Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Antibodies

The following antibodies were used in the present study: Primary antibodies against GAPDH (anti-rabbit), FLAG (anti-mouse), Immunoglobulin (IgG, anti-rabbit or anti-mouse), profilin-1 (anti-rabbit), tubulin (anti-mouse) and ubiquitin (anti-rabbit) were obtained from **Sigma Aldrich Chemicals** (St Louis, MO, USA). Antibodies against AKT (anti-rabbit), cleaved caspases-3, 8 and 9 (anti-rabbit), HA-tag (anti-rabbit), Myc-tag (anti-rabbit), p21 (anti-rabbit), phospho-p53 (anti-mouse), PTEN (anti-mouse), phospho-AKT (Ser473; anti-rabbit), phospho-GSK-3β (Ser9; anti-rabbit), phospho-IKKα/β (Ser177/181; anti-rabbit), phospho-IκBα (Ser32; anti-rabbit), and phospho-p65 (Ser276; anti-rabbit) were obtained from **Cell Signaling Technologies** (Danvers, MA, USA), whereas antibodies for cox-2 (anti rabbit), c-Rel (anti-rabbit), ICAM-1 (anti-rabbit), IKKα/β (anti rabbit), IκBα (anti-rabbit), Mdm2 (anti-rabbit), PARP-1/2 (anti-rabbit), Rel-B (anti-rabbit), p50 (anti-rabbit), p53 (anti-mouse), p65 (anti-rabbit) were obtained from **Santa Cruz Biotechnology** (Santa Cruz, CA, USA). HRP (Horse radish peroxidase) -conjugated secondary antibodies (anti mouse and anti-rabbit) were obtained from **Bangalore Genie** (Peenya, India). For immuno-fluorescence studies, secondary antibodies conjugated to Alexa Fluor (488 and 594, anti-mouse and anti-rabbit) were obtained from **Molecular Probes, Invitrogen** (Eugene, OR, USA).

2.1.2 Cell culture and Media

The cell lines used in the present study, HuT-78 (human T-cell lymphoma), MDA-MB-231 (human breast cancer) and MDA-MB-468 (human breast cancer) were obtained from American Type culture collection (Manassas, VA, USA). Human colon carcinoma cell lines HCT-116 (wild-type, $p53^{+/+}$) and HCT-116 (null, $p53^{-/-}$) were a kind gift from Prof. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Cells were cultured in DMEM or RPMI medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained in humidified incubator at 37ºC in 5% CO_2-95% air.

Media for mammalian cell culture (DMEM and RPMI), fetal bovine serum (FBS) and other reagents used in cell culture such as, PBS, Trypsin-EDTA, Antibiotic-antimycotic, Freezing medium, Geniticin, L-Glutamine, HEPES, etc. were

obtained from **Gibco, Invitrogen** (Carlsbad, CA, USA). For cell culture transfections, Lipofectamine-2000 and Opti-MEM were also obtained from **Life Sciences, Invitrogen** (Carlsbad, CA, USA).

Commonly used chemicals in cell culture based experiments such as all-trans retinoic acid (ATRA), arabinoside cytosine (AraC), carbobenzoxy-Leu-Leu-Leucinal (MG-132), cycloheximide (CHX), DMSO, doxorubicin, hydrogen peroxide (H_2O_2), lipopolysaccharide (LPS, *Escherichia coli* 055:B5), okadaic acid (OA), oleandrin, paclitaxel, phorbolmyristate acetate (PMA), vinblastine and vincristine were obtained from **Sigma Aldrich Chemicals**. Benzofuran was synthesized as reported earlier (Manna *et al*., 2010). Recombinant human TNFα, IL-1 and IL-8 were obtained from **PeproTech Inc.** (Rocky Hill, NJ, USA).

Growth media for bacteria culture, Luria Broth (LB) and Agar were obtained from **HiMedia laboratories** (Mumbai, India). Bacterial strain DH5α was used to make ultra-competent cells for transformation and plasmid isolation. Antibiotics, such as Ampicillin and Kanamycin used for selection of transformed colonies and culture were obtained from **Sigma Aldrich Chemicals**.

2.1.3 Plasmids

Wild type or H133S mutant of profilin-1 with either FLAG or un-tagged were cloned in pcDNA3.1 (+). Mdm2 gene upstream promoter region having p53 binding site was cloned in pLUC vector (designated as p53-Luc). The constructs of NF-κB-SEAP, p65 (RelA), wild type and dominant negative IKKβ (IKKβ-WT and IKKβ-DN, respectively) were a kind gift from Prof. Bharat B. Aggarwal (M. D. Anderson Cancer Center, Houston, TX). The constitutive active mutant of IKKβ, in which two serine residues are mutated to glutamic acid, at position 177 and 181 (referred as IKKβ-EE or IKKβ-CA) was gifted by Prof. Gourisankar Ghosh (University of California, San Diego, USA). FLAG or Myc tagged Full length and truncation mutants of PTEN were provided by Dr. M. Subba Reddy (CDFD, Hyderabad). For p53 gene knockdown studies, TP53 mission shRNA were obtained from Sigma Aldrich (St Louis, MO, USA). For PTEN silencing, retroviral vector based PTEN shRNA (shRNA#1-AGGCGCTATGTGTATTATTAT; shRNA#2-CCACAGCTAG-AACTTATCAAA; shRNA#3-CCACAAATGAAGGGATATAAA) was gifted by Dr. M. Subba Reddy (CDFD, Hyderabad).

2.1.4 Reagents and Buffers

For DNA isolation and purification, various kits such as Mini and midi-prep plasmid isolation, Gel extraction, PCR purification, etc., were procured from **Qiagen** (Hilden, Germany) or **HiMedia** (India). For RNA extraction, TRIzol was obtained from **Gibco BRL** (Grand Island, NY). cDNA was made from RNA by either Reverse transcriptase (**SuperScript III, Invitrogen**) or One step Access RT-PCR kit (**Promega**, Madison, WI). Reagents for PCR such as PCR 10X buffer, dNTPs, $MgCl_2$, Taq polymerase or AccuTaq were obtained from **Fermentas** or **Sigma Aldrich**. Recombination enzymes such as Restriction Endonucleases and DNA ligase used for recombinant DNA experiments (Bam-H1, Hind-III, Xho-I, Eco-RI, Not-I, and Sal-I) were obtained from **New England Biolabs** (Ipswich, MA, USA). Oligonucleotides used for various Gel shift assays *viz*. AP-1, NF-κB, p53 and Sp-1 were commercially synthesized from **XCelris** (Ahmedabad, India).

