identified because of their interaction with the 5′- and 3′- UTRs of *Leishmania braziliensis HSP70* transcripts. Among the identified proteins, *Lb*SCD6 (LbrM.25.2210) and *Lb*RBP42 (LbrM.30.3080) were further characterized in their RNA binding capacities [16]. These proteins were found to be true RBPs, and able to interact with structural RNAs motifs related to ARE elements [16]. Also, an *in silico* characterization showed the presence of RNA binding motifs: SCD6 possesses motifs present in members of the Sm-like protein family, such as a Lsm domain, in its N-terminal region, a FDF motif in its C-terminal region and several RGG motifs. Members of this family proteins have been described as proteins involved in translational repression and accumulation of mRNAs in ribonucleoprotein granules like PBs and SGs in humans (RAP55) [17], plants (DCP5) [18], nematodes (CAR—I) [19], *Drosophila* (TraI) [20], yeast (Scd6) [21], and other trypanosomatids as *Trypanosoma brucei* (SCD6) [22]. The high conservation of SCD6 in the evolutionary scale, along with its conserved localization in PBs and its interaction with different constituent proteins of these granules, suggest that SCD6 have a central role in the expression gene regulation in eukaryotes. On the other hand, the RBP42 in *T. brucei*, is an essential protein in multiple stages of this parasite life cycle. It is mainly located in the cytoplasm associated to polysomes, where interacts with transcripts coding to proteins involved in the energy metabolism [23]. The structure of *Lb*RBP42, as occurs in its mammalian homolog (G3BP) and in other trypanosomatids like *Trypanosoma cruzi* (*Tc*RBP42), presents a NTF2-like domain, which may be involved in nucleocytoplasmic transport, an RNA recognition motif (RRM), located in the C-terminal region, and several PxxP motifs [15,23–25]. Furthermore, in mammalian cells, this protein has been involved in the SGs assembly [26].

In summary, all these studies suggest that SCD6 and RBP42 might have an important role in controlling the translation, localization or stability of their RNA targets. To increase our knowledge on the functional role played by these proteins in *Leishmania*, this study was aimed at the identification of proteins interacting, either directly or indirectly, with SCD6 and RBP42 proteins in *L. braziliensis* promastigotes. For this purpose, immunoprecipitations with specific antibodies and proteomics analyses were conducted.

2. Materials and methods

2.1. Parasites culture

The *L. braziliensis* MHOM/BR/75/M2904 strain, provided by CIDEIM (Centro Internacional de Entrenamiento e Investigaciones Médicas - Colombia), was cultured *in vitro* at 26 °C in Schneider's insect medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% heat-inactivated fetal calf serum (Eurobio, Les Ulis, France), and 0.1 μg/ml 6-biopterin (Sigma Aldrich, St. Louis, MO, USA).

2.2. In vivo crosslinking and preparation of parasite lysates

This procedure was adapted from a method previously described by Das and co-workers [23]. In brief, 6×10^8 promastigotes were harvested from mid-log phase and washed twice with cold phosphate buffered saline (PBS), then parasites were resuspended in PBS. Cell suspensions (10 ml) were transferred to Petri dishes, placed on ice and UV-irradiated twice with 400 mJ/cm^2, separated 5 cm apart from the UV source. The samples were centrifuged at 4 °C for 15 min, and the pellet resuspended in 200 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM PMSF, 1% Triton X-100, 1× protease inhibitor cocktail (Roche, Mannheim, Germany)) and incubated on ice for 30 min; finally, cell lysis was completed by bath sonication for 10 min at 4 °C (Ultrasons, Selecta, Barcelona, Spain). Afterwards, the samples were treated with DNase I (100 μg/ml) and RNaseOUT (Invitrogen, Carlsbad, CA, USA), incubated at 37 °C for 15 min, centrifuged for 20 min at 4 °C and the supernatant immediately used in the immunoprecipitation step.

2.3. Immunoprecipitation of protein complexes

The anti-SCD6 and anti-RBP42 antibodies were produced after inoculation of the corresponding recombinant proteins in rabbits as described elsewhere [16]. The protein extracts (see above) were subjected to a pre-cleaning treatment. For this purpose, 50 μl of protein A magnetic beads (Invitrogen, Carlsbad, CA, USA) were extensively washed with a washing solution (PBS, 0.02% Tween-20, pH 7.4). Afterwards, the beads were incubated with 50 μl of the corresponding pre-immune serum (1:50 dilution) for 45 min at room temperature (RT). After washing thrice, the beads were incubated for 40 min at 4 °C with the protein extracts. Finally, the unbound proteins (cleared extracts) were used for immunoprecipitation with the specific antibodies.

