Role of TopBP1 in transformation of 3D breast acini cultures

Submitted by

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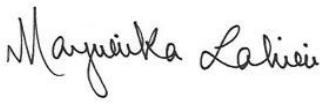
Certificate

This is to certify that this dissertation entitled '**Role of TopBP1 in transformation of 3D breast acini cultures**' towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by **Rintu M Umesh** at IISER Pune under the supervision of **Dr. Mayurika Lahiri**, Associate Professor, Biology Division, IISER Pune during the academic year 2015-2016.



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Declaration

I hereby declare that the matter embodied in the thesis entitled '**Role of TopBP1 in transformation of 3D breast acini cultures**' are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of **Dr. Mayurika Lahiri**, Associate Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

TopBP1 is an important mediator protein involved in the cell cycle checkpoint signaling pathway that is activated when there are single strand lesions on DNA. TopBP1 has structural and functional similarity to BRCA1 (a tumor suppressor gene). However, there are contradicting reports suggesting the role of TopBP1 either as an oncogene or a tumor suppressor gene. The primary objective of the study was to investigate the role of TopBP1 in cellular transformation of breast epithelial cells. 3D "on-top" culture of MCF10A cells was used as a model system because of its close similarity to the "*in-vivo"* glandular architecture. MCF10A cells over-expressing TopBP1 when grown on Matrigel® for 16 days formed acini with larger surface area than the control MCF10A cells. An increase in nuclear size was also observed. TopBP1 over-expressing cells showed disruption of basal polarity which is one the markers of transformation. An increase in collective cell migration was observed in TopBP1 over-expressing MCF10A cells. An increase in MMP activation, one of the key features of invading cells, was also observed, suggested the role of over-expressed TopBP1 in invasion. However, only slight difference was observed in the levels of the various EMT markers in TopBP1-MCF10A acini at protein level. There was no chemoresistance induced in TopBP1 over-expressing cells. Preliminary results suggest that TopBP1 can induce transformation of breast epithelial cells.

Abbreviations

TopBP1, DNA topoisomerase II β -binding protein 1; ECM, extracellular matrix; 3D, 3 Dimensional; EMT, epithelial to mesenchymal transition; DDR, DNA damage response; MMP, matrix metalloproteinases

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Introduction

DNA damage and checkpoint signaling pathway

DNA is prone to damage by various endogenous and exogenous agents. If the damage remains unchecked, it may lead to genomic instability leading to cancer (Abraham, 2001). Fortunately the cells have an elaborate checkpoint signaling mechanism to detect and repair the damage so that it ensures the faithful transmission of genetic material to the next generation. The canonical checkpoint pathway comprises of sensors, mediators, transducers and effector proteins (Figure 1). The Sensor proteins, ATM and ATR/ATRIP, senses double strand or single strand breaks respectively and activate Chk2 and Chk1 by phosphorylation through mediator proteins, like BRCA1,MDC1 (in Double Strand Break) (Cortez et al., 1999), Claspin and TopBP1 (in Single Strand Break pathway) (Harper and Elledge, 2007). Further the signal is relayed to effector proteins like Cdc25C, Nbs1, p53, to name a few (Banin et al., 1998; Brown et al., 1999; Petrini, 1999) which can induce cell cycle arrest to allow time for repair of damaged DNA or apoptosis depending upon the extent of damage. Defects in checkpoint activation can lead to accumulation of DNA damage which may eventually lead to cancer (Kastan and Bartek, 2004). TopBP1 is a major mediator protein involved in single strand break repair pathway (Wang and Elledge, 2002)

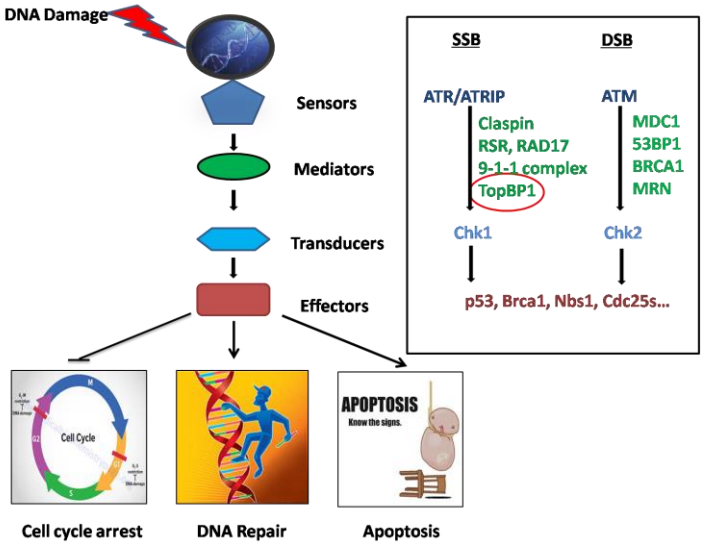


Figure 1: The canonical checkpoint repair pathway- Adapted from (Abraham, 2001)

TopBP1

TopBP1 or DNA topoisomerase II β -binding protein 1, is involved in activation of DNA damage checkpoint to maintain genome integrity (Xu and Leffak, 2010). TopBP1 gene is located at chromosome 3q22.1 and it is conserved across species (Wardlaw et al., 2014) (Figure 2).It was first identified through two independent screens in *Schizosaccharomyces pombe* (as rad4 (Schupbach, 1971), and cut5 (Hirano et al., 1986)) and later these two were identified to be the same (Saka et al., 1994). Further human TopBP1 was identified as an interactor of Topoisomesae II β in a two hybrid screen (Wang and Elledge, 2002) . The other known homologues of TopBP1 are Dpb11 (*S. cerevisiae*) (Araki et al., 1995), Mus101 (*D. melanogaster*) (Yamamoto et al., 2000), Mei1 (*A. thaliana*) and Xmus101 (*X. laevis*).

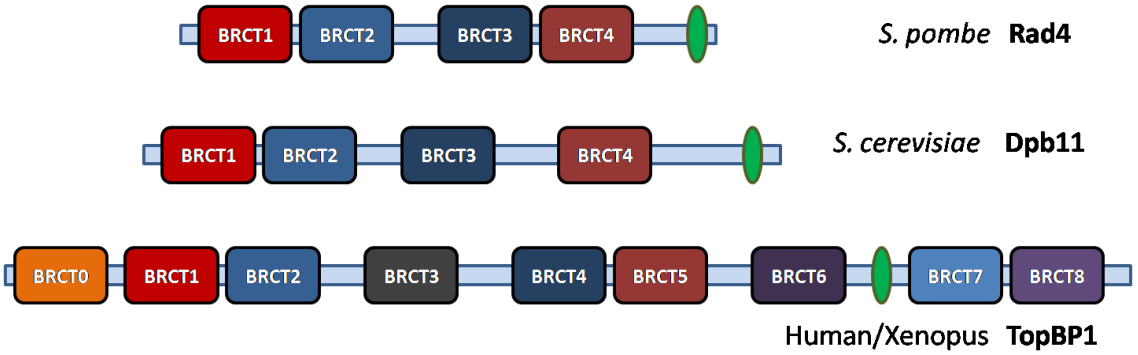


Figure 2: Domain structure of TopBP1 and its homologues: Highlighting BRCT domains and an AAD (green) of TopBP1. Image adapted from (Wardlaw et al., 2014)

TopBP1 has structural as well as functional similarities to BRCA1 (a tumor suppressor gene) (Karppinen et al., 2006; Yamane et al., 2003). It has nine BRCA1 C-Terminus (BRCT) domains through which it interacts with other proteins (Yu et al., 2003) and an ATR activation domain (AAD) which is necessary to stimulate the kinase activity of ATR (Kumagai et al., 2006) and thus checkpoint activation. It is a nuclear protein and has been found to co-localize with BRCA1 at the stalled replication forks (Yamane et al., 2002).

Other than its essential role in DDR, it is also involved in transcriptional regulation of genes involved in cell proliferation and apoptosis (E2F1 and c-Myc) (Liu et al., 2009; Liu et al., 2003). Also its interaction with BLM (Bloom Syndrome Helicase), has been shown to inhibit sister chromatid exchange thus maintaining genome stability (Wang

et al., 2013). At physiological levels, TopBP1 is required for G1/S transition in the cell cycle. However, aberrant expression of TopBP1 has been observed in breast cancer.

TopBP1 and Breast cancer

Breast cancer is the most common cancer and the most common cause of cancer mortality in women. It is caused by genetic as well as non-genetic factors which include chemicals, radiation, diet etc. Various studies have found inactivation of genes, like BRCA1, BRCA2, P53, PTEN, STK1 (Hemminki et al., 1998; Malkin et al., 1990; Meijers-Heijboer et al., 2002; Nelen et al., 1997) giving rise to increased susceptibility to breast cancer. Along with the similarity to BRCA1, recent studies suggest TopBP1 as one of the candidate breast cancer susceptible genes (Karppinen et al., 2006).

Immunohistochemical studies performed in breast cancer biopsy samples showed that TopBP1 is upregulated in breast cancer tissues and higher grade tumors and lower patient survival are associated with its over expression (Forma et al., 2012; Going et al., 2007; Liu et al., 2009) suggesting its role as an oncogene. p53, a tumor suppressor, which has been found to be mutated in almost half cancers, is actively repressed by TopBP1 (Liu et al., 2009; Liu et al., 2011). From this it can be speculated that the inactivation of p53 by TopBP1 may be the reason for the poor clinical outcome for breast cancer patients expressing high levels of TopBP1. PI(3)K/Akt and Rb are other common pathways deregulated in cancer (Cancer Genome Atlas, 2012) and these abnormalities can lead to TopBP1 oligomerization and over-expression respectively which finally results in inhibition of apoptosis and activation of proliferation (Liu et al., 2003; Liu et al., 2011).

However there are studies where no TopBP1 expression was observed in invasive breast cancer samples (Forma et al., 2012; Going et al., 2007). Also decreased levels of *TopBP1* mRNA were observed in high grade breast tumor samples which contradict its role as an oncogene (Going et al., 2007).

These reports along with the known functions of TopBP1 and its similarity to BRCA1 and availability of limited reports stating the possible role of TopBP1 in breast cancer

prompted us to investigate the role of TopBP1 in breast tumorigenesis using the three dimensional (3D) cultures as model system.

3 Dimensional cultures of breast epithelial cells as model

Mouse models and primary tumor tissues are the most widely used models to study breast cancer. However, it is difficult to study the cell biological and biochemical pathways involved in tumor formation using these models (Debnath et al., 2003). On the other hand, the monolayer cultures (2D) used to study the underlying mechanism in oncogenic transformation, does not recapitulate the 3D breast glandular structure. In this scenario, 3D cultures of epithelial cells that reproduce the *in-vivo* glandular architecture serve as a physiologically relevant model to study breast tumorigenesis (Hebner et al., 2008).

The functional unit of a mammary gland, acini, is composed of two cell layers: secretory cells with microvilli in the inner luminal layer which secrete milk covered by a layer of contractile myoepithelial cells whose contraction helps in the ejection of milk to the ducts (Vidi et al., 2013). These acini have centrally located hollow lumen surrounded by a layer of polarized cells (Hebner et al., 2008). The 3D acini formed *in-vitro* when grown on externally provided extra cellular matrix (ECM), recapitulates these structural features of acini in the mammary gland (Debnath et al., 2003). (Figure 3)

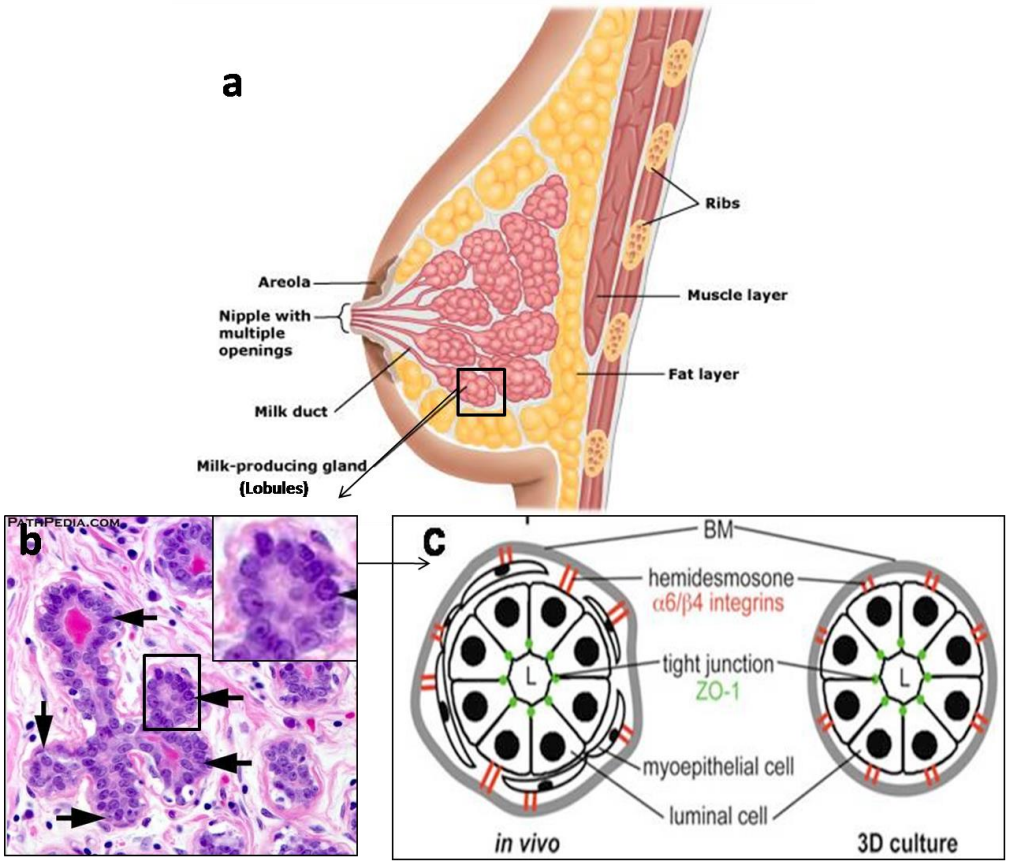


Figure 3: Anatomy of mammary gland- a) Schematic diagram of mammary gland with different lobules connecting to nipple **b)** Immunohistochemical staining of a section of lobule. **c)** Comparison of acini in mammary gland and acini formed in 3D culture. (Adapted from Vidi et al., 2013)

MCF10A cells (mammary non-tumorigenic epithelial cell line) have been largely used to study the morphogenesis of breast epithelial cells. A single cell suspension of MCF10A cells seeded on extracellular matrix (ECM; commercially available as Matrigel®) will proliferate and polarize to form a growth arrested 3D acini by day 16 (Figure 4). Reduced proliferation of MCF10A 3D acini after 16 days of morphogenesis, which is a distinguishing feature of *in-vivo* glandular epithelial, is of immense experimental value as it helps to investigate the role of oncogenes in morphogenesis of MCF10A (Debnath et al., 2003).

