Chapter 3
Material & Methods
3.1 Yeast Two Hybrid Assay

3.1.1 Materials

Yeast Two Hybrid Assay

Mouse macrophage cDNA library from WEHI-3 cell line cloned into Uni-ZAP XR vector (Catalog no. - 937306) and GAL4 Two-Hybrid Phagemid Vector Kit (Catalog no. - 211351) was procured from Stratagene. The pGEMT T/A cloning kit was obtained from Promega (Madison, USA). All the amino acids, Uracil, β-mercaptoethanol, NaH₂PO₄, Na₂HPO₄, KCl and D-Sorbitol were purchased from SRL Ltd., India. Adenine sulphate, Adenine hemisulphate, Calf thymus DNA, Lithium acetate, PEG 3350, Tris, EDTA, DMF, MgSO₄, SDS, X-gal, Chloroform, NaCl, Ampicillin, Tetracycline, Kanamycin, Chloramphenicol and Triton X-100 were bought from Sigma. Tryptone, Yeast extract, Agar, Yeast nitrogen base without amino acid and Casine hydrolysate were obtained from Himedia, Mumbai, India. The kits for plasmid Mini prep, Gel extraction, PCR purification and plasmid Maxi prep were purchased from Qiagen.

3.1.2 Methods

Preparation of prey in pADGAL4-2.1 vector and Y2H assays were performed according to the protocols described in the instruction manual for Uni-ZAP XR premade library and GAL4 Two-Hybrid Phagemid Vector Kit, respectively. A brief outline of Y2H assay has been described below.

3.1.2.1 The prey preparation for Y2H assay

The titer of ExAssist helper phage and the Uni-ZAP XR mouse macrophage cDNA library were checked, which was found to be 7.6 x 10¹⁰ pfu/ml and 1.56 x 10¹⁰ pfu/ml, respectively. The pfu counts were found to be at par with the recommended titer of ~10¹⁰ pfu/ml (ExAssist helper phage) and ~3 x 10¹⁰ pfu/ml (Uni-ZAP XR mouse macrophage cDNA library). The Uni-ZAP XR vector, designed to allow simple, efficient in vivo excision and recircularization of any cloned insert contained within the lambda vector to form pBluescript SK(-) phagemid. The pBluescript SK(-) phagemid was excised from the Uni-ZAP XR vector following the mass excision protocol provided by the supplier. The cell mixtures with the phagemids were collected and used for plasmid preparation. The plasmids thus obtained were digested with EcoRI and XhoI to visually observe the digestion profile of the cloned insert in pBluescript SK(-) vector. The cDNA fragments of size ~ 0.5 kb to 2.5 kb obtained from digestion, were gel extracted and ligated with pADGAL4-2.1 vector at EcoRI and XhoI sites. The ligation reactions were electroporated into DH5α cells. All the colonies
appearing on LB amp plates were collected, washed twice with LB broth and used for plasmid preparation. A total of 1 μg of insert of various sizes were ligated to the high quality pADGAL4-2.1 vector tested for self ligation. Mouse macrophage cDNA (~ 0.5 kb to 2.5 kb) cloned into pADGAL4-2.1 vector were used as the prey vector for Y2H assay.

3.1.2.2 The bait preparation for Y2H assay

On the basis of mycobacterium consortium database (www.doembi.ucla.edu/TB/PUBLIC/qs/qsearch.php?orf=Rv3810) primers (List of primers) were designed to amplify Δserp gene from the Mtb H37Rv genomic DNA using pfu enzyme. PCR amplified fragment was then treated with Taq enzyme with buffers at 72°C for 30 min. to add “A” at the ends of the Δserp fragments. The amplicon with “A” at the ends were cloned into pGEMT vector. The colonies were screened for clones with Sall site of the forward primer and the SalI site from MCS at the 3’ end. The insert (Δserp) from pGEMTΔserp and the vector (pBDGAL4cam) were prepared with SalI restriction enzyme and ligated with each other. The colonies were screened with PstI restriction enzyme for clones having insert in right orientation.

3.1.2.3 Transformation of pBDGAL4camΔserp and other control plasmids into YRG-2

YRG-2 yeast strain (Genotype - Mata ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS2::UASGAL1-TATA GAL1-HIS3 URA3::UAS GAL4 17mers(x3)-TATA CYC1-lacZ) was checked by streaking it onto SD minimal selection media without Histidine (His’), Tryptophan (Trp’), Leucine (Leu’) and Uracil (Ura’), respectively. YRG2 yeast strain, an auxotrophic mutant did not grow on His’, Trp’ and Leu’ SD selection media but were able to grow on Ura’ plate (Table 1). All the BD (Binding Domain) containing plasmids have tryptophan selectable marker gene for YRG2, while all the AD (Activation Domain) containing plasmids have Leucine as the selectable marker gene. The YRG-2 strain contains a dual selection system to detect protein-protein interaction based on HIS3 and lacZ reporter gene constructs. YRG-2 yeast competent cells were prepared as described in the instruction manual, Stratagene. The plasmid pBDGAL4camΔserp (bait) and other control plasmids (pBDGAL4, pBDWT, pBDMUT, pADWT, pADMUT and pLaminC) were transformed into YRG2 yeast strain individually by following the yeast transformation protocol (Instruction manual, Stratagene). The colonies thus obtained were maintained every week in SD agar plate with appropriate amino acid selection pressure.
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<table>
<thead>
<tr>
<th>Amino acid selection</th>
<th>Growth (+/-)</th>
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<tbody>
<tr>
<td>Trp⁻</td>
<td>-</td>
</tr>
<tr>
<td>Leu⁻</td>
<td>-</td>
</tr>
<tr>
<td>His⁻</td>
<td>-</td>
</tr>
<tr>
<td>Ura⁺</td>
<td>+</td>
</tr>
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</table>