In each experiment, four immunoprecipitation sets were prepared. After washing, protein A magnetic beads (100 μl) were incubated with 100 μl of antibody solution (1:50 dilution). Two sets were incubated with either anti-SCD6 or anti-RBP42 antibodies and the other two with the corresponding pre-immune sera. After incubation for 45 min at RT with shaking, the immunoglobulin-protein A complexes were covalently linked using the bis(sulfosuccinimidy)suberate (BS^3, Invitrogen, Carlsbad, CA, USA) reagent, according to manufacturer's instructions. After washing out the unbound antibodies, the magnetic beads were incubated with 100 μl of pre-cleaned protein extracts for 90 min at 4 °C in an orbital shaker. Finally, the magnetic beads were washed twice and

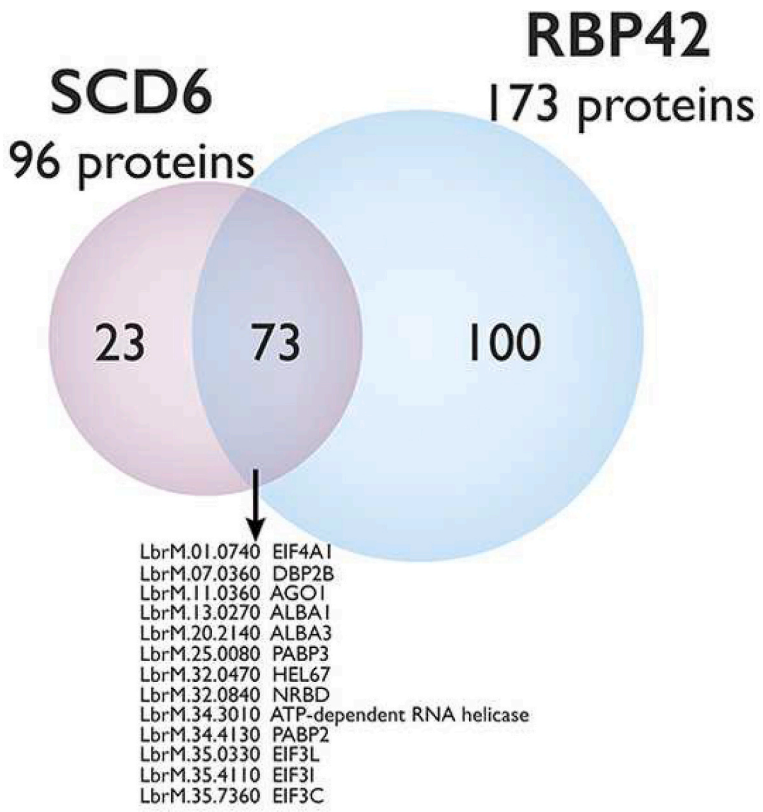

Fig. 1. Composition of the *Lb*SCD6 and *Lb*RBP42 interactomes. A total of 96 identified proteins interacting with *Lb*SCD6 protein, and 173 proteins interacting with *Lb*RBP42 protein were identified. The RBPs and proteins associated with RNA metabolism, common to both interactomes, are shown.

resuspended in 100 μl of Laemmli buffer [27] and heated for 10 min at 90 °C. The supernatants were collected and stored at -20 °C until proteomics analysis. Two biologically independent replicates of the experiment were carried out.

2.4. Proteomic analysis

Firstly, the proteins eluted from the beads (above) were concentrated by loading the samples onto 1.2-cm wide wells of a conventional SDS-PAGE gel (0.75 mm-thick, 4% stacking, and 10% resolving). The run was stopped as soon as the front entered 3 mm into the resolving gel; so that the proteins became concentrated in the stacking/resolving gel interface. The position of the band was visualized by Coomassie staining. Following this step, this part of the gel was excised, and placed into a microcentrifuge tube. The gel pieces were destained in acetonitrile: water (ACN:H_2O, 1:1), reduced and alkylated (disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 56 °C, and then thiol groups were alkylated with 10 mM iodoacetamide for 30 min at room temperature in darkness), and digested *in situ* with sequencing grade trypsin (Promega, Madison, WI) as detailed elsewhere [28]. After in-gel trypsin digestion for 12 h at 37 °C, whole supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore). The resulting peptides were analyzed by LC-MS/MS using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA).

Peptide identification from mass spectrometry data was carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). A search was performed against UniProt database (UniProt-Leishmania_braziliensis https://www.uniprot.org/). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. A probability cutoff corresponding to 1% false discovery rate (FDR) relative to a target-decoy database (reversed sequences) was applied. Protein identification by LC/MS/MS was carried out in the 'CBMSO PROTEIN CHEMISTRY FACILITY', that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001.

To determine the probability that a protein present in the specific-immunoprecipitations may be a casual discovery (null hypothesis), a binomial distribution test was used. For calculations, the number of peptides for each protein identified in the specific interactome (using each specific antibody) in contrast with the number of peptides present in the non-specific interactome (using the pre-immune serum) were counted. Only proteins supported by p values ≤ 0.05 were considered true constituents of the interactome (SCD6 or RBP42).

2.5. Bioinformatics analysis on selected proteins

Functional annotations were obtained from TriTrypDB [29] and classified according to Gene Ontology Consortium procedures [30,31].

3. Results and discussion

3.1. Characterization of SCD6 and RBP42 interactomes in L. braziliensis

In a recent study, we characterized the *L. braziliensis* RNA binding proteins SCD6 and RBP42 [16], which orthologous proteins in different organisms have been described as component of ribonucleoprotein (RNP) complexes involved in posttranscriptional regulatory mechanisms of gene expression. To further characterize the functional roles of SCD6 and RBP42 proteins in *L. braziliensis*, a proteomics approach was designed. Polyclonal antibodies, raised against *Lb*SCD6 or *Lb*RBP42 recombinant proteins, were used to immunoprecipitated proteins interacting with SCD6 and RBP42 in *L. braziliensis* promastigotes. During preparation of *L. braziliensis* protein extracts for the immunoprecipitation procedure, no RNase treatment was applied. Hence, the proteins

Table 1
Shared proteins in the *Lb*SCD6 and *Lb*RBP42 interactomes.

