PHOSPHORYLATION OF ANNEXIN A2 IS ESSENTIAL FOR ITS ASSOCIATION WITH EXOSOMES AND FOR MIGRATION, INVASION AND PROLIFERATION IN TRIPLE NEGATIVE BREAST CANCER

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- Priyanka Prakash Desai

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LIST OF ABBREVIATIONS:

TNBC – Triple-negative breast cancer

AnxA2 – AnnexinA2

GFP – Green fluorescent protein

DMFS – Distant metastasis free survival

OS – Overall survival

Tyr23 – Tyrosine 23

Ser11/25 – Serine 11/25

MMP-2/9 – Matrix Metalloproteinase-2/9

tPA- Tissue type plasminogen activator

CAL-148 – Centre Antoine Lacassagne - 148

CML – Chronic Myelogenous Leukemia

FBS – Fetal Bovine serum

WT – wild type

PBS – phosphate buffered saline

BSA – Bovine serum albumin

CD 81 – Cluster of Differentiation 81

Tsg 101 – Tumor susceptibility gene 101

ALIX - Apoptosis linked gene (ALG) 2 interacting protein X

GM130 – (Golgin A2; GOLGA2) *Cis*-Golgi matrix protein

Hsc70 – Heat shock cognate 70

SDS-PAGE – Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

kDa – Kilodalton

IP – Immunoprecipitation

IB- Immunoblot

ILV – Intraluminal vesicles

MVB – Multivesicular bodies

TME – Tumor microenvironment

CAFs – Cancer associated fibroblasts

PCa – Prostate cancer

CHAPTER 1: INTRODUCTION

INTRODUCTION

Triple-negative breast cancer

Triple-negative breast cancer (TNBC) is characterized by heterogeneous complex pool of diseases. It accounts for 15-20 % of total breast cancer cases [1]. According to the National Cancer Institute's Surveillance, Epidemiology and End Result Program (SEER), the new breast cancer cases are expected to be around 266,120, out of which 40,920 deaths are predicted to result from the disease [2]. TNBC has been associated with poor prognosis, high risk of relapse, low distant metastasis free survival (DMFS) and overall survival (OS) [3]. It lacks expression of progesterone, estrogen and HER2 (Human Epidermal Growth Factor-2) receptors and remains unresponsive to targeted therapies. Hence, it is characterized among the most aggressive type of breast cancer [4]. Recent data suggests that metastasis to different organs is associated with high mortality rate in TNBC. Our lab has previously shown that there is a reciprocal relationship between HER2 and AnnexinA2 (AnxA2), a calcium-dependent phospholipid binding protein, in TNBC which explains the role of AnxA2 in poor clinical outcomes [5]. We have also shown that AnxA2 present on the surface of the exosomes leads to increase in organ specific metastasis in TNBC. We have shown by in-vivo experiments that breast cancer cells can migrate to lung and brain in presence of exosomal AnxA2 and create favourable conditions for pre-metastatic niche formation [6]. Such metastasis is associated with different factors and intercellular communication between tumor cells [7].

AnnexinA2 (AnxA2)

The Annexins are a family of proteins that bind to negatively charged phospholipids in a calcium-dependent manner. They are identified in invertebrates, vertebrates, plants, fungi and protists [8].

There are 12 members in the family of annexins which are identified and named as AnxA1-11 and AnxA13. Annexins have been implicated in many functions like maintaining membrane cytoskeletal dynamics [9], signal transduction, RNA binding, epithelial and endothelial cell polarity, exocytosis-endocytosis and extracellular receptor activity [10]. Annexins have also been involved in many pathological conditions like cancer [11,12], diabetes, inflammation, cardiovascular diseases, autoimmune diseases such as Rheumatoid arthritis [13] and Systemic lupus [14,15].

Annexin A2 (also called as p36, annexin II, AnxA2, calpactin I, lipocortin II or placental anticoagulant protein IV) is a 36 kDa protein which is located on the chromosome number 15 [16]. It is highly expressed in many endothelial cells, macrophages, mononuclear cells and many types of tissues. It consists of two domains: a highly variable amino-terminal and a highly homologous carboxy-terminal domain. The carboxy-terminal core domain consists of four alpha helical structures each consisting of 70 amino acids which together results in tightly packed structure with a concave and convex sides [8]. This carboxy terminal has binding sites for heparin, mRNA [17], calcium, phospholipid and F-actin [8, 18]. The highly variable amino-terminal contains sites for post-translational modification and for protein-protein interactions which impart unique characteristics to AnxA2 [8]. It is located on the concave side of the core structure [19]. It has sites for post-translational modifications like Serine (Ser)1 for acetylation, Lysine-9 for sumoylation and Ser11, Ser25 and Tyrosine (Tyr)23 for phosphorylation [10]. Ser11, Tyr23 and Ser25 are the most important sites in AnxA2 N-terminal region for the process of phosphorylation. Ser11, Ser25

and Tyr23 have been associated with different function. Serine phosphorylation in

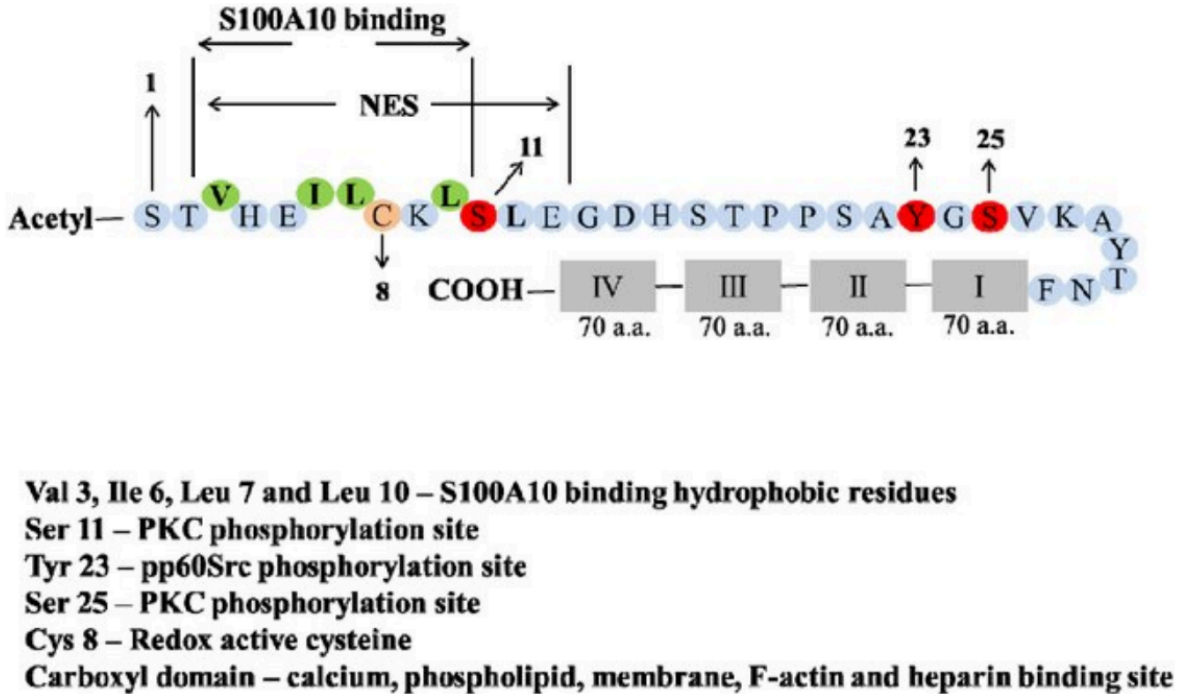

Figure 1: Domain structure of annexin A2. Annexin A2 structure consists of two domains : the amino-terminal domain and carboxyl-terminal domain. The amino-terminal domain has 32 amino acids (Ser1-Phe32). It is the site for post-translational modifications at amino acids, Ser-1 for acetylation and Ser-11, Tyr-23, Ser-25 for phosphorylation. It also has Cys8 as a redox active cysteine. The S100A10 binding site has Val-3, Ile-6, Leu-7 and Leu-10 making contacts with S100A10. The carboxyl-terminal domain has four alpha-helical domains each containing 70 amino acids with binding sites for heparin and RNA, calcium and phospholipid and as well as for F-actin. (*Bharadwaj et al, 2013, International Journal of Molecular Sciences*)

AnxA2 regulates recruitment and docking of secretory granules, association with p11 for formation of hetero-tetramer complex of $(AnxA2\text{-}p11)_2$ and sequestration of translationally inactive mRNA complexes [17, 20]. On another side, Try phosphorylation regulates actin

dynamics by regulating Rho/ROCK pathway which results in cell rounding. It also plays a role in AnxA2 association with endosomal compartments [21, 22]. Tyr23 phosphorylation in AnxA2 has been associated with many cancers like breast and pancreatic cancer. Tyr23 phosphorylation of AnxA2 has shown to be essential for invasion and proliferation and Stat3 phosphorylation in Human breast cancer SK-BR3 cells [23]. Studies have shown that Tyr23 phosphorylation and cell surface localisation of AnxA2 is necessary for invasion and metastasis in pancreatic cancer [9]. AnxA2 in its phosphorylated form at Tyr23 is necessary for in-vitro capillary formation and integrity[24]. Hence, we try and elucidate the phosphorylation of AnxA2 at Tyr23 in associating AnxA2 with exosomes and how exosomal AnxA2 impart metastatic phenotype to other breast cancer cells.

Exosomes

Exosomes are nano-sized vesicles which range in size from 40-120 nm with a density of 1.13-1.19 g/ml. It is formed during maturation of endosomes and during budding of late endosomes as intraluminal vesicles (ILVs) of multi-vesicular bodies (MVBs) [25]. Exosomes are released by different cell types and are present in all bodily fluids like breast milk, cerebrospinal fluid, amniotic fluid, blood, urine, saliva, etc [26-28]. Exosomes were first identified during the maturation process of reticulocytes where transferrin receptor gets exocytosed with the help of MVBs and gets released by 50 nm sized nanovesicles further named as exosomes [29]. Exosomes are mainly cytoplasm with enclosed lipid bilayer. Exosomes contains different proteins, carbohydrates and lipids. Exosomes contains different types of tetraspanins like CD9, CD81, CD63,CD82, co-stimulatory molecules CD86, adhesion molecules CD11b and CD54, heat shock proteins like HSP 70, HSP 90 and Hsc70, MHC class I and II molecules. Because exosomes are formed by

invagination of late endosomes, proteins involved in trafficking and vesicle formation like Apoptosis linked gene (ALG),2 interacting protein X(ALIX) and tumor suspectibility gene 101 (Tsg 101) are also present in the exosomes [30].

