Investigating the impact of Arginine methylation upon RBP16 in Leishmania major

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Abstract

Leishmaniasis is a neglected tropical disease, caused by the parasites of the genus *Leishmania* which are transmitted by the female phlebotomine sand flies during blood feeding. According to the World Health Organisation, between 12 and 15 million people in the world are infected, and 350 million are at risk in about 89 countries. Leishmaniasis is considered as a serious public health problem, while the most severe type, visceral leishmaniasis, leads to death if untreated.

As the kinetoplastid genome is co-transcribed in polycistronic arrays, the gene expression is regulated through post-transcriptional mechanisms. The proteins that participate in gene expression regulation at a post-transcriptional level are called RNA-binding proteins (RBPs). Their essential role for parasite viability and divergence from mammalian homologs has led to the investigation of RBPs as potential molecular targets for the treatment of leishmaniasis.

In *Leishmania major*, the RNA-binding protein, *Lmj*RBP16, is a mitochondrial protein that is methylated by protein arginine methyltransferase (PRMT), *Lmj*PRMT7, in the R/G rich C-terminal domain of the protein. *Lmj*PRMT7 plays an important role in pathogenesis as *Lmj*PRMT7 depletion results in an increased virulence while overexpression results in virulence attenuation. *Lmj*RBP16 is a substrate of *Lmj*PRMT7 which methylates the RBPs in arginines containing in the R/G rich domain. While RBPs are distinct from mammals *Lmj*PRMT7 shows homology to *Hs*PRMT7.

The arginine methylation has been shown to regulate both protein-protein and protein-RNA interactions. In the closely related *T. brucei* parasite, RBPs within the mitochondrion are associated with a variety of processes including RNA editing and stability, One mitochondrial RBP, *Tb*RBP16, is involved in the efficiency of RNA editing and it is associated with mRNA stability.

Accordingly, in this project, we investigated the trans regulator RBP16 in *L. major*, since its high similarity to *Tb*RBP16. In this project, the effort focused on the localization and the expression of *Lmj*RBP16 protein in different lifecycle stages and the identification of RNAs that are associated with *Lmj*RBP16 in the presence and absence of PRMT7-dependent monomethylation. We identified that

*Lmj*RBP16 is a mitochondrial protein in WT and *Δprmt7* cell lines in procyclic and metacyclic parasites. Also, *Lmj*PRMT7 is an epigenetic regulator of the parasites gene regulation which is involved in function and protein stability. Thus, we analysed the protein expression of *Lmj*RBP16 upon PRMT7 mediated methylation and no significance differences in protein expression levels was observed in both cell lines for procyclics and metacyclics. Then, RNA immunoprecipitation showed that there is a differential interaction between *Lmj*RBP16:RNAs in the different life cycle stages of *L.major* that needs further investigation.

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Declaration

I declare that this thesis is a presentation of the original work and I am the sole author. This work has not previously been presented for an award at this, or any other university. All sources are acknowledged as references.

CHAPTER I –Introduction

1.1.1 Leishmaniasis

According to the World Health Organisation (WHO), leishmaniasis is caused by a dimorphic protozoan of the genus *Leishmania*. Parasites are transmitted during blood meals of female sand-flies belonging to the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World). Final hosts are animals or human beings. The clinical spectrum of the disease is associated with a broad range of symptoms and levels of severity. Leishmaniasis affects mainly poor people in tropical and sub-tropical countries around the Mediterranean, East Africa, America, and Southeast Asia. Leishmaniasis is considered the second parasitic disease after malaria that leads to death without treatment and it is characterized as one of the most dangerous neglected tropical diseases.

1.1.2 Clinical manifestation

Leishmaniasis clinical manifestations depend on the parasite species and also on the host immunological defense (Ghorbani & Farhoudi, 2018). The most common form of the disease is the cutaneous leishmaniasis (CL) (figure,1B). CL symptoms are observed in areas of the body that are exposed and usually, are visible 1-4 weeks or up to a year after the sandfly bite (McGwire & Satoskar, 2014).CL can be cured in a short time after treatment however in some cases is healed spontaneously in a period of 3–9 months (Reithinger et al., 2007; Soto et al., 2002). Nevertheless, 1-10% of patients that have been cured of CL manifest symptoms of the mucocutaneous leishmaniasis (MCL) (figure 1D). (Burza, Croft, & Boelaert, 2018). MCL is caused by the extension or metastasis of parasites from local skin into the mucocutaneous tissue (Zijlstra, 2014). MCL symptoms include an invasion and destruction of the nasopharyngeal mucous or sometimes only mild local pruritus and swelling (Bravo & Sanchez, 2003). However, the most severe form is the visceral leishmaniasis (VL) in which the absence of treatment can lead to death. The metastasis of parasites and parasite-infected

macrophages to phagocytes within the reticuloendothelial system can cause hepatomegaly, splenomegaly and bone marrow suppression symptoms resulting from the proliferation of parasites in macrophages in the spleen, the liver, and the bone marrow (Burza et al., 2018) (figure 1C). VL is observed frequently in patients with AIDS or drug users but also to immunocompromised (Burza et al., 2014), undernourished individuals and preschool children (Harhay et al., 2011). Finally, VL treated patients after months or years display symptoms of Post-kala-Azar leishmaniasis (PKDL) (figure 1A) in which the parasites proliferate within the skin and cause diffuse macular, maculopapular or nodular lesions (Burza et al., 2018; Zijlstra, 2014).

Figure 1. Clinical manifestation of Leishmaniasis A. Post-kala-azar dermal leishmaniasis (PKDL), a severely debilitating lesion (Burza et al., 2018), **B.** Cutaneous leishmaniasis (CL), a skin ulcer (Torres-Guerrero, Quintanilla-Cedillo, Ruiz-Esmenjaud, & Arenas, 2017), **C.** Visceral leishmaniasis (VL), manifestation of a massive splenomegaly (van Griensven & Diro, 2012), **D.** Mucocutaneous leishmaniasis (MCL), lesion affecting the right nostril, upper lip, and philtrum (Burza et al., 2018).

1.1.3 Epidemiology and distribution

Leishmaniasis has been found in 98 countries and is endemic in Asia, Africa, America, and the Mediterranean region. According to WHO, ~12 million cases of leishmaniasis have been reported worldwide and it is estimated that ~350 million people are at risk, with an estimated worldwide annual incidence of 0.2–0.4 million cases of visceral leishmaniasis (VL) and 0.7–1.2 million cases of cutaneous leishmaniasis (CL) (Alvar et al., 2012) (Burza et al., 2018). In the case of CL, 90% of incidents occur in Brazil, Syria, Peru, Colombia, Afghanistan, Pakistan, Saudi Arabia, Algeria, Iran, , Morocco, Tunisia, and Turkey (Desjeux, 2004) (figure 2A). Leishmaniasis affects individuals of all ages and more than 50% of incidences refer to males (Khezzani & Bouchemal, 2017). MCL incidences have been found mainly in Latin America and less in Ethiopia and Sudan. Visceral leishmaniasis (VL) is endemic in more than 60 countries (Alvar et al., 2012). In South Asia, it is found in more than 60 percent of all VL cases. Generally, VL is endemic in Brazil, Ethiopia, India, Bangladesh, Kenya, Somalia, Sudan, Pakistan, Bangladesh, Nepal, and China (Burza et al., 2018) (figure 2B). The past decade has been noticed a decrease in VL incidences worldwide (Al-Salem, Herricks, & Hotez, 2016). In India, Nepal, and Bangladesh, an elimination in leishmaniasis cases was observed and so while the 50% corresponded to visceral leishmaniasis, in 2005 less than one case in 10.000 people per year reported (Burza et al., 2018). Then, while a steady proportion of visceral leishmaniasis has been reported in east Africa, in Ethiopia, an increase has been observed due to HIV infections and in 2006, up to 40% incidences were referred to as HIV and visceral leishmaniasis co-infection(Al-Salem et al., 2016) (van Griensven, Zijlstra, & Hailu, 2014). Post-kala-azar dermal leishmaniasis incidences range in Bangladesh and India and up to 5% of Indian patients with post-kala-azar dermal leishmaniasis never manifested symptoms of visceral leishmaniasis (Hirve et al., 2016). In east Africa, up to 50% of patients with PKDL have followed a treatment 1 year before. In Asia, post-kala-azar occurs in 5-10% of VL cases after 2-3 years of its treatment. Finally, in European countries incidences have been reported in Portugal, France, Italy,

Greece, Cyprus, Albania and Bulgaria (Pace, 2014) (figure 2A,2B). An increased percentage of leishmaniasis has been reported in non-endemic countries due to migration and international travellers (Burza et al., 2018).

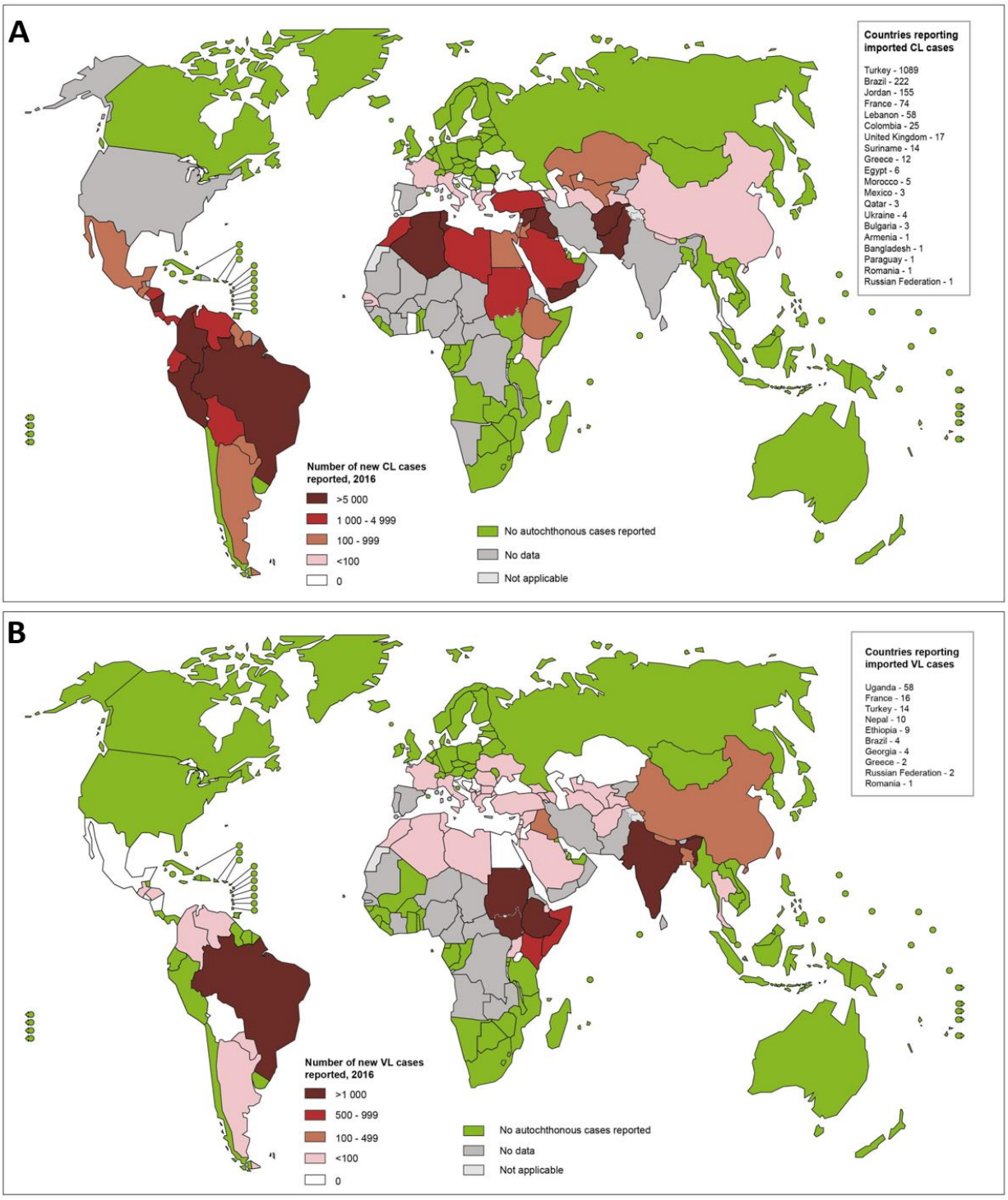

Figure 2.The geographical manifestation of VL and CL according to WHO [1]

A. The map presents the number of new CL cases in the year 2016. The dark brown colour corresponds to more than 5, ooo cases reported in Brazil, Colombia, Peru, Algeria, Yemen, Syria, Iraq, Afghanistan, Pakistan. The red-brown colour represents 1000-4,999 cases of CL found in countries Ecuador,

[1] Epidemiology maps for Visceral and cutaneous leishmaniasis are available on: https://web.archive.org/web/20181019040517/http://www.who.int/leishmaniasis/burden/en/ .

Venezuela, Guatemala, Bolivia, Morocco, Turkey, Sudan, and Saudi Arabia. The lighter brown colour corresponds in 100-999 cases of CL reported in Argentina, Mexico, Guyana, Israel, Kazakhstan, Egypt, and Jordan. Rose colour represents a number under 100 cases in Greece, France, Italy, and China.
B. The map presents the number of VL cases in 2016. Similarly, the dark brown colour corresponds to the highest number of VL (>1000) and corresponds to countries: Brazil, Soudan, Ethiopia, and India. Then the brown-red colour shows the countries Somalia and Kenya that represent 500-999 of VL cases. The lighter brown colour corresponds to the lowest number of VL cases (<100) and included countries are Colombia, Venezuela, Argentina, Uruguay, Guatemala, Morocco, Algeria, Nepal, Tunisia, Libya, Saudi Arabia, Syria, Jordan, Turkey, Cyprus, Greece, Georgia, Romania, Bulgaria, Ukraine, Italy, France, Afghanistan, and Thailand. In both maps, the white colour corresponds to zero cases of VL and Cl while the grey corresponds to no applicable data. Finally, green is shown the countries with reported cases of Leishmaniasis such as Canada, Australia, Russia, and the United States.

1.1.4 Sandfly vector and classification of *Leishmania* Parasites

The vector responsible for the transmission of Leishmaniasis is the sandflies of the genus *Phlebotomus* (figure 3A). and *Lutzomyia* (figure 3B). . Firstly, the genus *Phlebotomus* includes 13 subgenera. Also, the regions that *Phlebotomus* sandflies have been found, include Australian, Afrotropical, Malagasy, Oriental, Palaearctic and so they belong to the Old World sandflies. Usually, they are found in tropical and subtropical regions, with few trees and semiarid climates. It is also characteristic that they are found in different altitudes. The lowest was ~300 meters below sea level and the higher 3,600 meters above sea (Akhoundi et al., 2016). On the contrary, *Lutzomyia* genus consists of nearly 434 species and they belong to New World sandflies. They are mainly inhabits of forests in Central and South America (Akhoundi et al., 2016).

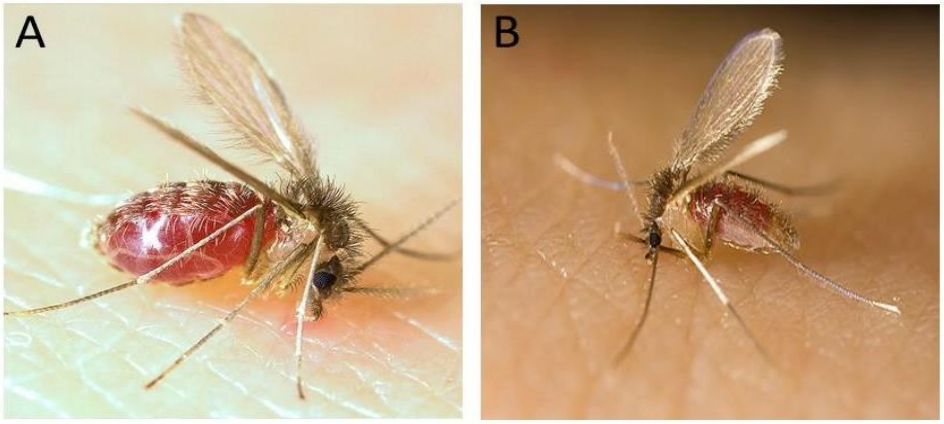

Figure 3. Vectors responsible for leishmaniasis transmission. A. *Phlebotomus* spp**., B** *Lutzomyia* spp. sandflies with bloodmeal-extended midgut regions (Von Stebut, 2015).

Leishmania species classification based on the ecological and geographical evolution of parasites as well as in molecular methods and analyses related to the evolution of the parasites relative to the host (Aaboud et al., 2017). The evolutionary relationship of *Leishmania* is shown to be closer to sandflies rather than the mammalian hosts (Akhoundi et al., 2016). Because of natural and ecological changes parasites were submitted over millions of years to selective pressure, and as a result, they followed a similar distribution. Also, the Palaearctic and the Neotropical region seems to be the origin of parasites and the fact that sandflies were fed firstly with reptile blood indicates that the original host of *Leishmania* might be reptiles (Noyes, 1998) (Tuon, Neto, & Amato, 2008). In figure 4, it is shown the modern taxonomy and classification of parasites *Leishmania* (Cupolillo, Medina-Acosta, Noyes, Momen, & Grimaldi, 2000).

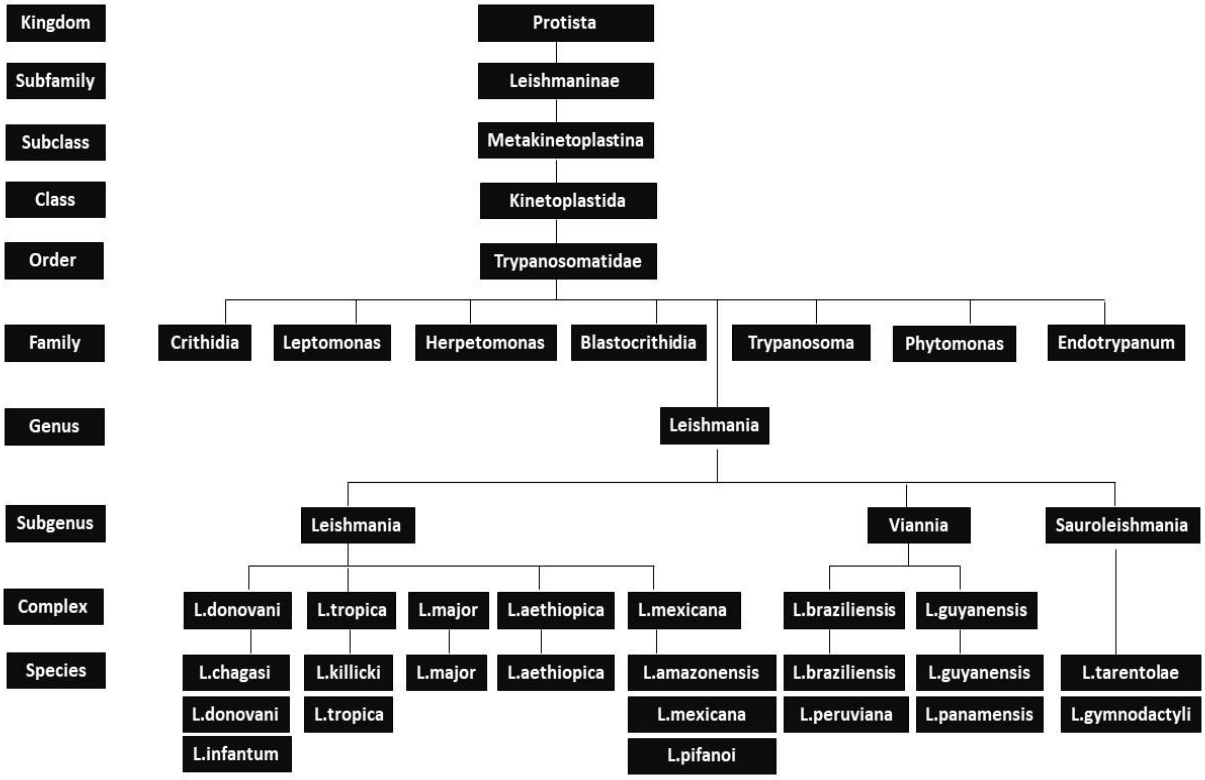

Figure 4. Classification of *Leishmania* species. A recent classification based on molecular data propose that *Leishmania* species adapted figure from (Akhoundi et al., 2016)

1.1.5 Life cycle of the parasite *Leishmania*

The life cycle of parasite *Leishmania i*nvolves the alternation between a vertebrate (mammal) and an invertebrate host (sandflies). Mammals are the final hosts and manifest the symptoms of the disease-caused by *Leishmania* parasites while the sandfly is responsible for the transmission of the parasite from host to host during the blood-feeding. First of all, morphological and functional changes play a key role in the survival of parasites inside the two hosts (Sunter & Gull, 2017). The morphological forms include the extracellular promastigote procyclic (PCF) and metacyclic (META) parasites in sandflies midgut and the intracellular amastigote (AMA) parasite within the mammals (figure 5).

At the beginning of the life cycle, the female sand-fly ingests blood from a mammal which is infected with *Leishmania* parasites (figure 5). During this stage, amastigotes are transferred into the posterior

'abdominal' midgut of sandfly (P. A. Bates & Rogers, 2004) and transform into procyclic promastigotes with ~20 µm length, a process called promastigogenesis, and the parasites multiply the next 24–48 hours by binary fission (Sunter & Gull, 2017). Then, in around 6-9 days the parasites complete their development in the sand fly. Then, during the metacyclogenesis, the procyclics differentiate in the anterior midgut into metacyclic promastigotes which have length around ~15 µm. Metacyclics are located behind the stomodeal valve (Sunter & Gull, 2017) and the life cycle is continued when during blood-feeding the sandfly transfers the infectious metacyclic parasites to the vertebrate host in the site of the bite. Finally, during amastigogeneses process the metacyclics inside the macrophages turn into amastigotes which are round and non-flagellated, with ~3-4 µm length (Sunter & Gull, 2017) (figure 5). The proliferation of amastigotes occurs within the phagocyte, and the parasites now behave as an obligate intracellular parasite of the phagolysosomal compartment until these are released to infect other macrophages (Kamhawi, 2006).

The *Leishmania* life cycle is characterized by a series of differentiation processes. In this case, alterations in transcriptomic and proteomic levels contribute to parasite's survival throughout the three main life cycle stages (PCF, META, AMA). Differentiation events seem to be associated with the environmental conditions (nutrient-rich environment within the insect vector and decreased nutrient availability in the mammalian host). Thus, during the life cycle of *Leishmania* species, it is reported a continuous change in absolute and relative RNA levels in response to an environmental stimulus (De Pablos, Ferreira, & Walrad, 2016). Interestingly, a poor correlation between RNA and protein levels is observed during the life cycle stages (De Pablos et al., 2016). Particularly, metacyclics, compared to procyclics, exhibit a decrease in the total turnover of RNA, protein, and lipid, because of the reduced nutrient availability in the anterior midgut. (Kloehn, Saunders, O'Callaghan, Dagley, & McConville, 2015. In procyclic stage, out of the total genes a percentage of ~40% corresponds to differentially expressed transcripts (DET) while in metacyclics a percentage of ~34% corresponds to DET. According to this data, it is suggested that specific changes in transcriptomic and proteomic during metacyclogenesis is provoked by the environment of the anterior gut (De Pablos et al., 2016) (figure

5). Then, in the first 4 hours of amastigogenesis, 30–40% of the transcriptome is differentially expressed while after 24 hours inside the phagolysosome of macrophages the percentage of DETs is 3% (figure 5) (De Pablos et al., 2016). Thus, it seems that the transformation from metacyclic to amastigotes is accompanied with dramatic changes in transcriptome (Dillon, Suresh, et al., 2015; Fernandes et al., 2016) . Thus, a further significant reduction in absolute RNA and protein turnover is observed in amastigotes . This observation is suggested to be a consequence of the *Leishmania* spp movement to mammalian host where the parasites meet decreased nutrient accessibility and so the conservation of resources occurs through reduction of the proliferative capacity (De Pablos et al., 2016). Hence, these observations strongly suggest that parasites differentiation processes are regulated at a post-transcriptional level.

Further evidence that kinetoplastid gene expression is post-transcriptional includes the organisation of the genes into large polycistronic gene clusters (PGCs) which are constitutively transcribed by Pol II. Accordingly, RNA binding proteins (RBPs) are presented as the main regulatory proteins that are involved in mRNA metabolism (Gehring, Wahle, & Fischer, 2017). However, RBPs expression levels do not correspond to expression levels of RNAs they interact with. Recently, isolation of the total proteome of *Leishmania* showed that 1,174 proteins out of 2,403 of the whole-cell proteome are differentially enriched at one life cycle stage (de Pablos et al., 2019). Specifically, in comparison to the whole-cell proteome, 69, 50 and 27 proteins are enriched in procyclic, metacyclic and amastigotes parasites respectively, and the majority of proteins that are present at each stage, they are present to all stages of the life cycle, too. However, this is not similar to RNA-binding proteins (RBPome) which are differentially enriched in all life cycle stages and also exhibit a stage-specific regulation (de Pablos et al., 2019). Particularly, the RBPome in PCF parasites seems to be enriched for factors essentially connected to translation, as it is the stage of high replication compared to quiescent metacyclic form. RNA binding proteins enriched in META compared to AMA suggests these RBPs are likely essentially connected to differentiation and amastigogenesis initiation (Supek, Bosnjak, Skunca, & Smuc, 2011).

Hence, the progression of each life cycle stage may be associated with essential but distinct RBPs and so trans-regulators could be considered as useful targets for leishmaniasis therapy.

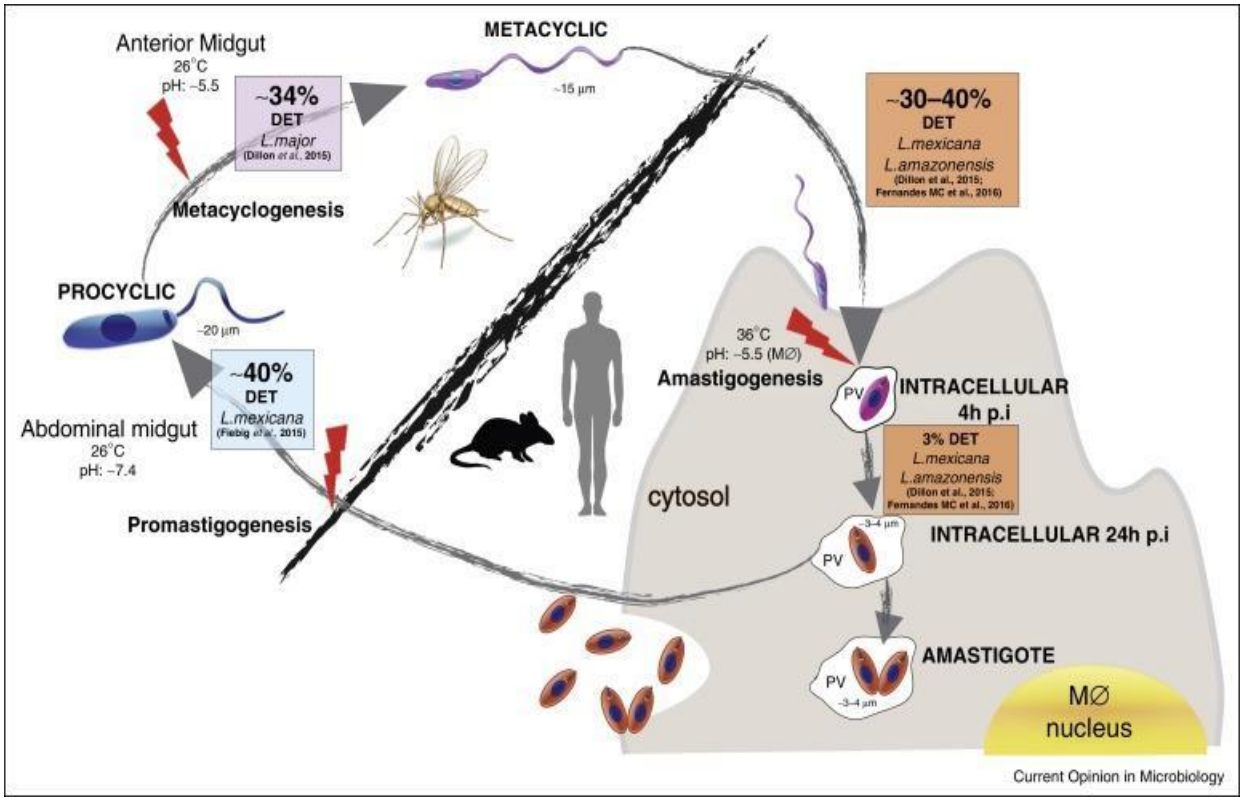

Figure 5. Life cycle of *Leishmania* parasites into the sand-flies and the mammalian hosts (De Pablos et al., 2016). The life cycle of parasites begins with the blood-feeding of infected female phlebotomine sand flies from mammalian skin. During this interaction, the sand fly transmits into mammalian the infective metacyclic promastigote forms. Inside the macrophage, the parasites are transformed into rounded amastigotes, a process called amastigogenesis. After a sandfly's blood meal, the amastigotes are transferred again into the midgut of sand-flies and differentiate into procyclic promastigotes. Finally, the procyclic parasites are differentiating in the anterior midgut into metacyclic form, a process called metacyclogenesis, then the parasites can infect another mammalian host. DET: differentially expressed transcripts, MØ: macrophage.

