To identify and characterize LdUrm 1-LdUba4 conjugation pathway in Leishmania donovani
Introduction

The post-translational modification of intracellular proteins by ubiquitin (Ub) and ubiquitin-like (UbL) proteins is a basic mechanism for regulating and fine-tuning all cellular processes. Indeed, this equips the metabolic machinery of cell to control stability, function and its localisation of many key protein and, therefore, they are instrumental in regulating cellular functions as diverse as protein degradation, cell signalling, vesicular trafficking and immune response. It is thus no surprise that pathogens in general, and parasites with multiple hosts in particular, have developed diverse strategies to either counteract or exploit the complex mechanisms mediated by the Ub and Ubl protein conjugation pathways.

Ubiquitin (Ub) and ubiquitin-like (Ubl) proteins regulate a wide array of cellular pathways by modifying the target protein substrates. Ub/Ubl-mediated signaling involves a cascade of enzymes including E1: Ubl-activating enzymes, E2: Ubl carrier proteins, and E3: Ubl ligases - mediated conjugation, transduced by proteins that recognize Ub/Ubl-modified substrates, and terminated by proteases which remove the Ub/Ubl from the substrate. E1 enzymes initiate pathway specificity for each cascade by recognizing and activating cognate Ubls, followed by catalyzing Ubl transfer to cognate E2 protein(s). Under certain circumstances, the E2 Ubl complex can direct ligation to the target protein, but most often requires the cooperative activity of E3 ligases. E3 ligases confer substrate specificity to the process by conjugating the activated Ub/Ubl to its selected target (Ciechanover and Ben-Saadon, 2004). Ub/ Ubls are ultimately transferred to the ε-amino group of a lysine residue in a target protein to yield an isopeptide-linked Ub/Ubl conjugate. UBL APG8 is an exception to this which does not attach to proteins by isopeptide link but forms an amide bond with an amino group of a lipid (Ichimura et al., 2000).

Ubls are known to be prevalent in eukaryotes, however are not extensively studied in prokaryotes. There exist mechanistic parallels between activation by Ub and prokaryotic sulfur carrier proteins viz ThiS and MoaD from Escherichia coli (Pedrioli et al., 2008). Unlike eukaryotic Ubls, these proteins serve as sulfur carriers in thiamine and molybdopterin synthesis pathways, respectively. Studies towards understanding the functional similarities between the prokaryotic and eukaryotic systems using ThiS and MoaD as query sequences, led to identification of a URM1 (Ub-related modifier 1) in S. cerevisae (Furukawa et al., 2000). Urm1 conjugates to its substrate protein molecule to yield high-molecular weight adducts, and

LdUrm1-LdUba4 conjugation pathway
the process is termed asurmylation. The process depends on Uba4, the putative Ubiquitin activating (E1) enzyme required for Urm1 activation. However, other components of the conjugation pathway, such as E2 and E3, have not been identified.

Studies towards understanding the physiological role of Urm1 have identified its substrates with diverse functions. These included (i) Ahp1, an alkyl hydroperoxide reductase with a role in oxidative stress protection in yeast (Goehring et al., 2003); (ii) Tot1/YLR384C10, an elongator subunit (Fichtner et al., 2003); (iii) ATPBD3, an uncharacterized Urm1 dependent enzyme required for thiolation of certain anticodon nucleosides (Schlieker et al., 2008); (iv) a functional crosslink with TOR (target of rapamycin) signaling pathway (Leidel et al., 2009); (v) thiolation of wobble uridine u34 of cytosolic tRNAs (Nakai et al., 2008). The components of this pathway involve URM1, UBA4, NFS1, NCS2, NCS6 and YOR251C in yeast (Bjork et al., 2007; Nakai et al., 2008; Dewez et al., 2008; Huang et al., 2008; Leidel et al., 2009).

Although most work on Urm1 has been done in yeast, the pathway seems to be conserved in other eukaryotes, as implied by experiments in C. elegans (Dewez et al., 2008; Leidel et al., 2008) and in humans (Schmitz et al., 2008), the findings suggested that Urm1 is highly conserved molecule and takes up a range of molecular functions across different species.

The discovery of the targets for the above mentioned effector molecules, vizUbls would be an exciting step towards understanding the functional role they play during infection. However, much remains to be learned about these fascinating molecules. What are the types of Ubl conjugates that are formed and the downstream pathways involved? What are the host enzymes (i.e. E2s and E3s) they engage to carry out their function? Finally and arguably most important to understand is their actual role in pathogenesis, what are the substrates they target? Understanding the mechanisms by which Ubls restrict their activity to specific cellular locations would also help to clarify their role in host-pathogen interactions. The high degree of sequence conservation from yeast to human clues functional significance for Urm1 in higher eukaryotes. In addition to broaden our understanding of Leishmania pathogenesis, the study on Urm1 and its conjugates will likely develop insights into biology of the pathogen’s ubiquitination pathway and may help to pave the ways towards development of novel therapeutic strategies.
In the present section of the study we demonstrate the presence of an active Urm1-Uba4 conjugation pathway in *L. donovani* on the basis of functional analysis and identify its putative molecular targets. The study helps to understand the mechanistic significance of Urm1 conjugation pathway in *Leishmania* pathogenesis and opens avenue for new drug targets.

**Reagents and chemicals**

Unless otherwise stated, all fine chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Antifade mounting reagent Vectashield was purchased from Vector Laboratories INC. US. Fetal Bovine serum (FBS) was obtained from Gibco® Life Technologies. pKSNeo vector was received as a gift from Hira L. Nakhasi, CBER, FDA, USA with permission from Greg Matlashewski, McGill University Canada. Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (GE Healthcare, UK). Platinum Taq polymerase Hi-Fi and restriction enzymes were procured from Invitrogen and New England Biolabs, UK, respectively.

**Experimental Procedures**

**Parasite culture and generation of stress phenotypes**

*A. Isolation and propagation of parasites*

Parasite isolates of *L. donovani* were prepared from bone marrow aspirates of KA patients originating from Bihar and reporting to Safdarjung Hospital (SJH), New Delhi using NM+30% FCS, as described before (Salotra *et al.*, 2001). The diagnosis of KA was confirmed by demonstrating the presence of LD bodies in the bone marrow. Informed consent was obtained from patients before collecting the bone marrow samples, according to the guidelines of the Ethical Committee, SJH. The Promastigotes (Pro) were cultured in Medium 199, 25mM HEPES N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], 100 IU and 100μg/ml each of penicillin G and streptomycin sulphate, respectively from 10,000IU and 10,000μg/ml combined stock, supplemented with 10% heat- inactivated FBS (fetal bovine serum) at 24°C and 7.4 pH. Promastigote forms of these parasites were routinely maintained in 15 ml sterile conical centrifuge tubes and transferred into fresh medium every 4-5 days, as necessary.
B. Generation of axenic amastigotes

Amastigotes were generated and grown in vitro by serially adapting them to grow at elevated temperatures and reduced pH conditions according to the method described elsewhere (Debrabant et al., 2004). Initially, the promastigote were adapted to grow in Medium 199 in presence of 25mM HEPES supplemented with 10% FBS, further supplemented with a final concentration of 2mM L- Glutamine, 100μM adenosine, 23μM Folic acid, 100 IU and 100μg/ml each of penicillin G and streptomycin sulphate, respectively from 10,000 IU and 10,000 μg/ml combined stock, 1X RPMI vitamin mix, 4.2mM NaHCO₃. The final medium was adjusted with 2N HCl (drop wise, while stirring) to pH 6.8 at 26⁰C, sterilized by filtration (0.22μM, Millipore) and stored at 4⁰C prior to use. Promastigote forms of these parasites were transferred into fresh medium every 4-5 days which is an essential need for amastigote development.

