Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Sources of chemicals

Agarose, β-mercaptoethanol, BSA, TEMED, IPTG, X-gal, ethidium bromide, lysozyme, formamide, Tris base, tricine, proteinase K, MOPS, Triton X-100, SDS, HEPES, DMSO, ampicillin, kanamycin, Acrylamide and Bis-acrylamide were all obtained from Sigma, Geneticin, FCS, MEM, biotin, PABA, Hemin, DTT, ATP, dNTP, alkaline phosphatase and PCR components were all obtained from GIBCO BRL (Invitrogen life technologies Ltd), USA; Protein estimation dye reagent and protein molecular weight marker for SDS-PAGE were obtained from Bio-Rad, USA; protease inhibitor cocktail were obtained from Roche molecular biochemicals, USA. RNase A, restriction enzymes, DNA molecular weight markers and T4 DNA Ligase were from New England Biolabs Inc, USA. Tryptone, Agar, Noble-agar and Yeast extract were from Difco Laboratory. Whatman filter papers were from Whatman International Ltd. Organic reagents like isopropanol, chloroform, and methanol were from Qualigens, other reagents are obtained from local suppliers of SRL, S.D.fine-chemicals Ltd. and MERCK India Ltd. and were of analytical grade. 2 X Sybr green master mix was obtained from Applied Biosystems, USA. ER-Tracker Blue-white DPX, FM 4-64 and DAPI were obtained from Molecular Probes Inc. USA.

2.1.2 Antibodies used

Polyclonal anti-DRG1 antisera were raised in mice as described in section 3.7. Anti-GP63, Anti-KMP11 monoclonal antibodies were obtained from Cedarlane laboratories Ltd, Canada. Polyclonal anti-GFP and anti-mRFP antibodies were obtained from Abcam, USA. Anti-BiP antibody is kind gift from Jay Bangs. Biotin-SP-conjugated Affinipure Goat Anti-Mouse IgG was obtained from Jackson Immuno Research Laboratories Inc, USA. Streptavidin-Cy3 conjugated tertiary antibody was obtained from Sigma, USA.

2.1.3 Bacterial strains

E. coli DH5α: F' end A1 hsd R17 (rK- mK-) sup E44 thi-1 rec A1 gyr A96 (Nal') rel A1 Δ (lac ZYA-arg F) u169 (φ80 lac Z8 M15).
Above described strain of *E.coli* was used for all transformations, plasmid isolations and for selection of recombinant clones.

*E. coli BL21 (λDE3)* (*E. coli* B F*, ompT, hsdS (rB*, mB*), gal, dcm*)

This strain was used for protein expression and purification studies

2.1.4 *Leishmania* strains

Wild type strain of *Leishmania donovani* (MHOM/IN/1983/AG83) was a kind gift from Dr. H. Majumdar, IICB, Kolkata. Promastigotes recovered from the spleen of golden hamsters infected with AG83 were named AG83Re and used for all subsequent experiments. AG83-GFP, AG83-DRG1-GFP, AG83-mRFP, AG83-DRG2-mRFP was generated for this study. Clonally identical attenuated line of *L. donovani* was generated previously in the lab by *in-vitro* passaging.

2.1.5 Plasmids

a) **TOPO-TA vector**: vector The TOPO-TA PCR2.1 vector from Invitrogen (Catalog No: K4600-40), has been engineered to be a linearized plasmid with 3’ deoxythymidine (T) overhangs that is activated by being covalently bonded to topoisomerase-I. The 3' A overhangs of the PCR product complement the 3' T overhangs of the vector and allow for fast ligation with the already present topoisomerase I. The plasmid can then be transformed into competent bacterial cells

b) **pMOS vector**: Dephosphorylated pMOS Blue cloning vector from Amersham Pharmacia Biotech Ltd (Catalog No: RPN 5110), allow the cloning of blunt end, 5'- or 3'- overhangs and PCR amplified product. Inserts are converted into blunt, phosphorylated product in one step and ligated into blunt dephosphorylated pMOS Blue vector.

c) **pET28a+**: pET28 vector is the kind gift from Dr Usha K Srinivas, CCMB, Hyd. It is a bacterial plasmid designed to enable the quick over expression of desired protein with His tag that will use for the purification. This plasmid contains several important elements – a N-terminal His Tag /thrombin /T7 Tag, an optional C-terminal His Tag sequence, a *lacI* gene which codes for
the lac repressor protein, a T7 promoter which is specific to only T7 RNA polymerase (not bacterial RNA polymerase) and a kanamycin resistance gene. Induction of protein was accomplished by adding the IPTG.

d) **pX-vector:** This *Leishmania* expression vector is the derivative of pX-βGAL from which β-Galactosidase cDNA has been removed by BamHI digestion and then relegated (kind gift from SM Beverley). This vector backbone has the 5' and 3' polypyrimidine tracts having signals for transsplicing of miniexon and polyadenylation required for gene expression in *Leishmania*.

e) **pX-GFP+ vector:** This vector have EGFP cDNA to express the GFP in *Leishmania* and also carry the same backbone and expression machinery as in pX-vector (kind gift from SM Beverley).

f) **Aktin-mRFP vector:** This vector is the derivative of actin-GFP from Clontech, USA in which GFP has been replaced with mRFP cDNA between BamHI and BglII site (kind gift from Dr Veena Parnaik, CCMB, India).

**2.1.6 Media**

**Luria-Bertani (LB Medium):** 1% Bactotryptone, 1% NaCl and 0.5% bacto-yeast extract were dissolved in milli-Q water. pH was adjusted to 7.4 using 10 N NaOH and then autoclaved.

**LB Agar:** LB media reconstituted with 2% Bactoagar was used for pouring LB plates.

**SOB media:** LB media supplemented with 250 mM KCl. Before using the media 19g of MgCl2 was added per 100ml LB media.

**MacConkey agar media:** MacConkey agar is a selective medium that inhibits the growth of Gram-positive bacteria due to the presence of crystal violet and bile salts. MAC is also a differential, meaning that it differentiates or distinguishes between groups of bacteria on the basis of a color change reaction. MacConkey's contains two additives that make it differential; neutral red (a pH indicator) and lactose (a disaccharide). Bacteria, that has lac Z gene intact, eat the media's lactose, and, in the process, create an acidic end product that causes the pH indicator, neutral red, to
turn pink. Non-lactose fermenting bacteria which have disruption in lacZ gene will be colorless or referred as recombinant bacteria.