Protein category	*L. braziliensis* gene ID	Annotation
RNA metabolic process		
1	LbrM.07.0360	ATP-dependent RNA helicase DBP2B (DBP2B)
2	LbrM.07.0940	Splicing factor ptsr1-like protein
3	LbrM.11.0360	Argonaute-like protein (AGO1)
4	LbrM.13.0270	ALBA-domain protein 1 (ALBA1)
5	LbrM.17.0020	Eukaryotic translation initiation factor 3 subunit a (EIF3A)
6	LbrM.20.2140	ALBA-domain protein 3 (ALBA3)
7	LbrM.25.0080	Poly(A)-binding protein 3 (PABP3)
8	LbrM.27.1440	KH domain containing protein, putative (ZC3H41)
9	LbrM.32.0470	ATP-dependent RNA helicase HEL67
10	LbrM.32.0840	Nuclear RNA binding domain (NRBD1)
11	LbrM.34.3010	ATP-dependent RNA helicase, putative
12	LbrM.34.4130	Polyadenylate-binding protein (PABP2)
Translation		
13	LbrM.01.0460	Ribosomal protein S7
14	LbrM.01.0470	Ribosomal protein S7, putative
15	LbrM.01.0740	Eukaryotic initiation factor 4A-1 (EIF4A1)
16	LbrM.03.0920	40S ribosomal protein S19 protein
17	LbrM.04.0740	60S ribosomal protein L10, putative
18	LbrM.07.0560	Putative 60S ribosomal protein L7a
19	LbrM.07.0750	40S ribosomal protein S9, putative
20	LbrM.11.0760	40S ribosomal protein S5
21	LbrM.11.0990	40S ribosomal protein S15A, putative
22	LbrM.13.1000	40S ribosomal protein S4
23	LbrM.15.0990	40S ribosomal protein S3
24	LbrM.15.1040	60S ribosomal protein L6
25	LbrM.16.0480	60S ribosomal protein L21, putative
26	LbrM.17.0090	Elongation factor 1-alpha
27	LbrM.20.2340	40S ribosomal protein S19 protein
28	LbrM.20.3240	60S ribosomal protein L21
29	LbrM.20.5810	Ribosomal protein S11 homolog
30	LbrM.21.1680	40S ribosomal protein S11, putative
31	LbrM.21.1280	60S ribosomal protein L9, putative
32	LbrM.22.1420	60S ribosomal protein L14, putative
33	LbrM.24.2150	40S ribosomal protein S8
34	LbrM.26.0160	60S ribosomal protein L7, putative
35	LbrM.28.2760	40S ribosomal protein S17
36	LbrM.29.1150	Ribosomal protein L1a, putative
37	LbrM.29.2430	60S ribosomal protein L13
38	LbrM.32.0970	60S ribosomal protein L18a, putative
39	LbrM.32.2950	Ribosomal protein L27
40	LbrM.32.4140	60S ribosomal protein L8, putative
41	LbrM.33.0840	40S ribosomal protein S6
42	LbrM.33.1110	40S ribosomal protein S3
43	LbrM.34.1920	40S ribosomal protein S6
44	LbrM.35.0270	Elongation factor 2
45	LbrM.35.0330	Eukaryotic translation initiation factor 3 subunit l (EIF3L)
46	LbrM.35.4110	Eukaryotic translation initiation factor 3 subunit i (EIF3I)
47	LbrM.35.5370	40S ribosomal protein SA
48	LbrM.35.7360	Eukaryotic translation initiation factor 3 subunit c (EIF3C)
Regulation of gene expression		
49	LbrM.11.0600	Hypothetical protein, conserved (RNA binding capacity)[a]
50	LbrM.21.0490	RNA binding protein
51	LbrM.34.2130	RNA-binding protein, putative (DRBD2)
Protein folding		
52	LbrM.28.1300	Luminal binding protein 1 (BiP)
53	LbrM.28.2970	Heat-shock protein hsp70
54	LbrM.28.2980	Heat-shock protein hsp70
55	LbrM.28.2990	Heat-shock protein hsp70
56	LbrM.29.0780	Heat shock protein 90 (LPG3)
57	LbrM.33.0330	Heat shock protein 83-1 (HSP83-1)
58	LbrM.33.0340	Heat shock protein 83-1 (HSP83-2)
59	LbrM.33.0350	Heat shock protein 83-1 (HSP83)
Metabolism		
60	LbrM.05.0510	ATP synthase F1, alpha subunit
61	LbrM.14.1330	Enolase
62	LbrM.15.1100	Tryparedoxin peroxidase
63	LbrM.24.1690	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
64	LbrM.25.2220	3-oxo-5-alpha-steroid 4-dehydrogenase
65	LbrM.25.2510	ATP synthase subunit beta, mitochondrial (ATPB)
66	LbrM.27.1920	Phosphoenolpyruvate carboxykinase [ATP], glycosomal
67	LbrM.29.2480	ATP-dependent 6-phosphofructokinase, glycosomal (PFK)
68	LbrM.30.2950	Glyceraldehyde-3-phosphate dehydrogenase, glycosomal
Cellular process		
69	LbrM.26.1880	Uncharacterized protein
70	LbrM.28.2950	Receptor for activated C kinase 1
Undetermined		
71	LbrM.13.1130	Hypothetical protein, conserved
72	LbrM.14.0900	tc40 antigen-like
73	LbrM.26.1260	Heat shock protein 70-related protein

[a] This capacity has been found in the *T. brucei* orthologue (Tb927.11.6440; TriTrypDB).

identified in this study may be interacting either directly (protein-protein interactions) or indirectly (protein-RNA-protein interactions) with *Lb*SCD6 and *Lb*RBP42.