The parasites were gradually adapted to grow at 26⁰C in potassium buffered (~140mM) RPMI based medium. This medium was formulated to contain the following salts at a final concentration of: KCl (15mM); KH₂PO₄ (114.6mM); KH₂PO₄.3H₂O (10.38mM); MgSO₄.7H₂O (0.5mM) and NaHCO₃ (24mM). Other constituents of the medium were 1X RPMI vitamin mix solution, 1X RPMI amino acid mix, 4mM L- glutamine, 100μM adenosine, 23μM folic acid, 100IU and 100μg/ml each of penicillin G and streptomycin sulphate, 1X phenol red, 22mM D-Glucose and 25mM 2-(N-morpholino) ethane sulfonic acid. To this, 1L of potassium based–basal medium, 256 ml of heat-inactivated FBS (25.6% v/v final serum concentration), was added. The pH of the medium was adjusted to 5.5 with 2N HCl (dropwise, while stirring). The medium was sterilized by filtration using 0.22μM filter and stored at 4⁰C prior to use. These parasites were grown in 25cm² tissue culture flasks at 26⁰C and the ratio of culture fluid volume to the total surface area (cm²) of the culture flask (i.e. 1:5) was stringently maintained at all subsequent culture conditions. The temperature was gradually increased to adapt the parasites to grow at 37⁰C. Subsequent to several passages under these conditions, the parasites transformed and grew as intermediate forms. As a final step towards the generation of axenic amastigotes, these intermediate form parasites were subsequently grown in potassium based RPMI-1640+MES/pH5.5 at 37⁰C in a humidified atmosphere containing 5% CO₂. Once adapted, these parasites were able to grow as axenic amastigotes and were routinely shuttled as promastigotes and axenic amastigotes.
C. Evaluation of the status of urmylation in Leishmania

To examine urmylation levels during stress, promastigote cultures in late log phase were subjected for 1 h to different stress conditions viz serum depletion (M199 without FBS supplement), low pH (pH 5.0), heat stress (42°C), H₂O₂ (50 mM) or UV irradiation (Van der Veen et al., 2011).

Patients’ samples

Two serum samples each of confirmed VL, Healed VL (HVL) hailing from the endemic area and two healthy individuals from non-endemic zones, were included in this study. Diagnosis was confirmed by either microscopic demonstration of the Ld bodies and/or by PCR of bone marrow aspirate (BMA) samples for VL/HVL. Two BMA samples from confirmed VL cases were included in the study for isolation of RNA and subsequent RT-PCR assay. The causative parasites were characterized by genotyping with ITS1-RFLP assay (Schonian et al., 2003) as *L. donovani* in VL. The study was approved by and carried out under the guidelines of the Ethical Committee of Safdarjung Hospital, New Delhi, with informed consent was obtained from all patients or their guardians. Two serum samples obtained from the blood of healthy volunteers was used as controls.

Isolation of Genomic DNA

Genomic DNA was isolated from promastigotes according to the methods described in the manual for GENOMIC DNA isolation kit from Promega Corporation, USA.

Isolation of RNA

The cells were collected at identical growth points (log phase promastigotes and axenic amastigotes) and grown under identical conditions to contain variations in the quality of RNA. Total RNA was isolated from promastigotes, axenic amastigotes and hamster derived amastigotes using Trizol reagent. Briefly, the cells (1-1.5X10⁹) were lysed in Trizol, at room temperature and to the homogenized samples, 0.2 volumes of chloroform was added followed by centrifugation at 12,000 x g for 15 min. The aqueous phase was collected and 0.5 volumes of isopropyl alcohol were added to precipitate total RNA. The total RNA was pelleted by centrifugation and washed in 70% ethanol (in autoclaved DEPC treated water) and stored at –70°C till further use. Approximately 130-140μg of the total RNA was cleaned up to eliminate salts and contaminating genomic DNA by preferential binding of RNA to the RNeasy columns before the labeling reaction, using RNeasy mini kit. The purified RNA was quantified.
spectrophotometrically by checking the absorbance at 260nm. The quality and integrity of RNA was checked using agarose/formaldehyde gel electrophoresis. To 1μg RNA, 0.5μl 10X MOPS buffer, 4μl 12.3 M formaldehyde, 4μl formamide and 0.5μl ethidium bromide were added. The samples were heat denatured at 66°C for 10 mins, mixed with 2X RNA loading buffer and loaded onto the gel. Electrophoresis was carried out at 65V for 3hrs and products visualized under UV transilluminator on 0.6% formaldehyde agarose gel.

**Expression analysis of LdUrm1 and LdUba4 at RNA and protein level**

RNA was prepared from *Leishmania* promastigote & amastigote cultures and BMA (bone marrow aspirates) of two confirmed VL cases. Subsequently, reverse transcriptase PCR (RT-PCR) was performed with the primers designed for *Leishmania* specific URM1 and UBA4 genes (Table 5.1) and their relative abundance at RNA level was predicted using appropriate endogenous controls (at promastigote and amastigote stages and in patient samples). Likewise, subsequent to generation of polyclonal antibodies against recombinant Urm1 and Uba4 proteins of *Leishmania*, the respective expression analysis was performed for both the proteins at both the stages of *Leishmania* parasite by Western Blotting. Further, the presence of the antibodies against the two proteins was tested in the serum samples of VL, healed VL, and Healthy individuals. Promastigote cell culture lysate and purified recombinant proteins (Urm1 and Uba4) probed with respective polyclonal antibodies (pAbs) were taken as positive control in the Western blots.

**Cloning, protein expression and antibody production**

Gene sequences for putative LdUrm1 (309 bp, accession no: LdBPK_342690.1) and LdUba4 (1389 bp, accession no. LdBPK_271570.1) were amplified from *L. donovani* genomic DNA using gene specific primers (Table 5.1). The genes were cloned in T/A cloning vector pGEMT Easy (Promega) and sequenced. LdUrm1 was sub-cloned in BamHII/XhoI backbone of pPROEXHTc (Invitrogen) and expressed as N-terminal GST tagged LdUrm1 in *E. coli*. Similarly LdUba4 was subcloned between Neol and XhoI sites of pET28a (+) and subsequently expressed as LdUba4-6XHIS. Cells were harvested and proteins were purified using GST-Sepharose beads and Ni2+ -NTA resin for GST-LdUrm1 and LdUba4-6XHIS respectively (Gupta *et al*., 2009). The proteins were concentrated using 10 kDa and 40 kDa cutoff centricon (Amicon, Millipore) for recombinant LdUrm1 and LdUba4 respectively and purity was assessed on SDS-PAGE. Protein concentrations were determined by BCA
Protein assay kit (Novagen). Purified LdUrm1 and LdUba4-6XHIS were subsequently used to raise respective polyclonal antibodies in rabbits with the help of Merck-Millipore.

