Studies on copper-loading protein and its role in virulence of *Leishmania donovani*

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CERTIFICATE

The research work embodied in this thesis **"Studies on copper-loading protein and its role in virulence of *Leishmania donovani*"** has been carried out in Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi-110067, India. This investigation is original and has not been submitted, so far, in part or full, for any degree or diploma of any university.

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Dedicated to my parents and Family…….

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Kavita Bharati

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LIST OF ABBREVIATIONS

β-ME	:	β-mercaptoethanol
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
AIDS	:	Acquired immune deficiency sundrome
ATCC	:	American Type Culture Collection
BCS	:	Bathocuproine disulfonic acid
BPS	:	Bathophenanthroline disulfonic acid
BOD	:	Biochemical Oxygen Demand
bp	:	Base pair
BSA	:	Bovine serum albumin
Cp	:	Ceruloplasmin
cDNA	:	Complementary DNA
Conc	:	Concentration
Cu	:	Copper
DAPI	:	4', 6-diamidino-2phenylinodole
DIG	:	Digoxigenin
DFO	:	Desferrioxamine
DMT1	:	Divalent metal transporter 1
DNA	:	Deoxyribonucleic acid
DTT	:	Dithiothreitol
ECL	:	Enhanced chemiluminescence
EDTA	:	Ethylene diamine tetra acetic acid
FBS	:	Fetal bovine serum
Fe	:	Iron
GFP	:	Green fluorescent protein
Hp	:	Hephaestin
HIV	:	Human immunodeficiency virus

HMA	:	Heavy metal associated domain
hr	:	Hour
IPTG	:	Isopropylβ-D-thiogalactoside
IRE	:	Iron response element
Kb	:	Kilo base
kDa	:	Kilo Dalton
LB	:	Luria Bertani medium
LD	:	*Leishmania donovani*
LDATP7	:	*Leishmania donovani* ATP7
LDMCO	:	*Leishmania donovani* multicopper oxidase
LIP	:	Labile iron pool
Li –Acetate	:	Litium acetate
LPG	:	Lipophosphoglycan
LPS	:	Lipopolysaccharide
M	:	Molar
M199	:	Medium199
mg	:	Miligram
ml	:	Millilitre
mM	:	Milimolar
min	:	Minute
MCO	:	Multicopper oxidase
mRNA	:	Messenger RNA
MQ	:	MilliQ water
N-terminal	:	Amino terminal
Ni^{2+}-NTA	:	Nickel-nitrilotriacetic acid
NaVan	:	Sodium vanadate
NRAMP	:	Natural resistance-associated macrophage protein
ROS	:	Reactive oxygen species
PAGE	:	Poly acrylamide gel electrophoresis

PEG	:	Polyethylene glycol
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PKDL	:	Post-kala-azar dermal leishmaniasis
PMSF	:	Phenylmethylsulfonyl fluoride
PV	:	Parasitophorous vacuole
PVDF	:	Polyvinylidene difluoride
RNA	:	Ribonucleic acid
RPMI	:	Roswell Park Memorial Institute medium
Rpm	:	Revolution per minute
RT	:	Room Temperature
RT-PCR	:	Reverse transcriptase polymerase chain reaction
Sec	:	Second
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulfate
SOD	:	Superoxide dismutase
SSC	:	Saline-Sodium Citrate
TBE	:	Tris borate, EDTA buffer
TGN	:	trans - Golgi network
TE	:	Tris EDTA
Tf	:	Transferrin
TfR	:	Transferrin receptor
Tris	:	Tris (hydroxylmethyl) – aminomethane
UV	:	Ultraviolet
Ura	:	Uracil
VL	:	Visceral leishmaniasis
WCL	:	Whole cell lysate
YPD	:	Yeast peptone dextrose
YNB	:	Yeast nitrogen base

Synopsis

Leishmaniases are diseases caused by protozoan parasites of the genus *Leishmania* order kinetoplastida. These are amongst most neglected tropical diseases with 2 million new cases occurring annually, 1.5 million of which are affected by cutaneous leishmaniasis and around 0.5 million by visceral leishmaniasis. *Leishmania* can cause a variety of diseases in human ranging from dermal lesion to visceral infection that is the most fatal form of the disease caused by *Leishmania donovani.* About 90 % of the world cases of visceral leishmanisis found in India, Bangladesh, Nepal, Sudan and Brazil. *Leishmania* parasite alternates its life between two different forms. One is elongated, flagellated, motile form found in the midgut of Phlebotomus sand fly vector and another is round, non flagellated, nonmotile form found in the phagolysosomal vacuoles of macrophages and reticuloendothelial cells of the vertebrate host. These forms are known as promastigote and amastigote, respectively.

Iron is an essential micronutrients for biological system, required as cofactor in many enzymes involved in maintaining homeostasis like oxygen metabolism, energy metabolism as well as in virulence. Biological significance of this metal is due to its involvement in redox reactions. Deficiency, overload or free form of the metal in cells can implicate several pathological conditions. This redox-active metal plays a significant role in host-parasite interaction as it is important for survival of both the pathogen and host. During infection an activated macrophage tries to limit access of iron to pathogen as a part of its defense mechanism, whereas the parasite needs to acquire iron from host iron pool for its survival and virulence. To do that intracellular pathogen needs to increase its iron uptake capacity. The mechanism of iron uptake from bacteria to higher mammals is mainly divided into two mechanisms, either by a siderophore/transferrin specific receptor pathway and/or by an iron oxidase/permease complex. Whereas, iron uptake by siderophores or by transferrin has been known for a longer period of time, the uptake of iron mediated by a multicopper oxidase (MCO) has been reported comparatively recently, first in yeasts (Askwith *et al.*, 1994; Stearman *et al.*, 1996) and later in mammalian cells (Mukhopadhyay *et al.*, 1998; Attieh *et al.*, 1999) as well as in algae *Chlamydomonas reinhardtii* (Herbik *et al.*, 2002), which underscores the importance of this pathway in evolution.

Iron uptake system in case of *Leishmania* is not well understood so far. It has been reported that *Leishmania amazonenesis* contains two identical genes lit1 and lit2 related to *Arabidopsis thaliana* irt1/irt2 genes of the ZIP family of Fe^{2+} transporter

(Huynh *et al*., 2006). These genes express only in its amastigote form after infecting into macrophages. But iron acquisition mechanism of *Leishmania* either in its promastigote form or during early stages of infection in macrophages is not known so far. Since, host macrophages actively sequester iron to limit availability to *Leishmania* the intracellular parasite may utilize high affinity iron uptake system to function in very low concentration of iron. Our laboratory found a unique multicopper oxidase in *Leishmania donovani*, which is regulated by cellular iron content and is essential for growth, virulence and survival of the parasite in both promastigote and amastigote forms (unpublished data; thesis of Dr. Sunil Solanki). Its role in virulence also confirmed in *in vivo* mice model as single allele LD-knock out (+/-) is less effective for infection and pathogenesis in balb/c mice. *Leishmania donovani* multicopper oxidase (LDMCO) has four copper binding sites and its activity and expression depends on availability of copper.

Copper is an essential trace element required in many metalloenzymes to maintain homeostasis of organisms. Copper serves as catalytic cofactors in redox chemistry of proteins required for growth and development. Despite being essential, copper is highly toxic because of its potential to produce reactive oxygen species (ROS) by Fenton chemistry. Copper is found in the mammalian ferroxidases ceruloplasmin and hephaestin as well as in other enzymes including cytochrome c oxidase, superoxide dismutase, tyrosinase, and lysyl oxidase. Like mammalian multicopper oxidases (MCO), copper is an important cofactor of all the MCOs in evolution. Copper depletion causes malfunction of MCOs to affect iron homeostasis. MCO is loaded with copper by a membrane copper transporter and is well characterized in yeast *Saccharomyces cerevisiae* as CCC2, a member of P-type ATPase family. In mammals the homologues of CCC2 are known as ATP7A and ATP7B or Menkes and Wilson disease gene products, respectively. In eukaryotes, copper-transporting P-type ATPases are involved in copper and iron homeostasis. Defective copper transporters are responsible for human Menkes disease and Wilson disease. These copper ATPases are members of the P_{IB} subfamily of P-type ATPases. These proteins hydrolyze ATP and use the γ-phosphate to form an acyl-phosphorylated intermediate, which is subsequently dephosphorylated by an intrinsic phosphatase activity. This cycle of phosphorylation and dephosphorylation provides the energy for cation translocation across lipid bilayers (Lutsenko and Kaplan, 1995).

Copper uptake and metabolism is mostly studied in yeast and mammalian system, but it is still unknown in *Leishmania*, an early eukaryote. Since our laboratory identified multicopper oxidase as essential for survival and virulence of LD, the present study was undertaken to identify the copper loading protein (putative LDATP7) and its functional characterization for better understanding of iron-copper relation in survival and virulence of the protozoan parasite *Leishmania donovani.*

The available gene database of *Leishmania major* and *Leishmania infantum* suggests presence of a putative copper transporting ATPase like gene on chromosome 33 of *Leishmania*. It shows 30% homology to *Saccharomyces cerevisiae* and 31.6 % homology to human at the amino acid level. P-type ATPases contain ATP binding, phosphorylation, phosphate domain and cation channel. Phosphorylation domain contains conserved aspartic acid residue, which undergoes phosphorylation during the transport cycle. This protein also possesses a conserved proline residue involve in energy transduction and ion binding. N terminal contains heavy metal associated domains Cys-X-X Cys motif involved in copper binding.

In this study LDATP7 gene was identified and cloned from *Leishmania donovani*. The initial effort to express the recombinant LDATP7 in bacterial system was unsuccessful but the N- terminal ~56 kDa peptide was expressed and purified to raise the antibody against LDATP7. Immunofluoresences studies revealed that in promastigote stage this protein is localized in flagellar pocket. This is important to note that LDMCO also localizes in flagellar pocket suggesting that LDATP7 and LDMCO may interact with each other for maintaining iron homeostasis in this parasite.

To understand the function of the LDATP7, single-allele knock out was created by targeted gene replacement using drug resistance gene Hygromycin and Neomycin. In result LD showed reduced growth rate and less ferroxidase activity in comparison to Wild type LD. Several attempts to knock-out both alleles were unsuccessful suggesting an essential role of LDATP7 in survival of LD. The episomal expression of LDATP7 in single allele knockout reversed the effect of gene deletion. Simultaneous expression of episomal expression of LDATP7 was also needed for knocking-out both the alleles. This strongly indicates that functional LDATP7 is essential for survival of LD and functional activity of LDMCO. The role of LDATP7

was further established by complementing Δccc2 yeast mutants in iron depleted conditions in which high affinity iron uptake was non functional due to the presence of copper-less apo form of yeast ferroxidase Fet3. This confirmed the function of LDATP7 as to incorporate copper in LDMCO for appropriate function in iron homeostasis.

In summary, in this study for the first time the role of copper transporting P- type ATPase LDATP7 in *Leishmania donovani* has been established. Interestingly, role of copper in leishmanial biology has not been explored *per se*. The current study also reconfirmed that LDMCO is unique in the family of ferroxidases in terms its direct role in the survival and virulence of an organism. Similarly, the role of any P-type ATPase in the survival of any other organism has not been revealed before. This study further extended the evolutionary conserved role of copper in maintaining iron homeostasis in protozoa that was not known before. More importantly, since LDMCO-LDATP7 axis is essential for the growth and virulence of *Leishmania donovani,* an effective inhibitor of this system should have potential for an alternative therapy for Kala-azar and related diseases.

Review of Literature

Leishmania and leishmaniases

Leishmaniases are a group of parasitic disease caused by different species of protozoan parasite *Leishmania*. Leishmaniases are vector borne disease transmitted by biting of female sand fly to vertebrate host. Almost 33 species of *Leishmania* transmitted by certain species of sand fly genus Lutzomyia in new world and Phlebotomus in old word. More than 20 different species of *Leishmania* are known to be pathogenic to humans. *Leishmania* is a member of kinetoplastida order and Trypanosomatidae family. *Leishmania* is a diploid organism which asexually divides by binary partition but there are a few recent reports on sexual reproduction in invertebrate sand fly stage (Akopyants *et al.*, 2009; Rougeron *et al.*, 2009).

According to World Health Organization (WHO) report 12 million people are infected and more than 2 million new cases of leishmaniases occur every year in 88 countries (http://www.who.int/health-topics/leishmaniasis.htm) of the tropical and subtropical region and 350 million people are at risk. The number of leishmaniases cases occurring outside of the endemic countries has been increasing due to tourism, military operations and the movement of immigrants from endemic countries (Palvia, 2010). Moreover, the rise of leishmaniasis is due to multiple factors including the AIDS and other immunosuppressive syndromes epidemic (Cruz, 2006), difficulties in controlling vectors, a lack of effective vaccines, international conflicts and the development of resistance to chemotherapy.

The disease has been around for a long time but *Leishmania* was first characterized during 1900-1903 when William Leishman, a British medical doctor and Charles Donovan, an Irish investigator found that splenic macrophages, of dum-dum fever patients in India, were heavily infected with trypanosomes like organisms. Initially these organisms called Leishman-Donovan bodies until Ronald Ross named it *Leishmania donovani* and proposed that these are intracellular form a new parasite. Charles Donovan suggested the link with kala- azar (Black fever) disease and Charles Bentley confirmed it by discovering *Leishmania donovani* in patients of kala-azar. In 1924 John Sinton discovered that distribution of specific species of sand fly is associated with visceral leishmaniasis. Swaminathan in 1942 shows that sand fly is responsible for transmission of *Leishmania* to human (Swaminathan *et al.*, 1942).

*Leishmani*a has been found as an opportunistic pathogen in AIDS patients with between 25 – 70% of adult visceral leishmaniasis in southern Europe (Montalban *et al*., 1990; Dedet *et al*., 1993). The co-infection of *Leishmania* and HIV has been observed to produce an atypical response to therapy (Corte ´s *et al*., 1997; Pe´rez-Molina *et al*., 1997) as well as unusual clinical presentations (Pe´rez-Molina *et al*., 1997). Leishmaniases is treated with pentavalent antimonial drugs namely sodium stibogluconate, meglumine antimonite which are first line drugs. Amphotericin-B, allopurinol antibiotics are second line of treatment. Treatment failure occurs as primary unresponsiveness occurring at the beginning of treatment or secondary unresponsiveness because of a relapse which might be fatal in most of the cases.

Leishmaniases is a spectral disease which comprise of distinct clinical manifestations depending on species of *Leishmania* and status of host's immune system. These manifestations are mainly divided in four forms cutaneous, mucocutaneous, visceral and post kala azar dermal (PKDL) (Figure A). Figure B shows the geographical distribution of different forms of leishmaniases.

Cutaneous : Cutaneous leishmaniasis is self healing form of disease. The sand fly bite causes skin ulcers at bite site, usually on exposed parts of the body, the face, neck, arms and legs. Different species produce cutaneous leishmaniasis in children and adults, primarily *L. major*, *L. tropica* and *L.(L) aethiopica* (old world cutaneous leishmaniasis); *L. infantum* and *L. chagasi* (Mediterranean and Caspian sea regions); and *L. mexicana*, *L. (L) amazonenesis*, *L. braziliensis*, *L. (V) panamensis*, *L. (V) peruviana and L. (V) guyanensis* (new world cutaneous leishmaniasis). Usually this form of disease is self limiting, but the time to lesion resolution varies between species and between individuals. Some species are also noted for causing non-healing cutaneous disease. Diffuse cutaneous leishmaniasis caused by *L. aethiopica*, *L. amazonensis* and *L. mexicana* occurs in anergic hosts with poor immune responses. Diffused cutaneous leishmaniasis occurs when lesions spread in large part of the body. This form of the disease is restricted to a few foci in Ethiopia, Kenya, Venezuela and the Dominican Republic suggest an important role for the genetics of the parasite as well as the genetics of the host in determining the disease phenotype. Infection is characterized by a primary lesion, which spreads to involve multiple areas of the skin with large numbers of parasites present in lesions (Kedzierski *et al.*, 2006).

Mucocutaneous : Mucocutaneous leishmaniasis (also known as espundia), usually caused by *L. braziliensis*. It begins as cutaneous form and initial skin lesions may cure, but the late development of metastatic lesions can lead to partial or total destruction of the mucous membranes. It causes an extensive disfiguring deterioration of mucous membranes of the nose, mouth and throat cavities and in extreme cases can result in impaired breathing. It can also involve destruction of cartilages. Mucocutaneous leishmaniasis may also arise after inadequate treatment of some *Leishmania* species, and if left untreated can lead to severe disfigurations or even death. Mucocutaneous leishmaniasis is widespread in South America and is about 5% of the cases which developed cutaneous leishmaniasis by *L. braziliensis*.

Visceral : Visceral leishmaniasis is the most fatal form of the disease also known as kala azar (black fever). It caused by *L. donovani* in the Indian subcontinent, Asia, and Africa (in adults and children), and by *L. infantum* or *L. chagasi* in the Mediterranean region, southwest and central Asia, and South America (primarily in young children); other species (e.g., *L. tropica* in the Middle East, *L. amazonensis* in South America) are occasionally viscerotropic. They infect the visceral organs and result in syndromes comprised of fever, weight loss, splenomegaly, hepatomegaly, pancytopenia, and hypergammaglobulinemia and anemia. If left untreated, the disease has a high mortality rate mainly due to immunosuppression and secondary infections (Murray *et al.*, 2005).

Post-kala-azar dermal leishmaniasis (PKDL) : This type of unusual syndrome appears within a few years of complete cure of visceral leishmaniasis. It is characterized by skin lesions which are macular, maculopapular or nodular, and usually spread from the perioral area to other areas of the body. PKDL patients are considered a major source of parasites for new infections because of large number of organisms in the skin are accessible to sandfly bites. In India, PKDL development was found in patients who were treated with sodium stibogluconate (SSG) and pentamidine, either with irregular or incomplete treatment of VL as well as with full course. Recent reports of PKDL in India are the one which developed after successful treatment of visceral leishmaniasis with miltefosine (Das *et al.*, 2009).

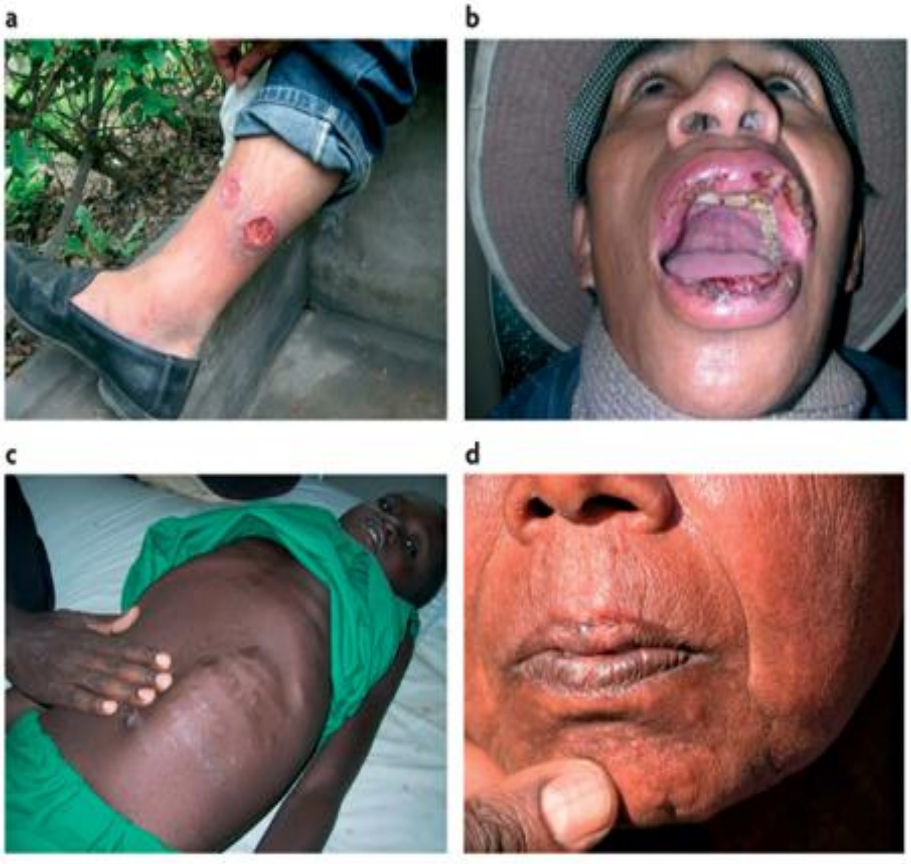


Figure A. Different forms of Leishmaniasis. *(a) Cutaneous (b) Mucocutaneous (c) Visceral (d) PKDL (Adapted from Chappuis et al., 2007).*

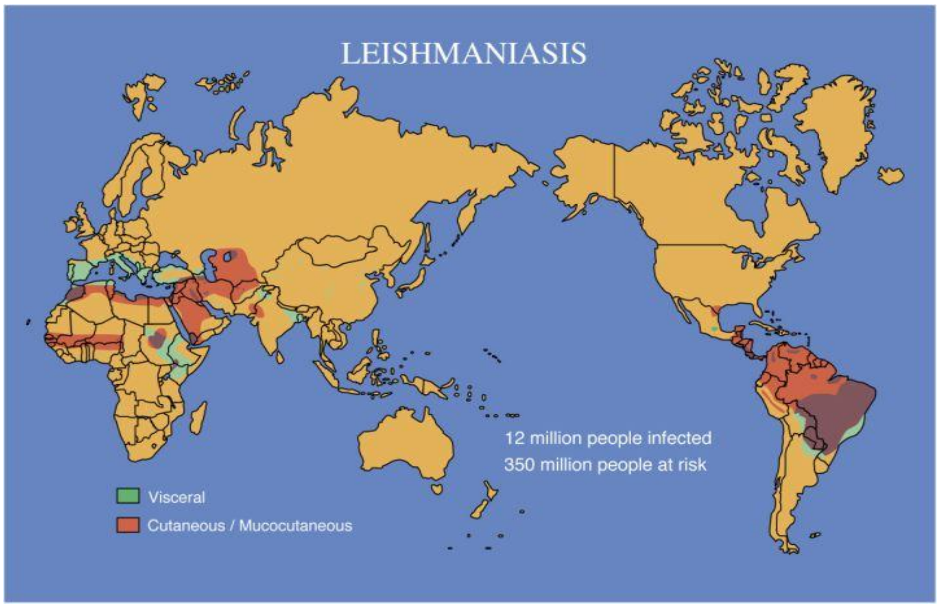


Figure B. Geographical distribution of Leishmaniasis. Source: http://www.who.int/ leishmaniasis/ disease_epidemiology/en/index.html

Life cycle of *Leishmania*

Leishmania has a digenetic life cycle (Figure C). The life cycle of the parasite includes an extracellular phase within the digestive tract of the invertebrate vector, promastigote and an intracellular stage inside the macrophages of a vertebrate host, amastigote (Lainson and Shaw, 1987) (Figure D). During development in the sandfly, promastigotes undertake a process known as metacyclogenesis, when procyclic promastigotes, which are dividing, non-infective forms differentiate into non-dividing infective metacyclic promastigotes (Sacks, 1989). *Leishmania* promastigotes has developed resistance mechanisms to protect itself from sandfly digestive system (Bates, 2008). Metacyclic promastigotes injected into host during blood meal of sand fly. The metacyclic promastigote are phagocytised directly by macrophage or after cycling through a wave of neutrophils (Zandbergen *et al.,* 2004). The reports show that promastigotes of *Leishmania donovani* and *Leishmania major* inhibit lipophosphoglycan (LPG) mediated phagolysosome biogenesis to evade the microbicidal phagolysosomal environment (Desjardins and Descoteaux 1997; Lodge *et al.,* 2006; Scianimanico *et al*., 1999; Spa¨th *et al*., 2003; Vinet *et al*., 2009). The surface of *Leishmania* parasite is enriched with glycophosphatidylinositol (GPI)-anchored macromolecules which are different in composition in different stages of life cycle (Ilgoutz and McConville, 2001). Promastigotes in sand fly vector stage contains extensive glycocalyx, comprised of complex glycoconjugates such as lipophosphoglycan (LPG) and a small number of glycoproteins such as GP63 (Joshi *et al*., 2002; Yao *et al*., 2003). Both LPG and GP63 are required for parasite survival in the vector. The LPG undergoes extensive modification during parasite metacyclogenesis (Saraiva *et al*., 1995). The surface of amastigote is comparatively less complex and contains lipid-anchored glycoconjugates, the glycoinositolphospholipids (GIPLs) (Ralton and McConville, 1998), while these have been shown to be non-essential for parasite survival in the mammalian host (Zufferey *et al*., 2003).

Phagocytosis of *Leishmania* proceeds through a subset of lipid-enriched membrane microdomains called caveolae, which are enriched in cholesterol, ganglioside M-1 (GM-1), GPI anchored proteins and caveolins-1, -2 and -3 (Harris *et al.*, 2002, Rodriguez *et al.*, 2006) . To withstand the oxidative stress generated by macrophages *Leishmania* contains unique low-molecular-mass thiols and redox proteins to

efficiently regulate the redox homeostasis (Irigoin *et al*., 2008). *Leishmania* parasites lack catalase and classical selenocysteine containing glutathione peroxidases for scavenging hydrogen peroxide and other hydroperoxides (Flohe *et al*., 1999; Schlecker *et al*., 2005; Castro and Tomas, 2008). Instead they contain trypanothione [T (SH) 2] for hydrogen peroxide detoxification (Fairlamb *et al*., 1985).

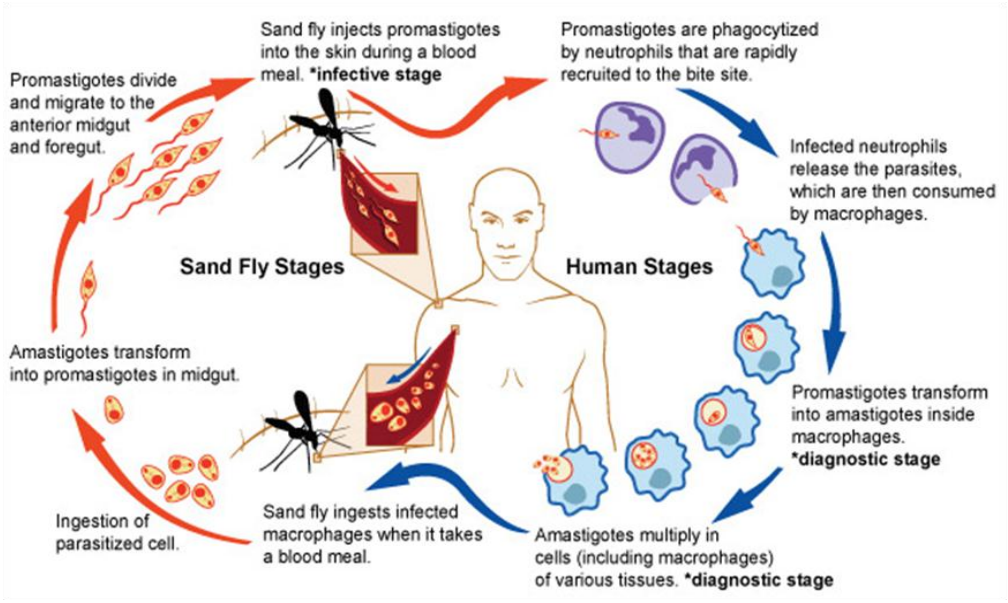


Figure C. Life cycle of *Leishmania.* *Sand fly satge and human stages of Leishmania showing infective stage (metacyclic promastigote) and diagonistic stage (amastigote transformation and multiplication). (Adapted from* http://www.niaid.nih.gov/topics/leishmaniasis/pages/lifecycle.aspx*).*

Within the macrophage parasite lives in parasitophorous vacuoles which fuses with lysosome to make phagolysosome. In the acidic conditions of phagolysosomes promastigotes transforms into round, nonflagellated amastigotes. Amastigotes keeps on multiplying within macrophages which cause bursting of infected macrophages and released infective amastigotes. These free amastigotes subsequently infects surrounding macrophages of reticuloendothelial cells and skin and continue to multiply. The female sand fly takes up the infective form of the parasite (amastigotes) during the blood meal from skin lesions or peripheral parasitemia and transmits it to new host. Other than this insect route, *Leishmania* can be transmitted via placental (Meinecke *et al.*, 1999), semen (Symmers, 1960) and by injection needles (Amela *et al.*, 1996). It is the commonest mode of transmission. Leishmaniasis can also be acquired by laboratory infections (Herwaldt *et al.*, 1993) and be transmitted

through the route of transfusion (Andre, 1958; Kostman *et al.*, 1963; Cohen *et al.*, 1991).