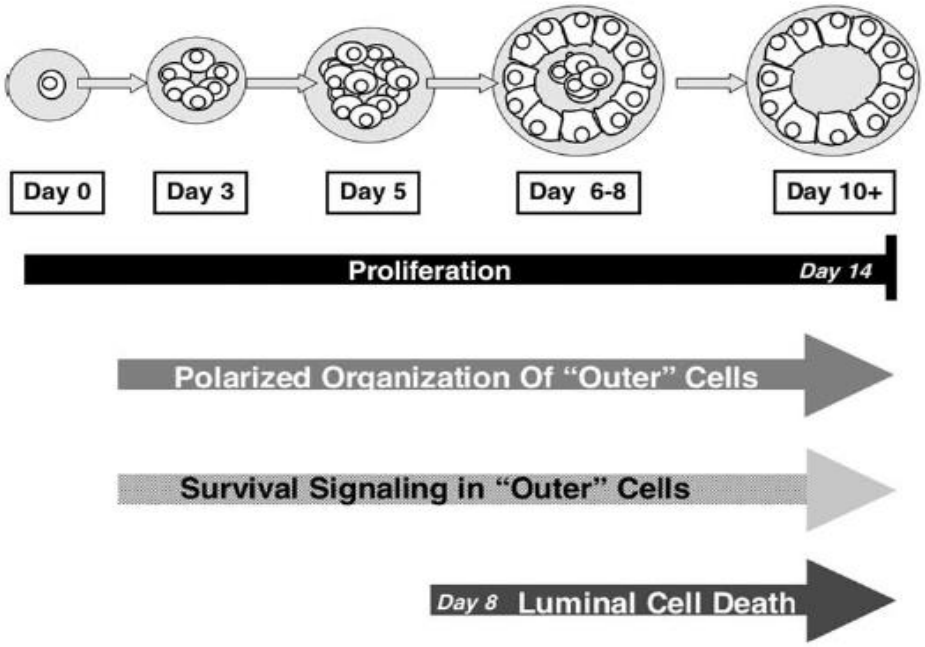


Figure 4: Schematic showing morphogenesis of MCF10A acini (Reproduced from Debnath et al., 2003).

Previous studies have shown that polarized MCF10A acinar structure can be disrupted by mutation of a single or multiple gene activity (Chen et al., 2010; Guo et al., 2010; Henry et al., 2011) which can form acini with filled lumen, multiple lumen, and protrusion like phenotype suggesting EMT (Figure 5). For example, mutations that induced over-expression of BCL2 (an apoptotic inhibitor) and reduction of BIM (a pro-apoptotic protein) created acini with filled lumen phenotype (Debnath et al., 2002; Reginato et al., 2005). Multi acinar structures with filled lumen are formed by ERBB2 (an oncoprotein) activation (Debnath et al., 2002). Deregulation of ERK along with matrix metalloproteinase (MMP) activation promotes formation of acini with protrusion like phenotype indication EMT (O'Brien et al., 2004) .These acini phenotypes resemble the architectural disorders of mammary gland during various stages of breast cancer (Debnath and Brugge, 2005). These key features make 3D culture system a good model to study the role of a gene(s) in tumorigenesis.

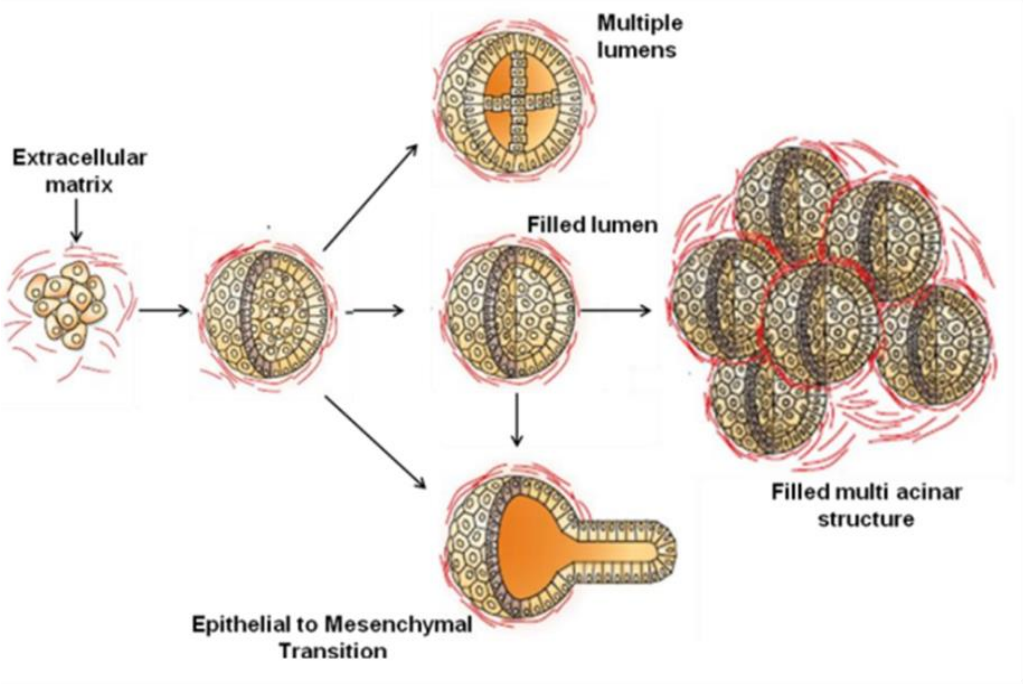


Figure 5: Schematic showing disrupted acini formed by transformed breast epithelial cells-Image adapted from (Debnath and Brugge, 2005; Debnath et al., 2002; Debnath et al., 2003)

Aim

To study the role of TopBP1 in transformation of 3D breast acini cultures

Objectives

- Generation of stable cell line of MCF10A over-expressing TopBP1 (TopBP1-MCF10A) and empty vector mVenus (mVenus-MCF10A)
- Investigate the phenotypic changes induced upon TopBP1-over-expression
    1. Morphometric analysis
    2. Apico-basal Polarity markers
    3. Cell- cell junction markers
    4. Invasion and Migration
    5. Epithelial to mesenchymal transition (EMT)
    6. Chemoresistance.
- Elucidate the mechanism by which TopBP1 induces transformation

Materials and Methods

Cell lines

MCF7 was purchased from European Collection of Cell Cultures (ECACC). HEK293T and MCF10A are generous gift from Dr. Jomon Joseph (National Centre for Cell Science, Pune) and Prof. Raymond C. Stevens (The Scripps Research Institute, California) respectively.

Plasmids

CSII-EF-MCS plasmid was a gift from Dr. Sourav Banerjee, NBRC, Manesar India. pCAG-HIVgp and pCMV-VSV-G-RSV-Rev plasmids were purchased from RIKEN BioResource Centre.

Table 1: MCF10A medium components- prepared in high glucose Dulbecco's Modified Eagle Medium (DMEM-Lonza) without sodium pyruvate

Components	Growth Medium (concentration)	Assay Medium (concentration)	Resuspension Medium
Horse serum (Invitrogen)	5%	2%	20%
Insulin (Sigma-Aldrich)	10 µg/ml	10 µg/ml	-
Hydrocortisone (Sigma-Aldrich)	0.5µg/ml	0.5µg/ml	-
Choleratoxin (Sigma-Aldrich)	100ng/ml	100ng/ml	-
Epidermal growth factor (EGF-Sigma-Aldrich)	20 ng/ml	5 ng/ml	-
Matrigel® (BD Biosciences)	-	2%	-
Pencillin-Streptamycin (Invitrogen)	100 units/ml	100 units/ml	100 units/ml

Cell culture

HEK293T and MCF7 cells were grown on 100mm 2D tissue culture treated dishes (Eppendorf or Corning) with high glucose containing Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 10% heat inactivated fetal bovine serum (FBS- Invitrogen) and 100 units/ml of penicillin- streptomycin (Invitrogen). MCF10A

cells were grown as monolayer in Growth medium (Table 1). Cells were incubated at 37ºC humidified 5% CO_2 incubators (Eppendorf or Thermo Scientific). MCF10A cells were re-suspended in Resuspension Medium (Table 1), DMEM without sodium pyruvate with 20% horse serum, while sub-culturing.

Transduction of MCF10A cells using lentiviral particles

4ml MCF10A Growth Medium containing 800 µl of concentrated viral supernatant which consists of viral particles with mVenus-CSII-EF-MCS-TopBP1 or mVenus-CSII-EF-MCS plasmid along with 8 µg/ml polybrene (Sigma) was added onto MCF10A cells for viral transduction. Additional 6 ml of Growth Medium was added 6 hours post transduction. Cells were supplemented with fresh medium after 24 hours of transduction.

MCF10A 3D "on-top" culture

0.5 X 10^4 cells of mVenus-MCF10A and TopBP1-MCF10A suspended in Assay Medium (Table 1), was seeded on each well of an 8 well chamber coverglass (Nunc Lab tek, Thermo Scientific) pre-coated with 50 µl of Matrigel®. Cells were maintained at 37ºC humidified 5% CO_2 incubators for 16 days and replenished with fresh Assay Medium with 2% Matrigel® and 5 ng/ml of EGF every 4 days.

Immunoflourescence for 2D cultures

After one PBS wash, cells were fixed with 4 % formaldehyde (Fisher Scientific) for 20 minutes in dark at room temperature (RT). Cells were washed thrice with 1X PBS for 10 minutes each prior to permeabilization with ice cold 0.5% Triton X-100 (USB Corporation) in 1X PBS at 4ºC for 10 minutes. Further, wells were washed thrice with PBS-Glycine (100mM glycine in 1X PBS) and blocked with 10% FBS in IF buffer (1X PBS, 0.05% [w/v] sodium azide, 0.1% [w/v] BSA, 0.2% [v/v] Triton-X-100 and 0.05% Tween 20) for 1 hour. Primary antibodies of required dilution (Table 2) prepared in 10% FBS in IF buffer was added onto the cells and incubated for 16 hours at 4ºC. Cells were washed thrice with IF buffer for 20 mints and incubated with Alexa Flour® secondary antibodies prepared in 10% FBS in IF for 1 hour at RT. After incubation, cells were washed once with 1X IF buffer and twice with 1X PBS for 10 minutes. 0.5 µg/ml of Hoechst 33342 (Invitrogen) in PBS was added to cells and incubated for not more than 5 minutes and immediately washed with 1X PBS for 10 minutes.

Table 2: Antibodies used for Immunoflourescence (IF) and Immunoblotting

Primary Antibody	IF (2D)	IF (3D)	Immunoblotting
GFP, anti-Rabbit (Abcam)	1:200	1:100	1:2500
Laminin V, anti-Mouse (Millipore)		1:100	
TopBP1, anti-Rabbit (Bethyl)	1:200	1:100	1:1000
α6 integrin, anti –Rat (Millipore)		1:100	-
E-cadherin, anti-Mouse (BD)			1:7500
N-cadherin, anti-Rabbit (Abcam)			1:1000
Vimentin, anti-Mouse (Abcam)			1:10000
β-catenin, anti- Rabbit (Abcam)			1:2000
Fibronectin, anti-Mouse (BD Biosciences)			1:20000
Cytokeratin 14, anti-Mouse (Abcam)			1:1000
Cytokeratin 19, anti-Rabbit (Abcam)			1:1000
GAPDH, anti-Rabbit (Sigma)			1:40000 1:10000 (for GZ lysates)
Phalloidin (Invitrogen)	1:100	1:100	
Secondary Antibodies			
Alexa Flour® goat anti-mouse (Jackson Immuno Research)	1:200	1:200	
Alexa Flour® goat anti-Rabbit (Jackson Immuno Research)	1:200	1:200	
Alexa Flour® goat anti-Rat (Jackson Immuno Research)		1:200	
Anti –Mouse HRP (Jackson Immuno Research)			1:10000
Anti –Mouse HRP (Jackson Immuno Research)			1:10000

Excess PBS was removed and Slow Fade® Gold antifade reagent (Molecular Probes) was added to mount the cells. Later cells were imaged under 63X oil immersion objective of Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, GmbH).