**Molybdenum blue assay for LdUrm1 conjugation**

To establish that LdUrm1 is adenylated by LdUba4, a spectrophotometric molybdenum blue assay was performed to quantify Ub/Ubl conjugation by monitoring pyrophosphate released in the first enzymatic step, an ATP dependent charging of E1 (Berndsen and Wolberger, 2011). Briefly, a reaction with 50mM Tris –Cl (pH 7.5), 10 mM MgCl₂, 12.5μM LdUba4, 25μM LdUrm1, 2mM ATP and 2 units of inorganic pyrophosphatase was carried out in 500 μl reaction volume at 37° C. At designated time points, a 50μl aliquots were taken, quenched with 150μL of quench solution (QS: 8% L-ascorbic acid and 0.67% ammonium molybdate) and subsequently developer solution (DS: 50 μL of 2% sodium citrate –trihydrate, 2% sodium (meta) arsenite in 2% acetic acid) was added. The absorbance was measured at 850nm. Further, to determine the reaction constants under the steady state condition, reactions were carried out using a range of LdUrm1 concentrations from 0-800μM and the velocity of the reaction determined. The values were plotted as Lineweaver Burk plot. Standard curve was generated using different concentrations of sodium phosphate in buffer containing 20 mm HEPES, pH 7.5, 100 mMNaCl. The data was plotted as absorbance vs concentration of phosphates (Abs/ μM). Using the slope of the phosphate standard curve, μM of Urm1 transferred per second were determined by the following formula:

Eq. 1 \[ \text{Absorbance }_{850nm} / \text{slope of std curve (Abs/μM)} = \mu \text{M phosphate formed.} \]

Eq. 2 \[ \mu \text{M phosphate formed} / 2 = \mu \text{M pyrophosphate formed} = \mu \text{M Urm1 transferred.} \]

The absorbance was determined on multimode microplate reader Infinite® 200 PRO (Tecan Group Ltd., Switzerland). Statistical analysis was performed on ORIGIN and kinetic parameters were determined.

**Table 5.1: Primer sequences used to amplify LdUrm1 and LdUba4 from *L. donovani*.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>LdUrm1</td>
<td>LdUrm1F</td>
<td>5’AggatcGCATGCAGATGACGCACAGAAAATC3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>LdUrm1R</td>
<td>5’Gcctag GCC GCCGTGCAGATGAGTAGAT 3’</td>
<td>XhoI</td>
<td></td>
</tr>
<tr>
<td>LdUba4F</td>
<td>5’AcatggGCATGCTCAACTCGGCCCTCTACG 3’</td>
<td>NcoI</td>
<td></td>
</tr>
<tr>
<td>LdUba4R</td>
<td>5’GcctagCGTCGTGGGTGGCGGTCTCTTT 3’</td>
<td>XhoI</td>
<td></td>
</tr>
</tbody>
</table>
**Immunolocalization of LdUrm1 and LdUba4 in L. donovani**

*Leishmania* cells were harvested in log phase, washed, and fixed with 4% paraformaldehyde. 100ul of the fixed cells suspension was allowed to attach on the poly- L-lysine charged coverslips. Cells were permeabilized with 0.25% Triton-X 100 in PBS for 10 min and blocked with 3% (w/v) bovine serum albumin (BSA) in 1X PBS for 1h. Followed by probing with α-LdUrm1/α-LdUba4 antibody (1:500 dilution) for 1h at 25°C. Cells were washed thrice with PBS and incubated with FITC labeled α-rabbit IgG (1:500 dilution) for 1 h at 25°C. After washing thrice with PBS the cells were stained with DAPI (2ug/ml in PBS) for 10 min at room temperature to stain both nucleus and kinetoplast. The coverslips were subsequently washed twice with PBS and mounted inverse on a drop of Vectashield (Vector Laboratories) placed on the microscope slides (Gannavaram et al., 2011). Cells were examined for fluorescence under the microscope (Nikon Eclipse TE2000-U). The images were further processed using Adobe Photoshop 5.5.

**Immunoprecipitation, Immunoblotting and Mass Spectrometry**

The lysate for immunoprecipitation was prepared from *L. donovani* culture in buffer containing 10 mMTrisHCl, 100 mMNaCl, 5 mM EDTA, 0.5% Triton X-100, 1x protease inhibitor mixture and 5% (v/v) glycerol at pH 7.5. After centrifugation at 13,000 × g at 4°C, the supernatant was incubated with α-LdUb4, α-LdUrm1, or preimmune antisera cross-linked to Protein- A Sepharose beads (Pierce) by disuccinimidylsulphate (DSS) with end-to-end shaking at 4°C for 12h. The beads were washed several times with Lysis/ Wash buffer (Pierce, Life Technologies), eluted with glycine-HCl buffer at pH 2.8, and examined on 12% SDS-PAGE followed by silver staining/ coomassie blue staining. The gels were further analyzed by Western blotting or subjected to MALDI-TOF/TOF analysis (Merck Millipore) to identify urmylated proteins. Western blots of the eluted immunoprecipitates were probed with α-LdUba4 and/or α-LdUrm1 antibodies.

**ATP mediated activation of LdUrm1 by LdUba4.**

LdUba4 and LdUrm1 recombinant proteins were co-incubated in presence or absence of 2 mM ATP under conditions as described elsewhere (Schmitz et al., 2008). The reaction mixture was incubated with α-LdUba4 immobilized Protein A agarose beads. The eluates were analysed on non-reducing SDS-PAGE followed by Western blotting with α-LdUba4 to demonstrate the interaction between LdUrm1 and LdUba4 under *in vitro* and *in vivo* conditions.
Preparation of mutant parasites with episomal expression of LdUrm1WT and LdUrm1AG in Leishmania

The Leishmania expression plasmid pKSNEO (Zhang et al., 1996) was used to express full-length (LdUrm1++)/truncated (LdUrm1AG) genes of L. donovani. The nucleotide sequence of the gene was confirmed by cloning into pGEMT®Easy TA cloning vector (termed pGLdUrm1). Sequence-confirmed pGLdUrm1 was used as template to amplify LdUrm1 with SpeI restriction site and N terminal HA tag using primers; OEUrm1F/R and OEUrm1AGF/R for LdUrm1++ and LdUrm1AG respectively as mentioned in Table 5.2.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer Name</th>
<th>Primer sequence</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>LdUrm1++</td>
<td>OEUrm1F</td>
<td>5’GGactagtTACCCATACGATGGTCCAGATACGCCGACGACAGAAAATC 3’</td>
<td>SpeI</td>
</tr>
<tr>
<td></td>
<td>OEUrm1R</td>
<td>5’GaactagtGCCGCCGTGCAGAGTAGATACAAACTCTCGAATGCAGATGACGCACAGAAAATC 3’</td>
<td>SpeI</td>
</tr>
<tr>
<td>LdUrm1AG</td>
<td>OEUrm1AGF</td>
<td>5’GGactagtTACCCATACGATGGTCCAGATACGCCGACGACAGAAAATC 3’</td>
<td>SpeI</td>
</tr>
<tr>
<td></td>
<td>OEUrm1AGR</td>
<td>5’GaactagtGCCGCCGTGCAGAGTAGATACAAACTCTCGAATGCAGATGACGCACAGAAAATC 3’</td>
<td>SpeI</td>
</tr>
</tbody>
</table>

Restriction sites included in the primers are underlined. HA tag is marked bold in the primers designed for transfection studies.

This was followed by transfection in L. donovani cells.

Following steps are involved in over-expression of genes in Leishmania:

1. DNA construction

The DNA encoding full length LdUrm1 gene was obtained by PCR from L. donovani genomic DNA isolated from the cloned line designated by the World Health Organization as MHOM/SD/62/1S-C12D (LDS 1). It was then sub cloned into pGEMT®Easy TA cloning vector. Ligation reaction was set up as follows.

\[ \text{pGEMT®Easy TA cloning vector (50ng)} \quad \text{1.0 µl} \]
The ligation reaction mixture was incubated at 14°C for 16 hrs. *E. coli* DH5α competent cells were prepared and the ligated product was transformed and subjected to blue-white / Ampicillin resistant (Amp'r) selection. The recombinant white Amp'r colonies were screened for the presence of gene of interest (insert) by digestion with SpeI enzyme. Further the confirmed colonies were sequenced in an automated sequencer (ABI3730) using M13 forward and reverse sequence for correct orientation and fidelity of the PCR product.