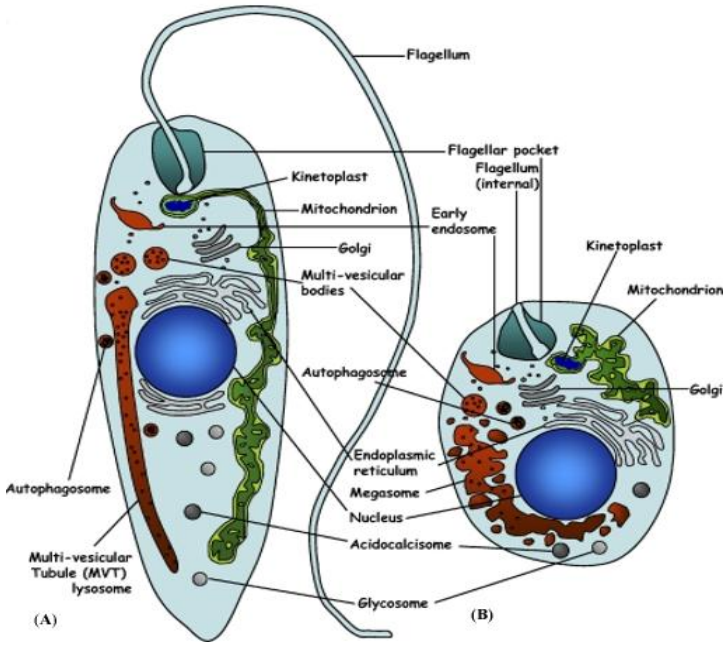


Figure D. Different forms of *Leishmania*. *Schematic representation of internal organization of promastigote (A) and amastigote (B) forms (Adapted from Besteiro et al., 2007).*

Role of iron in biological systems

Iron (Fe) is the second most abundant metal and comprises 5% of earth's crust. It is most versatile transition metal found in different oxidation states forms ranging from –II to +VI but mainly as II and III. Ferrous (Fe^{2+}) form is extremely water soluble, while Ferric (Fe^{3+}) form is quite insoluble in water (Ksp = 10−39M and at pH 7.0, [Fe3+] = 10−18M) and significant concentrations of water-soluble Fe^{3+} species can be attained only by strong complex formation. Iron (III) prefers hard oxygen ligands while iron (II) favours nitrogen and sulfur ligands to bind. In biological media, the hydrated ferrous ion is readily found where as hydrated ferric ion is relatively rare, although significant concentrations of $Fe(H_2O)_6^{3+}$ will be present at very low pH values.

Iron is important for the survival, replication and differentiation of the cells of animals, plants and almost all microorganisms with the exception of non–pathogenic *Lactobacilli* and pathogenic *Borrelia burgdorferi* (each of which use manganese in

place of iron.) Iron plays important role in various cellular processes such as electron transport, cellular respiration, synthesis of DNA, RNA and proteins, cell proliferation and differentiation and regulation of gene expression (Andrews *et al*.,1999; Boldt, 1999; Conrad *et al*.,1999; Gerlach *et al*., 1994; Wessling-Resnick, 1999), oxidation-reduction (alcohol dehydrogenase, dihydroorotate dehydrogenase, cytochrome oxidase) (Bezkorovainy, 1980; Scheibel, 1980), glycolysis (Sheibel and Adler, 1980), pentose shunt (Baily-Wood *et al*.,1975; Wrigglesworth and Baum, 1980) and proteolysis (Cook *et al.*,1961). Cellular iron deficiency arrests cell growth and leads to cell death because the requirement of iron in ribonucleotide reductase enzyme and other enzymes that are involved in cell division (Hoffbrand *et al.*, 1976). Iron is an essential component of oxygen carrier proteins such as hemoglobin, myoglobin, and leghemoglobin where it acts like an indispensable cofactor in their oxygen binding capacity (Jameson and Ibers, 1994). Inorganic iron is also important in redox reactions in the iron-sulfur clusters of many enzymes like nitrogenase and hydrogenase. Non-heme iron proteins includes the enzyme methane monooxygenase that oxidizes methane to methanol (Rocklin *et al.*, 1999; Wallar *et al.*, 1996), ribonucleotide reductase that reduces ribose to deoxyribose which is involved in DNA biosynthesis (Hoffbrand *et al.*, 1976), hemerythrins that is involved in oxygen transport and fixation in marine invertebrates (Kurtz *et al.*, 1977) and purple acid phosphatase that is involved in hydrolysis of phosphate esters (Stenkamp *et al.*, 1984). Iron is a cofactor of iron superoxide dismutase in microorganisms that plays an important role in protection against the oxidative damage (Paramchuk *et al.*, 1997). Thus, due to its involvement in many necessary functions, iron becomes an essential nutrient for the cell. Iron has an importance in biological systems as *E. coli* employ almost 50 genes to proteins involved in iron uptake, six distinct siderophore-mediated Fe^{3+} transport systems, one for iron uptake from ferric citrate, and one Fe^{2+} transport system, yet it synthesizes only one siderophore: enterobactin. In the case of blue-green algal blooms in the lakes the algal species with higher iron chelating capacity grows more than other species with low iron chelating capacity (Murphy *et al.*, 1976). In the case of mammalian tumor growth density of transferrin receptors which are required for iron uptake is detrimental factor for the tumor growth. The biological significance of iron is mainly because of its chemical properties as a transition metal. Abundance of iron also has a role in its biological importance although iron is not readily available because it mainly found as complexes. Ferrous and ferric form of

iron readily undergoes electron transfer and acid base reactions which render iron the capability to participate in variety of catalytic and other reactions. The efficiency of Fe (II) as an electron donor and of Fe (III) as an electron acceptor, with a redox potential compatible with the constrains of the cellular environment, is a fundamental feature form any biochemical reactions and renders iron to an essential mineral and nutrient. However, this very property turns iron into a potential biohazard, because under aerobic conditions, iron can readily catalyze the generation of noxious radicals. Iron's toxicity is largely based on Fenton and Haber-Weiss chemistry (Figure E),Where catalytic amounts of iron are sufficient to yield hydroxyl radicals ($OH^{\cdot}$) from superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), collectively known as reactive oxygen intermediates (ROIs) (Halliwell and Gutteridge, 1990). These free radicals are highly reactive species and may promote oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. Though it contributes to phagocyte mediated microbicidal activity (Miller and Britigan, 1997) of cell that may be helpful in fighting against pathogens but very harmful if remains uncontrolled.

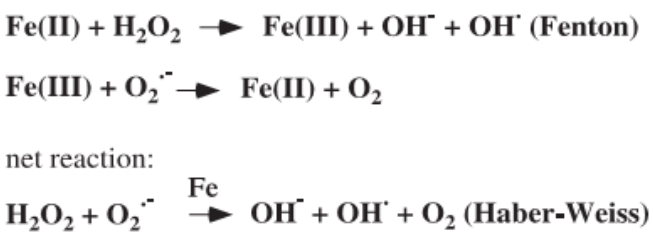


Figure E. Fenton and Haber-Weiss Reaction

Iron metabolism in mammalian system

Iron represents approximately 35 and 45mg/kg of body weight in adult men and women, respectively (Andrews, 1999 a, b: Bothwell *et al*., 1995). About 60-70% of total body iron is present in haemoglobin in circulating erythrocytes, about 10% is present in forms of myoglobins, cytochromes, iron - sulphur clusters and other iron containing enzymes, remaining 20-30% of iron is stored as ferritins and hemosiderins in hepatocytes and reticuloendothelial macrophages (conard *et al*., 1999).

In human iron deficiency causes anemia while an excess of iron is toxic and causes hereditary hemochromatosis, Parkinson's disease and Freidreich ataxia (Bleackley *et al.,* 2009). Since both cellular iron overload and iron deficiency causes cell death, the levels of reactive iron must be carefully controlled and limited. Most

pathologic consequences of systemic iron over load result from chronic iron accumulation in tissues. Thus an organism must sense its internal iron load to make a homeostatic balance of iron within it. Dietary iron in the form of haem and non-haem absorbed by duodenum. Inside the enterocytes iron is excised from haem-porphyrin ring by action of haem oxygenase.

Dietary non-haem Fe is largely present as less soluble and non-absorbable Fe^{3+} form and must be reduced to Fe^{2+} before it becomes bioavailable. This reduction is catalysed by both the dietary reducing agents (e.g. ascorbic acid) and an intestinal ferri-reductase (probably duodenal cytochrome b (DCYTB) (McKie *et al*., 2001). Reticuloendothelial macrophages engulf old erythrocytes by phagocytosis and subsequent lysosomal degradation in order to release haem- bound iron to recycle most of the iron. Free haem-bound iron in the form of haemo- or haptoglobin is taken up by macrophages through the haemoglobin-scavenger receptor (CD163) (Hentze *et al*., 2004). Fe^{2+} generated by these reducing mechanisms can be transported into the cell by divalent metal transporter 1 (DMT1) (Fleming *et al*., 1997; Gunshin *et al*., 1997), which is also present on the luminal membrane of duodenal enterocytes (Trinder *et al*., 2000).). If the body stores are adequate, Fe can be reoxidised to Fe^{3+} and stored in the enterocytes as ferritin. More recently, a mitochondrial ferritin has been identified, although its biological role is unclear. In the case of metabolic requirement Fe will enter a labile intracellular pool and be processed for transport out of the cell via a basolateral membrane export protein known variably as IREG1, ferroportin1 or MTP1 (Abboud and Haile, 2000; Donovan *et al*., 2000; McKie *et al*., 2000). Fe^{2+} leaving the enterocyte is immediately oxidised to Fe^{3+} either by plasma located ceruloplasmin or membrane bound hephaestin (Vulpe *et al*., 1999), and loaded on to transferrin (TF) for onward transport in the blood. Iron saturated form of TF (holo TF) contains two Fe^{3+} ions. The holo-TF binds to Transferrin receptor (TFR) and undergoes receptor mediated endocytosis. The TF-TFR complex delivers iron to the early endosome, where it is released for transport into the cytoplasm, probably through DMT1. The components apo TF and the TFR are recycled to the cell surface. To ensure iron availability and to eliminate the toxicity of free iron in addition to its accessibility for invading pathogens, mammals have evolved a strictly regulated system for iron homeostasis. Figure F depicts the iron metabolism in mammalian system.

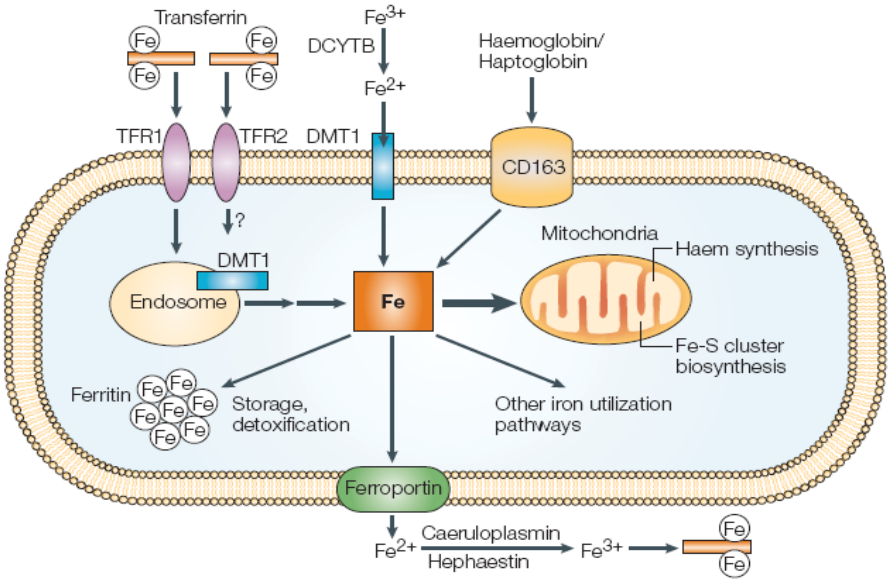


Figure F. Iron metabolism in the mammalian system. *The iron-saturated form of transferrin, holotransferrin, binds to the transferrin receptor (TFR), which is expressed on the surface of the cell. Iron is transferred to the early endosome, where it is exported into the cytoplasm, probably through DMT1. In the intestinal lumen, ferric iron (Fe3+) is reduced to ferrous iron (Fe2+) by the cytochrome-b-like reductase DCYTB. DMT1 then facilitates the absorption of Fe2+ from the lumen into the cytoplasm. In the cytoplasm, iron is stored bound to ferritin. In macrophages, haem-bound iron - in the form of haemoglobin or haptoglobin - is taken up by the cell through the haemoglobin scavenger receptor (CD163). Iron is exported from the enterocyte by a permease known as ferroportin. For circulation in the body, Fe2+ is oxidized to Fe 3+ by either the intestinal membrane-associated oxidase, hephaestin, or the plasma-located oxidase, caeruloplasmin. In the cell, the synthesis of haem and Fe-S clusters takes place in the mitochondria. (Adapted from Schaible et al., 2004).*

Iron metabolism is meticulously regulated and this is ensured, in part, by two iron regulatory proteins (Rouault, 2006). These molecules bind to iron-responsive elements (IREs) of mRNAs that encode proteins involved in iron uptake (TfR1 and DMT1), utilization (erythroid d-aminolevulinic acid synthase), storage (ferritin) and export (ferroportin-1). Hepcidin, a circulating peptide hormone, is a key player in the regulation of systemic iron homeostasis. Hepcidin regulates cellular iron efflux by binding to ferroportin-1 and inducing its internalization (Nemeth *et al.*, 2004). Hepcidin is induced by dietary iron overload, as well as by lipopolysaccharides from Gram-negative bacteria.

Role of iron in host and parasite interaction

Iron is a ubiquitous element in biological systems and intimately involved in many aspects of immunity (Doherty *et al.*, 2002; Farthing, 1989; Weiss, 2002;

Oppenheimer, 2001) and is a key regulator of host-pathogen interactions. Iron is essential for growth of almost all microorganisms including the pathogenic one. Iron acquisition is the fundamental step in the development of a pathogen. Iron is important for microbicidal responses of host because of its participation in generation of toxic oxygen and nitrogen intermediates, by Fenton reaction. The competition for iron between the pathogen and the host is an exciting and complex phenomenon, which includes a variety of different tactics on both sides. Both host and pathogen possess complex mechanisms to acquire iron and control flux and availability which lead to ongoing battle for iron. Host cell withholds iron by positioning of iron binding proteins, lactoferrin and transferrin in all body fluids except urine (Weinberg, 1984, 1990). Iron binding proteins lactoferrin and transferrin maintains the low iron state. Host cell uses different methods of iron sequestration to limit the growth of invading pathogens. The low iron environment is essential for the efficient functioning of natural defense mechanisms of host cell. The pathogens have to adapt the low iron environment to multiply in the host. Pathogens can acquire iron from the host mainly by two methods. One is using siderophores, which are low molecular weight compound and have high affinity for the host protein- iron complexes and are able to remove iron from these complexes. Second, can directly contact with the host iron source and utilizes the iron by releasing it from the host protein by enzymatic method (Philpott, 2006; Wandersman and Delepelaire, 2004). Some microbes synthesize several different siderophores while others synthesize only receptors for ferric-siderophore complex. These organisms rely on siderophores produced by other organisms. In pathogens there is high degree of genomic investment in systems for iron acquisition. These systems are present in vast range and have very low iron-binding constants which permit them to be effective even in highly iron-depleted environments (Ratledge and Dover, 2000).

Bacteria of the enteric genus produce enterochelin, which has the highest affinity of any siderophore. *Shigella, Escherichia, Yersinia, Neisseria*, and *Hemophilus* species have transport systems for transferrin or lactoferrin so that they can directly use heme and hemoproteins as a source of iron. Low iron condition in the host signals pathogenic bacteria to induce virulence factors e.g. Shiga-like toxin and haemolysins (haemolysins could also help to obtain iron from lysed cells). *Mycobacterium tuberculosis* resides within the phagosome of macrophage and prevents phagosome

lysosome fusion and secretes siderophore mycobactin and exochelin which helps in iron acquisition (Olakanmi, 2002). *Listeria monocytogenes* contains TF like siderophores that must have higher affinity for iron than ferritin (Bockmann *et al*., 1996). It has been found that a *Yersinia* high pathogenicity island encodes an iron uptake system, which is widely distributed among pathogenic Enterobacteriaceae and are responsible for the increased virulence of some strains of *Escherichia coli* (Schubert *et al*., 2002). Pasteurellaceae and Neisseriaceae have an entirely different method of iron acquisition, a direct interaction of the bacterial cell with the iron binding protein.

It has been reported that iron overload is associated with increased frequency and aggravates the outcome of many microbial and viral infection (Drakesmith and Prentice, 2008). *In vitro* studies show increased virulence in case of increased iron availability. Human with iron overload disease e.g. hemochromatosis complicates bacteremias with *Yersinia* and *Vibrio* species (Kelly *et al.*, 1987). There are also reports that malaria may be less frequent and less severe in iron deficient patient (Nyakeriga *et al*., 2004). McFarlane and coworkers suggested that in patients with low serum transferrin concentration, iron therapy resulted in a high transferrin saturation that promoted bacterial infection. (McFarlane *et al.*, 1972). NRAMP1, a late endosomal membrane protein in murine macrophages, contribute in innate defense response against various phagosomal pathogens, including *Salmonella typhimurium*, *Mycobacterium bovis*, *bacillus* Calmette–Guerin (BCG) and *Leishmania major* (Canonne-Hergaux *et al.*, 1999). Mutations in mouse Nramp1 gene increase the susceptibility to infection. NRAMP1 functions as a divalent-metal-ion transporter and is believed to deplete late endosomal compartments of iron by transporting it into the cytoplasm (Hackam *et al.*, 1998).

Role of copper in iron metabolism

In biological system, copper (Cu) presents in two oxidation states cuprous ($Cu^{+)}$ and cupric (Cu^{2+}). Cu^{2+} is fairly soluble, while solubility of Cu^{+} is in the sub-micromolar range. Cu^{2+} can readily accept an electron from strong reductants such as reduced glutathione and ascorbate (Galhardi *et al*., 2004). In biological systems three spectroscopically distinct classes of copper found which designated as Type 1, 2 or 3. These can be found alone or in combination. Copper is able to function as a critical

catalyst in metabolism. The copper proteins have a diverse range of function and tissue expression which shows an important role for copper in mammalian systems. Cu is an essential cofactor in a number of critical enzymes in metabolism, including superoxide dismutase (Cu/Zn-SOD), cytochrome c oxidase (COX), lysyl oxidase and ceruloplasmin (Cp) (Tainer *et al*., 1983; Linder and Hazegh-Azam, 1996; Kaplan and OHalloran, 1996; Rucker *et al*., 1998). Copper/zinc- superoxide dismutase (Cu/Zn-SOD) is an antioxidant required for the dismutation of superoxide radicals to hydrogen peroxide. Cu/Zn-SOD expressed ubiquitously and represents 1% of the total cell's proteins (Pardo *et al*., 1995). Genetic defects in Cu/Zn-SOD are associated with amyotrophic lateral sclerosis (ALS) (Waggoner *et al.*, 19992). Cytochrome c oxidase is essential for mitochondrial respiration. Tyrosinase has role in melanin production. Lysyl oxidase is required for collagen and elastin cross linking. The multicopper oxidases ceruloplasmin and hephaestin represent a family of copper-containing proteins which regulate the efficiency of iron transport. Dopamine β-hydroxylase has role in catecholamine production. Peptidylglycine α-amidating monooxygenase (PAM) is necessary for neuropeptide and peptide hormone processing.

There is a close relationship between the biology of copper and iron. Copper deficiency alters body iron metabolism. Central component of the physiological link between iron and copper is multicopper oxidase (MCO). Copper deficiency or defects in copper transporters; disrupt copper incorporation into many copper proteins including the multicopper ferroxidases (Yuan *et al*., 1995; 1997). The MCOs has been identified in several eukaryotes. Fet3p (Askwith *et al*., 1994; Blackburn *et al*., 2000; de Silva *et al*., 1995, 1997; Hassett *et al*., 1998) and Fet5p (Spizzo *et al*., 1997; Urbanowski and Piper, 1999) in *Saccharomyces cerevisiae* represent the ferroxidases found in lower eukaryotes. Homologues of Fet3 are present in the genomes of the yeasts *Schizosaccharomyces pombe* Fio Ip (Askwith and Kaplan, 1997), *Candida albicans* CaFet3p, and *Arxula adeninivorans* Fet3 homologue. In mammalian system ceruloplasmin (Cp) and Hephaestin (Hp), and zyklopen play essential and non overlapping roles in liver and intestinal and placental iron export, respectively (Eisenstein, 2000; Chen *et al*., 2010). *Drosophila melanogaster* express three proteins that contain all of the copper liganding motifs common to the multicopper oxidases but contains only one possible ferroxidase motits (Lang *et al*., 2012). Similarly F21D5.3 protein from

Caenorhabditis elegans and the three proteins from *Arabidopsis thaliana* all are clearly multicopper oxidases but are otherwise similar to neither the mammalian nor the yeast ferroxidase enzymes. These proteins may be functional laccase or amine oxidase homologues and play no role in the iron metabolism of their respective organisms.

The MCO depends on a copper ATPase, found in the membrane of specific vesicular compartment, for copper incorporation. These copper ATPase transporters depend on copper chaperone which gets copper from Cu^{+} specific plasma membrane transporter (CTR1) (Figure G). Copper homeostasis is tightly regulated. Dysfunction in any of these steps leads to the impact on copper incorporation into MCO which will affect iron homeostasis.

For copper there is no physiological store therefore the body levels are maintained by balancing the dietary absorption, distribution and utilization, with biliary excretion of excess copper. Copper is important for haemoglobin formation in rats feeding on milk based diet (Hart *et al*., 1928). Copper deficiency causes lowering of body iron content and hampering iron distribution to tissues in growing piglets (Gubler *et al*., 1956). Thus a copper deficiency is associated with anemia in animals and in human anemia, neutropenia and bone abnormalities. In domestic animals copper overload is associated with haemolysis. Anemias resulting from copper or iron deficiency display remarkable similarity in haematological features (Smith and Medlicott, 1944; Cartwright *et al*., 1956) which suggests the presence of a common pathway in the aetiology of these diseases. Initially, it was suggested that the common factor in disease progression is a coppper-dependent catalytic process. This lead to the isolation of ceruoplasmin. Ceruloplasmin act as a ferroxidase, converts Fe^{2+} to Fe^{3+} (Curzon and O'Reilly, 1960), and increase the rate of loading of iron onto transferrin (Osaki *et al*., 1966). This copper-dependent anemia was unresponsive to iron supplementation but was corrected on administration of ceruloplasmin (Lee *et al*., 1968). Ceruloplasmin is crucial for the mobilization of iron from the body stores for its metabolic utilization (Osaki and Johnson, 1969). There are reports showing that absence of ceruloplasmin does not produce marked changes in copper metabolism. But causes gradual accumulation of iron in the liver and other tissues (Harris *et al*., 1995).

Copper deficiency alters body iron metabolism via effects on the ferroxidase activity of ceruloplasmin. In liver copper is incorporated into ceruloplasmin via ATX1 homologue/ ATP7B dependent pathway. Thus multicopper oxidase-dependent ferroxidase activity is essential for eukaryotic iron homeostasis (Askwith *et al*., 1996; Harris *et al*., 1995; 1998., Wessling-Resnick, 1999). Ceruloplasmin contains 95% of the total plasma copper but is not a copper transport protein and is responsible for tissue iron efflux. More recently, the key role of ceruloplasmin in iron metabolism has been confirmed in studies on human patients and mice displaying disrupted ceruloplasmin production leads to aceruloplasminaemia, an autosomal recessive disorder that results in imbalances in body iron metabolism, with symptoms that include high serum ferritin (indicative of high tissue iron levels) and mild anaemia (Miyajima *et al*., 1987). Other studies, found that copper deficiency does not affect Cp mRNA expression (McArdle *et al*., 1990; Mercer *et al*., 1991) but dramatically decreases enzymatic activity. A defect in Hp leads to decreased intestinal iron efflux as observed in sex linked anemia (sla) mice (Vulpe *et al*., 1999).

Two possible copper transport mechanisms have been identified in intestinal cells, human Ctr1 (Lee *et al*., 2000) and DMT1 (Gunshin *et al*., 1997), but the relative roles of these two transport proteins in overall copper transport is unclear. Copper absorption and excretion are tightly regulated to maintain a relatively constant body copper content (Turnlund *et al*., 1989, 1998). In the light of these findings a number of research groups have studied the effects of copper loading or deficiency on the expression of Ctr1 and DMT1 in various model systems. Human Ctr1 belongs to a family of high-affinity copper transporters found in a diverse range of organisms from mammals to yeast and plants (Sharp, 2003). In yeast Ctr1 is tightly regulated at the transcriptional level by the copper content of the local environment (Dancis *et al*., 1994). Recent work suggests the existence of competition between copper and iron for transport via DMT1 (Tandy *et al*., 2000; Tennant *et al*., 2002; Arredondo *et al*., 2003). In copper deficient rats there is a decrease in ferritin protein level in enterocytes that leads to a reduced mucosal non-haem iron content (Thomas and Oates, 2003). In Caco-2 cells, (polarised intestinal epithelial cells) induction of copper deficiency stimulates iron uptake across the apical membrane (Zerounian and Linder, 2002). This finding is in contrast to those of previous animal studies, which shown effect of copper deficiency on the uptake step in iron absorption (Lee *et al*., 1968).

Interestingly, when iron deficiency is induced in Caco-2 cells Cu uptake is increased (Linder *et al*., 2003) and furthermore, when these cells are exposed to high copper levels iron uptake is markedly decreased (Tennant *et al*., 2002), suggesting that the absorption of these two metals may be closely related. Furthermore, when dietary copper levels are elevated the expression of the IRE-containing isoform of DMT1 is decreased. The combined effect of these first two stages is to decrease both iron and copper uptake into the enterocyte. In order not to compromise iron status it is believed that basolateral iron efflux is upregulated. IREG1 levels are increased by exposure of cells to high copper (possibly as a result of transcriptional regulation of the gene) leading to increased efflux of copper from the cell. Copper absorption and excretion are tightly regulated to maintain a relatively constant body copper content (Turnlund *et al*., 1989, 1998). Alga *Chlamydomonas reinhardlii* exhibits an adaptive metabolism of switching copper containing plastocyanin to iron containing cytochrome c6 in copper deficient conditions (Merchant *et al*., 1991). A recent study shows the *Yersinia* siderophore yersiniabactin (Ybt) which is meant for Fe binding can also bind with Cu^{2+} in human urine. This Cu^{2+} binding protects uropathogens during infection (Chaturvedi *et al*., 2012).