**Table 1: Growth characteristics of YRG2 yeast strain on SD amino acid selection media.** (-) sign no growth & (+) sign growth.

#### 3.1.2.4 Y2H assay

To fish out the mouse macrophage cDNA clone in pADGAL4-2.1 (~0.5 to 2.5 kb, prey), step wise yeast transformation protocol was followed (Instruction manual, Stratagene). Competent cells of single transformants of YRG-2 yeast with pBDGAL4camΔssea were prepared and pADGAL4-2.1 (with mouse macrophage cDNA ~ 0.5 - 2.5 kb) phagemids were transformed into it. The transformed cells were allowed to grow on SD Trp⁻, SD Leu⁺, SD Leu⁻Trp⁻ and SD Leu⁻Trp⁻His⁻ selection plates at 30°C. Similarly, control transformation experiments were also performed having combination like YRG-2 with pBDGAL4 (intact protein, positive control), YRG-2 with pBDWT + pADWT (perfect positive interaction control), YRG2 with pBDMUT + pADMUT (weak positive interaction control), YRG2 with pADWT + pLaminC (negative control strong interaction) and YRG2 with pADMUT + pLaminC (negative control weak interaction). The colonies appearing on SD Trp⁻ and SD Leu⁺ showed the authenticity of the transformed yeast; colonies appearing on SD Leu⁻Trp⁻ showed the transformation efficiency; while the colonies on Leu⁻Trp⁻His⁻ selection were the putative positive interacting clones. Since, HIS3 reporter gene has a tendency of leaky expression, therefore, the colonies appearing on the triple selection plates were filter lifted and LacZ assay or the filter lift assay was performed on them (Instruction manual, Stratagene). The blue colonies on the filter were carefully aligned with the original plate. The blue colonies were again streaked onto Leu⁻Trp⁻His⁻ SD plates and filter lift assay was performed again. This step was done to reduce the number of false positive in Y2H assay and to remove the yeast clones which have lost the plasmids in the due process.
The plasmid DNA was isolated from the $\text{His}^+\text{-lacZ}^+$ yeast clones by plasmid DNA isolation protocol (Instruction manual, Stratagene). The crude plasmid isolated from each clone were then transformed into DH5α electrocompetent cells and plated onto LB amp plates. The plasmid DNA was isolated from single colony using Qiagen plasmid mini prep kit and checked for the presence of insert DNA on digestion with EcoRI and XhoI. The inserts were scored on the basis of their size and then sent for sequencing. The sequenced genes were then analyzed using softwares like Gene runner, Blastn (www.ncbi.nlm.nih.gov), tBlastx (www.ncbi.nlm.nih.gov), ORF finder (www.ncbi.nlm.nih.gov) and webcutter. The primers were designed (Appendix II) to amplify full-length cDNA sequences for the Y2H positive clones from pBluescript SK(-) phagemid containing mouse macrophage cDNA library. These sequences were cloned into the cloning vector (pTZ57R/T or pGEMTeasy).

### 3.1.2.5 Genomic DNA isolation from Mycobacterium

Genomic DNA from $M$tb and $M$. smegmatis was prepared by the method described by Kremer et al. (2005). The bacterial cells were scrapped from the $M$tb slant culture using sterile loop and washed once with 500 µl of TE buffer I. The pellet was then resuspended in 200 µl of TE buffer I. The bacilli were heat killed at 80 °C for 1 h on a dry bath. The eppendrof tube was properly sealed with parafilm and some weight was kept on top of the tube to prevent it from opening during the process of heat killing. The killed bacteria was pelleted down at 7,700g for 10 min. and then resuspended in 200 µl TE buffer II containing 26 µl of Lysozyme (10 mg/ml) and 2 µl of RNase A (1mg/ml). The suspended bacteria was incubated for 2 h at 37 °C. After incubation, 60 µl of 10% SDS and 1 µl of Proteinase K (20 mg/ml) were added and further incubated for 1 h at 60 °C. To the eppendrof tube 660 µl of 5M NaCl and 550 µl of CTAB/ NaCl were added to the suspension and further incubated at 60 °C for 30 min. The cell debrises were pelleted down by centrifugation at 12,000 rpm, 20 min. at room temperature. The clear supernatant was taken carefully into fresh tube and then extracted first with equal volume of Phenol : Chloroform and then with chloroform alone. Finally the genomic DNA from the aqueous phase was precipitated with 0.7 volumes of Isopropanol followed by washing with 70% ethanol. The pellet was then dried and suspended in 100 µl of TE buffer II. All the process till the lysis of bacteria were carried out in Bio safety Level III hood.
3.1.2.6 Agarose gel electroporosis

