# Studies on identification and *in vivo* function of novel drug target enzymes of *Leishmania donovani* using biomolecular approaches

***A Thesis Submitted By***

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***In Partial Fulfillment of the Requirements for the Degree of***

**Doctor of Philosophy**

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**Indian Institute of Technology Guwahati**
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**August 2016**

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***Dedicated***
***To***
***My parents and teachers***
***for always supporting me in my endeavors and***
***molding me into a person I am today***

**INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI**

**DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING**

## STATEMENT

I hereby declare that the matter embodied in this thesis entitled **"Studies on identification and *in vivo* function of novel drug target enzymes of *Leishmania donovani* using biomolecular approaches"** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India under the supervision of **Prof. Vikash Kumar Dubey.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred. Further, the data in the thesis are collected by me. I certify that there is no fabrication or manipulation of data in the thesis.

*Date: August, 2016*

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**DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING**

# CERTIFICATE

It is certified that the work described in this thesis **"Studies on identification and *in vivo* function of novel drug target enzymes of *Leishmania donovani* using biomolecular approaches"** by **Ms. Ruchika Bhardwaj (**Roll No: 126106002), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

**Prof. Vikash Kumar Dubey**
(Thesis Supervisor)

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***Ruchika Bhardwaj***
*August, 2016*

# ABBREVIATIONS

| | | |
|---|---|---|
| AFC | : | Alpha factor converting enzyme |
| ATP | : | Adenosine-5'-triphosphate |
| BLAST | : | Basic local alignment search tool |
| BSA | : | Bovine serum albumin |
| CAAXII_DKO | : | CAAX prenyl protease II double knockout |
| CAAXII_SKO | : | CAAX prenyl protease II single knockout |
| CAAXII_TKO | : | CAAX prenyl protease II triple knockout |
| CDK | : | Cyclin dependent kinase |
| CKO_HP | : | LdBPK_070020 complemented cells |
| CLSM | : | Confocal laser scanning microscope |
| DAB | : | 3,3’-diaminobenzidine |
| DAPI | : | 4',6-diamidino-2-phenylindole |
| DHFR | : | Dihydrofolate reductase |
| DHS | : | Deoxyhypusine synthase |
| DKO_HP | : | LdBPK_070020 double knockout cells |
| DNA | : | Deoxyribonucleic |
| DPH | : | Diphenylhexatriene |
| DTT | : | Dithiothreitol |
| ELISA | : | Enzyme-linked immunosorbent assay |
| FA | : | Fluorescence anisotropy |
| FACS | : | Fluorescence-activated cell sorting |
| FBS | : | Fetal bovine serum |
| FESEM | : | Field emission scanning electron microscope |
| FITC | : | Fluorescein isothiocyanate |
| GFP | : | Green fluorescent protein |
| GR | : | Glutathione reductase |
| HRP | : | Horseradish peroxidase |

| | | |
|---|---|---|
| HYG | : | Hygromycin |
| ICMT | : | Isoprenylcysteine carboxylmethyltransferase |
| IFA | : | Indirect fluorescent antibody |
| MAP | : | Mitogen-activated protein |
| ML | : | Mucocutaneous leishmaniasis |
| MOPS | : | 3-(N-morpholino)propanesulfonic acid |
| NAC | : | *N*-acetylcysteine |
| NCBI | : | National center for biotechnology information |
| NEO | : | Neomycin |
| NTD | : | Neglected tropical diseases |
| PAC | : | Puromycin |
| PBS | : | Phosphate saline buffer |
| PCR | : | Polymerase chain reaction |
| PHLEO | : | Phleomycin |
| PI | : | Propidium iodide |
| PKDL | : | Post kala azar dermal leishmaniasis |
| PS | : | Phosphatidylserine |
| RCE | : | Ras and a factor converting enzyme |
| RNA | : | Ribonucleic acid |
| ROS | : | Reactive oxygen species |
| SDS | : | Sodium dodecyl sulfate |
| SKO_HP | : | LdBPK_070020 single knockout cells |
| SMT | : | D-sterol methyltransferase |
| TP | : | Tryparedoxin peroxidase |
| TryR | : | Trypanothione reductase |
| TryS | : | Trypanothione synthase |
| TX | : | Tryparedoxin |
| UTR | : | Untranslated region |
| VL | : | Visceral leishmaniasis |
| WT | : | Wild type cells |

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# CHAPTER I

## Leishmaniasis: cause, cure, current status and future prospective*

### 1.1 ABSTRACT

Leishmaniasis is a parasitic disease caused by a protozoan belonging to the species *Leishmania*. The disease is recognized among neglected diseases by WHO. The disease affects mainly the below poverty line population, as a result of which it is usually ignored by the government, media and pharmaceutical companies. Based upon the causative species and clinical manifestations, the disease is broadly divided into three types of infections *viz.*, cutaneous, mucocutaneous and visceral. Among all three, visceral leishmaniasis is the most dangerous form because if left untreated the consequences could be fatal. Visceral leishmaniasis is the most common form of infection in India, and Bihar is the state that accounts for 90% of the cases. The current drug scenario is not very good due to high host toxicity, low efficacy, high cost and growing incidents of drug resistance. Therefore the search for new and efficient drugs is continuous. Drug discovery is a multistep process. The initial key steps involve identification and validation of drug target before moving forward with drug candidate identification and optimization. Multi-target drug(s) as well as combinatorial therapy with drug components with different targets are considered to be a good approach to tackle drug resistance.

*Part of the review is submitted for publication

## 1.2 INTRODUCTION

A diverse set of communicable diseases prevalent in tropical and subtropical regions of the world are grouped under neglected tropical diseases (NTD) by the World Health Organization (http://www.who.in). Leishmaniasis, a vector borne parasitic disease, is identified as one of the NTDs.

### *1.2.1 History of leishmaniasis*

The origin of the disease leishmaniasis is unknown but its existence has been dated back to first century AD (*Momen and Cupolillo, 2000).* Alexender Russell gave the first detailed description of the disease in 1756 (*Hide et al., 2007*). Surgeon major Cunningham was the first to identify the parasite (*Cunningham, 1885),* while Peter Borovsky was the first to refer the parasite as protozoa and was able to identify the parasite and host tissue relation (*Hoare, 1938*). In India, the disease was popularly known as "dum dum fever", due to the historically high incidences of this disease in a place called Dum Dum, near Kolkata in India. Nowadays the disease is referred to as Kala-azar (*kālā* meaning black and *āzār* meaning fever) in the Indian subcontinent. In the year 1903, William Boog Leishman and Charles Donovan independently identified the parasite in the splenic tissue from the casualties of "dum-dum fever" and concluded the organism to be a new divergence of *Trypanosome* (*Leishman, 1903; Donovan, 1903*). But it was Ronald Ross who, in 1903, established the link between the disease and a new parasite, which was given the name *Leishmania donovani (Ross, 1903).* A collage of research articles and pictures of initial forefather of leishmaniasis is illustrated in **Figure 1.1**. In 1904, *Leishmania* was identified in children suffering from infantile splenic anaemia, by Cathoire and Laveran. This parasite was named as *Leishmania infantum* by Nicolle. By 1912, Carini had identified *Leishmania* in mucosal lesions of patients suffering from leishmaniasis in Brazil. And in 1922, Bramachari identified and described PKDL (Post kala azar dermal leishmaniasis) in India (*Bramachari, 1927*). The role of infected female sandflies in transmission of leishmaniasis to humans was first described by Swaminathan in the year 1942.

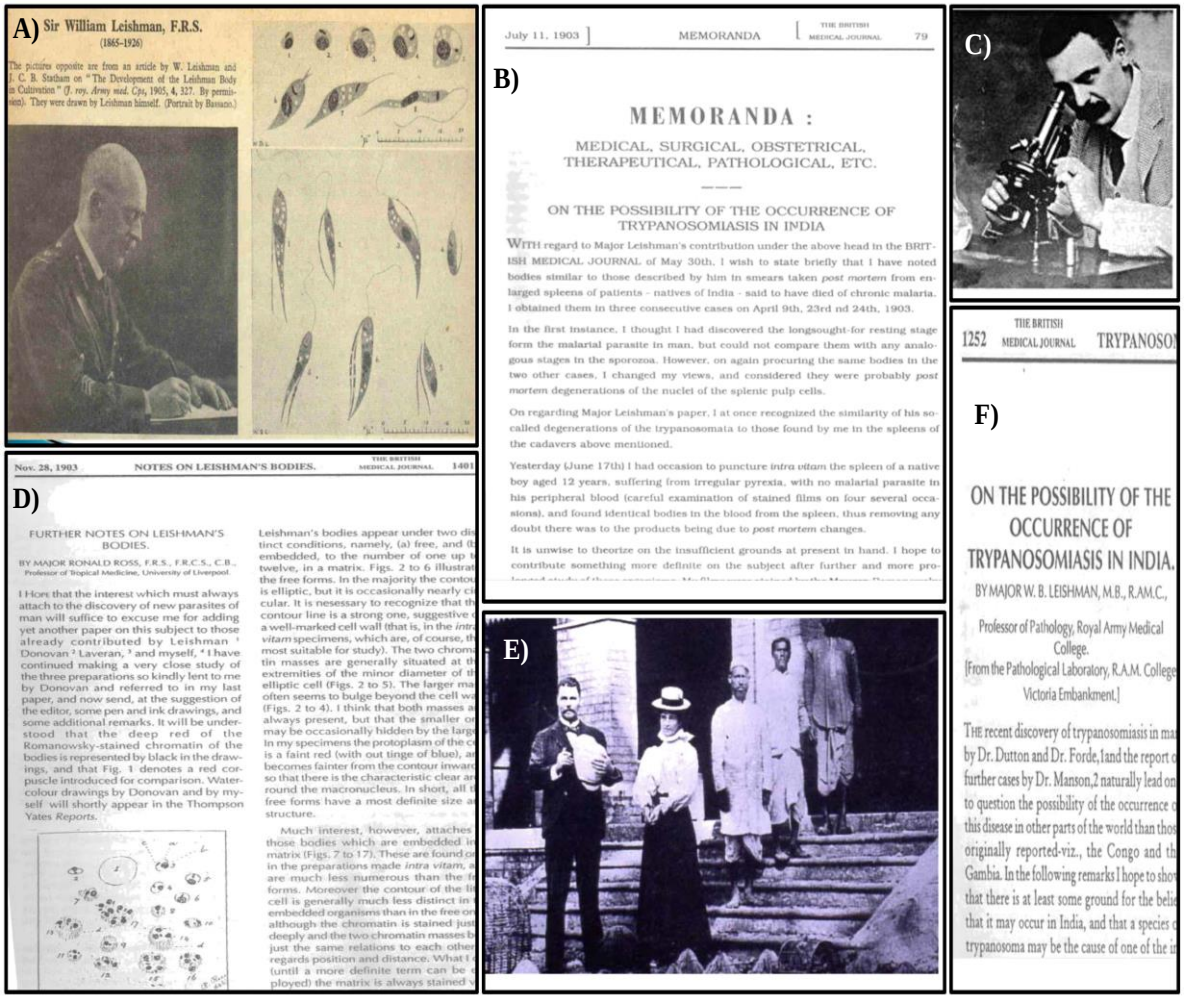


**Figure 1.1: Some early articles and forefathers of leishmaniasis: (A)** Sir William Leishman and his illustrations of the parasite. **(B)** Article by Prof. Charles Donovan published in British Medical Journal. **(C)** Prof. Charles Donovan. **(D)** Article by Prof. Ronald Ross in which he jointly accredits Leishman and Donovan for the discovery of the parasite. **(E)** Prof. Ronald Ross. **(F)** Research article by Sir William Leishman in The British Medical Journal. (Source: http://www.who.int/global_health_histories/seminars/presentation35a.pdf)

### *1.2.2 Leishmania: the causative agent*

Kinetoplast is a unique structure consisting of circular DNA within a single large mitochondrion which is closely associated with the flagellar basal body. All the species characterized by the possession of a kinetoplast, come under the family *Trypanosomatidae*. *Leishmania* boasts of over thirty different species that come under this family. *Leishmania donovani* is the most prevalent species in India. The parasite has two developmental forms depending upon the host it resides in. The first form i.e. amastigotes, are small spherical

bodies devoid of an exterior flagella. The size ranges from 2-4μm in diameter, putting them among the group of smallest nucleated cells known. The promastigotes form on the other hand posses a more elongated and tapered body, with the presence of exterior flagella. The size of this form ranges from 5-14μm in length and by 1.5-3.5μm in width. Labeled diagram of both the forms of *Leishmania* is shown in **Figure 1.2**.

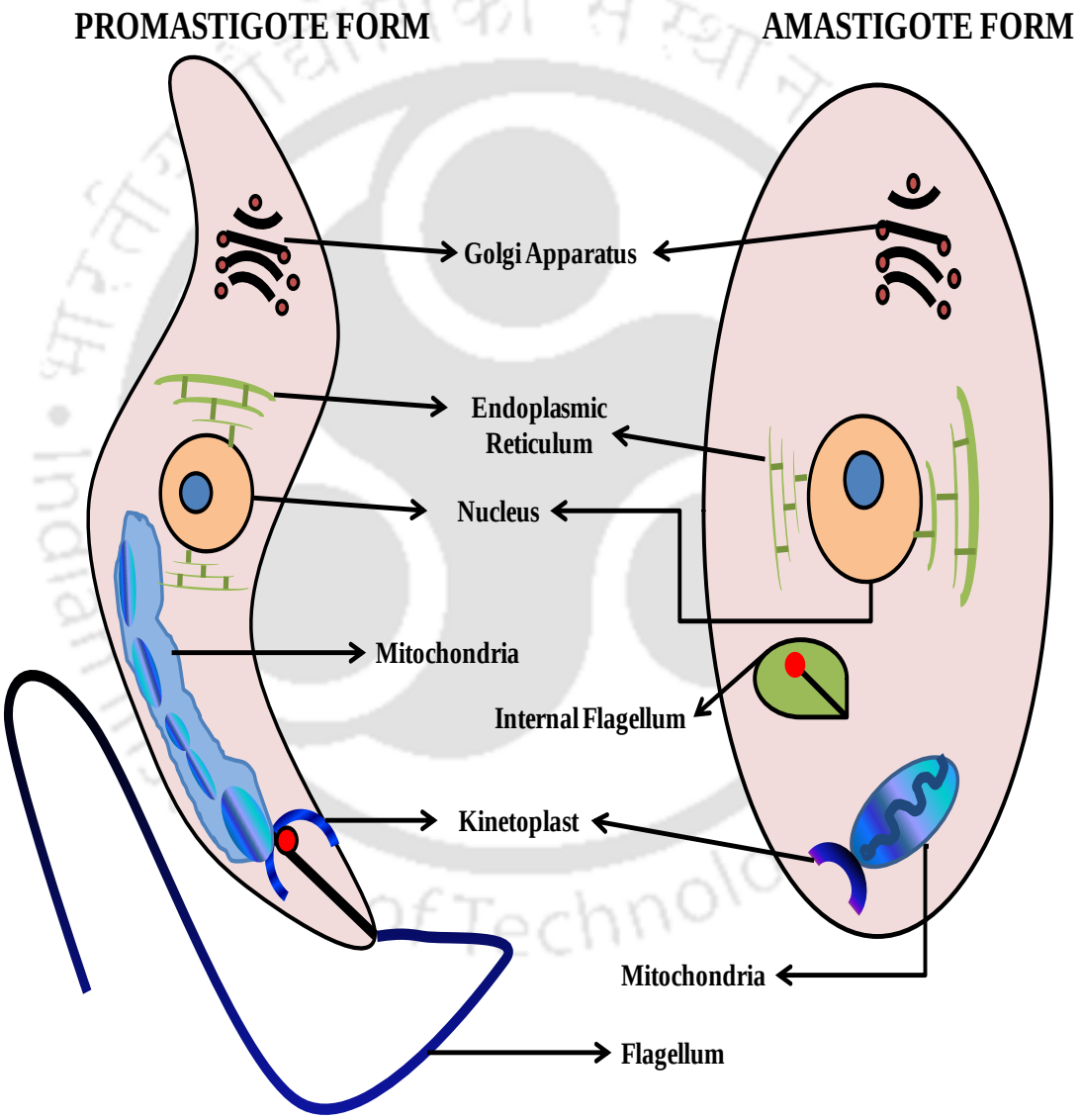


**Figure 1.2: Two different forms of *Leishmania* parasite:** The promastigote form in prevalent in the vector sandfly. It has an elongated body with exterior flagella for locomotion. The amastigote form of the parasite is found in mammalian host. It is more ovoid shape and lacks exterior flagella.

### *1.2.3 Vector and host range*

The parasite has a digenetic life cycle, hence altering between an insect vector and a mammalian host. Female sandflies are the vectors for this disease. These are small insects ranging from 1.5- 2 mm body length and usually reside in tropical and subtropical regions. There are over 700 species of sandflies, out of which only 30 are identified as transmitters of leishmaniasis. Among these 30 species, only two are proven responsible for human infections. Sandfly belonging to the species *Lutzomyia* affects the New World (Central and South America) **(Figure 1.3)**, while sandfly belonging to the species *Phlebotomus* affects the Old World (Middle East Asia and Africa) (*Sharma and Singh, 2008).* When it comes to the mammalian hosts, all *Leishmania* species infect humans. Dogs, rodents, sloths, pigs, bats, fox etc. serve as reservoirs for the parasite. List of various vector and host range along with the causative *Leishmania* species is given in **Table 1.1**.


B)

| | | |
|---|---|---|
| Kingdom | : | Animalia |
| Phylum | : | Arthropoda |
| Class | : | Insecta |
| Order | : | Diptera |
| Family | : | Psychodidae |
| Subfamily | : | Phlebotominae |
| Genus | : | *Lutzomyia* |

**Figure 1.3: Vector for leishmaniasis: (A)** *Lutzomyia longipalpis* taking a blood meal from a human host. **(B)** Scientific classification of *Lutzomyia* sandfly (Source: en.wikipedia.org).

**Table 1.1:** Various species of the *Leishmania* parasite, corresponding hosts, diseases and vectors. Data compiled from web resources http://parasite.org.au/para-site/text/leishmania-text.html

| ***Leishmania species*** | ***Vertebrate host*** | ***Disease*** | ***Insect vector*** |
|---|---|---|---|
| | | ***CUTANEOUS LEISHMANIASIS*** | |
| *L. aethiopica* | Humans, Hyraxes | Diffuse or Dry Cutaneous | *Phlebotomus* |
| *L. tropica minor* | Humans, Dogs, Rodents | Dry Cutaneous | *Phlebotomus* |
| *L. tropica major* | Humans, Dogs, Rodents | Wet Cutaneous, Oriental Sore | *Phlebotomus* |
| *L. peruviana* | Humans, Dogs | Uta, Cutaneous | *Lutzomyia* |
| *L. mexicana mexicana* | Humans, Rodents | Chicleros Ulcer, Cutaneous | *Lutzomyia* |
| *L. mexicana amazonensis* | Humans, Rodents | Diffuse, Cutaneous | *Lutzomyia* |
| *L. mexicana pifanoi* | Humans, Rodents | Cutaneous, Mucocutaneous | *Lutzomyia* |
| *L. braziliensis* | Humans, Rodents, Sloths | Espundia, Mucocutaneous | *Lutzomyia* |
| | | ***VISCERAL LEISHMANIASIS*** | |
| *L. donovani donovani* | Humans, Dogs, Foxes | Kala Azar, Dum-Dum Fever, Old World Visceral | *Phlebotomus* |
| *L. donovani infantum* | Humans, Dogs | Infantile, Visceral | *Phlebotomus* |
| *L. donovani chagasi* | Humans, Foxes, Cats | New World visceral | *Lutzomyia* |

### *1.2.4 Life cycle*

The parasite *Leishmania* shuffles between two hosts throughout its life. The promastigote form resides inside the vector i.e. sandfly, while the amastigote form resides in the mammalian host. It is speculated that pH and temperature are major factors governing the promastigote to amastigote or vice-versa transition. But co-occurrence of both forms in patients suffering from cutaneous leishmaniasis has been reported as well (*Haouas et al., 2014*). When an infected sandfly takes a blood meal from an uninfected human, it injects the promastigote form into the host. Once inside the host, the parasite is exposed to many host defence system and are ultimately phagocytosed by the macrophage cells. Inside the macrophage, the parasite resides in parasitophorous vacuole which fuses with lysosomes to form acidic compartments containing hydrolytic enzymes (*Lynn et. al., 2011*). The promastigotes further transform into amastigotes and continue to proliferate inside the infected macrophage. Ultimately macrophage lysis occurs, and the oval bodies are released into the blood stream, where they are further taken upon by uninfected macrophages, hence leading to spreading of infection. When an uninfected sandfly takes a blood meal from an infected mammalian host, it intakes the amastigote form. These amastigotes further differentiate into procyclic promastigotes in the vector's midgut. The procyclic promastigotes multiply and migrate to fly's proboscis in metacyclic form (*Pearson and de Queiroz, 1996*). This conversion of procyclic stage to metacyclic stage is known as metacyclogenesis and the gene involved in this process are Mat-1-a, SHERP and HASP (*Muskus et al., 2002*; *Silva et al., 1987*). When an infected sandfly takes a blood meal from any other animal, after carrying the parasite from human, then the stage is called as zoonotic. Here infected animals like dog, fox, rodent, pig, etc. act as reservoirs of the parasite. An illustration depicting the back and forth cycle of the parasite between the vector and mammalian host is shown in **Figure 1.4**.

### *1.2.5 Types of Leishmania infection*

Leishmaniasis is associated with wide spectrum of clinical manifestations, ranging from self recuperating cutaneous leishmaniasis to life threatening visceral leishmaniasis *(Murray et al., 2005).* Based upon these clinical manifestations and causative species of these manifestations, leishmaniasis is broadly divided into three types of infection. Cutaneous leishmaniasis is identified as an ulcerative self healing skin infection while mucocutaneous

leishmaniasis is a much more advanced form of cutaneous leishmaniasis. It mainly affects the oral and nasal mucosal regions leading to disfiguration of the face. The most dangerous form of *Leishmania* infection is visceral leishmaniasis. If left untreated, the subsequent consequences are usually fatal. A total of 21 different *Leishmania* species are responsible for these three different types of infections (*MacMorris-Adix, 2008*).

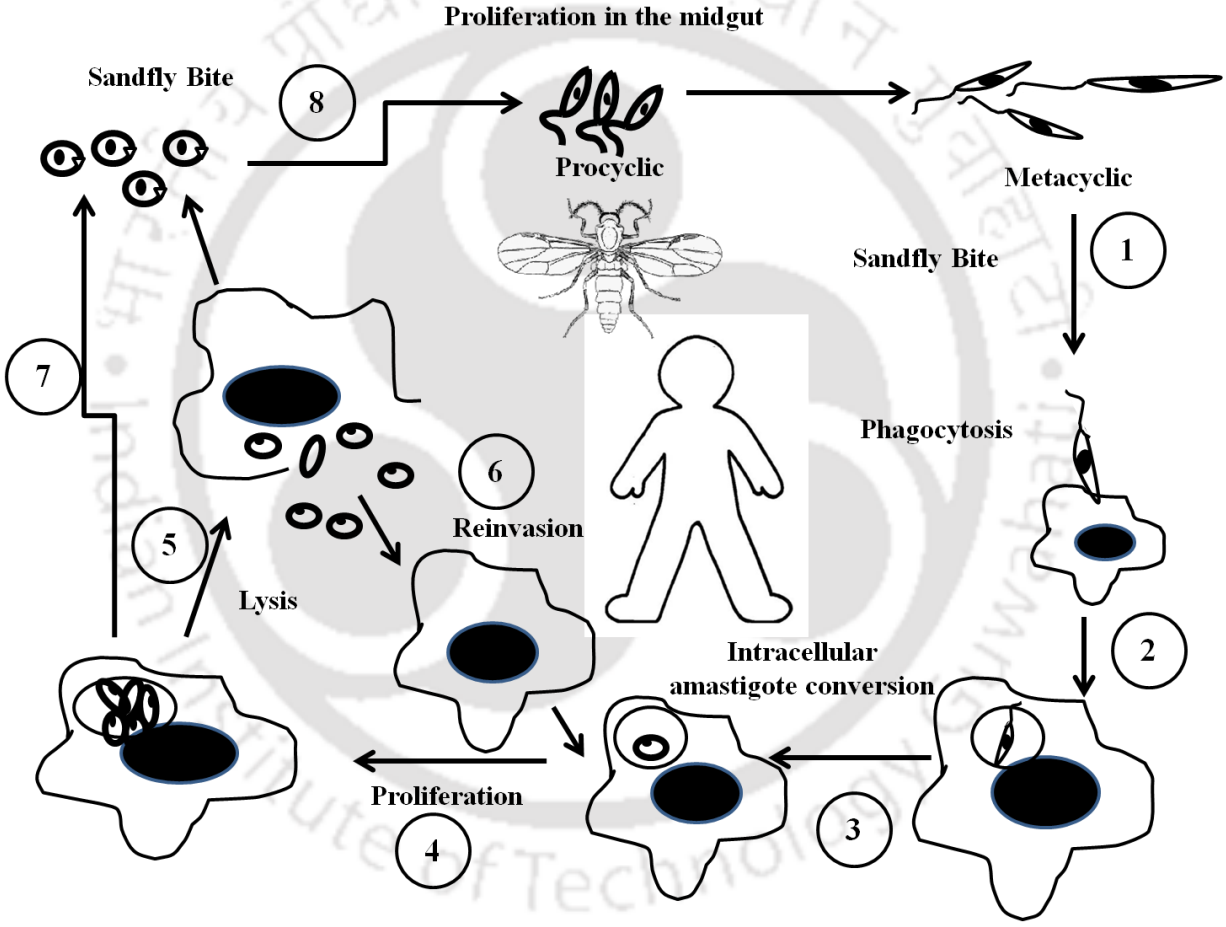


**Figure 1.4: Life cycle of *Leishmania* parasite: (1)** Promastigotes are transferred to the vertebrate host as the infected sand fly takes a blood meal. **(2)** The promastigotes are phagocytosed by macrophages and transformed into amastigotes. **(3)** The amastigotes undergo repeated cycles of binary fission within the macrophage and continue proliferation till macrophage lysis occurs. **(4)** Amastigotes are released from the infected macrophage and re-initiate the replicative cycle in new macrophages. **(5)** When an uninfective sandfly takes a blood meal from an infected mammalian host, it takes up the amastigote form of the parasite. **(6)** The amastigote form transform into procyclic promastigotes in the midgut of the sandfly. **(8)** Promastigotes quit dividing and undergo a terminal differentiation into metacyclic promastigotes which are infective for the vertebrate host.

*1.2.5.1 Cutaneous Leishmaniasis (CL):* Formation of skin ulcers on the site of sandfly bite is the characteristic of this infection **(Figure 1.5A)**. Usually the exposed body parts like arms, legs and face are the site of infection. The number of lesions may vary from a few to sometimes up to 200 leading to disability and invariable scars *(Reithinger et al., 2007).*

*1.2.5.2. Mucocutaneous Leishmaniasis (ML)*: This form of *Leishmania* infection leads to lesion formation on facial region, including nose, throat cavities and mouth **(Figure 1.5B)**. This ultimately causes partial or total destruction of mucous membranes.

*1.2.5.3 Visceral Leishmaniasis (VL):* The most notorious form of *Leishmania* infection is characterized by a set of synchronized symptoms. These symptoms include swelling of spleen and liver, anemia, irregular bouts of fever, substantial weight loss and darkening of the skin, which has given it the name of kala-azar, in India **(Figure 1.5C)**. The disease usually takes a lethal turn of events, if left untreated. Post kala-azar dermal leishmaniasis (PKDL) is a complication of visceral infection and is characterized by a maculopapular and nodular rash. This infection may occur in patients who have recovered from visceral leishmaniasis or are otherwise well *(Zijlstra et al., 2003).*

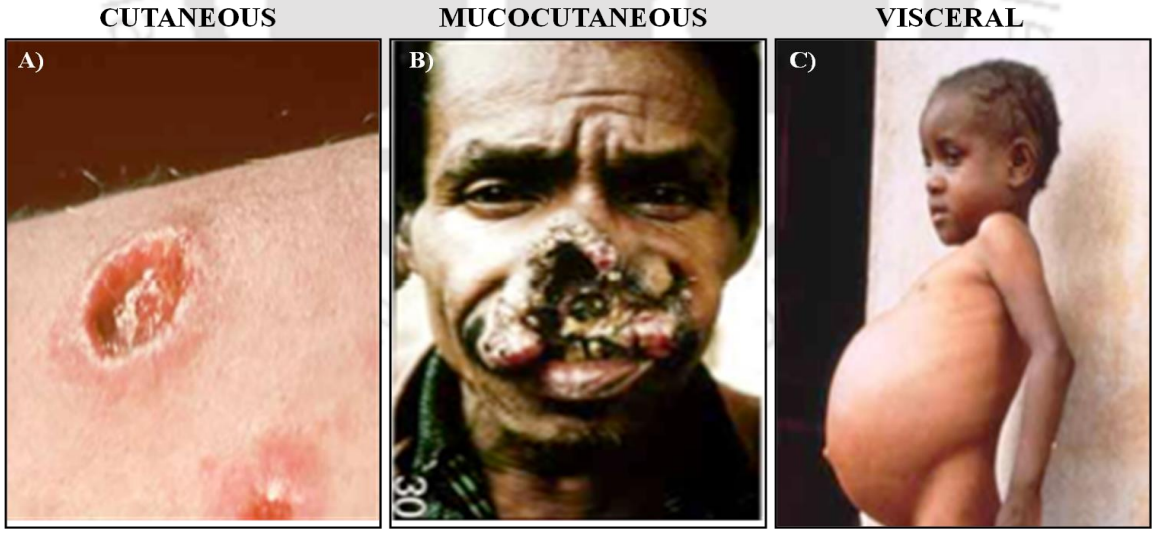


**Figure 1.5: Types of leishmaniasis infections:** Depending upon the clinical manifestation and causative species, leishmaniasis is broadly divided into three types of infections. **(A)** Cutaneous leishmaniasis is the most common form of infection. **(B)** Mucocutaneous leishmaniasis is the advanced form of cutaneous leishmaniasis. **(C)** Visceral leishmaniasis is the most dangerous form among the three types. (Source: http://web.stanford.edu/class/humbiol53/immuneEvasion/index.html)

### *1.2.6 Global impact of leishmaniasis*

The complex epidemiology and ecology, improper case management and scantiness of current incident data are among a few reasons for the negligence of leishmaniasis during discussions of tropical disease priorities *(Hotez et al., 2004)*. A map showing the global distribution of this disease is shown in **Figure 1.6A**.

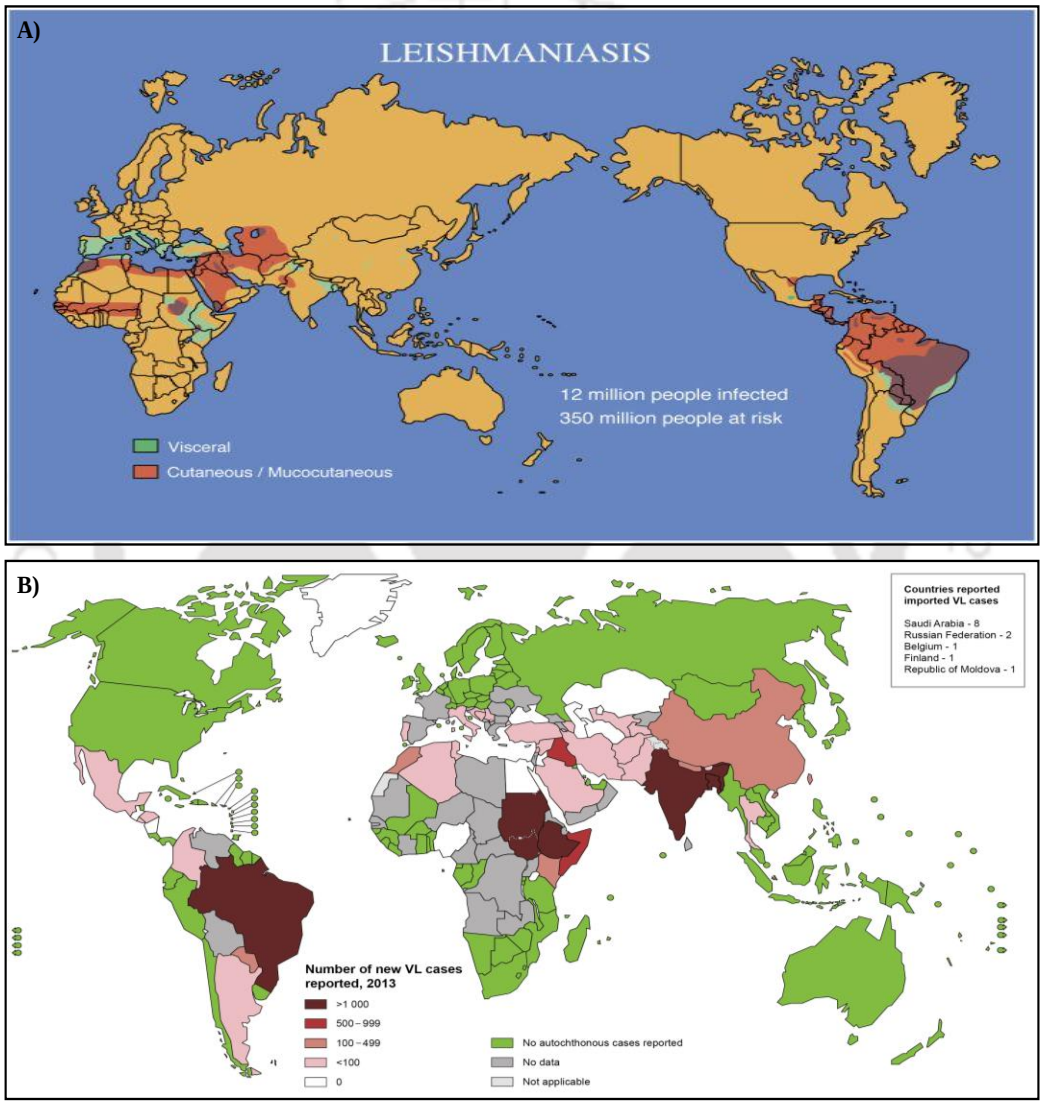


**Figure 1.6: Geographical distribution of the disease: (A)** Geographical distribution of different types of leishmaniasis infection worldwide. (Adopted from: www.wehi.edu.au/.../images/handman/world_map.jpg.) **(B)** Status of endemicity of visceral leishmaniasis all over the world. Source: (Adopted from: http://who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_VL.png?ua=1)

**Table 1.2:** Geographical distribution of the parasite. List of various types of leishmaniasis infections, causative species and their worldwide distribution.

| ***S.No.*** | ***Disease Manifestation*** | ***Causative species*** | ***Geographic Distribution*** |
|---|---|---|---|
| 1 | Visceral Leishmaniasis | *L. donovani* (Asia) | Northeast India, Nepal Bangladesh, Burma |
| 2 | Visceral Leishmaniasis | *L. infantum* | Mediterranean basin, Middle East, China, Central Asia |
| 3 | Visceral Leishmaniasis | *L. donovani* (Africa) | Sudan, Kenya, Horn of Africa |
| 4 | Visceral Leishmaniasis | *L. chagasi* | Central America, Northern South America, esp. Brazil |
| 5 | Cutaneous Leishmaniasis | *L. major* | Deserts in Middle East, Pakistan, North Africa |
| 6 | Cutaneous Leishmaniasis | *L. major* | Sub-Saharan Savanna, Sudan |
| 7 | Cutaneous Leishmaniasis | *L. tropica* | Towns in Middle East, Mediterranean basin, Asia |
| 8 | Cutaneous Leishmaniasis | *L. aethiopica* | Highlands of Kenya, Ethiopia |
| 9 | Cutaneous Leishmaniasis | *L. mexicana* | Yucatan, Belize, Guatemala |
| 10 | Cutaneous Leishmaniasis | *L. amazonensis* | Tropical forests of South America |
| 11 | Mucocutaneous Leishmaniasis | *L. braziliensis* | Tropical forests of South and Central America |

This disease can prejudice the economic growth and development of a nation, due to its association with malnutrition, population displacement and lack of resources. This disease is prevalent in Africa, Asia and Latin America. List of various *Leishmania* infections and their worldwide distribution is given in **Table 1.2**. According to recent WHO reports, approximately 0.2 to 0.4 million new cases of VL and 0.7 to 1.2 million new cases of CL occur annually worldwide *(WHO, 2013).* More than 90% of global VL cases are solely accounted by six countries *viz.*, Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan **(Figure 1.6 B)**. While 70-75% of global CL incidences are accounted by ten countries *viz.*, Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Peru, Sudan and Syria. Economic development has escorted the migration of human population from rural to urban areas in search of work. This further has led to the expansion of reservoir system of *Leishmania* and sandfly (*Thakur, 2000).* In addition to this, the population of new host i.e. immunodeficient HIV-infected patient is also increasing. The presence of the parasite outside reticuloendothelial system in HIV infected patients makes them a reservoir and source of infection for the vectors. Hence a set of newer challenges in the areas of diagnosis, treatment and disease control have materialized due to the surfacing of leishmaniasis in newer geographical areas as well as newer hosts.

