Chapter 2

Materials and Methods
2.1 Materials:

2.1.1 Sources of chemicals:

Curcumin, Colcemid, Agarose, BSA fraction V, β-mercaptoethanol, IPTG, X-gal, Triton X-100, Tween-20, SDS, TEMED, PMSF, leupeptin, aprotenin, penicillin, kanamycin, streptomycin, lysozyme, ethidium bromide, Ponceau S, formamide, calcium chloride, Tris base, sodium deoxycholate, MOPS, HEPES, DEPC, trypsin and RNase A were obtained from SIGMA Chemicals. Rhodamine phalloidin was from Molecular probes. PMOS Blue-Blunt cloning kit, Hybond C extra membrane and Low molecular protein marker for SDS-PAGE were obtained from Amersham Biosciences. Glycerol, FBS and DMEM were obtained from Invitrogen-Life Technologies. Acrylamide and Bis-acrylamide were obtained from Serva Chemicals. MMLV reverse transcriptase and RNase free DNase were from Promega. Restriction enzymes, T4 DNA ligase, DNA Molecular Wt. Marker were from New England Biolabs. Agarose gel elution reagents and miniprep columns for the preparation of high quality transfection grade plasmid were from Qiagen. Western lightning chemiluminescence reagents, dNTPs and PCR component were obtained from Roche life sciences. Bradford’s reagent obtained from Bio-Rad. Tryptone agar and yeast extract were from Difco laboratory. Whatmann filter papers were from Whatmann International Ltd. X-ray films were from Kodak. Intensifying screen was obtained from Dupont. Other reagents were from local suppliers like Qualigens, SRL, SD fine-chemicals Ltd., and Merke India Ltd. and were of analytical grades. Primers (oligonucleotides) were purchased from BioServe, India.

2.1.2 Antibodies:

Antibodies against Hsp25 (rabbit polyclonal), Hsp70 (mouse monoclonal), Hsp90 (mouse monoclonal) and Hsc70 were from Stressgen, BrdU (Mouse monoclonal) and Actin were (mouse monoclonal) from Sigma, β-tubulin (mouse monoclonal) from Upstate, α-tubulin (rabbit polyclonal), Hsp47 (mouse monoclonal), p21 (mouse monoclonal) and p53 (mouse monoclonal) were from Santacruz, SSEA1 (mouse monoclonal IgM) from Hybridoma technology, MBP was from NEB, Nanog (mouse monoclonal) was from Calbiochem.
Secondary antibodies for western blotting and immunofluorescence were from Amersham life sciences, Vector labs, Molecular probes and Jackson Lab.

2.1.3 Bacterial strains:

E coli DH5α:
[Genotype: F- recA1 endA1 hsdR17 (rK mK) (lacZYA-argF) U169 (φ80lacZΔ M15) supE44 thi-1 gyrA96 relA]
This strain was used for all routine transformations, plasmid isolations, selection of recombinant plasmids, etc.

E. coli BL21 DE3:
[E. coli B F , ompT, hsdS (rB , mB ) , gal, dcm.]
This strain was used for protein expression studies.

2.1.4 Plasmid and expression vectors:
a. pMOS Blue cloning vector from Amersham Pharmacia Biotech Ltd, was used for cloning PCR products.
b. pEGFP N- and C- series vectors were used for expressing gene of interest as a fusion protein with GFP.
c. pMALC2 vector for expressing protein in bacterial system.
d. pUAST vector for making transgenic fly.
e. pFPC19-FLAG vector for expressing gene of interest in S2 cells.

2.1.5 Cell lines:
a. P19: Mouse embryonal carcinoma cell line
b. Cos1: African green monkey (Cercopithecus aethiops) kidney cell line transformed by SV 40, established from CV-1 Simian cells
c. Schneider line 2 (S2) cells: A Drosophila embryonic cell line derived from Oregon strain from late stage embryos (20-24h).

2.1.6 Bacterial media and antibiotics:

LB (Luria Bertani) broth: 1% bacto-tryptone, 1% NaCl, 0.5% bacto-yeast extract, pH 7.0.
LB agar: LB medium containing 1.5% agar.
Ampicillin: A 1000X stock solution of 100mg/ml ampicillin was made in sterile-distilled water. Working conc. was 100 µg/ml.

Kanamycin: A 1000X stock solution of 50mg/ml ampicillin was made in sterile-distilled water. Working conc. was 50 µg/ml.

2.1.7 Commonly used solutions/medium:

30% Acrylamide solution: Dissolved 29.5g of acrylamide and 0.8g of N,N'-methylene bisacrylamide in 60ml of milli-Q water, warmed the solution to 37°C to dissolve the chemicals, volume was made upto 100ml and stored at 4°C in an amber coloured bottle.

DAPI: A 10mg/ml stock of 5-bromo-4-chloro-indolyl phosphate (DAPI) was constituted in DMF and stored at -20°C.

DEPC treated water: Water for RNA isolation was treated with 1% DEPC, left overnight and subsequently autoclaved to remove excess DEPC.

DNase-free RNase A: 20mg/ml stock was made in sterile double distilled water, boiled for 20min to inactivate the trace amounts of contaminating DNase, aliquated and frozen.

Chloroform : Isoamyl Alcohol: 24:1 (v/v) of chloroform and isoamyl alcohol.

IPTG: A 200mg/ml stock of isopropyl β-D-thiogalactoside in sterile double distilled water.

6X Agarose loading dye: 0.25% bromophenol blue and 0.25% xylene cyanol in 30% glycerol.

10X PBS: Dissolved 80g of NaCl, 2g KCl, 14.4g Na2HPO4 and 2.4g KH2PO4 in 1L of milli-Q water, autoclaved and stored at room temperature. 1X PBS was obtained by diluting 10X PBS.

SDS-PAGE Sample buffer (1X): 50mM Tris-HCl pH 7.5, 3% SDS, 10% glycerol, 4mM EDTA, 10% β-mercaptoethanol and 0.01% bromophenol blue. Usually 4X stock were made.

10X TBE: 108g of Tris base and 55g of Boric acid were dissolved in 800ml milli-Q water. 20ml 0.5M EDTA (ph 8.0) was added in it and final volume was made to 1L.