**Complete Homem media (1X):** Homem media was prepared by dissolving 11 g sodium pyruvate; 1.5 g D-glucose; 2.2 g NaHCO₃; 0.1 mg Biotin; 1 mg PABA in 1 liter of MEM containing Hanks salts, L-glutamate and non-essential amino acids; 25 ml of 1 M HEPES buffer, pH 7.5 was added to it; pH was adjusted to 7.4. The media was filter sterilized using a 0.2 μm bottle top filter apparatus from Nalge Nunc International and stored at -20°C. 100 ml of FCS was heat inactivated at 56°C for 30 minutes and stored at -20°C. A 1000X stock of Hemin was prepared by dissolving 60 mg Hemin in 10 ml of 1 N NaOH, filter sterilized using a 0.2 μ syringe filter from Nalge Nunc International and stored at -20°C. 100 ml of FCS and 1ml of Hemin stock solution was added to 900 ml of Homem media to make Complete Homem media and was stored at 4°C.

**Noble agar Homem:** A 2X solution of Complete Homem media was prepared by lyophilizing the Homem Media (1X) and re-constituting into the half volume. This 2X media were filter sterilized and stored at 4°C. A 2% solution of Agar Noble was prepared and mixed with an equal volume of 2X Complete Homem media before pouring into petri plates. For antibiotic selection 40-200 μg/ml G418 was added just before pouring plates. Plates were equilibrated with CO₂ for 30 minutes in a 5% CO₂ incubator. These plates were stored at 4°C for not more than a month.

**2.2 Buffers and Solutions**

For all buffers, the final concentrations are given and stock solutions are indicated in brackets.

**2.2.1 Buffers for DNA and RNA gel electrophoresis**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE-buffer (10X stock solution)</td>
<td>90.0 mM Tris-base, pH 8.3</td>
<td>Tris-base, pH 8.3</td>
</tr>
<tr>
<td></td>
<td>90.0 mM</td>
<td>Boric Acid</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>EDTA</td>
</tr>
</tbody>
</table>

**Gel-loading buffer (FM), native** 75.0 % (v/v) Glycerol
Stock solution

0.1 mM EDTA
0.1 % Bromophenol blue
0.1 % Xylene cyanol FF

Gel-loading buffer (FA).

80.0 % Formamide

Stock solution

1.0 mM EDTA
0.1 % (w/v) Bromophenol blue
0.1 % (w/v) Xylene cyanol FF

2.2.2 Buffers for protein gel electrophoresis

Acrylamide solution (40:1)

30.0 % (w/v) Acrylamide
0.75 % (w/v) Bisacrylamide

Stacking gel buffer

125.0 mM Tris-HCl, pH 6.8

(4X stock solution)

0.1 % (w/v) SDS

Separating gel buffer

375.0 mM Tris-HCl, pH 8.8

(4X stock solution)

0.1 % (w/v) SDS

Loading buffer

125.0 mM Tris-HCl, pH 6.8
20.0 % Glycerol
10.0 % β-Mercaptoethanol (Added freshly)
4.0 % (w/v) SDS
0.02 % (w/v) Bromophenol blue

Running buffer

25.0 mM Tris-OH, pH 8.3

(10X stock solution)

192.0 mM Glycine
0.1 % (w/v) SDS

Coomassie blue staining solution

40.0 % Methanol
10.0 % Acetic acid
0.1 % (w/v) Coomassie brilliant blue R-250

Fast Destaining solution

40.0 % Acetic acid
10.0 % Methanol
### 2.2.3 Buffers for molecular biological methods

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligase buffer</td>
<td>20.0 mM</td>
<td>Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>(10X stock solution)</td>
<td>5.0 mM</td>
<td>MgCl2</td>
</tr>
<tr>
<td></td>
<td>5.0 mM</td>
<td>DTT</td>
</tr>
<tr>
<td></td>
<td>50.0 µg/ml</td>
<td>BSA</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>ATP (added separately)</td>
</tr>
<tr>
<td>Alkaline phosphatase-(SAP) buffer</td>
<td>50.0 mM</td>
<td>Tris-HCl, pH 8.5</td>
</tr>
<tr>
<td>(10X stock solution)</td>
<td>0.1 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td>Proteinase K buffer</td>
<td>50.0 mM</td>
<td>Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>(10X stock solution)</td>
<td>10.0 mM</td>
<td>CaCl2</td>
</tr>
<tr>
<td></td>
<td>0.5 % (w/v)</td>
<td>SDS</td>
</tr>
</tbody>
</table>

### 2.2.3 Buffer for plasmid DNA preparations

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol buffer</td>
<td>80.0 % (w/v)</td>
<td>Phenol</td>
</tr>
<tr>
<td></td>
<td>50.0 mM</td>
<td>Tris-OH</td>
</tr>
<tr>
<td></td>
<td>0.1 %</td>
<td>hydroxy quinol</td>
</tr>
</tbody>
</table>

### 2.2.5 Solutions for plasmid DNA purification with QIAGEN columns

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>50 mM</td>
<td>Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>10.0 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td></td>
<td>50.0 mM</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>10.0 µg/ml</td>
<td>RNase A (added freshly before use)</td>
</tr>
<tr>
<td>P2</td>
<td>0.2 M</td>
<td>NaOH</td>
</tr>
<tr>
<td></td>
<td>1.0 %</td>
<td>SDS</td>
</tr>
<tr>
<td>P3</td>
<td>3.0 M</td>
<td>Sodium Acetate</td>
</tr>
<tr>
<td></td>
<td>2.0 M</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>QBT</td>
<td>50.0 mM</td>
<td>MOPS, pH 7.0</td>
</tr>
<tr>
<td></td>
<td>750.0 mM</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>15.0 %</td>
<td>Ethanol</td>
</tr>
</tbody>
</table>
Wash buffer (QC Buffer)
- 0.15 % Triton X-100
- 50.0 mM MOPS, pH 7.0
- 1.0 M NaCl
- 15.0 % Ethanol

Elution buffer (QF Buffer)
- 50.0 mM MOPS, pH 8.2
- 1.25 M NaCl

TE-buffer
- 10.0 mM Tris-HCl, pH 8.0
- 0.1 mM EDTA

2.2.6 Buffer for Genomic DNA isolation

Lysis Buffer
- 150.0 mM NaCl,
- 10.0 mM EDTA,
- 10.0 mM Tris-HCl
- Adjust pH 7.5 and add
- 0.4 % SDS
- 200 ug/ml Protienase K (Just prior to use)

2.2.7 Buffers for purification of 6X-His-tag proteins under denaturing conditions

Lysis buffer (Buffer B)
- 100.0 mM NaH2PO4
- 10.0 mM Tris-HCl, pH 8.0
- 8.0 M Urea
- Adjust to pH 8.0 using NaOH. Should be adjusted immediately prior to use.