1.1.6 Therapy

The first-line drug for cutaneous and visceral leishmaniasis treatment is the pentavalent antimony (Sb). The function of Sb is not well known but it is suggested that it inhibits glycolysis and fatty acid oxidation (Berman, Gallalee, & Best, 1987). Another suggestion is that Sb might affect macrophage activity against or affect the parasite's molecular processes (Ghorbani & Farhoudi, 2018). Even though

pentavalent antimonials are a very common drug for leishmaniasis treatment there are a lot of side effects and also parasites display increasing resistance (de Moura et al., 2016). Some of the side effects include cardiotoxicity, pancytopenia and electrolyte abnormalities and renal toxicity elevation of the liver and pancreatic enzymes (Ghorbani & Farhoudi, 2018). Nevertheless, the side effects have been prevented by providing a lower dose of the Sb which results in rapid action, lower costs and also a limitation of the disease in a short time (Ghorbani & Farhoudi, 2018).

A second line drug to treat leishmaniasis is amphotericin B (AmB). As it is known, AmB has an affinity to ergosterol and as a result, it binds to membrane sterols and forms ion channels that increase membrane permeability (Gray et al., 2012). With the pentavalent antimony, amphotericin exhibits side effects such as nephrotoxicity, hypokalaemia, fever, myocarditis and sometimes death (Ghorbani & Farhoudi, 2018). However, the toxicity of the drug has been minimized by the formation of liposomes to contain AmB. AmB is contained within the liposomes and as a result, the drug Is more stable and renal insufficiency and electrolyte abnormalities are reduced (Ghorbani & Farhoudi, 2018). However, as a result of this advance, the drug is more expensive and limited in use in poor countries.

Another drug for leishmaniasis treatment is miltefosine which is a synthetic phospholipid analogue and can be incorporated into the plasma membrane of cells (Geilen et al., 1994). Specifically, miltefosine interacts with enzyme phosphocholine cytidylyltransferase which inhibits the biosynthesis of phosphatidylcholine (Geilen, Wieder, & Reutter, 1992). Low amounts of phosphatidylcholine results in apoptosis (Z. Cui et al., 1996). Also, it is reported that miltefosine affects the replication machinery of parasites by reducing the RNA and protein synthesis (Azzouz, Maache, Garcia, & Osuna, 2005). Also, its effects on the mitochondrion function by producing reactive oxygen species which leads to parasite's death (Vincent, Racine, Legare, & Ouellette, 2015). However, parasites also display resistance to miltefosine. Some strains of parasites do not contain a Miltefosine Sensitivity Locus (MLS) and therefore patients relapse after treatment (Carnielli et al., 2018).

Chemotherapy is one of the approaches for leishmaniasis treatment which displays also disadvantages. The factors that affect the chemotherapy of Leishmaniasis include therapeutic failure (TF) and environmental factors. TF is associated with host-, parasite-, and drug- related factors but also with co-infection cases such as HIV. The most important reason for therapy failure is the drug resistance (DR) in parasites *Leishmania* which is a consequence of parasite biological factors such as the genome plasticity of *Leishmania* parasites and several molecular modifications that occur in stress conditions such as changes in membrane's lipids. The drug efficacy (DE) is also an important factor as the patients that have been cured, exhibit again after months or years, the symptoms of the Leishmaniasis (figure 6) (Ponte-Sucre et al., 2017).

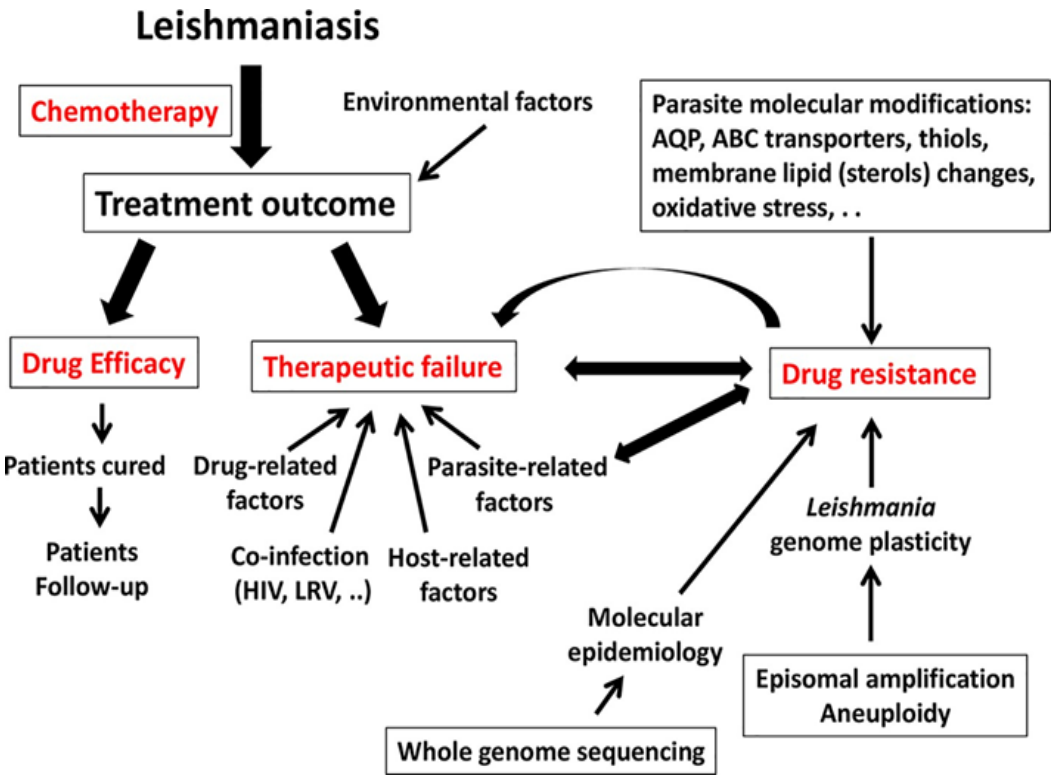

Figure 6. Schematic diagram of the factors that affect the treatment outcome of Leishmaniasis (Ponte-Sucre et al., 2017)**.** Therapeutic failure (TF), Drug resistance (DR), Drug efficacy (DE). The outcome of chemotherapy treatment is dependent on the environmental factors, DE: the patients that cured, the TF: associated factors with parasites, host and drug, co-infection and DR due to the *Leishmania* genome plasticity, aneuploidy, parasite molecular modifications.

Apart from the chemotherapy approach of leishmaniasis treatment, recent studies have focused on the development of vaccination for disease elimination (Ghorbani & Farhoudi, 2018) (figure 6). However, vaccines do not exist as the immune response against the parasites is unclear. For this reason, the understanding of parasites pathogenesis is necessary for the development of a new vaccine which will be more effective and will provide protection against the clinical forms of leishmaniasis (Ponte-Sucre et al., 2017).

The fact that the most infectious diseases occurred in poor and developing countries reflects the difficulties in leishmaniasis treatment. The main problems are that therapy is expensive and there are no facilities to support the drug maintenance and the effective use of the drugs. For these reasons, the discovery of new therapeutic targets that are more effective against leishmaniasis is an urgent is issue.

To conclude, many studies have a focus on molecules, as potential drug targets, which are involved in distinctive biochemical and metabolic pathways essential for parasite survival during the differentiation stages. Viable molecular drug targets may include the RNA-binding proteins (RBPs), which bind to mRNAs forming messenger ribonucleoprotein (mRNP) complexes. This dynamic association plays a key role in mRNA metabolism and function (Hieronymus & Silver, 2003). More specifically, RNA-binding proteins mediate the maturation and stability of mRNAs and subsequently affect the transcript levels within the cell. So, it is reflected that RBPs affect the fate of mRNAs, at the post-transcriptional level, and thereby impact and regulate kinetoplastid gene expression. Finally, many kinetoplastid RBPs do not exhibit homology to other systems. Thus, *Leishmania* RBPs seem to be interestingly distinct from mammals, which is the final host and the organisms manifest the symptoms of the disease. Consequently, recent investigations focus on trans-regulators as targets for the elimination of Leishmaniasis, as they are presented to be involved in the differentiation and survival of parasites in different environments throughout their life cycle.

1.2 Regulation of gene expression in trypanosomatids protozoan parasites

1.2.1 Genome organization

Trypanosomatids genome consists of large polycistronic gene clusters (PGCs) which contain 5-50 protein-coding genes arranged sequentially on the same strand of DNA. These structures are reminiscent of that in bacterial operons. The organization of long arrays in the open reading frames (ORF) of Kinetoplastids was observed firstly on *L. major* chromosome 1, (Martinez-Calvillo, Vizuet-de-Rueda, Florencio-Martinez, Manning-Cela, & Figueroa-Angulo, 2010). Chromosome 1 is the first completely sequenced chromosome in trypanosomatids and contains 85 genes. Totally, the genes are organized into two divergent PGCs and so the bottom strand contains 32 genes while the top strand contains 53 genes (Myler et al., 1999).

Further to the *Leishmania* genome, the publication of the complete genome of *T.brucei* (El-Sayed et al., 2005; Melville, Leech, Navarro, & Cross, 2000) and other organisms of the same class confirmed that in all the trypanosomatid chromosomes the majority of genes are organized into PGCs. However, comparing the *Leishmania spp* to the close relative *T. brucei*, it seems that there are differences in genome size. Specifically, *L. major* nucleus contains relatively small chromosomes and so 36 in total chromosomes consist of 32.8 megabases (Mb) of DNA (Ivens et al., 2005; Wincker et al., 1996). On the contrary, *T.brucei* has 11 large chromosomes and consists of 26Mb of DNA (genome) (Berriman et al., 2005; Melville et al., 2000).

Despite the differences in genome size, there is a strong conservation of gene order (synteny) for protein-coding genes (Berriman et al., 2005) nevertheless, synteny is not observed in the majority of tRNA clusters. Moreover, kinetoplastids lack introns but there is only one isotype of tRNA genes, the tRNA-Tyr, that is found to contain an intron (Boelaert et al., 2004; Padilla-Mejia et al., 2009).

Interestingly, kinetoplastids are diploid and in some cases, it is reported that some chromosomes are aneuploid (Bastien, Blaineau, & Pages, 1992). According to reports, gene copy number variation (CNV) in *Leishmania spp* is associated with drug resistance due to the increased expression of individual genes (Ubeda et al., 2008) (Ubeda et al., 2014). In this point, it is worth noting that many kinetoplastids lack the RNA interference (RNAi) machinery which plays a key role in eukaryotic gene expression. The absence of RNAi is observed in both *Leishmania major* and *Trypanosoma cruzi,* but it is active in *Trypanosoma brucei* and *Leishmania braziliensis* (Lye et al., 2010). Thus, it is hypothesized that the loss of RNAi may cause increased genome plasticity and changes in virulence.

However, despite the differences between *T. brucei* and *Leishmania spp*, it seems that the basic mechanism of gene regulation is largely conserved. Interestingly, common orientation of clustered genes does not indicate a common regulatory pattern as well, however, they are co-transcribed. Thus, the organization of kinetoplastids genome into large polycistronic gene clusters (PGCs) and the few encoded potential regulatory transcription factors (C. E. Clayton, 2002) imply that the regulation of gene expression has to be executed at the post-transcriptional level (Campbell, Thomas, & Sturm, 2003).

1.2.2 Transcription initiation

To begin with, the eukaryotic cell has three classes of RNA polymerase (pol), I, II and III and each RNA polymerase generates different RNAs. Particularly, Pol II transcribes the messenger RNAs (mRNAs) and most of the small nuclear RNAs (snRNA). Pol I produces the 18S, 5.8S, 28S ribosomal rRNAs and finally Pol III generates small RNAs such as snRNAs, tRNAs and 5S RNAs (C. E. Clayton, 2002). In kinetoplastids, Pol I synthesize rRNAs but also are found to be associated with promoters of the VSG genes in *T. brucei*. Pol III transcribes small RNAs and tRNAs similarly to other eukaryotes (C. E. Clayton, 2002) (Figure 7). Finally, RNA polymerase II of kinetoplastid displays high homology with other eukaryotes and it Is responsible for transcription of genes that are included in PGCs) (Figure 7). Hence, primary

polycistronic transcripts are generated by Pol II and they are further processed by trans-splicing and polyadenylation (Gilinger & Bellofatto, 2001) (Anish, Hossain, Jacobson, & Takada, 2009).

Then, a comparative analysis of transcription initiation in trypanosomatids with other eukaryotes revealed differences. Firstly, trypanosomatids characteristic is the lack of promoters. The only promoter of pol II reported before is the one regulating the expression of the SL RNA (Spliced Leader, SL), a small transcribed sequence which is further analysed in section 1.2.4 (Gunzl et al., 1997; Saito, Elgort, & Campbell, 1994).

On the contrary, in other eukaryotes, a particular gene possesses its promoter for transcription initiation. The transcription start site (TSS, +1) and the immediate flanking sequences are found in the promoter region (Boelaert et al., 2004). Polymerase II recognizes 40 bases of the promoter region that contain the *core promoter elements*: initiator (Inr), TATA box, and downstream promoter element (DPE) (Boelaert et al., 2004). The recruitment and assembly of the preinitiation complex (PIC) are directed by the core promoter elements. PIC consists of Pol II and the general transcription factors: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH (Boelaert et al., 2004; Juven-Gershon, Hsu, Theisen, & Kadonaga, 2008).

To most eukaryotes, *L. major* chromosome 1 mapping showed that the transcription of an entire chromosome is driven by a single region. More specifically, divergent transcription units are separated by dispersed start sites, and for each direction, there is a clear overlap between initiation sites (Figure 9(1)). The transcription initiation occurs bi-directional, in the region between the two divergent PGCs (oppositely-oriented) (Martinez-Calvillo et al., 2003) (Figure 9 (1-3)). The initiation start sites are GT-rich sequences found within a <100- bp region (Anish et al., 2009) and do not contain any core promoter elements or a TATA box. Interestingly, most chromosomes have at least two PGCs and transcription units can be over 100 kb long (Jackson, Quail, & Berriman, 2008) (Figure 7).

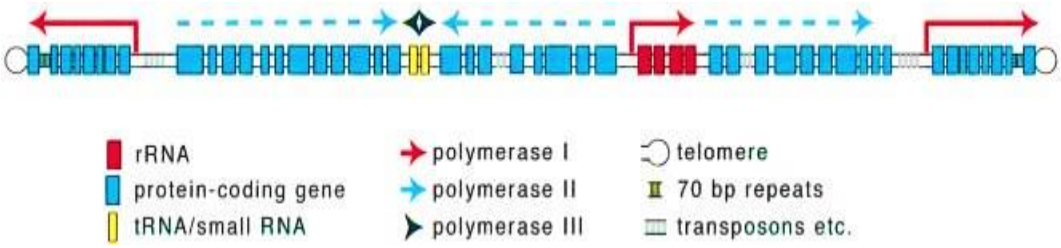

Figure 7. Illustration of a typical *T.brucei* chromosome (C. E. Clayton, 2002). The diagram shows typical features of a non-specific chromosome. It illustrates the diploid chromosome of *Trypanosomatids* in which known regions are transcribed from the correspondent's RNA polymerases.

Despite the differences between kinetoplastids and other eukaryotes in transcription initiation, recent investigations showed the existence of bidirectional promoters of transcription in mammals (Trinklein et al., 2004). This is usually observed in closely located TSSs which lack the TATA box and are highly enriched in GC. Accordingly, it is suggested that GC -rich regions drive the orientation of the promoters (Carninci et al., 2006; Huppert & Balasubramanian, 2007). Finally, although common characteristics exist between mammals and other eukaryotes with the ''strand-switch region'' from chromosome 1 of genus *L. major*, kinetoplastids exhibit similarities to other eukaryotes as well. For example, *T. cruzi* and *T. brucei* divergent ''strand-switch'' regions found to be enriched with histone modifications, a sign of activated genes and transcription initiation in other organisms (Siegel et al., 2009) (Respuela, Ferella, Rada-Iglesias, & Aslund, 2008). Finally, *L. major* chromosome I contains the transcriptional factors, TRF4 and SNAP50, although this chromosome is not characterized by any typical Pol II core promoter elements or TATA box, (Aaboud et al., 2017).

1.2.3 Transcription termination

In Kinetoplastids, termination of transcription by pol II can occur in upstream of the SL RNA array or tRNA genes or genes that are transcribed by pol-I (McAndrew, Graham, Hartmann, & Clayton, 1998; Reynolds et al., 2016; Siegel et al., 2009). However, the mechanism of pol II termination has yet to be described in detail. Some evidence showed that the existence of the ''base *J*'' (beta-D-glucosyl-

hydroxymethyluracil), a specific modification that is associated with termination in a species-specific way. Base *J* was first observed in *Trypanosoma brucei* nuclear DNA before being subsequently found in all kinetoplastid and unicellular flagellates but not in other protozoa or metazoa. Generally, base *J* is present in *Leishmania* (van Luenen et al., 2012), in *T.brucei* and *T.cruzi. T.cruzi* is found to be also associated with the initiation, and in *T.brucei,* J has not been found in procyclic forms (Cliffe, Siegel, Marshall, Cross, & Sabatini, 2010) (Ekanayake et al., 2011). In *L. major*, loss of base J showed an increase in the generation of antisense RNAs at the end of each polycistronic transcript unit (PTU) while in *T. brucei* J deletion resulted in upregulation of downstream genes expression within a PTU (Schulz, Zaringhalam, Papavasiliou, & Kim, 2016). In all organisms, base *J* is found mainly in the telomeric repeats. However, in both *T. brucei* and *T. cruzi*, base *J* has also been observed in repetitive DNA sequences. In *Euglena,* base *J* is also found outside telomeres (Borst & Sabatini, 2008).

In addition to base *J*, investigations in *T.brucei* showed the existence of histones H3V and H4V in the pol II termination region (Siegel et al., 2009). These histones are found also in telomeres where the chromatin is compacted and not transcribed (Muller et al., 2018). Additionally, identification of histones H3V and H4V in the regions of the genome that probably transcription terminates, indicates that the chromatin structure may play a key role in termination as well (Siegel et al., 2009). Further investigations in *L. major* chromosome 3 showed that tRNAs may be involved in termination as they were found between two convergent PGCs (Padilla-Mejia et al., 2009). Finally, RNA-binding proteins association to termination observed in RPB33 protein found in regions where pol II converges. Additionally, the depletion of RPB33 results in increased levels of transcripts originating from silent regions in normal conditions (Fernandez-Moya, Carrington, & Estevez, 2014).

In other eukaryotes, the pol II termination of transcription is associated with polyadenylation, a mechanism called ''torpedo''. Simply the sites of poly(A) adenylation seem to suffice for termination, however, it is enhanced by the 5'-> 3' exoribonuclease Rat1/Xrn2 (Loya & Reines, 2016). In

kinetoplastids, nuclear XRND shows homology to Rat1/Xrn2 and although it has an essential role, its detailed function has yet to be known (Li et al., 2006).

1.2.4 Processing of RNA

After transcription termination, the primary transcript (pre-mRNAs) is submitted to distinct processes for the generation of a translatable mature mRNA. Firstly, the individual mRNAs are created through endonuclease cleavage of precursor RNAs by RNAase (C. Clayton, 2019). Then, as in most eukaryotic, the precise excision of the introns that are found in between exons results in the generation of a mature mRNA. This process occurs through a mechanism called splicing, and it can be distinguished in *trans*- and *cis*-splicing. *Cis*-splicing is a classic mechanism to generate mRNAs in mammals. In *cis*-splicing, encompassed exons are joined in one pre-mRNA molecule. *Trans*-splicing occurs when two independent transcripts are joined together (Konarska, Padgett, & Sharp, 1985; Solnick, 1985) (figure 9 (3)).

Trans-splicing was first reported in kinetoplastids and then in yeast exons (Mayer & Floeter-Winter, 2005). However, *cis*-splicing exists in kinetoplastids as well, in the gene encoding the poly (A) polymerase (Mair et al., 2000). *Trans*-splicing in mammals is involved in the generation of protein diversity and subsequently, this mechanism has been adapted to a particular model of gene expression.

In *Trypanosomes*, the process of *trans*-splicing exhibits similarity to the *cis*-splicing of mammals. In mammals, *cis*-splicing occurs accordingly: the 5' splice site consensus is AG/**GU**RAGU in which R is the splice site and indicates 'purine' while bold is the dinucleotide 5' intron boundary (Mayer & Floeter-Winter, 2005). The 3' splice site consensus is Y**AG**/G in which the splice site is the **Y** and indicates 'pyrimidine' while bold is the dinucleotides 3' intron boundary (Mayer & Floeter-Winter, 2005). Around the branch point, there is the sequence of conserved nucleotides, CUR**A**Y, in which **Y** indicates

the pyrimidine, **R** the purine and **A** the branch (Mayer & Floeter-Winter, 2005). Also, between the branch point and the 3' splice site, it is observed a polypyrimidine tract of which the length varies (figure 8, def).

In kinetoplastids, characteristics of *trans*-splicing are the 39-nucleotide leader sequence (Spliced Leader, *SL*) or mini-exon at the 5' terminal of all mRNAs which is included in a small transcribed leader sequences of 135-147 nt, called SL-RNA (Ullu, Matthews, & Tschudi, 1993). This sequence was found in all mRNAs that encoding ''variant surface glycoproteins'' (VSGs) of *T. brucei* and later in all mRNAs of the organism and other organisms of the same class (Parsons, Nelson, Watkins, & Agabian, 1984). More specifically, the canonical GU at exon-intron boundary of 39 nt is observed in SL RNA substrate. Additionally, in pre-mRNA substrate, it is observed the 3' AG splice site (Liang, Haritan, Uliel, & Michaeli, 2003) (Benz, Nilsson, Andersson, Clayton, & Guilbride, 2005). This splice site is localised at the non-translated 5' part of the pre mRNA where the branch point is the conserved adenosine (A) residue (Mayer & Floeter-Winter, 2005) (figure 8a). Similarly, to mammals, between the 3' acceptor and the branch point, a polypyrimidine tract exists. Finally, the position of the conserved AG dinucleotide and the splice acceptor at the 3' splice site and an upstream polypyrimidine region site, is an important characteristic for this process (Benz et al., 2005; Hummel, Gillespie, & Swindle, 2000; Siegel, Tan, & Cross, 2005) (figure 8).

Comparing the ribonucleoprotein machinery, spliceosome, of trypanosomatids with other eukaryotes some significant similarities are indicating that splicing regulation may have arisen early in eukaryotic evolution. In mammals, components of the spliceosome are Sm core proteins. Also, in *cis*-splicing are involved the small ribonucleoproteins (U1, U2, U4, U5, U6 snRNPs) and approximately 300 distinct protein. Similar to mammals, kinetoplastids spliceosome components are the Sm core proteins (Jae et al., 2010; Tkacz et al., 2010). Briefly, the snRNPs U2, U4, U5, and U6 are involved in *trans*-splicing, however the SL RNA takes the place of the U1 snRNP which is implicated in *cis*-splicing of two transcripts (Palfi, Schimanski, Gunzl, Lucke, & Bindereif, 2005).

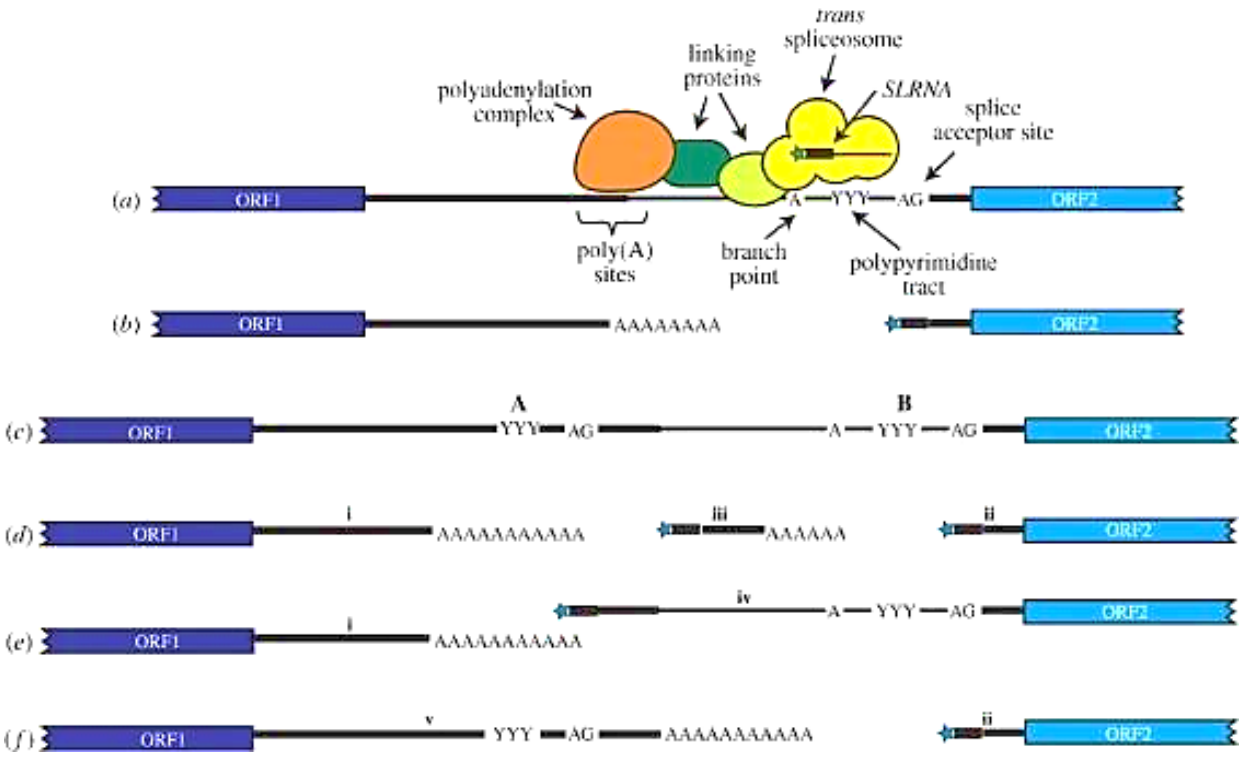

Figure 8. Schematic diagram of mRNA processing in *T. brucei* (C. Clayton, 2019). (*a*) This diagram shows two different open reading frames, ORF1 and ORF2. In (a) line is shown the proteins that are involved in this process. Two hypothetical linking proteins are bound to mRNA and interact with polyadenylation and splicing complexes. In (b) line is shown the simplest process of single splicing signal in a pre-mRNA. Line (c) shows two splicing signals, A and B of which only one protein will be produced If both signals are not used. This protein will have more than one open reading frame in mRNA. Line (d) shows the generated RNAs from both A and B signal sites: the RNA1 (i) with a short 30-UTR ORF1, the RNA2 (ii) with 50-UTR and also a processed RNA (iii) from intergenic region lack open reading frame. In (e) line is shown the generation of RNA with a long 50-UTR ORF2 (iv) including splicing signal (b) which is resulted by the site A of line (c). Finally, in (f) line, utilization of site B from (c) line results in the generation of mRNA with a long 30-UTR ORF1 (v) with the polypyrimidine tract included.