TBS: 20mM Tris.HCl pH 8.0, 150mM NaCl

1X TE: 10mM Tris.HCl, 1mM EDTA (pH 8.0)

X-Gal: 20mg/ml stock of 5-bromo-4-chloro-3-indolyl-βD-galactoside (X-Gal) in dimethyl formamide.
Ponceau S: A 10X stock of Ponceau S was made containing 2% Ponceau S, 30% trichloroacetic acid and 30% sulfosalisyllic acid in water.

Tris-saturated Phenol: Phenol needs to be equilibrated to pH 8.0 for DNA purification since DNA partitions into the organic phase at acidic pH. Melted phenol was mixed with equal volumes of 0.5M Tris.HCl pH 8.0 and 0.1% hydroxyquinoline, stirred and allowed to phase separate at room temperature. Following this, the upper aqueous phase was removed and equal volumes of 0.1M Tris.HCl pH 8.0 was mixed with the phenolic layer and again left for phase separation. This step was repeated until pH of aqueous solution reached 8.0. Once the phenol was equilibrated and the final aqueous phase had been removed, it was stored in light-tight bottles at 4°C after mixing with 0.1 volume of 0.1M Tris.HCl pH 8.0.

2.1.8 Tissue culture medium:
DMEM medium preparation:

For incomplete medium preparation, 13.4 gm DMEM and 3.7gm NaHCO₃ was dissolved in 800 ml Mili-Q water, pH was set at 7.4 using conc. HCl and made the volume 1Litre using MiliQ water. It was filter sterilized by passing through the 0.22μm-sterilized filter paper, stored at 4°C in sterile bottles. For complete medium preparation, 10% FCS and 1 X antibiotics solution was added in this. For preparation of 100 X Antibiotic solution, required amount of antibiotics (600mg Penicillin and 500mg Streptomysin) were weighed and dissolved in 100 ml of 1 X PBS.

0.5% Trypsin-EDTA:

Dissolved 0.5g Trypsin and 0.5g EDTA in 80ml of 1X PBS and adjust the pH 7.4. Made the final volume 100ml with 1X PBS filtered.

Freezing Medium:

50% FBS and 10% DMSO in incomplete DMEM. This solution was prepared freshly, filter sterilized and stored at 4°C.

Preparation of stock Curcumin solution:

10mM stock curcumin (Aldrich, M.Wt 368.39) solution was prepared in Methanol. For this, 3.6839 mg curcumin was dissolved in 1ml of methanol and distributed in 1.5 ml microfuge (200μl aliquot) and properly wrapped with parafilm. These vials were stored at -20°C.
2.1.9 List of fly stocks used for analysis:

**Wild type stock:** Canton-S (CS)

**GAL4 stocks:**

**UAS-Lines:**
UAS-mCD8-GFP (Lee and Luo, 1999), UAS-P35 (Bloomington Stock Centre), UAS-Cyc E (Richardson et al., 1995), UAS-CycD, UAS-String, UAS-P21, UAS-Cdk2 (Meyer et al., 2000), UAS-Cdk1 (Meyer et al., 2000), UAS-Cdk4 (Meyer et al., 2000), UAS-Cdk4-D175N (Meyer et al., 2000), UAS-E2F, UAS-mRb (Meyer et al., 2000).

**Alleles and deficiencies lines:**
w^{118}, Cyclin E deficiency [Df (2L) Sco^{25}/Cy0 (BS #6086) and Df (2L) el80f1/CyO (BS #3211)], Cyclin E allele [CycE^{4R95} (BS #6637)], Cdk1 allele [cdc2^{E1-24} (BS #6641) and cdc2^{R47} (BS #6643)], Dacapo allele [dap^4 (BS #6639)], Cdk4 allele [Cdk4^{3} (BS #6644)] (Meyer et al., 2000), DP allele [Dp^{49Fk-1} (BS #5553), Dp^{a1} (BS #7277)], E2F1 allele [E2f^{2} (BS #7274), E2f^{3G0332} (BS #13016), P{PZ}E2^{E7172} (BS #11717)], Stg allele [P{PZ}stg^{01453b} (BS #11533)], Stg Df [Df (3R) 01215 (BS #5424)].
### 2.1.10 List of Primers:

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21 (F)</td>
<td>5'-CCC GTG GAC AGT GAG CAG TTG-3'</td>
<td>Used for Semiquantitative PCR to amplify P21 (Fragment size 362bp)</td>
</tr>
<tr>
<td>P21 (R)</td>
<td>5'-TGT CAG GCT GGT CTG CCT CC-3'</td>
<td></td>
</tr>
<tr>
<td>tPA (F)</td>
<td>5'-CAA GCA ATG TGC CCC ACA AGA GAG-3'</td>
<td>Used for Semiquantitative PCR to amplify tPA (Fragment size 554bp)</td>
</tr>
<tr>
<td>tPA (R)</td>
<td>5'-TGG CAG CTC ACG AAG ATG ATG-3'</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>5'-GTG TCC ACT GGC GAT GTG AAG GTG AAC GTG G-3'</td>
<td>Used for Semiquantitative PCR to amplify Cytokeratin K14 (Fragment size 330bp)</td>
</tr>
<tr>
<td>K14 (F)</td>
<td>5'-GTG TCC ACT GGC GAT GTG AAG GTG AAC GTG G-3'</td>
<td></td>
</tr>
<tr>
<td>K14 (R)</td>
<td>5'-GCT GCC GCA GTA GCG ACT CTA CTG T-3'</td>
<td></td>
</tr>
<tr>
<td>AFP (F)</td>
<td>5'-CCT GTG AAC TCT GGT ATC AG-3'</td>
<td>Used for Semiquantitative PCR to amplify AFP (Fragment size 410bp)</td>
</tr>
<tr>
<td>AFP (R)</td>
<td>5'-GCT CAC ACC AAA GCG TCA AC-3'</td>
<td></td>
</tr>
<tr>
<td>Activin β6 (F)</td>
<td>5'-GCA AGA ATG TGC TGA TCA AC-3'</td>
<td>Used for Semiquantitative PCR to amplify Activin β6 (Fragment size 426bp)</td>
</tr>
<tr>
<td>Activin β6 (R)</td>
<td>5'-GTC AAT TTG ACG TGG TTT CC-3'</td>
<td></td>
</tr>
<tr>
<td>Collagen α4 (F)</td>
<td>5'-AGG CAG GTC AAG TTC TAG CG-3'</td>
<td>Used for Semiquantitative PCR to amplify Collagen α4 (Fragment size 463bp)</td>
</tr>
<tr>
<td>Collagen α4 (R)</td>
<td>5'-CAA GCA TAG TGG TCC GAG TC-3'</td>
<td></td>
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<tr>
<td>β-Actin (F)</td>
<td>5'-ATC TGG CAC ACC TCT TGC AAT GAG CTG CG-3'</td>
<td>Used for Semiquantitative PCR to amplify β-Actin (Fragment size 838bp)</td>
</tr>
<tr>
<td>β-Actin (R)</td>
<td>5'-CTG CAT CCT GCT TGC TGA TCA ACA TCT GC-3'</td>
<td></td>
</tr>
<tr>
<td>Brachyury (F)</td>
<td>5'-TGCTGCTGTGACCTATACAC-3'</td>
<td>Used for Semiquantitative PCR to amplify Brachyury (Fragment size 947bp)</td>
</tr>
<tr>
<td>Brachyury (R)</td>
<td>5'-TCC AGG TGC TAT ATA TGT CC-3'</td>
<td></td>
</tr>
<tr>
<td>Laminin B1 (F)</td>
<td>5'-ACCACTGTGCCCTTGACAGGC-3'</td>
<td>Used for Semiquantitative PCR to amplify Laminin B1 (Fragment size 662bp)</td>
</tr>
<tr>
<td>Laminin B1 (R)</td>
<td>5'-CACAGGCTGCAAGTCATGAC-3'</td>
<td></td>
</tr>
<tr>
<td>C1 A(F)</td>
<td>5'-ATT CAT ATG GCT GCG CAG GC-3'</td>
<td>Used to amplify C1 (ORF) and cloned in pBSKS vector, and to confirm the integration of C1 in transgenic fly.</td>
</tr>
<tr>
<td>C1 A(R)</td>
<td>5'-ATT CTC GAG GGG AAT TGT CTT TAG GGA-3'</td>
<td></td>
</tr>
<tr>
<td>ScoA</td>
<td>5'-ATA CTC GAG ATG GAG CTG TGC AGG CCC TTT G-3'</td>
<td>Used to amplify C1(ΔPro) and cloned in pMOS vector.</td>
</tr>
<tr>
<td>ScoB</td>
<td>5'-ATA GGA TCC GCA GCA GGA GCA GGT GAA G-3'</td>
<td></td>
</tr>
</tbody>
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### 2.1.11 List of constructs generated:

<table>
<thead>
<tr>
<th>Name of constructs</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-pBSKS</td>
<td>C1 (Acc No. NM_025858) was amplified using C1 A(F) and C1 A(R) primers and cloned into pBSKS vector in Smal site.</td>
</tr>
<tr>
<td>C1-pEGFP C1</td>
<td>C1 fragment was released from C1-pBSKS and subcloned between Xho I and Bam HI site of pEGFPC1 vector.</td>
</tr>
<tr>
<td>C1-pEGFP N3</td>
<td>C1 fragment was released from C1-pBSKS and subcloned between Hin D III and Sal I site of pEGFPC1 vector.</td>
</tr>
<tr>
<td>C1 (ΔCys)-pEGFP N1</td>
<td>Nco I and Sau3A site was used for making this construct, which involved digestion with both the enzyme, end filling and subcloned in pEGFPN1 vector</td>
</tr>
<tr>
<td>C1 (ΔPro)-pMOS</td>
<td>C1 (ΔPro) was amplified using ScoA and ScoB primers and cloned in pMOS vector</td>
</tr>
<tr>
<td>C1 (ΔPro)-pEGFP C2</td>
<td>C1 fragment was released from C1 (ΔPro)-pMOS and subcloned between EcoRI and Sal I site of pEGFPC2 vector.</td>
</tr>
<tr>
<td>C1 (ΔPro)-pMALC2</td>
<td>C1 fragment was released from C1 (ΔPro)-pEGFP C2 and subcloned between Bam HI and Hin DIII site of pMALC2 vector.</td>
</tr>
<tr>
<td>C1-FLAG-pFPC19</td>
<td>Bam HI digested pFPC19FLAG vector was end filled and further digested with Xbal. C1-pBSKS was digested with Ndel, end filled and further digested with Kbal and released fragment was ligated with digested vector.</td>
</tr>
<tr>
<td>C1-p[UAST]</td>
<td>C1 fragment was released from C1-pBSKS and subcloned between EcoRI and XbaI of p[UAST] vector</td>
</tr>
</tbody>
</table>

**Full length C1/Scotn protein:**
MAAPAPSLWLTTTTTTTTTTPPPPGAHGELCRPFGEEDNSIPVFCPDFCCGSCSNQYCCSDVLRKIQWNNE MCPEPESRFSTPEETPEHLSALKFRSSFDSPMSGFGATVAIGVTIFVFIATITICFTSCCCCLYKMCC CPQRPVVTNNTTTTVHAPYPQPQQPQPQPYPGPTYQYHPMPQPPGMPAAAPYTQYPYLYAQLTGP PYPYHESLAGASQPPYNPTYMDSLKTIP

**C1 (ΔCys) Protein: Contains 1-26aa, 95-23, Deleted region Δ27-95**
MAAPAPSLWLTTTTTTTTTTPPPPGAHGSSFDSPMSGFGATVAIGVTIFVFIATITICFTSCCCCLYKMCC PQRPVVTNNTTTTVHAPYPQPQQPQPQPYPGPTYQYHPMPQPPGMPAAAPYTQYPYLYAQLTGP PYPYHESLAGASQPPYNPTYMDSLKTIP

**C1 (ΔPro) Protein: Contains 1-132aa, Deleted region Δ133-235**
MAAPAPSLWLTTTTTTTTTTPPPPGAHGELCRPFGEEDNSIPVFCPDFCCGSCSNQYCCSDVLRKIQWNNE MCPEPERSRFSTPEETPEHLSALKFRSSFDSPMSGFGATVAIGVTIFVFIATITICFTSCCCCLYKMCC PQRPVVTNNTTTTVHAPYPQPQQPQPQPYPGPTYQYHPMPQPPGMPAAAPYTQYPYLYAQLTGP PYPYHESLAGASQPPYNPTYMDSLKTIP
2.2 Methods:

2.2.1 Cell biology protocols:

2.2.1.1 Sterilization:

All glassware and solutions were autoclaved at 121°C at 15 psi for 30 min. The solutions used for cell culture were filtered through 0.22µm seitz filters and all plasticware was gamma sterilized, and in addition, microwaved at 200 watts for 2 min before use. Glassware for RNA work was baked at 180°C overnight in addition to autoclaving.