Wash buffer (Buffer C)
- 100.0 mM NaH2PO4
- 10.0 mM Tris-HCl, pH 8.0
- 8.0 M Urea
- Adjust to pH 6.3 using HCl. should be adjusted immediately prior to use.
<table>
<thead>
<tr>
<th>Elution buffer (Buffer E)</th>
<th>100.0 mM</th>
<th>NaH2PO4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.0 mM</td>
<td>Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>8.0 M</td>
<td>Urea</td>
</tr>
</tbody>
</table>

Adjust to pH 4.5 using HCl. Should be adjusted immediately prior to use.

### 2.2.8 Buffer for Transfection in Leishmania

<table>
<thead>
<tr>
<th>Electroporation Buffer</th>
<th>21.0 mM</th>
<th>HEPES,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>137.0 mM</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>5.0 mM</td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>0.7 mM</td>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td></td>
<td>6.0 mM</td>
<td>D-Glucose</td>
</tr>
</tbody>
</table>

Adjust pH 6.4 and filter sterilize.

### 2.2.9 Buffers for Southern and Northern Hybridization

<table>
<thead>
<tr>
<th>Solution I (Hydrolysis)</th>
<th>0.25 M</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution II (Denaturation)</td>
<td>1.5 M</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>0.5 M</td>
<td>NaOH</td>
</tr>
<tr>
<td>Solution III (Neutralization)</td>
<td>1.0 M</td>
<td>Tris</td>
</tr>
<tr>
<td></td>
<td>1.5 M</td>
<td>NaCl pH8.0</td>
</tr>
<tr>
<td>Pre-hybridization buffer</td>
<td>6.0 X</td>
<td>SSC</td>
</tr>
<tr>
<td></td>
<td>0.5 %</td>
<td>SDS</td>
</tr>
<tr>
<td></td>
<td>5.0 X</td>
<td>Denhardt’s</td>
</tr>
</tbody>
</table>

50-100 μg/ml Sonicated Salmon sperm DNA

| Hybridization Buffer           | 6.0 X     | SSC     |
|                                | 0.01%     | EDTA    |
|                                | 0.5 %     | SDS     |
|                                | 5.0 X     | Denhardt’s |

50-100 μg/ml Sonicated Salmon sperm DNA
<table>
<thead>
<tr>
<th>Wash Buffer</th>
<th>0.2 X</th>
<th>SSC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td></td>
<td>0.1 %</td>
<td>SDS</td>
</tr>
</tbody>
</table>

**2.2.10 Buffers for RNA Isolation by AGPC Method**

All solution in DEPC water

<table>
<thead>
<tr>
<th>Denaturing Solution</th>
<th>4.0 M</th>
<th>Guanidinium thiocyanate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Solution D)</td>
<td>25.0 mM</td>
<td>Sodium Citrate pH 7</td>
</tr>
<tr>
<td></td>
<td>0.5 % (w/v)</td>
<td>N-Sorkosyl</td>
</tr>
<tr>
<td></td>
<td>0.1 M</td>
<td>2-mercaptopethanol (just prior to use)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sodium Acetate (pH 4.0)</th>
<th>2.0 M</th>
<th>Sodium Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 % ethanol</td>
<td>75.0 ml</td>
<td>Absolute Alcohol</td>
</tr>
<tr>
<td></td>
<td>25.0 ml</td>
<td>DEPC water</td>
</tr>
</tbody>
</table>

**DEPC water Saturated Phenol**

**2.2.11 Buffers for western blot analysis**

Transfer buffer

<table>
<thead>
<tr>
<th>Phosphate buffer saline</th>
<th>137 mM</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10X stock)</td>
<td>2.7 mM</td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>4.3 mM</td>
<td>Na$_2$HPO$_4$</td>
</tr>
<tr>
<td></td>
<td>1.4 mM</td>
<td>KH$_2$PO$_4$</td>
</tr>
</tbody>
</table>

Adjust the pH to 7.0 with HCl.

**2.2.12 Buffers for ultra competent cell preparation**

<table>
<thead>
<tr>
<th>RF1 Buffer</th>
<th>100mM</th>
<th>RbCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50mM</td>
<td>MnCl$_2$.3H$_2$O</td>
</tr>
<tr>
<td></td>
<td>30mM</td>
<td>Potassium acetate</td>
</tr>
<tr>
<td></td>
<td>10mM</td>
<td>CaCl$_2$.2H$_2$O</td>
</tr>
<tr>
<td></td>
<td>15 % (w/V)</td>
<td>Glycerol</td>
</tr>
</tbody>
</table>
Adjust the pH to 5.8 with 0.2 M acetic acid.
Sterilize by filtration.

**RF2 Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS</td>
<td>10 mM</td>
</tr>
<tr>
<td>RbCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>CaCl2.2H2O</td>
<td>75 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15 % (w/v)</td>
</tr>
</tbody>
</table>

Adjust the pH to 6.8 with NaOH. Sterilize by filtration.

### 2.2.13 Antibiotics solutions

- **Ampicillin**
  - 60 mg/ml
  - Ampicillin
  - Stored at -20°C

- **Kanamycin**
  - 50 mg/ml
  - kanamycin
  - Stored at -20°C

- **Geneticin (G418)**
  - 20 mg/ml
  - Geneticin in 100 mM HEPES pH 7.4 & stored at -20°C

- **Hygromycin**
  - 20 mg/ml
  - Hygromycin in 1X PBS pH 7.4 & stored at -20°C

**Tris-saturated phenol**: Melted phenol was mixed with equal volume of 0.5 M Tris-HCl, pH 8.0 and 0.1% 8-hydroxyquinoline and phase separation was allowed at room temperature. The upper phase was removed and the process repeated with 0.1 M Tris-HCl, pH 8.0. This step was repeated till the pH of the aqueous solution was equilibrated to 8.0 and then stored in an amber-colored bottle at 4°C after mixing with 0.1 volumes of 0.1 M Tris-HCl, pH 8.0.