### *1.2.7 Diagnosis of leishmaniasis*

The shared clinical features of leishmaniasis with other commonly occurring diseases like malaria, typhoid etc., have made the diagnosis of this disease a bit complex. The first and the foremost laboratory diagnosis technique involve examination of tissue specimens by light microscope. The specimen is stained with Leishman or Geimsa Blue stain, such that the cytoplasm appears pale blue in the background of small oval bodies measuring 2 to 3µm, which are identified as amastigotes (*Chulay and Bryceson, 1983*). Monoclonal antibodies tagged with a fluorophore are also used for speciation of the parasite. DNA hybridization is another technique used for the diagnosis of leishmaniasis. Conserved sequences found in circular DNA of kinetoplasts are used for detection via designing primers against them and carrying out PCR-based diagnostic approach *(Adhya et al., 1995).* A PCR-ELISA technique, was developed which was able to detect a minimum of 0.1 promastigote or 1fg of genomic material and showed sensitivity higher than IFA (indirect fluorescent antibody) test or

microscopy *(Sanchez et al., 2001)*. Immunodiagnosis is the most current detection technique, which employs both antigens as well as antibodies based detection. KATEX, a new latex agglutination test, is used for the detection of *Leishmanial* antigen from the urine of the patient. This test showed 100% specificity and sensitivity ranging between 68 and 100%, during preliminary trials and performed better than any other serological tests *(Attar et al., 2001)*. Antibodies in patients suffering from VL caused by *Leishmania donovani* have shown specificity towards a recombinant antigen, rk39. This antigen is highly conserved in the kinesin region and is derived from *Leishmania chagasi*. This antigen has demonstrated 100% specificity and is 100% sensitive for the diagnosis of both VL and PKDL by ELISA *(Rajasekariah et al., 2001; Kumar et al., 2001)*. Various commercially available kits are demonstrated in **Figure 1.7**.

### *1.2.8 Current drug scenario*

*1.2.8.1 Current Treatment:* Antiparasitic pentavalent antimonials, such as sodium stibogluconate and meglumine antimonite, were the first line of therapy. Mode of administration of these drugs was usually intravenous or intramuscular, which was not only a painful procedure, but required good medical facilities. These drugs have numerous adverse effects due to antimony toxicity and many *Leishmania* species have acquired resistance against them (*Rubiano et al., 2012*). The second line of therapy includes intravenous or intramuscular administration of Amphotericin B deoxylate. This drug associates with ergosterol, leading to formation of transmembrane channels that further lead to $K^+$ leakage and cell death. Liposomal form of Amphoterecin B is considered as a drug of choice for the treatment of VL but it is not approved for treatment of CL and ML. Countries that count on mainstream treatment by antimonial agents are prevented by the use of liposomal drugs due to cost issues *(CDC, 2014)*. Amphotericin B is nephrotoxic in nature. Further emergence of drug resistance against Amphotericin B is overshadowing its regular use. Miltefosine is the only available oral drug against leishmaniasis. Its mode of action is not very clear but studies report that it causes mitochondrial dysfunction and cytochrome c oxidase inhibition which leads to cell asphyxiation. This drug was initially developed as an antineoplastic agent but was later found to show anti-leishmanial activity. It is a preferred choice for chemotherapy in India due to drug resistance against traditional chemotherapy. This drug is approved by CDC

for treatment of CL, ML and VL in adults and children aged at minimum 12 years, but not for pregnant or breastfeeding females *(CDC, 2014)*. **Figure 1.8** illustrates the chemical formula of the current available drugs against leishmaniasis.

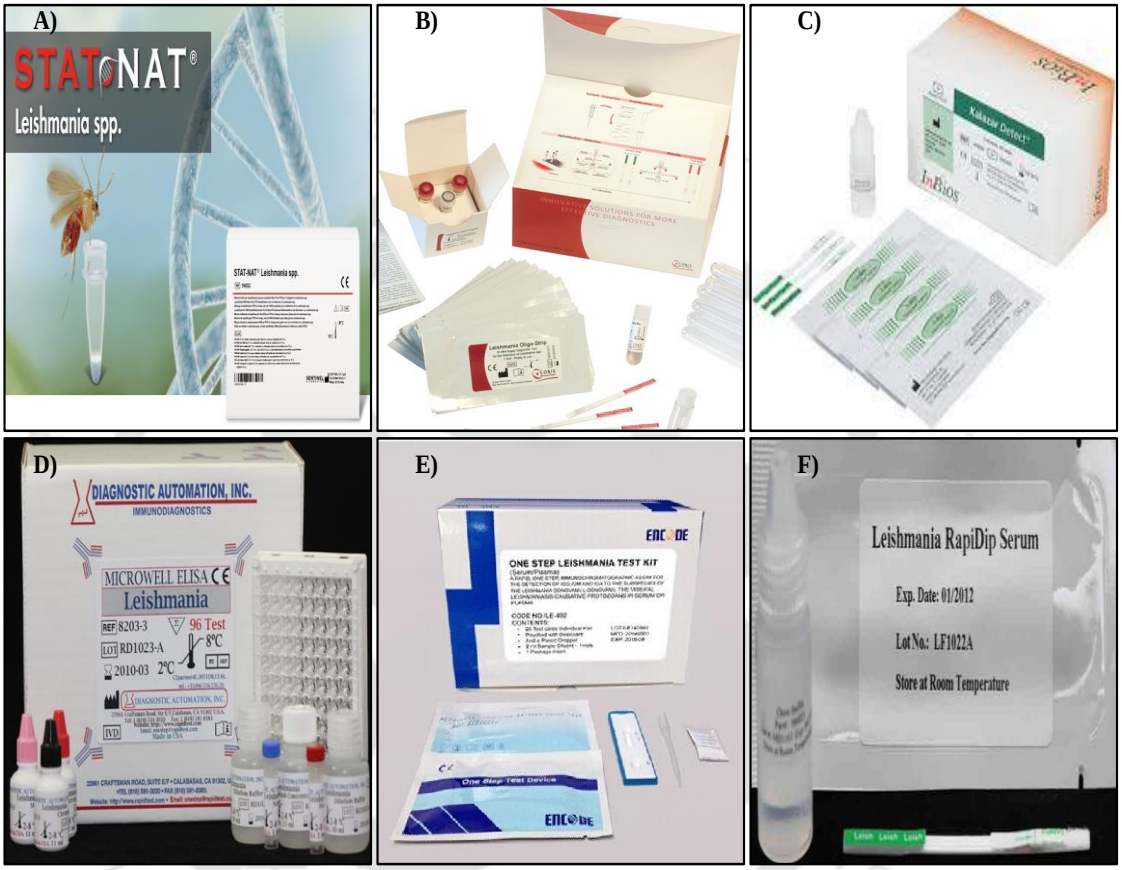


**Figure 1.7: Various commercially available diagnostic kits: (A)** STATNAT® *Leishmania* spp kit is a commercially available diagnostic kit patented by Sentinel Diagnostics. It detects almost all the species of *Leishmania*. It is real –time PCR based diagnostic kit (Source: http://www.sentinel.it). **(B)** *Leishmania* OligoC-TesT is based on a test which amplifies 18 S ribosomal gene of *Leishmania,* followed by one step oligochromatographic membrane assay. Its detection limit is 1fg of genomic DNA/PCR **(Source:** http://www.corisbio.com**). (C)** InBios' Kalazar Detect™ is a diagnostic kit for visceral leishmaniasis. It employs qualitative detection of antibodies to members of *L.donovani* complex in human serum (Source: http://www.inbios.com ). **(D)** AccuDiag™ Leishmania ELISA Kit employs qualitative screening of IgG antibodies against visceral Leishmaniasis in human serum (Source: http://www.rapidtest.com). **(E)** The *Leishmania* Ab Rapid Test employs qualitative detection of antibodies (IgG, IgM and IgA) to the subspecies of *Leishmania donovani* , in human serum, plasma or whole blood. It is a lateral flow immunoassay (Source: http://www.bio-equip.cn). **(F)** *Leishmania* RapiDip Test is a membrane based qualitative immunoassay used for detection of visceral leishmaniasis. The membrane is pre-coated with rK39 on the test line region, while chicken anti-protein A is present on the control line region. It employs colorimetric detection technique (Source: http://www.rapidtest.com).

*1.2.8.2 Advances in the development of new treatments:* Use of drug carriers like functionalized carbon nanotubes have also been tested. Amphotericin B attached to functionalized carbon nanotubes showed significantly higher anti-leishmanial activity than conventional amphotericin B treatment in *Leishmania donovani* infected hamsters *(Prajapati et al., 2011).* Another study reported lower toxicity and improved antileishmanial effect for betulin, a pentacyclic triterpenoid, conjugated functionalized carbon nanotubes *(Saudagar et al., 2014).* Further, use of multidrug combination has significantly reduced treatment duration, side effects and individual doses *(Croft and Olliaro, 2011).* Studies done on combination of miltefosine with amphotericin B or paromomycin have reported to be highly efficient and helpful for the treatment of antimony-resistant VL infections *(Seifert and Croft, 2006).* Another study showed lower side effects, short duration of treatment and less severity of the disease on combination of amphotericin B in liposomal formulation, miltefosine and paromomycin *(Sundar et al., 2011).*

*1.2.8.3 Natural Drug Candidates:* Plant, mineral and animal sources have been used as traditional medicines since ancient times to treat human diseases. Approximately 80% residents of developing countries rely mostly on traditional medicinal practices in order to meet their foremost medical needs *(Kim, 2005).* Thus many research laboratories around the world are exploiting the role of these natural products in order to seek bioactive molecules. Many studies have reported plants to be a valuable source of bioactive compounds with antileishmanial properties. Diverse group of chemicals like phenolic compounds, terpenoids and alkaloids derived from various crude extracts and fractions have shown antileishmanial activity *(Salem and Werbovetz, 2006; Rodrigues, 2011; Wink, 2012).* A list containing compounds from both natural and synthetic sources is given in **Table 1.3**.

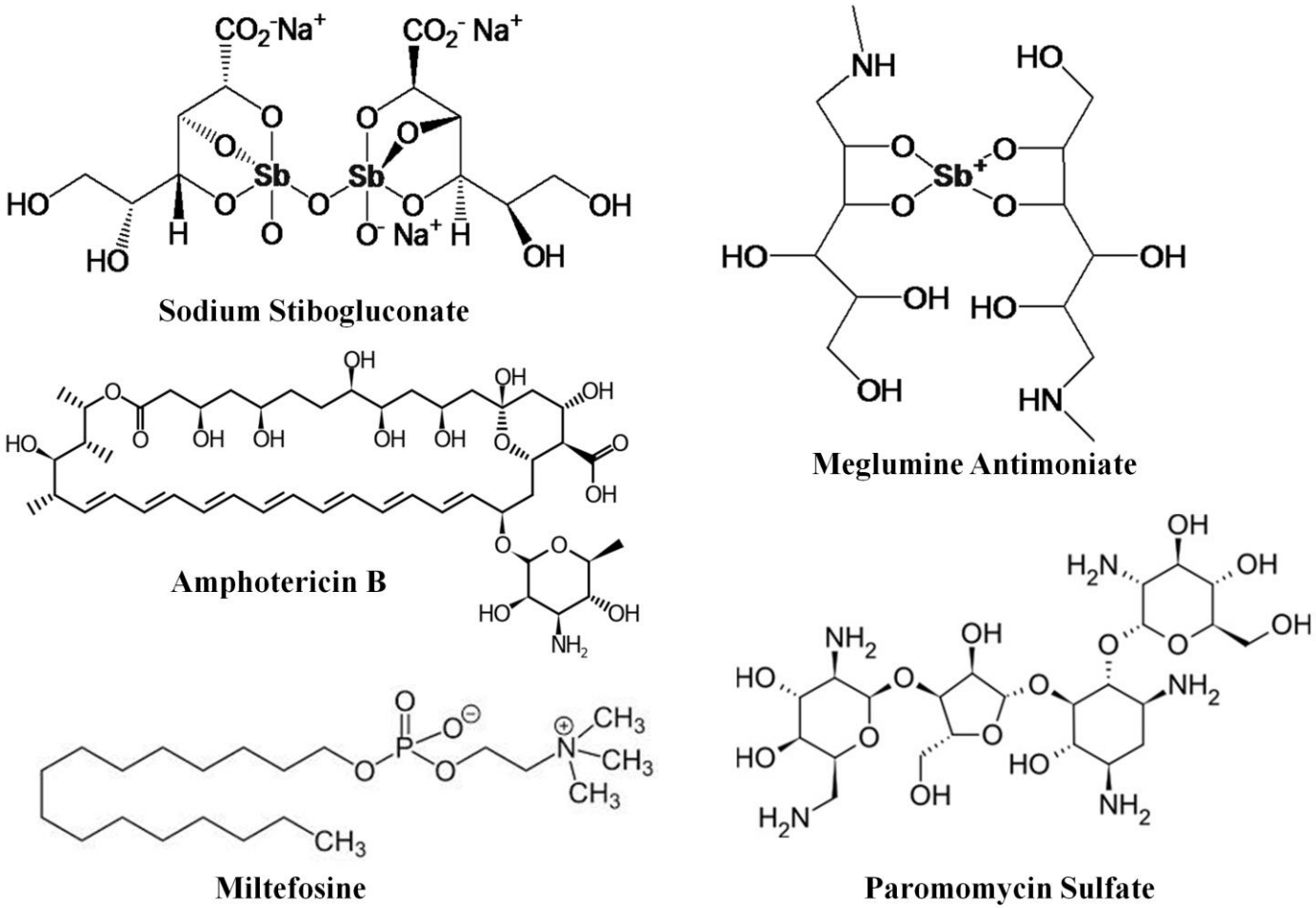


**Figure 1.8: Available drugs against leishmaniasis:** Sodium stibogluconate and meglumine antimoniate are antimony based drugs. Paromomycin sulfate is an amino-glycosidic aminocyclitol. Amphotericin B is included in the second line of drug treatment. Miltefosine is the only oral drug available. These drugs suffer from severe drawbacks in terms of toxicity, efficacy, emergence of resistance etc. Chemical structures are taken from web sources.

**Table 1.3:** Some antileishmanial compounds in the literature**.** List of synthetic and natural compounds having antileishmanial properties.

| ***Compound*** | ***Source*** | ***Reference*** |
|---|---|---|
| **SYNTHETIC** | | |
| Azasterols | Inhibitors of 24-methyltransferase | *Magaraci et al., 2003* |
| Nicotinamide | Inhibitor of certain III NAD-dependent deacetylase | *Sereno et al., 2005* |
| 3-substituted quinolines | Potential activators of macrophages | *Tempone et al., 2005* |
| Triazole SCH 56592 | Inhibitor of ergosterol synthesis pathway | *Al-Abdely et al., 1999* |
| 9, 9-dimethylxanthene tricyclics | Inhibitors of trypanothione reductase | *Chibale et al., 2000* |
| Edelfosine and Ilmofosine | New alkyl-lysophospholipid derivatives | *Azzouz et al., 2005* |
| N-acetyl-l-cysteine | Precursor of glutathione | *Chagas et al., 2008* |
| Perifosine | New alkyl phospholipid derivatives | *Cabrera-Serra et al., 2007* |
| Doxorubicin and Mitomycin C | Competitive inhibitors and subversive substrates of TryR enzyme | *Shukla et al., 2011* |
| 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester | Inhibitor of trypanothione synthetase and trypanothione reductase | *Saudagar et al., 2014* |
| **NATURAL** | | |
| Canthin-6-one alkaloids | *Zanthoxylum chiloperone* | *Ferreira et al., 2002* |
| Licochalcone A | *Chinese licorice* | *Chen et al., 2003* |
| Trichothecenes | *Holarrhena floribunda* | *Loukaci et al., 2000* |
| 2', 6'- dihydroxy-4'methoxychalcone | *Piper aduncum* | *Caio et al., 1999* |
| Maesabalide III | *Maesa balansae* | *Maes et al., 2004* |
| Coronaridine | *Peschiera australis* | *Delorenzi et al., 2004* |
| Parthenolide | *Tanacetum parthenium* | *Tiuman et al., 2005* |
| Plumbagin | *Pera benensis* | *Fournet et al., 1992* |
| Iridoid glucosides | *Nyctanthes arbortristis* | *Shukla et al., 2011* |
| Betulin | *Betula albosinensis* | *Saudagar and Dubey, 2014* |
| Hypericin | *Hypericum perforatum* | *Singh et al., 2015* |

### *1.2.9 Drug discovery strategy*

Development of a new drug is a complex process which can take 12-15 years to start from an original idea and to end with the launch of a finished product. There are a basic set of steps followed during any drug discovery process as shown in **Figure 1.9**. Two major reasons for failure of any drug during a clinical drug trail are: one, the drug does not work or two, the drug is not safe. Hence target identification and validation is the most crucial step of any drug discovery process. Wide spectrum of biological entities like proteins, RNA, genes etc. can be used as targets. A good target should be 'druggable' meaning, the binding of the target to the drug should lead to alteratios in the function of the target with therapeutic benefit to the patient. Drug targeting is further divided into three different types as shown in **Figure 1.10**. It is always advised to go for more than one target as it reduces the chances of drug resistance *(Oldfield and Feng, 2014).* Once the targets are identified and validated, the next step involves hit discovery process. This step involves identification and validation of lead candidates against the validated targets. Then comes the preclinical trials, followed by clinical trials and lastly product marketing and long term monitoring. The current antileishmanial drugs are shadowed by their high toxicity, severe side effects, long term use etc. Further these drugs are unable to completely eliminate the parasites from infected individuals and show only species-specific activity. Therefore there is an urgent requirement for new drugs against leishmaniasis. High performance techniques are being employed in order to identify chemotherapeutic targets. Transcriptomics and proteomics tools are used in order to study parasite and host cell interaction. This helps out with identification of new targets for drug discovery against leishmaniasis. The availability of complete *Leishmania* genome has opened up a broad window of opportunity which not only helps in better understanding of parasite biology but also subsequent identification of novel drug targets.

*1.2.9.1 Multidrug targeting approach:* Efforts towards new drugs discoveries as well as advancement in high throughput techniques have been made and are still ongoing, to fight against leishmaniasis. But still there are no new breakthrough drugs available even after increasing developments in drug discovery. Mostly the current available drugs against leishmaniasis are single target drugs. Studies have pointed out towards the fact that single target drugs do not show desirable effect on the entire system that is to be targeted. The

organism can easily compensate the specific target and cope up with the inhibition effect of the drug (*Lu et al., 2012*). Recent advancement in drug discovery are employing multidrug or combinatorial drug targeting, in which the drugs target more than one bio-molecule inside the pathogen. This not only increases the efficacy to the drugs but also helps it to cope up with the drug resistance issue.

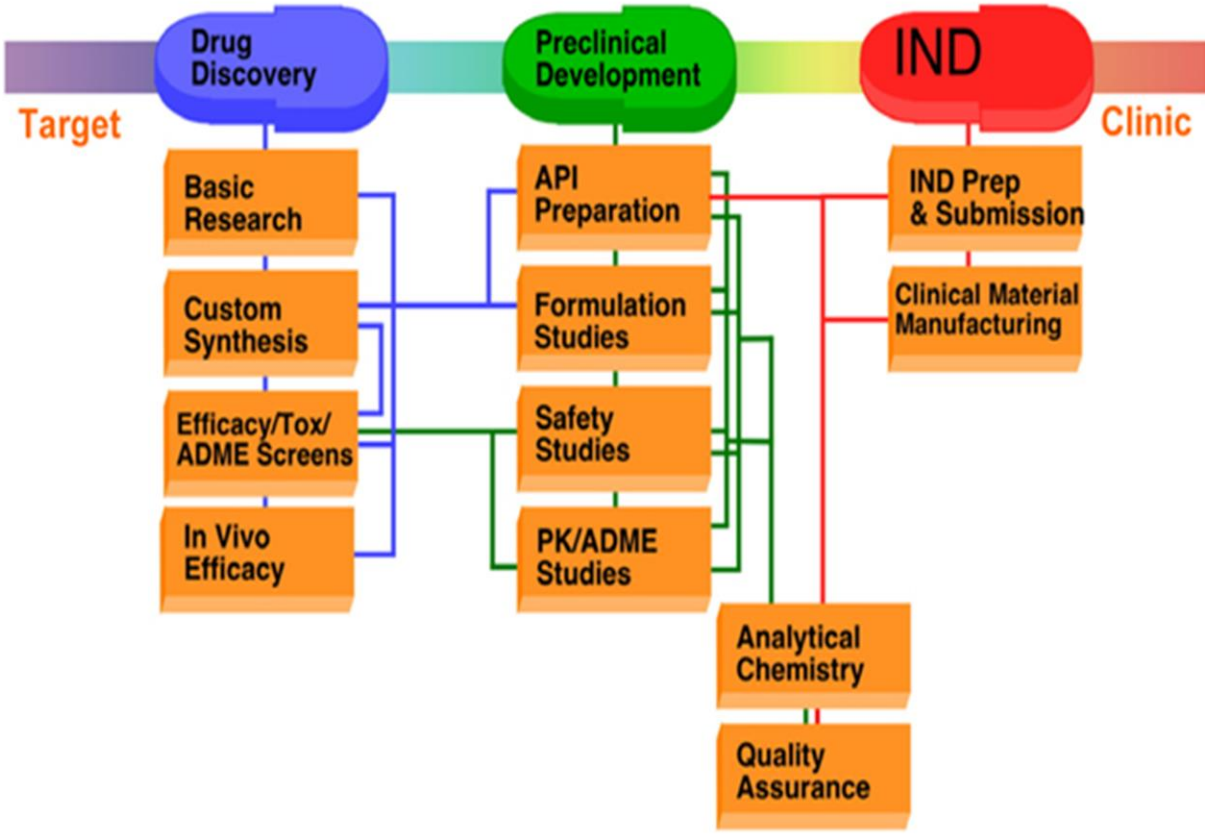


**Figure 1.9: Strategic drug discovery:** Comparative genome and proteome analysis of pathogen and host are done to identify a target inside the pathogen. Once the target is identified, experiments are done to validate it. This is followed up by identification of a drug candidate against the target and then optimization of the candidate. Once the lead drug candidate is validated, pre-clinical studies are done followed by clinical studies, product manufacturing and marketing. The last step is long term monitoring of the drug post marketing (Adpoted from BMC Neurology. The journal allows unrestricted use, distribution and reproduction of original work. *Reference: BMC Neurol. 2009, 9 Suppl 1:S2.).*

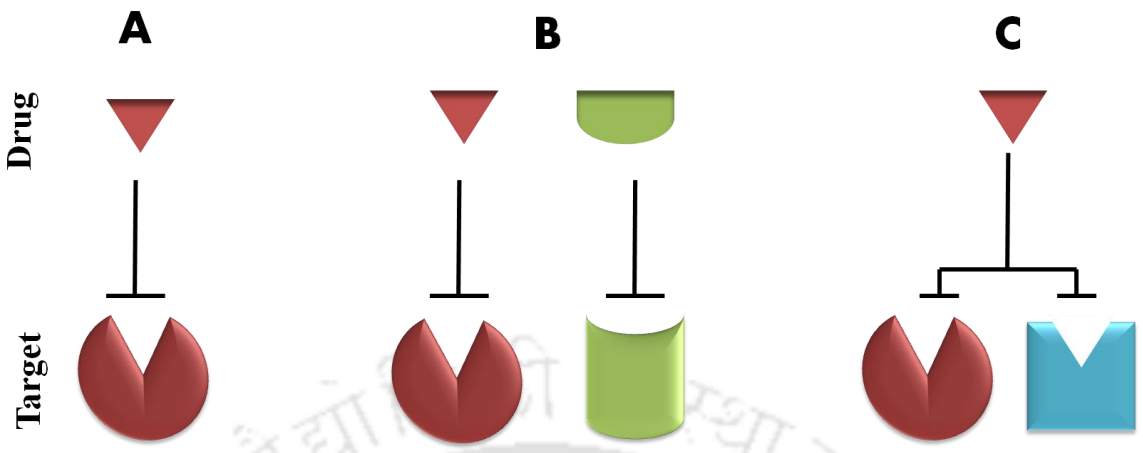


**Figure 1.10: Types of drug targeting: (A)** Single drug targeting: Individual drug developed against a single target. **(B)** Combinatorial targeting: Two or more drugs combined and developed against two or more targets. **(C)** Multi drug targeting: A single drug developed against more than one target. (Revised from *Oldfield and Feng, 2014, Biochem. J.* 393, 227–234)

### *1.2.10 Potential targets against leishmaniasis*

Biochemical and metabolic pathways, essential for parasite survival, are the focus when it comes to identifying new drug targets against leishmaniasis. The targets identified in the parasite should either be absent in human host or should have significantly lower structural and functional identity with human counterpart. Further simultaneous targeting of more than one biomolecules will always have an upper hand on single drug targeting.

*1.2.10.1 Sterols:* Sterols are important components of cell membrane which are not only responsible for its maintenance but also play a vital role in conducting various cellular functions. Ergosterol and 24-methyl sterols are major membrane sterols for *Leishmania* while these sterols are absent in mammalian cells. Targeting of squalene synthase (SQS), an enzyme that catalyzes the first step of sterol synthesis, has been done using inhibitors like zaragozic acids and quinclidines. ER_119884 and E5700, derivatives of quinuclidine have proven to be potent antileishmanial compounds *(Fernandes et al., 2008)*. Terbinafine, an allyamine, has shown to inhibit squalene epoxidase, causing parasitic growth inhibition, accompanied by changes in structural organization of mitochondrion *(Van-Santos et al., 1995)*. Bisphosphonates is another class of inhibitors that act upon farnesyl diphosphate synthase, which is a key enzyme for isoprenoid pathway *(Martin et al., 2001; Docampo and*

*Moreno, 2008).* D-sterol methyltransferase (SMT), a key enzyme in ergosterol biosynthesis, has also proven to be a potent target. This enzyme is present in trypanosomatids, but absent in human host. Effects of azasterols, known inhibitors of SMT, have been studied on *Leishmania* and *Trypanosoma*. These compounds have shown anti-proliferative effects on the parasites with $IC_{50}$ values ranging from submicromolar to nanomolar range *(Magaraci et al., 2003; Lorente et al., 2004).*

*1.2.10.2 Peptidases: Leishmania* genome boasts of 154 peptidases which include serine, cysteine, aspartic, threonine and metallopeptidases. Inhibition of the proteasome of *Leishmania mexicana* has led to impaired growth of the parasite under *in vitro* conditions *(Robertson, 1999).* Studies on inhibitor K11777, which is specific to cathepsin-L like cysteine peptidase, have reported the importance of these peptidases for parasite growth *(Mahmoudzadeh-Niknam and McKerrow, 2004)*. The effect of serine peptidase inhibitors like N-tosyl-l-lysyl-chloromethylketone and benzamidine, on *Leishmania amazonensis* parasite have shown changes in parasite morphology and reduced viability *(Silva-Lopez et al., 2007).*

*1.2.10.3 MAP kinases:* Mitogen-activated protein (MAP) kinases are important regulators of various cellular processes like cell proliferation and differentiation. Null mutants of LmxMKK and LmxMPK, which are a set of MAP kinases identified in *Leishmania mexicana*, did not show any major effect on cell viability of the parasite, but null mutants of another MAP kinase LmxMPK, showed inhibition of parasite proliferation inside the parasitophorous vacuole *(Wiese, 1998).*

*1.2.10.4 Cyclin dependent kinases:* Cyclin dependent kinases (cdks) play a major role in important cellular processes like cell cycle, apoptosis, transcription, etc. Disruption of CRK3 (Cdk related kinase3) in *Leishmania mexicana* led to change in cellular ploidy *(Hassan et al., 2001).* Inhibitors belonging to indirubin class have been found to be potent against CRK3 activity *(Grant et al., 2004).* LdGSK 3 (glycogen synthase kinase) in combination with CRK3 has been explored as drug targets in *Leishmania donovani (Xingi et al., 2009).*

*1.2.10.5 Metacaspases:* The crucial role of metacaspases in apoptotic like cell death of *Leishmania* parasite has been well established. Studies are still required to understand the complete function of leishmanial metacaspases due to their important role in chromosome segregation and parasite survival. LdMCA1 and LdMCA2 are two types of metacaspases

found in *Leishmania donovani* and both contain characteristic terminal proline rich domains *(Lee et al., 2007)*. LmjMCA, a metacaspase found in *Leishmania major*, has been reported to play an important role in segregation of nucleus and kinetoplast *(Denise et al., 2006)*. Studies have reported that metacaspases trigger apoptotic like cell death in $H_2O_2$ treated *Leishmania*. Further the hypersensitivity of *Leishmania* parasites towards $H_2O_2$ induced apoptotic like cell death increases when metacaspases are overexpressed inside the parasite *(Gonza'lez et al., 2007)*.

*1.2.10.6 Trypanothione pathway:* Trypanothione pathway is not only unique to trypanosomatids but it also plays a crucial role in maintaining the redox homeostasis inside the parasite **(Figure 1.11)**. Trypanothione is the key molecule of this pathway and its synthesis is catalyzed by two major enzymes, namely, trypanothione synthase (TryS) and trypanothione reductase (TryR). Trypanothione synthase utilizes glutathione and spermidine for the synthesis of trypanothione, while trypanothione reductase reduces this molecule in the presence of NADPH *(Fairlamb et al., 1985)*. This reduced trypanothione further reduces tryparedoxin (TX) followed by reduction of tryparedoxin recycling enzyme tryparedoxin peroxidase (TP), which plays crucial role in regulating the oxidative stress inside the parasite. Knockout of TryR showed reduced infectivity and survival within intracellular macrophages *(Dumas et al., 1997)*. Even though TryR has sequence as well as structural similarity with glutathione reductase (GR), its human counterpart, but its active site is still different from GR. The presence of five non-conservative changes in active site imparts overall negative charge to accommodate trypanothione disulphide hence making the enzyme specific towards its substrate *(Zhang et al., 1996)*. Many chemical compounds have shown inhibitory effect against TryR like, polyamine derivatives, tricyclics and aminodiphenyl sulphides *(Werbovetz, 2000)*.

Furthermore, anti-tumor agents, doxorubicin and mitomycin C were assessed as inhibitors against trypanothione reductase. These inhibitors destabilized physiological function of TryR by increasing ROS species. This increased ROS species further lead to apoptotic like cell death of *Leishmania donovani* promastigote *(Shukla et al., 2011)*. Antileishmanial activity of betulin was tested under *in vitro* conditions after establishing its role as an inhibitor of trypanothione synthase *(Saudagar and Dubey, 2011)*. The parasite

showed apoptotic response involving mitochondrial membrane damage, DNA fragmentation and activation of caspase-like proteases *(Saudagar and Dubey, 2014)*. Further a chemically synthesized compound 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester (PS-203), was found to inhibit both TryR and TryS *(Saudagar and Dubey, 2013)*. Studies under *in vitro* as well as *in vivo* conditions were carried out *(Sauadagar et al., 2014)*. The inhibition of these two enzymes by PS 203 leads to increased ROS species followed by apoptotic like cells death of *Leishmania donovani* parasite. No significant toxicity against host cells or animal models was found.

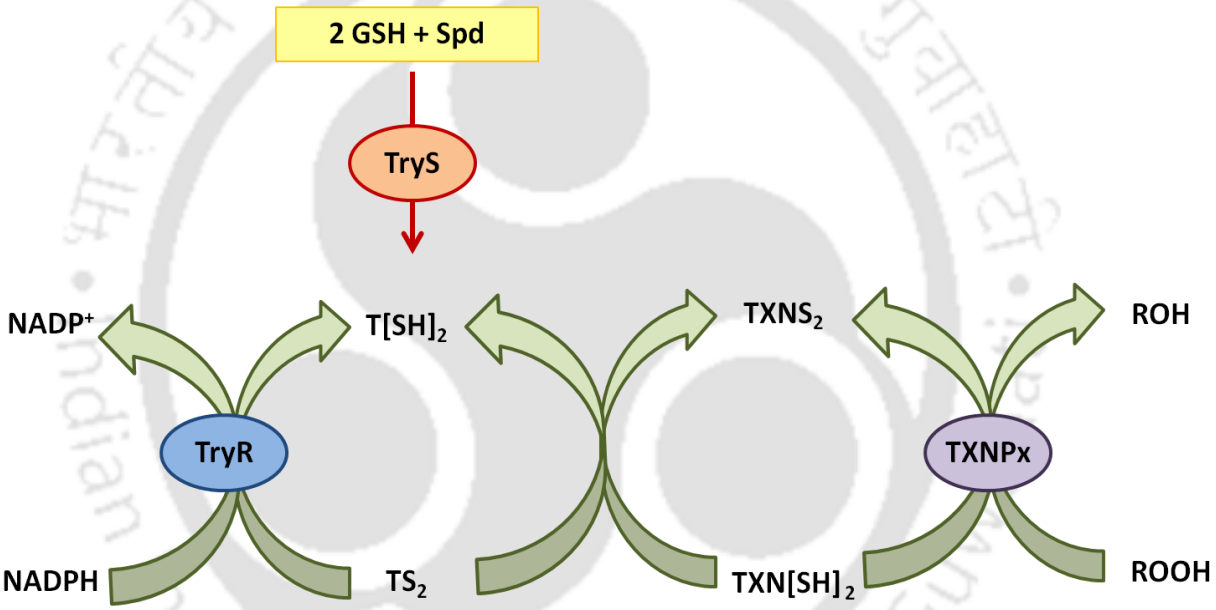


**Figure 1.11: Trypanothione pathway:** The host defense mechanism produces hydroperoxides (ROOH), which are detoxified by the *Leishmania* parasite to their respective alcohols (ROH). The parasite employs tryparedoxin-dependent peroxidases ($TXNP_X$) for this job. Thiol redox chain composed of TryR (trypanothione reductase), T $[SH]_2$/ $TS_2$ and tryparedoxin ($TXN[SH]_2/TXNS_2$), utilize NADPH to derive reducing equivalents for the conversion of ROOH to ROH. Here $[SH]_2$ and $S_2$ refer to the dithiol [reduced] and disulfide [oxidized] forms respectively. TryS (trypanothione synthase) utilizes two molecules of glutathione (GSH) and one molecule of spermidine (Spd) to synthesize T $[SH]_2$.