Mechanistically, in *trans*-splicing, a trimethyl cap of the spliced leader (SL) to the 5' end is required for the process (Ullu & Tschudi, 1991). Pseudouridylated *SL* RNA has been also reported but it is not necessary for the function (Hury, Goldshmidt, Tkacz, & Michaeli, 2009). As occurs in other organisms, capping and methylation of *SL* RNA occurs co-transcriptionally (Mair et al., 2000). In *T. brucei*, a cap-binding complex (CBC) has been reported to bind the trimethyl cap. CBC consists of five proteins: first three unknown, then Importin and finally CBP20, (Allahverdiyev et al., 2005). Then, instead of a lariat

intermediate which is formed in *cis*-splicing, a branched Y-structure intermediate has been observed in *trans*-splicing. In this case, upstream of the polypyrimidine tract, the *SL* RNA intron is 20-50 joined to an A residue in the intergenic region (figure 8a).

Finally, in addition to *trans*-splicing, the process of mature mRNAs is completed by the adding of a poly (A) tail, (Lucke, Jurchott, Hung, & Bindereif, 2005; Patzelt, Perry, & Agabian, 1989) (figure 9(5)). Polyadenylation sites are found within a short region 100–400nt in the 3' mRNAs and upstream of the next polypyrimidine *trans*-splicing signal (Dreyfuss, Kim, & Kataoka, 2002; Matthews, Tschudi, & Ullu, 1994; Schurch, Hehl, Vassella, Braun, & Roditi, 1994). Polyadenylation site is species-specific and located upstream from the active polypyrimidine tract (figure 8b). This site has a specific signal, the processing apparatus 'measures' which is dependent also on the species (C. Clayton & Michaeli, 2011; Dillon, Okrah, et al., 2015; Kolev et al., 2010).

The intergenic regions might also contain splicing signals and so it is suggested that acceptor sites are not found only at the beginning and the end of the transcription units (C. Clayton & Shapira, 2007). The production of non-translatable RNAs is directed by the multiple polypyrimidine tracts in the intergenetic sites (Benz et al., 2005) (figure 8c). Despite the suggestion of coordinated regulation of genes in trypanosomatids, it is suggested that nearby coding transcripts are processed independently due to the existence of *trans*-splicing signals in mRNAs which lack downstream open reading frames (Benz et al., 2005) (figure 8d). The generation of ORF-less processed RNAs explains how most of 3' mRNA in a polycistronic is polyadenylated. Finally, composition and the length of the polypyrimidine tract are two characteristics that determine the constitutive efficiency of a splicing signal (Siegel et al., 2005) (figure 8e,f). Consequently, the degradation of mRNAs that occurs faster than splicing, determines the poorly expression of a transcript (Hartmann, Hotz, McAndrew, & Clayton, 1998; Lopez-Estrano, Tschudi, & Ullu, 1998).

1.2.5 Degradation of mRNA

Similar to mammals and yeasts, kinetoplastids mRNA degradation follow the path of deadenylation as Poly (A) binding proteins are expressed that have poly (A) nuclease activity (deadenylase) (Hotchkiss et al., 1999; Bates et al., 2000) (figure 9(6)). In mammals, the stabilization or destabilization recognition sign of mRNAs is found in 3' untranslated regions (3' UTR) and contain the AU rich elements (AREs). Regulatory proteins recognize AREs and proceed to inhibit or promote deadenylation (Caponigro and Parker, 1996). In ARE-containing mRNAs, the poly (A) tails are trimmed to a short oligo (A) and this results in mRNA 3' decapping. Subsequently, degradation of mRNAs occurs in 5'-3' direction by cytoplasmic exonucleases and by the exosome, a large exonuclease complex which acts in direction 3'→5' (Butler, 2002).

In Trypanosomes, four potential exoribonucleases have been reported, XRNA, XRNB, XRNC, and XRND and it is suggested that they are similar to Xrn1. It is not known the function of XRNB and XRNC. However, it is known that XRNA is involved in mRNA degradation in both nucleus and cytoplasm (Kerr, Maitland, Bell, White, & Hunter, 2017) (Figure 9(10-13)). Interestingly, XRND is a nuclear exoribonuclease and is involved in mRNA degradation, despite the fact that in cytoplasm takes place the degradation of that most mRNA. The decay of mRNAs in trypanosomatids resembles AU-rich elements in mammals (Bloom et al., 2017), and subsequently, degradation occurs in U-rich elements (UREs) in the 3'-untranslated regions (3'-UTR) of the transcripts. However, in kinetoplastid, transcripts degradation is independent of deadenylation (C. Clayton, 2019).

In kinetoplastids, mRNA degradation is regulated by trans-regulatory factors that bind to RNAs (Dreyfuss et al., 2002). The regulation of the URE and RNA stability is controlled by proteins that are characterized as RNA-binding proteins (RBPs) (Lueong, Merce, Fischer, Hoheisel, & Erben, 2016). Interaction of various RBPs with mRNAs occur in 3' untranslated region (3'-UTRs). Specifically, the RNA binding domain (RBDs) of RBPs interact with specific sequence and/or structural motifs of RNAs (Kolev, Ullu, & Tschudi, 2014). This interaction results in changes in mRNAs localisation, stability, and

translation (Lueong et al., 2016). Evidence from some *Leishmania* species showed that they lack the RNAi machinery and although in *T.brucei* is active, its role is preferably the suppression of viruses and retroposons (Lye et al., 2010). Subsequently, it is proposed that the fate of mRNAs is dependent on the combinational interaction with proteins. The interaction of mRNA and proteins is called 'messenger ribonucleoprotein particle' (mRNP) (Linker et al., 2005).

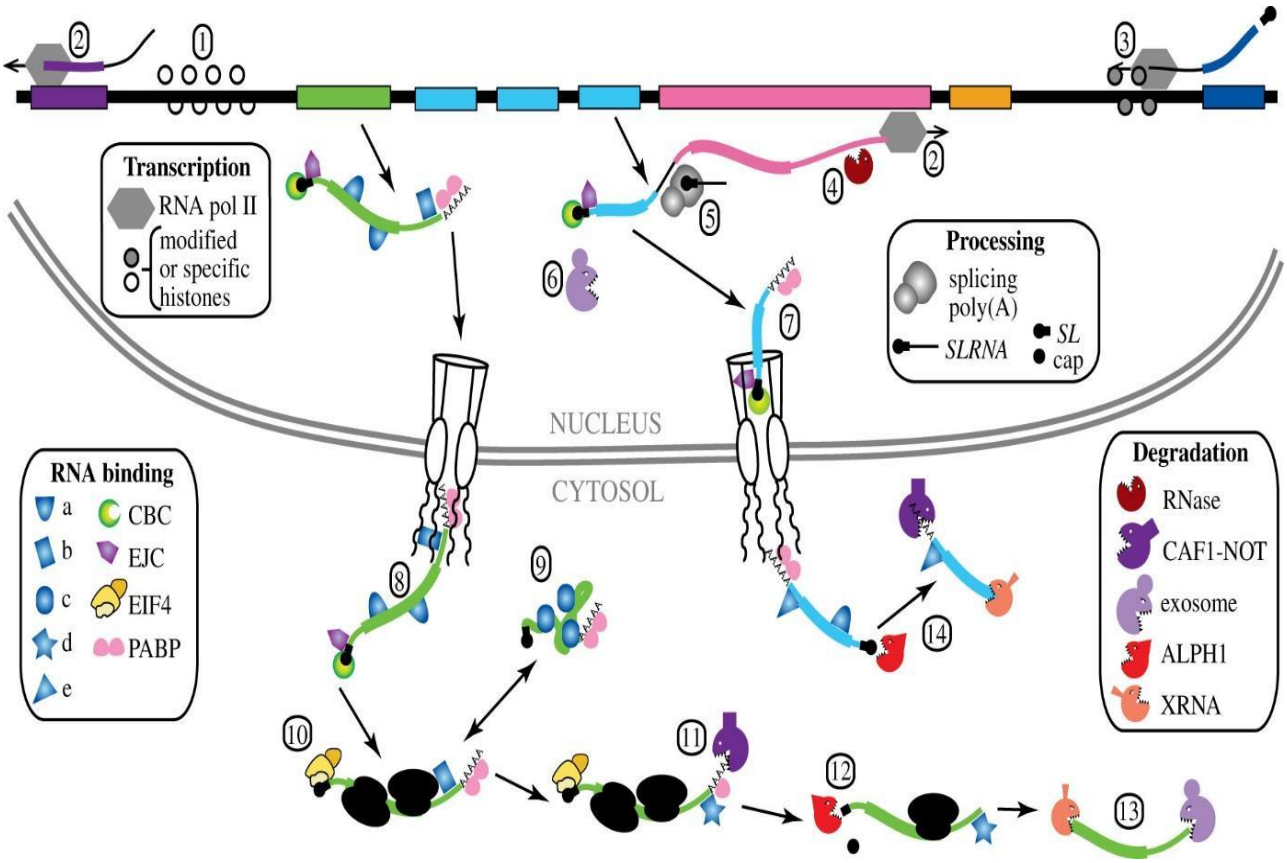

Figure 9. Diagram of gene expression in kinetoplastids. Adapted from (C. Clayton, 2019). The thick line is shown in the genome with multiple coloured mRNAs. In each mRNA, the thicker line represents the coding regions while the less thick line with the same colour corresponds to the untranslated regions. In the beginning, RNA pol II recognizes the initiation regions due to modified histones (1). Then Transcription elongation by pol II occurs (2), and finally the termination (3). Then, RNase involvement results in the endonuclease cleavage of precursor mRNA (4) and pre-mRNA is submitted to trans-splicing and polyadenylation (5). The incomplete mRNAs are degraded by the exosome (6). Complete mRNAs are exported to the cytoplasm by interacting with poly-A) binding protein (PABP), exon junction complex (EJC) and nuclear cap-binding complex (CBC) (7). The mature mRNA interacts

with proteins on the coding region and 30-untranslated regions (8). The mRNAs then can interact with the RNA-binding protein c, a silencing or aggregating protein and the complex condensates into granule (9). Apart from that mRNAs can be translated by the bound of translation factors EIF4E, EIF4G and EIF4A (10) Or mRNA is submitted to the deadenylation process after the replacement of protein by the destabilizing RNA binding protein d which (11). Then ALPH1 action results in decapping (12). And finally, mRNA is degraded by the XRNA and the exosome (13). Protein e promotes the decapping and results in a rapid decay (14).

1.3 RNA-binding proteins

As it is mentioned, the genome of Trypanosomes is co-transcribed in polycistronic arrays and thus the gene expression is regulated through transcriptional and post-transcriptional mechanisms (Haile & Papadopoulou, 2007) (Tsigankov, Gherardini, Helmer-Citterich, & Zilberstein, 2012). In this regulation, many kinds of proteins participate. Among these are RNA-binding proteins (RBPs) that are implicated in multiple events within the Kinetoplastids cells. More particularly, RBPs play a key role in mRNA fate as they bind to precursor, mature and decaying mRNA and regulate their fate and stability (Lueong et al., 2016).

RNA interactions are dynamic and change constantly in response to stimuli. Interaction of RNA-binding proteins with RNAs and the subsequent control of gene expression is dependent on RBPs repertoire and activity which influence the transcript stability and/or translational efficiency. However, RBPs activity is associated with several factors such as post-translational modifications and cofactor binding (Arif, Jia, Moodt, DiCorleto, & Fox, 2011; Clingman et al., 2014).

1.3.1 RNA-binding Domain

In kinetoplastids, there are distinct groups of mRNA binding proteins that are categorized according to their RNA-binding domain. In table 1, it is described the main categories of RNA-binding domains in kinetoplastids and general functional characteristics:

Category of RBPs according to RNA-binding domain	Characteristics and functions
RNA recognition motifs (RRMs)	RBPs consist of approximately 90 amino acids residues that include the octapeptide RNP1 sequence motif: (K/R) G (F/Y) (G/A) FVX (F/Y) (Dreyfuss et al., 2002). However, apart from RNP1 there is also the known RNP2 motif and both are involved in RNA binding through the central part of the RRM beta-sheet. RNP usually exists in many copies to enhance the binding specificity and are involved in the whole life of RNA processing (Lunde, Moore, & Varani, 2007).
Poly (A) binding domain (PABP1 and 2)	These proteins have been reported to bind to the poly (A) tail and interact with initiation translational factors to increase the translation efficiency (E. J. Bates, Knuepfer, & Smith, 2000; Batista, Teixeira, Donelson, Kirchhoff, & de Sa, 1994; Hotchkiss, Nerantzakis, Dills, Shang, & Read, 1999)
Zinc finger domain	Proteins contain the zinc finger domain (Brown, 2005). Zinc finger domains have been found in both N' and C' terminus. In the N' terminus, protein binds specifically to 39-(UAU) repeats in the mRNA UTRs, and the C-terminal domain regulates the stability.
hnRNP K-homology domain (KH)	Proteins with this motif are involved in several RNA processes such as transcriptional regulation, and translational control (Valverde, Edwards, & Regan, 2008).
serine arginine-rich domains	Proteins with this domain are involved in splicing (El-Sayed et al., 2005)
glutamine-rich or glycine-rich regions	These proteins have G- or U-rich RNA binding specificity and are involved in posttranscriptional control of gene expression promoting RNA stability (De Gaudenzi, D'Orso, & Frasch, 2003)
Alba domain	This domain has found in proteins involved in mRNA stabilization (Ferreira et al., In Submission, 2020).
Pumilio domain	PUF proteins are involved in mRNA degradation and rRNA maturation through the recognition of a putative motif, UUGUAC (Chritton & Wickens, 2010).
DEAD-box	RNA helicases contain a DEAD-box with the conserved sequence Asp-Glu-Ala-Asp (Ivens et al., 2005). These are the mediators of conformational change in RNA. This results in the unwinding of RNA duplexes or the disruption of RNA-protein interactions (Q. Yang & Jankowsky, 2005).

Table 1. List of mRNA binding proteins that are categorized according to their RNA-binding domain.

1.3.2 The mRNA-bound proteome of *Leishmania major*

The mRNA-bound proteome (mRBPome) has been isolated in large-scale and the regulatory candidates have been identified in many different organisms. In *Leishmania spp*, trans-regulators are characterized as primary gene regulators, however, not many investigations resulted in the validation or characterization of these regulators. Recently, the *L. mexicana* mRBPome isolated during different life cycle stages of parasites as well as the whole-cell proteome (de Pablos et al., 2019). More specifically, for mRBPome isolation procyclic promastigotes (PCF), metacyclic promastigotes (META) parasites and amastigotes (AMA) parasites derived from *in vivo* lesion (AMA (LD)) and 24-hour post-infection macrophages (AMA (MØ)) were used. The investigation included both post-UV cross-linked (XL) and non-cross-linked (nonXL) cultures for RBPome validation. UV irradiation results in an RNA-RBPs complex which is covalently cross-linked *in vivo*. Thus, the exposure of parasites to UV irradiation aimed to optimize the *in vivo* RBP: RNA (mRNP) crosslinking, providing mRNAs superior integrity while the heat stress was negligible (de Pablos et al., 2019).

From the whole cell proteome of *L. mexicana*, 2,403 proteins were identified of which 1,174 proteins seem to be enriched in one life stage. Additionally, 1,407 RBPs were identified for XLs samples of which 294 considered stage-specific proteins, while 1,403 RBPs from nonXL samples were also identified and 216 of these are referred to as stage-specific.

In vivo investigation of the mRNA-bound proteome in *T.brucei* bloodstream forms isolated 155 RBPs (Lueong et al., 2016). Relative *in vivo* studies of mRNA-bound proteome for *L. donovani* in amastigotes resulted in the isolation of 128 RBPs of which 49 showed no significant homology to human RBPs identified before (Nandan et al., 2017). Finally, similarly to *Leishmania*, RNA interactome studies showed that *Homo sapiens*, *Mus musculus,* and *Saccharomyces cerevisiae* have in total 1,914, 1,393 and 1,273 RBPs, respectively (Hentze, Castello, Schwarzl, & Preiss, 2018).

The isolation of the whole cell and mRBPome of *L. mexicana* revealed that mRNA-binding proteins are differentially regulated according to the lifecycle stage in both experimental procedures, XL and nonXL. More particularly, proteomics analysis showed that in PCF-XL samples the RBPome is appeared more enriched, comparing with the META-XL proteome, for factors that are essentially connected to translation, and it is consistent with the observed high division rate in promastigote procyclic stage. Comparison between META-XL and AMA (MØ) displayed a large distinction in these two stages, despite the short time (24hr) of parasites proliferation in macrophages (figure 8 B). Interestingly, the overall RBPome of AMA (LD) and AMA (MØ) is similar. Thus, it is suggested that RBPs are implicated in the differentiation process of *Leishmania major* parasites and regulation is stage-associated (de Pablos et al., 2019).

Further investigations focused on the expression of specific peptides and the levels of associated transcripts in procyclic and metacyclic promastigotes (De Pablos et al., 2016). Interestingly, the levels of RBPs are often higher than mRNAs and so the abundance of RBPs ensured the binding to transcripts and their further regulation. However, bioinformatics analysis from (de Pablos et al., 2019) showed that the ''whole cell expression of an RNA binding protein in *L. mexicana* specific lifecycle stage does not guarantee its association with target mRNAs''.

Analysis of RBPs expression revealed that their levels are variable in comparison with transcript expression. RBP16 is an example of this. RBP16 levels do not show a correspondence to encoding transcripts (de Pablos et al., 2019). More specifically, the RNA binding activity of RBP16 does not correspond to the protein's RNA transcript levels, as transcript and protein levels of RBP16 do not show a relative close correlation (de Pablos et al., 2019). Taking into consideration that RBPs are substrates for PTMs, it is assumed that post-translational modifications of RBPs play a key role in their functions. In *Leishmania*, stage-specific regulation of RBPs has been reported by PTMs and so PTMs change the regulation of RBPs activity at the post-translational level.

Accordingly, recent investigations focused on the impact of protein arginine methyltransferase 7 (PRMT7) showed that RBP16 is post-translational modified by PRMT7 and its methylation affects its stability in a stage-specific association. Furthermore, the RNA-binding protein Alba3 is also a substrate of PRMT7 and its methylation results in differentially mRNA-binding affinity. Particularly, Alba3 interacts with the mRNA *δ-amastin* facilitating its stabilization upon methylation however, Alba3 association or stabilization with *nmt* transcript is not impacted (Ferreira et al., 2019). Hence, arginine methylation regulators, such as PRMT7, modulate trans regulators and result in differential impact upon transcript targets. Additionally, as the gene regulation occurs in a post-transcriptional, it seems that the primary regulatory role belongs to trans regulators. So, the impact of PRMTs in RBPs function is important for the investigation of the transcriptional control in trypanosomatids.

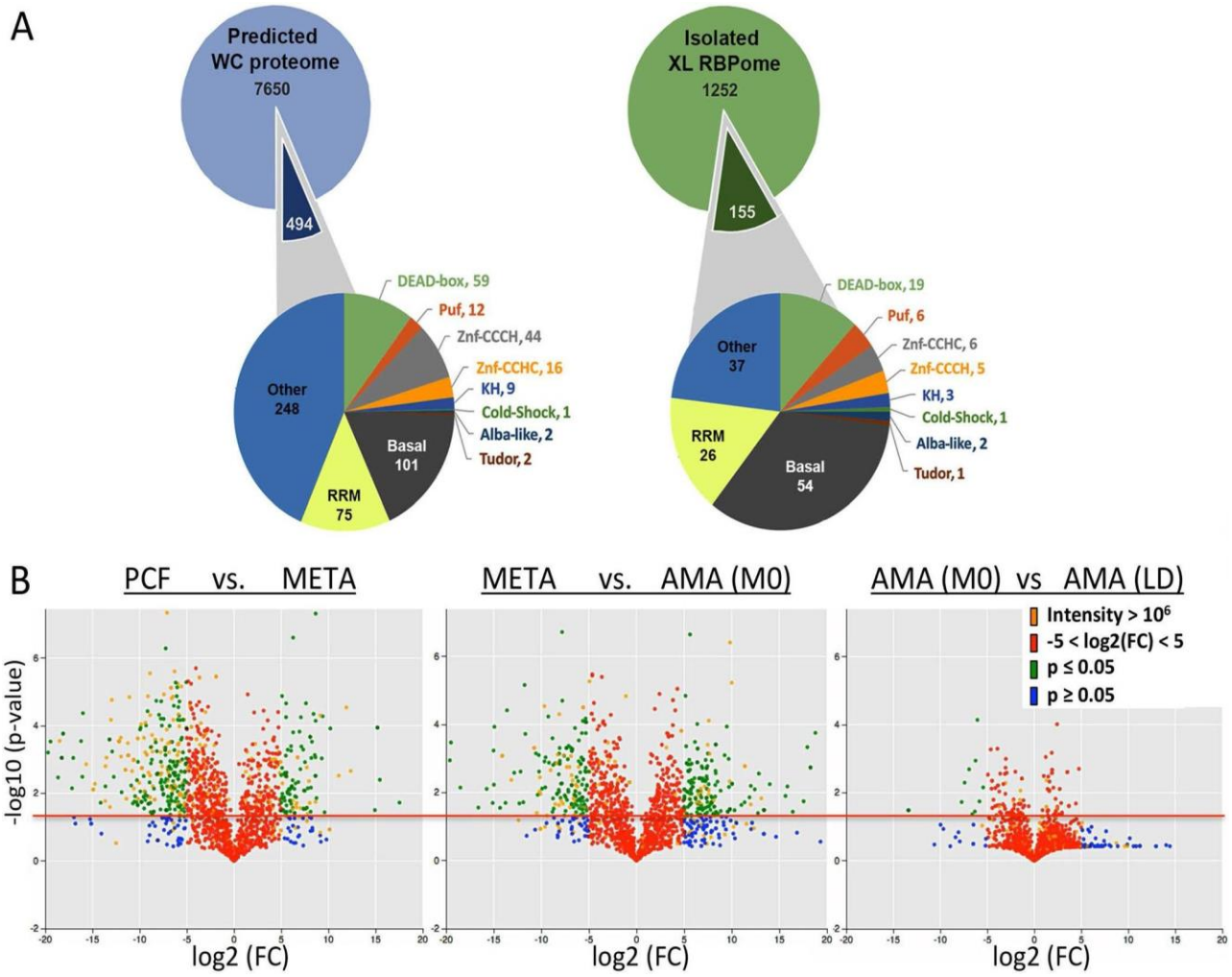

Figure 10. Diagram of whole-cell and RBPome comparisons from life cycle stages of *L. mexicana* (de Pablos et al., 2019). **A** figure shows the predicted whole-cell (WC) proteome of parasites and the isolated RBPome. The RBPs number was identified according to RNA-binding domains. Specifically, out of 8144 genes, 494 proteins contain RBDs in the WC RBPome, while in the XL-RBPome, the number of RBPs is 155, out of 1407 proteins. The sign "Other" corresponds to domains that are not highly conserved or less abundant domains within RBPs. "Basal" corresponds to proteins homologue to splicing and translational machinery. The **B** illustration corresponds to Volcano plots that show the RNA binding protein enrichment in each life cycle stage. Specifically, proteins that are found above red lines are remarkably enriched in one life cycle stage. Above the red line boundary, proteins are shown with green and below with blue. Highly expressed factors are shown with orange circles.

1.3 Kinetoplastids RNA binding protein 16 (RBP16)

Trypanosomes RBP16 is a mitochondrial RNA-binding protein which is reported firstly in the closely related *T. brucei*. Briefly, Hayman and Read purified this protein after an investigation which aimed to identify molecules that bind gRNAs in the mitochondrion of *T. brucei* (Hayman & Read, 1999). According to immunoprecipitation experiments, *Tb*RBP16 was represented as an abundant gRNA-binding protein which interacts with different gRNAs through the oligo(U) tail (Pelletier, Miller, & Read, 2000). Hence, further investigations of these proteins led to the suggestion that *Tb*RBP16 is an RNA-binding protein that controls RNAs stability in the mitochondrion (Goulah & Read, 2007). Additionally, its function is dependent on post-translational modifications and more particularly Arg methylation of *Tb*RBP16 by PRMT1 plays a key role in all these processes as it regulates the protein-protein and protein-RNA interactions (Goulah & Read, 2007).

In the mitochondrion, *Tb*RBP16 is associated with the 30% of native guide RNAs (gRNAs) but also with several mitochondrial mRNAs and this association strongly proposes that *Tb*RBP16 may is involved in the process and regulation of RNA editing (Ammerman, Fisk, & Read, 2008). Another association of *Tb*RBP16 that has been reported is 9S and 12S ribosomal RNA (rRNAs) which suggests regulatory function of RBP16 in RNA translation (Pelletier et al., 2000) (Hayman, Miller, Chandler, Goulah, & Read, 2001) In all cases, mRNAs, gRNAs and rRNAs, RBP16 binds the RNA with a similar affinity, through the oligo (U) tails (Miller & Read, 2003) Moreover, the fact that the *Tb*RBP16 is not a stable component of editosome proves that its function is limited to RNA editing itself rather than regulation of the process of editing (Ammerman et al., 2008). Consequently, *Tb*RBP16 is an important protein for the mitochondrial gene expression in *Trypanosome brucei* and it is implicated in crucial regulating functions including the polycistronic transcript cleavage, polyadenylation, RNA stability/editing as it has been referred. However, the Arg methylation of RNA-binding proteins affects these processes as it regulates the protein-protein and protein-RNA interactions (Goulah & Read, 2007).

In *Leishmania major*, *Lmj*RBP16 investigated recently from Ferreira et al., 2019, of which the role is dependent on the post-translational modifications by PRMT7. Initially, as *Lmj*RBP16 displays high homology to *Tb*RBP16, it was hypothesized that its role might be similar to that in *T. brucei*. However, cytosolic *Lmj*RBP16, in the absence of PRMT7, found to be unstable in a metacyclic life cycle. On the contrary, mitochondrial *Lmj*RBP16 is a stable protein for both life cycle stages, procyclin and metacyclic. Then, *Lmj*RBP16 is identified as a hypomethylated protein upon the loss of function of PRMT7. However, in the isolation of mRNA-bound proteome RBP16 was found to bind RNAs and so its function might be similar to *Tb*RBP16 (de Pablos et al., 2019).

RBP16 consists of two domains, an N-terminal cold shock domain (CSD) and a C-terminal arginine and glycine-rich region. The CSD domain shows homology to bacterial cold shock proteins and Y-box mammalian proteins (Hayman et al., 2001). In bacteria, the cold shock family proteins are referred to as RNA chaperones and they are associated with RNA by preventing non-desirable RNA secondary structures (Jiang, Hou, & Inouye, 1997).

In eukaryotes, some of Y-box proteins have been implicated in transcriptional and post-transcriptional regulation of gene expression, For example, in eukaryotes, the Y-box protein, Yb-1 is implicated in RNA stability and transcription control, in translation and RNA packaging (Raffetseder et al., 2003; Sommerville, 1999). However, *Lmj*RBP16 exhibits homology to the Y-box 1 (Yb-1) of mammals. Yb-1 is localized both in cytoplasm and nucleus and its function is relevant to the events of these environments. Yb-1 was found to have nucleic acid activity binding to both DNA and RNAs and involved in transcription, stress response, regulation of mRNAs splicing and apoptosis (Kohno, Izumi, Uchiumi, Ashizuka, & Kuwano, 2003) (Lyabin, Eliseeva, & Ovchinnikov, 2014) (Lyabin et al., 2014). It is also considered a mediator of mRNA translation initiation and the translation is affected by the Yb-1/mRNA ratio (Skabkin et al., 2004).