The proteins specifically immunoprecipitated (interactomes) by the *Lb*SCD6- or *Lb*RBP42- antibodies were 96 and 173 proteins, respectively (see Supplementary File for complete lists of the immunoprecipitated proteins in each set of experiments). Remarkably, 73 proteins were shared in both interactomes, amounting for the 76% and 42% of the proteins identified in the *Lb*SCD6 and *Lb*RBP42 interactomes, respectively (Fig. 1). These findings would be indicating that in some extend both proteins are forming part of the same protein complexes in the parasite. This hypothesis is reinforced by the identification of each protein (SCD6 or RBP42) in their reciprocal interactomes.

3.2. Functional description of the proteins identified as common components of the SCD6 and RBP42 interactomes

As *L. braziliensis* gene annotations are somewhat obsolete, functional annotations and GO terms were recovered from the information available for the *T. brucei* orthologues in the Tritryp database (TritrypDB.org), which is actively curated. Table 1 lists the names for the 73 proteins identified in both interactomes; the proteins were grouped according to functional categories. Many of the identified proteins (16.44%) are RNA-interacting proteins involved in several RNA metabolism processes. This group includes proteins such as an Argonaute-*like* protein whose orthologues in mammalian cells have been described as a constituent factor of PBs, cytoplasmic aggregates that include translational repressors and components of the mRNA decay machine [32]. This association is remarkable, considering that parasites of the genus *L. braziliensis* possess an active machinery of the iRNA pathway [33]. Recently, the *T. brucei* argonaute 1 was identified as constituent of RNP granules in parasites under starvation conditions [34]. The presence of Alba 1 and 3 is also noticeable. These proteins are known to complex together in trypanosomatids [35,36]. Additionally, Alba 3 in *T. brucei,* has been described as a constituent of RNA granules associated with nutritional stress and involved in translational regulation [35]. Two RNA-helicases were also found; both proteins have a DEAD/DEAH box helicase domain. In trypanosomatids, this family of helicases has been involved in several steps of RNA metabolism, from transcriptional regulation to mRNA decay, including nuclear RNA surveillance, ribosome biogenesis, pre-mRNA splicing, mRNA export, translation

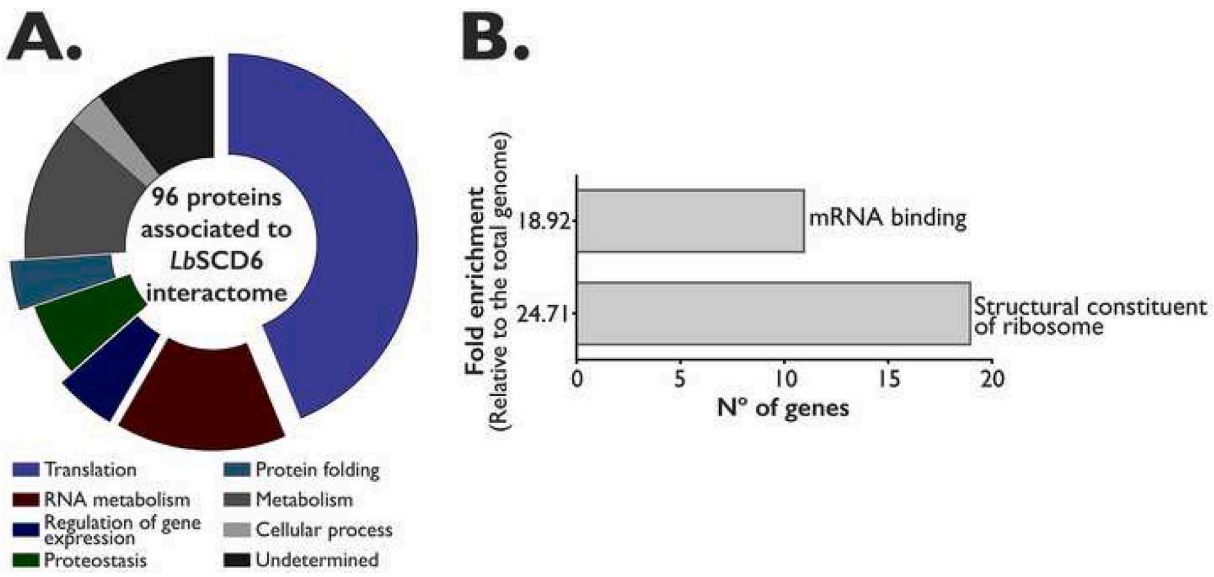

Fig. 2. Classification of the identified proteins in the *Lb*SCD6-interactome. (A) Functional classification according to biological processes. Within the translation category, both structural components of the ribosome and translational regulatory factors are included. The cellular process category includes mitotic cell cycle, cytokinesis and regulation of cytokinesis processes. (B) Proteins grouped into categories were analyzed for molecular function GO term enrichment. Only GO categories having enrichment above 3-fold (and a p value ≤ 0.05) are shown.