Immunoflourescence for 3D culture

3D cultures were washed with 1X PBS and fixed with 4% paraformaldehyde (VWR International) with 0.06% gluteraldehyde in PBS for 20 minutes at RT. Cells were washed thrice with 1X PBS for 10 minutes each prior to permeabilization with ice cold 0.5% TritonX-100 in 1X PBS at 4ºC for not more than 10 minutes (extreme care should be given while pipetting out the solution after permeabilization).Further, wells were washed thrice with PBS-Glycine (100mM glycine in 1X PBS) and blocked with primary blocking solution containing 10% goat serum (Abcam) in IF buffer for 1 hour followed by secondary blocking (primary blocking with 1% F(ab')2 fragment goat anti-mouse IgG (Jackson Immuno Research)) for 1 hour. Primary antibodies of required dilution (Table 2) prepared in primary blocking solution was added onto the cultures and incubated for 16 hours at 4ºC. Cultures were kept at RT for 15-20 minutes prior to washes with IF buffer (3 times, 20 minutes each). Respective Alexa Flour® secondary antibodies prepared in primary blocking solution was added to the cultures and incubated for 1 hour. After incubation, cells were washed once with 1X IF buffer for 20 minutes and twice with 1X PBS for 10 minutes each. 0.5 μg/ml of Hoechst 33342 in PBS was added to cells and incubated for not more than 5 minutes and immediately washed with 1X PBS for 10 minutes. Excess PBS was removed and Slow Fade® Gold antifade reagent was added to mount the cells. Later cells were imaged under 63X oil immersion objective of Zeiss LSM 710 laser scanning confocal microscope.

Immunoblotting

For immunoblot experiments, cell lysates were prepared in 2X sample buffer (Tris with SDS, bromophenol blue, dithiotreitol, and glycerol, pH 6.8) and were separated on 10% or 6%SDS-PAGE gels at 120V in a SE 260 mini-vertical gel electrophoresis unit (GE Healthcare)**.** Resolved proteins were transferred onto Immobilon-P polyvinyldifluoride (PVDF) membranes (Millipore) in TE22 mighty small transphor unit (GE Healthcare) at 250mA for 3 hours or 150mA for 16 hours. The blots were

blocked in 5% (w/v) skimmed milk (SACO Foods, US) prepared in TBS-Tween (25mMTris (pH 7.6), 150 mM NaCl and 2 mM KCl and 0.1% Tween 20) for 1 hour at room temperature (RT) and probed with the respective primary antibody for 1 hour (GAPDH) at RT or overnight at 4ºC. Blots were washed thrice with TBS-T and probed with HRP-conjugated suitable secondary antibody (anti mouse or anti-rabbit accordingly) for 1hour at RT. After 3-4 washes with TBS-T, blots were developed with Immobilon reagent (Millipore). Images were acquired on ImageQuant LAS4000 gel documentation system (GE Healthcare).

Wound healing Assay

Ibidi Culture Inserts, which have a defined cell free space manufactured from biocompatible silicon were placed in a 35mm dish. 5 X 10^5cells of mVenus-MCF10A or TopBP1-MCF10A suspended in Growth Medium was added on to both sides of the insert. 16 hours post seeding, cells were treated with 10 μg/ml mitomycin C (Sigma) for 2 hours. The medium was aspirated and cells were washed with PBS before replenishing it with fresh medium. Insert was removed and the wound was visualised at 0, 6,12,18 and 24 hrs under 10X objective of ECLIPSE TS100 (Nikon) microscope. After calculating the wound area using ImageJ software, percentage wound closure was calculated at different time points using the formula

$$\%\, WoundClosure = \frac{Initial\ wound\ area - Final\ wound\ area}{Initial\ wound\ area}\ X\ 100$$

Gelatin Zymography

Assay medium of TopBP1-MCF10A and mVenus-MCF10A 3D cultures were collected 16 days post seeding and stored at -80º C. Meanwhile the 3D cultures were washed with PBS, lysed using gelatin zymograph (GZ) lysis buffer (25mM Tris-HCl (pH 7.5), 100mM NaCl, 1% NP-40) and immunoblotting for GAPDH was performed, which served as loading control. 10 μl of 87 μl 3D medium + 13 μl GZ sample buffer (denaturing buffer) mixture was resolved on an 8% SDS-PAGE containing 0.06% gelatin [w/v] as substrate at 125V at 4ºC (SDS buffer should be changed if the current reduces below 10mA). Gel was washed with distilled water prior to incubation with 1X Renaturing buffer (2.5 % v/v of Triton X-100 in water) at

RT for 30 minutes with constant shaking. After washing with 300 ml of distilled water, gel was incubated in 100 ml 1X Developing buffer (0.5 M Tris-HCL pH-7.8, 2M NaCl, 0.05M CaCl2 and 0.2% Brij35) for 30 minutes with constant shaking. The gel was incubated at 37ºC for 48 hours with fresh Developing buffer. Later, the gel was stained over night with the staining solution (0.1% w/v Coomassie blue (Sigma-Aldrich), 50% v/v Methanol (Fisher Scientific) and 10% v/v acetic acid (Fisher Scientific) and destained with destaining solution (40% v/v methanol and 10% v/v acetic acid) for 1 hour. The gel images were acquired on ImageQuant LAS4000 gel documentation system (GE Healthcare).

MTT assay

1 X 10^4 cells of mVenus-MCF10A or TopBP1-MCF10A suspended in Growth Medium was seeded per well of a 96 well plate. 16 hours post seeding, cells were treated with different concentrations of Camptothecin (0 µM,0.02 µM, 0.07 µM, 0.21 µM, 0.63 µM, and 1.25 µM) or Doxorubicin (0 µM, 1 µM, 3 µM, 15 µM and 75 µM) and incubated for 24 hours at 37ºC. After removing the drug containing medium, 0.5 mg/ml thiazolyl blue tetrazolium (MTT;Sigma-Aldrich) in DMEM was added on to the cells and incubated at 37ºC after covering with aluminum foil. 4 hours post MTT addition, MTT-DMEM medium mixture was aspirated from the wells and 100 µl of Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the purple MTT-formazan crystals. The absorbance was recorded at 570nm using Varioskan Flash (Thermo Scientific).

Cloning of TopBP1 into mCherry-CSII-EF-MCS vector

3 µg of mCherry-CSII-EF-MCS and mVenus-CSII-EF-MCS-TopBP1 plasmid DNA were digested using HpaI (New England Biolabs -NEB) and NotI HF (NEB) restriction enzyme at 37ºC. 3 hours post digestion 3 µl of Calf intestinal phosphate (CIP; NEB) was added to mCherry-CSII-EF-MCS and incubated both the plasmids at 37ºC for another 1 hour. Digested plasmids were then resolved on a 0.8% agarose gel and the band corresponding to the size of TopBP1 (~4.3kb) and mCherry-CSII-EF-MCS vector (~9kb) was gel extracted and purified using gel extraction kit (Sigma). TopBP1 was ligated into mCherry-CSII-EF-MCS vector with an insert to vector ration of 3:1 using T4 DNA ligase (NEB) at 16ºC over night. 5 µl of ligation mixture was used to transform DH5α competent cells. Plasmid DNA was isolated

from the obtained colonies and screened the colonies by digesting with HpaI and NotI HF to check for insert release.

Lentiviral preparation and transduction using lipofectamine mediated transfection

2.5 X 10^5 HEK293T cells were seeded on a 12 well dish for viral production. Cells were incubated at 37ºC for 16 hours prior to transfection. 5 µl of Lipofectamin 2000 (Invitrogen) was added to 120 µl of OPTIMEM (Invitrogen) and incubated at room temperature for 5 minutes. Meanwhile 0.5 µg of mCherry-CSII-EF-MCS or mCherry-CSII-EF-MCS-TopBP1 plasmid along with 0.5 µg of pCAG-HIVgp and 0.25 µg pCMV-VSV-G-RSV-Rev packaging plasmids were mixed and OPTIMEM was added to make up the volume to 125 µl. 125 µl of lipofectamine-OPTIMEM mixture was added to the DNA-OPTIMEM mixture and incubated for 20 minutes at RT. In the meantime, DMEM was removed and cells were pre-conditioned with 1 ml of OPTIMEM for 20 minutes. 250 µl of OPTIMEM was added to the reaction mixture and added to the wells. 500 µl DMEM containing 30% FBS was added to the cells 24 hours post transfection. 5 X 10^5 MCF7 or MCF10A cells were seeded on a 35 mm dish for transduction. Viral supernatant was collected 48 hours post transfection and filtered through a 0.45 µm filter to get rid of the cell debris. Filtered viral supernatant containing media along with 1 ml fresh media was added to the MCF7 or MCF10A cells. 20 µg polybrene was added to the cells to increase the transduction efficiency. Cells were replenished with fresh medium 48 hours post transduction. Transduced MCF7 cells were lysed using 2X sample buffer and immunoblotting was performed to check for TopBP1 over-expression.

Statistical Analysis

IMARIS software was used to calculate the surface area and volume of TopBP1-MCF10A acini as well as nuclei. Results were analyzed and plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Mann Whitney U test was used to test the significance of difference of various parameters in morphometric analysis.

Results

Generation of MCF10A cells over-expressing TopBP1

MCF10A cells over expressing TopBP1 (mVenus-CSII-EF-MCS-TopBP1) were generated by lentiviral transduction. Viral particles containing mVenus CSII-EF-MCS TopBP1 or mVenus CSII-EF-MCS plasmid was added to MCF10A cells along with polybrene. mVenus expression was observed in transduced cells after 18hrs of transduction. These cells were then fixed and stained for anti-GFP antibody to confirm the expression of mVenus (Figure 6a). Further, ectopic expression of TopBP1 with mVenus tag was verified in TopBP1-MCF10A cells using western blotting (Figure 6b).

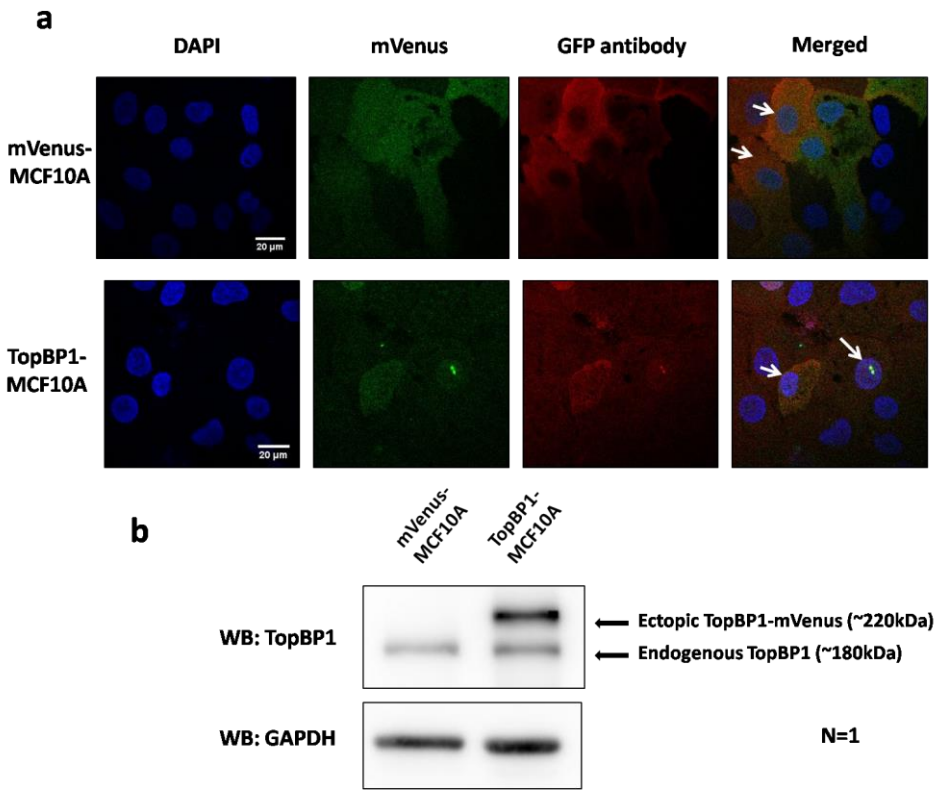


Figure 6: Ectopic expression of TopBP1 in TopBP1-MCF10A cells (2D)

a) MCF10A cells expressing mVenus [Green] (upper panel) and TopBP1- mVenus (lower panel) **b)** Western blot analysis of TopBP1 over-expression in TopBP1-MCF10A cells (2D).