For protein extraction, protease inhibitors such as aprotinin, leupeptin, PMSF, NaF, $NaVO_4$, etc. were obtained from **Sigma Aldrich**. Bradford reagent for estimation of protein concentration was obtained from **Bio-Rad** (Rockford Illinois, USA). For Immunoblotting, PVDF membrane, X-ray films and chemi-luminiscent detection reagent (ECL prime) were obtained from **GE Healthcare** (Little Chalfont, UK). For Immunofluorescence, vectashield-mounting medium with DAPI and Propidium Iodide (PI) were obtained from **Molecular Probes**, Invitrogen. For detection of cytotoxicity, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye, SDS and DMF (Dimethylformamide) were obtained from **Sigma Aldrich**. Live and dead cell assay kit was obtained from **Molecular Probes.**

Various chemicals required for preparation of regular buffers and solutions *viz*. Tris, Glycine, SDS, Sodium Chloride, Potassium Chloride, HEPES, Disodium Phosphate, Nonidet P-40, Tween 20, TritonX100, Formaldehyde, Glycerol, Agarose, Acrylamide, Bis-acrylamide, APS, TEMED, BSA, etc. were obtained from **Sigma Aldrich**.

The procedure of preparation of buffers and reagents used in the present studied are described below:

Table 2.1: Commonly used buffers and solutions

I. General buffers

(a) Phosphate Buffered Saline (PBS)

Components	Final conc.	For 10X stock (1L)
NaCl	137 mM	80g
KCl	2.7 mM	2g
Na_2HPO_4	10mM	1.44g
KH_2PO_4	2.0 mM	0.24 g
pH adjusted to 7.4 with HCl		

(b) Tris Buffered Saline (TBS)

Components	Final conc.	For 10X stock (1L)
Tris-Cl	50 mM	60.5 g
Sodium Chloride (NaCl)	150 mM	87.6 g
pH adjusted to 7.4 with HCl		

II. For Immunoprecipitation (IP) and Immunoblotting (IB)

(a) Cell lysis buffer A (For IP)

Components	Final conc.	For 100 ml
Tris (pH 7.4)	50 mM	2 ml
NaCl	150 mM	2 ml
NP-40	1%	5 ml
EDTA	0.5 M	200 µl

(b) Cell lysis buffer B (For IB)

Components	Final conc.	For 100 ml
HEPES (pH 7.9)	1M	2 ml
NaCl	1M	2 ml
NP-40	10%	5 ml
EDTA	0.5 M	200 µl

(c) 6X Protein loading buffer (Lammeli buffer)

Components	Final conc.	For 100 ml
1.5 M Tris-Cl pH 6.8	60 mM	4 ml
SDS	2%	2 g
Glycerol	10%	10 ml
β-mercaptoethanol	5%	5 ml
Bromophenol Blue	0.01%	0.01 g

(d) Resolving polyacrylamide gel

Components	For 8% gel (ml)	For 10% gel (ml)	For 12% gel (ml)
Water	4.6	4	3
30% Acrylamide	2.7	3.3	4
1.5 M Tris (pH 8.8)	2.5	2.5	2.5
20% SDS	0.1	0.1	0.1
10% APS	0.1	0.1	0.1
TEMED	0.006	0.004	0.004

(e) Stacking polyacrylamide gel

Components	For 10 ml
Water	6.8 ml
30% Acrylamide	1.7 ml
1M Tris (pH 6.8)	1.25 ml
20% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

(f) Running buffer

Components	Final conc.	For 1X buffer (1L)
Tris base	25 mM	14.4 g
Glycine	192 mM	3.03 g
SDS	0.1% (w/v)	1 g

(g) Transfer buffer

Components	Final conc.	For 1000 ml
Tris base	25 Mm	14.4 g
Glycine	0.2 M	3.03 g
Methanol	20% (v/v)	200 ml

(h) TBS-T

Components	Final conc.	For 1000 ml
TBS	q.s.	1000 ml
Tween 20	0.1%	1 ml

(i) Blocking buffer

Components	Final conc.	For 100 ml
Fat free milk	5%	5 g
TBST	q.s.	100 ml

(j) Stripping buffer

Components	Final conc.	For 1000 ml
1.0 M Tris-Cl (pH 6.7)	62.5 mM	62.5 ml
β-mercaptoethanol	100 mM	5.0 ml
SDS	2%	20 g

III. For Immunofluorescence (IF)

(a) 4% Formaldehyde fixative

Components	Final conc.
Formaldehyde	4%
PBS	q.s.

(b) Permeabilisation buffer: 0.2% Triton X100

Components	Final conc.	For 10 ml
Triton X100	0.2%	0.02 ml
PBS	q.s.	10 ml

(c) Blocking buffer: 2% BSA

Components	Final conc.	For 10 ml
BSA	2%	0.2 g
PBS	q.s.	10 ml

IV. For Cell fractionation

(a) Cytoplasmic extraction buffer (without protease inhibitors)

Components	Final conc.	For 100 ml
1 M HEPES	10mM	1 ml
2M KCl	10 mM	0.5 ml
0.5 M EDTA	0.1 mM	0.02 ml
0.1 M EGTA	0.1 mM	0.1 ml

(b) Nuclear lysis buffer (without protease inhibitors)

Components	Final conc.	For 100 ml
1 M HEPES	10 mM	1 ml
5 M NaCl	0.2 M	4 ml
0.5 M EDTA	0.5 mM	0.1 ml
0.1 M EGTA	0.5 mM	0.5 ml

V. For DNA electrophoresis

(a) TAE

Components	Final conc.	For 50X (IL)
Tris-Base	40 mM	242 g
Acetic acid	20 mM	57.1 ml
0.5 M EDTA	1 mM	100 ml

(b) Agarose gel

Components	0.8% gel	1% gel	2% gel
Agarose	0.8 g	1 g	2g
TAE	100 ml	100 ml	100 ml

(c) 6X DNA loading dye

Components	Final conc.	For 100 ml
Glycerol	30%	30 ml
BromophenolBlue	0.25%	0.25 g
Xylene Cyanol	0.25%	0.25 g