regulation, RNA silencing, and RNA editing [37]. Moreover, many of these proteins (*eg*, rck/p54, DHX36 or Dhh1) have been described as components of both SGs and PBs in different mammalian cells, yeasts and trypanosomatids [38–40]. The polyA-binding proteins PABP2 and PABP3 were also co-immunoprecipitated. The *Leishmania* genome codes for 3 polyA-binding proteins (PABP1, PABP2 and PABP3), being PABP3 exclusive for the *Leishmania* genus [41]. Immunoprecipitation assays have shown a direct association, RNA independent, between *Lm*PABP2 and *Lm*PABP3. In addition, these two proteins are detected in both cytoplasm and nucleus when transcription is inhibited, which suggest that these proteins could be involved in mRNA processing and export processes in *Leishmania* [41]. Additionally, the PABP2 protein has been described as a stabilizing factor of transcripts primarily associated with core factors of the SG [40,42] and constituent factors of PBs [43,44]. Interestingly, the presence of PABP2 and SCD6 in P granules was demonstrated in the *Caenorhabditis elegans* germline [45]. In *Crithidia fasciculata*, the PABP2 homolog has been found to be part of a complex that recognizes a cycling element in the UTRs of several mRNAs, whose levels vary periodically during the cell cycle [46]. This protein was also reported as a factor interacting with the 3' UTRs of *HSP70 I* and *II* transcripts in *L. braziliensis*. PABP3 also was described as interacting with the 3' UTR region of the *L. braziliensis HSP70-I*. Therefore, both proteins are postulated as possible regulatory factors for these transcripts in this parasite [15]. Another identified protein was the NRBD protein, this protein seems specific of trypanosomatids. NRBD has been involved in ribosomal RNA metabolism in *T. brucei*, while the homolog in *T. cruzi* is associated with the translational machinery [47,48]. Also, the RNA binding protein ZC3H41 was identified in both interactomes. In *T. brucei*, this protein interacts with mRNA molecules and is a component of RNP granules [49,50].

In addition, a large number (49.31%) of the identified proteins is associated with the translation machinery; among them, four components of the translation initiation 3 complex (EIF3C, EIF3I, EIF3L, and EIF4A1) and two elongation factors (EF1-alpha and EF2) were shared by both interactomes. The EIF3 complex has been described as a component of the SG in arsenite-stressed mammalian cells, recruited alongside with other components of the 43S preinitiation complex [51]. In agreement, it is remarkable that several of the small and large ribosomal subunits proteins were found common components of both interactomes (*Lb*SCD6 and *Lb*RBP42). In some way, this may be unexpected considering that the ribosomal proteins are not associated with the formation of cytoplasmic granules or involved in the processes of RNA metabolism in *T. cruzi* [40]. However, Shrivastava and collaborators found a high percentage of ribosomal proteins associated with nutritional stress granules in *Leishmania*, suggesting that these granules could serve for storage of ribosomes and ribosomal subunits that were stalled due to this stress [52]. On the other hand, studies in *D. melanogaster* and *Saccharomyces cerevisiae* have documented interactions between ribosomal proteins of the EIF3 complex and RBPs [53–56]. It should be note that EIF3 is a key participant in the assembly of the translation initiation machinery in *Leishmania amazonensis* [57]. Another remarkable finding is the presence of EIF4A1 in the interactomes. This protein is a DEAD-RNA helicase, whose family includes several proteins mainly involved in RNA metabolism, that interact specifically to *Leishmania* EIF4G as component of the EIF4F complex in *Leishmania* [58].

Other interesting group of proteins identified corresponds to transcriptional regulators of gene expression (4.11%) (Table *1*). Thus, the DRBD2 protein in *T. brucei* has been described as a transcriptional repressor [59], whereas the orthologues to LbrM.11.0600 and LbrM.21.0490 in *T. brucei* have been described as post-transcriptional activators, increasing either the stability or the translational efficiency of transcripts [49].

Several molecular chaperones, belonging to the HPS70 and HSP83/90 families, were identified in both interactomes (10.96%). This finding was not surprising since these proteins are important components of the molecular machineries involved in nearly every cellular process [60].

Finally, a fraction of the identified proteins (12.33%) maybe categorized as metabolic proteins. Nevertheless, the presence of these proteins, also referred as moonlight factors, is often observed in studies aimed to the identification of RNA binding proteins and for some of them a direct interaction with RNA has been demonstrated [61–64]. For instance, enolase has been detected in the nucleus of mammalian cells, where it participates in the transcriptional regulation of genes involved in cellular morphology and proliferation processes [65]. Additionally, in mammals and yeasts, mitochondrial import of specific tRNAs depends on enolase [66]. Finally, in bacteria, enolase has been reported as a major component of the degradosome, a nucleoprotein structure involved in regulating mRNA stability [67]. Another example is

Table 2
Proteins involved in mRNA processing, specifically associated with *Lb*SCD6 or *Lb*RBP42.