1.4.1 RNA editing in Trypanosomatids

In trypanosomes mitochondrion, gene regulation is characterized by a unique and essential RNA processing mechanism called kinetoplastid RNA (kRNA) editing (Miller & Read, 2003). kDNA is a huge DNA network which consists of catenated circular DNAs. There are two types of kDNA circles: *i*) the *maxicircles* that encode common genes of mitochondrion such as the genes for respiratory chain and *ii*) the *minicircles* that encode *trans-acting guide* RNAs (gRNAs) which are the genetic information for kRNA editing. Dozens of maxicircles exhibit variety in size which ranges from 20 to 40 kb among the species, On the contrary, the size of the thousands of minicircles ranges from 0.5 to 2 kb (Jensen & Englund, 2012). Maxicircles DNA is equivalent to the mitochondrial genomes of other eukaryotes transcripts are submitted to editing before the translation. In this process, associated guide RNAs (gRNAs) are encoded mostly in the minicircles but some are encoded in the maxicircle (Simpson, Douglass, Lake, Pellegrini, & Li, 2015).

More particularly, the pre-mRNAs undergo post-transcriptional modifications by the insertion and the deletion of uridine residues at numerous editing sites in the pre-mRNAs, a required process so as translatable open reading frames to be created (Hajduk & Ochsenreiter, 2010). To begin with, gRNAs consist of 30-50 nucleotides. The mature gRNAs consist of a poly-uridine tail with ∼20 nucleotides at the 3’ end which is post-transcriptionally added (Blum & Simpson, 1990). During editing, mRNA-gRNA interactions are stabilised through the interaction of gRNA poly (U) tail with the 5’ regions of the editing domain of pre-mRNA (Blum, Bakalara, & Simpson, 1990). Furthermore, the editing occurs, by the formation of gRNA 5’end anchor duplex at the relative editing domain of the pre-mRNA 3’ region (Ammerman et al., 2008). Cognate pre-edited mRNA and gRNAs binding occurs in anchor region at the 5' end of the gRNA, a sequence of 8-12 nucleotides (figure 15). The editing is regulated by a multiprotein complex termed editosome and the transcript maturation occurs in a 3’-5’ direction (Panigrahi, Allen, Stuart, Haynes, & Gygi, 2003; Stuart & Panigrahi, 2002). The mismatches between the RNA molecules are recognized by endonucleases as target sites. The insertion of U residues

catalysed by a uridylyltransferase (TUTase) which adds uridine residues in the 3’ hydroxyl of the 5’ endonuclease cleavage product of the mRNA (Militello & Read, 1999). U residues deletion is catalysed by a U-specific exonuclease. This exonuclease removes the uridine residues from the 3' end of the 5' cleavage product (Aphasizhev et al., 2003). In the end, RNA ligases piece together all the 5’- and 3’-end fragments (Hajduk & Ochsenreiter, 2010).

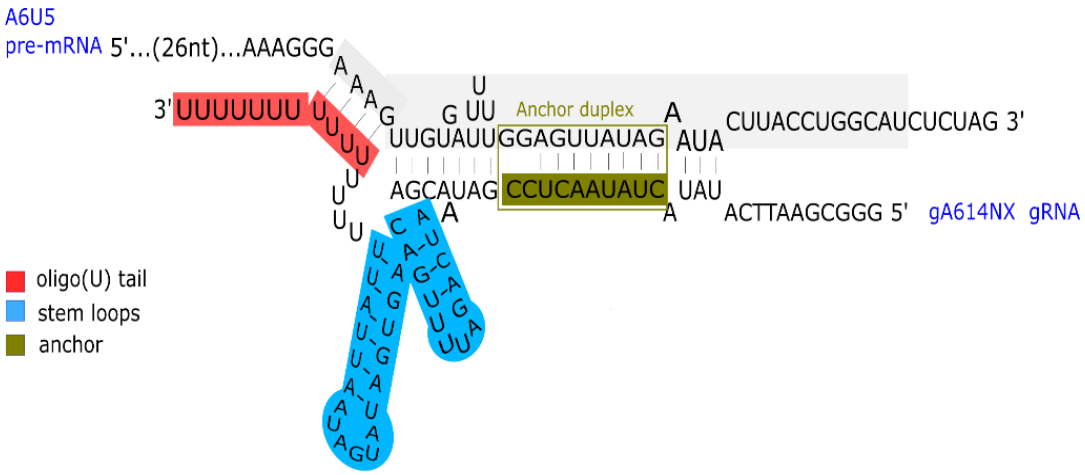

Figure 11. Diagram of the annealed A6U5 pre-mRNA (79nt)/gA6 (Aaboud et al.) NX substrate pair in *T.brucei* (adapted from (Ammerman et al., 2008)). It is shown the editing site, A6U5 of the ATPase 6 (A6) pre-mRNA which interacts with a cognate gA6[14]NX gRNA. RBP16 stimulates the annealing *in vitro* and so this hydridic is formed by the anchor duplex annealing of the pre-mRNA 3’-end and the 5’ -end of gRNA and the interaction of the gRNA 3’ oligo (U) tail with the upstream purine of pre-mRNA.

1.4.2 Protein structure of RBP16

T. brucei RBP16 protein (TbRBP16) in a mature form is a 141 amino acid peptide. The cold shock domain (CSD) in the N’terminal is a conserved nucleic acid binding motif. CSD comprises a 5-stranded b-barrel including RNP1 and RNP2 motifs (Graumann & Marahiel, 1998). In the C’ terminus, the arginine/glycine-rich region, resembling the RGG RNA-binding motif (Figure 12).

As it is mentioned, RBP16 belongs to the Y-box family and found to have high homology with the Y-box proteins. Y-box proteins display high similarity in the cold shock domains with more than 90%

identity, yet no significant homology is reported for the other protein regions (Bloom et al., 2017). Apart from the CSD domain, a characteristic of Y-box proteins is the high content of alanine and proline which corresponds to A/P domain in the N' terminus and the large C-terminal domain (CTD) (Bloom et al., 2017) (Figure 13).

Human Y-box 1 protein is a protein of 36KDa with the proportion of 11% Pro, 11.7% Arg, 12% Gly and 8.3% Glu (Minich, Maidebura, & Ovchinnikov, 1993). These characteristics define the function of the YB-1 protein. Firstly, Yb-1 interacts with nucleic acids and secondly interacts with other proteins and forms homomultimers and complexes (Kloks et al., 2002).

L.major RBP16 protein (*Lmj*RBP16) has a high similarity to TbRBP16, it is 142 amino acids peptides and the CSD domain in a length of amino acids 19-85 near the N' terminus. Besides, prediction tools of mitochondrial targeting sequences for *Lmj*RBP16 revealed one domain referred to as a cleaved mitochondrial import sequence in N' terminus. The R/G rich domain is found at the C' terminus which consists of the RGG/RG motif (Figure 12).

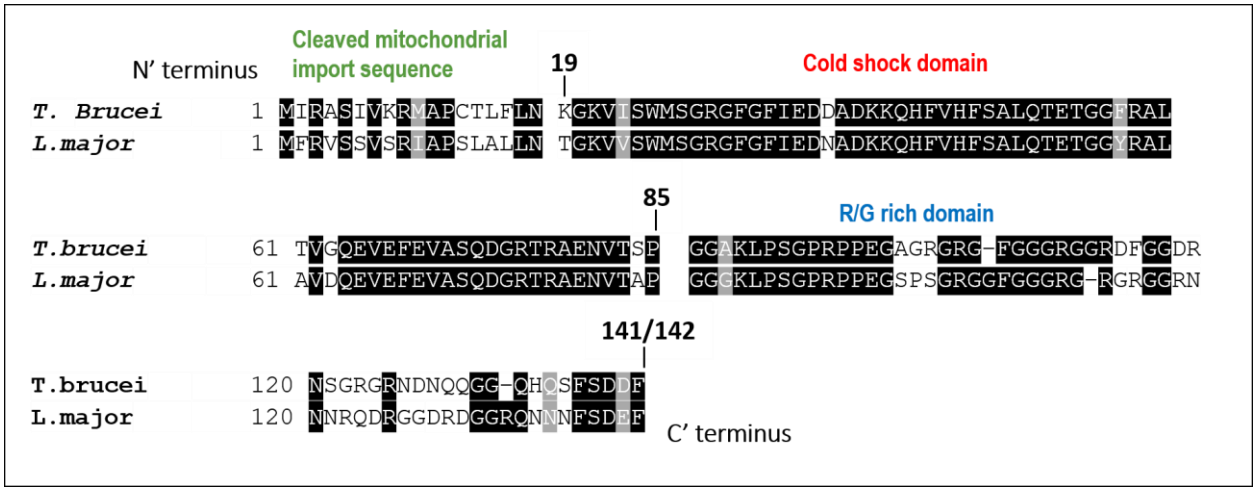

Figure 12. Alignment sequence of *T. brucei* and *L. major* RBP16. Sequences derived from TriTryDB with accession numbers: *Lmj*F.28.0825 for *L. major and Tb*927.11.7900 for *T. brucei.* For protein alignment comparison, online tool CLUSTAL and TCOFFE were used.[2]

[2] The RBP16 sequence for two organisms is derived from TriTryDB. Available on: https://tritrypdb.org/tritrypdb/app/record/gene/LmjF.28.0825 https://tritrypdb.org/tritrypdb/app/record/gene/Tb927.11.7900.
For the two sequence alignment the online tool CLUSTAL was used. Available on: https://www.ebi.ac.uk/Tools/msa/clustalo/
For further analysis of RBP16 homology analysis the online tools TCOFFE and EMBNET were used. Available on: http://tcoffee.crg.cat/apps/tcoffee/do:regular , https://embnet.vital-it.ch/software/BOX_form.html

In *T. brucei*, RGG repeats comprise the RGG RNA-binding motif. This is non-sequence-specific for the interaction with RNA and displays low-affinity (Miller & Read, 2003). Evidence revealed that the RGG motif facilitates the overall RNA affinity of RBP16. However, sequence-specific RNA binding can be promoted by the RGG domain which also mediates protein-protein interactions (Miller & Read, 2003).

Remarkably, mammalian Y-box proteins contain the aligned sequences of three RGG motifs, RGGDFRGGAPGDRGG. This is called ''RGG box'' and is characterised as an evolutionarily conserved motif found in different species (Thandapani, O'Connor, Bailey, & Richard, 2013). Interestingly, between RGG sequences are found aromatic residues. These are considered key residues for the hydrophobic stacking of proteins with RNA bases (Kiledjian & Dreyfuss, 1992). However, positively charged residues are not found within the RGG/RG motif, such as lysine and histidine. This proposes that the positive charge of arginine does not completely contribute to motif properties (Thandapani et al., 2013) (Figure 13).

Both in *T. brucei* and mammals, several cellular processes are associated with proteins that contain the RGG/RG motif. As a result, RGG/RG motif is a key regulatory motif (Miller & Read, 2003) and participates in different paths of regulation via posttranslational modifications, including arginine methylation. Briefly, some of the regulations that proteins with the RGG/RG motif are involved in are transcriptional regulation, pre-mRNA splicing, translational repression, and apoptosis.

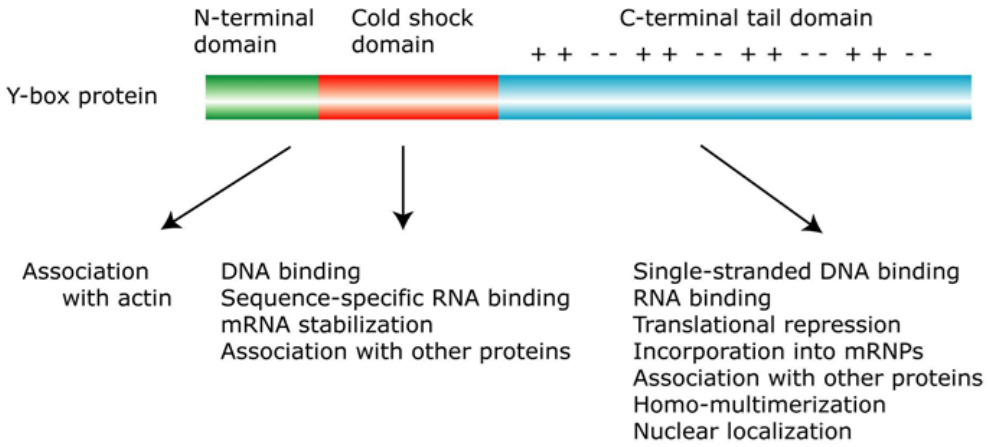

Figure 13. Schematic diagram of the Y-box protein structure (Matsumoto & Bay, 2005). Arrows display the function of Y-box proteins according to each domain. Green corresponds to the N-terminal domain, orange to the Cold shock domain and blue to the C' terminal domain. The ++ and – indications represent the alternating clusters of basic and acidic/aromatic amino acids.

The CSD domain of TbRBP16 shows homology to bacterial cold shock proteins and has been already referred to mammalian Y-box protein (Hayman & Read, 1999). Generally, Y-box protein structure has three domains as referred above: The N-terminal domain, the cold shock domain (CSD) and the C terminal tail domain (Figure 13).

In T.brucei, the CSD domain is a highly conserved motif that binds nucleic acid (Graumann & Marahiel, 1998). The specificity of TbRBP16 to bind to RNAs is found in the affinity of the CSD domain for poly (U). Thus, the RGG domain, which shows a preference in binding poly (G) homopolymers, stimulates the RNA binding protein capacity of CSD by increasing the stability of RBP16-RNA interactions (Miller & Read, 2003).

Generally, in bacteria, CspA and CspB proteins belong to the cold shock family proteins and bind to RNA with low affinity but broad specificity (Bae, Xia, Inouye, & Severinov, 2000). In eukaryotes, some of Y-box proteins are implicated in regulation of gene expression such as the Y-box protein, Yb-1. Yb-1 displays high homology to trypanosomes RBP16 and is implicated in transcription, RNA stability, translation, and RNA packaging (Lyabin et al., 2014).

1.4.3 RBP16 displays homology to mammals Y-box proteins, Yb-1

On the contrary to trypanosomes RBP16, Y-box 1 protein found to be localized in the cytoplasm. Nevertheless, evidence of their involvement in transcriptional regulation suggests that it might be localized in the nucleus as well (Lyabin et al., 2014). Localisation of Yb-1 to the nucleus occurs due to non-canonical nuclear localization signal which is found in the C' terminus. Additionally, the CSD seems to also play a role in nuclear localisation of Yb-1 (Bader & Vogt, 2005). Finally, the movement of Yb-1 from the nucleus to the cytoplasm and the opposite can be associated with the control of transcription and translation regulation (Soop et al., 2003).

Y-box proteins' post-translational modifications (PTMs) include phosphorylation, acetylation, and ubiquitination (Eliseeva, Kim, Guryanov, Ovchinnikov, & Lyabin, 2011). Kinases ERK2, GSK3β Yb-1 are involved in phosphorylation of Yb-1. Moreover, phosphorylation of Yb-1 occurs by kinase Akt (V. Evdokimova et al., 2006; Sutherland et al., 2005) and kinase RSK (Stratford et al., 2008). These modifications facilitate the binding of Yb-1 to VEGF gene promoter (Coles et al., 2005). Ubiquitination of Yb-1 by 26S proteasome results in complete proteolysis while the 20S proteasome action is responsible for a limited degradation (Lutz, Wempe, Bahr, Zopf, & von Melchner, 2006).

1.4.3.1 Function of Yb-1

The function of Yb-1 corresponds to its localization. In the nucleus, the transcription process of various genes is associated with Yb-1 (Lyabin et al., 2014). To begin with, Yb-1 is involved in the stimulation and inhibition of transcription (Kohno et al., 2003). Regulation of transcription by Yb-1 occurs through a direct interaction between the promoters that contain Y-box specific regions (5'-CTGATTGGC/TC/TAA-3'), and the protein (Sakura, Maekawa, Imamoto, Yasuda, & Ishii, 1988).

The interaction of Yb-1 with DNA results in gene expression changes. Yb-1 acting like a nucleic acid chaperone can melt double helices. As a result, DNA binding to transcription factors that have a higher

affinity to double-stranded DNA is not promoted (Dolfini & Mantovani, 2013). For instance, the inhibition of the VEGF gene occurs through the Yb-1 interaction with the non-coding strand of promoter DNA (Coles et al., 2005). This interaction is being enhanced by phosphorylation of Yb-1 by ERK2 and GSK3β kinases that increase the efficiency of inhibition (Coles et al., 2005). Interestingly, this mechanism can have the opposite results. The stimulation of transcription can occur after the binding of transcription factors that are involved in the inhibition of transcription. This occurs through the DNA polymerase and gene promoter single-stranded interaction with Yb-1 (En-Nia et al., 2005).

Furthermore, Yb-1 DNA repair activity is supported by its transmission to the nucleus under stress conditions (En-Nia et al., 2005; Lasham et al., 2003). In the nucleus, Yb-1 is involved in mRNA-splicing at a co-transcriptional level. For instance, Yb-1 interacts with proto-oncoproteins TLS and EWS and thus can be associated with the transcription complex. Briefly, the C- terminal of these proteins interacts with Yb-1 and the spliceosomal protein and the N-terminal binds the hyperphosphorylated RNA polymerase II (Chansky, Hu, Hickstein, & Yang, 2001; Dutertre et al., 2010). Yb-1's role in splicing is to direct splicing factors after the recognition of specific sequences within pre-mRNA. Hence, Yb-1 is considered as a splicing regulator of mRNAs rather than a core component of the spliceosome (Lyabin et al., 2014).

In the cytoplasm, Yb-1 is involved in mRNA translation/stability (Eliseeva et al., 2011). Yb-1 and poly(A) binding protein (PABP) are considered as the major protein of polysomal mRNPs. Yb-1 is also found in free cytoplasmic mRNPs and its amount reported to be twice as large as that in polysomal mRNPs (Minich & Ovchinnikov, 1992). Yb-1 regulation includes the interaction of both CSD and CTD with RNA and results in mRNP unwinding (Skabkin et al., 2004). This occurs after the exposure of mRNA termini to translation initiation factors and ribosomes. Remarkably the reason for this interaction is a relatively low Yb-1/mRNA ratio specific to polysomal translated mRNPs. In contrast, at a high ratio of Yb-1/mRNA, Yb-1 interacts preferably with mRNA upon CSD, while the CTDs might interact with each other. Thus, a large multimeric Yb-1 complex is formed (Skabkin et al., 2004). This function prevents

mRNA end to interact with the translation initiation apparatus and exonucleases (Skabkin et al., 2004). Consequently, mRNA lifetime and the translation are affected by Yb-1/mRNA ratio which translation is inhibited by high ratios and is activated by low ratios.

Finally, in mRNAs stability, CSD plays a key role in the prevention of degradation/stabilization. Similar to translation regulation, the mRNA stability is regulated by the high Yb-1/mRNA ratio (V. M. Evdokimova et al., 1998). In this case, the mRNA can be released from polysomes and proceed to the translation. According to evidence, the stability of mRNA regulated by Yb-1 is mainly dependent on the Yb-1/RNA ratio and it is not associated with AU rich 3'-UTR elements. Thus, mRNA stabilization upon Yb-1 is efficient for both long-lived and short-lived mRNAs such as TNF mRNA (V. Evdokimova et al., 2001). Also, Yb-1 can protect mRNAs from degradation. For example, renin mRNA stability occurs after the binding of Yb-1 to CU rich elements in the 3' UTR together with other proteins. Thus, Yb-1 appears to compete with other RNA-binding proteins for the interaction with mRNAs and also it is reported that Yb-1 binding is regulated by phosphorylation (Eliseeva et al., 2011).

Yb-1 found to be involved in cell proliferation. Interaction of Yb-1 with mRNAs coding for cyclins, growth and translational factors supports its ability to suppress their translation and affect the proliferation through the translational regulation level (V. Evdokimova et al., 2006). mRNAs inhibition is prevented by Yb-1 phosphorylation. Phosphorylated Yb-1 is not able to act on these molecules and so cell growth and proliferation are promoted (V. Evdokimova et al., 2006). Furthermore, Yb-1 is also involved in cell cancer apoptosis (Chatterjee et al., 2008). Evidence showed that Yb-1 interacts directly with the apoptotic factor p53 which activates genes of the cell cycle or pro-apoptotic genes (Jackson et al., 2008). Yb-1 affects the transcription or stability of the p53 gene and subsequently influences the modulation of the pro-apoptotic effect (Homer et al., 2005).

To conclude, similarly to RBP16, Yb-1 is presented as a protein that is involved in multiple cellular functions such as transcription regulation, splicing, translation, cell signalling, and stress response. Yb-

1 phosphorylation and its interaction with other proteins affect its affinity to mRNA binding. Also, the expression levels of Yb-1 both in nucleus and cytoplasm affect translation and gene expression, However, Yb-1 regulation is not considered as a global model of cell regulation.

1.5 RNA binding proteins are primary substrates of arginine methyltransferases enzymes

RNA-binding proteins are key regulators of gene expression in trypanosomatids. Thus, RBPs are regulated at a protein level by PTMs, such as protein methylation, phosphorylation, glycosylation, and acetylation resulting in turnover rate or protein stability (Rothbart & Strahl, 2014).

Furthermore, the regulation of gene expression in trypanosomatids occurs at the post-transcriptional level. The post-translational modifications of proteins can affect and alter gene expression in response to extracellular stimuli. A characteristic example is the phosphorylation or modification of latent cytosolic transcription factors by the action of PK (PK)/phosphatase signalling cascades, resulting in activation of transcription factors in the nucleus. In trypanosomatids, phosphorylation by PKs and phosphatases seems to be important for the parasite, differentiation, proliferation, cell cycle control and resistance to stress (Brivanlou & Darnell, 2002).

In addition to phosphorylation, recent investigations have focused on the arginine methylation. Arg methylation affects protein interaction and is associated with parasites metabolism, transcriptional regulation, mRNA splicing, virulence and signal transduction (Bedford & Clarke, 2009; Bedford & Richard, 2005; Migliori et al., 2012; Yu, 2011) (Ferreira et al., 2014).

In mammals and other eukaryotes, PRMTs' main role is the transcriptional regulation as they can modify histones. Protein modification allows the interaction with other proteins and this subsequently results in changes in chromatin structure and transcription process (Bedford & Clarke, 2009; Y. H. Lee & Stallcup, 2009). Hence, methylation can participate in gene activation or transcriptional repression

(Y. H. Lee & Stallcup, 2009). Additionally, the interaction of PRMTs with other proteins results in the inhibition or signalling and repairing of damaged DNA (Raposo & Piller, 2018).

Generally, histones modifications include acetylation by acetyltransferases, phosphorylation, and ubiquitination by ubiquitin ligases and kinases in the N-terminal tail (Rothbart & Strahl, 2014). However, according to many reports arginine methyltransferases targets can be both histones and non-histones (Raposo & Piller, 2018). To date, in mammals, more than 100 non-histone proteins contain methylated arginine sites (Wei, Mundade, Lange, & Lu, 2014). The most common substrates for PRMTs are the RNA-binding proteins (RBPs). Significantly, in *T. brucei*, over 850 proteins that are arginine methylated have been reported and most of them are RBPs (Fisk & Read, 2011).

The role of methylated RBPs includes the processing, folding, stabilization and /or destabilisation of RNAs/mRNAs. RBPs are considered as the largest group of proteins that are modified by arginine methylation (Pahlich, Zakaryan, & Gehring, 2006; Pelletier et al., 2001). This observation is a result of the GAR motif presence, in the glycine-arginine-rich (GAR) domains of RBPs (Kim et al., 1997; Liu & Dreyfuss, 1995; Najbauer, Johnson, Young, & Aswad, 1993). Arginine residue is a positively charged residue and is characterised as a mediator of the hydrogen bonding and amino-aromatic interactions (Bedford & Clarke, 2009). The addition of the methyl group to arginine residues renders the residue more hydrophobic and so the RNA-protein interactions are promoted. Overall, arginine methylated RBPs are associated with enzymes that are involved in modification and metabolism but also with signal transduction, DNA replication and repair.

1.5.1 Protein arginine methyl transferases (PRTMs) in mammals

As mentioned above, Arg methylation is catalysed in arginine residues by protein arginine methyltransferases (PRTMs). In arginine methylation, a methyl group from S-adenosyl-L-methionine (AdoMet) is transferred to the guanidino nitrogen atoms of arginine residues (Bedford & Richard,

2005). One or two methyl groups to a nitrogen atom on the arginine can be transferred by PRMTs. PRMTs are classified as type I, type II type III or type IV enzymes group and each correspond to the final product of arginine modification: i) monomethylate the proteins, ω-NG, monomethylated (MMA) (Zurita-Lopez, Sandberg, Kelly, & Clarke, 2012), b) ω-NG, NG-asymmetrically demethylated (aDMA) (Peng & Wong, 2017), iii) ω-NG, NG-symmetrically demethylated (sDMA) (Bedford & Clarke, 2009). The formation of an MMA intermediate can be catalysed by both type I and II. Type I PRMTs (PRMT1, 3, 4, 6 and 8) can act further and produce the aDMA formation. The type II PRMTs (PRMT5, PRMT7) catalyse the production of sDMA ((Bedford & Richard, 2005) (figure 14). Evidence showed that yeasts *Saccharomyces cerevisiae* and *Candida albicans* have also Type IV PRMTs, and there is a suggestion that this class may exist in plants as well (McBride et al., 2007; Niewmierzycka & Clarke, 1999). Type IV PRMTs act by transferring a single methyl group to the internal (guanidino) nitrogen of arginine residue.

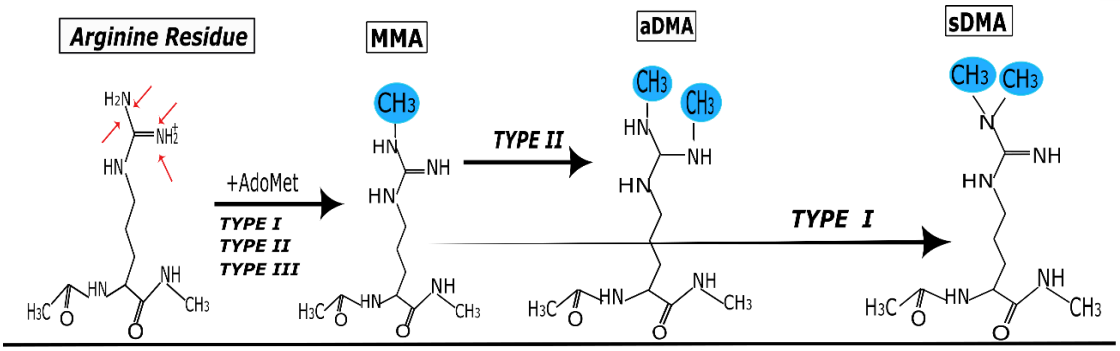

Figure 14. The arginine methylation in protein arginine residue from the PRMTs (adapted from (Raposo & Piller, 2018). The figure shows the three different formations of methylation by the three types of PRMTs group. Each group corresponds to the final product of arginine modification. The monomethylate the proteins (MMA), asymmetrically demethylated (aDMA) and, symmetrically demethylated (sDMA). The formation of an MMA intermediate is catalysed by Type I and II and then type I can also catalyse aDMA. sDMA is catalysed by Type II PRMTs.

Mammals PRMTs are distributed in 11 different classes (PRMT1-11) however only nine have been found to exhibit methyltransferase activity (Katz, Dlakic, & Clarke, 2003; Krause et al., 2007). In

humans, the main methyltransferase that modifies ~90% of arginine residues are the type I enzyme HsPRMT1 (Tang et al., 2000) (Yu, 2011).

PRMT1 isolated firstly in an attempt to purify the responsible enzyme for histone lysine methylation (Gary, Lin, Yang, Herschman, & Clarke, 1996) (Lin, Gary, Yang, Clarke, & Herschman, 1996). Interestingly, loss of function of HsPRMT1 revealed that the same arginine residues become targets for PRMTs. More particularly, loss of aDMA activity results in a dramatic increase of MMA methylation levels and it seems that enzymes for MMA act as primary enzymes in this case (Dhar et al., 2013). Hence, aDMA methylation seems to block Type II and III PRMTs activity which is activated after null mutants of PRMT1 (Di Lorenzo & Bedford, 2011).