**Chloroform: Isoamyl alcohol**: 24:1 (v/v) mixture of chloroform:i.soamyl alcohol.
2.3 Methods:

2.3.1 Preparation of Ultra competent cells

Ultra competent cells were prepared by the protocol described by Hanahan, as it gave higher transformation efficiency (Hanahan et al., 1983). A single colony of *E.coli* DH5α was grown overnight at 37°C and a 1% inoculum of this culture was grown in 120 ml of SOC medium supplemented with 1 M MgCl₂ and MgSO₄ at 18°C with vigorous shaking (200 rpm) till an OD₆₀₀ of 0.45-0.60 was attained. The culture was then chilled on ice for 15 min and harvested at 3000 rpm for 10 min at 4°C. The pellet was resuspended in 10 ml RF1 buffer (1/3 of culture volume) and incubated on ice for 15 min. Cells were centrifuged and resuspended in 9.6 ml of RF2 buffer (1/12.5 of culture volume). The cells were incubated on ice for 15 minutes, aliquoted, snap frozen in liquid nitrogen, and stored at -70°C.

2.3.2 Transformation

Competent cells were thawed on ice. 5-10 μl of ligation mixture or 10 ng of pure plasmid was mixed and incubated on ice for 30 minutes. Heat shock was given at 42°C for 90 seconds and the cells were held on ice for 2 minutes. 800 μl of LB media was added and kept for recovery at 37°C for 60 min. Cells were then spread on LB agar plates containing required amounts of antibiotic (e.g. 60 μg/ml ampicillin or 50 μg/ml kanamycin). For plasmids containing *lac Z* gene providing pink white selection e.g. pMOS or TOPO-TA plasmids, cells were spread on MacConkey agar plates. Plates were incubated at 37°C for colonies to grow.

2.3.3 Selection of recombinant clones

For pMOS or TOPO-TA plasmids recombinants were identified by pink white selection. Self ligated non-recombinant plasmids with only vector backbone give pink colonies while recombinant clones in which the insert has disrupted the *lac Z* gene are white. For expression plasmids recombinants were identified by picking random colonies isolating plasmid and checking it with an appropriate restriction digestion pattern or by PCR amplification of insert.
2.3.4 Glycerol stock preparation and revival

400 µl of sterile 100% glycerol was added to 600 µl of fresh overnight culture in a screw cap cryotube and mixed well on ice. It was frozen immediately at -80°C. To revive a glycerol stock the frozen stock was picked with a sterile tooth-pick and then placed on a pre-warmed LB plate with required antibiotic and streaked. Then the bacteria are allowed to grow overnight at 37°C to obtain colonies.

2.3.5 Plasmid isolation

Plasmid DNA, on a small scale was prepared by alkaline lysis method (Maniatis et al., 1982), briefly, the cells were lysed using NaOH and SDS and DNA was extracted from the lysate using phenol-chloroform. The DNA pellet was then precipitated with ethanol and finally resuspended in sterile water containing RNase A for removal of contaminating RNA. Plasmids for transfection were prepared using QIAGEN-tip 100 (midi) columns according to manufacturer's instructions. The procedure involves alkaline lysis of cells followed by affinity column purification to obtain a high purity plasmid with very low amounts of endotoxin. For screening plasmids were digested with restriction enzymes immediately following RNase A treatment, for sequencing plasmids or fragments were washed thoroughly with 70% ethanol.

2.3.6 Restriction endonuclease digestion

Typically 1 µg DNA was digested with 2-5 unit of restriction enzyme using compatible buffer in a 20 µl volume. The buffers and incubation conditions were used as per the manufacturer's recommendation. Digested products were visualized by resolving on an agarose gel.

2.3.7 Agarose gel electrophoresis

After adding appropriate amounts of DNA loading dye, the DNA samples were resolved, depending on size, in 0.8-1.0 % agarose gels made in 1X TBE. For visualization 0.25 µg/ml of ethidium bromide was added to the gel while preparing. 1X TBE was used as running buffer.
2.3.8 Genomic DNA isolation

Cells were washed thoroughly in PBS and pelleted by centrifugation at 3000 rpm for 10 minutes at room temperature. The pellet was then resuspended in an appropriate volume of lysis buffer (150 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.5, 0.4% SDS, 200 μg/ml Protienase K) vortexed hard and incubated at 37°C till pellet dissolved completely. DNA was then extracted with phenol chloroform. DNA was precipitated overnight with 100% ethanol at -20°C. It was then washed with 70% ethanol and resuspended in sterile water containing 25 μg/ml RNase A. Concentration of DNA was estimated spectrophotometrically by measuring absorbance at 260/280 nm.

2.3.9 Phenol chloroform extraction

Phenol- chloroform extraction was carried out to remove protein from nucleic acid preparations. Equal volumes of a 1:1 (v/v) ratio of phenol chloroform was added to DNA solutions, vortexed and centrifuged at 13,000 rpm for 10 min at room temperature. The top aqueous layer was removed and extracted with an equal volume of 24:1 chloroform: isoamylalcohol. DNA was then precipitated from the aqueous layer using isopropanol.

2.3.10 Isolation of RNA from Leishmania cells

Cells were washed thoroughly in PBS and pellet by centrifugation at 3000 rpm for 10 minutes at room temperature. Cell pellet were dissolved in 1 ml of solution D per 1 x 10⁸ cells and vortexed hard. Following solutions were added sequentially to 1 ml of lysate: 0.1 ml of 2 M sodium acetate, pH 4.0, mix thoroughly by inversion; 1 ml water-saturated phenol, mix thoroughly by inversion; 0.2 ml of chloroform/isoamyl alcohol (49:1), shake vigorously by for 10 s and cool on ice for 15 min for phase separation. Aqueous phase, which contains mostly RNA, was transferred very carefully to a clean tube and RNA was precipitated overnight with isopropanol at -20°C. pellet was then washed with 70% ethanol in DEPC water and resuspended in DEPC treated water. RNA was incubated for 10-15 min at 60 °C to ensure complete solubilization. The integrity of total RNA was checked by running
an aliquot of the RNA sample on a denaturing 1.2 % agarose gel containing formamide and EtBr was added directly to the gel to visualize the RNA bands.