Protein category	*L. braziliensis* gene ID	Annotation
SCD6		
RNA metabolic process		
1	LbrM.06.0240	5′-3′ exoribonuclease A (XRNA)
Protein folding		
2	LbrM.26.0650	10 kDa heat shock protein
Regulation of gene expression		
3	LbrM.29.0880	High mobility group protein TDP-1 (TDP1)
4	LbrM.30.3080	RNA-binding protein 42 (RBP42)
RBP42		
RNA metabolic process		
1	LbrM.21.1700	ATP-dependent RNA helicase SUB2 (SUB2)
2	LbrM.25.2210	SCD6.10
3	LbrM.29.2890	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain) (RBP6)[a]
4	LbrM.35.5350	Hypothetical protein, conserved (PUF11)[a]
Translation		
5	LbrM.03.0890	Eukaryotic translation initiation factor 2 subunit alpha (EIF2A)
6	LbrM.07.0710	Eukaryotic translation initiation factor 3 subunit h (EIF3H)
7	LbrM.08.0600	Eukaryotic translation initiation factor 2 beta subunit (EIF2B)
8	LbrM.17.1450	Eukaryotic translation initiation factor 3 subunit b (EIF3B)
9	LbrM.25.2400	Eukaryotic translation initiation factor 3 subunit F (EIF3F)
10	LbrM.28.2510	Eukaryotic translation initiation factor 3 subunit E (EIF3E)
11	LbrM.30.3030	Eukaryotic translation initiation factor 3 subunit d (EIF3D)
12	LbrM.34.4980	Polyadenylate-binding protein 1 (PABP1)
Regulation of gene expression		
13	LbrM.14.0460	N-terminal conserved domain of Nudc./CS domain containing protein (NUDC1)
14	LbrM.21.0890	CCR4-NOT transcription complex subunit 1 (NOT1)
15	LbrM.27.0850	Ras-related protein RAB1A (Rab1A)
16	LbrM.31.0080	Hypothetical protein, conserved (ZC3H34)[a]
17	LbrM.35.3430	14-3-3 protein 1 (14-3-3-I)

[a] The names in parentheses correspond to the *T. brucei* orthologue (TriTrypDB).

glyceraldehide-3-phosphate dehydrogenase (G3PD), a well-known housekeeping protein because of its role in glycolysis; however, it also acts in other cellular processes [review in [68]]. Thus, G3PD has been involved in regulation of gene expression by modifying mRNA stability [69].

3.3. Overview of proteins identified in the SCD6 interactome

As above mentioned, 96 proteins were identified as components of the SCD6 interactome (Supplementary file). According to their functional annotations, these proteins were grouped in seven categories (Fig. 2A). Possible enrichments of some categories of this interactome regarding the whole proteome were analyzed by the PANTHER tool (http://www.pantherdb.org/). Again, as the *L. braziliensis* genes are not incorporated in the PANTHER database, firstly a search for the *T. brucei* orthologues at TriTrypDB was conducted, as *T. brucei* proteins are incorporated in the PANTHER database. Fig. 2B shows the functional categories in which an enrichment higher than three-fold (p value ≤ 0.05) was obtained. This analysis showed a significant enrichment in the SCD6 interactome of proteins categorized as structural constituents of ribosome and mRNA binding proteins.

Apart from those proteins mentioned in the previous section common to both interactomes, the SCD6 interactome contains additional proteins involved in different aspects of mRNA metabolism, the presence of proteins like 5′-3′ exoribonuclease A (XRNA), TDP1 and RBP42 were highlighted (Table 2). XRNA plays a critical role in mRNA degradation in trypanosomes [70]. It has been described as component of different granules like PBs and nuclear periphery granules in *T. cruzi* and *T. brucei* [40,51,71–73], and in heat- or starvation-induced granules of *T. cruzi*, *T. brucei* and *L. amazonensis* [40,51,52]. TDP1 has been described as a potential post-transcriptional regulator factor associated with mRNA translational increase or stability in *T. brucei* [49]. Finally, the *Lb*RBP42 protein was found associated with SCD6 interactome, bolstering the functional relationship between these two proteins. In *T. brucei,* RBP42 is associated with polysomes and binds to diverse mRNAs [23], suggesting a role in the stabilization of the mRNA targets. Additionally, the SCD6-RBP42 interaction has been observed in RBP9-mRNP complexes in *T. cruzi* when the parasites are under nutritional stress [74].