Horizontal submarine gel electrophoresis (Sambrook et al., 2001) was used for DNA analysis. The gel was prepared by weighing appropriate amount of agarose powder in 1X TAE buffer and melted in microwave oven. Melted agarose was cooled to 50 °C and then ethidium bromide (0.5 μg/ml) was added before casting it into the casting tray fitted with a comb. Solidified gel was placed in an electrophoresis tank filled with 1X TAE buffer and DNA samples mixed with 6X gel loading dye were loaded into the wells. The electrophoresis was carried out at a constant voltage based on the formula 5V/cm of the distance between the electrodes. The DNA samples resolved on the agarose gel were viewed under UV transilluminator.

3.1.2.7 Plasmid DNA isolation by alkaline lysis

A single colony was inoculated into 10.0 ml LB having appropriate antibiotics and incubated at 200 rpm, 37 °C, for 12 h. The cells were pelleted down at 12,000 rpm for 2 min. and resuspended in 100 μl of ice cold solution I, incubated for 5 min at room temperature. 200 μl of freshly prepared solution II was added, mixed gently and incubated further for 5 min at room temperature. After incubation, 150 μl of ice-cold solution III was added and incubated for 15 min on ice, followed by centrifugation at 12,000 rpm, room temperature for 10 min. The aqueous phase was taken out slowly without disturbing the pellet. Equal volume of Tris saturated phenol pH 8.0 was added to the aqueous phase, mixed and centrifuged at 12,000 rpm for 5 min at room temperature. Aqueous layer was removed without disturbing interface and transferred to the fresh eppendorf tube. The trace of phenol was removed by adding 500 μl of chloroform to the aqueous phase, mixing and centrifugation at 12,000 rpm at room temperature for 5 min. After centrifugation aqueous layer was taken out in a fresh eppendorf tube. Plasmid DNA was precipitated by adding one volume of iso-propanol, mixed and centrifuged at 12,000 rpm at room temperature for 10 min. The supernatant was decanted by inverting the tube. The pellet was washed with 1 ml of 70% ethanol, by centrifugation at 12,000 rpm for 10 min at room temperature. Supernatant (70% ethanol) was removed carefully and the pellet was dried at 37 °C. The plasmid DNA was resuspended in 30 μl of TE (pH 8.0). DNA quantity was estimated spectrophotometrically at 260 nm and 280 nm and stored at 4 °C.
3.1.2.8 **PCR condition**

The PCR reagents from Qiagen were used for amplification. A reaction was set up according to the conditions given below. The reaction mixture contained:

- **10X Taq polymerase buffer**: 5 μl
- **dNTP mix**: 200 μM
- **Primers (Forward and Reverse)**: 0.5 μM each
- **Taq DNA polymerase**: 1.0-2.0 units
- **Template DNA**: 10-25 ng
- **5X Q buffer**: 10 μl
- **Autoclaved Milli Q**: to 50 μl (final volume)

The amplification conditions are given below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Condition</th>
<th>Duration/Size</th>
</tr>
</thead>
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<tr>
<td>Initial denaturation</td>
<td>94 °C for 3-5 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C for 30 sec</td>
<td>34 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-65 °C for 30 sec (primer dependent)</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C for 1-2 min (size dependent)</td>
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</tr>
<tr>
<td>Final extension</td>
<td>72 °C for 10 min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

Stored at 4 °C until use.

The PCR products were checked by Agarose gel electrophoresis.

3.1.2.9 **Preparation of competent cells by CaCl₂ method**

25 ml of LB was inoculated by 250 μl of inoculum from an o/n culture of *E. coli* strain DH5α or the required strain. The cells were grown to the log phase (OD₆₀₀ 0.5-0.6) at 37 °C and harvested at 5,000 rpm (fixed angle rotor) for 5 min at 4 °C. Cell pellet was resuspended in 10 ml of chilled solution I and placed on ice for 1 h. After incubation, cells were centrifuged for 5,000 rpm at 4 °C for 5 min. Finally, cells were resuspended in 2.0 ml of chilled solution II. An aliquot of 200 μl of competent cells were transferred to the pre-chilled eppendorf tubes and stored at −70 °C until use.

3.1.2.10 **Transformation by heat shock method**

The ligation mixture or plasmid was added to DH5α or any other *E. coli* strain competent cells and incubated on ice for 10 min. Heat shock was given to the competent cells at 42 °C for 90 seconds. The cells were quickly plunged into ice for 5 min. LB media (800 μl) without antibiotic was added and incubated in an incubator shaker at 37 °C, 200
rpm for 1 h. The bacterial cells were then plated onto the LB plate containing appropriate antibiotic. These plates were then incubated at 37 °C, O/N.

