“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.”

Albert Einstein
5. MATERIALS AND METHODS

5.1 Sources of antibodies, chemicals and c-DNA constructs

Chemicals, antibodies, kits and cDNA constructs were obtained from the following sources:

**Abcam, UK**
Rabbit polyclonal anti-NRP-1, anti-CD31 and anti-procaspase 9 antibodies were purchased from Abcam.

**Amersham Pharmacia (GE Healthcare), UK**
Salmon sperm DNA was obtained from GE Healthcare.

**BD Biosciences, USA**
Growth factor reduced matrigel and all plasticwares related to cell and tissue cultures were purchased from BD Biosciences.

**Bangalore Genei, India**
DNA Taq polymerase, 10 X Taq buffer, M-MuLV Reverse transcriptase, 5X M-MuLV Reverse transcriptase buffer, dNTPs, T4 Polynucleotide kinase, T4 Polynucleotide kinase buffer, 1 Kb and 100 bp DNA ladders were purchased from Bangalore Genei.

**Bio-Rad, USA**
Bradford protein assay kit and N.N, N’, N’-tetramethylethylene diamine (TEMED) were purchased from Bio-Rad.

**Biogenex, India**
Supersensitive polymer-HRP IHC detection kit was obtained from Biogenex.

**Board of Radiation and Isotope Technology (BRIT), India**
[γ-³²P]-ATP (5000 Ci/μmol) was purchased from BRIT, Hyderabad, India.
**cDNA constructs**

Wild type PTEN (wt PTEN) and dominant negative PTEN (dn PTEN) in EGFP-C2 vector was a kind gift from Dr. Nicholas Leslie (University of Dundee, Dundee, UK). Human Sema 3A cDNA in pCEP4 expression vector (H-Sema 3A-AP) was a generous gift from Prof. Alex Kolodkin (John Hopkins University School of Medicine, Baltimore, Maryland, USA). Wild type FOXO 3a (wt FOXO 3a) in pCI-neo and triple mutant FOXO 3a (FOXO 3a TM, a constitutive active form of FOXO 3a) in pECE vector was gifted from Dr. Boudewijn M.T. Burgering (University of Medical Center Utrecht, Stratenum, The Netherlands).

**Calbiochem, USA**

Anti-goat IgG, LY294002, PD98059 and SB203580 were purchased from Calbiochem.

**Cell Signaling Technologies, USA**

Rabbit polyclonal anti-pPTEN, anti-pFOXO 3a, anti-FOXO 3a, anti-pp38, anti-procaspase 3 and anti-procaspase 8 were obtained from Cell Signaling Technologies.

**Chemicon International, USA**

Cy3-conjugated anti-rabbit IgG and Cy2-conjugated anti-goat IgG were purchased from Chemicon International.

**Corning Costar, USA**

Transwell chambers and cell culture plates were purchased from Corning Costar, USA.

**Dharmacon International, USA**

Small interfering RNA (siRNA) specifically targeting Sema 3A, NRP-1, FOXO 3a and corresponding control siRNA were obtained from Dharmacon International. Dharmafect 1 and 4 transfection reagents were also purchased from Dharmacon International.
**Difco Laboratories, USA**
Yeast extract, Bacto Tryptone, Bacto Miller Luria Bertani agar and Miller Luria Bertani broth were obtained from Difco Laboratories.

**Fischer Scientific, USA**
Glycine, Tris-base and sodium chloride were purchased from Fischer Scientific.

**Greiner Bio-One, USA**
Plastic tubes (15 ml and 50 ml) and micropipette tips were obtained from Greiner Bio-One.

**ICN Biochemicals, USA**
Ammonium per sulphate, chloroform, citric acid, dimethyl sulphoxide (DMSO), glycerol, ethylenediamine tetra-acetic acid (EDTA), formaldehyde, nonidet P-40 (NP-40), paraformaldehyde, potassium chloride, trisodium citrate, sodium dodecyl sulphate (SDS), sodium orthovanadate, sodium phosphate, sodium acetate, trypan blue and tween-20 were purchased from ICN Biochemicals.

**Invitrogen, USA**
TRizol reagent, see blue plus prestained protein marker, lipofectamine 2000, ultra pure agarose, Dulbecco’s Modified Eagle’s Medium (DMEM), Leibovitz 15 (L-15), Minimum Essential Medium Eagle, MEM (E) media and fetal bovine serum (FBS) were purchased from Invitrogen.

**Merck, Germany**
Disodium hydrogen phosphate, ethanol, isopropyl alcohol, hydrogen peroxide, giemsa and sodium dihydrogen phosphate were purchased from Merck.

**Millipore, USA**
Nitrocellulose membrane filters (0.22 micron) and chromatin immunoprecipitation (ChIP) assay kit were obtained from Millipore.
Qualigens, India
Methanol and Xylene were purchased from Qualigens.

Qiagen, USA
Plasmid isolation kit was purchased from Qiagen.

R&D Systems, USA
Human recombinant Sema 3A, VEGF and anti-NRP-1 neutralizing antibody were purchased from R&D Systems.