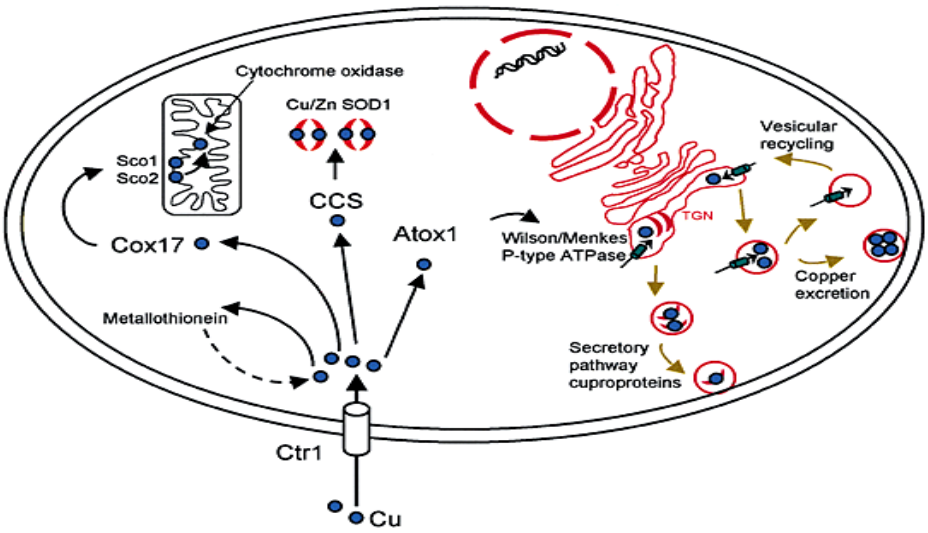


Figure G. Copper trafficking pathways within a mammalian cell. *The three principle copper chaperones, Cox17, CCS and Atox1 are shown along with the respective protein targets, cytochrome c oxidase, SOD1 and the Wilson and Menkes copper transporting ATPases. The mechanisms regulating initial copper uptake into the cell via the membrane transporter Ctr1 and the subsequent distribution of this metal to each chaperone remain unknown. (Adapted from Bartnikas et al., 2001).*

Role of Iron in *Leishmania* biology and infection

During its life cycle *Leishmania* encounters various hostile conditions such as oxidative stress due to heme digestion in blood meal (Vincent, 1989) and mid gut protease in the sand fly (Kamhawi, 2006), complement lysis in the blood upon transmission (Dominguez *et al*., 2003) reactive oxygen and nitrogen species during phagocytosis of host macrophage (Murray and Nathan, 1999). To combat the oxidative burst produced by host macrophages the parasites contain metalloenzyme superoxide dismutase (SOD) to detoxify O_2^- by converting it into H_2O_2 and H_2O (Fridovich, 1978). Superoxide dismutases contain Cu/Zn, Mn or Fe metals as their cofactors. Trypanosomatids are devoid of Cu/Zn and Mn dependent SOD instead they express Fe dependent SOD (Dufernez *et al*., 2006; Wilkinson *et al*., 2006). Several species of *Leishmania* including *L. chagasi, L. donovani* (Getachew and Gedamu, 2007*), L. aethiopica* (Genetu *et al*., 2006) and *L. major* (Ivens *et al.*, 2005) possesses SOD genes in their genome. *Leishmania* contains SOD B1 and SOD B2 which are developmentally regulated. *Leishmania* possesses a mitochondrial ascorbate-dependent peroxidase (APX) a hemoprotein, which can metabolize hydrogen peroxide but not organic peroxides (Wilkinson *et al*., 2002; Adak and Datta, 2005). *Leishmania* APX appears to have a role in the protection of mitochondrial membrane lipids from oxidative stress. Uptake of iron is required for antioxidant function of SOD and other essential metabolic reactions.

Iron is required for *in vitro* growth of both the intracellular and extracellular forms of *Leishmania* species (Chang and Hendricks, 1985). Iron is mostly supplied in the form of hemin or other heme-containing compounds *in vitro*. However, the iron sources in mammalian host or insect vector have not been defined. *Leishmania* species are defective in the heme biosynthetic pathway and thus require exogenous heme for growth (Chang and Chang, 1985). *Leishmania mexicana* shows the presence of receptors that facilitates the binding of heme or other metalloporphyrin compounds (Galbraith *et al.*, 1988) and heme-bound iron to promastigotes. It has been reported that *Trypanosoma brucei and* three species of *Leishmania* comprise of transferrin receptors ((Schell *et al.*, 1991, Voyiatzaki and Soteriadou, 1990, 1992)

Leishmania chagasi promastigotes can acquire iron from hemin, ferrilactoferrin, or ferritransferrin and other physiologic or nonphysiologic iron chelates (Britigan *et al.,*

1994). This capacity to utilize several iron sources facilitates the organism to survive in the diverse environments and adapt different growth conditions. Promastigotes preferentially took up iron in a reduced rather than an oxidized form, suggesting that extracellular iron must be reduced prior to internalization. An NADPH-dependent iron reductase activity has been detected in *Leishmania*, which indicates reductase-dependent iron uptake from transferrin and lactoferrin. Wilson *et al.*, 2002 showed the presence of a parasite associated or secreted reductase that reduces ferric to ferrous iron, decreasing its affinity for the extracellular chelate and allowing it to be readily internalized by the parasite. *Leishmania* encounters diversity of environments during its life cycle it is able to use several iron sources transferrin, lactoferrin or haemin.

Leishmania donovani promastigotes binds to haemoglobin in a saturable manner via a specific receptor, the binding is independent of the presence of transferrin, haemocyanin or myoglobin (Sengupta *et al.*, 1999). After initial binding to the sites localized within the flagellar pocket, haemoglobin is rapidly internalized (Sengupta *et al.*, 1999).The endocytosis of haemoglobin is regulated by small GTP-binding proteins of the Rab family (Singh *et al.*, 2002). Furthermore, the fusion of early endosomes containing haemoglobin with late endosomes requires a signal mediated through the cytoplasmic tail of the haemoglobin receptor (Singh *et al.*, 2002). During the intracellular stage of life cycle *Leishmania* has to deal with harsh environment of mammalian phagolysosome where divalent metal ions removed by the natural resistance-associated macrophage protein1 (Nramp1) transporter (Forbes and Gros, 2001). To deal with the iron restricted environment amastigotes possess a highly effective iron-uptake system. Recently a *Leishmania* iron transporter (LIT) member of the ZIP (Zrt, Irt-like protein) family of ferrous iron transporters LIT1 was described in *Leishmania amazonensis* (Huynh *et al.*, 2006). This transporter is found only in amastigotes and is essential for the development of pathogenic lesions in mice, but is dispensable for growth and differentiation in axenic culture (Huynh *et al.*, 2006). Further a ferricreductase (LFR1) has been identified in *Leishmania amazonensis* that encodes a membrane protein with ferricreductase activity and acts in concert with the ferrous iron transporter LIT1 (Flannery *et al.*, 2011). The ferric reductase activity of LFR1 is required for the differentiation of *L. amazonensis* into forms capable of initiating infections in the mammalian host and also can be detected on the cell surface of several *Leishmania* species. Till date, no efficient iron uptake system meant

for both promastigotes and amastigotes was reported. A multicopper oxidase mediated high affinity transport is involved in iron transport in promastigotes form and immediate after entry into macrophages (Solanki *et al.*, unpublished data) has been identified in *Leishmania donovani.* It has been reorted that *Leishmania donovani* depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth (Das *et al.*, 2009).

P-type ATPase

The ion motive ATPases has been divided into three different classes the F- type ATPaes (ATPases of bacteria, chloroplast and mitochondria) required for ATP synthesis while V-type ATPases (vacuolar and lysosomal) and P- type ATPases use energy derived from ATP hydrolysis to generate ion gradients. P-type ATPases are well documented in animal, higher plants and fungi.

P- type ATPases are divided into five families (P_I-P_V) which are further divided into subfamilies based on particular ion specificity, membrane topology, sequence and several regulatory domains (Kuhlbrandt, 2004). The P_I group is divided into P_{IA} bacterial Kdp-like ATPases, and P_{IB} heavy metals (Cu^+, Ag^+, Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}) (Axelsen and Palmgren, 1998; Rensing *et al.*, 1999). Group P_{II} includes sarcoplasmic reticulum (SR) Ca^{2+}-ATPases, plasma membrane Ca^{2+}-ATPases, Na^+/K^+ and H^+/K^+-ATPases. Group P_{III} contains H^+ and Mg^{2+} transporters. Group P_{IV} members are lipid transporters. Substrate specificity of group P_V is unknown. The salient features of P- type ATPase include six to ten transmembrane α-helices, one ATP binding domain, and the presence of a highly conserved sequence (DKTGT) in the large cytoplasmic loop. P- type ATPases transports cations against their concentration gradient by using ATP driven energy. Their catalytic cycle involves conformational transition states (E1 -E2) and formation of a covalent acylphosphate intermediate in Aspartate residue of DKTG consensus sequence (Figure H) (Axelsen and Palmgren, 1998; Inesi *et al.*, 1985; Glynn, 1985; Lutsenko and Kaplan, 1995). The formation of a phosphorylated intermediate is a signature property of these transporters, which imparts name to this family. The members of P_{1B} subfamily ubiquitously found in bacteria (Rogers *et al.*, 1991; Okkeri and Haltia, 1999; Rensing *et al.*, 2000), archaea (Mandal *et al.*, 2002), yeast (Rad *et al.*, 1994; Catty *et al.*, 1997), plants (Tabata *et al.*, 1997; Thomine *et al.*, 2000; Eren and Arguello 2004;

Gravot *et al*., 2004) and animals (Bull *et al*., 1993; Vulpe *et al*., 1993; Wu *et al*., 1993; Lutsenko and Petris, 2003)) and are vital for transition metal homeostasis.

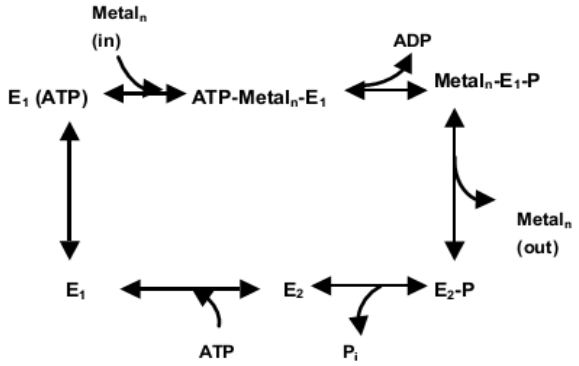


Figure H . Scheme of the catalytic cycle of PIB -type ATPases. *E1and E2 are conformations of the ion pump with ion-binding sites facing the cytoplasm and extracellular medium, respectively. The metal ion is translocated using the energy of generated by ATP hydrolysis, which triggers conformational changes of the enzyme. Enzyme phosphorylation and ephosphorylation occur on the cytoplasmic side of the protein. (Adapted from Voskoboinik et al., 2001).*

Wilson disease Protein and CCC2 protein: Copper transporting P-type ATPase

Copper is an essential trace element but excess of copper can be toxic to the cell therefore there is a need of tightly regulated homeostasis of copper. The knockout of *Arabidopsis thaliana* Cu -ATPase genes produce significant physiological alterations and in some cases found lethal (Hirayama *et al.*, 1999; Shikanai *et al*., 2003). In bacteria, these proteins proved to be necessary to confer tolerance and viability when exposed to high copper levels (Rensing and Grass, 2003; Rensing *et al.*, 2000).The two extensively studied and well characterized copper transporting proteins ATP7A and ATP7B which are related with human autosomal recessive disorders Menkes and Wilson disease, respectively (Hsi and Cox, 2004; Lutsenko and Petris, 2003). ATP7A and ATP7B belong to the P_{1B} subfamily of cation transporting P- type ATPases. Both ATP7A and ATP7B transports copper (Voskoboinik *et al.*, 2001, 1998; Hung *et al.*, 1997; Payne *et al.*, 1998) and copper transport is ATP dependent (Voskoboinik *et al.*, 2001, 2001).

In human dietary copper is absorbed through the intestinal mucosa and absorption depends on copper levels. This copper binds to albumin and macroglobulins called

tarnscuprein in serum (Linder *et al*., 1998) and transported to liver which is major regulator of copper status in body. In hepatocytes, in normal copper levels the copper gets incorporated into cerluoplasmin in trans - Golgi networks. The copper containing ceruloplasmin then secreted into the blood (Terade *et al*., 1998; Hellman and Gitlin, 2002). In the case of excess copper levels the excess copper is excreted into the bile through biliary excretion method. The delivery of copper to ceruloplasmin or to biliary excretion is copper induced trafficking which is carried out by ATP7B or Wilson protein. ATP7B found to be localized to the trans-Golgi network (TGN) of hepatocytes and has been shown to undergo copper-dependent trafficking to an undefined vesicular compartment (Hung *et al*., 1997).

Wilson disease and Menkes disease both are caused by disruption in copper transport but the proteins expressed in different tissues and have different physiological roles. ATP7A express in all tissues while ATP7B majorly express in liver, kidney, placenta, lower regions of brain, heart and lungs (Vulpe *et al*., 1993; Tanzi *et al*., 1993; Bull *et al*., 1993). Menkes disease is characterized by defect in copper transport in many tissues but normal copper level in liver. Copper enters into the intestinal cells but further transport is hampered which results severe copper deficiency. Wilson disease is more common genetic disorder of copper metabolism with a frequency of 1 in 30,000 live births. Wilson disease protein (ATP7B) majorly expressed in hepatocytes (Danks, 1995) and plays crucial role in copper incorporation into apo-cerulpolasmin (Schaefer and Gitlin, 1999) and biliary excretion of copper. In Wilson disease ATP7B is unable to incorporate copper into ceruloplasmin in liver cells fail to excrete copper from liver to bile. This leads to toxic accumulation of copper into liver, brain, cornea and kidney which cause liver cirrhosis and progressive neurological damage during the childhood and to early adulthood. A case has been reported of a patient having symptoms of extremely uncommon combination of Wilson disease and visceral leishmaniasis in Bihar, India (Pandey *et al*., 2007).

The most prominent feature of Wilson disease protein (ATP7B) is presence of six repeats of metal binding motifs (GMXCXXC) in N-terminal domain. These metal binding domains (MBDs) are evolutionary conserved but the number of motifs varies between proteins. CCC2p the yeast orthologue of ATP7A/ATP7B contains two MBDs at N-terminal (Vulpe *et al.*, 1993; Yuan *et al.*, 1995). The two cysteine residues in MBDs involve in metal binding (Lutsenko *et al.*, 1997; Gitschier *et al.*,

1998) and bind at least six atoms of copper (Lutsenko *et al.*, 1997; Didonato *et al.*, 1997). Copper binds either as Cu^{+} or Cu^{2+} but after binding found only as Cu^{+}. ATP7B protein contains other conserved domains the TGEA motif (phosphatase domain), the DKTGT motif (phosphorylation domain), the TGDN motif (ATP binding domain) and the sequence MXGDGXNDXP that connects the ATP binding domain to the transmembrane segment. Presences of a conserved intramembranous CPC, CPS or CPH motif (CPx motif); a conserved histidine- proline dipeptide (HP locus) 34 to 43 amino acid carboxy-terminal to the CPx motif, the SEHPL motif and eight transmembrane segments (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996) are the salient features of P_{IB} type ATPases. Figure I represent schematic diagram of conserved domains of yeast CCC2.

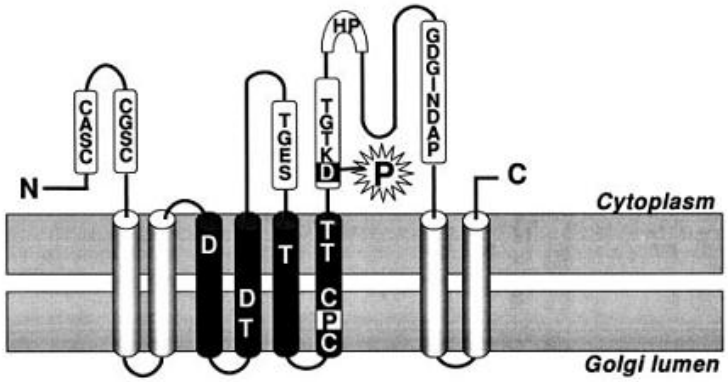


Figure I. Schematic representation of *Saccharomyces cerevisiae* CCC2p (Cu^{+} transporter P_{IB} type ATPase). *The N terminal cytoplasmic domain contains two metal metal binding consensus sequences CSAC and CGSC. Eight transmembrane domains, P- type ATPase consensus sequences DKTGT where Asp627 is the phosphorylated amino acid, ion channel CPC, HP locus and phosphates domain TGES (Adapted from Lowe et al., 2004)*

The yeast ATP7B homolog Ccc2 protein (Yuan *et al*., 1995) is a part of the pathway required for high-affinity iron uptake. The CCC2 protein delivers copper to protein acceptors in the lumen of the Golgi complex (Fu *et al.*, 1995). Copper delivery to the trans Golgi network (TGN) lumen is essential for iron metabolism in yeast, as the iron transporter Ftr1p must be activated by Fet3p in the TGN, (Figure J) which requires copper as a cofactor (Yuan *et al.*, 1995). The role of CCC2 in iron metabolism in yeast is similar to that in mammalian ATP7B with respect to ceruloplasmin, a protein synthesized in the hepatocyte TGN (Terada *et al.*, 1998).

Animal models of Wilson disease include the toxic milk mouse (Voskoboinik *et al.*, 2001) and the Long-Evans Cinnamon (LEC) rat (Wu *et al.*, 1994). Both rodent models develop hepatocellular damage from abnormal copper accumulation associated with mutations in the Wilson disease P-type ATPase but neither exhibit the neurologic symptoms associated with the human disease.

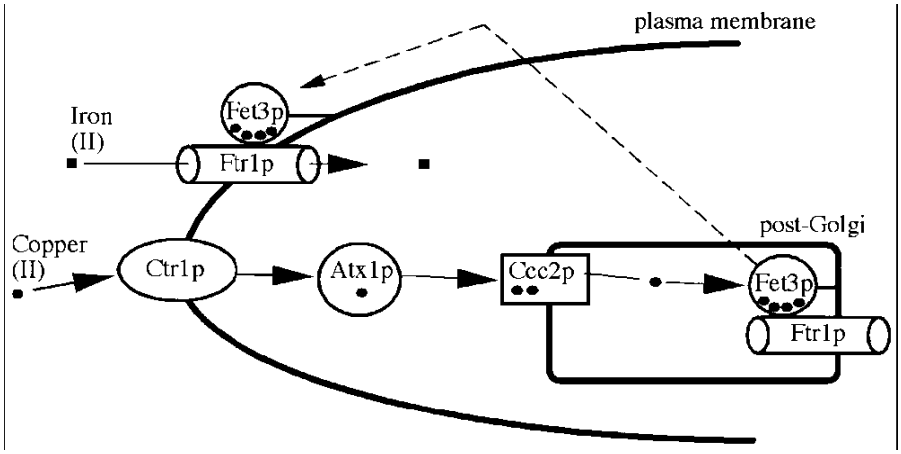


Figure J. Model of copper and iron uptake in yeast. *Depiction of iron and copper uptake in Saccharomyces cerevisiae (Adapted from Yuan et al., 1995)*

Studies of prokaryotic copper ATPases *Enterococcus hirae* CopA and CopB (Wyler-Duda *et al.*, 1996; Wunderli-Ye *et al.*, 2001), *E. coli* (Rensing *et al.*, 2000) and *Archeoglobus fulgidus* CopA (Mandal *et al.*, 2002) gives the thought for functional aspects of heavy metal transporte PI- ATPases. A functional assay has been developed for ATP7B on the basis of the complementation of the yeast mutant ccc2. Expression of ATP7B in ccc2 mutant cells complements the mutation and restores the cells' ability to grow on iron-limited medium. Similar functional assays have been reported and used to study ATP7A (Payne and Gitlin, 1998), ATP7B (Hung *et al*., 1997; Iida *et al*., 1998), and CUA-1, the *Caenorhabditis elegans* homologue of ATP7A/ATP7B (Sambongi *et al*., 1997; Yoshimizu *et al*., 1998), BnRAN1 the *Brassica napus* homologue of ATP7A/ATP7B (Southron *et al.*, 2004), RAN1 from *Arabidopsis thaliana* (Hirayama *et al.*, 1999).

Rationale for the study

During the intracellular stages of life cycle *Leishmania* faces harsh conditions both in the phagolysosome of macrophages and in the sand-fly gut environment. Parasite has to adapt with these diverse environments for its survival and growth. In that case, it has to acquire nutrients from host. Iron is one of the most essential nutrients needed for almost all of the organisms including *Leishmania*. It needs very well orchestrated but diverse strategies to acquire iron from the diverse host environment. In mammalian stage of its life cycle, LD resides in the phagolysosomes of host macrophages. Divalent metal ions are removed from phagolysosomes through Nramp1 transporter (Forbes and Gros, 2001) to the cytosol of the host leading to a challenging iron depleted condition for *Leishmania*. Thus in such iron restricted environment intracellular *Leishmani*a must equipped with high affinity iron uptake system. A ZIP family iron transporter LIT1 has been described in amastigote stage of *L. amazonenesis*. Leishmanial iron transporter (LIT1) is detectable only in amastigotes that grow intracellularly and is essential for the development of pathogenic lesions in mice, but dispensable for growth and differentiation in axenic culture (Huynh *et al.*, 2006). *Leishmania* requires a high affinity iron uptake system to cope with iron scarcity in its host and the expression of LIT1 is only detectable after about twelve hour of infection (Huynh *et al.*, 2006), which offers a more important question regarding iron transport in promastigotes form and immediate after entry into macrophages. Recently our laboratory has detected a multicopper oxidase mediated high affinity iron uptake system in *Leishmania donovani* (Solanki *et al*., unpublished data), which was found to be essential for the survival of both promastigote and amstigote stages of *Leishmania*. Multicopper oxidase is a copper containing protein requiring copper for its proper functioning. LDMCO has four copper binding sites and its activity and expression depends on availability of copper. Copper is an essential cofactor of MCO and its deficiency leads to malfunction of MCO, which can affect iron homeostasis of the parsite that may finally resulting into its affected survival, growth and virulence. Incidentally, copper homeostasis is even less understood than iron homeostasis in *Leishmania*, while it is well understood in yeasts and mammalian systems. Deficiency of copper was shown to cause iron deficiency in yeast (Dancis *et al.*, 1994a) and mammals (Hellman and Gitlin, 2002). Multicopper oxidase homolog in yeast and mammals require a copper transporting P-

type ATPase for incorporation of copper for its proper functioning. P-type ATPases like CCC2 in yeast (Weissman *et al.*, 2002; Marvin *et al.*, 2004) and ATP7B in mammals (Kramer, 2003) are reported to load copper in Fet3p and ceruloplasmin, respectively. There are reports of role of copper transporting P-type ATPase CtpA from *Listeria monocytogenes* in establishing infection in its virulence (Francis and Thomas, 1997 a,b). The identification of MCO in LD suggests a role of copper in maintaining iron homeostasis of the protozoan parasite *Leishmania donovani*.

The gene database of *Leishmania major* and *Leishmania infantum* suggest the existence of a putative copper transporting ATPase like gene present in chromosome 33 of these parasites. It shows 30% homology to *S. cerevisiae* and 31.6 % homology to human at the amino acid level. The current study has been undertaken to study the role of copper loading protein (putative LDATP7) on functional activity of LDMCO as well as its role in virulence of *Leishmania donovani*. To fulfill the above mentioned aims the current study entitled **"Studies on copper-loading protein and its role in virulence of *Leishmania donovani*"** was undertaken with the following objectives:

Objectives

1. To identify, clone and characterize copper transporter P- type ATPase from *Leishmania donovani*.
2. To study the role of putative LDATP7 in survival and virulence of LD.
3. To study the regulation of putative LDATP7 by copper availability of *Leishmania donovani.*

Materials and Methods

Materials

Tissue culture chemicals:

M199 and RPMI-1640 media, Bovine serum albumin (BSA), Penicillin-G, Streptomycin, Hygromycin B, G418 disodium salt, Phleomycin, Sodium bicarbonate, Phosphate buffer saline (PBS) are procured from Sigma Aldrich, Co., USA., Fetal bovine serum (FBS) from Biowest. Unless mentioned specifically, all reagents were obtained from Sigma-Aldrich. Tissue culture plastic wares were obtained from Corning.

General Chemicals:

Ampicillin, Kanamycin, Sodium dodecyl sulphate (SDS), Tris [hydroxylmethyl] aminomethane (Tris), Dimethyl sulfoxide (DMSO), Ammonium persulphate (APS), Acrylamide, N-N'- methylene -bis-acrylamide, Coomassie brilliant blue R-250, Coomassie blue G-500, Xylene cyanol, β-mercaptoethanol, Calcium chloride, Agarose, Formamide, 2-mercaptoethanol, Ethidium bromide, Formaldehyde, Diethylpyrocarbonate (DEPC), Phenol, Phenyl methyl sulphonyl fluoride (PMSF), N,N,N′,N′-Tetramethylethylenediamine (TEMED), Triton-X-100, Tween-20 Complete Freund's adjuvant, Incomplete Freund's adjuvant, Bathophenanthroline disulfonate (BPS), Bathocuproine disulfonate (BCS), Ferrozine-3-(2-Pyridyl)-5,6-dophenyl-1,2,4-triazine-4',4'-disulphonic acid sodium salt, IPTG, Ethylenediaminetetraacetate (EDTA), Ferrous ammonium sulphate, Sodium azide, Glycine, Lysozyme, Imidazole, Ni-NTA resin, HEPES, Trichloroacetic acid (TCA), Percoll were all purchased from Sigma Chemical Co., USA.

Analytical grade: Chemicals that were purchased locally are listed below:

Methanol, Glycerol, Ethanol, Chloroform, HCl, Isoamyl alcohol, Isopropanol, Glacial acetic acid, Toluene purchased from Qualigens, India. KCl, KH_2PO_4, K_2HPO_4, $MgCl_2$, Sodium chloride, NaH_2PO_4, Na_2HPO_4, Sodium hydroxide, Boric acid, Hydrogen peroxide (H_2O_2), Sodium acetate, Sulphanilamide, SSC buffer, Urea, Skimmed Milk powder, Orthophosphoric acid purchased from Merk, Germany. LB, Agar, Glucose, Yeast Extract, Peptone, Yeast Nitrogen base, drop out mix from Hi-Media laboratories.

Molecular biology reagents:

Restriction enzymes, Taq DNA polymerase, Pfu Taq Polymerase, ligases, DNAseI, other modifying enzymes etc. were purchased from Fermentas, Promega, New England Biolabs (NEB) USA. Salmon sperm DNA, TriPure- reagent for RNA isolation and protease inhibitor cocktail were from Roche.

Bacterial strains:

E. coli strain DH5α strain (Gibco BRL, Ltd., USA) used for all the cloning experiments for generation of new constructs and plasmid propagation. *E. coli* strain BL-21(DE3) and BL-21 Codon Plus (DE3)-RIL strains used for expression of the recombinant LDATP7 in expression vector system.

Yeast strains (*Saccharomyces cerevisiae*):

S. cerevisiae strains used were parental strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and ccc2 knockout strain (Mat a knockout strain, clone ID- 3629), YEp352 yeast expression vector purchased from Thermo Scientific Open Biosystems.

Molecular weight markers:

Protein molecular marker, 100 bp DNA ladder and Mass ruler were obtained from Fermentas Inc, MD, USA.

Oligonucleotides:

Sequence specific oligonucleotides were custom synthesized from Sigma Genosys, India.

Antibodies:

HRP conjugated anti-mouse IgG, anti-rabbit IgG used in western blotting purchased from Santa-Cruz, Biotechnology, USA. Alexa flour 488 goat anti rabbit, Alexa fluor 594 goat anti mouse IgG used in immunofluoresences purchased from Invitrogen.

Radioactive chemicals:

^{3}H-Thymidine was purchased from BERC, India.

Kits used:

RT-PCR was performed with MMLV Reverse transcriptase from Epicentre biotechnologies. Real time PCR was done by using High capactiv cDNA reverse transcriptase kit and Power SYBR green PCR master mix from Applied Biosystems. Mini prep kit for plasmid DNA isolation and purification, and QIA quick Gel Extraction kit was purchased from Qiagen, Germany. Genomic DNA extraction kit was purchased from Promaga, DIG High Prime DNA Labeling and Detection Starter kit II used for southern blotting purchased from Roche.

Others:

Bradford's reagent purchased from Biorad. Membranes filter papers were purchased from Whatman, USA. PVDF membranes were purchased from MDI and Millipore. Charged nylon for nucleic acid blotting from Millipore. Hybond N+ membranes were purchased from Amersham Biosciences, USA. X-ray films, developer and fixer were purchased from Kodak, India Photographic Company Ltd.

Animals:

BALB/c female mice of 4-12 weeks old (National Centre for Laboratory Animal Sciences, Hyderabad, India) were used for the experiments and for the propagation of the virulent strain of *Leishmania donovani*. For experimental use of the animals prior approval from the Institutional Animal Ethics Committee was taken (Reg: 19/1999 CPCSEA, 03.10.1999).

Methods

Growth medium for bacterial culture:

Luria-Bertani (LB) broth

For 1L of LB media 20g of LB powder was added and sterilized by autoclaving.

LB-agar plate

To each liter of LB, 20 g of agar was added and sterilized by autoclaving. The media was cooled to 60°C and the desired antibiotic was added and poured into 90 mm

petriplate (25-30 ml per plate). The plates were kept for at least 30 min under the laminar flow for solidification and drying.

Antibiotic solutions

Ampicillin solution was made by the addition of 100 mg of ampicillin into 1ml sterile water. Kanamycin solution was prepared by adding 50 mg of Kanamycin in 1ml of sterile water.