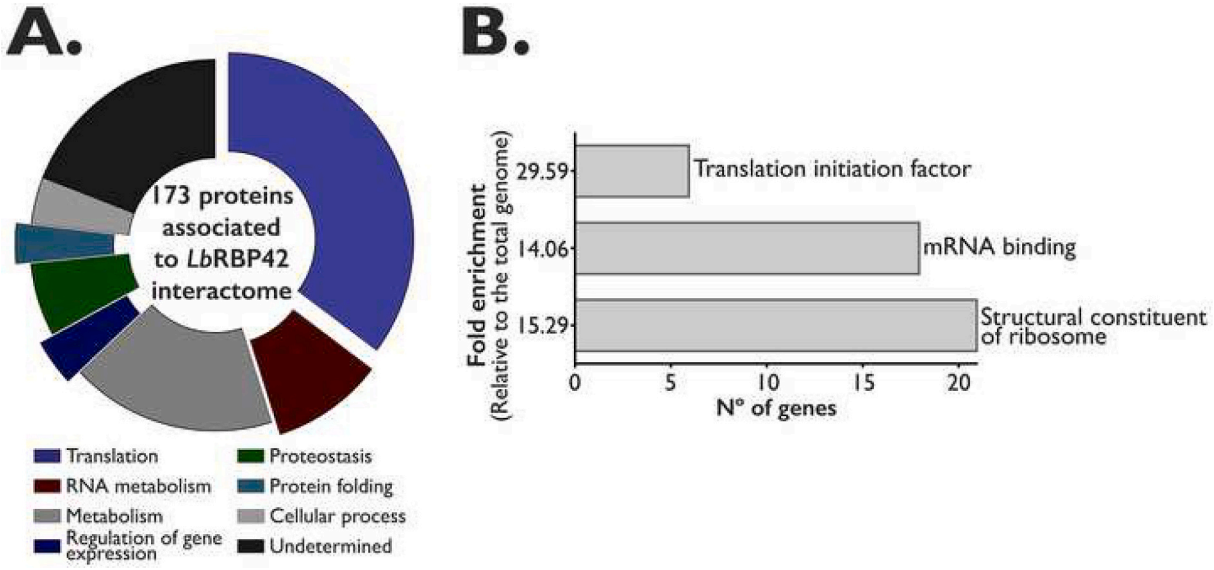

Fig. 3. Classification of the identified proteins in the *Lb*RBP42-interactome. (A) Functional classification according to their biological process. The cellular process category includes as subcategories the regulation of cytokinesis, transport, DNA replication, mitochondrion organization and mitotic cell cycle. (B) For description, see legend for Fig. 2B.

Table 3
Proteins identified as component of RNPs granules in trypanosomatids.

Component	Cytoplasmic Granule					Organism	Ref
	PB	SSG	OSG	HSSG	NPG		
ALBA1		x				*L. amazonensis, T. brucei*	40, 52
ALBA3		x	x			*T. brucei, T. cruzi*	40, 74
PABP 1		x	x	x		*T. brucei, T. cruzi*	40, 53, 74
PABP 2	x	x	x	x	x	*L. amazonensis, T. cruzi, T. brucei*	40, 50, 52, 53, 72, 74
DRBD2		x	x			*L. amazonensis, T. brucei, T. cruzi*	50, 52, 74
RBP6		x				*L. amazonensis, T. cruzi*	40, 52
NRBD1		x	x			*L. amazonensis, T. cruzi*	52, 74
PUF11		x				*T. cruzi*	74
ZC3H41		x	x			*T. brucei, T. cruzi*	50, 74
ZC3H34		x				*T. brucei*	50
DBP2		x				*T. cruzi*	74
HEL67 (VASA)					x	*T. brucei*	72
SUB2		x				*T. brucei*	50
LbrM.34.3010		x				*T. brucei*	50
EIF4A1		x				*T. brucei*	50
AGO1		x				*T. brucei*	50
XRNA	x	x		x	x	*T. brucei, T. cruzi*	40, 50, 53, 71, 72
EIF3A		x				*T. brucei*	50
EIF3B		x		x		*T. brucei, T. cruzi*	50, 53, 74
EIF3C		x				*T. brucei*	50
EIF3D		x				*T. cruzi*	40
EIF3I		x				*T. cruzi*	40
EIF3L		x				*T. brucei*	50
EIF2-alpha		x		x		*T. brucei*	50, 53
EIF5A		x				*L. amazonensis*	52
EF1-alpha		x				*T. brucei*	50
EF2		x				*T. brucei*	50
MKT1		x				*T. brucei*	50
NOT1		x				*T. brucei*	50
TDP1		x				*L. amazonensis*	52
tc40 antigen-like		x				*T. brucei*	50
Chaperones proteins		x				*L. amazonensis, T. brucei*	50, 52
40S ribosomal proteins		x	x			*L. amazonensis, T. cruzi*	52, 74
60S ribosomal proteins		x				*L. amazonensis*	52
DRBD3		x				*T. cruzi, T. brucei*	40, 50
PUF6	x	x				*T. brucei, T. cruzi*	50, 74, 83
PBP1		x				*T. brucei*	50
UBP1	x	x				*T. brucei, T. cruzi*	40, 50
UBP2		x				*L. amazonensis, T. brucei, T. cruzi*	40, 50, 52
ZC3H39		x				*T. brucei, T. cruzi*	50, 74
EF1-gamma		x	x			*T. brucei, T. cruzi*	40, 50
EIF3G		x				*T. brucei*	50
EIF3K		x				*T. brucei*	50
EIF4E3		x		x	x	*L. amazonensis, T. brucei*	50, 52, 53, 72
EIF4E4		x		x		*T. brucei*	50, 53
EIF4G5		x				*T. cruzi*	74

Proteins highlighted in gray correspond to proteins significantly associated with *Lb*SCD6 and/or *Lb*RBP42 interactomes. Non-highlighted proteins were found to be present, even though with a number of peptides below the threshold (5 peptides), in the *Lb*SCD6 and/or *Lb*RBP42 interactomes. PB: Processing bodies. SSG: Starving induced stress granules. OSG: Oxidative stress granules. HSSG: Heat shock induced stress granules. NPG: Nuclear periphery granules.

3.4. Overview of proteins identified in the RBP42 interactome

As mentioned above (Fig. 1), the RBP42 interactome was composed by 173 proteins (listed in Supplementary file). They were classified in seven functional categories according to their adscription to biological processes (Fig. 3A). Substantial enrichments occur in proteins belonging to the translation machinery (structural ribosomal proteins and initiation factors) and RNA metabolism (Fig. 3B). Table 2 lists those proteins, uniquely associated with *Lb*RBP42 that are involved in mRNA metabolism, translation and translational regulation expression processes. In the category of mRNA metabolism, we identified proteins like SUB2, an RNA helicase, component of the transcription/export complex involved in the mRNA export pathway, participating in nuclear mRNA metabolism [75]. The LbrM.29.2890 protein, homolog to *T. brucei* RBP6, was also identify. RBP6 is an RNA binding protein with affinity to RNA containing an AU-rich element; their over-expression results in down-regulation of particular *T. brucei* mRNAs, while a lack of RBP6 leads to up-regulation of ARE-containing transcripts [76]. Also, a homolog of *Tb*PUF11 was identify. PUF proteins are RNA-binding proteins controlling mRNAs localization, stability and translation [77]. This PUF11 protein has been recently identify in the RBP9-mRNA complex of *T. cruzi* epimatigotes subjected to nutritional stress [74].