VI. For preparation of Ultra competent cells

(a) Inoue buffer

Components	Final conc.	For 100 ml
0.5 M PIPES pH 6.7	10 mM	2 ml
$CaCl_2.2H_2O$	15 mM	0.22 ml
KCl	250 mM	1.865 g
$MnCl_2.4H_2O$	55 mM	1.088 g
pH adjusted to 6.7 with 1M Potassium Hydroxide (KOH) Filter sterilization		

VII. For Electrophoretic Mobility Shift Assay (EMSA)

(a) Native EMSA PAGE

Components	For 6.6% gel (50 ml)
Water	28.66 ml
30% Acrylamide	11 ml
5X EMSA buffer (pH 8.5)	10 ml
10% APS	400 µl
TEMED	40 µl

(b) 10X Binding buffer

Components	Final conc.	For 10 ml
HEPES (pH 7.9)	200mM	2 ml
EDTA (pH 8.0)	4mM	80 µl
DTT	40mM	40 µl
Glycerol	50%	5 ml
Water	--	2.88 ml

(c) 5X EMSA buffer

Components	Final conc.	For 5 l
Tris	0.25 M	151.4 g
Glycine	2.0 M	750.0 g
EDTA (pH 8.5)	0.01 M	100 ml
pH adjusted to 8.5		

(d) 6X EMSA sample loading dye

Components	For 10 ml
Xylene Cyanol FF	25 mg
Bromophenol Blue	25 mg
Milli-Q water	7.0 ml
Glycerol	3.0 ml

VIII. For Cytotoxicity assays

(a) MTT reagent

Components	Final conc.	For 10 ml
MTT	5 mg/ml	50 mg
PBS	--	10 ml

(b) MTT Extraction buffer

Components	Final conc.	For 250 ml
SDS	20%	50 g
DMF	50%	125 ml
Water	-	125 ml

2.2 METHODS

2.2.1 Maintenance of cell lines

In present thesis, various cell lines have been used as mentioned earlier. Cells were either cultured in DMEM or RPMI medium containing 10% fetal bovine serum (FBS) along with antibiotics such as penicillin (100 U/ml), and streptomycin (100 µg/ml). In general, cells were grown in tissue culture T-75 flask upto 85-90% confluency. Cells are washed with PBS, followed by trypsinization with 0.05% Trypsin EDTA solution. Cells were detached from the surface either by gentle tapping or gentle pipetting or incubated for 5 minutes at 37°C. Culture medium containing serum was then added to inactivate trypsin. After careful mixing, cells were transferred to a 15 ml tube and centrifuged at 800 rpm for 5 minutes. The cell pellet was re-suspended in a fresh culture media containing FBS. The cell viability was checked by trypan blue staining, followed by counting in Neubauer cell-counting chamber. Appropriate number of cells was then either sub-cultured in the ratio of 1:4 to 1:6 or seeded in culture dishes as per the experimental requirements. Cells were maintained in humidified incubator at 37ºC in 5% CO_2-95% air, throughout the experiment.

2.2.2 Transient transfection in adherent cells

Transfection of plasmid DNA in cells was performed using Lipofectamine 2000 reagent as per manufacturer's protocol provided with the reagent. Briefly, 0.5 to 1 million cells were seeded in a 60 mm or 100 mm tissue culture dish. After 12 h of seeding, transfections were performed. 6-12 µg DNA was mixed in 500-1500 µl of Opti-MEM in one polypropylene tube and simultaneously, 15-30 µl of Lipofectamine 2000 was mixed in similar volumes of Opti-MEM in another tube and incubated at room temperature for 10 minutes. Opti-MEM containing DNA and Lipofectamine 2000 were then mixed and incubated for 30 minutes at room temperature for the formation of DNA-lipid complex. Meanwhile, the cells were washed with sterile PBS and 4-10 ml of Opti-MEM was added in the plate. DNA-lipid complexes were then added to each dish for 6h. After that, the medium containing complexes was removed and complete medium (DMEM containing FBS) was added. Expression of transgene was evaluated 24-48 h after transfection either by immunoblotting or immunofluorescence or by RT-PCR followed by PCR.

2.2.3 Generation of profilin stable MDA-MB-231 cells

Briefly, cells were cultured on 35 mm culture dishes (Corning) and were transfected with pcDNA3.1 (+) empty vector or pcDNA3.1 (+) profilin-1 clone (encodes for neomycin resistance for selection in mammalian cells) using Lipofectamine 2000 reagent. The following day, cells were trypsinized and plated in four 10 cm culture dishes separately for each cell clones in selective media containing 800μg/ml of Geneticin, G418 (whose concentration was obtained from kill curve based on MTT assay). Over the time, it will select only those cells that have stably incorporated the plasmid into their genomic DNA. Cells were selected in G418 for about two weeks until colonies appeared. Meanwhile, cells were regularly washed with sterile PBS to remove dead cells from culture dishes. Colonies were picked using a pipette with a sterile tip by lowering it to the surface of the colony of interest, followed by gentle scraping and rapid sucking. About 20 colonies were picked, followed by dilution plating in 96-well plates until single cell per well was obtained to establish a pure colony. Once colonies have reached to fair confluency, they were further expanded by dilution plating and screened for stable expression of profilin by immunoblotting and semi-quantitative RT-PCR. Once the clonal cell line is established, aliquots were freezed for future use and rest were maintained in lower concentration of G418 with proper track of passage number.

2.2.4 Wound healing assay

Wound healing assay (Liang *et al*., 2007) was employed to study the difference in migration rates of profilin-stable compared to parental cells. Briefly, cells were cultured upto 90-95% confluent monolayer and a scratch was created through the cell monolayer with sterile needle. Cell debris was then removed by washing with PBS before adding the media. Images of the open gap created by this "wound" were then captured at three random locations immediately (0 h) and then at the same locations after regular interval using phase contrast microscopy until they are closed by migrating cells. Captured images were then used to quantify wound closure by the percentage change in the wound area per unit time and averaged for three locations for each experimental condition. During the course of the experiment, cells were maintained in 0.1% FBS containing DMEM media to ensure that wound closure was due to the migration of cells rather than divison of cells.