Competent *E. coli* cell preparation

Single colony of *E.coli* strain DH5α was inoculated in 5 ml of Luria – Bertani (LB) medium and incubated at 37°C, overnight. The 2 ml culture was transferred to a 500 ml culture flask with 200 ml of LB medium in laminar hood. The cells were grown up to optical density 0.4 to 0.6 at A_{600} and then kept on ice for 30 minutes. The cells were centrifuged at 4500 rpm at 4°C. The pellet was suspended in 2 ml 0.1 M $CaCl_2$ and kept on ice for 1 hour. The cells were centrifuged at 3000 rpm for 20 minutes. The cell pellet was suspended in 10% glycerol solution prepared in 0.1M $CaCl_2$. The mixture was kept into sterile cryovials as aliquots with 50µl each and stored at -80°C.

Transformation

The ligation mix to be transformed was added into 50 µl of competent cell. The mixture was incubated on ice for 30 minutes. Then heat shock was given at 42°C for 90 seconds and incubated on ice for 5 minutes. 800 µl of autoclaved LB was added to cells in a laminar hood and incubated at 37°C for 1 hour on shaking condition. From this 100 µl of suspension was plated on LB ampicillin plates. The plates were incubated at 37°C overnight, and the colonies were monitored next day.

Plasmid preparation

For analytical plasmid preparations, the Qiaprep Plasmid Mini-prep Kit (Qiagen) was used according to the manufacturer's instructions. 5 ml LB medium containing the appropriate antibiotic was inoculated with a single bacterial colony from a selective agar plate or from stored bacterial glycerol stocks. The colony was grown overnight at 37°C at shaking condition. The culture was centrifuged (1 min, 13000 rpm) and pellet was re-suspended in 250 µl buffer P1. 250 µl buffer P2 was added and mixed slowly

for up to 5 minutes. Then 350 µl buffer P3 was added with immediate mixing. The samples were centrifuged for 10 minutes at 13000 rpm. The supernatant was applied on the spin column with centrifugation at 13000 rpm for 1 minute. The column was washed with 750 µl buffer and DNA was eluted with 50-100 µl elution buffer or deionized water.

Growth media for Tissue culture:

M199 medium

The M199 medium was purchased from Sigma-Aldrich. To the final 500ml of medium 5ml of penicillin and streptomycin (100 units/ml penicillin, 100mg/ml streptomycin) and 50ml of FBS were added.

RPMI 1640 medium

The RPMI 1640 medium was purchased from Sigma-Aldrich. To the final 500ml of medium 5ml of penicillin and streptomycin (100 units/ml penicillin, 100mg/ml streptomycin) and 50ml of FBS were added.

Cell Lines and Culture Conditions:

Macrophage culture

Mouse macrophage cell line J774A.1 (American Type Culture Collection) were cultured in RPMI1640 (Sigma) supplemented with 10% heat inactivated fetal bovine serum, FBS (Biowest), 100 units/ml penicillin, 100 mg/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C (Heraeus BB 15).

Parasite culture

The *Leishmania donovani* (MHOM\IN\1983\AG83) was used for all the experiments. For *in vitro* culture, promastigotes were maintained in M199 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin at 22^{o}C in BOD incubator. Subculturing was done on every fourth or fifth day when the promastigotes reached stationary phase of growth. Culture and maintenance of the virulent parasites by passaging through BALB/c mice were performed as described before (Mukhopadhyay *et al*., 2000).

Briefly, $1x10^7$ stationary phase *Leishmania* promastigotes were taken to infect 2- 4 weeks old BALB/c mouse through tail vein. After 3-4 weeks of infection, spleen was removed aseptically, homogenized under sterile condition and suspended in M199 medium supplemented with 30% heat-inactivated fetal bovine serum, 100 unit ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. This suspension was incubated in the BOD chamber at 22º C. After the freshly transformed promastigotes are emerged, they were maintained in M199 medium supplemented with 10% FBS, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin at 22° C. On every fourth to fifth day subculturing was performed after promastigotes attained stationary phase of growth. Axenic amstigotes were obtained after shifting the parasite to 37ºC, 5% CO_2 for 48 h.

Isolation of genomic DNA from *Leishmania donovani*

Genomic DNA was isolated by Promega Wizard genomic DNA purification kit as per manufacture's protocol, 10 ml of 5 day old (stationary phase) culture of *Leishmania donovani* was pelleted at 3000 rpm for 10 minutes. The pellet was resuspended in 1ml PBS (1X) and was transferred into 1.5 ml micro-centrifuge tube. Cells were pelleted down again. 300 µl of nucleus lysis solution was added to the pellet and mixed gently by pipette. Then, 100 µl of protein precipitation solution was added and vigorously, vortexed for 20 second. After keeping on ice for 5 minutes, the sample was centrifuged at 12000 rpm for 3 minute. DNA containing supernatant was transferred to a sterile 1.5 ml micro centrifuge tube containing 300 µl of isopropanol. Mixing was done by gently inverting the tube until the thread like strand of DNA formed a visible mass. Centrifuge was done at maximum speed for 2 minute. Supernatant was removed and 300 µl of 70% ethanol was added to the pellet. Centrifuge was done at maximum speed for 2 minute. Ethanol was removed and pellet was kept for drying. 50 µl of DNA rehydration solution was added (as per manufacture's protocol). RNAse treatment was given whenever needed. Genomic DNA was stored at 2-8 ^oC

Cloning of LDATP7 in pET 28a vector

Both *Leishmania donovani* and *Leishmania infantum* show a very high homology between DNA and protein sequences (> 95%), so it was assumed that primers designed from *Leishmania infantum* might also amplify the sequence from *Leishmania donovani.* Accordingly, putative copper transporting P-type ATPase

LDATP7 was amplified from genomic DNA of *Leishmania donovani*. The primer sequences used were:

FORWARD PRIMER

5’ AGC CTG GGA TCC ATG GGC GCG ACG ACT GCT CAA GTG 3’

Bam H1

REVERSE PRIMER

5’ AGC CTG AAG CTT TCA CGT GAA ATA GAA ATC GCG TTC GCG 3’

HindIII

Reaction mixture contains

Reagent	Amount
Template (Genomic DNA)	100 ng
Forward primer	50 pmol
Reverse primer	50 pmol
dNTPs	200 µM
DMSO	1.5%
$MgCl_2$	1.5 µl (from 25 mM stock)
Taq polymerase	1 unit
Pfu	1 unit
MQ H_2O	As per required
Reaction volume	50 µl

PCR reaction was carried out in 0.2ml PCR tubes using Applied Biosystems thermal cycler. The PCR conditions were as follows.

Step	Temperature	Time
Initial denaturation	94°C	5 min
Denaturation	94°C	1 min
Annealing	60°C	30sec
Extension	72°C	3 min
Final extension	72°C	7 min
Hold	4°C	∞

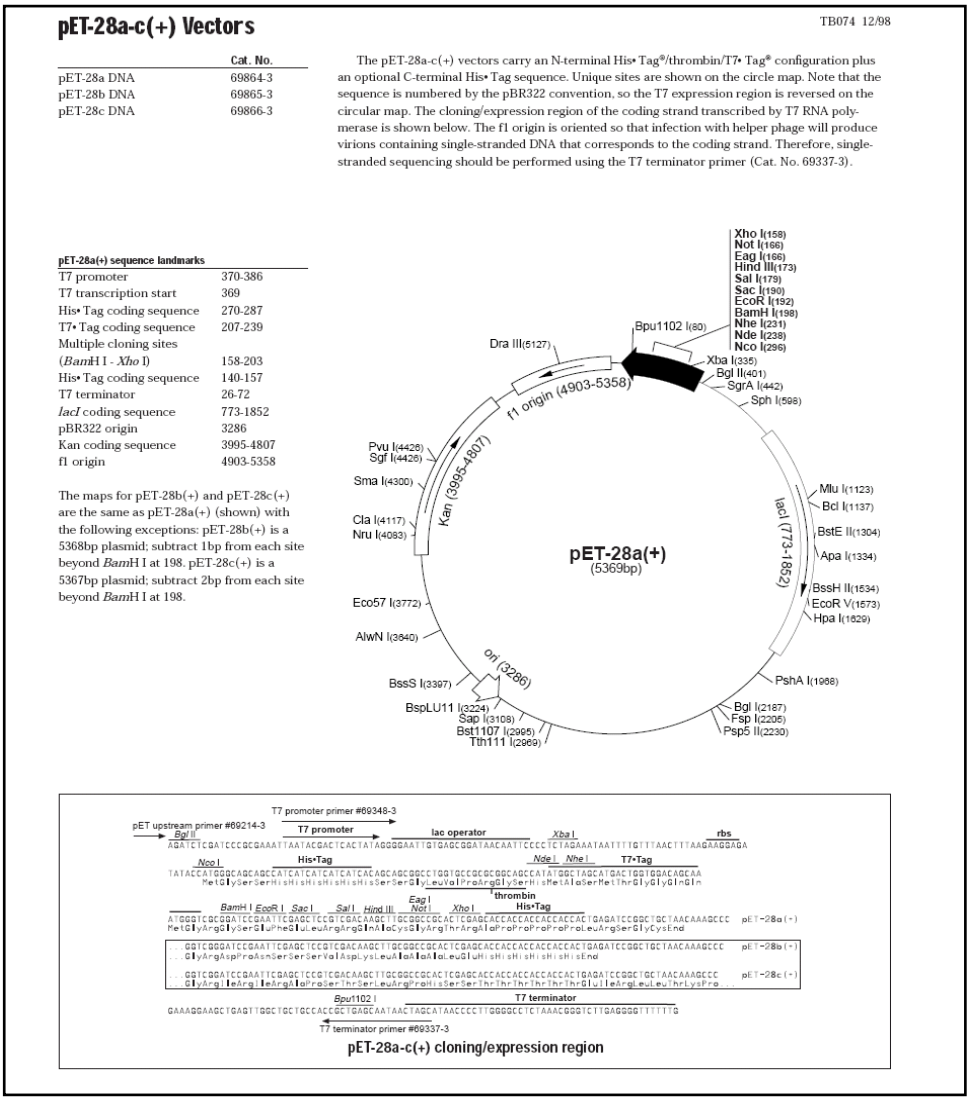
30 cycles

The 3492 bp product was amplified and cloned into pET-28a (+) expression vector. The ligated product transformed into *E. coli* DH5α strain. The transformants were selected on kanamycin LB agar plates. The screening of positive clones was done by colony PCR and finally confirmed by restriction mapping. Sequence analysis gave the final confirmation about the right product. Homology search by using NCBI BLAST tool shows 98% homology with *Leishmania infantum* and 96 % homology with *Leishmania major*.

Expression vectors for LDATP7 expression

pET-28a (+) vector

The putative 3492 bp LDATP7 was cloned into pET-28a (+) vector from Novagen between the BamH1 and Hind III sites using the selection marker Kanamycin. The pET-28a (+) vector carries an N-terminal His Tag/thrombin/T7 Tag configuration of about 2 kD plus an optional C-terminal His Tag sequence. The stop codon was not removed from LDATP7 ORF so that expressed protein should not have C-terminal His tag sequence. In this vector after the cloning, the coding strand is transcribed by T7 RNA polymerase in *E. coli* strain BL 21. This strain contains T7 RNA polymerase gene inserted into its genome under the control of a promoter, which is inducible by IPTG. IPTG induces the promoter and produces T7 RNA polymerase in high amount that in turn transcribes the cloned sequence. By this approach it was expected to get a very high expression of the cloned gene. The resultant protein should be tagged with His and that could be purified from other proteins of bacteria by affinity chromatography by using Ni-NTA resin.



pET-28a (+) Cloning/ Expression vector

Cloning and expression of N terminal 1417bp LDATP7

The recombinant pET-28a (+)-LDATP7 plasmids were transformed into BL21 or BL21 codon plus strains of *E. coli* and positive clones were checked for the expression of protein with different doses (0.5mM to 1mM) of IPTG. But several attempts failed to get the full expression of full length 123 kDa protein. The pET-28a (+)-LDATP7 clone was unable to express so it was decided to express only N-terminal 1417 bp of LDATP7. The LDATP7 gene contains Not I restriction site at 1413 position and the vector also contains Not I site. So, the pET-28a (+)-LDATP7 clone was digested with Not I, re-ligated and selected as mentioned above. Thus the resulted clone contains only N terminal 1413 bp in the vector. The *E. coli* BL21 containing the N terminal 1413 bp cloned in pET 28a (+) when treated with IPTG was

found to express an inducible protein close to the calculated molecular weight of 56 kDa. Due to very high expression and because of its being membrane protein, most of the protein fractions was found in the inclusion body.

Purification of His tagged LDATP7

The primary culture of *E. coli* BL 21 containing the cloned LDATP7 into pET28a (+) vector was grown in 10 ml LB in presence of Kanamycin (50µg/µl) overnight at 37^{o}C. 200 ml of LB was inoculated with the 2 ml of primary culture in the presence of Kanamycin (50 µg/µl) and incubated at 37^{o}C until the OD appeared about 0.6 (approximately three hours). Then the culture was induced by addition of IPTG (1mM). The culture was now incubated at 28^{o}C for 5 hours. Cells were pelleted down quickly after the incubation on ice for 30 minutes. 10 to 20 ml of PBS was added and cells were re-suspended and pelleted down again. Cell pellet was kept at -20^{o}C overnight after PBS wash. Pellet could be stored at this stage.

The cell pellet was put on ice from -20^{o}C for 30 min and then suspended in 2ml of PBS containing Lysozyme (20 µg/µl) and protease inhibitor cocktail. The solution was incubated on ice for 1 hour or until the cells lysed.

After cells were lysed, sonication was done at amplitude of 4 for 30 seconds in ice. This step of sonication was repeated for four times or until the solution loses its viscosity with an interval of one minute on ice. The solution was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 4^{o}C for 20 min at 13,000 rpm in a microfuge. Most of the protein was found within the inclusion bodies so the supernatant was discarded and pellet was resuspended again into 1ml of 1XPBS containing 8M Urea and 1X protease inhibitor cocktail and was kept for one hour in ice. The solution was centrifuged again at 4^{o}C for 20 min at 13000 rpm speed. Now the supernatant was taken and mixed with the active Ni- NTA beads slowly and the mixture was incubated at 4^{o}C rotating slowly for 1 hour. Beads were activated by incubating with nickel sulfate solution for 10 min on ice and washed twice with 1XPBS at 1500 rpm for 30 second. After 1 hour of binding step, the mixture was centrifuged at 1500 rpm for 30 second. The supernatant was discarded and the pellet was washed 5-6 times with 1XPBS containing 20µM of imidazol at 1500 rpm for 30 second each. The protein was eluted with PBS containing 200 µM of imidazole.

Preparation of antiserum against LDATP7

To generate polyclonal antibodies against LDATP7 protein female New Zealand White rabbits (4 months old) were used for immunization. Partially purified protein was subjected to SDS-PAGE, protein bands were visualized by soaking the gel in ice cold 0.1M KCl and desired protein band was excised from the gel. The excised gel was homogenized in 500ul of 1X PBS (~250 μg protein) and equal volume of Freund's complete adjuvant was added to form an emulsion. The emulsified mixture was injected as primary immunization dose. First and second booster doses of 200 μg proteins in Freund incomplete adjuvant were administered at an interval of every two weeks from the date of primary immunization. After second and third booster doses 5ml blood was collected after a gap of one week and was incubated at 37^oC for 3 hours and then overnight at 4^oC for coagulation. The residual serum was collected from cell debris following centrifugation at 13,000 rpm, 15 min, 4^o C. Aliquots of serum stored at -80^oC for long term storage.

For colocalization studies LDATP7 antiserum was raised in mice also. For this 4-6 week old Swiss albino mice were used. For primary immunization 15 μg of LDATP7 protein with Freund's complete adjuvant was used. First and second booster dose of 10 μg protein along with Freund's incomplete adjuvant was administered at an interval of 10 days. The blood was collected after a gap of 4 days from the second and third booster doses. The serum was isolated and stored as described above.

To ensure the specificity of LDATP7 in western analysis anti-LDATP7 antibody was also raised commercially by IMGENEX INDIA PVT. LTD. ORISSA. The peptide antibody raised in rabbit using the LDATP7 epitopes c ASQLQRPSETSKSTE and c SVTVRPASDDNGKVSS which shows 67% and 56% hydrophilicity, respectively.

Immunofluorescence microscopy for localization and colocalization of LDATP7 and LDMCO protein in LD

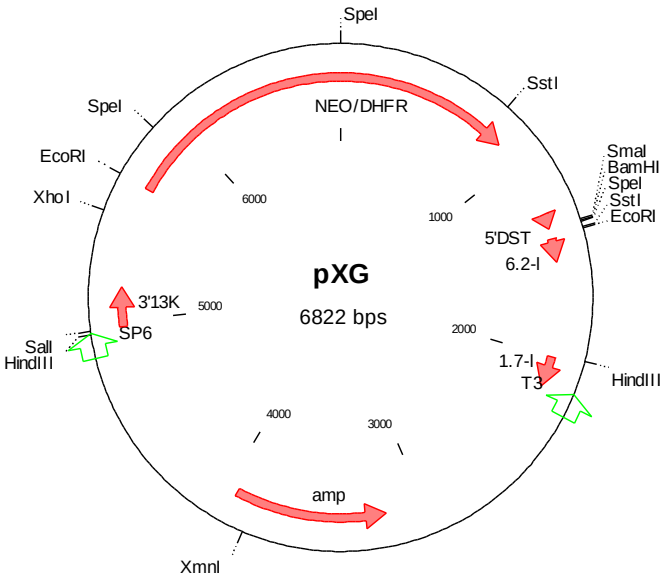
$1X10^6$ promastigotes were pelleted down and a smear of LD was made on cover slip and let it dry for 30 min. Cover slip was kept in a 30 mm dish and 2 ml of chilled methanol was added slowly over the cover slip. After 10 min of incubation methanol was pipette out and cover slip was dried again. After this the cover slip was put in 3 ml of 1X PBS containing 1% BSA for 2h. After this incubation the cover slip was washed thrice with 1X PBS for 5 min without shaking. Then the cover slip was put in

rabbit raised anti-LDATP7 primary antibody (1:100 in 1X PBS + 1% BSA) for 1 h at RT or overnight at 4^{o} C. Five washes with 1X PBS was given for 5 min each without shaking. Then the cover slip was put in the secondary antibody solution 1:1000 Cy3 or Alexa 488 anti rabbit +1:1000 Hoechest (nuclei stain) in 1X PBS + 1% BSA for 1h. Five washes with 1X PBS were given for 5 min each without shaking. The cover slip was mounted in 10% glycerol on slide for microscopic examination. For colocalization studies of LDATP7 and LDMCO proteins in LD promastigotes primary antibodies anti LDMCO (raised in rabbit used at dilution 1:200 in 1X PBS + 1% BSA) and anti LDATP7 (raised in mice used at dilution 1:100 in 1X PBS + 1% BSA) were used simultaneously. For secondary antibody Alexa 488 conjugated anti rabbit and Alexa 594 conjugated anti mice and Hoechest was used as mentioned above.

Generation of LDATP7 gene knockouts

The generation of knockout constructs was based on the pXG-HYG and pXG-NEO vectors (kind gifts from Dr. S. Beverly, Washington University) that consist of backbone of the vector pSP6-T3 and a cassette comprising a gene encoding resistance to hygromycin B (hyg) or G418 (neomycin) and flanking dihydrofolate reductase-thymidylate synthase (DHFR) sequences that are required for proper gene expression. To disrupt the LDMCO locus, the flanking regions of LDATP7 DNA sequences were cloned into pXG-HYG and pXG-NEO such that the hyg expression cassette (2,837 bp) and neo expression cassette (2,687 bp) were flanked at both ends by these sequences. This strategy permitted subsequent excision of the entire insert, excluding vector DNA, for transfection into the parasites to promote homologous recombination with chromosomal LDATP7 sequence. To knock out LDATP7 two strategies were used; one, where the complete LDATP7 ORF was deleted and another one where only N terminal 1640 bp was disrupted. For strategy one, the 1000 bp 5'UTR region of LDATP7 amplified by using forward primer 5'AGC CTG AAG CTT TCG CAG TCT CTC CGT GCT GTC 3' and reverse primer 5' AGC CTG CTC GAG CGT TGT GGA TAA AGG ACA GGA ACC 3' containing HindIII and XhoI restriction site, respectively. The 1000 bp of 3'UTR region was amplified by using forward primer 5' AGC CTG CCC GGG TGA CAC GGC AGG AAT GCT GAC 3' and reverse primer 5'AGC CTG GGA TCC TCA CGT GAA ATA GAA ATC GCG 3' containing SmaI and BamHI restriction sites, respectively. The 5' UTR and 3' UTR were cloned into pXG HYG and pXGNEO vectors.

In another strategy 5' UTR and 1852 bp of C terminal LDATP7 was amplified using primers forward 5' AGC CTG CCC GGG TGA CAC GGC AGG AAT GCT GAC 3' and reverse 5' AGC CTG GGA TCC TCA CGT GAA ATA GAA ATC GCG 3' containing Sma I and BamHI, respectively. These constructs cloned in pXG HYG and pXG NEO vectors to get LDATP7 knockout constructs. The vectors were digested with HindIII and BamHI, and the knockout fragment containing the selectable gene marker was purified. The procedures used in the transfection of LD promastigotes were described before. Wild type parasites were transfected with 10 µg of the linearized knockout constructs by electroporation, and transformants were selected at 45 µg of hygromycin B/ml or 50 µg/ml of G418/ml. By this strategy we generated half knock out of LDATP7, one with the selection marker of hygromycin B and another with selection marker G418. It is known that continuous subpassage of parasites may result in a decrease in virulence; therefore, the half knock outs and wild type strains were electroporated and subpassaged in parallel the same number of times under the same culture conditions.



pXG: ***Leishmania*** **expression vector with** ***NEO*** **marker Strain B1288**

Preparation of vector for LDATP7 over expression

For gene complementation studies, the entire coding region of the LDATP7 gene was cloned into the BamHI site of the pXG-Phleo expression vector (kind gift of Dr. Subrata Adak, IICB, Kolkata.) to generate the pXGPhleo-LDATP7 vector. The primer sequences used were same as used for amplification of LDATP7 gene but both

the primers with BamHI restriction sites. Cloning was done as mentioned above with the additional step of treating BamHI digested pXG- Phleo vector with Calf Intestine Phosphatase (CIP) to remove 5' phosphatase group thus preventing from self ligation. The clone was confirmed by sequencing and transfected to LD by electroporation and transformants were selected at 10 µg of Phleomycin/ml of media and maintained at 20 µg of Phleomycin/ml.

Transfection

Leishmania was cultured on M199 medium supplemented with 10% fetal bovine serum up to late log phase. Cells were collected by centrifugation and suspended at a density of 10^8 cells/ml in HEPES buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4, 6 mM glucose, pH 7.4). Electroporation cuvettes 2mm gap (BTX Harvard apparatus) were used for transfection. The transfection mode was set on 500V at Low Voltage mode of RESISTANCE & CAPACITANCE. The parameters were set as Capacitance at 1300µF, Resistance at 25 ohms, Chamber Gap 4 mm, Charging Voltage at 450V, Field Strength of E 2.25 kV/cm and a desired Pulse Length of 7 milli Second. Alternatively, the mode was set on 2500V at high Voltage mode of RESISTANCE & CAPACITANCE. The altered parameters were like Capacitance at 25 µF, Resistance at 25 ohms, the Chamber Gap was 4 mm, and a desired Pulse Length of 2 milli Second.

Procedure: - Cells were collected by centrifugation and suspended at a density of $1x10^8$ cells/ ml in HEPES buffered saline (approximately 400 µl) and kept on ice for 10 minutes and plasmid DNA (1mg/ml in 10 mM Tris-1mM EDTA, pH 7.4) was added to it before electroporation. One pulse was provided in case of low voltage mode and two pulses in 10 sec gap in case of high voltage mode. Transfected parasites were kept on ice for 10 minutes and then transferred to 5 ml of drug-free medium. After 24 hours the appropriate antibiotic solution was added for selection.

Determination of DNA and RNA concentration

DNA and RNA concentration were determined spectrophotometrically and calculated from the absorbance measured at 260 nm.

Conc. of DNA = A_{260} X 50 µg/ml

Conc. of RNA = A_{260} X 40 µg/ml

Southern blot analysis

The Digoxigenin (DIG) labeled probes for LDATP7, Hyg and Neo genes were generated by PCR amplification using primer sets forward primer 5' ACC CTG CTC TTG CAT CAC TC 3' reverse primer 5' AGC GTG AGC AGG TAG ACA AC 3' for LDATP7 gene, forward primer 5' GAG GGC GTG GAT ATG TCC TGC G 3' reverse primer 5' TGG GAA TCC CCG AAC ATC GCC TC 3' for Hyg gene, forward 5'GTG GAG AGG CTA TTC GGC 3' reverse primer 5' GGC GAT AGA AGG CGA TGC 3' for Neo gene according to protocol provided by the manufacturer (Roche). Genomic DNA was isolated from wild type and LDATP7 knockout LD cells as the method described above. The genomic DNA was digested overnight with BglII restriction enzyme. 5 μg of digested DNA was separated on 0.8% agarose gel. After running, the gel was trimmed and treated with denaturation buffer (1.5M NaCl, 0.5M NaOH) for 15-30 min then with neutralization buffer (3 M NaCl, 0.5 M Tris- Cl pH7.0) for 15-30 min and finally washed with 20X SSC at room temperature (25°C) for 15 minutes with constant and gentle shaking. DNA was transferred to the presoaked positively charged nylon membrane using 20X SSC overnight by capillary transfer method. Transferred DNA was fixed to the membrane by UV- crosslinking. The blots were then hybridized with DIG labeled PCR probes. The hybridized probes were immunodetected with anti-digoxigenin-AP and visualized with chemiluminescence substrate CSPD according to the manufacturer's protocol (Roche).

Infection of macrophage

J774A.1 macrophage cells were seeded on poly-L-lysine coated petri dish and grown up to 50-60 % confluence level and then infected with stationary phase *L. donovani* promastigotes at a ratio of 1:10 macrophage: parasite. After appropriate time of infection, macrophages were washed three times with phosphate buffered saline (PBS) to remove free parasites and fresh RPMI medium was added for further experiments.

Intracellular amastigotes isolation

The intracellular amastigotes were obtained from infection of J774A.1 macrophages as described earlier (Chang, 1980). After a definite time of infection, cells were washed with PBS once and flush the respective groups using a cell scrapper to

dislodge adherent macrophage. Maximum macrophages were removed and suspend in a 1.5ml micro-centrifuge tube (which will resist freeze cycle with liquid nitrogen). The macrophage content was collected and centrifuged at 1000 rpm for 10 min (Rotor radius = 6cm, Biofuge, Heracus). The pellet was resuspended in 2-3 ml of PBS and dispersed well. The tube was placed into liquid nitrogen until it freezes or kept in -80° C for one hour. Then it was put on 37°C water bath. This step was repeated for four times. A 90-40-20% percoll gradient was prepared previously in 15 ml centrifuge tube. All three layers should be distinctly visible. The cells were discharged slowly on the top of the gradient and spun at 3000 rpm for one hour. Without disturbing the gradient, there will be three layers separating out. The band at the interface of 90% and 40% percoll was collected. The content of these two entities was verified on a microscope. The appropriate amastigote population was picked out and washed once with PBS to remove percoll.

Counting of LD

The LD promastigotes and amastigotes were counted on a neobar chamber in light microscope at 40X magnification. LD was counted from all four 16-big squares and an average was considered for further calculations. The formula used is:

No. of cells = Average counting by neobar chamber X dilultion factor X 10^4 cells/ml

Whole cell lysate preparation

After the requisite treatment as indicated in different experiments, *Leishmania donovani* cells were centrifuged at 3000 rpm for 10 minutes and supernatant was discarded without disturbing the cell pellet. The cell pellet was resuspended in 1 ml PBS and centrifuged again at 3000 rpm for 10 minutes. Supernatant was discarded and the cell pellet was suspended in 100- 200 µl of lysis buffer containing 50 mM Tris-Cl pH-8.0, 0.5% sodium deoxycholate, 1% Triton-X-100, 150 mM NaCl, 0.1% SDS, 1mM PMSF, 2mM NaVan and 1X protease inhibitors. Cells were lysed by sonicating twice at 4 amplitude for 15 seconds with a gap of 1 min. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. Cell extract was collected in different tubes. Concentration of the protein was estimated by measuring absorbance at 595nm using Bradford reagent (Bio-Rad) and this was compared with a standard curve of bovine serum albumin (optical density plotted against various concentrations of BSA).

