Study of a *Leishmania infantum* zinc transporter – what is its role in zinc homeostasis?

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Abstract

Nutrients are essential for survival and infectivity of any pathogen. *Leishmania*, intracellular parasites responsible for a group of neglected diseases denominated leishmaniasis, are no exception. However, in order to survive and succeed in the infection process, *Leishmania* need not only to acquire zinc but also to maintain its homeostasis. The Molecular Parasitology Group at IBMC began to study the mechanisms used by these protozoans in the acquisition of zinc and described the first zinc transporter in *Leishmania*: *Li*ZIP3. This protein mediates internalization of the metal from the extracellular milieu into the cell and is highly regulated by zinc availability as its expression is reduced upon zinc supplementation and increased upon zinc deprivation. More recently, the Group started to study a metal transporter, *Li*CDF, present at the membrane of acidocalcisomes (cation- and phosphate-storage organelles). The hypothesis is that *Li*CDF transports excessive cytosolic zinc into acidocalcisomes, a process that may be co-regulated with the expression of *Li*ZIP3. Thus, the present study intends to unravel the relation between *Li*CDF and *Li*ZIP3, two proteins that may be players of the same pathway aiming at maintaining zinc homeostasis.

This study made use of two lines of parasites episomically expressing either *Li*ZIP3 (pXG_*Li*ZIP3) or 9E10_*Li*CDF (pTEX_9E10_CDF, an N-terminal 9E10-tagged version of *Li*CDF). These parasites were analysed, by western blotting, for the effect of the overexpression of one of the transporters on the expression of the other. We noticed that *Li*ZIP3 expression is higher in parasites overexpressing *Li*CDF. However, the effect of zinc, addition or removal, or of *Li*ZIP3 overexpression on *Li*CDF is still unclear. Given the difficulties in detecting the wild type *Li*CDF with the mono- and polyclonal antibodies developed in this work, parasites expressing 9E10_*Li*CDF flanked by the gene's own 3' UTR were produced, allowing the study of endogenous *Li*CDF with a commercial antibody. We subjected this line of parasites to both zinc supplemented or depleted media and, from preliminary results, we hypothesize that *Li*CDF regulation is zinc-dependent, and that its expressional behaviour is closely related to that of *Li*ZIP3, both orchestrating a fine-tuned response to maintain zinc balance in the cytosol.

Keywords: *Leishmania*, zinc transporter, zinc homeostasis, zinc-dependent regulation,

acidocalcisomes, Cation Diffusion Facilitator family.

Resumo

Os nutrientes são essenciais para a sobrevivência e infectividade de qualquer patogéneo. *Leishmania*, parasitas intracelulares responsáveis por um grupo de doenças negligenciadas denominadas de leishmaniose, não são excepção. Contudo, para sobreviver e para levar a cabo um processo de infecção eficaz, *Leishmania* precisa não só de adquirir zinco bem como de manter a sua homeostasia. O Grupo de Parasitologia Molecular do IBMC iniciou o estudo dos mecanismos utilizados por protozoários para a aquisição de zinco e descreveu o primeiro transportador de zinco em *Leishmania: Li*ZIP3 (Carvalho 2012). Esta proteína medeia a internalização do metal a partir do espaço extracelular para o interior da célula e é altamente regulada pela sua disponibilidade: a sua expressão é reduzida aquando da suplementação de zinco ao meio e é aumentada com a depleção do mesmo. Recentemente, o Grupo dedicou-se ao estudo de um transportador de metais, *Li*CDF, presente na membrana de acidocalcissomas (organelos de armazenamento de catiões e fosfatos). O Grupo sugeriu que *Li*CDF é responsável pelo transporte de zinco citosólico em excesso para o interior de acidocalcissomas. Este processo poderá ser co-regulado com a expressão de *Li*ZIP3. Assim, o presente trabalho pretende descortinar a relação entre *Li*CDF e *Li*ZIP3, duas proteínas que poderão ser peças fulcrais no processo de manutenção da homeostasia do zinco.

Este estudo fez uso de duas linhas de parasitas a expressar episomicamente *Li*ZIP3 (pXG_*Li*ZIP3) ou 9E10_*Li*CDF (pTEX_9E10_CDF, uma versão do *Li*CDF com um epítopo 9E10 no N-terminal). Estes parasitas foram analisados, por western blotting, de forma a verificar qual o efeito da sobre-expressão de um dos transportadores na expressão do outro. Observámos que a expressão do *Li*ZIP3 está aumentada nos parasitas que sobre-expressam o *Li*CDF. Contudo, o efeito do zinco, em excesso ou defeito, e da sobre-expressão do *Li*ZIP3 na expressão do *Li*CDF continua pouco clara. Devido à dificuldade em detectar o *Li*CDF em parasitas selvagens com anticorpos mono- e policlonais desenvolvidos durante este trabalho, foi necessário produzir parasitas a expressarem 9E10_*Li*CDF sob o controlo da respectiva 3' UTR, permitindo assim estudar a regulação endógena deste transportador com um anticorpo comercial. Submetemos esta nova linha de parasitas a meio suplementado ou depletado em zinco e, com base em resultados preliminares, inferimos que a regulação de *Li*CDF é

dependente de zinco e que a sua expressão está intricadamente relacionada com a do *Li*ZIP3, permitindo a orquestração de uma resposta para a manutenção do equilíbrio de zinco no citosol.

Palavras-chave: *Leishmania,* transportador de zinco, homeostasia de zinco, regulação dependente de zinco, acidocalcissomas, família de Facilitadores de Difusão Catiónica.

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List of Abbreviations

Bp – Base Pairs

BSA - Bovine Serum Albumin

CDF - Cation Diffusion Facilitator

CL - Cutaneous Leishmaniasis

CTD - C-Terminal Domain

DAT - Direct Agglutination Test

DCL - Diffuse Cutaneous Leishmaniasis

DDT - Dichlorodiphenyltrichloroethane

DMSO – Dimethyl Sulfoxide

DNA - Deoxyribonucleic acid

DTT - Dithiothreitol

E64 – trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane

EDTA - Ethylenediamine tetraacetic acid

ELISA - Enzyme-Linked Immuno Sorbent Assay

FBSi – heat-inactivated fetal bovine serum

G418 - Neomycin

GP63 - Glycoprotein of 63kDa

GPI - Glycosylphosphatidylinositol

HEPES - *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

HIV - Human Immunodeficiency Virus

IBs - Inclusion Bodies

IFAT - Indirect Fluorescent Antibody Test

IPTG - Isopropyl β-D-1-thiogalactopyranoside

IR – Intergenic region

kDa - kilo Dalton

LB – Lisogeny Broth

*Li*CDF - *Leishmania infantum* Cation Diffusion Facilitator

*Li*GAPDH - *Leishmania infantum* glyceraldehyde 3-phosphate dehydrogenase

*Li*ZIP3 - *Leishmania infantum* ZRT/IRT-like Protein 3

MCL - Muco-Cutaneous Leishmaniasis

NP-40 - Nonidet P-40

NTD - Neglected Tropical Disease

ORF - Open reading frame

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate-Buffered Saline

PCR - Polymerase Chain Reaction

PKDL - Post-Kala Azar Dermal Leishmaniasis

PMSF - phenylmethylsulfonyl fluoride

RNA - Ribonucleic acid

SDS - Sodium dodecyl sulfate

TB - Terrific Broth

TCA - Trichloroacetic Acid

TPEN – tetrakis-(2-Pyridylmethyl)ethylenediamine

Tris - tris(hydroxymethyl)aminomethane

UTRs - untranslated regions

VL - Visceral Leishmaniasis

WHO - World Health Organization

ZIP - ZRT/IRT-like-Proteins

Introduction

I) Leishmaniasis

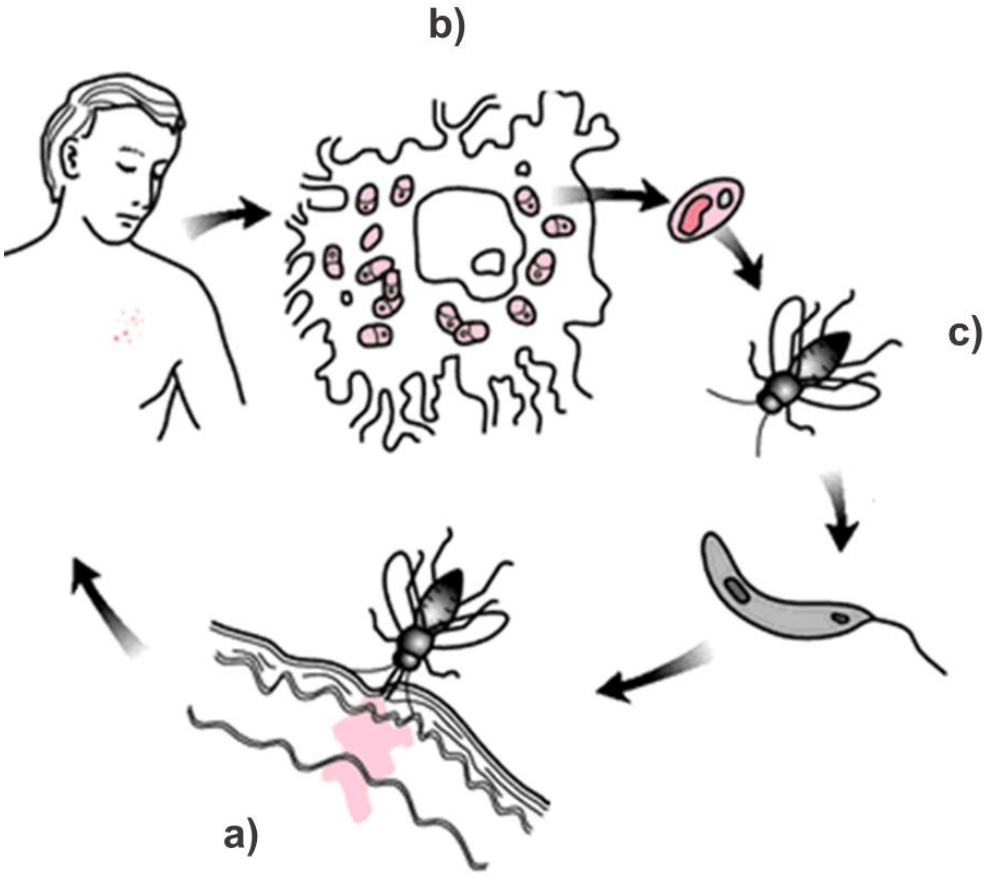
i) A neglected disease

Leishmaniasis is a disease caused by intracellular protozoan parasites of the Genus *Leishmania,* Family Trypanosomatidae, Order Kinetoplastida. So far, there are at least 20 different mammal-infecting species of *Leishmania*. The first records of leishmaniasis date back to 7th Century BC (Mason-Bahr 1996, reviewed in Cox 2002) but it was Alexander Russel who first made clinical records in 1756, naming it as Aleppo Boil. Nowadays, there are still many different designations to this same group of pathologies such as Dum-Dum Fever, Kala-Azar, white leprosy and espundia (Hide, Bucheton *et al.* 2006, reviewed in Dawit G. 2013). Leishmaniasis is considered a Neglected Tropical Disease (NTD) along with pathologies such as trypanosomiasis, filariasis, schistosomiasis, onchocerciasis, among others (Yamey and Torreele 2002). These diseases are mostly limited to the poor people in the rural areas of developing countries and are associated with low hygiene conditions, malnutrition and immunosuppression. NTDs affect more than 1 billion people worldwide and, although being the focus of high-quality research all over the world, little is actually translated in effective methods of control and eradication of such pathologies. A study conducted in 2002 revealed that of the 1393 new drugs marketed between 1975 and 1999, only 16 were aimed at neglected diseases (Trouiller, Olliaro *et al.* 2002), a gap that is justified by the expected low pharmaceutical companies' yields generated by the sales of NTD-related medicines (reviewed in Matlashewski, Arana *et al.* 2011).

ii) Life cycle of *Leishmania*

During their life cycle, *Leishmania* present two basic stages: the promastigote (extracellular, motile, elongated cell) in the invertebrate host and the amastigote (intracellular, non-motile, round-shaped cell) in the macrophages of vertebrates. Parasites are transmitted between hosts through the bite of infected female sandflies. During the sandfly blood meal, the infective stage of *Leishmania* (metacyclic promastigotes) is readily injected into the vertebrate host as the insect punctures the mammal. At the inoculation site, the transmitted metacyclic promastigotes are taken up by mono- and polymorphonuclear (neutrophils, eosinophils, basophils and mast cells)cells . Ultimately, they are phagocytized by macrophages and differentiate into the

amastigote stage in the phagolysossome. In these conditions, the parasite may multiply, setting the path for the infection of new macrophages. When another sandfly feeds on the infected mammalian host, it will acquire amastigotes and the cycle may begin again (Fig.1) (Bates 2007, Beattie and Kaye 2011, Kaye and Scott 2011).



Adapted from Mahon and Manuselis, 2000.

Figure 1 – *Leishmania* life cycle. a) During the sandfly bite, promastigotes are inoculated in the vertebrate host (in this case, human); b) promastigotes (motile forms) are phagocytised by macrophages and, inside these cells, they differenciate into amastigotes (non-motile forms) which survive and multiply; c) the life cycle finishes when a new sandly takes its bloodmeal from the infected vertebrate host, ingesting amastigotes**.**

iii) Clinical manifestations

Leishmaniasis presents itself in 3 distinct clinical syndromes: cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL, also known as espundia) and visceral leishmaniasis (VL, also known as Kala-Azar) (reviewed in Chappuis, Sundar *et al.* 2007). Cutaneous leishmaniasis is the most common form of the disease. Its

incubation time oscillates between two and eight weeks, although longer periods have been noted (Markle and Makhoul 2004). Despite presenting the same set of symptoms, CL can be caused by distinct species depending on the autochthonous variant of the disease. Old World CL is mainly originated by *Leishmania tropica* (urban areas) and *Leishmania major* (dry, desert areas), while the New World variant is caused by either *Leishmania leishmania (e.g.*, *Leishmania mexicana, Leishmania amazonensis, Leishmania chagasi*) *and Leishmania viannia* (*e.g.*, *Leishmania panamensis, Leishmania braziliensis, Leishmania guyanensis*) subgenera (reviewed in Markle and Makhoul 2004). Generally, CL physically translates into lesions, open or closed sores, at the site of the sandfly bite, usually arms, legs and exposed areas of the skin (David and Craft 2009, Maurer, Dondji *et al.* 2009) often causing deep plunging ulcers which heal in the course of months or years without any clinical treatment. Though painless, open lesions can be infected by bacteria originating painful sores. Occasionally, swollen glands (regional lymphadenopathy), satellites lesions and nodular lymphangitis can also be present. Moreover, when the treatment of a primary CL lesion is ineffective or in cases where the patient is immunosuppressed [such as human immunodeficiency virus (HIV)-infected patients], CL can evolve into a state of diffuse cutaneous leishmaniasis (DCL), in which lesions are present all over the patient's body. Due to its diffuse appearance and prevalence, DCL is commonly mistaken by leprosy (reviewed in David and Craft 2009). Some *Leishmania* species present in Central and South America are capable of spreading from the skin throughout the mouth and upper respiratory tract, where they infect macrophages from the naso-oropharyngeal mucosa. This process generally takes place years after the primary CL infection had been healed and also in cases where the treatment was inefficient or absent. This phenomenon originates muco-cutaneous leishmaniasis (MCL). In a more advanced state, MCL causes evident and disfigurating lesions from ulcerative destruction of the nose, mouth and pharynx. Opposing to CL, MCL does not heal spontaneously. This condition is found in New World areas where *L. braziliensis* and *L. panamensis* dominate as agents (reviewed in Chappuis, Sundar *et al.* 2007, McGwire and Satoskar 2013).