2.3.11 Quantification of nucleic acid

The nucleic acid concentration was determined by measuring the absorbance at 260 nm using Nanodrop spectrophotometer ND1000. Empirical relationship of 50 µg double stranded DNA, 33 µg of single stranded DNA (primers for PCR) and 40 µg of single stranded RNA was taken to be equal to 1.0 OD_{260}. Purity was checked by taking the ratio of the absorbance at 260 nm to 280 nm.

2.3.12 Gel elution and PCR purification

Gel purification and PCR purification of different DNA fragments were performed by using QIAGEN Gel elution kit and PCR purification kit as per manufacturer's instructions.

2.3.13 Ligation

DNA fragments and cDNAs obtained by PCR amplification or restriction digestion were cloned into appropriate vectors by ligation. A 10µl reaction containing 100 ng vector, 3-fold molar excess of insert DNA (according to size), 1 µl of 10X ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM DTT, 1 mM ATP), and 3 units of T4 DNA ligase enzyme. Ligations were carried out overnight at 16°C. For some of the ligation reactions, LigaFast ligation kit (Promega) was used and ligation was carried out as per manufacturer's instructions.

2.3.14 DNA sequencing

All DNA sequencing was done using automated DNA sequencing (ABI Prism, Model 3700 DNA Analyzer). Dideoxy dye terminator cycle sequencing ready reaction kit from Perkin-Elmer Cetus was used. 50 ng of plasmid and 1-2 pmole of primer in a total volume of 3.2 µl were mixed with 1.8 µl of the Big-dye-Terminator sequencing kit (Taq polymerase and fluorescent-tagged dNTPs) from Perkin-Elmer and PCR was carried out in Gene Amp PCR System 9600 Thermal Cycler using 'top-heating'. The following conditions were used:
Denaturation 96°C 10 seconds
Annealing 50°C 5 seconds
Extension 60°C 4 minutes

A total of 35 cycles and a final extension were done for 5 minutes. The reactions were then ethanol precipitated for 10 min on ice and washed twice with 70% ethanol to remove salt, as mentioned previously. The samples were resuspended in 10 µl High-dye-Formamide from Perkin-Elmer and loaded in capillaries.

2.3.15 Sequence analysis

Sequences were subjected to BLAST-N or BLAST-P at http://www.ncbi.nlm.nih.gov/BLAST/. Transmembrane domain predictions were performed using TMpred server at http://www.ch.embnet.org/software/TMPRED form.html and Wolf PSORT http://www.wolfpsort.org. Protein and DNA multiple sequence alignments were obtained using ClustalW (www.ebi.ac.uk/tools/clustalw). Sequences used in this study were obtained from the GeneDB databases for _L. major_, _L. infantum_ and _L. braziliensis_. DRG1 homologs in other Leishmania species were identified using BLAST at GeneDB database at http://www.genedb.org. The 5' upstream and 3'downstream sequences flanking _LdDRG1_ and _LdDRG2_ were generated by primer walking in which a sequential series of primers were designed and used to sequence both strand of genomic DNA flanking up to 2kb from Start codon of and Stop codon of both genes. Then sequence was assembled by using the software automated DNA sequences assembler in which _LdDRG_ locus sequences from _L. infantum_ were taken as a template. Sequences generated in this study for _L. donovani_ were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov). GenBank accession numbers are GQ214330 and GQ214331.

2.3.16 Oligonucleotide synthesis

All oligonucleotides used in this study were designed by using PRIMER 3 software available online and synthesized by BIOSERVE, Hyderabad. List of the primers used in this study is provided in the last section of Methods and Material as Table I-IV. For every primer melting temperature (Tm), 3'complementarity, hairpin
loop and all potential self-annealing sites were checked by using online software (http://www.basic.northwestern.edu/biotools/oligocalc.html). The following criteria were taken into consideration while designing the QRT-PCR primers:

1. Amplicon length: 150-200 bp
2. Optimal primer length: 20 bases
3. Tm: 58-60
4. % GC: 30-80
5. Last 5 nt at 3' end should contain no more than 2 (G+C) residues
6. Avoid runs in identical nt, if present, should be fewer than 4 consecutive G residue

2.3.17 Polymerase chain reaction (PCR)

A conventional PCR was carried out with the heated lid option in a thermal cycler (PTC-200, MJ Research). PCR for DNA amplification was done as per the method of Saiki et al., 1985. For routine PCR amplification 10 ng DNA (30 ng genomic DNA) was used as template in a 50 μl reaction. PCR was done in a reaction mix containing 1X PCR buffer from Invitrogen (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.001% w/v gelatin), 200 μM each of the dNTPs, 10 pmole each of primers and 1 unit of Taq DNA polymerase (Invitrogen). 34 step cycles were carried out after initial denaturation at 94°C for 5 min. Each step cycle consisted of denaturation, 94°C for 1 min, annealing (4°C below Tm of primer) for 1 min and extension, 72°C for 1-3 min depending upon the length of the amplicon. A final extension was given at 72°C for 7 min for completion of truncated products.

2.3.18 Random primer labeling of DNA

Double-stranded DNA was radiolabeled using random primers in a hexamer labeling kit as per the manufacturer's instructions or by PCR labeling method. About 50-100 ng of double-stranded DNA was denatured in a volume of 20 μl by boiling for 5 min and quick chilling on ice. This was followed by the sequential addition of 5 μl of random primers solution, 5 μl of 10X reaction buffer, 5 μl each of dATP, dGTP, dTTP, 40 μCi of α-[^32P]-dCTP and 2 units of Klenow enzyme. The reaction volume
was made up to 50 µl and the reaction was carried out at 37°C for 2 hrs. The enzyme was inactivated at 75°C for 10 min and the probe was separated from the unincorporated nucleotides by Sephadex G-50 spun column chromatography, denatured in boiling water for 5 min and rapidly chilled on ice and then use for hybridization.