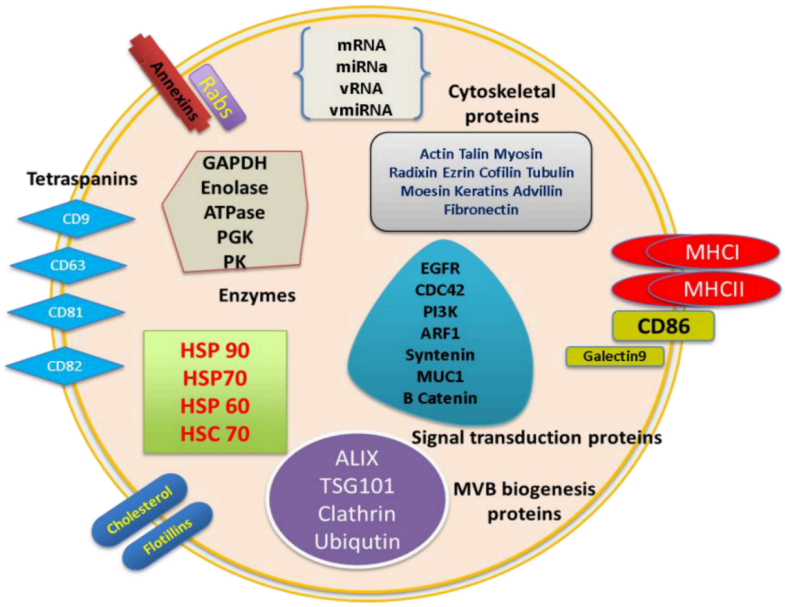

Figure 2: Structure and composition of exosomes. Exosomes consists of different proteins like tetraspanins like CD9, CD81, CD63,CD82, co-stimulatory molecules CD86, adhesion molecules CD11b and CD54, heat shock proteins like HSP 70, HSP 90 and Hsc70, MHC class I and II molecules. It also contains mRNA, miRNa and DNA. Proteins involved in exosome secretion like ALIX and Tsg 101 are also present in the exosomes (*Chahar et al, 2015, Viruses*)

Rab family proteins like Rab27a and Rab27b are also present in exosomes which are important for exosome secretion [31]. Exosomes also contain signal transduction proteins like G-proteins and protein kinases [32]. All these proteins are used as markers for exosome confirmation to distinguish from other vesicles. Exosomes are involved in lots of physiological states both beneficial and pathological. Exosomes functions are involved in transferring protein or nuclear

material from one cell to another, propogation of pathogens, elimination of unnecessary proteins, antigen presentation and many more.

Exosomes and Cancer

Exosome release is a normal process, but its high amount of secretion with varied pool of protein expression could potentially lead to oncogenic transformation in a cell. Transfer of miRNAs have been greatly studied for few years. Esophageal squamous cell carcinoma and glioblastoma cells have exhibited increase in miRNAs with increased aggressiveness and tumorigenesis. Increase in miR-17, miR-19a, miR21 has shown positive correlation with progression of metastatic melonoma [33, 34]. Exosomes isolated from prostate cancer (PCa) patients also showed higher expression of miR-141 [35]. In glioma, oncogenic receptor EGFRvIII was reported to be transported between the cells via exosomes which transferred transforming phenotype and EGFRvIII-dependent transcription [36]. Tumor microenvironment (TME) consists of different factors like extracellular matrix components, immune cells and tumor associated vasculature. Cancer associated fibroblasts (CAFs) are known to secrete different components in TME which help the microenvironment to modulate conditions for tumor cells to grow and metastasize. These CAFs secrete exosomes to modulate TME [37]. Cancer cells use different strategies to suppress immune response in TME. Cancer cells create hypoxic conditions to suppress immune response in TME. It has been shown that hypoxic PCa exosomes enhance invasiveness and stemness in PCa cells and also causes neoplastic changes in fibroblasts in TME [38] Early expression of exosomal miR-105 has been reported in metastasis. Suppression of miR-105 inhibits angiogenesis and the process of breast cancer metastasis. Hence, miR-105 could be used as a non-invasive early predictor or diagnostic marker in cancer [39]. It is reported that exo-AnxA2 released in TME leads to organ specific

metastasis in TNBC, wherein, breast cancer cells migrate to lung and brain by triggering macrophage-mediated activation of p38MAPK, NF-κB, and STAT3 pathways. Exo-anxA2 also increases secretion of IL-6 and TNF-α in TME [6]. Thus, studying AnxA2 phosphorylation and whether it leads to association of cell surface AnxA2 with exosomes becomes a crucial step towards studying TNBC metastasis. So, here we hypothesize that phosphorylation of AnxA2 is pivotal for its association with exosomes and for promoting migration, invasion and proliferation in triple-negative breast cancer cells.

CENTRAL HYPOTHESIS:

Our **central hypothesis** is "Phosphorylation of Annexin A2 is essential for its association with exosomal surface and for promoting migration, invasion and proliferation in triple negative breast cancer cells".

Scientific premise: AnxA2 phosphorylation at Tyr23 has been implicated in many cancers [40-43]. Several studies have suggested the role of AnxA2 in migration, invasion, proliferation, adhesion and angiogenesis [44, 45]. AnxA2 has been shown to be present on the surface of the exosomes by the proteomic profiling of Exocarta data [46]. It has been shown that Exo-AnxA2 is essential for pre-metastatic niche formation and the process of angiogenesis in TNBC [6]. So, here we try and elucidate the function of Tyr23 present in the N-terminal region of AnxA2 and its function in associating AnxA2 with exosomes and for promoting metastatic phenotype in cancerous cells.

So here we test our hypothesis with 2 aims:

Specific aim 1: To determine whether Tyr23 phosphorylation at the N-terminus region of AnxA2 increases its translocation to the surface and promotes migration , invasion and proliferation in MDA-MB-231 TNBC cells.

Specific aim 2: To determine whether Tyr23 phosphorylation at the N-terminus region of AnxA2 increases its association with the exosomal surface (exo-AnxA2) and whether increased exo-AnxA2 promotes migration, invasion and proliferation in TNBC.

CHAPTER 2: MANUSCRIPT

Phosphorylation of Annexin A2 is essential for its association with exosomes and promoting migration, invasion and proliferation in triple negative breast cancer

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Abstract:

Exosomes are membrane enclosed small vesicles that range from 40 – 120 nm in size and participate in cell-cell communication by transferring proteins to other cells. Annexin A2 (AnxA2), a calcium-dependent phospholipid binding protein, is present on the surface of the exosomes. AnxA2 phosphorylation plays an essential role in many physiological conditions by forming a heterotetrameric complex with p11 or S100A10 on the cell surface. We demonstrate here that the phosphorylation at Tyrosine (Tyr)-23 in the N-terminal region of AnxA2 is consequential for its association with the cell surface. This association increases the migratory, invasive and proliferative capacity of MDA-MB-231 triple negative breast cancer (TNBC) cells. An increase in cell surface AnxA2 further leads to a stronger association of AnxA2 with the exosomal surface. We also demonstrate that AnxA2 enriched exosomes promote proliferative and invasive characteristics of a different recipient cell [CAL (Centre Antoine Lacassagne) – 148]. These results demonstrate that Tyr23 phosphorylation of AnxA2 is pivotal for its association with exosomes and for imparting more malignant characteristics to the other breast cancer cells. Thus, AnxA2 could be used as a targeting approach for developing a treatment of TNBC.

Keywords: Tyrosine 23, phosphorylation, Annexin A2, exosomes, breast cancer

INTRODUCTION:

According to the National Cancer Institute's Surveillance, Epidemiology and End Result Program (SEER), in 2018, the estimates for new breast cancer cases are expected to be 266,120 and of those, 40,920 deaths are forecasted to result from the disease [2]. Despite advances in the treatment of breast cancer, some breast cancer subtypes still have a poor prognosis. Triple-negative breast cancer (TNBC) which represents up to 15-20 % total cases of breast cancer, is one of the most aggressive breast cancer types. It remains unresponsive to anti-hormonal and HER2 (Human Epidermal Growth Factor-2)-targeted therapies as it lacks estrogen, progesterone and HER2 receptors [4]. In order to study mechanisms that lead to the development of TNBC, we have conducted studies on the role of Annexin A2 in TNBC.

The Annexins are a family of proteins that bind to negatively charged phospholipids in a calcium-dependent manner. They are identified in invertebrates, vertebrates, plants, fungi and protists [8]. There are 12 members in the family of annexins which are identified and named as AnxA1-11 and AnxA13. Annexins have been implicated in many functions like maintaining membrane cytoskeletal dynamics [9], signal transduction, RNA binding, epithelial and endothelial cell polarity, exocytosis-endocytosis and extracellular receptor activity [10]. Annexins have also been involved in many pathological conditions like cancer, diabetes, inflammation, cardiovascular diseases, autoimmune diseases such as Rheumatoid arthritis [13] and Systemic lupus [14,15].

Annexin A2 is highly expressed in many cells and tissues. It consists of two domains: a highly variable amino-terminal and a highly homologous carboxy-terminal domain. The carboxy-terminal core domain consists of four alpha helical structures each consisting of 70 amino acids[8]. This carboxy terminal has binding sites for heparin, mRNA [17], calcium, phospholipid and F-actin [8,18]. The highly variable amino-terminal contains sites for post-translational modification

and for protein-protein interactions which impart unique characteristics to Annexins [8]. It has sites for post-translational modifications like Serine (Ser)1 for acetylation, Lysine-9 for sumoylation and Ser11, Ser25 and Tyrosine (Tyr)23 for phosphorylation [10]. Tyr23 has been associated with many cancers like breast and pancreatic cancer. Try23 phosphorylation of AnxA2 has shown to be essential for invasion and proliferation and Stat3 phosphorylation in Human breast cancer SK-BR3 cells [23]. Studies have shown that Tyr23 phosphorylation and cell surface localisation of AnxA2 is necessary for invasion and metastasis in pancreatic cancer [9]. AnxA2 in its phosphorylated form at Tyr23 is necessary for in-vitro capillary formation and integrity [24].