The most severe clinical display of *Leishmania* infection is visceral leishmaniasis (VL). Given the severity of the physical manifestation of this pathology, if untreated, it leads to the patient's death. After an initial incubation period of 2 to 6 months, the first signs of systemic infection start to arise in the form of fever, weight loss and extreme fatigue. VL clinical presentation is similar in the various endemic areas, excluding a few characteristics inherent to a certain geographical area or species. On a general note, parasites infect the reticulo-endothelial system and cause hepatomegaly, creating a

permanent inflammatory state, hyper-spleenism, as well as anemia, a condition commonly associated with this pathology (reviewed in Chappuis, Sundar *et al.* 2007). VL is caused by *Leishmania donovani*, predominant in East Africa and Indian subcontinent, and *L. infantum,* found in Europe, North Africa and Latin America (here known as *L. chagasi*). *L. donovani* life cycle is characterized by its anthroponotic profile, *i.e.* humans are the main reservoir of parasites, whereas *L. infantum* is defined as zoonotic, *i.e.* rodent animals and canids are the main reservoirs (reviewed in Chappuis, Sundar *et al.* 2007). In some cases, after VL treatment, post-kala azar dermal leishmaniasis (PKDL) occurs. This is characterized by macular, macular-papular or nodular rashes populated by parasites, thus, being a dangerous, highly infectious state contributing to the anthroponotic cycle (Addy and Nandy 1992). It affects mainly patients in Sudan and India and, generally, immunosuppressed individuals in *L. infantum*-endemic areas (Zijlstra, Musa *et al.* 2003).

iv) Epidemiology

Leishmaniasis is ranked by World Health Organization (WHO) as the ninth highest disease burden of all infectious diseases worldwide. More than 98 countries, mostly located in large areas of the tropics, subtropics and Mediterranean Basin, are endemic regions, putting at risk more than 350 million people. VL incidence is higher in 6 countries: Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil. Worldwide, it is estimated that 2 million new cases of leishmaniasis arise each year (0.5 million of VL and 1.5 million of CL) but these numbers may not be accurate as data are sparse in these developing countries (WHO, 2010). Despite being often associated with developing countries and poverty, other factors are currently contributing to the increase of VL numbers worldwide VL incidence such as climate changes and migration (Antinori, Schifanella *et al.* 2012) but also by lack of control measures and, mainly, HIV-VL co-infection (Desjeux 2001). HIV-VL co-infection has been reported in over 35 countries worldwide. As an immunossupressive disease, HIV leads to diminished cellular and humoral responses to leishmaniasis (Moreno, Canavate *et al.* 2000). This immunological vulnerability also constrains chemotherapy applied to VL patients, thus, contributing to the spreading of the disease (Ritmeijer, Dejenie *et al.* 2006).

v) Diagnosis

Confirmation of a clinical diagnosis of leishmaniasis relies on the microscopical detection of amastigotes, through Giemsa staining of the smear or touch preparation, in tissues such as liver, spleen, skin lesions, bone marrow or lymph nodes (Singh 2006). Confirmation can also be obtained by culturing *Leishmania,* although each species require different culture media, or by inoculation in small animals or rodents (Dawit G. 2013). Nevertheless, these methods require biopsy of the tissues (which may be painful), trained personnel and a microscope as well as expensive surgical material (reviewed in Dawit G. 2013). Serological tests are also a valuable tool for diagnosis and they present some advantages as they do not require invasive procedures (such as biopsy) and allow a very early diagnosis since they detect antibodies present in the blood. Enzyme-Linked Immuno Sorbent Assay (ELISA) is one of the most sensitive tests available. It allows testing a large number of samples at the same time and it can be performed either in laboratories or in the field (Martin-Sanchez, Lopez-Lopez *et al.* 2001). Indirect Fluorescent Antibody Test (IFAT) and Direct Agglutination Test (DAT), both based on the recognition of immobilized parasites by antibodies present in the patient's blood can also be used. Indeed, DAT is cheap and easy to perform, presenting high sensitivity (Dawit G. 2013). These serological tests are becoming a staple for diagnosing leishmaniasis, however, these are only suitable for VL and not for the CL variant as the latter induces a cellular response instead of a humoral one. One of the disadvantages of these serological tests is associated to immunosuppressed individuals: their sera will yield false-negative results. On the other hand, as the serum antibodies prevail in the body after successful treatment, false-positives may also be obtained (Silva, Romero *et al.* 2011). Polymerase Chain Reaction (PCR) has been put to use in leishmaniasis diagnosis. This technique proves to be rapid and highly sensitive, allowing diagnosis in cases with low parasite numbers, however, it demands trained personnel and costly equipment (reviewed in Dawit G. 2013).

Although so many diagnosis techniques are available, misdiagnosis is still a very common phenomenon. CL is commonly mistaken for over-infected mosquito bites, tropical, traumatic and venous stasis ulcers or foreign-body reactions. PKDL is also wrongly diagnosed as shypillis or leprosy. VL shares symptoms (fever or organomegaly) with a number of conditions, making it difficult to perform an accurate diagnosis (reviewed in Dawit G. 2013).

vi) Prevention and treatment

Prevention measures such as large-scale spraying programs and insecticide-impregnated household items (from soap to bednets) are currently in use. However these actions are creating cases of sandfly resistance to insecticides, especially to DDT (dichlorodiphenyltrichloroethane) (Sharma and Singh 2008). Given that canids, especially dogs, are relevant to zoonotic VL, the control of these reservoirs is primordial. This can be achieved by culling of infected dogs, which is nowadays ethically questionable, and by treatment of infected animals. Nevertheless, the latter is not effective as relapses are common (Alvar, Molina *et al.* 1994). A close surveillance of endemic areas and active case detection, both in humans and dogs, followed by treatment, is a valuable, and more acceptable, strategy for controlling the dissemination of leishmaniasis (reviewed in Dawit G. 2013).

In what concerns to treatment, namely chemotherapy, few options are available: pentavalent antimonials, amphotericin B, paromycin, pentamidine, miltefosine, imiquimod and azoles. Since the 1940's, pentavalent antimonials (stibogluconate and meglumine antimoniate) are the preferred treatment for leishmaniasis (humans and animals). Their exact mechanism of action is still unclear but it probably consists in an amalgam of factors related to both the macrophage action and to its influence on molecular processes of the parasite (Baiocco, Colotti *et al.* 2009). Frequent usage of antimonial therapies causes severe side effects such as cardiotoxicity, electrolyte abnormalities, pancytopenia and elevation of liver and pancreatic enzymes, thus requiring close monitoring of patients (McGwire and Satoskar 2013). Amphotericin B works by binding to *Leishmania*'s membrane ergosterol, destabilizing the membrane. A liposomal version of the drug is already available under the name AmBisome and, despite causing less side effects, its cost is highly prohibitive (Barratt and Legrand 2005). Concerning to paromycin and pentamidine, they interfere with biosynthesis at different levels, deeply affecting *Leishmania*'s anabolism: paromycin works by blocking protein synthesis by binding to 16S ribosomal RNA (ribonucleic acid) while pentamidine interferes with biosynthesis of macromolecules such as RNA, DNA (deoxyribonucleic acid), phospholipids and proteins. Miltefosine, originally intended as an anti-neoplastic drug, is highly administrated to *Leishmania*-infected patients and, currently, it is the only one in use in India and East Africa against VL. However, given its teratogenic effects, it is not indicated for pregnant women. Other drugs such as imiquimod and azoles as well as heat therapy (heating lesions up to 50ºC) and cryotherapy (applying liquid nitrogen

directly onto the lesion), combined or not, are also used to treat leishmaniasis (reviewed in McGwire and Satoskar 2013).

II) *Leishmania*

i) Morphology

Leishmania parasites are unicellular eukaryotic organisms. They share a number of morphologic characteristics with trypanosomatids, the causing agents of diseases such as Sleeping Sickness and Chagas disease (*Trypanosoma brucei* and *Trypanosoma cruzi*, respectively). Noteworthy characteristics of these parasites include: the single mitochondrion that extends between the two ends of the cells (de Souza, Attias *et al.* 2009); the kinetoplast, a vacuole-like space filled with electron dense material, containing the mitochondrial DNA organized in mini- and maxi-circles, which is responsible for encoding guide RNAs; glycosomes (equivalent to the peroxisomes found in mammallian cells) containing H_2O_2 producing oxidases; the flagellar pocket, which is an invagination of the plasma membrane, absent of microtubules, and that is responsible for receptor-mediated endocytosis of large molecules; the flagellum, sprouting from the flagellar pocket, that is crucial for the organism's motility and is present in the promastigote stage; a set of very large lysosomal compartments, called megasomes, that are acidic, electron dense, membrane-bound structures containing cysteine proteinase and acid phosphatase (reviewed in de Souza 2002); and the acidocalcisomes, vacuolar-like structures relevant to the maintenance of homeostasis in the cell (Rohloff, Montalvetti *et al.* 2004) and that are rich in orthophosphate (Pi), PPi and poly P complexed with different molecules such as basic amino acids, and cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+} and Fe^{3+}) (reviewed in Docampo, de Souza *et al.* 2005).

ii) Nutritional Demands

Nutrition is of uttermost importance for pathogens. In order to thrive and cause an effective infection, parasites scavenge and internalize nutrients obtained from the host. They should also be capable of metabolizing drugs and other agents to escape toxicity, as well as being able to cope and to adapt to environmental changes occurring during transition between hosts. All living organisms display different ways of obtaining

nutrients: they can either be substrate-unspecific or –specific. In the latter, nutrient trafficking (either to internalize or excrete molecules) requires particular receptors and transporters. These proteins display high specificity towards target molecules and their expression may differ between environments, allowing the pathogen to fully adapt to new conditions and efficiently infect the host (reviewed in Landfear 2011). The term "nutrient" covers a broad range of distinct groups of molecules including metals. These, in particular transition metals, are a class of nutrients of great importance to living organisms. Their functional roles in biological systems can be loosely classified into non-catalytic, and redox and non-redox catalytic functions. Moreover, they are widely recognized as cofactors for a number of enzymes. The importance of transition metals, such as Fe, Mn and Zn, is so evident that, in order to prevent infection by pathogenic organisms, hosts are capable of both actively constrain the pathogen's access to these elements or forcibly expose it to them, thus influencing the progression of infection. These strategies are named nutritional immunity (reviewed in Hood and Skaar 2012). Intentional metal deprivation is a valuable defense strategy: calprotectin is an effective anti-bacterial agent as it chelates Mn^{2+}, inhibiting bacterial defenses against damaging superoxides (Kehl-Fie, Chitayat *et al.* 2011). On the other hand, exposure of the pathogen to damaging concentration of metals occurs, for example, in the events following the engulfment of either *Mycobacterium tuberculosis* or *Escherichia coli,* when macrophages release Zn^{2+} from their intracellullar stores to the phagolysosome, where the pathogen perseveres (Botella, Peyron *et al.* 2011). The survival of the parasite depends on the expression of an efflux system capable of excreting Zn^{2+} accumulating in its cytosol, ensuring the maintenance of physiologically safe levels of zinc (Botella, Peyron *et al.* 2011).

III) Zinc Homeostasis

The aforementioned phenomenon illustrates the relevance of zinc in pathogenesis. This ion is the second most abundant transition metal in most living systems and it plays a significant role in both structure and catalysis in proteins (Hantke 2005). Concerning *Leishmania*, the acquisition and control of zinc concentration are quintessential for its survival and growth. For instance, GP63 (Glycoprotein of 63kDa) is a perfect example for the importance of zinc in *Leishmania*. This protein is a zinc metalloprotease, attached to the parasite's cell membrane by a GPI

(Glycosylphosphatidylinositol) anchor and it is a well-known virulence factor (reviewed in Olivier, Atayde *et al.* 2012). Studies in *L. amazonensis* and *L. major* promastigotes show that GP63 cleaves C3b, allowing the parasite to dodge the host's complement-mediated lysis (Brittingham, Morrison *et al.* 1995). It can also protect the parasite against anti-microbial peptides (Hallé, Gomez *et al.* 2009) and interact with fibronectin receptors on macrophages, allowing the parasite to adhere onto its hosts cells (Brittingham, Chen *et al.* 1999). In amastigotes, GP63 has a protective role against the harsh environment parasites face inside the macrophage. It does so by modifying several intracellular pathways such as crucial signal transduction cascades in the macrophage (reviewed in Olivier, Atayde *et al.* 2012).

Zinc, although not being redox-active under physiological conditions, when in excess, can exert toxic effects both on the host and the parasite (Koh, Suh *et al.* 1996). Therefore, zinc must be supplied in such a way that nutritional demands are met and toxicity avoided. Proteins from the ZIP (ZRT/IRT-like Proteins) (Zhao and Eide 1996, Grotz, Fox *et al.* 1998, Eide 2004) and CDF (Cation Diffusion Facilitator) (Nies and Silver 1995, Palmiter and Findley 1995, Palmiter and Huang 2004) families are partially responsible for maintaining the desirable balance.

Cation Diffusion Facilitator (CDF) proteins are responsible either for exporting zinc out of the cell or internalizing it into organelles; on the other hand, ZIP proteins mediate the influx of zinc from the extracellular space or the efflux from organelles, increasing the cytosolic concentration of the ion (reviewed in Kambe, Suzuki *et al.* 2006).

i) ZIP family

The first two identified members of this family were Zrt1 of *Saccharomyces cerevisiae* and Irt1 of *Arabidopsis thaliana* (reviewed in Gaither and Eide 2001). These transporters are predicted to have 8 predicted TMDs (Transmembrane Domains) with both the amino (N-) and carboxyl (C-) terminals facing the extracellular milieu or the lumen of the organelles. Another prominent characteristic is the long loop present between TMD III and IV, which is a highly variable region among ZIP members harboring a histidine-rich cluster (Guerinot 2000). These histidine residues are thought to be a metal binding domain essential to the role of ZIP proteins (Gaither and Eide 2001, Milon, Wu *et al.* 2006). As for TMD IV and V, they are amphipathic and it is proposed that together form a cavity through which divalent metals are transported (Eide 2006).

From previous work developed at this same laboratory, a zinc ZIP transporter denominated ZIP3 was identified in *L. infantum.* The expression levels of this transporter

are closely dependent on zinc status: an evident upregulation of *Li*ZIP3 expression occurs when zinc levels are reduced in the medium and a downregulation whenever the metal becomes available. This zinc-mediated downregulation was found to require a short-lived mRNA-destabilizing protein and to be dependent on the 3' untranslated regions (3' UTR) of the *Li*ZIP3 mRNA (Carvalho 2012). Nevertheless, the mechanism through which this regulation occurs is yet to be elucidated.

ii) CDF Family

This Family of transporters was first described in 1995 by Nies and Silver as a novel family of heavy metal transporters, which they baptized as CDF (Cation Diffusion Facilitators) Family, present in both prokaryotes and eukaryotes (Nies and Silver 1995). In 1997, Paulsen and Saier, based on thirteen sequenced proteins belonging to this family, predicted the topology of the transporter: six putative transmembrane spanners with higher levels of conservation on the four N-terminal TMDs (Paulsen and Saier 1997). CDF proteins transport transition metals such as Cd, Co, Fe, Mn, Ni and Zn, using the proton motive force (reviewed in Zeytuni, Uebe *et al.* 2014) and are sub-divided into three groups according to the metals they transport: Zn, Fe/Zn and Mn (Montanini, Blaudez *et al.* 2007). Structurally, CDF transporters are either homo-oligomeric complexes (Bloss, Clemens *et al.* 2002, Blaudez, Kohler *et al.* 2003) or hetero-oligomeric (Ellis, Macdiarmid *et al.* 2005, Suzuki, Ishihara *et al.* 2005). There is already some evidence that these transporters can interact with other proteins either to regulate their activity (Jirakulaporn and Muslin 2004) or to release the metal (Murgia, Vespignani *et al.* 1999). Some regions are highly conserved throughout the whole family, especially the amphipathic TMDs I, II, V and VI, which are most likely involved in metal transport (Haney, Grass *et al.* 2005). Just like the ZIP family, most CDF transporters contain a histidine-rich region which is thought to the responsible for metal binding (Suzuki, Ishihara *et al.* 2005, Zeytuni, Uebe et al. 2014). This region can be found either between TMD IV and V or at the N- and/or C- termini (Montanini, Blaudez et al. 2007). All CDF transporters are dimers in which the C-terminal domains (CTDs) are projected into the cytoplasm (Paulsen and Saier 1997). CTDs structures present a fold similar to metallochaperone (Zeytuni, Uebe et al. 2014). Each CTD has a distinctive cation efflux domain present in most members of the Metallochaperone Family, a well-described group of proteins responsible for delivering metals across the cytoplasm to target proteins such as metal transporting P-type ATPases (O'Halloran and Culotta 2000). Futhermore, insight on coordination of zinc with CDF transporters has been given in the context of the YiiP protein (found in *E. coli*): the

binding-sites are located on different faces of metallochaperone-like domain, allowing both transport regulation and metal trafficking in the same module. An auto-regulatory mechanism has been proposed in which zinc cations, when in higher concentrations, bind and stabilize the formation of the active dimer (Lu and Fu 2007, Lu, Chai *et al.* 2009).