3.1.2.11 Preparation of electrocompetent cells

1.0 liter of LB was inoculated with 1 ml of inoculum from an o/n culture of E. coli strain DH5α or the required strain. The cells were grown to the log phase (OD₆₀₀ 0.5-0.6) at 37 °C and harvested at 5,000 rpm (fixed angle rotor) for 10 min at 4 °C. Cell pellet was washed twice with (first wash 1.0 litre and second with 500 ml) chilled autoclaved double distilled water. Third wash was given with 50 ml of chilled autoclaved 10% Glycerol in double distilled water. Finally, the pellet was resuspended in 1 ml of chilled autoclaved 10% Glycerol in double distilled water and stored in 50 µl aliquots at -80 °C until use.

3.2 GST Pull-down assay

3.2.1 Materials

The M15 bacterial expression host and pQE30 plasmid expression vector were purchased from Qiagen. The BL21(DE3) bacterial expression host and pET41a plasmid expression vector were purchased from Novagen. The Ni-NTA resin was procured from Qiagen while the GST-sephrose high performance beads were procured from Amersham. Q-sephrose ion exchange resin, Imidazole, Ammonium persulphate and Coomasie brilliant blue R250 was purchased from ICN. Acrylamide, Bis acrylamide, Triton X-100, Tween 20, TEMED, BSA, SDS, EDTA, PMSF, Benzamidine, orthovanadate, Reduced glutathione and Glycerol were obtained from Sigma chemicals. Agarose and IPTG were purchased from Bangalore Genei. The nitrocellulose membranes (PROTRAN BA83 and Westran S) were procured from Whatman, while Snake skin pleated dialysis tubings from Pierce and the protein concentrators from Millipore, respectively. Freund's complete and incomplete adjuvant was procured from Gibco BRL. All the restriction enzymes, other DNA modifying enzymes and protein ladders (SM-0671, SM-0431 and SM-0441) were purchased from MBI Fermentas.

3.2.2 Methods

3.2.2.1 Cloning of Δsserp into pQE30 and spt2 into pET41a vector

The vector (pQE30) and the insert (Δsserp) were prepared by treating pQE30 and pBDGAL4camΔsserp vectors with Sall restriction enzyme. The CIAP treated pQE30 vector was then ligated with Δsserp. The pQE30Δsserp was screened with PstI. The spt2 gene was
PCR amplified (Appendix II) and cloned into pTZ57R/T cloning vector. The pET41a vector and pTZ57R/Tspt2 were digested at EcoRI and XhoI site. The prepared pET41a vector and the spt2 were ligated and transformed into DH5α. The colonies were screened with BamHI. The primary Anti-His antibody was purchased from sigma while secondary anti bodies and other western reagents were procured from Amersham.

3.2.2.2 Overexpression and purification of ΔssErp

The recombinant plasmid pQE30Δsserp was over expressed in M15 cells. A single colony of the desired clone was allowed to grow overnight in LB broth in presence of 50 µg Kanamycin/ml and 100 µg Ampicillin/ml at 37°C, 200 rpm. The bacterial cells were sub cultured into 1.0 liter broth (1% inoculum) with appropriate antibiotics. At O.D₆₀₀ of 0.6, cells were induced with 0.5mM IPTG for 6.0 hours at 30°C, 200 rpm. The induced cells were harvested, resuspended and sonicated in PBS (pH 7.4). The cell lysate was centrifuged at 12,000 rpm, 4°C for 30 min. The supernatant was loaded onto Ni-NTA column, equilibrated with Lysis buffer. The column was washed thrice with Lysis buffer, Wash buffer I and Wash buffer II. The protein was eluted in Elution buffer with 250 mM Imidazole. The purity of Ni-NTA affinity purified ΔssErp protein was checked by Commassie staining 10% SDS-PAGE and also by probing with Anti-His antibodies after transfer onto nitrocellulose membrene.

The ΔssErp protein purified with Ni-NTA column was dialysed in the Dialysis buffer to remove imidazole and to reduce the NaCl concentration from 300 mM to 50 mM. The dialysed ΔssErp was further purified by passing it through anion exchange column (Q sepharose). Protein purified with Ni-NTA column was loaded onto Q sepharose, pre-equilibrated with 20 mM NaH₂PO₄, 50 mM NaCl and 1 mM EDTA buffer (pH 8.0). The column was washed with 10 ml of same buffer but with varying concentration of NaCl (50, 100, 200, 400 and 600 mM). All the wash fractions and flow through were collected to check the purity and presence of ΔssErp protein on 10% SDS-PAGE. Only 7th and 8th wash fraction were checked on the 10% SDS-PAGE as the packing volume of the Q sepharose column was 5 ml (the new buffer comes out only after the old buffer has passed through the column and its volume depends on the volume of the packing material used).
3.2.2.3 Raising polyclonal antibodies against ΔssErp in rabbit