Ectopic expression of TopBP1 in TopBP1-MCF10A 3D acini

To investigate the phenotypic changes induced upon TopBP1 over-expression,TopBP1-MCF10A cells cultured on Matrigel® for 16 days were analyzed using immunoflourescence. TopBP1 positive cells were identified using mVenus flourescence. To ascertain positive cells, an anti-GFP staining was done. An overall acinar staining of GFP similar to mVenus flourescence was observed in 3D cultures (Figure 7a). Western blotting with TopBP1-MCF10A 3D lysates confirmed the ectopic expression of TopBP1 in 3D acini (Figure 7b).

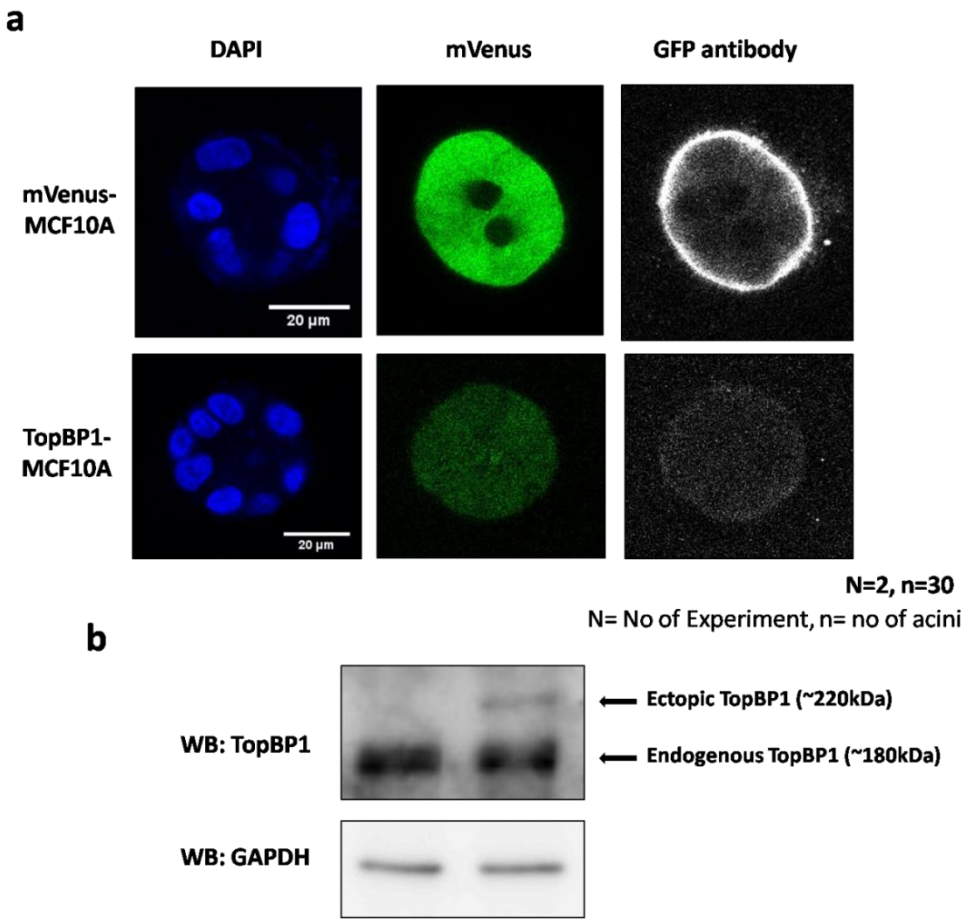


Figure 7: Ectopic expression of TopBP1 in TopBP1-MCF10A 3D acini

a) MCF10A cells expressing TopBP1-mVenus or mVenus grown as 3D "on-top"cultures. **b)** Western blot analysis of TopBP1 ectopic expression in TopBP1-MCF10A 3D acini.

TopBP1 expression and localization in MCF10A 3D acini

TopBP1 is a nuclear protein, however cytoplasmic localization of TopBP1 was observed in high grade breast tumor samples (Forma et al., 2012; Going et al., 2007). In our study, TopBP1-MCF10A acini showed an all over staining across the acini (Figure 7). To understand the expression pattern of TopBP1 in 3D acini, we performed immunostaining of MCF10A cells cultured on Matrigel® for 16 days with TopBP1 antibody. An overall expression of TopBP1 expression was observed in MCF10A 3D acini.

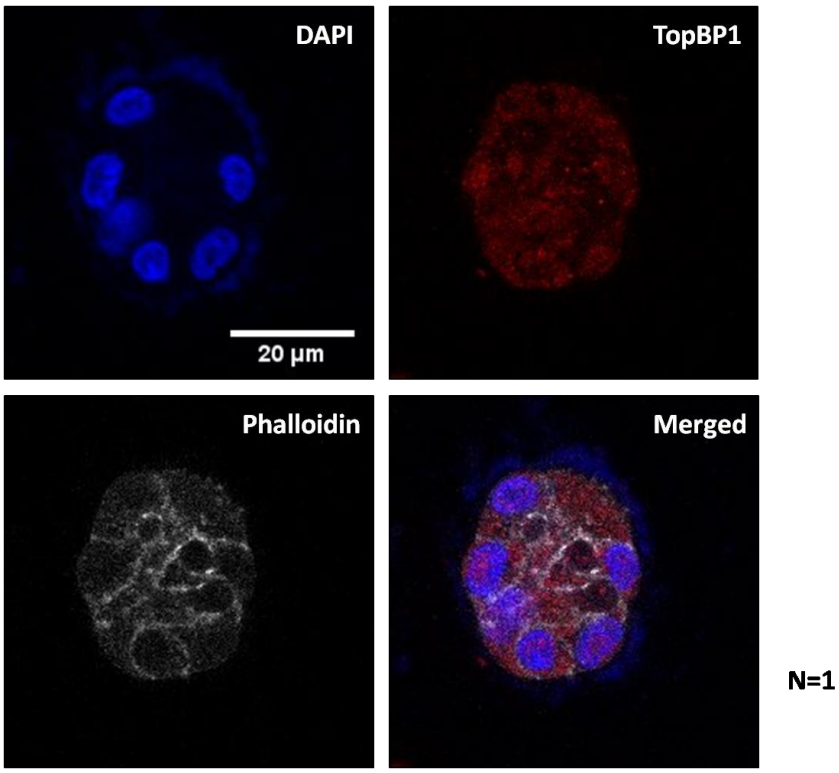


Figure 8: TopBP1 expression in MCF10A 3D acini-
MCF10A acini were fixed and immunostained for TopBP1 (red) and Phalloidin (grey).

TopBP1 over-expression led to increase in acinar as well as nuclear size

Surface area and volume of TopBP1-MCF10A and mVenus-MCF10A acini were calculated using IMARIS software. Morphometric analysis revealed that TopBP1 over-expression resulted in increased acinar surface area compared to mVenus-MCF10A acini (Figure 9a). However, no significant difference in volume was observed between the mVenus and TopBP1 MCF10A acini (Figure9b). The increase in surface area without an increase in volume can be a result of difference in the acinar shape. The 3D projection of mVenus and TopBP1 acini revealed that TopBP1-MCF10A acini had a more elongated and flattened structure compared to mVenus-MCF10A acini which were more spherical in shape (Figure 10a). This change in shape was further confirmed by calculating the sphericity of the acini. It was observed that TopBP1 over-expression led to decrease in sphericity compared to mVenus which had a sphericity of 1 (Figure 10b). No significant diffence in the number of cells per acini was observed between the mVenus and TopBP1 acini (Figure 11). In addition to that the surface area and volume of the nuclei of the cells in the TopBP1-MCF10A 3D acini were observed to be higher compared to that of mVenus-TopBP1 (Figure 12a and 12b).

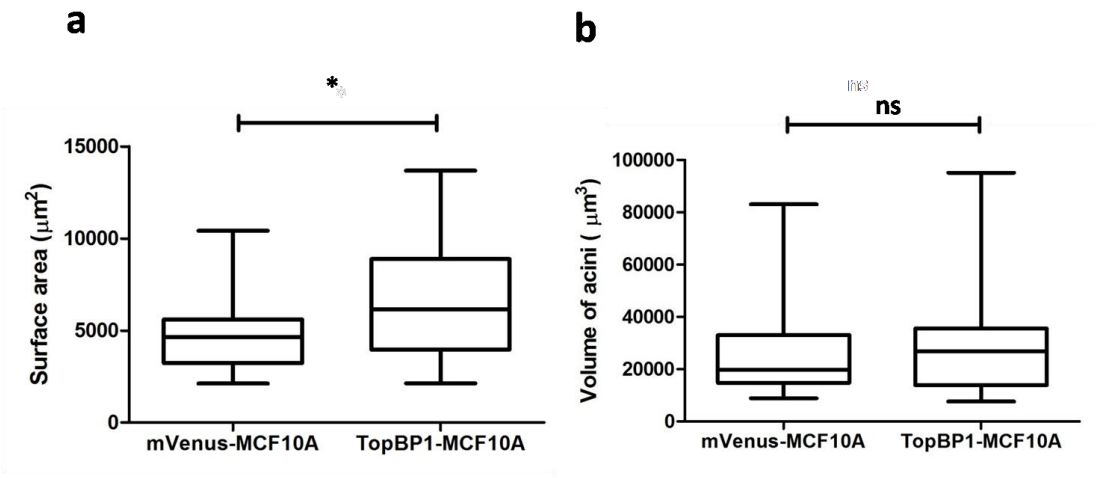


Figure 9: TopBP1 over-expression led to increase in acinar size- a) Surface area and **b)** volume of TopBP1-MCF10A and mVenus-MCF10A 3D acini were calculated using IMARIS software and represented as box plots. 30 acini from 2 independent experiments were analysed. Statistical analysis was done using Mann Whitney test (* p = 0.0350)

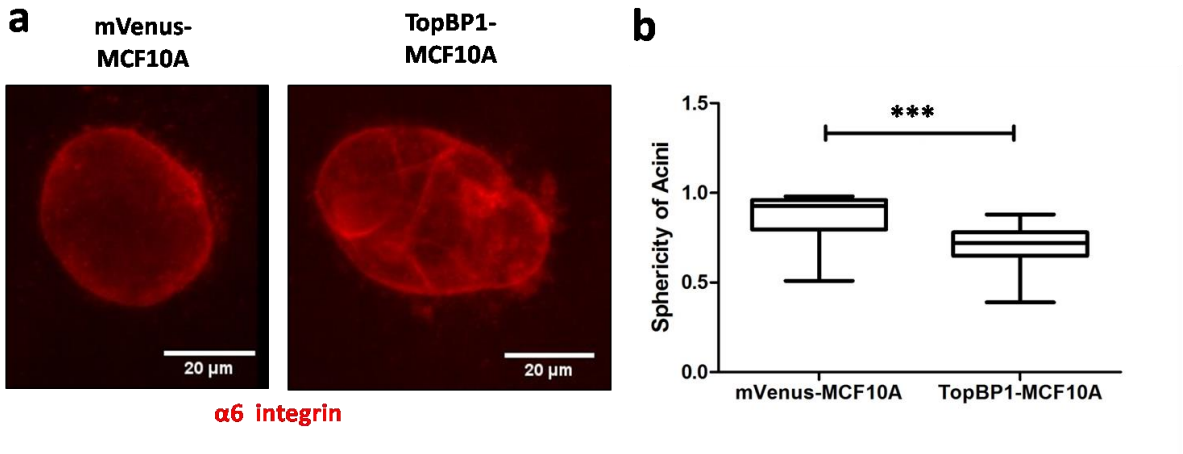


Figure 10: TopBP1 over-expression resulted in decrease in sphericity of the MCF10A acini- a) Representative images of 3D projection of mVenus and TopBP1-MCF10A acini showing variation in shape (generated using ImageJ software) **b)** sphericity of TopBP1-MCF10A and mVenus-MCF10A 3D acini were calculated using IMARIS software and represented as box plots. 30 acini from 2 independent experiments were analysed. Statistical analysis was done using Mann Whitney test (*** p< 0.0001)

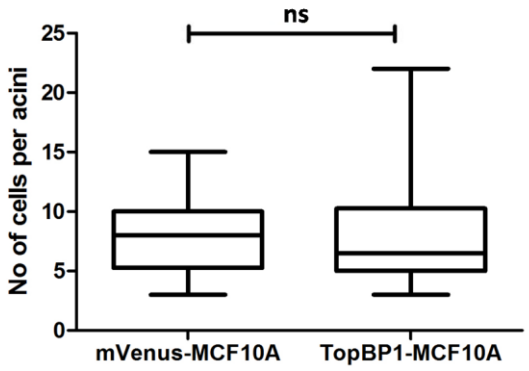


Figure 11: TopBP1 over-expression did not lead to increase in number of cells per acini- Number of cells per acini of TopBP1 and mVenus 3D acini were counted with the aid of IMARIS software. 30 acini from 2 independent experiments were analysed. Statistical analysis was done using Mann Whitney test. (ns, p = 0.4928)