2.2.5 Cell proliferation assay

The method described earlier by Gillies and co-workers was slightly modified and followed (Gillies *et al*., 1986). Briefly, parental and profilin-stable cells were seeded in triplicates at a density of 20,000 cells per well of a 24-well culture plates. Each day after seeding, cells were washed with PBS and stained with 0.2% crystal violet in 2% ethanol for 15 minutes. Vigorous washing was done with PBS to remove excess dye. Crystal violet dye was then eluted using 1% SDS solution with extensive pipetting and diluted 10 fold. Absorbance of the extracted dye was then determined at 570 nm in a spectrophotometer. Absorbance data based on triplicate set of samples for each experimental condition were then averaged for each time point to generate a growth curve.

2.2.6 Preparation of whole cell, cytoplasmic and nuclear lysates

In order to extract the total cell homogenate, the culture media was removed and cells were washed with ice cold PBS. The cells were then gently scrapped and pelleted by centrifugation at 3000 rpm for 3 minutes at 4°C. The cell extraction buffer was added to the cell pellet and placed on rotor kept in cold room for 30 minutes for cell lysis. Lysed cells were then centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was collected as cellular lysate. Protein concentration was estimated using Bradford assay, described below. For a typical Western blot assay, 30-70µg of protein was loaded on the SDS-PAGE.

For the preparation of cytoplasmic lysate, ice-cold hypotonic cytoplasmic extract buffer was added in the cell pellet and gently mixed with the pipette in a microfuge tube. The cell suspension was incubated on ice for 30 min to allow them to swell. After incubation, freshly prepared 10% NP-40 was added and vortexed vigorously for 15 seconds to rupture the plasma membrane. The contents were then centrifuged at 13000 rpm for a minute at 4°C and supernatant containing the cytoplasmic lysate was transferred to another pre-chilled microfuge tube and stored at –70°C. The pellet was then further processed for extraction of nuclear lysate. For this, ice-cold nuclear extract buffer was added to the pellet and incubated on ice for 45 min with intermittent vortexing after every 10 min of incubation. Finally, cell suspension was centrifuged for 5 min at 14000 rpm. The supernatant containing nuclear lysate was stored at –70°C for further experiment.

2.2.7 Estimation of protein concentration in cellular lysates

Bradford method (Bradford, 1976) was used to determine the quantity of protein in various samples in a 96-well plate. Bradford's reagent was prepared by diluting Bradford dye with water in the ratio of 1:5. For estimating the concentration of protein in a particular sample, 50 µl volume reaction was set and 200 µl of freshly prepared Bradford's reagent was added. The complex gives a purplish color whose intensity is proportional to the amount of protein present in the sample. A standard curve was also generated using increasing concentrations of BSA (50 µg/ml, 100 µg/ml and 200µg/ml). Cell lysates of test samples were diluted to 1:50 in the same volume. Each sample (including blank and standards) was taken in duplicates. The concentration of protein was measured using the ELISA reader at 570 nm. The unknown protein concentration (X) was calculated as follows:

$$X = \frac{\text{BSA Std 1(conc)}/OD_1 + \text{BSA Std 2(conc)}/OD_2}{2}$$

where,

OD_1& OD_2: Optical densities of Standard (Std) 1 & Standard (Std) 2, respectively.

BSA: Bovine serum albumin

X×50 (dilution factor)/1000 = Y

Concentration of unknown protein (µg/µl) = Y × OD

2.2.8 Immunoblotting (Western Blotting)

Immunoblotting was performed as essentailly described by Lee (Lee, 2007). Equal amounts of protein were resolved on a denaturating SDS-polyacrylamide gel (8-12%). After completion of the run, the gel was transferred onto PVDF membrane and placed in the blotting cassette. The cassette was then put into the mini transblot apparatus and transfer was done for 2-3 hours at a constant voltage of 80 V, depending on the size of the protein. Post transfer, membrane was rinsed in TBS containing 0.1% Tween-20 (TBST) and blocked with 5% non-fat milk in TBST for 1 h at 37ºC, on a gentle shaking rotator. After blocking, membrane was rinsed thrice in TBST and incubated with primary antibody diluted in TBST (ranging from 1:1000 to 1:10000, depending upon antibody used) for either 3h at room temperature or overnight in the cold room. The membrane was then washed thrice with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted

in 5% fat free milk solution in TBST (1:7000) for 45 min at room temperature and then washed thrice. The detection of signal was performed with ECL detection reagent (Amersham Biosciences) followed by detection of signal either on X-ray film (Hyperfilm-ECL, Amersham Biosciences) or in a chemidoc system (Proteinsimple, California, USA). The blot was reprobed with anti-tubulin or anti-GAPDH antibody to ensure equal loading of extracted protein.

2.2.9 Co-Immunoprecipitation (Co-IP)

Co-Immunoprecipitation assays were performed essentially as described by Lee (Lee, 2007). For a typical immunoprecipitation assay, cells were washed with ice-cold PBS and scapped in ice-cold microfuge tube. Then, cells were lysed with NETN buffer (containing 1 μg/ml each of leupeptin, aprotinin, 10mM each of NaF and phenylmethylsulfonyl fluoride (PMSF)) on shaking rotator in cold room for 30 min. After centrifugation, the whole cell lysate (500 μg-1 mg) obtained was incubated with 1 μg of antibody of interest (or with isotype control) on shaking rotator in cold room for 3 h, followed by addition of 10-20 μl of Protein Sepharose A/G beads (Santa Cruz) for 1 h. The immuno-complexes bound to beads were then pelleted at low speed centrifugation (2500 rpm for 3 min) and washed three times with NETN buffer. The proteins bound to beads were resolved by SDS-PAGE and immunoblotting was performed according to standard protocol described earlier.

2.2.10 Immunofluorescence

Immunofluorescence assay was carried out as described by Bhattacharyya *et* al., 2010.Adherent cells were grown either on cover slips. After treatment, cells were fixed with 3.7% paraformaldehyde solution in PBS for 15 min and permeabilised with 0.5% Triton X-100 at room temperature for 10 min, followed by blocking in PBS containing 2% BSA for 1 h. Post blocking, cells were incubated with a primary antibody in PBS (1:200 to 1:500) for 2 h. After washing, cells were incubated with fluorescent-conjugated secondary antibody in PBS (Alexa Fluor 488 or 594 goat anti-rabbit or antii-mouse, 1:1000) for 30 min. After final wash with PBS, nuclei were counterstained with DAPI containing mounting medium (Vectashield, USA). All the steps were performed at room temperature, unless otherwise stated. Images were obtained using either the laser scanning confocal LSM510 (Carl Zeiss, Oberkochen, Germany) or fluorescence inverted (Olympus 1X51, Tokyo, Japan) microscopes.