Aims of the work

Aims of the work

Maintenance of zinc homeostasis is of utmost importance for *Leishmania.* Previous work performed at the Molecular Parasitology Group at IBMC depicted the contribution of the *L. infantum* ZIP3 membrane-bound transporter for zinc acquisition and its zinc-dependent regulation. In a membrane proteome analysis, aiming at identifying other metal transporters, a protein annotated in the *L. infantum* genome as member of the CDF family was selected for further studies. This protein, which was denominated *Li*CDF, shares 53% identity with an acidocalcisomal CDF member identified in *T. cruzi* (Ferella, Nilsson *et al.* 2008), and, according to TriTrypDB, these genes are syntenic. From a Real Time Reverse Transcription-PCR (qRT-PCR) analysis from parasites grown in normal or zinc-supplemented medium, it was observed that zinc causes an increase in *LiCDF* mRNA level. This is the opposite of what is seen for *LiZIP3* mRNA. Therefore, the aim of my work was to shed light on the crosstalk existing between *Li*ZIP3 and *Li*CDF, which, we hypothesize, play a role on the homeostasis of zinc in *Leishmania*. More specifically, my objective was to characterize *Li*CDF function and study its regulation by zinc.

Materials and methods

Materials and methods

Reagents

When not indicated, enzymes were purchased from Fermentas – Thermo Fischer Scientific and Klenow large fragment was used to produce blunt ends.

Culture of promastigotes

Promastigotes of *L. infantum* (strain MHOM/MA/67/ITMAP-263) were cultured at 25˚C in RPMI complete medium, *i.e.* RPMI 1640 GlutaMAX^{TM}-I medium supplemented with 10% heat-inactivated fetal bovine serum (FBSi), 50 U/mL penicillin, 50 µg/mL streptomycin (all from Gibco) and 20 mM HEPES (Sigma) pH 7.4. For each assay, parasites were first synchronized through 4-5 daily passages at 1x10^6/mL and, at the beginning of each experiment, promastigotes were seeded at 1x10^6/mL. Parasite numbers were evaluated by cell counting in a hematocytometer.

Production of anti-Δ*Li*CDF polyclonal and monoclonal antibodies

To produce anti-*Li*CDF mono- and polyclonal antibodies, the *LiCDF* gene (451 amino acids) was PCR-amplified from wild type *L. infantum* genomic DNA using Taq polymerase together with sense primer 5'- ccgcgcacatATGTCACCTGAGCAACCGAG-3' (with *Nde*I restriction site underlined and clamp sequence in lower case) and antisense primer 5'- caccgctcgagTCACATGTAGTGGCTCAAAATC-3' (with *Xho*I restriction site underlined and clamp sequence in lower case). The resulting sequence was then inserted into the *Nde*I and *Xho*I restriction sites of the expression vector pET28a (+) His$_6$-tag (Novagen) in frame with an N-terminal His$_6$-tag to produce pET28a (+) His$_6$_*Li*CDF (Fig. S1). For the generation of pET28a (+)_His$_6$-tag_Δ*Li*CDF vector (Fig. S2), the aforementioned plasmid containing the entire *Li*CDF gene was digested with *Sal*I and

*Nde*I restriction enzymes and ligated. The correct amplification of DNA fragments was verified by sequencing.

The pET28a (+)_His_6-tag_Δ*Li*CDF vector was introduced into BL21(+) *E. coli* cells so that the Δ*Li*CDF (115 aa) with an N-terminal tag composed of 6 histidine residues could be produced. Protein expression was induced with 0,2mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 26ºC for 6h. The culture was pelleted, resuspended in 500mM NaCl and 20mM Tris (Buffer A), pH 7.6 and lysed by sonication. The soluble fraction was then applied to a HiTrap™ Chelating HP (Amersham Biosciences) column and eluted with an imidazole gradient (5 to 500 mM in Buffer A) at a flow rate of 2 ml/min. Collected fractions were then submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to assess for the presence of the 12 kDa Δ*Li*CDF peptide. Finally, fractions containing Δ*Li*CDF were pooled, purified from an acrylamide gel. The gel fragments containing the Δ*Li*CDF were mechanically macerated and the peptide was eluted with 5Mm DTT and 0,02%SDS, overnight, followed by liofilization and acetone precipitation. It was then solubilized in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4 (PBS). Polyclonal and monoclonal antibodies were produced following standard procedures (Hendriksen and de Leeuw 1998, Cooper and Paterson 2001).

Construction of plasmids for parasite transfection

To construct pTEX_9E10_*Li*CDF (Fig. S3), the *LiCDF* gene was removed from the previous pET28a (+)_His_6-tag_*Li*CDF by restriction with *Nde*I, blunt ended with Klenow, followed by digestion with *Xho*I. Simultaneously, an expression vector with a previously cloned *L. infantum* gene, pTEX_9E10_*Li*ZIP2, was digested with *Hind*III, blunt ended with Klenow, and further digested with *Xho*I (this allowed removal of *LiZIP2* gene). Finally, the *LiCDF* gene was introduced in the pTEX_9E10 plasmid, and the final construct pTEX_9E10_*Li*CDF was obtained. Another construct was assembled to express *Li*CDF and simultaneously mimic its endogenous regulation. To achieve this, the 3' UTR and the downstream intergenic region (IR) of the gene were introduced into the pTEX plasmid containing the *Li*CDF open reading frame with the 9E10 epitope at its N-terminus. Enzymes *Xho*I and *Sal*I were used to remove a fragment of 284bp from the 3' end of the gene in the pTEX_9E10_*Li*CDF. Simultaneously, pGEMT-easy_5'UTR_9E10_*Li*CDF_3'UTR, a previously developed plasmid containing both 5' and 3' UTRs as well as the downstream IR, was digested with *EcoR*I, then blunt-ended

with Klenow and digested with *Sal*I to obtain the final portion of the gene and the 3'UTR and IR (Δ*Li*CDF_3'UTR). The fragment was then introduced in the digested pTEX_9E10_*Li*CDF vector to produce the pTEX_9E10_*Li*CDF_3'UTR construct (Fig. S4). PCR amplified fragments were analysed by DNA sequencing.

Transfection of *L. infantum* parasites

Promastigotes in the logarithmic phase of growth were electroporated using Bio-Rad Gene Pulser as described elsewhere (Beverley and Clayton 1993). Briefly, logharitmic promastigotes were washed and diluted in transfection buffer with 3-15 µg of plasmid DNA (*pTEX_9E10_LiCDF*, *pTEX_LiCDF_9E10*, *pTEX_9E10_LiCDF_3'UTR*) and electroporated at 450V and 350-400µF. Cultures were plated in agar containing 15µg/mL G418 and isolated colonies were further cultured in liquid medium with 15 to 150µg/mL of G418. Southern blotting (following standard procedures) or plasmid rescue were performed to verify the correct transfection of parasites.

Plasmid rescue

To recover the plasmid from transfected parasites, these were subjected to DNA extraction as described elsewhere (Teixeira, Russell *et al.* 1994). Purified DNA was quantified using Nanodrop 1000 Spectrophotometer and digested with *EcoR*I overnight at 37ºC. DNA was then precipitated and ligated with T4 DNA Ligase overnight, at 4ºC. Ligation product was introduced into DH5-α cells and these were plated on agar, with ampicillin. PCR-positive clones were selected and, upon DNA extraction, plasmids were digested with *Spe*I and *EcoR*I/*Sal*I.

Zinc measurement

To analyse zinc levels in parasites, cultures were seeded at $1x10^6$/ml in RPMI complete medium for 72h, after which 50µM Zn were added for 1.5h. Parasites were then collected, washed twice in cold PBS, dissolved in 65% nitric acid and incubated for 2h at 65ºC. Concentration of zinc was determined by atomic absorption spectrometry (flame atomization) using the Atomic Absorption Spectrometer PU 9200X (Philips).

Indirect immunofluorescence assays (IFATs)

For localization assays using immunofluorescence techniques, parasites were harvested, washed twice in cold PBS, fixed in 4% paraformaldehyde (w/v) and placed onto poly-lysine slides. Cells were left to dry, rehydrated with PBS and further incubated with 0.1 M glycine for 15 minutes. Parasites were then permeabilized with 0.1% Triton X-100 in 0.05 M glycine and, incubated with a blocking solution containing 1% bovine serum albumin (BSA, Sigma) in PBS. Probing with antibodies was performed in a humid chamber at room temperature for 1 hour. Following primary antibody, samples were incubated with Alexa Fluor® 488 or 568-conjugated (Molecular Probes) for 1 hour at room temperature. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) at 5 µg/mL concentration. For co-localization assays, acidocalcisomes were detected using anti-*Tb*VP1. Promastigotes were visualized in a confocal inverted microscope Leica DMI6000-CS (Leica Microsystems) with a 63x glicerol-immersion objective. Images were acquired using the LAS 2.6 (Leica Microsystems) software.

Western blotting

Parasites were counted in a Newbauer chamber, harvested and then centrifuged at 3000 *xg* for 10 minutes at 4ºC. Following two washes with ice-cold PBS, pellets were ressuspended at $5x10^6$/mL or $7.5x10^6$/mL in 4% SDS, 50 mM Tris-HCl pH 7.4 containing protease inhibitors E-64 (0,01µg/mL), Pepstatin (0,02µg/mL), EDTA (0,5µg/mL), and PMSF (0,5µg/mL). Extracts were sonicated and stored at -80ºC until further use. Prior to western blotting, samples were prepared in Laemli sample buffer with 4 M Urea and 1% β-mercaptoethanol to ensure protein denaturation. Samples were then resolved on 12% SDS-PAGE, without prior boiling, and transferred onto nitrocellulose Hybond-C Extra membranes (Amersham Biosciences), these were incubated in a blocking solution of 5% skim milk and probed with the primary antibodies. Detection was performed with the BIO-RAD Clarity™ chemiluminescence kit (BioRad Laboratories).

Results

Results

*1. Li*CDF bioinformatics analysis

*Li*CDF [*LinJ.31.2470*, TriTrypDB, http://tritrypdb.org/ (Aslett, Aurrecoechea *et al.* 2010)] codifies a 451 amino-acids protein, predicted to weight 48KDa. According to PFAM database [www.pfam.sanger.ac.uk (Finn, Bateman *et al.* 2014)], *Li*CDF belongs to the Cation Efflux Superfamily. Regarding its topology, according to different prediction software, *Li*CDF contain 5-6 TMDs: TMHMM [http://www.cbs.dtu.dk/services/TMHMM/ (Krogh, Larsson *et al.* 2001)] indicates that *Li*CDF contains 5 TMDs while PHOBIUS [http://phobius.sbc.su.se/ (Kall, Krogh *et al.* 2004)] predicts 6 TMDs. Aiming to disclose more information on its three-dimensional structure, we resorted to SwissModel [http://swissmodel.expasy.org/ (Biasini, Bienert *et al.* 2014)]. *Li*CDF tertiary structure prediction was not very conclusive as the analysis retrieved only 3 hits, all below 20% identity percentage, thus, with poor homology to the *Li*CDF sequence. The best hit was the YiiP Zinc Transporter monomer, which belongs to the Cation Diffusion Facilitator (CDF) family of proteins.

CDF transporters have been characterized as containing a histidine-rich loop between TMD IV and V or at the N- and/or C-termini and a particular signature sequence (see below, Montanini, Blaudez et al. 2007). To evaluate if *Li*CDF possessed these characteristics, we elaborated a 2-dimensional representative diagram of the protein using Protter [http://wlab.ethz.ch/protter/start/ (Omasits, H. Ahrens *et al.* 2013)]. It can be observed in Figure 2 that *Li*CDF possesses a histidine-rich loop between TMDs IV and V, as well as a series of zinc-binding residues as described in Huang and Tepaamorndech (2013) and the signature sequence proposed by Montanini, Blaudez *et al.* (2007).

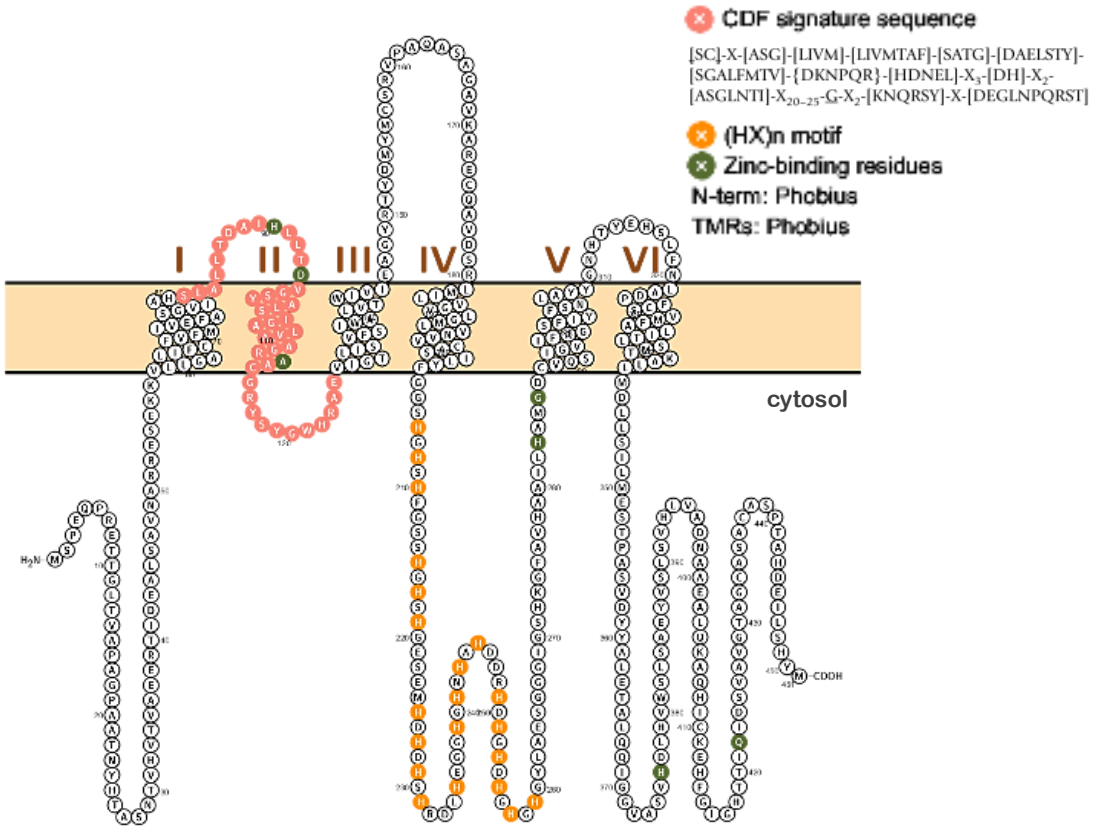


Figure 2 - Representative 2-dimensional diagram of *Li*CDF. *Li*CDF FASTA sequence was introduced in Protter server and a 2-D model was obtained. Accordingly to the caption on the superior right corner of the figure: pink round-shaped residues correspond to the CDF signature sequence as predicted by Montanini, Blaudez et al. (2007); orange round-shaped residues highlight the characteristic CDF rich-histidine loop found between the TMDs IV and V; green round-shaped residues are zinc-binding amino acids, common to a number of CDF transporter sequences, as described in Huang and Tepaamorndech (2013). N-terminal and TMDs were predicted using PHOBIUS server. Pink colored bar represents the plasmatic membrane in which *Li*CDF is located.

As previously described (Montanini, Blaudez et al. 2007), CDF transporters are subdivided into three distinct groups, according to the metal elements they transport: group I (zinc-CDF), group II (iron/zinc-CDF) and group III (manganese-CDF). Trying to unveil in which of the CDF groups *Li*CDF would fit, we performed a protein sequence alignment using *Li*CDF and 2 members of each of the three groups, which share the aforementioned CDF signature sequence (Fig. 3). Results show that *Li*CDF and group I CDF transporters display the same conserved residues found in all members of group I CDF proteins, indicating that *Li*CDF is probably responsible for zinc transport.