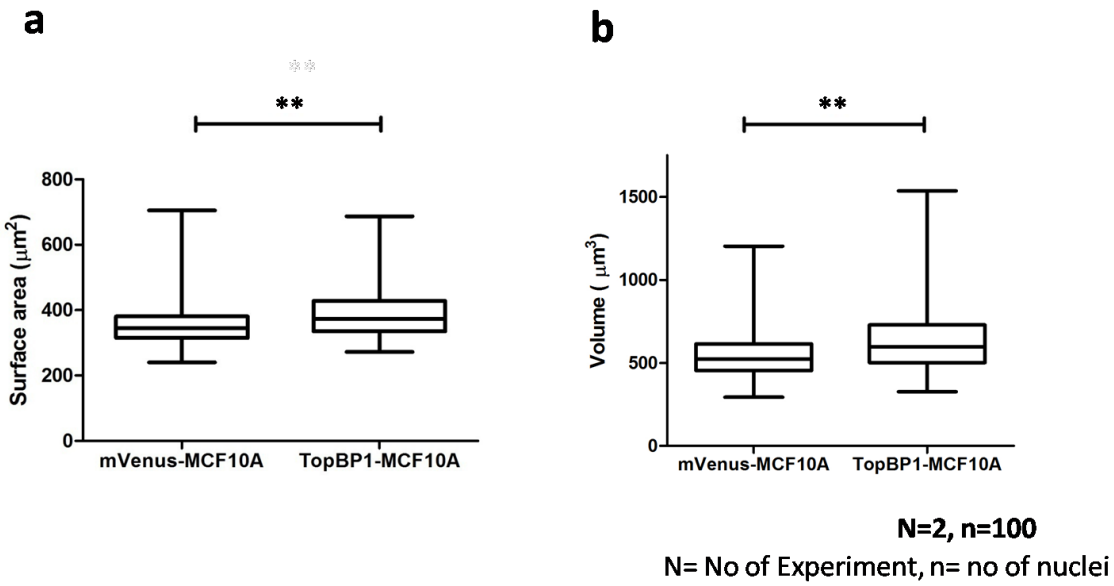


Figure 12: TopBP1 over-expression lead to increase in nuclear size- a) Surface area and **b)** volume of nuclei of cells in TopBP1 and mVenus MCF10A 3D acini were calculated using IMARIS software. 100 nuclei from 2 independent experiments were analysed. Statistical analysis was done using Mann Whitney test. (** p< 0.030)

TopBP1 over-expression disrupted basal polarity in MCF10A 3D acini

To investigate the effect of TopBP1 over-expression on polarity, TopBP1-MCF10A cells grown on Matrigel® were stained using basal polarity markers like α6 integrin and Laminin V. Loss or discontinuous staining of α6 integrin at the basal region of acini was observed in 56% of TopBP1- MCF10A acini (Figure 13a and 13b). 70% of TopBP1-MCF10A acini showed a loss or dicontinuous staining of Laminin V (Figure 14a and 14b). Preliminary results suggests that TopBP1 over-expressions leads to disruption of basal polarity.

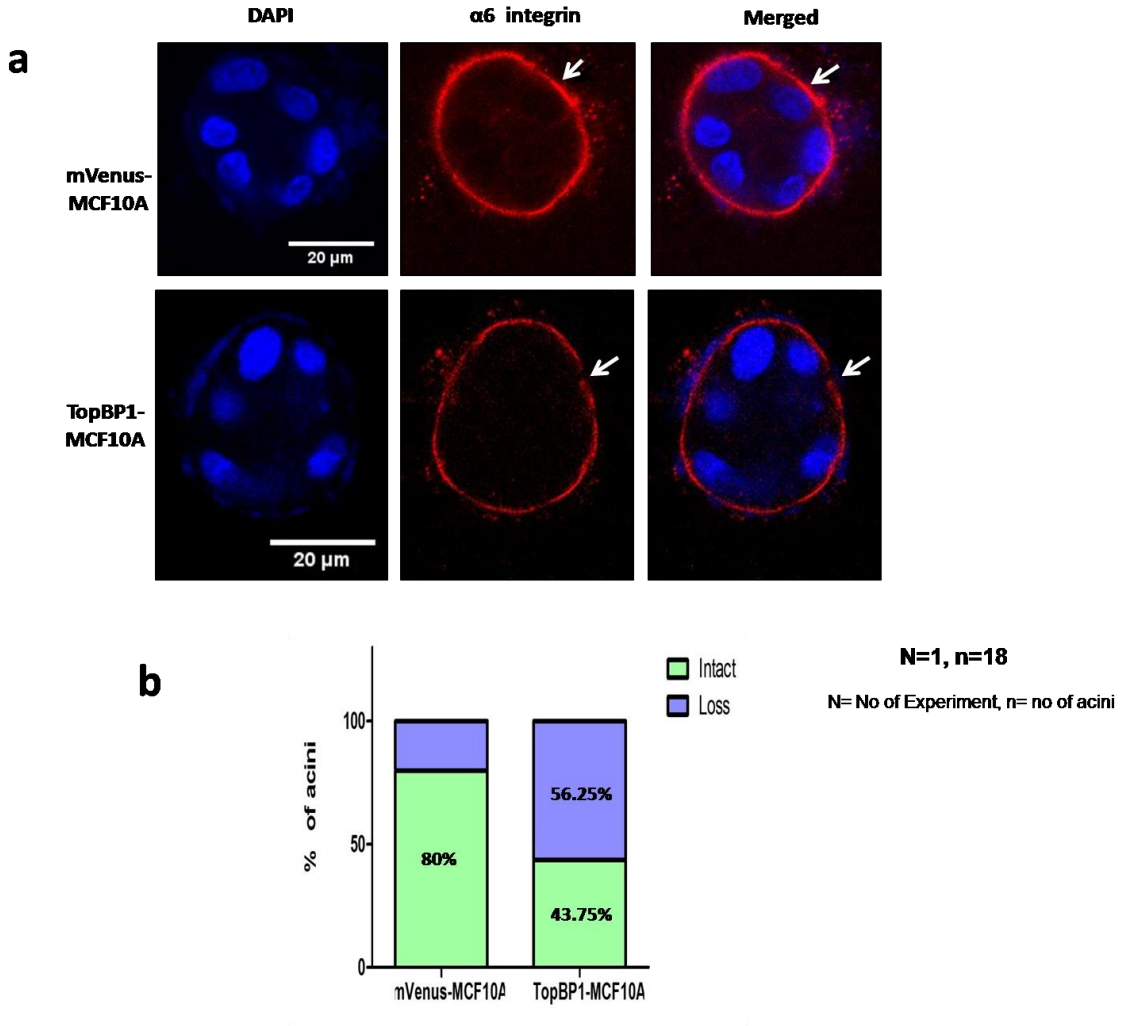


Figure 13: TopBP1 over-expression leds to loss of α6 integrin in MCF10A 3D acini- a) Representative images of TopBP1-MCF10A 3D acini showing loss of α6 integrin **b)** Images were quantified and % of acini showing loss of α6 integrin was represented as bar diagram.

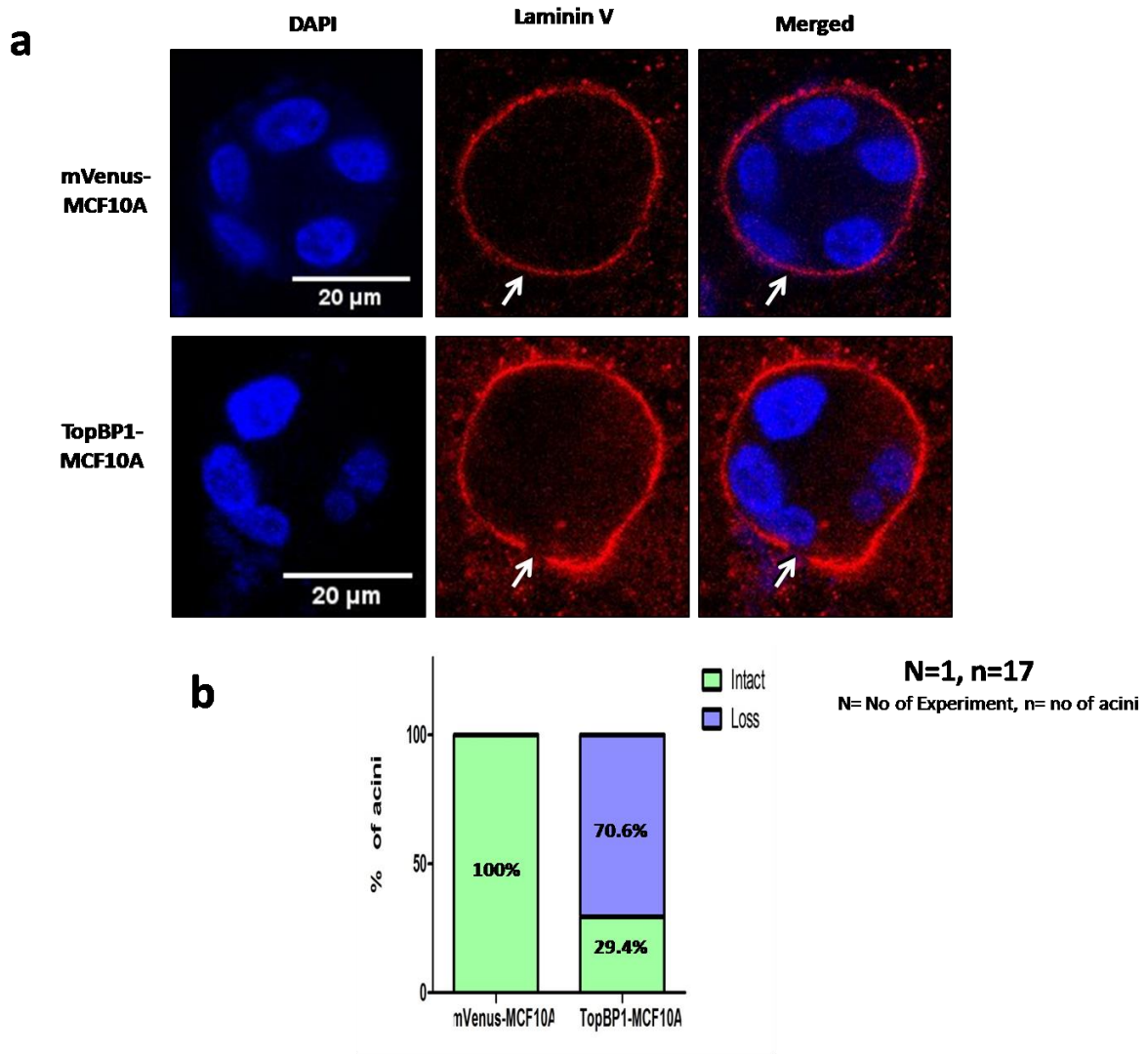


Figure 14: TopBP1 over-expression led to Laminin V loss in MCF10A 3D acini- a) Representative images of TopBP1-MCF10A 3D acini showing loss of Laminin V. **b)** Images were quantified and % of acini showing loss of Laminin V was represented as bar diagram.

<u>TopBP1 over-expression may induce EMT in MCF10A 3D acini</u>

To investigate the role of TopBP1 over-expression in epethelial to mesenchymal transition (EMT), cell lysates of TopBP1-MCF10A 3D cultures grown on Matrigel® for 16 days were collected and analysed using immunoblotting. TopBP1 over-expression led to slight increase in E-cadherin (an epithelial marker) and reduction in N-cadherin (a mesenchymal marker). EMT markers like Vimentin showed a slight increase however Fibronectin remained unchanged (Figure 15). An upregulation of β-catenin (a cell-cell junction marker) was also observed upon TopBP1 over-

expression. TopBP1 over-expression also led to a reduction in the protein levels of intermediate filaments like cytokeratin 14 and cytokeratin 19 . Preliminary results suggests that TopBP1 over-expression may induce EMT in MCF10A 3D acini. However, looking at the expression of these protein markers using immunoflourescence will be required to conclude the same.