2.2.11 Cycloheximide (CHX) chase assay

CHX pulse chase assay was performed as essentially described by Zhou (2004). Cycloheximide (CHX), a protein biosynthesis inhibitor was used to determine the half-life and stability of protein of interest. CHX blocks translation elongation step, thereby halting the synthesis of new proteins and therefore, time course degradation of protein can be studied. Briefly, parental and profilin-stable cells were seeded in 35 mm culture dishes and treated with CHX (50 μg/ml) the following day. Cells were harvested at different time points and level of protein was determined by immunoblotting.

2.2.12 RNA interference

Retroviral based system was used for silencing of PTEN. BOSC23 packaging cells were grown in 100 mm culture dishes upto 80-85% confluency. Retroviral RNA vector containing either scrambled control shRNA or pool of PTEN shRNA along with a PCL-Ampho helper plasmid were co-transfected using Lipofectamine 2000 reagent to generate viral particles. After 48 h, supernatant containing viral particles were used to infect MDA-MB-231 cells in the presence of polybrene (8μg/ml).

For p53 gene knockdown, TP53 mission shRNAs obtained from Sigma Aldrich (St Louis, MO, USA) were transfected using Lipofectamine 2000 (Invitrogen, USA) and non-targeting shRNAs (Sigma) were used as controls. The cellular homogenates were prepared 36-48 h post transfection and were subjected to immunoblotting to check the levels of protein knockdown.

2.2.13 *in vivo* Ubiquitination assay

Uniquitination assay was performed as described by Choo and Zhang, 2009. Ubiquitination is an enzymatic process of the covalent attachment of polypeptide ubiquitin on specific lysine residues of protein, which is then degraded by proteasome complex. MG132 (carbobenzoxy-Leu-Leu-Leucinal), a proteolytic activity inhibitor of proteasome complex, is widely used to assess the stability of protein in vivo. Briefly, parental and profilin-stable cells were treated with 10 μM MG132 for 6 h. The whole cell extracts prepared in NTEN lysis buffer were then subjected to immunoprecipitation with anti-ubiquitin antibody. The analysis of ubiquitination was performed by immunoblotting with anti-PTEN antibody.

2.2.14 Reporter gene transcription assays

Secretory alkaline phosphatase (SEAP) assay: For SEAP assay, the culture supernatant was analyzed for SEAP activity essentially as per the Clontech kit protocol (Palo Alto, CA). Briefly, cells were transiently co-transfected with Lipofectamine 2000 transfection reagent, 0.5 μg of required plasmid DNA(s) with the protein of interest or empty vector, 0.5 μg of reporter plasmid containing NF-κB binding site cloned upstream of heat-stable SEAP (designated as NF-κB-SEAP) and 0.5 μg of green fluorescence protein (GFP) expression plasmid (Clonetech) in Opti-MEM media. After 6 h of transfection, cells were washed and cultured for 12 h in complete media, followed by treatment with different inducers. GFP positive cells were then counted to ensure similar transfection efficiency. At the end of treatment, cell culture-conditioned medium was harvested and 25 μl of medium was mixed with 20 μl of 5X buffer (0.5 M Tris, pH 9 and 0.5% bovine serum albumin) in a total volume of 100 μl in a 96-well plate followed by incubation at 65°C for 30 min. The plate was chilled on ice for 2 min and 50 μl of 1 mM 4- methylumbelliferylphosphate (MUP, substrate) was added to each well and incubated at 37 °C for 2 h. The activity of SEAP was assayed on a 96-well fluorescent plate reader (Fluoroscan, Lab Systems, MA) with excitation set at 360 nm and emission at 460 nm. The average number (± SD) of relative fluorescent light units for each transfection was then determined and reported as fold activation with respect to empty SEAP-transfected cells.

***Luciferase (Luc) assay*:** The cell pellet was lysed and extract was analysed as per Promega kit protocol. Briefly, cells were co-transfected with Lipofectamine with 0.5 μg of reporter plasmid containing p53 binding site cloned upstream of luciferase (designated as p53-luciferase) and 0.5 μg of GFP constructs. After 6 h of transfection, cells were washed and cultured for 12 h, followed by treatment with different inducers of apoptosis. GFP positive cells were then counted. Cells were pelleted down and lysed using the lysis buffer. The samples were freeze-thawed twice by storing them at -70^{o}C to ensure total lysis. The supernatant, obtained by centrifuging the same at 11,000 rpm for 2 min was transferred to a fresh tube. About 100 μl of the substrate (Firefly luciferin, Promega) was added to the supernatant and light emission was measured in luminometer by using a delay time of 2 sec and read time of 10 sec. The values were calculated as fold of activation over vector-transfected value.

2.2.15 Assays for Apoptosis

***Cytotoxicity assay*:** The drug-induced cytotoxicity was measured by the 3-(4,5-Dimethylthiazolyl-2)-2,3-diphenyltetrazolium bromide (MTT) assay as essentially described by Mosmann *et al*., 1983. Briefly, $5x10^4$ cells/well were seeded in 96-well plate. After 12 h, cells were treated in triplicates with different agents for different concentrations and time (in a final volume of 100 µl). After completion of treatment, 25 µl of MTT solution (5 mg/ml in PBS) was then added and incubated for 2 h. The cytotoxicity was evaluated by uptake and cleavage of yellow MTT dye to purple formazan crystals by dehydrogenase activity in mitochondria of the living cells. Thereafter, 100 µl of extraction buffer (20% SDS in 50% dimethlylformamide) was added. After an overnight incubation at 37ºC, the absorbance at 570 nm was measured using 96-well multiscanner autoreader (Bio-Rad) with the extraction buffer as blank. Absorbance values were normalized to untreated cells and represented in percent cell viability for different concentrations or treatments.