Roche Molecular Biochemicals, Germany
Ethidium bromide (EtBr), protease inhibitor cocktail and phenyl methyl sulfonyl fluoride (PMSF) were purchased from Roche.

Santa Cruz Biotechnology, USA
Rabbit polyclonal anti-pAkt, anti-Bax, anti-Bak, anti-Bcl-2, anti-Bim, anti-cyclin D1, anti-pERK1/2, anti-ERK-1/2, anti-Ki67, anti-PARP, anti-p38, anti-pPTEN, goat polyclonal anti-actin, anti-Akt, anti-Cox-2, anti-Sema 3A, anti-MelCAM, anti-FOXO 3a (supershift), mouse monoclonal anti-PTEN, anti-tubulin, anti-Bad, anti-horseradish peroxidase-conjugated IgG antibodies, western blot luminol reagent, mounting medium, bovine serum albumin and Sema 3A/NRP-1 blocking peptides were purchased from Santa Cruz Biotechnology.

Sigma Chemicals Co., USA
Acrylamide, N,N-methylene bisacrylamide, DTT (Dithiothreitol), sodium deoxycholate, bromophenol blue, β-mercaptoethanol, HEPES, PIPES, propidium iodide (PI) and [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] (MTT) were obtained from Sigma. shRNA specific to Sema 3A in pLKO vector was purchased from Sigma.

US Biologicals, USA
Comassie R-250 and Triton-X 100 were purchased from US Biologicals.
Zymed Laboratories, USA

Rabbit polyclonal anti-VEGF antibody was obtained from Zymed.

Miscellaneous

Human breast cancer cells (MCF-7 and MDA-MB-231) were obtained from American Type Culture Collection (Manassas, VA). Human umbilical cord vein endothelial cells (HUVECs) and EGM-2 media were purchased from Lonza (USA). The non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were obtained from the Experimental Animal Facility (EAF), NCCS, India. MDA-MB-231-luc cells were purchased from Xenogen Corporation (Alameda, CA). D.L-sulforaphane (SFN) was a generous gift from Professor S.V. Singh, University of Pittsburgh, School of Medicine, PA, USA. All other chemicals used were of analytical grade.

5.2 Maintenance of cell lines

MDA-MB-231 and MCF-7 cells were cultured in L-15 and DMEM respectively supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air (MCF-7) and 100% air (MDA-MB-231) at 37°C. HUVECs and MDA-MB-231-luc cells were maintained in EGM-2 and MEM(E) media respectively supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

5.3 Preparation of LB agar containing Ampicillin and Kanamycin

Luria Bertani (LB) agar was prepared by dissolving 35 g Luria agar in 1000 ml of distilled water and sterilized by autoclaving at 121°C for 20 min at 15 psi. Once temperature of LB agar reached 42°C, 50 μg/ml of Ampicillin or 25 μg/ml of Kanamycin was added to the agar and poured into plates and allowed to solidify at room temperature. The plates were incubated at 37°C for 16-18 h to check the sterility and then used for further experiments.
5.4 Preparation of competent cells

Reagents and buffers

Buffer A (100 ml)
- 1 M CH₃COOK 3 ml
- 1 M KCl 10 ml
- 1 M CaCl₂ 1 ml
- Glycerol 15 ml
- 1 M MnCl₂ 5 ml

Buffer B (50 ml)
- 1 M MOPS 0.5 ml
- 1 M KCl 0.5 ml
- 1 M CaCl₂ 3.75 ml
- Glycerol 7.5 ml

Methodology

A single colony of *E.coli* strain (DH5α) was inoculated in 5 ml of Luria-Bertani (LB) broth containing 20 mM MgSO₄ and grown for 16-18 h at 37°C at 200 rpm. 100 ml of LB broth was inoculated using 1 ml primary culture and grown till optical density reaches from 0.3 to 0.6 at A₆₀₀. Growth of the bacterial culture was arrested by placing them on ice for 30 min while continuously stirring to maintain uniform temperature. The culture was aseptically transferred to prechilled and sterile oakridge centrifuge tubes. The bacterial cells were centrifuged at 4000 rpm for 10 min at 4°C and the pellet was resuspended in 20 ml of chilled buffer by gentle vortexing. The suspension was incubated on ice for 60 min. The cells were again centrifuged at 4000 rpm for 10 min at 4°C. Aliquots of 100 μl were transferred to sterile cryovials and stored at -80°C.

5.5 Transformation

1-5 μg of plasmid DNA was added to 100 μl aliquot of competent cells, mixed well by tapping and then incubated for 30 min on ice followed by heat shock at 42°C for 90 sec. The mixture was further incubated for 2 min on ice. 850 μl of LB without antibiotic was added to the mixture and incubated at 37°C for 1 h with continuous shaking at 220 rpm. The cells were pelleted down and plated on LB agar.
plate containing specific antibiotic. The plate was incubated overnight at 37\(^\circ\)C and colonies were monitored on the next day.