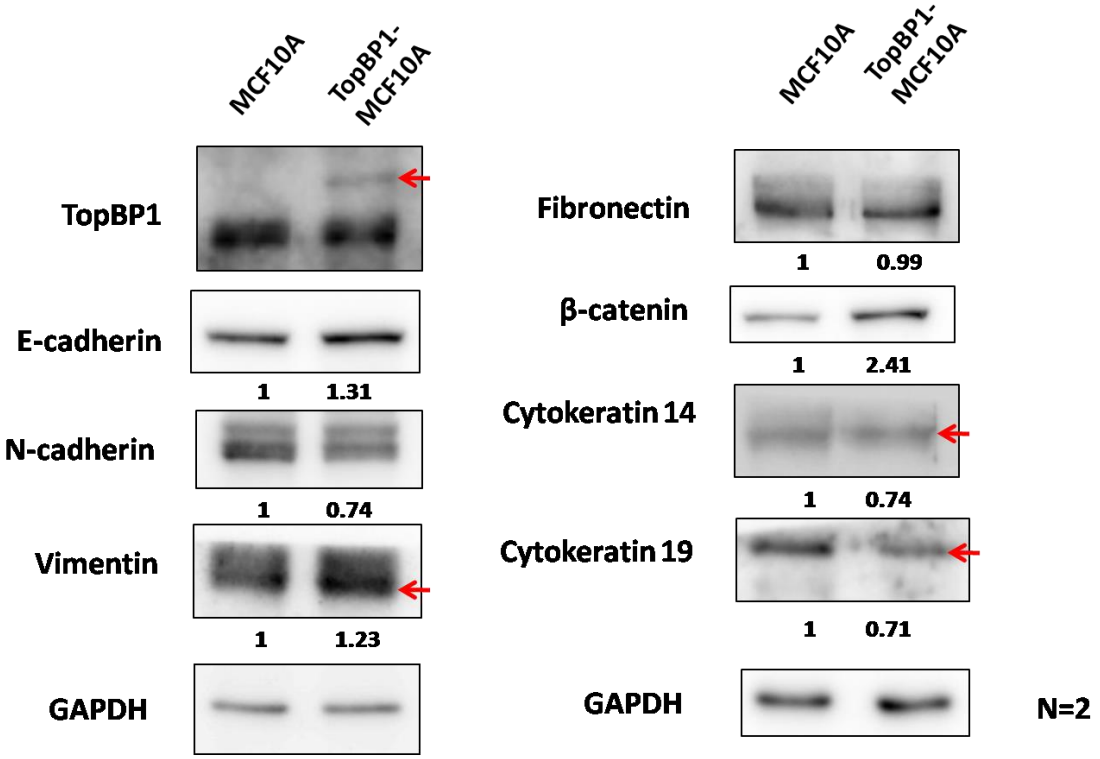


Figure 15: TopBP1 over-expression may induce EMT in MCF10A 3D acini- Immunoblots of protein lysates prepared from MCF10A 3D acini over-expressing mVenus or TopBP1 against an array of EMT markers. Quantification was done with respect to GAPDH

TopBP1 over-expression induced enhanced cell migration in MCF10A cells

Wound healing assay was performed to understand the role of TopBP1 over-expression in migration of MCF10A cells. MCF10A cells expressing mVenus or TopBP1 were seeded on either sides of the Ibidi silicon inserts which have defined cell-free gap . 16 hours post seeding, cells were treated with mitomycin C for 2

hours. Inserts were removed and the wound was visualised at 0, 6,12,18 and 24 hours. It was observed that TopBP1-MCF10A cells showed increased wound closure compared to mVenus-MCF10A (Figure 16a and 16b), suggesting that TopBP1 over-expression leads to increased collective migration in MCF10A cells.

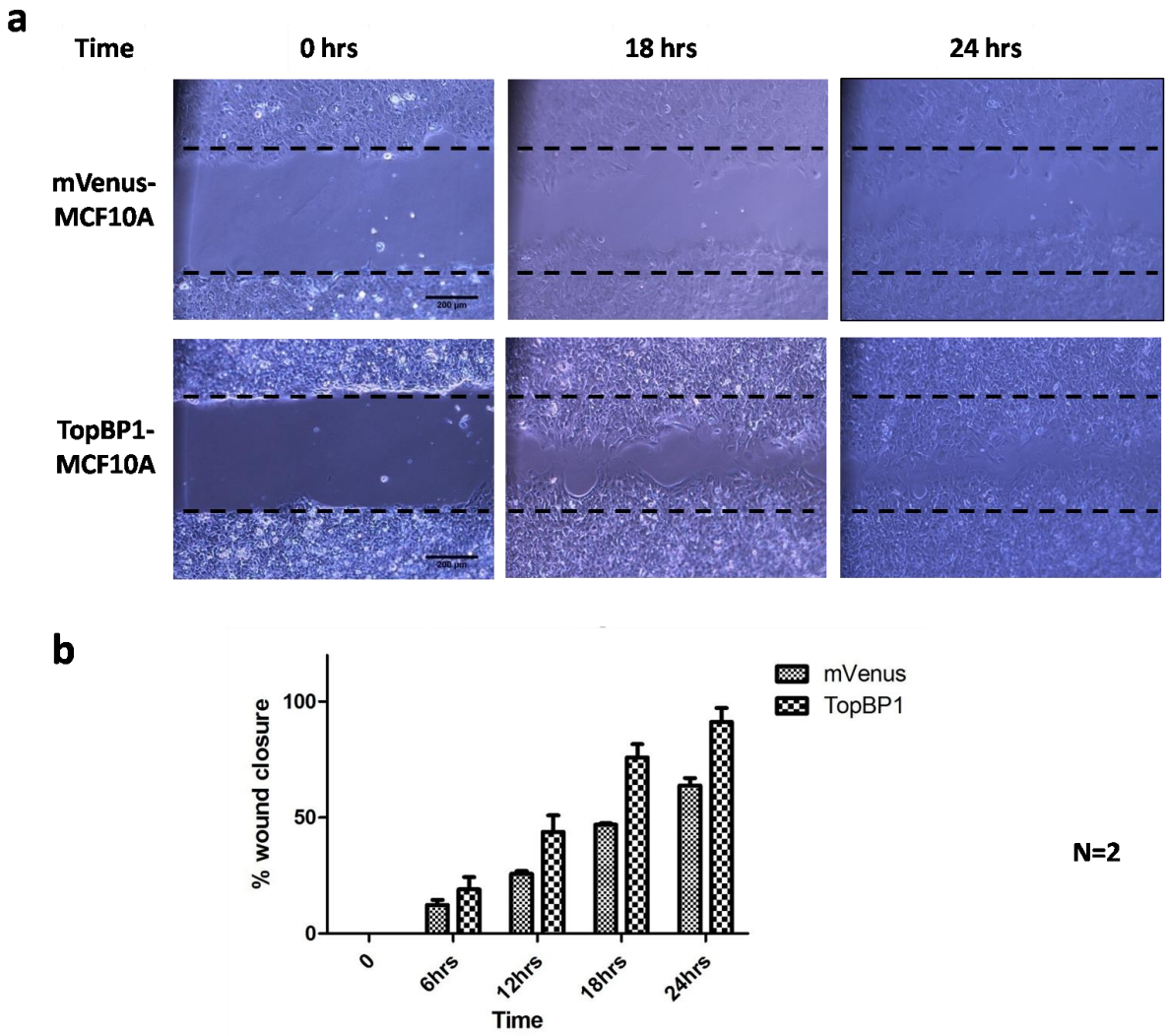


Figure 16: TopBP1 over-expression induces enhanced collective cell migration in MCF10A cells- a) Representative images of wound healing assay demonstrating collective cell migration of MCF10A cells stably expressing mVenus or TopBP1-mVenus at different time points. Images were acquired in ECLIPSE TS100 Nikon microscope at 10X magnification (scale bar-200 µm). **b)** Quantification of percentage (%) wound closure of the MCF10A cells expressing TopBP1 or mVenus.

TopBP1 over-expression induces MMP activation in MCF10A 3D acini

To study the role of TopBP1 in invasion, gelatin zymography was performed. Invading cells secrete endopeptidases like matrix metalloproteinases (MMP) into its surrounding to degrade the extracellular matrix proteins (Woessner, 1991). MMP2 and MMP9 are two such proteinases that have gelatinase activity. Media was collected from TopBP1-MCF10A and mVenus-MCF10A 3D cultures grown on Matrigel® for 16 days and loaded on to a SDS-PAGE gel which contained gelatin as a substrate. Meanwhile the 3D cultures were lysed and a parallel immunoblot for GAPDH was performed, which served as loading control. After renaturing and developing, TopBP1-MCF10A media showed more clearance of the gelatin than mVenus-MCF10A cells indicating the presence of active MMP2 and MMP9 in the media (Figure 17) . This data indicates that TopBP1 over-expression may lead to invasion.

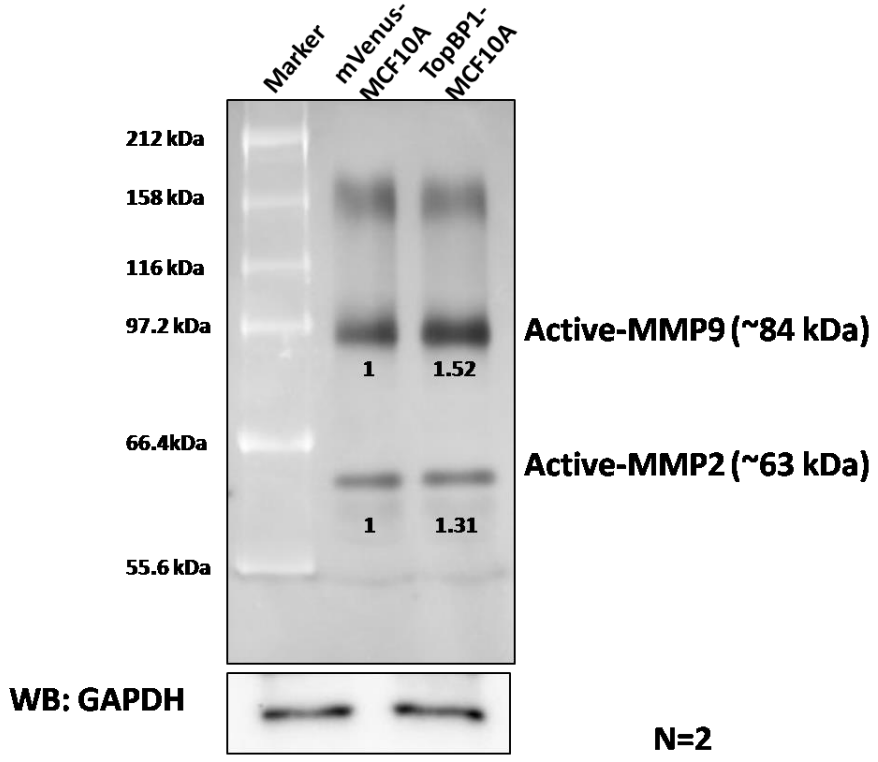


Figure 17: TopBP1 over-expression induces MMP activation in MCF10A 3D acini- a) Gelatin zymograph gel showing clearance of gelatin at the size of active MMP9 (~84 kDa) and MMP2 (~67 kDa) [inverted image]. **b)** Cell lysates from which media was collected was probed for GAPDH (western blotting) as loading control.

TopBP1 over-expression did not induced chemoresistance in MCF10A cells

To investigate whether TopBP1 over-expression induces chemoresistance in MCF10A cells, MTT assay was performed. MCF10A cells over-expressing TopBP1 or mVenus were treated with different concentrations of Camptothecin (0 µM,0.02 µM, 0.07 µM , 0.21 µM, 0.63 µM, and 1.25 µM) or Doxorubicin (0 µM, 1 µM, 3 µM, 15 µM and 75 µM) (Lim et al., 2013). MTT was added 24 hours post treatment and percentage cell viability was calculated. No significant difference in percentage cell viability was observed between mVenus-MCF10A and TopBP1-MCF10A cells upon Camptothecin (Figure18a) or Doxorubicin (Figure18b) treatment suggesting that chemoresistance was not induced by TopBP1 over-expression in MCF10A cells.

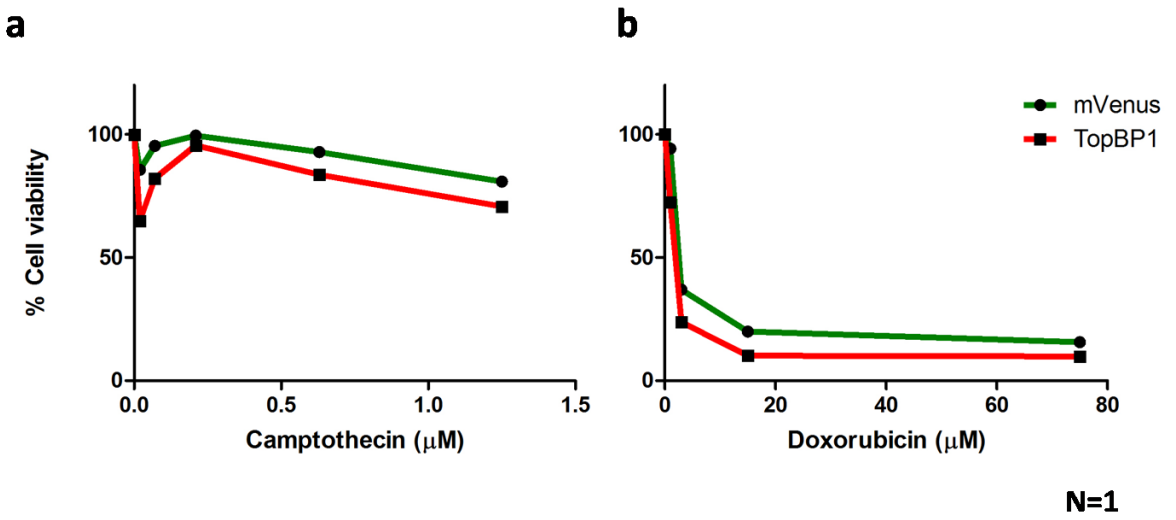


Figure 18: TopBP1 over-expression do not induces chemoresistance in MCF10A cells- % cell viability of TopBP1 or mVenus MCF10A cells calculated from MTT assay after treatment with different doses of **a)** Camptothecin or **b)** Doxorubicin

Cloning of TopBP1 into mCherry-CSII-EF-MCS vector

The flourescence of mVenus in TopBP1-MCF10A was very faint which caused difficulty in identifying the TopBP1 over-expressing acini during immunoflourescence studies. Therefore an attempt was made to cloneTopBP1 into mCherry-CSII-EF-MCS vector (Figure 19a) since mCherry is more photostable compared to mVenus (Shaner et al., 2005). mCherry-CSII-EF-MCS and mVenus-CSII-EF-MCS-TopBP1 plasmids were digested using HpaI and NotI restriction enzymes (Figure 19b and

19c), gel extracted and ligated using T4 DNA ligase. The colonies obtained after tranformation of the ligation mixture into DH5α cells were screened for insert release. Clone #1 showed a release of 4.3kb band corresponding to the size of TopBP1 (Figure 19d).