2.2.1.2 Cell culture:

Cell lines (P19 and COS1) were maintained in DMEM supplemented with 10% FBS and antibiotics (50µg/ml Penicillin and 60µg/ml Streptomycin) at 37°C in a humidified 5% CO₂ containing incubator. Cells were harvested for subculturing or for performing experiments using Trypsin-EDTA (0.1% each in PBS). Culture was never allowed to grow beyond 80% confluency. For cryopreservation, Cells were trypsinized, resuspended in a growth medium and counted. Cells were collected by centrifugation and resuspended at a density of 2 X 10⁶ cells/ml in freezing medium (Freezing medium: DMEM medium with 50% FCS and 10% DMSO). Cells resuspended in freezing medium were distributed in freezing vials (1ml aliquot) and stored at -70°C overnight in an insulated container (for slow freezing). Frozen vials were transferred to liquid nitrogen for long-term storage.

2.2.1.3 Immunofluorescence:

Treated or untreated cells were fixed with 4% formaldehyde for 10 min and permeabilized in permeabilization solution (0.1% Triton X-100 and 0.02% tween-20 in PBS) for 10 min. For centrosome detection, cells were fixed with ice cold 80% methanol for 15 min at 4°C, after that cells were permeabilized. Blocking was done in blocking solution (2% BSA in PBS) at room temperature for 1 hr. Cells were incubated with primary antibody, diluted in washing buffer (washing buffer: 0.2% BSA and 0.02% tween-20 in PBS) for 1 h at room temperature. Cells were washed with washing buffer for 30 min (3 washing, 10 min each) and incubated with secondary antibody conjugated (diluted in washing buffer) with appropriate fluorophore for 45 minutes at room temperature. Cells were washed with
washing buffer for 1h (6 washing, 10min each) and then mounted in mounting medium containing DAPI (counter stain) and examined by fluorescent/confocal microscopy.

2.2.1.4 BrdU incorporation assay:

Heat shocked cells, grown on glass coverslips were treated with 25 μM BrdU for 4hr at regular culture conditions. After incubation medium was removed and cells were washed with PBS twice and fixed in 100% ethanol for 30min at room temperature. To denature the DNA, cells were treated with 2N HCl containing 0.5% triton X-100 and 0.5% tween-20 for 30min at room temperature. Cells were washed with PBS (3 washes, 5min each) and neutralization was done with NaBH₄ (1mg/ml) and again washed with PBS (3 washes, 5min each). Blocking was done in blocking buffer (10% horse serum in PBS containing 0.5% triton X-100) at room temperature for 2hrs. Cells were incubated with anti-BrdU antibody (1:200 dilution in blocking buffer) for 2hr at room temperature. Cells were washed with blocking buffer at least for 1h (6 times, 10min each) and incubated with biotinylated secondary antibody for 45min at room temperature, washed with blocking buffer for 30min (3 times, each for 10min) and treated with alexa 594 conjugated avidin for 30min. Again washed with blocking buffer for 60min (6 times, each for 10min). Cells were mounted in mounting medium and analyzed by fluorescent microscope.

2.2.1.5 Rhodamine phalloidin staining:

For detection of actin microfilaments, rhodamine phalloidin was used. Cells grown on coverslips were washed twice with 1 X PBS and fixed with 3.5% Formaldehyde for 10-15 min at room temperature. Cells were permeabilized with 0.5% Triton-X-100 and 0.05% Tween-20 (In PBS) for 10 min and stained with rhodamine phalloidin (diluted in PBS 1:200, according to manufacturer instruction) for 60 min. Cells were washed for 1h (six washing, 10min each) and mounted in mounting medium containing DAPI.

2.2.1.6 Transfection:

Transfection into mammalian cells was done using lipofectamine plus reagent according to manufacturer’s instructions. Cells were grown on glass coverslip and transfection was done when cells were about 50% confluent. 0.3μg -1.0μg DNA was used
for one 22mm coverslip. Plasmid DNA was pre-complexed with the 1μl of lipofectamine plus reagent in the presence of 10μl of incomplete DMEM medium and incubated for 15 minutes. In another tube 1μl of lipofectamine was diluted with 10μl of medium, mixed and incubated for 15 minutes at room temperature. Both of them were then mixed together and incubated at room temperature for 30min. Mean while the growth medium of adherent cells were removed and washed with incomplete DMEM medium. The volume of DNA lipid complex were made 100-150 μl with incomplete DMEM and poured on cells, previously washed with incomplete DMEM and kept at 37°C in CO₂ incubator for 4 hours. After 4 hours, 1ml growth medium containing serum was added in this.

2.2.1.7 Embryoid body formation assay:

Cells were grown in suspension (10^5 cells/ml) in bacterial grade petriplates for 8 days. Every after 48h, the entire medium (leaving the attached cells in the petriplate) was taken into 15 ml tubes, kept for 10min without shaking to allowed EBs to settle down. Supernatant was removed and new complete medium was added, mixed by inversion and transferred to new bacterial grade petriplates. Rounded EB like structure started seeing after 4th days.

2.2.1.8 Soft agar assay for colony formation:

Soft agar assay was done in 35mm dishes. 0.5% aragose [1% agarose melted and cooled to 40°C, and mixed with equal volume of complete 2X DMEM medium and poured (1.5ml in one 35mm dish)] was used as a bottom layer. On top of bottom layer, cells mixed in 0.35% agar was plated. For that 0.7% agar melted and cooled to 40°C, mixed with equal volume of complete 2X DMEM medium and required number of cells (5,000-10,000 cells/35mm plate) and poured (1.5ml) on to base agar layer. Cells were grown in regular cell culture conditions (37°C, 5% CO₂ in humidified incubator) for 21 days.