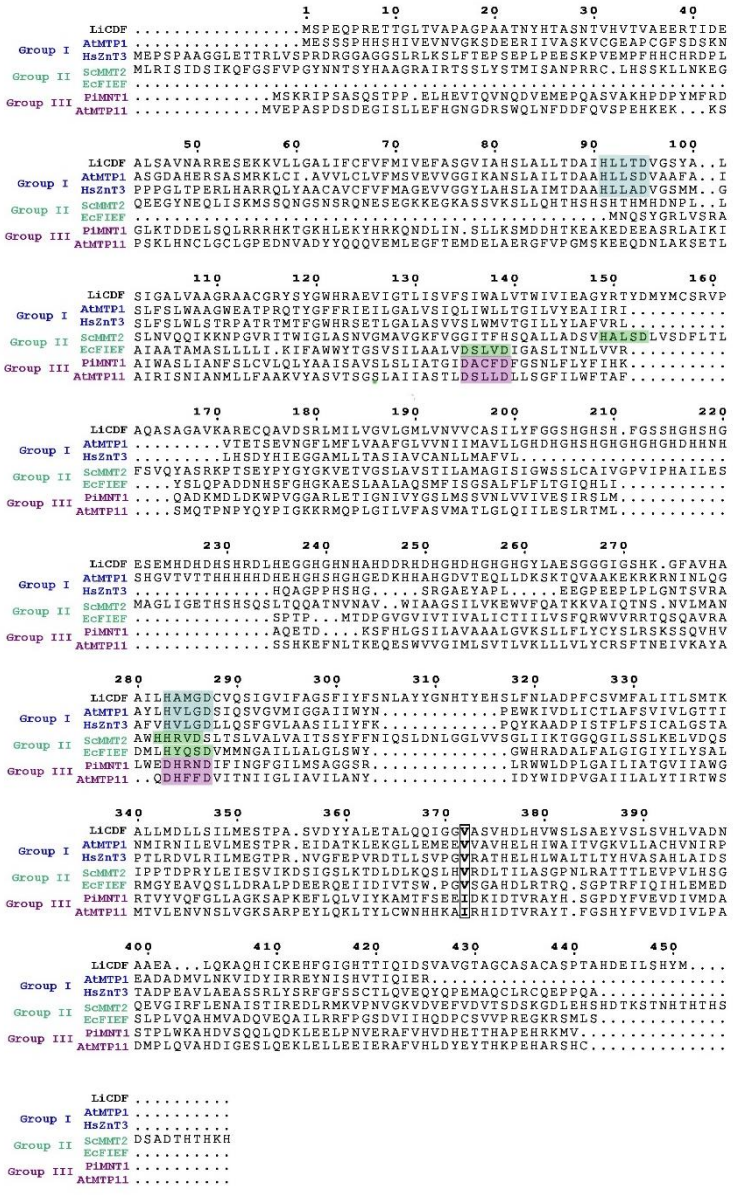


Figure 3 - *Li*CDF shows high similarity to other Group I CDF Transporters. Sequence alignment between *Li*CDF and representative members of each of the three groups illustrates the higher resemblance between *Li*CDF and group I CDF transporters: in blue, zinc-CDF. group I (Zinc-CDF) - *At*MTP1, *Hs*ZNT3 (both belonging to ZnT2-like cluster); in green, group II (Iron/Zinc – CDF) – *Ec*FIEF and *Sc*MMT2; in purple, group III (Manganese – CDF) – *Pi*MNT1 and *At*MTP11. Blue panel highlights conserved regions found in members of group I; green and purple panels refer to conserved regions in group II and III (Montanini, Blaudez et al. 2007).

To characterize *Li*CDF, we proceeded then to the determination of its localization in the cell and to study its expression pattern in response to zinc. To accomplish this, specific antibodies against *Li*CDF were produced.

2. Localization and expression of *Li*CDF

2.1 Antibody production

To produce antibodies recognizing *Li*CDF, the corresponding gene, *LinJ.31.2470*, was amplified from the *L. infantum*'s genomic DNA and cloned into the commercial vector pET28a with a His_6 tag at its N-terminal end. BL21-CodonPlus *E. coli* (BL21+) cells were transformed with the obtained plasmid and different expression conditions were evaluated in a series of small-scale induction screening assays. However, none proved to be adequate for efficient expression of the *Li*CDF recombinant protein in the soluble fraction and with the required yield. This was probably due to the hydrophobic nature of TMDs, resulting in protein aggregation. Therefore, we continued the antibody production strategy using a bioinformatically predicted soluble polypeptide. We cloned the last 288 nucleotides of the *LiCDF* gene (corresponding to 115aa) in pET28a_His_6 vector and performed a new series of small scale trials in BL21+ cells. We observed expression of recombinant Δ*Li*CDF (approximately 12 kDa) in the insoluble fraction of the bacterial extract. To reduce protein aggregation, which could result from a fast and high-level expression of recombinant protein (Mayer and Buchner 2004), we decided to compare several expression conditions: BL21+ *versus* Rosetta cells; different growth media, Lisogeny Broth (LB) *versus* Terrific Broth (TB); different temperatures and induction periods, 26ºC, 6h *versus* 37ºC, 3h; and different inducing agent (IPTG, Isopropyl β-D-1-thiogalactopyranoside) concentrations, 0.2 *versus* 1.0 mM. The highest yield was achieved with BL21+ cells, grown and induced in TB medium, for 6h at 26ºC with 0.2 mM IPTG. Although it was not possible to produce high levels of soluble protein, the highest yield was achieved with BL21+ cells, grown and induced in TB medium, for 6h at 26ºC with 0.2 mM IPTG. The soluble recombinant protein was, then, purified in Aktaprime Plus using a His-Trap column and eluted proteins were run in a preparative polyacrylamide gel to remove contaminants (Fig. 4). Animals at the IBMC and IMHT Animal Facility were immunized, following the established procedures, to produce polyclonal and monoclonal antibodies, respectively.

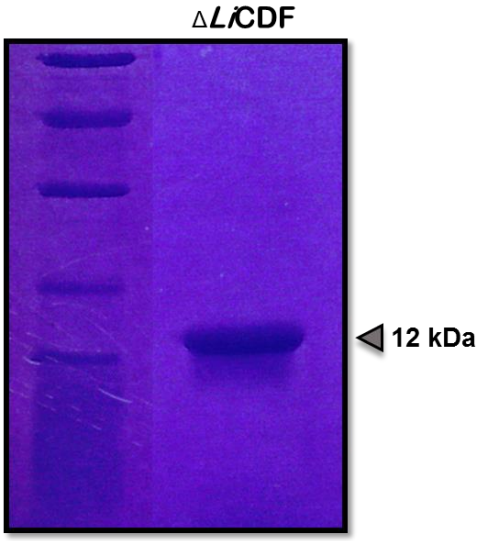


Figure 4 – Coomassie gel demonstrates Δ*Li*CDF peptide enrichment after purification.

2.2 Antibody testing

Sera collected from immunized animals were tested for the ability to recognize the recombinant Δ*Li*CDF and the *Li*CDF from protein extracts of wild type parasites. Given the likely relation between *Li*CDF and *Li*ZIP3, we also analysed the expression of *Li*CDF in parasites overexpressing *Li*ZIP3 (pXG_*Li*ZIP3) with different selective drug pressure. As shown in Figure 5, the polyclonal antibody (P*) barely detected *Li*CDF in wild type parasites, although it was able to recognize the recombinant protein, Δ*Li*CDF. Moreover, *Li*CDF expression could be seen in protein extracts from parasites overexpressing *Li*ZIP3 with the predicted molecular weight, 48 kDa. Two conclusions were, then, inferred: *i) Li*ZIP3-overexpressing parasites display higher levels of *Li*CDF than wild type parasites, and *ii)* the antibody does not efficiently detect *Li*CDF levels in wild type parasites. We tested several conditions to improve detection efficiency. *i)* We questioned whether the use of the chaotropic agent urea (at 4 M when loading the sample) would destroy a structural epitope. To assess this we prepared the samples in Phosphate-Buffered Saline (PBS) solution. *ii)* To check if temperature could somehow alter the epitope recognition, samples were prepared for SDS-PAGE at room temperature, 65ºC or 94ºC. *iii)* To achieve milder lysis conditions that could possibly favor epitope recognition, the nonionic, non-denaturing detergent Nonidet P-40 (NP-40) (1% in PBS) was used instead of Tris-HCl 4% SDS lysis buffer, an anionic denaturing detergent. *iv)* We also tried to renature proteins, removing the SDS anionic detergent, by incubating the polyacrilamide gel in ddH_2O 2,5% Triton X-100 for 1 hour. *v)* Finally,

we questioned whether protein levels *per* lane were too low to be detected so we enriched protein extract. All these attempts proved to be unsuccessful, as we were still unable to gather consistent results concerning *Li*CDF expression.

Regarding the monoclonal antibody, tests in Doutora Ana Domingos' lab, in Lisbon, using recombinant Δ*Li*CDF and protein extracts from wild type parasites showed positive reactions, but only after two days of exposure to the developing reagent (Fig. 6A). Although we could detect bands when we performed the western blotting and the development in the same conditions as those done in Lisbon, the signals had too low intensity and we could not observe Δ*Li*CDF (Fig. 6B). Therefore, we concluded that the monoclonal serum could not be used in subsequent experiments.

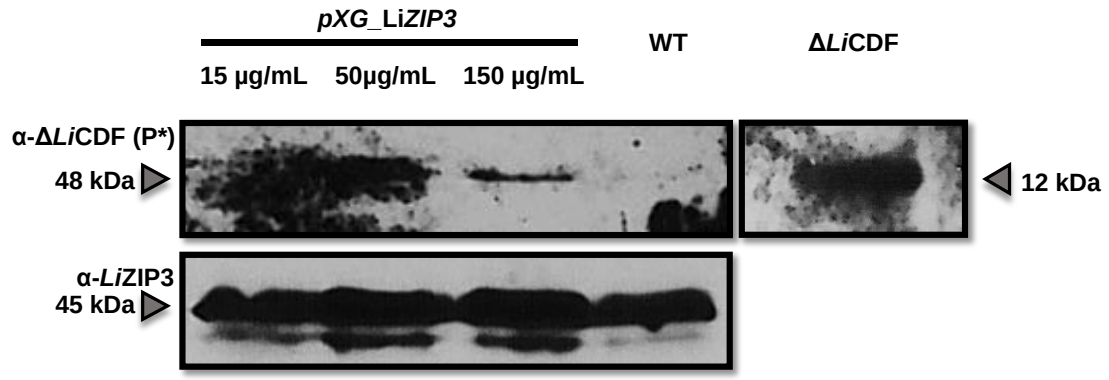


Figure 5 - *Li*ZIP3 overexpressing parasites (pXG_*Li*ZIP3-transfected) have noticeable higher levels of *Li*CDF, however, the latter is not detected in wild type parasites using anti-Δ*Li*CDF polyclonal sera (P*). pXG_*Li*ZIP3-transfected parasites were subjected to increasing concentrations of G418 (15 µg/mL, 50 µg/mL and 150µg/mL) and protein extracts were analyzed by SDS-PAGE (12%) and western blotting alongside untransfected (wild type, WT) parasites. *Li*CDF and *Li*ZIP3 expression was assessed using the antibodies raised against each of the proteins. Panel on the right demonstrates the affinity of anti-Δ*Li*CDF polyclonal sera towards the truncated version of *Li*CDF (Δ*Li*CDF, 1µg).

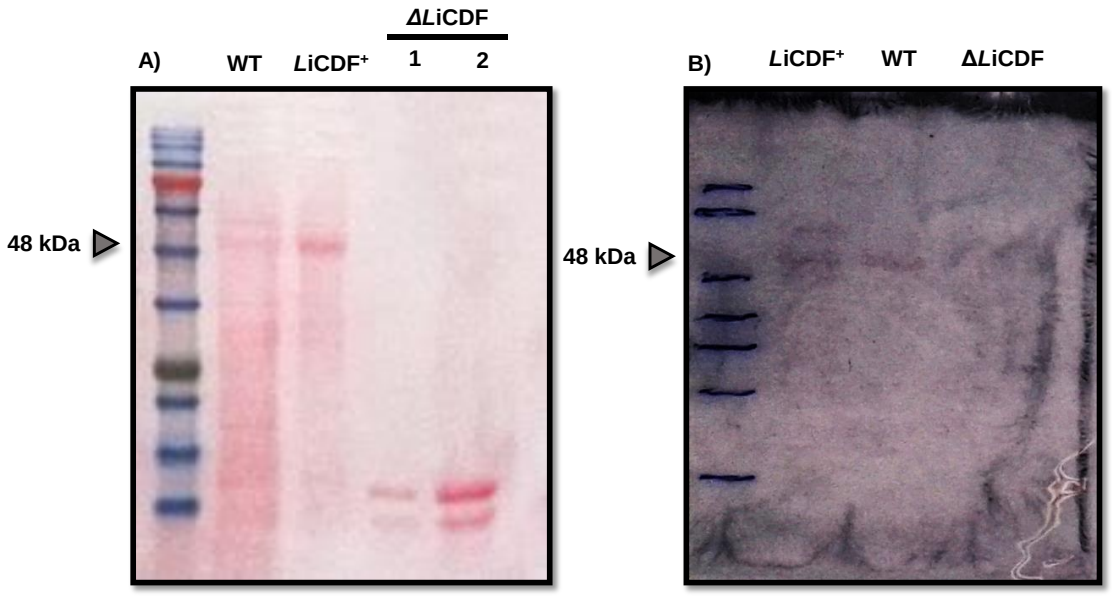


Figure 6 – *Li*CDF is inefficiently detected with monoclonal anti-Δ*Li*CDF antibody. Both panels show PVDF membranes hybridized with anti-Δ*Li*CDF monoclonal antibody. **Panel A)** SDS-PAGE followed by western blotting, performed at Doutora Ana Domingo's laboratory, shows detection of *Li*CDF in both wild type parasites and pTEX_9E10_*Li*CDF transfected ones (*Li*CDF+, cultured with G418 150µg/mL) with the expected MW of 45kDa. As a positive control, samples of Δ*Li*CDF recombinant protein were run side by side: sample 1 contains 1µg of Δ*Li*CDF, sample 2 contains twice as much. **Panel b)** SDS-PAGE followed by western blotting performed at our laboratory, shows two bands in extracts from pTEX_9E10_*Li*CDF transfected parasites (cultured with G418 150µg/mL), and one of smaller weight in wild type parasites extracts. However, no differences in expression were noticeable. No band was detected in Δ*Li*CDF lane (2 µg of protein were loaded).

2.3 Developing a 9E10 tagged version of *Li*CDF

As an alternative strategy to study *Li*CDF in *Leishmania*, we developed a tagged version of the protein so that *Li*CDF could be detected using an efficient commercial antibody. In a first instance, we opted for an N-terminal fusion protein, because, as it had already been described in other organisms, the C-terminal fragment is important for both the function and oligomerization of CDF family transporters (Fukunaka et al., 2009; van der Zaal et al., 1999). Therefore, we cloned the 9E10 tag upstream the *Li*CDF open reading frame (ORF) and transfected the parasites with the pTEX_9E10_*Li*CDF vector. Three clones of parasites expressing 9E10_*Li*CDF were subjected to different selective drug pressures and analysed by western blotting to address protein expression (Fig. 7). Two bands, at approximately 45 and 48 kDa, could be observed when the anti-9E10 antibody was used. The 45 kDa is the result of a non-specific reaction, since it is also present in wild type extracts (please see WT in Figure 7), which do not express the 9E10

tag. The band of 48 kDa corresponds to the 9E10-*Li*CDF protein, which meets the predicted molecular weight. As expected, parasites grown with higher drug pressure (100µg/mL G418) express higher levels of tagged-*Li*CDF than those cultured with low concentrations of selective drug. Thus, this result confirms the viability of the strategy. It should be noted that 9E10_*Li*CDF-expressing parasites grown with 100µg/mL G418 present, in general, a round shape, which is different from the more elongated morphology of those parasites grown with 30µg/mL. Additionally, those parasites grow slower than the ones subjected to lower drug pressure.

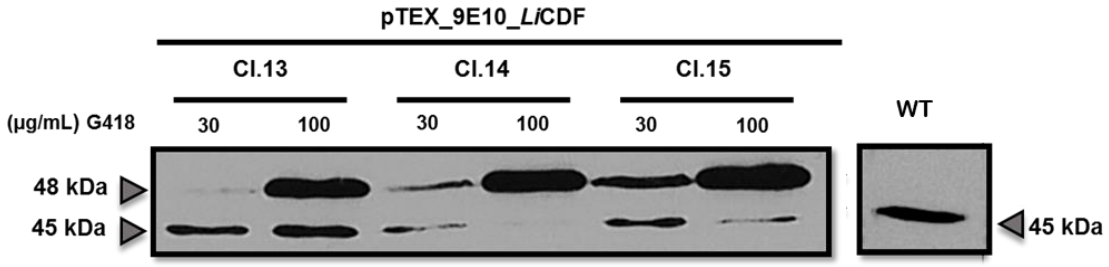


Figure 7 - 9E10_*Li*CDF expression is intensified when pTEX_9E10_*Li*CDF-transfected parasites are subjected to increasing G418 concentrations. Total protein extracts of pTEX_9E10_*Li*CDF clones 13, 14 and 15, selected in agar plates, were run in 12% SDS-PAGE gel and western blotting probing was performed with commercial anti-9E10 antibody. Blots show increased expression of the tagged protein as the parasites are subjected to higher concentrations of G418. Panel on the right, corresponding to the WT parasites (which does not possess the 9E10 tag), shows that the band of 45kDa detected with the anti-9E10 antibody results from an unspecific reaction.