Within the category of proteins involved in the translation proteins, the subunits B, D, E, F, and H of the EIF3 complex were identified. This is an interesting finding considering that most of this protein complex has been also co-immunoprecipitated together with *Lb*RBP42 protein. This EIF3 complex in trypanosomatids is composed by twelve subunits (EIF3A-L) [57,78], and in the *Lb*RBP42 interactome, we found nine of them (EIF3A,B,C,D,E,F,H,I,L). However, it should be noted that subunits EIF3G and EIF3K were also identified in this interactome, although their statistical significances were below the threshold value. This is because, although these proteins are only present in the antibody assay and not in the control (pre-immune serum), the numbers of peptides of the identified proteins was low (less than five peptides which were set as the threshold; see Supplementary file for the complete list of proteins identified in this study). Additionally, we identified other proteins that have been described to be associated with the EIF3 complex, as are the EIF2 subunits A and B, forming the 40S complex [57]. Finally, the PABP1 was also identified. This protein influences transcripts stability, allowing the interaction of the mRNA polyA tail with the EIF4E4/EIF4G3 complex, forming in turn, the major translation initiation complex [79]. In addition, *T. brucei* PABP1 has been shown to stimulate translation of reporter mRNAs [49,59].

Other proteins involved in RNA metabolism that we identified were NOT1, LbrM.31.0080, 14-3-3-I and NUDC1. NOT1 is a component of CCR4/CAF1/NOT complex, which is mainly involved in deadenylation of mRNAs [80,81]. Although being below the statistic threshold, the NOT10 protein was also identified. This protein is important in the association of CAF1 with NOT complex in *T. brucei* [82]. Additionally, both NOT1 and NOT10 proteins were found be enriched in SG under starvation conditions in *T. brucei* [50]. LbrM.31.0080 is a zinc finger (CCCH type)-containing protein that, in *T. brucei* (named ZC3H34), together with 14-3-3-I and NUDC1 proteins, have been described as post-transcriptional activator factors enrichment in starving SG in this parasite [49,50].

3.5. Comparison of SCD6 and RBP42 interactomes regarding the composition of cytoplasmic granules in trypanosomatids

Considering the plausible hypothesis that SCD6 and RBP42 proteins would be components of the ribonucleoprotein granules involved in the regulation of gene expression in *L. braziliensis*, we determined which proteins present in the SCD6 and/or RBP42 interactomes had been previously described as component of different cytoplasmic granules described in trypanosomatids. As shown in Table 3, for a large number of proteins found in the *Lb*SCD6 and *Lb*RBP42 interactomes, their orthologues have been described as component of different RNP granules. Given the variety of RNP granules described in trypanosomatids and the identification of proteins ascribed to the different classes of granules, it is hard to conclude a preferential association of SCD6 and RBP42 to a particular set of granules. However, for *Lb*SCD6, it can be postulated a preferential associated with PB granules, given its specific association with XRNA, a protein predominantly presents in PB [32]. Additionally, SCD6 was found to be associated with DEAD/DEAH helicases, which are marker for this kind of granules.

On the other hand, when the proteins associated with *L. braziliensis* RBP42 are considered, the high proportion of factors engaged with the translation process is striking. Among them, the presence of the multiprotein EIF3 complex and several elongation factors are noticeable, as well as two components of the EIF4E complex (Table 3). Also, the UBP1 and UBP2 proteins were also identified, although with a number of peptides below the threshold (see Supplementary file). All of them have been described as common components of SG induced by starvation [40,50,52]. Moreover, most of these factors have been described as SG components induced by different mechanism (Table 3). Although the role of these structures is not fully understood in these parasites, SG granules are considered to be structures for translational silencing and storing of transcripts, when translation initiation is impaired [83]. Thus, these structures would serve as mRNA triage, which guide to the mRNA targets to sites of re-initiation, degradation or storage [84]. Although our assays did not use parasites under stress conditions, the experimental interactome of *Lb*RBP42 suggests that the protein may contribute to SG (or SG-like) formation, given its association with components of the translational machinery; thus, RBP42 in *L. braziliensis* would be involved in modulating mRNA stability, as previously described for its homolog in *T. brucei* [23].

4. Conclusions

In this work, we describe a list of candidate proteins of the interactomes for *Lb*SCD6 and *Lb*RBP42 proteins. Given the high proportion of proteins common to both interactomes, a functional relationship between both proteins may exist. Additionally, the results of this study provide insights into the protein networks in which *Lb*SCD6 and *Lb*RBP42 would be involved; in particular, mRNA metabolism and translation processes are highlighted. Remarkably, many of the proteins identified in the interactomes have been described too as constituents of mRNPs granules in trypanosomatids. In summary, we postulate that both SCD6 and RBP42 are components of RNP granules linked to the translational machinery and to the regulation of mRNA stability in *Leishmania*. However, further studies are needed to precisely establish the interactions between these two proteins and the other proteins found in the interactomes described in this work.

Data availability

No data was used for the research described in the article.

Declaration Of Competing Interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.104066.

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