AnxA2 is also present on the exosomes, and exosomes have been reported to impart metastatic characteristics to tumors in melanoma and pancreatic cancer. In pancreatic cancer, exosomes could initiate pre-metastatic niche formation in liver [47]. In melanoma, *Peinado et al* showed that exosomes educate bone marrow progenitor cells to impart metastatic behavior to primary tumors through mesenchymal-epithelial transition [48]. In Chronic Myelogenous Leukemia (CML), cancer cell-derived exosomes modulate bone microenvironment by activating epidermal growth factor receptor (EGFR) in stromal cells [49]. Exosomal AnxA2 also leads to angiogenesis in triple-negative breast cancer and organ specific metastasis to brain and lung [6]. Moreover, HS5 mesenchymal stem cells, a source of bone marrow microenvironment, treated with LAMA84 CML cell-derived exosomes, increase AnxA2 expression and adhesion of leukemic cells to the stromal monolayer and increase the growth and survival of CML cells [50].

We studied Annexin A2 and its N-terminal Tyr23 phosphorylation for its association with exosomes and its ability to promote oncogenic phenotype of recipient cells. For this study, we generated phosphomimetic (AnxA2-Y23E-GFP) and non-phosphomimetic (AnxA2-Y23F-GFP) mutants in MDA-MB-231 cell line and isolated exosomes from the same. We treated another

triple-negative breast cancer cell line with the above mutant cell-derived exosomes to see the impact of phosphomimetic and non-phosphomimetic AnxA2 bearing exosomes on the migration, invasion and proliferation in those cells.

Materials and Methods:

Cell lines model used:

MDA-MB-231 TNBC cell line was purchased from Animal Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified eagle's medium (DMEM) (Hyclone) containing 10 % FBS , 4.05mM glutamine, 100IU penicillin, 100IU streptomycin and 0.25ug/ml Amphotericin B . CAL-148 (Centre Antoine Lacassagne – 148) breast adenocarcinoma TNBC cell line was purchased from ATCC and was grown in DMEM containing 10 ng/ml of Human Epidermal Growth factor (EGF) (Gibco), 10 % FBS, 4.05mM glutamine, 100IU penicillin, 100IU streptomycin and 0.25ug/ml Amphotericin B. MDA-MB-231 empty vector (EV), AnxA2-Y23E-GFP cells where Tyr 23 was interchanged to glutamic acid for constitutive phosphorylation (Phosphomimetic) and AnxA2-Y23F-GFP cells where Tyr23 was interchanged to phenylalanine for non-phosphorylation (non-phosphomimetic) were generated by transfecting pEGFP-N1 plasmids bearing AnxA2 mutant genes for Tyr23. Stable transfectants were selected using Geneticin with concentration of 700 μg/ml (Gibco) for two weeks and were later maintained in 200 μg/ml of Geneticin. All the cell lines were tested for mycoplasma infection with MycoAlert PLUS from Lonza (Basel, Switzerland). All the cell lines were confirmed to be mycoplasma free prior to use.

Plasmids and constructs:

The cDNA of AnxA2 was cloned into pEGFP-N1 plasmid backbone for expression of a full length AnxA2. GFP was tagged to the C-terminus of Wild type (WT) and mutated AnxA2 gene. The N-terminal phosphomimetic and non-phosphomimetic mutant at Tyr23 were generated by QuikChange site-directed mutagenesis kit (Stratagene) by interchanging Tyr23 to glutamic acid

and phenylalanine, respectively. In this report, the plasmids are referred to as pEGFP-N1-EV (Empty vector) , pEGFP-N1-AnxA2Y23E, and pEGFP-N1-AnxA2Y23F.

Exosome Isolation:

MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP cells were grown till the cells achieve 70% confluency in T-175 flask. Medium containing 10% FBS was washed by Phosphate Buffered Saline (PBS)(Hyclone). Minimum amount of medium supplemented with 2 % exosome depleted serum (Gibco) was added onto the cells and were grown for 48 hours. After 48 hours, medium was collected and subjected to ultracentrifugation in fixed angle 45Ti rotor (Beckman Coulter). Cells were spun at 2000 x g for 10 minutes to remove cells and cell debris. Supernatant was collected and was spun at 18000 rpm for 1 hour. Media was filtered through 0.2 um syringe filter (corning) and spun at 40000 rpm for 2 hours to pellet out exosomes. Pelleted exosomes were resuspended in 1 ml PBS and stored at -80^{0}C. The exosomes were named as per their source of derivation, exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP.

Western blots and antibodies:

AnxA2 mutant cells were lysed in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 50mM Tris-HCl (pH-8.0), 0.1% SDS] supplemented with protease Inhibitor Cocktail Set I (Millipore). Cell lysates were sonicated and was centrifuged at 4800 rpm for 15 minutes at 4^{0}C. Protein concentration was measured by BCA protein assay kit (Thermo scientific). Cell lysates were subjected to SDS PAGE and were transferred to a nitrocellulose membrane (iBlot, Invitrogen). Membrane was blocked with 5% Bovine Serum Albumin (BSA) (sigma) solution for 2 hours and probed with primary antibodies in 2.5 % BSA solution for overnight at

4^0C: Anti-AnxA2 primary mouse monoclonal antibody (BD Biosciences, 1:1000 dilution), rabbit polyclonal anti-GFP (Abcam, 1:1000 dilution), mouse monoclonal anti-tissue type plasminogen activator (tPA) (Abcam, 1:1000 dilution), rabbit polyclonal anti-MMP-2 (Bioss, 1:1000 dilution), rabbit polyclonal anti-MMP-9 (Abcam, 1:1000 dilution) and mouse monoclonal anti Beta-actin antibody (Cell Signaling, 1:1000 dilution). Blots were further washed with TBST three times for 10 minutes each. Further, membrane was probed with specific HRP-conjugated secondary antibody (Southern Biotech) in 2.5 % milk for 1 hour at RT and washed three times with TBST . Blots were developed by Immobilon Western Chemiluminescent HRP substrate (Millipore).

For exosome lysates, lysates were prepared in the same way as described above for cell lysates and western blot was carried out. 10 μg of exosome lysates from exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP were separated using a 4-12% Bis-tris NuPAGE gel (Life Technologies Corporation) and protein was transferred to a nitrocellulose membrane. Membrane was blocked with 5% BSA (sigma) solution for 2 hours and probed with primary antibodies in 2.5 % BSA solution for overnight at 4^0C: Anti-CD 81 (Santacruz Biotechnology) (1:1000 dilution), anti-Tsg 101 (BD Transduction laboratories) (1:1000 dilution), anti-Hsc 70 (Enzo) (1:1000 dilution), anti-GM 130 (BD Transduction laboratories) (1:1000 dilution), anti-Lamin A/C (Santacruz Biotechnology) (1:1000 dilution) and anti-cytochrome c (Santacruz Biotechnology) (1:1000 dilution). Thrice blots were washed by TBST. Further, membrane was probed with specific HRP-conjugated secondary antibody (Southern Biotech) in 2.5 % milk for 1 hour at RT and was washed three times with TBST. Blots were developed by Immobilon Western Chemiluminescent HRP substrate (Millipore).

Immunoprecipitation of AnxA2 present on cell surface and exosomal surface:

1 x 10^6 MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP cells were grown in a 10mm petri dish till cells achieve 70-80 % confluency. Cells were treated with 1 ml of 1X Versene (Gibco) for 10 minutes. Supernatant was collected and centrifuged at 1000 rpm to remove the cells. The resultant supernatant was considered as Versene elute [51]. Versene elute was incubated with anti-AnxA2 primary mouse monoclonal antibody (BD Biosciences) for 2 hours at 4^0C followed by addition of Protein A/G rich agarose-PLUS bead solution (Santacruz Biotechnology) to the elute and treated overnight at 4^0C. Pellet was collected by centrifuging at 3000 rpm for 5 minutes at 4^0C. Pellet was washed twice by 100 µl of RIPA buffer. Finally, pellet was resuspended in 10 µl of sample loading buffer with B-mercaptoethanol and boiled for 10 minutes. Samples were subjected to SDS-PAGE followed by Western blot. Membrane was probed with anti-AnxA2 primary mouse monoclonal antibody in 2.5 % BSA solution overnight at 4^0C, followed by HRP-conjugated secondary goat anti-mouse antibody in 2.5 % BSA for 1 hour at RT. Membrane was washed thrice with TBST for 10 minutes each and developed by using Immobilon Western Chemiluminescent HRP substrate.

Isolated exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP were treated with 1 ml of 1X Versene wash for 10 minutes. 2 µl of AnxA2 mouse monoclonal primary antibody was added to the versene eluate. Further, 10 µl of Protein A/G rich agarose bead solution was added to the elute and was treated for overnight at 4^0C. Pellet was collected by centrifuging at 3000 rpm for 5 minutes at 4^0C. Pellet was washed twice by 100 µl of RIPA buffer. Finally, pellet was resuspended in 10 µl of sample loading buffer with B-mercaptoethanol and sample was boiled for 10 minutes. Sample was subjected to SDS-PAGE followed by Western blot as described above.

Flowcytometry of cells and exosomes:

MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP cells were cultured and was trypsinised by using 0.5 % Trypsin solution. 0.4 x 10^6 cells were used for flowcytometric analysis. Further, cells were fixed by 4 % paraformaldehyde (Affymetrix USB) for 30 minutes at room temperature (RT) and spun at 1000 rpm for 10 minutes. Cells were blocked by using 5% BSA solution for 10 minutes. Cells were probed with AnxA2 mouse monoclonal antibody (1:200 dilution) and isotype antibody (normal mouse IgG) (Santacruz Biotechnology, 1: 400 dilution) in 2.5 % BSA for 30 minutes at RT. Cells were spun at 1000 rpm for 10 minutes. Alexa fluor-594 antibody diluted in 2.5 % BSA was added to the cells for 30 minutes at RT. Cells were again spun at 1000 rpm for 10 minutes. Further, cells were resuspended in 500 µl PBS. Samples were analyzed on BD Biosciences LSR II cytometer.