Immunoblot analysis

Proteins were denatured at 95° C by mixing with appropriate volume of 2X SDS-PAGE gel loading buffer and loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (percentage of the gel was determined according to the protein size analyzed) under reducing condition. After resolving, proteins were transferred to the PVDF membrane (Millipore), blocked with 5% non-fat milk in TBST buffer and incubated with specific anti-LDATP7 antibody solution (1:1000 anti LDATP7 in TBST + 1% BSA) for 1h. 5 washes were given with TBST buffer for 5 min each. After the washes membrane was incubated with horseradish peroxidase-conjugated solution (1:2500 secondary antibody in TBST + 1% BSA) for 1h. After the incubation the blots were washed for 5 times with TBST buffer and 3 times with TBS buffer. Finally, proteins were visualized by enhanced chemiluminescence (ECL) following the manufacturer's protocol (Amersham Biosciences).

Ferroxidase assay

Ferroxidase activity was performed at 25°C by using whole cell lysate of LD cells. Buffer used for whole cell lysate preparation contains 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1mM PMSF, 2 mM NaVanadate. Whole cell lysates were prepared as described earlier. Ferrous ammonium sulfate as electron donor and 3-(2-pyridyl)-5, 6-bis (4-phenylsulfonic acid)-1, 2, 4-triazine (ferrozine) as a chelator to specifically detect the ferrous iron remaining at the end of the reaction. Each assay mixture (1 ml) contained 100 mM sodium acetate buffer, pH 5.2, 0.03 mM ferrous ammonium sulfate and reaction was started by addition of enzyme. Samples were incubated at room temperature for 10 min and reaction was quenched by adding ferrozine to 0.3 mM, and the rate of Fe(II) oxidation was determined by measuring the absorbance of residual Fe(II)- ferrozine ($E570 = 7.26\ mM^{-1}\ cm^{-1}$). Sodium azide inhibited reactions and heat denatured protein samples were used as control. Sodium azide is a classic complex forming agent and is a known inhibitor of multicopper oxidase mediated ferroxidase activity.

DNA synthesis assay

DNA synthesis was measured by incorporation of (^{3}H) thymidine into trichloroacetic acid (TCA) precipitable material. 1x 10^7 *Leishmania* cells were incubated at 22^o C for 48 h. (^{3}H) thymidine was added to culture as 0.4 µCi/ml concentration and cells were

incubated for 12 h at 22^{o} C. After the incubation cells were pelleted down and washed with 5% TCA (ice cold) three times. 0.5 ml of 0.25 M NaOH was added to the pellet and pellet was mixed slowly on a shaker platform for 10 min at room temperature. 50µl of 6 M HCl was added to neutralize the solution to minimize artifact due to chemiluminesence in the scintillation counter. Equal volumes of samples were added in Poly Q vials containing 10 ml toluene based Cocktail-O solution. Radioactivity was measured by using a scintillation counter (Beckman).

RNA Isolation and semi-quantative Reverse Transcriptase-PCR (RT-PCR) from LD

Total RNA was isolated from LD, using TriPure reagent (Roche), according to the manufacturer's protocol. In the case of intracellular amastigotes, RNA was obtained directly from the infected macrophages. RNA (4µg) was reverse transcribed using the first Strand cDNA synthesis kit for RT-PCR (Epicentre) with specific reverse primer in a 25µl reaction. 10% of the cDNA solution was taken for doing PCR to quantify the RNA.

Quantitative real time PCR

For quantitative real time PCR total RNA was prepared as mentioned above. cDNA was prepared by using High capacitv cDNA Reverse Transcription Kit (Applied Biosystems, ABI) in a 20µl reaction using 4-5 µg of total RNA. PCR was carried out in ABI 7500 thermocycler (Applied Biosystems) by using Power SYBR Green PCR Master Mix (ABI) and LDATP7 specific primers forward primer 5' CAA GGT TGG GGA AGA GAC GAT GAT CG 3'and reverse primer 5' TTG TCA GAC CGT GCC CGT TCT CCT CC 3' according to manufacturer's protocol. Amplification of 18S rRNA was used as endogenous control using primer sequence forward 5'CAA CCT CGG TTC GGT GTG TG 3' and reverse 5' ACT GGG CAG CTT GGA TCT CG 3'. Melting curve analysis was done to confirm amplification of the specific PCR products.

Site directed mutagenesis of conserved domains of LDATP7

LDATP7 possesses conserved domains of P IB type ATPase transporter. Mutation in these domains can lead to improper function of LDATP7. Site directed mutagenesis was performed by using megaprimer method. It comprises of two steps of PCR, first

step PCR was done to introduce the mutation and the resulting amplicon was used as megaprimer to amplify the complete ORF. Primers were designed to introduce mutations in first Heavy Metal Associated (HMA) domain (5' TTG AGC TGA TCC CCT ACT CGT CAT CC 3') 24GMTCNSC30 to 24GMTSNSS30 two conserved cysteine residues were replaced with serine residues. Aspartate residue of phosphorylation domain 796DKTGT800 was replaced with glutamic acid 796EKTGT800 using primer 5' TGC GTC GTG CTC GAA AAG ACG GGC AC 3'. Mutations were confirmed by DNA sequencing analysis.

Preparation of constructs for yeast complementation assay

Saccharomyces cerevisiae CCC2 which is a functional homolog of Wilson disease gene also shows the homology with LDATP7. To assess the function of LDATP7, Δccc2 complementation assay was performed according to standard protocol. For this the LDATP7 gene was cloned into YEp352, a yeast expression vector containing the URA3 auxotrophic selection marker. To generate YEp352-LDATP7 construct pET-28a (+)-LDATP7 plasmid was digested with BamHI and HindIII and cloned into YEp352 plasmid. The site directed mutants of conserved domains of LDATP7 were also cloned in YEp352 vector. *S. cerevisiae* ccc2 gene was amplified from *S. cerevisiae* genomic DNA using primer forward primer 5' AGC CTG TCT AGA ATG AGA GAA GTG ATA CTT GC 3' and reverse primer 5' AGC GTC CTG CAG TTA CAA GAC TTC TTC GTT TG 3' containing Xba I and PstI restriction sites, respectively. The amplified product was cloned in YEp352. These constructs and empty vector were transformed in ccc2 knockout strain according to standard Lithium acetate method described below. The transformants were selected on minimal media (0.67% yeast nitrogen-based medium without amino acids; 2% glucose; 2% agar; 0.2 g dropout mix containing all amino acids except uracil. And transformants were maintained in YPD media (1% Yeast extract, 2% Peptone, 2% Glucose and 2.5% Agar).

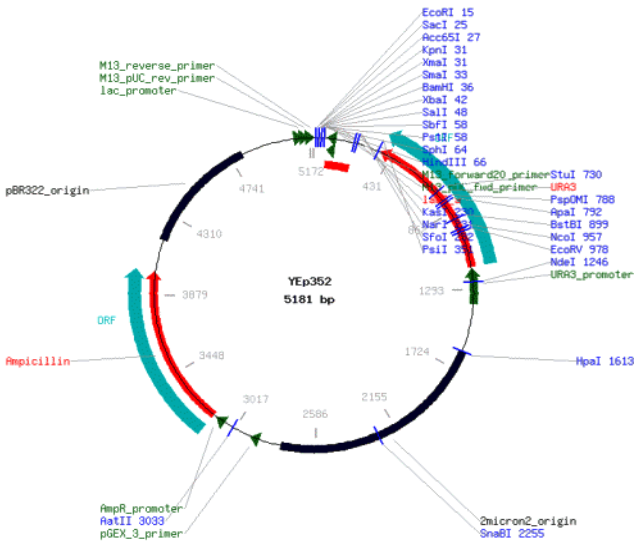
Yeast Transformation

Yeast cells were inoculated in 10 ml YPD media and grown overnight at 30^{o} C with shaking at 200 rpm. 2.5 ml of this primary culture was inoculated in 50 ml YPD and grown at 30^{o} C, 200 rpm until optical density reaches up to 0.4-0.6. Cells were collected by centrifugation at 5000 rpm at 4^{o} C and washed with 1 ml TE buffer (10

mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0). Finally cells were resuspended in 0.4 ml TE buffer and 0.4 ml of 0.2 M Li-Acetate, mixed vigorously by vortexing and incubated at 30^{o} C for 1 hour with shaking at 150 rpm. 0.1 ml from this mixture was taken and 1-5 µg plasmid DNA (experimental, vector DNA used as positive control and without DNA was negative control) was added. The mixture was incubated at 30^{o}C for 30 min without shaking. Then 0.1 ml of 70% PEG was added and mixed with blunt tips. Incubated at 30^{o} C for 1 hour without shaking. Heat shock was given at 42^{o} C for 5 min and centrifuged for 1min at 4000 rpm at room temperature. Cells were washed twice with sterile MQ water to remove residual PEG. Finally resuspend in 100 µl sterile MQ water and plated on YNB -Ura agar plate.

Yeast complementation assay

The positive transformants were selected for the yeast Δccc2 complementation assay. Selected cells were grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2.5% agar) plate for overnight at 30^{o} C then resuspended in 0.9 % saline and adjusted to A_{600}=0.1 absorbance units. These cells serially diluted five times 1, 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000. Five µl of each serial dilutions was spotted onto the YPD agar plate as control and YPD supplemented with 140 µM of iron chelator bathophenanthrolinedisulfonic acid (BPS) to create iron limited condition and grown for 2 days at 30° C before imaging.



Yeast expression vector Yep352

Statistical analysis

All data are expressed as Mean ± Standard Deviation (S. D.) and are represented as data of at least three different sets of experiments. Mean and standard deviation values were calculated with the help of Microsoft Excel.

Introduction

Iron is the fourth most abundant metal on earth's crust. At neutral pH and aerobic condition it exists as an oxidized ferric form. Ferric form is highly insoluble thus is not readily available for biological systems. Iron is required for most of the organisms as it is involved in various metabolic processes. Thus organisms have evolved different ways for acquiring iron from their available sources. Bacteria and some eukaryotes synthesize siderophores that binds to ferric form and mobilize it for its high affinity uptake (Champomier-Vergès *et al.*, 1996). In yeast multicopper oxidases are involved in high affinity iron uptake. Multicopper oxidase (MCO) is copper containing enzyme, which oxidizes ferrous into ferric and is a component of an oxidase- permease system that serves the high affinity of iron uptake in yeast (Stearman *et al.*, 1996). In *Saccharomyces cerevisiae* high affinity iron uptake system is comprised of Fet3 as MCO and Ftr1 as permease (De Silva *et al.*, 1997 and Hassett *et al.*, 1998). In plants MCO is found in the form of laccase, which is also known as p-diphenol: O_2 oxidoreductase (Keilin *et al.*, 1939). In Mammalian system ceruloplasmin is the Fet3 homolog and has a central role in iron homeostasis (Harris *et al.*, 1999). Furthermore, basolateral surface of the intestine utilizes a membrane MCO hephaestin (Vulpe *et al.*, 1999) for iron transport. Another MCO, FLP is involved in iron homeostasis in green algae *Chlamydomonas reinhardtii* (Herbik *et al.*, 2002). MCOs have been described to have a variety of different physiological functions for bacteria, including manganese oxidation, copper tolerance and iron oxidation (Huston *et al.*, 2002 and Cooksey, 1994). These reports show that multicopper oxidases are evolutionary conserved and involved in high affinity iron uptake system.

Protozoan parasites are known to be pathogenic for most of the mammals. *Leishmania donovani* (LD) is the causing agent of visceral leishmaniasis or kala-azar (Lukes *et al*., 2007). Amongst different leishmaniases, visceral leishmaniasis (VL) is the most fatal form if left untreated. VL accompanied by fever, weight loss and parasitic invasion of the blood and reticulo-endothelial system such as enlarged lymph nodes, spleen and liver (Desieux, 2004b). Control of leishmaniases is hampered by the lack of a safe vaccine, limitation of frontline drugs, and the emergence of drug resistant strains (Santos *et al*., 2008).

Iron plays an important role for survival and proper growth of LD (Soteriadou *et al*., 1995; Solanki *et al*., unpublished data). Like any other organisms *Leishmania*

donovani also requires iron for its metabolic processes. Iron is also required for antioxidant enzyme iron superoxide dismutase that plays an important role in protection against the oxidative damage inside macrophage resulting from activation of the host NADPH oxidase (Paramchuk *et al.*, 1997). Iron uptake is essential for survival of pathogens including LD. There are few studies to understand the iron uptake mechanism in *Leishmania* species. Some studies proposed the presence of a glycoprotein that can bind transferrin in *Leishmania infantum* membrane (Soteriadou, 1995). *Leishmania chagasi* was shown that it could acquire iron from lactoferrin and transferrin (Wilson *et al.*, 1994). These studies are done in *in vitro* promastigote forms of *Leishmania*. *Leishmania* faces iron scarcity in their host. LD spends part of its life cycle as promastigotes in the midgut of sand fly (pH=7.5) and the other part as amastigotes within the phagolysosomes (pH=5.0) of host macrophages (Hyde, 1990), where iron is not readily available. Moreover, macrophage is known to limit the access of iron to the pathogen as a defense strategy against the pathogen (Weinberg, 1992), so amastigotes might face larger challenge to accumulate iron. To survive in this iron restricted condition *Leishmania amazonensis* expresses LIT1 protein exclusively in amastigote stage (Huynh *et al*., 2007). LIT1 is a divalent metal transporter with preference to iron. Previous work from our laboratory has found a ferroxidase mediated iron uptake system in *Leishmania donovani*. It has been observed that a unique multicopper oxidase (LDMCO) in LD is essential for its iron uptake, growth, virulence and survival in promastigote and early stages of infection in macrophages (Solanki *et al*., unpublished data).

Multicopper oxidase contains copper which forms a trinuclear copper centre and serves as electron acceptor to oxidize iron. Copper deficiency was found to cause iron deficiency in yeast (Dancis *et al.*, 1994a) and humans (Hellman and Gitlin, 2002). Copper transporter P- type ATPases are found in yeast as CCC2 (Weissman *et al.*, 2002 and Marvin *et al.*, 2004) and in mammals as ATP7A/ATP7B (Kramer, 2003) that load copper into yeast MCO Fet3 and mammalian ceruloplasmin, respectively. Thus copper deficiency may lead to improper function of these enzymes. MCOs are regulated by cellular iron deficiency. In yeast under iron deficient conditions a transcription factor Aft1 is up-regulated to regulate a region of genes comprised of an iron reductase (fre1), copper loader (CCC2), iron transporter permease (ftr1) and MCO (fet3) (Yamaguchi-Iwai *et al.*, 1995, 1996; Hassett *et al.*, 1998). In mammals

during iron deficiency transcription factor hypoxia-inducible factor-1 (HIF-1) is activated to transcriptionally regulate ceruloplasmin (Mukhopadhyay *et al.*, 2000).

Copper is required for the proper functioning of any of the multicopper oxidases present across the evolution and linked with iron metabolism (Askwith *et al.*, 1994, 1996; Mukhopadhyay *et al.*, 1998). *Leishmania donovani* multicopper oxidase contains four copper centres that influence its activity and expression (Solanki *et al*., unpublished data). Interestingly, depletion of copper was shown to affect the activity of mammalian ferroxidase ceruloplasmin (Cp) without affecting its expression (Holtzman *et al.*, 1970). In hepatic cells P-type ATPase loads copper into Cp and copper depletion inhibits this mechanism (Scheinberg *et al.*, 1952; Tanzi *et al.*, 1993; Yamaguchi *et al.*, 1993). In yeast, Cu is required for high-affinity iron uptake (Askwith, 1994; Stearman, 1996). Mutations either in the Cu loader (Dancis *et al*, 1994b; Yuan, 1995) or Cu deficiency (Askwith, 1994) resulted in impaired iron uptake.

Since, LDMCO is important for even in essential iron uptake in *Leishmania donovani*; it would be interesting to understand the copper loading system in LDMCO. There is so far not much attempt has been made to understand copper homeostasis in protozoan parasite biology. *Leishmania* genome database shows the presence of Fe-SOD but Cu/Zn SOD is absent. The yeast homolog of MCO Fet3 receives copper from copper transporting P- type ATPase CCC2 in trans-Golgi network. The mammalian ceruloplasmin requires Wilson disease protein (ATP7B) for incorporation of copper in the trans -Golgi lumen of hepatic cells. In yeast and mammalian systems the copper homeostatic pathways are well understood. Copper homeostasis is tightly regulated to ensure the availability and minimize the potential toxicity. It includes copper transporters for uptake of copper, copper chaperons for delivery of copper ions to specific copper containing proteins.

The P- type ATPases are evolutionary conserved proteins having a fundamental role in homeostasis and biotolerance of transition metal ions in a wide range of organisms. They transport specific cations across the membrane using ATP driven energy. The members of P- type ATPase family shares some common features like presence of a transmembrane domain, a phosphorylation domain, a nucleotide-binding domain, and an actuator domain (Toyoshima *et al*., 2000; Morth *et al*., 2007; Pedersen *et al*., 2007).

The known copper P-type ATPases fall into two different classes. One class, belonging to the PIB-1or PIB-2 subgroups, is characterized by a Cys-Pro-Cys (CPC) metal-binding sequence in Transmembrane (TM) 6 and a Cys-X-X-Cys motif in the N-terminal metal binding domain (MBD) (Argüello, 2003). This class includes the CopA copper pumps of *Archaeoglobus fulgidus* and *Enterococcus hirae*. In prokaryotes, these pumps are believed to serve the uptake of copper (Odermatt *et al*., 1994; Francis and Thomas, 1997). The other class is represented by the CopB copper pump of *A. fulgidus*, which belongs to the P1B-3 subgroup and is thought to be active in copper extrusion rather than uptake (Odermatt *et al*., 1994; Bissig et al., 2001). This class has the characteristic putative metal-binding motif Cys-Pro-His (CPH) in TM6 (Argüello, 2003), a histidine-rich N-terminal MBD, and the characteristic sequence motifs GYN in TM7 and MS-X-ST in TM8 (Axelsen and Palmgren, 1998). Eukaryotic copper-ATPases are unique among the P-type ATPases because they do not bind copper directly from the cytosol, where the amounts of free copper are extremely low (Rae *et al*., 1999) but receive the metal ion from a small cytosolic protein called a metallochaperone through direct protein-protein interactions (Lockhart and Mercer, 2000; Larin *et al*., 1999; Walker *et al*., 2002; Pufahl *et al*., 1997).

Copper transporting P- type ATPase has been reasonably studied as Wilson ATPase (Hung *et al*., 1997; Forbes and Cox, 1998), the Menkes ATPase (Payne and Gitlin, 1998), the *Caenorhabditis elegans* copper ATPase (Yoshimizu *et al*., 1998), *E. coli* CopA (Fan and Rosen, 2002) and *E. hirae* CopB (Bissig *et al*., 2001).

The present study was taken up to explore the presence and importance of a copper loading protein (putative LDATP7) on functional activity of LDMCO as well as its role in virulence of *Leishmania donovani*. Copper transporting P-type ATPase is an evolutionary conserved protein known to transport copper through trans- Golgi membrane where copper gets incorporated into multicopper oxidase. With the help of available *Leishmania infantum* genome a putative copper transporter in LD has been identified. The precise role of LDATP7 in loading copper in to LDMCO and thus in general iron uptake of this parasite was determined by creating genetically modified LD. Like LDMCO, the LDATP7 has been also found as essential for survival and growth of LD.

Results

Effect of Iron and copper chelation on *in vitro* growth of *Leishmania donovani* (LD) promastigotes

To determine the effect of iron and copper chelation on *in vitro* growth of LD, promastigotes were treated with different concentrations (0-4mM) of BPS (iron chelator) or BCS (copper chelator) and number of cells were counted after every 24 hour and compared with the growth of untreatd parasite. Figure 1 shows that copper chelation is more effective in blocking the growth of the parasite than iron chelation. This experiment suggests the essential requirement of copper for maintaining homeostasis in LD.

Copper deficiency affects *in vitro* growth of *Leishmania donovani* (LD)

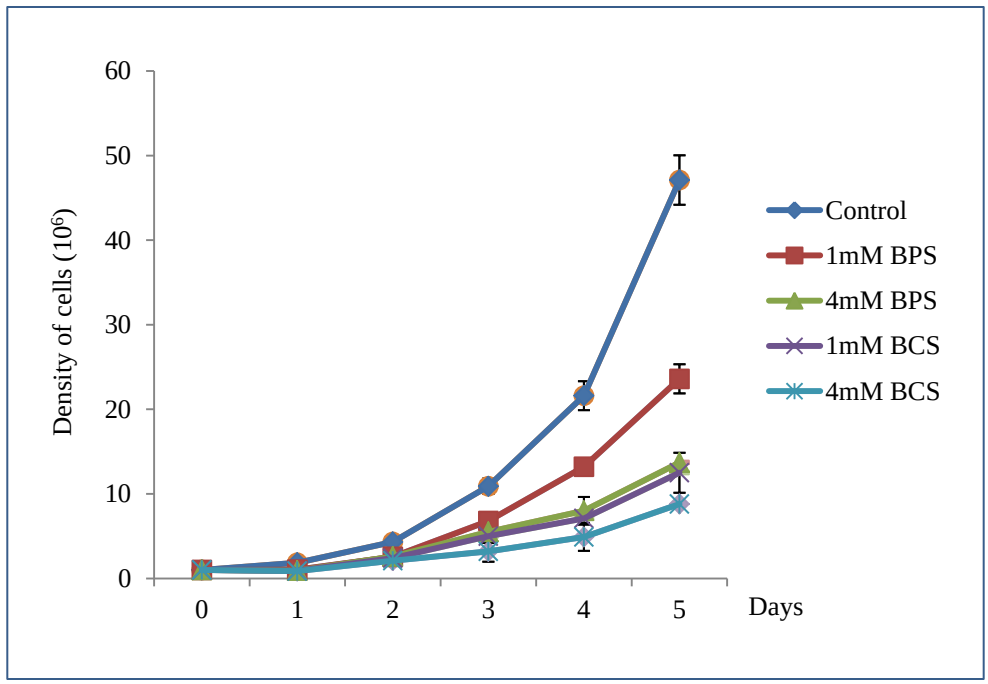
Bathocuproine disulfonate (BCS) is a Cu^{+} specific chelator, which chelates copper from the growth media. Different concentrations of BCS were used to test the *in vitro* growth rate of LD. The result showed that increasing concentration of BCS affected LD growth in a dose dependent manner (Fig. 2A). This was further measured by ^{3}H-thymidine uptake assay. The result suggests reduction of DNA synthesis due to depletion of copper (Fig. 2B). The result also underscores the importance of copper in LD growth. Since, copper is the cofactor of LDMCO, understanding copper loading mechanism is important and following experiments are designed to identify and characterize the copper loading P-type ATPase in LD.

***Leishmania major* and *Leishmania infantum* contains putative copper transporter P-type ATPase**

The genome databases of *Leishmania major* and *Leishmania infantum* have been published (Ivens *et al.*, 2005; Peacock *et al*., 2007). Gene database search for copper transporter shows that both *L. major* and *L. infantum* contain putative copper transporting ATPase like gene on chromosome 33. It comprises 3492 bp encoding 1163 amino acids. This putative ATPase like protein also bears the signature motifs of copper transporting P- type ATPase.

Cloning and Sequencing of *Leishmania donovani* copper transporting P-type ATPase

Leishmania major, *Leishmania infantum* and *Leishmania donovani* show a high (~ 96 %) homology in their nucleotide sequences in general. Thus to clone LDATP7



Density = average no. of cells X dilution X 10^4 cells /ml

Figure 1. Effect of Iron and Copper chelation on LD growth. 1X10^6 *Leishmania* parasites were grown in M199 media containing 10% FBS, 1% PS and treated with or without different concentrations of iron chelator (BPS) or copper chelator (BCS). Parasite numbers were counted after every 24 hours with a haemocytometer. Data is shown as mean ± S.D of three independent experiments.

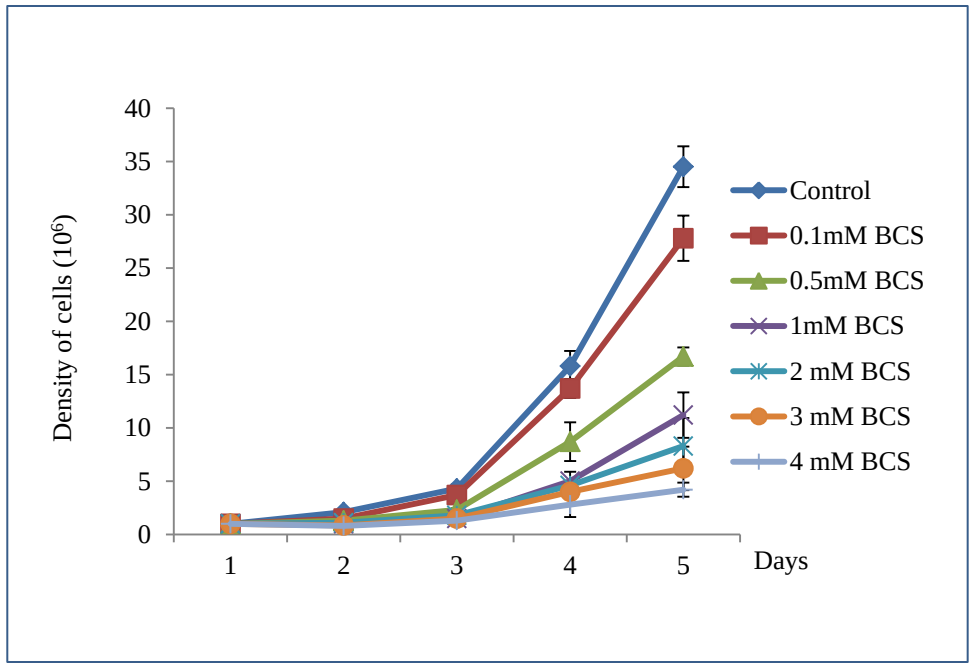


Figure 2A. Effect of copper chelation on the growth of *Leishmania donovani*. LD was grown in culture media M199 containing 10% FBS, 1% PS and treated with different concentrations of copper chelator (BCS). Parasite numbers were counted after every one day. Data is shown as the mean ± S.D of three independent experiments.

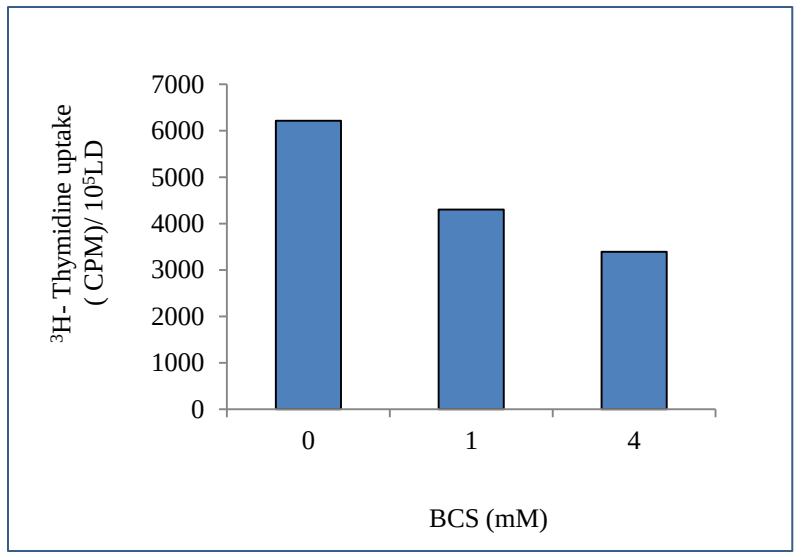


Figure 2B. ^{3}H-Thymidine uptake assay. LD was treated with different concentrations of BCS for 48 hours and then incubated with ^{3}H-Thymidine at 0.4µCi/ml concentration for 12 hours. Cells were collected and radioactivity was measured. The result represents one of the three independent experiments with similar results obtained.

from LD genomic DNA primers were designed from *L. infantum* gene because it belongs to *Leishmania donovani* complex (the genome database of LD has been published after this experiment was performed). Figure 3A shows the strategy for cloning of LDATP7 from *L. donovani* into pET28a (+) vector. PCR amplification provided a 3492 bp product (Figure 3B), which was cloned in to pET 28a (+) *E. coli* expression vector. Restriction mapping (Figure 3C) and DNA sequencing analysis confirmed the right clone.