**5.6 Plasmid preparation**

Plasmid isolation was done using QIAGEN plasmid Midi kit as follows:

**Buffer composition**

**Buffer P1 (Resuspension buffer: 4 ml)**
50 mM Tris HCl, pH 8.0
10 mM EDTA
100 μg/ml RNase A

**Buffer P2 (Lysis buffer: 4 ml)**
200 mM NaOH
1% SDS (w/v)

**Buffer P3 (Neutralization buffer: 4 ml)**
3 M CH\(_3\)COOK, pH 5.5

**Buffer QBT (Equilibration buffer: 4 ml)**
750 mM NaCl
50 mM MOPS, pH 7.0
15% Isopropanol (v/v)
0.15% Triton X-100 (v/v)

**Buffer QC (Wash buffer: 20 ml)**
1 M NaCl
50 mM MOPS, pH 7.0
15% Isopropanol (v/v)

**Buffer QF (Elution buffer: 5 ml)**
1.25 M NaCl
50 mM Tris.Cl, pH 8.5
15% Isopropanol (v/v)

**TE buffer**
10 mM Tris.Cl, pH 8.0
1 mM EDTA
Methodology

A single colony of transformed cells or plasmid bearing host bacterial cells was picked from freshly streaked ampicillin/kanamycin plate and inoculated in 2-5 ml of LB broth containing specific antibiotic and incubated at 37°C for 8 h with vigorous shaking at 300 rpm. The primary culture was diluted (1:500) in LB broth and further grown at 37°C for 16 h at 300 rpm. The bacterial cells were harvested by centrifugation at 6000 x g at 4°C for 15 min. The pellet was resuspended in Buffer P1 and mixed thoroughly. The cells were lysed using Buffer P2 at room temperature for 5 min. Chilled Buffer P3 was added to the mixture and incubated for 15 min on ice and then centrifuged at 20,000 x g at 4°C for 30 min. The supernatant was collected and applied to QIAGEN-column which was pre-equilibrated using Buffer QBT. The column was again washed using Buffer QC. The plasmid DNA was eluted using Buffer QF. The eluted DNA was subjected to ethanol precipitation and air-dried prior to resuspending in TE buffer.

5.7 Agarose gel electrophoresis

Reagents and buffer

5X TBE (Tris-borate buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>54 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>27.5 g</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

The volume was made upto 1 litre with distilled water.

Casting 1.2% agarose gel

1.2 g of agarose was added to 100 ml of 0.5X TBE buffer. The mixture was heated for melting and poured into the casting tray with the combs. 1µl of ethidium bromide (10 mg/ml) was added prior to pouring of gel.

5.8 Preparation of glycerol stocks

The transformed *E. coli* cells containing different plasmids were inoculated in LB ampicillin or kanamycin broth and were grown till O.D reaches to 0.6. To 0.85 ml of culture, 0.15 ml of sterile glycerol was added to a final concentration of 15% and 1 ml of the same was aliquoted into sterile cryovials which were snap-frozen in liquid nitrogen and stored at -80°C. The glycerol stocks were revived by
streaking (without thawing) on Luria agar plate and single isolated colonies were obtained from the plate.

5.9 Mammalian cell transfection

MDA-MB-231 or MCF-7 cells (5x10⁵) were seeded 16 h prior to transfection. Cells were transfected with specific plasmid DNA (1 μg) or siRNA (80 μM) according to manufacturer’s instruction. Briefly, plasmid DNA or siRNA was diluted in 50 μl serum free media and in another tube 5 μl lipofectamine 2000 or Dharmafect transfection reagent was diluted in 50 μl media and incubated separately for 5 min. Then plasmid DNA or siRNA were mixed with lipofectamine 2000 or Dharmafect transfection reagents respectively and further incubated for 20 min. The DNA: lipofectamine or siRNA: Dharmafect complex were added to 900 μl of antibiotic/serum free media and then applied onto the cells. After 6 h of incubation, media was replaced with L-15/DMEM media supplemented with 10% FBS. The cells were grown for 48 h and used for further experiments. For stable transfection, cells after 2 days of transfection were subjected to continued selection with puromycin (1 μg/ml) or hygromycin (400 μg/ml) for 15 days. Later on, hygro- and puro-resistant colonies were expanded for further studies.

5.10 Whole cell lysate preparation

**Cell lysis buffer composition (20 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris HCl (pH 7.4)</td>
<td>1ml</td>
</tr>
<tr>
<td>3M NaCl</td>
<td>1ml</td>
</tr>
<tr>
<td>1% Sodium deoxycholate</td>
<td>200 mg</td>
</tr>
<tr>
<td>1% Nonidet P-40</td>
<td>200 μl</td>
</tr>
<tr>
<td>1% Triton-X-100</td>
<td>200 μl</td>
</tr>
<tr>
<td>100 mM Phenyl methyl sulfonyl fluoride</td>
<td>400 μl</td>
</tr>
<tr>
<td>1M Dithiothreitol</td>
<td>100 μl</td>
</tr>
<tr>
<td>Protease inhibitor cocktail (as per manufacturer’s instruction)</td>
<td></td>
</tr>
</tbody>
</table>

**Methodology**

The cells were washed twice using PBS and resuspended in required amount of ice-cold lysis buffer and incubated on ice for 30 min followed by centrifugation at
12,000 rpm for 20 min to collect the supernatant. Protein estimation in the whole cell lysates was performed using Bradford’s method.