For analyzing exosomal surface AnxA2, 50 µg of exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP was mixed in each tube with 1 µl of 4um Aldehyde sulphate latex beads (Invitrogen). Tubes were placed on spinner at 4^0C overnight for proper binding of exosomes to beads. Samples were spun down at 2000 x g for 10 minutes at RT. 100 µl of 100 mM Glycine (Sigma) solution was added for 30 minutes to stop the binding reaction. Samples were spun down at 2000xg for 10 minutes. 100 µl of 2 % BSA was added as a blocking solution to the bead conjugated exosomes for 2 hours at RT. Samples were centrifuged at 2000xg for 10 minutes. 1:200 diluted AnxA2 mouse monoclonal antibody and 1:800 diluted isotype antibody (normal mouse IgG) prepared in 0.5 % BSA solution was added for 30 minutes at RT following centrifugation at 2000xg for 10 minutes. 1:3200 diluted secondary Alexa-fluor-594 goat anti-mouse antibody prepared in 0.5 % BSA solution was added for 30 minutes at RT in dark. Samples were spun down at 2000xg for 10 minutes. Pelleted bead conjugated exosomes were resuspended in 500 µl PBS.

Samples were subjected to BD Biosciences LSR II cytometer and were analyzed for surface AnxA2-GFP expression.

Immunofluorescence of cell surface AnxA2:

MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP mutant cells were cultured on coverslips. Cells were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 30 minutes followed by washing thrice with PBS for 10 minutes each. Blocking was performed by adding 5% Goat serum (Vector laboratories. Inc.) for half an hour. Serum was removed and anti-GFP rabbit polyclonal primary antibody (Cell signaling) diluted in PBS was added for overnight at 4^{o}C. Cells were washed thrice with PBS for 10 minutes each. Alexa fluor-595 secondary antibody (Invitrogen) was added to the cells and incubated for 2 hours at RT in dark. Finally, cells were washed thrice with PBS for 10 minutes each and visualized under Confocal microscope.

Scratch wound assay:

MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP mutant cells were grown in 6 well plate till cells attend 80 % confluency in 10 % FBS containing DMEM medium. Cells were kept in serum free media for 12 hours. Wound was created with 10 µl tip. Wells were washed with PBS to remove floating cells. Wounded area was marked for taking pictures of specific area for given time points. Percentage of wound healing was calculated by Image J. Experiment was carried out in triplicates and was repeated three times .

Transwell Invasion assay:

24-well plate was used with 8 um pore size inserts (Corning) for the experiment. 100 µl of serum free medium was added into the upper chamber. 200 µl of $4x10^4$ [52] MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP mutant cells/serum free DMEM medium was added into the upper chamber and 750 µl of only DMEM medium with serum was added into the lower chamber. Cells were Incubated at 37^0C for 24 hours and fixed with 4% paraformaldehyde for 10 minutes at RT. Later, once cells were washed with PBS and fixed in absolute methanol for 20 minutes at RT. With one PBS wash, cells were stained with 0.25 % Crystal violet stain for 30 minutes. Stain was removed and washed with PBS. Non-migrated cells were scraped off by cotton swab. Further, cells were viewed under the inverted microscope. Cell counting was performed by image J by selecting 3 fields each cell type. Experiment was carried out three times in duplicates.

CAL-148 cells were treated with 50 µg of exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP for 48 hours at 37^0C. 24-well plate was used with 8 um pore size inserts for the experiment. 100 µl of serum free medium was added into the upper chamber following 200 µl of 1.25 $x10^5$ cells/serum free medium. 750 µl of only serum containing medium was added into the lower chamber. Further, experiment was performed same as for the mutant cells described above. Experiment was carried out three times in duplicates.

Cell proliferation assay:

5000 cells/well MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP MDA-MB-231 mutant cells were grown in 96-well plate in 10 % FBS containing medium. Cells were grown for 24-48 hours till they form colonies. Media was removed and washed twice with PBS. Cells were fixed with 200 µl of 4 % paraformaldehyde for 20 minutes. Again cells were washed twice with

PBS. Cells were stain with 0.25 % Crystal violet solution for one hour. Further, three representative images were taken by choosing three different fields of each cell type. Experiment was carried out three times in triplicates.

5000 cells/well MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP MDA-MB-231 mutant cells were grown in 96-well plate in 10 % FBS containing DMEM medium. Cells were treated with MTT at different timepoints of 24, 48 and 72 hours. 10 µl of 5 mg/ml MTT solution was added for 2 hours. Cells were centrifuged at 1000 rpm for 10 mins. 100 µl of DMSO was added and cells were incubated for 30 minutes. Spectrophotometric reading was taken at 590 nm. Experiment was carried out in triplicates.

CAL-148 were treated with 40 µg of EV, Y23E and Y23F mutant MDA-MB-231 cell derived exosomes for 48 hours at 37^0C. 5000 cells/well CAL-148 treated cells were seeded in 96-well plate in 10 % FBS containing specific cell type media. Cells were grown for 24, 48 and 72 hours till they form colonies. Further steps were carried out as described in the above MTT assay for mutant cells.

Statistical analysis:

The two tailed unpaired t-test was used to calculate the statistical difference between the phosphomimetic and non-phophomimetic cells. Data are presented as mean ± standard error of the mean. * denotes a p-value of less than 0.05,** denotes a p-value of less than 0.01 and *** denotes a p-value of less than 0.001

Results:

Generation of phosphomimetic and non-phosphomimetic Annexin A2 expressing MDA-MB-231 cell lines:

The pEGFP-N1 plasmid (Figure 1-a) containing AnxA2 WT and Tyr23 mutated AnxA2 gene were generated by site directed mutagenesis. All the plasmids were confirmed for the incorporation of the WT and mutated gene by restriction enzyme digestion of the plasmids by specific restriction enzymes like NotI, XhoI, XbaI and NdeI. Plasmid digestion with NotI and XhoI showed the insert approximately of 1500 bp corresponding to the AnxA2-EGFP gene and the higher band corresponding to empty vector in pEGFP-N1-AnxA2-Y23E and pEGFP-N1-AnxA2-Y23F. Plasmid digestion with XhoI and XbaI showed the release of the insert with an empty vector band of 4700bp (supplementary Figure 1a). When, the same plasmids were digested with NdeI enzyme, pEGFP-N1-AnxA2WT showed the DNA band release of approximately 2000 bp and no bands were seen in the plasmid-bearing mutated AnxA2 gene confirming the identity of the WT and mutated AnxA2 gene bearing pEGFP-N1 plasmids. (supplementary Figure 1b). Further, transfected cells were sorted for top 10 % GFP positive cells and verified for GFP fluorescence by fluorescence microscopy. More than 90 % of cells were seen to be GFP positive (Figure 1-b). Further, mutant cell lysates were subjected to western blot and immunoblotted with anti-AnxA2 (Figure 1-c) and anti-GFP (Figure 1-d) antibodies. When immunoblotted with anti-GFP or anti-AnxA2 antibody, cells expressing empty vector (EV) showed only GFP and only endogenous AnxA2 and no AnxA2-GFP band was seen at 61 kDa, whereas, mutant cells AnxA2-Y23E-GFP (Y23E) and AnxA2-Y23F-GFP (Y23F) showed AnxA2-GFP band at 61 kDa, when immunoblotted with both the antibodies.

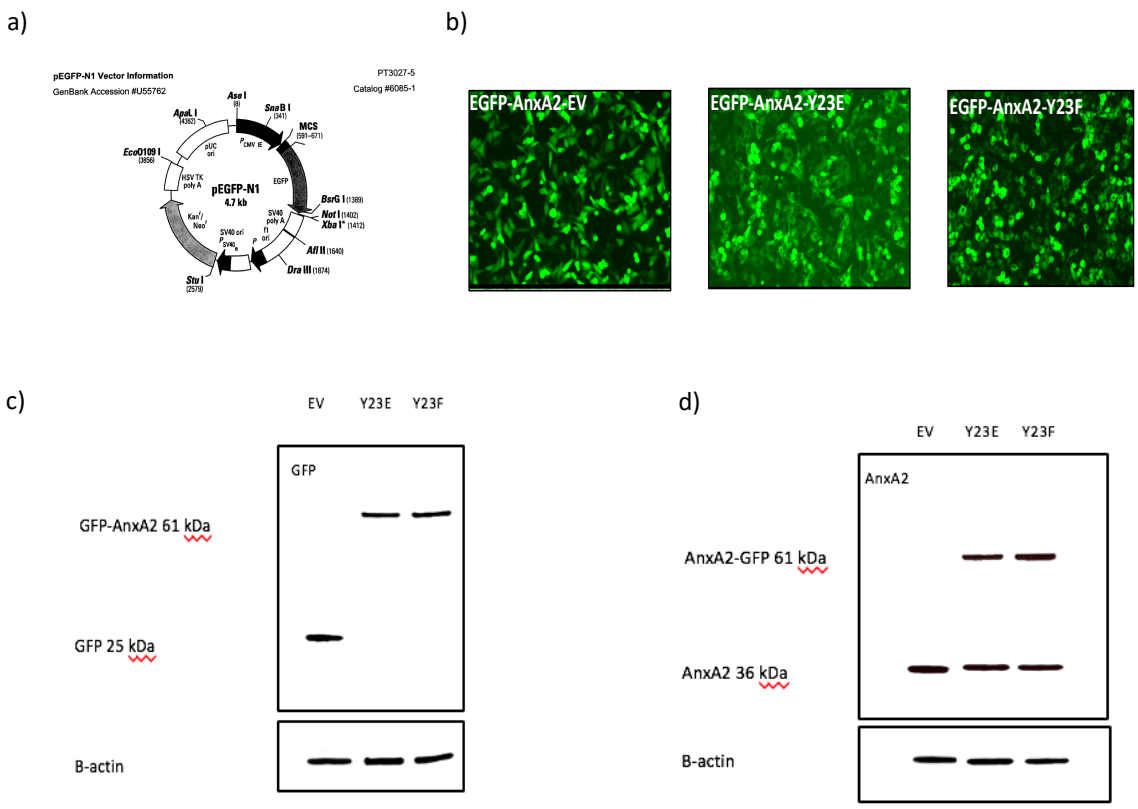


Figure 1: Generation of mutant MDA-MB-231 cell lines by transfecting with pEGFP-N1-EV, pEGFP-N1-AnxA2-Y23E and pEGFP-N1-AnxA2-Y23F vectors. a) pEGFP-N1 plasmid (4.7 kb) used for creating MDA-MB-231 EV, AnxA2-Y23E-GFP and AnxA2-Y23F-GFP cells. b) MDA-MB-231 cells expressing pEGFP-N1-EV, pEGFP-N1-AnxA2-Y23E and pEGFP-N1-AnxA2-Y23F after antibiotic selection and after selecting top 5% GFP positive cells. Western blot analysis of cell lysates from MDA-MB-231 EV (EV), AnxA2-Y23E-GFP (Y23E) and AnxA2-Y23F-GFP (Y23F) cells c) Immunoblot with anti-GFP antibody showing only GFP band at 25 kDa in EV cells and 61 kDa AnxA2-GFP in AnxA2-Y23E-GFP (Y23E) and AnxA2-Y23F-GFP (Y23F) cells and d) Immunoblot with anti-AnxA2 antibody showing only endogenous AnxA2 band at 36 kDa in MDA-MB-231-EV cells and 61 kDa AnxA2-GFP band in AnxA2-Y23E-GFP (Y23E) and AnxA2-Y23F-GFP (Y23F) cells.