***Determination of nuclear fragmentation*:** The morphology of live and dead cells was observed by staining the nucleus with DNA intercalating dye, propidium iodide (PI). Briefly, cells were treated with several apoptotic inducers for different concentration or time. Thereafter, cells were washed with PBS and fixed in ice-cold 80% methanol for overnight at 4°C. Following day, cells were washed and suspended in 100 µl of PI solution (0.1% Triton, 0.2 mg/ml RNase A and 50 µg/ml PI in PBS) for 30 min in dark. Cells were then mounted on slides and viewed under fluorescence microscope (in 560 nm filter) to determine morphology of intact or fragmented nucleus.

***Live & dead assay*:** The cytotoxicity of various drugs was determined using the commercially available Live/Dead assay kit (Molecular Probes, Eugene, OR). Live cells have intact membrane and active cellular metabolism, which allow Calcien-AM to permeate inside and get cleaved into green fluorescent compound, Calcein (ex/em ~495 nm/~515 nm) due to intrinsic cellular esterase activity. On the other hand, Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). Hence, the cell viability can be assayed by either flow cytometry or fluorescence microscopy. For imaging, cells with different drugs treatments were washed with PBS

and stained with 'Live & Dead' cell assay reagent (5 μM ethidium homodimer, 5 μM calcein-AM) for 30 min at room temperature. Red (as dead) and green (as live) cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). For flow cytometry, cells were transiently transfected with either empty vector or various constructs. After 12 h, cells were treated with a combination of CHX (cycloheximide, 25 μg/ml) and TNF (5 nM) for 24 h. Cells were washed, trypsinised and then subjected to flow cytometry (FACS Aria, BD Biosciences) using Live-Dead Cytotoxicity assay kit (Invitrogen). Live versus dead cells were analysed using FlowJo software.

2.2.16 Radioactive labelling of oligonucleotides

The oligonucleotides of different transcription factors such as NF-κB, AP-1, p53 and SP-1 were 5′-end labelled using radioactive γ^{32}-ATP (obtained from BRIT, BARC, Mumbai, India) and Polynucleotide kinase, as per manufacturers' protocol. The reaction mixture containing the different components, described in the table 2.2 below was added in a microfuge tube.

Table 2.2: Various components of oligonucleotide labeling reaction mixture

Chemicals	Volume pipetted
Oligonucleotides (1 pmol/μl)	3μl
10X T_4 Polynucleotide kinase buffer	1μl
Distilled water	2.5μl
γ^{32}-ATP	3μl
T_4-Polynucleotide kinase	0.5μl
Total reaction volume	10μl

The mixture was then incubated at 37ºC for 45 min to radiolabel the oligonucleotide. Simultaneously, the Sephadex G-50 column was prepared in 1 ml syringe. The reaction mixture was loaded on the column and the eluting fractions were collected in the microfuge tube by loading 200 μl milli-Q water on the top of the column. After collecting 5-6 fractions, the tubes were analysed using a GM counter for the amount of radioactivity. The fractions having specific activity between 3.5-4.5 X 10^6 cpm/pmoles were pooled. To this, 100 pM of the complimentary strand of the oligonucleotide was added and heated at 95°C for 5 minutes. The mixture was allowed to anneal at room temperature for 1 h and further used in Gel shift assay.

2.2.17 Gel shift assay

For detection of protein- nucleic acid interaction, an electrophoretic mobility shift assay (EMSA) was conducted as described by Hellman and Fried (2007). Breifly, 8-10 µg of nuclear extract protein was incubated with binding reaction-mixture containing either ^{32}P end-labeled double-stranded oligonucleotides (NF-κB, AP-1, p53 or SP-1) or unlabeled oligonucleotide as shown in table 2.3. The reaction mixture was incubated at 37°C for 45-60 min. After completion of reaction, 6µl of 6X DNA-loading dye was added and mixed well by gentle tapping.

Table 2.3: Binding conditions for DNA-protein complexes in EMSA

Stock solutions	Volume pipette
Distilled water	10µl
10X Binding buffer	2µl
Poly dI:dC (1µg/µl)	2µl
^{32}P-labelled oligonucleotide	4µl
10% NP-40	2µl
Total reaction volume	20µl

The DNA-protein complexes were then separated from free oligonucleotides on 6.6% native PAGE gel. The samples were loaded into a native PAGE gel, which was pre-run at constant current (40-50 mA) for 15-30 minutes. Electrophoresis was performed at constant current (80-100 mA), till the bromophenol blue dye front reached 1-2 cm from bottom of the gel. The glass plate was carefully removed without disturbing the gel and the Whatmann filter paper no. 3, cut to the size of the gel, and was placed over it. The paper was pressed gently and the gel, which was now firmly stuck on the paper, was covered with saran wrap and kept for vacuum drying on the gel-dryer at 80^{o}C for 1 h. After drying, gel was exposed on a Phospho-imager screen for 12-24 h and scanned on Phospho-imager to detect the band of interest.

To determine the specificity of the transcription factor binding or sub-unit interacting to the desired oligonucleotide, super-shift assay was performed. For this, 8-10 µg of nuclear extracts were first incubated with desired antibodies (concentration varies for different) or their isotype control for 1h at 25°C, followed by incubation with binding reaction mixture. The various oligonucleotide sequences used in the present study are listed in Table 2.4 below.

Table 2.4: List of double-stranded oligonucleotides used in the present study

Transcription Factor	Consensus Binding sites (5'→ 3')
NF-κB	TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGG CGTGG
AP-1	CGCTTGATGACTCAGCCGGAA
Sp-1	ATTCGATCGGGGCGGGGCGAGC
p53	CCAGGCAAGTCCAGGCAGG
Oct-1	TGTCGAATGCAAATCACTAGAA

2.2.18 Isolation of total RNA from cultured cells

Total RNA was isolated from the treated cells using TRIzol method, essentially described by Donald and his co-worker (Donald *et al.,* 2010). TRIzol is a single-phase solution of guanidinium isothiocyanate and phenol that can concomitantly denature proteins and other biological material. Addition of chloroform to this leads to phase separation: proteins remains in organic phase whereas, DNA and RNA resolves to interphase and aqueous phase, respectively.