*1.2.10.7 Glycolytic pathway:* Trypanosomatids solely depend on carbon source available in the host, when it comes to energy metabolism. Glycolysis is the only process which supplements the parasite with energy, hence blocking it could be fatal for the parasite.

Presence of glycosomes, compartments containing the glycosomes, in *Leishmania* parasite provides it a unique feature hence resulting in a large phylogenetic distance with mammalian host. Compounds that bind to glycolytic enzymes are obtained by structure based drug designing since 3-D structures of various trypanosomatid enzymes like, glyceraldehydes-3-phosphate dehydrogenase (*Vellieux et al., 1993),* triosephosphate isomerase *(Wierenga et al., 1991),* phosphoglycerate kinase *(Rigden et al., 1999),* fructose-1,6-bisphosphate aldolase *(Chudzik et al. 2000)* etc., are available. N6-(1-naphthalenemethyl)-20-(3-methoxybenzamido) adenosine, one of the analogs of adenosine showed growth inhibition in *Leishmania mexicana* with an IC50 of 0.28µM *(Aronov et al., 1999).*

*1.2.10.8 DNA topoisomerases:* Topoisomerases are essential for the removal of torsional stress in DNA by introducing protein-bridge DNA breaks on either one (type I) or both (type II) the DNA strands. These enzymes are omnipresent and are major drug targets in cancer as well as bacterial chemotherapy *(Schneider et al., 1991; Heisig, 2001).* The presence of mini and maxicircles in kinetoplasts mitochondria, in *Leishmania* parasite, makes the role of topoisomerase, especially type II, essential. Overexpression and increased activity of topoisomerase II was observed in arsenite resistant strains of *Leishmania donovani (Singh et al., 2005).* Drugs like novobiocin, etoposide and fluoroquinolones, which target topoisomerase II, can be used for inhibition of cell growth and inactivation of genetic integrity *(Rosypal et al., 2010).*

*1.2.10.9 Hypusine pathway:* Hypusine (Ne-(4-amino-2-hydroxybutyl) lysine), is an unusual amino acid derived from polyamine spermidine. Post translational modification of eukaryotic initiation factor 5A (eIF5A), results in synthesis of hypusine. Recent studies have reported the occurrence of this pathway in *Leishmania donovani* parasite. Deoxyhypusine synthase (DHS) catalyzes the first step of hypusine synthesis *(Chen and Dou, 1988; Murphey and Gemer, 1987).* The parasite contains two DHS domain containing proteins viz., DHS-like gene (DHSL20) and DHS34 gene, of which only the latter was found to be functionally active under *in vitro* conditions. Further it was not possible to obtain null mutants of DHS 34, hence pointing out towards the importance of this protein for parasite survival *(Chawla et al., 2010).* Most potent inhibitors of human enzymes like $GC_7$ and other spermidine analogs were

not effective against recombinant DHS34 hence establishing the difference in the spermidine binding sites of both the enzymes.

*1.2.10.10 Polyamines:* Polyamines like putrescine, spermidine and spermine play an important role in growth as well as differentiation, from promastigote to amastigote form, of the parasite *(Tavares et al., 2005)*. These molecules also help out the parasite with down regulation of the lipid peroxidation generated by oxidant compounds, hence making the surrounding harmonious for parasite survival *(Vannier-Santos et al., 2008)*. The regulatory mechanisms responsible for unchanged polyamine pool during initial phase and environment transition of parasite from vector to host can be exploited for future drug targeting *(Roberts et al., 2007)*. Antileishmanial activity has been found for inhibitors of polyamine biosynthetic pathways. Adment DC inhibitor is well established cure for animal leishmaniasis, but has yet to be tested on humans and requires further studies *(Heby et al., 2007)*.

*1.2.10.11 Glyoxalase system:* The main function of glyoxylase system is detoxification of the parasite by removing methylglyoxal, which is a by-product of glycolysis and is toxic as well as mutagenic in nature *(Cooper, 1984)*. **Figure 1.12** illustrates the various steps involved in this pathway. Glyoxalase I (lactoyl glutathione lyase) and II (hydroxyacyl glutathione hydroxylase) comprise the glyoxalase system and glutathione is used as a cofactor. In case of trypanosomatids, glyoxalase system is trypanothione dependent, making this pathway unique for the parasite. Glyoxalase I and II have already been characterized from *Leishmania donovani (Padmanabhan et al., 2005; Padmanabhan et al., 2006)*. Sequence analysis showed the lack of basic residues in active site of glyoxalase II, in *Leishmania donovani* which is otherwise conserved in the human homolog. Thioester of trypanothione is the positively charged substrate for *Leishmania donovani* gyloxalase II. This substrate cannot be accommodated in the active site of human glyoxalase II, hence making it a potent drug target.

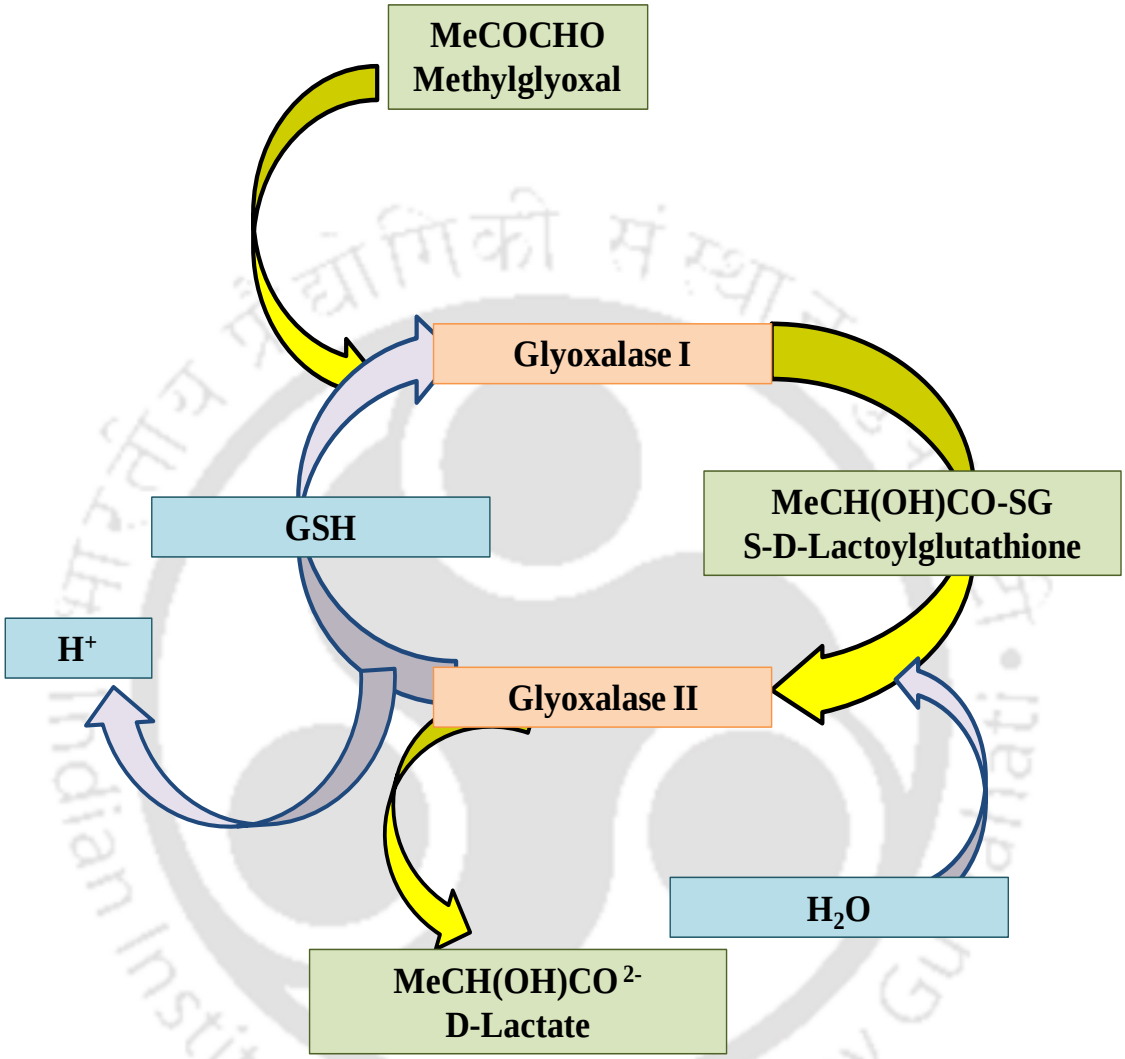


**Figure 1.12: Glyoxalase pathway:** The glyoxalase system consists of two consecutive enzymatic reactions by employing two enzymes viz. glyoxalase I and glyoxalas II. This system prevents the accumulation of methylglyoxal, a product of glycolytic biological systems, to a toxic level. The glyoxalase sytem, catalyses the conversion of methylglyoxal to D-lactate and S-D-lactoylglutathione is the intermediate.

### *1.2.11 Conserved hypothetical proteins: potential targets*

First complete genome of a cellular life form was published in 1995 (1.8Mb genome of *Haemophilus influenza*, RdKW20 strain). It has been over a decade now and genomes of more than 150 organisms have been sequenced and sequencing of many more is in progress. The beginning of genome era faced inconsistency in the accuracy of genome annotation *(Brenner, 1999)*. The so-called '70% hurdle" is still an issue because functions of only ~50±70% genes are predicted in any given genome *(Bork, 2000)*. The remaining genes are further subcategorized into two types (i) conserved hypothetical genes: encode for conserved proteins with unknown functions and (ii) hypothetical or non-characterized genes: do not have any known homologs and their role in encoding an actual protein is still ambiguous. Conserved hypothetical proteins, encoded by conserved hypothetical genes, pose a challenge for not only functional genomics, but also to biology in general *(Galperin, 2001)*. The complete understanding of model organisms like *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* etc., is not feasible as long as there are hundreds of conserved hypothetical proteins in these models. **Figure 1.13** illustrates a few key points to identify a conserved hypothetical protein.

Pathogens reduced their genomic size in order to adapt to parasitic lifestyle. They reduced the genome size through loss of various genes which encoded for many metabolic enzymes, membrane permeases and transcriptional regulators. The genes that have been conserved in distantly related organisms are expected to be important for cell survival *(Arigoni et al., 1998)*. These genes can further be exploited as potential drug targets *(Galperin and Koonin, 1999)*. When a conserved protein is found in several genomes this does not necessarily mean that its function is completely unknown *(Natale et al., 2000)*. General prediction of a conserved hypothetical protein can be based upon several factors like conserved sequence motif, sequence similarity to a protein which has been characterized previously, presence of diagnostic structural features etc. *(Galperin and Frishman, 1999)*. Various approaches have been exploited in order to predict the function of a conserved hypothetical protein. Protein function can be predicted by using the information derived from phylogenetic and gene expression profiles, sequence similarity, protein-protein interactions etc.

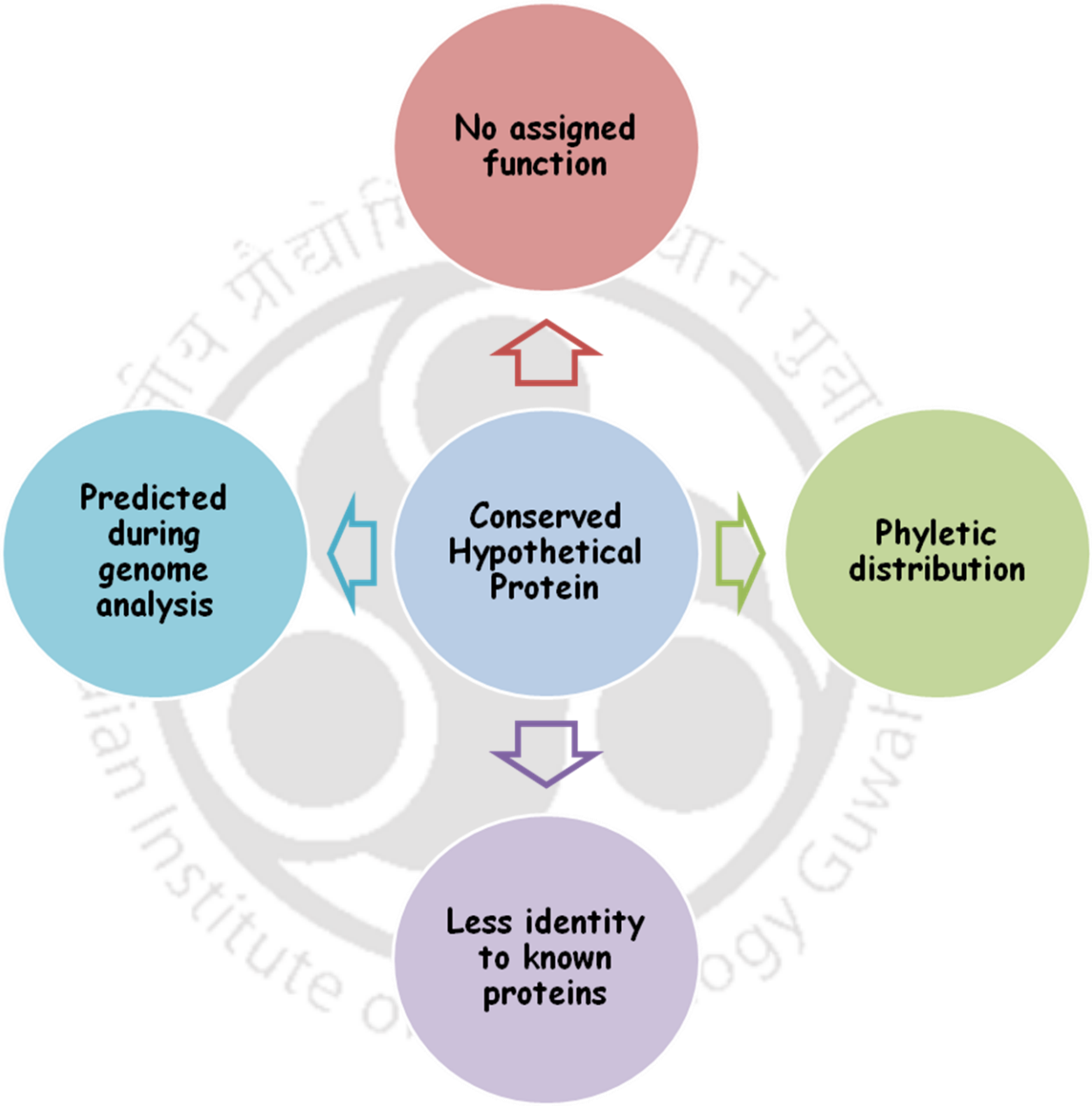


**Figure 1.13: Conserved hypothetical protein:** A conserved hypothetical protein is a protein whose existence was predicted during genome analysis. These proteins are called hypothetical because their function is not yet being assigned. These proteins have significantly lower identity with functionally annotated proteins. Conserved hypothetical proteins have a wide phyletic distribution.

### *1.2.12 Criteria for prioritization of a conserved hypothetical protein as a target*

Certain set of standards are set in order to prioritize a conserved hypothetical protein for targeting.

*1.2.12.1 Phyletic distribution:* The ubiquitous nature of a conserved hypothetical protein is an attractive trait when it comes to selecting traits for experimental analysis. Majority of conserved hypothetical genes are either 'nearly ubiquitous', primarily in parasites, or are universal, like in case of bacteria and archaea. These ubiquitous genes are known to play an important role in cellular processes like translation and transcription or some other processes with are closely related to translation and transcription *(Koonin, 2003).* When genes demonstrate partial phyletic patterns matching to those of essential genes, then it is highly probable that both share similar functions *(Galperin and Koonin, 2000).* Following this line of action, various discoveries have been made like an alternative lysyl-tRNA synthetase, thymidylate synthase, shikimate kinase and several enzymes of thiamine biosynthesis *(Ibba et al., 1997; Myllykallio et al., 2002; Daugherty et al., 2001; Morett et al., 2003).* More narrowly represented conserved hypothetical genes can be used as excellent targets for determination of particular phenotypes or individual taxa. For example conserved hypothetical genes that are shared by cyanobacteria and plants but are absent in bacteria, could play a role in photosynthesis *(Raymond et al., 2002).*

*1.2.12.2 Essentiality:* Biological importance or essentialities of quite a number of conserved hypothetical proteins are known due to recent genome-wide analyses of knockout phenotypes in various organisms *(Kobayashi et al., 2003; Gerdeset al., 2003; Zhang et al., 2004).* Proteins belonging to same family, including the conserved hypothetical proteins, are often regarded as essential for survival of more than one organism. However, essentiality for growth in an organism is a complex phenomenon and it is the function rather than a particular gene that is responsible for survival of an organism *(Koonin, 2000).*

*1.2.12.3 Protein structure:* Another criteria for prioritization of a conserved hypothetical protein as a target is the availability of a 3 D structure (X- ray or NMR) of the protein. Various structural genomic projects are discovering new structures, as a result of which many families of conserved hypothetical proteins have at least one representative with a known 3 D

structure *(Vitkup et al., 2001; Frishman, 2003)*. Even though the 3 D structure prediction does not help out in complete functional prediction but it still provides various clues. This further helps in narrowing down the range of probable functions of conserved hypothetical proteins *(Kolker et al., 2004)*.

*1.2.12.4 Expression and binding information:* Experimental data on expression of a given gene and the binding properties of protein encoded by it is essential. This helps out with prediction of the expression profile of a conserved hypothetical protein under certain conditions like oxidative stress, nutritional stress, ultraviolet radiation etc *(Tao et al., 1999; Price et al., 2001; Liu et al., 2003; Kolker et al., 2003)*. This information still won't help with the complete function prediction of a conserved hypothetical protein, but it can be prioritized as a target depending upon its stress response.

*1.2.12.5 Other criteria:* Another set of factors that determine if a conserved hypothetical protein can be used as a target are, solubility of the protein, whether or not it can be expressed highly as a recombinant protein, if yes then can it be easily purified, etc. The properties predicted by sequence analysis including its size, behavior etc. can be only validated by experimental analysis.

### *1.2.13 Prenylation pathway: modification of Ras proteins*

Organisms have developed a vast range of co-translational as well as post translational modification for enhancement of protein functions in response to external stimuli and internal adjustments *(Ambrogelly et al., 2007)*. Prenylation comes under one such post translational modification, which is responsible for the modification of Ras proteins. This pathway is known to play a crucial role in regulating various groups of Ras proteins which are ultimately responsible for carrying out biological functions in eukaryotes *(Zhang and Casey, 1996; Gelb et al., 2006; Wang and Casey, 2016)*. Majority of Ras proteins that have to be prenylated contain the CAAX moiety. The prenylation is initiated by attachment of a 15-carbon (farnesyl) or a 20- carbon (geranylgeranyl) isoprenoid lipid to the cysteine residue of CAAX moiety. Farnesyl transferase (FTase) or geranylgeranyl transferase I (GGTase I) are responsible for this step respectively and farnesylation is the term for this enzymatic step. Proteins are further processed by CAAX prenyl protease I or CAAX prenyl protease II,

which simply removes the –AAX residue. This is followed up by capping of isoprenoid-modified cysteine residue with a methyl group. Isoprenylcysteine carboxylmethyltransferase (ICMT) is the enzyme responsible for this step *(Ashby, 1998; Winter-Vann and Casey, 2005).* The **Figure 1.14** shows the various steps involved in protein prenylation pathway.

Prenylation increases the capacity of the Ras proteins to interact with cellular membranes, by providing a hydrophobic C terminus to these proteins. CAAX protein sequence and structure along with prenylated motif determine the distinct localization of these proteins, which can either be in a specific area of the plasma membrane or could be endomembrane. It further helps the Ras proteins by enhancing its stability and facilitating specific protein-protein interactions.

*1.2.13.1 Biological roles of prenylation pathway:*

*1.2.13.1(A) Protein trafficking:* Each protein owns a characteristic pattern of cellular localization and membrane trafficking due to the combined effect of unique CAAX structure and prenylation modification. Apart from attachment of Ras proteins to the membrane, the protein-protein interactions involved in subcellular trafficking of CAAX protein are among best characterized role of prenylation pathway *(Wang and Casey, 2016*. The Ras proteins are modified in the cytosol and then on the ER but their ultimate site of function is cell membrane. Various binding or chaperone proteins facilitate the trafficking of these proteins from one place to the other. For example, in case of RHO GTPases, the prenylation modification is crucial determinant of their interaction with RHOGDIs, a group of cellular proteins, which further influence the localization, GTP-binding properties and activities of proteins belonging to RHO family *(Hoffman et al., 2000; Jaffe and Hall, 2005).* Further studies on interaction of Ras proteins with prenyl-binding protein called phosphodiesterase- δ (PDEδ), have reported the regulatory role of PDE δ in membrane release and distribution of Ras as well as other farnesylated proteins *(Bhagatji et al., 2010; Wang and Casey, 2016).*

*1.2.13.1(B) Cellular Roles:* The role of farnesylated Ras proteins in carcinogenesis has attracted a lot of attention in the field of cancer biology *(Malumbres and Barbacid, 2003).* Further the active role of geranylgeranylated NRAS and KRAS in cancer and other pathological conditions have been established *(Doll et al., 2004).* Suppression of FTase led to better understanding of Ras membrane association and signaling. Knockout of FTase in

mice led to blockage in proliferation of fibroblasts and conditional inactivation of FTase in mice with KRAS-induced lung cancer resulted in impaired tumor growth which led to improved survival *(Liu et al., 2010)*. Further FTase inhibitors (FTI) resulted in G1 and G2-M phase arrest in cell cycle of different types of cancer cells *(Ashar et al., 2001)*.

*1.2.13.1(C) Impact of RCE1 mediated proteolysis:* Studies done on RCE-knockout mice confirmed its exclusive role in carrying out proteolysis of majority of CAAX proteins *(Kim et al., 1999)*. RCE1 knockout mice died during late gestation or within the first week of life, with no apparent organogenesis defects. Further post-developmental phenotypes were studied as well, where removal of RCE 1 in specific tissues, most notability in the heart, resulted in dilated cardiomyopathy at early stage, ultimately leading to death after 10 months *(Bergo et al., 2004)*. It should be noted that the CAAX protein(s) which were affected by limiting RCE1 activity have not been identified. The RCE1 dysfunction shows tissue specific effects and some RCE1 substrates will be more affected than the others by incomplete processing. Studies done on removal of RCE1 in the retina led to retinal degeneration. This was reported to be linked with defective transport of PDE6, a prenylated protein, from the cell body to the outer segments of photoreceptor cells, while the other prenylated proteins in the retina remained unaffected *(Christiansen et al., 2011)*.

*1.2.13.1(D) Impact of ICMT-catalysed methylation:* ICMT is responsible for catalysis of carboxyl methylation of prenylated proteins and its function has been established by gene disruption studies. Null mutant mice of ICMT died around embryonic day 11.5 with a defect in liver development *(Bergo et al., 2001; Lin et al., 2002)*. Further inhibition studies done on ICMT for different types of cancer cells resulted in G1 phase cell cycle arrest and cell death *(Wang et al., 2008)*. A link between protein prenylation and autophagy was established when inhibition of ICMT promoted robust autophagy and cell death linked to autophagy. Although this mechanism is still unclear, but multiple ICMT substrates like RAC3 GTPase, have been known to regulate autophagy *(Zhu et al., 2011)*. Recent studies have pointed out towards the suppression of mitochondrial respiration during ICMT inhibition, which ultimately leads to an energy-depleted state followed by an increase in autophagy *(Teh et al., 2014)*.

### *1.2.14 CAAX prenyl proteases*

CAAX prenyl proteases are categorized into two different type *viz.*, type I and type II. A flow chart showing the main differences between the two types of CAAX prenyl protease is shown in **Figure 1.15**. CAAX prenyl protease I, also known as alpha-factor converting enzyme (AFC1), is a metalloprotease. It has a conserved HEXXH motif (H: histidine, E: glutamate, X: any amino acid).

CAAX prenyl protease II, also known as Ras and a-factor converting enzyme (RCE1), lacks the conserved motif (*Boyartchuk et al., 1997; Schmidt et al., 2000; Tam et al., 2001*). Further its enzymatic nature is controversial. Some studies have reported it to be cysteine protease based upon inhibition studies. Further the presence of cysteine residue near the active site of RCE1p was reported and mutation of these cysteine residues resulted in deactivation of the enzyme *(Dolence et al, 2000)*. While in defense of these claims, few studies have reported CAAX prenyl protease II as metalloprotease (*Pei et al, 2001; Pei et al, 2011*). No significant sequence or structure similarity was found on comparison of type I and type II CAAX prenyl protease. Interestingly, both the proteases i.e. CAAX prenyl protease I and II show distinct but overlapping substrate specificity. Both proteases were found to have proteolytic activity against a-factor CAAX sequence CVIA. However when CAAX sequence was substituted by CAMQ only AFC1 was able to proteolyse it while RCE1p was able to proteolyse CTLM substituted a-factor CAAX sequence. Similarly yeast Ras2 protein, CIIS, was proteolysed by RCE1p but not by yeast AFC1p (*Boyartchuk et al, 1997; Trueblood et al, 2000*).

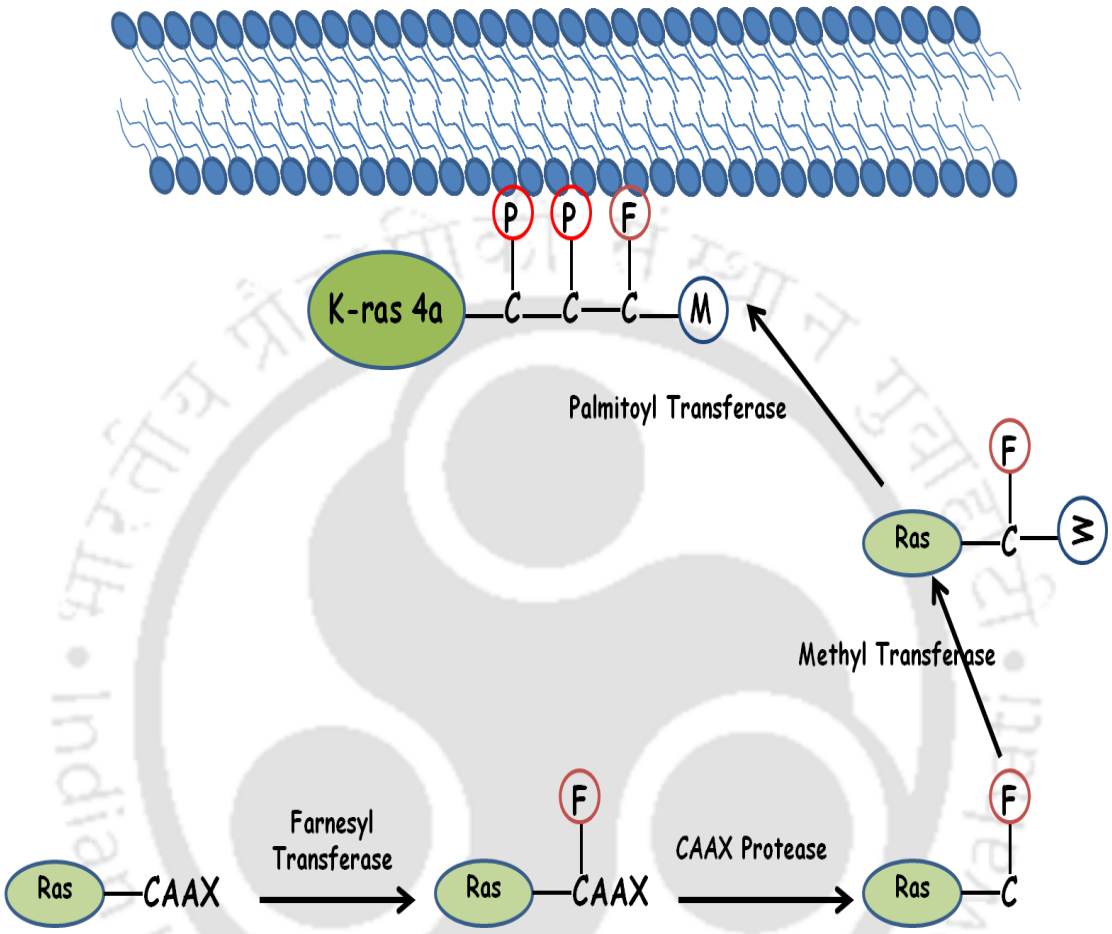


**Figure 1.14: Prenylation pathway:** Prenylation is a post translational modification pathway for many signal proteins. It involves maturation of proteins sepecially belonging to the Ras superfamily. A farnysyl or geranylgeranly group is added to the cysteine of the CAAX moiety attached to the Ras protein. The -AAX is further cleaved by CAAX prenyl protease. Farnesylated cysteine is further modified by addition of methyl group. This is followed up by further modification till Ras maturation occurs and the matured molecule then mobilizes to the cell membrane. (Revised from: *Friday and Adjei, 2005, Biochem Biophys Acta*; 1756, 127–144)

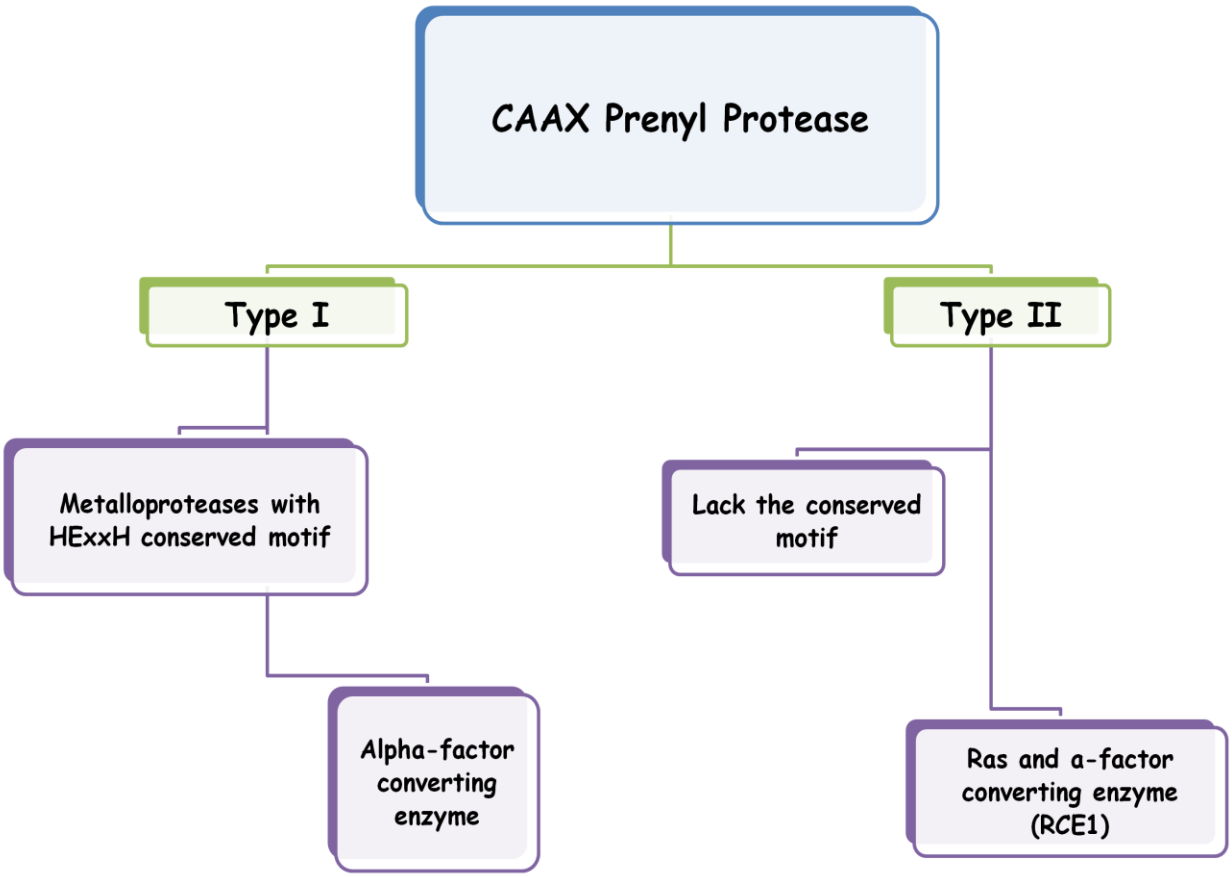


**Figure 1.15: Two types of CAAX prenyl protease:** There is no significant sequence similarity between CAAX prenyl protease I and CAAX prenyl protease II. Interestingly these proteases have distinct but overlapping substrate specificity. *Leishmania donovani* genome codes for both the proteins.

### *1.2.15 Relevance of current research*

The available drugs for leishmaniasis have several limitations in terms of drug toxicity, mode of administration, high cost etc. (*Rijal et al., 2013).* Currently there are not many antileishmanial drugs in the pipeline. Moreover the drug resistance of the parasite against the current available drugs is adding up to the struggle of overcoming this disease. Publication of genome of *Leishmania donovani* has opened a new avenue for drug development (*Downing et al., 2011*). One of the preliminary steps in any drug discovery is identification of novel drug target(s), followed by validation and optimization. In our study we have identified two targets *viz.* a conserved hypothetical protein and a functionally annotated protein, because multi targeting is always superior to single target. For both the proteins, we utilized the gene knockout strategy in order to predict the enormity of these proteins for the survival of *Leishmania donovani* parasite. LdBPK_070020 was the conserved hypothetical protein picked out, because this protein was conserved throughout the other species of *Leishmania* as well. Moreover sequence similarity with human genome was minimalistic. For functionally annotated protein, the prenylation pathway of the parasite was targeted and CAAX prenyl protease II was chosen as a target. This selection was made due to its lower similarity with human counterpart and its essential role in localization of various signal molecules which are switches to major cellular processes inside the cell.

**The work put forward in this thesis is divided into four experimental chapters:**

**Removal of LdBPK_070020 expression from *Leishmania donovani* by employing gene knockout strategy:** Conserved hypothetical protein LdBPK_070020 was studied as a potential target and gene knockout strategy was employed to study its importance for parasite survival. LdBPK_070020 is conserved throughout other species of *Leishmania* and its similarity to human genome is significantly low. The gene knockout strategy employed was based upon homologous recombination. Knockout cassettes were prepared followed up by sequential removal of gene copies from *Leishmania* genome. The knockout strains were confirmed by PCR and western blot. Complemented cells were also prepared by giving back the protein expression to gene knockout mutants in form of episomal expression.