Sequence analysis and homology search of LDATP7

Sequence analysis gives the coding sequence of LDATP7 (Figure 4A) which should provide a protein containing amino acid sequence (Figure 4B) using ExPASy translational tool. NCBI BLAST conserved domains search shows presence of three heavy metal associated domains in amino terminal of protein in which the first shows GMTCRGC, second shows GMSCTSC and third shows GMPCASC sequence. Further it contains an E1-E2 ATPase domain and a haloacid dehalogenase-like hydrolase domain. PROSITE scan results show presence of heavy metal associated domains at 17-84, 244-310, 351-416 amino acids. E1-E2 ATPase phosphorylation site DKTGT is present at 796-802 position. It contains other signature motifs of PIIb type ATPase transporter proteins like CPC ion channel, TGES phosphatase domain. Multiple alignments were done by using EMBL-EBI Clustal W2 software. LDATP7 protein aligned with Menkes disease protein (CAB94714.1), Wilson disease protein (AAB52902.1), *S. cerevisiae* (NP_010556.1), *L. major* (XP_001685970.1), *L. infantum* (LinJ33_V3.2210) *T. brucei* (Tb927.11.1260) (Figure 4C). Transmembrane domain search by using TMHMM Server v.2.0 predicts presence of nine transmembrane domains at 378-400, 446-465, 480-502, 515-537, 541-563, 700-722, 737-759, 1100-1122, 1127-1149 positions (Figure 4D).

Expression and purification of recombinant LDATP7

The 3492 bp gene encodes 1163 amino acids containing protein with apparent molecular weight of 123 kD and isoelectric point (pI) at pH 6.9. Several attempts were failed to express such a large protein in pET28a (+) expression system. So, for antibody production it was decided to express the N-terminal 1412 bp gene encoding 56 kD peptide. The N-terminal 1412 bp gene was cloned into pET28 a (+) expression vector and for expression transformed in *E. coli* BL21 strain. During the attempt of

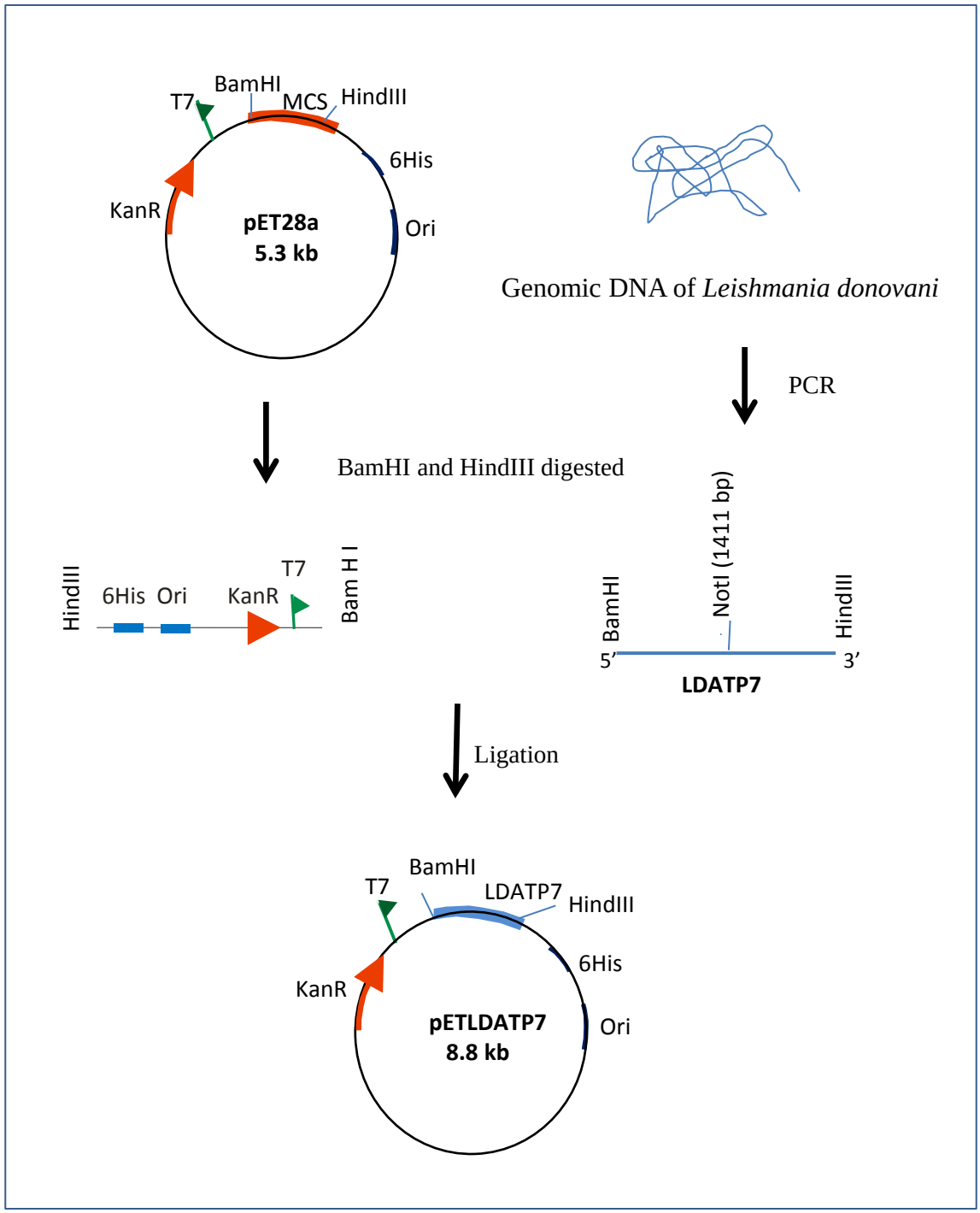


Figure 3 (A) Schematic representation of construction of recombinant pET28-LDATP7

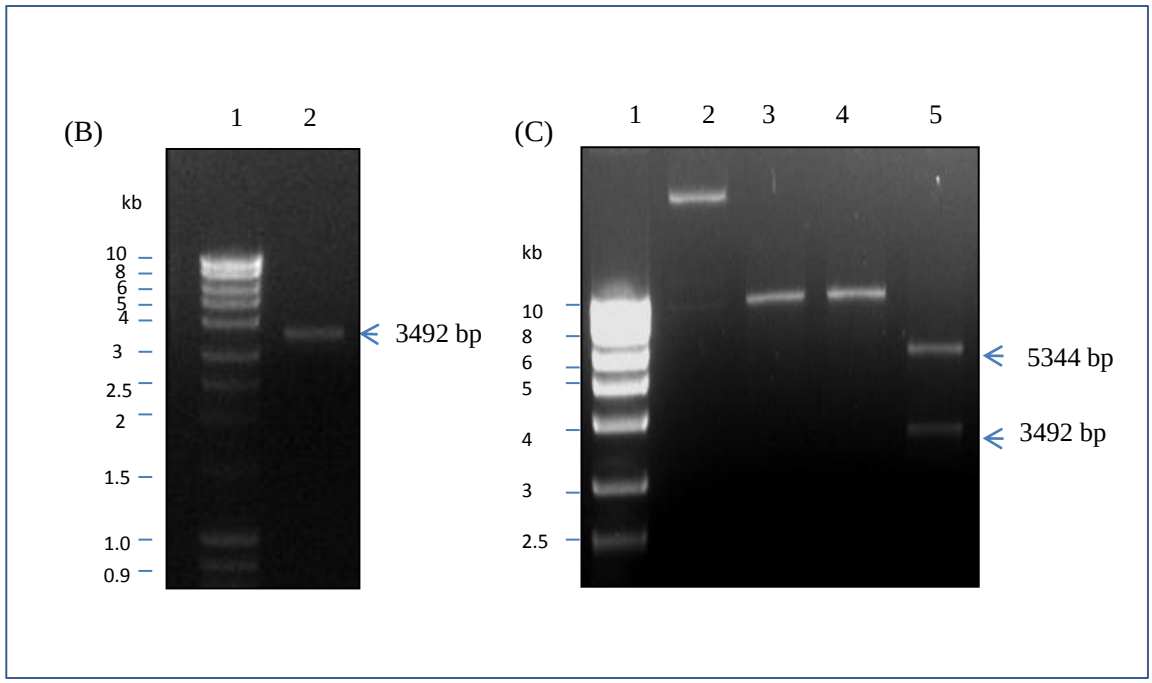


Figure 3. Cloning of LDATP7. **(B)** Agarose gel analysis of PCR amplification of putative LDATP7 from genomic DNA of LD. Lane1. marker (Mass ruler DNA ladder mix), lane2. 3492 bp amplified LDATP7 gene. **(C)** Restriction digestion to confirm positive clone. The 3492 bp LDATP7 gene was cloned in pET28a (+) vector. Lane 1. marker (Mass Ruler DNA ladder mix), lane 2. uncut plasmid containing LDATP7, lane3. positive clone cut with BamHI, lane 4. positive clone cut with HindIII, lane 5. positive clone cut with BamHI and HindIII.

Figure 4 A. DNA sequence of LDATP7 cloned from *Leishmania donovani.* Sequencing data confirms the gene contains 3492 bp nucleotides.

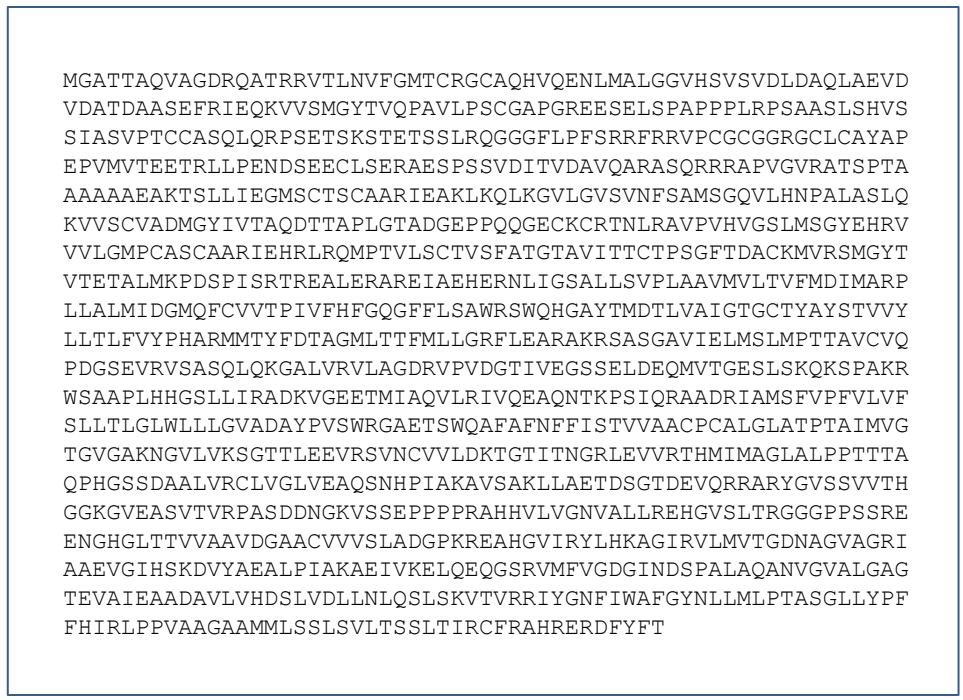


Figure 4 B. Predicted Amino acid sequence of LDATP7 cloned from *Leishmania donovani.* Amino acid predicted by using ExPASy proteomics tools.

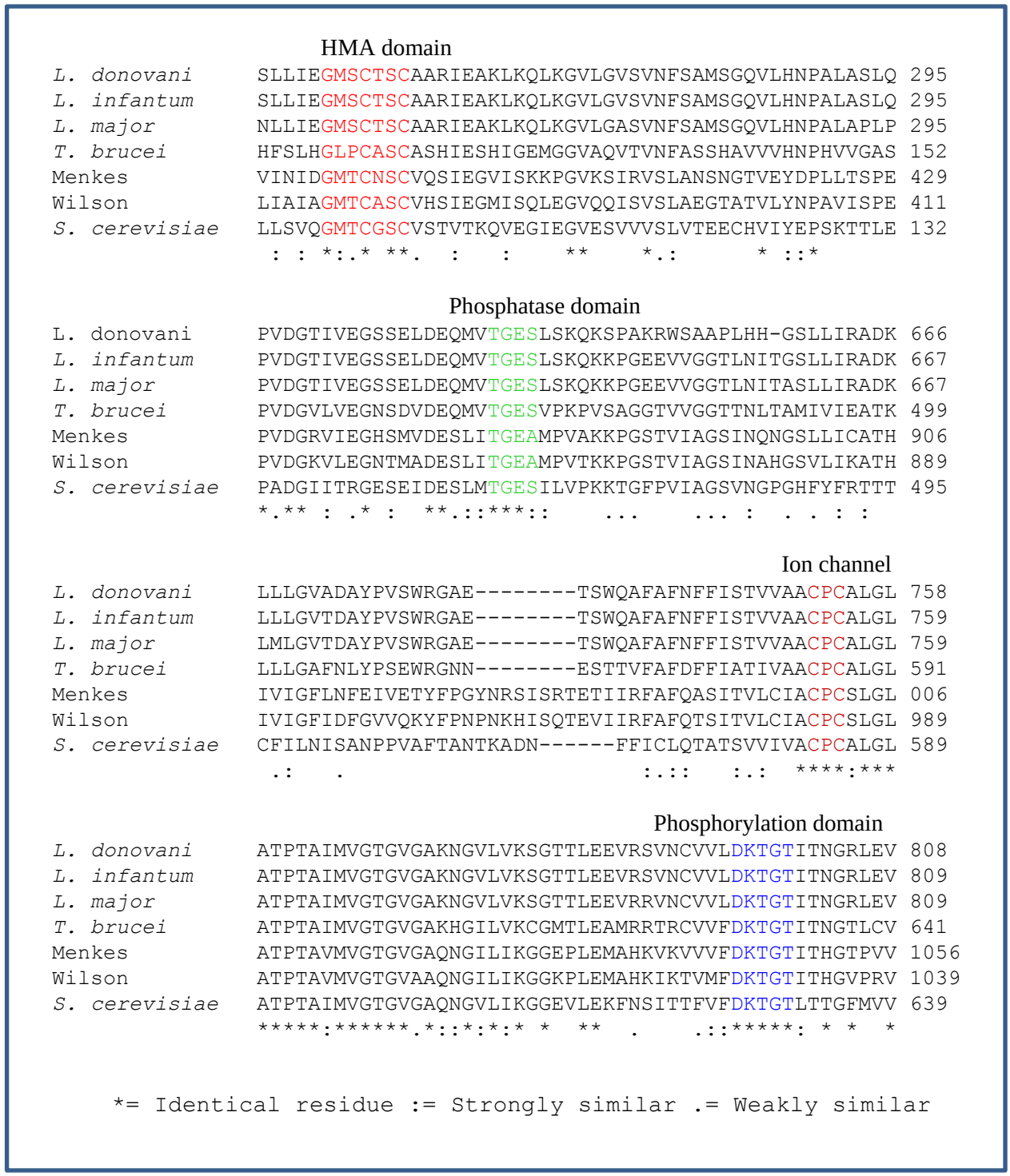


Figure 4 C. Amino acid sequence alignment of the conserved domains of *Leishmania donovani* LDATP7 protein with other copper transporter P_{II}B type ATPases. LDATP7 protein aligned with Menkes disease protein (CAB94714.1), Wilson disease protein (AAB52902.1), *S. cerevisiae* (NP_010556.1), *L. major* (XP_001685970.1), *L. infantum* (LinJ33_V3.2210), *T. brucei* (Tb927.11.1260), using EMBL-EBI ClustalW2 mutiple alingment software. Colored sequences show conserved domains.

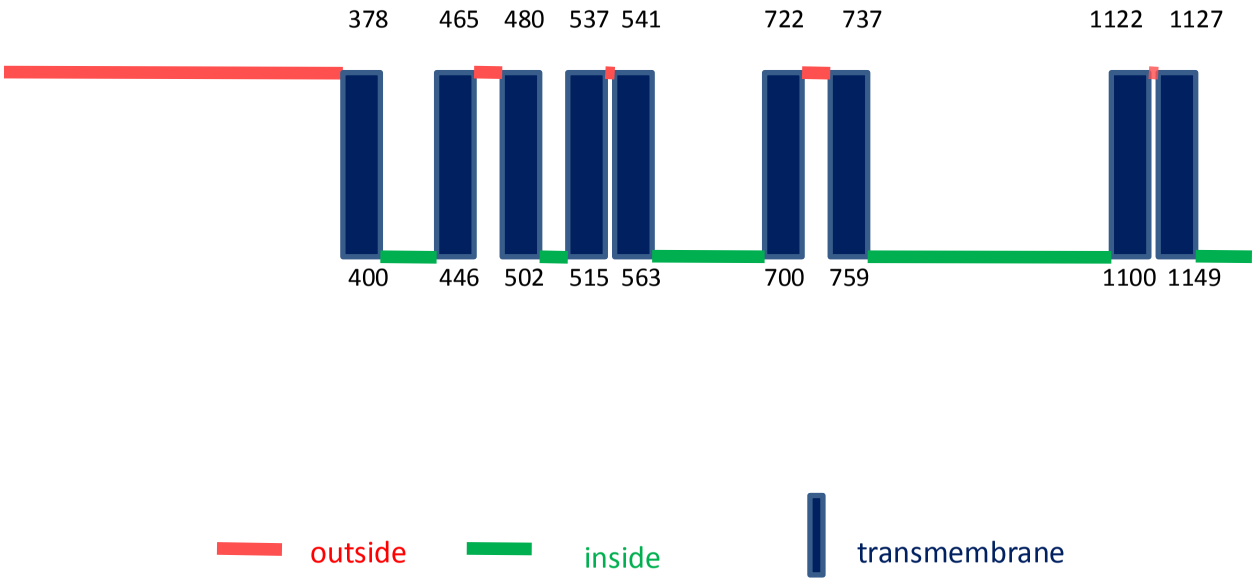


Figure 4D. Membrane topology prediction. Schematic presentation of Transmembrane domains predicted by using TMHMM Server 2.0 shows presence of nine transmembrane helices .

the expression of this N-terminal peptide inclusion bodies were formed as shown in Figure 5A, which was solubilized by using 8M urea. The solubilized protein was purified and used for injecting rabbit to raise antibody. Specificity of the antibody was verified by Western blot analysis for pre-immune serum and anti-LDATP7 against recombinant protein and LD WCL (whole cell lysate) (Figure 5B).

Localization of LDATP7 in promastigote stage of LD

In yeast CCC2 was found in the trans-Golgi network (Yuan *et al.*, 1997). The Wilson disease protein was also found to be localized in trans-Golgi network of hepatocyte (Hung *et al.*, 1997). Amino acid sequence analysis of LDATP7 using TMHMM Server v.2.0 software predicts the presence of nine transmembrane domains. This suggests that LDATP7 is a membrane bound protein. To determine the localization of LDATP7 in LD promastigote immunofluoresence studies were performed by using anti-LDATP7 antibody raised in mice and Alexa 594 conjugated anti mouse IgG as secondary antibody. Figure 6 shows the presence of LDATP7 in flagellar pocket. Position of nucleus and kinetoplastid DNA were confirmed by using Hoescht3342 (Fig. 6).

Generation and molecular characterization of LDATP7 mutants

To determine the physiological role of LDATP7 and interrelation between LDATP7 and LDMCO in *Leishmania donovani*, it was decided to manipulate the LDATP7 gene. LDATP7 knock out parasites were generated by replacing one or both alleles using homologus recombination method. *Leishmania* is an asexual diploid organism, thus requires two rounds of sequential replacement for the two alleles using dominant selectable marker. To achieve the goal two different targeting constructs were used containing 1000 bp of 5' UTR and 1000 bp of 3' UTR of LDATP7 surrounding the NEO or the HYGRO selectable marker (Figure 7A). In another strategy to replace the allele construct was made by cloning 5'UTR and C-terminal 1852 bp of LDATP7 surrounding NEO or HYGRO selectable markers. This would disrupt the N-terminal 1640 bp of LDATP7 gene. The first allele was eliminated with either of the selectable marker. Heterozygotes were selected on plates containing 30µg/ml hygromycin or 40µg/ml G418 for HYGRO and NEO selectable markers, respectively. These concentrations of antibiotics were found enough to kill untransfected cells. The allelic

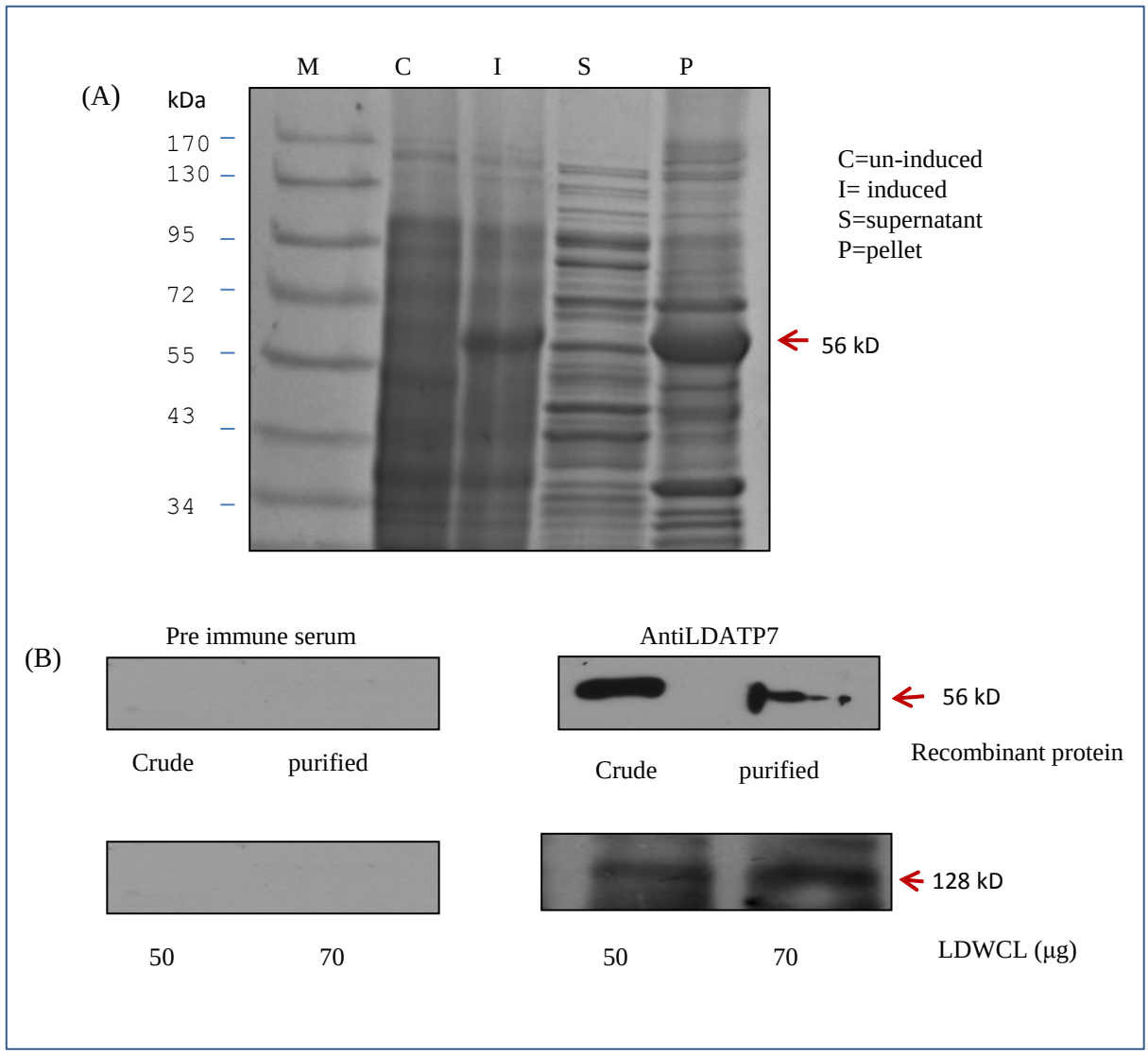


Figure 5. Expression of N-terminal 56 kDa LDATP7 recombinat product and antibody generation. (A) SDS-PAGE shows induction of recombinant protein product (Coomassie staining). **(B)** Western blot analysis shows specificity of anti LDATP7 antibody. *E. coli* expressed LDATP7 recombinant protein (upper panels) and LD whole cell extract (bottom panels) were incubated with either pre immune serum or anti LDATP7 antibody.

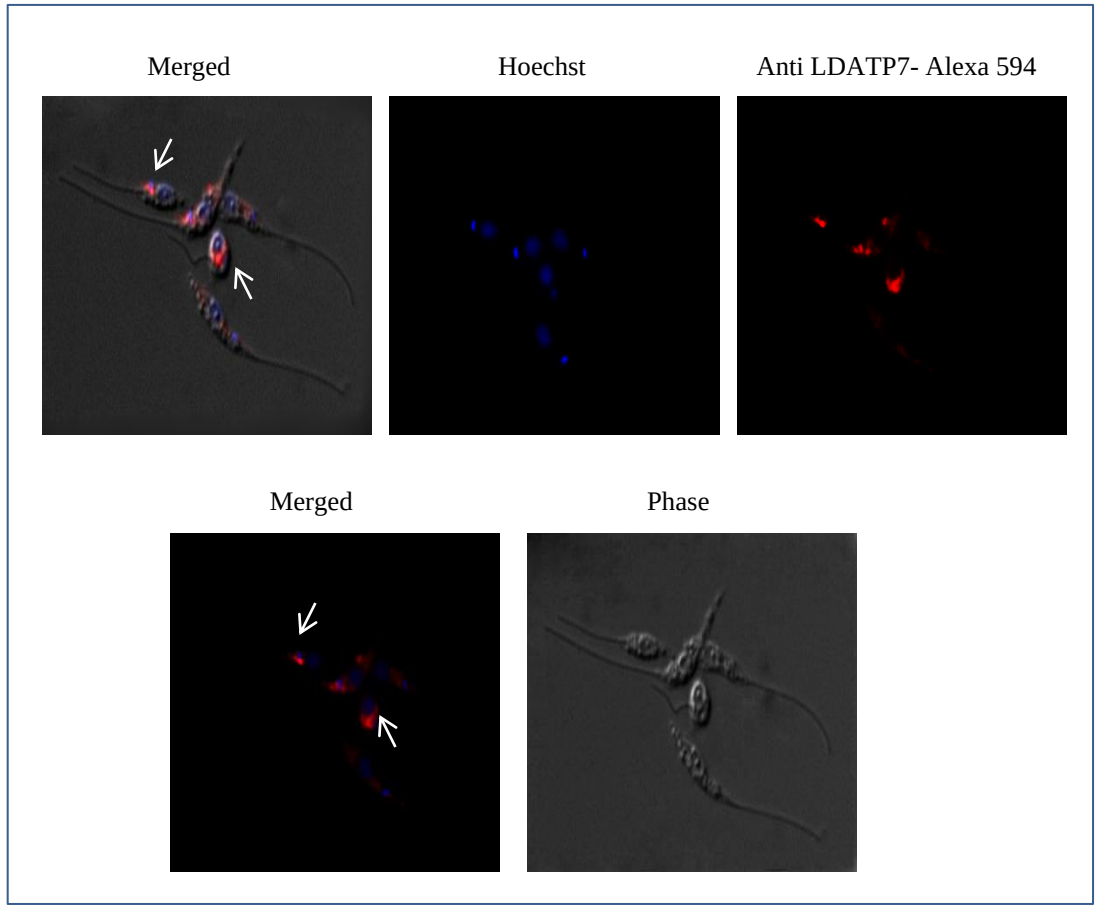


Figure 6. Localization of LDATP7 in *Leishmania donovani* promastigotes. Immunofluoresence analysis of LDATP7 in LD promastigotes . Alexa 594 conjugated anti mice antibody was used as secondary antibody.