5.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

I. Reagents and solutions

30% Acrylamide/0.8% bis-acrylamide solution

Acrylamide 30.0 g
N, N- Methylene bisacrylamide 0.8 g
The volume was made upto 100 ml with deionized water. The solution was stored in dark.

4X Tris-HCl, pH 6.8 (0.5 M Tris-HCl, Stacking solution)

6.05 g was dissolved in 40 ml of deionized water; pH was adjusted to 6.8 with 1 N HCl. The volume was made upto 100 ml. The solution was stored at 4°C.

4X Tris-HCl, pH 8.8 (1.5 M Tris-HCl, Separating solution)

18.17g Tris-base was dissolved in 70 ml deionized water, pH was adjusted to 8.8 with 1N HCl and the volume was made upto 100 ml. The solution was autoclaved and stored at 4°C.

10% Sodium dodecyl sulphate (SDS) solution

10 g SDS was dissolved in 80 ml deionized water, the volume was made upto 100 ml with deionized water and stored at room temperature.

10% (w/v) Ammonium persulphate (APS) solution

0.1g APS was dissolved in 1ml deionized water. The solution was freshly prepared every time before use.

5X SDS-PAGE sample buffer

1M Tris HCl, pH 6.8 2.5 ml
Glycerol 5 ml
SDS 1 g
β- Mercaptoethanol 0.5 ml
Bromophenol blue 0.05 g
Volume was adjusted to 10 ml with deionized water.
10 X Electrophoresis buffer

Glycine 144.2 g
Tris base 30.3 g
SDS 10.0 g

Volume adjusted to 1000 ml with deionized water.

**II. Gel composition**

<table>
<thead>
<tr>
<th>Resolving Gel (9 ml)</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/ 0.8% bis-acrylamide</td>
<td>2.25 ml</td>
<td>3 ml</td>
<td>3.75 ml</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl(pH 8.8)</td>
<td>2.25 ml</td>
<td>2.25 ml</td>
<td>2.25 ml</td>
<td>2.25 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>4.5 ml</td>
<td>3.75 ml</td>
<td>3 ml</td>
<td>2.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>90 μl</td>
<td>90 μl</td>
<td>90 μl</td>
<td>90 μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>120 μl</td>
<td>120 μl</td>
<td>120 μl</td>
<td>120 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 μl</td>
<td>20 μl</td>
<td>20 μl</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

Stacking gel (5ml)

30% Acrylamide/0.8% Bis-acrylamide 1.0 ml
4 X Stacking solution 2.5 ml
Deionized water 6.5 ml
10% SDS 100 μl
10% Ammonium per sulphate 50 μl
TEMED 10 μl

**III. Coomassie blue staining solutions**

Coomassie blue 0.25%
Acetic acid 10%
Methanol 50%

The volume was made upto 100 ml with deionized water.
IV. Destaining solution
Acetic acid  7.5%
Methanol  10%
The volume was made upto 100 ml with deionized water.

V. Reagents and Solutions for western blot

1X Transfer buffer
Glycine  14.42 g
Tris base  3.03 g
Methanol  200 ml
Volume adjusted to 1000 ml with deionized water.

10X Phosphate buffered saline (PBS) (pH 7.4)
KCl  2.0 g
NaCl  80 g
Na₂HPO₄  14.4 g
KH₂PO₄  2.4 g
The pH was adjusted to 7.4 with 1N HCl and the volume was made upto 1000 ml.
The solution was autoclaved and stored at 4°C.

10X TBS (pH 7.4)
Tris base  30 g
NaCl  80 g
KCl  2 g
The chemicals were dissolved and pH adjusted to 7.4 with 1N HCl and the volume was made upto 1000 ml.

1X TBS-T
Tween-20 was added to a final concentration of 0.1% in 1X TBS.

Blocking solution
5% non-fat dry milk in TBS-T or 5% BSA in TBS-T.

Stripping buffer
62.5 mM Tris-HCl, pH 6.7
2% SDS
100 mM β-mercaptoethanol
Methodology

The protein concentration from cell lysates was measured by Bio-Rad protein assay kit. The samples containing equal amount of total proteins were mixed with 5X sample buffer. The samples were boiled at 95°C for 5 min. The proteins were resolved on SDS polyacrylamide gel using mini-gel electrophoresis system (Bio-Rad) with constant voltage of 60 V till the dye front reaches the bottom of the gel. For western blot analysis, proteins were electrotransferred to nitrocellulose membrane using a mini trans-blot electrophoretic transfer system (Bio-Rad). The membrane was blocked with 5% milk or BSA in 1X TBS-T for 1-3 h at room temperature. The blots were washed thrice with 1X TBS-T for 10 min each and incubated with separate primary antibodies at appropriate dilution in 0.5% milk or BSA in 1X TBS-T. The blots were incubated overnight in primary antibodies at 4°C and washed thrice with 1X TBS-T. Blots were then incubated with HRP-conjugated anti-rabbit/ mouse/goat IgG antibody for 1 h at room temperature and later washed thoroughly with 1X TBS-T. The membrane was exposed to luminol detection reagents (solutions A and B in 1:1 mixture) for appropriate time periods and specific protein band was detected by autoradiography. For reprobing, the membranes were incubated with stripping buffer at 50°C for 30 min and thoroughly washed. The membrane was then blocked and immunodetected with separate primary and secondary antibodies as described above.