**Understanding the importance of LdBPK_070020 for the parasite biology by studying the knockout mutants:** The subsequent effects, after removal of LdBPK_070020 from *Leishmania donovani* were studied by analyzing the knockout mutants using various biochemical and biophysical tools. Change in cell morphology, increased ROS levels, change in mitochondrial membrane potential and subsequent cell death via apoptosis were the events observed after complete removal of LdBPK_070020 expression. Complementation of the gene (LdBPK_070020) by episomal expression resulted in partial recovery of the effects. The data suggests that LdBPK_070020 knock out results in impaired mitochondrial function.

**Localization studies of conserved hypothetical protein LdBPK_070020 inside the parasite:** In order to identify whether the role of LdBPK_070020 was directly related to mitochondria or the protein was indirectly affecting the working of various mitochondrial proteins, we went forward with localization studies. The results illustrated that the protein was present in the nucleus and kinetoplast of the parasite. The protein is possibly altering mitochondrial function indirectly by regulating expression of other proteins. ***It is important to mention that at the time of the thesis submission LdBPK_070020 was annotated (Inferred from Electronic Annotation) as putative Isy1-like splicing family protein.***

**Deciphering the role of CAAX prenyl protease II as a target in *Leishmania donovani*:** For the second potential drug target, CAAX prenyl protease II was selected. It is a key enzyme of prenylation pathway. Gene knockout strategy was employed in this case as well to identify its importance for parasite survival. Removal of CAAX prenyl protease II expression did not kill the parasite but the cell growth rate and macrophage infectivity was significantly lower compared to the wild type. Improper localization of Ras proteins (signal proteins) and increased G1 to S phase transition in cell cycle was observed in CAAX prenyl protease II knock out parasites.

# CHAPTER II

## Removal of LdBPK_070020 expression from *Leishmania donovani* by employing gene knockout strategy*

### 2.1 ABSTRACT

The genome of *Leishmania donovani*, the causative agent of visceral leishmaniasis, codes for approximately 30% of conserved hypothetical proteins. Studies on 'conserved hypothetical' proteins are expected to reveal not only new and crucial aspects of *Leishmania* biochemistry, but it could also lead to discovery of novel drug candidates. Conserved hypothetical protein, LdBPK_070020, is a 31.14 kDa protein, encoded by an 810 bp gene. BLAST analysis of LdBPK_070020, performed against NCBI *non-redundant database*, showed 80-99% similarity with conserved hypothetical proteins of other *Leishmania sp.* Further the sequence similarity of the gene encoding for LdBPK_070020 was significantly lower, when compared to human genome. Gene knockout strategy was used for removal of LdBPK_070020 expression from parasite. There are two copies of gene encoding for LdBPK_070020 in the *Leishmania donovani* genome, which were knocked out via homologous recombination. Further complemented cells were prepared by giving back the expression of LdBPK_070020 to null mutants, in the form of episomal expression. The knockout of LdBPK_070020 was confirmed by PCR and western blot analysis.

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## 2.2 INTRODUCTION

Leishmaniasis is a neglected tropical disease (WHO). The causative agent of leishmaniasis is a protozoa belonging to genus *Leishmania,* of the order Trypanosomatida (*Desjeux, 2004; Singh et al., 2006*). The parasite has a digenetic life cycle and it is transmitted to mammalian host through vector sandfly (*Phlebotomus*). The *Leishmania sp.* exists in two forms, infective promastigote in the midgut of sandfly and amastigote stage in macrophages of mammalian host. Depending upon the clinical manifestations and the causative species, leishmaniasis is divided into three forms namely, visceral, mucocutaneous and cutaneous. Out of all the different forms of infection, visceral leishmaniasis is the most dangerous form. If left untreated, the consequences on the patient suffering from visceral leishmaniasis usually turn out to be fatal. Visceral leishmaniasis is the most prevalent form of leishmaniasis infection in India and is commonly known as Kala-azar. *Leishmania donovani* is the causative agent of visceral leishmaniasis in India (*Desjeux, 2004; Singh et al., 2006*). The disease is endemic in 88 countries of the 5 continents (Africa, Asia, Europe, North America and South America). As per the recent World Health Organization (WHO) statistics, about 350 million people are under the risk of leishmaniasis and about 12 million people are affected *(http://www.who.int, 2014).* Approximately 1.3 million new cases are reported annually including 0.20 to 0.40 million cases of visceral leishmaniasis, the most deadly form of this disease *(http://www.who.int, 2014).* Countries like India, Brazil, Sudan, Nepal and Bangladesh account for about 90% cases of visceral leishmaniasis, while in India, the state of Bihar alone accounts for 90% cases *(http://www.who.int, 2004).* However, increase in human migration across the globe has increased the risk of spreading of the disease in non endemic areas as well. The current drug scenario against this disease is overshadowed by high drug toxicity, major side effects, high cost etc. Moreover emergence of resistance among various *Leishmania sp.* towards currently available drugs, is adding on to the problems of currently available drugs. Hence the need of new drugs with high efficacy and lower side effects is now a matter of urgency. Identification of a drug target inside the parasite is one of the crucial steps in drug discovery process *(Hughes et al., 2011). Leishmania* genome codes for approximately 30% conserved hypothetical genes, which further encode for proteins with wide phyletic distribution. We have selected LdBPK_070020, a conserved hypothetical protein present in *Leishmania donovnai,* as a potential drug target. This protein is conserved

throughout the other species of *Leishmania* parasite and has significantly lower similarity with human proteome. Gene knockout strategy, based upon homologous recombination, was employed to remove the expression of LdBPK_0720020 from the parasite.

## 2.3 MATERIAL AND METHODS

### *2.3.1 Chemicals and cell lines*

Gene specific primers of LdBPK_070020 hypothetical protein, PCR clean up kit (Qiagen), Plasmid isolation kit (Sigma, USA), *Bam*HI, *Bst*EII, *Hind*III, *Sal*I and *Nhe*I (NEB, USA), Gel extraction kit (Qiagen), T4 DNA ligase (NEB), Ampicilin, Hygromycin B (Himedia), Geneticin (Gibco), Phleomycin (Sigma, USA), Genomic DNA isolation Kit (Quiagen). Protein A purified mouse-anti-hypothetical protein LdBPK_070020 antibody was custom supplied by Abgenex Pvt Ltd, India. α-tubulin antibody was purchased from ThermoFisher (catalog No. MA1-19162). The *Leishmania donovani* strain (BHU-1081) was obtained from Prof. Shyam Sunder, Banaras Hindu University, India and all the *Leishmania* expression vectors, used for preparing knockout cassettes as well as complementation studies were donated by Beverley Lab, Washington University Medical School, USA.

### *2.3.2 Parasite cell culture and maintenance*

*Leishmania donovani* cells were maintained according to the protocol already established in our laboratory *(Saudagar et al., 2013; Das et al., 2013)*. In brief, cells were grown and maintained at 25 °C in complete M199 media, which is composed of M199 liquid media (pH 7.4), supplemented with 15% fetal bovine serum (FBS), 100µg/ml penicillin and 100µg/ml streptomycin. For selection and maintenance of knockout parasites, cells were grown in complete M199 media supplemented with respective antibiotics required for the selection process, i.e., Geneticin G148 (20 µg/ml for selection and 50 µg/ml for maintenance) Hygromycin B (100µg/ml for selection and 120 µg/ml for maintenance) and Phleomycin (5 µg/ml for selection and 10 µg/ml for maintenance) as reported earlier *(Beverley and Clayton, 1993)*.

### *2.3.3 Preparation of molecular constructs for knockout as well as complementation*

The pXG series of vectors are *Leishmania* expression vectors constructed by Beverley Lab, Washington University Medical School, USA *(Beverley and Clayton, 1993; Ha et al., 1996; Flannery et al., 2011)*. The schematic representation of LdBPK_070020 locus and molecular constructs used for gene replacement by homologous recombination is shown in Figure 2.1. Based on the selection marker present in these vectors they are differentiated, for e.g. pXG B1288 (Neo), has genetecin as selection marker (Neo), pXG B3318 (Hyg) has Hygromycin as selection marker and pXG 3324 (Phleo) has phleomycin as selection marker . All these vectors also posses Tn5 *Kan* marker, for selection in *E. coli* cells. These vectors were used for the preparation of knockout cassettes as well as the complementation cassette. Two knockout cassettes were constructed, since LdBPK_070020 is diploid, i.e., two copies of this gene are present on chromosome number seven. Before the construction of the cassettes, the pXG vectors, namely, pXG 1288 (Neo) and pXG 3318 (Hyg), were modified using site directed mutagenesis, in order to disband the extra *HindIII* site present at position 1981/1985. This was done, because *HindIII* was required as a single cutter in pXG vector so that 5' UTR of LdBPK_070020, could be cloned between *HindIII* and *Sal I* site present at position 4954/4958 and 4969/4973. Primer employed for this purpose was: primer1: 5'Pho-CACACACAAAGCTGCCTTGCACACAACG-3'.

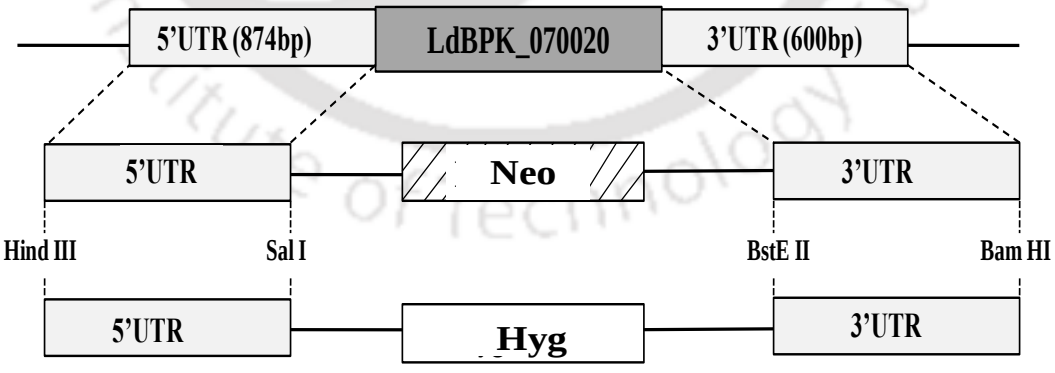


**Figure 2.1:** Schematic representation of LdBPK_070020 locus and molecular constructs used for gene replacement by homologous recombination.

Next step involved was PCR amplification of 0.874 Kbp upstream flanking region (5'UTR) and 0.6 Kbp downstream flanking region (3'UTR) from the genomic DNA of *Leishmania donovani* using primers: primer2: 5'-CCAAGCTTCGTCCTTCAGGGTTTGGTG C-3', primer3: 5'-GCGTCGACCTCGAATAACGGCCTCGCC-3',primer4: 5'-GGGTTACCG TGCTGCTGACATGGACTTG-3' and primer5: 5'–CGGATCCGAG CCAAGGTGCAAA GACG-3'. Primers 2 and 3 were used to amplify 5'UTR and primers 4 and 5 were used to amplify 3'UTR. The amplified products were further double digested: *Hind* III and *Sal* I for 5'UTR and *BstE II* and *Bam HI* for 3'UTR and were cloned on either side of pXG B1288 and pXG B3318 vectors, respectively **(Figure 2.1)**. In order to prepare complementary vector, 810 bp LdBPK_070020 gene was amplified from genomic DNA of *Leishmania donovani* and cloned in pXG 3324 (Phleo) vector using *Bam HI* and *Xma I* as restriction sites. Primers employed were: primer6: 5'- CCCCCGGGATGCAGGATAGACTAAGGG -3' and primer7: 5'-CGGGATCCTCAACTTTTCCCACGAAGCG-3'.

### *2.3.4 Generation of LdBPK_070020 knockout Leishmania donovani strains and complementation cells*

Gene knockout by homologous recombination in *Leishmania* was performed using method established by Beverley group *(Beverley and Clayton, 1993).* In brief, the knockout cassettes (3' and 5' UTR cloned B3318 or B1288 vector), were linearized by *Nhe*I digestion (single cutter). These linearized cassettes were further transfected into WT cells via electroporation *(Beverley and Clayton, 1993).*The WT cells were transfected with empty cassettes as a positive control, while just WT cells were electroporated without any vector and were used as a negative control. Approximately $10^7$cells/ml were washed twice with PBSG buffer (10mM $NaH_2PO_4$, 10mM $Na_2HPO_4$, 145mM NaCl and 2% glucose) and the pellet was further re-suspended in 360µl of electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM $Na_2HPO_4$, 6mM glucose), to which about 40 µl of ~5-10mg/ml linearized DNA was added. This solution was then transferred to ice cold sterile electroporation cuvettes and incubated on ice for about 10 minutes. The cuvettes were further placed in BioRad shockpod and conditions for electroporation were set (Exponential protocol: Voltage_450V, Capacitance_500 F and the resulting time constant should be around 4.5ms). After the electroporation the cuvettes were further kept on ice for 10 minutes and later the cells were transferred to complete M199 media. After 24 hrs of incubation at 25°C, the cells were

transferred to selection media. The selection of mutated cells was performed in such a way that, both control and test samples were grown in selection media (specific antibiotics dependent upon the selection marker present in the pXG vector).After 5 days, the control cells (WT) died in selection media, while the test survived, which were further maintained in selection media. For experimental purposes, the mutants were transferred to normal M199 complete media 24 hrs priors to the experiment, so that the growth conditions for all the test and control samples are same. After the selection and confirmation of first single knockout (SKO_HP), the cells are taken up for second round of electroporation and further selection was carried out for double knockout cells (DKO_HP). The same protocol was followed for complementation, where the DKO_HP cells were transfected with modified pXG B3324 (Phleo) vector containing LdBPK_070020 gene, in order to revert back the protein expression. The concentrations of antibiotics employed for selection purpose of both mutated and complemented cells were, 20µg/ml of G148 (Genetecin) for pXG 1288 selection, 100µg/ml Hygromycin B for pXG 3328 and 5µg/ml Phleomycin for pXG 3224 selection.

### *2.3.5 Generation of antibodies against LdBPK_070020*

Antibodies against LdBPK_070020 were prepared in collaboration with Abgenex Pvt. Ltd. (Project ID: CP-06-15). The purified recombinant LdBPK_070020 protein, obtained by bacterial expression, was injected in mice. The antibodies obtained were then affinity purified using Protein A. Indirect ELISA was further performed. The Protein A purified LdBPK_070020 antibodies (200ng) were tested against 200ng custom antigen and pre-bleed was used as a control. The results obtained were plotted in the form of a bar graph. The result of indirect ELISA are shown in **Figure 2.2** ( experiment was done by Abgenex Pvt. Ltd.)

### *2.3.6 Confirmation of knockout by employing PCR*

The knockout of LdBPK_070020 gene was confirmed by PCR. Genomic DNA of single (SKO_HP) and double (DKO_HP) knockout cells was isolated. These genomic DNAs were further used as templates to carryout PCR. Following sets of primers were used for the PCR reactions: primer8: 5’-GCGCTTCTGCATGTGCCTCTTATCCC-3’, primer9: 5’-CGCGCCGTGTCTCTATGGATGCCG-3’, primer10: 5’-CGCCGCCTCCTTTCACCCGTCATAG-3’, primer 11: 5’-CGCCTCCGCCTCACGCTCAAACCGAAC-3’, primer12: 5’-GC

CGGTGATGCAGGCCTGG-3', primer13: 5'-GGAGGGAGGAATGAGGTGAGC-3', primer14: 5'-GACGCCCTCCTCCTCCCTC-3', primer 15: 5'-CCGCCTCACGCTCAAA CCGAAC-3'. The genomic DNA of WT cells was used as control.

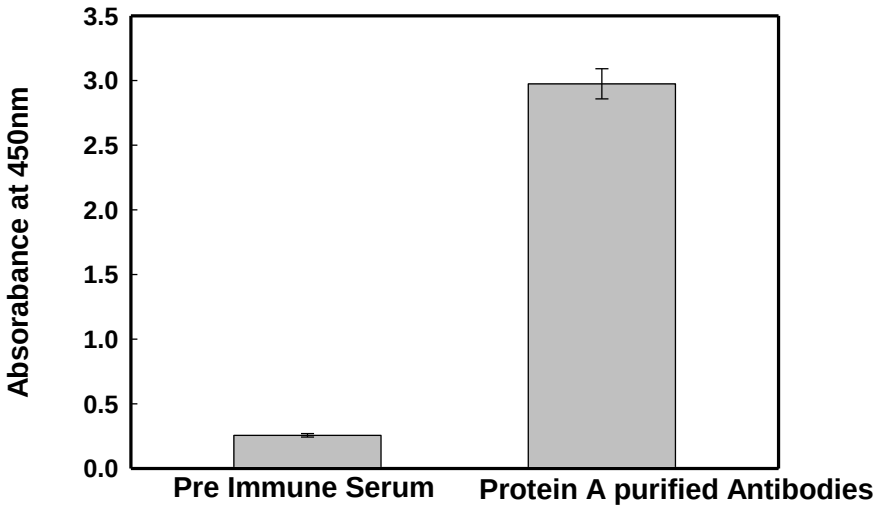


**Figure 2.2: Indiect ELISA:** Protein A purified antibodies (200ng) tested against 200ng of custom antigen, and reading was taken at 450nm. Pre immune sera were used as a control in place of primary antibodies at 1:1000 dilutions. Plate was read after 3 min of enzyme reaction.

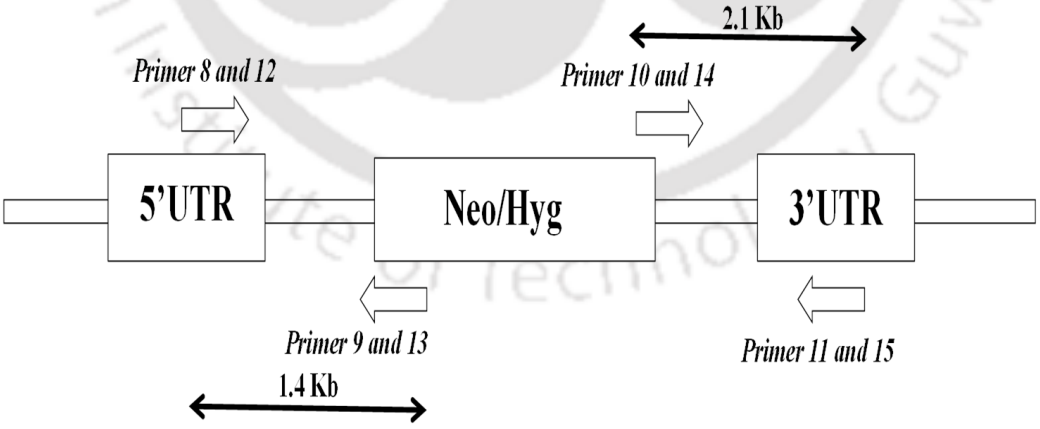


**Figure 2.3:** Diagrammatic representation of the primers employed in PCR confirmation of knockout of LdBPK_070020 gene. Primer set 8 and 9 amplify 1.4 Kb fragment and primer set 10 and 11 amplify 2.1 Kb fragment of Neo replaced region, while primer set 12 and 13 and primer set 14 and 15 amplify 1.4Kb and 2.1 Kb fragment of Hyg replaced region.

The primers were designed in such a way that they covered some part of the UTRs and some region of the antibiotic resistance marker gene which has replaced the original LdBPK_070020 gene. Primer set 8 and 9 amplified 1.4 Kbp region of 5'UTR and Neo gene, while primer set 10 and 11 covered region of 3'UTR and Neo gene. Set 12 and 13 covered 1.4 Kbp region of 5'UTR and Hyg gene and set 14 and 15 covered 2.1 Kbp region of 3'UTR and Hyg gene. The diagrammatic representation of primer sets and the regions which will be amplified using them is given in **Figure 2.3**.

### *2.3.7 Confirmation of knockout by Western blotting*

For western blot studies, wild type (WT) *Leishmania* promastigotes, SKO_HP promastigotes, DKO_HP promastigotes and CKO_HP promastigotes were harvested and washed three times with cold PBS (pH-7.5). Cell lysis was performed by dissolving the cells in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1mM DTT, 15 µl/ml protease cocktail inhibitor, pH 7.4) followed by sonication for 3 min ( Pulse On: 2 sec, Pulse Off: 5 sec). For the removal of cell debris, the lysed cells were centrifuged at 10,000 rpm for 45 min at 4 $^0$C. Supernatant was collected, followed by total protein estimation by Bradford method *(Bradford, 1976)*. Equal amount of protein (250µg), from the collected supernatant of all the four populations of *Leishmania* promastigotes, was separated on SDS PAGE gel (12 % running and 5 % stacking). Prestained dye was used as a marker. Based upon the separation, the gel was cut into two parts and transferred on polyvinylidene fluoride membrane. After transfer, blocking step was performed where the membrane was incubated with 5% skimmed milk solution, overnight. This was followed by overnight incubation of membrane with antibodies against mouse anti-α-tubulin (1:5000) (Thermo Scientific) and mouse anti-hypothetical protein LdBPK_070020 (1:500) at 4 $^0$C. The membrane was further incubated with anti-mouse horse radish peroxidase conjugated secondary antibodies (1:1000) (Thermo Scientific). For immunodetection, the membrane was treated with chromogen, 3,3'-diaminobenzidine (DAB) followed by addition of 1% $H_2O_2$ *(Krajewski et al., 1996)*. Here, α-tubulin was taken as endogenous control *(Mukherjee et al., 2012).*

## 2.4 RESULTS

### *2.4.1 Knockout cassettes and complementation vector were successfully prepared*

Two copies of LdBPK_070020 gene are present in *Leishmania* genome hence two vectors (pXG B1288 and pXG B3318) were employed for replacement of both copies. The 5'UTR (600bp) and 3'UTR (874 bp) of LdBPK_070020, were respectively cloned in pXG 1288 and pXG 3318 vectors **(Figure 2.4 A and Figure 2.4 B)**. The proper insertion of 5' UTR in both the vectors was confirmed by double digestion with *Bst*EII and *Bam*HI, which showed a release of 600bp. Further the proper insertion of 3'UTR in both the vectors was confirmed by double digesting the vectors with *Hin*dII and *Sal*I which showed a release of 874 bp **(Figure 2.4 C and Figure 2.4 D)**. For complementation studies, pXG Phleo vector was modified by cloning LdBPK_070020 gene (810bp) in it. Double digestion of clone with *Bam*HI and *Xma*I showed a release of 810 bp confirming the insertion of LdBPK_070020 gene in pXG Phleo vector **(Figure 2.4 E)**. The generated knockout cassettes and complementation vectors were further confirmed by nucleotide sequencing.

### *2.4.2 Knockout of Leishmania cells was successfully confirmed by PCR*

After the preparation of knockout cassettes (3' and 5' UTR cloned 3318 or 1288 vector), the vectors were linearized and transfected into wild type cells (WT). After the selection of single knockout cells (SKO_HP), second round of electroporation and selection was carried out for double knockout cells (DKO_HP). For complemented cells (CKO_HP), the modified complemented pXG Phleo vector was transfected in DKO_HP cells. SKO_HP and DKO_HP cells were confirmed by isolating genomic DNA of the respective cells and performing PCR. The primers were designed in such a way that they covered some region of the UTRs and some region of the antibiotic resistance marker gene which replaced the original LdBPK_070020 gene **(Figure 2.5 A)**. A band of 1.4Kb (5' UTR region and Neo/ Hyg gene) and 2.1 Kb (3' UTR region and Neo/ Hyg gene) was obtained for SKO_HP and DKO_HP cells, while no band was obtained for genomic DNA isolated from WT **(Figure 2.5 B)**. Hence from the PCR results obtained, it can be inferred that, both the copies of LdBPK_070020 gene was successfully knocked out from *Leishmania donovani* genome.

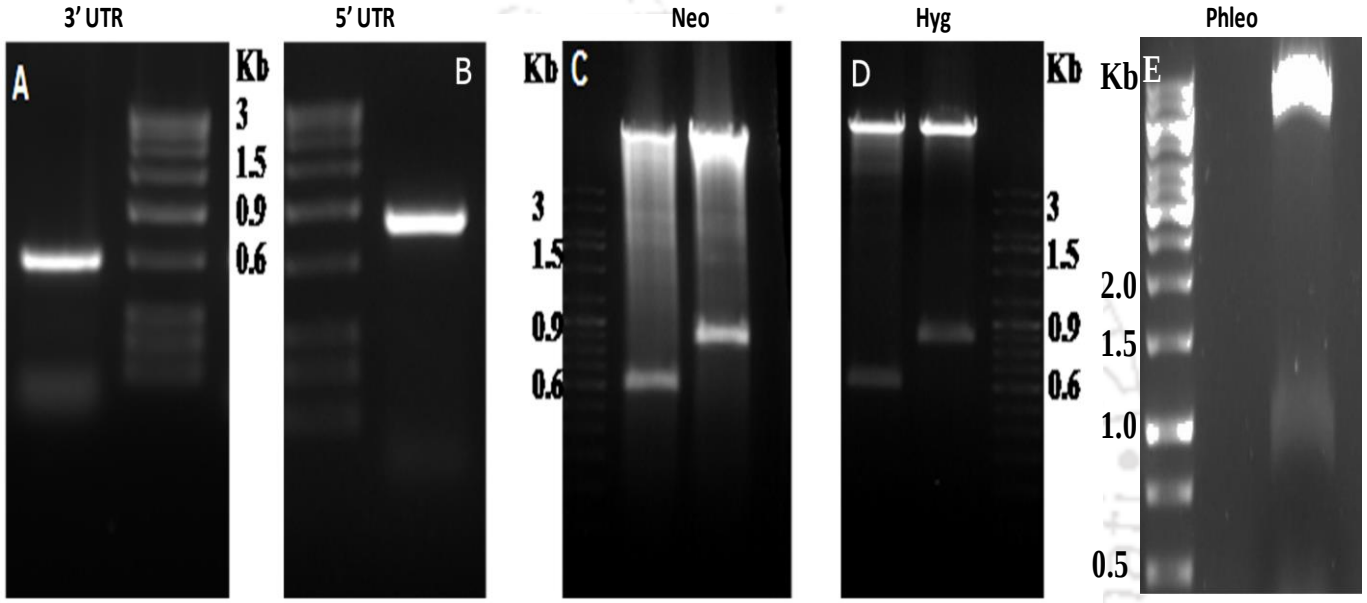


**Figure 2.4: Generation of knockout cassettes and complementation vector: A)** PCR amplification of 5'UTR (600bp) from LD1801 genomic DNA obtained at Tm 65°C **B)** PCR amplification of 3'UTR (874bp) from LD1801 genomic DNA obtained at Tm 62°C. **C)** Confirmation of 3'UTR (*Bam*HI and *Bst*EII) and 5' UTR (*Hind*III and *Sal*I), cloned in vector pXG1288, by double digestion. **D)** Confirmation of 3'UTR (*Bam*HI and *Bst*EII) and 5' UTR (*Hind*III and *Sal*I), cloned in vector pXG3188, by double digestion. **E)** Confirmation of LdBPK_070020 gene cloned in pXG phleo vector by double digestion (*Bam*HI and *Xma*I). The results were further confirmed by sequencing results.

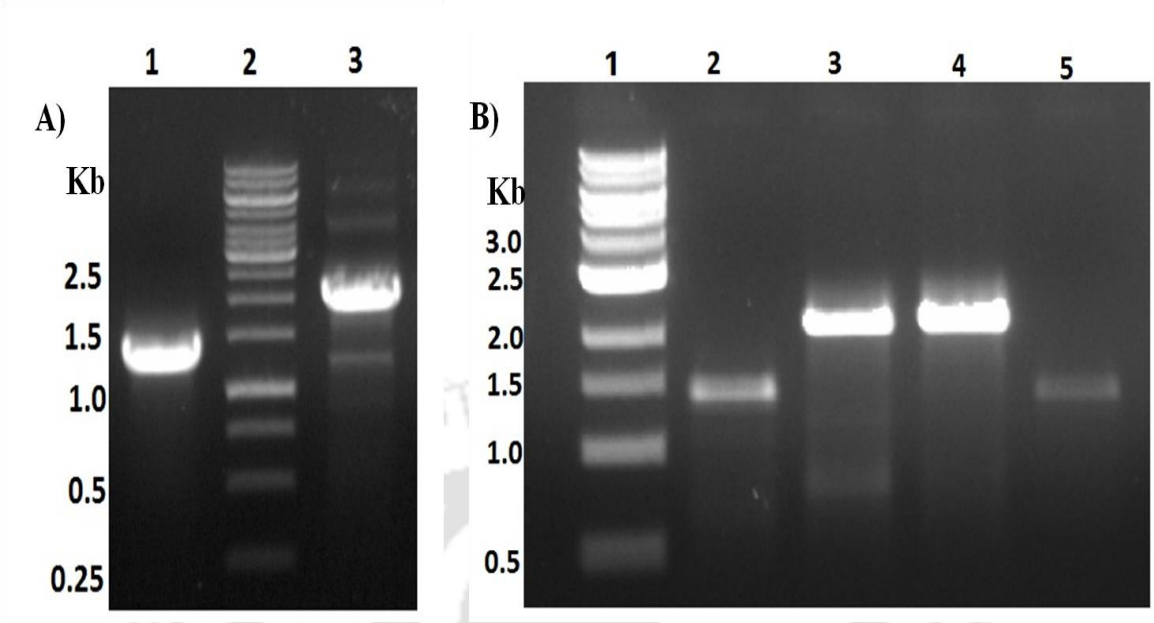


**Figure 2.5: Knockout confirmation by PCR: A)** PCR confirmation of SKO_HP: Genomic DNA of SKO_HP is used as DNA template. Lane 1 represents 1.4 Kb (5'UTR+Neo) amplified product of PCR employing primer set 8 and 12 and lane 3 represents 2.1 Kb (3'UTR + Neo) amplified product of PCR employing primer set 10 and 14. Lane 2 is 1Kb DNA ladder. **B)** PCR confirmation of DKO_HP: lane 1 represents 1Kb molecular marker, while lane 2 and 5 represent amplified product from genomic DNA of DKO_HP, using primer set 8 and 9 and set 12 and 13, which amplify 1.4 Kb region of 5'UTR+Neo and 5'UTR+ Hyg, respectively. While lane 3 and 4 represent 2.1Kb amplified product from DKO_HP genomic DNA using primer set 10 and 11 and set 14 and 15, which encompasses the 3'UTR + Neo region and 3'UTR + Hyg region, respectively.

### *2.4.3 Removal of LdBPK_070020 was successfully confirmed by western blot studies*

Western blot was performed using equal amount of cell lysates of all the different populations of cells (wild type, SKO_HP, DKO_HP and CKO_HP). Antibodies raised against LdBPK_070020 were used as test, while alpha tubulin was employed as an endogenous control. No protein band was observed in case of DKO_HP lane, but bands were observed in WT, SKO_HP and CKO_HP lane **(Figure 2.6)**. Though the band intensity was highest in case of WT, while lower in SKO_HP and lowest in CKO_HP. The results hint towards the low episomal expression of protein in CKO_HP cells.

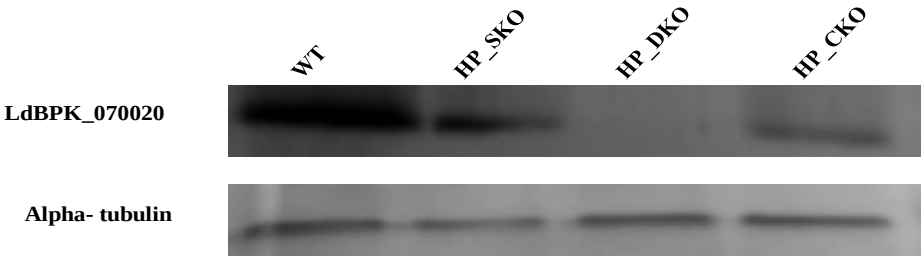


**Figure 2.6: Western blot results are represented:** Equal amount of proteins (250µg) from WT, SKO_HP, DKO_HP and CKO_HP lysates were used for the blotting experiment. Mouse anti-LdBPK_070020 antibodies were used for immunodetection. Alpha tubulin was used as an endogenous control. After the SDS_PAGE, gel was cut into two parts, one with LdBPK_070020 (31.1 kDa) and other with α-tubulin (49.7 kDa). These two gel parts were transferred on different blots for respective antibody treatment *i.e.* anti-LdBPK_070020 or α-tubulin.

## 2.5 DISCUSSION

*Leishmania donovani* does not have a strict chromosome ploidy and aneuploidy or tetraploidy is a common occurrence in the chromosomes. In case of chromosome number 7 of *L. donovani*, which contains the gene LdBPK_070020, the ploidy of the chromosome is diploid. Since two copies of gene were present, therefore two knockout cassettes were required for the complete knockout of LdBPK_070020. The removal of LdBPK_070020 from *Leishmania donovani* was successful by employing gene knockout strategy based upon homologous recombination. Replacement of both the copies of LdBPK_070020 with antibiotic resistance marker genes (Neomycin and Hygromycin) was confirmed by PCR studies. Respective amplifications, indicating the exchange of genes were found in case of SKO_HP and DKO_HP genomic DNA templates. But no bands were observed when WT genomic DNA was used as a template. Western blotting was performed to further confirm the removal of LdBPK_070020 expression from *Leishmania donovani* parasite. No protein band in case of DKO_HP clearly indicated the removal of LdBPK_070020 expression. But interestingly, in case of CKO_HP, the protein band observed was very faint compared to WT. Earlier studies report that the expression of integrated gene is higher than gene expressed via an expression vector, in which the expression is episomal *(Taheri et al., 2014).* The CKO_HP cells suffer from mosaicism i.e., each cell will have varied protein expression levels due to random segregation of complementation vectors during cell division

# CHAPTER III

## Understanding the Importance of LdBPK_070020 for the Parasite Biology by Studying the Knockout Mutants *

### 3.1 ABSTRACT

The currently available drugs against leishmaniasis have limitations. There is an urgent need for discovery of novel drug targets for better and more effective drugs. We have taken up studies on conserved hypothetical protein to get insight into its function as well as its role in the parasite growth and infectivity. *Leishmania donovani* genome codes for approximately 30% genes as conserved hypothetical proteins. We focused our studies towards conserved hypothetical protein, LdBPK_070020, which is a 31.14 kDa protein, encoded by an 810 bp gene. BLAST analysis of LdBPK_070020, performed against NCBI non-redundant database showed 80-99% similarity with conserved hypothetical proteins of *Leishmania* belonging to other species. The sequence similarity of LdBPK_070020 gene with human genome was significantly low. Further, BLAST analysis also revealed a 30-42% similarity with pre-mRNA splicing factor ISY1 of other organisms. To get insight into functional role of LdBPK_070020, we compared the changes in growth and other cellular characteristics after removal of LdBPK-070020 expression from *Leishmania donovani*. The results have indicated towards important role of LdBPK_070020 in the parasite survival and growth.

