Role of ASXL1 in Tumorigenesis and EMT in Breast Cancer

Doctoral Thesis

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Here I declare that my doctoral thesis entitled “Role of ASXL1 in Tumorigenesis and EMT in Breast Cancer” has been written independently with no other sources and aids than quoted.

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Abbreviations

%	Percentage
$(NH4)_2SO_4$	Ammonium Sulphate
°C	degree Celsius / centigrade
µg	microgram
µl	microliter
µM	micromolar
APS	Ammonium persulfate
ASXL	Additional Sex Combs Like
bam	Binary Version of sam files
BAP1	BRCA1 Associated Protein 1
BGP	β-glycerophosphate
BGS	Bovine Growth Serum
bigwig	Binary Version of wiggle files
bp	base pair
BSA	Bovine Serum Albumin
CDH1	E-cadherin
CDH2	N-cadherin
cDNA	Complementary DNA
Cfp1	CxxC finger protein 1
ChIP	Chromatin Immunoprecipitation
ChIP-seq	ChIP followed by high-throughput sequencing
CO_2	Carbon dioxide
CpG	Cytosine phosphate Guanine
CSCs	Cancer Stem Cells
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco modified eagle's Minimal Essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNMT	DNA Methyltransferase
dNTP	Deoxy ribonucleotide triphosphate
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
EDTA	Ethylene Diamine Tetra Acetic acid
EGF	Epidermal Growth Factor
EMT	Epithelial-to-mesenchymal transition
EMT-TF	EMT-transcription factor
EZH2	Enhancer of zest homolog 2
FBS	Fetal Bovine Serum
bFGF	basic Fibroblast Growth Factor
FN1	fibronectin
g	gravity
GO	Gene Ontology
h	hour
H1	histone 1
H2A	Histone 2A
H2Aub1	Monoubiquitinated histone 2A
H2B	Histone 2B
H2Bub1	Monoubiquitinated histone 2B
H3	Histone 3
H3K27me3	Histone 3 trimethylated at position lysine 27
H3K4me3	Histone 3 trimethylated at position lysine 4
H4	Histone 4
HAT	Histone acetyltransferase
HMT	Histone methyltransferase
HNRNPK	heterogeneous nuclear ribonucleoprotein k
HRP	Horse Radish Peroxidase
HSC70	Heat Shock 70 KDa protein
IAA	Iodacetamide

IF	Immunofluorescence
IgG	Immunoglobulin G
IGV	Integrative Genomics Viewer
K_2HPO_4	Dipotassium phosphate
kb	kilo base pairs
KCl	Potassium Chloride
KDa	Kilo Dalton
kg	kilogram
KH_2PO_4	Monopotassium phosphate
LB	Lysogeny Broth
LiCl	Lithium Chloride
LSD1	Lysine-Specific Demethylase 1
m	milli (10^{-3})
M	molar, mol/L
MACS	Model-based Analysis of ChIP-seq
MCF10A	Michigan Cancer Foundation-10A
MCF12A	Michigan Cancer Foundation-10A
MET	Mesenchymal-to-epithelial transition
mg	milligram
$MgCl_2$	Magnesium Chloride
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
n	nano (10^{-9})
n.s.	non-significant
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium Chloride
NAF	Sodium Fluoride
NaH_2PO_4	Sodium dihydrogen phosphate

NaN_3	Sodium Azide
NCBI	national center for biotechnology information
NEM	N-ethylmaleimide
NES	Normalized Enrichment Score
$NiCl_2$	Nickel Chloride
nm	nanometer
NP-40	Nonidet P40
p300	e1a binding protein p300
pAdj	Adjacent p value
PBS	phosphate buffered saline
PBST	phosphate Buffered Saline with Tween-20
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
PHD	plant homeodomain
PI3K/Akt	Phosphotidylinositol-3-Kinase and Protein Kinase B
PRC1	Polycomb repressive Complex 1
PRC2	Polycomb repressive Complex 2
PTEN	phosphatase and tensin homolog
qRT-PCR	Quantitative real-time PCR
RIPA	Radio-Immunoprecipitation Assay buffer
RNA-seq	Sequencing of rt-transcribed RNA
RNF20	Ring Finger Protein 20
RNF40	Ring Finger Protein 40
RT	Room Temperature
RT-PCR	Reverse transcription PCR
sam	Sequence Alignment map
SDS	Sodium dodecyl Sulfate
SDS-PAGE	SDS- polyacrylamide Gel Electrophoresis
sec	second
siControl	negative control sirna

siRNA	small interfering RNA
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TBST	Tris-buffered saline and tween 20
TE	Tris-EDTA
TEMED	Tetra methyl ethylene diamine
TGF-β	transforming growth factor beta
TJP3	Tight junction protein 3
TR	Transcribed Region
Tris	Tris(hydroxymethyl)aminomethane
TSS	Transcription Start Site
U	unit (enzyme activity)
ub	ubiquitin
V	voltage
v/v	volume per volume
VIM	Vimentin
vs.	versus
w/v	weight per volume
Wdr	WD repeat domain
ZEB1	Zinc finger E-box binding homeobox 1
ZO1	Zonula occludes1

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Abstract

Breast cancer is one of the types of cancer that occurs in women frequently and most of the cancer deaths are due to metastasis. During cancer metastasis, the tumor epithelial cells lose their cell-cell contacts, acquire mesenchymal, migratory and invasive characteristics through epithelial-to-mesenchymal transition (EMT). Although many studies shed light on the mutations of ASXL family members in several cancers but the mechanism of action and the regulation remained poorly understood. ASXL1 is considered as a novel type of tumor suppressor in myeloid malignancies, which acts through the regulation of cell proliferation.

In this study, we showed that the depletion of ASXL1 in normal mammary epithelial cells leads to the loss of epithelial characteristics and gain stem cell-like, migratory and metastatic properties, which is characterized by the increased expression of the mesenchymal markers and decreased expression of the epithelial markers. Global RNA-seq transcriptome analysis revealed an enrichment of gene signatures associated with a mammary stem cell phenotype and EMT pathways upon ASXL1 knockdown. We also examined the genome-wide binding of ASXL1 via ChIP sequencing and identified a preferential occupancy of ASXL1 near transcriptional start sites of a subset of ASXL1 target genes. From our results, we found PTEN as a target gene and the depletion of PTEN promotes EMT phenotype.

Depletion of ASXL1 decreases the enrichment of H3K4me3 at the promoters of target genes. ASXL1 is physically interacting with methyltransferase SET1 complex members (Cfp1 and Wdr82) and it might be required for the maintenance of the cellular expression of SET1 complex members (Cpf1 and Wdr82). Taken together, our results suggest that ASXL1 is a tumor suppressor and negatively regulates EMT.

ASXL1 might promote transcription of a subset of ASXL1 affected genes either by directly binding to the target genes or through the active mark, H3K4me3 on the promoters. Further studies may provide additional insight which may be useful for developing new therapeutic targets for preventing or treating breast cancer metastasis.

1. Introduction

Breast cancer is one of the most common among all cancers in women and the second most frequent cause of cancer-related deaths in females worldwide. And more importantly, around 90% of them are caused by invasion and metastasis of the tumor cells. It also occurs, but rarely, in men. It accounts 23% of total cancer patients and 14% of all cancer deaths (Jemal, Bray et al. 2011). The breast is made up of glands which contain lobules and ducts. The most common type of breast cancer is ductal carcinoma.

Cancer development generally begins with a series of molecular events that alter the properties of normal cells, which leads to abnormal cell growth and division. Normal cells consist of a sophisticated system to check and control cell overgrowth, which is dysregulated in the cancerous cells making them to proliferate in an uncontrolled and indefinite manner. The loss of growth control is a result of the accumulation of genetic abnormalities like mutations, deletions, translocation or amplification. The mutated or malfunctioning genes can be grouped into three classes, such as oncogenes, tumor suppressors and DNA repair genes. Oncogenes are a mutated version of proto-oncogenes which normally enhance cell division and prevent cell death. In contrast, tumor suppressors have opposite functions like inhibiting cell division or causing cell death. Aberrations in DNA repair genes cause inappropriate DNA repair, which leads to accumulation of mutations and cancer. Although the affected genes may differ between tumors, the cancer cells share most of the characteristics, such as changes in cell morphology, insensitivity to anti-growth signals, evasion of apoptosis, continuous division and gain of migratory, invasive and metastatic properties. (Hanahan and Weinberg 2011, Sandoval and Esteller 2012).

Traditionally, cancer was considered as a genetic disease, but it is now realized that a combination of epigenetic abnormalities and genetic alterations work together to promote cancer development and progression (Jones and Laird 1999, Feinberg, Ohlsson et al. 2006, Jones and Baylin 2007).

1.1. Chromatin Structure and organization

Eukaryotic cells contain approximately two-meters-long genomic DNA, which is compacted and packed in the form of chromatin into 10 µm nucleus. However, it can be accessible to the cellular processes such as gene expression and regulation at the same time (Felsenfeld and Groudine 2003).

Chromatin is a complex of DNA, histones and other proteins. It is a highly ordered structure made up of arrays of nucleosomes that forms the genetic material of a cell (Burgoyne, Mobbs et al. 1976). The nucleosome is the repeating unit of chromatin that comprises of approximately 147 base pairs of DNA wrapped in about 1.67 turns around the octamer of core histones. The core histones that form the octamer are H2A, H2B, H3 and H4 and each present as two copies within the nucleosome (Kornberg 1974, Fletcher and Hansen 1996, Luger, Mader et al. 1997, Suganuma and Workman 2011). Core histones contain positively charged lysine and arginine residues and their basic nature that allows them to be wrapped by the negatively charged DNA, which help in maintaining the stability of nucleosome. Within the histone octamer, histone proteins are known to dimerize through a structural feature called, histone fold, which constitutes three alpha-helices. In addition to the histone fold, histone possesses N-terminal tails that project out of the nucleosome and are known to contribute to the overall structural organization of the chromatin due to their

propensity to be modified post-translationally (Fletcher and Hansen 1996, Luger, Mader et al. 1997).

Each nucleosome is joined to the other through a linker DNA (180-240 bp) which is in turn bound by a fifth type of histone called linker histone H1. The linker histones are highly basic and bind to the DNA in the nucleosome core particles through their globular domains and the linker DNA with their tails (Das, Lucia et al. 2009, Vempati, Jayani et al. 2010, Suganuma and Workman 2011). The linker histones play a major role in the secondary level of chromatin organization by connecting adjacent nucleosomes to form a fiber-like structure, chromatin (Allan, Cowling et al. 1981, Fletcher and Hansen 1996, Dutnall and Ramakrishnan 1997, Luger, Mader et al. 1997, Thomas 1999) (Fig.1).

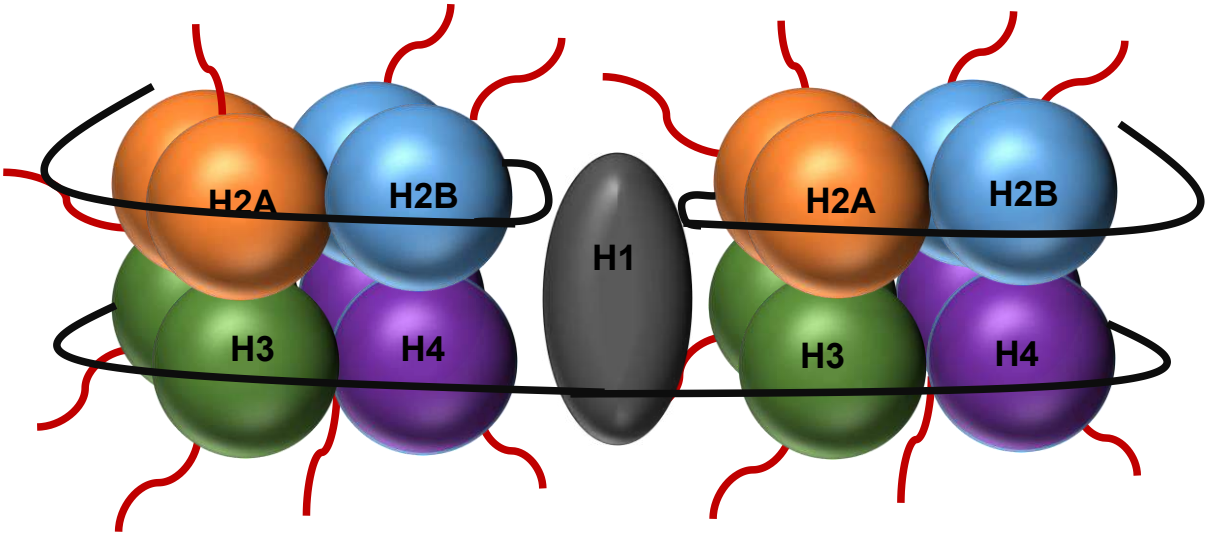


Figure. 1: The structure of the nucleosome. The basic structure of chromatin, nucleosome comprises of an octamer of histones H2A, H2B, H3 and H4 as dimers. Histones are wrapped around by 147 base pairs of DNA. The two nucleosomes are joined together by linker DNA and H1 binds to adjacent nucleosomes. The histone tails undergo post-translational modifications.

The most striking property of chromatin is the way it is packed within a cell. Nucleosome along with linker DNA form linear 11 nm fiber-like structure, where

nucleosomes are arranged as “beads on a string” which is further compacted into a thicker fiber with 30 nm diameter (Kornberg 1974, Marsden and Laemmli 1979, Turner 1993). The 30-nm fiber achieves a compaction of roughly 50-fold from the original DNA length, which is further packed to form the higher-order structures (Fig. 2) (Henikoff 2000, Richards and Elgin 2002, Felsenfeld and Groudine 2003).

Chromatin can be divided into two major states, “euchromatin” and “heterochromatin” Heterochromatin is a highly condensed form of chromatin that is inaccessible to DNA binding factors and transcriptionally inactive. In contrast, euchromatin is less condensed, open and easily accessible, comprises a high density of genes that are either actively transcribed or repressed (Owen-Hughes and Bruno 2004, Grewal and Elgin 2007). Heterochromatin is also known as a chromatin state that functions in controlling chromosomal stability and preventing mutations and translocations (Huang, Fan et al. 2004, Muegge 2005, Talbert and Henikoff 2006).

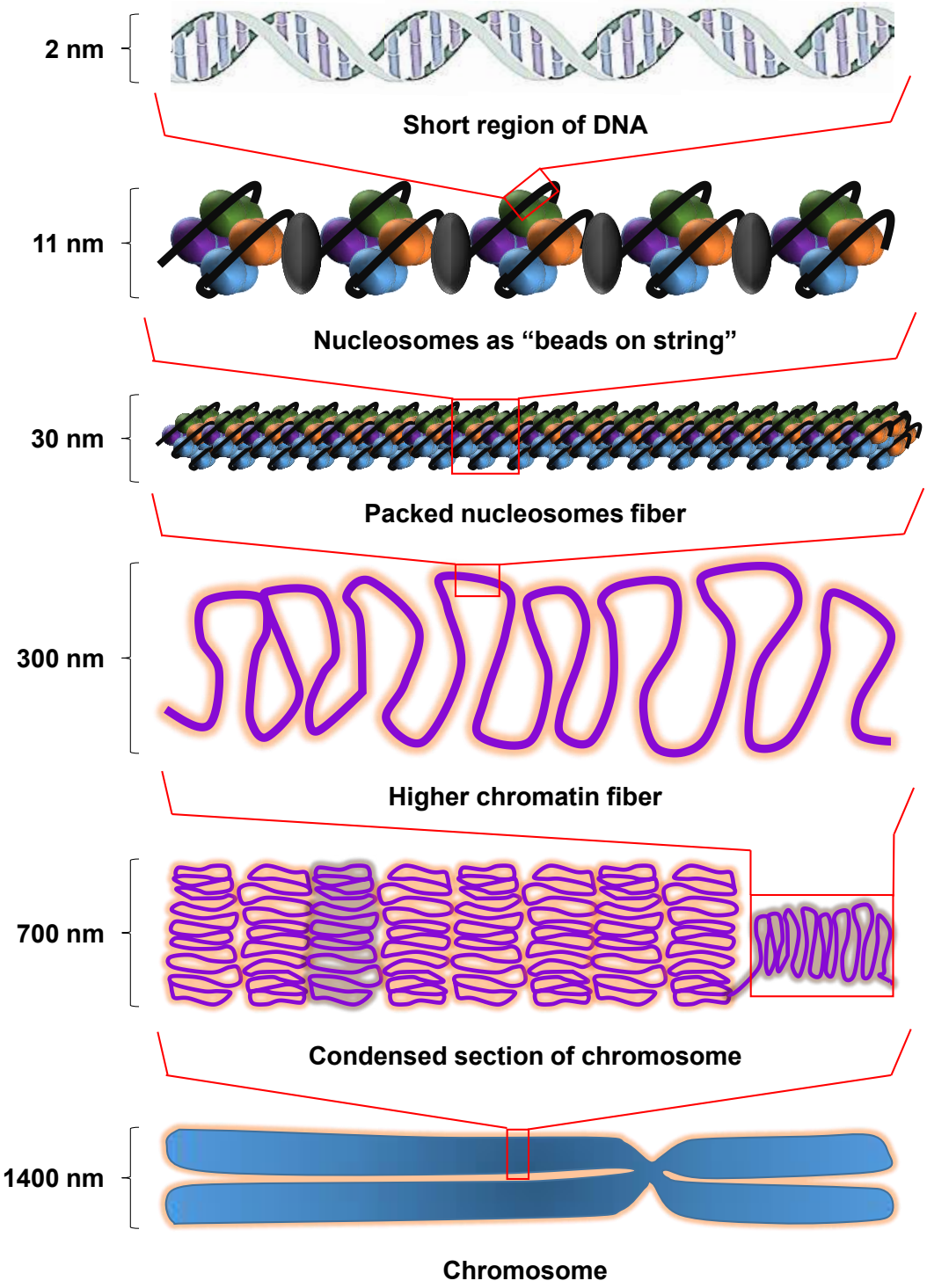


Figure. 2: Chromatin organization. DNA is wrapped around a histone octamer to form nucleosome and the two nucleosomes are connected by linker DNA to form 11 nm chromatin fiber. The chromatin fibers are condensed to form 30 nm fiber and then compacted to form higher order structures. Modified from (Felsenfeld and Groudine 2003)

1.2 Histone modifications

In general, the core histones are subjected to different post-translational modifications in their N- and C-terminal tails, which are unstructured and freely accessible on the surface of the nucleosome core to various enzymatic machineries to act at specific amino acid residues (Luger, Mader et al. 1997, Jenuwein and Allis 2001, Rando and Chang 2009). The post-translational modifications include methylation of lysine and arginine residues, acetylation of lysine residues and phosphorylation of serine and threonine residues, which are well studied. There is also poorly understood category of the post-translational modifications, which includes ubiquitination and sumoylation of lysine residues (Fig.3) (Fischle, Wang et al. 2003, Osley 2004, Yang 2005, Johnsen 2012). Acetylation and methylation are the most elaborately studied modifications. Their functional consequences and the domains that recognize them are also well studied along with the enzymes that carry out these modifications. These modifications extend the histone's role in the regulation of processes like replication, transcription, recombination and repair besides scaffolding the chromatin structure (Jenuwein and Allis 2001).

The combination of post-translational modifications of histones creates a complex set of signals defined as "histone code". Before the histone code was proposed, histones were simply thought as proteins required for DNA compaction. Now it has become evident that the DNA sequence alone can not decide all the outcomes of chromatin and there are other factors that lead to various chromatin-templated events.

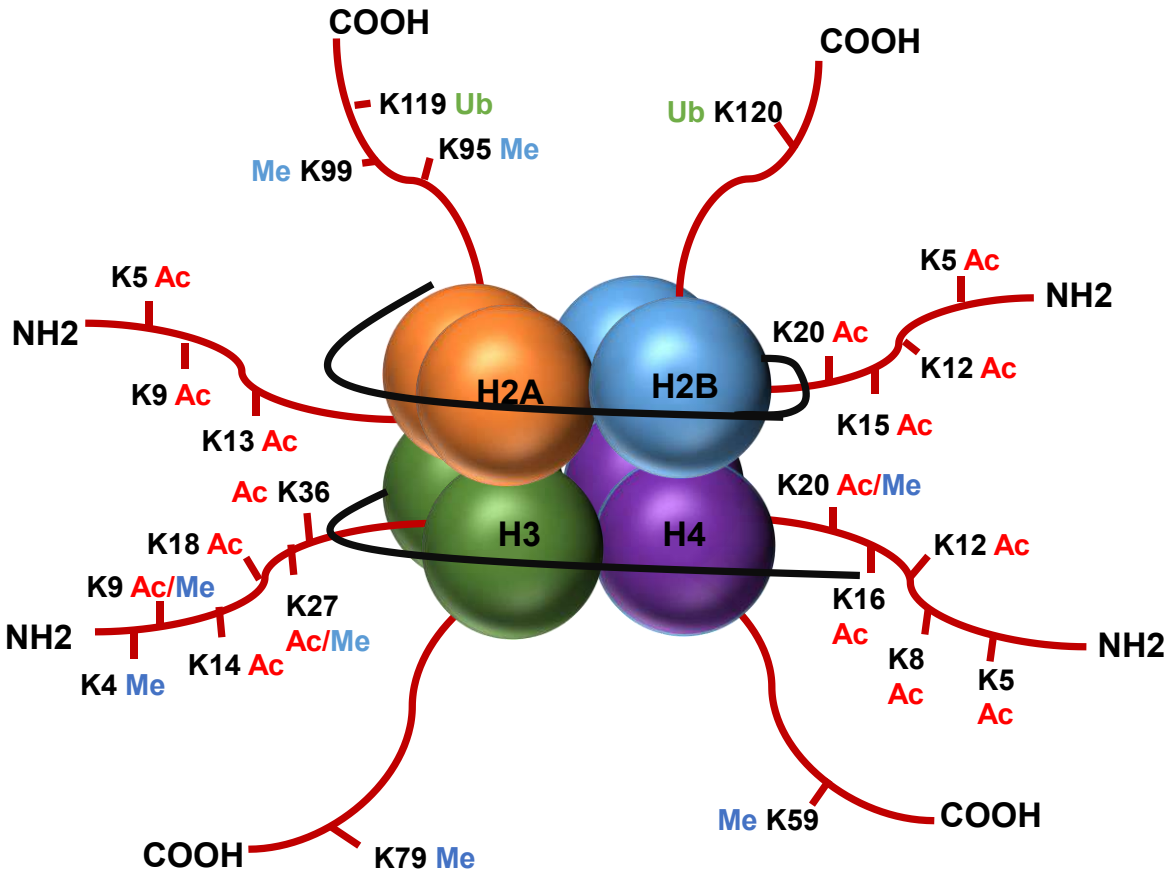


Figure. 3: Main post-translational histone modifications. Different types of post-translational modifications occur at the NH2- terminal and COOH- terminal tails of the histones which define the chromatin state. Histone modifications include acetylation, methylation, ubiquitination, phosphorylation and sumoylation. Some of the modifications are shown here. Modified from (Cota, Shafa et al.)

The “histone code” provides a specific binding site for selected effector proteins and can determine the cell fate by regulating key processes such as cell cycle, apoptosis and signal transduction (Strahl and Allis 2000, Jenuwein and Allis 2001, Latham and Dent 2007). Some histone modifications are associated with transcriptional silencing (H3K27me3) (Karpiuk, Najafova et al.) and others with activation (H3K4me3) (Strahl and Allis 2000, Bannister, Schneider et al. 2002). This function is mediated either directly by changing the physical or chemical properties of the chromatin fiber or indirectly by recruiting the chromatin-modifying proteins that can activate or repress the transcription.

1.2.1 Chromatin modifying enzymes

Epigenetic modifications (DNA and histones) play an important role in the regulation of hetero and euchromatin. Epigenetic modifications remodel the chromatin either directly by influencing the nature of interactions with the DNA or by employing other non–histone proteins which regulate cell-specific gene expression or repression. The epigenetic regulators alter the chromatin structure and regulate its accessibility to the replication and transcriptional machinery (Luger, Mader et al. 1997, Horn and Peterson 2002, Narlikar, Fan et al. 2002). These modifications can be achieved by the specific types of enzymes and co-factors and they can be majorly classified into the following categories: “epigenetic writers” and “epigenetic erasers”. Epigenetic writers are the enzymes that catalyze the addition of chemical groups onto either histone tails or the DNA such as acetyl or methyl groups or ubiquitin moieties (e.g. histone lysine acetyltransferases, histone lysine/arginine methyltransferases, and ubiquitin ligases, DNA methyltransferases) (Campos and Reinberg 2009, Cedar and Bergman 2009). Epigenetic erasers are a group of enzymes that can remove the histone modifications (e.g. histone demethylases and histone deacetylases) (Kangaspeska, Stride et al. 2008, Metivier, Gallais et al. 2008). Misregulation of factors that mediate the deposition or removal of histone modifications leads to epigenetic imbalance, which causes cancer initiation, progression and metastasis.

1.2.2 Chromatin binding proteins

“Epigenetic readers” are a special class of proteins that recognize and are recruited to the specific modifications on histones or nucleotides. Some of the well-studied examples are- bromodomain for acetylated lysine, chromodomain, PHD-finger

domains and WDR40 for methylated lysine or arginine residues. (de la Cruz, Lois et al. 2005).

Chromatin is a highly dynamic structure that is constantly remodeled to provide accessibility to several factors facilitating important biological processes. In addition to the chromatin modifiers, there are chromatin remodeling enzymes and histone chaperones. that alter the chromatin architecture by removing, adding, moving or replacing histones in the chromatin (Strahl and Allis 2000, Loyola and Almouzni 2004, Taverna, Li et al. 2007, Wilson and Roberts 2011). Chromatin remodeling is known to participate in various DNA transaction processes like replication, recombination, transcription, repair and chromatin assembly. The chromatin remodeling complexes are classified into following families, such as SWI/SNF (switching defective/sucrose non-fermenting) family, the ISWI (imitation SWI) family, the NuRD (nucleosome remodeling and deacetylation)/Mi-2/CHD (chromodomain, helicase, DNA binding) family and the INO80 (inositol requiring 80) family (Morrison and Shen 2009, Hargreaves and Crabtree 2011).

1.3 Cancer and metastatic progression

Cancer metastasis is a complex and multistep process in which tumor cells disseminate from the primary tumor, migrate and survive during circulation, invade and adapt to the microenvironment of distant secondary site to form new tumors (secondary tumors) that result in the 90% of deaths in the cancer patients (Zajicek 1996, Gupta and Massague 2006).

1.3.1 Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is first identified in the development of the embryo and it plays an essential role in the early developmental processes such as gastrulation, mesoderm formation and neural crest development. During EMT, polarized, immotile epithelial cells transform their plasticity into a migratory mesenchymal phenotype by transient dedifferentiation (Wang and Zhou 2013, Lamouille, Xu et al. 2014). To undergo EMT, the epithelial cancer cell loses the expression of E-cadherin and cell-cell adhesion proteins such as claudin, occludins and Zonula occludes 1 (ZO1) (Huang, Guilford et al. 2012). The adherent junctions are destabilized by degradation of epithelial cadherin (E-cadherin) and repression of cytokeratins. The down-regulation of E-cadherin is compensated by increased expression of mesenchymal neural cadherin (N-cadherin), fibronectin, Vimentin and facilitate cell migration and invasion (Maschler, Wirl et al. 2005, Kim, Litzenburger et al. 2007, Wheelock, Shintani et al. 2008, Yilmaz and Christofori 2009, Huang, Guilford et al. 2012).

EMT is also involved in wound healing, tissue regeneration and organ fibrosis (Wang and Zhou 2013). In cancer, the differentiated epithelial tumor cells convert into differentiated mesenchymal cells through the EMT process. EMT phenotype is associated with a reduction in tumor growth, increased resistance to apoptosis, increased motility and invasiveness and enhanced metastatic ability (Kang and Massague 2004, Eccles and Welch 2007).

During EMT, a set of genes, associated with cell adhesion, differentiation, migration and invasion, are transcriptionally altered which is achieved by several transcription factors, SNAL1, SLUG, TWIST1, ZEB1, ZEB2 and basic helix-loop-helix factors.

Depletion of E-cadherin is an important characteristic of EMT during the development and cancer progression. SNAL1 repress CDH1 gene transcription by directly binding to the E-boxes of CDH1 promoter. In addition to SNAIL1, several other transcription factors are also capable of repressing E-cadherin transcription. SLUG, a close relative of SNAL1 and two other members of ZEB family- ZEB1 and ZEB2 also bind to the CDH1 promoter and downregulate the E-cadherin expression (Hennig, Behrens et al. 1995, Giroldi, Bringuier et al. 1997, Batlle, Sancho et al. 2000, Cano, Perez-Moreno et al. 2000, Comijn, Berx et al. 2001, Hajra, Chen et al. 2002, Eger, Aigner et al. 2005). These transcription factors act as transcriptional repressors and suppress the expression of E-cadherin and ZO-1, leading to the dissolution of cell-cell adhesions and tight junctions. Repression of epithelial markers is balanced by upregulation of mesenchymal markers, such as N-cadherin, Vimentin and fibronectin. N-cadherin upregulation stimulates the tumor metastasis and its expression is associated with poor survival. Vimentin is an intermediate filament which is involved in the migration and invasiveness and its expression is considered as a charcteristic of epithelial cells undergoing EMT (Franke, Grund et al. 1982, Nakajima, Doi et al. 2004). Altogether, these changes enable the cells to switch from an epithelial phenotype to mesenchymal phenotype by regulating expression of epithelial markers to promote cell migration and invasion.