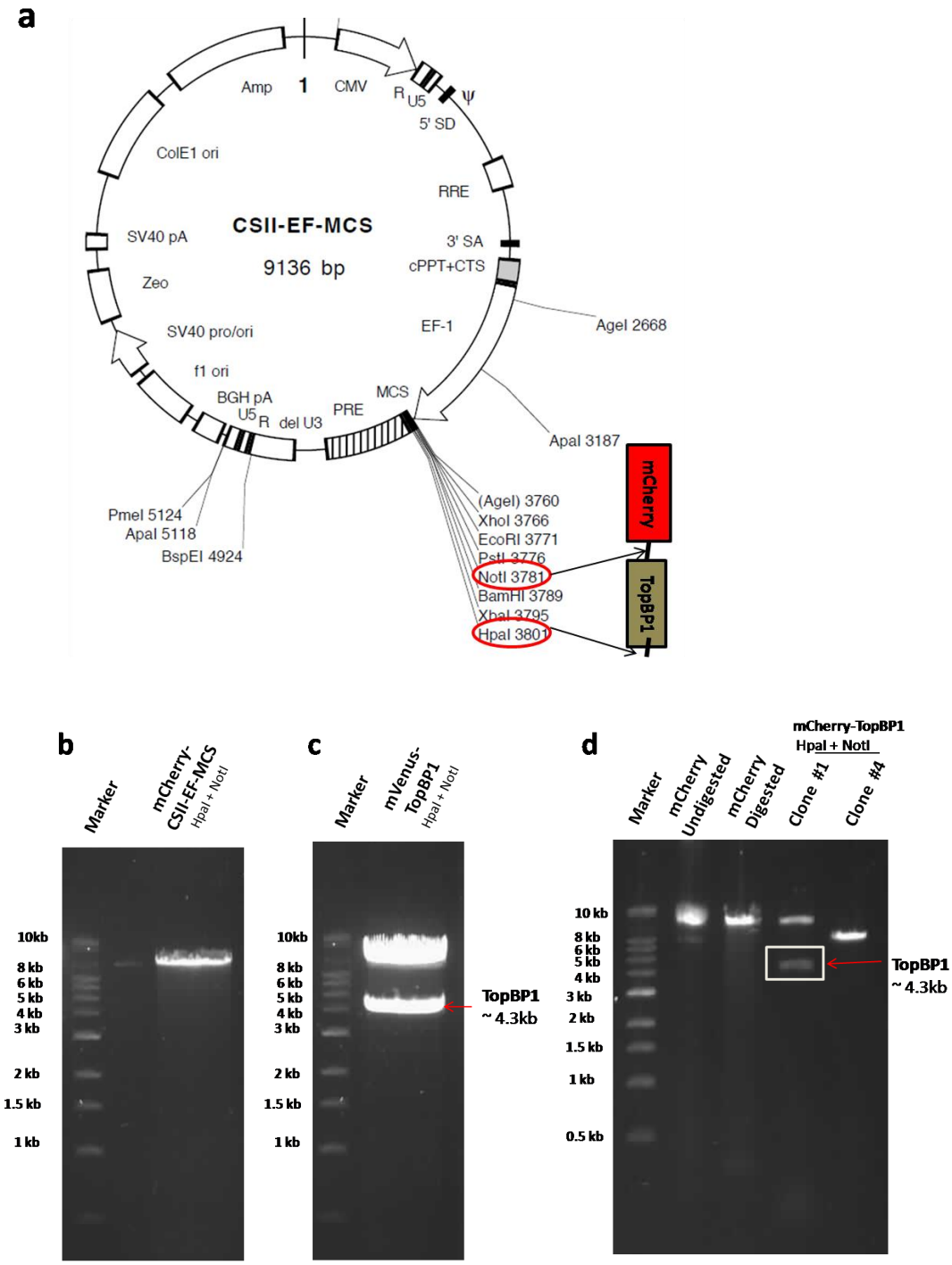
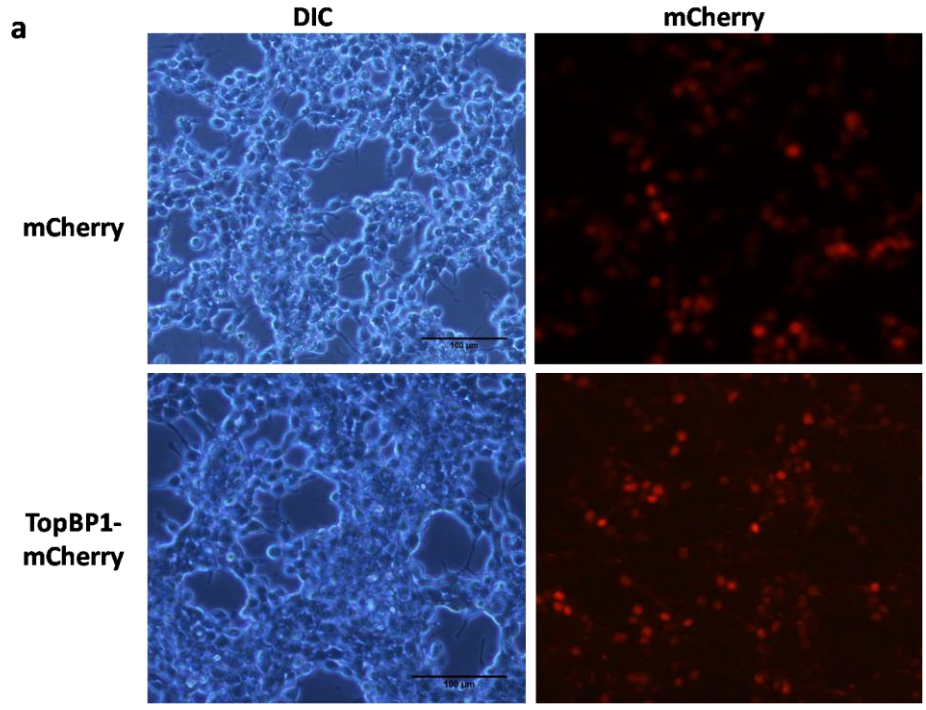


Figure 19: Cloning of TopBP1 into mCherry-CSII-EF-MCS vector-a) Vector map of mCherry-CSII-EF-MCS-X vector with TopBP1 insertion sites. Restriction digestion of **b)** mCherry-CSII-EF-MCS-X and **c)** mVenus-CSII-EF-MCS-TopBP1 using HpaI

and NotI HF restriction enzymes. **d)** Screening of Clone#1 and Clone#4 by restriction digestion using HpaI and NotI.

Expression of mCherry-CSII-EF-MCS-TopBP1 in MCF7 after stable transduction

To confirm the expression of TopBP1-mCherry, mCherry-CSII-EF-MCS-TopBP1 clone #1 obtained (Figure 19d) was used to transfect HEK293T cells along with its packaging plasmids pCMV-VSV-G-RSV-Rev and pCAG-HIVgp by lipofectamine mediated transfection for lentiviral production. mCherry fluorescence was observed in HEK293T cells 24 hours post transfection (Figure 20a). The viral supernatant obtained was used to transduce MCF7 cells for generation of stable MCF7 cell line expressing TopBP1-mCherry. Transduced MCF7 cells showed mCherry fluorescence 48 hours after transduction (Figure 20b). Western blot analysis of transduced MCF7 cell lysate using anti-TopBP1 antibody confirmed the expression of ectopic TopBP1-mCherry in MCF7 cells (Figure 20c).



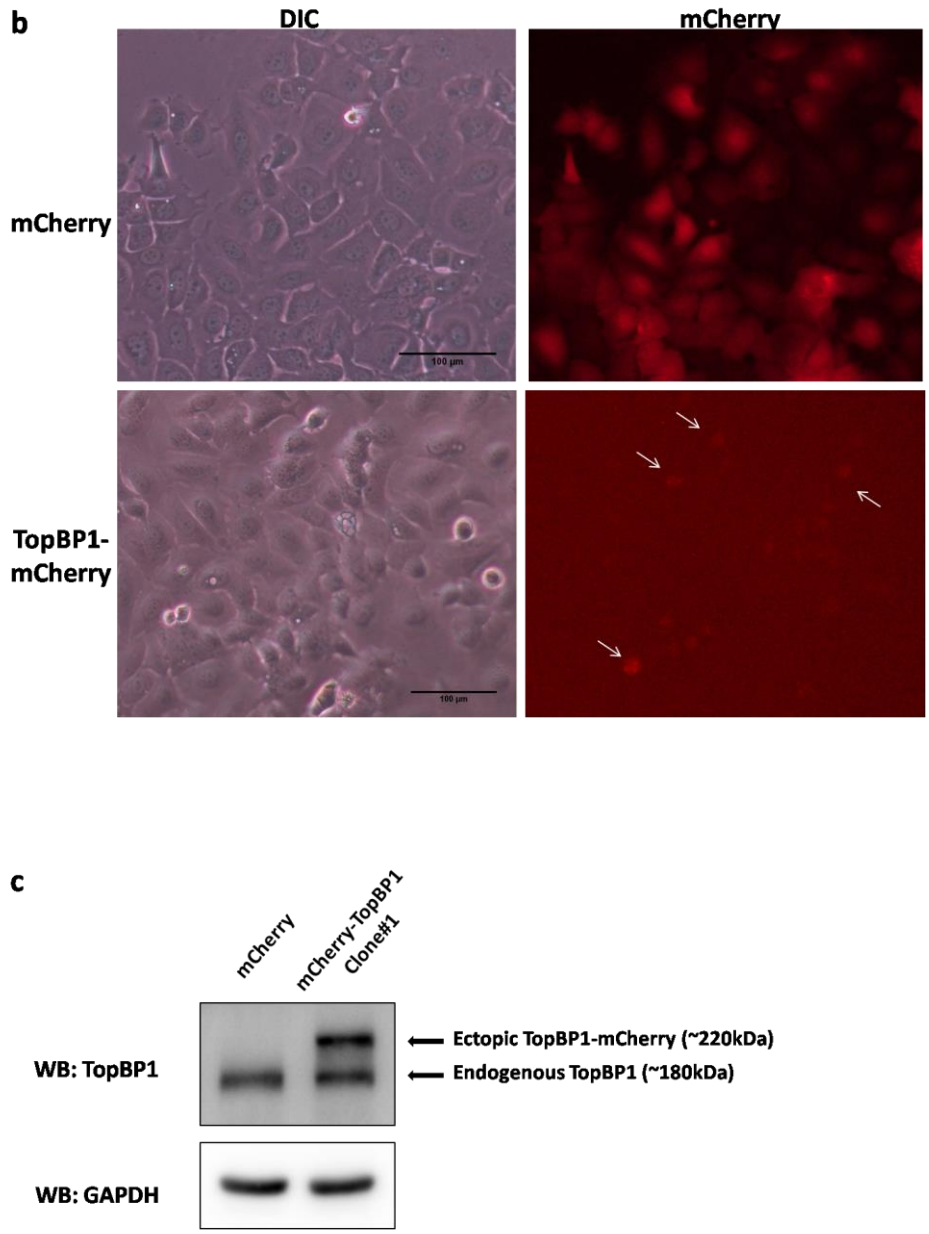


Figure 20: Expression of TopBP1-mCherry in MCF7 cells- a) Representative image of mCherry fluorescence in HEK293T cells transfected with mCherry or mCherry-TopBP1 for lentiviral generation. **b)** mCherry fluorescence in MCF7 cells tranduced with the generated lentiviral particles. Images were acquired in ECLIPSE TS100 Nikon microscope at 10X magnification (scale bar-100 µm). c) Immunoblot analysis of TopBP1-mCherry expression in transduced MCF7 cells.

To generate MCF10A cells expressing TopBP1-mCherry, lentiviral particles were prepared by transfecting HEK293T cells using the mCherry-CSII-EF-MCS-TopBP1 clone #1 along with the packaging plasmids. mCherry fluorescence was observed in HEK293T cells 24 hours post transfection (Figure 21). The viral supernatant collected after 48 hours of transfection was used to transduce MCF10A cells. However, no mCherry fluorescence was observed even after 96 hours of transduction.