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## 3.2 INTRODUCTION

Neglected tropical diseases are responsible for affecting almost one sixth population of the world every year with a high death rate. Leishmaniasis, a parasitic disease, is also characterized among neglected tropical diseases. The disease is endemic in 88 countries of the 5 continents (Africa, Asia, Europe, North America and South America). As per World Health Organization (WHO) statistics, about 350 million people are under the risk of leishmaniasis and about 12 million people are affected *(WHO, 2012).* The causative agent of leishmaniasis is a protozoa belonging to genus *Leishmania*, of the order Trypanosomatida (*Desjeux, 2004; Singh et al., 2006*). The parasite has digenetic life cycle i.e., in addition to the primary host, the parasite requires an additional secondary host for perpetuating its existence. The *Leishmania* sp. exists in two forms, infective promastigote type in the midget of sandfly (phlebotominae) and amastigote type in the macrophages of mammalian host *(Sharma and Singh, 2008; Shukla et al., 2010)*. Depending upon the causative species of *Leishmania* and the clinical manifestation of the infection, the disease is broadly segregated into three types, namely, visceral, mucocutaneous and cutaneous. Out of all the different forms of the disease, visceral leishmaniasis, commonly known as Kala-azar in India, is the most fatal and severe form and is caused by *Leishmania donovani* and *Leishmania infantum (Desjeux, 2004; Singh et al., 2006*). Countries like India, Brazil, Sudan, Nepal and Bangladesh account for about 90% cases of visceral leishmaniasis, while in India, Bihar is the state that alone accounts for 90% cases *(WHO, 2012)*. However, increase in human migration across the globe has increased the risk of diffusion of the disease in non endemic areas as well. The available drugs against leishmaniasis have several limitations in terms of side effects, drug resistance, etc. *(Shukla et al., 2011; Rijal et al., 2013; Das et al., 2013)*. Currently there are not many antileishmanial drugs in the pipeline and moreover the growing resistance of the parasite for the current available drugs is adding up to the struggle of overcoming this disease. Novel drugs against the disease, utilizing new targets, are desired to surmount the issue related to drug resistance and other shortcomings. Genome publication of *Leishmania donovani* has opened a new avenue for drug development *(Downing et al., 2011).* The genome analysis shows considerable number of genes coding for ‘conserved

hypothetical' proteins that are functionally not characterized *(Downing et al., 2011)*. The experimental characterization of these 'conserved hypothetical' proteins is expected to provide fundamental insight into the *Leishmania* biology.

Studies on these 'conserved hypothetical' protein could also lead to discovery of novel drug candidates. LdBPK_070020 is a 31.1 kDa protein, expressed by an 810 bp gene present on the seventh chromosome of the *Leishmania* genome. In case of chromosome number 7 of *L. donovani*, which contains the gene LdBPK_070020, the ploidy of the chromosome is diploid. BLAST analysis of LdBPK_070020 was performed against the *non-redundant database* maintained by NCBI. Apart from showing almost 80–99% similarity with conserved hypothetical proteins belonging to other *Leishmania* species, the protein also shows 30–42% similarity with pre-mRNA splicing factor ISY1 of other organisms including human. Most of the genes of *Leishmania donovani* or any other Kinetoplastids do not have introns. But sequences of 39 bp mini-exon gene, highly conserved in Kinetoplastids, are reported, which act as spliced leaders and are important for the maturation of pre-mRNA by the process of trans-splicing. This further is responsible for differential protein expression in the parasite *(Fernandes et al., 2001; Thomas et al., 2005)*. Though we could not study this aspect, but role of LdBPK_070020 in pre-mRNA maturation by trans-splicing of mini-exon cannot be ruled out. The current research was undertaken to explore the role of LdBPK_070020 in parasite survival, using gene knockout, and elucidate its significance for *Leishmania donovani*. A series of experiments, on knocked out strain, were done and both phenotypic and biochemical changes were compared with the wild type strain. Our results showed that LdBPK_070020 plays an important role in the survival of the pathogen, hence insinuating it towards a potential drug target.

## 3. 3 MATERIAL AND METHODS

### *3.3.1 Chemicals and cell lines*

$H_2$DCFDA dye, Annexin V-FITC apoptosis detection kit and ATP estimation kit (Life Technologies), Mitocapure Dye (Calbiochem), FURA-2AM (Sigma, USA), DPH powder and Tetrahydrofuran (Himedia). The *Leishmania donovani* strain (BHU-1081) was obtained from Prof. Shyam Sunder, Banaras Hindu University, India.

### *3.3.2 Parasite cell culture and maintenance*

The procedure for parasite culture maintenance was followed as per reported in our publications *(Saudagar et al., 2013; Saudagar, 2014; Das et al., 2013)*. Parasites were grown and maintained in M199 liquid media (pH 7.4); supplemented with 15% heat inactivated fetal bovine serum (FBS) and 100µg/ml penicillin and 100µg/ml streptomycin. While single knockout (SKO_HP) cells were maintained in complete M199 media supplemented with 50µg/ml Geneticin G148 and double knockout (DKO_HP) cells were maintained in 50µg/ml Geneticin G148 and 120µg/ml Hygromycin B supplemented complete M199 media (Beverley et al., 1993). Complemented (CKO_HP) cells were maintained in complete M199 media supplemented with 50µg/ml Geneticin G148, 120µg/ml Hygromycin B and 10 µg/ml Phleomycin. The parasite is continually regenerated in fresh media. All data were collected after transferring the cells from media containing different selection antibiotics to normal complete M199 media. This transfer was done to provide consistent environment for all the different populations of cells and also to prevent any discrepancy in the data.

### *3.3.3 Comparative study of the growth rate and outer morphology of WT with SKO_HP, DKO_HP and CKO_HP*

The growth curves of different parasite cells (WT, HP_SKO, HP_DKO and CKO_HP) were prepared in order to see whether the knockout of LdBPK-070020 is having any effect on the proliferation and growth of the cells *(Verma et al., 2011)*. In brief, the mutant and complemented cells, after selection in respective selection media, were transferred to normal M199 complete media, 24 h prior to the experiment. After 24 h, equal number of cells ($\sim 10^5$ cells/ml), from all the four populations, i.e., WT (normal LD_BHU 1081 strain), SKO_HP, DKO_HP and CKO_HP, were inoculated in 10 ml of M199 complete media (in duplicates). Counting of the cells was done under a compound microscope (Motic® AE31), using a hemocytometer, once a day, at a fixed time. This protocol was followed for all the four population of the cells and then the data was plotted (No. of cells v/s No. of days) in order to obtain the growth curve. Further to observe any change in the morphology of knocked out cells, FESEM images were taken. The sample preparation for the four populations of cells involved fixing of $\sim 10^6$ cells/ml with 2.5% glutaraldehyde and placing

around 10 µl of these cells on aluminium foil covered glass piece followed by overnight drying in a vacuum desiccators *(Lam et al., 2002)*. The samples were then subjected to double gold coating before mounting them for FESEM imaging.

### *3.3.4 Analysis of reactive oxygen species generated in knockout cells*

ROS generation analysis was done using procedure reported in our earlier publications *(Shukla et al., 2011; Das et al., 2013; Saudagar et al., 2013; Saudagar and Dubey, 2014)*. In brief, ~$10^7$ cells/ml (Control [−], Control [+], HP_SKO, HP_DKO, HP_DKO + NAC and CKO_HP) were washed twice with filtered PBS (pH7.5) and re-suspended in about 200 µl of M199 media. For positive control, cells (~$10^7$ cells/ml) were treated with 100 µM $H_2O_2$ *(Armstrong et al., 2002)* and incubated for 30 min, while for HP_DKO + NAC, HP_DKO cells (~$10^7$ cells/ml) were incubated for 45 min in 20 mM of *N*-acetylcysteine (NAC) at 25 °C. This was followed by PBS wash, after which the cells were incubated with 10 µM of CM-$H_2$DCFDA (a cell permeable fluorescent probe) for 30–45 min at 25 °C in the dark. The fluorescence was measured using BD FACSCalibur flow cytometer and data was analyzed using CellQuestPro software. In order to measure the fluorescence, the cells were excited at a range of 492–495 nm, and the emission (517–527 nm) was collected by optical detector FL1, which has a 525 nm band pass filter. The data was then represented in the form of overlapping histograms.

### *3.3.5 Studying the change in mitochondrial membrane potential ($\Delta\psi_m$)*

Next step was to determine the change in mitochondrial membrane potential of all the four populations. The mitochondrial membrane potential was assessed by using MitoCapture™ apoptosis detection kit (Calbiochem). MitoCapture™ is a cationic dye which aggregates in mitochondria, with unaltered potential, hence divulging red fluorescence, but in cells with altered $\Delta\psi_m$ the dye accumulates in the cytosol hence divulging green fluorescence *(Takai et al., 2011; Saudagar et al., 2013)*. The sample preparation of WT, HP_SKO, HP_DKO and HP_CKO cells was followed as per the instructions provided in the kit (using MitoCapture™ apoptosis detection kit, Calbiochem). In brief ~$10^7$ cells were washed with PBS (pH 7.5) twice and then re-suspended in 1 ml incubation buffer containing MitoCapture™ reagent (dilution of the reagent was done as per the instruction in the kit). After incubating the cells

at 37 °C, for 30 min in the dark, the cells were washed again, and data was collected using FACSCalibur flow cytometer and analyzed using CellQuestPro software.

### *3.3.6 Analysis of apoptosis of knockout cells*

Apoptosis of HP_DKO cells was analyzed, as reported in our earlier publications *(Das et al., 2013; Saudagar et al., 2013)*, using Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit from Life Technologies. A concentration of $\sim 10^7$ cells/ml (Control [−], Control [+], HP_SKO, HP_DKO, HP_DKO + NAC and CKO_HP), were washed twice with PBS (pH7.5). For positive control WT cells were treated with 50 μM Miltifosine, with an incubation period of 12 h and for pre NAC treatment HP_DKO cells, HP_DKO cells were treated with 20 μM NAC, for 45 min at 25 °C, prior to staining of cells. After washing, the cells were stained with Annexin V-FITC and propidium iodide (PI) according to the instructions given by the manufacturer. The fluorescence was further detected and analyzed in FACSCaliber flow cytometer and CellQuestPro software, respectively. Moreover, uniform gating was done to fix the population of cells, to be analyzed.

### *3.3.7 Real Time PCR*

The variation in expression levels of various genes involved in maintaining redox homeostasis **(Table 3.1)** as well as genes responsible for the expression of various proteases **(Table 3.2)** inside the *Leishmania* parasite was determined by quantifying its expression levels using Real Time (Quantitative) PCR. The foremost step involved was extraction of total RNA, using RNeasy Mini kit (Qiagen), of all the four populations of promastigotes i.e. WT, HP_SKO, HP_DKO and HP_CKO. After which RNA quantification was done using nanodrop (Eppendorf), so that equal concentration of RNA is used for the cDNA preparation using AMV First Strand cDNA synthesis kit (NEB) *(Tavares et al., 2011)*. After obtaining cDNA, it was subjected to Real Time PCR (7500 Real Time PCR System Applied Biosystems), where the cDNA template was amplified and simultaneously quantified using Power SYBR® Green PCR Master mix *(Carter et al., 2006)*.

### *3.3.8 Quantifying the cellular ATP content*

Comparative study of cytosolic ATP content was done using ATP estimation kit (Life Technologies), which followed luciferin - luciferase bioluminescence based assay. In brief, ~$10^6$ cells (WT, HP_SKO, HP_DKO and CKO_HP) were mixed with standard reaction solution, containing DTT (1 mM), d-luciferase (0.5 mM) and firefly luciferase (1.25 µg/ml). A standard curve of ATP was prepared by using varying ATP concentrations (0–50 pM), and the ATP concentrations of different cell types was then measured by comparing luminescence intensities obtained from these samples with that plotted on the standard curve *(Mukherjee et al., 2002; Verma et al., 2011; Mukherjee et al., 2012).* The luminescence intensities for both the standard and sample were measured in BioTek® Synergy HT/FLx800 96-well plate reader.

### *3.3.9 Intracellular $Ca^{2+}$ estimation*

In order to estimate the intercellular $Ca^{2+}$ concentration, a fluorescent probe, FURA 2AM, was used *(Das et al., 2013; Gupta et al., 2006).* Briefly, ~$10^7$ cells/ml (WT, HP_SKO, HP_DKO and CKO_HP) were washed twice (1000 g for 5 min) with wash buffer (5.5 mM glucose, 116 mM NaCl, 0.8 mM $MgCl_2$, 5.4 mM KCl, and 50 mM MOPS, pH 7.4) and then incubated for 6 h at 25 °C, in the same buffer containing 8 µM FURA 2AM and 15% sucrose. After incubation, the cells were again washed twice with the wash buffer and resuspended in the same buffer. This was followed by fluorescence measurement, with emission at 340 nm and excitation at510 nm.

### *3.3.10 Measurement of membrane fluidity*

Change in membrane fluidity was measured by measuring the change in fluorescence anisotropy (FA) using Diphenylhexatriene (DPH) as a fluorescent probe, which binds to the membrane *(Ghosh et al., 1990; Chakraborty et al., 2005).* FA is indirectly proportional to membrane fluidity hence determining the FA of all the four populations of cells will help determining the membrane fluidity. Concisely, a stock solution of DPH (2 mM) was prepared in tetrahydrofuran. For working aqueous solution, DPH from the stock is mixed to the rapidly stirring PBS (pH 7.2), such that the final concentration of DPH becomes 2 µM. For labeling, approximately $10^7$ cells/ml (WT, HP_SKO, HP_DKO and CKO_HP) were washed twice and

dissolved in PBS (pH 7.2) and mixed with aqueous working solution of DPH in 1:1 ratio. The cells were further incubated at 37 °C for 2 h and later washed thrice with PBS followed by measurement of fluorescence anisotropy (FA). For the measurement of fluorescence, sample was excited at 365 nm and the emission was recorded at 430 nm. FA was computed using the following equation: $FA = [(I_{//} - I_{\perp})/(I_{//} + 2I_{\perp})]$, where $I_{//}$ and $I_{\perp}$ are fluorescence intensities, oriented, respectively, parallel and perpendicular to the direction of the excited light *(Shinitzky and Berhenholtz., 1974)*. The microviscosity parameter was calculated using the following equation: microviscosity = $[(r_0/r) - 1]^{-1}$, here r is the FA of the sample while $r_0$ is the maximal limiting FA value observed for DPH, which is 0.362 *(Ghosh et al., 1990)*.

**Table 3.1:** The following table enlists genes responsible for the expression of various enzymes involved in the Try S and Try R redox metabolism of *Leishmania donovani.* Primers were designed for these genes, which were used for measuring their expression levels by performing Real Time PCR.

| S. no. | Abbreviation | Gene ID | Name | Primer Set | |
|---|---|---|---|---|---|
| 1 | ThioR | LdBPK_010270 | Thioredoxin putative | ThioR-F | GTGGACGCAGACAACAACAC |
| | | | | ThioR-R | CGCCTCCTCTCGTATCTTTG |
| 2 | Sper S | LdBPK_040570 | Spermidine synthase putative | Sper S-F | CGGACTACGACGAGTTTGTG |
| | | | | Sper S-R | TCTTGCTCTGCTGAATCACG |
| 3 | TDR | LdBPK_141580 | Thiol-dependent rductase 1 | TDR-F | CGTAGAGGTGGAGCCAATGT |
| | | | | TDR-R | GACAAAGCGGACGAGAGAAC |
| 4 | TPP | LdBPK_151100 | Tryparedoxin peroxidase | TPP-F | CCGACAAGACCAAGAGCATC |
| | | | | TPP-R | ACGAACTGAAAAGCCTCCAG |
| 5 | GGCS | LdBPK_181660 | γ-Glutamylcysteine synthetase | GGCS-F | AACACGGTTACCCTCAGCAC |
| | | | | GGCS-R | AAAGTCACAAAGGTGGTGCC |
| 6 | GlutR | LdBPK_201020 | Glutaredoxin putative | GlutR-F | CTCATCTCCGCCACCTACTG |
| | | | | GlutR-R | GTCGTATCCGCCAAGGTACT |
| 7 | GSS | LdBPK_252500 | Glutathionyl spermidine synthase putative | GSS-F | TAGTTGACAGTGACGGCGAC |
| | | | | GSS-R | ATGTCAGAGAGCGTCGGACT |
| 8 | TR | LdBPK_291240 | Tryparedoxin | TR-F | TCCCTTACTCCACCAGCTTC |
| | | | | TR-R | CTCCTCGTCCCAAGAGATCA |
| 9 | GIT | LdBPK_353060 | Glyoxalase I trypanothione-dependent glyoxalase I | GIT-F | CATATTGCTATCGGGGTGGA |
| | | | | GIT-R | GTGCCCTGCTCCTTCATATC |
| 10 | GltP | LdBPK_363160 | Glutathione peroxidase putative | GltP-F | CTATGCGACGCTTATCGTGA |
| | | | | GltP-R | CACCCGTAGCATCTGAAACA |
| 11 | DHFR | LdBPK_060890 | Dihydrofolate reductase-thymidylate | DHFR-F | CCTTTCAACATCGCCTCCTA |
| | | | | DHFR-R | AACTGACGCTCCTCCTTGAA |

**Table 3.2:** List of proteases whose under/ over expression levels were quantified using Real Time PCR and further comparative studies were done between the obtained results of WT, HP_SKO, HP_DKO and HP_CKO.

| S. no. | Abbreviation | Gene ID | Name | Primer Set | |
|---|---|---|---|---|---|
| 1 | SerP | LdBPK_120920 | Serine peptidase, putative | SerP-F | GATCGATCACTCCTACACTCTGC |
| | | | | SerP-R | GTTACGACACGCTCTCGAAGTATC |
| 2 | ZnMP | LdBPK_341130 | Mitochondrial ATP-dependent zinc metallopeptidase, putative | ZnMP1-F | GATCCAGTTTAGCACCTACTACCC |
| | | | | ZnMP1-R | GAACGTAAACCGACTCTTCTCC |
| 3 | MAP2 | LdBPK_210960 | Methionine aminopeptidase 2, putative | MAP2-F | CACCTCATGAACCTGAAC |
| | | | | MAP2-R | CGAGGTAGATCGTGTGTT |
| 4 | ZnMP2 | LdBPK_191620 | ATP-dependent zinc metallopeptidase, putative | ZnMP2-F | GCTACGAACTTTGTGGAC |
| | | | | ZnMP2-R | CGGCTAAGATAGTGGTTG |
| 5 | MAP | LdBPK_190540 | Methionine aminopeptidase, putative | MAP1-F | CTGTGCCAAAGGAGATAG |
| | | | | MAP1-R | GGCGTTGTTGTAGTCTTC |
| 6 | Mcas | LdBPK_351580 | Metacaspase, putative | MCas-F | TCGACCTGTACAAGCCCTTC |
| | | | | MCas-R | CGGTACGTGGACTGGGTAAC |
| 7 | UbH | LdBPK_310150 | Ubiquitin hydrolase, putative | UbH-F | GAGAGCGGCTACTATGACCTGT |
| | | | | UbH-R | GCCACTTGTCTGCTTTCTTACC |
| 8 | CCP | LdBPK_040430 | Calpain-like cysteine peptidase, putative | CCP-F | CGATCTACTACGTCAACGACTACG |
| | | | | CCP-R | GACGATGTTATCACCGATCTCC |
| 9 | CPA | LdBPK_191460 | Cysteine peptidase A | CPA-F | GCAGACAGCCTACTTCCTCAAT |
| | | | | CPA-R | CGTAGTAGTTGGGGTTCAGGTACA |
| 10 | CPB | LdBPK_070600 | Cysteine peptidase B | CPB -F | CCCTCTTATAGACGCACTTACCAG |
| | | | | CPB -R | GGACACACTCCTCGTTGATGAT |
| 11 | CPC | LdBPK_290860 | Cysteine peptidase C | CPC-F | GGCTACAAGAGTGGAGTGTACAAG |
| | | | | CPC-R | GGATCAGGAAGTAGCCTTTGTC |
| 12 | DHFR | LdBPK_060890 | Dihydrofolate reductase-thymidylate | DHFR-F | CCTTTCAACATCGCCTCCTA |
| | | | | DHFR-R | AACTGACGCTCCTCCTTGAA |

### *3.3.11 Statistical analysis*

Student's unpaired *t*-test was performed using SigmaPlot software. The test was employed to determine the statistical significance of differences between two groups. Differences with p values of <0.05 and < 0.005 were considered significant.

## 3.2 RESULTS

### *3.4.1 LdBPK_070020 is essential for the progression of the parasite under in vitro conditions*

In order to investigate the role of LdBPK_070020 for the subsistence of the parasite, the gene was knocked out by homologous recombination and the knockout was confirmed by PCR and western blot. After obtaining the knockouts, through selection in antibiotic containing selection media and confirmation by PCR and western blotting, it was observed that compared to the WT and SKO_HP cells, DKO_HP cells survived only for 3–4 weeks, in normal M199 complete media, after which cell death occurred. A comparative study of the growth curves of all the three populations indicated, that compared to WT and SKO_HP cells, DKO_HP cells grew at a slow rate before diminishing, while the growth rate of SKO_HP cells was slightly less than WT cells but better than HP_DKO cells **(Figure 3.1 B)**. For reverting back the expression of LdBPK_070020, modified *Leishmania* expression vector pXG 3324 (Phleo), which contained LdBPK_070020 gene, was introduced in DKO_HP cells (in the 2nd week) for the episomal expression of the conserved hypothetical protein LdBPK_070020. It was observed that the growth rate of complemented double knockout cells (CKO_HP) was slow and almost similar to HP_DKO cells, but unlike DKO_HP cells which die after almost 3–4 weeks, HP_CKO cells had persistent but sluggish growth. A stark change in the morphology of the knockout parasite was observed compared to the WT cells. Usually, a body of a WT cell is elongated and tapered followed by long flagella as shown in **Figure 3.1 A1**. But a change in the shape and flagellar length of the parasite was observed, after removal of LdBPK_070020 expression. Compared to WT cells, DKO_HP cells had a more globular body and smaller flagella **(Figure 3.1 A3)**. While the bodies of SKO_HP and CKO_HP cells were less globular compared to DKO_HP cells, but still CKO_HP cells had shorter flagella like DKO_HP cells **(Figure 3.1 A2 and A4)**.

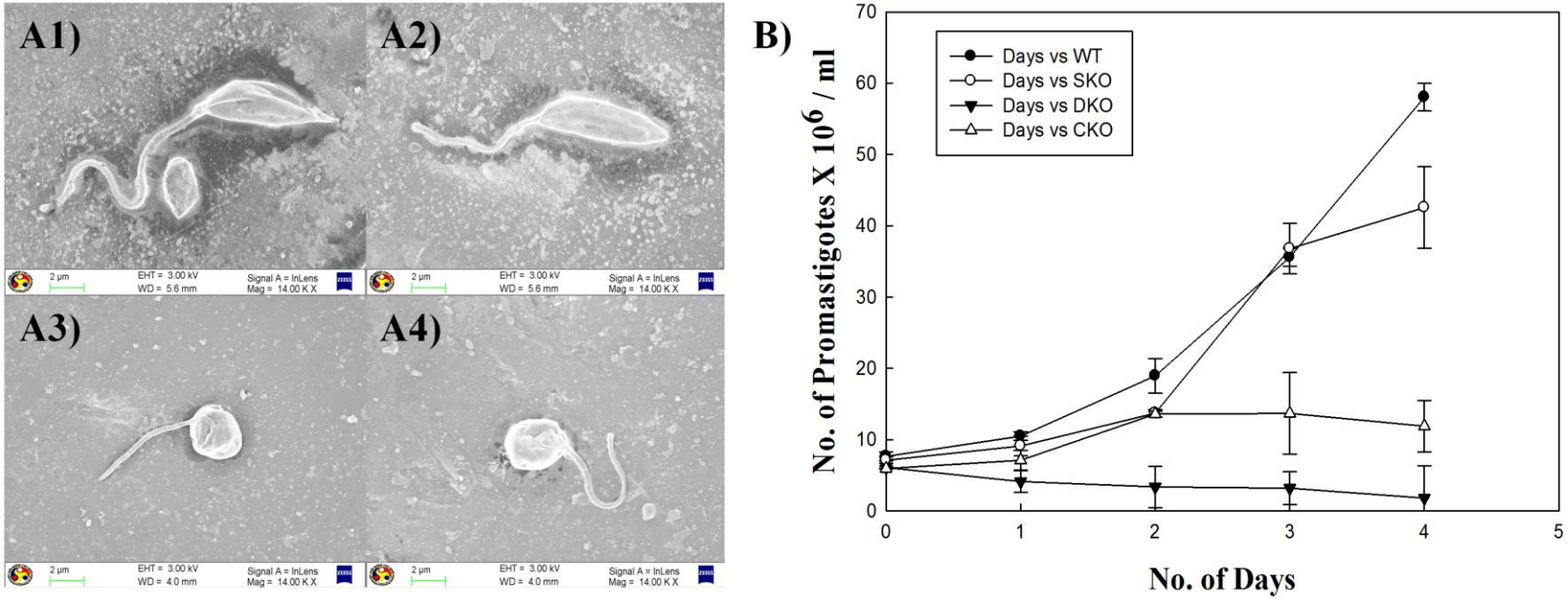



**Figure 3.1: A)** Change in the morphology: **A1** is the FESEM image of WT cells showing healthy elongated and tapered body with a long flagella, **A2** is the FESEM image of SKO_HP, showing cell morphology almost similar to WT, **A3** is the FESEM image of DKO_HP demonstrating a much globular body and a shorter flagella and **A4** is the FESEM image of CKO_HP demonstrating the cell morphological features between SKO_HP and DKO_HP. **B)** Comparative growth curve analysis: It can be inferred from the growth curve of WT, SKO_HP, DKO_HP and CKO_HP that DKO_HP has protracted growth compared to the other three populations of cells, in brief growth rate of WT > SKO_HP > CKO_HP >> DKO_HP.

### *3.4.2 Generation of ROS is observed on removal of LdBPK_070020 expression and complementation of LdBPK_070020 impedes the effect to some extent*

After selection of knocked out parasite, they were transferred to fresh media. After 24 h of incubation in fresh media, all analysis on knocked out parasite were done. In order to check whether the knockout of LdBPK_070020 have prompted any disturbance in the redox equilibrium of the parasite, a comparative study on the approximation of ROS generation was done between WT, HP_SKO and DKO_HP. A positive control (100 μM $H_2O_2$ treated WT cells) was also used for the studies along with pre NAC (20 μM) treated HP-DKO cells. ROS estimation of CKO_HP cells was done in order to assess, if complementation of LdBPK_070020 can revert back the redox equilibrium inside the parasite. The cells were labeled with CM-$H_2$DCFDA dye and fluorescence signal generated was measured by flow cytometer as explained in method section and data obtained was plotted in the form of a histogram as shown in **Figure 3.2**. An increase in DCF fluorescence signal was observed in HP_DKO cells compared to WT, indicating generation of ROS, while the signal was low for HP_SKO cells. A weak signal was observed for the pre NAC treated HP_DKO cells, as NAC is a ROS scavenging molecule, preventing the accumulation of ROS in HP_DKO cells. An impediment in the fluorescence signal of HP_CKO cells was observed indicating lower ROS levels in the parasites compared to HP_DKO cells.

### *3.4.3 Change in the expression levels of various genes involved in redox metabolism, observed in knockouts of LdBPK_070020*

There was a clear observation of increased ROS production in HP_DKO cells. Hence it was evident to see how the redox metabolism of the parasite is being affected. This was determined by checking the over or under expression of various genes involved in maintaining the redox homeostasis, inside the promastigote **(Figure 3.3)**. Few genes, responsible for the expression of various enzymes included in TryR and TryS redox metabolic system of *Leishmania* parasite, were picked and their expression levels were quantified using Real Time PCR. Almost all the genes picked showed altered expression levels due to removal of LdBPK_070020. After the removal of LdBPK_070020, there was almost 5 fold increase observed in expression of tryparedoxin peroxidase and glutaredoxin,

and 2.5 fold increase in thiol dependent redoxin. While genes responsible for expression of spermidine synthase, glutathionyl spermidine synthase and glyoxlaseI trypanothione dependent glyoxylase I were down-regulated for the same.

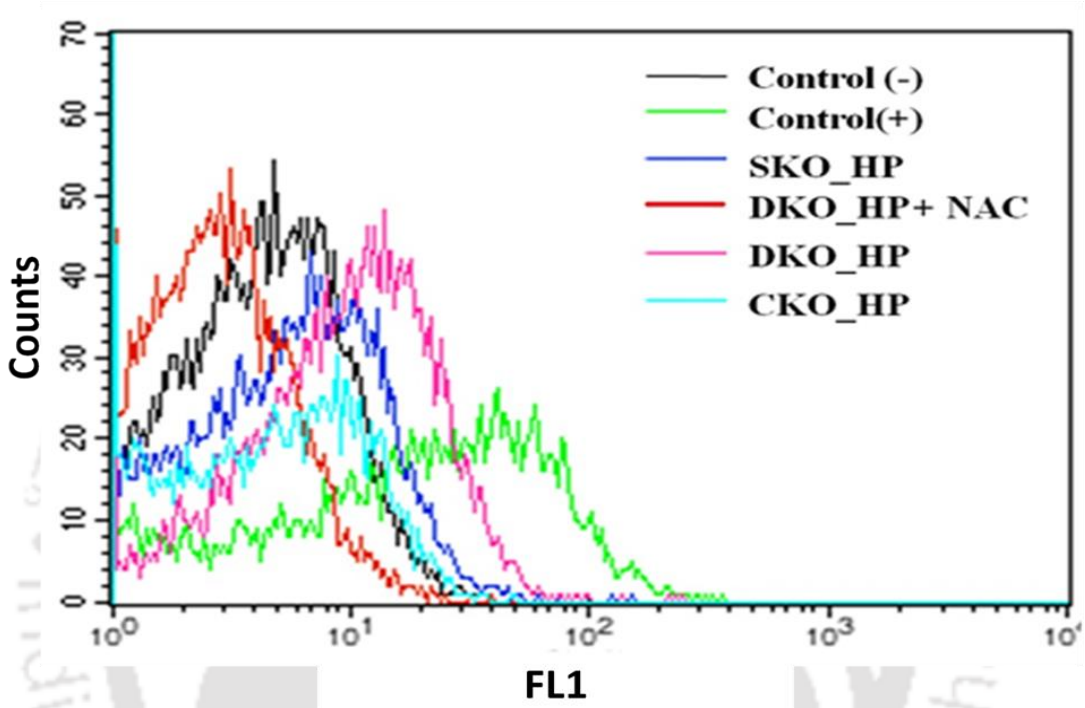



**Figure 3.2: Generation of Reactive Oxygen Species:** Reactive oxygen species inside the cells were measured using DCF fluorescence signal. All the samples were given a 30-45 min treatment with 10µM CM-$H_2$DCFDA dye in the dark at 25ºC. For positive control, WT cells were treated with 100 µM $H_2O_2$ (Armstrong et al., 2002) and incubated for 30 min and for Pre NAC- HP-DKO, cells were given a 45 min treatment with N-acetyl cysteine (ROS scavenging molecule) before being labeled with the dye. The resultant fluorescence was then collected using flow cytometer and the data is represented in the following figure.

### *3.4.4 Knockout of LdBPK_070020 prompts the change in mitochondrial membrane potential ($\Delta\Psi_m$)*

After selection of knocked out parasites, they were transferred to fresh media. After 24 h of incubation in fresh media, assessment of mitochondrial membrane potential ($\Delta\Psi_m$) was done, using MitoCapture™ dye, a cationic dye. The dye aggregates in the mitochondria, with retained potential, hence imparting red fluorescence, while the cells with altered $\Delta\Psi_m$ will impart green fluorescence due to accumulation of the dye in the cytosol. An evaluation of the fluorescence data obtained indicated a major alteration of $\Delta\Psi_m$ in HP_DKO cells compared

to WT cells. Data obtained was plotted in the form of dot-plot as shown in **Figure 3.4**. While in case of HP_SKO and HP_CKO, the latter demonstrated a minor alteration of ΔΨm.

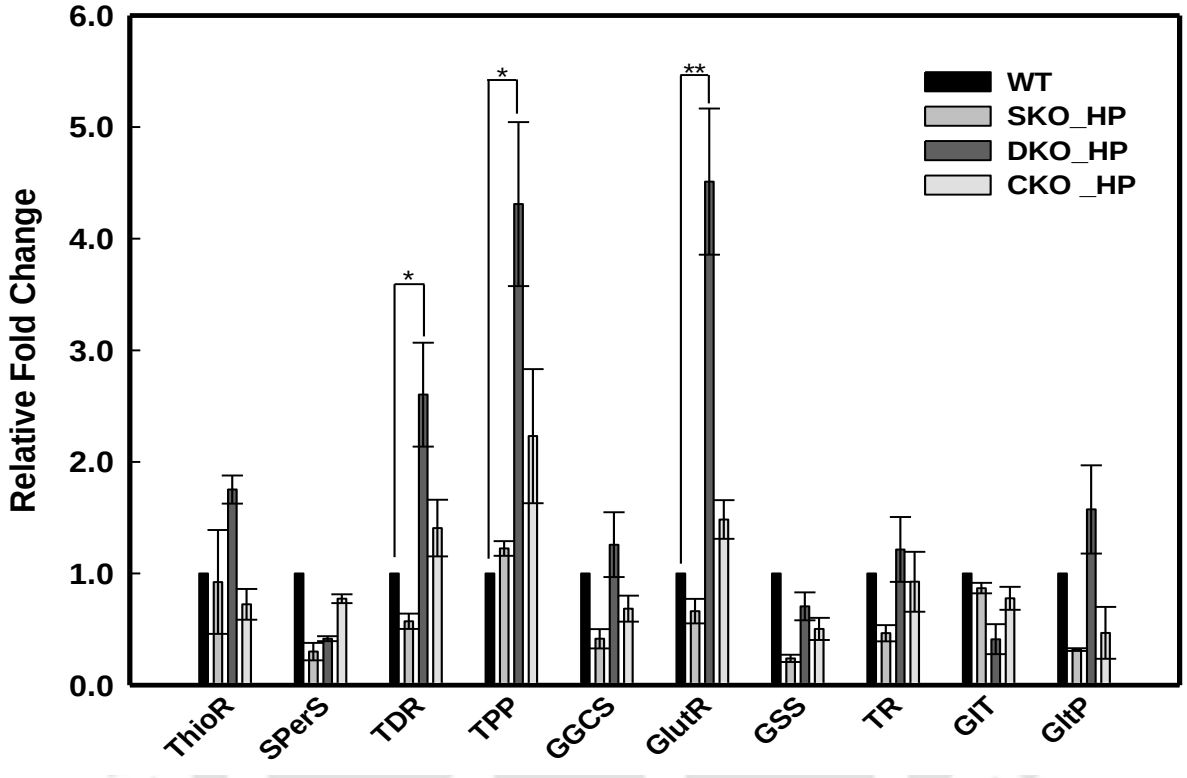



**Figure 3.3: Real Time PCR analysis of redox genes:** Expression levels of various genes involved in maintaining the redox metabolism inside the cells was quantified by performing Real Time PCR. Total RNA of all the four populations (WT, SKO_HP, DKO_HP and CKO_HP) were isolated, quantified and transcribed to cDNA, which was subjected to simultaneous amplification and quantification using Power SYBR® Green PCR Master mix. (* denotes p value < 0.05 and ** denotes p value < 0.005).