1.3.2 Mesenchymal-to-Epithelial transition

Mesenchymal-to-Epithelial transition (MET) is a reverse-EMT process in which motile, multipolar or spindle- shaped mesenchymal cells are transformed into polarized epithelial cells. In general, this process occurs after the cell reaches the destination followed by their differentiation into specific tissue resident cells during

embryo development or organogenesis (De Craene and Berx 2013, Nieto 2013). But in the case of cancer, the migrated mesenchymal tumor cells revert back into epithelial cells through MET process and form a secondary tumor (Peinado, Olmeda et al. 2007, Yang, Chen et al. 2009, Martin, Dwyer et al. 2010, Sun, Zhao et al. 2010).

EMT and MET consist the opposite processes by which cells switch between epithelial and mesenchymal phenotypes and play a vital role both in embryo development and organogenesis and in tumor metastasis. As the critical EMT event is the down-regulation or repression of E-cadherin, the reexpression of E-cadherin is an important hallmark of MET. (Fig.4). Both EMT and MET are dynamically balanced to maintain tissue homeostasis and development of embryo and metastatic cancer (Prindull 2005, Ricci-Vitiani, Lombardi et al. 2007, Thiery, Acloque et al. 2009)

MET participate in the stability of distant metastases by making the cancerous cells regain epithelial properties and integrate into distant organs (Micalizzi, Farabaugh et al. 2010, Heerboth, Housman et al. 2015). MET is also an essential developmental process and well-studied in kidney organogenesis, cardiogenesis, hepatogenesis and somitogenesis (Nakajima, Yamagishi et al. 2000, Nakaya, Kuroda et al. 2004, Li, Zheng et al. 2011).

1.3.3 Cancer stem cells and metastasis

Cancer stem cells (CSCs) or tumor-initiating cells represent a small percentage of tumor cells that have an ability to self-renew and differentiate into cancer cells (Lapidot, Sirard et al. 1994, Jaggupilli and Elkord 2012).

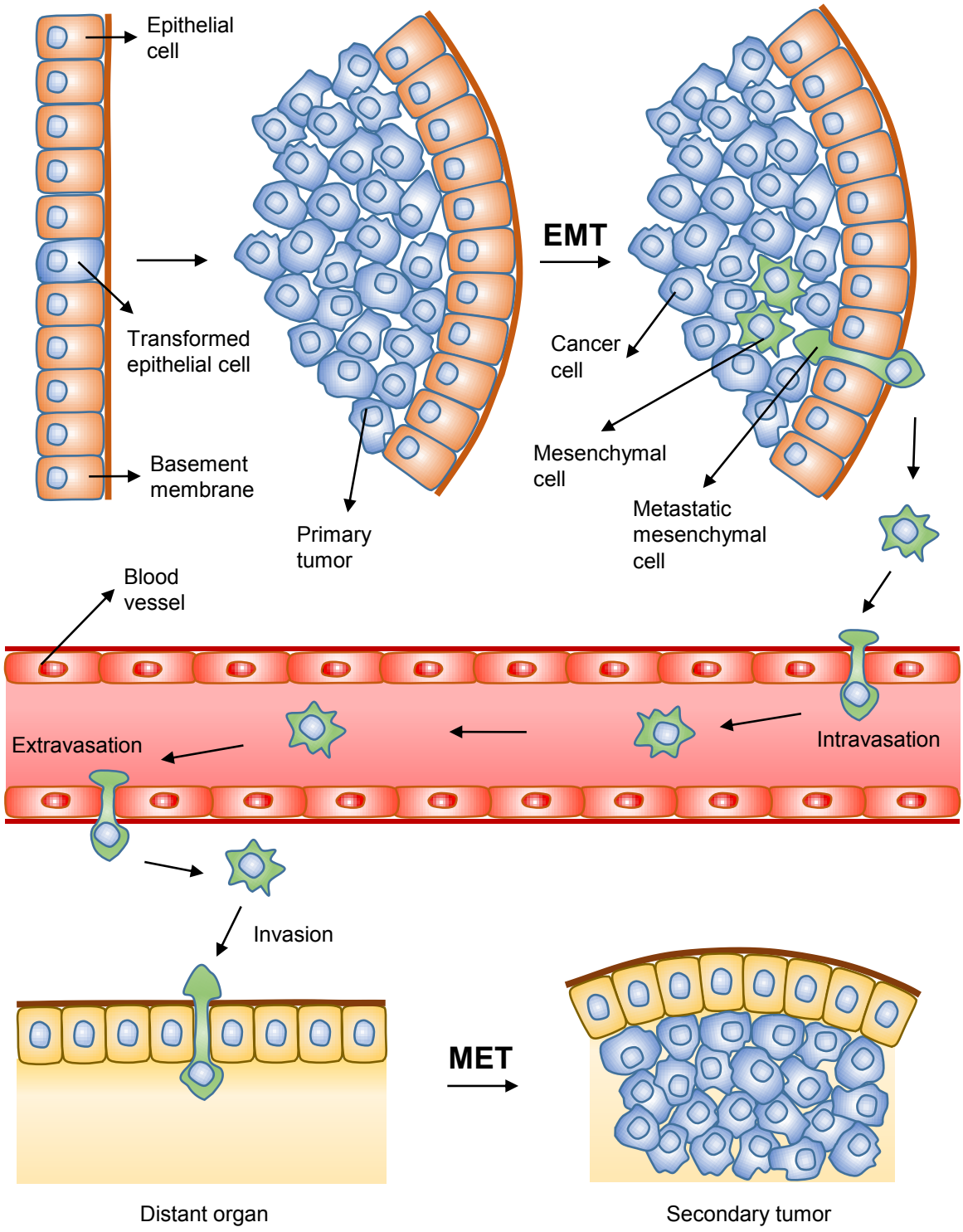


Figure. 4: EMT and MET in carcinogenesis and tumor metastasis. The transformed epithelial cell divide, grow and form a primary tumor. The epithelial cells are converted to mesenchymal cells through EMT process. Then the tumor cells disseminate from the primary tumor and enter into circulation (intravasation). The disseminated tumor cells exist the blood vessels (extravasation) and invade into the distant organ and the mesenchymal cells revert back into epithelial cells through MET to form a secodary tumor. Modified from (Peinado, Olmeda et al. 2007).

Similar to the normal stem cells, CSCs can also develop into different types of cancer cells in the tumor and hence termed as cancer stem cells. CSCs are capable of dictating invasion, oncogenesis, metastasis, tumor growth and cancer recurrence. Differentiated cancer cells were shown to proliferate faster than CSCs, but have the little tumor-initiating capacity (Chen, Kasai et al. 2012). CSCs can form new tumors when injected into immunodeficient mice (Chen, Yang et al. 2012). CSCs are first identified in leukaemia and have been reported in different cancers including breast (Al-Hajj, Wicha et al. 2003), colon (Ricci-Vitiani, Lombardi et al. 2007), prostrate (Gu, Yuan et al. 2007), ovarian (Zhang, Balch et al. 2008), pancreas and other tissues (Lapidot, Sirard et al. 1994, Visvader and Lindeman 2008).

CSCs can be identified by both CSC-specific cell surface marker expression and also by functionality assays, such as sphere formation and aldehyde dehydrogenase (ALDH) activity assays. The surface marker expression profile of CSCs, that promotes breast cancer metastasis, is $CD44^{high}/CD24^{low}$ (Al-Hajj, Wicha et al. 2003). Signal transduction pathways of stem cell regulation also play an important role in carcinogenesis e.g. Notch, Sonic hedgehog (Gigi, Geiger et al.), and Wnt signaling. The traditional therapies used against cancer such as chemotherapy and radiotherapy are inefficient due to drug resistance of cancer cells and self-renewal properties of CSCs, which leads to cancer recurrence (Kopper and Hajdu 2004, Dragu, Necula et al. 2015).

1.4 Epigenetics in cancer

Cancer is initiated by genetic alterations such as mutations or by epigenetics, such as tumor suppressors and oncogenes, which regulate cell survival, proliferation and homeostatic functions. Epigenetic mechanisms play an indispensable role in normal

development and maintenance of tissue-specific gene expression. (Egger, Liang et al. 2004, Sharma, Kelly et al. 2010). Disruption of epigenetic processes alters gene expression and malignant cellular transformation (Sharma, Kelly et al. 2010). The post-translational modifications of histones play a crucial role in regulating the accessibility of epigenetic regulators and transcription factors to the specific regions of chromatin. Tumorigenesis is hypothesized that alteration in the epigenetic modifications leads to inappropriate expression or silencing of genes (oncogenes or tumor suppressors) (Chi, Allis et al. 2010).

Following is a brief overview of different epigenetic modifications, the enzymes involved in maintaining their dynamics and their functional relevance.

1.4.1 DNA methylation

DNA methylation is one of the fundamental and most intensely studied epigenetic modifications, which plays an important role in regulating gene expression and stable gene silencing. DNA methylation is associated with histone modifications and regulate the genomic function by changing chromatin architecture. DNA methylation is a covalent modification of DNA that occurs at the cytosine residues in CpG dinucleotides, which form large clusters called CpG islands. DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs). CpG islands occupy approximately 60% of human gene promoters (Bird 2002, Takai and Jones 2002, Wang and Leung 2004).

Cancer cells exhibit aberrant DNA methylation patterns such as hypo and hypermethylation, which can drive malignant transformation. Tumor suppressor genes are often silenced in tumor cells due to hypermethylation whereas oncogenes are hypo methylated (Gonzalo 2010, Zhang, Cardarelli et al. 2011). DNMTs are

overexpressed in breast cancers. It was reported that DNA methylation silences several genes in breast cancer and thus affects several pathways involved in breast tissue homeostasis, including cell cycle regulation, tumor susceptibility, carcinogen detoxification and cell adhesion (Widschwendter and Jones 2002).

E-cadherin (CDH1) was reported to be downregulated by DNA methylation promoting an invasive phenotype in prostate and other epithelial cancers. It was shown that hypermethylation of CpG island in the promoter region of the CDH1 gene alters the expression of E-cadherin, leading to loss of tissue integrity which is an important step in tumor progression. (Caldeira, Prando et al. 2006, Keil, Abler et al. 2014). DNA methylation and histone modifications cooperate with each other to repress the target gene expression. E-Cadherin expression is significantly downregulated by suppressing demethylation of histone H3 on lysine 9 (H3K9me2) and DNA methylation by an interaction of EMT-TF, SNAIL with G9a and DNA methyltransferases (DNMTs) and recruits to the promoters (Dong, Wu et al. 2012). It was also reported that DNA methylation is involved in the generation of CSCs (Esquela-Kerscher and Slack 2006, Volinia, Calin et al. 2006). Altogether, alteration in DNA methylation plays an important role during the EMT induction.

1.4.2 Histone modifications and their functional consequences

Alteration in the post-translational modifications of histones contributes to the repression of tumor suppressor genes and the activation of oncogenic genes. Several histone readers and writers are mutated or transcriptionally altered in tumors. Histone modifications like acetylation, methylation and ubiquitination were shown to be associated with several disorders and cancers (Fraga and Esteller 2005, Seligson, Horvath et al. 2009).

1.4.2.1 Histone acetylation

Histone acetylation is a reversible, very dynamic and well-characterized modification which is involved in chromatin structure, transcription, DNA repair and cancer progression (Wang, Allis et al. 2007, Choudhary, Kumar et al. 2009). Histone acetylation is a chemical reaction carried by various histone acetyltransferases (HATs), which transfer the acetyl group from acetyl coenzyme A (acetyl-CoA) to the ε-amino group of the lysine residues of histone proteins. Histone acetylation is generally associated with a more open chromatin structure and active transcription. The acetylation of histones neutralizes the positive charge of lysines, which can potentially reduce the interaction between DNA and histones and thereby increases DNA accessibility to the transcription factors (Imhof, Yang et al. 1997). Histone acetylation regulates many cellular processes and the unique acetylation marks either alone or in combination lead to distinct outcomes (Grunstein 1997, Tropberger and Schneider 2010).

The most prominent members of the acetyl transferases are the GNAT (Gcn5-related N-acetyltransferases), CBP/p300 and MYST (MOZ/YBF2/SAS2/TIP60/HBO1) families. Broadly each of these enzymes is capable of modifying different lysine residues of histones. HATs can act as tumor suppressors and control cell cycle progression and proliferation. They can also function as oncogenes and activate malignant genes by altering acetylation profile and contribute to cancer (Di Cerbo and Schneider 2013). The HAT, p300 promotes EMT and tumor progression by affecting the regulation of SNAIL and ZEB1 in colon cancer (Pena, Garcia et al. 2006). The HATs such as KAT2A, KAT2B and KAT5

acetylate the oncogene c-MYC and leads to cancer progression (Patel, Du et al. 2004).

1.4.2.2 Histone deacetylation

Deacetylation is a reverse reaction to acetylation, catalyzed by Histone deacetylases (HDACs), which restores the positive charge of the lysine residues. Histone deacetylation potentially results in chromatin compaction and transcriptional repression (Yang and Seto 2007). Eighteen mammalian HDACs are reported till now and are categorized into four major classes based on their sequence homology and cofactor dependency. Class I includes HDACs 1, 2, 3 and 8 (similar to yeast Rpd3), Class II consists HDAC 4, 6, 7, 9 and 10 (similar to yeast Hda1), Class III includes sirtuins, SIRT1-7(similar to yeast Sir2) and class IV consists of HDAC 11 (Ropero and Esteller 2007).

Loss of histone acetylation is not only involved in carcinogenesis but also in tumor invasion and metastasis (Yasui, Oue et al. 2003). Like HATs, HDACs are also important for breast cancer development. HDAC1 plays an important role in TGFβ1 induced EMT and inhibition of HDAC1 suppress TGFβ1 induced EMT (Yoshikawa, Hishikawa et al. 2007, Lei, Zhang et al. 2010). HDAC3 interacts with a component of histone methyltransferase complex, WDR5 and induces hypoxia-mediated EMT by regulating acetylation and methylation patterns on EMT genes. SIRT1 was shown to interact with ZEB1 and repress CDH1 expression by deacetylation of its promoter (Wu, Tsai et al. 2011, Byles, Zhu et al. 2012). HDAC1/2 complex cooperates with either SNAIL or ZEB1 and recruited to the CDH1 promoter and represses its expression.

1.4.2.3 Histone methylation

Histone methyltransferases (HMTs) transfer methyl groups from S-adenosyl methionine to the side chain of lysine and arginine residues. As methyl groups do not carry any charge on them, histone methylation does not alter the overall charge of the histone proteins. Lysines residues may be mono-, di-, or tri-methylated whereas arginines may be mono-, symmetrically or asymmetrically dimethylated (Bedford and Clarke 2009). The methyltransferases are classified into arginine (PRMTs) or lysine (KMTs) methyltransferases depending on the methylation of the amino acid residue. SET domain is the catalytic subunit of all histone methyltransferases (HMTs) such as G9a, EZH1/2, SUV39H1/H2, except for the DOT1L, H3K79 methyltransferase, and transfer methyl group (Miller, Krogan et al. 2001, Wang and Shang 2013). Lysines 4, 9, 27, 36 and 79 of histone H3 and 20 of histone H4 are methylated by lysine methyltransferases. Histone methylation is known to be associated with either transcriptional activation or repression depending upon the position of the amino acid residue modified. For example, trimethylation of histone H3 at K4 (H3K4me3), K79 (H3K79me3) and K36 (H3K36me3) are considered as active marks, whereas H3K9me2, H3K9me3 and H3K27me3 are considered as transcriptional repressive marks (Kouzarides 2007).

Histone methylation is important for proper genome programming during development. Misregulation of methylation machinery can lead to diseased states such as cancer. Several studies reported that altered histone methylation might play a role in cancer, tumor recurrence and poor survival (Albert and Helin 2010, Chi, Allis et al. 2010). Aberrant regulation of G9a (H3K9 methyltransferase) was identified in several cancers. EMT-TF, SNAIL cooperates with histone methyltransferase G9a

and DNMTs to the CDH1 gene promoter to modulate CDH1 expression (Dong, Wu et al. 2012). It is also reported that Suv39H1 can trimethylate H3K9 on the CDH1 promoter (Serrano-Gomez, Maziveyi et al. 2016). Other methyltransferases, MMSET can di- or trimethylate H3K36 at the TWIST promoter whereas SET8 methylates H420 at the CDH1 and CDH2 promoters (Serrano-Gomez, Maziveyi et al. 2016). Mesenchymal markers are marked with H3K4me3 by WDR5, part of MLL and SET1 HMT complex upon hypoxia (Wu, Tsai et al. 2011). The PRC2 complex which contains methyltransferase along with EED and SUZ12 plays a crucial role in transcriptional silencing by a repressive mark, H3K27me3 in carcinogenesis and EMT (Orlando 2003, Herranz, Pasini et al. 2008)

1.4.2.4 Histone demethylation

Since methylation is a very specific and stable mark, it was initially thought to be an irreversible modification until the discovery of the lysine demethylase, LSD1 or KDM1A which can remove mono and dimethyl groups from K4 of histone H3 (H3K4me1/2), leading to transcription repression (Shi, Lan et al. 2004). JMJD6, a jumonii domain containing protein is the first arginine demethylase, which can demethylate methyl group from R2 and R3 of histone H3 (Chang, Chen et al. 2007). LSD1 is recruited to the epithelial gene promoters and repress the gene expression by removing the methyl groups from the H3K4me2 by interacting with EMT-TF, SNAIL (Lin, Ponn et al. 2010, Amente, Lania et al. 2013, Ferrari-Amorotti, Fragliasso et al. 2013). A histone H3K4 demethylase, KDM5B increases the expression of transcription factors ZEB1 which further downregulates the E-cadherin expression and upregulates the mesenchymal marker genes (Enkhbaatar, Terashima et al. 2013). Two other demethylases, JMJD3 (KDM6B) and JMJD2B (KDM4B) can

demethylate H3K27me3 and H3K9me3 respectively and were shown recently to promote EMT (Ramadoss, Chen et al. 2012, Zhao, Li et al. 2013).

1.4.2.5 Histone ubiquitination

Ubiquitination is a basic biochemical process of covalent attachment of one or more ubiquitin molecules to lysine residues of proteins or to existing ubiquitin molecules on a protein. Ubiquitination is catalyzed by the sequential action of three enzymes, E1 ubiquitin-activating, E2 ubiquitin-conjugating and E3 ubiquitin-ligase enzymes (Hershko and Ciechanover 1998, Sadowski and Sarcevic 2010). Ubiquitination controls the protein functions, such as degradation and subcellular localization (Pickart and Eddins 2004). Monoubiquitination can regulate DNA repair and gene expression but polyubiquitination through UbK48 targets proteins for proteasome degradation (Passmore and Barford 2004).

H2A monoubiquitination is carried by PRC1 complex, which comprises RING1A and RING1B (Wang, Wang et al. 2004, Cao, Tsukada et al. 2005). H2B monoubiquitination is performed by RNF20/40 heterodimer (Zhu, Zheng et al. 2005). Ubiquitination plays a vital role in PRC-mediated silencing (Niessen, Demmers et al. 2009). Both histones, H2A and H2B are monoubiquitinated at K119 (H2Aub1) and K120 (H2Bub1) respectively. H2Aub1 is associated with heterochromatin and prevents transcription elongation, whereas H2Bub1 is localized to euchromatin and promotes transcription elongation (Wang, Wang et al. 2004, Cao, Tsukada et al. 2005, Xiao, Kao et al. 2005, Shukla, Stanojevic et al. 2006, Minsky, Shema et al. 2008). H2Bub1 physically disrupt chromatin structure and makes DNA accessible to transcription factors and DNA repair proteins and plays a vital role in transcription, DNA damage response and also stem cell differentiation.

Decreased H2Bub1 levels were reported to be associated with tumor progression and tumor invasion (Shema, Tirosh et al. 2008, Prenzel, Begus-Nahrmann et al. 2011, Johnsen 2012). Global H2Bub1 levels are either decreased or absent in advanced cancers such as breast, lung, colorectal and parathyroid cancers. The components of the PRC1 complex ubiquitinates H2A and promotes EMT by upregulating SNAIL through regulating PI3K/Akt/GSK-3β signaling pathway and also targets other EMT-TFs, Twist1 and ZEB1 (Song, Li et al. 2009, Wellner, Schubert et al. 2009, Yang, Hsu et al. 2010).

1.4.2.6 Deubiquitination

As other histone modifications, ubiquitination is also a reversible process and the removal of ubiquitin molecules is termed as deubiquitination which is performed by deubiquitinases (DUBs). There are around 100 DUBs encoded by the human genome, which are classified into 5 families. They are ubiquitin C-terminal hydrolases (Firestein, Bass et al.), ubiquitin-specific proteases (USPs), ovarian tumor domain DUBs, machado Joseph domain DUBs and JAB1/MPN metalloenzyme. The activity of these enzymes affects the half-life, activity and localization of multiple proteins, which in turn regulate cell homeostasis, protein stability and signaling pathways. DUBs can also be categorized into oncogenes or tumor suppressors as they regulate the activity of the other proteins involved in tumor development and progression (Fraile, Quesada et al. 2012). EMT-TFs such as Snail/Slug, ZEB1/ZEB2, and Twist1 protein levels are tightly controlled by the ubiquitin-proteasome system (UPS) and several E3 ubiquitin ligases are shown to play crucial roles in the regulation of EMT. Genetic aberrations and alterations of these ligases have been detected in human cancer (Liu, Yang et al. 2011). USP22

(Ubiquitin-specific-protease-22), an H2B deubiquitinating enzyme is upregulated in tumors with a stem cell-like phenotype exhibiting a poor patient outcome (Glinsky, Berezovska et al. 2005, Zhang, Varthi et al. 2008, Inoue, Itoh et al. 2016). BAP1 (BRCA1-Associated Protein 1), an H2A deubiquitinating enzyme, is reported as a tumor suppressor and its expression showed a negative correlation with tumorigenesis in lung cancer (Shen, Wang et al. 2016). Another report showed that H2A DUB, USP3 depletion induces mesenchymal cellular phenotype in A549 epithelial lung cells (Nicassio, Corrado et al. 2007, Buus, Faronato et al. 2009).

1.5 Role of signaling pathways in EMT

Several molecular signaling pathways play an important role in cancer development, progression and maintenance of CSC phenotype. Cell signaling pathways such as Wnt-β-catenin, Notch, transforming growth factor β (TGFβ), Sonic Hedgehog (Gigi, Geiger et al.), PI3K/Akt and Hypoxia are involved in the progression of EMT. These pathways are activated by various dynamic stimuli from the microenvironment, including cytokines, hypoxia etc.

1.5.1 TGF-β signaling pathway

Transforming growth factor-β (TGF-β) signaling is a well-studied pathway and plays a crucial role in diverse cellular processes like proliferation, apoptosis, differentiation and development. Misregulation of TGF-β leads to defects in developmental processes, organ fibrosis and cancer. TGF-β acts as a tumor suppressor in normal cells. Interestingly, it functions as a tumor promoter in advanced cancers. (Derynck and Akhurst 2007, Guo and Wang 2009, Nagaraj and Datta 2010). The signaling is initiated by TGF-β binding to the transmembrane receptors that have serine/threonine kinase activity. After TGF-β binding to the TGF-β receptor II and

TGF-β receptor I is recruited into the complex and phosphorylated. This leads to the phosphorylation of the C-terminus of the receptor-regulated Smad proteins (R-Smads). The activated R-Smads interact with other Smads (Co-Smads), Smad 4 and the Smad complex is translocated into the nucleus where it directly binds to the DNA and regulates TGF-β target gene expression along with other DNA binding factors, like ZEB, SNAIL and Twist (Massague 2000, Shi and Massague 2003, Zavadil and Bottinger 2005, Gomis, Alarcon et al. 2006, Fuxe, Vincent et al. 2010). This results in the decreased expression of epithelial markers, E-cadherin and cytokeratins and upregulation of mesenchymal markers, Vimentin, N-cadherin and fibronectin.

TGF-β is a potent driver of cancer progression through EMT induction which in turn is associated with metastatic cancer (Wood, Parsons et al. 2007, Jones, Zhang et al. 2008, Lamouille, Xu et al. 2014). TGF-β stimulation in normal and transformed mammary epithelial cells generates cells with the capacity to propagate new tumor (Mani, Guo et al. 2008). TGF-β induced EMT can also regulate cancer cells to de-differentiate and gain CSC properties (Caja, Bertran et al. 2011, Fernando, Malfettone et al. 2015) . Mutations in the genes encoding TGF-β receptors and Smads can also result in carcinogenesis (Samanta and Datta 2012, Katsuno, Lamouille et al. 2013).

1.5.2 Notch signaling pathway

Notch signaling pathway is important for maintaining a balance between cell proliferation and apoptosis. Notch signaling regulates several cellular processes like cell proliferation, differentiation, development and cell death. It also affects the development and function of several organs. It also plays a role in neuronal function,

embryonic development, angiogenesis, cardiac homeostasis and bone regeneration. It is also essential for the development of normal mammary gland (Miele and Osborne 1999, Miele 2006, Miele, Miao et al. 2006, Pannuti, Foreman et al. 2010, Ranganathan, Weaver et al. 2011). Activation of Notch signaling starts with the interaction between ligands such as Delta-like (Delta-like-1, -3 and -4) and Jagged (Jagged1 and Jagged2) and Notch receptors (Notch1-4) (Nichols, Miyamoto et al. 2007, Van de Walle, De Smet et al. 2011). After binding to the ligand, the Notch receptor's intracellular domain (NCID) is cleaved and released, which is then translocated to the nucleus and regulates the transcription complexes (CBF1/CSL/RBPjk/Su(H)) (Komatsu, Chao et al. 2008).

Alteration in the Notch signaling pathway is associated with a range of multiple human disorders from developmental syndromes to adult onset diseases and cancer. Overexpression of Notch1 and their ligand jagged1 was observed in breast cancer, bladder cancer leukemia and prostate cancer (Miele and Osborne 1999, Miele 2006, Wang, Banerjee et al. 2006, Wang, Zhang et al. 2006). Overexpression of Notch-1 induces Snail and promote EMT. Notch stimulates the Slug promoter, which results in the upregulation of Slug and EMT initiation (Timmerman, Grego-Bessa et al. 2004, Niessen, Fu et al. 2008). Dysregulation of this pathway causes several malignancies such as T-cell leukemia and breast cancer (Gallahan and Callahan 1997, Grabher, von Boehmer et al. 2006)

1.5.3 PI3K/Akt signaling pathway

Phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays a vital role in regulating several biological processes such as cell proliferation, cell growth, metabolism, apoptosis and also maintaining the characteristics of the tumor cell. (Kanamori,

Kigawa et al. 2001). PI3K/Akt pathway is a multistep process, which is modulated by various proteins, including PI3K, phosphoinositide-dependent kinase 1 (PDK), phosphatase and tensin homolog (PTEN) and heat-shock protein 90 (HSP90). PI3K phosphorylates phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) which recruits Akt to the membrane. At the membrane, Akt is activated by PDK1 which leads to the translocation of Akt to the nucleus. Akt mediates several cellular processes such as apoptosis, cell proliferation, transcription, and cell migration and it also plays a vital role in promoting cell survival.

Dysregulation of PI3K/Akt pathway is implicated in several diseases such as diabetes, cancer, cardiovascular and neurological disorders. Genes in the PI3K/Akt pathway are frequently altered in several human cancers. Akt1 is frequently activated in breast, prostate and ovary cancers. Akt2 is overexpressed in ovarian, pancreatic, breast and thyroid cancers (Cheng, Godwin et al. 1992, Bellacosa, de Feo et al. 1995, Ringel, Hayre et al. 2001, Testa and Bellacosa 2001). PTEN is frequently mutated or depleted in various cancers (Grille, Bellacosa et al. 2003, Mayer and Arteaga 2016). Akt has been shown to repress the expression of E-cadherin and upregulate the expression of EMT inducers, Snail, Slug, BMI-1 and EZH2 which in turn promote EMT and CSC phenotype (Huang, Zhang et al. 2011, Dong, Konno et al. 2014). As a result of these somatic alterations, PI3K/Akt pathway is aberrantly activated and is associated with cellular transformation, tumorigenesis, cancer progression, and drug resistance.

1.5.4 Wnt signaling pathway

Wnt is an extremely conserved developmental signaling pathway. Wnt proteins are secreted ligands that act as local mediators to regulate many aspects of development in all animals. The Wnt signaling pathway regulates cell proliferation, morphology, cell-contacts, migration and structural remodeling (Cadigan and Nusse 1997, Reya and Clevers 2005). Wnt signaling is important for activating multiple intracellular pathways related to cell proliferation, differentiation and polarity. This pathway propagates through either canonical pathway or non-canonical pathway (Bhanot, Brink et al. 1996, Yang-Snyder, Miller et al. 1996, He, Saint-Jeannet et al. 1997). The wnt proteins interact with the transmembrane receptor, Frizzled (FZD) (along with co-receptor LRP), which activates Disheveled (Dvl) results in the formation of the activated receptor complex. The complex then triggers removal of the multifunctional kinase GSK-3β from a regulatory complex (APC/Axin/ GSK-3β). Stabilized β-catenin then translocates to the nucleus, where it binds to LEF/TCF transcription factors to activate transcription of the target genes (Pandur, Maurus et al. 2002, Logan and Nusse 2004).