5.12 Preparation of nuclear and cytoplasmic extracts

Reagents and solutions

Hypotonic buffer (pH 7.9): 50 ml

1 M HEPES, (pH 7.9) 0.5 ml
3 M MgCl₂ 25 μl
2.5 M KCl 0.2 ml
0.1 M PMSF 0.1 ml
1 M DTT 25 μl

Nuclear extraction buffer (pH 7.9): 50 ml

1 M HEPES, (pH 7.9) 1.0 ml
3 M MgCl₂ 25.0 μl
3 M NaCl 6.7 ml
0.5 M EDTA 20.0 μl
Glycerol 1.25 ml
0.1 M PMSF 0.25 ml
1 M DTT 25.0 μl

Methodology

Equal number of MCF-7 or MDA-MB-231 cells transfected with either siRNA or plasmid DNA were subjected to different treatment conditions for indicated time periods. Cells were washed and harvested in ice-cold PBS and then resuspended in appropriate amount of hypotonic buffer and allowed to swell on ice for 10 min and later homogenized. The samples were centrifuged at 3,300 g for 15 min at 4°C to collect the supernatant as cytoplasmic extract. The nuclear pellets were resuspended in ice-cold nuclear extraction buffer and incubated on ice for 30 min and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as nuclear extract. The protein concentration was measured using Bio-Rad protein assay. Equal amount of nuclear extracts were used for EMSA.

5.13 Electrophoretic mobility shift assay (EMSA)

5.13.1 Labeling of oligonucleotide

Reagents and buffers

TE Buffer
10 mM Tris-HCl (pH 8.0)
1 mM EDTA

The double stranded FOXO 3a oligonucleotides were labeled using T4 polynucleotide kinase. The labeling reaction was performed as follows.

Labeling reaction

Consensus oligonucleotide (1.75 pmol/μl) 2 μl
T4 polynucleotide kinase (10 X buffer) 2 μl
$[\gamma-^{32}P]$ ATP (1000 μCi/100 μl) 3 μl
Nuclease free water: 11 μl
T4 polynucleotide kinase (5-10 μg/μl) 2 μl

This reaction mixture was incubated at 37°C for 1 h. The reaction was stopped by heating at 68°C for 10 min. The reaction mixture was diluted to 50 μl using nuclease free water prior to use.
Components of 8% native gel (10 ml)

- 40% Acrylamide: 2 ml
- 2% Bis-acrylamide: 0.32 ml
- 10X TGE: 0.5 ml
- 50% Glycerol: 1 ml
- Deionized water: 6.18 ml
- 10% APS: 100 μl
- TEMED: 10 μl

The non-denaturing 8% polyacrylamide gel was prepared and allowed to polymerize for 15 min. Pre-electrophoresis was carried out for 1 h at 80V at 4°C. Meanwhile, 5-8 μg nuclear extracts treated or transfected under various conditions were incubated with labeled oligonucleotides under the following conditions:

### 5.13.2 Protein-DNA binding reaction (20 μl)

- Nuclear extract: 5 μg
- Binding buffer: 2 μl
- Salmon sperm DNA (2.5 μg/μl): 1 μl
- BSA (1mg/ml): 1 μl
- Labelled probes: 2 μl

The total volume was made up to 20 μl with nuclease free water. The binding reaction was incubated at room temperature for 30 min and the DNA-protein complexes were resolved on 8% native gel at 25 mA for 30 min. The gel was dried and autoradiographed.

### 5.14 Reverse transcription-PCR (RT-PCR)

MDA-MB-231 cells were treated with Sema 3A for 0-24 h at 37°C in 6 well plates and RNA was extracted using Trizol reagent (GIBCO BRL, Grand Island, NY). cDNA synthesis was performed using M-MuLV Reverse Transcriptase (Bangalore Genei) with 5 μg RNA at 37°C for 1 h as follows:

**cDNA synthesis (25 μl)**

- RNA: 4-8 μg
- Oligo dT (100 pmol/μl): 1 μl
- 5X Reverse transcriptase (RT) buffer: 5 μl
- 10 mM dNTP Mix: 1 μl
Reverse transcriptase (RT) enzyme (5 U/μl) 2 μl
Water made upto 25 μl
The reaction mixture was incubated at 37°C for 1 h. The samples were stored at -20°C prior to use.