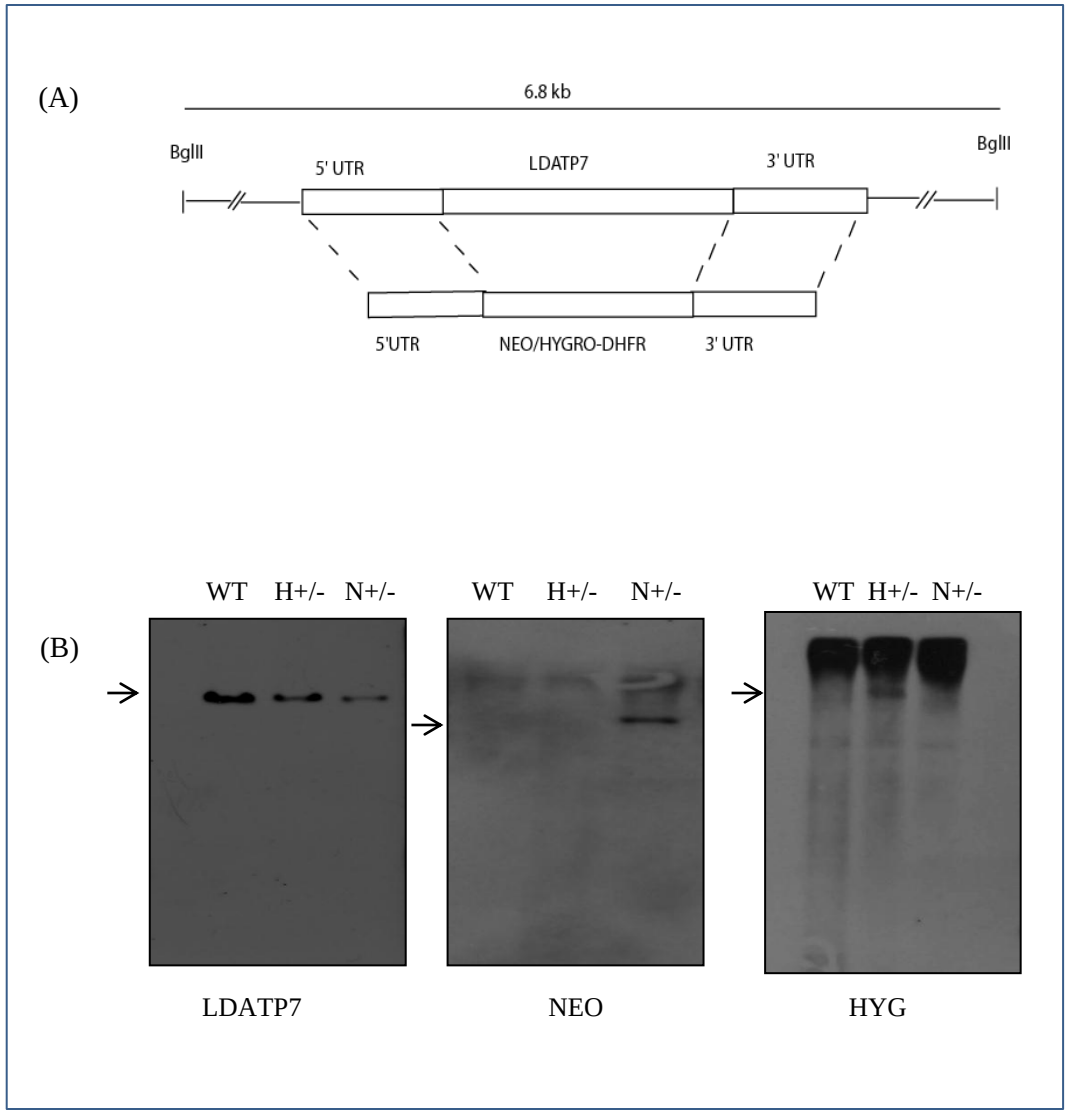


Figure 7. Targeted replacement of *Leishmania donovani* LDATP7 gene. (A) Systemic representation of the strategy used to replace LDATP7 gene from LD. **(B)** Southern blot analysis of wild type and LDATP7 single allele knock outs. Genomic DNA was isolated from wild type (WT) and single neo (N+/-) and hygro (H+/-) replacements and digested with BglII, seperated through 0.8% agarose gel, blotted and hybridized with DIG labeled DNA probe.

replacement of LDATP7 was monitored by southern blotting using LDATP7, HYGRO and NEO specific probes (Figure 7B). Restriction mapping of LDATP7 shows that BglII digestion followed by Hybridization with LDATP7 probe will generate a ~ 6.8 kb hybridization signal. RT-PCR was also used to confirm the integration of HYGRO and NEO markers and replacement of single allele (Figure 7C, 7D). To further check the orientation of the integrated gene PCR was performed from genomic DNA of heterozygous mutants using specific primers (Figure 7E). LDATP7 single allele knock outs were also confirmed by immunoblot studies, revealing almost half expression of LDATP7 protein in comparison to wild type LD (Figure 7F). The LDATP7 heterozygous mutants were maintained at 60µg/ml of Hygromycin or 65 µg/ml of Neomycin. In order to make homozygous mutant of LDATP7 the single allele knockout was targeted for the replacement of second allele. But several attempts resulted in failure of getting complete knock out LDATP7 LD, as selection in presence of both the antibiotic was unable to grow the cells. This might suggest the necessity of LDATP7 gene for survival of LD.

Growth rate studies of LDATP7 mutants

The wild type and LDATP7 single allele LD promastigotes were grown in M199 media with 10% FBS at 22º C. The growth was measured by counting parasites for five successive days. Both the single allele knockouts show about half growth compared to wild type LD (Figure 8). The retardation of growth in absence of one LDATP7 allele further implicates the importance of LDATP7 in the growth of LD.

Characterization of single-allele knockout parasites by determining ferroxidase activity of Wild type and mutants

Copper is essential for ferroxidase activity of LDMCO (unpublished data from our laboratory). The effect of deletion of copper loading gene on MCO activity was examined by ferroxidase assay. WT and single-allele mutant LD were harvested in late log phase and whole cell lysate was used for assaying the ferroxidase activity. The mutant shows reduced ferroxidase activity in comparison to WT (Figure 9). Repetition of experiment showed similar results suggesting the requirement of LDATP7 in copper-loading to LDMCO.

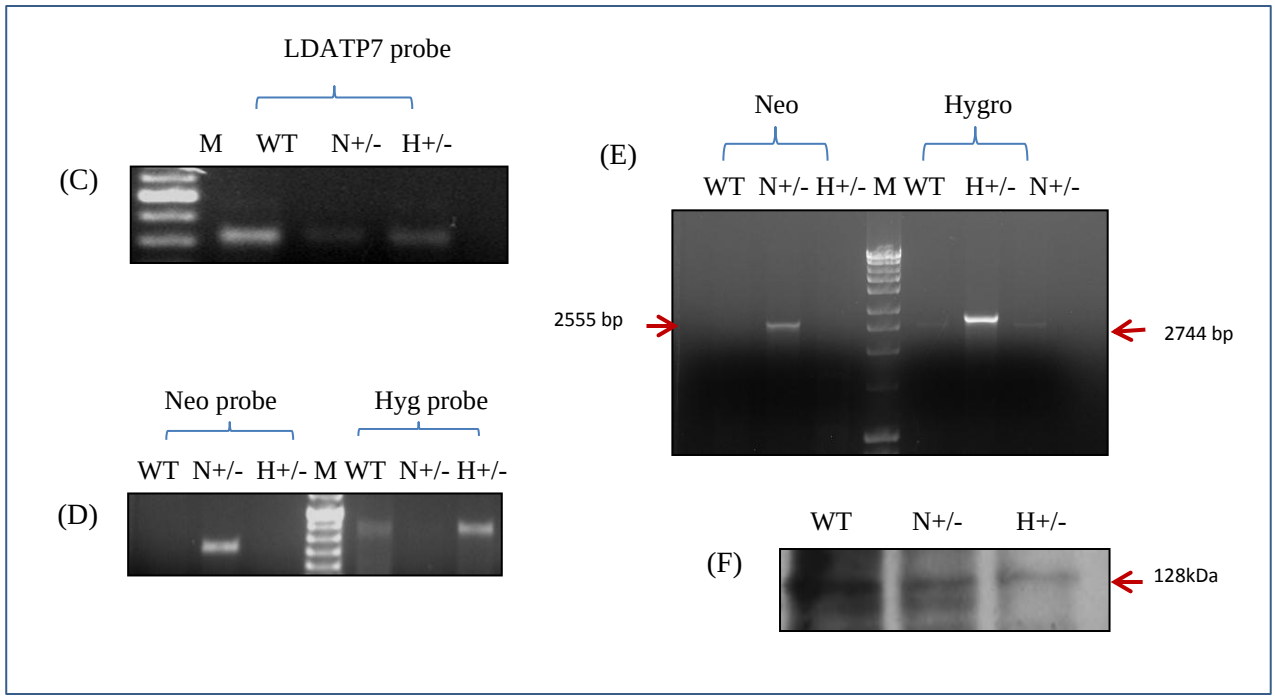


Figure 7. RTPCR analysis for the wild type and LDATP7 single allele knockout . Total RNA was isolated from WT and single allele knockout LD and RTPCR was performed using **(C)** LDATP7 **(D)** Neo and Hygro specific primers **(E)** PCR to verify the integration orientation of replacement cassette . PCR was performed from genomic DNA using specifically designed primers. **(F)** Western blot analysis to show the protein expression level of wild type LDATP7 and single allele Knockout LD using anti LDATP7 antibody.

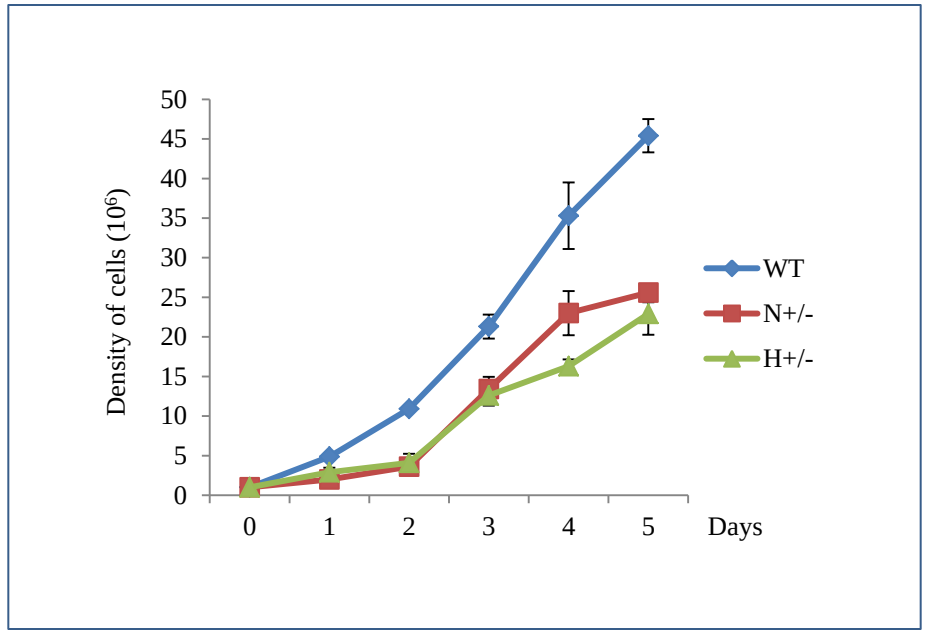


Figure 8. Growth rate analysis of wild type and LDATP7 single allele knockout (S-KO). Growth rate analysis for wild type and LDATP7 half knockouts. Late log phase cells inoculated at $1x10^6$ cells/ml density in M199 media with 10% FBS, 1% PS and cells were counted after 24 hour interval. Data is shown as mean ± S.D of three independent experiments.

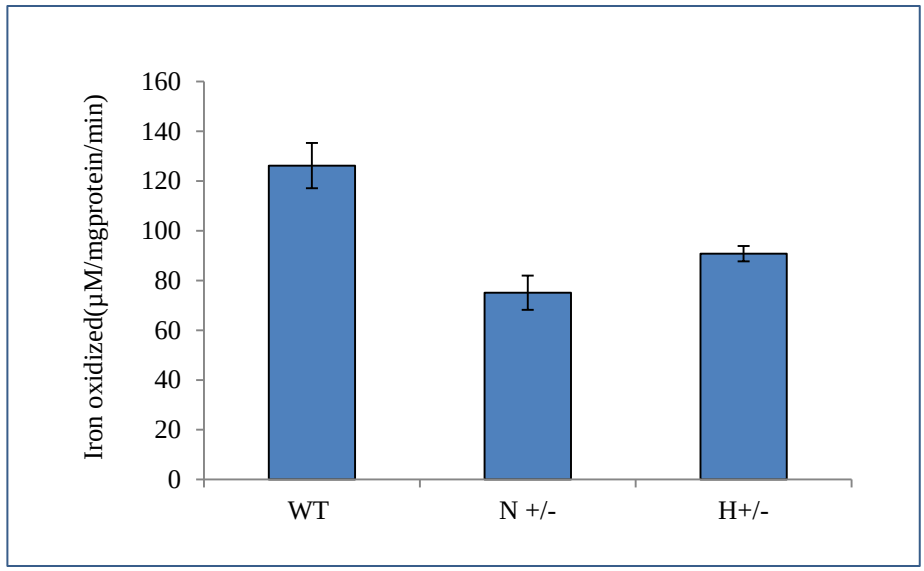


Figure 9. Effect of LDATP7 gene deletion mutation on ferroxidase activity of MCO. Ferroxidase activity of wild type and LDATP7single allele knock out mutants. 10µg of whole cell lysate was used for spectrophotometric analysis. Data is shown as mean ± S.D of three independent experiments.

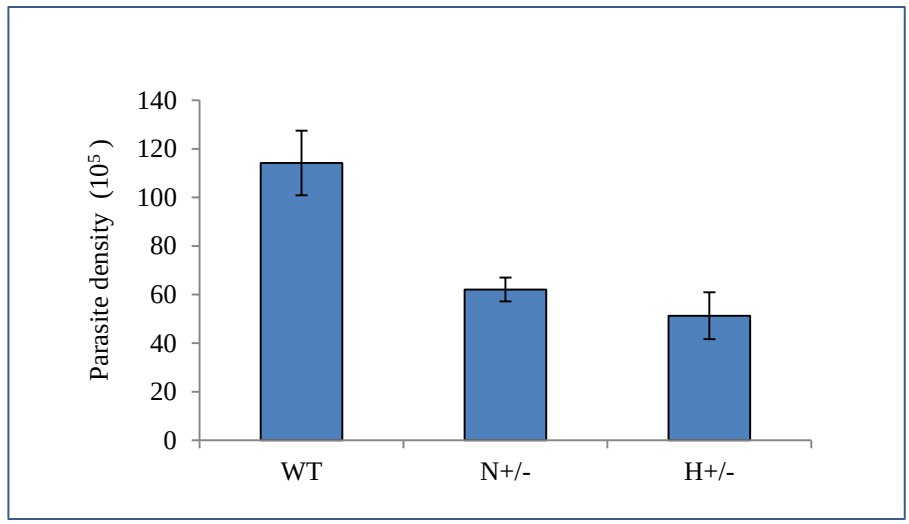


Figure 10. Growth of wild type, LDATP7 single allele knockout (S-KO) LD amastigotes isolated from J774A.1 by Percoll Gradient Method. J774A.1 macrophages were infected with wild type and single allele KOs LD. After 16 hrs of infection intracellular amastigotes were isolated from macrophages by percoll gradient method. Infectivity was estimated by counting amastigotes using haemocytometer. Data is shown as mean ± S.D of three independent experiments.

Role of LDATP7 in virulence by estimating infectivity of wild type and LDATP7 single-allele knockout

J774A.1 macrophage cells were taken at a density of $1x10^6$ cells in 100mm dish and infected with LD with an MOI of 1:10 as macrophage: LD for 16 hour. Infected macrophages were then lysed by freeze-thaw method and amastigotes were isolated by percoll gradient method. Infectivity was estimated by counting amastigotes using hemocytometer. Results show that single allele knockout is less infective in comparison to wild type (Figure 10).

Colocalization of LDATP7 and LDMCO

The earlier data showed (unpublished data, thesis of Dr. Sunil Solanki) presence of LDMCO in the flagellar pocket of LD promastigote by immunofluoresence studies and episomal expression of GFP-MCO construct. The colocalization of LDATP7 and LDMCO were verified by immunofluoresence microscopy. Anti-LDATP7 antibody and anti-LDMCO antibody were raised in mice and in rabbit respectively. Alexa 488 conjugated anti rabbit IgG and Alexa 594 conjugated anti-mouse IgG were used as secondary antibody. Hoescht 3342 was used for staining nuclei and kinetoplast DNA. It was found that LDATP7 and LDMCO were colocalized in the flagellar pocket (Figure 11). It strongly suggests the interrelationship of LDMCO and LDATP7.

Complementation of Δ ccc2 *Saccharomyces cerevisiae* mutants with LDATP7

Saccharomyces cerevisiae contains LDATP7 gene homolog ccc2. CCC2 protein in *S. cerevisiae* loads copper into high affinity iron uptake multicopper oxidase protein Fet3. Thus to further confirm the function of LDATP7 in loading copper into MCO, yeast complementation assay was performed. LDATP7 and *Saccharomyces cerevisiae* CCC2 (positive control) were cloned in yeast episomal vector (YEp352). *S. cerevisiae* Δ ccc2 cells were transformed with constructs containing either LDATP7 or CCC2 or empty vector. Transformed strains were spotted on YPD and YPD containing 140 µM BPS (iron chelator) agar plates. In iron deficient condition Δ ccc2 showed highly retarded growth, which was complemented by LDATP7 or CCC2 (positive control) but not with empty vector (Figure 12). HMA-1 and phosphorylation domains are known to be needed for transporting copper in to P-type ATPases. So mutations were introduced in HMA-1 and phosphorylation domains in LDATP7 by site directed mutagenesis and cloned into YEp352. These functional domain mutants of LDATP7

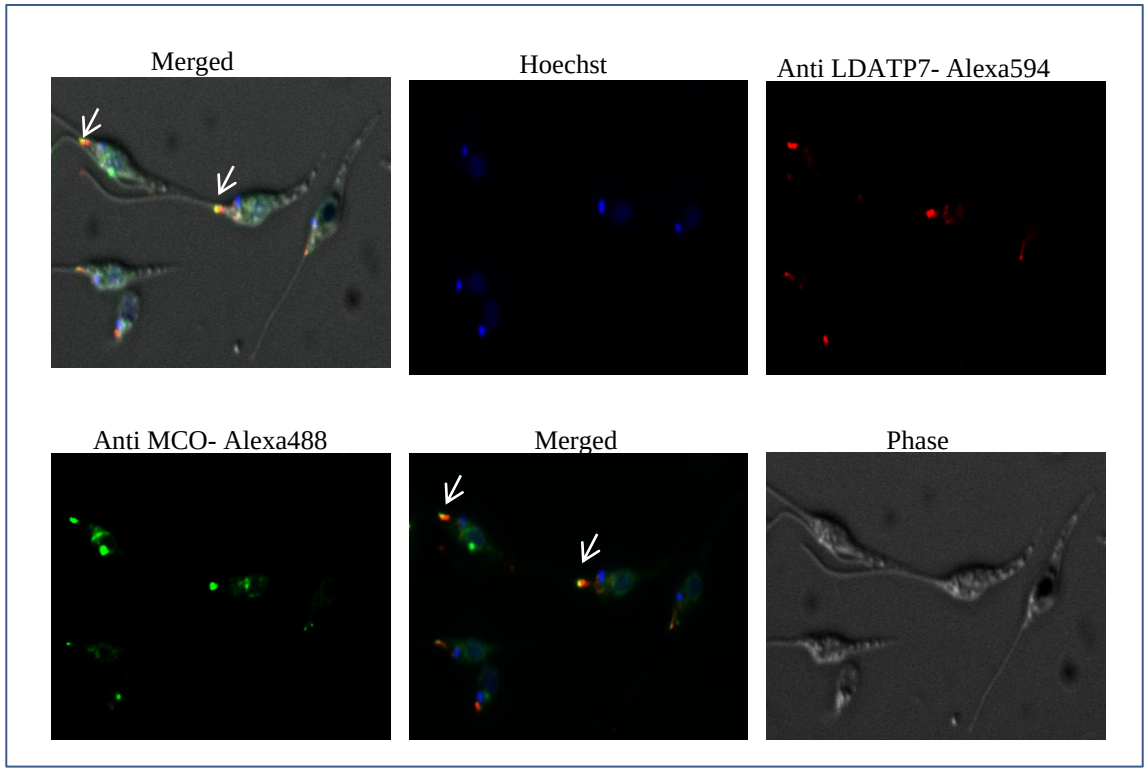


Figure 11. Colocalization of LDATP7 and LDMCO. Proteins in LD promastigote stage were shown by immunofluoresence using Alexa 488 (green) and Alexa 594 (red) conjugated secondary antibodies. White arrows indicate the colocalized LDMCO and LDATP7.

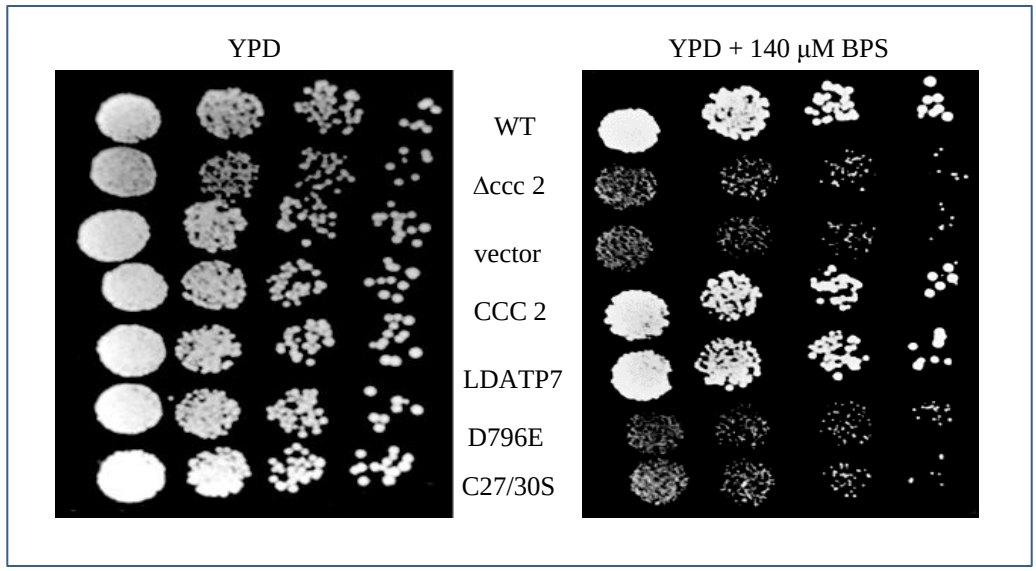


Figure 12: Complementation assay in *Saccharomyces cerevisiae*. LDATP7, *Saccharomyces cerevisiae* CCC2 and functional domain mutants of LDATP7 (D796E and C27/30S) were cloned in Yeast episomal expression vector (YEp352). *S. cerevisiae* Δ ccc2 cells were transformed with these constructs and empty vector. Transformed strains were analysed by spot assay on YPD and YPD containing 140 μM BPS (iron chelator) agar plates. The plates were photographed after 2 days of incubation at 30° C.

used to further confirm the role of LDATP7 in loading copper into LDMCO. Results showed complementation with LDATP7 Heavy Metal Associated domain (HMA-1) and phosphorylation domain mutants were unable to rescue the Δccc2 yeast cells in iron deficient conditions confirming LDATP7 has an essential role in functioning of LDMCO.

LDATP7 double allele knock out is survived only by episomal expression of LDATP7

Leishmania is a diploid organism, thus to generate LDATP7 null mutant two rounds of targeted gene replacement is required. Several attempts were made to create LDATP7 null mutant by deleting both the alleles but every attempts were failed as none of the parasite was survived when both the alleles were knocked out. Moreover, the earlier data showed the growth of single allele knock out was actually half of the wild type LD suggesting essentiality of LDATP7 for survival of LD (Figure 8). To confirm further the essentiality of the LDATP7 on the survival of the parasite, the gene was cloned in leishmanial episomal expression vector pXG Phleomycin. The LDATP7 half knock outs were transfected with pXG-Phleo LDATP7 and cells were selected using 40 µg/ml Neomycin/Hygromycin and 20 µg/ml Phleomycin. These LDATP7 half knockouts with LDATP7 overexpression were then subjected to the replacement for second allele. The episomal LDATP7 overexpressed parasites were then tested by measuring the growth rate. Results showed that LDATP7 overexpression can rescue the effect of LDATP7 double allele replacement. LDATP7 overexpression in wild type (WT), LDATP7 single allele knockout and LDATP7 null mutant results in increased growth rate compared to respective controls (Figure 13). Similarly, the effect of LDATP7 over expression on ferroxidase activity was also examined. LDATP7 overexpression increases the ferroxidase activity compared to all respective controls (Figure 14).

Expressions of LDATP7 gene during early infection stages at RNA level

J774A.1 macrophage cells were taken at a density of $1x10^6$ cells in 100mm dish and infected with LD with MOI of 1:10 as macrophage: LD for different period of time (1-8h). Total RNA was isolated and reverse transcribed into cDNA and used for gene specific PCR amplification. 18S rRNA was used as loading control for *Leishmania*. The quantitative real time PCR result showed that during early hours of infection

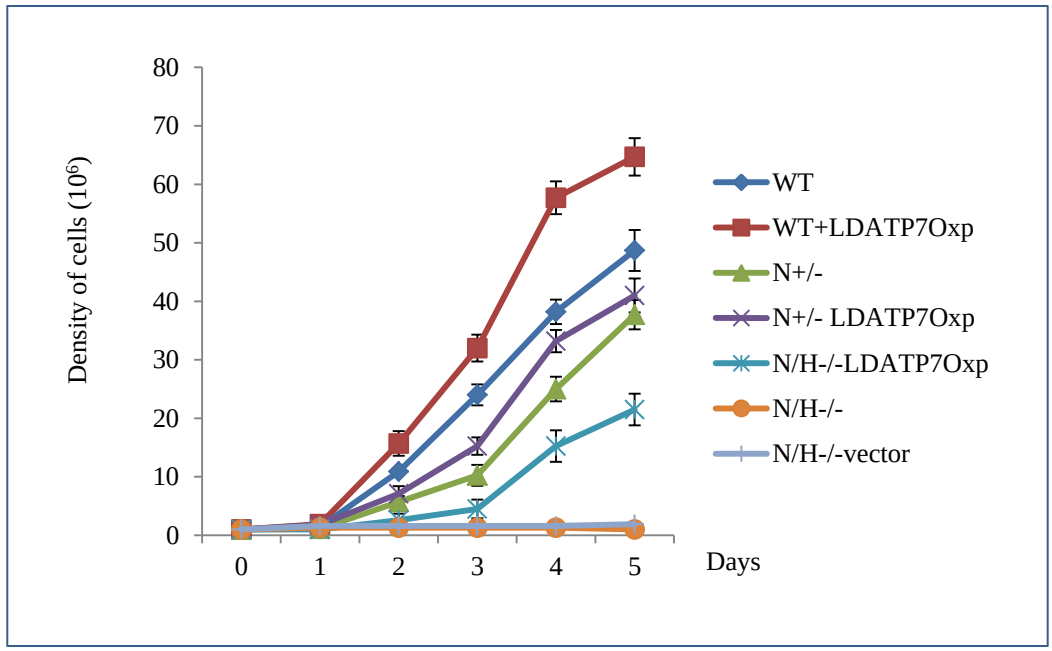


Figure 13. Effect of LDATP7 episomal expression on WT, single allele knockout and null mutant on growth of LD. Wild type and LDATP7single allele knock out mutants and LDATP7 null mutant transfected with plasmid containing episomal expression cassette. Late log phase cells inoculated at $1x10^6$ cells/ml density in M199 media with 10% FBS, 1% PS and cells were counted after 24 hour interval. Wild type (WT), WT transfected with LDATP7 overexpression cassate (WT+LDATP7 Oxp), LDATP7 single allele knockout (N+/-), single allele knockout with LDATP7 overexpression (N+/- LDATP7Oxp), LDATP7 null mutant with LDATP7 overexpression (N/H-/-LDATP7 Oxp), LDATP7 null mutant (N/H-/-), LDATP7 null mutant transfected with pXG Phleo vector only are presented in the data. Data is shown as mean ± S.D of three independent experiments.