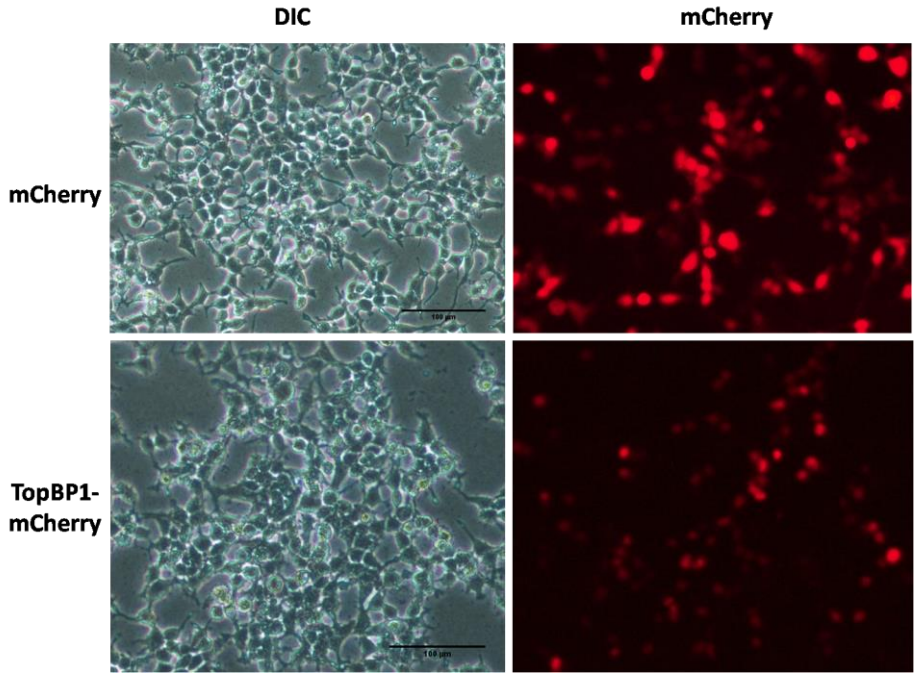


Figure 21: Expression of TopBP1-mCherry in HEK293T cells - Representative image of mCherry fluorescence in HEK293T cells transfected with mCherry or mCherry-TopBP1 for lentiviral generation for transduction of MCF10A cells.

Discussion

Cancer is caused by variation in gene(s) which may give rise to increase in cellular proliferation, decrease in apoptosis, increase in cell migration and invasion and/or EMT (Hanahan and Weinberg, 2011). TopBP1, DNA topoisomerase II β -binding protein 1, a mediator protein in the DNA damage checkpoint pathway, has been reported by various groups to be a breast and ovarian cancer susceptible gene. (Forma et al., 2012b, Karppinen et al., 2006). TopBP1 was seen to be upregulated in high grade breast cancer biopsy samples with a few outliers where no TopBP1 expression was observed (Forma et al., 2012a; Going et al., 2007; Liu et al., 2009). Increased levels of TopBP1 protein but decreased levels of *TopBP1* mRNA were also reported in high grade breast tumors (Forma et al., 2012a). All these contradicting reports made it difficult to comment on its role as an oncongene or tumor suppressor. Since it is a predicted potential breast cancer susceptibility gene, understanding its role in breast tumorigenesis is of immense clinical value. We herein demonstrate that TopBP1 over-expression can induce transformation of 3D breast acini cultures suggesting its role as an oncogene.

Analysis of the cellular and nuclear pleomorphism and extent of architectural disorder are the preliminary diagnostic and prognosis determination method for breast carcinoma (Debnath and Brugge, 2005). 3D cultures of breast epithelial cells as well as cancer cells recapitulate most of the features of these cells *in-vivo*. Detailed analysis of these characteristic features can aid in ascertaining the role of perturbed genes. The morphometric analysis of TopBP1-MCF10A acini revealed that TopBP1 over-expression induced changes in the acinar and nuclear morphology. TopBP1 over-expressing cells formed acini with slightly larger surface area but similar volume than the control acini. This anomaly was speculated to be a result of change in shape of the acini upon TopBP1 over-expression; wherein sphericity calculations revealed that TopBP1-MCF10A acini were elongated and flattened as compared to control. Apart from this, no significant increase in number of cells per acini was observed in TopBP1-MCF10A compared to mVenus control. In addition to this, nuclear morphology also appeared to be disrupted, with the TopBP1 over-expressing cells having larger nucleus; which is supported by reports from various groups which demonstrate a change in nuclear morphology in cancer (Debnath and

Brugge, 2005)(Zink et al., 2004). In support of this observation, TopBP1 is known to play major role in maintaining genomic stability, ensuring proper DNA replication and G1/S transition in cell cycle (Kim et al., 2005); perturbation in its level can distort the DNA synthesis and cell cycle which may alter the nuclear content. However further validation is required to conclude the same. FACS analysis and metaphase spread will provide valuable insights about this phenotype.

De-regulation of apico-basal polarity is one of the characteristics of epithelial cancer (Debnath and Brugge, 2005). In invasive cancer, the cells lose their contact with neighboring cells and break down the basement membrane to invade into other tissues (Moreno-Bueno et al., 2008). Here, preliminary results suggest that TopBP1 over-expression leads to disruption of basal polarity. The loss of basal polarity markers like α6 integrin and Laminin V indicates the reduced interaction of acini with ECM which can be an indication of invasive phenotype. However, further analysis of various cell-cell junction and apical markers are required to conclude the role of TopBP1 in apico-basal polarity disruption.

EMT is a process through which epithelial cells dedifferentiate and acquire mesenchymal phenotype that can increase their motility and invasiveness. Disruption of basal polarity can lead to reduced interaction with ECM which can be followed by EMT, one of the early steps in invasion. TopBP1 over-expression in MCF10A 3D acini resulted in increase in the protein levels of mesenchymal markers like vimentin and β-catenin where fibronection remained unchanged. Slight reduction in the level of epithelial markers like cytokeration 14 and cytokeratin 19 was also observed. However N-cadherin and E-cadherin showed an opposite result than expected (Bae et al., 2015) where N-cadherin (mesenchymal marker) was down-regulated and E-cadherin (epithelial marker) was up-regulated. Given that the over-expression of TopBP1 is not too high; the effect in the levels of EMT markers seen can be significant. Further analysis using immunoflouresence and semi-quantitative PCR is required to confirm the role of TopBP1 in induction of EMT.

Further, we investigated the migratory and invasive potential of TopBP1-MCF10A cells by wound healing and gelatin zymography respectively. During invasion and metastasis, cells move either as single entities (Theveneau and Mayor, 2011) or collectively as small groups (Deisboeck and Couzin, 2009) and secrete

endopeptidases like matrix metalloproteinases (MMP) into its surrounding to degrade the extra cellular matrix proteins (Woessner, 1991). Wound healing assay can be used to investigate collective cell migration. Our study showed that upon TopBP1 over-expression the collective migration of MCF10A cells were enhanced. Single cell migration assay has to be done to investigate the role of TopBP1 over-expression in solitary migration. MMP acitivation was also observed in TopBP1-MCF10A 3D acini medium which suggested a role of TopBP1 in invasion. However, invasion assays like collagen-Matrigel® assay or DQ collagen assay need to be performed to conclude the same.

The analysis of four breast cancer databases by Lin's group clearly demonstrates that higher levels of TopBP1 are associated with an increased risk of relapse or death. This suggests that TopBP1 may be inducing chemoresistance in the patient who have undergone chemotherapy. Also recent studies have shown that TopBP1 over-expression leads to radioresistance of lung cancer brain metastasis (Choi et al., 2014) and chemoresistance in C33A cells to cisplatin (Liu et al., 2011). However, our study showed TopBP1 over-expression did not induced chemoresistance to camptothecin or doxorubicin in MCF10A cells.

Thus, the preliminary study demonstrates the possible role of TopBP1 in tumorigenesis. Further studies will be done to establish the same.

Future Plans

In our study, the role of TopBP1 in polarity disruption and EMT requires further validation using immunofluorescence assay. MCF10A cells stably expressing TopBP1-mCherry are required to do the same. Firstly, TopBP1-mCherry-MCF10A stable cell line needs to be generated. An attempt to generate the cell line was made. However the inherent difficulty in transducing MCF10A cells along with the low viral titer led to unsuccessful transduction. Therefore, transduction of MCF10A needs to be carried out by increasing lentiviral titer as well as the concentration of polybrene to increase the transduction efficiency.

Our results suggest that TopBP1 over-expression leads to transformation of breast epithelial cells. Now, it is of great importance to elucidate the mechanism by which TopBP1 induces transformation. With the available literature, we have tried speculating a pathway, as depicted in Figure 22. Studies from Lin's group showed the role of TopBP1 in inhibition of p53 and E2F1 transcriptional activity which then leads to inhibition of apoptosis and G1 arrest (Figure 22) (Liu et al., 2009; Liu et al., 2003). TopBP1 can also lead to increase in proliferation and reduction in apoptosis by inducing mutant p53 (mut p53) gain of function (Figure 22) (Liu et al., 2011). Previous reports have shown that polarity disruption in MCF10A cells can be induced by mutant p53 gain of function through EMT (Zhang et al., 2011). So we can speculate that TopBP1 induced transformation might be through induction of mut p53 gain of function. However, in our study, p53 protein levels remained unchanged upon TopBP1 over-expression (Figure 22). It can be speculated that the TopBP1 over-expression that is obtained from our results is not sufficient to induce inhibition of p53. We need to validate this hypothesis by either increasing TopBP1 over-expression in MCF10A cells by multiple transduction, or by over-expressing TopBP1 in MCF10AT1, a premalignant breast epithelial cell line (MCF10A with an H-Ras mutation) and then investigate the p53 levels. Further analysis of p53 and E2F1 downstream targets upon TopBP1 over-expression is required to validate the speculated pathway for TopBP1 induced transformation.

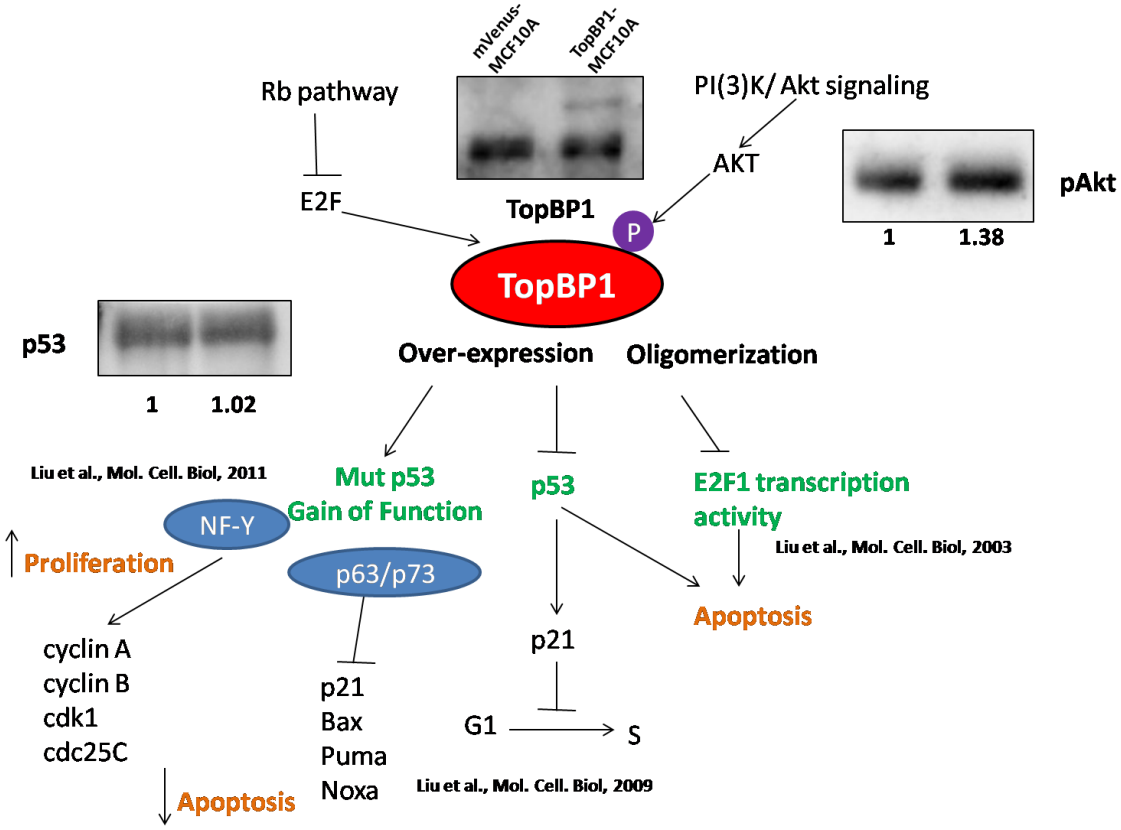


Figure 22: Speculated pathways involved in TopBP1 induced transformation

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