Phosphorylation of AnxA2 at Tyr23 increases cell surface expression of AnxA2 in MDA-MB-231 cells expressing phosphomimetic AnxA2 compared to cells expressing non-phosphomimetic AnxA2:

For studying the effect of constitutive phosphorylation on AnxA2 surface localisation in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells, flowcytometric analysis of cell surface AnxA2 was carried out. Results showed high association of AnxA2-GFP on MDA-MB-231 AnxA2-Y23E-GFP cell surface compared to AnxA2-Y23F-GFP cells (Figure 2-a). Immunofluorescence also confirmed the results showing increased cell surface GFP staining in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells (Figure 2-b). Immunoprecipitation of cell surface AnxA2 also showed increased cell surface AnxA2-GFP expression on phosphomimetic compared to non-phosphomimetic cells (Figure 2-c).

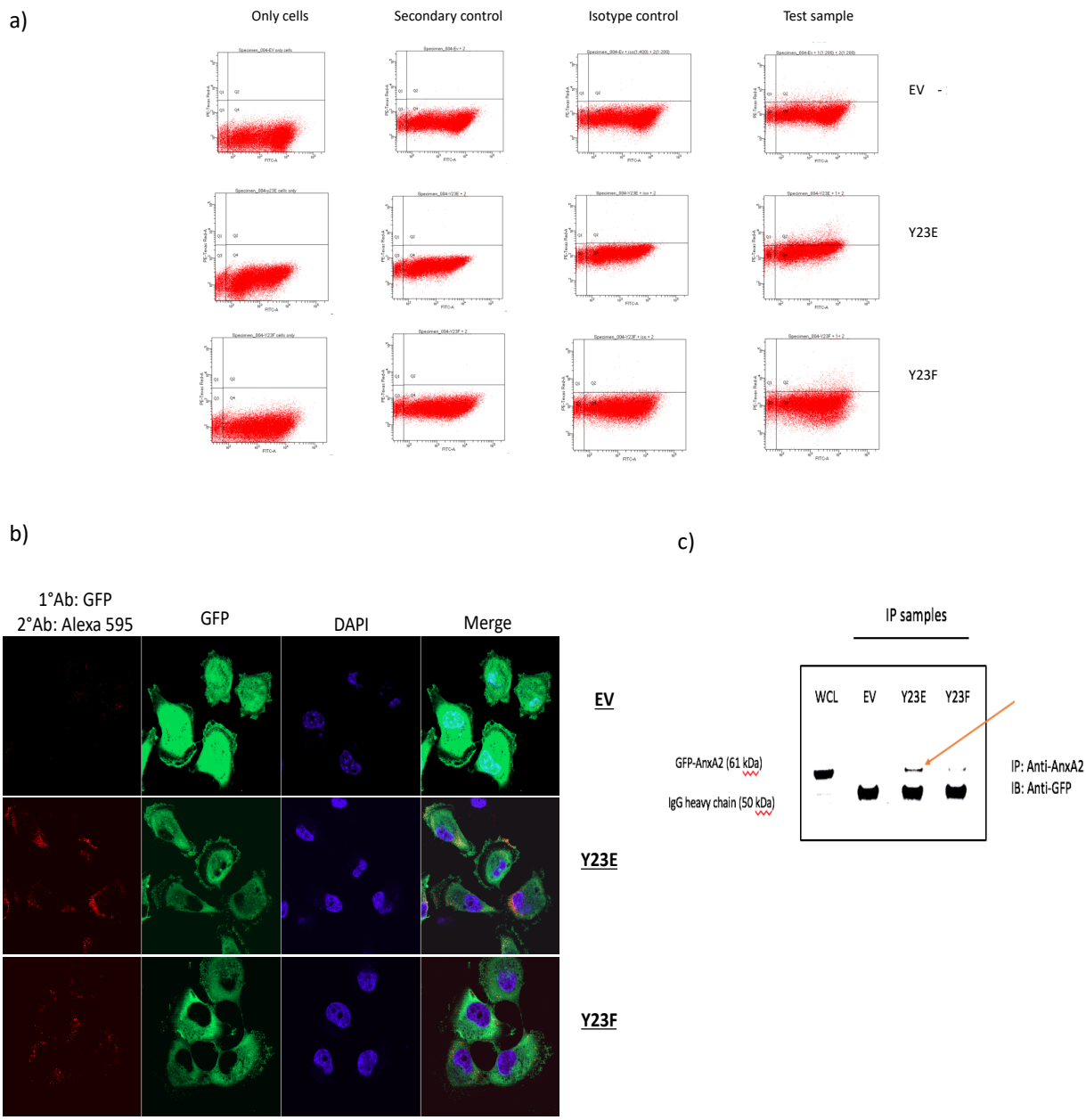


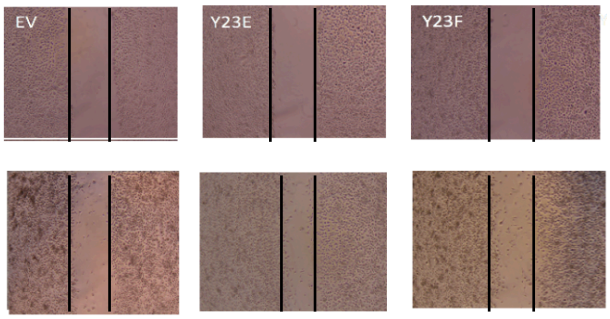
Figure 2: Phosphorylation of AnxA2 at Tyr23 increases cell surface expression of AnxA2 in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells. a) Flowcytometric analysis of cell surface AnxA2 in MDA-MB-231 EV (EV), AnxA2-Y23E-GFP (Y23E) and AnxA2-Y23F-GFP (Y23F) cells showing increase in cell surface AnxA2 in AnxA2-Y23E-GFP compared to AnxA2-Y23F-GFP cells. b) Immunofluorescence assay by immunostaining cells with polyclonal anti-GFP antibody showing increase in cell surface AnxA2 in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells. c) Immunoprecipitation (IP) study by using

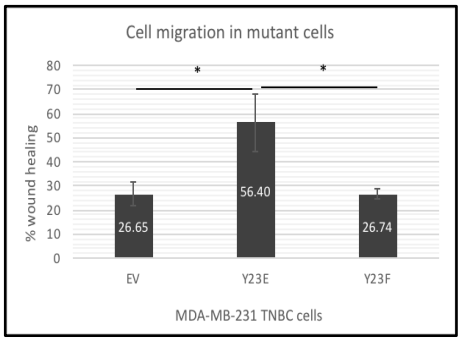
monoclonal anti-AnxA2 antibody and immunoblotting (IB) with polyclonal anti-GFP antibody also showed increase in cell surface AnxA2 in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells.

MDA-MB-231 cells expressing phosphomimetic AnxA2 increase migration, invasion and proliferation compared to cells expressing non-phosphomimetic AnxA2:

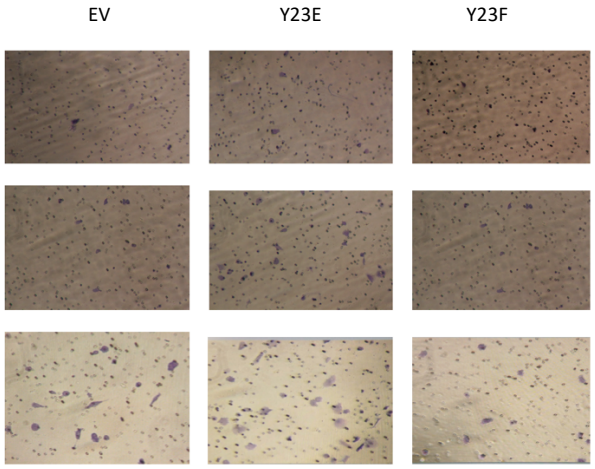
Generation of successful mutant cells were confirmed by AnxA2 specific functional assays. As AnxA2 has been implicated in migration, invasion and proliferation, we carried out migration, invasion and proliferation specific in-vitro assays. Scratch wound assay for migration showed that MDA-MB-231 AnxA2-Y23E-GFP cells migrated more compared to AnxA2-Y23F-GFP cells (Figure 3-a). Wound healing in AnxA2-Y23E-GFP cells was found to be 29.66 % more compared to AnxA2-Y23F-GFP cells. For invasion study, transwell invasion assay showed significant increase in invasive capacity of AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells (Figure 3-b). Moreover, MDA-MB-231 AnxA2-Y23E-GFP cells had more proliferative rate than AnxA2-Y23F-GFP cells (Figure 3-c). Western blot analysis of mutant cell lysates showed increase in t-PA, active MMP-9 and active MMP-2 in AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP and EV cells (Supplementary figure 2).