Before starting the experiment, area was sanitized with RNAZap to remove any contamination of DNases. After treatment, culture media was gently removed from the dish without disturbing the cell monolayer. TRIzol reagent was added directly on to the dish and cells were allowed to suspend in it by repeated pipetting. The cellular homogenate was then transferred into a microfuge tubes. For each ml of TRIzol used, 200µl of chloroform was added and vortexed for about 30 seconds, followed by centrifugation at maximum speed of 13,000 rpm for 10 minutes. The upper aqueous phase was transferred into a fresh micro-centrifuge tube and 500µl of ice-cold iso-propanol was added to precipitate RNA. The RNA was pelleted by centrifugation at 13,000 rpm for 30 minutes at 4°C. The supernatant was decanted and the pellet was allowed to wash with 1 ml of ice-cold 70% ethanol followed by centrifugation at maximum speed for 10 minutes. Finally, the supernatant was removed and the pellet was allowed to air-dry for about 5-10 minutes and solubilized in 50 µl RNase free deionized (DEPC-treated Milli-Q) water and quantified by spectrophotometry for further use.

2.2.19 Reverse transcriptase (RT)-PCR

Total RNA, isolated by TRIzol method was reverse transcribed into cDNA by One step Access RT-PCR kit (Promega, Madison, WI), as per manufacturer's protocol. Briefly, 1 µg of RNA, 1 µl oligo-dT (500ng), 1 µl 10 mM dNTP and nuclease free water was added in a PCR tube. This reaction mixture was incubated at 65°C for 5 minutes in a thermo cycler and then quickly transferred to ice. To the mixture, 4 µl 5X first strand buffer, 1 µl 0.1M DTT, 1 µl RNase-OUT (40 U/µl) and 1 µl (200 units/µl) of SuperScript III were added. The contents were mixed by gentle vortexing and incubated at 37°C for 60 minutes in a thermo cycler. Finally, the reaction was stopped by increasing the temperature to 72°C for 10 minutes on thermo cycler. The cDNA thus prepared, was used as a template for PCR.

PCR was then performed for either amplification of the gene of interest or relative expression of desired genes by the using gene specific primers (Table 2.5). Products were separated by agarose gel electrophoresis (2%) and visualized by ethidium bromide staining.

Table 2.5: List of RT- PCR primers used in the study

S.No.	Gene	Primer sequence (5 → 3')		Size of the product
1.	Profilin-1	F	ACGCCTACATCGACAACCTC	117 bp
		R	TGATGTTGACGAACGTTTTCC	
2.	ICAM-1	F	AGGCCACCCCAGAGGACAAC	406 bp
		R	CCCATTATGACTGGGGCTGCTA	
3.	Cox2	F	TTCAAATGAGATTGTGGGAAAAT	305 bp
		R	AGATCATCTCTGCCTGAGTATCTT	
4.	Angiopoietin-1	F	GCCTACACTTTCATTCTTCCAGA	500 bp
		R	TCTTCCTTGTGTTTTCCTTCCAT	
5.	IL-8	F	GCAGCTCTGTGTGAAGGTGCA	186 bp
		R	CAGACAGAGCTCTCTTCCAT	
6.	VEGF	F	ATGAACTTTCTGCTGTCTTGGGT	344 bp
		R	TGGCCTTGGTGAGGTTTGATCC	
7.	PTEN	F	GGACGAACTGGTGTAATGATATG	567bp
		R	TCTACTGTTTTTGTGAAGTACAGC	
8.	Actin	F	CCAACCGTGAAAAGATGACC	616 bp
		R	GCAGTAATCTCCTTCTGCATCC	
9.	GAPDH	F	ACCTGCCAAATATGATGAC	192 bp
		R	TCATACCAGGAAATGAGCTT	

2.2.20 Polymerase Chain Reaction (PCR)

The PCR amplification of desired genes were carried out using Taq polymerase reaction kit obtained from Fermentas. Typically, a reaction mixture containing primers with plasmid containing gene of interest or c-DNA was prepared as described in Table 2.6.

Table 2.6: Various components of PCR reaction mix

Components	Stock Concentration	Volume/reaction (50 µl reaction)
10 X Buffer	As per manufacturer	5 µl
DMSO	100%	1 µl
dNTP mix	10 mM	2.5 µl
Forward primer	10 pmole/µl	2.0 µl
Reverse Primer	10 pmole/µl	2.0 µl
Template DNA	10-50 ng/µl	0.5 µl
Taq. Polymerase	5 U/ml	0.5 µl
Nuclease free water	-	q.s. 50 µl

The reaction mixture was then placed on a thermo-cycler with the required cycling conditions for amplification of the desired gene, as described in Table 2.7. The amplified products were purified by PCR purification kit (Qiagen) and either quantified by spectrometry or separated by agarose gel electrophoresis.

Table 2.7: Cycling conditions for PCR

Steps	Temperature	Time	No of cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	1 min	30
Annealing	55-60°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	10 min	1

2.2.21 Spectrophotometric estimation of nucleic acids

The quantity and purity of nucleic acids were determined by measuring the absorbance at 260 and 280 nm using automated NanoDrop instrument. The concentration of nucleic acids was calculated by taking 1 OD at 260 nm = 50 µg/ml for DNA and 40 µg/ml for RNA. The purity of nucleic acids was checked by their A260/A280 ratio.

2.2.22 Agarose gel electrophoresis

For preparing agarose gels, appropriate amount of agarose (0.8-2%) was dissolved by boiling in TAE buffer, until clear slurry was formed. It was then poured in a casting tray containing a comb for desired number of wells. The gel was allowed to solidify and shifted to horizontal electrophoresis tank containing TAE buffer with 1 µg/ml ethidium bromide. Appropriate volumes of 6X DNA loading dye were added in the samples and subjected to electrophoresis at constant voltage (generally at 80 V), along with appropriate DNA ladder, until band were resolved. The gel was visualised under UV-light in a Gel-Doc or UV-transilluminator.

2.2.23 DNA sequencing

Automated DNA sequencing on plasmid templates or on PCR products was carried out with dye terminator cycle sequencing kits from Perkin-Elmer on an automated sequencer (model 377, Applied Biosystems), following the manufacturer's instructions.