Type I methyltransferases are also HsPRMT3, 6, 8 and they methylate substrates that contain GAR motifs (Pahlich, Bschir, Chiavi, Belyanskaya, & Gehring, 2005). These motifs are mostly targeted for aDMA and sDMA methylation by PRMTs. Nevertheless, the type II methyltransferase *Hs*PRMT5 which realizes symmetrically methylation can act in the same substrates (GAR motifs) as PRMT1, 3, 6 and 8 9 (Cheng, Cote, Shaaban, & Bedford, 2007). However, *Hs*PRMT5 can also act by methylating symmetrically dimethylated arginine residues within PGM motifs which are proline-, glycine- and methionine-rich (Cheng et al., 2007).

Similarly, to *Hs*PRMT5, *Hs*PRMT7 methylates arginine of the GAR motif. However, *Hs*PRMT7 found to methylate arginines in unknown regions of substrates (Pahlich et al., 2006). Thus, as type I PRMTs is reported to have also monomethylated activity, *Hs*PRMT7 additional activity results in heavily MMA methylation of proteins (Zurita-Lopez et al., 2012). MMA is considered as a precursor of aDMA and sDMA. However, it is not known if the same arginine residues within GAR and PGM motifs are targets for PRMTs that catalyse aDMA and sDMA (Cheng et al., 2007).

Finally, some enzymes have substrates that are not known, such as *Hs*PRMT4. On the contrary, *Hs*PRMT3 methylates substrates which contain motifs in C' terminal and so it is revealed that the

structure of the substrate plays an important role in methylation and the region determines the specific methylation (Pahlich et al., 2005).

To conclude, in humans the arginine methylation has a significant biological role. The balance of Arg methylation is necessary for the function and the living of the organisms (D. Y. Lee, Teyssier, Strahl, & Stallcup, 2005) (Wysocka, Allis, & Coonrod, 2006) (Wysocka et al., 2006). However, aberrant modifications have been reported in different diseases and result in the alternation of PRMTs activity and function (Gao et al., 2016; Y. Yang et al., 2010). Thus, it is suggested that while arginine methylation modifications are catalysed by PRMTs, other enzymes act by removing the methylation marks. These enzymes are characterized as demethylases that reverse the arginine methylation. Subsequently, it seems that there is a dynamic cyclic process of arginine methylation and demethylation (Ng, Yue, Oppermann, & Klose, 2009).

1.5.2 Protein arginine methyltransferases in Trypanosomatids

In *T. brucei* genome but also in *Leishmania major* five putative PRTMs have been identified. The fact that is the largest number of PRMTs found in single-cells eukaryotes indicates that arginine methylation is highly important for these organisms (Fisk et al., 2010).

Among the PRMTs of *T.brucei*, homology exists in *Tb*PRMT1 and *Tb*PRMT5 with humans *Hs*PRMT1 and *Hs*PRMT5 (Fisk & Read, 2011). Similarly, to mammals in *T.brucei* PRMT1 is the primary PRMTs and it is associated with proteins that contain the GAR domain (Pelletier, Pasternack, & Read, 2005). It is known that there are protein substrates that are methylated by different PRMTs simultaneously. For example, the pre-mRNA binding protein SmB is methylated by both type I and type II PRMTs, PRMT4 or PRMT5 and also Histone H4 by PRMT1 or PRMT5 (Pahlich et al., 2006). *Tb*PRMT5 belongs to type II PRMT with substrates that include both GAR domain and non-GAR domain targets like PGM motifs (Pasternack, Sayegh, Clarke, & Read, 2007). Hence, it seems that protein-protein interaction displays

different biological functions and a proteomic function broadened by aDMA and sDMA methylation (Bedford & Richard, 2005).

In mammals, several mechanisms of PRMTs regulation have been reported, including protein-protein interactions and phosphorylation (Pahlich et al., 2006). In the case of *Tb*PRMT1, external or/and developmental stimuli can negatively regulate the access to specific substrates. Subsequently, *Tb*PRMT7 catalyses monomethylation (Lott, Zhu, Fisk, Tomasello, & Read, 2014). Nevertheless, access to the same substrate may result in different consequences for the substrate target. For instance, in mammals, monomethylation of the histone protein H3 R2 results in gene activation, while aDMA demethylation of the same residue results in transcriptional repression ((Schurter et al., 2001) (Guccione et al., 2007) (Migliori et al., 2012).

Additionally, *Tb*PRMT1 is associated with other PRMTs (Pelletier et al., 2005). Particularly, interdependence is shown between *Tb*PRMT1 and *Tb*PRMT3 as the loss of one of the results of this enzyme in the instability and degradation of the other (Lott et al., 2014). The simultaneous depletion of both *Tb*PRMT1 and *Tb*PRMT3 resulted in a change to the global levels of aDMA (Lott et al., 2014). Interestingly, the fact that *Tb*PRMT3 activity was not detectable *in vitro*, suggests that *Tb*PRMT3 is an active enzyme only after the formation of an enzymatic complex with *Tb*PRMT1 (Willert & Phillips, 2012). Thus, *Tb*PRMT1 and *Tb*PRMT3 homology and codependence relationships exhibit that the inactive enzyme stimulates the function of an active enzyme upon binding (Lott et al., 2014).

As mentioned above PRMT1 is the major type I PRMT in *T. brucei* and RBP16 is arginine methylated by PRMT1. Arg methylation occurs on three residues in C' terminus that contains the RG-rich motif, at a post-transcriptional level (Miller & Read, 2003). Generally, in *T. brucei* asymmetrically demethylation is a characteristic of arginines that is found within the RGG RNA-binding domains (Miller & Read, 2003). The preferred sequence for asymmetric arginine methylation is the G/FGGRGGG/F and in *Tb*RBP16 this sequence is found in the R/G rich domain (Bedford, 2007). *Tb*PRMT1 depletion experiments revealed that arginine methylation is a regulator of mitochondrial gene expression with

broad aspects and the methylation of *Tb*RBP16 affects the RNA stability and editing and subsequently the mitochondrial gene expression (Goulah & Read, 2007). Other substrates of *Tb*PRMT1 are DRBD3 and SCD6, and both they act also as stabilizing factors of mRNAs. Arginine methylation of DRBD3 by *Tb*PRMT1 affects mRNAs fate and it is associated with stress granules in cytoplasm during starvation(Estevez, 2008). *Tb*SCD6 is involved in cytoplasmic mRNA metabolism and it is found to be a component of processing bodies (Rajyaguru, She, & Parker, 2012). However, *Tb*RBP16 is a mitochondrial RNA-binding protein that is widely investigated in *T.brucei* as a substrate of *Tb*PRMT1, and so this study is focused on the function of RBP16 in the mitochondrion of trypanosomes.

In *Leishmania major*, recent investigations from Warlad lab showed that *Lmj*RBP16 is a substrate for *Lmj*PRMT7 arginine methyltransferase. In kinetoplastids, PRMT7 is a cytosolic enzyme and so it methylates mainly RBPs (Fisk et al., 2009).On the contrary, in humans, PRMT7 is found to nucleus where substrates are histones proteins. *Trypanosomes* PRMT7 catalyses only MMA forms and it is the only type III PRMT that has been reported (Fisk et al., 2009). However, the type of *Hs*PRMT7 is still not known as it is considered to catalyse either type III or Type II (Bedford, 2007).

MMA methylation in trypanosomes seems to be a very common methyl activity. PRMT7 is considered as an enzyme with a strong impact in the cell as it is active at least 30 times more than human homolog concerning the ability to transfer the methyl group per hour and microgram (Fisk & Read, 2011). However, there is evidence that type III *Tb*PRMT7 may act synergistically with other PRMTs that belong in type I and II. According to this, one hypothesis is that *Tb*PRMT7 first catalyses MMA methylation and then allows other PRMTs to proceed to subsequent catalysation of aDMA and sDMA methylation (Fisk & Read, 2011). Additionally, it is suggested that trypanosomes PRMTs have a variety of abilities to recognize MMA methylations and act further catalysing the aDMA or sDMA demethylations (Fisk & Read, 2011). This hypothesis originates from mammals PRMTs which are reported to have either ''processive or distributive enzymatic mechanisms'' (Lakowski & Frankel, 2008, 2009; Osborne, Obianyo, Zhang, Cheng, & Thompson, 2007).

Further investigations on *T. brucei* revealed that *Tb*PRMT7 knockdown results in the non-reduction of MMA methylation. First of all, loss of *Tb*PRMT7 showed that the expression levels of *Tb*PRMT1, *Tb*PRMT3, or *Tb*PRMT6 are not affected (Lott et al., 2014). This observation is consistent with the theory that PRMT7 interacts with other PRMTs, not as a substrate for DMA mark by type I and II but catalysing the synthesis of the MMA intermediate (Lott et al., 2014).

Moreover, the fact that PRMT7 catalyses MMA formation expected a reduction in MMA methylation and subsequently a decrease in aDMA levels (Lott et al., 2014). However, *Tb*PRMT7 knockdown revealed a non-significant reduction of MMA and aDMA levels. Thus, it seems that there are non-canonical PRMTs that are not active in the presence of *Tb*PRMT7 (Lott et al., 2014). This is supported by the evidence of previous studies in the mitochondrion of *T. brucei* in which 168 proteins found to be arginine methylated by non-characterized PRMTs (Fisk et al., 2013).

Evidence for noncanonical PRMTs have been observed in other organisms as well and it seems that their homologs in trypanosomes. For example, *T.brucei* mitochondrial protein shows homology to the human NDUFAF7 which is associated with the complex formation, NDUFAF7 in the mitochondrion and symmetrically methylate the NDUFS2 subunit of complex 1 (Willert & Phillips, 2012). Another homology evidence referred to a SPOUT methyltransferase protein of *S. cerevisiae*, and *T. brucei* is reported to contain five SpoU domain-containing proteins (Sirum-Connolly & Mason, 1993) (Cavaille, Chetouani, & Bachellerie, 1999) (Jackman, Montange, Malik, & Phizicky, 2003; Meyer et al., 2011). In *S. cerevisiae*, Yor021c is a SPOUT protein that forms MMA on the ribosomal protein Rps3 (Meyer et al., 2011).

1.5.3 Function of RBP16 in *T. brucei* upon Arginine methylation

To begin with, TbRBP16 is a Y-box protein that contains the CSD and this domain that exists in other homologs of eukaryotes but also in prokaryotes as CSPs which is the factor of melting and annealing

of DNA or RNA strands (Bae et al., 2000). Thus, investigations focused on the role of domains in RBP16 annealing activity and the differences in the affinity of the CSD domain and the R/G rich domain (Miller & Read, 2003).

CDS domain displays a high affinity for RNA and so the RBP16 is associated with gRNAs with specificity through the oligo(U) tail. On the contrary, the R/G rich domain has a low-affinity, and its function is dependent on protein's specific domain which is required for RNA annealing (Miller & Read, 2003). However, it is interesting that the RGG domain does not have a high affinity but can promote the RNA annealing by destabilizing secondary structures (Miller & Read, 2003). As a result, experiments proved that RBP16 does not lose the annealing activity and this means that RBP16 has also RNA chaperone activity as the sequence-specific interactions are not necessary. Finally, another characteristic of RNA chaperones apart from annealing is the unwinding capacity and in fact, TbRBP16 has both abilities (Ammerman et al., 2008).

Secondly, RBP16 in *Trypanosoma brucei* is a mitochondrial RNA-binding protein which is encoded in the nucleus. Thoroughly, investigations by the laboratory of Laurie K. Read proved that RBP16 is associated with the 30% of native guide RNAs (gRNAs) but also with several mitochondrial mRNAs proposes that RBP16 is involved in the process and regulation of RNA editing (Pelletier et al., 2000). Another association of RBP16 that has been reported is 9S and 12S mitochondrial ribosomal RNA (rRNAs) which suggests the regulatory function of RBP16 in RNA translation. In all cases, mRNAs, gRNAs and rRNAs, RBP16 binds the RNA with a similar affinity, through the oligo (U) tails (Pelletier & Read, 2003).

In mitochondrion of *T. brucei* the majority of native RBP16 is present in a complex with other macromolecules and does not form homomultimer (Goulah & Read, 2007). *Tb*RBP16 is present in 5S and 11S complexes and it is shown that while the 5S complex contains RBP16 associated with other proteins the 11S complex contains both gRNAs and additional proteins (Goulah & Read, 2007). As a

result, the association of RBP16 with gRNAs proposes that the protein plays an important role in RNA editing. Further investigations based on mutants of PRMT1 showed that loss of methylation might result in disruption of protein-RNA interactions. Thus, Arg methylation is necessary for both 5S and 11S macromolecular interactions, as the non- methylate RBP16 can form only the 5S complex which might interact with different RNA components (Goulah & Read, 2007). Moreover, the fact that the lack of 11S formation after depletion of PRMT1 suggests that *Tb*PRMT1 mediates the assembly or stability of this complex or other complex components through methylation of RBP16.

Further investigations of RBP16 methylation from Read et al (2007) revealed the specific interaction between RBP16-RNA and its effect in their stability (Goulah & Read, 2007). First, it is reported that methylated RBP16 decreases its association with gRNAs while non-methylated RBP16 increases the interaction with mRNAs. Subsequently, this observation strongly suggests that methylation of RBP16 is required for RBP16 RNA binding capacity and facilitates mRNA stabilization. On the contrary, RBP16-gRNA binding capacity is decreased when RBP16 is methylated and the fact that the RBP16 interacts with other proteins is suggested that methylation is required for additional protein-protein interaction and this results in the limitation of RBP16-gRNA interaction (Goulah & Read, 2007).

Specific mitochondrial transcripts that are known to interact with RBP16 are the ATPase subunit 6 (A6), the cytochrome oxidase subunit I (COI), the apocytochrome b (CYb) and the NADH dehydrogenase subunit 4 (ND4) (Pelletier & Read, 2003). The determination of RBP16-RNAs interaction affected by arginine methylation occurred through PRMT1 loss of function and in all cases the binding capacity reduced in a proportion that ranged from 53%-100% (Goulah & Read, 2007). However, the association between non-methylated-RBP16 and gRNAs increased remarkably. Subsequently, RBP16 specificity and binding affinity is affected by methylation. Methylation influences directly the RNA binding capacity or indirectly protein-protein interaction.

Besides, RBP16 shows homology with Y-box proteins, so it suggested that it has a similar reported function (Hayman & Read, 1999). Y-box proteins are involved in translation as its RNA chaperone

activity results in destabilisation of RNA secondary structures at low temperatures. Also, Y-box proteins such as mammals Yb-1 have been reported to act by promoting RNA-RNA annealing (Skabkin, Evdokimova, Thomas, & Ovchinnikov, 2001). These hypotheses led Read et al (2008) to investigate the regulation of *Tb*RBP16 in order to determine its annealing ability and its function as RNA chaperone. In vitro analysis demonstrated RBP16 is involved in the stimulation of gRNA binding to a cognate pre-mRNA. Even though RBP16 has a high RNA-binding affinity with gRNAs oligo(U) tail, the tail is not required for RBP16's annealing ability (Ammerman et al., 2008).

In summary, RBP16 structure and arginine methylation are shown to be determinant characteristics that affect RBP16 protein-protein and RNA-protein interactions (Goulah & Read, 2007). Mitochondrial mRNAs stability and editing are affected by RBP16 which modulates the RNAs through its chaperone activity. Additionally, it is suggested that RBP16 is involved in kinetoplastid RNA editing of mitochondrial RNAs and possible in gene regulation at the post-transcriptional level. The fact that TbRBP16 is not found to be a stable component of the editosome (Stuart et al., 2002) shows that its function is potentially to regulate the RNA editing rather than to catalyse the process of RNA editing (Ammerman et al., 2008) (Pelletier & Read, 2003). Finally, RBP16 has been found to stimulate the ''annealing of a cognate gRNA/pre-mRNA pair and is independent of its high-affinity gRNA-binding capacity'' (Ammerman et al., 2008).

1.5.4 PRMT7 a key enzyme for *Leishmania major* virulence

As mentioned again, PRMT7 catalyses the formation of MMA and does not proceed with sDMA catalysis. *Lmj*PRMT7 and *Tb*PRMT7 comparison revealed that these proteins are 48. 6% identical (ID) and 68. 9% similar (SIM) (Ferreira et al., 2014). Analysis of human homologs showed that *Lmj*PRMT7 is ~~more~~ similar to *Hs*PRMT7 (Ferreira et al., 2014). More particularly, by the comparison of the methyltransferase domain of these enzymes, it is shown there is 24.6% identity and 42.3% similarity

between *Lmj*PRMT7 and *Hs*PRMT7 while the alignment displayed only 11.9% identity and 19.9% similarity between *Lmj*PRMT7 and *Hs*PRMT10 (Ferreira et al., 2014).

Interestingly, a significant difference of *Lmj*PRMT7 with *Hs*PRMT7 is found in the AdoMet-binding domain, a key motif, conserved across PRMTs (Bedford & Clarke, 2009). In *Homo sapiens*, *Hs*PRMT7 has a duplicated AdoMet-binding domain, and so has one methyltransferase domain in both C' and N' terminal. However, the overall activity of the full-length enzyme is dependent on N'- terminal AdoMet domain rather than the C' terminal domain. *Lmj*PRMT7 has one AdoMet domain in C'-terminal and the ''THW loop'', the other conserved motif of arginine methyltransferase domain, is found to be modified in *Lmj*PRMT7.However, the fact *Lmj*PRMT7 exists as homodimer inside the parasites reveals that these structures might mimic the double domain of mammals. *Tb*PRMT7 has only one AdoMet-binding domain in C'-terminal as in *Leishmania* spp. and shows high similarity to the active N'-terminal of *Hs*PRMT7 (27,7% identity and 44,9% similarity).

Experiments from Ferreira et al. (2014) focused on the protein expression levels of PRMT7 in different life cycle growth stages of *L.major* virulent (LV39) and attenuated (CC1) strains(figure 15). Significantly, differences were noticed in the expression between the two strains in which levels of PRMT7 in the early procyclic stage found to be high and then decreased in the stationary and late stationary stage. Interestingly, the attenuated cell lines were detected until the fourth day of culture while the virulent strains were not detected after the 2 days (figure 15A).Moreover, the CC1 stain line was noticed to be able to rescue the log-like levels of *Lmj*PRMT7 within 4-8 hours when metacyclics transferred into a fresh culture(figure 15B). Subsequently, the gene of *Lmj*RPRMT7 is expressed in higher levels in CCI cell line compared to LV39. Similar to endogenous null mutants and overexpression of *Lmj*PRMT7 do not have important differences in proliferation and survival which suggests that PRMT7 is not an essential protein for parasites growth (figure 15C,D).

Similar to early-log promastigote stages, the expression levels of PRMT7 are higher in amastigotes than in the stationary phase. Thus, the further importance of PRMT7 in parasites virulence was also

investigated in null mutants and overexpression *Lmj*PRMT7 parasites (figure 16). For this purpose, *in vitro* macrophages of the peritoneal exudate and in vivo BALB/c mice lesions were infected by *L. major* parasites from both cell lines LV39 and CC1. In both cases, it is shown that the *Δlmprmt7* parasites display an increase in virulence while the overexpressing *Lmj*PRMT7 parasites revealed a decreased proportion of macrophage infection, as the results showed the diminish of macrophage infection and the progress of lesion size was attenuated (figure 16A,B). Subsequently, these results showed that the altered expression of *Lmj*PRMT7 does not affect the proliferation and differentiation of parasites. Thus, *Lmj*PRMT7 plays a key role in virulence and considered a significantly important enzyme that is implicated in pathogenesis. Thus, it is hypothesized that *Lmj*PRMT7 might interact with proteins of the host and so it is implicated in the regulation of amastigotes pathways and proliferation (Ferreira et al., 2014).

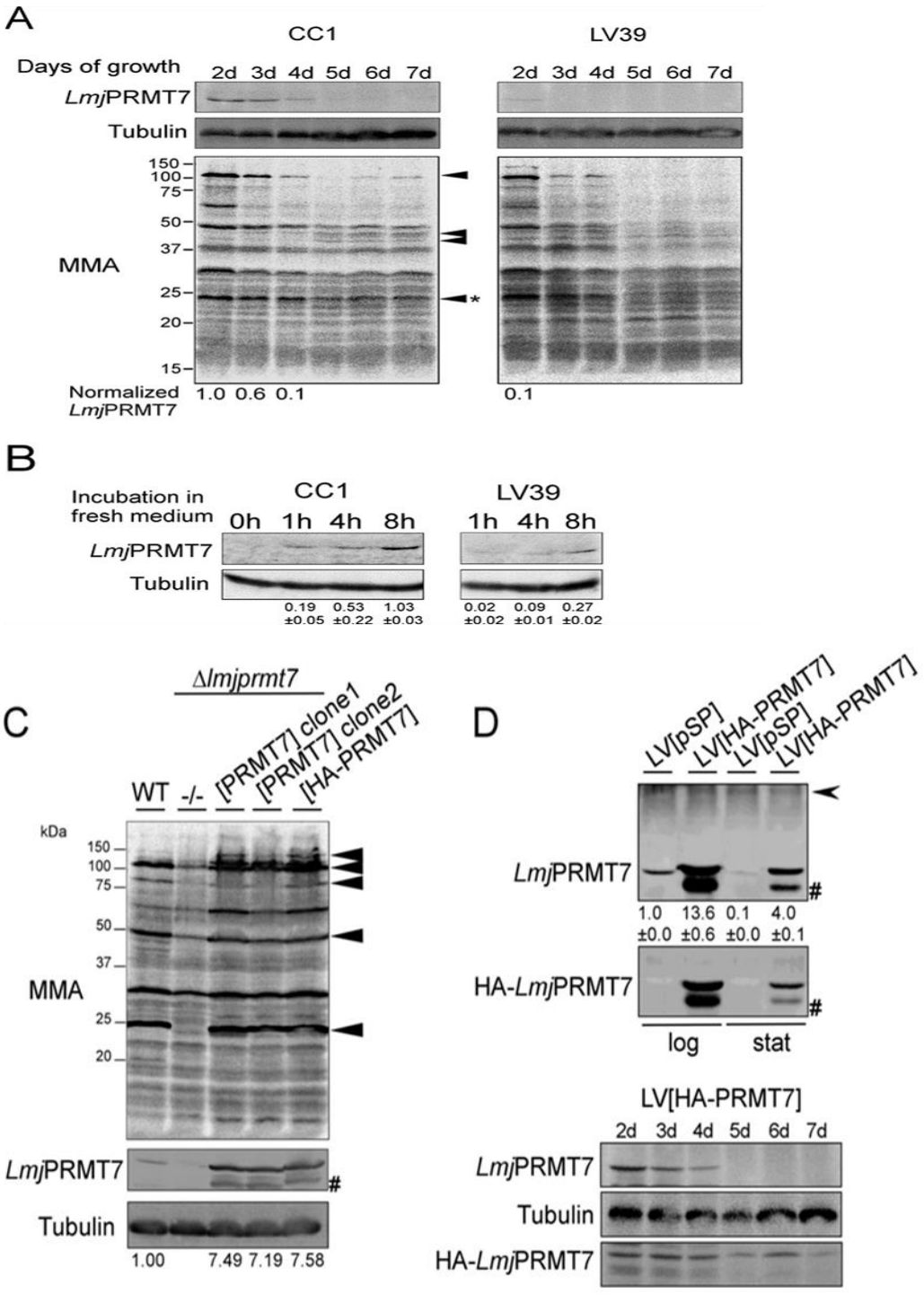

Figure 15. Experimental evidence of the *Lmj*PRMT7 expression and monomethyl arginine levels in procyclic promastigote phase in *L.major* (Ferreira et al., 2014). A. WT LV39 and CC1 strains from promastigote phase analysed using the polyclonal anti-*Lmj*PRMT7 antibody. The monoclonal Me-R4-100 antibody was used for the identification of MMA levels. Immunoblot analysis showed higher levels of *Lmj*PRMT7 expression during day 2 and then a reduction in days 5-7. The MMA modifications levels in promastigote phase decrease for proteins above 35KD, in late log and stationary phases**. B** immunoblot shows the increase of *Lmj*PRMT7 levels after 4-8 hours, which resulted in the transfer of

promastigotes from late-stationary-phase into a fresh medium M199 resulted. **C** shows the levels of MMA modification comparing the WT, with *Δlmprmt7* null mutant (Δlmjprmt7 (−/−), [PRMT7] expression by the ectopic copy and HA-tagged protein [HA-PRMT7]. MMA levels in *Δlmjprmt7 (−/−)* cell lines are decreased compared with WT and cell lines and [PRMT7] clones. In **D** immunoblot, LV39 (LV[HA-PRMT7]) that overexpress the PRMT7, were used for the analysis of *Lmj*PRMT7 levels compared to the control vector (LV [pSP]) in log and stationary growth phases. Results showed a higher level of protein in the log phase which decreases in the stationary phase, similar to endogenous protein.

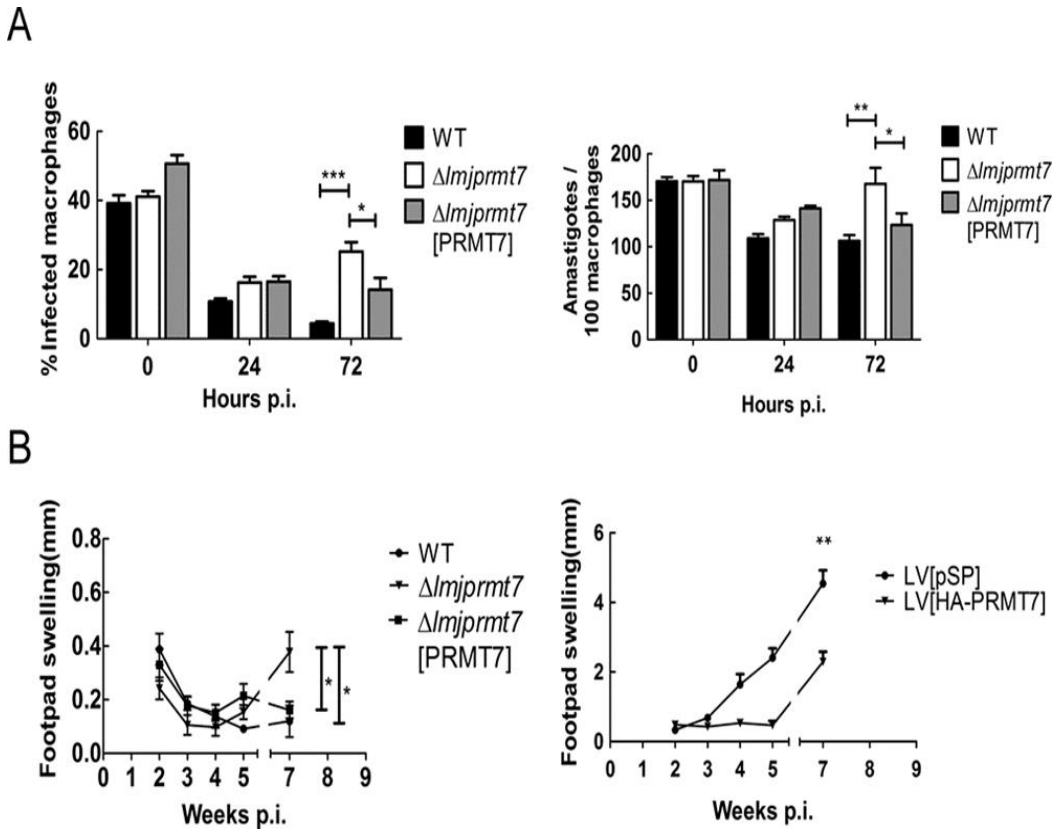

Figure 16. Experimental evidence of *Lmj*PRMT7 virulent levels in intracellular amastigotes of *L.major* CCI and LV39 cell lines (Ferreira et al., 2014). **A** diagram shows the increase of infectivity of *Δlmprmt7* compared to WT CCI strains and *Δlmprmt7[PRMT7]* after the infection of peritoneal exudates macrophages. **B** diagram shows the lesion in BALB/c mice infected with *Δlmprmt7*, WT LV39 cell lines and *Δlmprmt7 [PRMT7]* (on the left). Remarkably, 7 weeks after infection the mean lesion size exhibits an increase in *Δlmprmt7* cell line. In contrast, overexpression of PRMT7 cell line (*LV[HA-PRMT7]*, showed that virulence is decreased compared with the strains bearing the vector alone *(LV[Psp])* (on the right).