II. Insertion of full length gene in pKSNEO vector

Sequence confirmed plasmid DNA of pGEMT@Easy TA-fused with full length insert was used as template using respective gene primers with SpeI site and HA tag for sub cloning into the *Leishmania* specific expression plasmid vector pKSNEO (Zhang *et al.*, 1996). The vector pKSNEO consists of a 1.6-kb 5' upstream flanking region of the A2 gene, the entire Neo gene and a 1.8-kb fragment containing the A2 3’ UTR and a synthetic pyrimidine tract (Zhang *et al.*, 1996). The sequence and multiple cloning site of the pKSNeo vector is shown in Fig 5.1.

[pKS NEO] expression plasmid

![Diagram of the pKS NEO expression plasmid](image)
Fig 5.1: Multiple cloning site of pKSNEO expression vector

The oligos were designed to introduce a hemagglutinin tag (HA tag) at the N-terminus of the fusion protein and SpeI restriction sites at the either end (Table 5.2). The corresponding inserts with flanking SpeI restriction sites was sub-cloned into the SpeI/SpeI backbone of pKSNEO vector using T4 DNA ligase and transformed in E. coli DH5α cells.

III. Restriction digestion

Plasmids were prepared from bacterial colonies containing the respective constructs along with pKSNEO vector and digested with SpeI enzyme to release the insert.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid (pKSNeo + Insert)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>SpeI enzyme (1U)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>NEB buffer 2</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>3.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.0 µl</strong></td>
</tr>
</tbody>
</table>

The orientation of the gene in the plasmid pKSNeo was confirmed before transfection by using the following primers:

- NeoFp: CACATATCTGCTATAACCTGC
- NeoRp: GCATCTAGATCATCGATCGC
- McsInt: ATGCATTCCTGCACTCTGACAGC

The plasmid constructs with correct orientation of the inserts were further used to transf ect Leishmania cells to obtain strains expressing wild-type (LdUrm1++) and mutant (LdUrm1ΔG) forms of the LdUrm1 gene.
IV. Transfection of construct in *L. donovani*

a) Preparation of cold DNA

20μg plasmid DNA was re-precipitated in 3M sodium acetate and 2 volumes of isopropanol, washed with 80% ethanol, dried and dissolved in 20μl sterile TE buffer and the plasmid was kept in ice until transfection.

b) Electroporation

2X10⁸ mid log phase *L. donovani* cells were harvested, washed once with electroporation buffer and resuspended in ice-cold electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mMNa₂HPO₄,6mM glucose, pH 7.4). 0.5 ml cells were taken in 2mm cuvette and 20μl cold plasmid DNA was added to it for transfection. The DNA was electroporated into *Leishmania* cells at 450 V, 500μF, 800π resistance. Following electroporation, cells were recovered in M199+10% FCS for 24hrs. After 24hrs, cells were washed once with M199 and resuspended in M199 + 10% FCS + 50 µg/mL G418 as selection marker (Papadopoulou *et al*., 1992). The drug concentration was gradually increased to a final concentration of 200µg/ml for the selection of mutant cell lines. Parasites transfected with the empty vector pKSNeo were prepared as mock controls.

Results:

Identification of Urm1 and Uba4 protein homologues in *L. donovani*

Based on blast search in the TritrypDB using *S. cerevisae* Urm1 and Uba4p (Furukawa *et al.*, 2000) as query sequences, *L. donovani* genome was found to contain homologous sequences corresponding to both Urm1 (LdBPK_342690.1) and Uba4p (LdBPK_271570.1) that we designated as *LdUrm1* and *LdUba4* respectively. The ORFs of *LdUrm1* and *LdUba4*, comprised of 309 bp and 1389 bp, encoding 103 and 463 amino acids (aa) with calculated molecular masses of 11.3 and 50.9 kDa, respectively. The details of phylogenetic and bioinformatics analysis are given in Chapter 4.

Cloning, Protein Expression and Generation of antibodies.

PCR amplicons corresponding to the size of putative *LdUrm1* [Fig. 5.2A] and *LdUba4* [Fig. 5.2B] were obtained using primer sets *LdUrm1F/R* and *LdUba4F/R* respectively (Table 5.1). The amplicons were cloned and expressed as GST-*LdUrm1* of 37 kDa and 6X-HIS-*LdUba4* of ~50 kDa and purified using GST sepharose column and Ni²⁺-NTA affinity resins.
respectively. The purity of the proteins was assessed on SDS-PAGE as single bands for GST-LdUrm1 (37 kDa) and 6XHIS-LdUba4 (50 kDa) [Fig. 5.2C, 5.2D]. GST tag was removed from GST-LdUrm1 to obtain ~11 KDa protein and the purity of LdUrm1 was confirmed on SDS-PAGE [Fig. 5.2E]. Recombinant LdUba4 and LdUrm1 proteins were used to raise polyclonal antibodies in rabbits by standard methods and the specificity of the polyclonal antibodies assessed on Western blots.

**Fig 5.2:** PCR amplification, cloning and recombinant protein preparation. A. PCR amplicon corresponding to putative LdUrm1 was amplified from L. donovani genomic DNA using appropriate primer sets. B. PCR amplification of putative LdUba4 sequence. C. Expression of GST-LdUrm1 after subcloning LdUrm1 into pPROExHTCexpression vector in the BamHI/XhoI backbone. D. Recombinant LdUba4 (rLdUba4) protein expressed as LdUba4-6XHIS using pET28a (+) expression vector. E. Purity of LdUrm1 after removing GST tag. C, D, and E show Coomassie stained gels.

**Expression profiles of LdUrm1 and LdUba4 in promastigote and amastigote stages**

RNA expression profile of LdUrm1 and LdUba4 was assessed by RT-PCR at promastigote and amastigote stages of the parasite to evaluate if the expression is stage specific. The data revealed that there was no difference in the expression of LdUrm1 and LdUba4 at the RNA level [Fig 5.3A, B]. Subsequently, the protein expression profiles of LdUrm1 and LdUba4 were assessed at promastigote and amastigote stages of the parasite to check the expression pattern of these proteins at the two stages of the parasite. The data revealed that there was no difference in the expression of LdUrm1 [Fig 5.3C] and LdUba4 [Fig 5.3D] proteins in the promastigote and axenic amastigote forms.
Evaluation of LdUrm1 and LdUba4 transcript expression in VL bone marrow samples

RT-PCR was done with the RNA isolated from the bone marrow samples of the VL patients (n=2) to evaluate if the transcripts for the above two proteins expressed. It was observed that LdUrm1 transcripts were detectable in the bone marrow samples [Fig 5.3A] while LdUba4 transcripts were not [Fig 5.3B].

**Fig 5.3:** Expression of Urm1 and Uba4 at RNA and protein levels. RT-PCR was done using cDNA preparations made from RNA of promastigote, axenic amastigotes and BMA of VL patients to assess the expression of LdUrm1 and LdUba4 transcripts. (A) Urm1 expression was seen at RNA level in Pro, ax ama and bone marrow samples. (B) Uba4 expression was seen at pro and ax ama stages of the parasite but was absent in the bone marrow samples. (C) Promastigote and Axenic amastigote cell culture lysate were assessed for the expression of endogenous LdUrm1 and (D) endogenous LdUba4 proteins. α-tubulin was used as loading control. Tubulin and HPRT were used as the loading controls in RT-PCR for parasite and patient samples respectively.

Detection of antibodies against LdUrm1 and LdUba4 in the serum samples.

We assessed the antibody expression against LdUrm1 and LdUba4 proteins in the serum samples collected from VL patients, healed VL and healthy volunteers. The western blot analysis showed that there was a clear cut expression of antibodies against LdUrm1 [Fig 5.4A]
and LdUba4 proteins [Fig 5.4B] not only in the serum samples of the VL, healed VL but in healthy individuals as well.