Wnt pathway plays a vital role in both developmental and cancer-related EMTs (Heldin, Vanlandewijck et al. 2012). Wnt signaling also plays a basic role in reprogramming and maintenance of CSC phenotype that is activated by EMT. Mutations in Wnt genes or components of Wnt pathway results in cancer progression, including tumor initiation, tumor growth, cell senescence, cell death, differentiation and metastasis. Aberrant Wnt signaling was reported to be associated with several tumors (Anastas and Moon 2013, Kim and Kahn 2014). β-catenin, along with TCF/LEF binds to the promoter region of SNAIL1 and activates its transcription

(Easwaran, Pishvaian et al. 1999, ten Berge, Koole et al. 2008). Abnormal activation of Wnt/β-catenin pathway also strongly correlates with carcinogenesis and cancer progression through maintaining cancer initiating cells (CICs) (Nguyen, Vanner et al. 2012).

1.6 Additional Sex Comb-Like (ASXL), transcription regulator

The Additional Sex Combs-like (ASXL) genes such as ASXL1, ASXL2 and ASXL3 are the mammalian homologues of the Drosophila gene, Addition of sex combs (Asx) (Fisher, Berger et al. 2003, Katoh and Katoh 2003, Katoh and Katoh 2004). The Asx protein has dual roles in the transcriptional regulation of homeotic and non-homeotic genes as a cofactor of both the Polycomb group (PcG) repressor complex and the Trithorax-group (trxG) activator complex and is involved in the transcriptional repression and activation of target genes in a context-dependent manner (Sinclair, Milne et al. 1998, Gildea, Lopez et al. 2000, Brock and van Lohuizen 2001, Dietrich, Moore et al. 2001, Grimaud, Negre et al. 2006, Halachmi, Schulze et al. 2007, Petruk, Smith et al. 2008). ASXL1 and ASXL2 are expressed in a wide variety of mammalian tissues but ASXL3 expression is confined to the brain and the eye (Fisher, Randazzo et al. 2006, Bainbridge, Hu et al. 2013, LaFave, Beguelin et al. 2015).

1.6.1 Structure of ASXL1 protein

The ASXL family proteins share common domains that include, an ASXN helix-turn-helix domain in the N-terminal region, an ASXH globular domain in the N-terminal adjoining region, ASXM1 and ASXM2 domains in the central part and a PHD domain in the C-terminal region. The full length human ASXL1 codes for a nuclear protein of 1541 amino acids and weighs about 170 kDa. The ASXN domain is a DNA binding

domain and it is structurally similar to the Forkhead-box (FOX) domain of FOXA3, FOXK1, FOXO1 and FOXO4, which regulates transcription and DNA repair during embryogenesis and carcinogenesis. (Sanchez-Pulido, Kong et al. 2012, Katoh 2013). The ASXH domain directly binds to the H2A deubiquitinating enzyme BAP1, an ubiquitin carboxy-terminal hydrolase (UCH) (Scheuermann, de Ayala Alonso et al. 2010) and Lysine Specific Demethylase-1A (LSD1/KDM1A) (Lee, Cho et al. 2010, Sanchez-Pulido, Kong et al. 2012). The ASXM1 and ASXM2 domains are protein-protein interacting domains, which directly binds to the nuclear hormone receptors (NHRs) such as RAR, RXR, ER, AR, GR and TR as well as to the NHR-coactivator NCOA1. ASXM2 domain consists of the LVxxLL motif. (Cho, Kim et al. 2006, Grasso, Wu et al. 2012). And the PHD domain is a histone- or DNA-binding domain of chromatin regulators and transcription factors (Sanchez and Zhou 2011, Li and Li 2012, Liu, Qin et al. 2012). The functional divergence between ASXL1 and ASXL2 is due to the presence of CBX5 (HP1*α*)-binding PxVxL motif in ASXL1 and ASXL3, but absent in ASXL2 (Lee, Cho et al. 2010) (Fig.5).

Figure. 5: Schematic representation of ASXL1 protein structure. Human ASXL1 encodes a 1541 amino acid protein. The ASXN and PHD domains bind to modified histones or DNA. The ASXH, ASXM1 and ASXM2 domains are binding modules for BAP1, LSD1 (KDM1A), NCOA1 and NHRs. CBX5 * indicates that CBX5 binds to ASXL1, but not to ASXL2 (modified from Katoh 2013).

ASXL1 contains a number of putative motifs, such as nuclear localization sequences (NLSs), PEST (proline (P), glutamate (E), serine (S) and threonine (T)) sequences , cyclin interaction substrate recognition sites and phosphorylation sites for cyclin-dependent kinases (CDKs) (Fisher, Randazzo et al. 2006).

1.6.2 Functions of ASXL1

ASXL family members are scaffolding proteins that recruit epigenetic regulators and transcription factors to specific genomic loci with specific histone modifications. ASXL proteins have divergent functions in developing tissues. ASXL1 is highly expressed in the developing and adult hematopoietic cells and depletion of ASXL1 leads to misregulation of hematopoietic progenitor proliferation and differentiation (Fisher, Pineault et al. 2010, Abdel-Wahab, Gao et al. 2013, Wang, Li et al. 2014). ASXL1 is essential for the regulation of Hox gene expression during embryogenesis

in mice (Fisher, Lee et al. 2010). ASXL1 is required for the maturation of lung and development of eye and cardiovascular system. ASXL1 interacts with polycomb proteins and transcription activators and repressors and regulate epigenetic marks and transcription (Cho, Kim et al. 2006, Boultwood, Perry et al. 2010, Scheuermann, de Ayala Alonso et al. 2010). ASXL1 is important for normal and myeloid differentiation. Both ASXL1 and ASXL2 are essential for the anteroposterior patterning of the mesoderm and homeotic transformation of the axial skeleton. ASXL1 and its paralog ASXL2 play an important role in the regulation of peroxisome proliferator-activated receptors (PPARs) and play opposite roles in adipocyte differentiation. ASXL2 promotes adipocyte differentiation whereas ASXL1 blocks in mouse 3T3-L1 cells (Park, Yoon et al. 2011). It was reported that loss or deletion of ASXL1 in osteoblasts and their progenitors leads to dramatic bone loss and decreased a number of bone marrow stromal cells (BMSCs) (Zhang, Xing et al. 2016).

1.6.3 ASXL1 and interacting proteins

ASXL proteins are known to be the members of Enhancer of Trithorax and Polycomb (ETP) group proteins and are activators and repressors of gene transcription, depending on their interacting partners and cellular context. ASXL1 along with BAP1 is a part of the Polycomb repressive deubiquitinase complex (PR-DUB) which deubiquitinates monoubiquitinated H2AK119 mark generated by PRC1 complex and is an important Polycomb Repressor group protein, responsible for repressing genes controlling cell differentiation. Both ASXL1 & 2 regulate the de-ubiquitinating activity of BAP1 (Scheuermann, de Ayala Alonso et al. 2010, Sahtoe, van Dijk et al. 2016). ASXL1 is associated with the components of the polycomb repressive complex 2

(PRC2), such as SUZ12 and EZH2, which increases the repressive mark, histone H3K27 methylation. Inhibition or loss of ASXL1 function leads to a decrease in H3K27me3 levels (Lee, Cho et al. 2010, Sugimoto, Muramatsu et al. 2010). ASXL1 also interacts with HP1α/CBX5, a component of the heterochromatin repressive complex through PxVxL motif and regulates the activity of LSD1 (Lysine-Specific Demethylase 1) (Lee, Cho et al. 2010, Beisel and Paro 2011). Apart from BAP1, ASXL1 is also known to interact with the UTX (Ubiquitously Transcribed Tetratricopeptide Repeat, X chromosome) and LSD1, histone demethylases as well as the MLL (Mixed-Lineage Leukemia) and EZH2 (Enhancer of Zeste Homologue 2) histone methyltransferases (Gelsi-Boyer, Brecqueville et al. 2012).

1.6.4 ASXL1 in cancer

Mutations of ASXL1 are associated with several cancers. ASXL family members have dual functions, as they act either as tumor suppressors or as oncogenes in a context-dependent manner. Truncation mutations in the ASXL1 gene are frequently observed in myeloid malignancies, whereas amplification, translocation or point mutations of the ASXL1 gene have been observed in other types of human cancers (Gelsi-Boyer, Trouplin et al. 2009). Nonsense point mutations or frame-shift mutations of ASXL1 occur in hematological malignancies such as myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) (Carbuccia, Murati et al. 2009, Gelsi-Boyer, Trouplin et al. 2009, Quesada, Conde et al. 2012). ASXL1 mutations lead to aggressive disease and the patients have poor over survival rate. Whole-exome sequencing analyses revealed that ASXL1 is mutated in 2.9% of CLL cases (Quesada, Conde et al. 2012), whereas meta-analyses revealed that ASXL1 is

mutated in 45.3% of CMML cases, 30% of secondary AML cases, 16.2% of MDS cases, 34.5% of MPN cases, and 6.5% of de novo AML cases (Gelsi-Boyer, Brecqueville et al. 2012). ASXL1 is most frequently mutated in colorectal cancer (CRC) cell lines with microsatellite instability (MSI) (Williams, Bird et al. 2010). ASXL1 is also mutated in several other cancers such as castration-resistant prostate cancer, breast cancer, liver cancer and head and neck squamous cell carcinoma (Li, Zhao et al. 2011, Stransky, Egloff et al. 2011, Grasso, Wu et al. 2012). ASXL1 is amplified and overexpressed in 5.1% of cervical cancers (Scotto, Narayan et al. 2008). ASXL1 mutations are associated with adverse outcome in leukemia patients (Katoh 2013, Aumann and Abdel-Wahab 2014). Mutations in ASXL1 leads to depletion of ASXL1 protein or truncated protein expression. The truncated proteins lead to gain-of-function as they retain the N-terminal BAP1 interacting domain and leads to enhanced H2AK119Ub deubiquitinating activity.(Scheuermann, de Ayala Alonso et al. 2010, Balasubramani, Larjo et al. 2015)

1.7 Aim of the project

ASXL family members are known to be mutated in several cancers and their role in cancers was poorly characterized. Many reports showed that ASXL proteins have epigenetic regulatory functions by recognizing and replacing several histone modifications by interacting with demethylases, methyltransferases and deubiquitinases. In this study our aim is to elucidate the role of ASXL1 during tumor progression and metastasis, especially in epithelial-to-mesenchymal transition (EMT) and decipher its epigenetic mode of action. To understand the ASXL1 mediated effects on tumorigenesis, two approaches were followed. In our first approach, ASXL1 was either depleted or ectopically overexpressed in normal mammary cells

and breast cancer cells. In the other approach, RNA-seq and ChIP-seq analyses were performed to elucidate its molecular mechanism of action.

2. Materials

2.1 Technical equipment

EQUIPMENT	COMPANY
2100 Bioanalyzer	Agilent Technology, Santa Clara, USA
5100 Cryo 1°C Freezing Container	Thermo Fisher Scientific, Waltham, USA
Agarose gel chamber	Harnischmacher Labortechnik, Kassel
Balance	Sartorius AG, Goettingen
Bandelin Sonoplus Sonicator	Bandelin electr. GmbH & Co. KG, Berlin
Biological Safety Cabinet " Hera Safe "	Thermo Fisher Scientific, Waltham, USA
Bioruptor® Plus Sonicator	Diagenode SA, Liege, Belgium
C1000™ Thermal Cycler	Bio-Rad Laboratories GmbH, Muenchen
Centrifuge (Megafuge 1.OR)	Thermo Fisher Scientific, Waltham, USA
Centrifuge 4°C (5417R)	Eppendorf AG, Hamburg
Centrifuge 4°C (Fesco 21)	Thermo Fisher Scientific, Waltham, USA
CFX96™ Optical Reaction Module	Bio-Rad Laboratories GmbH, Muenchen
Confocal microscope LSM510 META	Leica, Germany
Counting chamber (Neubauer)	Brand GmbH & Co. KG, Wertheim
DynaMag™ 2	Life Technology, Carlsbad, USA
DynaMag™ 96 side	Life Technology, Carlsbad, USA
Eclipse TS100	Nikon, Tokyo, Japan
Electrophoresis & Electro transfer Unit	Bio-Rad Laboratories GmbH, Muenchen
Freezer -150°C (MDF-C2156VAN)	Panasonic, Kadoma, Japan
Freezer -20°C	Liebherr GmbH, Biberach
Freezer -80°C "Hera freeze"	Thermo Fisher Scientific, Waltham, USA
Gel Imager Gel iX Imager	Intas Science Imaging GmbH, Goettingen
HERA cell 150i CO2 Incubator	Thermo Scientific, Waltham, USA
Incubator (bacteria culture)	Infors AG, Bottmingen
Incubator (bacteria)	Memmert GmbH & Co. KG, Schwabach
Incubator (cell culture) "Hera cell 150"	Thermo Fisher Scientific, Waltham, USA
Inverse Microscope "Axiovert 40 CFL"	Carl Zeiss MicroImaging GmbH, Goettingen
Isotemp® water bath	Thermo Fischer Scientific, Waltham, USA
Magnet stirrer "MR3001"	Heidolph GmbH & Co. KG, Schwabach
Microwave	Clatronic International GmbH, Kempen
Mini Trans-Blot™ Cell	Bio-Rad Laboratories, Hercules, USA

Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories, Hercules, USA
Mr.Frosty® Cryo Freezer	Thermo Fischer Scientific, Waltham, USA
Nano Drop® ND-1000	Peqlab Biotechnology GmbH, Erlangen
pH meter inoLab®	WTW GmbH, Weilheim
Pipette Aid® portable XP	Drummond Scientific Co., Broomall, USA
Pipettes “Research” Series	Eppendorf AG, Hamburg
Power supply “Power Pack P25T”	Biometra GmbH, Goettingen
PowerPac™ Basic Power Supply	Bio-Rad Laboratories, Hercules, USA
PowerPac™ HC Power Supply	Bio-Rad Laboratories, Hercules, USA
Qubit® 2.0 Fluorometer	Invitrogen GmbH, Karlsruhe
Refrigerator	Liebherr GmbH, Biberach
Repeat pipette	Gilson Inc; Middleton, USA
Scanner Epson V700 Photo	Seiko Epson, Suwa, Japan
ScanScope XT	LMS Co., Ltd., Tokyo, Japan
Shaker “Rocky”	Schuett Labortechnik GmbH, Goettingen
Table centrifuge (VWR Mini Star)	VWR International, LLC, South Korea
Test tube rotator	Schuett Labortechnik GmbH, Goettingen
Thermo mixer C	Eppendorf AG, Wessling-Berzdorf
Ultrapure Water System “Aquintus”	Scientific Industries, Inc., Bohemia, USA
Vacuum pump (BVC Control)	Vacuubrand GmbH and Co. KG, Germany
Vortex-Genie 2	Electro Scientific Industr. Inc; Portland, USA
Water bath “TW 20”	noLab® WTW GmbH, Weilheim
X- Ray Cassettes	Rego X-ray GmbH, Augsburg

2.2 Consumable materials

MATERIAL	COMPANY
96 Multiply® PCR plate white	Sarstedt AG & Co., Nuembrecht
96-well Multiplate PCR plate white (Pena-Llopis, Vega-Rubin-de-Celis et al.)	Bio-Rad Laboratories GmbH, Muenchen
Cell scraper (16 cm, 25 cm)	Sarstedt AG & Co., Nuembrecht
Cellstar 6- and 12-well cell culture plate	Greiner Bio-One GmbH, Frickenhausen
Cellstar PP-tube 15 and 50 ml	Greiner Bio-One GmbH, Frickenhausen
Cellstar tissue culture dish 100×20 mm	Greiner Bio-One GmbH, Frickenhausen

Cellstar tissue culture dish 145×20 mm	Greiner Bio-One GmbH, Frickenhausen
Cryo Tube™ Vial (1.8 ml)	Thermo Fisher Scientific, Waltham, USA
DNA loBind Tube (0.5 and 1.5 ml)	Eppendorf AG, Wessling-Berzdorf
Falcon® assay plate, 96 well	VWR Int., LLC, West Chester, USA
Gel blotting paper (Whatman paper)	Sartorius AG, Goettingen
Glass coverslips (18 mm)	Gebr. Rettberg GmbH, Goettingen
Hybond™-PVDF Transfer Membrane	GE Healthcare Europe GmbH, Muenchen
Microtube 0,5 ml, 1.5 ml, 2 ml	Sarstedt AG & Co., Nuembrecht
Microtube 1.5 ml, conical	VWR International GmbH, Darmstadt
NORM-JECT Syringes of different volume	Henke Sass Wolf GmbH, Tuttlingen
Parafilm® “M”	Pechiney Plastic Packaging, Chicago, USA
PCR plate white (96-well Multiplate®)	Bio-Rad Laboratories GmbH, Muenchen
PET track-etched cell culture inserts	BD Bioscience, Franklin Lakes, NJ, USA
Petri dish 92×16 mm	Sarstedt AG & Co., Nuembrecht
Pipette filter tips	Sartorius AG, Goettingen
Pipette tips	Greiner Bio-One GmbH, Frickenhausen
Protan® Nitrocellulose transfer membrane	Whatman GmbH, Dassel
Shandon Coverplate	Thermo Fisher Scientific, Waltham, USA
Syringe filter, Ca-membrane	Sartorius AG, Goettingen
Tissue microarrays US	Biomax,Inc., Rockville, MD, USA
Ultralow attachment plates	Corning Life sciences, NY, USA
X-ray films “Super RX”	Fujifilm Corp., Tokyo, Japan

2.3 Chemicals

CHEMICAL	COMPANY
Acetic acid	Carl Roth GmbH & Co. KG, Karlsruhe
Adefodur WB developing concentrate	Adefo-Chemie GmbH, Dietzenbach
Adefodur WB fixing concentrate	Adefo-Chemie GmbH, Dietzenbach
Agar	USB Corporation, Cleveland, USA
Agarose	Biozym Scientific GmbH, Oldendorf
Agencount® AMPure® XP Beads	Beckman Coulter Inc. Brea USA
Albumin Fraction V (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe

Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium sulfate $(NH4)_2SO_4$	Carl Roth GmbH & Co. KG, Karlsruhe
Ampicillin	AppliChem GmbH, Darmstadt
Aprotinin	Carl Roth GmbH & Co. KG, Karlsruhe
Bovine Serum Albumin (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe
Bromophenol blue	Sigma-Aldrich Co., St. Louis, USA
Calcium Chloride ($CaCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Cholera Toxin	Sigma-Aldrich Co., St. Louis, USA
Colorless co-precipitant	Bioline, Luckenwalde
Co-precipitant Pink	Sigma-Aldrich Co., St. Louis, USA
Crystal violet	Carl Roth GmbH & Co. KG, Karlsruhe
DePeX mounting media	VWR International GmbH
Diethylpyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe
Dimethyl sulfoxide (DMSO)	AppliChem GmbH, Darmstadt
di-Sodium hydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe
Dithiothreitol (DTT)	GIBCO®, Invitrogen GmbH, Darmstadt
DMEM	GIBCO®, Invitrogen GmbH, Darmstadt
DMEM/F12	Carl Roth GmbH & Co. KG, Karlsruhe
dNTPs	Th. Geyer GmbH & Co. KG, Renningen
Epidermal Growth Factor (EGF)	Carl Roth GmbH & Co. KG, Karlsruhe
Ethanol absolute	Carl Roth GmbH & Co. KG, Karlsruhe
Ethidium bromide	Sigma-Aldrich Co., St. Louis, USA
Ethylene diamine tetraacetic acid (EDTA)	Thermo Scientific HyClone, Logan, USA
Fetal Bovine Serum (FBS)	Sigma-Aldrich Co., St. Louis, USA
Formaldehyde	Carl Roth GmbH & Co. KG, Karlsruhe
Glycerol	Sigma-Aldrich Co., St. Louis, USA
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe
Horse Serum	Sigma-Aldrich Co., St. Louis, USA
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Hydrocortisone	Sigma-Aldrich Co., St. Louis, USA
Immobilon™ Western HRP substrate	Merck,Millipore KGaA, Darmstadt
Insulin	Sigma-Aldrich Co., St. Louis, USA
Iodacetamide	Sigma-Aldrich Co., St. Louis, USA
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe

Leupeptin	Carl Roth GmbH & Co. KG, Karlsruhe
Linear Acrylamide	Thermo Fischer Scientific, Waltham, USA
Lithium chloride (LiCl), 8M	Sigma-Aldrich Co., St. Louis, USA
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
Methanol	M. Baker B.V., Deventer, Netherlands
Monopotassium phosphate	Carl Roth GmbH & Co. KG, Karlsruhe
N-ethylmaleimide (NEM)	Sigma-Aldrich Co., St. Louis, USA
Nickel chloride ($NiCl_2$)	Sigma-Aldrich Co., St. Louis, USA
Nonidet™ P40 (NP-40)	Sigma-Aldrich Co., St. Louis, USA
Opti-MEM	GIBCO®, Invitrogen GmbH, Darmstadt
PBS tablets	GIBCO®, Invitrogen GmbH, Darmstadt
Pefabloc SC Protease Inhibitor	Carl Roth GmbH & Co. KG, Karlsruhe
Penicillin-Streptomycin solution	Sigma-Aldrich Co., St. Louis, USA
Peptone	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium chloride (KCl)	AppliChem GmbH, Darmstadt
Potassium dihydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe
Protein-A Sepharose CL-4B	GE Healthcare, Uppsala, Sweden
Protein-G Sepharose 4 Fast Flow	GE Healthcare, Uppsala, Sweden
RNAiMAX	New England Biolabs, Frankfurt am Main
RNase inhibitor	Invitrogen GmbH, Karlsruhe
Roti®-Phenol	Carl Roth GmbH & Co. KG, Karlsruhe
Rotiphorese® Gel 30	Carl Roth GmbH & Co. KG, Karlsruhe
Rotipuran® Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Rotipuran® isoamylalcohol	Carl Roth GmbH & Co. KG, Karlsruhe
Sepharose™ CL-4B	GE Healthcare, Uppsala, Sweden
Skim milk powder	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium azide	AppliChem GmbH, Darmstadt
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium deoxycholate	AppliChem GmbH, Darmstadt
Sodium dodecylsulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium Fluoride (NaF)	AppliChem GmbH, Darmstadt

Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium pyruvate (Na-Pyr)	USB Corporation, Cleveland, USA
ß-Glycerolphosphate (BGP)	Carl Roth GmbH & Co. KG, Karlsruhe
SYBR Green I	Carl Roth GmbH & Co. KG, Karlsruhe
TEMED	GIBCO®, Invitrogen GmbH, Darmstadt
Tris	Carl Roth GmbH & Co. KG, Karlsruhe
Triton X-100	Carl Roth GmbH & Co. KG, Karlsruhe
TRIzol® Reagent	AppliChem GmbH, Darmstadt
Trypsin-EDTA (0.05%)	Invitrogen GmbH, Karlsruhe
Tween-20	GIBCO®, Invitrogen GmbH, Darmstadt
Xylene	AppliChem GmbH, Darmstadt
Yeast extract	USB Corporation, Cleveland, USA
α, α-Trehalose Dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe

2.4 Kits and Reagents

KIT and REAGENT	COMPANY
Agilent High Sensitivity DNA Kit	Agilent Technology, Santa Clara, USA
Bioanalyzer DNA High sensitivity kit	Agilent Technology, Santa Clara, USA
Immobilon Western Chemiluminescent HRP Substrate	Millipore, Billerica, USA
ISOLATE II PCR and Gel Kit	Bioline USA Inc; Taunton, USA
LipofectamineTM 2000	Life technology, Carlsbad, USA
LipofectamineTM RNAiMAX	Life technology, Carlsbad, USA
Microplex Library PreparationTM V2 Kit	Diagenode SA, Liege, Belgium
Microplex Library PreparationTM Kit	Diagenode SA, Liege, Belgium
NEBNEXT® UltraTM Library Prep Kit	New England Biolabs, Ipswich, USA
NucleoBond® Xtra Midi	Macherey-Nagel Inc; Bethlehem, USA
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, St. Leon--Rot
QIAprep® Spin Miniprep Kit	Qiagen GmbH, Hilden
Qubit dsDNA HS assay	Life technology, Carlsbad, USA
SuperSigmal® West Femto Maximum	Thermo Fisher Scientific, Waltham, USA
SuperSignal® West Dura	Thermo Fisher Scientific, Waltham, USA

2.5 Nucleic acids

2.5.1 Vectors and expression constructs

Name	Reference
pCMV6-Flag-ASXL1	(Abdel-Wahab, Adli et al. 2012)
pSG5-HA-P2A-ERT2-Hygromycin	This study
pSG5-HA-P2A-ASXL1-Hygromycin	This study

2.5.2 siRNA Oligonucleotides

For transfections, the individual siRNAs (- #4) against the respective genes were pooled in a 1:1:1:1 ratio.

siRNA	Target sequence 5'-3' direction	Source	Cat. No.
siASXL1#1	GAUCGUCAGUCCUUUCGUA	Dharmacon	D-012856-01
siASXL1#2	GGAUUCAACUUUCACGUAU	Dharmacon	D-012856-02
siASXL1#3	GCAGUGCACUAAAUAACGA	Dharmacon	D-012856-03
siASXL1#4	GAAAGUGUACGUCAGAUCU	Dharmacon	D-012856-04
Non-targetting siRNA-5	-	Dharmacon	D-001210-05-05
siPTEN SMARTpool	-	Dharmacon	L-003023-00-0002

2.5.3 Cloning primers

Gene Name	Primer Sequence (5' – 3')	Reference
ASXL1 F	GCTGACGCGGCCGCAATGAAGGACAAACAGAAGAAG	This study
ASXL1 R	GCTGACGCTAGCTCTCACCACAAGGCACAATACACAG	This study

2.5.4 RT PCR primers

Reverse transcription primers were ordered from Sigma-Aldrich, Germany.

2.5.4.1 Quantitative PCR primers (qPCR)

Quantitative PCR primers were designed using the NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Gene Name	Primer Sequence (5' – 3')	Reference
ARID1B F	GCAAGGTGTGAGTGGTTACTG	This study
ARID1B R	GGACTGGGACGGCAGATACT	This study
ASXL1 F	GGTCCTGTCTCAGTCCCTCA	This study
ASXL1 R	ATAACCACGGGGTCAGAGGT	This study
CD24 F	GCTCCTACCCACGCAGATTT	This study
CD24 R	GAGACCACGAAGAGACTGGC	This study
CDH1 F	CTTTGACGCCGAGAGCTACA	This study
CDH1 R	AAATTCACTCTGCCCAGGACG	This study
CDH2 F	GGGTCATCCCTCCAATCAAC	This study
CDH2 R	ACCTGATCCTGACAAGCTCT	This study
DLL1 F	GCAAGCGTGACACCAAGTG	This study
DLL1 R	AAGTTGAACAGCCCGAGTCC	This study
FN1 F	CCCTGGTGTCACAGAGGCTA	This study
FN1 R	GAGAGAGAGCTTCTTGTCCTGTC	This study
HNRNPK F	ATCCGCCCCTGAACGCCCAT	(Karpiuk, Najafova et al. 2012)
HNRNPK R	ACATACCGCTCGGGGCCACT	(Karpiuk, Najafova et al. 2012)
PTEN F	GTGGCGGAACTTGCAATCCT	This study
PTEN R	CGGCTGAGGGAACTCAAAGT	This study
TJP3 F	CAGAGCATGGAGGATCGTGG	This study
TJP3 R	TCAGGTTCTGGAATGGCACG	This study
VIM F	GGCTCGTCACCTTCGTGAAT	This study
VIM R	CAGAGAAATCCTGCTCTCCTCG	This study

2.5.4.2 ChIP Primers

Gene Name	Primer Sequence (5' – 3')	Reference
PTEN TSS F	AACCCTCCTAGGTCTCCTCG	This study
PTEN TSS R	TCGGTTCTCAGAGACCACCT	This study

TJP3 TSS F	GGCCGATTGACTGTTTCCAG	This study
TJP3 TSS R	GTTCTGGGGTCCTGGGTC	This study
ARID1B TSS F	GGGAGTAATGCGAGCGAAGT	This study
ARID1B TSS R	TCCTCTCTAGCCCGGATATGG	This study

2.6 Proteins, enzymes and standards

2.6.1 Antibodies

2.6.1.1 Primary antibodies

Primary antibodies for western blot, ChIP and immunofluorescence were used in the respective dilutions and concentrations. The antibody dilutions for immunoblot analysis were supplemented with 0.01% sodium azide.