Subsequently, we addressed the protein localization in *L. infantum* by immunofluorescence. Given that *Li*CDF is homologous to the *Trypanosoma cruzi* CDF (EAN89594.1) previously seen to localize to acidocalcisomes (Ferella, Nilsson et al. 2008), we used an antibody against the acidocalcisome membrane vacuolar pyrophosphatase (*Li*VP1) (Ulrich, Jimenez *et al.* 2011), kindly gifted by Dr. Roberto Docampo (Center for Tropical and Emerging Global Diseases, Univ. of Georgia, Athens). As depicted in Figure 8, 9E10_*Li*CDF co-localizes with *Li*VP1, showing that *Li*CDF is present in acidocalcisomes. We obtained the same results with parasites expressing the C-terminal 9E10 tagged-version of *Li*CDF (see lower panel in Fig. 8).

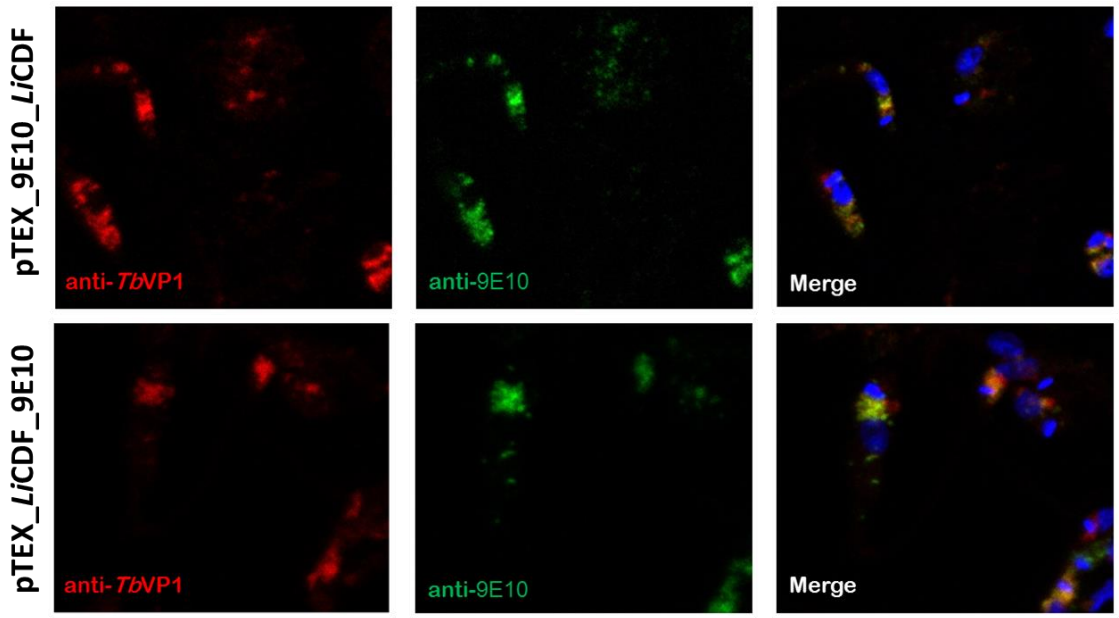


Figure 8 – Both 9E10_*Li*CDF and *Li*CDF_9E10 co-localize with *Li*VP1, confirming its presence in acidocalcisomes. 9E10_*Li*CDF expressing parasites (subjected to 15µg/mL G418) and *Li*CDF_9E10 expressing parasites (subjected to 15µg/mL G418) were harvested and analised by IFAT. Simultaneous probing with anti-9E10 (green) and anti-*Tb*VP1 (acidocalcisomes marker, red) antibodies shows co-localization between the two.

Having defined *Li*CDF molecular weight and localization, our study focused on the interplay between *Li*CDF and *Li*ZIP3 in zinc homeostasis in *Leishmania*.

2.4. Cross-talk between *Li*CDF and *Li*ZIP3

To analyse how *Li*CDF and *Li*ZIP3 are related, we cultured wild type parasites, parasites overexpressing 9E10_*Li*CDF (pTEX_9E10_*Li*CDF) and parasites overexpressing *Li*ZIP3 (pXG_*Li*ZIP3) and analysed the expression of each transporter, by western blotting.

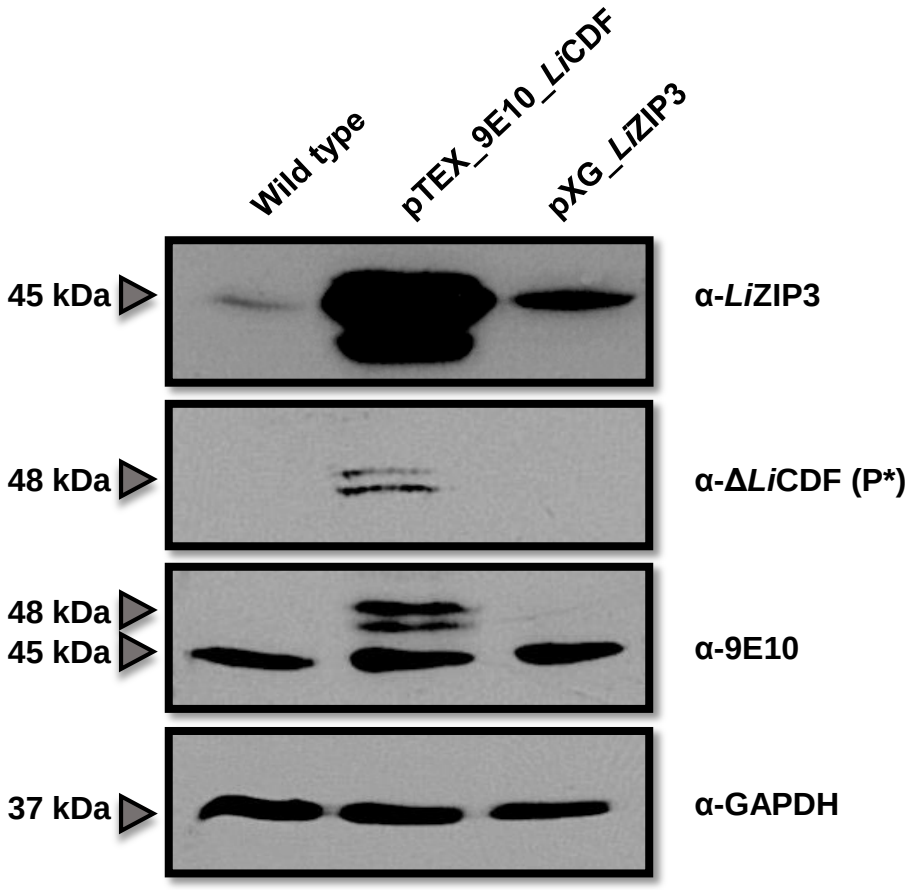


Figure 9 – Parasites overexpressing *Li*CDF display increased *Li*ZIP3 expression. Expression of *Li*ZIP3 and *Li*CDF was analysed in protein extracts of wild type (WT), pTEX_9E10_*Li*CDF (80µg/mL G418) and pXG_*Li*ZIP3 (80µg/mL G418) parasites, by western blotting. *Li*CDF was visualized using the polyclonal serum raised against the Δ*Li*CDF recombinant protein and with the anti-9E10 antibody. Expression of *Li*GAPDH was used as loading control. The 45kDa band in the anti-9E10 blot is the result of unspecific binding as it is detected in wild type parasites.

As expected, parasites transformed with pXG_*Li*ZIP3 express more *Li*ZIP3 than wild type ones. Additionally, the anti-Δ*Li*CDF polyclonal serum was able to recognize *Li*CDF but only in parasites transformed with pTEX_9E10_*Li*CDF, demonstrating that the *Li*CDF levels of expression in wild type and in pXG_*Li*ZIP3 parasites are too low to be detected. Interestingly, the anti-9E10 and anti-Δ*Li*CDF sera detected two closely migrating bands. This pattern might indicate that *Li*CDF is processed either by cleavage of the C-terminal end, maintaining the 9E10 tag at the N-terminal and originating the lower molecular weight band, or by post-translational modifications, which would yield the higher band. We can observe that overexpression of 9E10_*Li*CDF induced high *Li*ZIP3 expression. This result led us to hypothesize that pTEX_9E10_*Li*CDF cells excessively mobilize zinc into acidocalcisomes, causing zinc restriction in the cytosol

and, therefore, lead to an augmented *Li*ZIP3 expression and increased cellular zinc uptake. If this is correct, 9E10_*Li*CDF-overexpressing parasites should have an overall zinc content higher than wild type and pXG_*Li*ZIP3 cells. To test this, the zinc content of wild type, pTEX_9E10_*Li*CDF and pXG_*Li*ZIP3 parasites was analysed, by atomic absorption spectroscopy, at timepoints 0 and 1.5h after zinc addition to the culture medium. The results depicted in Figure 10, cleared out that, indeed, zinc levels were higher in 9E10_*Li*CDF-overexpressing parasites, suggesting that *Li*CDF and *Li*ZIP3 work in a synchronous way promoting zinc homeostasis in the parasite.

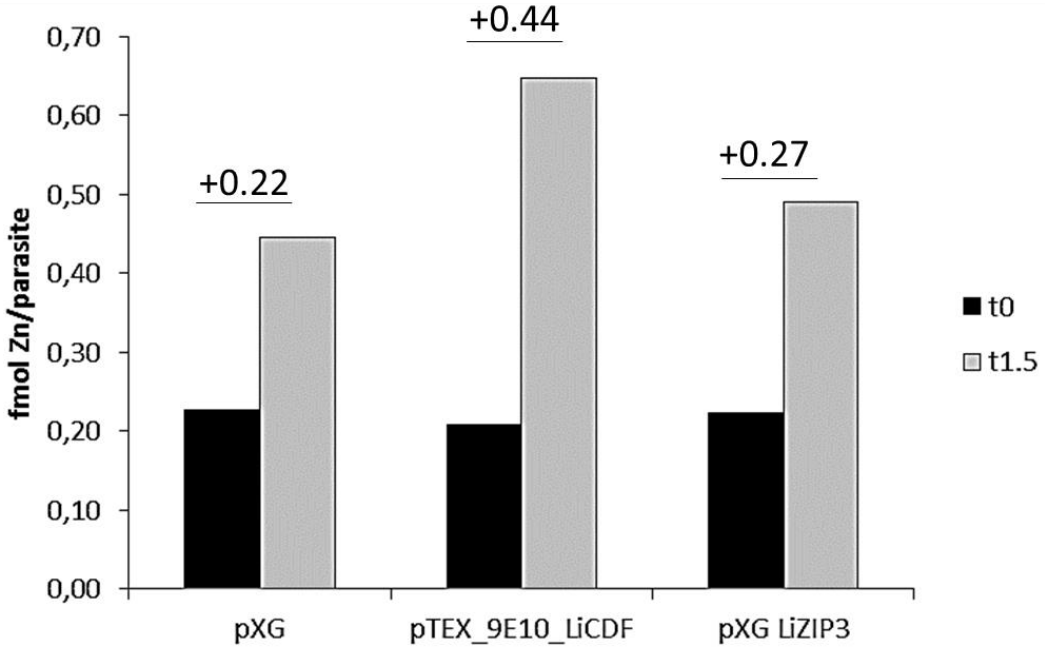


Figure 10 - Parasites overexpressing *Li*CDF contain more zinc than *Li*ZIP3-overexpressing ones and controls (pXG, parasites transfected with the empty vector). Parasites, pXG, pTEX_9E10_*Li*CDF and pXG_*Li*ZIP3, were grown for 72h and zinc was added for 1,5h. The total zinc content of zinc in the three parasite lines was measured by atomic absorption spectrometry. Values on top of each bar depict the increase seen in zinc content after zinc supplementation.

Expression and localization of *Li*CDF and *Li*ZIP3 were also analysed by immunofluorescence in the different parasite lines (Figure 11). Untransfected (wild type, WT) parasites were used as control for basal expression of both transporters. Interestingly the polyclonal anti-Δ*Li*CDF serum was able to recognize *Li*CDF in wild type cells, showing the same punctate, acidocalcisome-like, pattern as the anti-9E10 antibody in *Li*CDF-overexpressing parasites. Moreover, similarly to what was observed in the

western blot, parasites overexpressing 9E10_*Li*CDF display, in comparison to wild type cells, increased of expression of both *Li*CDF and *Li*ZIP3, though, in this IFAT essay, the increase of expression of the latter is not as striking as the one seen in western blotting essays. In what concerns to the expression of *Li*CDF in *Li*ZIP3-overexpressing parasites, a difference in intensity in comparison to wild type cells is not obvious.

These results suggest that *Li*CDF over-expression causes a disruption in zinc homeostasis, to which the parasite responds by increasing *Li*ZIP3 expression (at the membrane), allowing the reposition of zinc in the now-zinc-depleted cytosolic environment. This effect is not as visible when *Li*ZIP3 expression is artificially increased.

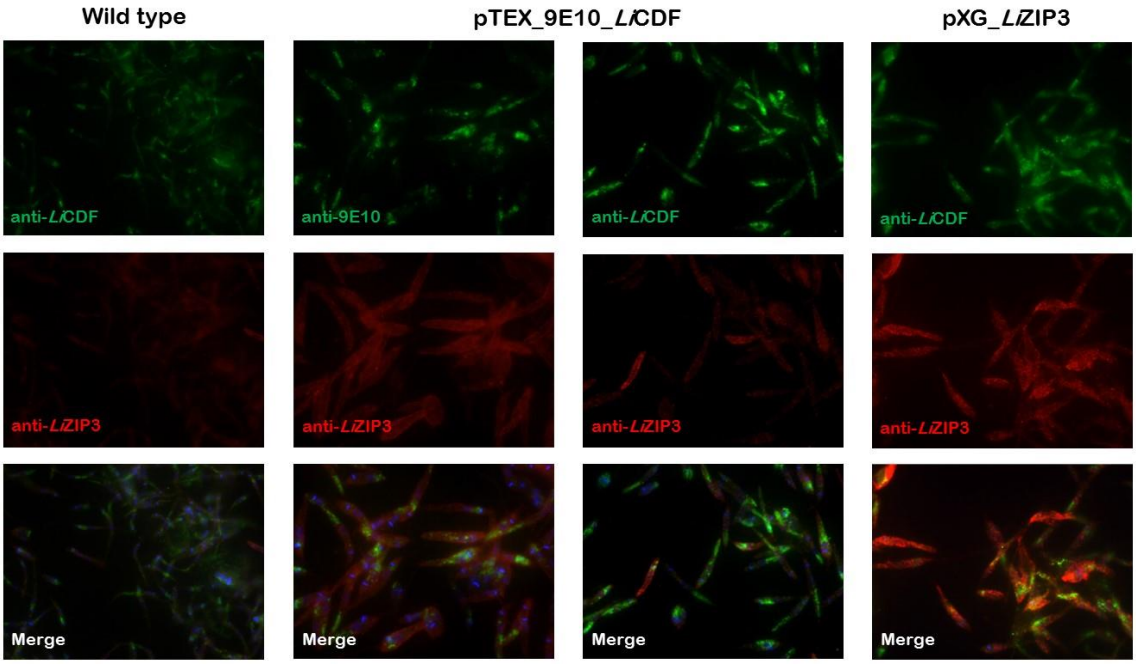


Figure 11 - *Li*CDF-overexpressing parasites display higher expression of *Li*ZIP3 and parasites over-expressing *Li*ZIP3 have increased levels of *Li*CDF. Untransfected (wild type, WT), 9E10_*Li*CDF and *Li*ZIP3-overexpressing parasites were analysed by IFAT for the expression and localization of each of the transporters. *Li*CDF and *Li*ZIP3 are visualized in green and red, respectively.

2.5. Zinc-mediated regulation of *Li*CDF

Previous work from the Group showed that, similarly to *Li*ZIP3, *Li*CDF is regulated at the mRNA level by zinc, although in opposing directions (*Li*ZIP3 mRNA decreases in response to zinc supplementation while *Li*CDF mRNA increases) (Carvalho 2012). Therefore, we investigated zinc-mediated *Li*CDF regulation at the protein level. Given that the study of *Li*CDF regulation by zinc depended on the visualization of the protein

by western blotting and that the efficiency of its detection was too low in wild type parasites, we engaged a different approach intended to produce parasites where *Li*CDF could be detected by western blotting while simultaneously subjected to its endogenous regulation by zinc. As observed in the previous sections, the anti-9E10 antibody is efficient in recognizing the tagged transporter. Therefore, we proceeded to the production of parasites expressing 9E10_*Li*CDF from *i)* the genomic context, *i.e.* by inserting the tag into one of the chromosome alleles, and from *ii)* an episome containing the 3'UTR and intergenic region (IR) of *Li*CDF cloned downstream the tagged gene (pTEX_9E10_CDF_3'UTR). Expression of *Li*CDF from the genomic context or from an episome maintaining the endogenous 3'UTR would allow for the regulated expression of the transporter as it has been reported that sequences in the 3'UTR of *Leishmania* and related trypanosomatids' genes play an important role in post-transcriptional regulation in these parasites (Boucher, Wu *et al.* 2002, McNicoll, Muller *et al.* 2005, Requena 2011).