1.5.5 *Lmj*PRMT7 is presented as an epigenetic regulator that affects *Lmj*RBP16 stability

*Lmj*PRMT7 is a protein with an indirect and direct role inside the cell, suggesting that it causes changes in the interaction between hosts and parasites. In *T. brucei* PTMR7 is found to methylate bovines histones and RNA-binding proteins that are involved in RNA editing. Also, the fact that PRMT7 in trypanosome exists as a homodimer, it seems that its function may differ from that in mammals with the duplicate domain. However, *Lmj*PRMT7 has not been reported to be localized in the nucleus as occurs in mammals and subsequently does not involve in histone methylation but RNA-binding proteins are its primary targets.

The hypothesis that the *Lmj*PRMT7 acts in specific substrates and results in the alternation of arginine methylation in the cell suggested after the reduction of protein methylation in null mutants *Δlmjprmt7* (Ferreira et al., 2014). It is proposed that the changes of PRMTs levels of *Leishmania major* may affect the overall arginine methylation of proteins and so results in inhibition of parasites killing inside the host. As experimental evidence showed, substrates of *Lmj*PRMT7 are associated with parasite virulence, after it was known that AdoMet/AdoHcy ratio in *Leishmania* strictly controls its survival.

As has been mentioned above, RBP16 identified as a PRMT7 target in *Leishmania major* which directly modulates the protein in the RGG motif. Proteomics analysis by methyl-SILAC between WT and loss of *Lmj*PRMT7 revealed that there are in total 247 unique MMA peptides of which 40 are hypomethylated in the absence of *Lmj*PRMT7 (Ferreira et al., 2020). This is considered as a large part of MMA-target (3% of total *L. major* proteome), as arginines are found to be dimethylated by PRTMs. Accordingly, *Lmj*RBP16 identified as a hypomethylated protein after the loss of *Lmj*PRMT7 and the fact that the hypomethylated substrates contain the RG/GGR motifs proposes that it is a target motif for *Lmj*PRMT7. Additionally, mutations in the PRMT7 catalytic domain and double E loop revealed that both are essential for RBP16 methylation in RG/RGG motif (Ferreira et al., 2020).

As *Lmj*PRMT7 revealed to be important for monomethylation of RBP16, further investigations focused on the biological impact of the loss of function of this methyltransferase enzyme on its substrates (Ferreira et al., 2020). Firstly, the RBP16 endogenous tag in the N' terminus exhibits a reduction of expression levels in the stationary phase. Complementary to RBP16 other targets of *Lmjprmt7* the endogenous N-terminal HA tagging HA-Torus revealed reduction in expression levels upon *Δlmjprmt7* at both log and stationary phase of proliferation. Also, a third target of *Lmjprmt7,* the endogenous N-terminal HA tagging, HA-DDX3, displayed instability similar to RBP16 as a production of enzymatic cleavage observed at stationary phase however, in stationary phase it is slightly upregulated. Subsequently, this observation showed that *Δlmjprmt7* affects the protein expression and exhibits coordinated control of expression at the stage-specific level (Ferreira et al., 2020). Then, this observation was confirmed by comparing the levels of translation of the *rbp16* transcript with the levels of the half-life of the protein. Indeed, half-life levels of HA-RBP16 reduced at the stationary phase for the *Δprmt7* cell lines. Hence, PRMT7 is proposed as an enzyme that post-translationally controls the stability/degradation of protein targets in a stage-specific level (Ferreira et al., 2020).

1.6 Project aims and hypothesis

This project aimed to investigate how the arginine methylation of PRMT7 versus the absence of PRMT7 methylation affects the function of the RNA-binding protein, RBP16 and which is the association of RBP16 with mRNAs in *L. major*. For this purpose, knockdown cell lines for PRMT7 were previously generated and used in comparison with the wild type. More particularly, for the aims of this project the following experiments carried out:

i) Transfect WT and Δprmt7 cell lines with the C-terminal HA tag of RBP16 generated

ii) Utilize the anti-TbRBP16 and anti-HA antibodies to investigate the expression and localization of RBP16 in WT and Δprmt7

iii) Cross linking RNA immunoprecipitation (CLIP) carried out to identify the RNA targets of RBP16

Evidence of the close relative *T. brucei* showed that TbRBP16 is involved in the efficiency of RNA editing and mRNA stability in the mitochondrion of this organism. These processes are affected by arginine methylation of *Tb*RBP16 upon PRMT1, as it regulates the protein-protein and protein-RNA interactions. In mitochondrion of *T. brucei* RBP16 is associated with mRNAs and gRNAs and its methylation positively and negatively affects their stability, respectively. Accordingly, in *L. major* it is hypothesized that *Lmj*RBP16 is localized in the mitochondrion and associated with RNAs. Also, after the identification of RBP16 as a substrate for PRMT7, we hypothesize that the depletion of PRMT7 could affect the association and the stability of RNAs similarly to *Tb*RBP16.

Finally, as it is known that the *Leishmania* genome is transcribed in polycistronic arrays and thus genes are regulated through post-transcriptional mechanisms, we aimed to investigate if RBP16 is a significant molecular target that plays a key role in gene expression of *L. major*.

CHAPTER II. Materials and methods

2.1 *Leishmania* manipulation

2.1.1. *Leishmania major* culture

Leishmania major promastigotes from strain CCI were cultured at 26 °C, in M199 (Gibco) media supplemented with 40 mM HEPES, 10% FBS, 100 U µg/ml, 100 µM adenine, 0, 0005% Hemin and pH 7,2. For the experiments and the transfectants generation was used WT and *Δprmt7* parasites strain line. PRMT7 null parasites (Δprmt7) were previously generated (Ferreira et al., 2014). Parasites were counted in Beckman coulter machine which is considered as the most reliable laboratory method for counting cells. For the count, an electrolyte is used that contains the cells and travels through a small narrow hole connected to an electric current. Cells are poorly conductive particles, and so when they are included in the electrolyte, they cause changes in voltage. More specifically, the cells cause an increase in electrical resistance and then a decrease in electrical current. Changes in the electrical current are related to the concentration of cells. Accordingly, 100µl of culture were diluted in isoton. The counting occurred in triplicate and blank isoton were used in between each measurement as a control.

For parasites storage, 1 ml of parasites from logarithmic procyclic stage were mixed with 1ml freezing media and incubated in – 80 °C before they transferred for permanent storage in liquid nitrogen. Then if needed, parasites defrosted from liquid nitrogen. For this, parasites left at room temperature for a few minutes before they transferred into the M199 media of culture and finally incubated in 26 °C.

2.1.2 Parasites transfection

To investigate the role of RBP16, it was necessary to create the appropriate transfectants strains of parasites. For this reason, a C' terminal tagging construct used, generated before in Walrad lab (Figure 17) (unpublished) according to the 500 base pair homology regions for homologous recombination into the genome. Procyclic promastigotes from both *L. major* strains were submitted to

electroporation for genome integration of linear C' terminal HA-RBP16 tagging construct, using the Amaxa Nucleofector II protocol as it is provided by the manufacturer. Procyclic parasites were mixed with 2pg of plasmid DNA and spread on semi-sold agar plates in M199 media in the presence of the antibiotic Neomycin (Sigma, G418). Expression of neomycin phontransferase (NEO) contributed to selection of transfected cells . After 2 weeks of incubation, transfectants WT-RBP16-HA and *Δprmt7*-RBP16-HA were selected from 1% agar plates with 10 mg/ml NEO and cultured in supplemented M199 media under NEO pressure.

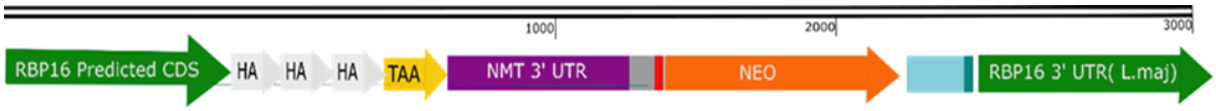

Figure 17. Schematic diagram of RBP16 C-terminal HA tag constructs.[3] 3000base pair construct. GREEN on the left: predicted coding sequence (CDS), GREY: 3x HA tag sequence, YELLOW: termination codon-TTA, PURPLE: NMT 3' untranslated region(3'-UTR) , ORANGE: gene coding for neomycin antibiotic resistance(NEO), GREEN on the right: three prime untranslated region (3'-UTR) of RBP16.

2.2 DNA Manipulation Protocols

2.2.1 Genomic DNA extraction

Leishmania major genomic DNA from promastigote procyclic parasites of cell lines WT, *Δprmt*7, and HA-RBP16-WT and HA-RBP16-*Δprmt*7 was extracted using the DNAzol reagent and followed in instructions of the manufacturer (Invitrogen). Parasites collected in tubes and centrifuged. The pellets were resuspended with 20µl of lysis buffer, 10 µl of protease containing buffer and 70 µl of MilliQ H_2O. The mixes were incubated in 75 °C for 5 minutes and in 95 °C for 10 minutes. Finally, 500 µl MilliQ H_2O were added, the mixes were centrifuged for 1 minute and the supernatants were transferred in a new

[3] The RBP16 sequence was derived from : https://tritrypdb.org/tritrypdb/app/record/gene/LmjF.28 .
Schematic diagram was created using Inkscape and SnapGene software. Available on: https://www.snapgene.com/ https://inkscape.org/release/inkscape-0.92.4/

tube and stored in -20 °C. DNA was tested via PCR reaction to confirm the genome integration in transfectants.

2.2.2 Ethanol precipitation

The extracted DNA was incubated with 10% of 3M sodium acetate and 200% of ethanol for 30 minutes and temperature <4 °C. After a 15-minute centrifugation, the pellet was resuspended with 70% ethanol and centrifuged at 10,000g for 15 minutes. The pellet was aired dry in a sterile environment and resuspended in 10µl MilliQ H_2O.

2.2.3 Conventional PCR

The Polymerase Chain Reaction (PCR) technique was used for genome DNA amplification of the WT-RBP16-HA and *Δprmt*7-RBP16-HA to confirm the C-terminal HA-tagging integration to the genomic DNA of *L. major* parasites. For this PCR, the following protocol was used: PCRBIO Rapid Extract PCR kit according to manufacturer's instructions (PCR biosystems). The transfectants were validated with PCR using 5'FLR and 3'FLR primers. Forward: GCAACCAAGCTTATGTACCCATACG,

Reverse: GCTAACTGCATGCGGAGTGGTGCTGATCGAAGCAC.

2x PCRBIO HS Taq Mix Red polymerase		25µL
Primer forward		1 µL
Primer reverse		1 µL
MilliQH_2O		22 µL
DNA extracted template		1 µL
Conditions		
95 °C	1min	
95 °C	15 sec	30x
56 °C	15sec	
72 °C	30min	
68 °C	5min	
12 °C	∞	

Table 2. PCR conditions for testing the integration of HA tag into the genome.

2.2.4 Gel electrophoresis

PCR was analysed in 1% agarose gel. Syber Safe stain (Invitrogen) was used for gel preparation for samples loading purple dye (Biolabs). The DNA ladder was 1Kb (PCR bio). For gel preparation, TBS 1x buffer was used.

2.3 Cross-linking Immunoprecipitation (CLIP)

2.3.1 Crosslinking

This method was used to fix RNA-protein complexes in living cells and subsequently to purify the RNA targets (Figure 18). The 254nm UV exposure results in the formation of covalent bonds between RNA and RNA binding proteins which are in close contact and interact through an RNA recognition motif. In this project crosslinking was performed with UVC using the LT40 "Minitron" system (UV03 Ltd; (Granneman, Petfalski, Swiatkowska, & Tollervey, 2010) for 120 s at 1.6mJ/cm^2 (de Pablos et al., 2019). Accordingly, cells from procyclic and metacyclics WT and *Δprmt7* cell lines were cultured in triplicate in 250ml and 1x10^6 parasites/ml inoculum in supplemented media M199 to be collected and submitted to crosslinking. The cell harvesting occurred in logarithmic (2^{nd} day) and stationary phase (5^{th} day). Finally, 4×10^8 crosslinked procyclic and metacyclic promastigotes were centrifuged at 2000 g for 10 min at 4°C, and they resuspended in 1 ml of IP lysis buffer (KCl 50 mM, Tris10 mM, pH 7.5, $MgCl_2$ 2mM, 1% Triton X-100, glycerol 10%, PMSF 4 mM, DTT1 mM, 1× Roche Complete protease inhibitor cocktail (Roche Applied Science).

2.3.2 Immunoprecipitation

Lysis of cross-linked parasites was carried out with the utilisation of G30 needle on ice until and the complete cell disruption was observed in microscope. After the total extract centrifuged for 4 minutes in 0 °C and 13,000g. The supernatants which correspond to S10 lysis cells were collected and

transferred into an RNAse-inhibitor eppendorf (Thermo scientific). For the blocking step, the same amount of Protein-G conjugated Dynabeads (Invitrogen) were used for all samples. Initially, the beads were placed in a MagRack6 (GE-Healthcare) and were washed twice in PBS-Tween 0.05% (PBS-T). The samples were incubated while rotating for 45 minutes at 4°C. After the beads were incubated with the same amount of 100 µl *T. brucei* anti-RBP16 rabbit antibody in dilution 1:1000 for 45 minutes, rotating in 4°C. Then, magnetics protein G beads were added in all samples and they have incubated again for 45 minutes, rotating in 4°C. Finally, the samples were placed in MagRack6, the supernatant (flow-through) was collected. Then, the beads resuspended in the lysis buffer and six washes followed. Finally, the beads separated to 90% used for RNA elution and 10% used for protein elution.

2.3.2.1 Protein elution

The eppendorfs placed in MagRack6 and beads resuspended in 25 µl Glycine 0,1M. All the samples were incubated in a rotating machine for 15 minutes. After the supernatant collected a new RNAase-inhibitor eppendorf and the process repeated twice. Finally, the supernatants from two steps mixed (50µl) and diluted with 16,6 µl Laemlli buffer (final concentration, 20%, β-mercaptoethanol). The beads left from the above protein elution were used to elute boiled protein. The protein eluted with 66µl Laemlli buffer (20%, β-mercaptoethanol) boiling for 5 minutes in 95°C.

2.3.2.2 RNA elution

For RNA elution Protein G beads were treated with Trizol for 5 minutes at room temperature. Finally, the eppendorfs placed in magnetics and the supernatant was collected into a new RNAase-inhibitor eppendorfs and followed the protocol for RNA extraction as manufacturers recommend from Direct-zol Miniprep (ZYMO Research):

- In all RNA samples added an equal amount of ethanol 100% and mixed thoroughly. The mix transferred into Zymo-Spin IIC Column in a collection tube and centrifuged at 10.0000g for 30 seconds.
- The samples treated with DNase I. For this, 400 µl of RNA Wash buffer is added in columns and centrifuged. Then, 5 µl DNase I and 75 µl of DNA digestion buffer were added in columns and the samples incubated at room temperature for 15 minutes.
- After incubation, 400 µl of Direct-zol RNA PreWash added and the column centrifuged at 10.0000g for 30 seconds.
- Then 700 µl of RNA Wash buffer added to the column to remove completely the wash buffer and followed the centrifuge for 2 minutes at 10.0000g.
- For the elution of RNAs the column transfer into RNase-free tube and 50µl DNase/RNase free water added directly to the column and centrifuged 10.0000g for 30 seconds.
- Finally, RBP16-associating RNAs are sent for sequencing for identities and relative quantification.

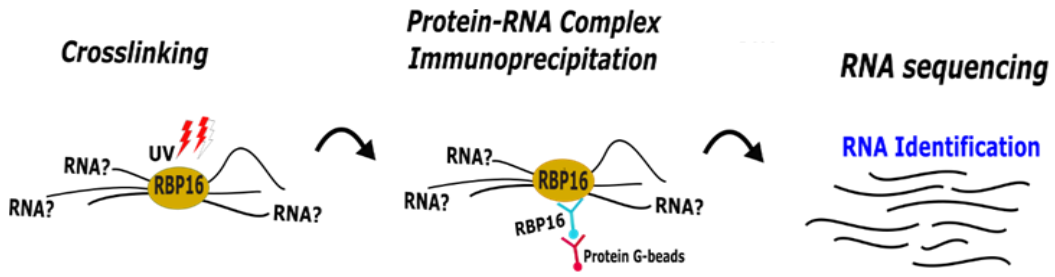

Figure 18. Schematic diagram of Crosslinked immunoprecipitation (CLIP) and isolation of RNA targets for RNA sequencing[4]. RNA is excited *in vivo* by UV irradiation at 254nm. This excitation transfers to nearby molecules and forms covalent bonds between RNA and nearby proteins. Antibodies are used to isolate RBP16 and associating RNAs.

[4] Crosslinked immunoprecipitation process was illustrated by using the software Inkscape. Available on: https://inkscape.org/release/inkscape-0.92.4/

2.4 Protein studies

2.4.1 Protein extraction

Promastigotes at the log and stationary growth phase (10^7 cells per lane) were extracted from WT CC1 and *Δprmt7* CC1, WT-RBP16-HA and Δprmt7-RBP16-HA cell lines and were sedimented at 2,000 g for 10 minutes. The cells were lysed in 2x SDS sample buffer and boiled for 5 min before loading on SDS-PAGE gels.

2.4.2 Immunoblot

The protein samples loaded in 15% of SDS-PAGE (Acrylamide 30%, Tris (2,5M. pH 8, 8), 10% SDS, Ammonium persulfate (APS) 10%, TEMED) and run in 1x SDS-Tris Glycine running buffer for 2 hours in 100V. After, proteins are transferred in PVDF membrane and run in Transfer Buffer: 25x Semidry, ethanol and DdH_2O) at 20 V for 1 h using a Novex Semi-Dry blotter (Thermo). Blots were blocked in 5% milk TBS-T (20 mM Tris, 150 mM NaCl, pH 7.4, 0.05% Tween) for 1h at room temperature. After washing 3x with TBS-T the membrane incubated overnight with the primary antibody rabbit anti-TbRBP16 [1:2,000], and mouse anti-HA 1:10,000] (Invitrogen). Membrane washed again with TBS-t 3x and incubated with secondary antibody HRP anti-rabbit and HRP anti-mouse (Invitrogen) for 1 hr at room temperature. Finally, the membranes were washed and incubated in ECL Prime (GE Healthcare) detection solutions for 5 minutes before ECL Hyperfilm exposure (GE Healthcare). Films were developed, scanned and images were processed using GIMP.

For NMT staining, the membrane was incubated for 3hr with NMT anti-rabbit, diluted in 1% milk in TBS-T and incubated for 3 hours at room temperature. Then it washed with TBS-t and incubated for 1hr at room temperature with the secondary antibody. Finally, the membrane was imaged with ECL Reaction Buffer.

2.4.2.1 Immunoblot for eluted RBP16

Eluted protein from immunoprecipitation samples run in 15% SDS-PAGE following the same steps as described above for RBP16 immunoblotting.

Antibodies	**Suspension**
mouse anti-HA	1:10000,5% (w/v) milk in 0,1 TBST
anti-mouse HRP	1:10000, 5% (w/v) milk in 0,1 TBST
rabbit α-RBP16 *T. brucei*	1:2000, 5% (w/v) milk in 0,1 TBST
anti-rabbit HRP	1:10000, 5% (w/v) milk in 0,1 TBST
rabbit anti-NMT	1:10000, 3hr 1% (w/v) milk in 0,1 TBST
anti-rabbit HRP	1:2000, 1% (w/v) milk in 0,1 TBST

Table 3. List of antibodies utilised for Western blots.

2.5 Microscopy

2.5.1 Immunofluorescence

Promastigote procyclic parasites (logarithmic phase) were collected to examine RBP16 localisation:

- For this $2x10^6$ parasites were centrifuged and the pellet (parasites) resuspended in 100 µl of 500nM MitoTracker™ Green FM (Molecular Probe). The parasites were incubated for 30 minutes at 26 ^oC.
- After incubation 100µl of PBS is added and parasites centrifuge for 10 minutes at 32000rpm.
- The pellet resuspended with 100 µl 4% paraformaldehyde for 20 min (PFA). Parasites were fixed at room temperature for 20 minutes.
- Then the mix was top up with PBS and centrifuged for 10 minutes at 32000rpm, the supernatant was removed, the pellet resuspended with 200µl PBT (2% Tritonx100+Glycine 0,1Min PBS) and incubate for 20 minutes at room temperature.
- Fixed cells centrifuged for 10 minutes at 32000 rpm, resuspended in 100 µl of PBS and attached to poly-lysine-coated coverslips. After the left amount removed and the slides washed with 1% BSA in PBS 3 times for 10 minutes

- Then, followed incubation of 10 µl of the primary antibody anti-HA mouse [1:500] (Sigma) or rabbit anti-TbRBP16 [1:500] in 2% in the 0, 1% Triton, 1% BSA in PBS. Incubation lasts 1 hour, at room temperature.
- After the primary antibody incubation, the slides washed 3 times with PBT for 10 minutes,
- For the secondary antibody incubation used anti-mouse or anti-rabbit Alexa Fluor 594 [1:1,000] (Invitrogen) in PBT (0, 1 % Triton, 1%BSA in PBS). The samples incubated for 1 hr and then washed 3 times with PBT and mounted with Fluoroshield™ (Sigma).
- Finally, DAPI staining was added for nuclear staining.
- Images were acquired on an Axio Observer microscope (Zeiss) and images were processed using ZEN 2.3 SP1 (black, Carl Zeiss) and GIMP.

2.5.2 Metacyclic parasites enrichment

Parasites from stationary phase were collected and tested for RBP16 localization, according to the protocol for metacyclic parasites enrichment (Spath & Beverley, 2001):

- From WT and Δprmt7 and WT-HA-RBP16 and *Δprmt7*-HA-RBP16 cell lines cultures, 20ml were collected and centrifuge for 10 minutes in 3200 rpm and the pellet were resuspended in 3ml DMEM 1x.
- In a 15ml falcon, 3ml Ficoll 20% added and then another 3ml of 10% Ficoll. Finally, the top was covered with the 3ml of resuspended parasites in DMEN 1x.
- The mixture was centrifuge for 10minutes at room temperature, acceleration 1 and 1800g.
- Then the metacyclic parasites were collected from the top of the falcon (3ml), transferred into a new falcon, and topped up until 15ml with DMEN 1x.
- Centrifuge for 10 minutes at 3200rpm and resuspend the pellet in 1x PBS.
- The cells were incubated in Mitotracker following the above protocol.

3. CHAPTER III. Results

3.1 C' terminal RBP16-HA tag negative affects the proliferation of transfectants *Δprmt7* cell lines compared with the WT cell lines

Initial experiments from Ferreira et al. (2019), focused on the expression of the N' terminal HA tagged RBP16. The N'-terminal HA tagging of RBP16 in *L. major* resulted in HA-RBP16 cytosolic localization and its methylation affects the stability of the molecule. Cytoplasmic localized HA-RBP16 is not the expected localization, as *T. brucei* RBP16 is a mitochondrial protein. Also, online prediction tools revealed a mitochondrial cleaved sequence of RBP16 which might be the reason why RBP16 protein targeting for transmission to the mitochondrion is not recognized. For this reason, we aimed to identify the differences between the endogenous N' terminal HA-RBP16 and the C' terminal RBP16-HA.

Consequently, a C-terminal HA-tagging strategy and construct for RBP16 was generated. The C-terminal RBP16-HA tag construct is generated to integrate the tagging sequence to the natural chromosome and be expressed in the protein RBP16 of *L. major*. For this purpose, the plasmid pPOTv2 used to be amplified and digested with the amplified products of *L. major* gene sequence of RBP16 contained the 3xHA tag sequence. The 3xHA tag is placed before the stop codon TAA and after the coding sequence of RBP16 so as to be expressed in the C' terminal of the RBP16 protein . Then, parasites from WT and *Δprmt7* CC1 *L. major* Friedlin cell lines submitted to transfection. The parasites that survived, had integrated the plasmid DNA into their genome and as a result, they exhibited resistance to the antibiotic neomycin.

The successful survival of parasites in the presence of the antibiotic neomycin, DNA amplification was followed to test the DNA integration into the genome of parasites. For this reason, DNA extraction from the procyclic promastigote phase occurred to be used for PCR reaction. In Figure 19, the PCR results confirm the C-terminal-HA tagging construct integration in both cell lines and the amplification

of WT and *Δprmt7* DNA resulted in the same size as plasmid construct. The fact that the genome DNA (gDNA) in figure 19 is blank (negative control) reveals that the tagging plasmid construct is integrated in WT and *Δprmt7* cell lines.

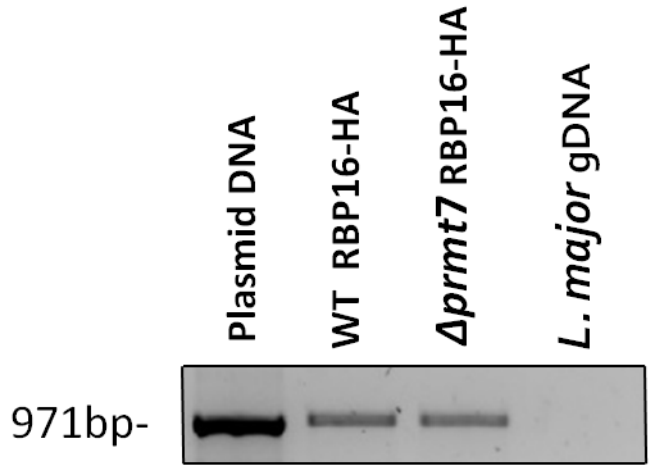

Figure 19. PCR reaction for testing the integration of RBP16-HA plasmid construct into the genome DNA of *L. major*. The plasmid DNA used as a positive control for transfectants DNA, while the WT genomic DNA was used as the negative control.

In Figure 20, it is shown the proliferation of parasites in 8 days of culture. As it is shown, *Δprmt7*-RBP16-HA has a lower concentration compared with *WT*-RBP16-HA in the culture media and RBP16 C' terminal HA tag negatively affects the proliferation of null mutants. On the contrary, in parental cell lines, the *Δprmt7* CC1 are displayed at a higher concentration than the WT in culture. However, the growth rate of two transfectant cell lines showed that for both the transfection is sustainable and the growth curve is similar to the normal parasites growing in culture.