2.2.1.9 Metaphase chromosome preparation:

Exponentially growing cells (60-70% confluent) were treated with colcemid (final conc. 0.1μg/ml, stock conc. 10μg/ml) for 2hr at regular cell culture conditions (37°C, 5% CO₂ in humidified incubator). Cells were trypsinized and collected in a 15ml centrifuge
tube. Centrifugation was done at room temperature for 5 min at 1000 rpm. Pellet was dislodged and 10 ml hypotonic solution (0.075M KCl) was added slowly and incubated at 37°C for 10-15 min. After incubation, 5 ml fixation solution (3:1 Methanol: Glacial acetic acid) was added in this, incubated for 5 min and centrifuged at 1000 rpm for 5 min. Pellet was resuspended in fixation solution and incubated for 1 h. Again it was centrifuged at 1000 rpm to collect the cells and resuspended in 0.5 ml fixation solution. Properly washed slides (washed with 70% acetic acid) were used for metaphase preparation. The cell suspension was dropped onto glass slides, air dried, stained with DAPI and analyzed by fluorescent microscope.

2.2.1.10 Alkaline phosphatase assay:

Cultured cells were were washed with 1X PBS and fixed with ice cold 4% paraformaldehyde for 15 minutes at 4°C. Alkaline phosphatase staining solution containing 100 mM NaCl, 100 mM Tris-HCl pH 9.5, 5 mM MgCl2, 1 mg/ml NBT, 0.1 mg/ml BCIP was added and incubated at 37°C for 2 h.

2.2.1.11 MTT assay:

MTT assay was done in 96 well plate. 2,500 cells were plated in each well and treatment was given. Cells were washed with incomplete medium and 50 µl of MTT solution (5 mg/ml in incomplete medium) was added in each well (treated and untreated cells). Incubation was done at regular cell culture conditions (37°C, 5% CO2 in humidified incubator) for 2 hrs. Cells were washed with PBS. To dissolve formazan crystals, 200 µl DMSO was added in each well and colour intensity was estimated using spectrophotometer (Elisa reader) at OD590.

2.2.1.12 Mitotic Index calculation:

Cells were stained for β-tubulin and DAPI. Random field was selected under the microscope. In each field, all the cells and mitotic cells were counted, and percentage of mitotic cells were calculated.
2.2.1.13 PI exclusion test for cell death analysis:

For cell death analysis, untreated and treated cells were trypsinized and stained with PI (1µg/ml) for 5min in PBS, and cell death was determined by flow cytometry (FACS Caliber, BD biosciences) using Cell Quest software.

2.2.1.14 Cell doubling time calculation:

5X10^5 cells were plated in 25cm^2 flasks and at time point 24h, 48h and 72h cells were trypsinized and counted by haemocytometer. Cell doubling time was calculated from the exponential growth phase. Experiments were done in triplicate and were repeated three times.

2.2.1.15 FACS analysis:

Untreated and treated cells were trypsinized, washed with PBS and fixed with 80% methanol. These cells can be stored at 4°C for 10-15 days. After collecting all the samples, methanol was removed and cells were stained with PI solution (50 µg/ml PI, 20 µg/ml RNaseA, 0.1% sodium citrate and 0.1% triton X-100 in PBS) and analyzed in a FACS Caliber Cytometer (Becton-Dickinsom, San Jose, CA) using Cell-Quest software.

2.2.1.16 Heat treatment:

Heat shock is given at 40-50% confluence. 40-50% confluent flask was submerged in water bath set at required temperature. After that flask or petriplates was transferred to regular cell culture conditions (37°C, 5% CO2 in humidified incubator).

2.2.1.17 Curcumin treatment:

40-50% confluent flask was used for giving curcumin treatment. Required amount of curcumin was added to complete medium to achieve the required concentration of curcumin, mixed and flask medium was replaced by this medium. Every after 24hrs, this medium was replaced by new curcumin containing medium. After incubation time was over, flask was washed with PBS and complete DMEM medium without curcumin was added.
2.2.1.18 Colocalization analysis:

Colocalization analysis was done LSM510 META software.

2.2.2 Drosophila protocols:

2.2.2.1 Routine Fly techniques:

Fly stock were grown on standard corn meal with sugar and agar (Ashburner, 1989) and maintained at 25°C unless specified otherwise. The wild type strain used was Canton-S and other fly strains in this work and their sources are listed in the section ‘List of fly stock used’. Detailed information on all the line is available at the Flybase: http://flybase.bio.indiana.edu/

Recombinant chromosomes and combination of GAL4 drivers, UAS lines, different mutations and/or markers were made by standard genetic techniques. FM7a was used as a first chromosome balancer, CyO as a second chromosome balancer and TM2 and TM6B as the third chromosome balancer. In a standard fly cross, males and virgin females were crossed with the ratio of around 1:3 (male : female). Finally five to ten females were used.

2.2.2.2 Genomic DNA isolation:

A single fly was squished with the help of pipette tip in 20μl of squishing buffer [10 mM Tris.HCl pH 8.2, 1 mM EDTA, 25 mM NaCl, and 200μg/ml Proteinase K] in a 0.5ml microfuge tube. Incubated at 37°C for 30min. Proteinase K was inactivated by heating to 95°C for 10min., centrifuged and supernatant was used for PCR reaction.

From (http://wheat.pw.usda.gov/~lazo/methods/lazo/dnafly.html)

2.2.2.3 Generation of transgenic fly line:

Embryo collection:

Egg laying plates were prepared by boiling 30g of sugar, 25g of agar and 17g of Yeast in 1L of water. After it was cooled down to 55°C, propionic acid (5ml/l), orthophosphoric acid (1ml/l) and p-methyl benzoate (5ml of 5% solution in 1L) were added. The medium was poured into petriplates and allowed to set overnight. A layer of yeast paste was coated just before using these plates for egg collection. The plates were then taped to the bottom of the cylinder chamber used for egg collection. Desired genotype (w¹¹¹⁸) flies were
kept inside this egg collection chamber. The plates were changed every after 40-60min and eggs were collected from these plates. Collected eggs were washed with tap water and decorionated in 3% sodium hypochlorite solution (4-6min) rinsed thoroughly in water and aligned on the coverslip with the help of glue and used for microinjection.