Antibody	Source	Cat. No.	Clone	WB	IF	ChIP
ASXL1	Santacruz	sc-98302	H-105	1:250		1 µg
E-Cadherin	Cell Signalling	3195	24E10	1:1000	1:200	
N-Cadherin	Cell Signalling	#13116	D4R1H	1:1000		
CD24	Hans Peter	-	SWA11	1:2		
Vimentin	Santacruz	sc-6260	V9	1:1000	1:200	
ZO-1	Cell Signalling	8193	D7D12	1:1000		
ZEB1	Sigma	HPA027524	-	1:500		
BAP1	Santacruz	sc-28383	C-4	1:500		1 µg
AKT	Cell Signalling	9272		1:1000		
P-AKT	Cell Signalling	4060		1:1000		
H3K4me3	Diagenode	-	pAb-003-050	1:1000		1 µg
H3K27me3	Diagenode	-	pAb-195-050	1:1000		
H2Aub1	Diagenode	- C15410002		1:1000		
H2Bub1	Hybridoma	-	7B4	1:10		
H2B	Cell Signalling	2934	53H3	1:5000		
HSC-70	Santacruz	sc-7298	B-6	1:10,000		

IgG (non-specific)	Abcam	Ab37415	-			1 µg
WDR82	David G.Skalnik	-	-	1:500		
Cfp1	David G.Skalnik	-	-	1:250		

2.6.1.2 Secondary antibodies

Antibody	Source	Cat. No.	WB	IF
Goat Anti-Mouse IgG-HRP	Santacruz	sc-2005	1:10,000	
Goat Anti-Rabbit IgG-HRP	Santacruz	sc-2004	1:10,000	
Alexa Fluor®594 Goat Anti-Mouse IgG	Life Technologies	A11005		1:500
Alexa Fluor®488 Goat Anti-Rabbit IgG	Life Technologies	A11008		1:500

2.6.2 Enzymes

Enzyme	Company
Phusion Polymerase	New England Biolabs, Frankfurt am Main
Proteinase K	Life Technology, Carlsbad, USA
Restriction enzymes	New England Biolabs, Frankfurt am Main
T4 DNA Ligase	New England Biolabs, Frankfurt am Main
Reverse Transcriptase (M-MuLV)	New England Biolabs, Frankfurt am Main
RNase A	Qiagen GmbH, Hilden
Taq DNA Polymerase	Prime Tech, Minsk, Belarus
RNase Inhibitor	New England Biolabs, Frankfurt am Main

2.6.3 Molecular weight standards

Standard	Company
Gene Ruler^{TM} DNA-Ladder	Fermentas GmbH, St. Leon-Rot
Page Ruler^{TM} Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot

2.7 Buffers and Solutions

2.7.1 ChIP Buffers

Nelson Buffer (modified)

Component	Final concentration
NaCl	150 mM
EDTA (pH 8.0)	20 mM
Tris (pH 7.5)	50 mM
NP-40 (v/v)	0.5 %
Triton-X-100 (v/v)	1 %
NaF	20 mM

Gomes Lysis Buffer

Component	Final concentration
NaCl	150 mM
NP-40 (v/v)	1 %
Sodium deoxycholate	0.5 %
Tris-HCl pH 8.0	50 mM
EDTA	20 mM
NaF	20 mM
SDS	0.1%

Weinmann Lysis Buffer

Component	Final concentration
Tris-HCl pH 8.0	50 mM
EDTA	10 mM
SDS (w/v)	1 %

Gomes Wash Buffer

Component	Final concentration

Tris-HCl pH 8	100 mM
LiCl	500 mM
NP-40 (v/v)	1 %
Sodium deoxycholate (w/v)	1%
EDTA	20 mM
NaF	20 mM

TE Buffer

Component	Final concentration
Tris-HCl pH 8.0	10 mM
EDTA pH 8.0	1 mM

Cross-linking buffer

Component	Final concentration
Formaldehyde	1 %
PBS	1x

Proteinase inhibitors

Component	Stock concentration	Final concentration
$NiCl_2$	1 mM	1:1000
Pefabloc	1 mM	1:100
IAA	10 µM	1:10000
BGP	10 mM	1:100
NEM	1 mM	1:100
Aprotinin/Leupeptin	1 ng/µl	1:1000

2.7.2 Western blot Buffers

SDS separating gel (X %)

Acrylamide	X %
Tris-HCl pH 8.8	375 mM

SDS (w/v)	0.1 %
APS (v/v)	0.1 %
TEMED	0.04 %

SDS stacking gel (5 %)

Acrylamide	5 %
Tris-HCl pH 6.8	125.5 mM
SDS (w/v)	0.1 %
APS (v/v)	0.1 %
TEMED	0.1 %

Transfer buffer

Western salts (10X) (v/v)	10 %
Methanol (v/v)	20 %

Western salts (10X)

Glycine	1.92 M
Tris-HCl pH 8.3	250 mM
SDS	0.02 %

6X Laemmli buffer

Tris-HCl pH 6.8	0.35 M
Glycerol	30 %
SDS (w/v)	10 %
DTT	9.3%
Bromophenol blue	0.02 %

SDS Running Buffer

Glycine	200 mM
Tris-HCl	25 mM

SDS (w/v)	0.1 %

TBS 10X pH 7.6

Tris-HCl	100 mM
NaCl	150 mM

TBS-T

TBS + 0.1 % (w/v) Tween-20

RIPA buffer

PBS	1 X
NP-40 (v/v)	1 %
Sodium deoxycholate	0.5 %
SDS (w/v)	0.1 %

Blocking solution

TBST	1 X
Skimmed milk (w/v)	5 %

Proteinase inhibitors

Component	Stock concentration	Final concentration
Pefabloc	1 mM	1:100
BGP	10 mM	1:100
NEM	1 mM	1:100
Aprotinin/Leupeptin	1 ng/µl	1:1000

10X PBS (pH 7.4)

NaCl	730 mM
KCl	27 mM

$NaH_2PO_4{}^*7H_2O$	14.3 mM
KH_2PO_4	14.7 mM

PBS-T

PBS + 0.1 % (w/v) Tween-20

PCR-Mix 10X

Component	Final concentration
Tris-HCl pH 8.8	750 mM
$(NH_4)_2SO_4$	200 mM
Tween-20	0.1 %

RT-PCR Master Mix

Component	Concentration
PCR-Mix	1X
$MgCl_2$	3 mM
SYBR Green	1:80,000
dNTPs	0.2 mM
Taq-polymerase	20 U/ml
Triton X-100	0.25 %
Trehalose	300 mM

TAE buffer (50X)

Tris	2 M
Acetic acid	1 M
EDTA	0.1 M

2.8 Cell culture medium

LB Agar

LB medium	1X
Agar	1.5 %

LB medium

Peptone	1 %
Yeast extract	0.5 %
NaCl	86 mM

DMEM cell culture medium

DMEM phenol red medium	
Fetal bovine serum	10 %
Penicillin	100 U/ml
Streptomycin	100 µg/ml

DMEM/F12 cell culture medium

Phenol red-free high-glucose DMEM/F12	
Horse serum	5 %
EGF	100 µg/ml
Hydrocortisone	1 mg/ml
Cholera toxin	1 mg/ml
Insulin	10 mg/ml
Penicillin	100 U/ml
Streptomycin	100 µg/ml

Cell freezing medium

DMEM	42 %
FBS	50 %
DMSO	8 %

PBS for cell culture

1 PBS tablet per 500 ml ddH_2O

2.9 Cells

2.9.1 Bacterial cells

Escherichia coli DH10BTM from Invitrogen GmbH, Karlsruhe

2.9.2 Human Cell lines

Cell Line	Species	Tissue Origin	Disease	Source
MCF10A	Human	Mammary gland/breast	Nontumorigenic epithelial cell line	ATCC (CRL-10317)
MCF12A	Human	Mammary gland/breast	Nontumorigenic epithelial cell line	ATCC (CRL-10782)
MDA-MB-231	Human	Mammary gland/breast	adenocarcinoma	ATCC (HTB-26)
MCF10A with stable pSG5 overexpression	Human	Mammary gland/breast	Nontumorigenic epithelial cell line	This study
MCF10A with stable pSG5-ASXL1 overexpression	Human	Mammary gland/breast	Nontumorigenic epithelial cell line	This study
MDA-MB-231 with stable pSG5 overexpression	Human	Mammary gland/breast	adenocarcinoma	This study
MDA-MB-231 with stable pSG5-ASXL1 overexpression	Human	Mammary gland/breast	adenocarcinoma	This study

Co-IP Lysis Buffer

Component	Final concentration
Tris-HCl pH 7.1	5 mM
NaCl	25 mM
Triton X-100	0.5 %
NaF	25 mM
Na_3VO_4	0.5 mM
DTT	0.2 mM

2.10 Software and online tools

NAME	SOURCE
useGalaxy	https://usegalaxy.org/
Galaxy Cistrome	http://cistrome.org/ap/root
DAVID GO analysis	http://david.ncifcrf.gov
Galaxy Deeptools	http://deeptools.ie-freiburg.mpg.de/
Kaplan-Meier plotter	http://kmplot.com/analysis/index.php?p=background
Bio-Rad CFX Manager 3.1	Bio-Rad Laboratories, Hercukes, USA
Integrative Genome Viewer 2	https://www.broadinstitute.org/software/igv/download
Gene Set Enrichment Analysis	http://software.broadinstitute.org/gsea/index.jsp
Image Lab Version 5.2 build 14	Bio-Rad Laboratories, Hercukes, USA
R statistical software	http://www.r-project.org
Oncomine database	http://www.oncomine.org/resource/main.html
Primer designing tool/NCBI primer-BLAST	www.ncbi.nlm.nih.gov/tools/primer-blast/

3. Methods

3.1 Cell culture

3.1.1 Cell culture of mammary cells

MCF10A and MCF12A (human breast epithelial) cells were cultured in phenol red-free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 5% horse serum, 0.5 µg/ml hydrocortisone, 10 µg/ml Insulin, 20 ng/ml human EGF (epithelial growth factor), 0.1 µg/ml Cholera toxin, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C under 5% CO_2 atmosphere. MDA-MB-231 (human breast cancer, adenocarcinoma) cells were cultured in high-glucose Dulbecco's modified Eagles medium (DMEM) and GlutaMAX supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C under 5% CO_2 atmosphere.

3.1.2 Reverse-transfection with siRNA

Reverse-siRNA transfections were performed using Lipofectamine™ RNAiMAX according to the manufacturer's instructions. For each 6-well transfection, 30 pmol of respective siRNAs were diluted with 500 µl of Opti-MEM and 5 µl of Lipofectamine™ RNAiMAX was added and mixed gently. The transfection mixture was then incubated for 20 min at RT. Meanwhile, MCF10A, MCF12A or MDA-MB-231 cells were washed with 1XPBS, trypsinized and diluted in "respective normal medium" without antibiotics. Neubauer counting chamber was used to count the cells and nearly 250,000 cells (MCF10A or MCF12A) or 300,000 cells (MDA-MB-231) from the diluted cells were added to each 6-well containing the siRNA-Lipofectamine™ RNAiMAX complexes. After 24 h, the medium was replaced with fresh medium

containing antibiotics. Cells were harvested after 72 h. For transfections in 10 cm plates, four times more cell number and transfection reagents were used.

3.1.3 Plasmid DNA transfection

A day before transfection, cells were plated in 10 cm plates to attain approximately 70-80% confluency on the day of transfection. The cells were washed twice with 1XPBS and the medium was replaced with the respective antibiotic free medium. Plasmid transfection was performed using Lipofectamine^{TM} 2000 according to the manufacturer's instructions. For each transfection, 8-10 µg of plasmid DNA was mixed with 2 ml of Opti-MEM in a reaction tube. In a second tube, 20 µl of Lipofectamine^{TM} 2000 was mixed with 2 ml of Opti-MEM and incubated for 5 min at RT. After combining the contents of both tubes and mixing gently by inverting, the samples were incubated for another 20 min at RT. The transfection mixture was then added to the respective plates containing cells and incubated for 4 h at 37°C. After 4 h, the transfection medium was removed, the cells were washed with 1XPBS and fresh medium with antibiotics was added.

3.1.4 Stable transfection with plasmid DNA

To create a stable cell line MCF10A or MDA-MB-231 cells were transfected with respective linearized plasmid DNA (using VspI) as described in section 3.1.2. After transfection, cells were grown for 4 h in media without antibiotics and then the media was replaced with media containing antibiotics. Then the cells were trypsinized, diluted and transferred to new plates. The cells were grown in a selective media containing Hygromycin (100 µg/ml). The cells were grown in a selection medium for 3-4 weeks and these cells were further used for the later experiments.

3.1.5 Migration assay

To monitor the migration potential of the cells, a transwell migration assay was performed. The stable cell lines (MCF10A and MDA-MB-231 with stable ASXL1 ectopic expression) or cells (MCF10A and MCF12A) transfected with respective siRNAs were used for this experiment. To study migration of the cells, 8.0 µm PET track-etched membrane cell culture inserts, pre-equilibrated with serum-free medium for at least 20-30 mins, were used. The cells were trypsinized and approximately 50,000 MCF10A and MCF12A cells and 20,000 MDA-MB-231 cells were seeded into the cell culture inserts with normal culture medium and cells were allowed to migrate through the membrane. The cells were cultured for further 48 h and the cells were gently scraped from the upper side of the membrane using a Q-Tip. 100% methanol was used for the fixation of the migrated cells for 10 mins. Migrated cells were then stained using crystal violet staining (1% (w/v) crystal violet dissolved in 20% (v/v) ethanol) for 10 mins. The inserts were rinsed twice with distilled water to remove the excess staining and were allowed to dry. The crystal violet stained membrane with the migrated cells was visualized under the microscope and the images were taken using Nikon light microscope.

3.1.6 Mammosphere formation assay

In vitro proliferative capacity of the single-cell suspension was determined by mammosphere formation assay. In this method, single cells were grown in non-differentiating and non-adherent conditions. The cell lines (stable cell lines or siRNA transfected) were trypsinized to form single-cell suspensions and were seeded at a density of nearly 2500 cells/ml in DMEM/F12 medium supplemented with 2% (v/v) B27 (serum-free supplement), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 20 ng/ml

basic fibroblast growth factor (bFGF) and 20 ng/ml human epidermal growth factor (EGF) were seeded into Corning® flat bottom ultra-low-attachment 96 well microplates (200 µl). The cells were fed every 48 h by addition of fresh medium and grown for 6 days. The mammospheres formed were counted and images were taken using Nikon light microscope. Images were analyzed (used Celigo) and quantified and represented as “relative number of spheres”. The statistical significance of the triplicates was calculated.

3.2 Molecular Biology

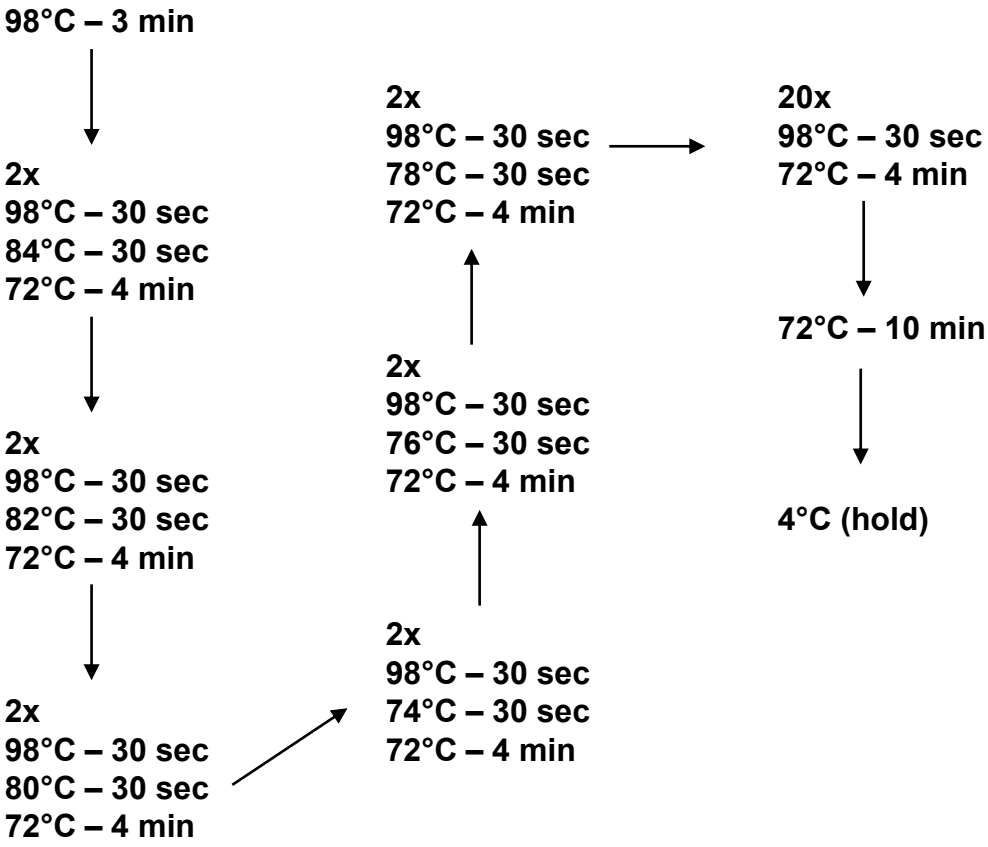
3.2.1 Molecular cloning

The ASXL1 construct used in this study was obtained by amplification of synthetic ASXL1 and cloning into pSG5–HA–P2A–ERT2–Hygromycin vector.

3.2.1.1 Polymerase chain reaction (Scott, Korfi et al.)

The pCMV6 plasmid with human ASXL1 (NM_015338) with an N-terminal FLAG tag was a generous gift from Dr. Ross L. Levine (Memorial Sloan-Kettering Cancer Center (MSKCC)) (Abdel-Wahab, Adli et al. 2012). This plasmid was used as a template for the amplification of hASXL1. The PCR reaction was set up as follows: 10 µl of 5x Phusion High-Fidelity buffer, 100 ng of plasmid template, dNTPs mix to a final concentration of 200 µM, 5 µM of sense and 5 µM of antisense primers, 1 unit of Phusion® High-Fidelity DNA Polymerase and the total volume was made up to 50 µl by adding water.

A PCR reaction was performed using the following gradient protocol.



The final PCR product was analyzed on a 1% agarose gel with appropriate molecular weight markers and purified by using agarose gel purification kit (Bioline).

3.2.1.2 Restriction enzyme digestion

To clone hASXL1 into a pSG5 vector, restriction enzyme digestion was performed to cut the hASXL1 PCR product and the vector using NotI and SpeI. The restriction digestion mixture was set up as follows: 2-4 µg of DNA, 3 µl of 10x restriction enzyme buffer, 5 units of each restriction enzyme, made up with ddH_2O to a total volume of 30 µl. The reaction was incubated at 37°C for overnight followed by heat inactivation of restriction enzymes at 95°C for 10 min. The digested PCR products or

vectors were separated on a 1% agarose gel and subsequently gel eluted for further use.

3.2.1.3 Agarose gel purification of DNA fragments

DNA fragments of interest (amplified PCR products, digested PCR products and vector) were purified by electrophoresis on a 1% low melting agarose gel in 1x TAE. The samples were loaded along with molecular size markers to aid in excising out the right sized band of interest in a UV transilluminator. The DNA in the gel piece was isolated using agarose gel purification kit (Bioline).

3.2.1.4 DNA ligation

In order to ligate the hASXL1 insert into the pSG5-HA-ERT2-P2A-Hygromycin vector, the ligation mixture was set up as follows: 100 ng of restriction enzyme digested vector DNA, 300 ng of restriction enzyme digested insert DNA (ratio vector: insert, approximately 1:3), 2 µl of 10x ligase buffer, 400 U of T4 DNA ligase and reaction volume was made up to 20 µl with ddH_2O. The ligation reaction was performed at 16°C overnight.

3.2.1.5 Heat shock transformation

For transformation, the competent bacterial (DH5α) cells were thawed on ice and incubated with DNA or ligation mixture on ice for 20-30 min with occasional mixing. After incubation, the cells were applied with heat shock at 42°C for 90 sec followed by chilling on ice for 3-5 min. The cells then were supplemented with 1 ml of LB medium and incubated at 37°C for 1 h followed by centrifugation. The supernatant was discarded and the cell pellet was resuspended in 200 µl of LB medium, which was then plated onto LB agar plates containing the appropriate antibiotic. The plates

were incubated at 37°C overnight in a bacterial incubator after which the colonies were picked for further screening.

3.2.1.6 Screening of recombinant clones and plasmid isolation

The bacterial colonies obtained in the above protocol were randomly picked and inoculated into 3 ml LB medium with ampicillin and grown to saturation. The culture was centrifuged at 12,000 rpm for one min at 4°C and the supernatant was discarded. The cell pellet was resuspended, lysed and neutralized using Macherey-Nagel buffers. The contents were spun at 11,000 g for 10 min at 4°C and the supernatant containing the plasmid was transferred to a fresh microfuge tube. The nucleic acids were precipitated by adding an equal volume of isopropanol at room temperature for 20 min and stored at -80°C for at least an hour. The DNA was pelleted by centrifuging at 11,000 g for 10 min.at 4°C. The pellet was washed with 70% alcohol, dried and resuspended in a minimum volume of autoclaved water. An aliquot of the DNA was then digested with suitable restriction enzymes to release the insert analyzed 1-1.5% agarose gel electrophoresis. The positive clones were further confirmed by DNA sequencing. The positive bacteria culture was used to inoculate 100 ml of LB medium supplemented with Ampicillin and incubated with shaking at 37°C overnight. On the next day, the cells were pelleted and the plasmid DNA was purified using the Pure Macherey-Nagel (MN) Plasmid Midiprep kit according to the manufacturer's instructions.

3.2.2 RNA isolation

RNA was isolated from the cultured cells using QIAzol® reagent according to the manufacturer's instructions. Culture medium was aspirated and cells were washed twice with 1XPBS. The cells were lysed by adding 500 µl of QIAzol® reagent to each

well (6 well plates), scraped and collected into 1.5 ml tubes. 100 µl of chloroform was added to the samples, the tubes were then vortexed approximately 20 sec and centrifuged at 12,000g for 20 min at 4°C. The upper aqueous phase was collected into a fresh 1.5 ml tube and then samples were precipitated with isopropanol at -80°C for 2 h. After 2 h the samples were centrifuged at 12,000g for 30 min at 4°C. The pellets formed were washed with 70% ethanol, dried and resuspended in 40 µl of DEPC water. RNA concentration was measured using a NanoDrop. The isolated RNA was used for cDNA synthesis or RNA-sequencing.

3.2.3 cDNA synthesis

For cDNA synthesis 1 µg of total RNA was mixed with 6 µl of a master mix containing 4 µl of 2.5 mM dNTP mix and 2 µl of 15 µM random primers. DEPC water was added to make the volume up to 16 µl. The mixture was then incubated at 70°C for 5 min followed by cooling on ice for few sec. To this, 4 µl of reverse transcription master mix containing 2 µl of 10x reaction buffer, 10 units of RNAse inhibitor, 25 units of reverse transcriptase M-MuLV and 1.625 µl of DEPC water was added to each sample. cDNA synthesis was performed at 42°C for 1 hour. Then the enzyme was inactivated at 95°C for 5 min. Finally, cDNA was diluted up to 50 µl with DEPC water.

3.2.4 Quantitative real-time PCR

Quantitative real-time PCR was performed with a final reaction volume of 25 µl. One µl of cDNA or ChIP DNA and 24 µl of master mix was used for each reaction. Each 24 µl of master mix contains 14 µl of the qRT-PCR mix, 1.5 µl of 5 µM primer mix (forward and reverse) and 8.5 µl of ddH_2O. qRT-PCR mixture consists of 20 mM $(NH_4)SO_4$, 3 mM $MgCl_2$, 200 µM dNTPs, 75 mM Tris-HCl (pH 8.8), 0.01% Tween-

20, 0.5 U/reaction Taq DNA Polymerase. 0.25% Triton X-100, 1:80,000 SYBR Green I and 300 mM Trehalose.

A two-step PCR reaction was performed using the following protocol.

95°C– 2 min

95°C– 15 sec
60°C– 1 min **x 40**

The PCR reaction was followed by a melting curve analysis from 60°C to 95°C with read every 0.5°C.

cDNA was quantified using a standard curve made from all cDNA samples. HNRNPK or 36B4 primers were used as an internal reference gene to normalize all qRT-PCR samples before the statistical analysis. The expression levels were represented as relative to the control samples and expressed as “relative mRNA expression”.

A standard curve made from the ChIP input DNA was used for the quantification of ChIP and ChIP input samples. ChIP samples were normalized to their corresponding input samples and represented as “% of input”.

3.2.5 Chromatin immunoprecipitation (ChIP)

3.2.5.1 Cross-linking and sonication

For ChIP experiments, cells were grown in either 10 cm or 15 cm plates. Protein-DNA complexes in cells were cross-linked with 1% formaldehyde in 1XPBS for 20 min (ASXL1 ChIP) or 10 min (histone modifications ChIP) at RT. 1.25 M glycine was

added in order to quench the formaldehyde and incubated for 5 min. Cells were washed twice with ice- cold 1XPBS and 1 ml of Nelson buffer containing protease inhibitor cocktail was added. Then the cells were scraped and collected into 1.5 ml tubes. The nuclear pellet was isolated by centrifugation at 12,000g for 1 min at 4°C and the supernatant was discarded. The nuclear pellet was washed with 1 ml of Nelson buffer and again centrifuged. The final nuclear pellet was resuspended in 200-250 µl of Gomes Lysis buffer and incubated on the rotor for 15 min at 4°C. Samples were then sonicated using Bioruptor® Pico (Diagenode) at high power with 30 sec on/off pulse for 15-30 cycles. Sonicated samples were centrifuged at 12,000 g at 8°C for 10 min.

3.2.5.2 Shearing check

To check the sonication efficiency of chromatin, the shearing check was performed before proceeding with a pre-clearing step. 10 µl of the sonicated chromatin sample was taken into a separate tube and 100 µl of Weinmann Lysis buffer (WB) and 1 µl of Proteinase-K (20 mg/ml) were added and incubated in a thermo-shaker at 65°C (800 rpm) overnight. Samples were then spun down and DNA was isolated by phenol/chloroform/isoamyl alcohol extraction as described below (for visualization pink precipitant was used). The isolated DNA pellet was dissolved in 15 µl of 10 mM Tris-HCl (pH 8) containing 100 µg/ml RNAse A and incubated for 1 h at 37°C (800 rpm). Then the DNA was mixed with the loading dye and allowed to run on 1.5% agarose gel at 100 V followed by analysis on gel documentation. The shearing of DNA was considered efficient if the DNA fragments were concentrated around 150-300 bp.

3.2.5.3 Pre-clearing and immunoprecipitation

For pre-clearing, 100 µl of 50% sepharose beads slurry was added to the chromatin extract and incubated on a rotor for 1 h at 4°C. The samples were centrifuged and the supernatant was transferred to a fresh tube. The samples were diluted using Gomes Lysis Buffer containing proteinase inhibitor cocktail. Chromatin samples were aliquoted and samples were either snap frozen in liquid nitrogen and stored in -80°C or proceeded for immunoprecipitation reaction. 10% of ChIP extract per corresponding ChIP sample was collected as input into a separate tube and snap frozen. For each immunoprecipitation reaction, 100 µl of chromatin extract was diluted to 500 µl with Gomes Lysis Buffer containing proteinase inhibitor cocktail and 1-2 µg of respective antibody was added and incubated overnight on a rotor at 4°C. Chromatin complexes were pulled down by adding 30 µl of 50% Protein-A sepharose slurry and incubated for 2 h at 4°C. After 2 h, samples were centrifuged at 2000g for 2 min at 4°C. The samples were washed with different ice-cold buffers as follows: twice with Gomes Lysis Buffer, twice with Gomes Wash Buffer, twice with Gomes Lysis Buffer and twice with TE buffer. The samples were mixed gently with the buffers during washings.

3.2.5.4 DNA isolation

Immunoprecipitated chromatin complexes and input samples were treated with 15 µl of RNAse A (10 µg) diluted in 10 mM Tris-HCl pH 8 (0.2 µg/ µl) and incubated at 37°C for 30 min. Protein bound to DNA was removed by treating the samples with 1 µl of Proteinase-K (20 mg/ml) and incubated at 65°C on a thermo-shaker overnight. The samples were centrifuged at 2,000g for 2 min at RT and supernatant was transferred to a separate tube. DNA was precipitated by adding 10 µl of 8 M LiCl and

4 µl of colorless co-precipitant (Bioline). Subsequently, 200 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added, vortexed for 30 sec and centrifuged at a maximum speed at 37°C for 2 min. The aqueous phase was transferred to a fresh tube and back extraction was performed by adding 200 µl of 10 mM Tris-HCl pH 8 + 0.4 M LiCl and vortexed for 30 sec. Samples were centrifuged at a full speed and the aqueous phase was collected and pooled with the first one. Precipitation was performed by adding 1 ml of 100% ethanol for 2 h at -80°C followed by centrifugation at 15,000g for 30 min at 4°C. DNA pellets were washed with 70% ethanol and centrifuged at 15,000g for 5 min. The pellets were dried and resuspended in 40 µl of water. 5 µl of ChIP DNA was aliquoted into a fresh tube and diluted for analysis by quantitative real-time PCR to check the efficiency of ChIP. The background (nonspecific) binding was determined by performing a ChIP with a non-specific IgG antibody. ChIP samples were normalized to input DNA samples, and displayed as “% of input”.