![Figure 5.4: Evaluation of LdUrm1 and LdUba4 antibody expression in the serum samples.](image)

**Fig 5.4:** Evaluation of LdUrm1 and LdUba4 antibody expression in the serum samples. **A.** Recombinant LdUrm1 protein and **B.** recombinant LdUba4 protein were run on SDS – PAGE and blotted on the nitrocellulose membrane and probed with the serum samples from VL, HVL and healthy individuals. rLdUrm1, purified recombinant LdUrm1 protein probed with α- LdUrm1 / r. LdUba4, purified recombinant LdUba4 protein probed with α- LdUba4 in addition to promastigote and axenic amastigote culture lysates probed with VL serum samples were taken as controls. K1 and K2, kala azar patient serum samples; HV1 & HV2, Healed VL serum samples; H1 and H2, Healthy volunteers (from non endemic zones) serum samples.

**Reconstitution of the LdUrm1-LdUba4 conjugation pathway in vitro**

To gather evidence for the existence of Urm1-Uba4 conjugation pathway in *Leishmania*, a reconstitution assay was performed using LdUrm1 and LdUba4 recombinant proteins (Schmitz et al., 2008). The E1 like enzymatic activity of LdUba4 to catalyze the formation of an LdUrm1-LdUba4 conjugate was demonstrated in the presence of ATP based on appearance of a high molecular weight band corresponding to LdUrm1-LdUba4 transient conjugate in vitro [Fig. 5.5B] and in vivo [Fig. 5.5C]. However, in the absence of ATP only a band corresponding to the size of LdUba4 (~50kDa) was seen on the blot [Fig. 5.5A]. Taken together, the data confirmed that LdUrm1 and LdUba4 interact to form the transient LdUrm1-LdUba4 intermediate in the conjugation pathway in *L. donovani*, and the process is energy-dependent.
**Fig 5.5: Co-immunoprecipitation assay depicting interaction between LdUrm1 and LdUba4.**

A. Recombinant LdUrm1 and LdUba4 do not interact in the absence of ATP evident by appearance of 50 kDa band on Western blot when probed with α-LdUba4 antibody. B. LdUrm1 and LdUba4 interact in presence of ATP to produce a high molecular weight (60 kDa) LdUrm1-LdUba4 conjugate in vitro depicted by asterisks (*). C. Co-IP with *L. donovani* cell lysate produced a band corresponding to LdUba4- LdUrm1 adduct (~60 kDa). A few bands of higher molecular weight seen on the blot are presumed to be adducts with LdUrm1 substrate(s). E1, E2, and E3 depict the first, second and third elute fraction respectively taken during Co-IP.

**Kinetic Analysis of LdUba4 as E1-like enzyme**

To evaluate the enzymatic activity of LdUba4, the kinetic parameters were determined as using Molybdenum blue assay. The method quantified Ub/Ubl conjugation by monitoring release of inorganic phosphates via pyrophosphate, the first enzymatic step of Ub/ Ubl transfer i.e. the ATP- dependent charging of E1 enzyme. The inorganic phosphate formed was quantitated by measuring the absorbance of molybdenum that absorbed visible light at 850nm wavelength. The apparent kinetic parameters were determined as $K_{m} = 95.92 \, \mu M$; $V_{max} = 0.1276 \, \mu M/sec$ and $K_{cat}(s^{-1}) = 0.01 \, s^{-1}$ [Fig. 5.6A, 5.6B, 5.6C]. Collectively, this result provided the first direct evidence to establish LdUba4 as E1-like enzyme capable to adenylate LdUrm1 forming LdUrm1-AMP and pyrophosphate subsequently.
Fig 5.6: Kinetic analysis of LdUba4 as E1-Like enzyme for LdUrm1: A. Standard Curve. The absorbance of each solution was plotted as absorbance versus concentration in µM. B. Velocity of the reaction. Reactions were carried out at 37° C and at designated time points, a 50µl aliquot of reaction was taken and quenched and developer solution added. Absorbance of the reaction was determined at 850nm. µM of PO₄⁻³ formed were plotted against time and velocity of the reaction was calculated. C. Reaction constants under the steady state condition. Lineweaver Burk plot using a range of LdUrm1 concentrations from 0-800uM to determine K_m and K_cat.

**Immunolocalisation of LdUrm1 and LdUba4 in L. donovani**

Subcellular distribution of LdUrm1 and LdUba4 was examined at both promastigote and amastigote stages by immunofluorescence assay. LdUrm1 was found, localized near flagellar pocket, distinctly anterior in position in defined puncta not coinciding either with nucleus or kinetoplast in both promastigote [Fig. 5.7A] or amastigote stages [Fig. 5.7C] of the parasite. LdUba4 on the other hand was found to be localized to cytoplasm in both the stages of the parasite [Fig. 5.7B, 5.7D]. These staining patterns were not observed when pre-immune serum was used instead of α-LdUrm1 antibody or α-LdUba4 antibody.

**Expression analysis of LdUrm1 in L. donovani under stress conditions**

Studies to understand urmylation in *S. cerevisae* has revealed that the process is regulated by oxidative stress (Van der Veen *et al.*, 2011). Therefore, we subjected *L. donovani* culture to different stresses and evaluated the status of urmylation in *Leishmania* under normal and various stress conditions. We observed that the number of proteins urmylated under different stress conditions were comparable to those under normal conditions in *L. donovani*.
Fig 5.7: Intracellular localization of LdUrm1/LdUba4 in *Leishmania* promastigote/axenic amastigote cells. To determine the localization of endogenous LdUrm1 and LdUba4 *Leishmania*, cells were fixed and permeabilized with Triton-X-100 as described in Materials and methods. LdUrm1 and LdUba4 were labeled with α-LdUrm1 and α-LdUba4 antibodies respectively and visualized using FITC-labelled goat anti-rabbit secondary antibody. A, C: Green shows the localization of LdUrm1 in promastigotes and amastigote (middle panel). B, D: Green shows the localization of LdUba4 (middle panel). Nucleus (n) and kinetoplast (k) of *Leishmania* were labeled with DAPI and appear blue (middle panel). Merged DAPI and FITC signals (right panel in each row). Immunofluorescence analysis was conducted using a fluorescence microscope Nikon Eclipse TE-2000-s at magnification, X100. Scale bar is 5 µM.
Identification of putative urmylation targets in *L. donovani*

Urmylated proteins were identified by mass spectrometry (MALDI-TOF/TOF) analysis of protein bands obtained following co-IP using α-LdUrm1 antibody [Fig. 5.8]. Proteins identified met strict data quality criteria: a Mascot score >65 (p< 0.05) and PMF (Peptide Mass Fingerprint) based search. Further results were refined by using blastp tool on TriTrypDB. This was not an exhaustive proteomic screening for the urmylated targets in *L. donovani* as our aim was to identify proteins that are constitutively urmylated in promastigotes under normal growth conditions. As we analyzed gel bands excised from 1D gel, it is conceivable that one or more proteins were LdUrm1 targets. A number of interesting proteins were identified as LdUrm1 targets in the proteomic screen (Table 5.3). These urmylated proteins included early endosome mediated vesicular trafficking associated proteins (rab-like GTPase activating protein, ras-related protein rab-5, phosphatidylinositol 3-kinase, vacuolar sorting-associated-like protein, calcium calmodulin protein kinase, sodium channel clathrin linker hypothetical protein); cytoskeletal proteins {Plectin-1; kinesin like putative, dyenin assembly factor, internalin leucine rich repeat (LRR), OSM3-like kinesin, plectin 1, ankyrin repeat protein}. In addition to these, an E3-like ubiquitin-protein ligase RSP5, HECT domain containing putative protein was also identified to which LdUrm1 binds in *L. donovani*. Our proteomic approach revealed *L. donovani* proteins whose orthologues have been shown to be monoubiquitinated in eukaryotes.
Fig 5.8: Identification of urmylated proteins in L. donovani by Mass Spectrometry. Cell lysate from log phase L. donovani cells were subjected to Co-IP using α-LdUrm1 antibody immobilized onto Protein-A agarose beads. Immunoprecipitates were run on SDS-PAGE and silver stained (left panel) or subjected to Western blotting using α-LdUrm1 antibody (right panel). Autoradiographs were aligned with silver stained gels, six bands chosen for analysis (indicated by arrows), and the LdUrm1 target proteins were identified by tryptic digestion followed by MALDI TOF/TOF. Lane M shows the molecular weight marker. * shows the position of band corresponding to the unconjugated LdUrm1 (~11 kDa) on both panels.