### *3.4.5 Removal of LdBPK_070020 restrains the survival of the parasite, ultimately leading to apoptosis*

The survival of the parasite is hindered from the knockout of LdBPK_070020. HP_DKO cells survive at a diminishing growth rate, with an ultimate compliance to cell death after 3–4 weeks. After selection of knocked out parasite, they were transferred to fresh media. After 24 h of incubation in fresh media, fraction of dead cells and mode of cell death were

evaluated using flow cytometric analysis after staining of the cells with annexin V-FITC and propidium iodide **(Figure 3.5)**. WT cells did not show any staining with annexin V-FITC and PI, while on the other hand positive control cells (50 μM Miltefosine treated WT cells) showed significant staining with both annexin V-FITC and PI, hence indicating apoptosis. An assessment of flow cytometric data of HP_DKO, points out to programmed cell death i.e. apoptosis, but NAC (n-acetylcysteine) pretreated HP_DKO cells do not show signs of apoptosis indicating role of induces ROS leading to apoptotic process. On the other hand HP_SKO and HP_CKO cells do not show any prominent staining with these two dyes.

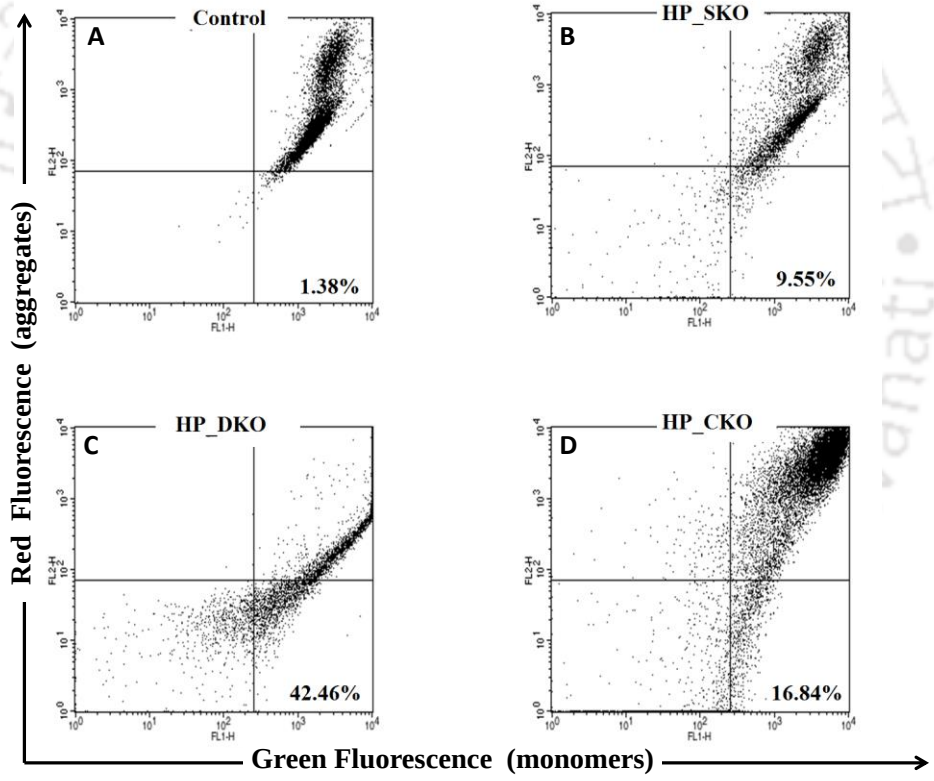



**Figure 3.4: Analyzing the change in mitochondrial membrane potential ($\Delta\Psi_m$):** Cells were stained with MitoCapture™ (Calbiochem), a mitochondria specific cationic dye. The dye gets aggregated in mitochondria, with normal potential ($\Delta\Psi_m$), hence imparting red fluorescence, but in case of cells with altered $\Delta\Psi_m$, the dye accumulates in the cytosol in its monomeric form, hence imparting the green fluorescence. The fluorescence was measured using flow cytometer and data was analyzed to observe a shift from red to green fluorescence. (A) WT cells FACS data showing a shift of 1.38% towards green fluorescence (B) HP-SKO cells showing a shift of 9.55% (C) HP-DKO cells showing a shift of 42.46% and (D) CKO_HP cells with a shift of 16.84%.

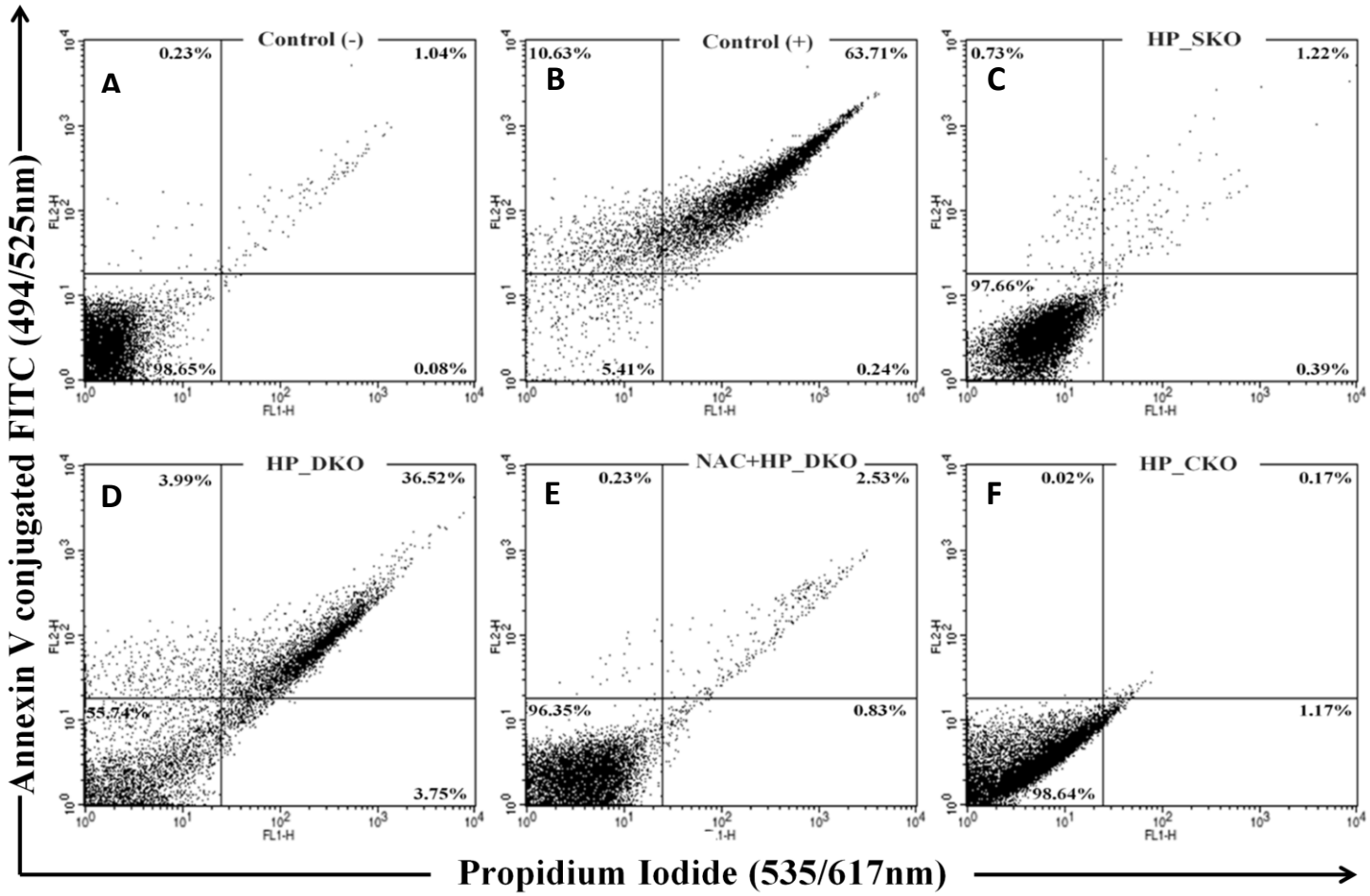



**Figure 3.5: Mode of cell death:** The cells were co-stained with annexin V-FITC and propidium iodide (PI), respectively prior to the flow cytometric analysis. (A) WT cells used as negative control (B) WT cells treated with 50µM Miltefosine (12 hr incubation), used as positive control (C) flow cytometric data of HP_SKO cells (D) flow cytometric data of HP_DKO (E) HP_DKO cells pre-treated with NAC (20µM) for 45 min prior to flow cytometric analysis (F) data analysis of HP_CKO cells. It can be clearly made out that one of the major reasons for cell death of HP_DKO cells is apoptosis.

### *3.4.6 Varied protease levels observed on removal of LdBPK_070020*

Various proteases, like cysteine, serine and metalloproteases, are requisite for the survival of *Leishmania* parasite *(Pereira et al., 2014)*. Any physiological change in the parasite will lead to an altered expression of these proteases. Hence the varied expression of few such proteases was studied using Real Time PCR **(Figure 3.6)**. A six fold increase in the expression levels of Metacaspase was observed in HP_DKO cells, while its levels were decreased in case of HP_SKO and HP_CKO cells. Various metalloproteases like ATP-dependent zinc metallopeptidase, putative and Mitochondrial ATP-dependent zinc metallopeptidase, putative too showed a prominent increase, in case of HP_DKO cells, while their expression was lower in HP_SKO and HP_CKO cells. But there was an eminent decrease in the expression of serine protease (putative) in all the three cell populations i.e. SKO_HP, DKO_HP and CKO_HP. For cysteine proteases, varied alterations were observed, like cysteine peptidase B showed lowered expressions in all three populations, while cysteine peptidase A showed increased levels for HP_DKO cells. There was decreased expression of cysteine peptidase C in case of SKO_HP and CKO_HP cells, but not much prominent change in case of HP_DKO cells.

### *3.4.7 Knockout of LdBPK_070020 stimulates an increase in intracellular ATP levels*

After selection of knocked out parasite and subsequent growth in fresh media for 24 h, determining intracellular ATP levels using luciferin–luciferase bioluminescence assay was performed. Compared to WT, a decrease was observed in the ATP concentration of HP_SKO. But the same trend was not followed for HP_DKO and HP_CKO cells. A shoot up in the levels of ATP was detected in both HP_DKO and HP_CKO cells, though the increase in ATP levels was lower in HP_CKO when compared to HP_DKO **(Figure 3.7 A)**.

### *3.4.8 Intracellular $Ca^{2+}$ levels ascend in the absence of LdBPK_070020 within parasite cells*

Intracellular $Ca^{2+}$ levels were measured using FURA- 2AM, a fluorescent probe. The fluorescence signal reflects the levels of $Ca^{2+}$ in WT, HP_SKO, HP_DKO and HP_CKO cells **(Figure 3.7 B)**. It was observed that compared to WT cells, HP_DKO and HP_CKO

cells showed tremendous amounts of intracellular $Ca^{2+}$, while that of HP_SKO was comparable to the WT levels.

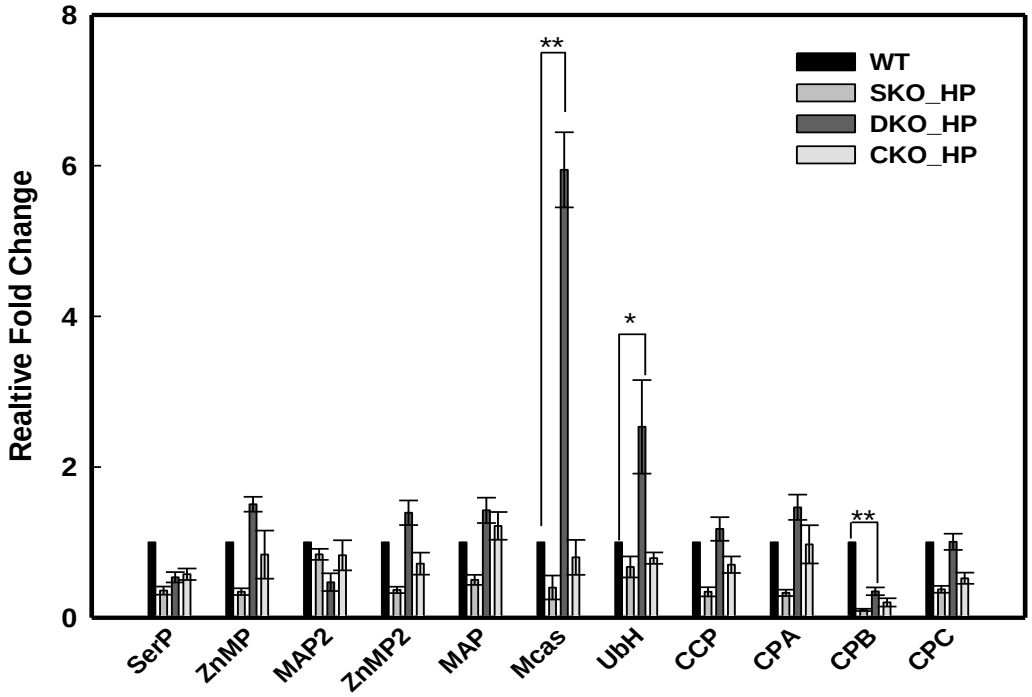



**Figure 3.6: Expression of various proteases during knockout:** The up regulation or down regulation of various protease activities inside the parasite was measured by performing Real Time PCR. The steps involved included total RNA isolation from WT, HP_SKO, HP_DKO and HP_CKO promastigotes, respectively, followed by quantification and cDNA synthesis. The cDNA synthesized was further subjected to quantification by Real Time PCR using Power SYBR® Green PCR Master mix. (* denotes p value < 0.05 and ** denotes p value < 0.005).

### *3.4.9 The membrane of the parasite becomes more rigid on removal of LdBPK_070020 due to decrease in membrane fluidity*

Membrane fluidity of the parasite is measured by measuring the fluorescence anisotropy (FA) using DPH as a probe and results obtained are shown in **Figure 3.8**. The FA signal emitted by WT and SKO_HP was almost similar, while that of HP_CKO was a bit less than the former. But the signal emitted by HP_DKO cells was elevated compared to all the other three populations. The microviscosity parameter for HP_DKO cells was deduced to be 8.165, while that for WT, HP_SKO and HP_CKO was 1.109, 1.080 and 0.942 respectively. Therefore on complete knockout of LdBPK_070020, the parasite membrane is becoming more microviscous or less fluid.

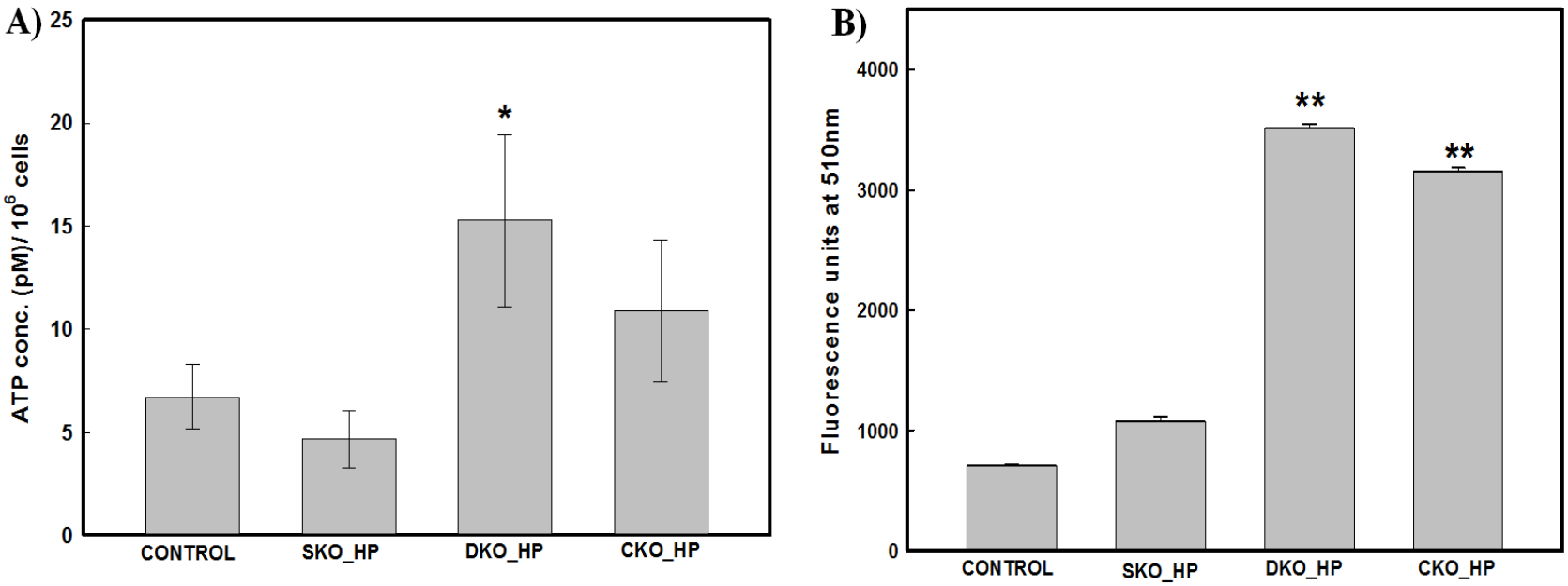



**Figure 3.7: A) ATP estimation:** The measurement of intercellular ATP levels was done using ATP determination kit. A standard curve of varying ATP concentrations was prepared by measuring the bioluminescence emitted by luciferin-luciferase reaction. This was followed up by measuring the bioluminescence of samples which consisted of approximately $10^6$ cells, incubated in the same reaction mixture used for the standard. The experiment was performed in triplicates and the data represented are the means and SDs of three independent experiments. (* denotes p value < 0.05). **B) Measurement of cytosolic $Ca^{2+}$ concentration:** Cells were washed and stained with FURA 2AM, proceeded with 6 hrs incubation, at 37°C. This was further followed with measurement of fluorescence at excitation of 340nm and emission of 510nm. The experiment was performed in triplicates and the data represented are the means and SDs of three autonomous experiments. (** denotes p value < 0.005).

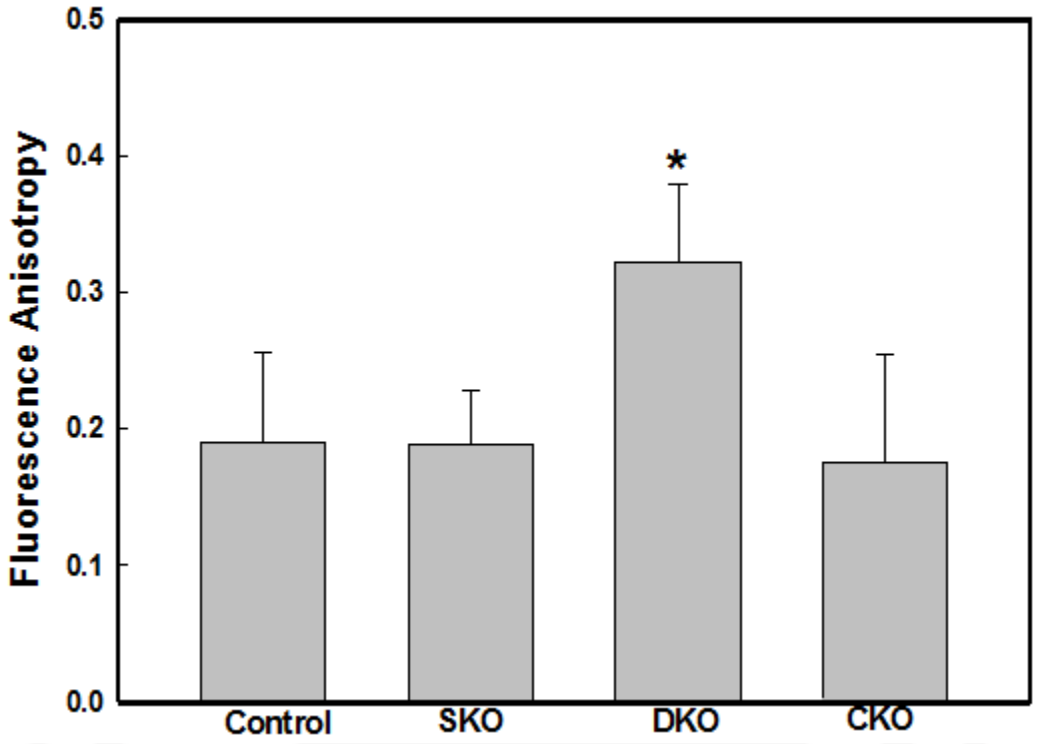



**Figure 3.8: Assessment of membrane fluidity:** The measurement of membrane fluidity was done by treating the cells ($10^6$ cells) with DPH, a fluorescent probe and measuring the fluorescence anisotropy (excitation: 360nm and emmision: 430nm), preceding 2-4 hrs incubation at 25°C. The experiment was performed in triplicates and the data represented are the means and SDs of three autonomous experiments. (* denotes p value < 0.05).

## 3.5 DISCUSSION

Any evidence of morphological or biochemical changes in WT cells electroporated with empty pXG vectors (positive control), were not found. WT cells electroporated with empty pXG vectors showed vigorous growth like normal WT cells in M199 complete media. Hence indicating that the presence of empty pXG vector is not responsible for change in parasite survival and it is the removal of LdBPK_070020 which is causing the change in parasite sustenance and survivability. It can be observed in our studies, that DKO_HP and CKO_HP cells are following an almost comparable trend of results. For example, the growth trend of both DKO_HP and CKO_HP cells is almost similar, but the only difference is that the CKO_HP cells survive with lower growth rate surpassing the survival of DKO_HP cells.

Similarly higher ATP and $Ca^{2+}$ levels were observed in CKO_HP compared to WT population. But unlike DKO_HP cells, CKO_HP cells do not show apoptosis, further ROS levels are lower, Mcas protease activity is lower, etc. Hence it can be inferred that the parasite is utilizing the episomally expressed LdBPK_070020, to somewhat revert back to its original state, but the recovery is not 100%. Consequently, the similarity in the morphology of DKO_HP and CKO_HP cells, is another aspect which can be attributed to the fact that the parasite has not completely progressed by episomal expression of the protein.

Further when it comes to morphological changes in HP_DKO cells, it can be inferred that either LdBPK_070020 is involved in maintaining the morphology of the parasite or its removal has put the cell under stress which has led to a change in the morphology of the parasite. Out of the two inferences, the latter seems to fit better into the picture, since high load of reactive oxygen species were observed in HP_DKO cells, which were scavenged after addition of NAC. Further real time analysis, of various genes involved in maintaining the redox homeostasis of the parasite, showed altered levels of expression, hence indicating the disturbance in the redox metabolism of the parasite. It is known that one of the major reaction pathways responsible for ROS production is electron transport chain reaction, which produces enough ROS required for microdomain signaling *(Brookes et al., 2004)* and occurs in inner and outer mitochondrial membrane. Removal of LdBPK_070020 leads to elevated ROS levels hence the next step was to determine the change in mitochondrial membrane potential which is responsible for maintaining the mitochondrial membrane permeability to various components including ROS. A prominent change in mitochondrial membrane potential was observed in HP_DKO cells suggesting altered regulation of ROS. Change in morphology, increased ROS levels, change in mitochondrial potential were a few observation made before the cell death of HP_DKO after 3–4 weeks. The mode of cell death was determined by labeling the cells with Annexin V conjugated FITC and PI, after 1.5 weeks of selection post electroporation and the resultant data showed apoptosis to be mode of cell death. Hence it could to be said to some level of assurance that LdBPK_070020 is important for the survival of the parasite, because removal of its expression is leading to cell death while complementing the expression back to the mutant parasite is reverting back its survival. Various other supplementary experiments were done to support the collateral damage caused by removal of LdBPK_070020. The change in expression levels of various

proteases inside the parasite were quantified and compared. High expression levels of metacaspase was observed in HP_DKO cells and it has been shown in earlier studies that metacaspases play a major role in oxidative stress induced cell death of *Leishmania* parasite *(Lee et al., 2007)*. Further decreased level of serine protease was observed for the mutant cells. Direct role of serine protease is not very clear with regard to the removal of LdBPK_070020, but cell death using serine protease inhibitors *(Silva-Lopez et al., 2007)* has been reported, hence indicating, decreased levels of serine protease lead to cell death. In case of cysteine peptidases A, B and C, peptidase B showed decreased expression levels, while peptidase A showed increased levels and no prominent change was observed in case of peptidase C.

Intracellular ATP estimation of all the four populations of cells was observed (WT, HP_SKO, HP_DKO and HP_CKO) and elevated levels of cytosolic ATP was observed in HP_DKO and HP_CKO compared to HP_SKO and WT. It has been reported that when cells are under apoptotic stimuli, during which caspase activation and other processes like DNA fragmentation is occurring, the cytosolic ATP levels remain high *(Zamaraeva et al., 2005)*. Further profusion in $Ca^{2+}$ ions was observed in HP_DKO and HP_CKO cells. Mitochondria are involved in maintaining the cellular $Ca^{2+}$ homeostasis by regulating its movement across the double membrane of the mitochondria into the matrix *(Brookes et al., 2004)*. Uptake of $Ca^{2+}$ is driven by mitochondrial membrane potential ($\Delta\Psi m$), and since the removal of LdBPK_070020 have changed $\Delta\Psi m$, hence the homeostasis is disturbed resulting in increased $Ca^{2+}$ levels in HP_DKO and HP_CKO cells. Change in membrane fluidity of HP_DKO cells was observed with increased microviscosity. It is well known that early stages of apoptosis involves translocation of phosphatidylserine from inner side to the outer layer of the plasma membrane *(Vermes et al., 1995)*, hence this change coupled with various other changes in plasma membrane, like disintegration of various cytoskeleton proteins, during apoptosis *(Jaruga et al., 1998)* are responsible for plasma membrane alterations leading to change in membrane fluidity. BLAST analysis has shown that LdBPK_070020 might be involved in some pre-mRNA splicing activity, which further affects the expression of various proteins involved in proper functioning of cell processes, in various cell organelles including mitochondria. In a gist, we can say that a vast array of changes in the parasite has been observed, after the removal of LdBPK_70020 expression. The changes are not only

phenotypic but various biochemical processes are been affected as well. Based on the results we cannot exactly pin out the specific function of the protein. But the observations are insinuating the fact that conserved hypothetical protein LdBPK_070020, is indispensible for parasite survival. The parasite has capitulated to the stress. It suffers from lack of LdBPK_070020 and even in CKO_HP cells, consisting of episomal expression, the resurgence is not complete. Hence it can be deduced that removal of the gene and the resulting consequences are not exactly extorting the information about the specific function of the protein but pulling out the value of the protein for the parasite. The disruption of this protein is hampering the harmonized orchestra, hence making the parasite off tune. We have earlier reported studies on redox metabolism of the parasite as target for novel drug discovery *(Singh et al., 2008; Shukla et al., 2011; Verma et al., 2012; Shukla et al., 2012; Saudagar et al., 2013; Saudagar and Dubey, 2014; ).* The hypothetical protein LdBPK_070020 also seems to be a promising durg target for anti-leishmanial drug discovery.

# CHAPTER IV

## Localization Studies of Conserved Hypothetical Protein LdBPK_070020 inside the Parasite*

### 4.1 ABSTRACT

*Leishmania donovani* genome consist of approximately 8000 genes which code for both functionally annotated and hypothetical proteins. Unknown proteins with wider phyletic distribution are referred to as conserved hypothetical protein. LdBPK_070020 is one of the conserved hypothetical proteins of *Leishmania donovani*. Its existence is reported in the *Leishmania donovani* genome published in 2011, but its location and function inside the parasite was still unknown. Our studies have suggested important function of LdBPK_070020 in the parasite survival and infectivity. In order to understand the role of the protein in the parasite, we have performed computational and biochemical studies to understand the localization of the protein. The findinge reported here clearly indicate that the conserved hypothetical protein, LdBPK_070020, is present inside the nucleus as well as the kinetoplast. The protein is possibly altering mitochondrial function indirectly by regulating expression of other proteins. However, precise mechanism still needs further investigation and can be future direction of the study.

*Part of the work is submitted for publication

## 4.2 INTRODUCTION

Leishmaniasis is a parasitic disease caused by various species of the protozoa *Leishmania*. The causative agent of this disease has a digenetic life-cycle. Throughout its life, the parasite shuttles between the vector sandfly and the mammalian host *(Murray et al., 2005)*. This disease is endemic in 88 countries across the globe *(WHO, 2012)*. Based on the clinical manifestations and causative species, leishmaniasis is broadly divided into three types of infections, viz. cutaneous, mucocutaneous and visceral *(Desjeux, 2004)*. Out of the three infections, visceral is the most treacherous form, because if neglected, the cases turn out to be fatal. Visceral leishmaniasis is the most common form of infection found in India, and is known as Kala azar here *(Singh et al., 2006)*. The causative agent of this disease in India is *Leishmania donovani*. The current status of drugs against leishmaniasis suffers from huge fall due to their low efficacy, side effects and the emergence of drug resistance *(Croft et al., 2006)*. The genome of *Leishmania donovani* has a substantial number of genes encoding for hypothetical protein *(Downing et al., 2011)*. Conserved hypothetical proteins are proteins that have wide phyletic distribution but are still not characterized functionally *(Galperin and Koonin, 2007)*. LdBPK_070020 is a conserved hypothetical protein present in *Leishmania donovani*. In order to get an insight into the possible role of the protein, we have performed LdBPK_070020 knocked out studies that showed impairment in cell proliferation and growth followed by death, hence indicating the indispensible role of this protein in parasite survival *(Bhardwaj et al., 2016)*. The study also reports impaired mitochondrial function in LdBPK_070020 knocked out parasite *(Bhardwaj et al., 2016)*. The current study was undertaken to understand the localization of the protein for better functional understanding.

## 4.3 MATERIAL AND METHODS

### *4.3.1 Material required*

Gene specific primers of LdBPK_070020 hypothetical protein, PCR clean up kit (Qiagen), Plasmid isolation kit (Sigma, USA), *Xho*I and *Bgl*II (NEB, USA), Gel extraction kit (Qiagen), T4 DNA ligase (NEB), Ampicilin (Himedia), Geneticin (Gibco), Genomic DNA isolation Kit (Bioline), DAPI (Sigma, USA), MitoTracker® Red CMXRos (ThermoFisher). The *Leishmania donovani* strain (BHU-1081) attained from Prof. Shyam Sunder, Banaras

Hindu University, India. Pgl1686_GFP_ATG *Leishmania* expression vector was kindly donated by Prof Jeremy C Mottram - University of Glasgow, Scotland, UK.

### *4.3.2 In silico sub cellular localization*

Four online tools were used for the prediction of subcellular localization of hypothetical protein. LocTree 3 (https://rostlab.org/services/loctree2/) *(Goldberg et al., 2014)* and CELLO v 2.5 (http://cello.life.nctu.edu.tw/) *(Yu et al., 2006)* were used to predict the subcellular localization. These tools are based on two level SVM (support vector machine) system and gene ontology information. TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM-2.0/) *(Krogh et al., 2001)* was used for the prediction of transmembrane helices. This tool is based on hidden Markov model. Signal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) *(Emanuelsson et al., 2007)* was used for presence and location prediction of signal peptide sequences. Its prediction is based upon combination of several neural networks.

### *4.3.3 Parasite cell culture and maintenance*

*Leishmania donovani* cells (MHOM/IN/2010/BHU1081) were obtained from Prof. Shyam Sundar (Banaras Hindu University, Varanasi, India). These cells were grown and maintained according to the protocol already established in our laboratory *(Saudagar et al., 2013; Das et al., 2013).* To be brief, cells were grown and maintained in complete M199 media at 25°C. M199 liquid media (pH 7.4), is supplemented with 15% fetal bovine serum (FBS), 100µg/ml penicillin and 100µg/ml streptomycin. For selection and maintenance of transfected parasites, cells were grown in complete M199 media supplemented with Geneticin G148 (20µg/ml).

### *4.3.4 Construction of Pgl1686_GFP_HP vector*

Vector Pgl1686_GFP_ATG was modified by replacement of *ATG* gene with *LdBPK_070020* gene. Firstly, *LdBPK_070020* gene was PCR amplified from genomic DNA of *Leishmania donovani*. The following primers were used to amplify 810bp LdBPK_070020 gene: FP_HP_GFP: GAAGATCTATGCAGGAT AGACTAAG and RP_HP_GFP: CCCTCG AGTCAACTTTTCCCACG. Further ATG gene was removed from the vector by sequential digestion of the vector using two restriction enzymes, *viz*., *Bgl*II and *Xho*I. The amplified gene was digested with the *Bgl*II and *Xho*I restriction enzymes as well. Further gene was

ligated in the modified vector, followed by transformation in *E. Coli DH5α* cells to increase the copy number. The proper replacement of ATG gene with LdBPK_070020 gene in Pgl1686 vector was confirmed by PCR, double digestion as well as nucleotide sequencing.

### *4.3.5 Transfection of Pgl_GFP_HP vector in Leishmania donovani cells*

For transfection of *Leishmania* cells with Pgl_GFP_HP vector, we employed the method established by Beverley group *(Ha et al., 2006; Bhardwaj et al., 2016)*. In brief, approximately $10^7$cells/ml were washed twice with PBSG buffer (10mM $NaH_2PO_4$, 10mM $Na_2HPO_4$, 145mM NaCl and 2% glucose) and further re-suspended in 360µl electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM $Na_2HPO_4$, 6mM glucose). To this suspension, about 40 µl of ~5-10mg/ml of Pgl_GFP_HP vector was added. The cell suspension was then transferred to ice cold electroporation cuvettes and electroporated in BioRad electroporater. Conditions for electroporation were set as follows: Exponential protocol: Voltage_450V, Capacitance_500 F and the resulting time constant should be around 4.5ms. Cells were later introduced to selection pressure after 24 hrs of transfection by supplementing the M199 complete media with 20µg/ml Genetecin G148.

### *4.3.6 Confocal laser scanning microscopy*

Localization studies of LdBPK_070020 protein was done, 6-8 days after incubation of transfected cells in selection media. For localization studies, the GFP expressing *Leishmania* cells were co-stained with organelle specific dyes and further analyzed using Confocal Laser Scanning Microscopy (CLSM) *(Patel et al., 2008; Leprohon et al., 2009)*. In brief, the Pgl_GFP_HP transfected cells were washed twice with PBS (pH 7.4) followed by fixation of cells. Cells were fixed by incubating them with 4% paraformaldehyde at 25ºC for 30 minutes. This was followed by rigorous washing with PBS (pH 7.4). After washing, the cells were then permeablized by treatment with 0.1% Triton X 100. This was followed with co-staining of cells with Mitotracker® Red CMXRos and DAPI simultaneously, according to the manufacturer's instruction. Slides were prepared using 50% glycerol and imaging was done in confocal laser scanning microscope (Leica DMi8) using a 63X and oil immersion objective.

## 4.4 RESULTS

### *4.4.1 In silico studies*

In order to initially conceptualize the location of conserved hypothetical protein LdBPK_070020, inside *Leishmania donovani* parasite, various online tools were used. TMHMM and Signal P predicted that the query protein sequence was neither a transmembrane domain nor a signal protein. In case of LocTree3, the software predicted 93% chance of LdBPK_070020 being nuclear. The score range for LocTree3 was between 0-100%, where 100% was the most dependable projection for subcellular localization. In case of CELLO v 2.5, localization site prediction was checked and results are shown in **Figure 4.1**.