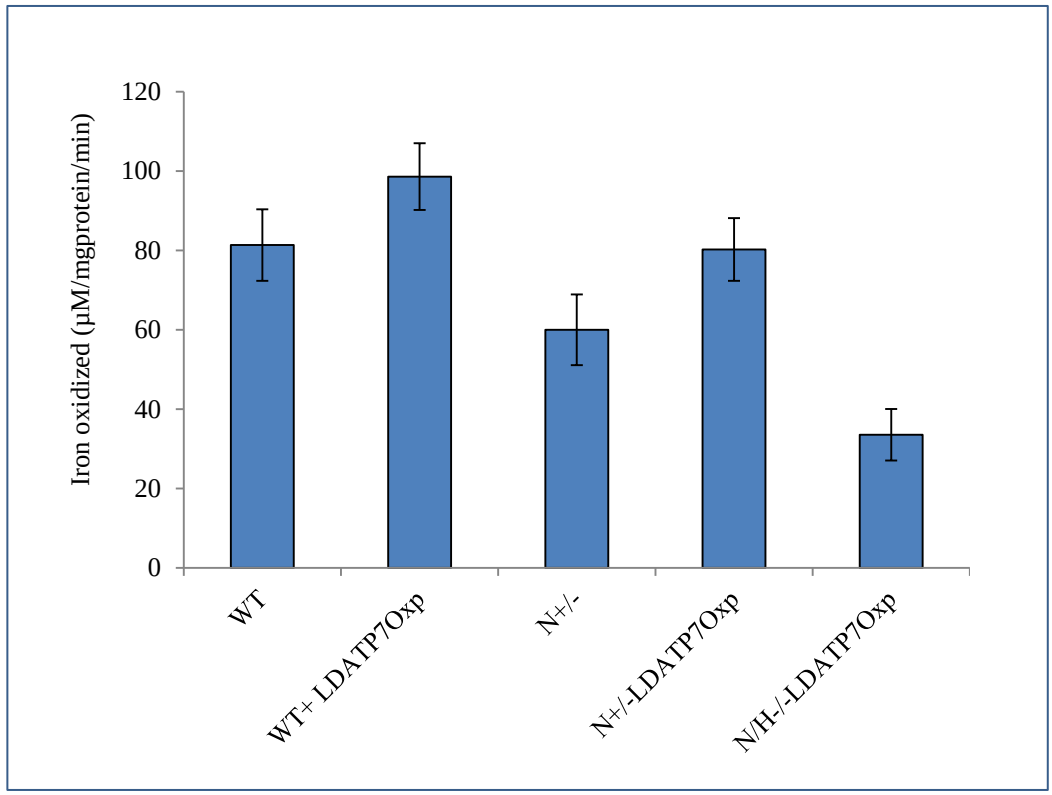


Figure 14. Effect of LDATP7 episomal expression on WT, single allele knockout and null mutant on ferroxidase activity of LDMCO. Ferroxidase activity of wild type and LDATP7 single allele knock out mutants and LDATP7 null mutant transfected with plasmid containing episomal expression cassette. 10µg of whole cell lysate was used for spectrophotometric analysis. Wild type (WT), WT transfected with LDATP7 overexpression cassate (WT+LDATP7 Oxp), LDATP7 single allele knockout (N+/-), single allele knockout with LDATP7 overexpression (N+/- LDATP7Oxp), LDATP7 null mutant with LDATP7 overexpression (N/H-/-LDATP7 Oxp) are shown in the chart. LDATP7 null mutant (N/H-/-) are not shown due to absence of any viable cell in this case. Data is shown as mean ± S.D of three independent experiments.

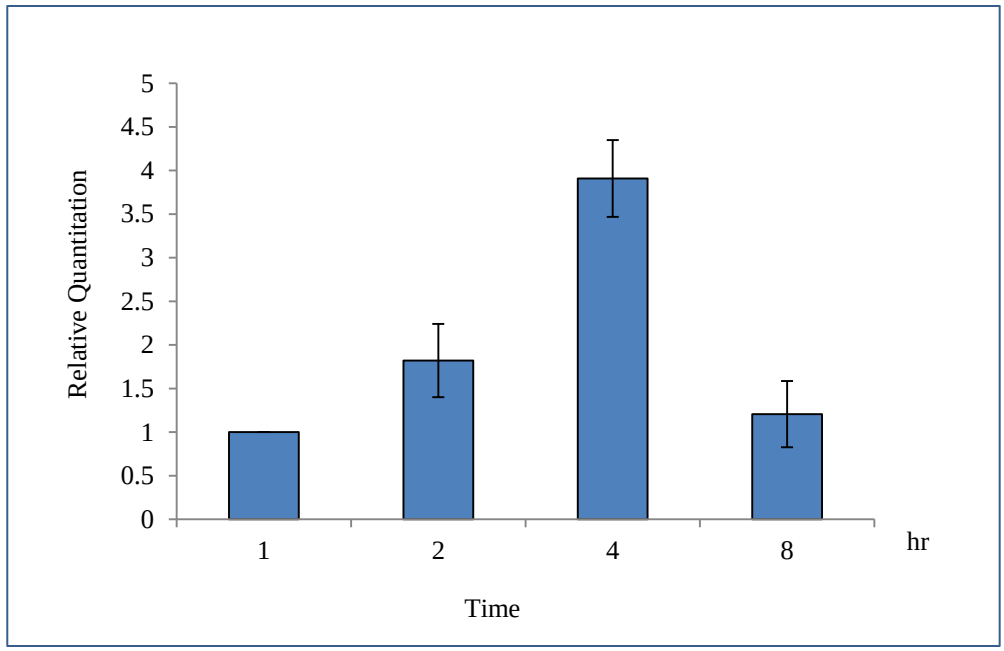


Figure 15. mRNA expression level of LDATP7 in LD infected J774.A macrophages. J774A.1 macrophages were infected with LD at 1:10 MOI for different time points. LDATP7 mRNA expression level was measured by quantitative real time PCR. All data were normalized using 18S rRNA as the endogenous control. Data shown as ± SD of three independent experiments.

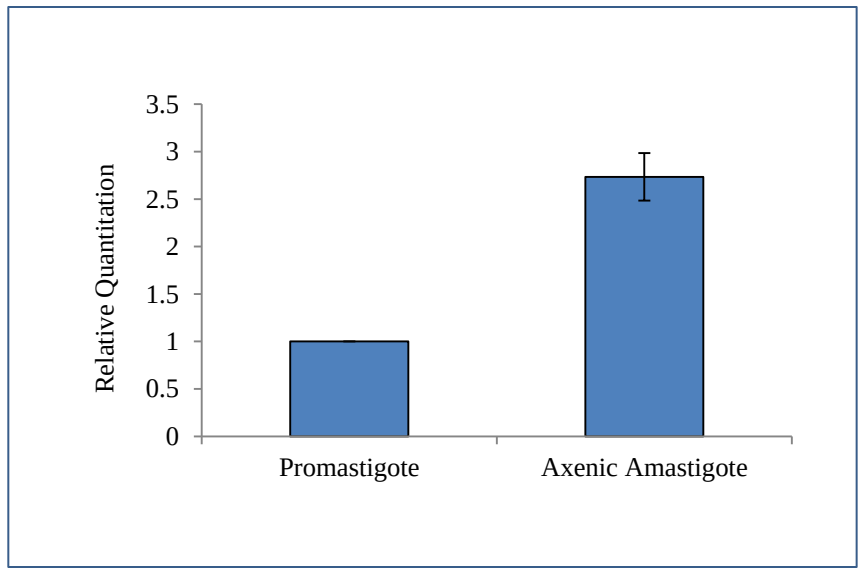


Figure 16. mRNA expression level of LDATP7 in different life stages of LD. LDATP7 mRNA expression level during promastigote and axenic amastigote stages of LD was measured by quantitative real time PCR. All data were normalized using 18S rRNA as the endogenous control. Data shown as ± SD of three independent experiments.

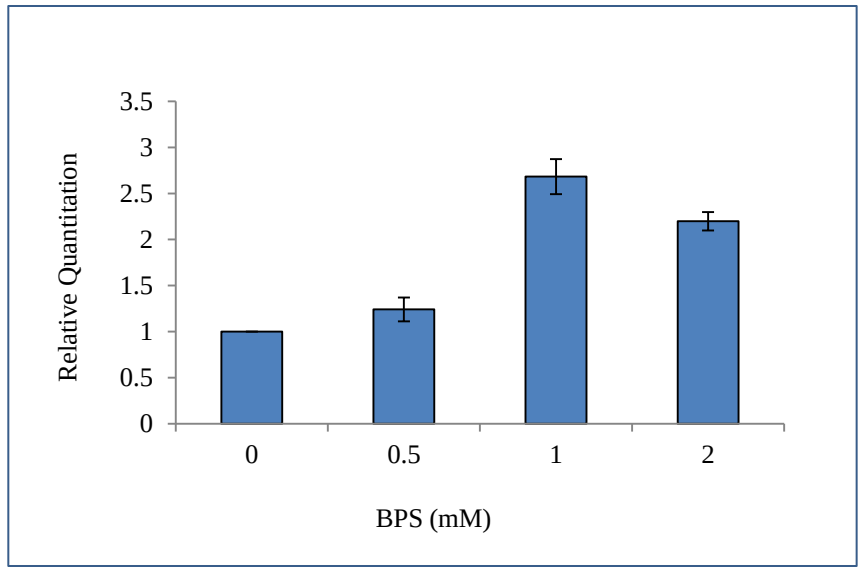


Figure 17. mRNA expression level of LDATP7 in response to different doses of iron chelator. LDATP7 mRNA expression level after treatment with different doses of iron chelator Bathophenanthroleine disulfonate (BPS) was determined by quantitative real time PCR. All data were normalized using 18S rRNA as the endogenous control. Data shown as ± SD of three independent experiments.

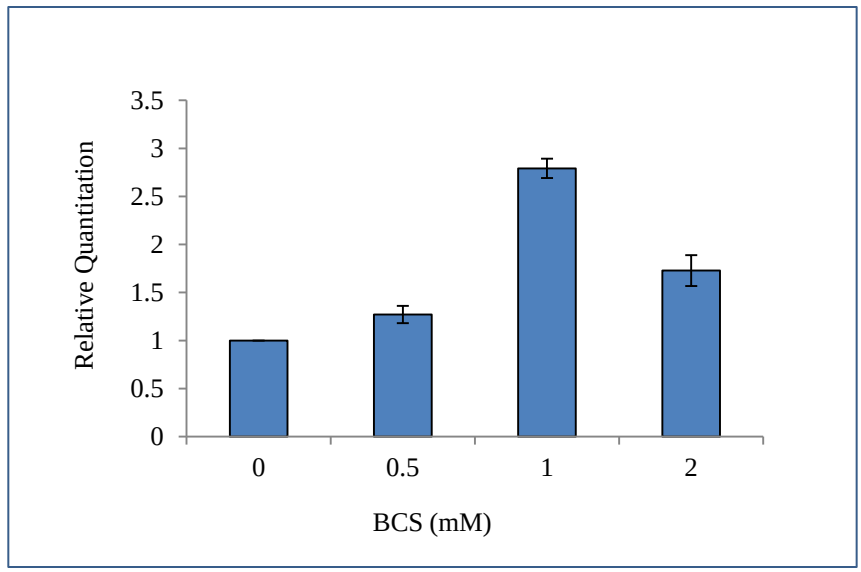


Figure 18. mRNA expression level of LDATP7 in response to different doses of copper chelators. LDATP7 mRNA expression level after treatment with different doses of copper chelator Bathocuproine disulfonate (BCS) was determined by quantitative real time PCR. All data were normalized using 18S rRNA as the endogenous control. Data shown as ± SD of three independent experiments.

LDATP7 mRNA expression was increased in time dependent manner suggesting higher expression of LDATP7 was needed for LD during infection into macrophages (Figure 15). The earlier report by Solanki *et al.* shows that LDMCO shows increased expression during early hours of infection to fulfill the need of iron in establishing infection. The increased expression of LDATP7 during early hours suggests the role of LDATP7 in activating LDMCO within macrophages.

LDATP7 mRNA increased in axenic amastigote stage of *Leishmania donovani*

Axenic amastigote was obtained by incubating metacyclic promastigotes at 37˚C, 5% CO_2 for 48 hour. This is a standard protocol to transform promastigotes in to axenic amastigotes. The total RNA was isolated from both axenic amastigote and promastigote forms. Expressions of LDATP7 mRNA were detected by quantitative real time PCR using gene specific primers. 18s rRNA specific primers were used for loading control. Figure 16 shows significant (almost 2.5 fold) increase in LDATP7 mRNA in amastigote stage than promastigote. This result further signifies the role of LDATP7 in LDMCO mediated iron uptake in amastigote stage of this parasite.

Increased LDATP7 mRNA in iron deprivation condition

To further verify the implication of LDATP7 on LD iron homeostasis promastigote form of the parasite was subjected to iron depletion by adding increasing concentrations of iron chelator BPS. In this condition parasites were grown for 72 hours. Then total RNA was isolated and quantitative real time PCR was performed using LDATP7 specific primers. 18S rRNA specific primers were used as loading control. Results showed more than 2-fold increase in LDATP transcript level when subjected with 1 mM BPS treatment (Fig 17). Further increase in concentration of BPS affected parasite growth in the given condition probably due to toxicity.

Increased LDATP7 mRNA in copper deprivation condition

The effect of copper deprivation on LDATP7 mRNA level was also examined by incubating LD with different doses of copper chelator (BCS) for 72 hours. Total RNA was isolated as per the protocol and quantitative real time PCR was performed using LDATP7 specific primers. 18S rRNA specific primers were used as loading control. The result showed a similar result like iron chelation (Fig 18). This result implicates further the role of copper on LDATP7 expression.

Discussion

Multicopper oxidases are complex copper-containing enzymes and usually contain a variety of different physiological functions like copper tolerance, manganese oxidation, and mainly iron oxidation (Huston, 2002 and Cooksey, 1994). They oxidize ferrous form of iron into the ferric form and help it to get inside the cell by oxidase-permease system of organisms in diverse areas of evolution. MCOs are long known with some prescribed *in vitro* functions like iron oxidation. Studies with yeast (Askwith *et al.*, 1994, 1996) defined its function as a ferroxidase *in vivo* and involved in high-affinity iron uptake. A similar function in iron uptake in iron deficiency was ascribed for mammalian homologue ceruloplasmin (Cp) (Muckhopadhyay *et al.*, 1998). Later MCOs are also found in bacteria (Kim *et al.*, 2001) and green algae (Herbik *et al.*, 2002) and implicated with respective iron homeostasis of these organisms. So far, presence of a multicopper oxidase in any trypanosomatid parasite or parasite in general was not reported. Study by Solanki *et al*., (unpublished data) identified and characterized a functional LDMCO ferroxidase describing the presence of a multicopper oxidase in parasite for the first time. In the current study the identification and functional characterization of the copper loading component of LD have been performed. Deletion of LDATP7 *in vivo* shows that the parasite fails to grow even in iron rich medium by affecting its ferroxidase activity. The LDMCO was found to be essential for the survival, growth and virulence of LD. The current study reveals a similar role of LDATP7 in leishmanial survival, growth and virulence. This finding extends the role of copper in maintaining iron homeostasis in trypanosomatid parasite like other kingdom of life.

Copper is an important cofactor of the evolutionary conserved family of ferroxidase enzymes linked with iron metabolism in yeast and mammals (Askwith *et al.*, 1994, 1996; Mukhopadhyay *et al.*, 1998). Interestingly, depletion of copper was shown to affect the activity of mammalian ferroxidase Cp without affecting its expression (Holtzman *et al.*, 1970). In hepatic cells P-type ATPase loads copper into Cp and in depletion of copper this mechanism is inhibited (Scheinberg *et al.*, 1952; Tanzi *et al.*, 1993; Yamaguchi *et al.*, 1993). In yeast, Cu is required for high-affinity iron uptake (Askwith, 1994; Stearman, 1996). Mutations either in the high-affinity Cu transporters (Dancis *et al.*, 1994b; Yuan, 1995) or Cu deficiency (Askwith, 1994) resulted in impaired iron uptake. Yeast MCO Fet3p plays a critical role in maintaining cellular iron homeostasis as during iron scarcity this component of high-affinity iron

uptake is transcriptionally up-regulated along with the permease Ftr1 and copper loader CCC2 to promote iron uptake to maintain homeostasis (Yamaguchi-Iwai *et al.*, 1995, 1996; Hassett *et al.*, 1998). The transcription factor Aft1p activates and regulates all these high-affinity iron uptake components (Yamaguchi-Iwai *et al.*, 1995, 1996; Hassett *et al.*, 1998). Similarly, in iron deficiency mammalian ferroxidase Cp is transcriptionally regulated by hypoxia-inducible factor-1 to maintain iron homeostasis (Mukhopadhyay *et al.*, 2000).

Since copper is important cofactor of LDMCO and other enzymes required for the proper growth of LD, the effect of copper chelation on *in vitro* growth of LD promastigotes was examined. A copper chelator bathocuproine disulfonic acid (BCS), which specifically chelates Cu (I) was used at concentration ranging from 0.1 mM to 4 mM. Copper chelation significantly inhibited the growth of the parasite. Effect of iron chelation on LD growth was also examined and it was interesting to found that copper chelation was more effective for the LD promastigote growth under the same dose of copper and iron chelator. The DNA synthesis assay confirmed the inhibitory effect of copper chelation on LD growth. Ferroxidase activity depends on the availability of copper and the P-type copper ATPase either in yeast or mammals was shown to be responsible for copper loading into ferroxidases (Weissman *et al.*, 2002; Marvin *et al.*, 2004). The reduced ferroxidase activity of single allele knock-out LD confirmed the role of copper in iron uptake activity of this parasite.

Role of copper in mammalian iron homeostasis has been reported for long. Reports show that copper deficiency impairs iron absorption in swine (Gulberg *et al*., 1952; Lee *et al*., 1968) and sex linked anemia (*sla)* mice model (Edwards and Bannerman 1970). On the other hand iron absorption is increased in rat hepatocytes under copper deficient conditions (Thomas and Oates, 2003).

Copper transporting P- type ATPase is a member of PIB type ATPase. Copper transporting P- type ATPase presents in prokaryotes and eukaryotes and plays crucial roles in accumulation and tolerance of heavy metals in biological systems (Williams *et al*., 2000; Silver 1996; Bull and Cox, 1994) and for delivery of copper to metalloenzymes (Petris *et al*., 2000).

In this study, a copper transporting p type ATPase of *Leishmania donovani* has been cloned and characterized, which is apparently responsible for copper incorporation

into LDMCO. *L. major* and *L. infantum* gene database search shows the presence of a putative copper transporting P- type ATPase in chromosome 33. *Leishmania infantum* and *Leishmania donovani* both are responsible for visceral leishmaniasis thus in comparison to *Leishmania major*, *L. infantum* shows close proximity to *L. donovani*. So to clone *L. donovani* copper transporting P- type ATPase primer were designed on the basis of *L. infantum* gene sequence. A single ~3492 bp PCR product was obtained, cloned and sequenced.

The cloned *Leishmania donovani* LDATP7 shows identity with different orthologs like 31% homology to *S. cerevisiae* CCC2 protein, 33 % homology to human Wilson's protein, 34% homology with Menkes disease protein, 45% identity with *T. brucei*, 92 % with *L. major* and 98 % with *L. infantum* at amino acid level. While it shows greater similarity in conserved domains like heavy metal associated domains, transduction domain ions channel phosphorylation, phosphatase and ATP binding regions. LDATP7 contains three heavy metal associated domains (HMA) in N-terminal region. *E. coli* CopA contains two HMAs (Rensing *et al*., 1999) while human Wilson and Menkes protein contain six heavy metal associated domains while several bacterial soft metal pumps contain single HMA in N terminal domain (Phung *et al*., 1994; Solioz and Odermatt, 1995). Reports suggest that HMAs of Menkes protein has role in copper binding and also required for intracellular trafficking in humans (Voskoboinik *et al*., 1999). While prokaryotes does not require intracellular trafficking. Protozoan parasite *Leishmania* is in between of these two and shows a gradation in number of HMAs. Trans-membrane prediction shows the presence of nine transmembrane helices.

Since, the experimental evidences suggest a role of LDATP7 in loading LDMCO, the localization studies were performed to know the localization of both these proteins in LD promastigotes. Immunofluoresence based study using antibody raised against LDATP7 revealed the presence of LDATP in flagellar pocket of LD. It has been shown that LDATP7 homologue in *S. cerevisiae* CCC2 is located in trans-Golgi network and Wilson's disease protein ATP7B and Menkes disease protein ATP7A are also present in trans-Golgi network of respective tissues. In some studies ATP7A has been shown to undergo copper regulated trafficking between trans- Golgi network and plasma membrane (Petris *et al*., 1996). The colocalization studies using antibodies raised against LDMCO and LDATP7 reveal that both these proteins are colocalized in

flagellar pocket of LD. It has been previously found by both immunofluoresence study and GFP-tagged LDMCO that LDMCO is also localized in flagellar pocket of LD which is important for iron uptake of this protozoan parasite. (Solanki *et al*., unpublished data). Flagellar pocket in *Leishmania* is known to be site of nutrient uptake. Thus, these findings further suggest the role of LDATP7 in leishmanial iron uptake by loading copper into LDMCO.

In order to assess the biological function of LDATP7, LDATP7 mutants were generated. The functions of several genes of *Leishmania* have been established by using sequential gene targeting procedures (Bello *et al*., 1994; Papadopoulou *et al*., 1994b, 1996; Mottram *et al*., 1996). Homologous recombination strategy was used to get single allele knockout mutants. The wild type parasites were transfected with HYG and NEO disruption cassettes separately. The effect of LDATP7 on the growth of the parasite was assessed by comparing the growth rate of wild type and the single allele knockout. Single allele knock-outs were obtained using both HYG and NEO disruption cassettes separately and both these mutants exhibited slower growth rate than comparable control parasites. The LDATP7 homolog CCC2 in yeast and Wilson's disease gene are known to incorporate copper in yeast Fet3 and mammalian ceruloplasmin respectively. Fet3 and ceruloplasmin are multicopper oxidases, which show ferroxidase activity and thus help in high affinity iron uptake to maintain iron homeostasis. Attempt was made to examine similar role of LDATP in LD to find that whether LDATP7 had any effect on ferroxidase activity of LDMCO. The result showed decreased ferroxidase activity in LDATP7 single allele knockouts compared to the wild type. This result strongly suggests that LDATP7 plays role in ferroxidase activity of LDMCO in *Leishmania donovani*.

There are emerging evidences that heavy metal transporting P-type ATPase may play role in virulence in host-pathogen interaction (Papp-Wallace and Maguire, 2006; Agranoff and Krishna, 1998). It has been reported that chromosomal deletion of ctpA copper transporting P-type ATPase in *Listeria monocytogenes* leads to impaired infectivity (Francis and Thomas, 1997). The effect of LDATP7 gene on infectivity of LD was also examined by counting intracellular parasites isolated from infected macrophages by percoll gradient method. The LDATP7 single allele knockout shows less number of intracellular amastigotes in comparison to wild type LD. It shows the LDATP7 plays a role in virulence of *Leishmania donovani*.

Loss of one LDATP7 allele by gene disruption was compensated by genetic complementation with an episomal copy of LDATP7. Several attempts to generate LDATP7 null mutants by two step gene replacement method were failed despite the successful disruption of the two alleles by gene targeting. But it was not able to find alive LD cells when both the alleles were knocked out. There are reports which show that in *L. major*, generation of dihydrofolate reductase-thymidylate synthase null mutant resulting into an extra chromosome number to keep one dhfr-ts allele intact (Cruz *et al*., 1991). Disruption of trypanothione reductase gene in *Leishmania* also resulted in to partial trisomy for the TR locus (Dumas *et al*., 1997). These reports led to proposal that such alterations in the genome could be because of the essential nature of the gene.

To verify the essentiality of LDATP7 gene in LD, the LDATP7 gene single allele knockouts were transfected with a plasmid carrying LDATP7 gene for episomal expression and then the second allele was knocked out. The genetic complementation before the second round of gene replacement gives the LDATP7 null mutants with two selection markers. The heterozygous mutants when complemented with an episomal copy of LDATP7 were able to overcome the effect caused by the deletion of the one allele of LDATP7 and also enable us to get the LDATP7 double allele mutant. The episomal expression of LDATP7 increased the growth rate of LD. LDATP7 when episomally expressed even in wild type LD increased its growth rate. Similarly, episomal expression of LDATP7 in single allele knockouts showed increased growth rate and LDATP7 double allele knockout were survived. LDATP7 episomal expression also increased the ferroxidase activity of LD comparatively. Thus, these results showed that the function of LDATP7 is essential for survival, growth and ferroxidase activity of *Leishmania donovani*.

Usually yeast complementation assay is used to assess the function of putative genes. Δccc2 yeast complementation assay was used to elucidate the activity of several copper P type ATPases as reported earlier (Sambongi *et al*., 1997; Forbes, 2000; Forbes and Cox, 1998; Payne and Gitlin, 1998). The CCC2 protein is localized to a late- or post- Golgi network in secretory pathway and delivers copper to multicopper oxidase Fet3 (Yuan *et al*., 1995, 1997). The copper loaded Fet3 is required for high affinity iron uptake in the plasma membrane (Askwith *et al*., 1994). Disruption of ccc2 gene in *Saccharomyces cerevisiae* strain produces copper deficient Fet3, which

unables to take part in high affinity iron uptake. To grow in iron deficient conditions a functional high affinity iron uptake system is required. The ability of a putative copper ATPase to rescue the Δccc2 in iron deficient condition is a measure of this protein in transporting copper in to MCO. Usually, in iron sufficient conditions iron uptake occurs through a CCC2 independent low -affinity iron uptake system.

In this study it has been found that LDATP7 was able to replace functionally the yeast CCC2 protein. LDATP7 provided the copper transporting activity required for copper delivery to Fet3 thus restoring its activity of high affinity iron uptake in yeast ccc2 mutant strain. It was also found that LDATP7 having mutation in some conserved domains was not able to rescue the yeast Δ ccc2 mutants. The site directed mutation in one of the three heavy metal associated domains (HMA), where both the Cysteine in CXXC is replaced with Serine failed to rescue the function of FET3. The replacement of the Cys by Ser was chosen because the substitution of oxygen to the sulfur of the Cys should reduce the affinity for copper without inducing many changes in the structure (Lowe *et al.,* 2004). Similarly, another mutation in LDATP7 was introduced in phosphorylation domain DKTGT, where Aspartate was replaced with Glutamate. This mutation impaired the formation of acyl- phosphate intermediate required for the activity of the protein thus was unable to complement the yeast CCC2 function. All these experiments strongly suggest the LDATP7 as a copper loader for LDMCO.

Previous results from this laboratory showed increased expression and activity of LDMCO in early stage of infection of the parasite into host macrophages. Since, macrophages are well known to sequester iron to control the survival and growth of intracellular parasites; parasites need to scavenge iron in iron scarce condition. This condition should be ideal to activate MCO for high affinity iron uptake from host iron pool, which was observed in this laboratory (unpublished data, Solanki *et al.*). Now, higher activity of MCO needs higher capacity of copper loading by LDATP7. So, when LDATP7 expression was examined an increased mRNA level of internalized LD was detected during the early stages of infection in to macrophages. Similarly, the axenic amastigotes showed increased expression of LDATP7 mRNA in comparison to promastigote stage. Axenic amastigote mimics the intracellular amastigote which is the virulent stage of *Leishamnia* and is more virulent than promastigote stage. It

suggests that LDATP7 expression is linked with the virulence of the LD and might help in establishment of infection.

To further verify that the LDATP7 links copper and iron homeostasis its mRNA expression level was examined in iron and copper deficient conditions using different doses of iron and copper chelators. Results showed that both iron and copper chelation affected the LDATP7 mRNA expression level.

In summary, the current study is the first to report an essential copper transporting ATPase in protozoan parasite *Leishmania donovani*. This study also extends the evolutionary conserved relation between copper and iron in trypanosomatids. The LDATP7 is a homologue of Wilson and Menkes disease gene that is also reported in yeast and other mammals; however, in none of these species it acts as an essential gene as has been detected for *Leishmania donovani*. This parasite affects millions all over the world including India. Most of the drugs available are either highly toxic or acquired resistance already by the parasite. The essentiality of this gene thus provides an opportunity to find a suitable drug against this Kala-azar causing parsite, which needs further research.

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