**PCR reaction (25 μl)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Taq buffer A</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 μl</td>
</tr>
<tr>
<td>Forward primer (10 pmol/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse primer (10 pmol/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl)</td>
<td>0.3 μl</td>
</tr>
<tr>
<td>Template DNA (cDNA)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Water made upto</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

**PCR reaction cycle**

- 95°C for 10 min
- 95°C for 30 sec
- 55°C for 1 min \( \times \) 35 cycles
- 72°C for 1 min
- 72°C for 10 min
- Hold at 4°C

Following primer sequences were used:

- MelCAM sense 5′AAG GAG AGG AAG GTG TGG GTG AAA 3′
- MelCAM anti-sense 5′ TCC AGG AAG AGG ATG CTG GTG TTT 3′
- β-actin sense 5′ GGC ATC CTC ACC CTG AAG TC 3′
- β-actin anti-sense 5′ GGG GTG TTG AAG GTC TCA AA 3′

The amplified products were resolved in 1.8% agarose gel.

**5.15 Chromatin immunoprecipitation (ChIP) assay**

**Reagents and buffers**

**SDS lysis buffer (10 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% SDS</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>200 μl</td>
</tr>
<tr>
<td>1M Tris (pH-8.0)</td>
<td>500 μl</td>
</tr>
</tbody>
</table>
### Dilution buffer (10 ml)
- 10% SDS: 10 µl
- 10% Triton X-100: 1.1 ml
- 0.5 M EDTA: 24 µl
- 1M Tris (pH 8.0): 167 µl
- 5M NaCl: 334 µl

### Low salt buffer (10 ml)
- 10% SDS: 100 µl
- 10% Triton X-100: 1 ml
- 0.5 M EDTA: 40 µl
- 1M Tris (pH 8.0): 200 µl
- 5M NaCl: 300 µl

### High salt buffer (10 ml)
- 10% SDS: 100 µl
- 10% Triton X-100: 1 ml
- 0.5M EDTA: 40 µl
- 1M Tris (pH 8.0): 200 µl
- 5M NaCl: 1 ml

### LiCl wash buffer (10 ml)
- LiCl: 0.106 g
- 10% NP-40: 1 ml
- Sodium deoxycholate: 0.1 g
- 0.5 M EDTA: 20 µl
- 1M Tris (pH 8.0): 100 µl

### Elution buffer (3 ml)
- 10% SDS: 60 µl
- 1M NaHCO₃: 0.3 ml
- Water: 2.64 ml

### Methodology
The chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer’s instructions using ChIP assay kit (Millipore, USA). Briefly, MDA-MB-231 cells were cross-linked in 1% formaldehyde solution for 10 min at room temperature. Cells were lysed in 300 µl of SDS lysis buffer and sonicated to
generate 200-1000 bp DNA fragments. The size of generated fragments was checked by analyzing them on 0.8% agarose gel. The cross linked chromatin in sonicated fractions was immunoprecipitated with anti-FOXO 3a antibody. DNA fragments were analyzed by PCR using specific primers which includes FOXO-3a binding site of MelCAM promoter (sense 5'TTC TGT GCT TGT TTG GTG G 3', anti-sense 5' CAA CCC CCT TCC TTC ACT T 3').

**PCR reaction (20 μl)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase free water</td>
<td>12.7</td>
</tr>
<tr>
<td>10X buffer (for enzyme)</td>
<td>2</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1</td>
</tr>
<tr>
<td>Forward Primer (10 pmol/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse Primer (10 pmol/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (5U/μl)</td>
<td>0.3</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1</td>
</tr>
</tbody>
</table>

**PCR reaction cycles**

- 95°C for 1 min
- 95°C for 30 sec
- 56°C for 30 sec
- 72°C for 1 min
- 72°C for 10 min

X 40 cycles

Hold at 4°C

PCR products were analyzed by electrophoresis using 2% agarose gel.

### 5.16 Immunofluorescence assay

**Reagents and Buffer**

- 1 X cold PBS (pH 7.4)
- 2% paraformaldehyde in 100 mM HEPES (pH 7.9)
- 0.1% glycine in 1X PBS
- 0.1% Triton X-100 in 1X PBS
- 2% BSA

**Methodology**

Equal number of cells were seeded onto sterile cover slips. After appropriate treatments, cells were fixed in ice-cold 2% paraformaldehyde for 15 min. Cells
were permeabilized using 0.1% Triton-X-100. The cells were then washed three times for 5 min each with PBS and incubated in 2% BSA for 1 h at room temperature to block the non-specific binding. Further, cells were incubated using specific primary antibody (1:30 dilution) overnight at 4°C followed by secondary antibody incubation (1:50 dilution) for 30 min at room temperature. The cover slips were mounted onto slides and visualized under confocal microscope (Zeiss).

5.17 Cell proliferation (MTT) assay
MDA-MB-231 or MDA-MB-231-Sema 3A stable cells (8x10^3) were seeded in 96 well plates and the growth kinetics of cells was determined for a period of 8 days. In separate experiments, MDA-MB-231 cells (2x10^4) were treated with Sema 3A (25-100 ng/ml) or SFN (5 µM or 10 µM) alone or pre-treated using various inhibitors (LY294002, PD98059 or SB203580) followed by combined treatment of Sema 3A (100 ng/ml) and SFN (10 µM) and subjected to cell viability assay. At the end of 24 h, media was removed and 0.5 mg/ml MTT solution was added. After 4 h incubation, formazane crystals formed were dissolved in isopropanol and absorbance was recorded at 570 nm.