Due to technical constrains in one of the cloning steps, it was not possible to produce, so far, the parasites expressing 9E10_*Li*CDF from the chromosome context. Nevertheless, we were able to transfect parasites with the pTEX_9E10_*Li*CDF_3'UTR vector. A stable polyclonal culture of parasites transfected with the episome was not readily obtained as it took much longer than the usual 1-2 weeks. Additionally, it was not possible to isolate colonies of these parasites, as no growth was observed in agar plates. Plasmid rescue showed that some parasites in polyclonal culture are correctly transfected (Fig. S5). This culture presented initially round shape and slow-growing cells, similarly to what had already been registered for 9E10_*Li*CDF expressing parasites. It should also be noted that, during PCR, a mutation (G1198A) was introduced in the final part of the *LiCDF* gene. This mutation results in an amino acid substitution (A400T) from a hydrophobic residue to a polar one, with uncharged side chains. Alignment with CDF family members shows that this mutated residue is not one of those important for zinc-related transport or regulation of CDF activity, however, one cannot discard the hypothesis of alterations in its structure caused by this mutation (Fig. 2).

We first analysed the expression of 9E10_*Li*CDF in the pTEX_9E10_*Li*CDF_3'UTR-transfected parasites using protein extracts from pTEX_Neo parasites as negative control (cells carrying the empty vector) and pTEX_9E10_*Li*CDF protein extracts as positive (note the absence of the 3' UTR) (Fig. 12). Detection with the anti-9E10 antibody showed that there is a specific band in both parasite lines that express the 9E10-tagged *Li*CDF.

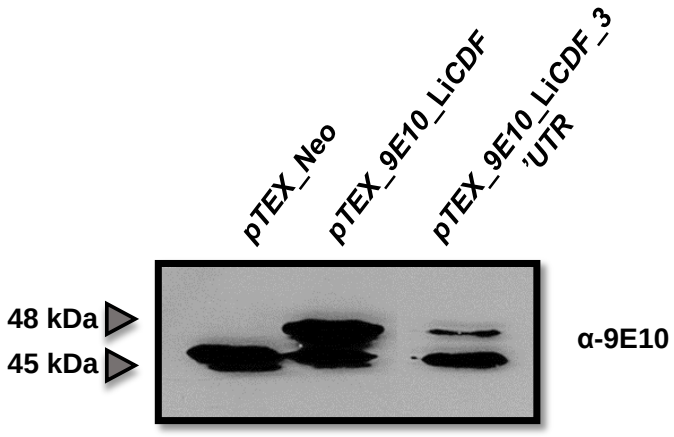


Figure 12- Parasite transfected with the pTEX_9E10_*Li*CDF_3'UTR vector express 9E10_*Li*CDF. Total proteins extracts from pTEX_9E10_*Li*CDF_3'UTR (15µg/mL G418) were subjected to hybridization with anti-9E10. Parasites carrying the empty vector (pTEX_Neo) were used as a negative control for 9E10_*Li*CDF expression and parasites transfected with pTEX_9E10_*Li*CDF (80µg/mL G418) as a positive one.

In order to study the pattern of expression of *Li*CDF, we grew pTEX_9E10_*Li*CDF_3'UTR parasites for 9 days, harvesting samples at each day, and analysed by western blotting.

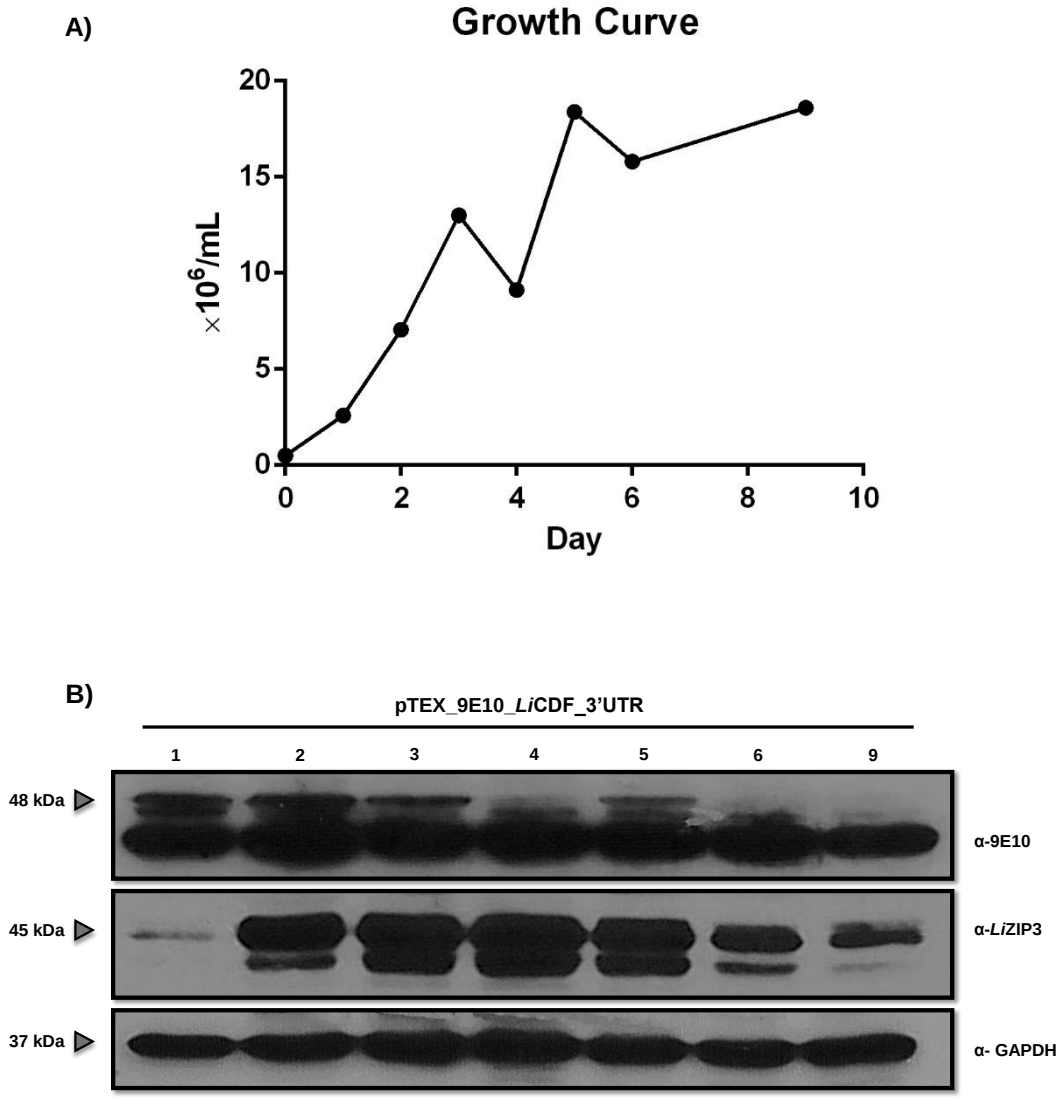


Figure 13 - Pattern of expression of *Li*CDF during the days in culture. A) Growth curve of pTEX_9E10_*Li*CDF_3'UTR-transfected parasites. Parasites were synchronized prior to the beginning of the experiment and, at day 0, cells were seeded at 1x10^6/mL. **B)** 9E10_*Li*CDF expression levels decrease during the days of culture. Parasites were harvested at every 24h and total protein extracts analysed by SDS-PAGE and western blotting. Expression of *Li*GAPDH is used as loading control.

As shown in Figure 13, *Li*CDF is mainly expressed in the first days of culture, in lag and log-growth phases, decreasing in the following days. Expression of *Li*ZIP3 increases from 24 to 48h, where *Li*CDF has its peak of expression, and also decreases at longer periods, though later than *Li*CDF. The increase in the expression observed in the two transporters in the first days of culture suggests that parasites internalize and store zinc in this period.

A more extensive assay was performed in order to determine the effect of zinc availability in *Li*CDF expression. This was done by assessing 9E10_*Li*CDF expression at several time-points after addition of zinc or TPEN, a zinc chelator.

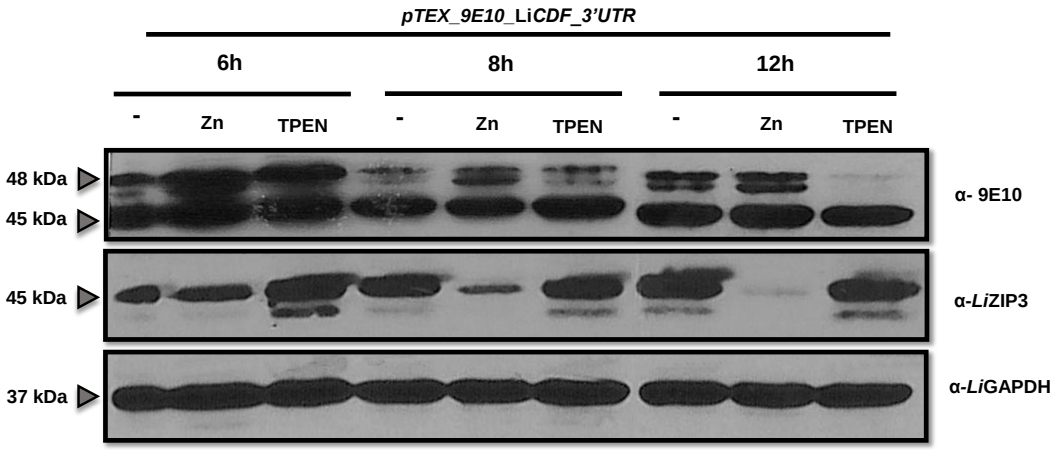


Figure 14 - *Li*CDF expression is increased when parasites are subjected to zinc-supplemented conditions. pTEX_9E10_*Li*CDF_3'UTR (15µg/mL G418) parasites were cultured for 48h and subjected to zinc supplementation (50µM $ZnCl_2$), zinc depletion (10µM TPEN) or kept as non-treated for a total 12h period. Parasites were harvested at 6h, 8h and 12h time-points. Membranes were subjected to hybridization with anti-9E10, anti-*Li*ZIP3 and anti-*Li*GAPDH.

From this assay, we can infer that *Li*CDF upregulation caused by zinc occurs at 6h upon addition of the metal to the culture medium and that its expression slightly decreases in the following period. Introduction of TPEN (zinc chelator) in the culture medium initially induces an increase in the expression of *Li*CDF in comparison to non-treated parasites but the levels of the transporter decrease from 6 to 12h. In what concerns to *Li*ZIP3, the expression levels of this transporter varied according to what had been previously seen (Carvalho 2012): zinc supplementation leads to decreased expression and zinc chelation to an increase in the transporter's levels.

These results show that, indeed, *Li*CDF and *Li*ZIP3 are regulated by zinc in opposing directions, suggesting that both transporters are players in the maintenance of zinc homeostasis.

Discussion

Discussion

Maintenance of nutrient homeostasis is an essential process in the cell, involving multiple mechanisms and molecules, all functioning in a synchronous pace to achieve equilibrium. Amongst the many classes of nutrients, transition metals are considered crucial for the well-being of many organisms, including *Leishmania* parasites. Such molecules play major roles in cells, assuming one of three distinct functions: non-catalytic, and both redox and non-redox catalytic (Andreini, Bertini *et al.* 2008). However, when in excess or limitation, they can cause damage to the cell and possibly result in death of the organism. In fact, this is the premise of a defense strategy called "nutritional immunity" in which the host deprives or exposes the invasive organism to nutrients (Hood and Skaar 2012).

Zinc is one of the many nutritional staples vital for *Leishmania* parasites to promote infection and thrive in the host. In other organisms, the acquisition and regulation of zinc are mostly dependent on ZIP and CDF families of transporters, each functioning in opposite directions. A *Leishmania* ZIP transporter, designated as *Li*ZIP3, has indeed been extensively described at the Molecular Parasitology Group, at IBMC, and proved to be deeply involved in the acquisition of zinc by *L. infantum* promastigotes (Carvalho 2012). Trying to disclose which other proteins could also be playing a part in zinc metabolism, the membrane proteome of parasites subjected to higher concentrations of zinc was analysed by mass spectrometry. This analysis revealed the involvement of a CDF transporter, whose expression appeared to be induced in these conditions. In the general context of the work performed at our group, the newly identified transporter, named *Li*CDF, posed itself as a relevant piece in the maintenance of zinc balance. This dissertation focuses on the characterization of the *Li*CDF transporter as well as on its interplay with the *Li*ZIP3 cell membrane-bound high affinity zinc transporter.

As a preliminary study, we analyzed *Li*CDF sequence (already available at TriTryp website) using bioinformatics tools, gathering some information about its predicted structure. Afterwards, we began our work by producing and purifying the recombinant *Li*CDF through the use of the *E. coli* heterologous system. We tested a number of different conditions, ranging from different IPTG concentrations and induction temperatures to cell strains, always retrieving very low yields of proteins. This could

partly be explained by the intrinsic hydrophobic quality of the transporter, as it is a transmembrane protein. Therefore, we proceeded to the production of a truncated version of the protein, containing the C-terminal region which is predicted to be projected into the cytosol. Obtaining better production yields, we were able to successfully immunize both rat and mouse at IBMC and IMHT (Lisbon), respectively. Extensive testing of the produced poly- and monoclonal anti-Δ*Li*CDF sera demonstrated that these antibodies were inefficient at detecting endogenous *Li*CDF in wild type parasites by western blotting. However, we observed that anti-Δ*Li*CDF polyclonal serum detects the protein in IFAT, suggesting that these antibodies recognize a structural epitope. Anti-Δ*Li*CDF monoclonal serum is yet to be tested in IFAT.

As we tried to evaluate the tie between *Li*CDF and *Li*ZIP3, we detected an astonishing increase of *Li*ZIP3 expression in *Li*CDF-overexpressing parasites (pTEX_9E10_*Li*CDF). This led us to conclude that *Li*CDF is perchance mobilizing zinc out of the cytosol inwards acidocalcisomes, where we showed it is located. Indeed, parasites overexpressing *Li*CDF have higher zinc content than wild type and *Li*ZIP3-overexpressing parasites. We hypothesize that, since *Li*CDF is at the membrane of acidocalcisomes internalizing zinc into these organelles, excessive expression of this transporter leads to severe zinc restriction in the cytosol and this, in turn, induces the expression of *Li*ZIP3 in order to increase the internalization of the metal into the cell. This hypothesis has yet to be confirmed. This can be done using different fluorescent probes that sense the amount of free zinc in the cytosol and of that present in acidocalcisomes. An alternative is using EDX mapping, in which X-ray excitation of a sample leads to emission of a different set of X-ray, according to the distinctive atomic structure of each element. Based on the profile of each set of peaks, it is possible to identify distinct elements as well as its quantity. It is curious, though, that, expression of *Li*CDF is not induced in *Li*ZIP3-overexpressing parasites. This might be explained by the levels of LiZIP3 regulation. Preliminary work performed in this Group suggests that *Li*ZIP3 present at the membrane is inactivated after addition of zinc. Therefore, although pXG_*Li*ZIP3 parasites are over-expressing *Li*ZIP3, part of the protein might be inactivated and the amount of zinc that is internalized is not sufficient to cause an increase in *Li*CDF.

To study the endogenous *Li*CDF behavior in response to zinc while assuring that the transporter was going to be detected by western blotting, we used a parasite line transfected with a vector containing the 9E10-tagged *Li*CDF ORF with the 3'UTR and IR of *Li*CDF cloned downstream the gene. The 3'UTR and the IR were introduced in the plasmid in order to maintain possible regulatory elements that, in *Leishmania* and related trypanosomatids, are present in the 3'UTR. Moreover, the IR contains all the signals

required to the correct processing of the mRNA, namely the addition of the poly-A. Therefore, we expected to have parasites expressing a detectable pool of *Li*CDF that was regulated by the same stimuli as the endogenous, chromosomic-derived *Li*CDF. The expression pattern obtained during the days of culture showed that, at the initial days of culture, *Li*ZIP3 and *Li*CDF were highly expressed. This may be justified by the parasite's need to grow and replicate, thus, displaying a high metabolic rate. As the culture reaches the stationary phase, levels of both transporters decrease, though *Li*CDF levels decay sooner than those of *Li*ZIP3. We can hypothesize that when parasites are subjected to high concentrations of zinc, such as that occurring when they are in lag and log-growth phases, cells take up the metal and store it in acidocalcisomes, increasing *Li*CDF and *Li*ZIP3 levels. As the metal becomes scarce in the medium, there is no reason to store zinc and *Li*CDF expression decreases. The fact that expression of *Li*ZIP3 is maintained at high levels after *Li*CDF levels have been reduced might reflect the high zinc demands these parasites have.