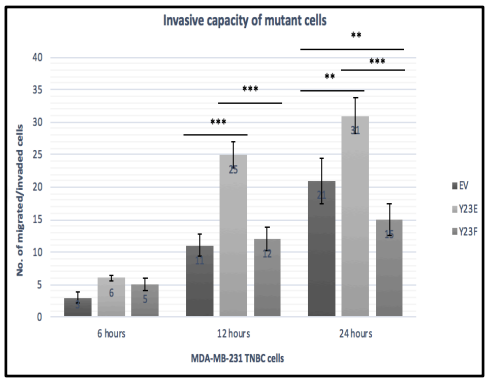
a)


EV
Y23E
Y23F

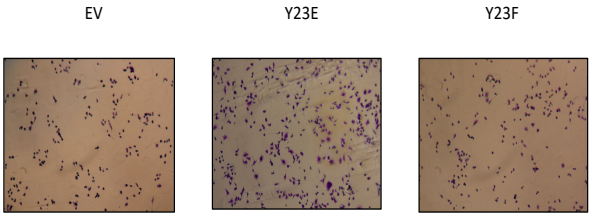

Cell migration in mutant cells
% wound healing
80
70
60
50
40
30
20
10
0
*
*
26.65
56.40
26.74
EV
Y23E
Y23F
MDA-MB-231 TNBC cells

b)


EV
Y23E
Y23F


Invasive capacity of mutant cells
No. of migrated/invaded cells
40
35
30
25
20
15
10
5
0
***
***
**
**
***
6
5
25
12
21
31
15
6 hours
12 hours
24 hours
MDA-MB-231 TNBC cells
EV
Y23E
Y23F

c)


EV
Y23E
Y23F

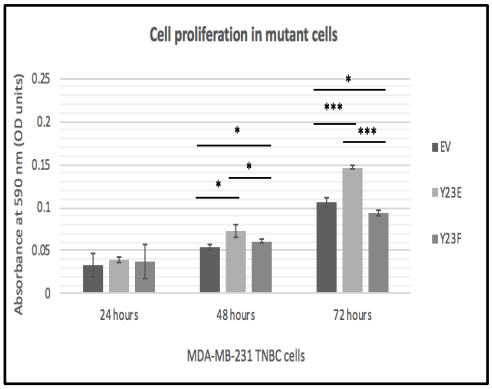

Cell proliferation in mutant cells
Absorbance at 590 nm (OD units)
0.25
0.2
0.15
0.1
0.05
0
*
*
*
***
***
*
24 hours
48 hours
72 hours
MDA-MB-231 TNBC cells
EV
Y23E
Y23F

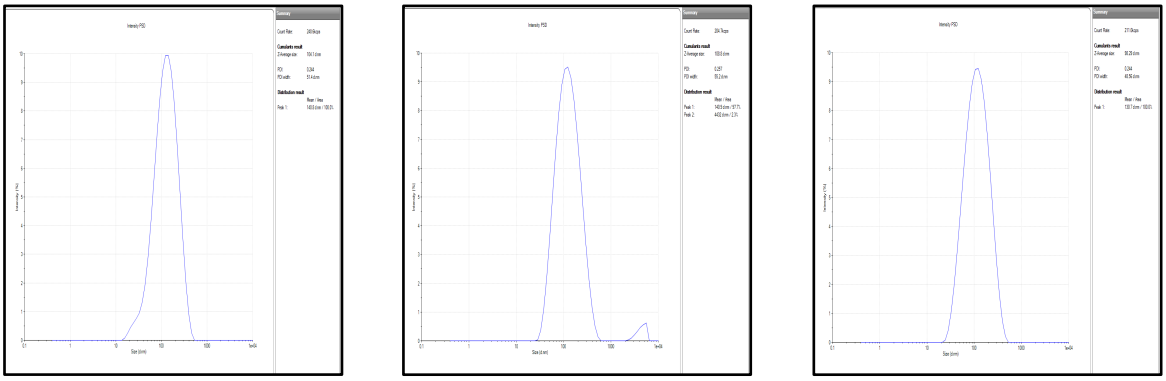
Figure 4: MDA-MB-231 AnxA2-Y23E-GFP cells increase migration, invasion and proliferation compared to AnxA2-Y23F-GFP cells. a) Migration of MDA-MB-231 EV (EV), AnxA2-Y23E-GFP (Y23E) and AnxA2-Y23F-GFP (Y23F) was studied by Scratch wound assay for 24 hours. MDA-MB-231 AnxA2-Y23E-GFP cells showed higher migratory capacity compared to EV and AnxA2-Y23F-GFP cells. b) Invasion was assessed by transwell invasion assay and MDA-MB-231 AnxA2-Y23E-GFP cells showed higher invasiveness compared to EV and AnxA2-Y23F-GFP cells after 24 hours. c) Cell proliferation assay with MTT assay as end-point assay showed highly significant increase in proliferative rate in MDA-MB-231 AnxA2-Y23E-GFP cells compared to EV and AnxA2-Y23F-GFP cells after 48 and 72 hours. Mean ± S.E.M.; *P < 0.05; **P<0.01; ***P<0.001 (Student's T-test).

Phosphorylation of AnxA2 at Tyr23 is essential for its association with exosome in triple negative breast cancer cells:

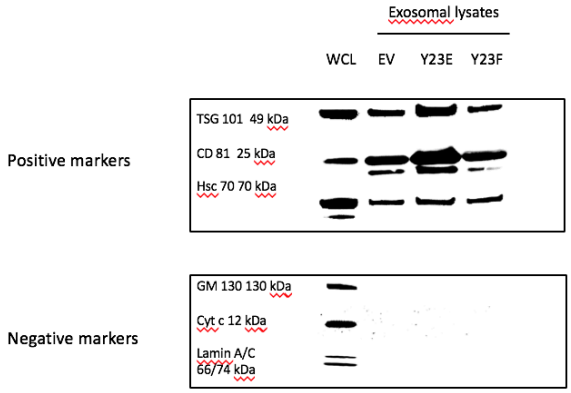
Successful isolation of exosomes by ultracentrifugation was confirmed by size analysis by Zetasizer. Exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP were isolated and was checked for the size analysis each time before subjecting them to the experimental purposes. Average size was found to be consistently between 100 ± 5 nm (Figure 4-a). Exosome lysates were found to be positive for positive markers like CD81, Tsg101 and Hsc70 and negative for negative markers like GM130, Lamin A/C and cytochrome c (Figure 4-b). This confirmed that exosomes isolated were with least contamination with other organelles. Exosomes were further subjected to flowcytometry by using 4um aldehyde sulphate latex beads and surface AnxA2-GFP was detected using mouse monoclonal anti-AnxA2 antibody. Exosomes derived from MDA-MB-231 AnxA2-Y23E-GFP cells showed 41.1 % AnxA2-GFP staining compared to 6.2 % staining in exosomes derived from AnxA2-Y23F-GFP cells (Figure 4-c). Immunoprecipitation of exosomal surface AnxA2-GFP was carried out using primary mouse monoclonal anti-AnxA2 antibody and was immunoblotted with rabbit polyclonal anti-GFP antibody. Results showed increased exosomal surface AnxA2-GFP on exosomes derived from MDA-MB-231 AnxA2-Y23E-GFP cells compared to exosomes derived from AnxA2-Y23F-GFP cells (Figure 4-d).

a)

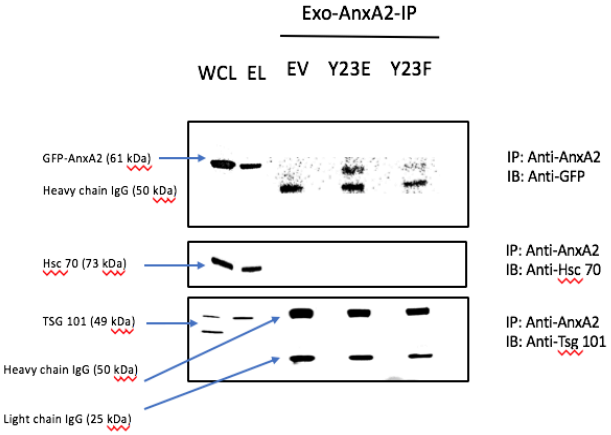
i) ii) iii)



b)


Exosomal lysates
WCL EV Y23E Y23F
Positive markers
TSG 101 49 kDa
CD 81 25 kDa
Hsc 70 70 kDa
Negative markers
GM 130 130 kDa
Cyt c 12 kDa
Lamin A/C 66/74 kDa

c)


Exo-AnxA2-IP
WCL EL EV Y23E Y23F
GFP-AnxA2 (61 kDa)
Heavy chain IgG (50 kDa)
IP: Anti-AnxA2
IB: Anti-GFP
Hsc 70 (73 kDa)
IP: Anti-AnxA2
IB: Anti-Hsc 70
TSG 101 (49 kDa)
Heavy chain IgG (50 kDa)
Light chain IgG (25 kDa)
IP: Anti-AnxA2
IB: Anti-Tsg 101

d)

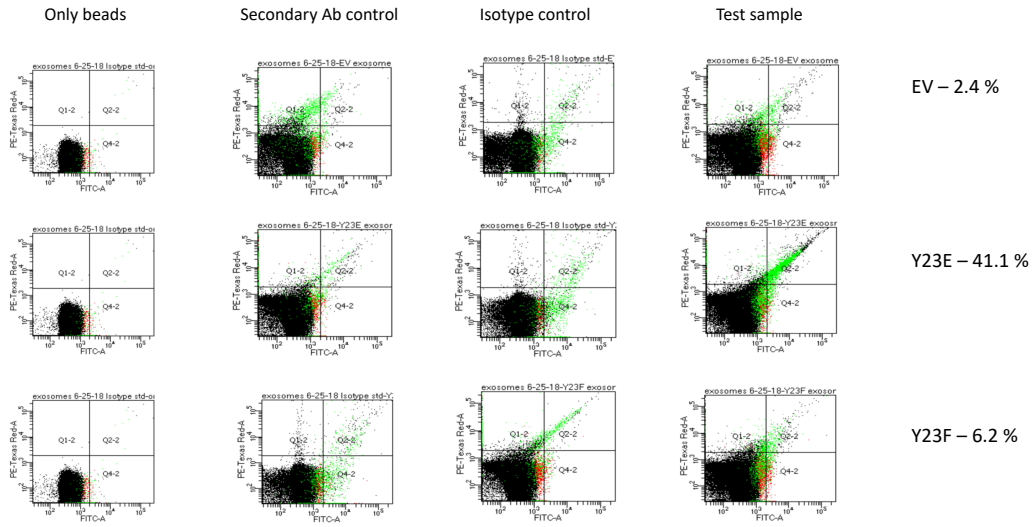

Only beads
Secondary Ab control
Isotype control
Test sample
EV – 2.4 %
Y23E – 41.1 %
Y23F – 6.2 %

Figure 4: Phosphorylation of AnxA2 at Tyr23 is essential for its association with exosome in triple negative breast cancer cells. a) Size analysis of exosomes derived from MDA-MB-231 i) EV, ii) AnxA2-Y23E-GFP and iii) AnxA2-Y23F-GFP cells by Zetasizer showing 100 ± 5 nm size of all the three types of exosomes. b) Confirmation of exosome isolation by Western blot using three positive markers (CD81, Tsg101, Hsc70) and three negative markers (GM130, cytochrome c and Lamin A/C) showing no contamination from other organelles in the exosome samples. c) Immunoprecipitation of exosomal surface AnxA2-GFP by using monoclonal anti-AnxA2 antibody and immunoblotting with polyclonal anti-GFP antibody showing increase in exosomal surface AnxA2 in exosomes derived from MDA-MB-231 AnxA2-Y23E-GFP (Y23E) cells compared to exosomes derived from AnxA2-Y23F-GFP (Y23F) cells. d) Confirmation of increase in exosomal surface AnxA2 in exosomes derived from MDA-MB-231 AnxA2-Y23E-GFP cells (41.1%) compared to AnxA2-Y23F-GFP cells (6.2 %) by flowcytometric analysis using monoclonal anti-AnxA2 antibody.