5.18 Matrigel colony formation assay
The matrigel colony formation assay was carried out to assess the anti-tumor activity of both Sema 3A and SFN. Briefly, 50 µl of growth factor depleted matrigel was coated on 96 well plates. MDA-MB-231 cells (1x10^3) were plated on matrigel coated plates and incubated at 37°C for 24 h. Cells were either treated with Sema 3A or SFN or in combination for 24 h. Existing media was replaced with fresh media containing 10% FBS on every alternate day. After 7 days of incubation, colonies were visualized under microscope (Nikon) and photographed.

5.19 DNA fragmentation assay
The cells were treated with Sema 3A with or without SFN for 24 h and lysed on ice in Lysis buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM EDTA and 1% NP-40 for 10 sec. Lysates were vortexed and cleared by centrifugation at 3000 rpm for 5 min. The supernatant was treated with 10% SDS and 50 mg/ml RNase at 37°C for 2 h followed by proteinase K treatment at 56°C for 2 h. The samples were
subjected to ethanol precipitation and the precipitate thus formed was resuspended in 50 µl TE and analyzed electrophoretically on 2% agarose gel containing ethidium bromide.

5.20 Cell cycle analysis
MDA-MB-231 cells were serum-starved for 12 h and subsequently treated in absence or presence of Sema 3A and SFN for 24 h. In separate experiments, cells were pretreated using various inhibitors (LY294002, PD98059 or SB203580) prior to treatment with Sema 3A in presence of SFN. Cells were washed twice with cold PBS and then centrifuged. The pellets were fixed in 75% (v/v) ethanol for 1 h at 4°C. The cells were washed once with PBS and resuspended in cold PI solution (50 µg/ml) containing RNase A (0.2 mg/ml) in PBS (pH 7.4) for 30 min in dark. Flow cytometry analyses were performed using FACS Calibur (Becton Dickinson, San Jose, CA). The sub-G1 population was calculated to estimate the apoptotic cell population.

5.21 Transwell migration assay
MDA-MB-231 cells (5x10^4) transiently transfected with wt PTEN or dn PTEN or siRNA specific to PTEN were added onto the upper chambers of Transwells in serum free media. In other experiments, cells were transiently transfected with wt FOXO 3a, FOXO 3a TM (triple mutant) or using siRNA specific to FOXO 3a were added onto the upper chambers of Transwells. Cells were then subjected to Sema 3A treatment and untreated cells were used as control. In separate experiments, cells were treated with Sema 3A alone or pre-treated with NRP-1 neuralizing antibody (5 µg/ml) or blocking peptide (20 µg/ml) or transfected with NRP-1 siRNA, then treated with Sema 3A and used in Boyden chamber migration assay. Sema 3A overexpressing stable cells were also used in the Transwell migration assay. At the lower chambers, 5% FBS was used as chemo attractant. After 16 h, the migrated cells to the lower surface of Transwells were fixed, stained with Giemsa and counted in 3 high-power fields (C/HPF) under an inverted microscope (Nikon). In other experiments, cells (1x10^5) were incubated with SFN (0-10 µM) or Sema 3A (100 ng/ml) or both for 8 h and added to upper chambers of Transwells. The migrated cells were photographed and counted. The
fold change in migration was quantified, analyzed statistically and represented as bar graph.

### 5.22 Wound migration assay

MDA-MB-231 cells (5x10^5) were seeded and transfected with either siRNA (PTENi, NRP-1i or FOXO 3ai) or different cDNA constructs (wt PTEN, dn PTEN, wt FOXO 3a or FOXO 3a TM). After 48 h, wounds of constant diameter were made on confluent monolayers and subjected to Sema 3A treatment. In separate experiments, cells were pre-treated using either NRP-1 neutralizing antibody or blocking peptide and then treated with Sema 3A and subjected to wound migration assay for 16 h. The wound assay was also performed using Sema 3A overexpressing MDA-MB-231 cells or MCF-7 cells treated with Sema 3A blocking peptide (20 µg/ml). In another set of experiments, serum starved MDA-MB-231 cells were treated with Sema 3A (100 ng/ml) or SFN (0-10 μM) or both in combination for 12 h. Wound photographs were captured using phase contrast microscope (Nikon). Wound closure was assessed by calculating the area covered by cells in the prescribed time periods and analyzed using Image-Pro plus 6.0 software.

### 5.23 Tumor-endothelial cell comigration/coinvasion assay

Tumor-endothelial interaction was performed in Boyden chambers or matrigel coated invasion chambers. NRP-1 expression in HUVECs was either silenced using siRNA specific to NRP-1 or NRP-1 neutralizing antibody (5 µg/ml) or blocking peptides (20 µg/ml) which were added to the upper chambers of Transwells. CM collected from MDA-MB-231 cells treated with Sema 3A was added in the lower chambers. After 16 h, invaded cells were stained with Giemsa and photographed at three high-power fields under an inverted microscope (Nikon) at magnification of 10 X. Cells were counted from three different images; mean was calculated, analyzed statistically and represented graphically.