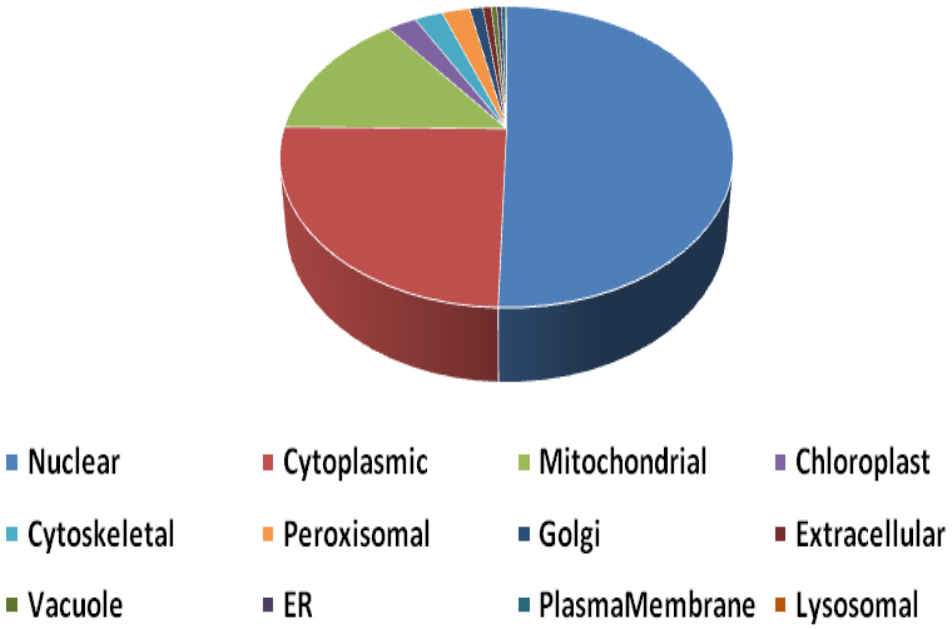



**Figure 4.1: CELLO v 2.5 data:** The resultant data from Cellov v 2.5 was illustrated in the form of a pie chart. The data clearly points out the likeliness of LdBPK_070020 to be located in the nucleus.

### *4.4.2 Construction of Pgl_GFP_HP vector*

The replacement of ATG in Pgl1686_GFP_ATG vector with LdBPK_070020 gene was successful **(Figure 4.2 A)**. The confirmation of replacement was done, by PCR and double digestion with *Bgl*II and *Xho*I **(Figure 4.2 B)**. Further nucleotide sequencing confirmed the replacement.

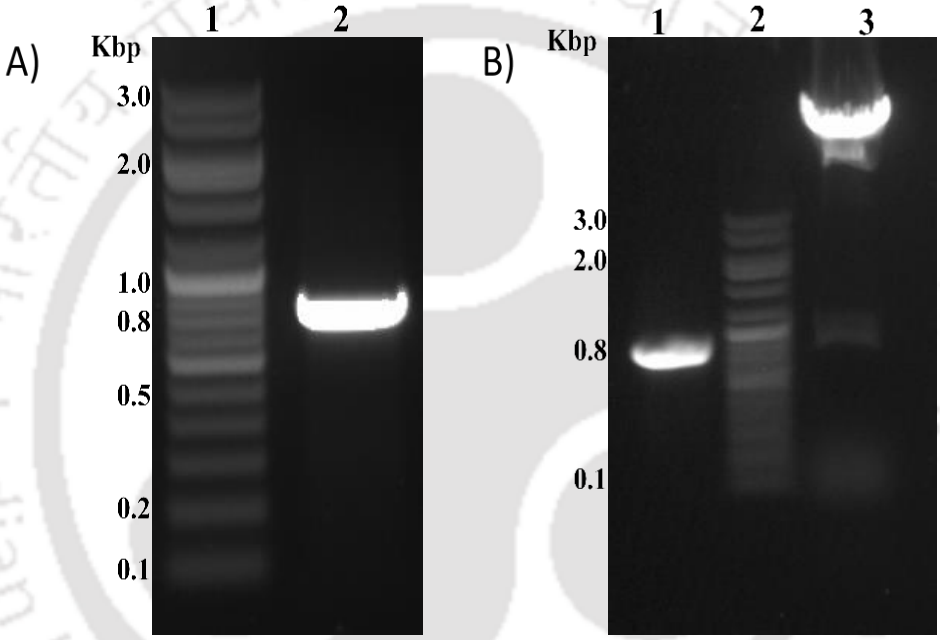



**Figure 4.2: Construction of PGL_GFP_HP vector: A)** PCR amplification of LdBPK_070020 using *Leishmania donovani* genome as a template. Lane 1 depicts 100bp DNA ladder and lane 2 represents amplified 810bp LdBPK_070020 gene band. **B)** The construction of PGL_GFP_HP vector was confirmed by PCR and double digestion. Lane 1 shows PCR amplified LdBPK_070020 using Pgl_GFP_HP as a template. Lane 2 represents 100bp DNA ladder while lane3 shows the release of 810 bp, after Pgl_GFP_HP construct was double digested with *Bgl*II and *Xho*I. Further the results were confirmed by DNA sequencing.

### *4.4.3 In vivo localization of LdBPK_070020*

The co-staining of modified *Leishmania* cells, with nuclear specific dye, DAPI and mitochondria specific dye, Mitotracker® Red CMXRos was done to investigate the location of GFP-tagged LdBPK_070020 protein. Slides of the stained cells were prepared and observed under confocal laser scanning microscope (CLSM). The cells were exposed to

sequential laser beams followed by imaging because GFP, DAPI and Mitotracker® Red CMXRos have different excitation and emission spectra. Sequential scanning was performed in CLSM to prevent cross talk. Cross talk is a phenomenon where emission spectrum of one fluorophore overlaps with the excitation spectra of another. DIC (Differential Interference Contrast) image was taken using Trans mode. DAPI image was taken using 405 laser line, while 488 laser line was used for GFP tagged LdBPK_070020 and 543 laser line for Mitotracker® Red CMXRos. The results are shown in **Figure 4.3**. It can be inferred from the image overlay that LdBPK_070020 is localized in the nucleus as well as the kinetoplast of the parasite.

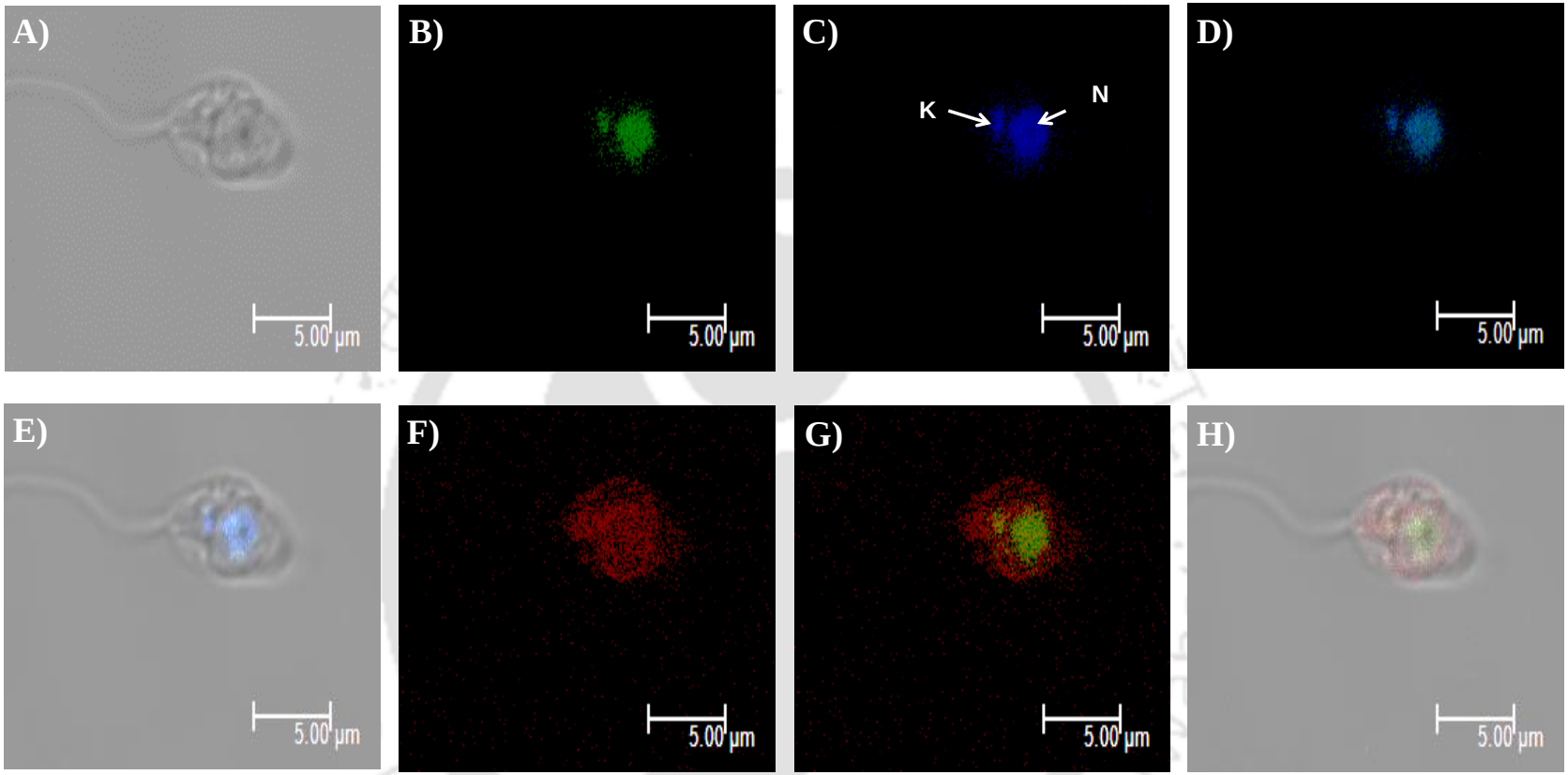



**Figure 4.3: Confocal Laser Scanning Microscopy:** LdBPK_070020-GFP-tagged cells were co-stained with organelle specific dyes and then observed under CLSM. **A)** DIC image *Leishmania donovani*. **B)** Cell exposed to 488 laser line, which illuminates the GFP_tagged LdBPK_070020 protein. **C)** Cell observed under 405 laser line after treatment with DAPI (1:1000, 1µg/ml). Blue colour observed indicates nucleus (**N**) and kinetoplasts (**K**) inside the cell. **D)** Overlay of images B) and C). **E)** Overlay of images A) and D). **F)** Cell stained with Mitotracker® Red CMXRos (1ng/ml) and observed under 543 laser line. **G)** Overlay of images B) and F). **H)** Overlay of images A) and G). Hence the results indicate the location of LdBPK_070020 inside the nucleus and kinetoplast. It can be inferred from the image overlay that LdBPK_070020 is localized in the nucleus as well as the kinetoplast of the parasite. ***Note:*** **The brightness of the image was increased to improve the resolution of the image in a printout.**

## 4.5 DISCUSSION

The combination of *in silico* and biochemical studies pointed out towards the existence of the protein in the nucleus as well as kinetoplast. Kinetoplast is specific to protozoa belonging to family Kinetoplastida and is a network of circular DNA. Kinetoplast contains multiple copies of mitochondrial genome. In our previous studies, we have reported that the knockout of LdBPK_070020 gene from *Leishmania donovani* results in impaired mitochondrial function *(Bhardwaj et al., 2016).* This let us to speculate that the localization of the protein may be mitochondrial and the protein might be directly involved in functioning of mitochondria. However, the current results suggested that the protein is present inside the nucleus as well as kinetoplast. These results were puzzling. Apparently, LdBPK_070020 which is localized in nucleus and kinetoplast may be involved in regulation of some protein(s), which are essential for mitochondrial function and thereby altering mitochondrial function indirectly.

Apart from defining the conserved role of LdBPK_070020 in other species of *Leishmania*, BLAST analysis of LdBPK_070020 also indicated 30-42% similarity with pre-mRNA splicing factor ISY1 of other organisms including human. Since *Leishmania* parasite has a polycistronic mRNA, it does not possess cis-splicing. But there have been reports of trans-splicing in *Leishmania (Dillon et al., 2015).* In trans-splicing 39 bp mini-exon gene, highly conserved in the Kinetoplastids, is spliced during pre-mRNA maturation and this is responsible for differential protein expression inside the parasite *(Fernandes et al., 2001).* Hence there is a possibility that LdBPK_070020 is an essential protein which might play an important role in trans-splicing, which is ultimately responsible for expression of various proteins. The finding of this study clearly indicates that the conserved hypothetical protein LdBPK_070020 is present inside the nucleus as well as the kinetoplast. The protein is possibly altering mitochondrial function indirectly by regulating expression of other proteins. We have earlier reported drug candidates altering enzymes of redox metabolism of the parasite (*Singh et al., 2008; Shukla et al., 2011; Verma et al., 2012; Shukla et al., 2012; Saudagar et al., 2013; Saudagar and Dubey, 2014;* ). The hypothetical protein LdBPK_070020 also seems to alter mitochondrial function. However, precise mechanism still needs further investigation***. It is important to mention that at the time of thesis submission LdBPK_070020 was annotated (Inferred from Electronic Annotation) as putative Isy1-like splicing family protein (www.genedb.org/gene/LdBPK_070020.1)***

# CHAPTER V

## Deciphering the role of CAAX prenyl protease II as a target in *Leishmania donovani* *

### 5.1 ABSTRACT

Prenylation pathway is responsible for post translational modification of various signal proteins, especially the ones belonging to Ras superfamily. CAAX prenyl proteases are known to be a key player in prenylation pathway. In the current study we have evaluated CAAX prenyl protease II as a possible drug target against *Leishmania donovani* parasite, the causative agent of visceral leishmaniasis. Gene knockout strategy was employed to target CAAX prenyl protease II and subsequent effects were studied. CAAX prenyl protease II knockout resulted in significant decrease in growth and infectivity as well as delayed G1 to S phase transition in cell cycle of the parasite. However, parasite could still survive, possibly due to existence for another isoform *i.e* CAAX prenyl protease I.

*Manuscript covering this part of the work is under preparation

## 5.2 INTRODUCTION

Leishmaniasis is a parasitic disease, which is recognized as neglected tropical disease by WHO. The causative agent of this disease is *Leishmania*, which is a dimorphic protozoan parasite. Leishmaniasis is associated with wide spectrum of clinical manifestations, ranging from self recuperating cutaneous leishmaniasis to life threatening visceral leishmaniasis *(Murray et al., 2005)*. Recent WHO statistics indicates the prevalence of this disease in 88 countries across 5 continents and 2 million new cases are occurring annually. The current drug scenario against this disease is not satisfactory, due to poor efficacy, host toxicity, high cost and emergence of resistance *(Croft et al., 2006)*. Hence there is an eminent requirement for drugs with higher efficacy, lower side effects and reasonable pricing. Drug discovery is a multistep progression involving target identification and validation before lead drug candidate identification and optimization *(Hughes et al., 2011)*. Therefore making target identification and validation as a key step for any drug discovery process.

It is well established that biological molecules in eukaryotes undergo post-translational modification for their functional stimulation and regulation. Prenylation pathway is one such modification which is involved in maturation of various important signal proteins, like the ones belonging to Ras superfamily. These modified proteins further act as molecular switches for various signaling pathways that control important processes like cell proliferation, cell differentiation, membrane trafficking etc. Prenylation involves attachment of an isoprenoid group, i.e. 15C farnesyl or 20C geranylgeranyl group, to a cysteine residue by thio-ether linkage. Small GTP-binding proteins containing CAAX motif (C: cysteine, AA: aliphatic amino acid, X: any amino acid) at or near its carboxyl terminus are target molecules for prenylation *(Zhang et al., 1996)*. CAAX prenyl protease is a key enzyme of prenylation pathway. There are two isoforms of this protease depending upon the substrate specificity and presence or absence of HEXXH (H: histidine, E: glutamate, X: any amino acid) conserved motif. CAAX prenyl protease I is an alpha- factor converting enzyme (AFC1) and posses HEXXH conserved motif *(Boyartchuk et al., 1997; Schmidt et al., 2000)*. While on the other hand CAAX prenyl protease II is a Ras and yeast a-factor converting enzyme (RCE1), which lacks the HEXXH conserved motif *(Dolence et al., 2000)*. Mislocalization of Ras proteins was observed in mouse embryonic fibroblast which lacked RCE1 (CAAX prenyl protease II) or ICMT (carboxyl methyl transferase) *(Michaelson et al., 2005)*.

Moreover some studies reported that RCE1 deficiency was lethal in late embryonic development in mouse hence indicating towards the physiological consequences of CAAX prenyl protease II *(Kim et al., 1999)*. Further knockout of CAAX prenyl protease II in *Trypanosoma brucei* resulted in impairment of parasite growth *(Gillespiea et al., 2007)*. Earlier studies on other organism clearly support importance of CAAX prenyl proteases. In addition, the sequence similarity of CAAX prenyl protease II encoded by *Leishmania donovani* is significantly lower than the human counterpart. In the current study, we have targeted CAAX prenyl protease II (LdBPK_262720) present in *Leishmania donovani* by removing its expression from the parasite using homologous recombination. Effect of CAAX prenyl protease II knockout was studied in *Leishmania donovani*. Further complementation studies were also done to see reversal of the effects observed after CAAX prenyl protease II knockout.

## 5.3 MATERIAL AND METHODS

### *5.3.1 Materials required*

Gene specific primers of CAAX prenyl protease II, PCR clean up kit (Qiagen), Plasmid isolation kit (Sigma, USA), *Bam*HI, *Xho*I, *Sac*I, *Xma*I and *Eco*NI (NEB, USA), Gel extraction kit (Qiagen), T4 DNA ligase (NEB), Ampicillin, Hygromycin B, Puromycin (Himedia) and Geneticin (Gibco), Phleomycin (Sigma, USA), Genomic DNA isolation Kit (Quiagen), propidium iodide, poly-L-lysine (Sigma, USA). Novex® ECL chemiluminescence substrate reagent kit (Thermo Fischer). Anti Ras antibodies produced in rabbit and anti-rabbit FITC conjugated secondary antibodies was purchased from Sigma-Aldrich, USA (Cat. No: SAB4301113 and F9887). Protein A purified rabbit-anti-CAAX prenyl protease II antibody was custom supplied Abgenex Pvt Ltd, India. Rabbit-α-tubulin antibodies (Cat. No. SAB3501072) were purchased from Sigma-Aldrich, USA and goat-HRP-anti-rabbit IgG secondary antibodies (Cat. No. 656120) were purchased from ThermoFisher. The *Leishmania donovani* strain (BHU-1081) was obtained from Prof. Shyam Sundar, Banaras Hindu University, India. Macrophage cell line J774A, was taken from National Centre for Cell Science, Pune, India. The *Leishmania* expression vectors, used for

preparing knockout cassettes as well as complementation vector were donated by Beverley Lab, School of Medicine, Washington UniversityUSA.

### *5.3.2 Parasite cell culture and maintenance*

*Leishmania donovani* cells (MHOM/IN/2010/BHU1081) were obtained from Prof. Shyam Sundar (Banaras Hindu University, Varanasi, India) and were maintained according to the protocol already established in our laboratory *(Das et al., 2013; Saudagar et al., 2014; Bhardwaj et al., 2016)*. In brief, the growth and maintenance of *Leishmania* cells was done at 25$^{o}$C in complete M199 media. The M199 liquid media (pH 7.4) was supplemented with 15% fetal bovine serum (FBS), 100µg/ml penicillin and 100µg/ml streptomycin. For the knockout selection process, parasites were grown in complete M199 media supplemented with respective antibiotics required for the selection process, i.e., Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin (10 µg/ml) and Phleomycin (5 µg/ml).

### *5.3.3 Preparation of molecular constructs for knockout as well as complementation*

PCR amplification of 0.617 Kbp upstream flanking region (5'UTR) and 0.448 Kbp downstream flanking region (3'UTR) was done using *Leishmania donovani* genomic DNA as template. Overall strategy for gene knockout is shown in **Figure 5.1**. Primers employed for this purpose were as follows: primer 1: 5'-CCTGCATTAGG CACAGACATGACGG- 3' and primer 2: 5' –CGCTCGAGGGAAGAACGGAT- 3', for 5'UTR amplification, primer 3: 5'-CGAGCTTCGGTACTATTATGAG- 3' and primer 4: 5'-CGGGATCATGGAGGT GGAG-3' for 3'UTR amplification. These amplified products were further cloned in *Leishmania* expression vectors pXG B1288 (NEO), pXG B3318 (HYG) and pXGB3325 (PAC) using *XhoI- Eco*NI and *BamHI-SacI* as restriction sites for 5' UTR and 3' UTR respectively. For complementation vector, 0.675 Kbp CAAX prenyl protease II gene was amplified from *Leishmania donovani* genomic DNA using the following set of primers: primer 5: 5'-CCCCCGGGATGTGCTGCCTTGTCAG- 3' and primer 6: 5'-CGGGATCCTCAGTAGC GCAGCAGCG- 3'. *BamHI* and *XmaI* restriction sites were used to clone the gene in pXGB3324 (PHLEO) vector.

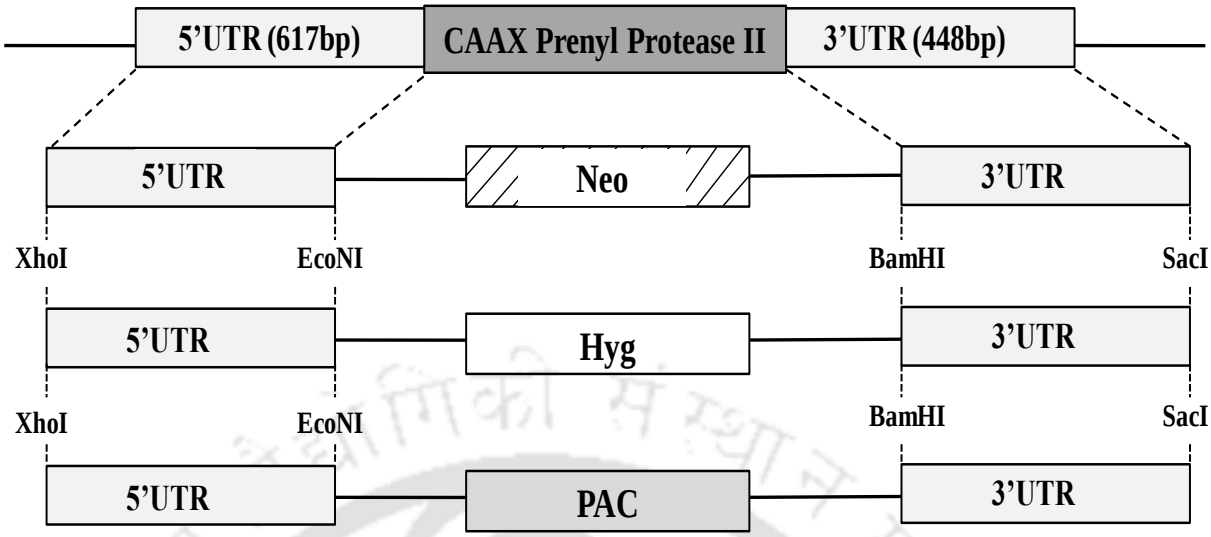



**Figure 5.1:** Schematic representation of CAAX prenyl protease II locus and molecular constructs employed for knockout by homologous recombination.

***5.3.4 Generation of knockout strains of CAAX prenyl protease II and complementation***

Removal of expression of CAAX prenyl protease II from the parasite was done by following gene knockout strategy based upon homologous recombination *(Beverley and Clayton, 1993; Bhardwaj et al., 2016)*. In brief, the knockout cassettes were linearized by *Eco*NI digestion and were further transfected into *Leishmania* cells via electroporation. Approximately $10^7$cells/ml were washed twice with PBSG buffer (10mM $NaH_2PO_4$, 10mM $Na_2HPO_4$, 145mM NaCl and 2% glucose) followed by re-suspension in electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM $Na_2HPO_4$, 6mM glucose). Further approximately 5-10mg/ml linearized knockout cassettes were added and cell suspension was transferred to ice cold electroporation cuvette. After 10 min of incubation on ice, electroporation was performed (Exponential protocol: Voltage_450V, Capacitance_500 F and the resulting time constant should be around 4.5ms), again followed by incubation on ice. The cells were then incubated in complete M199 media at 25°C for 24 hrs followed by transfer in antibiotic selection media. After first round of electroporation and selection, subsequent rounds of electroporation and selection were performed till we had all the three populations of knockout mutant viz., single knockout (CAAXII_SKO), double knockout (CAAXII_DKO) and triple knockout (CAAXII_TKO). The same method was employed for complemented

cells with slight alterations. Here CAAXII_TKO cells were transfected with CAAX prenyl protease II gene containing pXG B3324 (PHLEO) vector. The selection was performed in M199 complete media containing 20µg/ml of G148 for CAAXII_SKO cells, 20µg/ml of G148 and100µg/ml Hygromycin B for CAAXII_DKO cells, 20µg/ml of G148, 100µg/ml Hygromycin B and 10µg/ml Puromycin for CAAXII_TKO cells and finally 20µg/ml of G148, 100µg/ml Hygromycin B, 10µg/ml Puromycin and 5µg/ml Phleomycin for complemented cells. The selection was carried out for a week with continuous change in selection media. After the selection and confirmation of knockout as well as complemented cells, the cells were transferred to normal media for all subsequent experiments.

### *5.3.5 Generation of antibodies against CAAX prenyl protease II*

Antibodies against CAAX prenyl protease II were prepared in collaboration with Abgenex Pvt. Ltd. (Project ID: CP-49-15). Antibodies were obtained by immunization of rabbits with synthetic peptide (DAYRNCEDKGVAGNDRDEKER), which was derived from *Leishmania donovani* sequence. The antibodies obtained were then affinity purified using Protein A. Indirect ELISA was further performed. The Protein A purified CAAX prenyl protease II antibodies (200ng) were tested against 200ng custom antigen and pre-bleed was used as a control as shown in **Figure 5.2 (**the experiment was done by Abgenex Pvt. Ltd.).

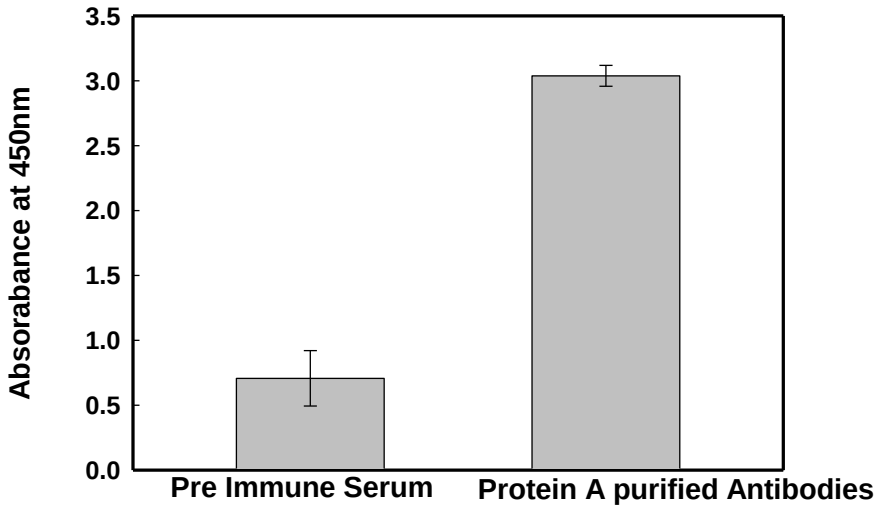



**Figure 5.2: Indirect ELISA:** Protein A purified antibodies (200ng), tested against 200ng of custom antigen and reading was taken at 450 nm. The O.D. obtained was plotted in bar graph. Pre-immune sera was used as control in place of primary antidodies at 1:5000 dilutions. Plate was read after 3 min of enzyme substrate reaction.

### *5.3.6 Confirmation of knockout cells*

Confirmed knockout was done by selection in Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin and Phleomycin (5 µg/ml). Further, complementation is confirmed by selection in Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin and Phleomycin (5 µg/ml). Further, it was substantiated by western blot analysis. For western blot, cell lysate of wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complemented *Leishmania* cells was obtained by performing cell lysis and further western blotting was done using the procedure reported earlier *(Bhardwaj et al., 2016)*. In brief, cells were harvested and washed in cold PBS (pH 7.4) followed by re-suspension in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1mM DTT, 15 µl/ml protease cocktail inhibitor, pH 7.4) and sonication ( Time duration: 3min, Pulse On: 2 sec, Pulse Off: 5 sec). Cell debris were removed by centrifugation. Protein was quantified using Bradford method and an equal amount of protein, was then separated on SDS PAGE gel (12 % running and 5 % stacking). Resolved proteins were transferred to polyvinylidene fluoride membrane and blocked by incubating it with 5% skim milk solution, overnight. This was followed by CAAX prenyl protease II antibodies (1:1000) treatment for 2-3 hrs, at RT and then goat-HRP-anti-rabbit IgG secondary antibodies (1:5000) treatment for 1 hr, at RT. Immunoreactive bands were observed using Novex® ECL chemiluminescence substrate reagent kit. The blot was then stripped using stripping buffer (100 mM glycine, pH 2.5, 200 mM NaCl, 0.1% Tween 20 (v/v) and 0.1% (v/v) β-mercaptoethanol). This was followed with treatment with mouse anti-α-tubulin (1:1000) for 2 hrs at RT and then rabbit-anti-mouse- IgG (H+L) peroxidase conjugated secondary antibodies (1:5000) treatment for 1 hr at RT. The bands were again visualized using chemiluminescent reagent.

### *5.3.7 Growth curve determination*

Comparative growth curve analysis of wild type *Leishmania* cells with knocked out mutants (CAAXII_SKO, CAAXII_DKO, CAAXII_TKO) and complemented cells was done in order to observe the effect on the growth of the parasite after removal of CAAX prenyl protease II. In short, ~$10^5$ cells/ml were inoculated in 10ml complete M199 media, in triplicates. This was followed by cell counting at a fixed time, using hemocytometer under an inverted light

microscope (Motic® AE31). The data obtained was then plotted (No. of cells v/s No. of Days) in SigmaPlot, in order to obtain a comparative growth curve.

### *5.3.8 Ras localization studies by immunofluorescence*

The effect on the localization of Ras protein within the *Leishmania* parasite due to removal of CAAX prenyl protease II was studied using immunofluorescence approach *(Dutta et al., 2011; Kaur et al., 2013).* Briefly, ~$10^7$ cells (wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complement cells) were harvested and washed in cold PBS (pH 7.4). Cells were then fixed by incubating them in 4% paraformaldehyde, on ice for 1hr. This was followed up by washing (10min wash X 3, PBS pH 7.4) and attachment of fixed cells on poly-L-lysine coated glass slides. The dried smear of cells, on the slide, was washed (5 min wash X 3, PBS pH 7.4). The next step was permeablization of attached cells, by incubating them with permeablization buffer (0.2% Triton X-100 and 5% FBS dissolved in PBS pH7.4) for 30 min. Again the cells were washed (5 min wash X 3, PBS pH 7.4) and the slides were incubated overnight in blocking buffer (2% BSA, 0.02% Tween-20 and 0.1% Triton X-100 dissolved in PBS pH 7.4) at 4°C. The next day cells were incubated with Rabbit-Anti-Ras antibodies (1:250) for 1hr at room temperature followed by washing (5 min wash X 3, PBS pH 7.4). After washing, cells were incubated with Anti-rabbit-FITC secondary antibodies (1:320) in the dark for 1 hr at room temperature. The slides were then observed under confocal laser scanning microscope (Leica DMi8).

### *5.3.9 Cell cycle analysis*

The cell cycle of knocked out population of cells (CAAXII_SKO, CAAXII_DKO, CAAXII_TKO), along with the wild type and complemented cells was assessed using flow cytometry *(Kaur et al., 2013).* In brief, ~$10^7$ cells/ml were washed with PBS (pH 7.4) and fixed. For fixing, 70% ethanol was added drop wise to the washed cell pellet, with constant stirring. The cells were then incubated with 70% ethanol overnight at 4°C. The subsequent day cells were washed and re-suspended in PBS (pH 7.4), followed by RNase A (100µg/ml) at 37°C for 1 hr. Next step involved incubation of cells with propidium iodide (5µg/ml) for 45 min followed by analysis using flow cytometer. The data obtained was processed using ModFit LT software for Win 7.

### *5.3.10 Macrophage infectivity assay*

In order to analyze the effect of removal of CAAX prenyl protease II on the infectivity of the parasite, macrophage infectivity assay was performed using method reported earlier *(Saudagar et al., 2013)*. In short, macrophage cell line J774A.1 was grown overnight in 6-well plate in DMEM media supplemented with 10% heat inactivated FBS, 100µg/ml penicillin and 100µg/ml streptomycin. Next day the non adherent cells were removed by PBS (pH 7.4) washing and fresh media was added. The macrophages present in different wells were infected with different populations of *Leishmania* promastigotes (wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complement cells) such that the parasite: macrophage ratio is maintained to 10:1. The 6-well plate was then incubated overnight at 37 ºC in 5% $CO_2$. Subsequent cell counting was done depending upon the different time periods. For cell counting, infected macrophages were stained with Geimsa dye. Randomly 200 cells were counted and infection index was measured. Infection Index = AXB, here A is percentage of infected macrophages (out of 200) and B is geometric mean of number of parasites per infected macrophage.

### *5.3.11 Statistical analysis*

All the data was expressed as mean ± standard deviation. Unpaired student's t- test was performed using SigmaPlot, in order to determine differences between two groups by analyzing statistical significance. Differences were considered significant at 0.0005 and 0.0001 level of confidence.

## 5.3 RESULTS

### *5.4.1 Confirmation of knockout of CAAX prenyl protease*

The removal of CAAX prenyl protease II expression from *Leishmania donovani* was confirmed selection in Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin and Phleomycin (5 µg/ml). Further, complementation is confirmed by selection in Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin and Phleomycin (5 µg/ml). The western blot experiments gave several non specific bands even after several attempts.

However, band corresponding to molecular mass of CAAX prenyl protease II hints towards removal of CAAX prenyl protease II **(Figure 5.3)**.