Pre-immune sera were collected from the rabbit 1 - 2 days before injecting the rabbit with the primary dose. Purified recombinant ΔssErp protein (~ 125 μg) was mixed with equal volume of Freund’s complete adjuvant (v/v) and injected subcutaneously into rabbit’s thigh. Booster dose was prepared by mixing equal volume of ΔssErp (125 μg) and Freund’s incomplete adjuvant (v/v) and was administered 30 days after the primary dose. On the 11th day after the first booster dose, blood was collected and antibody titer was determined by Enzyme Linked Immuno-Sorbant Assay (ELISA). Second booster dose was given 30 days after the first booster dose. Again on 11th day after the second booster dose, blood was collected and antibody titer was determined by ELISA.

3.2.2.4 ELISA

The antibody titer in the serum was analysed by ELISA using horse raddish peroxidase (HRP) linked secondary antibodies and Ortho Phenylene Diamine (OPD) as the chromogenic substrate. Purified ΔssErp antigen was coated on the ELISA plates with coating buffer at a concentration of 5 μg/ml, overnight at 4 ºC and blocked with blocking buffer (3% BSA in PBS) for 2 h at 37 ºC. Serial dilutions of primary ΔssErp antibody dilutions were made in the blocking buffer and incubated for 1 h at RT followed by 6 washes with PBST (PBS containing 0.1 % Tween-20). HRP conjugated secondary antibodies were diluted at 1:2000 in blocking buffer and then incubated for another 1 h at room temperature. After 6 washes with PBST plates were developed for 2 min by adding 50 μl of OPD solution at 1 mg/ml concentration in 0.1 M citrate buffer (pH 5.0) with 1 μl of 30% H2O2 as the catalyst. Reaction was stopped by adding 50 μl of 7.5% H2SO4 and measured the optical density at wave length 490 nm in the ELISA reader.

3.2.2.5 Overexpression and purification of GST tagged SPT2 fusion protein

The spt2 gene was cloned into pET41a vector at EcoRI and XhoI site. The clones were screened with BamHI. The pET41aspt2 plasmid was transformed into BL21(DE3) host and the expression of GST-SPT2 fusion protein was checked at 37ºC, 30ºC and 25ºC 6 hrs post induction with 1 mM IPTG. The induced cells were harvested, resuspended and sonicated in PBS (pH 7.4). The cell lysate was centrifuged at 12,000 rpm, 4°C for 30 min. The western blotting with Anti-His antibodies was performed to check the availability of GST tagged SPT2 protein in the soluble fraction. A single colony of the BL21(DE3) cells
with pET41aspt2 plasmid was allowed to grow overnight in LB broth in presence of 50 μg Kanamycin/ml at 37°C, 200 rpm. The bacterial cells were sub cultured into 1.0 liter broth (1% inoculum) with appropriate antibiotics and allowed to attain an O.D₆₀₀ of 0.6. These cells were then induced with 1.0 mM IPTG for 6.0 hours at 30°C, 180 rpm. The induced cells were harvested and resuspended in PBS (pH 7.4). After sonication the cell lysate was centrifuged at 12,000 rpm, 4°C for 30 min. The cleared lysate of BL21(DE3) cells were passed through GST sepharose column pre-equilibrated with PBS (pH 7.4). The GST sepharose column was charged with 2 volumes of PBS (pH 7.4). The cleared lysate in PBS (pH 7.4) was passed through the column. The GST sepharose column was washed with 5 volumes of PBS (pH 7.4). The purified GST-SPT2 protein was then eluted in 20 mM reduced Glutathione in 50 mM Tris buffer pH – 8.0. The quality of purified protein was checked by 10% SDS-PAGE.

3.2.2.6 GST Pull-down assay

Equimolar concentration of purified ΔssErp, GST-SPT2 and GST (over expressed and purified from pET41a) proteins were used for the GST-Pulldown assay. For GST-Pull down assay the proteins were flipped that is ΔssErp was acting as “prey”, while GST or GST-SPT2 was acting as “bait”. These proteins were added to the GST sepharose column simultaneously and incubated for 2 h at 4°C on the Rotospin for 360° mixing vertically. The beads were then washed thrice with ice cold PBS + 1% Triton x-100 buffer and finally eluted in 20 mM reduced Glutathione containing elution buffer. The eluted fractions were resolved on 10% SDS-PAGE. Western blotting was done with Anti-His and Anti-ΔssErp polyclonal antibodies.