Table 5.3: List of proteins identified as LdUrm1 targets in L. donovani

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Accession id</th>
<th>Title</th>
<th>Peptides identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>LdBPK_366330.1</td>
<td>rab-like GTPase activating protein, putative</td>
<td>DLARTFP;MAEEESF;SEGWKII FR;IALLK</td>
</tr>
<tr>
<td>2.</td>
<td>LdBPK_181140.1</td>
<td>ras-related protein rab-5, putative</td>
<td>LVGNK;IWDTAGGER;LVVYDTN;KIVMLG</td>
</tr>
<tr>
<td>3.</td>
<td>LdBPK_280030.1</td>
<td>origin recognition complex subunit 1 (ORC1), putative</td>
<td>RATQQEDVMR;KDLVCR;HHT MQIFGMPGTGK;GAQPTAVFL NGFVVQK;LGAVEPCLAQCA PR;LITISNSMELDAKTK;VG AIEPK</td>
</tr>
<tr>
<td>4.</td>
<td>LdBPK_201140.1</td>
<td>phosphatidylinositol 3-kinase, putative</td>
<td>YVLGLGDRHL;IPFRLTR;EEQVSFTEMEE;LYESLS;YELLCCWSSLGLADY;EIVLN;VIPIS</td>
</tr>
<tr>
<td>5.</td>
<td>LdBPK_110250.1</td>
<td>protein kinase, putative, calcium calmodulin protein kinase</td>
<td>PFTTAA;LYEVIND;DGLVYLHE</td>
</tr>
<tr>
<td>6.</td>
<td>LdBPK_170770.1</td>
<td>protein kinase, putative, serine/threonine kinase, putative</td>
<td>RFQCRVGLGRGGFACKYCE;IHR RMKHKHVVFRLRTFRD;VIHR DLKPNGMLD;TLDLKLVR;C GTPNYIAPEII;WSLGVL:mysql;VPD NARDLI;NVKIGDFGLAEL</td>
</tr>
<tr>
<td>7.</td>
<td>LdBPK_291060.1</td>
<td>Plectin-1; kinesin like putative</td>
<td>RLASLDTKRNALQ;AAVQALE NEKASL;KEALQAQLE</td>
</tr>
<tr>
<td>8.</td>
<td>LdBPK_181150.1</td>
<td>hypothetical protein, conserved; sodium channel clathrin linker hypothetical</td>
<td>PASVASFRKN;QQLHDALQQQS;LELRQQQQLVQYRQ;LRTL CDDNATLRNIQAGERRKRD;A AAAGLV;HTNGAPNEAPATVTE AEMEA;NKVLHEIKVDE;KLLM GANR;LQLYDRTARAAY;VQRN ASRMR;AEEKALQQKREV;ST</td>
</tr>
<tr>
<td>Protein ID</td>
<td>Description</td>
<td>Conserved Regions</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>LdBP_K240500.1</td>
<td>Hypothetical protein, conserved, dyenin assembly factor, internalin A, leucine rich repeat</td>
<td>GTDERVAAY;REEVKRMSELIR;LAVVAALP;RRRSSAA</td>
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</tr>
<tr>
<td>LdBP_K_280380.1</td>
<td>OSM3-like kinesin, putative</td>
<td>GYNATVFAYGQSGSGKT;SSRSHSLFT;VTSSFEDA;VDLAGSEK;LLKDSLGNN;NKEDE;KLEEKLVT;DKLVKKL</td>
<td></td>
</tr>
<tr>
<td>LdBP_K_303310.1</td>
<td>Hypothetical protein, conserved, plectin 1</td>
<td>IEGMEVIL;CQVLDDHGAD;NAQGRTPLHEA</td>
<td></td>
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<tr>
<td>LdBP_K_361570.1</td>
<td>Hypothetical protein, conserved</td>
<td>ALLVEPPQAAYLRRCA;SVCKRCEHT;QTQVLNRWESLQAALEH;NMYDLKIK;MLCRNL</td>
<td></td>
</tr>
<tr>
<td>LdBP_K_367190.1</td>
<td>Hypothetical protein, conserved</td>
<td>PVAPASPAPA;LPPTAVPL;VVVDEATAPHL;VVFGGARYSGASL;GAKRTTHL;TAGCAA;ARHYLKEAR;VMEKFKFDG;FKPRSHAPRQNAALPVT</td>
<td></td>
</tr>
<tr>
<td>LdBP_K_170800.1</td>
<td>Hypothetical protein, conserve</td>
<td>LRQWGWRF;ALRQTVVEL;LEFFISRSCRGAIFMD;VCSHGRPSFA;QWARKFLII;DQHAVHERVRLEFFLPVFVFVFD;AVQASNT;GLFHNLPVR</td>
<td></td>
</tr>
<tr>
<td>LdBP_K_170800.1</td>
<td>Hypothetical protein, conserve</td>
<td>LRQWGWRF;ALRQTVVE;LEFFISRSCRGAIFMD;VCSHGRPSFA;QWARKFLII;DQHAVHERVRLEFFLPVFVFVFD;AVQASNT;GLFHNLPVR</td>
<td></td>
</tr>
<tr>
<td>LdBP_K_354040.1</td>
<td>Hypothetical protein, conserved, mitochondrial</td>
<td>KERLQSQPPS;STLSSFGPSA;SLLTNPLE;VKTRIQVQRAV</td>
<td></td>
</tr>
</tbody>
</table>
### LdUrm1-LdUba4 conjugation pathway

| 18. | LdBPK_030030.1 | **D-3-phosphoglycerate dehydrogenase-like protein** | TRSVAEL.VI;RGKTVGIVGYG;N VILTPH |
| 19. | LdBPK_210530.1 | **vacuolar sorting-associated-like protein** | ELRTN;YYEL;IVPRLYLL;RGL FLRHFLLT;LWIRME;VLVGMN VVRVAQLD;EPLAQQYL; LIQVFPDEFHL;SLMERL;GAGG GV |
| 20. | LdBPK_310480.1 | **calpain-like cysteine peptidase, putative** | LLEL.D;QGP.FYVIPRIM;DFTL GML;KSTARGLRVSFVHLPDTC PTFR;KKRGGAPRVKAG; FVIPRV;VNGE |
| 21. | LdBPK_282610.1 | **vacuolar ATP synthase subunit b, putative** | VLSK;QELLATHIKELNES;YSV KPHLEYTTIR;GQVLEVDGTK; QAGLVK;EEVPGRR; DILAMK |
| 22. | LdBPK_100780.1 | **t-rna specific adenosine deaminase, putative** | DAFMRRAA;EGEVVPVG;SCI.AA RGRNQTNLQHHALAHAE;FIA VQ;HKRRRK |
| 23. | LdBPK_170850.1 | **hypothetical protein, conserved, pre mRNA splicing factor** | QCRREW;WSLEE;KWRLISE |
| 24. | LdBPK_270930.1 | **cysteine desulfurase, putative** | YGNPNS;EKARK;LDLKVLE;V KVDVN;GALYVRRR;SGSACTS A;LRALGVD;VSGGG |
| 25. | LdBPK_366860.1 | **protein-l-isoaspartate o-methyltransferase, putative** | GLIKT;TISAPHMHA;LDIGSGS G;VVGIEHVQEL;FDVHVGA;F VPLT;LVIPVG; |
| 26. | LdBPK_366600.1 | **ubiquitin-protein ligase, putative, E3 ubiquitin-protein ligase RSP5, HECT domain** | WYFEV;GLMQIGW;NGHGVG.D ;GDVVG.C;FFLNGR;PAVSL;VS MGQADS.V;SYY.YEV |
| 27. | LdBPK_362070.1 | **Phosphomannomutase, putative** | ILLFDV.DGLTPLPRNPETLDMK ;LGVGGSDFAK;CLHLIA.DLI PVQR;GTFVEFRNMFNVSPIG R;TIHFFGDK;EGGNDYEIYTDC R |