**Preparation of DNA for injection:**

DNA, prepared from Quigen column, was diluted in injection buffer [5mM KCl, 0.1mM phosphate buffer, pH6.8 (0.05mM K₂HPO₄, 0.05% mM KH₂PO₄)] to a final concentration of 400ng/µl and used for injection. The DNA mix was injected into the poleplasm of 0-1h old embryos (of genotype w¹¹¹⁸) by standard techniques.

**Selecting for transformants:**

pUAST vector, used for making transgenic fly, contains wild type copy of eye colour gene, white⁺ and the w¹¹¹⁸ fly, used for making transgenic fly, had mutant eye colour gene (eye colour white). Recombinant was selected on the basis of eye colour. The flies derived from injected embryos were crossed to w¹¹¹⁸ flies and the progeny of this cross were scored for the presence of eye colour marker. Progeny with eye colour, pale yellow to dark red, were considered as transgenic flies.

**2.2.2.4 Acridine orange (AO) staining:**

Dissected larvae were kept in Acridine orange solution (5µg/ml in PBS) for 5min. After incubation, they were washed with PBS for 10min (3 washes, 3-4min each). Disc were dissected and analyzed for apoptotic cells under fluorescent microscope (wavelength used for detection: 488nm).

**2.2.3 Molecular biology protocols:**

**2.2.3.1 Competent cell preparation:**

The competent cells were prepared according to Hanahan (1985) with minor modifications. A single colony of *E.coli* DH5α or BL21 was inoculated in 5ml of LB and incubated overnight at 37°C in shaking water bath. 1ml of this culture was taken to inoculate
100 ml of LB (1:100 dilution) medium and incubated at 37°C with vigorous shaking till optical density at 600nm reached 0.5. The culture was chilled on ice for 15 min and the cells were collected by centrifugation at 2000 rpm for 12-15 min at 4°C. The supernatant was drained thoroughly and cells were resuspended in 10ml of RF1 solution (100mM RbCl, 50mM MnCl2, 30mM Potassium acetate, 10mm CaCl2, 15% Glycerol). After incubating on ice for 15min, cells were collected as before, resuspended in 5ml of RF2 (10mM RbCl, 75mM CaCl2, 15% glycerol, 10mM MOPS pH6.8) and left on ice for another 15 minutes. Aliquots (200μl) of these cells were flash frozen in liquid nitrogen and stored at −70°C till needed.

2.2.3.2 Transformation:

The frozen competent cells were thawed on ice slowly just before use. Transforming DNA in a volume less than 10 μl was mixed with competent cells and incubated on ice for 15 minutes. The cells were subjected to heat shock for 90 seconds at 42°C, followed by rapid chilling on ice for 5 minutes. 800μl of LB medium was added to the cells and the cells were incubated for an hour at 37°C with gentle shaking. When supercoiled DNA was used for transformation, 50-100μl mix was plated and for a ligated mix, the entire mix was pelleted at 4000 rpm for 2 minutes, the supernatant was discarded, pellet was suspended in 100 μl of LB medium and plated on an LB plate containing required antibiotic and color development reagents (IPTG 10mM and X-Gal 20mg/ml). The plates were incubated overnight at 37°C and recombinants were picked up.

2.2.3.3 Plasmid isolation:

Small-scale preparation of plasmid DNA was done by alkaline lysis method as described by Sambrook et al (1989) with slight modifications. 5ml of LB medium containing 100μg/ml ampicillin was inoculated with a single colony of E. coli DH5α bearing the plasmid of interest and incubated overnight at 37°C with shaking at 200 rpm. Bacterial pellet from 3ml of overnight grown culture was resuspended in 100μl of ice-cold solution 1 (50mM glucose, 25mM Tris.HCl pH 8.0, 10mM EDTA pH 8.0) by vortexing. 200μl of freshly prepared solution II (0.2N NaOH, 1% SDS) was added and the contents
was mixed by inverting the tube 4-6 times. Then 150μl of ice-cold solution III (3M potassium-5M acetate) was added and mixed by inverting the tube 4-6 times. The tube was incubated on ice for 10min and centrifuged at 13,000rpm for 10min at 4°C. The clear supernatant containing the plasmid DNA was collected into a fresh 1.5ml microfuge tube leaving the cellular debris behind and precipitated with two volumes of absolute ethanol for 5min at room temperature. The precipitated plasmid DNA was pelleted by centrifugation at 13,000 rpm for 30min at room temperature, washed with 70% ethanol, air dried and dissolved in 25μl of autoclaved double distilled water or TrisEDTA (pH 8.0) containing 20μg/ml DNase free pancreatic RNase A. The plasmid DNA was quantitated and 1-2μg DNA was checked on a 0.8% agarose gel. It was stored at -20°C. The typical yield for a high copy plasmid from 3ml culture was about 12-16 μg and the DNA was suitable for routine procedures such as restriction digestion.

Transfection grade or sequencing grade plasmid was prepared using QIAGEN-tip20 (miniprep) and QIAGEN-tip 100 (midiprep) columns or using SNAP midiprep kit, Invitrogen, according to manufacturer’s instructions. The protocol involves alkaline lysis followed by column purification of DNA that yields high purity plasmids with relatively low level of impurities.

2.2.3.4 RNA isolation:

Adherent cells were washed three times with ice-cold 1X PBS and PBS was removed completely. Required amount of Trizole (1ml per 5 X 10^6 cells or 0.5 ml per T25 flask) was added to the flasks and cells were scraped using a policeman and all contents were transferred to a 1.5 ml microfuge tube and stored at -70°C until isolation. For isolation of RNA, the lysate were thawed at room temperature. Chloroform was added (for 1ml lysate, 200μl chloroform), mixed properly (Brief vertex), and incubated for 5 min at room temperature. The solution was centrifuged at 13,000 rpm for 15 min at 4°C. The aqueous phase was collected in a fresh tube and precipitated with equal volume of isopropanol (for 500μl supernatant, 500μl isopropanol was added) at room temperature for 5 min. RNA was obtained by centrifugation at 13,000 rpm for 15 min at 4°C. The pellet was washed to
remove salt using 70% ethanol, air dried in the hood and re-suspended in DEPC treated water.

2.2.3.5 Quantitation of nucleic acid:

The nucleic acid concentration was determined by measuring the absorbance at 260 nm (Sambrook et al., 1989). Empirical relationship of 50 μg double standard DNA, 33 μg of single standard DNA (as in primers for PCR) and 40μg of single standard RNA was taken to be equal to 1.0 OD_{260}. Purity of the preparation was estimated using ratio of absorbance at 260 nm to 280 nm. A ratio of 1.6-1.8 was considered good for DNA preparations and 1.6-2.0 was considered good for RNA preparations.