2.3.19 Measurement of radioactivity in nucleic acids

For routine checking of labeling efficiency, samples were counted by Cerenkov’s method as described in Scarpitta and Fisenne, 1996. An aliquot of 1 µl of labeled DNA was spotted on a Whatman 3 MM filter and counted in the [³H] channel of a liquid scintillation counter. The value was multiplied by four to correct for the difference in efficiency between the [³H] and [³²P] channels. For a more accurate measure of radioactivity, the filter was suspended in scintillation fluid and the sample was counted in the [³²P] channel.

2.3.20 Southern blotting

The DNA samples to be hybridized were digested with appropriate restriction enzymes and resolved on 0.8% to 1% agarose gels alongside DNA markers as described earlier. The gel was stained with ethidium bromide, photographed and the DNA was transferred to Hybond N+ membrane by vacuum blotting in a vacuum blotter (Amarsham Pharmacia, USA) for 1 - 1.5 hr in 400 mM NaOH. The blot was then rinsed in 5X SSC, air-dried and subjected to prehybridization, hybridization, washing and developing as described in section 2.3.22.

2.3.21 Northern blotting

Total RNA was resolved in 1% agarose gel containing 3.7 M formaldehyde made in 1X MOPS buffer (20 mM MOPS pH 7.0, 0.8 mM sodium acetate, 1 mM EDTA). RNA samples were denatured by mixing 4-5 µl RNA (10 µg), 3.5 µl formaldehyde, 2 µl 10X MOPS and 10 µl deionized formamide and incubating at 56°C for 15 min. 2.5 µl of 10X RNA gel loading dye was added to the samples and the samples were resolved in 1% agarose gel using 1X MOPS as the running buffer. The gel was then washed thrice with 100 ml of sterile double distilled water to remove formaldehyde.

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RNA was then stained with 1 ng/ml ethidium bromide in water for 10 min and photographed. The RNA was vacuum blotted (Amarsham Pharmacia, USA) onto Hybond N+ using 50 mM NaOH for 1 hour. The blot was then rinsed in 0.1X SSC air-dried and subjected to cross linking, prehybridization, hybridization, washing and developing as described in section 2.3.22.

2.3.22 Reverse northern blotting

Differentially regulated gene fragments were amplified by PCR using specific primers. 500 ng of each fragment DNA is diluted in 30 μl and mixed with 10 μl of 2 N NaOH. DAN was then denatured at 100°C for 5 min and then neutralized with 10 μl of 3 M sodium acetate buffer, pH 4.8. The final volume was adjusted to 110 μl. 50 μl of each sample were blotted in duplicate on nylon membranes in a dot-blot apparatus (Bio-Rad). Membranes were then UV-cross linked. Pre-hybridization, hybridization, washing and developing as described in section 2.3.23.

2.3.23 Hybridization for northern or southern blot and visualization of radioactive signal

Hybridizations were performed in 50% formamide; 6XSSC, 0.1% SDS and 0.25 mg/ml fish DNA at 63°C overnight. Finally, two post hybridization washes were performed in 0.1X SSC, 0.2% SDS at 65°C for 15min each and the signals on the filters were visualized by Fuji FLA-3000 phosphoimager system. Densitometric analysis was performed on the hybridization signals using Quantity One software (Bio-Rad). The values obtained from both the control and sample hybridizations were normalized. RNA ladder Millennium™ Markers-Formamide (Ambion Inc.) and DNA ladders were used to determine the approximate size of the transcripts and DNA fragment respectively.

2.3.24 De-probing Southern or Northern blots

To re-probe Southern or Northern blots, the bound radioactive probe was stripped from the N+ membrane by pouring hot de-probe solution (0.5X SSC and 0.1% SDS) on the blot in a glass tray and then boiling the blot containing tray carefully for 3-5 min. The de-probe solution containing the stripped radioactive probe was carefully discarded into a radioactive waste container and the blot
checked for counts using a Geiger counter without drying the blot and also the signals on the filters were checked by Fuji FLA-3000 phosphor imager system. The whole procedure was repeated several (2-3) times till the counts decreased to the desired basal level. A single blot was re-probed at least twice after de-probing using this procedure.

2.3.25 Protein expression and purification

The LdDRG1 ORF was cloned in pET28a+ bacterial expression vector and sequenced for confirmation of correct reading frame. E. coli BL21 λ DE-3 competent cells were transformed with recombinant plasmid and grown on LB plate containing 50 µg/ml kanamycin. A single colony was inoculated in 5 ml of LB medium containing 50 µg/ml kanamycin and grown overnight in a 50 ml flask at 37°C. For standardization of expression conditions, 10ml of LB medium containing 50 µg/ml kanamycin was inoculated with 1% (i.e., for 10ml 100µl overnight grown culture) overnight grown culture and incubated at 37°C with vigorous shaking till OD600 reached 0.6. Expression of recombinant protein was induced by adding IPTG to a final concentration of 1mM. The culture was grown and samples were collected after 3 hrs; cells were pelleted and re-suspended in 120µl of PBS with protease inhibitor. Equal amount of induced and uninduced samples are boiled in SDS-PAGE sample buffer. Optimum conditions for induction of 6XHis-DRG1 protein expression was standardized which were 37°C, 1mM IPTG conc. and 3 hr of induction.

Purification was done under denaturating condition as 95% of induced protein is going into insoluble fraction. Purification was performed by QIA Ni-NTA agarose as the instructions given in the manual. Briefly, induced cells from 100 ml culture were collected and pellet was resuspended in 4 ml of buffer B containing 8M urea. Lysates was centrifuged at 5000 rpm for 1 min to remove the cellular debris then 1 ml of 50% Ni-NTA slurry was added to this cleared lysate and kept for shaking for 60 min at room temperature. Resin was washed three times with buffer C. Finally elution was done three times by adding 500ul of buffer E (elution buffer).
2.3.26 Estimation of protein concentration

The protein content of the cell lysate was quantitated by the method described by Bradford (1976). Briefly, the sample to be estimated was made up to a volume of 160 µl with water in a microtitre plate. 40 µl of Bio-Rad reagent (Bradford dye concentrate) was added to the samples to be checked as well as to the standard curve samples, and mixed briefly for 5 min. The O.D.₅₉₅ was read in an ELISA reader. A curve was plotted using the known concentration samples, and the protein concentration of the unknown sample was estimated using this standard curve.