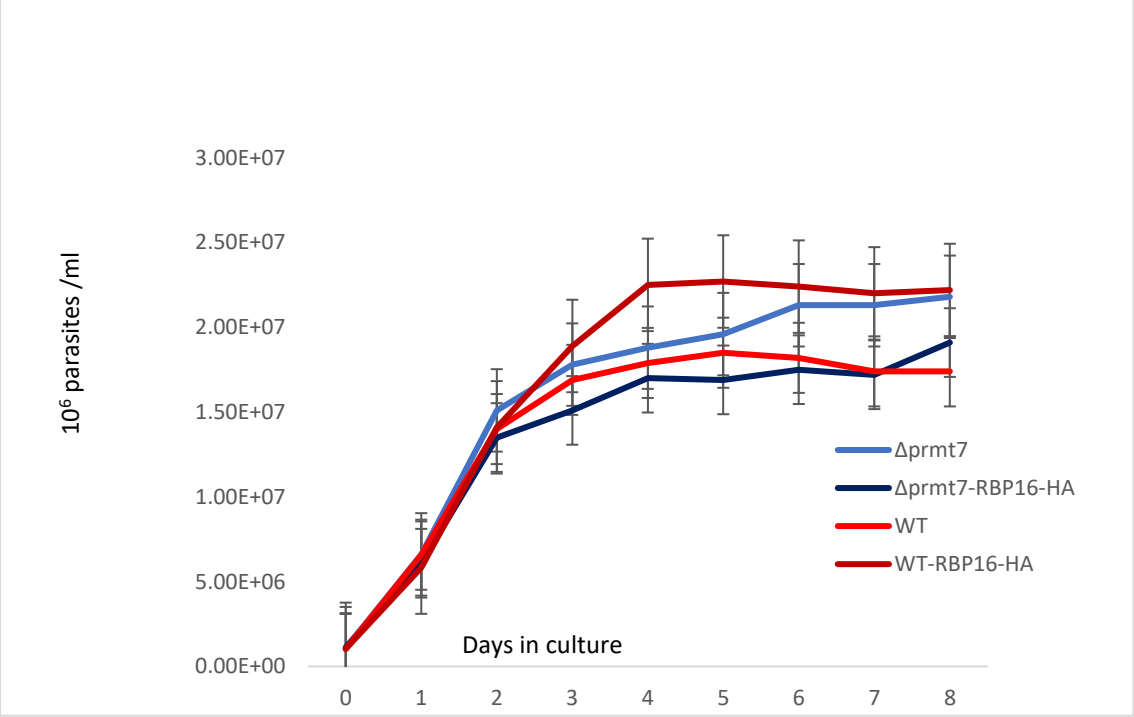

Figure 20. Illustrations of the growth rate of WT RBP16-HA and *Δprmt7* RBP16-HA parasites against days and concentration of parasites in growth medium M199. Error bars represent standard deviations from three independent experiments.

3.2 Localisation of endogenous *Lmj*RBP16 in WT and *Δprmt7* is similar to the relative *Tb*RBP16 and is found in the mitochondrion in both life cycle stages

After, the confirmation of the RBP16-HA tag at the C' terminus, immunofluorescence experiments were carried out to identify RBP16 protein localization of *Δprmt7* and WT cell lines. Previous immunofluorescence experiments in Walrad lab using procyclic promastigotes parasites that overexpressed plasmid of RBP16-HA tag in C-terminal showed that RBP16 of both *Δprmt7* and WT cell lines is localized in the mitochondrion. Accordingly, immunofluorescence RBP16-HA experiments were carried out to identify the mitochondrial localization of the RBP16 C-terminal HA tag as it has been observed initially in the *Tb*RBP16. However, this attempt was not as successful as the protein was not detectable. Additionally, the fact that in an immunoblot analysis the RBP16-HA protein was still not detectable it might suggest that the levels of protein are low (Figure 21). Consequently, in consideration of the anti-HA detection troubles in our experiments, the *T. brucei* anti-RBP16 antibody

was tested for this purpose as it displays high similarity (90%) to *Lmj*RBP16 protein (a kind gift from L. Read).

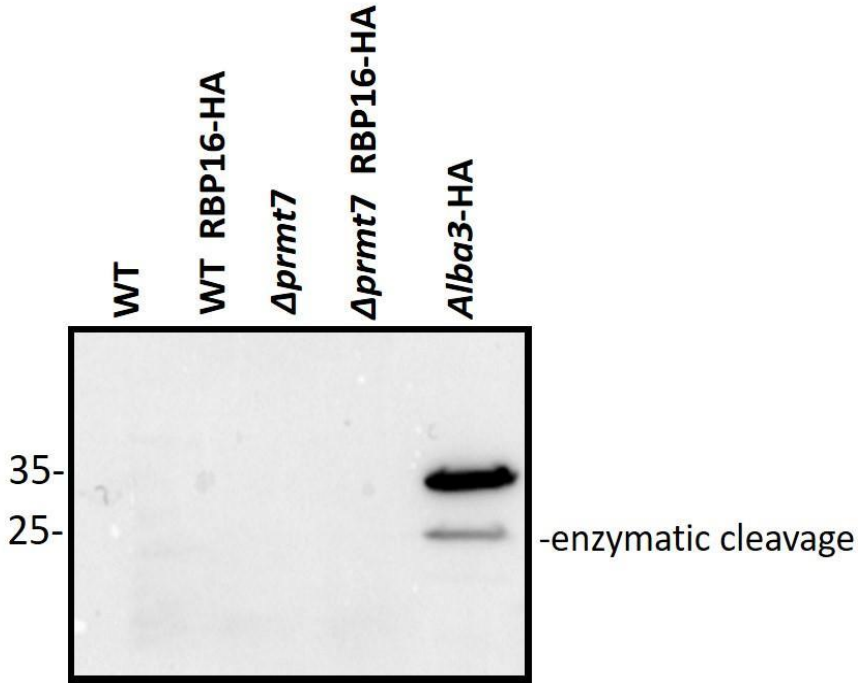

Figure 21. Immunoblot analysis of endogenous RBP16-HA in WT and depleted for *Δprmt7* cell lines. In immunoblot is shown the Alba3-HA protein in the corresponding molecular weight (35kDa) and an enzymatic cleavage in 25kDa. The RBP16-HA is not detectable in WT and *Δprmt7* cell lines. For the analysis the anti-HA antibody was used in dilution 1:10. 000.Alba3-HA protein was the positive control, WT and *Δprmt7* were the negative controls for the detection of HA-tagging in WT-RBP16-HA and *Δprmt7* RBP16-HA cell lines. Expected molecular weight of RBP16-HA: 18kDa

Immunoblot analysis for RBP16-HA expression was not carried out with the *T.brucei* anti-RBP16 antibody and for the following experiments the WT and *Δprmt7* CC1 cell lines were used. For this project, the endogenous RBP16 localization analysis was compared with the N-terminal HA-RBP16 tagging from (Ferreira et al., 2020). The localization of *L. major* RBP16 in WT and *Δprmt7* CC1 cell lines was identified using the anti-RBP16 antibody from the relative organism *T. brucei*. For our results to be representative and more convincing the images were collected from a confocal microscope and the signal of the antibody and mitotracker staining was quantified to show the co-localization of RBP16 with the mitochondrion.

In figure 22, it is shown an optical detection of RBP16 expression in procyclics (logarithmic phase) and metacyclic (stationary phase) parasites. It is shown that the subcellular localization of *Lmj*RBP16 using the anti-*Tb*RBP16 is the mitochondrion while the endogenously tagged HA-*Lmj*RBP16 is localized in cytoplasm and it is destabilized in metacyclics *Δprmt7*-HA-RBP16 cell lines in the stationary phase of promastigotes parasites.

For further analysis of RBP16 localization, fluorescence colocalization analysis focused on the distribution of signals in images. The overlap coefficiency as a measure of colocalization is shown in Table 4. As it is known, the scores that are approaching the 1 correspond to the colocalization. The lower scores indicate that the localization between mitochondrion (mitotracker) and protein (RBP16) differs. In summary, the transfectants HA-RBP16 cell lines displayed a lower colocalisation coefficient score compared with WT and *Δprmt7* CC1 cell lines. Finally, the metacyclics parasites were separated from procyclic in the culture and the representative metacyclics were used for co-localization analysis as described in chapter II.

anti-RBP16	Mitochondrion **Average Colocalization Coefficiency**		Protein Average Colocalization Coefficiency	
	Logarithmic	Stationary	Logarithmic	Stationary
WT	1.00	0.88	0,54	0,81
Δprmt7	0.88	0,65	0,76	0,70

anti-HA	Mitochondrion **Average Colocalization Coefficiency**		Protein Average Colocalization Coefficiency	
	Logarithmic	Stationary	Logarithmic	Stationary
WT HA-RBP16	0,15	0,63	0.54	0.32
Δprmt7 HA-RBP16	0,60	0	0,25	0

Table 4. **Quantification of RBP16 and mitochondria overlap.** The colocalization coefficient was generated via confocal software for RBP16 and mitochondria using anti-HA and anti-RBP16 versus the mitochondrial marker mitotracker.

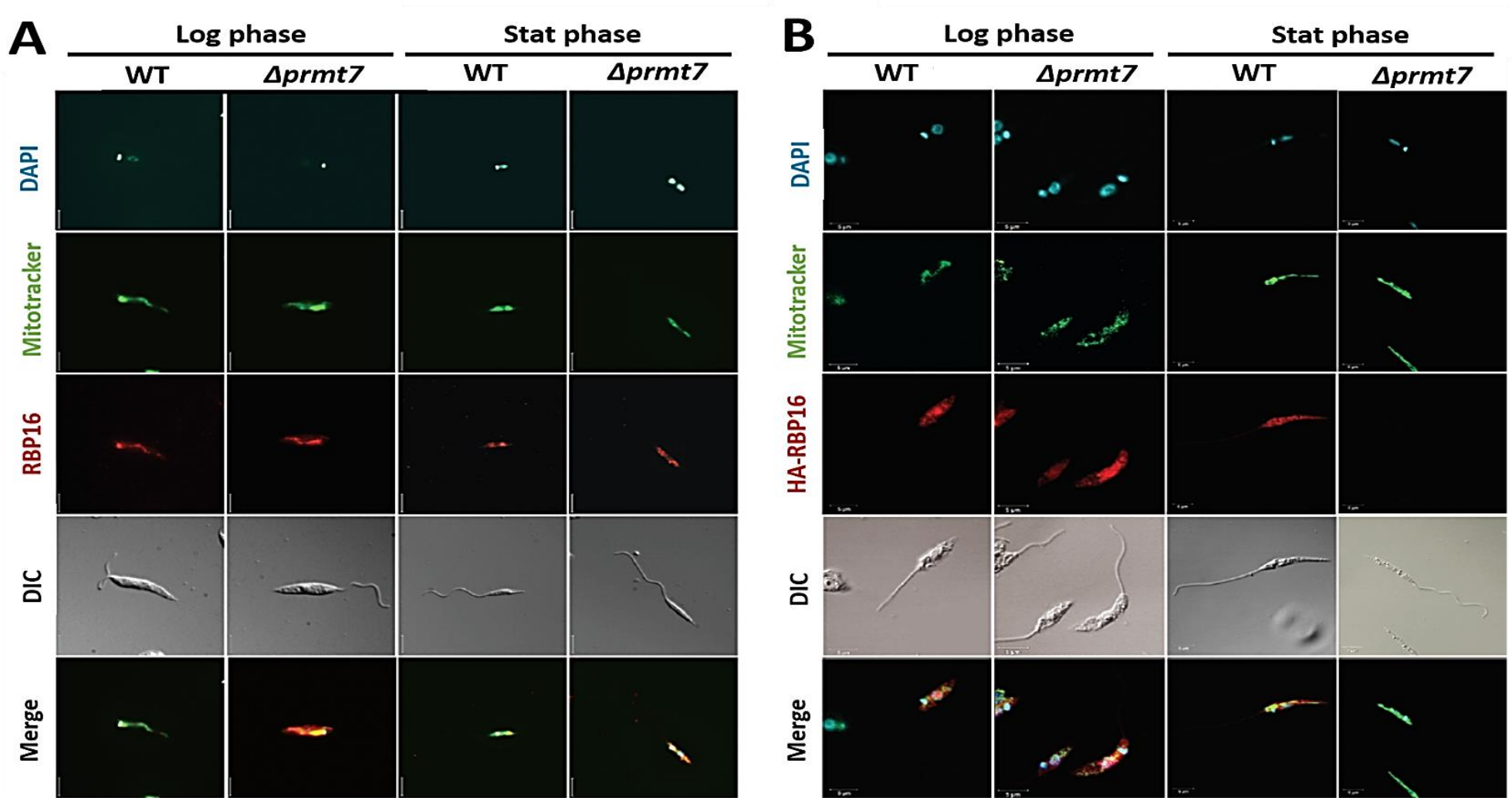
A
Log phase
Stat phase
WT
Δprmt7
WT
Δprmt7
DAPI
Mitotracker
RBP16
DIC
Merge
B
Log phase
Stat phase
WT
Δprmt7
WT
Δprmt7
DAPI
Mitotracker
HA-RBP16
DIC
Merge

Figure 22. Immunofluorescence detection of RBP16 localization in the logarithmic and stationary phase[5]. Blue (DAPI stain) shows the stained DNA in nuclear and kinetoplastid, Green (Mitotracker stain) shows the mitochondrion, the Red: (antibody fluorescence) shows the protein RBP16, DIC: Differential interference contrast, Merge: shows the nucleus, the mitochondrion and the protein together, line = 5µ. A. The picture shows the WT and *Δprmt7* CC1 cell lines in logarithmic and stationary phase. The green fluorescence exhibits the mitochondrion and the red reveals protein fluorescence. Together the green and the red fluorescence show the co-localisation of protein and mitochondrion in both life cycle stages. B. The pictures show the HA-RBP16 WT and HA-RBP16 *Δprmt7* CC1 cell lines in logarithmic and stationary phase. In log phase, the HA-RBP16 in both WT and *Δprmt7* is localised in the cytoplasm. In stationary phase, the HA-RBP16 in WT is again localised in cytoplasm while the HA-RBP16 in *Δprmt7* it is not detectable as it is destabilised.

[5] The illustration of localization analysis of RBP16 was made by using the software GIM2: https://gimp.en.uptodown.com/windows

3.3 Mitochondrial *Lmj*RBP16 exhibits protein stability in contrast to the cytoplasmic HA-RBP16

As it is mentioned, endogenously tagged HA-*Lmj*RBP16 is a cytosolic protein in the procyclic stage. However, in metacyclic parasites, the protein levels reduced significantly and RBP16 presented as a protein with low stability in the absence of PRMT7 in the stationary phase. For this reason, to test the expression levels and the stability of the protein, and immunoblot analysis was carried out to compare both life cycle stages. For this reason, the protein was extracted from procyclic parasites on the second day, (logarithmic phase) of culture and from the metacyclic parasites on the fifth day (stationary phase) of cell culture(figure 20). The analysis revealed that the protein is expressed in both life cycle stages with constant levels. This is strongly supported by using the *T. brucei* polyclonal anti-RBP16 antibody as its high similarity to *L. major* RBP16 simulates the expression of RBP16 inside the cell.

In figure 23, it is shown the 15kDa *Lmj*RBP16 protein for both procyclic and metacyclic life stages. Also, the second band in around 30kDa appears at a higher level. In the beginning, it was suggested that the protein might form a dimer. However, in *T. brucei*, RBP16 is referred to as not to be presented in homodimers but in complexes with other molecules (Goulah & Read, 2007). Thus, it was hypothesized that the 30kDa band might correspond in a cross-reactive and so antibody recognises an antigen similar to RBP16 band. Nevertheless, the existence of a homodimer could not be ignored; on the contrary it is a hypothesis that requires further investigations.

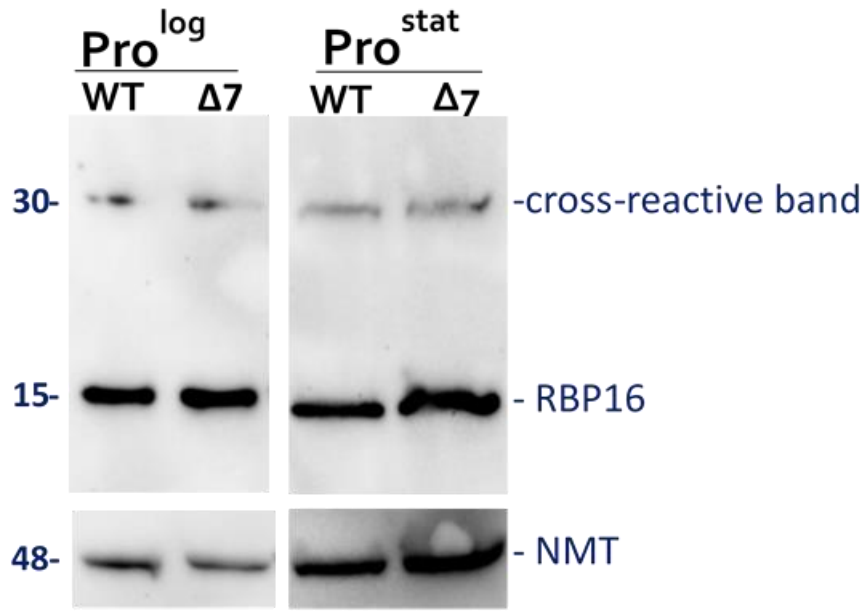

Figure 23. **Western blot analysis of RBP16 expression in promastigote procyclics and metacyclics parasites**. The *T. brucei* RBP16 antibody was used for RBP16 detection. The anti-NMT antibody is used for loading control.

3.4 *Lmj*RBP16 purification implies interactions with RNAs in a stage-specific level

Evidence from *Tb*RBP16 revealed that RBP16 is a mitochondrial protein with the RNA-binding domain and as a result, interacts with RNAs. In the mitochondrion of *T.brucei*, RBP16 found to be associated with several mRNAs and gRNAs (Pelletier & Read, 2003). Accordingly, in our investigations, we aimed to identify the candidate mRNA targets of *Lmj*RBP16. We hypothesized that *Lmj*RBP16 should have a relevant function within the cell of *Leishmania* after its high homology to *Tb*RBP16 and its identification as an RNA-binding protein in the isolation of the whole-cell proteome of *the Leishmania mexicana* (de Pablos et al., 2019).

*Tb*RBP16 function is regulated by the arginine methyltransferase PRMT1. Accordingly, loss of function of PRMT1 revealed the role of *Tb*RBP16 in RNA stability. Methylation of *Tb*RBP16 affects the RBP16-gRNA interactions opposite to RBP16-mRNA interactions. Hence, *Tb*RBP16 methylation increases its association with mRNAs and facilitates mRNA stabilisation (Goulah & Read, 2007).

Accordingly, to these data, the crosslinking immunoprecipitation experimental method was carried out for the isolations and identification of the RNA targets that interact with *L. major* RBP16 in procyclic and metacyclic life cycle stages. For this purpose, $4x10^8$ parasites were cultured and harvested in logarithmic phase/day 2 (PCF) and stationary phase/ day 5 (META) (Figure 24). The growth curve shows that the Wt have a lower proliferation compared to *Δprmt7* and so the absence of PRMT7 seems to promote replication during days 5-9 of culture.

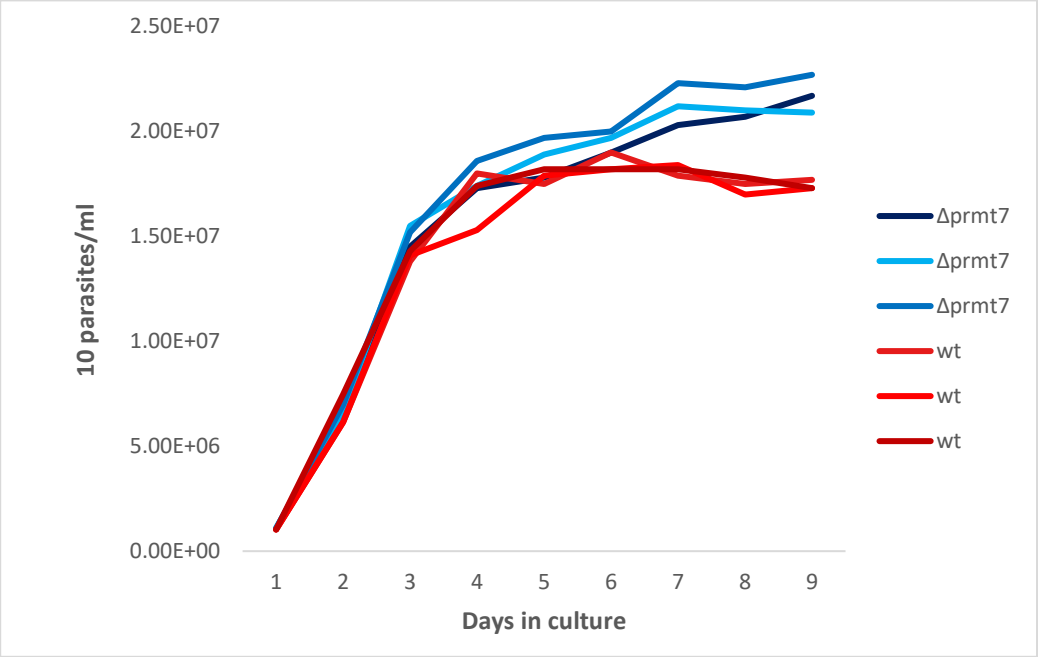

Figure 24. **Growth rates of WT and *Δprmt7* parasites against days and concentration of parasites in growth medium M199.** Concentrations of *L. major* parasite cultures in the presence (WT) and absence (*Δprmt7*) of PRMT7 levels. 3 replicates are displayed of each cell type.

Figure 25 presents the immunoblot analysis of procyclic RBP16 from both *Δprmt7 and* WT cell lines parasites. In the total extract of protein, multiple bands appear in WT western blot. There is no doubt that the 15kDa corresponds to endogenous RBP16. The 10kDa protein, which is shown not only in total extract but also in flow-through and eluted boiled RBP16 may be degraded RBP16 protein as a consequence of the UV procedure. This hypothesis comes off the fact that the anti-*Tb*RBP16 antibody

is polyclonal and so binds to different sites of the same protein, thus would likely bind *Lmj*RBP16 degradation products in addition to the full-length protein.

Then, strong evidence indicates a functional interplay between protein arginine methyltransferases in *Trypanosoma brucei* (Lott et al., 2014). In the beginning, we hypothesised that MMA and aDMA/sDMA formation could result in increasing the molecular weight of RBP16. However, the size of a methyl group is approximately 15Da, and so this could not significantly change the molecular weight of the protein. For this reason, we review the implications of UV irradiation exposure to our protein analysis. As RBP16 interacts with RNAs, we hypothesised that if RNA is bound tightly to RBP16, then the higher bands of 20-25kDa of RBP16 in our immunoblots may correspond to RBP16-RNA interactions. This could be a consequence of the irradiation exposure which results in alterations of RBP16 charge, as RNA is negatively charged, and proteins are positively charged.

In procyclic parasites, (Figure 25A) RBP16 is present in higher molecular weights, 20 and 25 kDa however bands are weaker than WT. This might indicate that depletion of PRMT7 results in decreased association with RNAs (Figure 25B).

In both Western blots(figure 25 A,B), the bands in flow through lanes are similar to the eluted protein and it is suggested that it corresponds to the protein that had not bound to the anti-RBP16 antibody. Additionally, for protein elution, two steps followed, in the one the protein boiled which is the classic way for protein elution in immunoprecipitation and the protein elution carried out without boiling step. This occurred after the hypothesis that RBP16 is a sensitive peptide in high temperatures. However, the non-boiled pathway was not effective for protein elution, as the protein is only detectable after the boiling elution pathway.

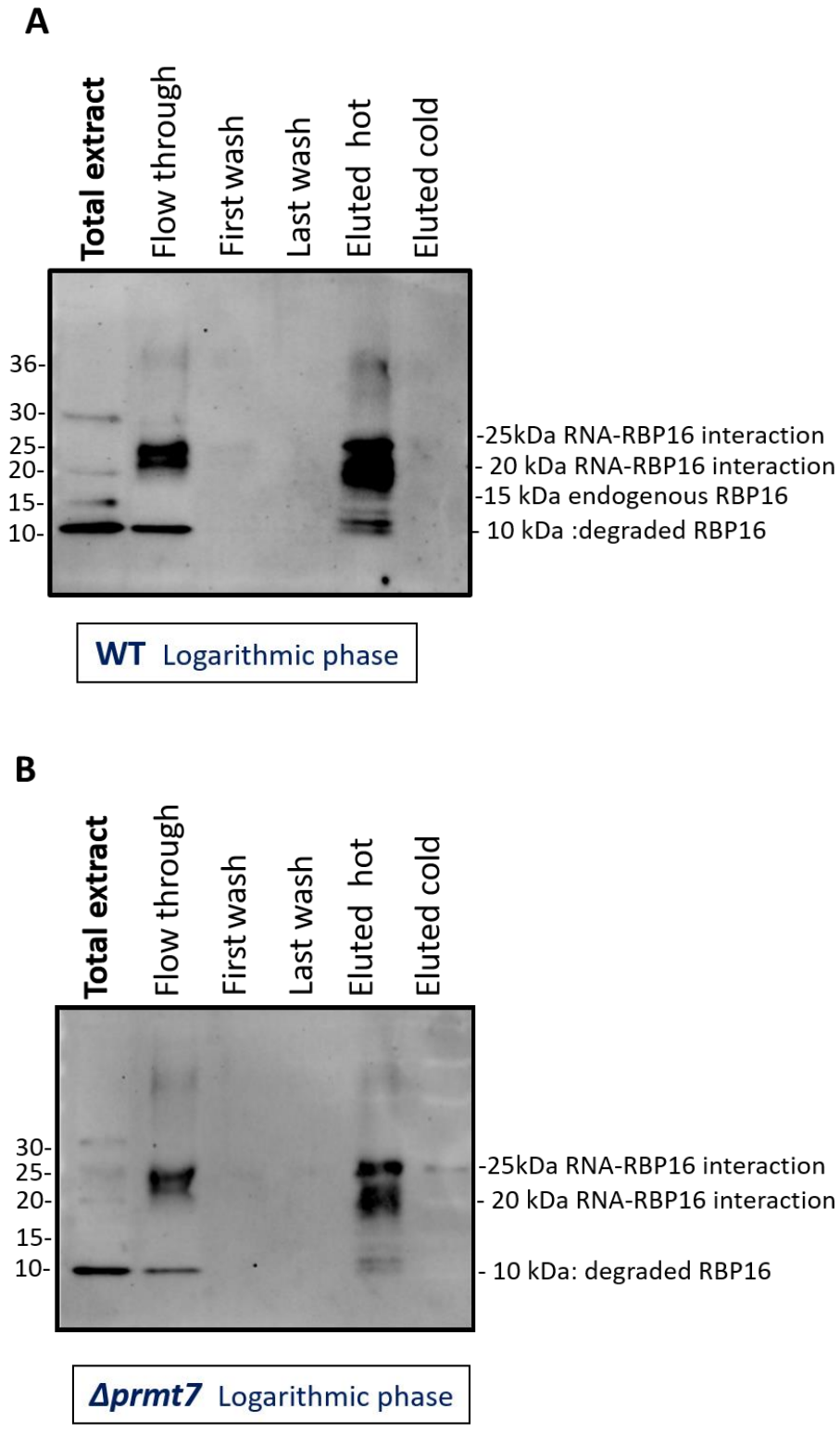

Figure 25. Immunoblot analysis of immunoprecipitated RBP16 in procyclic parasites of WT and *Δprmt7* cell lines. Both A and B immunoblots show the levels of immunoprecipitated RBP16 (eluted hot) compared with the total extract the flow-through, the first and the last wash of

immunoprecipitation process. On the left, it is shown the molecular weight of each band according to the protein ladder size (protein ladder size is 10-250kDa). The ''eluted cold'' lane corresponds to the protein that eluted by omitting the boiling at elution process,'' total extract'' corresponds to protein extraction from the whole cell, ''flow-through'' represents the protein before anti-RBP16 incubation and first and last washes correspond to the washing step with lysis buffer after the antibody incubation. The endogenous RBP16 molecular weight is 15kDa.The 10kDa corresponds to a degraded RBP16. **A** immunoblot corresponds to eluted RBP16 (eluted hot) from WT cell lines in logarithmic phase. The bands with size approximately 20-25 kDa show the RBP16 of which the size is shifted due to its interaction with RNA. The band that is observed in 30kDa in total extract lane is considered as a background band of lower affinity to the antibody as it is not present in the immunoprecipitation lanes. **B** immunoblot shows the immunoprecipitated RBP16 (eluted hot) of null mutants *Δprmt7* cell lines in logarithmic phase. RBP16 in 20 and 25 kDa corresponds to protein and RNA interaction.

Figure 26 presents the immunoblot analysis of immunoprecipitated RBP16 from the metacyclic life cycle stage of parasites. Similar to the procyclic stage, RBP16 is detectable in different molecular sizes. In total extract and flow through a 15kDa of protein corresponds again to endogenous RBP16 protein. In immunoblot analysis of immunoprecipitated RBP16, a higher band of 30kDa is detectable in this analysis as observed in previous immunoblot of this work (Figure 23), and so we conclude in the hypothesis that it might represent a cross-reactive band, as it is present only in the total extract and not in the eluted protein.