3.3 Protein biochemistry

3.3.1 Whole cell extract preparation

For total protein extraction, the cells were washed with 1XPBS and lysed in ice-cold RIPA buffer containing proteinase inhibitor cocktail (1 mM Pefabloc, 10 mM BGP, 1 mM NEM and 1 ng/µl Aprotinin/Leupeptin). The extracts were then sonicated for 15 cycles for 30 sec on/off pulse using a Bioruptor® Pico (Diagenode) at high power to shear genomic DNA.

3.3.2 SDS polyacrylamide gel electrophoresis

After sonication, the protein samples were boiled with Laemmli Buffer for 10 min at 95°C. The proteins were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels (SDS-PAGE) (Laemmli 1970). The resolving and stacking gel were prepared as described in the Materials section. Polyacrylamide gels were run in SDS running buffer at 100 V till the dye front reached the bottom of the gel.

3.3.3 Immunoblot analysis and detection

The proteins resolved according to their molecular weight by SDS-PAGE were later detected by immunoblot (Towbin, Staehelin et al. 1992) using specific antibodies against the protein of interest. Separated proteins were then transferred at 100 V to nitrocellulose membrane for 90 min. The membrane with transferred proteins was then incubated with blocking solution (5% skimmed milk in TBST) at room temperature for 1 h to block non-specific antibody binding. Then the membrane was incubated at 4°C for overnight with appropriate primary antibody diluted in blocking solution as described in the materials section. On the next day, membrane was washed thrice with TBST buffer and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody diluted in blocking solution at RT. Membrane was then washed thrice with TBST buffer and analyzed for HRP signals by using Enhanced chemiluminescence reagent and further exposed in western blot imager (Biorad) or on X-ray films

3.3.4 Co-immunoprecipitation

Cells were washed with 1XPBS and scraped in 1.0 ml of ice-cold Co-IP lysis buffer containing protease inhibitors and incubated for 1 h on a rotator at 4°C. The cells

were sonicated and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then transferred to a fresh 1.5 ml tube and resonicated followed by centrifugation. The supernatant was transferred to a fresh tube and 100 µl was collected to use as input. An appropriate amount of supernatant was incubated with 1-2 µg of antibody for overnight at 4°C on a rotator. The next day, 30 µl of 50% slurry of Protein A or G sepharose was added and incubated for 2 h on a rotator at 4°C. Beads were collected by centrifugation at 1000 rpm for 2 min, washed twice with lysis buffer and resuspended in 50–100 µl of lysis buffer. Beads were boiled in 6x Laemmli buffer, loaded on SDS-PAGE and proteins of interest were detected using specific antibodies.

3.3.5 Immunofluorescence

MCF10A cells were transfected with respective siRNAs and grown on chamber slides. The cells were washed with 1XPBS and then fixed with 4% paraformaldehyde (PFA) for 20 min at RT. Cells were washed thrice with 1x PBS to remove the residual PFA and permeabilized using 0.1% Triton X-100 for 10 min. After washing thrice with1XPBS, cells were blocked with 3% BSA (bovine serum albumin) for 20 min followed by overnight incubation with primary antibody diluted in 3% BSA. Next day, unbound antibody was washed away thrice with 1XPBS and incubated with Alexa-488 or Alexa-594 conjugated secondary antibodies diluted in 3% BSA. Cells were washed thrice with 1XPBS and the nuclei were stained with DAPI followed by 1xPBS wash three times. The samples were mounted with coverslips using the mounting medium. Images were taken using Leica inverted-2 confocal microscope followed by processing of images using the LSM Image browser.

3.4 Next generation sequencing

3.4.1 RNA sequencing

RNA sequencing was performed in duplicates. RNA integrity was checked by visualizing the RNA bands on a 1% formaldehyde-agarose gel before proceeding for library preparation. The RNA samples with proper integrity were used for library preparation.

3.4.1.1 Library preparation

RNA library preparation was performed using the NEBNext® Ultra™ Library Prep according to the manufacturer's instructions. 1 µg of total RNA was used for the downstream processing. From the total RNA, mRNA enrichment was performed using polyadenylated magnetic beads. The enriched mRNA was fragmented by incubating the samples at 94°C for 15 min in a thermocycler. Fragmented and primed mRNA was used for the first and the second strand cDNA synthesis. Double-stranded cDNA was purified using Agencourt AMPure XP beads and afterward end repair reaction (single base overhangs at 5′ end) which was then followed by adaptor ligation (sequencing adaptor) and purification steps. Purified adaptor-ligated cDNA samples were PCR amplified by using universal primer for all samples and a specific index primer for each sample which later allows the separation of individual sample loaded on the same lane of the sequencer.

3.4.2 Chromatin immunoprecipitation sequencing (ChIP-seq)

After confirming the efficiency of chromatin immunoprecipitation by quantitative real-time PCR, isolated ChIP DNA was used for sequencing. The concentration of isolated ChIP DNA was measured using a Qubit dsDNA HS assay on a Qubit® 2.0

Fluorometer before starting the library preparation. ChIP sequencing was performed in duplicates.

3.4.2.1 Library preparation

2-10 ng of ChIP DNA was used for the library preparation. DNA samples were resonicated to make fragments of 200-300 bp size using Bioruptor® Pico (Diagenode). Then fragmented DNA was used for the library preparation. Library preparation was performed using NEBNext Ultra DNA library preparation kit (New England Biolabs) for histone modifications and Microplex^{TM} Library preparation kit v2 for ASXL1 from Diagenode® according to the manufacturer's instructions. End prep reaction was set by adding end prep reaction buffer and end prep enzyme mix (total reaction volume 65 µl) to each sample and incubated at 20°C for 30 min and then at 65°C for 30 min. Adaptor ligation reaction was set up by adding following reagents to the samples: Blunt/TA ligase master mix (15 µl), ligation enhancer (1 µl), NEBNext adaptor for Illumina (2.5 µl) making the reaction volume to 83.5 µl. Then the samples were incubated at 20°C in a thermocycler for 15 min. 3 µl of USER enzyme was added to the samples and incubated at 37°C for 15 min. 0.9x AMPure XP magnetic beads were added to the adaptor-ligated DNA for size selection followed by two washes using 80% ethanol. Beads were dried and resuspended in 28 µl of 10 mM Tris-HCl pH 8.0. 23 µl of purified adaptor ligated DNA was PCR amplified by using specific index primers (barcode) for each library, which later allows the separation of individual sample loaded on the same lane of the sequencer. PCR amplified DNA was cleaned using AMPure XP beads and the DNA was resuspended in 33 µl of 10 mM Tris-HCl pH 8.0.

Each ChIP library DNA concentration was measured using Qubit® 2.0 Fluorometer and the fragment size of the libraries were analyzed using Agilent Bioanalyzer 2100 (High Sensitivity DNA assay). ChIP DNA libraries were pooled together to a final concentration of 10 nM and then diluted to 2 nM. The pooled libraries were sequenced using HiSeq 2500 (Illumina) sequencer at the Transcriptome Analysis Laboratory (TAL), Göttingen.

3.5 Bioinformatic analysis of RNA and ChIP sequencing data

3.5.1 RNA-sequencing data analysis

RNA sequencing raw data was obtained as Fastq files and the quality was checked using the FastQC tool on Galaxy. Then the Fastq files were then mapped to the human genome (hg 19) using the TopHat tool (settings were set to 'very sensitive) (Kim, Pertea et al. 2013). SortSam (version 1.126.0) was used to sort the coordinates from Picard tools on Galaxy. The resulted files were then used for read counting using the HTSeq tool (version 0.6.0) (Anders, Pyl et al. 2015). The output htseqcount files were used for DEseq package in R-script (Bioconductor version 3.2) (Love, Huber et al. 2014). The list of differentially regulated genes was used for Gene Set Enrichment Analysis (GSEA). GSEA was performed with standard parameters (1000 permutations of gene sets, Signal2Noise ranking metric) and significantly enriched pathways (c5.all gene sets) were selected (Subramanian, Tamayo et al. 2005). Gene Ontology (GO) analysis was performed using the DAVID (Database for Annotation, Visualization, and Integrated Discovery) software. DAVID is a web-based program for analyzing the list of genes associated with the biological annotation. The significantly enriched biological pathways were selected.

3.5.2 ChIP-sequencing data analysis

Raw ChIP sequencing data was obtained as Fastq files. The quality of the raw data was checked by running Fastq quality check (FastQC) tool on Galaxy (S.Andrews Babraham Institute). After checking the quality of the raw data, the data was further analyzed using publicly available servers (Galaxy, Galaxy/Cistome and Galaxy/deepTools).

3.5.3 Mapping and Peak calling

The Fastq files were mapped to the human genome (hg 19, GRch37) ((Langmead and Salzberg 2012) using Bowtie2 tool on Galaxy, which involves alignment of short DNA reads to the human genome. The end result of the Bowtie2 was in SAM (Sequence Alignment Map) format which was then converted to BAM (Binary Alignment Map) format using SAM-to-BAM conversion tool (Li, Handsaker et al. 2009). BAM files were then used for Model- Based Analysis of ChIP-seq 2 (MACS2) tool (Zhang, Liu et al. 2008), which helps in the identification of the peaks for the protein binding, commonly referred as peak calling with p-values ≤ 10e-5 and minimum FDR (q-value) cutoff for peak detection was set to 0.05.

3.5.4 Normalization and visualization of ChIP- sequencing data

The generated Bed file containing the peak location and Wiggle (Wig) file containing signal profile was further used to analyze the genome-wide enrichment of proteins or histone modifications. The total number of mapped reads were then normalized using the reads per kilobase per million (RPKM) option. Bigwig file was used to visualize the enrichment at the individual genomic regions using Integrative Genomics Viewer (IGV) software (Robinson, Thorvaldsdottir et al. 2011). Cis-

regulatory Element Annotation System (CEAS) was used to determine the enrichment of the ChIP regions at specific genomic locations compared to the whole genome (Shin, Liu et al. 2009). Bigwig files were used to generate aggregate profile plots over defined genomic regions (deep Tools and Cistrome).

4. Results

In this study, the role of Additional Sex Combs Like 1 (ASXL1) in Epithelial-to-mesenchymal transition (EMT) was examined. Our main goal was to reveal the potential role of ASXL1 as a tumor suppressor and understand the molecular mechanism involved in suppressing the stem cell phenotype in mammary epithelial and breast cancer cells. Cellular events such as cell migration, adhesion, invasion and stemness are essential for normal cells. Previous studies reported that ASXL1 is perturbed in different cancers but the mechanistic role of ASXL1 as a tumor suppressor and EMT suppression is not yet studied. We also focused on studying the epigenetic regulation of ASXL1 in EMT which is a potent driver for cancer progression and metastasis. For this we used cell lines derived from human breast tissue such as MCF10A, MCF12A and MDA-MB-231. MCF10A and MCF12A are normal mammary epithelial cells and MDA-MB-231 is breast cancer cells and they all were used as a model system to study EMT.

4.1 Role of ASXL1 in breast cancer

4.1.1 Expression of ASXL1 is downregulated in breast cancer

In order to demonstrate the functional role of ASXL1, we compared the expression of ASXL1 mRNA between normal and cancer tissues. For this purpose, we used publicly available 'Oncomine' database. The Oncomine database contains a huge collection of gene expression datasets for a wide range of cancers, which allows the users to examine the expression of individual genes in context to different cancers and also in comparison with normal tissues. Breast carcinoma datasets were utilized to check the expression of ASXL1 mRNA levels and found that ASXL1 mRNA levels

were significantly downregulated in breast carcinoma compared to the normal breast tissues in four independent datasets (Fig. 6). Reduced levels of ASXL1 expression in breast cancer samples further supports the tumor suppressor role of ASXL1 in breast cancer.

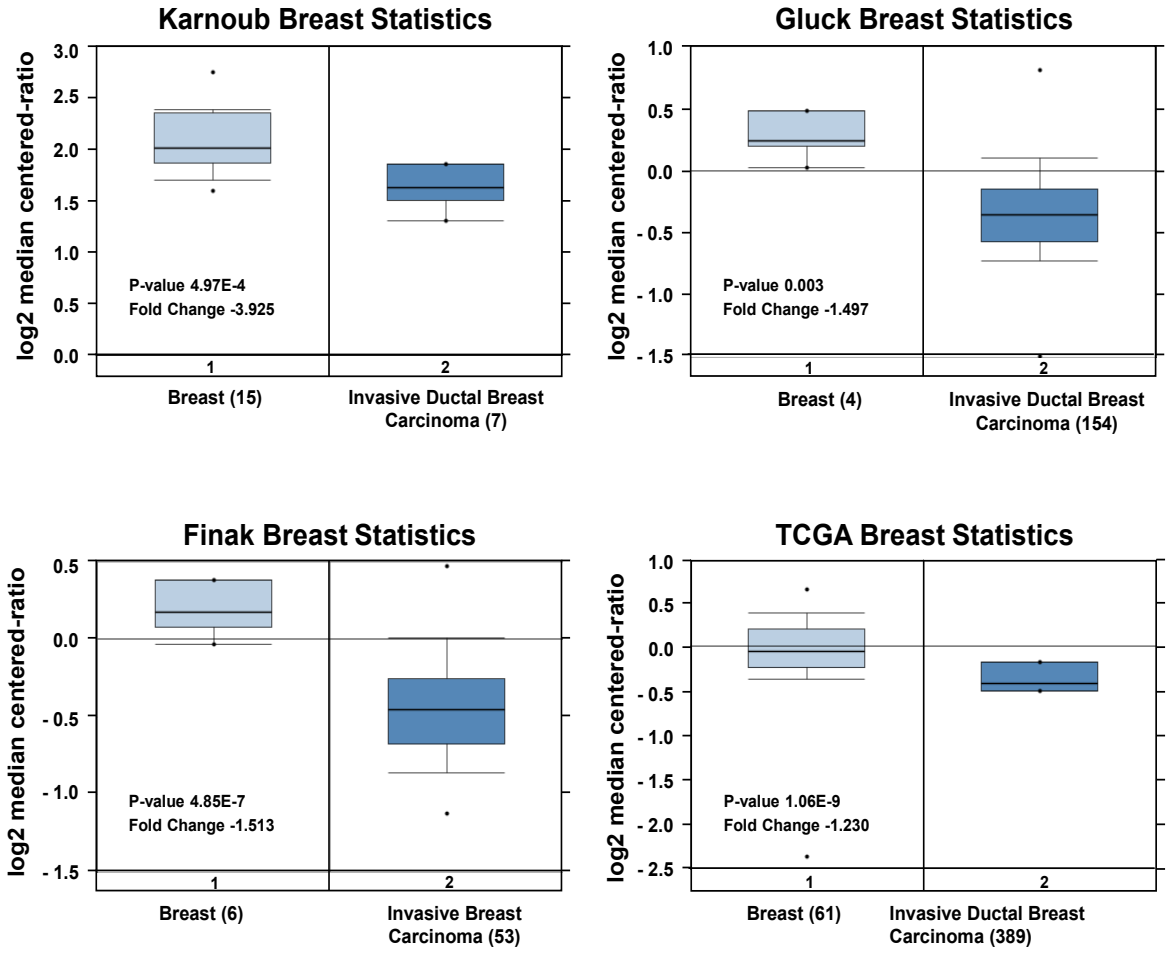


Figure. 6: ASXL1 gene expression in breast carcinoma. Expression of ASXL1 in breast cancer samples was found to be significantly downregulated when compared to the normal breast samples as depicted in four independent datasets. Publicly available Oncomine database was used to generate the above data.

4.1.2 ASXL1 expression and survival analysis

To investigate whether expression of ASXL1 can be used as a prognostic marker for the disease outcome in cancer patients, an online survival analysis tool, Kaplan-

Meier Plotter was used to elucidate the prognostic role of ASXL1 in breast cancer. The Kaplan-Meier plotter is a publicly available online software, which can be used to assess the effect of genes on survival using several cancer samples. Kaplan-Meier relapse-free survival for all breast cancer patients was analyzed and we observed that breast cancer patients with low ASXL1 expression levels have poor relapse-free survival (RFS) compared to patients with high ASXL1 (Fig. 7).

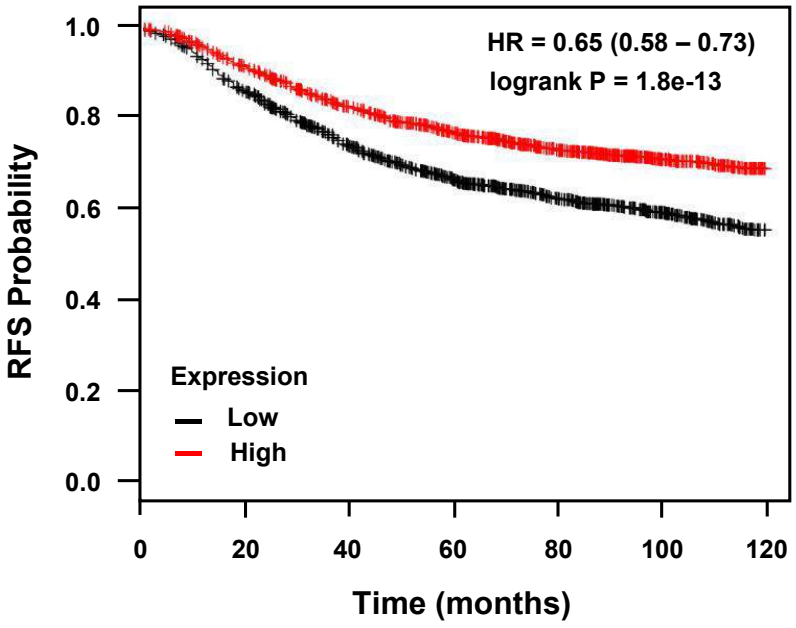


Figure. 7: ASXL1 expression level predicts the disease outcome. Kaplan - Meier plot for breast cancer showing that patients with low ASXL1 expression have significantly poor relapse-free survival rate compared to the patients with high ASXL1 expression.

4.2 ASXL1 and epithelial-to-mesenchymal transition

4.2.1 ASXL1 downregulation perturbs the expression of EMT pathway genes

As a first step towards understanding the biological functions of ASXL1 in mammary epithelial cells, we performed siRNA-mediated downregulation of ASXL1 in MCF10A cells (non-transformed mammary epithelial cells). As ASXL1 expression was perturbed in breast cancer patients, we employed MCF10A cell line. To perform

transcriptome-wide analysis, MCF10A cells were transfected with either non-targeting control siRNA or ASXL1 siRNA. After 72 h, total RNA was isolated and was used for mRNA sequencing. A thorough analysis of differentially regulated genes was carried out, where the genes are initially sorted based on fold change and significance values. For further analyses the genes that are differentially perturbed, up regulated (FC ≥ 1.0) or down regulated (FC ≤ 0.65) with a padj ≤ 0.05 were selected.

As assumed, a significant fraction of EMT regulated genes was affected by depletion of ASXL1 compared with control (ASXL1 vs Control). However, some genes are not affected by depletion of ASXL1, which implies that ASXL1 is not required for their regulation. Interestingly, a number of other genes were differentially regulated by ASXL1 loss. To understand the mechanistic role of the ASXL1-regulated genes, we employed DAVID (Database for Annotation, Visualization, and Integrated Discovery), a web-based Gene ontology application. DAVID was used to determine the GO terms related to transcriptome-wide regulation and several biological pathways regulating important functions such as cell adhesion, motility were identified upon ASXL1 depletion (Fig. 8A). One of the primary and crucial characteristics of epithelial and differentiated cells in the tissues is to adhere to the extracellular matrix and to the adjacent cells. However, during metastasis, the altered cells eventually lose cell to cell and also cell to extracellular matrix contacts, enabling them to migrate to distant sites.

To further understand the role of ASXL1-regulated genes, we employed GSEA (Gene Set Enrichment Analysis), which identifies the significantly enriched pathways, which are proposed for specific sets of genes. Using ‘c2.all’ curated gene set for

GSEA analysis, we could identify significantly enriched pathways associated with EMT and epithelial differentiation pathways in control conditions compared to ASXL1 depletion (Fig. 8B). GSEA analysis suggests that the ASXL1 loss results in an EMT-like phenotype, which may lead to metastasis. Furthermore, it also revealed an enrichment of gene signatures associated with a mammary stem cell phenotype and EMT pathways upon ASXL1 depletion. The graphical representation (enrichment plots) of pathways for epithelial differentiation and EMT identified from GSEA were shown in Fig. 8C.

A

GO_TERM	Enriched Pathways	Count	%List	P-Value	Benjamini (FDR)
SP_PIR_KEYWORDS	Proto-oncogene	20	5.3	1.10E-07	2.60E-05
GOTERM_BP_FAT	Response to wounding	34	9	9.20E-07	2.20E-03
GOTERM_BP_FAT	Regulation of cell proliferation	41	10.8	1.00E-05	4.10E-03
GOTERM_BP_FAT	Regulation of cell death	42	11.1	9.90E-06	4.80E-03
SP_PIR_KEYWORDS	Cell adhesion	20	5.3	5.70E-04	1.70E-02
GOTERM_BP_FAT	Biological adhesion	33	8.7	5.60E-04	4.90E-02
GOTERM_BP_FAT	regulation of cell-cell adhesion	5	1.3	1.90E-03	9.00E-02
GOTERM_BP_FAT	Locomotary behaviour	16	4.2	3.30E-03	1.20E-01
GOTERM_BP_FAT	Cell motion	23	6.1	3.40E-03	1.20E-01
GOTERM_BP_FAT	Angiogenesis	11	2.9	3.60E-03	1.20E-01
GOTERM_BP_FAT	Blood vessel development	15	4	3.00E-03	1.20E-01
GOTERM_BP_FAT	Response to hypoxia	10	2.6	5.90E-03	1.60E-01

B

Gene Set Name	Size	NES
BOSCO_EPITHELIAL_DIFFERENTIATION_MODULE	63	2.03
AMIT_SERUM_RESPONSE_120_MCF10A	61	1.94
CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_UP	415	1.90
CHARAFE_BREAST_CANCER_BASAL_VS_MESENCHYMAL_UP	114	1.84
JAEGER_METASTASIS_DN	246	1.84
KOBAYASHI_EGFR_SIGNALING_24HR_UP	92	1.84
SARRIO_EPITHELIAL_MESENCHYMAL_TRANSITION_DN	92	1.82
WANG_HCP_PROSTATE_CANCER	106	1.62
LIM_MAMMARY_STEM_CELL_DN	395	1.58
ONDER_CDH1_TARGETS_2_DN	438	1.43

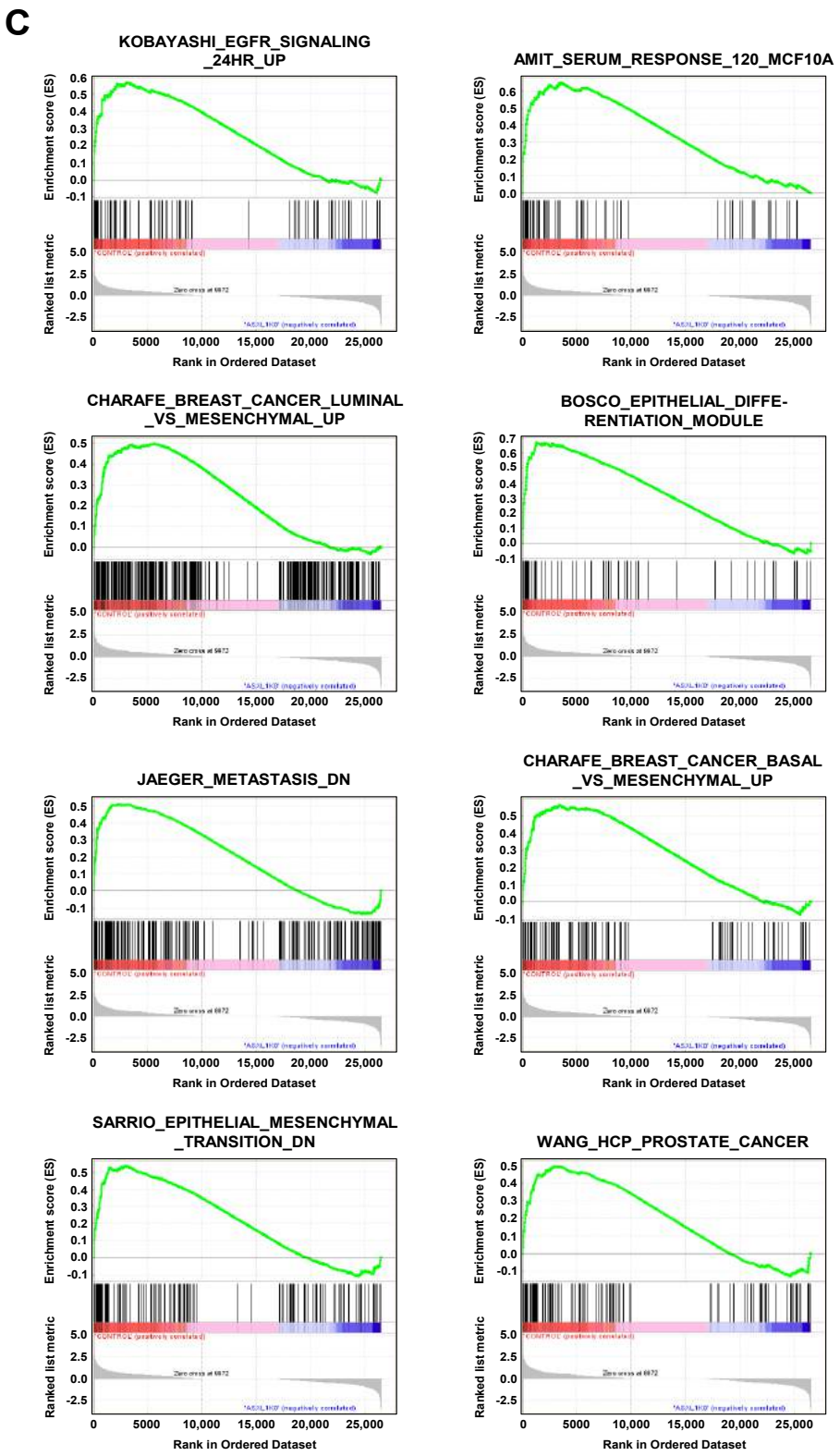
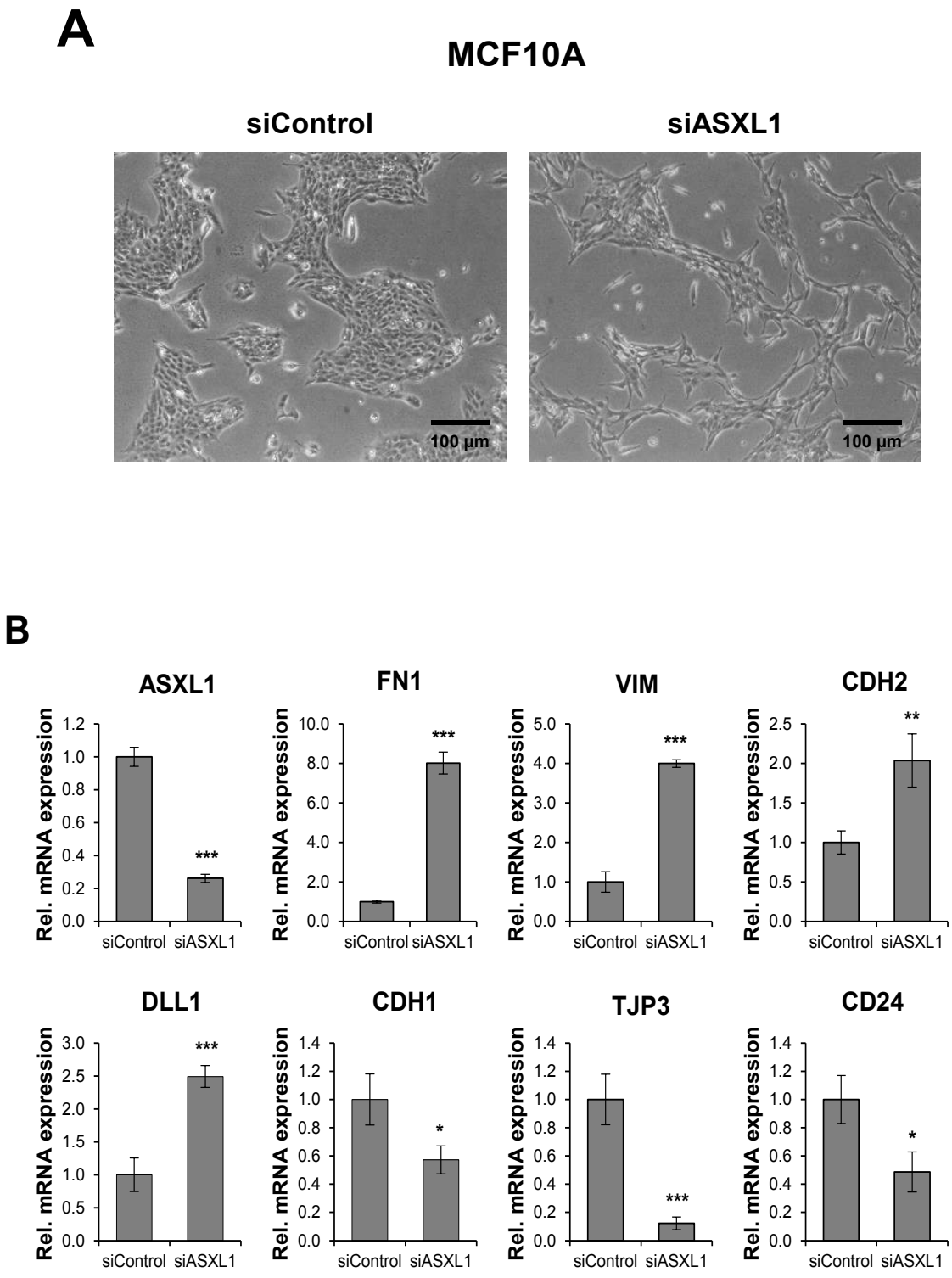


Figure. 8: ASXL1 regulate EMT and metastasis properties. (A) The upregulated and downregulated genes were used for DAVID Gene Ontology analysis. The significantly enriched biological pathways involved in cell migration and adhesion. (B and C) GSEA analysis recognizes EMT and epithelial differentiation pathways.