2.2.3.6 Agarose gel electrophoresis:

DNA and RNA samples were mixed with appropriate amounts of 6X loading dye (0.25% bromophenol, 0.25% xylene cyanol and 30% glycerol) and were resolved, using 0.8-2% agarose gels made in 1X TBE buffer. Sample was loaded and the gel was run at 5V/cm (calculating the distance between the two electrodes, not the length of the gel). Nucleic acids were visualized by adding 0.25 μg/ml ethidium bromide in the agarose gel and 0.05 μg/ml in the running buffer (1XTBE).

2.2.3.7 Restriction digestion:

Restriction digestion of DNA was carried out with 1-5 units/μg of DNA in 25μl of reaction volumes, using the buffers and incubation conditions recommended by the suppliers. The reactions were stopped by raising the reaction temperature to 85/65°C for 10-15 minutes or subjected to phenol:chloroform extraction.

2.2.3.8 Gel elution of DNA from agarose gel:

DNA fragment, resolved on agarose gel was purified using QIAquick Gel Extraction kit from Qiagen as per the manufacturer's instructions.

2.2.3.9 Ligation:

DNA fragments, obtained after gel purification of PCR products or restriction digestion, were ligated using T4 DNA ligase at 20°C for 8hr to overnight in ligation buffer.
(50mM Tris-HCl pH 7.6, 10mM MgCl₂, 10mM DTT and 5mM ATP). A molar ratio of vector to insert of 1:3 was generally used. When using the pMOS vector for cloning PCR products, the protocol suggested in the product literature was followed.

2.2.3.10 Kinase reaction for primers:

As 5' ends of primers used for PCR were not phosphorylated, amplified products had to be kinased before these could be used for ligation with dephosphorylated vector. About 10 units of T4 PNK were used for phosphorylation of approx 500 ng DNA in T4 PNK buffer (50mM Tris-HCl, pH 7.7, 10mM MgCl₂, 5mM DTT, 0.1mM spermidine hydrochloride, 0.1 mM EDTA) containing 10mM ATP pH 7.0 in a total volume of 30 µl. Samples were incubated at 37°C for 40 minutes and reaction was stopped by heating at 65°C for 15 minutes. Vector and ligase were added to the same mix and the ligation reaction was set.

2.2.3.11 Dephosphorylation of the vector DNA:

50 ng of Digested and purified vector DNA was incubated with 1 unit of SAP (Shrimp Alkaline Phosphatase) along with 1X SAP buffer (0.5M tris HCl, 50mM MgCl₂ pH 8.5) in a 20µl reaction volume at 37°C for 10 minutes. The reaction was stopped by heating the reaction mix at 65°C for 15 minutes.

2.2.3.12 Blunting of 5' and 3' overhangs:

Filling in of 5' overhangs and removing of 3' overhangs was done using either the Klenow fragment of E. coli polymarase I or by using T4 DNA polymerase. These enzymes possess the 3'→5' exonuclease but have lost the 5'→3' exonuclease activity. In both cases, the reaction was carried out with suitable buffer recommended by the manufacturers using 1 unit of enzyme per µg of DNA. However, Klenow reaction was carried out at 25°C for 15 min and the reaction mixture was supplemented with 33µM each of dNTPs. T4 DNA polymerase reaction requires a supplementation of 100mM each of dNTPs and was carried out at 12°C for 15min. The reaction was stopped by adding EDTA to a final concentration of 10mM and by heating 75°C for 20min. However, elevated temperatures, prolonged reaction times, excessive usage of enzymes and failure to supplement with enough dNTPs might results in recessed ends due to the 3'→5' exonuclease activity of both these enzymes.
2.2.3.13 DNA sequencing:

ABI Prism Model 3700 DNA Analyzer or model 3730DNA Analyzer were used for all DNA sequencing reactions. 200ng of DNA and 2.5-5 pmols of primer was constituted in a volume of 3.2 µl and mixed with 1.8µl of the big-dye terminator sequencing kit (Perkin-Elmer). PCR was carried out in the Gene Amp PCR System 9600 Thermal Cycler with denaturation at 96°C for 10sec, annealing at 50°C for 5 sec and extension at 50° for 4min, for a total of 35 cycles and final extension was done for 5min. DNA was ethanol precipitated and washed with 70% ethanol. The samples were then resuspended in 10µl High-dye-formamide (Perkin-Elmer) and 5µl of this was loaded in the capillaries.

2.2.3.14 Polymerase chain reaction (PCR):

PCR was done in a 20µl reaction volume containing template DNA (10ng for plasmid), 1X PCR buffer (10mM Tris.HCl pH9.0, 50mM KCl, 1.5mM MgCl2, 0.01% gelatin-Taq buffer 10A from Bangalore Genei), 0.2mM dNTPs mix, 200ng primers (both forward and reverse) and 1.5 units of Taq DNA polymerase. After and initial denaturation at 94°C for 2min, required number of cycles was carried out. Each cycle contained denaturation at 94°C for 30sec, annealing temperature specific for each primers for 1min and extension at 72°C for time, dependent on the length of amplified product (for amplifying 1kb region, 1min extension time was given).

2.2.3.15 Reverse transcription for semi quantitative PCR:

Genomic DNA free RNA was used for reverse transcription reaction. RNA from different samples was treated with RNase free DNase for 30min at 37°C and enzyme was removed by Phenol:Chloroform extraction. 2.5-5µg total RNA was used for a reaction. Reaction was performed in a 25µl total volume with 200u of MMLV RT enzyme. Reaction was done as recommended by supplier (Promega).

2.2.3.16 Cell lysate preparation:

Adherent cells were washed twice with ice cold PBS and appropriate amount (for 1million cells, 100µl lysis buffer or for 60mm dish, 0.5ml) of cell lysis buffer [50mM Tris.HCl (pH 8.0), 150mM NaCl, 1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate,
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100µg/ml PMSF, 1µg/ml Aprotenin, 1µg/ml Leupeptin] was added and incubated on ice for 20min. Cells were scraped using a rubber policeman and transferred to the microfuge tube. Cells were sonicated. Lysate was centrifuged at 14,000 rpm for 5 min and the supernatant was transferred to a fresh tube and protein content was estimated.