3.2.2.7 SDS-PAGE

SDS-PAGE was carried out, essentially according to Laemmli (1970). Prior to loading onto the gel, the samples were heated in a dry bath for 10 min. The samples were then analyzed on a discontinuous vertical SDS-polyacrylamide gel with a 5% (w/v) stacking and a 10% (w/v) resolving gel (Laemmli, 1970). Gels were run in a Laemmli buffer at constant current at 15 mA. Protein molecular weight markers were run concurrently on the gels for appropriate analysis.
3.2.2.8 Western blotting

The western blots were prepared by transferring the separated proteins from the polyacrylamide gel onto a nitrocellulose membrane in a Trans-blot apparatus (Bio-Rad) as described by Towbin et al., (1979). After electrophoresis the gel was sandwiched between filtered paper on one side and nitrocellulose membrane on other side. Additional sheets of filtered paper were added on both sides, fixed tightly in Trans-blot apparatus. Care was taken to avoid any trapped air bubbles between the gel and the nitrocellulose membrane. The apparatus was placed in such a way that the nitrocellulose membrane faces the anode. The transfer was carried out at 350 mA for 6 h at 4 °C. Complete transfer of proteins onto the membrane was visually checked by Ponceau S stain. The membrane was incubated in blocking buffer (5% skimmed milk in 1X PBST) for 2 h followed by incubation with corresponding primary antibody for 2 h at room temperature with gentle rocking. The membrane was washed with 1X PBST thrice of 10 min each. The membrane was then incubated with HRP-tagged or AP-tagged secondary antibodies and incubated for 1 h. The blot was again washed three times with 1X PBST, for 10 min each. For coloured reaction, BCIP/NBT substrate solution was used. For Co-IP studies, blots were developed by ECL method as per manufacturer’s instructions. After development the blots were rinsed with distilled water, dried and stored.

3.3 Co-Immunoprecipitation

3.3.1 Materials

The BHK21 fibroblast cell line was kind gift from Dr. Amog Anant Sahasrabuddhe, CDRI, Lucknow. The pcDNA2aFLAG and pcHA vectors were kind gift from Dr. Andrew Catling, USA. Anti-FLAG M2 beads, Sepharose CL-6B beads, Flag peptide, Trypsin (1:250), EGTA, NaF, Sodium pyrophosphate and Bradford’s reagent were purchased from Sigma chemicals. The Anti-HA antibody was obtained from Sigma and anti Actin Polyclonal antibody was purchased from Santa Cruz. The DMEM and FBS (North American type) were purchased from Gibco BRL while Lipofectamine and Plus reagent were procured for Invitrogen.

3.3.2 Methods

3.3.2.1 Cloning

The Flag tagged bait in the mammalian expression vector was prepared by digesting the pBDGAL4camAsserp vector with SalI, then treating the insert Asserp with Klenow
enzyme. Fragments, thus obtained were redigested with XbaI and gel extracted. The mammalian expression vector pcDNA2aFLAG was digested with EcoRV (Blunt) and XbaI (sticky). The ligation product was transformed into DH5α cells. The pcDNA2aFLAGΔsserp clones were screened with HindIII + XbaI. The HA prey for Co-immunoprecipitation were prepared by cloning spt2 into pcHA vector at NotI site. The pcHAΔserp clones were screened with BamHI restriction enzyme.

The full length erp gene was cloned into pcDNA2aFlag vector at HindIII and SphI site. The pcDNA2aFLAGΔserp clones were screened with EcoRI restriction enzyme. The msΔsserp gene was PCR amplified from the genomic DNA of M. smegmatis and cloned into pTZ57R/T cloning vector. The pcDNA2aFLAG vector and the msΔsserp gene were prepared by HindIII + EcoRI restriction enzyme and ligation was set. The clone pcDNA2aFLAGmsΔsserp was screened with HindIII + EcoRI.

The four deletion mutants of erp gene namely, ΔNerp, ΔCer, ΔPGLTSerp and ΔSrcer were prepared either by normal PCR or by overlap extension PCR (described in next section). These deletion mutants were first cloned into pTZ57R/T cloning vector and sequenced. The vector pcDNA2aFLAG was prepared by digesting it with EcoRV + XhoI, while the pTZ57R/T vector containing all the inserts were first digested with PstI and followed by Klenow treatment. Klenow treated pTZ57R/T vector with inserts were digested with XhoI enzyme. The blunt and sticky end ligation was set to ligate pcDNA2aFLAG and the deletion mutants of erp. The mutant clones were screened with HindIII and XhoI restriction enzyme.

Normal PCR or overlap extension PCR (described in next section) were used to generate four deletion mutants of spt2 gene namely, spt2ΔN, spt2ΔM1, spt2ΔM2 and spt2ΔC. These deletion mutants were sequenced after cloning into pTZ57R/T vector. These genes were cloned into pcHA vector at XhoI + Sall (spt2ΔN and spt2ΔM2) or at NotI site (spt2ΔM1 and spt2ΔC). These clones were screened with BamHI restriction enzyme.