4.2.2 Loss of ASXL1 enhances EMT phenotype

To elucidate the effect of ASXL1 expression on EMT phenotype, we performed several cell culture based assays in MCF10A and MCF12A cells. During EMT, epithelial cells lose their epithelial markers like E-cadherin and junction proteins and express mesenchymal markers like fibronectin, Vimentin and N-cadherin. This leads the cells to undergo morphological changes, lose cell-cell adhesion properties and become elongated. ASXL1 knockdown in MCF10A using siASXL1 transfection resulted in mesenchymal-like sporadic long spindle phenotype compared to si-control transfected cells with intact ASXL1 (Fig. 9A). Based on RNA-seq data, several epithelial and mesenchymal genes involved in EMT were selected for further validation. For this, gene expression analysis for EMT markers was performed by qRT-PCR and the results were depicted as “relative mRNA expression”. As expected, the epithelial markers (CDH1, TJP3) were significantly down-regulated while the mesenchymal markers (CDH2, VIM, FN1) were significantly upregulated in ASXL1 depleted cells compared to siControl transfected cells. We also checked the expression of CD24, a marker associated with a differentiated phenotype, which was reported to be absent in cancer stem-like cells (Al-Hajj, Wicha et al. 2003) (Ponti, Costa et al. 2005). In support of the other data, CD24 expression was significantly decreased upon ASXL1 depletion. Furthermore, DLL1, a ligand for Notch signaling was significantly up-regulated in ASXL1 depleted cells (Fig. 9B & C). These results suggest that ASXL1 is required for the expression of epithelial genes and for suppression of mesenchymal genes in basal-like mammary epithelial cells.


A
MCF10A
siControl
siASXL1
100 µm
100 µm
B
ASXL1
FN1
VIM
CDH2
DLL1
CDH1
TJP3
CD24
Rel. mRNA expression
siControl
siASXL1
***
**
*

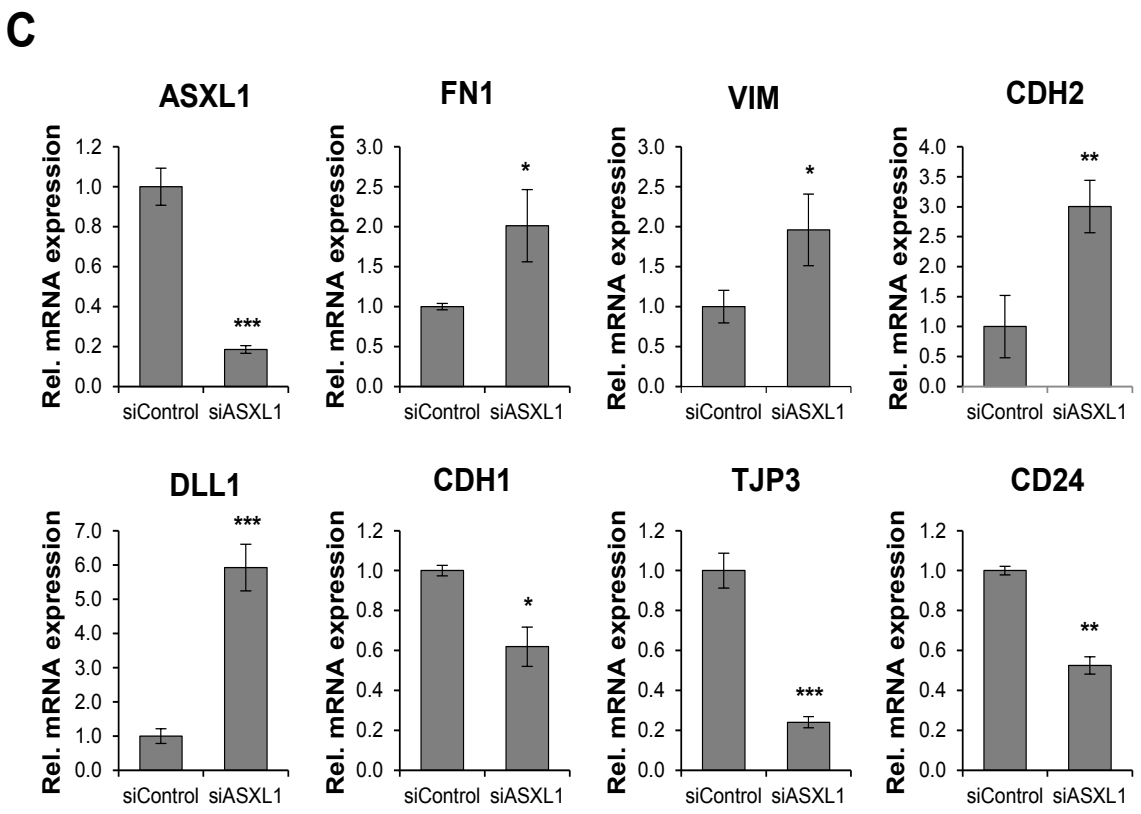
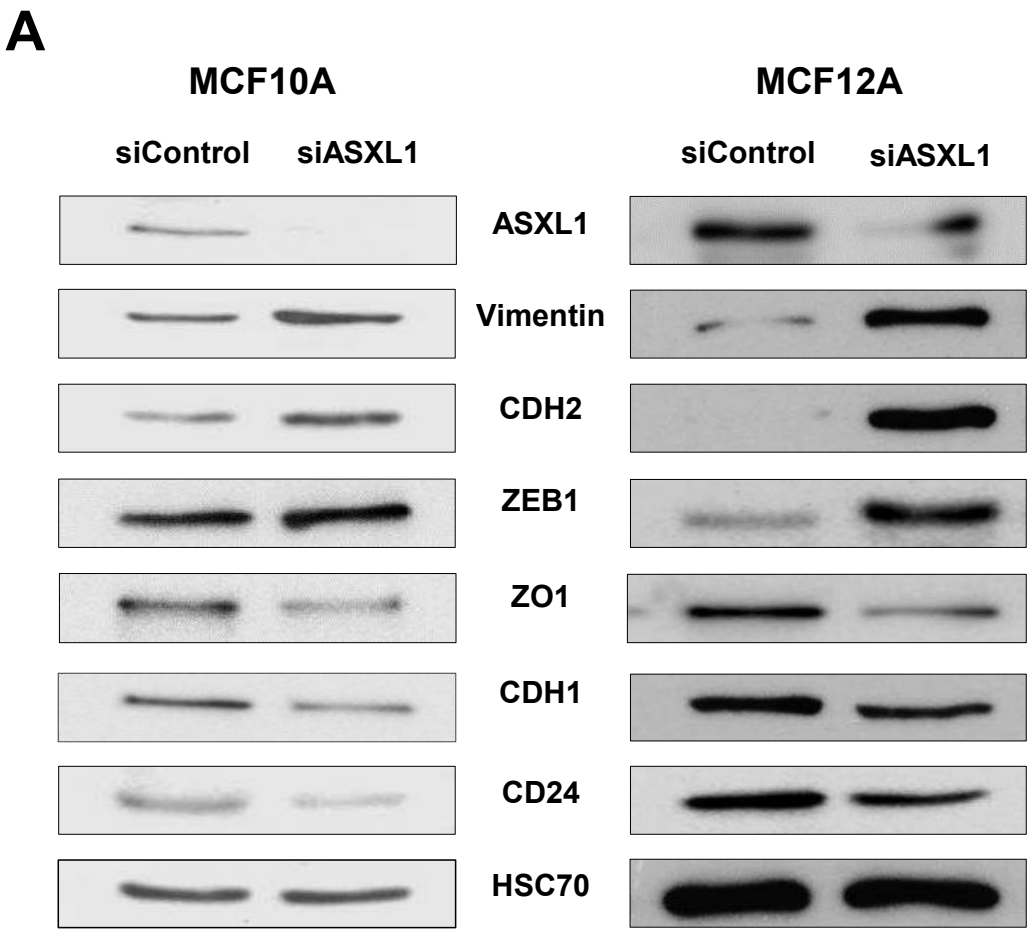


Figure. 9: Depletion of ASXL1 induces EMT phenotype. (A) Phase contrast images exhibiting the morphological changes of MCF10A cells upon ASXL1 knockdown. Scale bars are represented as 100 µm. (B) Gene expression levels of epithelial markers (CDH1 and TJP3) and mesenchymal markers (CDH2, FN1 and VIM) were analyzed by qRT-PCR in (B) MCF10A and (C) MCF12A cells and represented as "relative mRNA expression" normalized to HNRNPK expression level. Data are shown as mean ± SD. n=3. $^{***}p \leq 0.005$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

We also checked the expression of EMT markers by Western blot following the depletion of ASXL1 in MCF10A and MCF12A cells. Consistent with our gene expression analysis, ASXL1 depletion resulted in upregulation of mesenchymal markers, Vimentin, N-cadherin and ZEB1 and downregulation of epithelial markers, E-cadherin and ZO1 (Fig. 10A). To further support the findings of EMT marker's gene expression at RNA and protein level in ASXL1 depleted cells, we visualized selected EMT markers, by immunofluorescence staining and confocal microscopy. For this, we used MCF10A cells transfected with siControl and siASXL1 and stained with antibodies for the epithelial marker, E-cadherin and mesenchymal marker,

Vimentin 72 h post transfection. Confocal analyses of the immunofluorescence staining revealed a significant downregulation of the epithelial marker, E-cadherin and a strong upregulation of the mesenchymal marker, Vimentin in ASXL1 depleted cells (Fig. 10B). Taken together, these results revealed the involvement of ASXL1 in suppressing EMT.



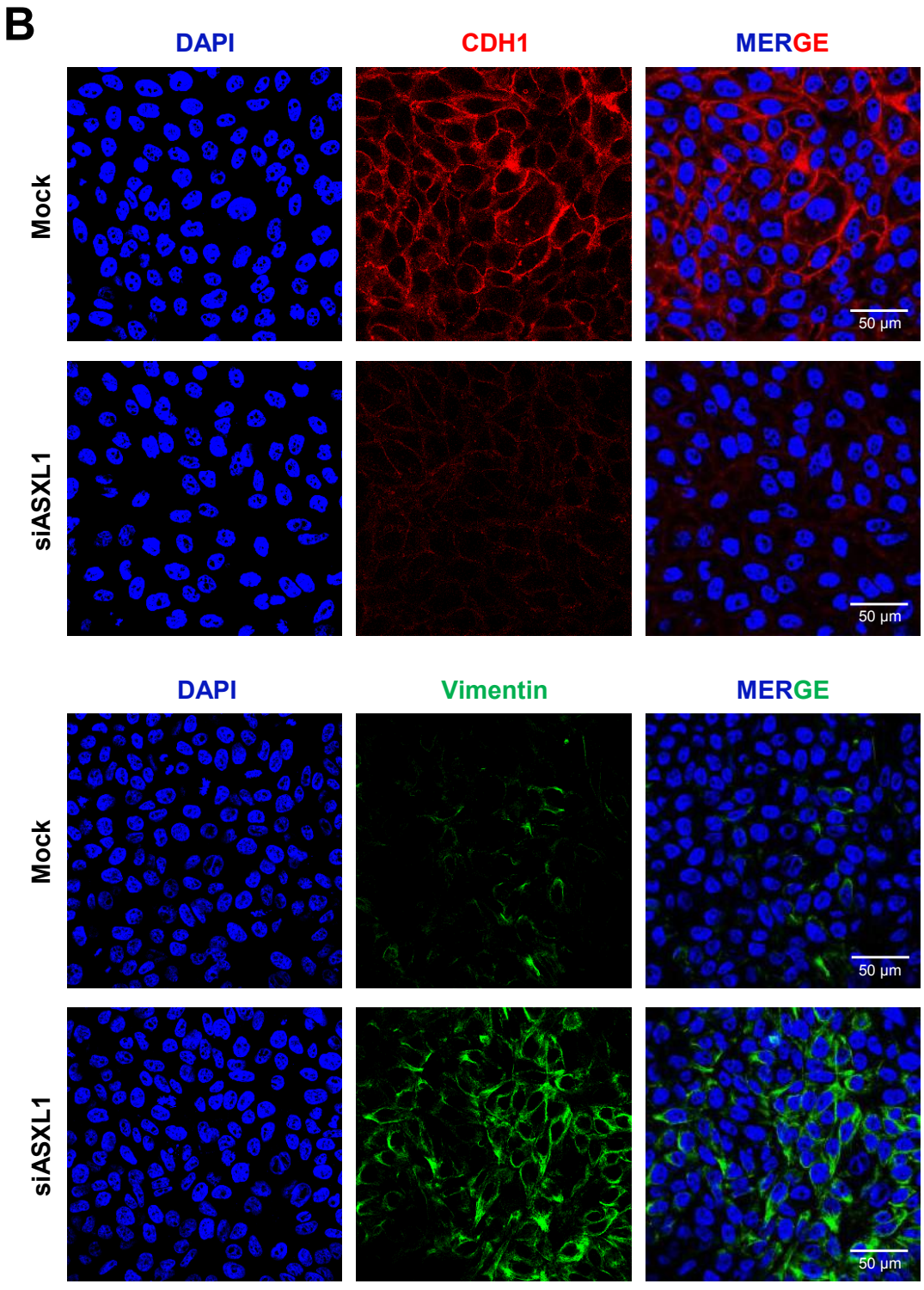


Figure. 10: ASXL1 loss enhances EMT phenotype. (A) Immunoblotting analysis of whole cell lysates depicting decreased expression of epithelial markers (E-cadherin and ZO1) and increased expression of mesenchymal markers (N-cadherin, Vimentin and ZEB1) upon loss of ASXL1 in MCF10A and MCF12A cells. ASXL1 immunoblot shows the knockdown efficiency. HSC70 was used as a loading control. (B) Immunofluorescence staining of epithelial marker, E-cadherin and mesenchymal marker. Vimentin in ASXL1 depleted cells

indicates enhanced EMT. DAPI was used to stain the nuclei. Scale bars are represented as 50 µm.

4.2.3 ASXL1 depletion enhances migratory properties

In cancer cells, acquiring EMT phenotype is the foremost step for migration which helps to transform differentiated cells into the stem-cell-like state (Mani, Guo et al. 2008) (Polyak and Weinberg 2009). One of the main features of EMT is to increase the migratory potential of cells to travel to distant places to develop a secondary tumor (metastasis). The earlier results revealed that the loss of ASXL1 enhances EMT-like phenotype. To understand whether this in turn enhances the migratory potential of the cells, we performed a transwell migratory assays in MCF10A and MCF12A cells. For this, the cells were transfected with either control or ASXL1 siRNAs and grown for 48 hours. After 48 hours, cells were seeded into 8.0 µm PET track-etched membrane cell inserts and allowed to migrate through the membrane for further 48 hours. Finally, the migrated cells on the membrane were visualized by crystal violet staining, which revealed that ASXL1 depletion leads to enhanced migratory potential compared to the control cells (Fig. 11). Together, these findings demonstrate that ASXL1 depletion is associated with migration.

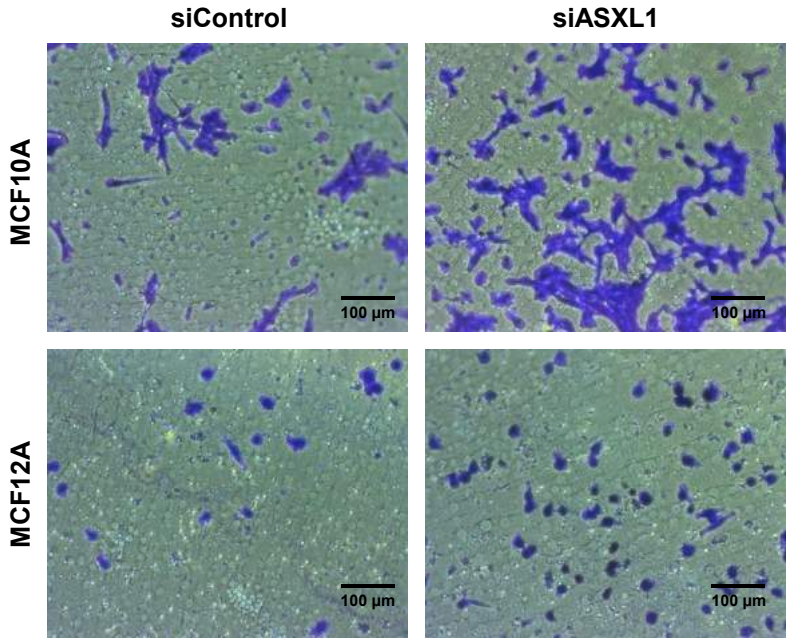


Figure. 11: Loss of ASXL1 promotes migratory potential. MCF10A and MCF12A cells were used for the transwell migration assay. Cells transfected with siASXL1 and siControl were seeded as single cells (50,000) into the upper Chamber of the insert after 48 hours. Cells are allowed to migrate for additional 48hrs and fixed with 100% methanol. The membrane was stained by using crystal violet. There is an increased migration in ASXL1 depleted in MCF10A and MCF12A cells. Scale bars are represented as 100 µm.

4.2.4 Loss of ASXL1 induces stem cell characteristics

In order to identify the enrichment of stem cell populations *in vitro*, the mammospheres culture in breast cancer has been widely used (Grimshaw, Cooper et al. 2008). The cells transfected with Control and ASXL1 siRNAs were seeded as single cells into low attachment plates and allowed to grow in non-adherent and non-differentiating conditions. Therefore, single cells possessing stem cell properties could give rise to 3D spheres called mammospheres the number of which were later counted. ASXL1 depletion significantly increased the formation of the number of mammospheres (Fig. 12A & B) which suggests an important role of ASXL1 in suppressing stem cell phenotype and maintaining epithelial phenotype.

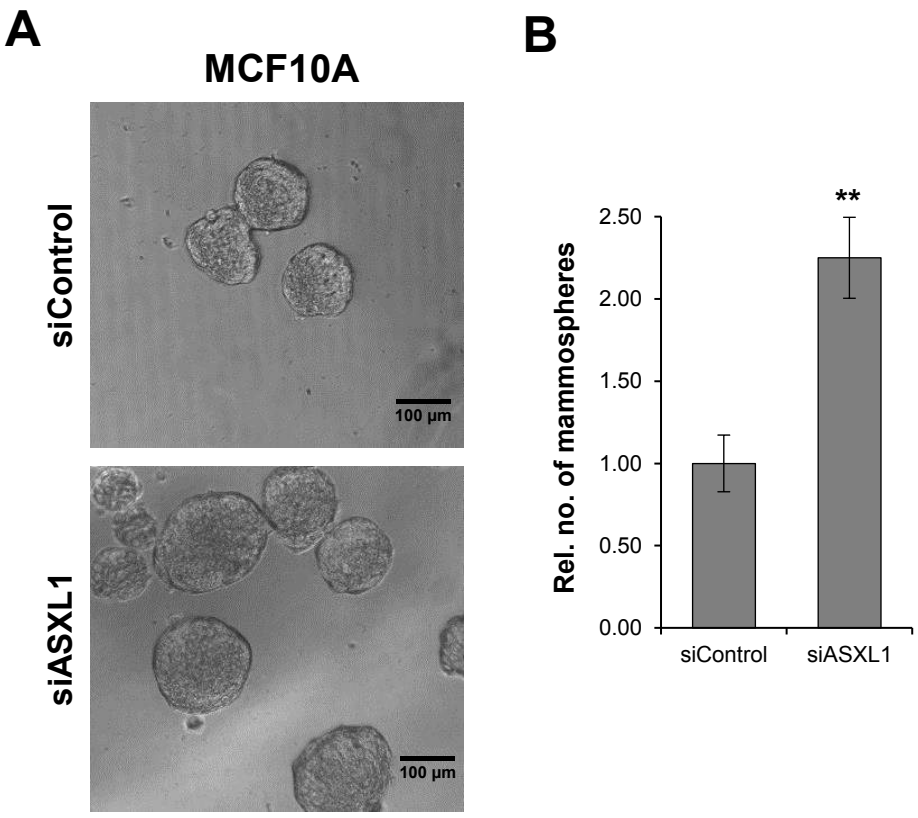


Figure. 12: ASXL1 depletion induces stem-cell properties. (A) Phase contrast images of mammospheres upon control and ASXL1 siRNAs transfection in MCF10A cells. 10x magnification. Scale bars are represented as 100 µm. (B) The mammospheres formed were quantified and represented as "relative number of mammospheres". The data indicated that there is an increase in mammospheres number upon ASXL1 knockdown. The data are represented as ± SD. n=3. $^{***}p \leq 0.005$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

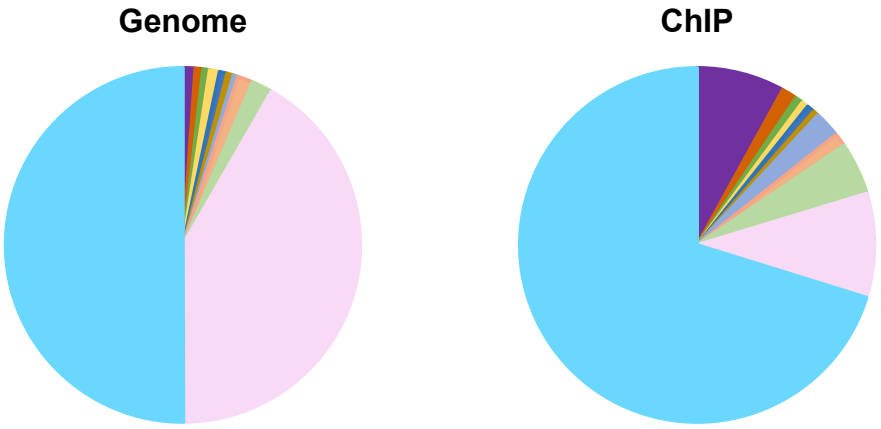
4.3 Genome-wide occupancy of ASXL1

Our results demonstrated that ASXL1 plays an important role in suppressing EMT phenotype in normal breast epithelial cells, which was supported by our transcriptome-wide analysis. However, additional studies are needed to further understand and characterize the functional and mechanistic role of ASXL1 in EMT during tumor progression and metastasis. Genome-wide occupancy studies were performed to understand the mechanism of action and determine the vital target genes of ASXL1. For this, we performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq).

4.3.1 Enrichment of ASXL1 on promoter regions

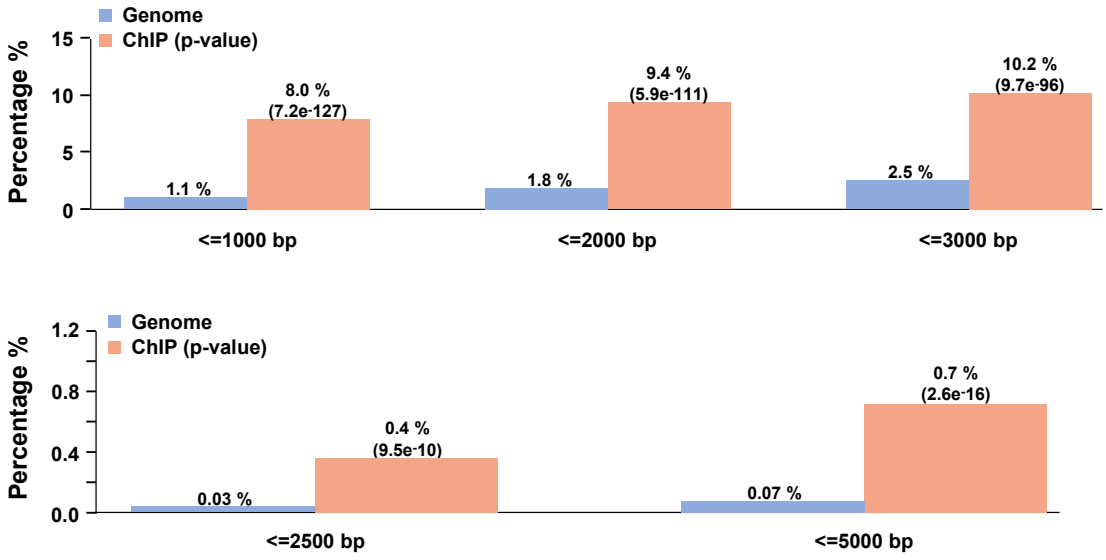
ChIP-sequencing of ASXL1 was performed in MCF10A to investigate the occupancy of ASXL1 on specific genome locations like promoters, introns and coding exons using a tool, CEAS (Cis-regulatory Annotation System). CEAS is a part of Cistrome package which is used to determine the relative enrichment of ChIP-binding regions at specific genome regions with respect to the whole genome. CEAS data pie chart depicts the distribution of ASXL1 enrichment in various genomic regions. CEAS analysis revealed that the majority of ASXL1 binding sites (70.2%) were confined to the distal intergenic regions compared to the whole genome (Fig. 13A & B). CEAS also revealed that a significant portion (8% and 9.5%, respectively) of ASXL1 binding sites were confined to promoter regions and introns compared to the whole genome. A small portion (4.8%) of binding sites was confined to coding exons. Furthermore, aggregate plot analysis was performed to observe the ASXL1 enrichment around the transcription start site (TSS) of the ASXL1 bound genes, which revealed that the highest ASXL1 enrichment near the TSS region of the genes and a low signal at the 5kb upstream and downstream stream of the TSS (Fig. 13C). Taken together these results show that ASXL1 is mostly recruited to promoter and distal intergenic regions of target genes genome-wide. Furthermore, a very few ASXL1 binding sites were enriched on introns and coding exons.

A



		Genome	ChIP
	Promoter (<=1000 bp)	1.1	8
	Promoter (1000-2000 bp)	0.7	1.4
	Promoter (2000-3000 bp)	0.6	0.7
	Downstream (<=1000 bp)	0.9	0.6
	Downstream (1000-2000 bp)	0.7	0.7
	Downstream (2000-3000 bp)	0.6	0.5
	5'UTR	0.4	2.4
	3'UTR	1.4	1.2
	Coding exon	1.9	4.8
	Intron	41.6	9.5
	Distal intergenic	50	70.2

B



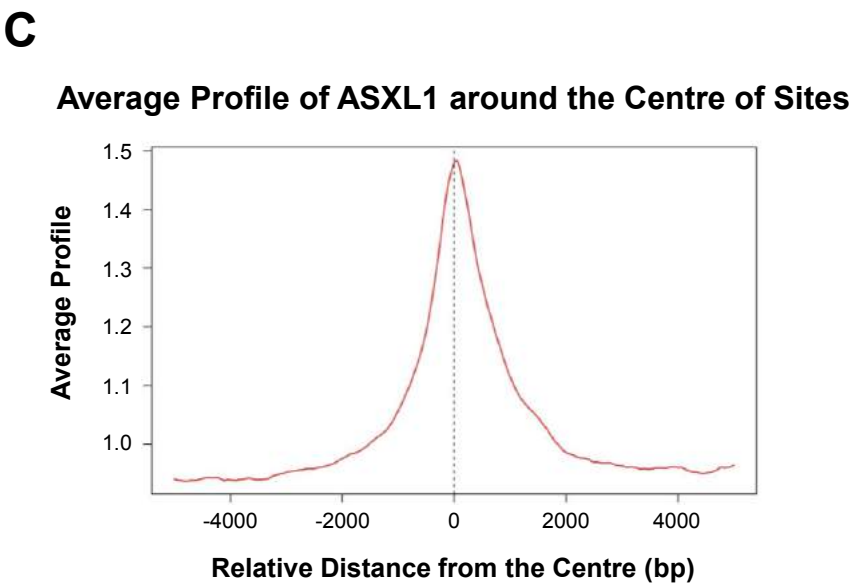


Figure. 13: ASXL1 enrichment in the genome. (A) Pie chart depicting the relative occupancy of ASXL1 on various genomic regions with respect to the whole genome. (B) Percentage enrichment of ASXL1 enrichment regions around the promoter relative to the genome. (C) Aggregate plot showing average ASXL1 enrichment ±5 kb around the transcriptional start site (TSS).