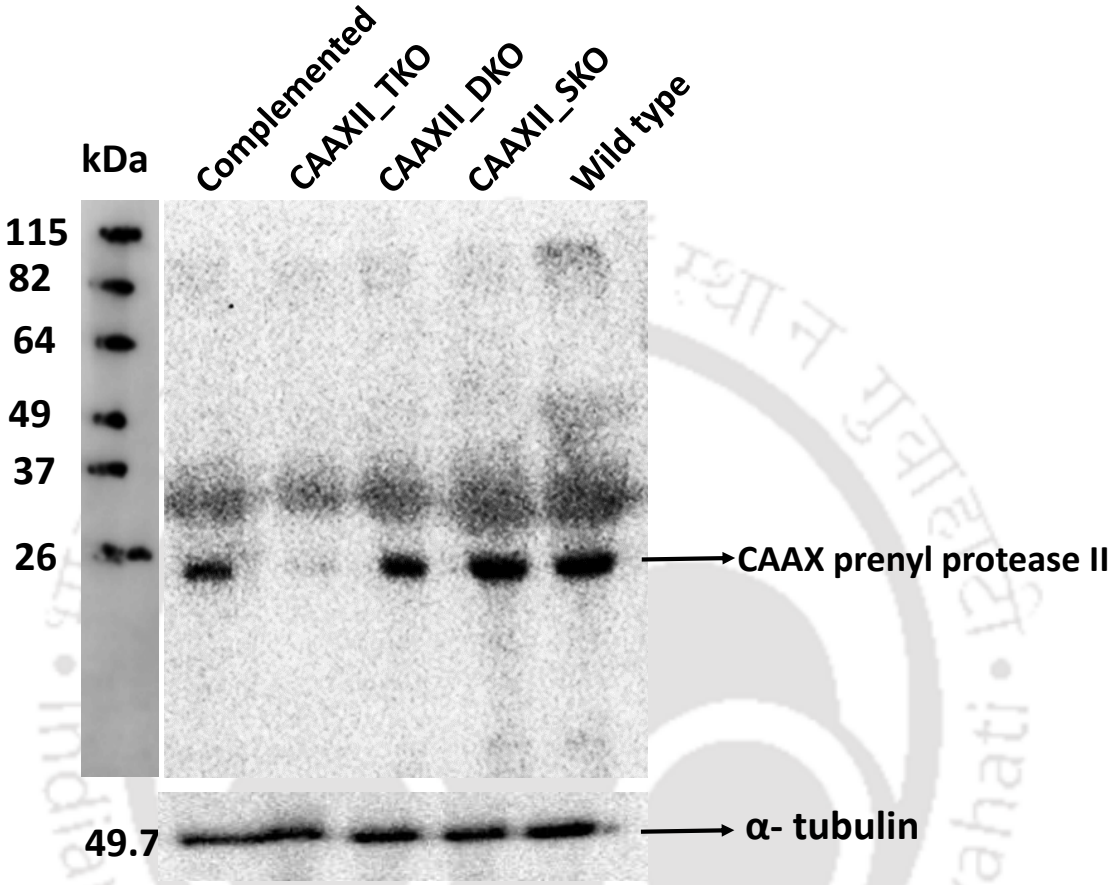



**Figure 5.3: Western Blot data to confirm knockout of CAAX prenyl protease:** Western Blot data to confirm knockout of CAAX prenyl protease. Equal amount of protein from wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complemented cell lysates was loaded on SDS PAGE gel. The protein bands separated on SDS-PAGE were then transferred to PVDF membrane. This membrane was then treated with anti-CAAX prenyl protease II antibodies and further subjected to HRP-conjugated secondary antibodies. Protein band for CAAX prenyl protease II (24.8 kDa) was absent for CAAXII_TKO lane and the intensity of the band for complemented cells lysate was less, pointing out towards the low episomal expression in complemented cells. ChemiDoc™ XRS+ System was used to take epi white illumination as well as chemiluminescent image of the blot. The protein ladder is depicted using epi white illumination image and shown as sliced lane. The protein bands developed are represented using chemiluminescent image of the blot. α-tubulin was used as an endogenous control. Some non- specific bands due to poor specificity of polyclonal antibody were ignored. The data together with selection in Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin and Phleomycin (5 µg/ml) gives confidence about knockout. Further, complementation data is also supported by selection in Geneticin G148 (20 µg/ml) Hygromycin B (100µg/ml), Puromycin and Phleomycin (5 µg/ml).

### *5.4.2 Removal of CAAX Prenyl Protease II led to significant stoop in cell growth curve*

After selection of knockout mutants, comparative growth curve analysis was done. The results obtained showed that compared to the wild type cells, CAAXII_TKO and CAAXII_DKO had significantly lower growth rate. On the other hand, growth rate of CAAXII_SKO and complemented cells was between wild type and CAAXII_DKO. The removal of CAAX prenyl protease II expression, inside the parasite, did not cause cell death. Though the cells were growing, but the growth rate was significantly slower compared to wild type cells **(Figure 5.4)**.

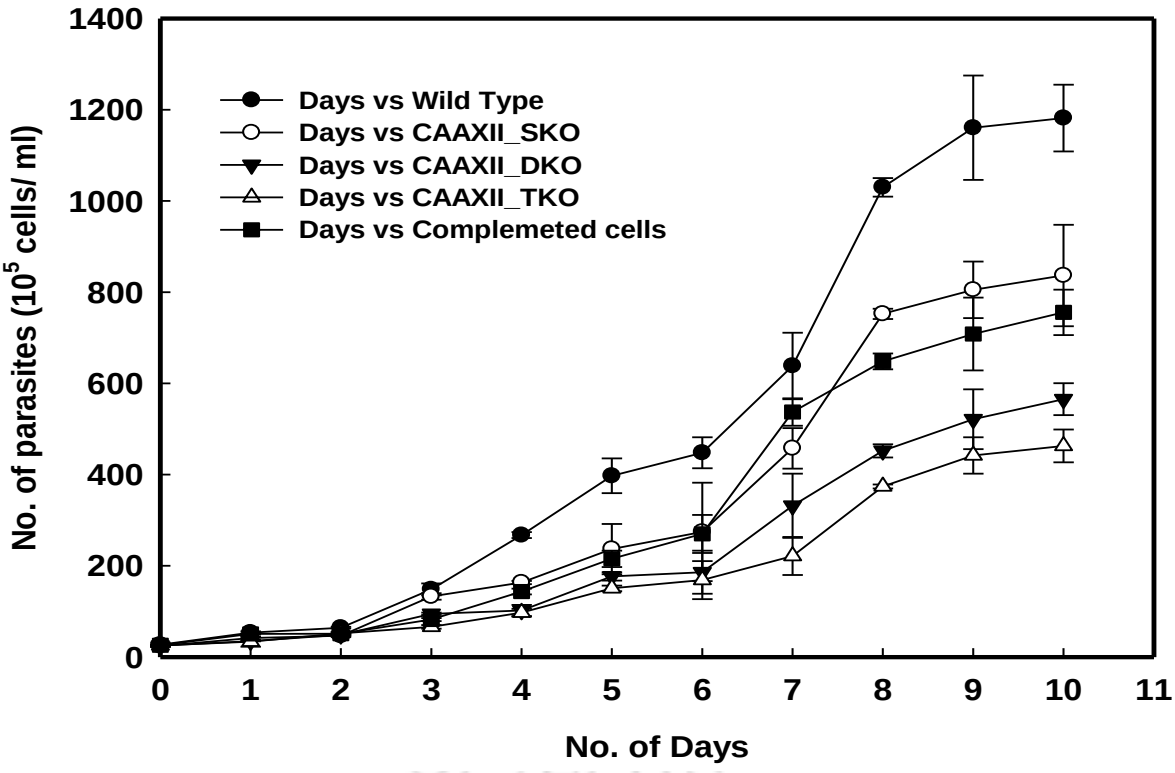



**Figure 5.4: Relative growth curve analysis:** The inference drawn from the comparative growth curve was that compared to wild type cells, the growth rate of CAAXII_TKO cells was significantly lower. There was a marginal difference between the growth rate of CAAXII_TKO versus CAAXII_DKO cells and CAAXII_SKO versus complemented cells. Though the growth rate of CAAX prenyl protease II knocked out cells was low compared to wild type cells, but the result was not fatal.

### *5.4.3 Diffused localization of Ras protein in the absence of CAAX Prenyl Protease II*

CAAX prenyl protease II is involved in the post translational modification of Ras proteins. A properly modified Ras protein will attach itself to the cell membrane. If the Ras protein is not modified, then attachment of the protein to the membrane is hindered and it stays in the cytoplasm. The anti-Ras antibodies used for this study were commercially available polyclonal antibodies, raised against synthetic peptide corresponding to immunogenic region of v-Ha-ras Harvey rat sarcoma viral oncogene homolog. Immunofluorescence studies were done to observe, how removal of CAAX prenyl protease II was affecting the localization of Ras proteins within the cell. The results obtained clearly indicated that with every copy knockout, the more and more Ras protein had a diffused distribution from the cytoplasm to the cell membrane **(Figure 5.5)**. Image J software was used in order to do a comparative analysis of diffused fluorescence signal within the cytoplasm of different cell populations. In case of WT cells the signal was more prominent in the cell membrane and very less signal observed in cytoplasm.

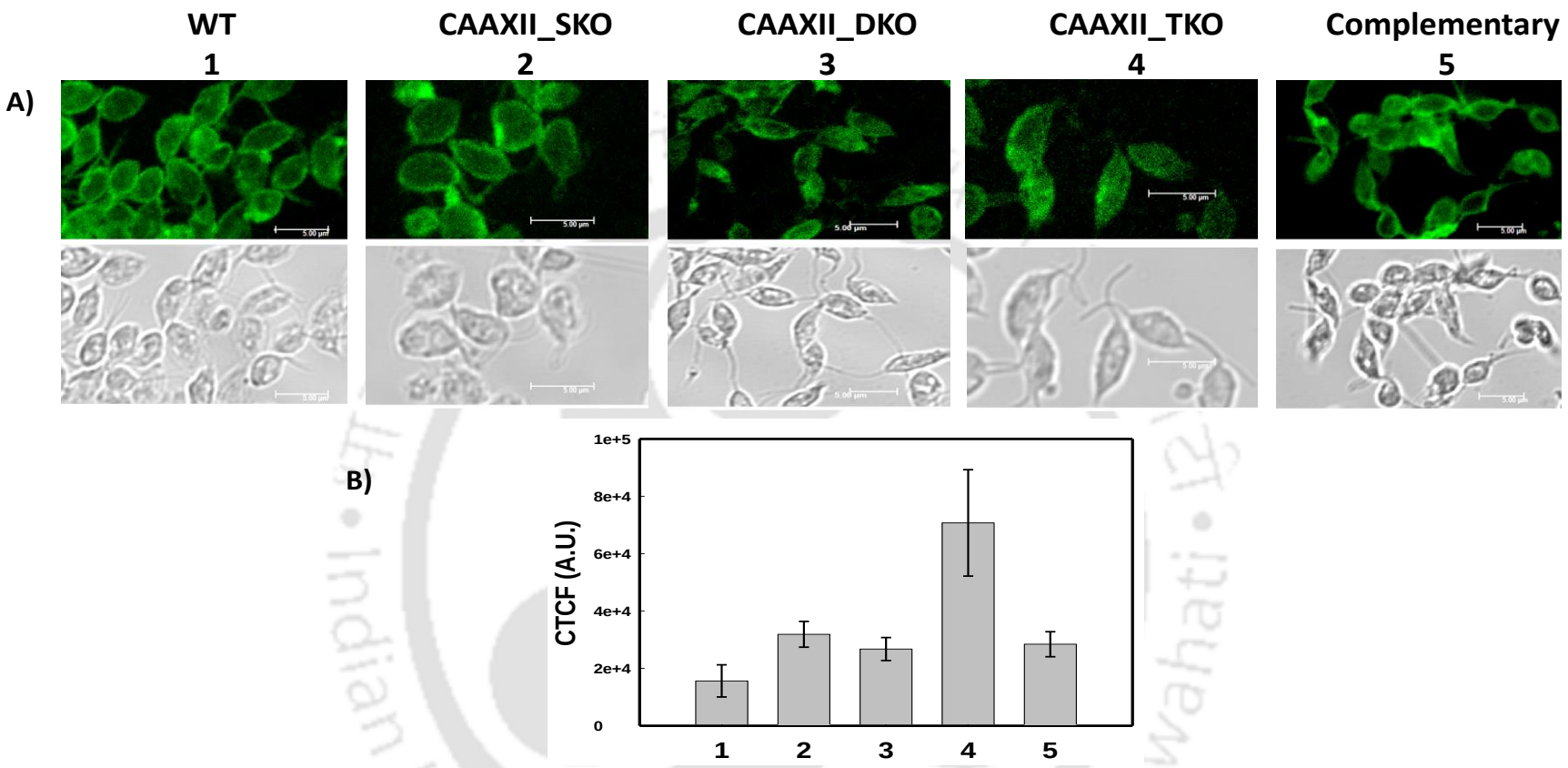


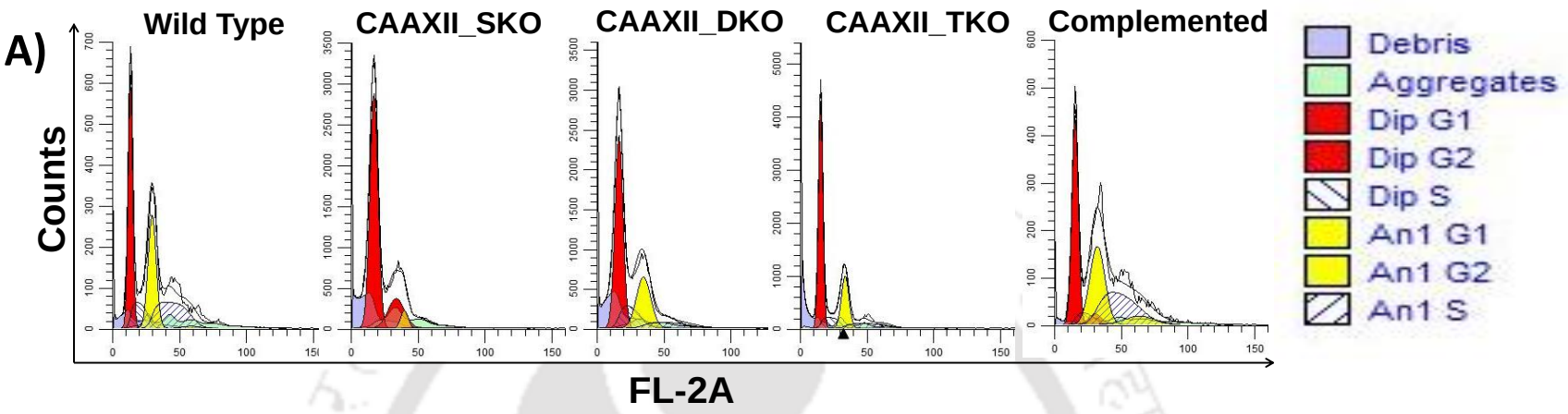

**Figure 5.5: Ras localization studies:** CAAX prenyl protease II play a key role in maturation process of Ras protein. After maturation Ras protein attaches itself to the membrane of the cell. Immunofluorescence studies were done in order to study the localization of Ras protein after CAAX prenyl protease II removal. Rabbit-anti Ras antibodies were used as primary antibodies, while anti-rabbit FITC conjugated antibodies were used as secondary antibodies for the immunofluorescence assay. In case of wild type cells, most of the Ras protein was concentrated in the cell membrane and reduced amount of Ras protein in the cytoplasm. Interestingly, with every knockout of CAAX prenyl protease II allele, the fluorescence signal diffused more and more from the cell membrane towards the cytoplasm. This indicated that the localization of Ras protein is affected due to removal of CAAX prenyl protease II. In case of complemented cells, some shift of fluorescence signal from cytoplasm to cell membrane was observed. The following data shown is a representative experiment of at least three experiments which gave similar results. **B)** Corrected total cell fluorescence (CTCF) is measured using Image J software. The software is used to measure the diffused fluorescence signal inside the cell i.e. cytoplasm. The membrane fluorescence was excluded in the analysis. The error bar represents standard error of fluorescence analyses of multiple cells. The brightness of the image was increased to improve the resolution of the image in a printout.

In case of CAAXII_SKO, CAAXII_DKO and complemented cells, both populations were observed i.e. cells with properly localized Ras protein and cells with diffused Ras localization. In CAAXII_TKO cells the fluorescence signal was diffused in the cytoplasm completely. Though Ras proteins were present in the cell membrane as well in CAAXII_TKO cells, but signal was significantly low. The data suggests that some Ras proteins localized in CAAXII_TKO cells even when CAAX prenyl protease II is completely knocked out might have been processed by other isoform of the protein (CAAX prenyl protease I).

### *5.4.3 Delayed transition from G1 to S phase observed during cell cycle analysis of knockout mutants*

The *Leishmania* parasite contains both aneuploidy and diploid cells in its population, hence both the cell types were considered for cell-cycle analysis of the population (Sterkers et al., 2014). Our results have pointed out that the removal of CAAX prenyl protease II from *Leishmania donovani* leads to mislocalization of Ras proteins. It is known that Ras proteins are molecular switches to various cellular processes. Cell division is one such process affected by the mislocalization of Ras protein. Cell cycle was analyzed and the data pointed out towards the delayed transition from G1 to S phase in knockout cells (Figure 5.6). On comparing both diploid and aneuploidy populations of cells, the transition of cells from G1 to S phase is significantly lower than control cells. A comparative table giving different percentage of cells in different cell cycle phases is shown in Figure 5.6 B.

A)




B)

| Type of Cells | Diploid | | | | Aneuploid | | | |
|---|---|---|---|---|---|---|---|---|
| | % population | G1 (%) | G2 (%) | S (%) | % population | G1 (%) | G2 (%) | S (%) |
| Wild Type | 46.14% | 76.15 | 0.00 | 23.85 | 53.86% | 52.65 | 1.81 | 45.54 |
| CAAXII_SKO | 94.65% | 79.74 | 20.09 | 0.17 | 5.35% | 94.73 | 0.00 | 5.27 |
| CAAXII_DKO | 67.50% | 78.97 | 0.00 | 21.03 | 32.50% | 77.04 | 0.00 | 22.96 |
| CAAXII_TKO | 68.20% | 85.45 | 0.00 | 14,55 | 31.80% | 71.23 | 0.00 | 28.77 |
| Complemented | 39.26% | 80.25 | 8.25 | 11.48 | 60.74% | 45.54 | 9.85 | 44.61 |

**Figure 5.6: Cell cycle analysis:** In order to determine the effect on cell cycle after removal of CAAX prenyl protease II, the cells (wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complemented cells) were labeled with propidium iodide and sample was read using flow cytometry and ModFit LT4.1 software was used for data analysis. **(A)** Data represented in the form of histogram. **(B)** Data represented in the form of a table containing values obtained from **A**. From both **A** and **B**, it can be observed that the transition from G1 to S phase was reduced in knockout cells, when compared to the wild type. The complemented cells too showed lower transition from G1 to S phase. This could be due the partial recovery of protein function, as the complemented cells possessed episomal CAAX prenyl protease II expression. The following data shown is a representative experiment of at least three experiments which gave similar results.

### *5.4.5 The infectivity of the parasite is reduced in the absence of CAAX Prenyl Protease II*

Infectivity of *Leishmania* promastigote after removal of CAAX prenyl protease II activity was analyzed by manually counting 200 Giemsa stained, infected macrophages (J774A.1). Infection index of all the infected macrophages at different time periods was calculated and plotted (Figure 5). After 12 hrs, the infection index for wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complement infected macrophages was same. The considerable change was observed after 24 hrs, where compared to wild type, the infection index for CAAXII_TKO was significantly lower. The same trend was observed for CAAXII_TKO after 36 hrs. The infection index for CAAXII_SKO, CAAXII_DKO and complement promastigotes was found to be lower when compared to wild type after 24 and 36 hrs respectively. Though the infection index of CAAXII_SKO and complemented cells was comparable to each other, but when compared to CAAXII_TKO and CAAXII_DKO cells, the infection index was high **(Figure 5.7)**.

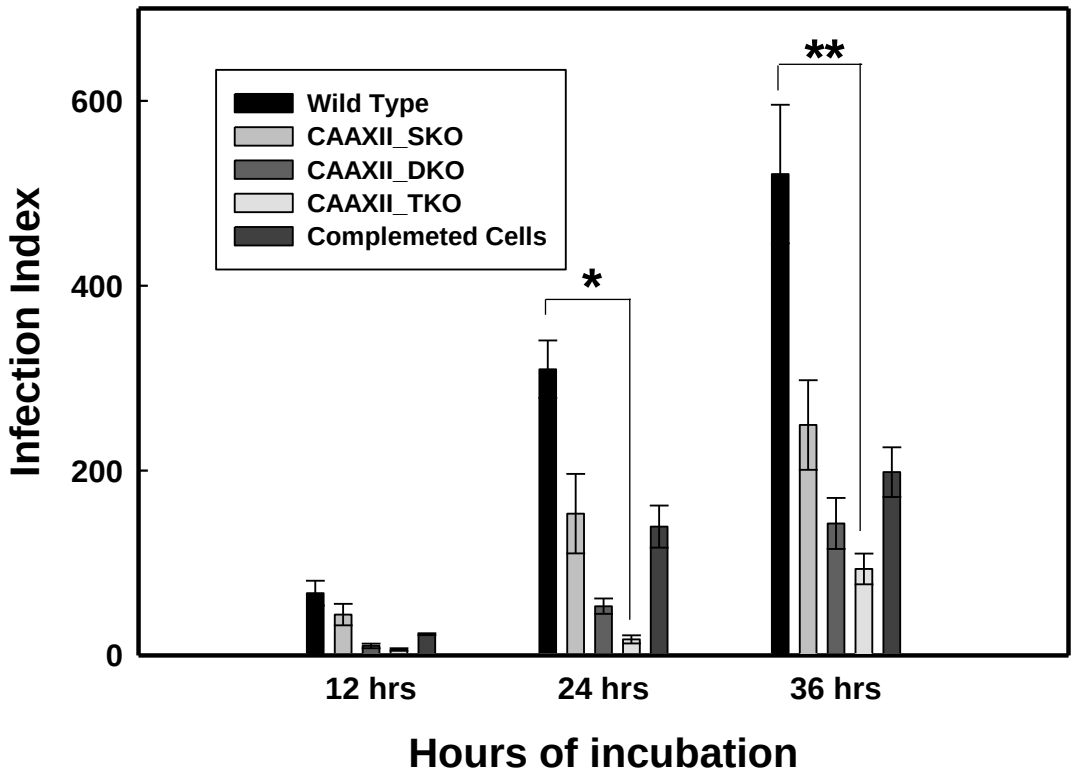



**Figure 5.7: Infectivity Assay:** Different time periods were used to study the infection of macrophages by *Leishmania* knockouts and complemented cells. Giemsa staining of macrophages was done for cell counting under inverted light microscope under 60X lens. The infection index (percentage of infected macrophage multiplied by geometric mean of number of amastigotes per infected macrophage) was calculated. Compared to the wild type, CAAXII_TKO and CAAXII_DKO cells showed significantly lower infection index, even after 36 hrs the infection index for CAAXII_SKO and complemented cells was low as well, when compared to wild type. Data with P values of < 0.0001 (*) and 0.0005 (**) were considered statistically significant.

## 5.5 DISCUSSION

CAAX prenyl protease II is known to play a key role in prenylation pathway, leading to maturation of signal proteins. Mature Ras proteins bind to the cell membrane of the parasite and act as molecular switches for various cellular processes *(Casey, 1992)*. Hence, we expected that complete removal of this protein should obstruct the prenylation pathway leading to events which would be catastrophic for the parasite. But interestingly, we discovered that removal of CAAX prenyl protease II was not fatal for *Leishmania* parasite. We observed reduced growth rate in knockout cells, but still parasite death did not occur. In case of complemented cells, the growth rate was lower than wild type cells. This could be due to the fact that the expression given back to the null mutants was in the form of episomal

expression. Hence the protein amount present in complemented cells helps out with only partial recovery from the loss *(Taheri et al., 2014)*. This is also substantiated by lower intensity western blot band of CAAX prenyl protease II in complemented cells compared to wild type **(Figure 1B)**. We also carried out Ras protein localization studies, which would determine the proper maturation of Ras protein. The immunofluorescence results showed that, compared to wild type cells, the null mutants (CAAXII_TKO cells) had diffused distribution of Ras protein inside the cytoplasm. However, Ras molecules were still sparsely distributed in the cell membrane. This indicated towards the presence of matured Ras protein, even if CAAX prenyl protease II was totally knocked out (CAAXII_TKO cells). Matured Ras proteins bound to the parasite cell membrane are known to play a key role in signaling cascades which lead to processes like cell proliferation and parasite infectivity. Following the line, we observed that the null mutants showed slower G1 to S phase transition for cell cycle and significantly reduced infectivity towards macrophages. We inferred from this study that knockout of CAAX prenyl protease II is disturbing the prenylation pathway of the parasite but not completely stopping it. This points out towards the presence of another protein, inside the parasite, which is partially helping it out to cope up with the loss. The genome analysis of *Leishmania donovani* points out towards the co-existence of both the isoforms of CAAX prenyl proteases i.e. Type I and Type II. Type I is known to be a metalloprotease *(Schmidt et al., 2000)*. The specific nature of Type II is still not clear. Some studies report CAAX prenyl protease II to be metalloproteases *(Pei and Grishin, 2001)*, while other suggests them to be cysteine proteases *(Dolence et al., 2000; Pie et al., 2011)*. Interestingly, studies have shown that both the proteases have distinct but overlapping substrate specificity *(Boyartchuk et al., 1997; Tam et al., 2001)*. Hence there could be a possibility that when the expression of CAAX prenyl protease II is removed from *Leishmania donovnai*, CAAX prenyl protease I is partially filling in for its duty. Thus, CAAX prenyl protease II alone may not be a very effective drug target. However, CAAX prenyl protease II as one of the targets in combination with other validated targets could be a very effective approach. It is worth mentioning that the combinatorial therapy targeting two distinct pathways is always a better approach toward management of infection.

# CHAPTER VI

## Summary of research performed

Our work provides clear evidence about key function(s) of hypothetical protein, LdBPK_070020, for survival of the parasite. LdBPK_070020 knocked out strain of *Leishmania donovani* could not survive due to defective mitochondrial function. Further, the finding reported in the thesis clearly indicates that the conserved hypothetical protein LdBPK_070020 was present inside the nucleus as well as the kinetoplast. The protein is possibly altering mitochondrial function indirectly by regulating expression of other proteins. CAAX prenyl protease II knockout also resulted in significant decrease in growth rate and infectivity of the parasite. However, parasite could still survive, possibly due to existence for another isoform CAAX prenyl protease I. Thus, CAAX prenyl protease II alone may not be a very effective drug target. However, CAAX prenyl protease II as one of the targets in combination with LdBPK_070020 could be a very effective approach.

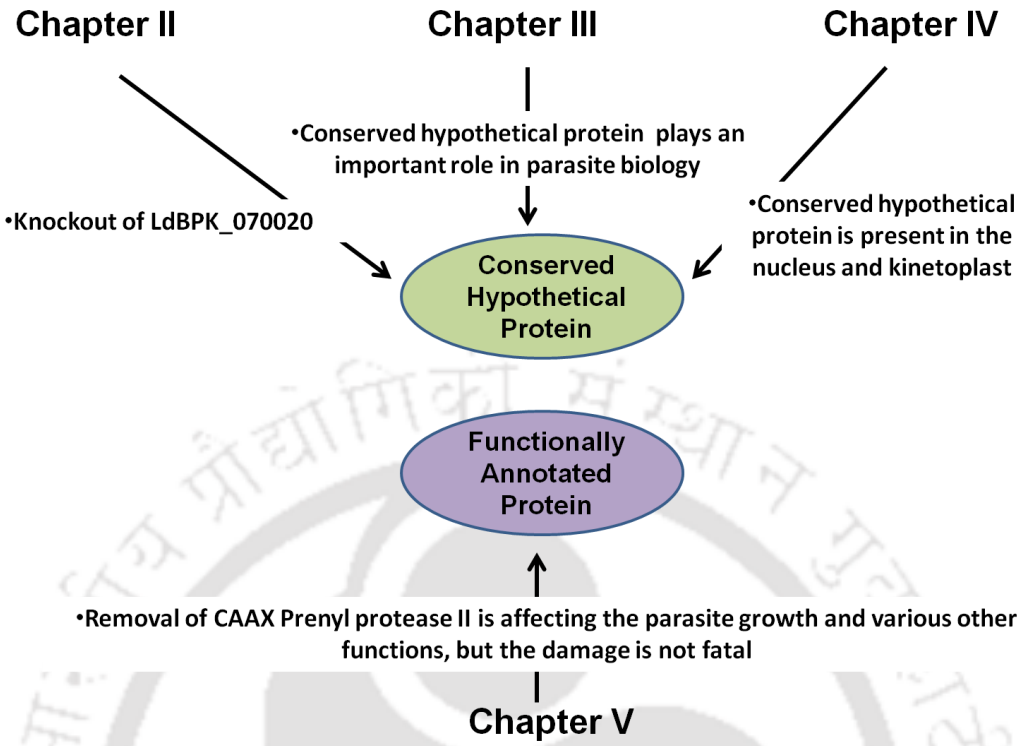



**Figure 6.1:** The figure shows overall outcome of the PhD work. The conclusions from the results obtained from experimental chapters are shown in the image.

### *6.1 Removal of LdBPK_070020 expression from Leishmania donovani by employing gene knockout strategy*

***Conclusion:*** The removal of LdBPK_070020 from *Leishmania donovani* was successful by employing gene knockout strategy. The complemented cells were successfully prepared as well. Knockout was confirmed by PCR and western blot. Western blot results pointed out towards the lower amount of LdBPK_070020 protein in CKO_HP cells as a result of episomal expression.

### *6.2 Understanding the importance of LdBPK_070020 for the parasite biology by studying the knockout mutants*

***Conclusion:*** We observe a vast array of changes in the parasite, after the removal of LdBPK_70020 expression. The changes are not only phenotypic but various biochemical

processes are also affected. It is very conclusive that the knock out of the gene results in alteration in mitochondrial function. However, the precise mechanism is still unclear. The protein may be localized in mitochondria and performs some key function or it may be localized in nucleus and regulates the expression of a key protein which is involved in mitochondrial function. Hence localization studies were done for more insight into the function of the parasite.

### *6.3 Localization studies of conserved hypothetical protein LdBPK_070020 inside the parasite*

***Conclusion:*** The finding of the study clearly indicates that the conserved hypothetical protein LdBPK_070020 is present inside the nucleus as well as the kinetoplast. This is very interesting, as it throws some light towards the probable function of the conserved hypothetical protein. We predicted in the earlier chapter that removal of LdBPK_070020 is altering the mitochondrial functions. As the protein is not located in mitochondria but present in the nucleus, hence the protein is altering mitochondrial function indirectly by regulating expression of other mitochondrial proteins.

### *6.4 Deciphering the role of CAAX prenyl protease II as a target in Leishmania donovani*

***Conclusion:*** We inferred from this study that knockout of CAAX prenyl protease II is disturbing the prenylation pathway of the parasite but not obstructing it. This points out towards the presence of another protein, inside the parasite, which is partially helping it out to cope up with the loss. The genome analysis of *Leishmania donovani* suggests co-existence of both the isoforms of CAAX prenyl proteases *i.e.* Type I and Type II. Hence it could be possible that when the expression of CAAX prenyl protease II is removed from *Leishmania donovnai*, CAAX prenyl protease I is partially filling in for its duty and the CAAX prenyl protease II knocked out parasite is somehow able to compete with the loss of CAAX prenyl protease II activity and survives.

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## Vitae

**Ruchika Bhardwaj** was born on 25th June, 1988 in Baijnath, Himachal Pradesh, India. She did her schooling from S.D Public School, Pitampura, New Delhi and completed her Higher Seconadry Education in 2006. She earned her B.Tech degree in Biotechnology from University School of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi in 2010 and received her M.Tech degree in Biotechnology from the Indian Institute of Technology Guwahati in 2012. Having qualified the Graduate Aptitute Test in Engineering (GATE) examination, she joined the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati for her doctoral research in July 2012. During this period she was recipient of reseach fellowship from IIT Guwahati.

### Journal Publications from PhD thesis

- **Ruchika Bhardwaj**, Sanjeev Kumar Singh and Vikash Kumar Dubey*. Localization studies on LdBPK _070020, a conserved protein, of ***Leishmania donovani.*** **Journal of Vector Borne Diseases** , Accepted [Publisher: Medknow@ Indian Council of Medical Research]

- **Ruchika Bhardwaj**, Ritesh Kumar, Sanjeev Kumar Singh, Chandrabose Selvaraj and Vikash Kumar Dubey*. Understanding the importance of conservative hypothetical protein LdBPK_070020 in *Leishmania donovani* and its role in subsistence of the parasite. Archives of Biochemistry and Biophysics, 2016, 596, 10-21.

- **Ruchika Bhardwaj**, Mousumi Das, Shalini Singh, Sanjeev Kumar Singh and Vikash Kumar Dubey*. Studying the importance of the prenylation pathway of Leishmania donovani by removing the expression of CAAX prenyl protease II. (2016) Manuscript under preparation.

### Journal Publications from collaborative work

- Shalini Singh, Ekta Kumari, Ruchika Bhardwaj and Vikash Kumar Dubey *. Molecular events leading to death of *Leishmania donovani* under spermidine starvation after hypericin treatment. . **Infection, Genetics and Evolution**, under revision.

- Shalini Singh, Shyamali Sarma , Shashank P Katiyar , Mousumi Das , **Ruchika Bhardwaj** , Durai Sundar Vikash Kumar Dubey***.** Probing molecular mechanism of hypericin induced parasitic death: An insight into role of spermidine beyond redox metabolism of Leishmania. **Antimicrobial Agents and Chemotherapy**, 2015, 59, 15-24.

- Shalini Singh, Sitrarasu Vijaya Prabhu, Venkatesan Suryanarayanan, **Ruchika Bhardwaj**, Sanjeev Kumar Singh and Vikash Kumar Dubey*. Molecular docking and structure based virtual screening studies of CAAX prenyl protease I &II. **Journal of Molecular Structure and Dynamics, 2016**,1-20,

## Other publications

- **Ruchika Bhardwaj**, Prakash Saudagar and Vikash Kumar Dubey*. Nanobiosciences: A contemporary line for Antiparasitic Drug. ***Molecular and Cellular Pharmacology,*** 2012, 4, 97-103.

## Conference and Workshop Proceedings during PhD work

- Ruchika Bhardwaj, and Vikash Kumar Dubey. Probing the significance of LdBPK_070020, a conserved hypothetical protein, for incessant survival of *Leishmania donovani.* Research Conclave' 16 organized by Students' Academic Board, IIT Guwahati, 2016.

- Participated in the hands on training program on Confocal Laser Scanning Microscopy, organized by Guwahati Biotech Park in association with Leica Microsystems, held on 3$^{rd}$ -5$^{th}$ February2016.
- Participated in conference on Bioinformatics and Computer Aided Drug Design organized by DBT-Bioinformatics Infrastructure Facility, Department of Biosciences and Bioengineering, IIT Guwahati, during 7$^{th}$ December 2015.
- Participated in Recent Development in Medical Biotechnology and Structure Based Drug Designing, organized by Department of Biosciences and Bioengineering, IIT Guwahati, held on 6$^{th}$ -7$^{th}$ December, 2015.
- Participated in Symposium cum workshop on Advances in Computational Biology and Computer Aided Drug Designing, organized by Bioinformatics Infrastructure Facility, Department of Biosciences and Bioengineering, IIT Guwahati, held on 24$^{th}$-26$^{th}$ June, 2015.
- Ruchika Bhardwaj and Vikash Kumar Dubey. Investigating the role of a conserved hypothetical protein LdBPK_070020 for the continued existence of *Leishmania donovani*. 83rd Annual Meeting of Society of Biological Chemists (India), 18$^{th}$ -21$^{st}$ December, 2014, Odisha, Bhubaneshwar.