Exo-AnxA2-Y23E-GFP treatment increases invasiveness and proliferative capacity of CAL-148 triple negative breast cancer cells:

Western blot of cell lysates of CAL-148 and MDA-MB-231 cells showed less expression of AnxA2 in CAL-148 cells (Supplementary figure 3). After treating CAL-148 with exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP for 48 hours, cell lysates were prepared and subjected to western blot. Further, lysates were stained with primary monoclonal anti-AnxA2 antibody which showed increase expression of AnxA2 in CAL-148 cells treated with exosomes (Figure 5-a). Exosome treated CAL-148 cells were then subjected to in-vitro transwell invasion and proliferation assay. Transwell-invasion assay showed increase in migratory and invasive capacity of CAL-148 cells treated with exo-AnxA2-Y23E-GFP as compared to exo-AnxA2-Y23F-GFP (Figure 5-b). Proliferative rate was also assessed by proliferation assay which showed increased proliferative capacity of CAL-148 cells treated with exo-AnxA2-Y23E-GFP compared to exo-AnxA2-Y23F-GFP (Figure 5-c).

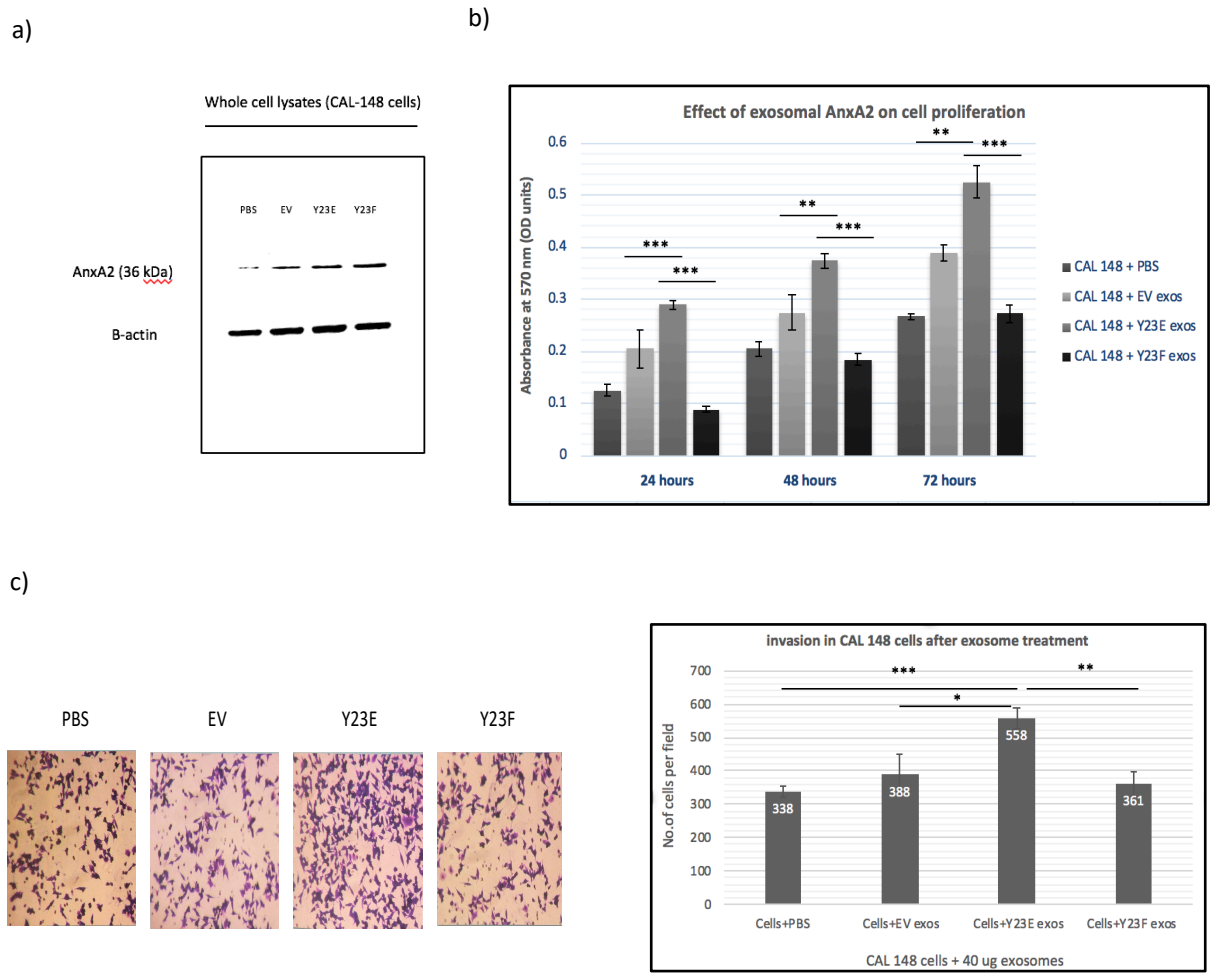


Figure 5: Exo-AnxA2-Y23E-GFP treatment increases migratory and proliferative capacity of CAL-148 triple negative breast cancer cells. a) Western blot of whole cell lysates derived from CAL-148 cells treated with exo-EV, exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP for 48 hours and immunoblotted with monoclonal anti-AnxA2 antibody showing uptake of exosomes by CAL-148 cells and increase expression of AnxA2 in cells treated with exosomes compared to PBS treated CAL-148 cells. b) Cell proliferation assay with end-point MTT assay for CAL-148 cells treated with 40 µg of exo-EV (EV), exo-AnxA2-Y23E-GFP (Y23E) and exo-AnxA2-Y23F-GFP (Y23F) for 48 hours. Cell proliferation was found to be increased in CAL-148 cells treated with exo-AnxA2-Y23E-GFP compared to CAL-148 treated with exo-AnxA2-Y23F-GFP at 24 , 48 and 72 hours. c) Invasion assay for CAL-148 cells treated with 40 µg of exo-EV,exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23F-GFP for 48 hours. Cell invasion was found to be increased in CAL-148 cells treated with exo-AnxA2-Y23E-GFP compared to CAL-148 treated with exo-AnxA2-Y23F-GFP after 24 hours. Mean ± S.E.M.; *P < 0.05; **P<0.01; ***P<0.001 (Student's T-test).

Discussion:

The role of AnxA2 has been implicated in many cancers including renal cell carcinoma, pancreatic, head and neck, brain and colorectal cancer [40-42]. Several studies have suggested the role of AnxA2 in migration, invasion, proliferation, adhesion and angiogenesis [44, 45]. AnxA2 has been shown to be present on the surface of the exosomes by the proteomic profiling of Exocarta data [46]. Exosomes, which are tiny vesicles of 50-120 nm are said to be involved in the progression of tumorigenesis. It has been shown experimentally in many cancers that exosomes can communicate with different cancer cells in order to phenocopy the metastatic potential of a cancer cell to another cell. [6, 47, 48]. Exosomal AnxA2 (exo-AnxA2) has been implicated in the process of metastasis in triple-negative breast cancer cells. In-vivo Matrigel plug assay showed that exo-AnxA2 increased angiogenesis when nude mice were injected with exosomes bearing AnxA2 as compared to AnxA2 inhibitory peptide-treated exosomes. In-vivo metastatic studies have shown that exo-AnxA2 increases breast cancer metastasis to brain and lung [6].

AnxA2 has a variable N-terminal region and its phosphorylation site like Tyr23 has been implicated in many cancers [12]. In our study, we elucidate the role of Try23 phosphorylation in the N-terminal region of AnxA2 and how it regulates the association of cell surface AnxA2 to exosomal surface. Here, we demonstrated that MDA-MB-231 AnxA2-Y23E-GFP cells (constitutive phosphorylation) increase cell surface localization of AnxA2-GFP compared to AnxA2-Y23F-GFP cells (non-phosphorylation). These cells were also checked for their migration, invasion and proliferation capacity due to constitutive phosphorylation in cells. We observed higher migratory, invasive and proliferative capacity in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells. Here, we show that AnxA2-Y23E-GFP cells have higher

expression of migration-invasion markers like tPA, active MMP-2 and -9 compared to AnxA2-Y23F-GFP cells.

For exosomal surface AnxA2 studies, we isolated exosomes and demonstrated that exosomes isolated from AnxA2-Y23E-GFP cells have higher AnxA2-GFP on the surface compared to exosomes derived from AnxA2-Y23F-GFP cells. As exosomes are said to be a communicable vehicle to transfer the phenotype of one cell to another, we treated one of the triple negative breast cancer cell line CAL-148, with a relatively less expression of AnxA2, with mutant exosomes. We observed that exosomes were able to enter the CAL-148 cells and transfer AnxA2. Moreover, CAL-148 cells treated with exosomes derived from AnxA2-Y23E-GFP cells showed more invasiveness and proliferative capacity compare to CAL-148 cells treated with exosomes derived from MDA-MB-231 AnxA2-Y23F-GFP cells.

In summary, we demonstrate that Tyr23 phosphorylation at the N-terminal region of AnxA2 is essential for its translocation to the cell surface in MDA-MB-231 triple negative breast cancer cells. Further, constitutive phosphorylation at Tyr23 leads to an association of AnxA2 with the exosomal surface. Exosomes with higher expression of surface AnxA2 leads to an increase in the invasive and proliferative capacity of other breast cancer cells. Thus, Tyr23 phosphorylation of AnxA2 is consequential for its association with the exosomal surface and for imparting more malignant phenotypic characteristics to other cancer cells.