4.3.2 ASXL1 occupancy on individual genomic regions

To examine the mechanistic role of ASXL1 in EMT, ChIP-seq analysis was performed in MCF10A cells. Genome-wide and single gene analyses show that ASXL1 largely occupies regions of the transcription start site (TSS). Remarkably, we observed a prominent ASXL1 peak around the promoter regions of ASXL1 affected genes (PTEN, TJP3 and ARID1B) (Fig. 14A). To confirm whether ASXL1 indeed directly bind to the above genes, we designed primers across the TSS, for ChIP-qRT-PCR analysis, where the ASXL1 peak was visualized. And, ASXL1 ChIP was performed in MCF10A cells with or without ASXL1 depletion followed by the confirmation of ASXL1 enrichment using ChIP-qRT-PCR. Consistent with the ChIP-seq results, ChIP-qRT-PCR analysis of ChIP samples showed a significant enrichment of ASXL1 binding on the TSS region of PTEN, TJP3 and ARID1B genes.

Furthermore, the ChIP-qRT-PCR analysis showed a significant decrease of ASXL1 binding on the TSS upon ASXL1 depletion, which proves the specificity of ASXL1 binding (Fig. 14B).

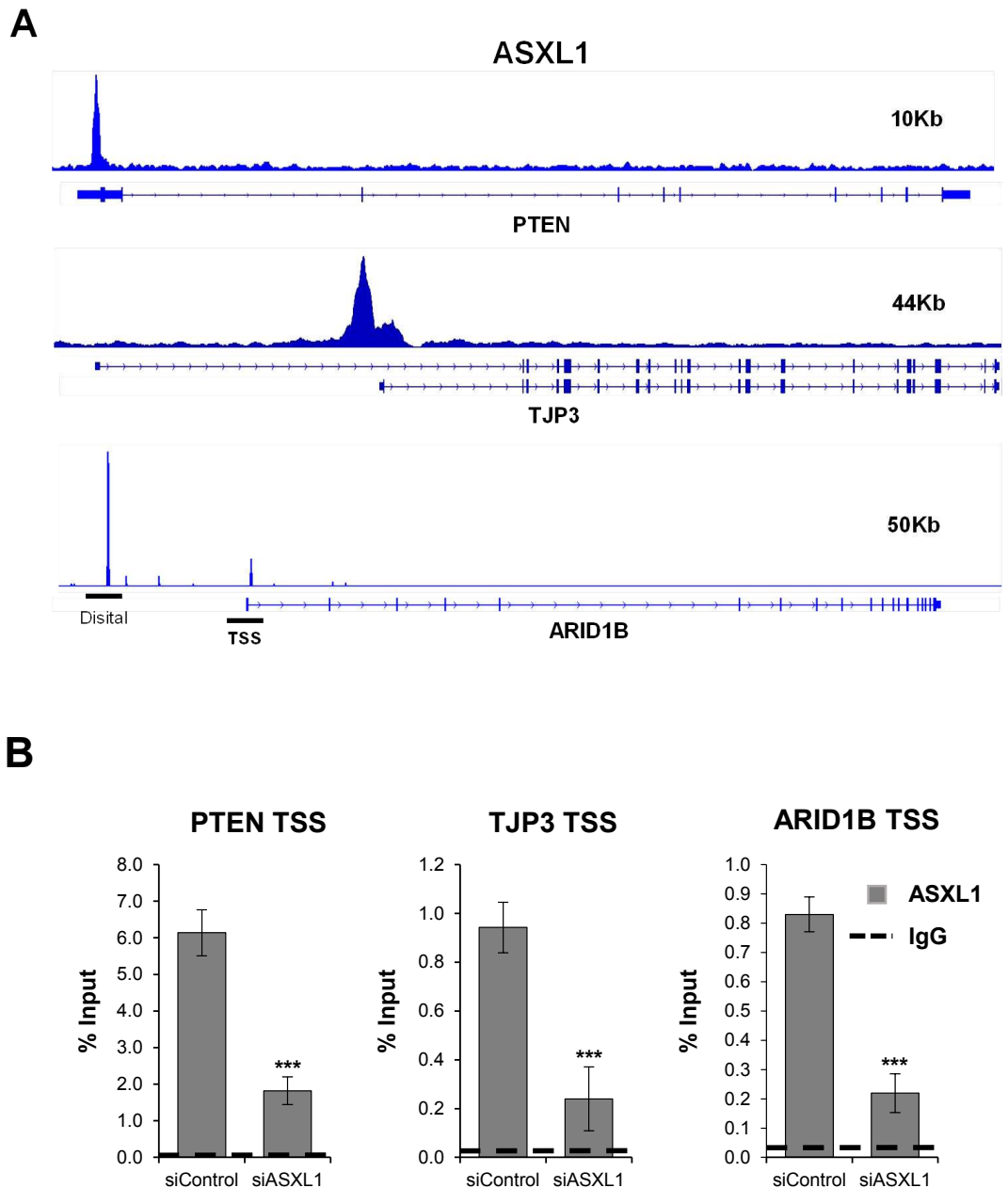
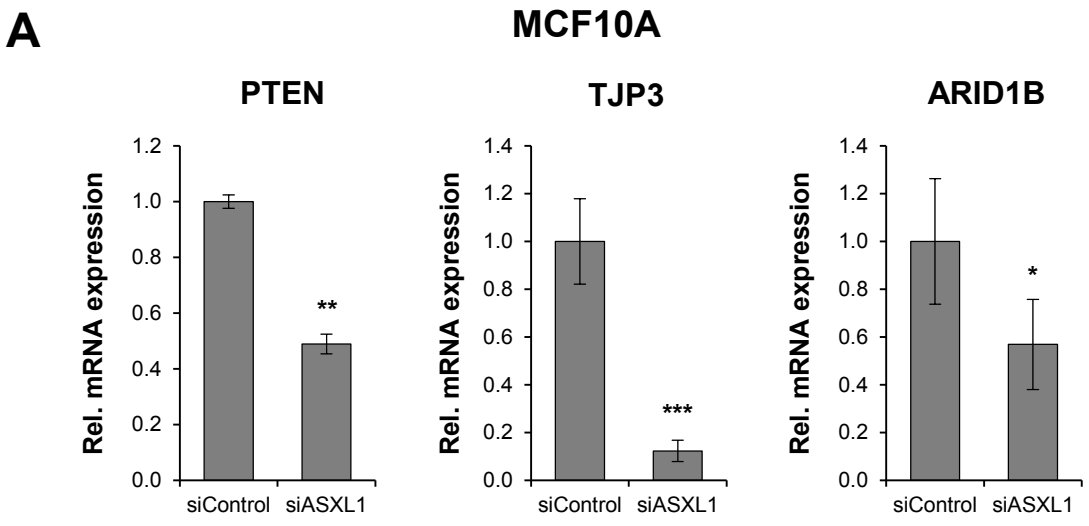


Figure. 14: ASXL1 enrichment on the promoters of single genes. (A) ChIP-seq profile of ASXL1 on PTEN, TJP3 and ARID1B genes. Significant peak was observed around the TSS. (B) ChIP analysis of ASXL1 enrichment on TSS of single genes in MCF10A cells with and without ASXL1 depletion. Immunoprecipitated DNA is compared to input and represented as

"% of Input". IgG antibody was used a negative control to measure the background signal level and displayed as a dotted line. Data are shown as mean ± SD. n=3.

4.4 ASXL1 occupancy correlate with gene expression

To determine whether ASXL1 play a direct role in regulating transcription of its target genes, the expression of these genes was studied in presence or absence of ASXL1. For this, MCF10A and MCF12A cells were transfected with siControl or siASXL1 followed by RNA isolation 72 h post transfection and cDNA preparation. This cDNA was used to assess the changes in expression of PTEN, TJP3 and ARID1B mRNA levels by qRT-PCR, which revealed that there was a significant decrease in gene expression of PTEN, TJP3 and ARID1B upon ASXL1 depletion in both MCF10A and MCF12A cells (Fig. 15A & B). These results confirm that ASXL1 directly regulates the expression of PTEN, TJP3 and ARID1B.



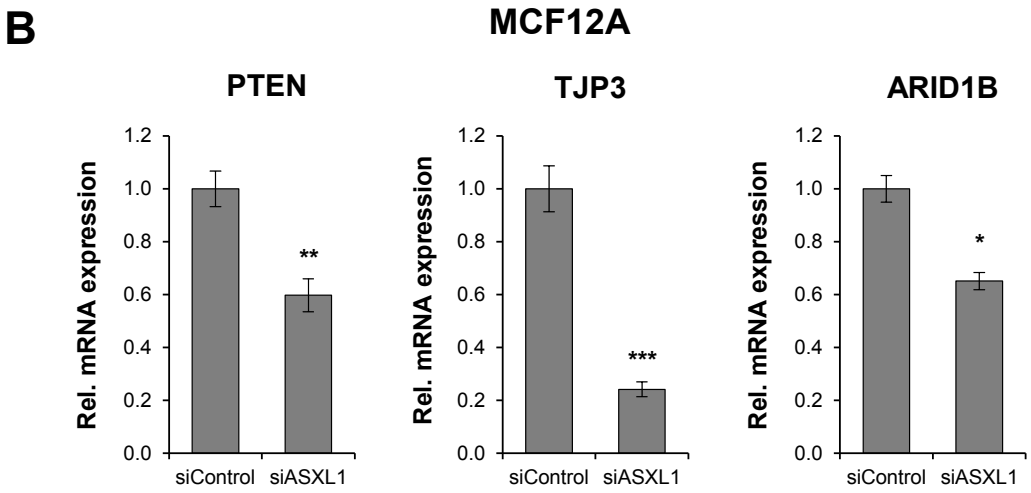


Figure. 15: ASXL1 directly regulates gene expression. Gene expression levels of ASXL1 bound genes (PTEN, TJP3 and ARID1B) were analyzed by qRT-PCR in (A) MCF10A and (B) MCF12A cells and represented as "relative mRNA expression" normalized to HNRNPK expression level. The qRT-PCR reveals that there is a significant decrease in expression of genes in ASXL1 depleted cells. Data are shown as mean ± SD. n=3. $^{***}p \leq 0.005$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

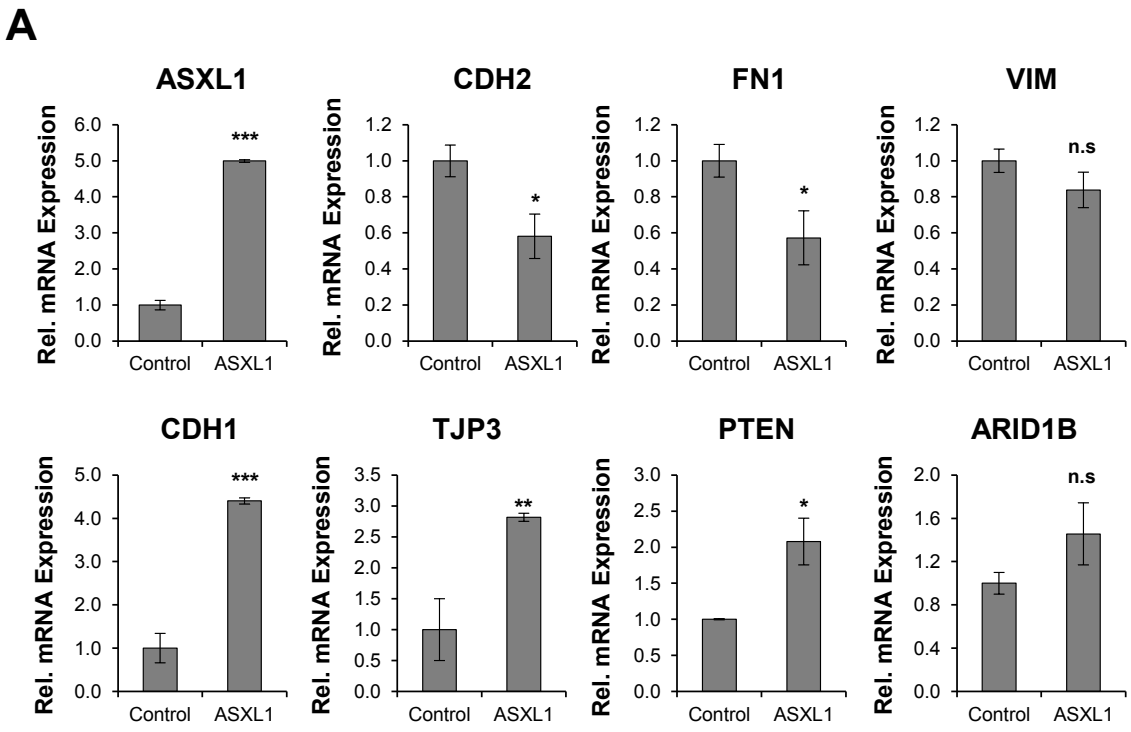
4.5 Generation of ASXL1 expressing stable cell line

To further validate the role of ASXL1 in EMT, MCF10A (normal breast epithelial cells) and MDA-MB231 (human breast cancer, adenocarcinoma) cells were stably transfected with linearized expression vector for ASXL1 or control vector and grown in Hygromycin selection medium for a few weeks. These stable cells were utilized for further studies.

4.5.1 Ectopic expression of ASXL1 reverses EMT phenotype

To reveal the effect of ASXL1 expression on EMT phenotype, we performed several cell culture based assays in the stable cell lines expressing ASXL1. As already shown, ASXL1 depletion results in downregulation of epithelial markers and upregulation of mesenchymal markers. We then checked whether overexpression of ASXL1 can reverse the effects of ASXL1 depletion i.e. increase in the expression of

epithelial markers and decrease in the expression of mesenchymal markers in MCF10A and MDA-MB-231 cells. For this, we isolated RNA and protein samples from the stable cell lines. The gene expression levels were analyzed by qRT-PCR and depicted as “relative mRNA expression”. The epithelial markers (CDH1, TJP3) were significantly up-regulated and mesenchymal markers (CDH2, VIM, FN1) were significantly down-regulated in ASXL1 expressing stable cells compared to the control cells. Furthermore, the genes bound by ASXL1 were also regulated by ASXL1 overexpression (TJP3, PTEN and ARID1B) (Fig.16A & B).



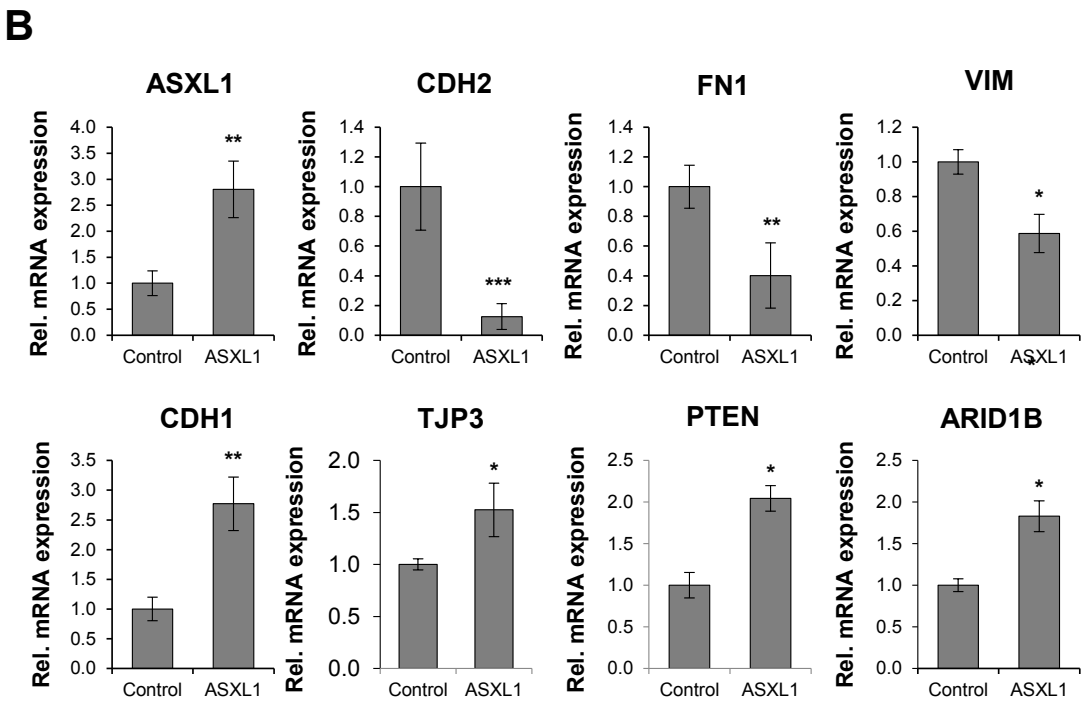


Figure. 16: Expression of ASXL1 induces MET phenotype. (A) Gene expression levels of epithelial markers (CDH1 and TJP3) and mesenchymal markers (CDH2, FN1 and VIM) were analyzed by qRT-PCR in (A) MCF10A and (B) MDA-MB-231 cells and represented as "relative mRNA expression" normalized to HNRNPK or 36B4 expression level. Data are shown as mean ± SD. n=3. $^{***}p \leq 0.005$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

To further support our findings from qRT-PCR data in the stable MCF10A and MDA-MB-231 cells, the changes in EMT markers expression were analyzed by Western blot analysis. It was observed that epithelial markers, E-cadherin and ZO1 were upregulated and mesenchymal markers, Vimentin and N-cadherin were downregulated (Fig. 17). Altogether, these results demonstrate that ASXL1 suppresses EMT and promotes mesenchymal-to-epithelial (MET) phenotype.

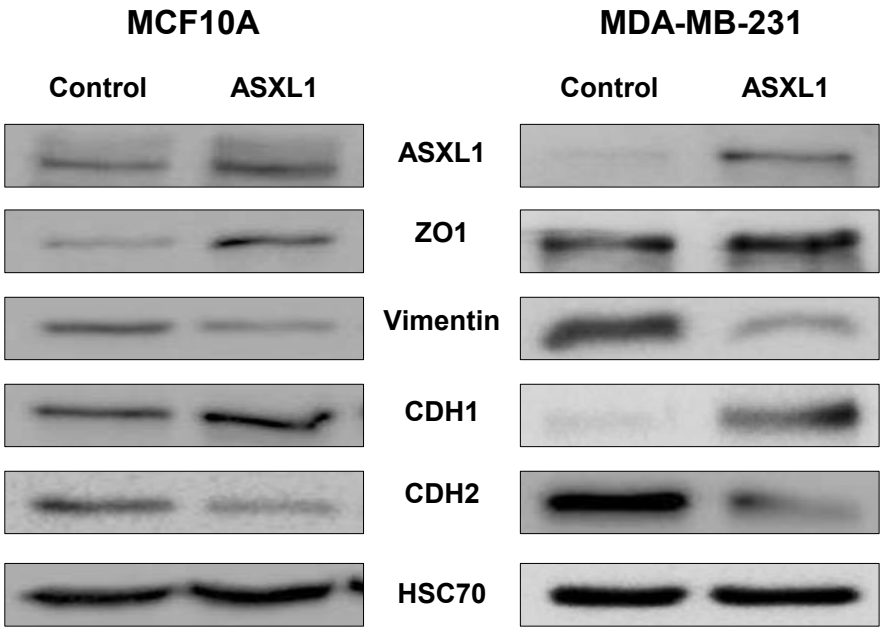


Figure. 17: ASXL1 expression reverses EMT phenotype. Immunoblotting analysis of whole cell lysates depicting increased expression of epithelial markers (E-cadherin and ZO1) and decreased expression of mesenchymal markers (N-cadherin, Vimentin and ZEB1) upon ectopic expression of ASXL1 in MCF10A and MDA-MB-231 cells. ASXL1 immunoblot shows the increase in ASXL1 expression. HSC70 was used as a loading control.

4.5.2 ASXL1 expression reduces migratory properties

From the earlier results, we demonstrated that ectopic expression of ASXL1 reverses EMT-like phenotype whereas ASXL1 depletion promotes it. To understand whether an ectopic expression can inhibit the cell migration, as it is one of the important characteristics of EMT or metastasis, we performed transwell migratory assays in MCF10A and MDA-MB-231 cells, stably transfected with ASXL1 expressing plasmid. For this the cells were trypsinized and 50,000 MCF10A and 15,000 MDA-MB-231 cells each were seeded into 8.0 µm PET track-etched membrane cell inserts and allowed to migrate through the membrane for 48 hours. Visualization by crystal violet staining of the membrane revealed that ASXL1 overexpression leads to a reduced migratory potential compared with the control cells (Fig. 18).

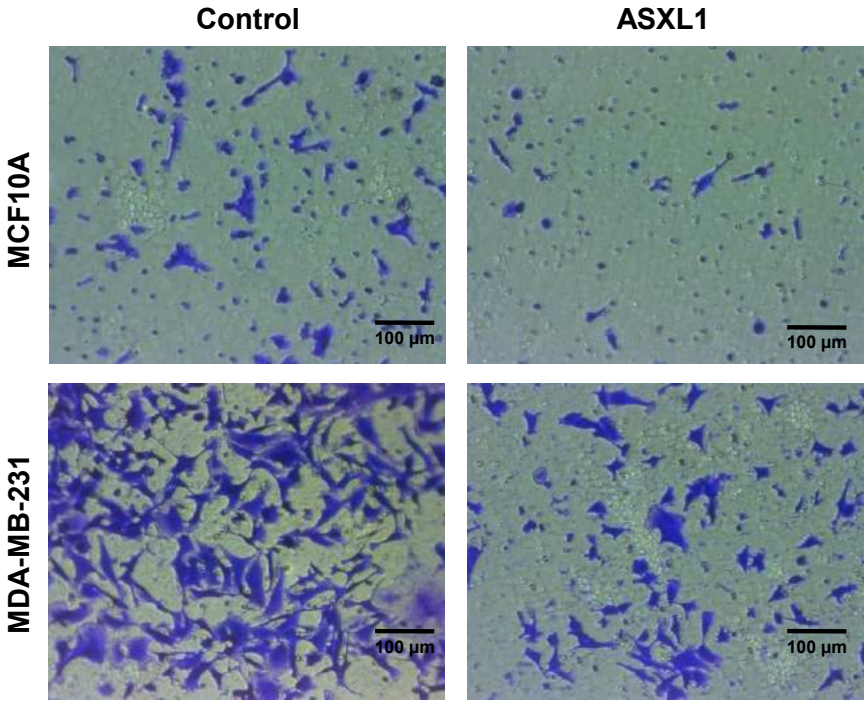


Figure. 18: Expression of ASXL1 suppresses migratory potential. MCF10A and MDA-MB-231 stable cells were used for the transwell migration assay. Stable cell lines, MCF10A (50,000) and MDA-MB-231 (15,000) were seeded as single cells into the upper Chamber of the insert. Cells are allowed to migrate for 48hrs and fixed with 100% methanol. The membrane was stained by using crystal violet. There is decreased migration in ASXL1 overexpressed (A) MCF10A and (B) MDA-MB-231 cells. Scale bars are represented as 100 µm.

4.5.3 ASXL1 expression reduces stem cell characteristics

To understand the role of ASXL1 on stem cell phenotype, the stable cell line, MCF10A was seeded as single cells into low attachment plates and allowed to grow in non-adherent and non-differentiating conditions. The mammospheres formed were counted and observed that the number of mammospheres were reduced upon ASXL1 overexpression which demonstrates that ASXL1 negatively regulates the mammospheres formation and anchorage-independent growth by suppressing the stem cell phenotype. (Fig. 19A & B).

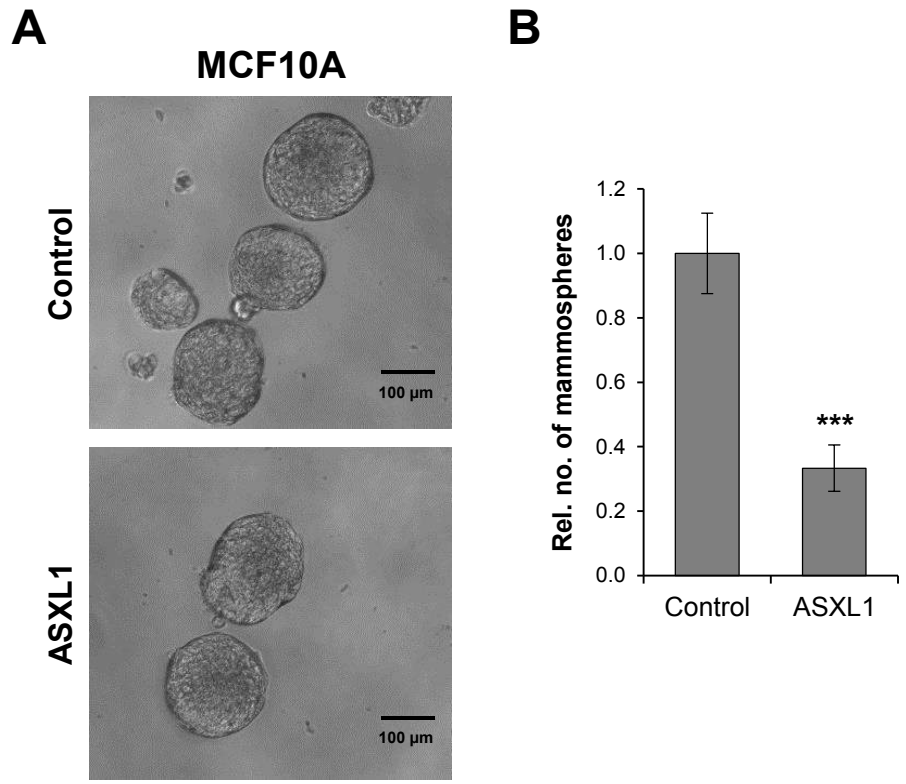


Figure. 19: Expression of ASXL1 depletes stem-cell properties. (A) Phase contrast images of mammospheres upon control and ASXL1 expressing stable cells (MCF10A). The scale bars are represented as 100 µm. (B) The mammospheres formed were quantified and represented as "relative number of mammospheres". The data indicated that there is a decrease in mammospheres number upon ASXL1 overexpression. The data are represented as ± SD. n=3. $^{***}p \leq 0.005$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

4.6 ASXL1 occupancy increased with ectopic ASXL1 expression

To investigate the effect of ASXL1 expression on ASXL1 occupancy, ASXL1 ChIP was performed in MDA-MB-231 cells with ASXL1 stable expression. ASXL1 enrichment was checked by ChIP-qRT-PCR, which showed a significant enrichment of ASXL1 occupancy on the TSS of TJP3, PTEN and ARID1B genes. Furthermore, the ChIP-qRT-PCR analysis showed a significant increase of ASXL1 binding on the TSS upon ASXL1 stable expression, which proves the specificity of ASXL1 binding (Fig. 20).

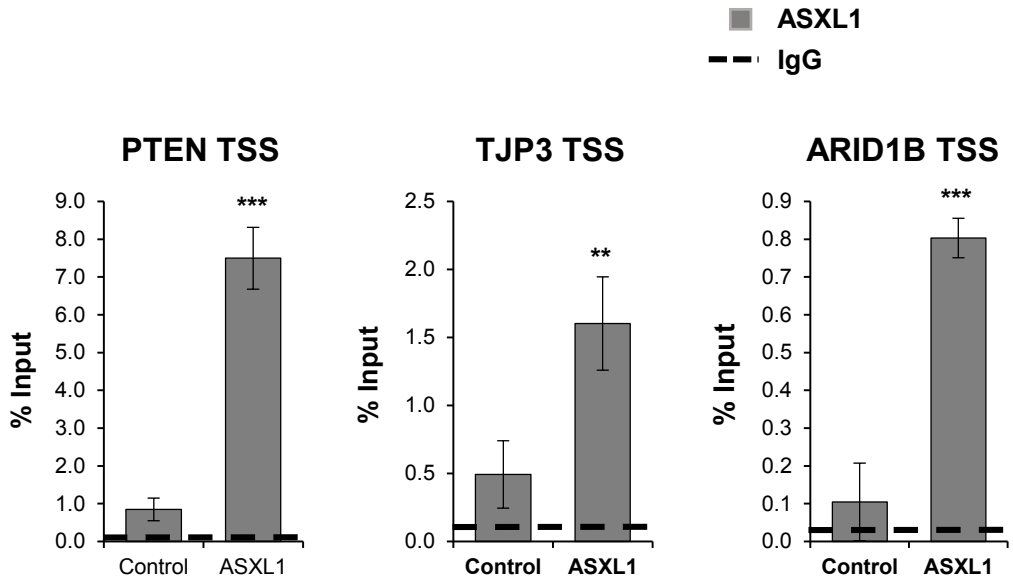


Figure. 20: Enrichment of ASXL1 increased with ectopic ASXL1 expression. ChIP analysis of ASXL1 enrichment on the promoter of PTEN, TJP3 and ARID1B genes in MDA-MB-231 stable cells with and without ectopic ASXL1 expression. Immunoprecipitated DNA is compared to input and represented as "% of Input". IgG antibody was used a negative control to measure the background signal level and displayed as a dotted line. Data are shown as mean ± SD. n=3.

4.7.1 ASXL1 regulates epithelial gene expression by promoting the expression of PTEN

To further elucidate the mechanism by which ASXL1 promotes EMT, we examined the genes regulated following perturbation of ASXL1. We found that the expression of PTEN was significantly down-regulated by ASXL1 depletion and also up-regulated by ectopic expression of ASXL1. PTEN is a tumor suppressor and a multifunctional enzyme belonging to the phosphatase family which is reportedly mutated in several cancers and also known to regulate cell migration (Bowen, Doan et al. 2009, Fournier, Fata et al. 2009).