### 3.3.2.2 Site directed mutagenesis by overlap extension PCR

The overlap extension PCR (Ho et al., 1989) is a method of site directed mutagenesis used mostly for generation of site specific internal mutations in a gene. Here a set of four primers are designed namely, forward primer (primer 1), reverse primer (primer 4), site specific internal mutagenic primer (primer 2) and internal complementary primer (primer 3) (Fig. 6). First step involves setting up of 2 separate PCR reactions. Reaction one involves primer 1 and primer 2 while reaction two involves primer 3 and primer 4, respectively. The
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PCR products thus obtained were purified and used as template for the third PCR reaction were overlap extension (a self priming step) was followed by amplification of the mutant gene by primer 1 and primer 4 (Fig. 6). The mutants thus obtained were cloned into pTZ57R/T and sequenced.

Fig. 6: Site directed mutagenesis by overlap extension PCR based on Ho et al. (1989).

3.3.2.3 DNA sequencing

The DNA sequencing reaction was run on ABI BigDye v3.1 cycle sequencing. The sequencing reaction mixture is described below –

- Terminator Ready Reaction mix. - 1μl
- Sequencing buffer (5X) – 1.5 μl
- Template (Plasmid) – 150 to 300 ng
- Primer – 4 pmol
- Autoclaved Milli Q water – to 10 μl final volume

The sequencing reaction was run with following condition

Initial denaturation 96 °C for 1 min. } 1 cycle
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<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>96 °C</td>
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<td>Hold</td>
<td>4 °C</td>
<td></td>
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</table>

After completion of the sequencing reaction the reaction was stopped further by adding 2 μl of 125 mM EDTA and mixed well. The DNA is precipitated by adding 50 μl of absolute ethanol, mixing the contents and incubating at RT for 15 minutes. The samples were centrifuged at 12,000 rpm, 20 min at RT. The supernatant was decanted and the pellet was washed twice with 70 % ethanol by centrifugation at 12,000 rpm for 10 min. The supernatant was aspirated and the pellet was air dried at RT. The pellet was either stored at -20 °C till further use or heated at 95 °C for 3 minutes with 10 – 12 μl of HiDi-Formamide and chilled on ice for 5 minutes immediately. After brief centrifugation the samples were loaded on the 96 well plates for DNA sequencing. The sequences were analysed on FinchTV, GENERUNNER and other sequence alignment tools.

3.3.2.4 Transfection into BHK21 fibroblast cell line

Baby Hamster Kidney 21 (BHK21) fibroblast cell line was maintained in the DMEM + 10% FBS. A day before transfection 1.8 million cells were seeded on 100 mm tissue culture dishes. The cells were allowed to grow in a humidified CO₂ incubator at 37°C, 5% CO₂ till the cells reached a confluency of 60 - 70%. Next day, DNA-Plus reagent-Liposome complex was prepared by mixing 3 μg of plasmid (1.5 μg of plasmid expressing flag tagged gene + 1.5 μg of plasmid expressing HA tagged gene) with 1.5 μl of plus reagent and 10 μl of Lipofectamine to a volume of 1.6 ml with DMEM. The mixture was incubated for 20 minutes and its final volume was made up to 5 ml with DMEM. The cells were washed twice with PBS (pH 7.4) and incubated with 5 ml of DNA-Plus reagent-Liposome complex for 5 hrs in CO₂ incubator. Two washes with PBS (pH 7.4) were given after 5 hrs. and the cells were further incubated for 20 h or more (depending on the experimental requirement) in DMEM + 10% FBS.

3.3.2.5 Co-Immunoprecipitation

The transfected BHK21 cells were taken out of the CO₂ incubator and chilled on ice. These cells were then washed 5 times with ice cold PBS (pH 7.4). Then 600 μl of FLAG lysis buffer with protease inhibitors (100 mM PMSF – 1: 100 dilution + 300 mM
Benzamidine – 1: 100 dilution + 200 mM Orthovanadate – 1: 1000 dilution) were added to each tissue culture dish and incubated for 5 min on ice. The cell lysate was collected in an eppendorf tube and the cell debris was cleared by centrifugation at 12,000 rpm, 4°C for 15 min. About 600 µl of cleared lysate was added to the tubes with the slurry of Anti-FLAG M2 beads and Sepharose CL-6B beads. The lysates along with M2 FLAG beads were mixed at 4°C for 2 h. The beads were washed with FLAG lysis buffer thrice and then the Co-immunoprecipitated proteins were eluted with (10 µg) FLAG peptide. The samples (both lysates and Co-immunoprecipitates) were resolved on 10% SDS-PAGE and probed by western blotting. Each Co-immunoprecipitation experiment was repeated at least three times.

3.4 Co-localization

3.4.1 Materials

The plasmids pECFP-N1 and pEYFP-N1 were procured from Clontech. The Paraformaldehyde, DABCO and Anti-GFP antibodies were purchased from Sigma chemicals. ER Tracker red dye was bought from Invitrogen. The glycine was obtained from SRL India.

3.4.2 Methods

3.4.2.1 Cloning

The erp, Δsserp, ΔNerp and spt2 genes were amplified through PCR (Appendix II) and cloned into pTZ57R/T cloning vector. The erp, Δsserp and ΔNerp genes were cloned into pECFPN1 vector at HindIII + Sall, Xhol + Sall and EcoRI + Xhol sites, respectively. The clones generated were screened with the same enzymes. While the spt2 gene was cloned into pEYFPN1 vector at EcoRI and Xhol site and screened with BamHI. The expression of these clones in BHK21 cells were checked by western blotting with Anti-GFP antibodies.