2.2.3.17 Protein estimation:

50µl of Bio-Rad reagent was mixed with 199µl double distilled water. 1µl of protein lysate was added, mixed properly and incubated at room temperature for 5 min. The colour was quantified using an ELISA reader recording the OD at 590 nm. The concentration of proteins was calculated using a standard curve generated using the same protocol and reagents but with known concentrations of Bovine Serum Albumin (BSA).

2.2.3.18 SDS Polyacrylamide Gel Electrophoresis:

SDS polyacrylamide gel electrophoresis was carried out by the method described by Sambrook et al., (1989) using a discontinuous buffer system. Stacking gel (0.125M Tris.HCl pH 6.8, 5% acrylamide and 0.1% SDS) and resolving gel (0.375M Tris.HCl pH 8.8, 8-12% acrylamide and 0.1% SDS) were polymerized using TEMED and Ammonium per sulphate. Electrophoresis was carried out in electrophoresis buffer (0.025M Tris, 0.192M glycine and 0.1% SDS) at 20mA constant current till the samples entered the stacking gel than at 40mA through the resolving gel.

2.2.3.19 Coomassie brilliant blue staining:

The gels were stained with 0.2% Coomassie brilliant blue R250 in methanol:Acetic acid:water (45:10: 45 v/v). The gels were de-stained with same solution without dye for 6 hours on the rocking platform with 2-3 changes.

2.2.3.20 Immunobloting:

The protein samples, resolved on the SDS-PAGE were transferred to the nitrocellulose membrane by semi-dry graphite method as described in Amersham semi-dry protein transfer apparatus instruction manual. The gel containing the protein, the C Extra Nitro cellulose membrane and 6 sheets of Whatmann 3M paper cut to the size of gel were
soaked in transfer buffer (39mM Glycine, 48mM Tris, 0.037% SDS and 20% methanol) and arranged as follows: 3 pieces of soaked paper were placed on the anode plate. The membrane was placed over it. The gel was aligned on the membrane and 3 more pieces of blotting paper were placed over it. Care was taken to remove air bubbles from the 3M sheets as they interfere with the transfer. The transfer was performed for 2 hrs using a current of 0.8mA/cm². The efficiency of transfer was subsequently checked by staining the membrane with Ponceau stain.

The membrane containing transferred protein was incubated with TBS containing 3%BSA for 1 hour. This procedure, called blocking, is used to block the free spaces on the membrane with a neutral protein - BSA. The blot was rinsed once with TBS, incubated with the recommended dilution of primary antibody in TBS containing 1%BSA for 1-2 hours. The blot was washed with TBST (0.1% Tween-20) for 30 minutes with 3 changes. The blot was incubated with either 1:10,000 dilution (HRP tagged) or 1:1000 dilution (ALP tagged) of secondary antibody in TBS with 1% BSA for 45 minutes –1 hour. It was then washed with TBST for 30-60 minutes with 4-5 changes of solution on a rocking platform.

For ECL method (HRP tagged Secondary antibody), the blot was removed from the wash solution and placed within a polythene cover. Developing solution mix (Roche) was spread on it using a pipette and incubated for one minute. Excess solution was removed from the blot and the blot within the polythene was exposed to an X-ray film that was subsequently developed. In the chromogenic method (ALP tagged Sec Ab), the blot was placed in ALP buffer containing NBT/BCIP (substrates for Alkaline Phosphatase) and kept on a rocker till clear bands appeared on the blot. The blot was rinsed with TBST and dried using blotting paper.

2.2.3.21 Protein expression and purification:

The gene of interest was cloned in pMALC2 vector and sequenced for confirmation of correct reading frame. BL21 competent cells were transformed with recombinant plasmid and grown on LB plate containing 100μg/ml ampicillin. A single colony was inoculated in 5ml of LB medium containing 100μg/ml ampicillin and grown overnight at 37°C. For standardization of expression conditions, 10ml of LB-Rich medium (10g/L tryptone, 5g/L yeast extract, 5g NaCl, 2g glucose) containing 100μg/ml ampicillin was inoculated with 1%
(i.e., for 10ml 100µl overnight grown culture) overnight grown culture and incubated at 37°C with vigorous shaking till $\text{OD}_{600}$ 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 at 2hr, 4hr, 8hr, 12 hr; cells were pelleted and re-suspended in 120µl of SDS-PAGE sample buffer. SDS-PAGE analysis was done using induced and uninduced samples to determine optimum expression time, conc. of IPTG and induction temperature. Optimum condition for induction of Scotin/C1 protein expression was standardized which were 30°C, 0.5mM IPTG conc. and cells collection after 3h of induction.

Purification was done following the instructions given in the manual (NEB). Briefly induced cells were collected, washed with column buffer (20mM Tris.HCl, 200mM NaCl, 1mM EDTA, 10mm β-mercaptoethanol) and kept overnight at -20°C. It was sonicated briefly, and centrifuged at 13,000rpm for 5min at 4°C. Supernatant was collected and passed through the amylose column, pre-equilibrated with column buffer. After that, column was washed with column buffer overnight at 4°C. Protein was eluted from the column with column buffer containing 10mM maltose.

2.2.3.22 Generation of polyclonal antibody:

Collection of control sera from animal:

Blood collected from the animal was incubated at 37°C for 1 hr and at 4°C for 2-3 hour. Serum (supernatant) was collected by centrifuging the sample at 2500-3000 rpm for 30 minutes. The serum was stored at -70°C in 100-150µl aliquots.

Polyclonal Antibody:

For raising polyclonal antibody against Scotin/C1 protein in the rat, 100µg of protein was diluted to 250µl in PBS and mixed with 250µl of Freund’s complete adjuvant. The mixture was made into an emulsion by passing through a 2ml syringe (1.5inch 19G needle) and intermittently keeping it at -20°C. This emulsion was injected into the rat subcutaneously. After 15 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 10-days interval and the antibody titer was
checked using dot blot. For determination of antibody titer 20ng protein was spotted in a row, air-dried and incubated with different concentrations of immune serum (1-100 to 1-50,000). Western blot experiments were carried out when the appropriate titer (minimum of 1:10,000) was obtained following booster doses.