In this western blot of stationary phase, it is observed a degraded RBP16 in 10kDa, similarly to the procyclic RBP16. However, additional bands are observed also in higher sizes in flow-through, first, wash and eluted protein lanes. Specifically, RBP16 is observed in sizes around 20,30 and 36 kDa in this life cycle stage. Again, we hypothesised that RBP16 charge might change due to the tight bound of RNAs to RBP16 and this implies alteration of RBP capacity to shift due to charge. Additionally, we hypothesised that RBP16 might interact with different RNAs in this life cycle stage, as there is an association between the charge and the length of RNA.

In this point, we can ignore the possibility of MMA-dependent regulation of protein-RNA interactions in a stage specific level. This hypothesis comes from the fact that in immunoblot analysis of RBP16

from both WT and *Δprmt7* showed alterations in interaction with RNAs compared with logarithmic phase.

To conclude, comparing the immunoblot analysis from both life cycle stages, we cannot ignore the possibility of additional RBP16 modifications by other PRMTs, as the increased methylation of RBPs results in mRNA stability (Miller & Read, 2003). Thus, the depletion of PRMT7 may unmask substrates for aDMA/sDMA formation, facilitating the interaction of RBP16 with RNA targets. However, this hypothesis needs additional analysis in order to identify the potential PRMTs that target RBP16. Finally, it is hypothesised that UV irradiation may play a role in stabilisation of RNA: protein interaction, an observation which needs further investigations.

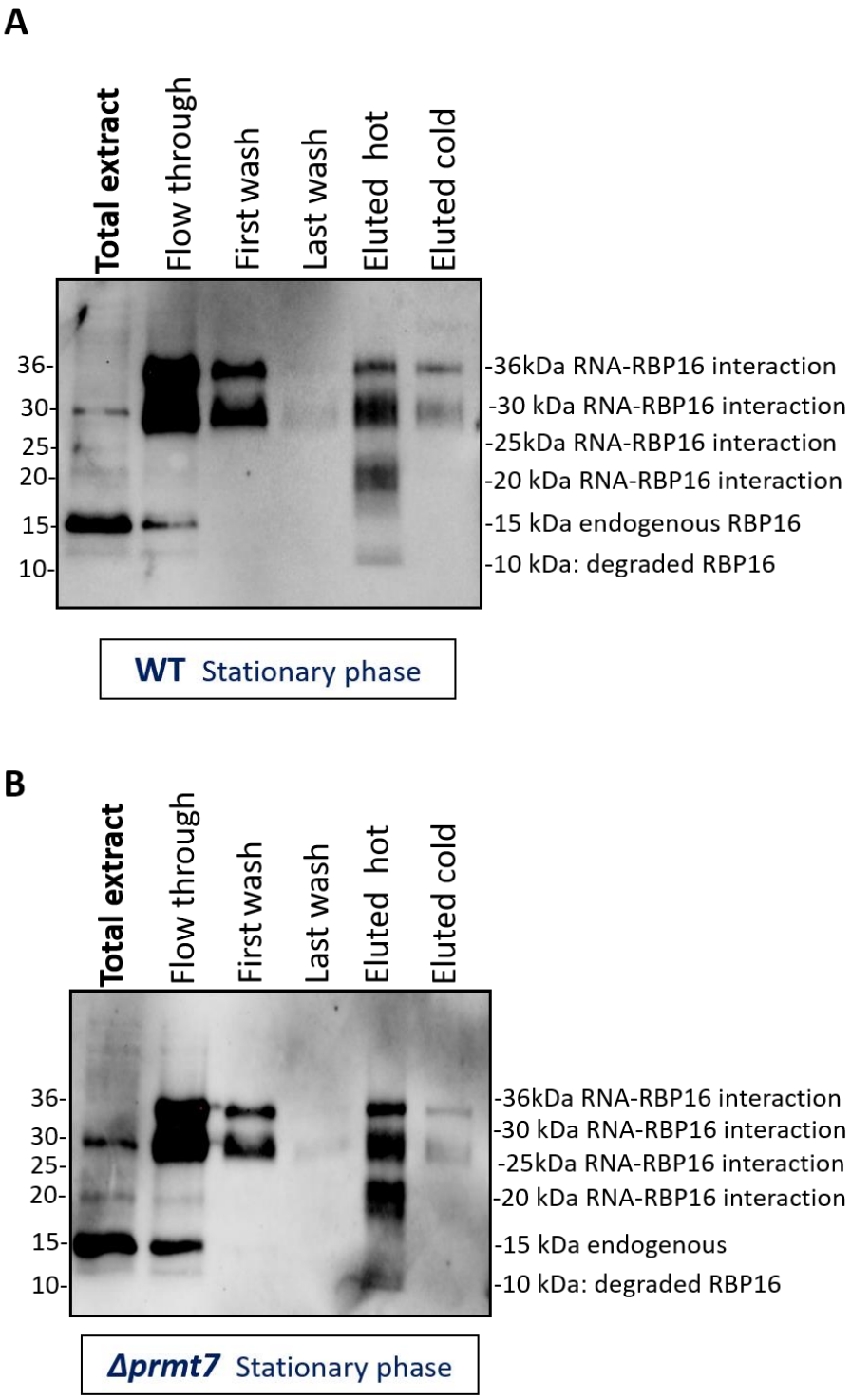

Figure 26. Immunoblot analysis of immunoprecipitated RBP16 from metacyclic parasites in WT and *Δprmt7* cell lines. Immunoprecipitated RBP16 (eluted hot) levels are shown in A and B immunoblots (eluted hot) compared with the total extract the flow-through, the first and the last wash. On the left, it is shown the molecular weight of each band according to the size of the protein ladder (protein ladder size is 10-250kDa).The ''eluted cold'' lane corresponds to the protein that eluted by omitting the boiling at elution process,'' total extract'' corresponds to protein extraction from the whole cell,

''flow-through'' represents the protein before anti-RBP16 incubation and first and last washes correspond to the washing step with lysis buffer after the antibody incubation. In both Western blots the 15KDa band in total extract and flow through corresponds to endogenous RBP16. The band that is observed in 30kDa in total extract lanes is considered as a background band of lower affinity to the antibody as it is not present in the immunoprecipitation lanes. **A** immunoblot analysis shows the immunoprecipitated RBP16 (eluted hot) of WT cell lines in stationary phase.RBP16 is shown to interact with RNAs that are tightly bound on it in different molecular weights (20-36kDa) **B** immunoblot shows the immunoprecipitated RBP16 (eluted hot) of null mutants *Δprmt*7 cell lines in stationary phase. eluted RBP16 interaction with RNA targets alters the charge of protein and subsequently is observed in higher sizes (20-36 kDa).

3.5 RBP16 association with RNA targets might be stage-specific regulated upon arginine methylation

After protein elution and analysis of RBP16 purification as described above, an RNA preparation for sequencing analysis and RNA targets identification were followed. RBP16 is a protein that interacts with several RNAs in the mitochondrion of relative *T. brucei*. The mRNA targets of TbRBP16 are the cytochrome c oxidase subunit I (COI), apocytochrome b (CYb), ATPase 6 (A6) and the NADH dehydrogenase subunits 4 (ND_4). Evidence for these RNA targets showed that methylation of TbRBP16 positively affects the stability of mRNAs.

Accordingly, it is expected that mRNAs targets of *Lmj*RBP16 could be similarly affected given its high homology with *Tb*RBP16. In a first attempt, RNA isolation in the procyclic life cycle stage was corresponded to 50-100 pg/µl RNA in 20 µl which was not a sufficient concentration to proceed to RNA sequencing. This result could support our hypothesis that UV irradiation procedure implies tightly bound of the associated molecules and so the isolation of RNA targets might be blocked.

Even though the identification of the potential RNA targets was unsuccessful in the procyclic stage, the stationary phase showed different results, as a higher concentration of extracted RNA was sufficient for RNA sequence. According to our hypothesis, the tightly bound of RNAs to RBP16 due to

UV irradiation procedure may be altered in stationary phase. In our results, we hypothesised that there might be a differential interaction of RBP16 with RNA targets which results in charge alterations, *in vitro*. This interaction might be dependent on arginine methylation which could promote the protein RNA binding in *Leishmania major* and results in mRNA stability. This observation could be additionally supported by the fact that UV irradiation might result in a tighter bound of different RNAs in *Lmj*RBP16 of WT and *Δprmt7* cell lines in both procyclic and stationary phase.

In summary, our results showed that RBP16 is a mitochondrial protein within *Leishmania major* and its stability in the cytoplasm is impacted by PRMT7 arginine methylation. Furthermore, we hypothesized arginine methylation of RBP16 might be increased in the stationary phase for both WT and cells depleted for PRMT7 compared to the logarithmic phase suggesting that additionally, PRMTs act directly to RBP16.However, function of *Lmj*RBP16 upon arginine methylation seems to need further investigations.

4. CHAPTER IV. Discussion

This project aimed to investigate the role of RBP16 upon arginine methylation by depletion of the methyltransferase enzyme PRMT7 in *Leishmania major* parasite. Also, one of the objectives was to identify potential candidate mRNA targets of RBP16 and how methylations affect protein-RNA interaction.

For this project, we used cell lines that are depleted for the PRTM7 and generated before in the Walrad lab and compared them with wild type (WT) cell lines. We successfully carried out the RNA targets isolation from RBP16 and submitted them to RNA sequencing only from the stationary phase. RNA sequencing data is not present as the process is ongoing. Then, despite multiple attempts, we failed to detect the endogenous C' terminal HA tagging RBP16 by immunoblot and immunofluorescence.

4.1 *Leishmania major* RBP16 is a mitochondrial protein with similar expression levels in procyclic and metacyclic parasites

In this project, we aimed to identify the localization of *Lmj*RBP16 and subsequently its expression in procyclic and metacyclic parasites. *T. brucei* RBP16 is the first Y-box protein found in the mitochondrion and interacts with several RNAs within mitochondrion (Hayman & Read, 1999). On the contrary, a mammalian homolog of RBP16, Yb-1 is found to be localized to both the nucleus and cytoplasm (Soop et al., 2003). According to our data, it seems that *L. major* RBP16 function displays high similarity to *Tb*RBP16 as there is a high similarity to *Tb*RBP16. In this project, we showed that *Lmj*RBP16 is indeed a mitochondrial protein in both life cycle stages examined, procyclic and metacyclic.

Initial experiments showed that N' terminal tagging HA-RBP16 localized to the cytoplasm. There, the protein stability is affected by depletion of PRMT7 in the metacyclic life cycle stage which is the

infectious stage of parasites. In contrast, anti-TbRBP16 revealed protein stability of RBP16 in the procyclic and metacyclic stages and RBP16 is not affected in *Δprmt7* cell lines when protein is localised in the mitochondrion.

Interestingly, these differences revealed the influence of PRMT7-dependent methylation upon the protein stability of RBP16. This support previous work demonstrating that post-translational modifying enzymes can regulate the fate of target proteins. As a result, the regulation of one modifying enzyme can act as an inhibiting factor of other enzymes. Thus, it is suggested that the loss of PRMT7 may allow other proteins to target RBP16 for protein degradation in metacyclic parasites, specifically in the cytoplasm. In this mechanism are included the ubiquitinating enzymes and the proteasome which has been reported to be inhibited by other proteins within the cells (Hu et al., 2015; Tikhanovich et al., 2015). Finally, Comparing the cytoplasmic HA-RBP16 and the mitochondrial *Lmj*RBP16 seems that the microenvironment also plays an important role for protein stability. Hence, *Lmj*RBP16 is considered as a mitochondrial protein with RNA-binding activity and so this reflects the importance for stability and maintenance of expression levels within the mitochondrion (Ferreira et al., 2020).

In contrast, the mammalian homologue of *Lmj*RBP16, Yb-1, seems to be localized both in cytoplasm and nucleus and its function is relevant to the events of these environments. However, there is no evidence of mitochondrial localization of Yb-1. All evidence suggests that mammals Yb-1 is a pleiotropic protein with a distinguished function within the cells (Kohno et al., 2003) (Lyabin et al., 2014) (Lyabin et al., 2014 (Skabkin et al., 2004).Similar to Yb-1, RBP16 is associated with regulation of gene expression however, Yb-1 function is regulated by phosphorylation (Eliseeva et al., 2011), while trypanosomes RBP16 is post-translationally modified by PRMTs.

4.2 RBP16 arginine methylation is not significantly affected by PRMT7 depletion

According to our results , the tightly bound of RNAs to RBP16 due to UV irradiation procedure revealed that a differential interaction of RBP16 with RNA targets might exist between WT and *Δprmt7* cell lines in procyclic and metacyclic parasites and this is associated with arginine methylation which could promote the protein RNA-binding activity in *Leishmania major* and results in mRNA stability.

(Ferreira et al., 2020) initial investigations focused on the impact of PRMT7 loss of function in its protein targets. 40 proteins found to be hypomethylated and *Lmj*RBP16 is among this group protein. Consequently, *Lmj*RBP16 is suggested to be a substrate for other methyltransferases enzymes as well. To our interest, 17 proteins found to be hypomethylated by the absence of PRMT7. Thus, it seems that there is an alternative PRMTs activity within *Leishmania*. Overall, comparing the evidence of *Leishmania* and other organisms, there is no doubt that PRMTs interact and act synergistically inside the cell but also compete for the same residues of substrates. Thus, PRMTs can be considered a complex regulatory system that needs further investigations in *Leishmania* organisms.

In *T. brucei* regulation of RBP16 is controlled by arginine methylation. Thus, *Tb*RBP16 is a substrate of PRMT1 and PRMT1 can catalyse asymmetrical demethylation but also can monomethylate protein targets (Bedford & Richard, 2005). Consequently, it seems that within the cells, MMA formation can occur from both PRMT1 and PRMT7 which can result in heavy monomethylation marks of proteins. Similarly, to *Hs*PRMT5, *Hs*PRMT7 found to methylates arginines in unknown regions of substrates indicating GAR motif are not the sole target sites for PRMT7 action (Pahlich et al., 2006). Also, in mammals type I PRMTs are reported to have monomethylated activity (Zurita-Lopez et al., 2012).

Hypothetically, PRMT1 which is a type I enzyme may catalyse MMA formation as well in *Leishmania*. Thus, the role of both *Lmj*PRMT1 and *Lmj*PRMT7 is important to explore, as depletion of PRMT7 might unmask arginine sites for PRMT1 and so the latter does not have to compete for PRMT7 same substrate.

More evidence from *T.brucei* showed a functional interplay between PRMTs (Lott et al., 2014). For this reason, it seems that the simultaneous depletion of PRMT1 and PRMT7 in *Leishmania major* will shed light on the function of these enzymes as both found to methylate trypanosomes RBP16. Hypothetically, RBP16 is a substrate for both PRMTs in *Leishmania* then we might expect a significant decrease and/or absence of RBP16 methylation. Accordingly, this can also have an impact on the fate of mRNAs targets as methylation affects mRNA and protein binding.

Furthermore, more studies are important to be done in *Leishmania major* to investigate the access in methylation targets to the same substrate. Generally, methylations occur within RG and RXR sites, however, there are exceptions. For example, *Hs*PRMT3, 6, 8 methylated substrates with GAR motifs (Pahlich et al., 2005). In addition to the target sites, the structure of the substrates plays a key role in the arginine methylation and so region determines the specific methylation.

4.3 Depletion of PRMT7 revealed a stage-specific regulation of RBP16 interaction with RNA targets

In previous studies, PRMTs are characterized as regulators of RNA affinity to targets RBPs (W. Cui, Yoneda, Ueda, & Kurokawa, 2018; Yu, 2011). In *T.brucei*, *Tb*RBP16 methylation upon PRMT1 directly affects protein-RNA binding and although no increase in RNA-RBP16 binding observed, loss of methylation resulted in reducing of mRNA-RBP16 interaction (Goulah & Read, 2007).

Similarly, in our results, we hypothesised that there is a differential interaction of RBP16 with RNA targets and might be dependent on differential arginine methylation activity which could promote the protein RNA binding in *Leishmania major* and so the mRNA stability. This observation would be additionally supported by the fact that UV irradiation might result in a tighter bound of RNA in *Lmj*RBP16 of WT and *Δprmt7* cell lines in both procyclic and stationary phase*, in vitro*. However, the fact that isolated RNAs were derived only from in stationary phases implies that RBP16 might interact

with different RNAs and this interaction is dependent on methylation, *in vitro*. These observations indicate *that Leishmania major* RBP16 might interact with specific RNAs and they may have a divergent function upon arginine methylation in a stage specific level.

In our results, RBP16 and mRNA levels might not be associated in procyclic parasites, as RNAs concentration is low. As described before, gene expression levels in trypanosomes and protein levels are low correlated (de Pablos et al., 2019). Interestingly, loss of function of PRMT7 would explain this observation in procyclic life cycle stage as RBP16 is a substrate for PRMT7. However, in metacyclic parasites, we observed that the associated RNA with RBP16 increased in both WT and *Δprmt7.* Thus, we hypothesized that the PRMT7 loss function might unmask substrates for other methyltransferases. Such PRMTs could be the PRMT1 and subsequently *Lmj*RBP16 might be marked by MMA and/or aDMA formation. Importantly, the interactions of PRMTs in *Leishmania* have to be investigated, as loss of function of PRMT7 could reveal the upregulation of other methyltransferases in *Leishmania* that act in the same substrate.

Interestingly, *Lmj*PRMT7 is presented as a key enzyme in virulence which is implicated in pathogenesis. In our project, we showed that there might be also a stage-specific regulation of RBP16-mRNA interaction upon the *Lmj*PRMT7 mutant. Hypothetically, increased methylation of RBP16 is associated with mRNA stabilization in the stationary phase which is the human-infectious form. Remarkably, as the increased virulence is associated with the increased division of parasites and survival within the cell, the mRNA stability of activated mitochondrial gene expression is required for energy-producing according to cell needs (Naderer & McConville, 2008). According to this, we hypothesized that the effects of null mutants *Δlmjprmt*7 in cell lines in the stationary phase may resemble a pro-environmental adaptation, preparing the parasites for the adaptation and proliferation within the host.

Moreover, we showed that protein levels of RBP16 do not exhibit any significant differences in the loss of function of PTMT7 in both mutants and wild type. This is in contrast to other substrates of

PRMT7 in *Leishmania* such as Alba20. Alba20 stationary levels reduced remarkably compared with the early-log phase however the protein levels in null mutants *Δlmjprmt7* cell lines exhibit stability in both life cycle stages. Thus, it is hypothesized that alters in virulence may be controlled by PRMTs and subsequently be relevant to protein expression levels of specific substrates. As it has been reported, PRMTs are associated with protein-protein interactions alterations (Lott et al., 2015; Poornima, Shah, Vignesh, Parker, & Rajyaguru, 2016). Thus, PRTMs are presented as target-specific regulators that may are involved in *Leishmania* pathogenesis.

Leishmania is presented as a unique organism to specifically investigate the impact of arginine methylation in RBPs. *Leishmania* PRTMs found in cytosolic and non-histones targets identified as substrates of PRTMs, suggesting that RBPs are the primary substrates in *Leishmania* (Ferreira et al., 2020). Similarly, homolog in mammals Yb-1, which is regulated by phosphorylation, RBP16 is post-translationally modified by PRTMs.

As trans-regulatory proteins are of high-interest proteins as they are considered as primary gene regulators, in *Leishmania* few have been identified in the whole-cell proteome (de Pablos et al., 2019). RBP16 is a trans regulator that is involved in post-transcriptional regulation and gene expression in trypanosomes. Additionally, the fact that its methylation from PRMT7 which is undoubtedly associated with *Leishmania* virulence, renders it as an interesting substrate for investigated *Leishmania* pathogenesis.

Thus, we showed that RBP16 is post-translationally might be modified from other PRMTs as there is functional interplay between PRMTs in trypanosomes. However, investigation of *Lmj*RBP16 post-translationally modifications is necessary to shed light on the function of this protein as little is known about PTMs that control RBP16. Finally, PRMT7 seems to act directly in post-translational level and indirectly in the post-transcriptional level by regulating the function and stability of post-transcriptional regulators in *Leishmania major*.

4.4 Future work

Overall, additional studies are necessary to progress these findings. For this reason, simultaneous depletion of PRMTs, such as PRMT1 and PRMT7 which targets the RBP16 in trypanosomes could shed more light on the function of these enzymes and the regulation of RBP16 protein. However, a parallel analysis of *Lmj*PRMT1 and 7 depletion and its effect in RBP16 expression levels could be an interesting observation in *Leishmania* parasites, to compare the evolutionary correlation between trypanosomes.

Also, we suggest that further analysis of RBP16 expression levels in the amastigote life cycle stage could reveal the association of RBP16 and parasites virulence upon arginine methylation, and subsequently its effect in gene expression in amastigote parasites.

As mentioned before, the transcriptomic analysis of RNAs that bound to *Lmj*RBP16 in stationary phase is ongoing. Preferably, we proceeded in this analysis as the identification of RNA targets could be useful for their comparison with the corresponding RNAs that found to interact with *Tb*RBP16. Methylation of RBP16 by PRMT1 results in a negative association with gRNA or positive association with mRNAs.Thus, the transcriptomic results might reveal if the regulation of gRNA and mRNA in *Leishmania major* is MMA-dependent but also the possibility of a divergent regulation of *Lmj*RBP16.

Finally, according to the transcriptomic analysis, the relative mRNA levels from the identified RNA targets can be further analysed and quantified by qRT-PCR.

5. Conclusion

This project aimed to investigate the RBP16 expression levels and their relation to post-transcriptional control through the mRNA stability of mRNAs targets. We conclude that similarly to *T. brucei*, *Leishmania major* RBP16 is a mitochondrial RNA-binding protein which is associated with mRNA-stability upon arginine methylation. Also, on the contrary to cytosolic localization, mitochondrial RBP16 is a stable protein in both life cycle stages.

Finally, we showed that increased methylation of the RBP16 trans regulator might result in a positive effect of mRNA stability in the stationary phase. Also, we concluded that although *Lmj*RBP16 is a substrate for PTRMT7 methyltransferase additionally PRMTs might act upon it and control protein's regulation in the post-transcriptional level.

Notably, the experiments of this project will be included in two publications of which the one has been already submitted and corresponds to (Ferreira et al., 2020).

6. Appendices

6.1 Solutions made in the laboratory

<table>
<tr><td>M199 5x
pH 7,4</td><td>5x M199 powder Gibco/Invitrogen
MiliQH2O
NaHco3 (Sodium biocarbonate)</td><td>27,45g
500ml
0,825gr</td></tr>
<tr><td>M199 1x</td><td>Hemin
Adenine
Hepes 1M
Pen/Strep
FBS
H_2O</td><td>10ml
20ml
1ml
5ml
50ml
500ml</td></tr>
<tr><td>Resolving 15%</td><td>H_2O
Acrylamide 30%
Tris (1M. pH 6,8)
10% SDS
Ammonium persulfate (APS) 10%
TEMED</td><td>6,8 ml
1,66 ml
1,26 ml
0,1 ml
0,1 ml
0,001 ml</td></tr>
<tr><td>Stacking gel</td><td>H_2O
Acrylamide 30%
Tris (1M. pH 6,8)
10% SDS
Ammonium persulfate (APS) 10%
TEMED</td><td>6,8 ml
1,66 ml
1,26 ml
0,1 ml
0,1 ml
0,001 ml</td></tr>
<tr><td>TBS 10x</td><td colspan="2">1L ddH_2O
12,2 gr Tris HCL
87,65 gr NaCl
pH 8,0</td></tr>
<tr><td>TBS-t 1x</td><td colspan="2">900 ml ddH_2O
100ml 10x TBS
1ml Tween 20</td></tr>
<tr><td>Lysis buffer</td><td colspan="2">10mM Tris HCl, pH 8.0
0.05mM EDTA
150mM NaCl
<table><tr><td>Complete with</td><td>1 tablet Roche EDTA-free Protease inhib. cocktail/≤15ml</td></tr><tr><td>For RNA IP</td><td>1mM DTT
100U/mL RNAsein
20U/mL RNAse-free DNAse</td></tr></table></td></tr>
</table>

Table 5. Solutions

6.2 Databases and links

https://tritrypdb.org/tritrypdb/

https://www.ebi.ac.uk/Tools/msa/clustalo/

https://gimp.en.uptodown.com/windows

https://inkscape.org/release/inkscape-0.92.4/

https://www.snapgene.com/

https://www.scopus.com/home.uri

http://tcoffee.crg.cat/apps/tcoffee/do:regular

https://embnet.vital-it.ch/software/BOX_form.html

https://web.archive.org/web/20181019040517/http://www.who.int/leishmaniasis/burden/en/

List of abbreviations

AmB: amphotericin B

AREs: AU rich elements

AMA: amastigotes

AMA (LD): amastigotes in vivo lesion

AMA (MØ): amastigotes 24-hour post-infection

aDMA: ω-NG, NG-asymmetrically dimethylated

A6: ATPase subunit 6

AdoMet: S-adenosyl-L-methionine

BSA: Bovine serum albumin

CL: cutaneous leishmaniasis

CBC: cap-binding complex

CSD: cold shock domain

CTD: C-terminal domain

COI: the cytochrome oxidase subunit I

CYb: the apocytochrome b

CNV: copy number variation

DCL: diffuse cutaneous leishmaniasis

DAT: direct agglutination test

DET: differentially expressed transcripts

DMEN: Dulbecco's Modified Eagle Medium

DPE: downstream promoter element

DR: drug resistance

DE: drug efficacy

ELISA: enzyme-linked immunosorbent assay

EJC: exon junction complex

FBS: Fatal bovine serum

gRNA: trans-acting guide RNAs

Hs: *Homo sapiens*

IFA: indirect fluorescent antibody

IP: immunoprecipitation

KH: K-homology domain

kRNA: kinetoplastid RNA

LAT: latex agglutination test

LST: *Leishmania* intradermal skin test

LCL: localized cutaneous leishmaniasis

Lmj: *Leishmania major*

MCL: mucocutaneous leishmaniasis

MMA: ω-NG, monomethylated

MLS: Miltefosine Sensitivity Locus

mRNP: messenger ribonucleoprotein particle'

mRBPome: mRNA-bound proteome

MST: Montenegro skin test

mRNA: messenger RNA

META: metacyclic promastigotes

Mb: megabases

ND4: NADH dehydrogenase subunit 4

NEO: neomycin phontransferase

nonXL: non-cross-linked

nt: nucleotide

OXPHOS: oxidative phosphorylation .66

ORF: open reading frames

PIC: preinitiation complex

pol: RNA polymerase

pre-mRNA: precursor RNA

PABP: poly (A) binding protein

PUM: pumilio

PCF: procyclic promastigotes

PKDL: Post kala-azar leishmaniasis

PM: peritrophic matrix

PSG: promastigote secretory gel

PCR: polymerase Chain Reaction

PGCs: polycistronic gene clusters

PTMs: post-translational modifications

PTU: polycistronic transcript unit

PRTMs: protein arginine methyltransferases

PBS: Phosphate buffered saline

PFA: paraformaldehyde

rRNA: ribosomal RNA

RBPs: RNA-binding proteins

RBDs: RNA-binding domains

RRMs: RNA recognition motifs

RNAi: RNA interference

RBP16: RNA binding protein 16

snRNA: small nuclear RNAs

SL RNA: small leader RNA

snRNP: small nuclear ribonucleoproteins

sDMA: ω-NG, NG-symmetrically dimethylated

Sb: pentavalent antimony

TSS: transcription start site

Tb: *Trypanosoma brucei*

TUTase: uridylyl transferase

T-BST: tris-buffered saline with tween

TBS: Tris-buffered saline

TNF mRNA: Tumor Necrosis factor mRNA

TSS: transcription start sites

tRNA: transfer RNA

TF: therapeutic failure

3’ UTR: 3’ untranslated regions

UREs: U-rich elements

VSG: variant surface glycoproteins

VL: visceral leishmaniasis

WHO: World Health Organisation?

XL: UV cross-linked

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