3.4.2.2 Slide preparation

For co-localization studies CHO K1 cell line was maintained in DMEM + 10% FBS. A day before transfection poly L-Lysine coated sterile cover slips were taken in six well plates. CHO K1 cell were seeded at 0.5 X 10^5 cell per well of six well plate. The cells were allowed to grow in a humidified CO₂ incubator at 37°C, 5% CO₂ till the cells reached a confluency of 40 - 50%. Next day transfection was performed as mentioned in section 3.3.2.c with 1 µg of plasmid per well. The transfected cells were observed in the
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fluorescence microscope and washed twice with sterile PBS (pH 7.4). For localization studies the cells were stained with ER tracker dye in sterile PBS (pH 7.4) for 15 min in the humidified CO₂ incubator at 37°C. The cells were washed again with sterile PBS (pH 7.4) and incubated with DMEM + 10% FBS for 2-4 h so that the cells regain its shape. After incubation the cells were washed again with sterile PBS (pH 7.4) and fixed with freshly prepared 4% Paraformaldehyde for 5-10 min. The reaction was quenched by removing the 4% Paraformaldehyde and washing the cells thrice with 5% Glycine in PBS (pH 7.4). The cover slip was removed carefully and placed on top of a glass slide with the surface of the cover slip with the cells facing upwards. An antifading agent DABCO in 10% glycerol (10 μl) was added on top and then covered with another cover slip. The cover slip was fixed on the glass slide with nail polish. For co-localization studies CFP and YFP tagged proteins were used as fluorophore, therefore, the cells were directly fixed.

3.4.2.3 Condition

Localization studies were carried out using Zeiss laser-scanning confocal microscope (LSM 510 META) in multi tracking mode to prevent interference of dyes. The images were obtained using multitracking mode with 63X/1.4 oil DIC objective lens [CFP excitation (458 nm); NFT (490 nm); emission detection (505-530 nm), band pass filter; ER tracker red dye excitation (543 nm); NFT (545 nm); emission detection (560 nm) long pass filter].

Co-localization studies using CFP and YFP tagged proteins were done using Nikon A1R confocal microscope in multi tracking mode for CFP and YFP proteins with Plan Apo VC 100X oil objective lens. The images were taken with CFP [excitation (457 nm) and emission detection (464-499 nm) band pass filter] and YFP [excitation (514 nm) and emission detection (525-555 nm) band pass filter].

3.5 Biological characterization of Erp and SPT2 interaction

3.5.1 Materials

The HEPES buffer, Palmitoyl CoA, Pyridoxal 5-phosphate and NH₂OH were purchased from Sigma. The L- Serine and Methylcellulose was bought from SRL while the CHCl₃ and Toluene were purchased from Qualigens. PPO and POPOP were procured from Spectrochem, India.
3.5.2 Methods

3.5.2.1 Cloning, over expression and purification of ΔNErp

The ΔNerp gene was cloned into pQE30 vector at SphI + SalI site. The clones generated were screened with the EcoRI + SalI. The plasmid construct pQE30-ΔNerp was over expressed and purified from M15 cells as described in section 3.2.2.2. The purified protein was western blotted and probed with Anti-Erp antibodies.

3.5.2.2 SPT activity assay

BHK21 cells were maintained in DMEM + 10% FBS. A day ahead of setting the SPT enzyme activity, 1.8 million cells were seeded in 100 mm tissue culture dishes. The cells were washed five times with ice cold PBS (pH 7.4). These cells were then scrapped in 500μl of 50mM HEPES buffer (pH 8.3) with protease inhibitors (PMSF, Benzamidine and Orthovanadate) and sonicated. The cell lysate was cleared by centrifugation at 12,000 rpm, 4°C for 15 min. The cleared cell lysate from all the tubes were then pooled and 200μl of this pooled lysate was used for SPT enzyme activity. The composition of SPT enzyme assay buffer is mentioned below.

To the 200 μl of BHK21 cell lysate, 50 μl of concentrated assay buffer was added and the tubes were incubated at 37 °C for 1 h. Immediately the reaction was stopped by adding 200 μl of 2.0N NH₄OH and mixed by inverting the tube. The lipids were extracted with 500 μl of CHCl₃ by vortexing and spinning the tubes at 12,000 rpm, 30 seconds. The top aqueous phase was removed and the lower organic phase was washed thrice with 0.5N NH₄OH in water. About 350 μl of organic phase was transferred to the scintillation tube and kept at RT for drying. To each scintillation vial, 2 ml of scintillation fluid was added and the ¹⁴C counts of the samples for the condensation of the radioactive L-(U-¹⁴C)-Serine with Palmitoyl CoA was detected by Perkin Elmer liquid scintillation counter.