Concerning the amino acid substitution that was introduced during construction of the pTEX_9E10_*Li*CDF_3'UTR vector, and, although it seems not to affect an important residue for CDF activity or regulation, we cannot discard the possibility that, in the *L. infantum* CDF, these amino acid might assume an essential role. Therefore, correction by site-directed mutagenesis is a priority. Another mutation in the 3'UTR and IR of the gene was identified, which can affect the transporter's regulation, and, as aforementioned for the ORF, this mutation should also be corrected. Additionally, pTEX_9E10_*Li*CDF_3'UTR presented us with serious difficulties during the initial period of culturing. We can consider that the size of the introduced plasmid (approximately 12kB) is challenging, requiring a longer period to replicate, and, therefore, conditions parasite growth. Moreover, future experiments will have to be performed in selected clones, as we were using a polyclonal culture in which some parasites were not correctly transfected,

The observation of a different morphology and growth rates in *Li*CDF-overexpressing parasites in relation to wild type cells was recurring. This might be linked to the function of acidocalcisomes in the regulation of cell volume (Moreno and Docampo 2009). Indeed, the high expression of *Li*CDF and the expected higher zinc content (measured by atomic absorption spectrometry) may be affecting the capacity of acidocalcisomes to function correctly.

Performing various assays with anti-9E10, we observed a two band pattern in hybridized blots. One might think that this is fruit of cleavage of the C-terminal end the protein or of post-translational modifications (PTMs). To discriminate between cleavage

of termini and PTMs, one could perform a western blotting of two lines of parasites each expressing the 9E10 tag at the N-terminal (produced in this study) or C-terminal (produced meanwhile in the lab). If the two bands are still present in both samples, cleavage can be dismissed. In what concerns to PTMS, a bioinformatics study shows that *Li*CDF might be N- and O-glycosilated. Nevertheless, several other PTMs occur in trypanosomatids (Leung, Riley *et al.* 2011, Frenal, Kemp *et al.* 2014, Hu, Yu *et al.* 2014) and, in particular, in *Leishmania* (Rosenzweig, Smith *et al.* 2008).

Conclusion

Organisms require a highly orchestrated functioning of transporters to achieve steady equilibrium and homeostasis. *L. infantum* is no exception as its life cycle requires dramatic changes in morphology and physiology. This work focused on zinc metabolism in these protozoan parasites, more precisely on the study of a metal transporter, *Li*CDF. The results obtained show that *Li*CDF is present in the membrane of acidocalcisomes and suggest that this transporter is responsible for zinc storage in these organelles when zinc is in excess: *Li*CDF appears to sense cytosolic zinc levels and, when opportune, mobilizes the cation inwards acidocalcisomes for storage; consequently cytosolic zinc levels decrease, contributing to the homeostasis of the parasite.

Additional studies are required to better understand *Li*CDF expression in response to zinc and the link between *Li*CDF and *Li*ZIP3. However, this work was the first step towards that knowledge and, besides creating tools for further analysis, unveiled part of the mechanisms involved in zinc homeostasis in *Leishmania*.

Future Work

As these results regarding *Li*CDF regulation by zinc are still preliminary, it would be important to, on a first stage, correct the cloned *Li*CDF sequence in the pTEX_9E10 vector. Afterwards, we can proceed with our expression status and see is *Li*CDF expression altered when *Li*ZIP3 is overexpressed. Also, we are still unsure whether *Li*CDF regulation is either dependent on the zinc-status of the medium or if it is exclusively influenced by cytosolic concentration of the cation. Finally, similarly to what has been done with *Li*ZIP3, it is relevant to identify the regulating sequence in the 3'UTR of the gene and how is this regulation mediated.

References

References

Addy, M. and A. Nandy (1992). "Ten years of kala-azar in west Bengal, Part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas?" Bull World Health Organ **70**(3): 341-346.

Alvar, J., R. Molina, M. San Andres, M. Tesouro, J. Nieto, M. Vitutia, F. Gonzalez, M. D. San Andres, J. Boggio, F. Rodriguez and et al. (1994). "Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy." Ann Trop Med Parasitol **88**(4): 371-378.

Andreini, C., I. Bertini, G. Cavallaro, G. L. Holliday and J. M. Thornton (2008). "Metal ions in biological catalysis: from enzyme databases to general principles." J Biol Inorg Chem **13**(8): 1205-1218.

Antinori, S., L. Schifanella and M. Corbellino (2012). "Leishmaniasis: new insights from an old and neglected disease." Eur J Clin Microbiol Infect Dis **31**(2): 109-118.

Aslett, M., C. Aurrecoechea, M. Berriman, J. Brestelli, B. P. Brunk, M. Carrington, D. P. Depledge, S. Fischer, B. Gajria, X. Gao, M. J. Gardner, A. Gingle, G. Grant, O. S. Harb, M. Heiges, C. Hertz-Fowler, R. Houston, F. Innamorato, J. Iodice, J. C. Kissinger, E. Kraemer, W. Li, F. J. Logan, J. A. Miller, S. Mitra, P. J. Myler, V. Nayak, C. Pennington, I. Phan, D. F. Pinney, G. Ramasamy, M. B. Rogers, D. S. Roos, C. Ross, D. Sivam, D. F. Smith, G. Srinivasamoorthy, C. J. Stoeckert, S. Subramanian, R. Thibodeau, A. Tivey, C. Treatman, G. Velarde and H. Wang (2010). "TriTrypDB: a functional genomic resource for the *Trypanosomatidae*." Nucleic Acids Research **38**(suppl 1): D457-D462.

Baiocco, P., G. Colotti, S. Franceschini and A. Ilari (2009). "Molecular basis of antimony treatment in leishmaniasis." J Med Chem **52**(8): 2603-2612.

Barratt, G. and P. Legrand (2005). "Comparison of the efficacy and pharmacology of formulations of amphotericin B used in treatment of leishmaniasis." Curr Opin Infect Dis **18**(6): 527-530.

Bates, P. A. (2007). "Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies." Int J Parasitol **37**(10): 1097-1106.

Beattie, L. and P. M. Kaye (2011). "Leishmania–host interactions: what has imaging taught us?" Cellular Microbiology **13**(11): 1659-1667.

Beverley, S. M. and C. E. Clayton (1993). "Transfection of *Leishmania* and *Trypanosoma* brucei by electroporation." Methods Mol Biol **21**: 333-348.

Biasini, M., S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. G. Cassarino, M. Bertoni, L. Bordoli and T. Schwede (2014). "SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information." Nucleic Acids Research **42**(W1): W252-W258.

Blaudez, D., A. Kohler, F. Martin, D. Sanders and M. Chalot (2003). "Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif." Plant Cell **15**(12): 2911-2928.

Bloss, T., S. Clemens and D. H. Nies (2002). "Characterization of the ZAT1p zinc transporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomes." Planta **214**(5): 783-791.

Botella, H., P. Peyron, F. Levillain, R. Poincloux, Y. Poquet, I. Brandli, C. Wang, L. Tailleux, S. Tilleul, G. M. Charriere, S. J. Waddell, M. Foti, G. Lugo-Villarino, Q. Gao, I. Maridonneau-Parini, P. D. Butcher, P. R. Castagnoli, B. Gicquel, C. de Chastellier and O. Neyrolles (2011). "Mycobacterial p(1)-type ATPases mediate resistance to zinc poisoning in human macrophages." Cell Host Microbe **10**(3): 248-259.

Boucher, N., Y. Wu, C. Dumas, M. Dube, D. Sereno, M. Breton and B. Papadopoulou (2002). "A common mechanism of stage-regulated gene expression in *Leishmania* mediated by a conserved 3'-untranslated region element." J Biol Chem **277**(22): 19511-19520.

Brittingham, A., G. Chen, B. S. McGwire, K. P. Chang and D. M. Mosser (1999). "Interaction of *Leishmania* gp63 with cellular receptors for fibronectin." Infect Immun **67**(9): 4477-4484.

Brittingham, A., C. J. Morrison, W. R. McMaster, B. S. McGwire, K. P. Chang and D. M. Mosser (1995). "Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis." J Immunol **155**(6): 3102-3111.

Carvalho, S. (2012). Studies on metal acquisition in *Leishmania infantum*, University of Porto.

Chappuis, F., S. Sundar, A. Hailu, H. Ghalib, S. Rijal, R. W. Peeling, J. Alvar and M. Boelaert (2007). "Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?" Nat Rev Microbiol **5**(11): 873-882.

Cooper, H. M. and Y. Paterson (2001). Production of Polyclonal Antisera. Current Protocols in Neuroscience, John Wiley & Sons, Inc.

Cox, F. E. (2002). "History of human parasitology." Clin Microbiol Rev **15**(4): 595-612.

David, C. V. and N. Craft (2009). "Cutaneous and mucocutaneous leishmaniasis." Dermatologic Therapy **22**(6): 491-502.

Dawit G., G. Z., Simenew K. (2013). "A Review on Biology, Epidemiology and Public Health Significance of Leishmaniasis." Journal of Bacteriology & Parasitology **4:2**.

de Souza, W. (2002). "Special organelles of some pathogenic protozoa." Parasitol Res **88**(12): 1013-1025.

de Souza, W., M. Attias and J. C. Rodrigues (2009). "Particularities of mitochondrial structure in parasitic protists (Apicomplexa and Kinetoplastida)." Int J Biochem Cell Biol **41**(10): 2069-2080.

Desjeux, P. (2001). "Worldwide increasing risk factors for leishmaniasis." Med Microbiol Immunol **190**(1-2): 77-79.

Docampo, R., W. de Souza, K. Miranda, P. Rohloff and S. N. Moreno (2005). "Acidocalcisomes - conserved from bacteria to man." Nat Rev Microbiol **3**(3): 251-261.

Eide, D. J. (2004). "The SLC39 family of metal ion transporters." Pflugers Arch **447**(5): 796-800.

Eide, D. J. (2006). "Zinc transporters and the cellular trafficking of zinc." Biochim Biophys Acta **1763**(7): 711-722.

Ellis, C. D., C. W. Macdiarmid and D. J. Eide (2005). "Heteromeric protein complexes mediate zinc transport into the secretory pathway of eukaryotic cells." J Biol Chem **280**(31): 28811-28818.

Ferella, M., D. Nilsson, H. Darban, C. Rodrigues, E. J. Bontempi, R. Docampo and B. Andersson (2008). "Proteomics in *Trypanosoma cruzi* - localization of novel proteins to various organelles." Proteomics **8**(13): 2735-2749.

Finn, R. D., A. Bateman, J. Clements, P. Coggill, R. Y. Eberhardt, S. R. Eddy, A. Heger, K. Hetherington, L. Holm, J. Mistry, E. L. L. Sonnhammer, J. Tate and M. Punta (2014). "Pfam: the protein families database." Nucleic Acids Research **42**(D1): D222-D230.

Frenal, K., L. E. Kemp and D. Soldati-Favre (2014). "Emerging roles for protein S-palmitoylation in *Toxoplasma* biology." Int J Parasitol **44**(2): 121-131.

Gaither, L. A. and D. J. Eide (2001). "Eukaryotic zinc transporters and their regulation." Biometals **14**(3-4): 251-270.

Grotz, N., T. Fox, E. Connolly, W. Park, M. L. Guerinot and D. Eide (1998). "Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency." Proc Natl Acad Sci U S A **95**(12): 7220-7224.

Guerinot, M. L. (2000). "The ZIP family of metal transporters." Biochim Biophys Acta **1465**(1-2): 190-198.

Hallé, M., M. A. Gomez, M. Stuible, H. Shimizu, W. R. McMaster, M. Olivier and M. L. Tremblay (2009). "The *Leishmania* Surface Protease GP63 Cleaves Multiple Intracellular

Proteins and Actively Participates in p38 Mitogen-activated Protein Kinase Inactivation." Journal of Biological Chemistry **284**(11): 6893-6908.

Haney, C. J., G. Grass, S. Franke and C. Rensing (2005). "New developments in the understanding of the cation diffusion facilitator family." J Ind Microbiol Biotechnol **32**(6): 215-226.

Hantke, K. (2005). "Bacterial zinc uptake and regulators." Curr Opin Microbiol **8**(2): 196-202.

Hendriksen, C. F. and W. de Leeuw (1998). "Production of monoclonal antibodies by the ascites method in laboratory animals." Res Immunol **149**(6): 535-542.

Hide, M., B. Bucheton, S. Kamhawi, R. Bras-Gonçalves, S. Sundar, J. L. Lemesre and A. L. Bañuls (2006). Understanding Human Leishmaniasis:The Need for an Integrated Approach. Encyclopedia of Infectious Diseases, John Wiley & Sons, Inc.**:** 87-123.

Hood, M. I. and E. P. Skaar (2012). "Nutritional immunity: transition metals at the pathogen-host interface." Nat Rev Microbiol **10**(8): 525-537.

Hu, H., Z. Yu, Y. Liu, T. Wang, Y. Wei and Z. Li (2014). "The Aurora B kinase in *Trypanosoma brucei* undergoes post-translational modifications and is targeted to various subcellular locations through binding to TbCPC1." Mol Microbiol **91**(2): 256-274.

Huang, L. and S. Tepaamorndech (2013). "The SLC30 family of zinc transporters - a review of current understanding of their biological and pathophysiological roles." Mol Aspects Med **34**(2-3): 548-560.

Jirakulaporn, T. and A. J. Muslin (2004). "Cation diffusion facilitator proteins modulate Raf-1 activity." J Biol Chem **279**(26): 27807-27815.

Kall, L., A. Krogh and E. L. Sonnhammer (2004). "A combined transmembrane topology and signal peptide prediction method." J Mol Biol **338**(5): 1027-1036.

Kambe, T., T. Suzuki, M. Nagao and Y. Yamaguchi-Iwai (2006). "Sequence similarity and functional relationship among eukaryotic ZIP and CDF transporters." Genomics Proteomics Bioinformatics **4**(1): 1-9.

Kaye, P. and P. Scott (2011). "Leishmaniasis: complexity at the host-pathogen interface." Nat Rev Microbiol **9**(8): 604-615.

Kehl-Fie, T. E., S. Chitayat, M. I. Hood, S. Damo, N. Restrepo, C. Garcia, K. A. Munro, W. J. Chazin and E. P. Skaar (2011). "Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of *Staphylococcus aureus*." Cell Host Microbe **10**(2): 158-164.

Koh, J. Y., S. W. Suh, B. J. Gwag, Y. Y. He, C. Y. Hsu and D. W. Choi (1996). "The role of zinc in selective neuronal death after transient global cerebral ischemia." Science **272**(5264): 1013-1016.

Krogh, A., B. Larsson, G. von Heijne and E. L. Sonnhammer (2001). "Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes." J Mol Biol **305**(3): 567-580.

Landfear, S. M. (2011). "Nutrient transport and pathogenesis in selected parasitic protozoa." Eukaryot Cell **10**(4): 483-493.

Leung, K. F., F. S. Riley, M. Carrington and M. C. Field (2011). "Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes." Eukaryot Cell **10**(7): 916-931.

Lu, M., J. Chai and D. Fu (2009). "Structural basis for autoregulation of the zinc transporter YiiP." Nat Struct Mol Biol **16**(10): 1063-1067.

Lu, M. and D. Fu (2007). "Structure of the zinc transporter YiiP." Science **317**(5845): 1746-1748.

Markle, W. H. and K. Makhoul (2004). "Cutaneous leishmaniasis: recognition and treatment." Am Fam Physician **69**(6): 1455-1460.

Martin-Sanchez, J., M. C. Lopez-Lopez, C. Acedo-Sanchez, J. J. Castro-Fajardo, J. A. Pineda and F. Morillas-Marquez (2001). "Diagnosis of infections with *Leishmania infantum* using PCR-ELISA." Parasitology **122**(Pt 6): 607-615.

Mason-Bahr, P. E. C. (1996). "Old World leishmaniasis." 206-217.

Matlashewski, G., B. Arana, A. Kroeger, S. Battacharya, S. Sundar, P. Das, P. K. Sinha, S. Rijal, D. Mondal, D. Zilberstein and J. Alvar (2011). "Visceral leishmaniasis: elimination with existing interventions." Lancet Infect Dis **11**(4): 322-325.