2.3.27 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out by the method described by Laemmli (1970) in a discontinuous buffer system. Gels of 1 mm thickness were cast and run on a vertical gel apparatus (Bio-Rad, USA). The 15% resolving gel and stacking gel 5% were polymerized by the addition of TEMED and freshly prepared ammonium persulphate solution. The protein samples were boiled in 1X Laemmli buffer for 3 min and loaded on the gel. Electrophoresis was carried out in electrophoresis buffer at a constant voltage of 150 V. After the run, the gel was stained with 0.25% Coomassie Brilliant Blue R250 in methanol: acetic acid: water (4:1:5) and boiled for 30 sec and keep it for 10-15 min on shaker and destained in 200 ml of methanol: acetic acid: water (4:1:5) and boiled for 30 sec and keep it on shaker until bands were clearly seen.

2.3.28 Generation of polyclonal antibody

For raising polyclonal antibody against recombinant 6X-His-DRG1 protein in the mouse, 100 µg/animal of protein was with equal volume of Freund's complete adjuvant. The mixture was made into an emulsion by passing through a 2 ml syringe (1.5 inch 19G needle) and intermittently keeping it at 4°C. This emulsion was injected into the mouse subcutaneously. After 14 days, the first booster dose was given in a similar manner, except that Freund's complete adjuvant was replaced with Freund's incomplete adjuvant. Subsequent booster doses were given after a 14-day interval and the antibody titer was checked using dot blot. For determination of antibody titer 20 ng of purified protein was spotted in a row, air-dried and
incubated with different concentrations of immune serum (1:1,000 to 1:5,000). Western blot experiments were carried out when the appropriate titer (minimum of 1:5,000) was obtained following booster doses.

2.3.29 Collection of immune sera

Mice were sacrificed and blood collected from the inferior vena cava using a 23 G needle and a 2 ml syringe. The blood was allowed to stand at room temperature for a couple of hours and then kept at 4°C for overnight to allow formation of a firm clot. Blood cells were removed by centrifugation and the sera were collected in a fresh tube. 0.2% NaN₃ was added and stored at 4°C.

2.3.30 Western Blot Analysis of LdDRG2::GFP Protein in Transfected Cells

*Leishmania* promastigote or amastigote of different stages of growth were harvested, washed in PBS and then resuspended in 1XPBS with 1X protease inhibitor cocktail (Roche) and then sonicated using VibraCell (Sonics & Materials Inc.) with 50% amplitude and three pulses for 30 sec each. 10µg of Protein lysate from each sample were separated on 15% SDS-PAGE and transferred onto nitrocellulose Hybond ECL membranes (Amersham Biosciences). Membranes were blocked with 5% nonfat dried milk in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The following antibodies were used: Anti-rabbit Polyclonal GFP antisera (Abcam; 1:3000), Anti-rabbit Polyclonal mRFP antisera (Abcam; 1:3000) anti-mouse polyclonal BiP antisera (1:10,000), anti-mouse or anti rabbit biotinylated antibodies (1:5000, Amersham) and avidin conjugated with horseradish peroxidase (1:10000, Amersham Biosciences). After incubation, membranes were washed three times with TBS-T. Immunodetection was carried out using the ECL western blotting detection system (GE Healthcare) according to the manufacturer's instructions and images were obtained using BIOMAX™-XBT x-ray film (Kodak).

2.3.31 Reverse-transcription (RT) reaction or cDNA synthesis

Reverse transcription was done with oligo dT using an RT-PCR kit as per the manufacturers' instructions. RT was carried out in a final concentration of 5 mM magnesium chloride; 1 mM each of dNTPs; 1X PCR buffer; 1 unit RNase inhibitor; 2.5
units superscript III™ and 1-2 μg total RNA. A master mix was prepared (20 μl per reaction) and final volume was made up with DEPC-treated water. 1-2 μg RNA and Oligo dT were incubated at 65°C for 5 min. RT was carried out in a step cycle comprising extension at 42°C for 1 hr. Inactivation of RT enzyme was done at 75°C for 15 min. further cDNA was stored at -20°C.

2.3.32 Quantitative Real-time PCR

All real-time PCR assays were performed on the ABI prism 7900 HT sequence detection system (ABI) as per the manufacturer’s instructions. A typical 10 μl reaction mixture contained 5 μl of 2x SyBR green dye (ABI), 1 pm of each primer, 1 μl of template and 2 μl of distilled water. All reactions were set up, in triplicate, in a 384-well optical reaction plate. The PCR conditions were as follows: 95 °C for 10min, 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s for 40 cycles. Following primers were used in QRT-PCR analysis; for DRG1; TV385 & TV273, for DRG2; TV385 & TV272; for GAPDH, TV366 & TV367 and for 18sRNA; TV457 & TV458. All the reactions were analyzed by software (SDS 2.2) provided with the instrument. The generation of amplification plots, standard curves and dissociation stage analysis was as per the manufacturer’s protocol. The calculation of the melting temperature of each amplicon (Tm) was done directly by software provided. The relative expression of the genes was calculated by using 2^−ΔΔCT formula using GAPDH as a normalizer (18s RNA for Cycloheximide treatment). The values reported are the mean of at least three biological replicates, each with three technical replicates. The standard deviation from the mean is shown as error bars in each group.

2.3.33 Leishmania Growth Condition

The conditions for promastigotes and amastigote cultures are as described as earlier (Bera et al., 2003). The promastigotes were routinely inoculated at a starting density of 10^6 cells/ml and grown in a 26°C incubator in tissue culture flasks or microtitre plates; 3 days and 7 days old cultures were designated as log phase and stationary phase respectively. For all experiments, cell numbers were estimated by direct counting in a hemocytometer under a light microscope.
2.3.34 In vitro conversion of promastigotes to amastigotes and vice versa

Promastigotes were grown for three days in Complete Homem media (10% FCS and pH 7.4). 2-4 X 10^6 cells/ml were taken from the above culture, pelleted down by centrifugation at 3000 rpm for 10 min, washed in PBS and resuspended in amastigote media (Complete Homem, 20% FCS, pH 5.4). These cells were grown at 37°C with 5% CO₂. Promastigotes convert into amastigotes within 3 days. Amastigotes were maintained in tissue culture by changing the media once in every 5 days. Amastigotes were reconverted back to promastigotes by resuspending them in Complete Homem media pH 7.4 with 10% FCS and incubating at 26°C for 3-4 days.