*Urm1 is essential for the growth of L. donovani*
Our proteomic data clued the fact that LdUrm1 mediated modifications are important to L. donovani metabolic machinery. To test this we prepared L. donovani cells overexpressing LdUrm1 (LdUrm1++) and L. donovani cell expressing non-conjugatable LdUrm1, (LdUrm1ΔG) by transfection [Fig 5.9 A] and confirmed the transfection by Western blotting [Fig 5.9 B]. Further we examined and compared the phenotypes of three different L. donovani strains: (i) one that over-expressed LdUrm1 viz LdUrm1++; (ii) other that expressed non-conjugatable form of LdUrm1 viz LdUrm1ΔG (iii) and the wild type i.e control strain transected with pKSNeo (empty vector) (LdUrm1KS). LdUrm1KS and LdUrm1++ showed normal and comparable growth, however LdUrm1ΔG showed slower growth [Fig. 5.9 C] suggesting negative dominant effect of the mutant protein. Despite reduced growth rate, the culture expressing truncated LdUrm1 (LdUrm1ΔG) survived for normal growth period probably due to the rescuing effect of the endogenous LdUrm1 over LdUrm1ΔG towards normal metabolism. LdUrm1ΔG also showed aberrant morphological phenotypes in liquid culture. They displayed variant sizes, forming cells with shortened length and often remaining attached at one end near flagellar pocket. Hence, LdUrm1ΔG cells displayed heterogenous behavior with respect to cell separation indicating that LdUrm1 is essential for the normal cell division in L. donovani.

**Fig 5.9**: Characterization of L. donovani cells overexpressing LdUrm1 (LdUrm1++) compared to L. donovani expressing non-conjugatable LdUrm1, (LdUrm1ΔG). A. Schematic presentation for the LdUrm1++ and LdUrm1ΔG construct cloned in pKSNeo. B. Appearance of LdUrm1++ and LdUrm1ΔG on Western blot when probed with α-HA antibody confirming transfection (upper panel; Lane 1 and 2). The blot when re-probed with α-LdUrm1 antibody showed elevated expression of LdUrm1 in LdUrm1++ (middle panel; Lane 1) compared to LdUrm1ΔG (middle panel; Lane 2). The amount of protein loaded onto gel was...
normalized using α-tubulin antibody (lower panel). C. Growth curve of LdUrm1++; LdUrm1AG and pKSNeo transfected L. donovani cells. Each data point on the curve represents the mean± standard deviation of three separate assays.

Discussion

In this report we have demonstrated the existence of an Urm1 conjugation pathway in the protozoan parasite Leishmania donovani including the enzymatic steps involving activation by Uba4 (E1) and identified its putative target proteins. Fig 5.10 shows the schematic presentation to depict Urm1 conjugation pathway and the enzymes involved in the pathway in L. donovani. Towards this end we cloned and expressed homolog of Urm1 in L. donovani, LdUrm1 and other components of its conjugation pathway viz. E1 enzyme, LdUba4. Sequence comparison of the cloned LdUrm1 protein along with Urm1 sequences reported from different organisms revealed that LdUrm1 contains highly conserved β-grasp fold and C-terminal di-glycine motif. In trypanosomatid parasites, it has been observed that the Ubls such as Ub, SUMO and Ufm1 usually have one or more aminoacid residues after the C-terminal glycine that need to be processed before conjugation (Gannavaram et al., 2011). However LdUrm1 is not synthesized as a precursor but exists in its processed form with pre-exposed di-glycine motif readily available to form LdUrm1 conjugates in the parasite, indicating that a C-terminal processing protease may not exist unlike in other Ubls. Interestingly, apicomplexan parasites such as Plasmodium are also known to contain Ubls (Atg8) that terminate in a C-terminal Glycine eliminating the need of processing prior to conjugation (Ponder and Bogyo, 2007). This structural diversity among different parasites is believed to be due to physiological adaptations that parasites have taken up in their respective environments during their complex life cycles. Additionally, LdUrm1 was found to be very closely related to the structure of TbUrm1 and homology modeling predicted the functional similarities on the basis of structural features. The final modeled structure obtained for LdUrm1 when subjected to validation analysis and compared with the solution structure of TbUrm1 (PDB: 2K9X) confirmed its stability (Zhang et al., 2009). LdUrm1 only differed in having a small fourth helix compared to TbUrm1. The above findings collectively supported the existence of this post-translational modifier in L. donovani. The high degree of homology of LdUrm1 with human Urm1 (hUrm1) underpin its role in cellular processes important to Leishmania biology.

Further, we raised experimental evidence for the existence of LdUrm1-LdUba4 conjugation pathway in Leishmania. We showed that LdUrm1 and LdUba4 recombinant
proteins interacted to form a high molecular weight transient conjugate corresponding to LdUrm1-LdUba4 adduct both in vitro and in vivo. This was accounted due to enzymatic reactions of LdUba4 (E1-like) onto LdUrm1 (with a free C-terminal di-glycine motif) in presence of ATP. Enzyme kinetic parameters determined for LdUba4 further verified the Ub activating enzymatic (E1-like) behaviour of LdUba4. E1 like activity of Uba4 has been demonstrated earlier against hUrm1 and MOCS2A (Schmitz et al., 2008). It is likely that Urm1 is transferred to its respective target proteins via conserved Rhodanese-like domain (RLD) in Uba4, since the involvement of an E2 protein has not been identified for Urm1 conjugation so far in any eukaryote (Schmitz et al., 2008). InterproScan revealed that LdUba4 also has conserved RLD, therefore possibly retained the E2 like activity. This is consistent with the reports that Cys397 of Uba4-RLD is essential for Urm1 protein ligation in yeast (Hochstrasser, 2005; Kerscher et al., 2006). The existence of E3 in the enzyme cascade of Urm1 conjugation pathway is yet to be elucidated in any system. However, E3 ligase independent conjugation of Ubls via E2 activity is still a distinct possibility (Hoeller et al., 2007). Additionally we also elucidated that LdUrm1 and LdUba4 did not express differentially in the two forms that is promastigote and amastigotes, both at transcript level and translational level inferring that the proteins are house keeping and showed constitutive expression. The high degree of sequence similarity of LdUrm1 and LdUba4 with respect to hUrm1 (44% and 38% respectively) justified the presence of antibodies against the two proteins in the serum samples of VL, HVL and healthy volunteers.