### 5.24 In vitro tube formation assay

Matrigel (growth factor reduced) was thawed at 4°C and each wells of a prechilled 96-well plate was coated with 50 µl matrigel and incubated at 37°C for 30 min and
HUVECs (1x10⁴ cells/well) were added. Cells were treated either with Sema 3A (100 ng/ml) or NRP-1 blocking antibody (5 µg/ml) or with the conditioned media collected from MDA-MB-231 cells transfected with Sema 3A cDNA followed by VEGF (50 ng/ml) treatment. After 12 h, photographs were captured using inverted microscope (Nikon), analyzed statistically using Image Pro plus 6.0 software and the mean tube length was measured and presented as mean tube length ± SE.

5.25 Chorioallantoic membrane (CAM) assay
The CAM assay was performed using white leghorn fertilized chicken eggs. Gelatin sponge inserts were loaded with media alone or with Sema 3A (100 ng/ml) or VEGF (50 ng/ml) or both and placed on the CAM of 4 day old fertilized white leg horn eggs. On day 6, photographs were taken using stereo microscope attached with camera and the extent of vessel growth was quantified using Angioquant software.

5.26 In vivo tumorigenicity experiments
In vivo tumorigenicity experiments were performed with the approval of Institutional Animal Care and Use committee (IACUC) of National Centre for Cell Science (NCCS). Briefly, MDA-MB-231 cells (1x10⁶) either non-transfected or stably transfected with Sema 3A were injected along with matrigel (1:1) orthotopically into female NOD/SCID mice (4-6 weeks of age) and inhouse in pathogen-free condition in the Experimental Animal Facility (EAF) of NCCS for 9 weeks. In separate experiments, MCF-7-empty vector or MCF-shSema 3A stable cells (2x10⁶) were used in in vivo tumorigenicity experiments. In other experiments, MCF-7 cells (2x10⁶) were injected into female NOD-SCID mice. Sema 3A blocking peptide (1 mg/Kg body weight) was injected twice a week into site of tumor upto 6 weeks. In other experiments, MDA-MB-231-Luc cells (1x10⁶) were injected into right mammary fat pad of 4-6 week old female NOD-SCID mice. After 7 days, Sema 3A (10 µg/kg body weight) or SFN (3.5 mg/kg body weight) alone and in combination were injected into the tumor sites twice a week till 4 weeks. The tumor volume was calculated using the formula, V= (Π/6 (d1x d2)³/²). After 4 weeks, mice were sacrificed by cervical dislocation, tumors were
excised and weighed. Tumor specimens were used for immunohistochemical and western blot analysis.

5.27 Analysis of human breast cancer clinical specimens
Human breast cancer specimens were collected as formalin fixed paraffin embedded (FFPE) blocks from Ruby Hall Clinic (Grant Medical Foundation, Pune, Maharashtra) with informed consent. The samples were analyzed by immunohistochemistry.

5.28 Immunohistochemistry

Reagents and Buffers
- Xylene
- Ethanol
- Isopropanol
- 1X PBS
- Triton X-100
- BSA
- Citrate buffer (1 M)
  - 14.7 g of Tri sodium citrate was dissolved in 300 ml deionized water and pH was adjusted to 6.0. The volume was made upto 500 ml with deionized water.
  - Citric acid - 10.51 g of citric acid was dissolved in 300 ml deionized water and pH was adjusted to 6.0. The volume was made upto 500 ml with deionized water.
  - Citrate buffer (0.01 M) was prepared by mixing 80 ml Tri sodium citrate and 20 ml Citric acid and volume made upto 1000 ml with deionized water.

Methodology
The tumor sections isolated from mice were deparaffinized with xylene, followed by rehydration of tissues using ethanol gradation. Antigen retrieval was done using Sodium citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) at 95°C for 20 min. The tissues were permeabilized in 0.01% Triton X-100 and blocked with 2% BSA for 1 h. The tumor sections were analyzed by immunofluorescence using specific antibodies. The human breast tumor tissues were sectioned onto poly-L-lysine coated slides, deparaffinized and dehydrated by heating in microwave for 5 min. The slides were then immersed in xylene for 5 min thrice. The step was repeated
three times with 100% isopropanol and finally rinsed with deionized water. Antigen retrieval was done by boiling in Tris-EDTA buffer (pH 9.0) at 95°C for 20 min and allowed to cool at room temperature. DAB staining was performed using IHC detection kit (Biogenex Life sciences) and the slides were processed according to manufacturer’s instructions. The intensity of tissue staining was scored by oncopathologist and an index of 0-3 was used. Correlation studies were performed using Mann Whitney test and Spearman rank correlation coefficient was also determined. All statistical analyses were two tailed and statistical significance for observed difference was set at p < 0.05.

5.29 Statistical analysis
Statistical analysis was performed by Student’s ‘t’ test using Sigma plot software. The statistical significance for observed difference was set at p<0.05. Wound migration analysis was performed using Image Pro Plus 6.0 software (Nikon). Clinical data analysis was performed using SPSS (Windows, version 21.0, Chicago Illinois).