2.3.35 Freezing and revival of Leishmania cells

Cells were washed in PBS and resuspended in Complete Homem media containing 7.5% DMSO on ice, aliquoted in cryotubes and frozen immediately at -80°C. After 24 hours the tubes are transferred to liquid N₂ for long-term storage. For reviving clones from the frozen stock cells were thawed on ice for 30 minutes and transferred to 5 ml of media and incubated at 26°C for 24 hours before adding antibiotic to the media.

2.3.36 Treatment of Leishmania with G418 and Hygromycin

Sensitivity of virulent and attenuated promastigotes towards the G418 and hygromycin were monitored indirectly by determining the growth of promastigotes cultured in the presence of various concentrations of the drug G418 and hygromycin. Promastigotes were grown for 4 days the at the starting densities of 10^6 cells/ml in the presence of G418 (10-200 μg/ml) and hygromycin (10-200 μg/ml). Cell densities were counted every 24 hr time interval.

2.3.37 Electroporation of Leishmania promastigotes

Electroporation of Leishmania cells was done as described in Kapler et al., 1990. Leishmania promastigotes were grown to a density of 3 x 10^7 cells/ml. Cells were washed in PBS and resuspended in electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose, 21 mM HEPES pH 7.5) at a density of 1 x
10^8 cells/ml and kept on ice. 400 μl of cells were aliquoted per eppendorf and 100 μg of DNA (of concentration not less than 1 μg/μl) was added such that final volume was 500 μl. Cells and DNA were mixed well and transferred to 0.2 mm cuvettes (Bio-Rad) on ice making sure that the liquid completely covered the metal plates. Cuvettes were placed in the chamber and a pulse of 450 volts (2.25 v/cm) was given at capacitance 500 mF. Cuvette was immediately placed on ice for 10 min. Time constant values were noted and recorded. 1 ml of complete Homen media was added to each cuvette and the cells were transferred to T25 flask containing 5 ml media. Cells were allowed to grow for 48 hours at 26°C. Transformants are selected in HOMEM containing G418 (20 μg/ml for virulent promastigote and 200 μg/ml for attenuated promastigotes) or Hygromycin (10 μg/ml for both) before plating in HOMEM-G418 plates for selection. Approximately 100 parasites/plate was placed on 1% agar plate containing the same growth medium supplemented with either the G418 (geneticin; Invitrogen) at 40 μg/ml for virulent promastigote and 400 μg/ml for attenuated promastigote or hygromycin (Invitrogen) at 20 μg/ml.

2.3.38 Selection of transfected clones

Electroporated cells were plated on pre-warmed Homem-agar-antibiotic plates at 10^2 cells per plate, spread gently and uniformly, parafilm sealed and incubated at 26°C. It takes 7 to 10 days for colonies to appear on the plate. Plates were re-equilibrated with CO₂ for 30 min after every 7 days. Colonies were picked by coring agar with a blue tip (P1000 tip) into 2 ml Complete Homem media in a 15 ml falcon tube. Clones were checked for expression of the transgene. Several clones were expanded and frozen. Clones isolated were maintained in liquid media containing 20 μg/ml or 200 μg/ml geneticin or 10 μg/ml hygromycin.

2.3.39 Treatment of Leishmania with cycloheximide

For cycloheximide experiments, log or stationary phase promastigote or amastigotes culture were collected by centrifugation and resuspended in fresh medium to achieve a culture density of 10^7 cells/ml. Cycloheximide was added to a final concentration of 5μg/ml and incubated for different time interval. This concentration was examined previously in the lab to inhibit the protein synthesis by more the 90% (personal communication).
2.3.40 Treatment of leishmania promastigotes with $\text{H}_2\text{O}_2$

Exponentially growing cells were collected by centrifugation and resuspended in fresh medium to achieve a culture density of $10^7$ cells/ml. Cells were dispensed in 24-well culture plates and appropriate concentrations of $\text{H}_2\text{O}_2$ were added. After 30 min cell motility was checked by visual inspection in a Neubauer haemocytometer and the number of motile cells versus the total number of cells was calculated and expressed as percentage motile cells.

2.3.41 Labeling of Intracellular Organelles in Live Cells with Vital Stain

Cells were labeled with the vital stain ER-Tracker Blue-White DPX (Molecular Probes Inc.) at a final concentration of 1uM. Endocytic organelles in promastigotes were labeled in live cells by adding vital stain FM 4-64 (final concentration 8uM) in medium. Cells were incubated for 2hr at 26°C. Non-internalized FM 4-64 was back-extracted by resuspending the cells in fresh medium. Similarly, DNA staining dyes was also added directly to the culture to the final concentration of 1 ug/ml for DAPI (Molecular Probes Inc.) or 0.5 ug/ml for ethidium bromide (Sigma) and incubated for 10-15 min at 26°C. After incubation, cells were gently pelleted by centrifugation at 3000 rpm for 5 min and resuspended in fresh medium and analyzed by confocal fluorescent microscopy. Visualization of dyes and fluorescence was according to the suppliers instructions. Excitation and emission range of different dyes used in this study are listed below:

<table>
<thead>
<tr>
<th>Dye or reporter gene</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>488</td>
<td>485-520</td>
</tr>
<tr>
<td>mRFP</td>
<td>580</td>
<td>610-650</td>
</tr>
<tr>
<td>ER-Tracker Blue DPX</td>
<td>345</td>
<td>400-455</td>
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<td>590-650</td>
</tr>
<tr>
<td>DAPI</td>
<td>345</td>
<td>400-455</td>
</tr>
</tbody>
</table>
2.3.42 Confocal Microscopy of Transfected Cells

Promastigotes were immobilized by mounting on and under poly-L-lysine coated coverslips and slides (Fischer Biosciences) or in 0.7% Low melting agarose (Sigma) for fluorescence microscopy. Samples were viewed with a Leica TCS SP5 confocal imaging system. Images were acquired with 100 X objectives, 5X zoom in 1024 × 1024 pixel format. Images were generated by Leica Application Suite Advanced Fluorescence software. Further, all images were cropped and processed for brightness/contrast using Adobe Photoshop CS3 software. For determining the distribution of cells showing different localization of DRG1-GFP transfectants, images were obtained with 100 X objectives, 2X zoom in 1024 × 1024 pixel format. Six different localizations was manually observed and recorded as a percentage of total GFP positive cells.