Maurer, M., B. Dondji and E. von Stebut (2009). "What determines the success or failure of intracellular cutaneous parasites? Lessons learned from leishmaniasis." Med Microbiol Immunol **198**(3): 137-146.

Mayer, M. and J. Buchner (2004). "Refolding of inclusion body proteins." Methods Mol Med **94**: 239-254.

McGwire, B. S. and A. R. Satoskar (2013). "Leishmaniasis: clinical syndromes and treatment." QJM.

McNicoll, F., M. Muller, S. Cloutier, N. Boilard, A. Rochette, M. Dube and B. Papadopoulou (2005). "Distinct 3'-untranslated region elements regulate stage-specific mRNA accumulation and translation in *Leishmania*." J Biol Chem **280**(42): 35238-35246.

Milon, B., Q. Wu, J. Zou, L. C. Costello and R. B. Franklin (2006). "Histidine residues in the region between transmembrane domains III and IV of hZip1 are required for zinc transport across the plasma membrane in PC-3 cells." Biochim Biophys Acta **1758**(10): 1696-1701.

Montanini, B., D. Blaudez, S. Jeandroz, D. Sanders and M. Chalot (2007). "Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity." BMC Genomics **8**: 107.

Moreno, J., C. Canavate, C. Chamizo, F. Laguna and J. Alvar (2000). "HIV--*Leishmania* infantum co-infection: humoral and cellular immune responses to the parasite after chemotherapy." Trans R Soc Trop Med Hyg **94**(3): 328-332.

Moreno, S. N. and R. Docampo (2009). "The role of acidocalcisomes in parasitic protists." J Eukaryot Microbiol **56**(3): 208-213.

Murgia, C., I. Vespignani, J. Cerase, F. Nobili and G. Perozzi (1999). "Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells." Am J Physiol **277**(6 Pt 1): G1231-1239.

Nies, D. H. and S. Silver (1995). "Ion efflux systems involved in bacterial metal resistances." J Ind Microbiol **14**(2): 186-199.

O'Halloran, T. V. and V. C. Culotta (2000). "Metallochaperones, an intracellular shuttle service for metal ions." J Biol Chem **275**(33): 25057-25060.

Olivier, M., V. D. Atayde, A. Isnard, K. Hassani and M. T. Shio (2012). "*Leishmania* virulence factors: focus on the metalloprotease GP63." Microbes Infect **14**(15): 1377-1389.

Omasits, U., C. H. Ahrens, S. Müller and B. Wollscheid (2013). "Protter: interactive protein feature visualization and integration with experimental proteomic data." Bioinformatics.

Palmiter, R. D. and S. D. Findley (1995). "Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc." EMBO J **14**(4): 639-649.

Palmiter, R. D. and L. Huang (2004). "Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers." Pflugers Arch **447**(5): 744-751.

Paulsen, I. T. and M. H. Saier, Jr. (1997). "A novel family of ubiquitous heavy metal ion transport proteins." J Membr Biol **156**(2): 99-103.

Requena, J. M. (2011). "Lights and shadows on gene organization and regulation of gene expression in *Leishmania*." Front Biosci (Landmark Ed) **16**: 2069-2085.

Ritmeijer, K., A. Dejenie, Y. Assefa, T. B. Hundie, J. Mesure, G. Boots, M. den Boer and R. N. Davidson (2006). "A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection." Clin Infect Dis **43**(3): 357-364.

Rohloff, P., A. Montalvetti and R. Docampo (2004). "Acidocalcisomes and the contractile vacuole complex are involved in osmoregulation in Trypanosoma cruzi." J Biol Chem **279**(50): 52270-52281.

Rosenzweig, D., D. Smith, P. J. Myler, R. W. Olafson and D. Zilberstein (2008). "Post-translational modification of cellular proteins during *Leishmania donovani* differentiation." Proteomics **8**(9): 1843-1850.

Sharma, U. and S. Singh (2008). "Insect vectors of *Leishmania*: distribution, physiology and their control." J Vector Borne Dis **45**(4): 255-272.

Silva, L. A., H. D. Romero, G. A. Nogueira Nascentes, R. T. Costa, V. Rodrigues and A. Prata (2011). "Antileishmania immunological tests for asymptomatic subjects living in a visceral leishmaniasis-endemic area in Brazil." Am J Trop Med Hyg **84**(2): 261-266.

Singh, S. (2006). "New developments in diagnosis of leishmaniasis." Indian J Med Res **123**(3): 311-330.

Suzuki, T., K. Ishihara, H. Migaki, K. Ishihara, M. Nagao, Y. Yamaguchi-Iwai and T. Kambe (2005). "Two different zinc transport complexes of cation diffusion facilitator proteins localized in the secretory pathway operate to activate alkaline phosphatases in vertebrate cells." J Biol Chem **280**(35): 30956-30962.

Suzuki, T., K. Ishihara, H. Migaki, W. Matsuura, A. Kohda, K. Okumura, M. Nagao, Y. Yamaguchi-Iwai and T. Kambe (2005). "Zinc transporters, ZnT5 and ZnT7, are required for the activation of alkaline phosphatases, zinc-requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane." J Biol Chem **280**(1): 637-643.

Teixeira, S. M., D. G. Russell, L. V. Kirchhoff and J. E. Donelson (1994). "A differentially expressed gene family encoding "amastin," a surface protein of *Trypanosoma cruzi* amastigotes." J Biol Chem **269**(32): 20509-20516.

Trouiller, P., P. Olliaro, E. Torreele, J. Orbinski, R. Laing and N. Ford (2002). "Drug development for neglected diseases: a deficient market and a public-health policy failure." Lancet **359**(9324): 2188-2194.

Ulrich, P. N., V. Jimenez, M. Park, V. P. Martins, J. Atwood, 3rd, K. Moles, D. Collins, P. Rohloff, R. Tarleton, S. N. Moreno, R. Orlando and R. Docampo (2011). "Identification of contractile vacuole proteins in *Trypanosoma cruzi*." PLoS One **6**(3): e18013.

Yamey, G. and E. Torreele (2002). "The world's most neglected diseases." BMJ **325**(7357): 176-177.

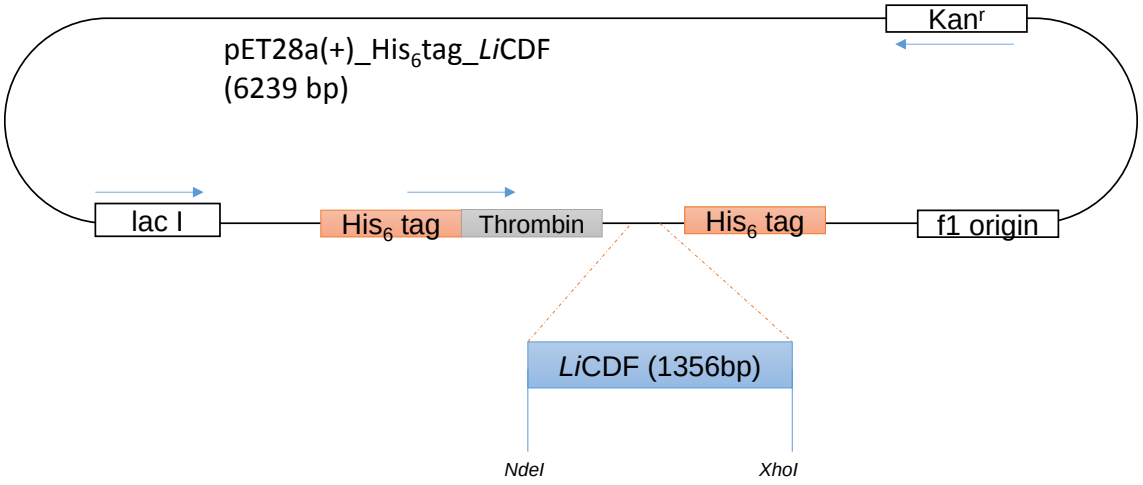
Zeytuni, N., R. Uebe, M. Maes, G. Davidov, M. Baram, O. Raschdorf, M. Nadav-Tsubery, S. Kolusheva, R. Bitton, G. Goobes, A. Friedler, Y. Miller, D. Schuler and R. Zarivach (2014). "Cation diffusion facilitators transport initiation and regulation is mediated by cation induced conformational changes of the cytoplasmic domain." PLoS One **9**(3): e92141.

Zhao, H. and D. Eide (1996). "The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation." Proc Natl Acad Sci U S A **93**(6): 2454-2458.

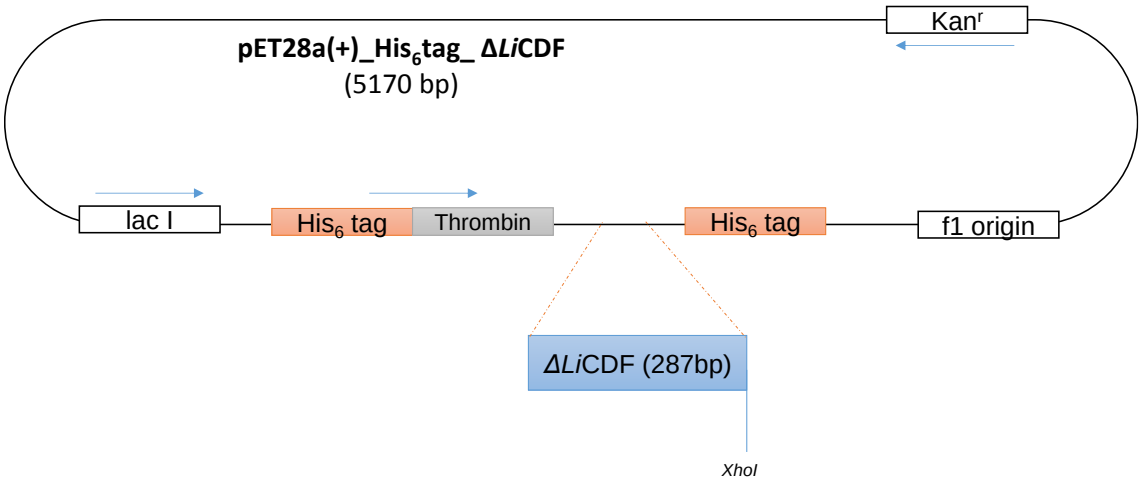
Zijlstra, E. E., A. M. Musa, E. A. Khalil, I. M. el-Hassan and A. M. el-Hassan (2003). "Post-kala-azar dermal leishmaniasis." Lancet Infect Dis **3**(2): 87-98. (2010).

WHO (2010). Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases. Geneva, Who.

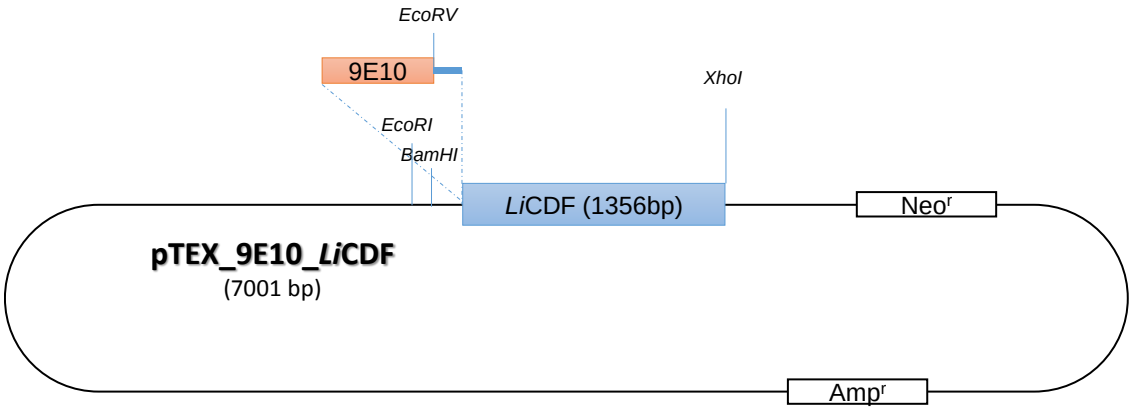
Supplementary Data



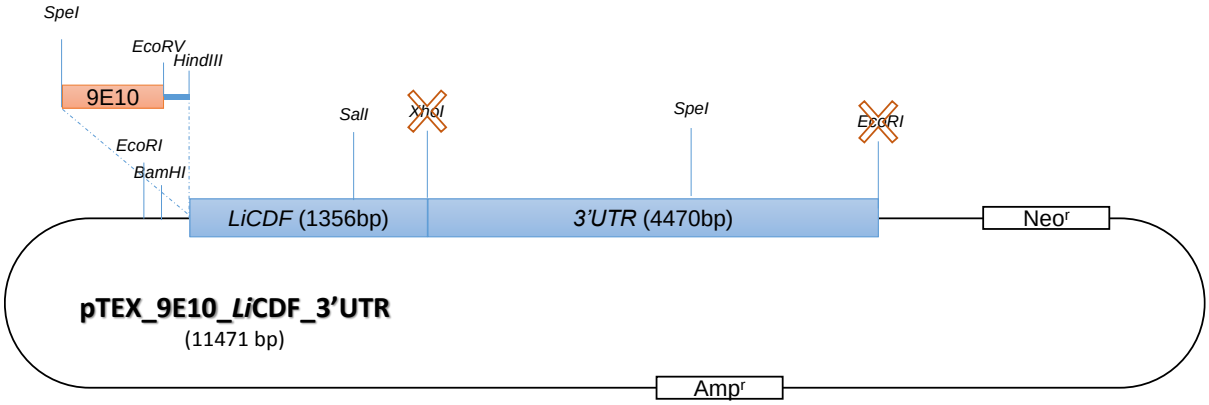
Supplementary Figure 1 - pET28a(+)_His$_6$tag_*Li*CDF plasmid construct.



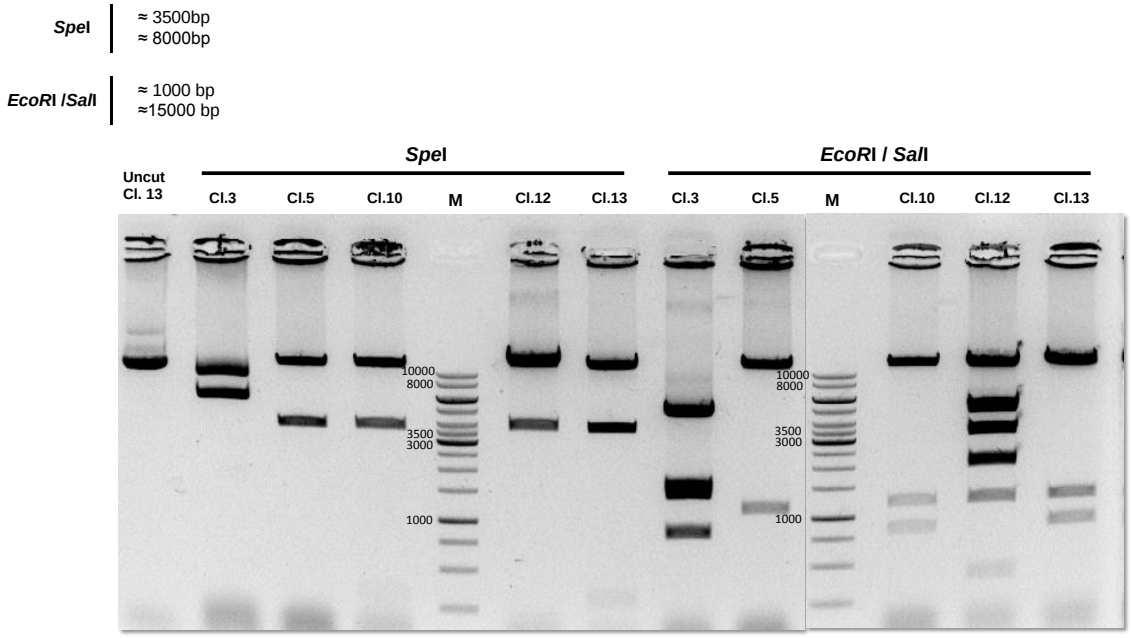
Supplementary Figure 2 - pET28a(+)_His$_6$tag_Δ*Li*CDF plasmid construct.



Supplementary Figure 3 – pTEX_9E10_*Li*CDF plasmid construct.



Supplementary Figure 4 – pTEX_9E10_*Li*CDF_3'UTR plasmid construct.



Supplementary Figure 5 – pTEX_9E10_*Li*CDF_3'UTR plasmid rescue and digestion of the resulting plasmids obtained from colonies 3, 5, 10, 12 and 13. On left top corner, the expected sizes are discriminated. Only colony 5 seems to meet the expected, the remaining tested clones do not match the same band pattern.