Disclosure statement:

The authors declare no conflict of interest.

Funding:

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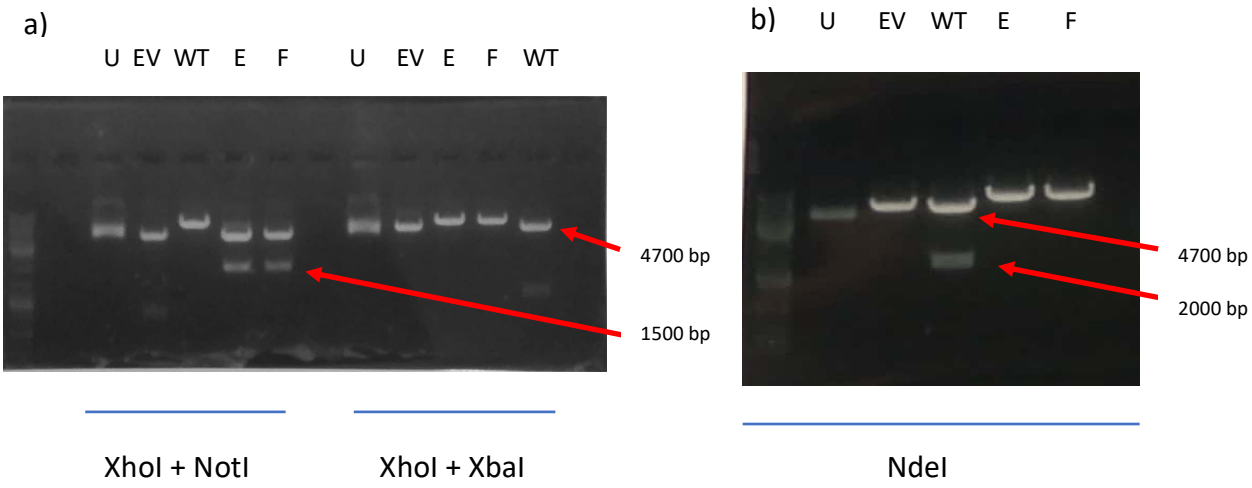
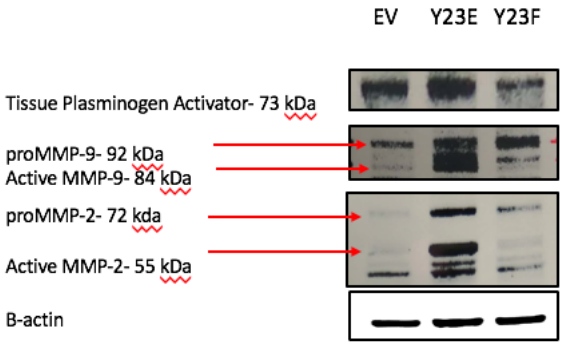
Supplementary figure 1:



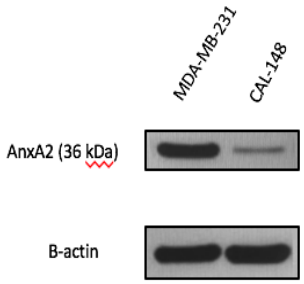
Supplementary figure 1: Plasmids confirmation for the incorporation of the WT and mutated gene by restriction enzyme digestion using specific restriction enzymes. All the plasmids were confirmed for the incorporation of the WT and mutated gene by restriction digestion of the plasmids using specific restriction enzymes such as NotI, XhoI, XbaI and NdeI. Plasmid digestion with NotI and XhoI showed the DNA bands of approximately 1500 bp and 4400 bp corresponding to the AnxA2-EGFP gene and the empty vector, respectively, in pEGFP-N1-AnxA2-Y23E and pEGFP-N1-AnxA2-Y23F. Plasmid digestion with XhoI and XbaI showed the release of the insert with an empty vector band of approximately 4700bp in pEGFP-N1-AnxA2WT (supplementary Figure 1a). When, the same plasmids were digested with NdeI enzyme, pEGFP-N1-AnxA2WT showed the DNA band release of approximately 2000 bp and no bands were seen in the plasmid-bearing mutated AnxA2 gene confirming the identity of the WT and mutated AnxA2 gene bearing pEGFP-N1 plasmids. (supplementary Figure 1b). (U- Uncut plasmid, EV- pEGFP-N1 empty vector, WT- pEGFP-N1-AnxA2WT, E- pEGFP-N1-AnxA2Y23E, pEGFP-N1-AnxA2Y23F)

Supplementary figure 2:



Supplementary figure 2: Western blot analysis of mutant cell lysates showing increase in t-PA, active MMP-9 and active MMP-2 in mutant cells. Western blot analysis of mutant cell lysates showing increase in t-PA, active MMP-9 and active MMP-2 in MDA-MB-231 AnxA2-Y23E-GFP (Y23E) cells compared to MDA-MB-231 AnxA2-Y23F-GFP (Y23F) and EV cells.

Supplementary figure 3:



Supplementary figure 3 : Western blot analysis of cell lysates derived from MDA-MB-231 and CAL-148 cells for studying AnxA2 expression. Western blot analysis of cell lysates derived from MDA-MB-231 and CAL-148 cells and immunoblotted with monoclonal anti-AnxA2 antibody showing less expression of AnxA2 in CAL-148 cells compared to MDA-MB-231 cells.

CHAPTER 3: DISCUSSION AND FUTURE DIRECTIONS

Discussion and Future directions:

AnxA2 present in a wide range of eukaryotic organisms, is associated with a multitude of different functions like membrane traffic, cytoskeleton dynamics and signal transduction. [10]. The role of AnxA2 has been associated with many cancers, and thus its functional studies becomes an important research focus [40-42]. Studying phosphorylation of AnxA2 has become an important topic as its multi-functionality regulates with complexity via post-translational modification (PTM) [10]. PTM, like phosphorylation at N-terminal region of AnxA2 at Ser11, Tyr23 and Ser25 represents important phosphorylation sites [53-56]. Several studies have suggested the role of AnxA2 in imparting metastatic phenotype like migration, invasion, proliferation, adhesion and angiogenesis to the cancerous cells [44, 45]. The phosphorylation site at Tyr23 has been linked to many cancer [21]. Proteomic profiling of Exocarta data has shown AnxA2 as one of the proteins present on the surface of the exosomes [46]. Exosomes which are tiny vesicles of 50-120 nm are said to be involved in the progression of tumorigenesis. It has been implicated in many cancers that exosomes can communicate with different cancer cells in order to phenocopy the metastatic potential of a cancer cell to another cell. [6, 47, 48]. Exosomal AnxA2 (exo-AnxA2) has been found to be active in the process of metastasis in triple negative breast cancer cells. In-vivo Matrigel plug assay showed that exo-AnxA2 increased angiogenesis when nude mice were injected with exosomes bearing AnxA2 compared to AnxA2 inhibitory peptide treated exosomes. In-vivo metastatic studies have also shown that exo-AnxA2 increases breast cancer metastasis to brain and lung [6].

In our study, we elucidate the role of Try23 phosphorylation in the N-terminal region of AnxA2 and how it regulates the association of cell surface AnxA2 to exosomal surface. Here, we demonstrated that MDA-MB-231 AnxA2-Y23E-GFP cells (constitutive phosphorylation)

increases cell surface localization of AnxA2-GFP compared to AnxA2-Y23F-GFP cells (non-phosphorylation). These cells were also checked for their migration, invasion and proliferation capacity due to constitutive phosphorylation in cells. We observed higher migratory, invasive and proliferative capacity in MDA-MB-231 AnxA2-Y23E-GFP cells compared to AnxA2-Y23F-GFP cells. Here, we show that AnxA2-Y23E-GFP cells have higher expression of migration-invasion markers like tPA, active MMP-2 and -9 compared to AnxA2-Y23F-GFP cells.

For exosomal surface AnxA2 studies, we isolated exosomes and demonstrated that exosomes isolated from AnxA2-Y23E-GFP cells have higher AnxA2-GFP on the surface compared to exosomes derived from AnxA2-Y23F-GFP cells. As exosomes are said to be a communicable vehicle to transfer the phenotype of one cell to another, we treated one of the triple negative breast cancer cell line CAL-148, with a relatively lower expression of AnxA2, with mutant exosomes. We observed that exosomes were able to enter the CAL-148 cells and transfer AnxA2. Moreover, CAL-148 cells treated with exosomes derived from MDA-MB-231 AnxA2-Y23E-GFP cells showed more invasiveness and proliferative capacity compare to CAL-148 cells treated with exosomes derived from AnxA2-Y23F-GFP cells.

In our study, we could see some expression of AnxA2-GFP on the surface of MDA-MB-231 AnxA2-Y23F-GFP cells and on the exosomes derived from the same cells. The possible mechanism could be the crosstalk between phosphorylation and acetylation [57], sumoylation or ubiquitination of AnxA2 which could lead to possible induction or stabilization of protein conformation leading to more access to the modification sites. Another reason for the AnxA2-GFP expression could be the phosphorylation of Ser25 which leads to reduced accessibility to Tyr23 for phosphorylation by a kinase and hence, phosphorylation of either residue will prevent

phosphorylation of other residue. Thus, with no Tyr23 phosphorylation, there could be increase in Ser25 phosphorylation [10, 17].

In summary, we demonstrate that Tyr23 phosphorylation at N-terminal region of AnxA2 is crucial for its translocation to the cell surface in MDA-MB-231 triple negative breast cancer cells. Further, constitutive phosphorylation at Tyr23 leads to an association of AnxA2 with exosomal surface. Exosomes with higher expression of surface AnxA2 leads to an increase in invasive and proliferative capacity of other breast cancer cells. Thus, Tyr23 phosphorylation of AnxA2 is consequential for its association with the exosomal surface and for imparting more malignant phenotypic characteristics to other cancer cells.

In future studies, we will carry out in-vivo experiments to study the effect of exosomes derived from phosphomimetic and non-phosphomimetic cells on the process of angiogenesis by in-vivo Matrigel plug assay in the nude mice. We will also study, how exo-AnxA2Y23E-GFP will help in adhesion and uptake of tumor exosome to distant metastatic sites compared to exo-AnxA2Y23F-GFP. Quantitative proteomic analysis of exosomes to determine how exo-AnxA2Y23E-GFP affects diverse cellular signaling at pre-metastatic niche sites will also be studied.

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