2.2.24 Isolation of plasmid DNA

Plasmid DNA was isolated from 10-100 ml of bacterial culture grown overnight by using commercially available Miniprep or Midiprep kits (Invitrogen) as per manufacturers' protocol. Briefly, bacterial cells were pelleted by centrifugation at 6000 rpm for10 min. The pelleted cells were re-suspended in 300 µl-4ml of resuspension solution containing RNase H. About 300 µl-4 ml of alkaline lysis solution was then added and mixed by gently inverting the tubes until clear and uniform lysate was appeared. Subsequently, 350 µl-5 ml of neutralizing solution was added, the tubes were inverted repeatedly and gently to ensure homogeneous mixing, followed by incubation for 5 min on ice. After centrifuging at 12,000 rpm for 15 min, supernatant was passed through commercially supplied filter columns by either gravity flow or high-speed centrifugation. During this step, plasmid DNA binds to the column and was recovered by passing elution buffer through the column. The plasmid DNA present in the elution buffer was collected into a fresh tube and 70% (w/v) of iso-propanol was added. The precipitated nucleic acids were then recovered by centrifugation at 12,000 rpm for 30 min. The pellet was washed once with 70% ethanol, air-dried and re-suspended in desired amount of TE-buffer. Finally, the purity of plasmid was observed on 1% agarose gel.

2.2.25 Digestion and elution of DNA

For construction of desired clones, 1-2 µg of DNA was used for restriction digestion. In a typical reaction, 2-5 units of restriction enzymes were used in the total reaction volume of 50 µl along with 5 µl of recommended buffer (supplied as 10X digestion buffer). The reaction mixture was incubated for 3-5 h at 37ºC. The digested DNA was then loaded along with DNA size marker and separated on agarose gel electrophoresis. The gel was visualized over a UV illuminator and section containing the desired DNA fragment was carefully sliced out. The sliced agarose gel was then processed using commercially available Gel Extraction kit (Qiagens) for this purpose.

2.2.26 Ligation and transformation of DNA

In a typical ligation reaction, a total of 100 ng of vector was used whereas; the concentration of insert varies from 300 to 500 ng. For 10 µl reaction volume, 1µl ligation buffer (provided by the manufacturer) and 0.5 µl of T4-DNA ligase were added in vector-insert mixture. The reaction was maintained at 16ºC for 14-16 h. After ligation, 2 µl of the ligation mixture (of total volume of 10 µl reaction) was added to a vial of ultra-competent DH5α bacterial cells and incubated in ice for 30 minutes. The ligation mixture was allowed to heat shock at 42ºC for 90 seconds followed by quick transfer on ice. About 1 ml of LB broth was added to the tube and incubated at 37ºC for 1 hour. The bacterial cells were then pelleted by centrifugation at 6000 rpm for 5 min and plated on LB plate containing appropriate antibiotic.

2.2.27 Preparation of Ultra competent cells

The highly efficient Ultra competent DH5α cells for transformation were prepared by Inoue method (Inoue *et al*., 1990). Briefly, a single bacterial colony was picked from LB agar plate, inoculated into 10 ml LB medium and incubated overnight at 37°C temperature with 200 rpm shaking. Following day, 1% of the pre-inoculum was sub-cultured in 100 ml LB-broth and incubated at 18°C until OD_{600} of 0.5-0.6 was reached. Culture was then kept on ice for 10 min with constant shaking. Cells were pelleted by centrifugation at 5000 rpm at 4°C for 10 min. Pellet was resuspended in 40 ml of ice-cold Inoue buffer. Bacterial suspension was kept on ice for 30 min, re-spun at 2000g at 4°C for 10 min. Pellet was resuspended in 8 ml of TB buffer in which 560 µl DMSO was added and left on ice for 10 min. Finally, 100 µl aliquots were made by snap freezing in liquid nitrogen and stored at -80°C.

2.2.28 Molecular docking

AutoDock tools 1.5.6 (Morris *et al*., 2009) and PyMOL (Sanner, 1999) was used to prepare and analyze the docking simulations.

***Preparation of protein structures for docking*:** The three dimensional structure of PTEN (PDB ID: 2PBD) (Lee *et al*., 1999) and profilin-1 (PDB ID: 1D5R) (Ferron *et al*., 2007) were obtained from protein data bank, PDB (www.rcsb.org). Prior to initiating the docking simulations, all non-protein molecules were removed from the intact PTEN and only the chain P was retained. In the same way, we retained ('chain A') for profilin-1. All the non-protein molecules were removed using Chimera (Pettersen *et al*., 2014). Protein PTEN was used as receptor and profilin-1 was used as ligand. Kollman united atom charges and polar hydrogen were added to the receptor protein. Receptor protein was kept rigid in docking process, assuming there is no induced conformational change upon ligand binding. In contrast to the protein, torsional flexibility was permitted for the ligands via the side-groups and backbone of protein was kept rigid. Atomic affinity and electrostatic potentials were computed for a grid box and positioned around the approximate centre of the binding site. The grid box size was set at 58.0 X 76.0 X 58.0 Å (x, y, and z) with the spacing between grid points at 1 Å and the center at 36.253 X 82.395 X 31.728 for x, y and z coordinates, respectively. All other docking parameters remained as the Autodock default settings.

Analysis of docking data: AutoDockVina (Trott and Olson, 2010) software was used for docking simulations. The resulting docked conformations were clustered into families of similar conformations, with the root mean square deviation (r.m.s.d.) clustering tolerance of 2.0 A°. As a rule, the lowest docking- energy conformations were included in the largest cluster. The process was repeated for each remaining conformation, until all the conformations belonged to a single cluster. A new reference conformation was defined every time a new cluster was created. Following this, the best-docked conformation is selected as the lowest energy pose in the most populated cluster, i.e., the cluster with the highest convergence out of the 100 trials. This differs from the practice of simply choosing the overall lowest energy pose. Thus, we avoid picking lower energy ranked poses belonging to sparsely populated clusters, which can be considered as 'chance-hits' given that the docking algorithm was unable to converge to similar poses in other independent trials.

2.2.29 Statistical analysis

All data were presented as mean ± standard deviation (SD)/standard error of mean (SEM) from three independent experiments. Statistical analysis was performed using Student's t-test and one-way ANOVA followed by a post hoc Tukey test wherever applicable. Results were analyzed and illustrated by SPSS statistical software package (SPSS for Windows, version 16). Comparisons are done within and between the test groups (i.e., parental cells and profilin-stable cells). Asterisk (*) symbol indicates statistical difference between parental and profilin-stable cells, whereas Number (#) and Dollar ($) signs indicate statistical difference within parental and profilin-stable cells, respectively. Significance of results was determined as $p \leq 0.01$ and $p \leq 0.05$ (*indicates $p \leq 0.05$, **indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and **** indicates $p \leq 0.0001$)