Leishmania promastigotes are highly polarized cells and have a flask-shaped invagination of the plasma membrane in the apical region of the cells known as the flagellar pocket from where the flagellum originates (Field and Carrington, 2009). Both exocytosis and endocytosis exclusively occur in Leishmania through this specialized invagination of the plasma membrane (Overath et al., 1997). Immuno-fluorescence studies using α-LdUrm1 antibody revealed that endogenous LdUrm1 was localized near kinetoplast in the anterior region of the cells. Additionally, MS analysis of LdUrm1 modified proteins identified Rab-like GTPase activating protein, Ras-related protein Rab-5 as one of the important target proteins. It has been established earlier that L. donovani specific Rab5b localizes to early endosome (Marotta et al., 2006). Moreover, it is a known fact that monoubiquitination of RasGTPase via Rab5 causes endocytosis of Ras (Zerial and McBride, 2001) into early endosome eventually sequestering the protein away from plasma membrane (de la Vega et al., 2011, Baker et al., 2013). It has been shown that Leishmania endocytoses hemoglobin (Hb) through a specific receptor located in the flagellar pocket (Singh et al., 2003; Krishnamurthy et al., 2005) and
bound Hb is rapidly internalized into discrete Rab5 positive early endosomal compartment and subsequently targeted to the lysosomal compartment in a Rab7 dependent way (Singh et al., 2003; Patel et al., 2008). Taken together, our identification of Rab5 and RabGTPase as LdUrm1 target clues possible role of LdUrm1 in early endosome mediated heme internalization in Leishmania. One of the known RabGTPases is Rabex 5 that recruits Rab5 to the membrane either directly or indirectly via interaction with a Rab5 effector Rabaptin-5 (Zhu et al., 2010). Rabex5 binds with ubiquitin which eventually recruits it to early endosome (Mattera et al., 2006). Rabex5 harbors an E3 ligase like activity that causes its self mono-ubiquitination (Mattera et al., 2006). Besides Rabs, we also identified other proteins associated with LdUrm1 in MS that included phosphatidylinositol 3-kinase vacuolar sorting-associated-like protein, calcium calmodulin protein kinase sodium channel clathrin linker hypothetical protein are known to be important constituents of vesicular trafficking involved in recruitment of clathrin onto early endosomes and finally to late endosomes (Larsen and Vincenzi, 1979; Kirchhausen, 2000; Simonsen et al., 2001; Lindmo and Stenmark, 2006; Hierro et al., 2007). These results indicated that the components of endocytic and intracellular trafficking machinery are well conserved in Leishmania and LdUrm1 mimics monoubiquitination (de la Vega et al., 2011) to target this pathway unlike other Ubls which typically target individual protein.

Additionally, we also identified a hypothetical protein with Leucine rich repeats (LRR) that showed homology to internalin A, a protein that belonged to Li. monocytogenes internalin family. The LRR-containing proteins of Leishmania possessed all the attributes found in LRR-containing bacterial virulence factors. Their striking similarity strongly suggested that LRRs are involved in the macrophage invasion process and contribute to the overall pathogenesis of Leishmania (Kedzierski et al., 2004). LRR containing proteins are even known to trigger monoubiquitination via a new class of E3 ligases viz NEL (Novel E3 ligases) as reported in Salmonella (Quezada et al., 2009). Intriguingly, our identification of a putative E3 ubiquitin-protein ligase RSP5, HECT domain containing protein in our MS data also raised the possibility of the existence of an E3 ligase enzyme in LdUrm1 conjugation pathway. Notably, E3 ligases that confer specificity of conjugation to substrate proteins have been extensively investigated as potential drug targets (Duncan et al., 2011).

Several observations suggested that urmylation is important to cell growth and differentiation in this clinically important protozoan parasite. Overexpression of LdUrm1 in L. donovani promastigotes did not affect the growth rate thereby indicating unaffected metabolic rate. Further, L. donovani mutants expressing LdUrm1<sup>ΔG</sup> episomally, showed retarded growth compared to the wild type strains during log phase. The C-terminal di-Glycine (GG) motif is
essential to LdUrm1 to establish conjugation with the Lysine residues of the target proteins. Therefore, it is possible that the deletion of GG from the C-terminal of LdUrm1 led to mutation that confers resistance against LdUrm1 mediated protein modification. Further, this non-conjugatable form of LdUrm1 (LdUrm1ΔG) is stable and could interfere with the function of the wild type LdUrm1 and hence reducing the growth of parasites. This therefore, may be accounted on the basis of dominant negative effect of the mutant protein in the cells as observed in our previous study with LdUfm1, a mitochondrial post translational modifier (Gannavaram et al., 2011). It has been elucidated that clathrin assembly is required for the formation of coated pits and coated vesicles during Hb endocytosis in Leishmania which subsequently dissociates from Hb-containing vesicles to form uncoated vesicles which presumably fuse with early endosomes (Singh et al., 2003) and ultimately Hb is delivered to lysosomes (Patel et al., 2008). Since LdUrm1 facilitates Rab mediated early endosome-endosome fusion, the competitive mutant protein (LdUrm1ΔG) eventually disturbs the cargo delivery to lysosomes via Rab 7. The other reason for the metabolically compromised state of mutant parasite may be defective heme uptake machinery. However, more elaborate studies are required to determine to what extent the heme uptake process in Leishmania is affected. Moreover, appearance of heterogenous morphologies of mutant parasite characteristically with small body length, smaller flagella, and slow division also suggested the role of LdUrm1 in the parasite’s cell division process. A reduction of cellular Urm1 levels is known to cause severe cytokinesis defects in HeLa cells that result in the accumulation of enlarged multinucleated cells (Schlieker et al., 2008). Alternatively, urmylation also appeared to be important for the regulation of cytoskeletal organization in L. donovani as identification of putative kinesin-like, dyenin and ankyrin proteins as urmylation targets was consistent with this view. Moreover, since the cytoskeletal organization is an integral part of membrane trafficking, the possibility remains that cytoskeleton is a virtual target for urmylation.

To our knowledge, this is the first report that elucidates the presence of complete Urm1 pathway along with its diverse set of substrates importantly with RabGTPases as one of the unique targets localized in early endosomes. Collectively, our data also indicated that protein urmylation is a dynamic process as Urm1 takes up a diverse range of activities from yeast to Leishmania to humans. This suggested that URM1 has evolved mechanistically to equip the metabolism of the different organisms with the specialized functions indicating itself as an essential gene in the protozoan parasite Leishmania.
Conclusions

The present study is the first demonstration of *Leishmania* specific ubiquitin related modifier-1 (LdUrm1), its conjugation pathway and its target proteins in *Leishmania donovani*. Overexpression studies revealed that the protein is essential for the balanced growth of the parasite thus could be important to parasite pathogenesis. Further, demonstration of the existence of early endosome associated LdUrm1 conjugation in *Leishmania* opens new avenues to explore this pathway in the context of pathogenesis and host-parasite interactions. Importantly, the Ub-proteasome pathway has been recognized as a viable therapeutic pathway in the treatment of cancer (Eldridge and Brien, 2010). Interestingly, the identification of LdUrm1 mediated protein modification pathways in *Leishmania*, with its distinct subset of diverse substrate proteins as demonstrated in this report present themselves as potential drug targets.

![Fig 5.10: Schematic presentation of Urm1 conjugation pathway and the enzymes involved in the pathway.](image)

The first step in the process of urmylation cascade is the ATP dependent charging of the E1 activating enzyme. E1 uses the ATP to adenylate the C-terminus of Urm1 forming Urm1-AMP and pyrophosphate. The Urm1-AMP bond is then cleaved by an active site cysteine present within E1, resulting in a bond between the C-terminus of Urm1 and the sulfhydryl of the cysteine. The nature of this bond is not known.