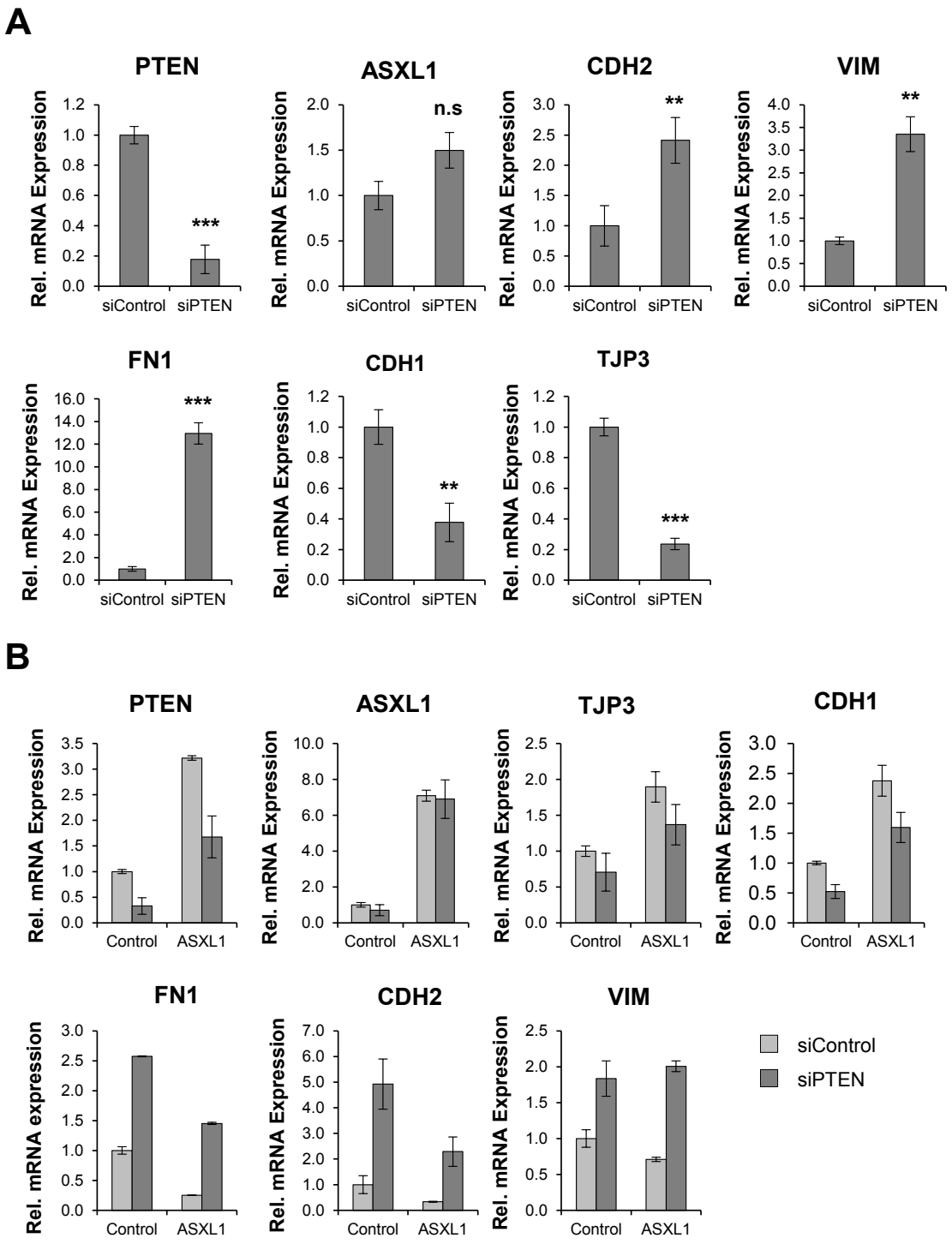


Figure. 21: PTEN expression and its effect on EMT markers. Gene expression levels of epithelial markers (CDH1 and TJP3) and mesenchymal markers (CDH2, FN1 and VIM) were analyzed by qRT-PCR in (A) MCF10A and (B) MDA-MB-231 stable cells and represented as "relative mRNA expression" normalized to HNRNPK expression level. Data are shown as mean ± SD. n=3. $^{***}p \leq 0.005$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

To elucidate the effect of PTEN on EMT phenotype, PTEN was depleted using siRNA in MCF10A and stable MDA-MB-231 cells followed by gene expression analysis of EMT markers using qRT-PCR. The epithelial markers, CDH1 and TJP3 were downregulated and mesenchymal markers, CDH2, VIM, and FN1 were upregulated in PTEN depleted cells compared to siControl transfected cells. However, ASXL1 expression was not effected by PTEN depletion. Furthermore, the presence of ASXL1 ectopic expression in MDA-MB-231 cells was able to counteract the upregulation of mesenchymal markers, CDH2 and FN1, but not VIM (Fig. 21A and B).

To further support the role of PTEN in suppressing EMT, the changes in EMT markers were validated by Western blot analysis in PTEN depleted cells. As expected, mesenchymal markers, Vimentin and N-cadherin were upregulated and epithelial marker, E-cadherin was downregulated (Fig. 22). Taken together, our studies showed that PTEN depletion has similar effects observed following ASXL1 loss.

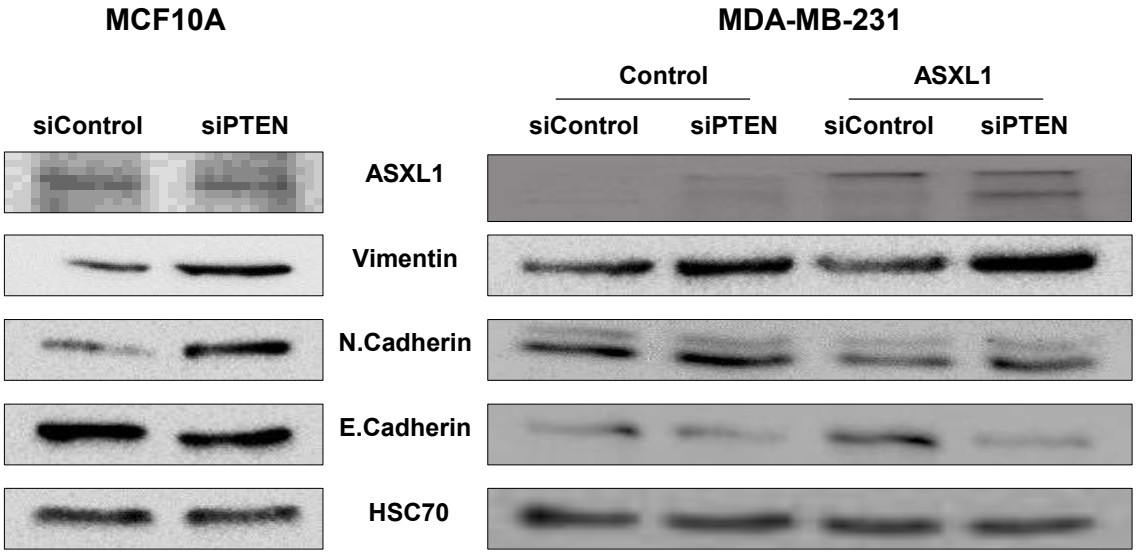


Figure. 22: ASXL1 loss enhances EMT phenotype. Immunoblotting analysis of whole cell lysates depicting decreased expression of the epithelial marker, E-cadherin and increased expression of mesenchymal markers, N-cadherin and Vimentin upon loss of PTEN in MCF10A and MDA-MB-231 stable cells. HSC70 was used as a loading control.

4.7.2 PTEN regulates AKT activation

PTEN is known to inhibit the PI3K–AKT signaling pathway by catalyzing phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) to phosphatidylinositol-4, 5-bisphosphate (PIP2) (Cully et.al, 2006). AKT signaling is associated with the epithelial-to-mesenchymal transition, epithelial differentiation, migration and metastasis (Larue and Bellacosa, 2005; Zhuang et al., 2012). To study whether PTEN depletion is associated with activation of the PI3K–AKT signaling pathway, an immunoblot for p-AKT was performed. In MCF10A cell lines, we observed an inverse correlation between PTEN expression and p-AKT levels. Furthermore, cells with depleted expression of ASXL1 and PTEN had increased p-AKT expression whereas cells with ASXL1 expression had decreased p-AKT expression (Fig. 23). With this, our results indicate that the depletion of ASXL1 and PTEN may lead to activation of PI3K-AKT pathway, which in turn promotes epithelial-to-mesenchymal transition.

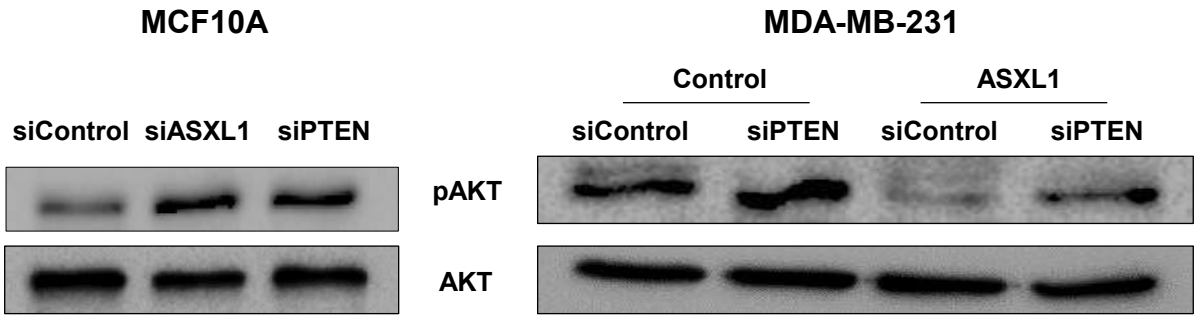


Figure. 23: PTEN depletion enhances AKT activation. Immunoblotting analysis of whole cell lysates depicting increased expression of p-AKT upon loss of ASXL1 and PTEN in MCF10A and decreased expression of p-AKT in ASXL1 expressing MDA-MB-231 stable cells. AKT was used as a loading control.

4.8 Depletion of ASXL1 regulates global levels of epigenomes

Previously, it was shown that ASXL1 binds to the polycomb repressive complex 2 (PRC2) members, specifically EZH2, EED, and SUZ12 and ASXL1 depletion inhibits trimethylation of H3K27 (H3K27me3). ASXL1 also associates with the H2A deubiquitinating enzyme BAP1 (Abdel-Wahab, Adli et al. 2012, Sahtoe, van Dijk et al. 2016). Thus, ASXL1 appears to be involved in both PRC2-mediated gene repression and counteracting PRC1 ubiquitination (Scheuermann, de Ayala Alonso et al. 2010, Abdel-Wahab, Adli et al. 2012, Chung, Schatoff et al. 2012, Dey, Seshasayee et al. 2012, Abdel-Wahab and Dey 2013). Furthermore, it was shown that H2Bub1 plays a tumor suppressor function and also reported to be decreased during tumor progression in breast cancer (Shema, Tirosh et al. 2008, Prenzel, Begus-Nahrmann et al. 2011). To understand epigenetic roles of ASXL1, we examined whether depletion of ASXL1 altered the global histone methylation and monoubiquitination levels in MCF10A cells. Western blot analysis showed decreased levels of global H3K4me3 and H2Bub1 and increased levels of H2Aub1 and no effect on H3K27me3 levels in ASXL1 depleted cells compared with control cells.

These results indicate that ASXL1 is required to maintain H3 methylation and H2A monoubiquitination (Fig. 24).

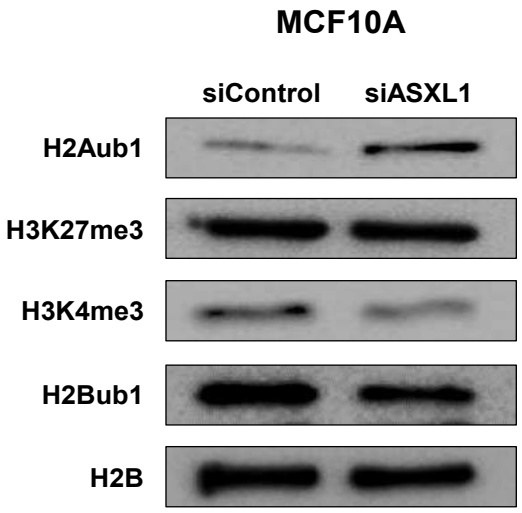


Figure. 24: ASXL1 depletion regulates histone methylation and monoubiquitination levels. ASXL1 depletion by siASXL1 decreases H2Bub1, H3K4me3 protein levels and increased H2AK119Ub protein levels. H2B was used as a loading control.

4.9.1 ASXL1 enhances the gene expression by epigenetic regulation

Methylation of lysine 4 on histone H3 (H3K4) at promoters is tightly linked to transcriptional regulation in human cells and also H3K4me3 is known to be an important histone modification coupled with transcription activation (Okitsu, Hsieh et al. 2010, Dong, Tsuji et al. 2015). In order to investigate the mechanisms by which ASXL1 regulates PTEN, ARID1B and TJP3 expression, we checked the average H3K4me3 signals (±5Kb) around the TSS region of ASXL1-bound genes genome-wide in an aggregate plot for all the ASXL1-bound genes. And, we observed H3K4me3 enrichment across the ASXL1 bound peaks. Published ChIP-seq data for H3K4me3 (SRR398029) (Choe, Hong et al. 2012) were used for the analysis (Fig. 25)

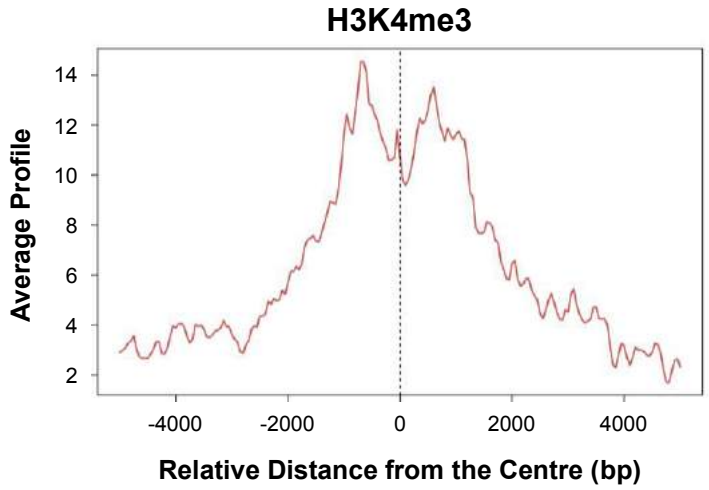
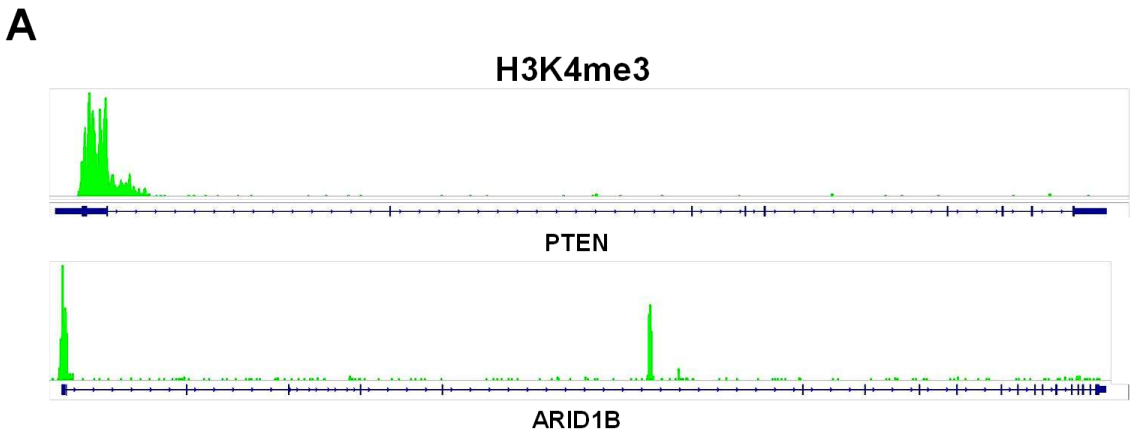


Figure. 25: H3K4me3 enrichment correlates with gene expression. Published H3K4me3 ChIP-seq was used for the aggregate plot analysis on the ASXL1-bound peaks genome-wide. The analysis was performed around the (±5Kb) TSS of the genes.

4.9.2 ASXL1 depletion leads to decreased H3K4me3 on ASXL1 bound genes

To investigate the occupancy of H3K4me3 on ASXL1 bound genes, H3K4me3 ChIP-seq data was used for the genome-wide and single gene analyses, which showed H3K4me3 enrichment around the transcription start site (TSS). Prominent peak was observed around the promoters of ASXL1 bound genes (PTEN and ARID1B) (Fig. 26A). To confirm the enrichment of H3K4me3 on ASXL1 bound genes, we performed H3K4me3 ChIP in MCF10A cells with or without ASXl1 depletion and also in MDA-MB-231 cells with stable ASXL1 expression. The enrichment of H3K4me3 was confirmed by ChIP-qRT-PCR. Consistent with the ChIP-seq results, qRT-PCR analysis of ChIP samples showed a significant enrichment of ASXL1 binding on the TSS region of ASXL1 bound (PTEN, TJP3 and ARID1B) genes. Furthermore, the qRT-PCR analysis showed a significant decrease of H3K4me3 enrichment on the TSS upon ASXL1 depletion whereas a significant increase of H3K4me3 enrichment on the TSS upon ASXl1 over-expression (Fig. 26B). Taken together, these results

confirm that ASXL1 promotes increased H3K4me3 occupancy on the genes and subsequent gene activation.



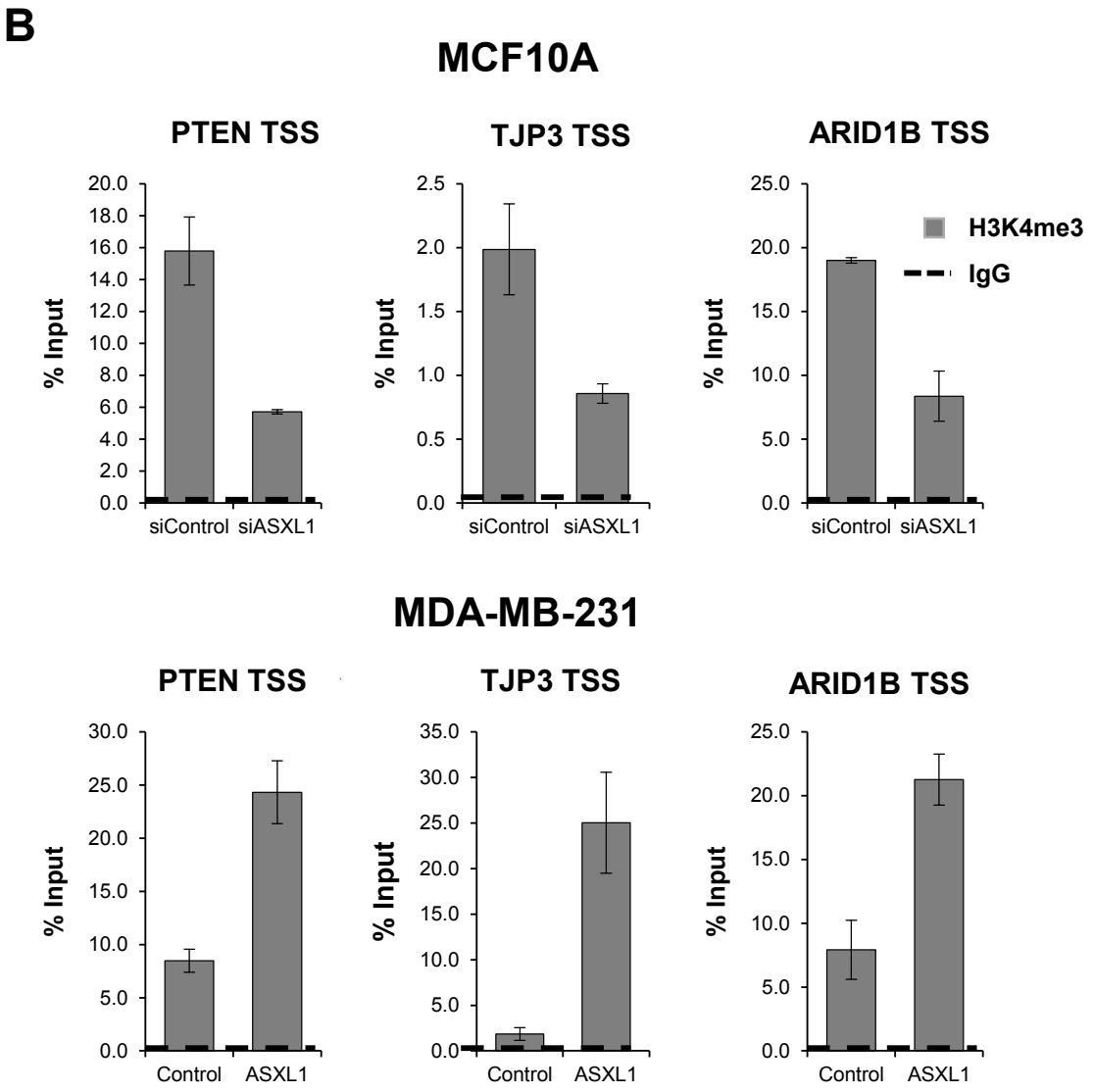


Figure. 26: H3K4me3 enrichment on the promoters of ASXL1 target genes. (A) ChIP-seq profile of H3K4me3 on PTEN and ARID1B genes. Significant peak was observed around the TSS. (B) ChIP analysis of H3K4me3 enrichment on TSS of single genes in MCF10A cells with and without ASXL1 depletion and with and without ASXL1 overexpression in MDA-MB-231 cells. Immunoprecipitated DNA is compared to input and represented as "% of Input". IgG antibody was used a negative control to measure the background signal level and displayed as a dotted line. Data are shown as mean ± SD. n=3.

4.9.3 ASXL1 cooperates with SET1 methylase complex to epigenetically enhance gene expression

To further explain how ASXL1 positively regulates the H3K4me3 level at the promoters of the ASXL1 bound genes, we performed co-immunoprecipitation of

ASXL1 to check the interaction with SET1 complex and BAP1. SET1 complex methylate the histone 3 lysine 4 (H3K4) (Thornton, Westfield et al. 2014). Recently, BAP1 was shown to interact with and deubiquitinate the transcriptional regulator host cell factor 1 (HCF-1) which is one of the members of SET1 complex (Lee, Tate et al. 2007, Narayanan, Ruyechan et al. 2007, Machida, Machida et al. 2009). We observed that ASXL1 physically interacts with H3K4-specific methyltransferase SET1 complex members (Cfp1 and Wdr82) and furthermore we also found that BAP1 also interacts with Wdr82 (Fig. 27)

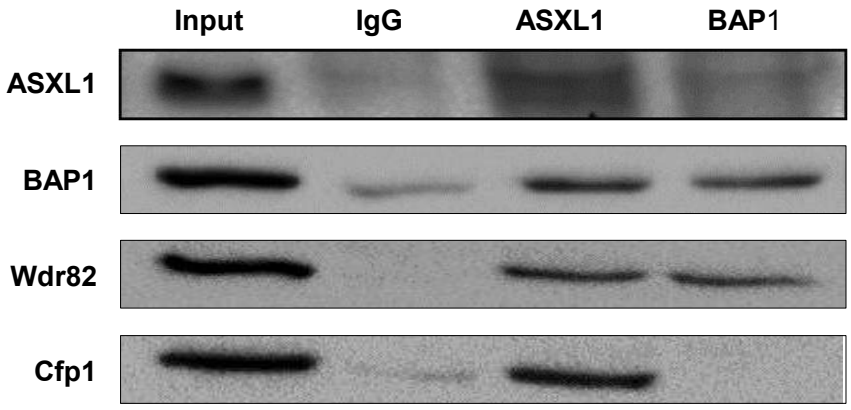


Figure. 27: ASXL1 and BAP1 associate with SET1 complex members. Immunoprecipitation followed by immunoblotting showed that ASXL1 interacts with Wdr82 and Cpf1 whereas BAP1 interacts with only Wdr82.

4.9.4 Depletion of ASXL1 leads to decreased expression of SET1 complex members

It was reported that the coactivator HCF-1 is required for recruitment of Setd1A and MLL1 and BAP1 is known to deubiquitinate HCF-1. From our Co-IP results, we found that ASXL1 and BAP1 were interacting with SET1 complex members. We performed immunoblotting to check the expression of Wdr82 and Cfp1 in ASXL1 depleted cells and also in ASXL1 overexpressing cells (Fig. 28). In ASXL1 depleted cells, we observed that Wdr82 expression was significantly decreased whereas Cfp1

expression was partially decreased, which may affect the stability of SET1 complex and decreased methylation. In ASXL1 overexpressing cells there was increased Wdr82 expression whereas Cfp1 expression was not affected. Previously, it was shown that Wdr82 depletion leads to decreased Setd1A and Cfp1 expression (Lee and Skalnik 2008). Hence, Wdr82 is required for the targeting of Setd1A-mediated histone H3-Lys4 trimethylation near transcription start sites.

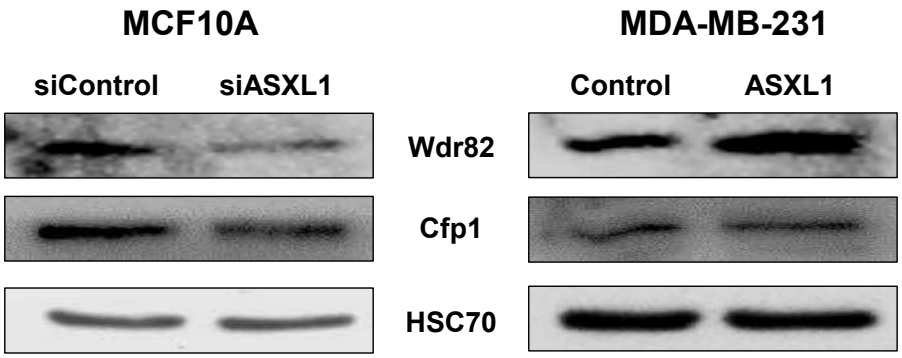


Figure. 28: ASXL1 depletion regulates expression of SET1 complex members. The expression of SET1 complex members, Cfp1 and Wdr82 was checked by immunoblotting.

5. Discussion

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females and most of them are caused due to tumor invasion and metastasis. Genetic and epigenetic alterations in the normal cells lead to the formation of the malignant cells. The malignant cells lose control over the cellular processes including cell morphology, proliferation, migration, cell death and eventually forms a tumor. The cellular plasticity, the fundamental characteristic required by the cells during embryo development, plays an essential role in cancer development and metastasis, in which the epithelial tumor cells undergo enormous cellular modifications and transdifferentiate into mesenchymal cells by activating the epithelial-to-mesenchymal transition (EMT) process (Boyer and Thiery 1993, Hay 1995, Vicente-Duenas, Gutierrez de Diego et al. 2009). In order to metastasize to distant sites, these cells reorganize their cytoskeleton, breakaway from tumor and migrate, invade and colonize to form secondary tumor by undergoing mesenchymal-to-epithelial transition (MET) (Chambers, Groom et al. 2002, Bacac and Stamenkovic 2008, Tsai and Yang 2013). Epigenetic processes play a vital role in the normal development and tissue-specific gene expression and its maintenance. However, the aberrant epigenetic state leads to an altered gene expression which can transform a normal cell into the malignant cell (Sharma, Kelly et al. 2010, Tam and Weinberg 2013). ASXL1 is one among the frequently mutated genes in malignant myeloid diseases (Gelsi-Boyer, Brecqueville et al. 2012). ASXL1 was reported to be one of the components of various chromatin modifying complexes which regulate gene expression. (Abdel-Wahab, Adli et al. 2012). In this study, we focused on characterizing the role of ASXL1 in breast cancer with an aim to reveal the

mechanism involved in ASXL1 mediated tumorigenesis and metastasis and to establish new therapeutic targets for preventing cancer progression and metastasis in various cancers.

5.1 The role of ASXL1 as a potential tumor suppressor

The development of cancer is a multistep and complex process and is initiated by the inactivation of tumor suppressor genes or the activation of oncogenes, which can eliminate the control on cell growth and proliferation and eventually transform a normal cell into a cancerous cell (Hahn and Weinberg 2002). Tumor suppressor genes function by keeping a constant check on cell proliferation and inducing apoptosis to maintain the tissue homeostasis, which is a native suppression of tumor formation. So, inactivation or loss of tumor suppressors results in loss of control over cell growth which is considered as one of the most important hallmarks of cancer development (Hanahan and Weinberg 2011). Based on the context-dependent functions of ASXL family members as transcription activators or repressors, these proteins are proposed to be tumor suppressive or oncogenic, which depends on the genes they activate or repress (Katoh 2013). ASXL1 is mutated in several types of cancers such as lung squamous and bladder cancer (Futreal, Coin et al. 2004, Kandoth, McLellan et al. 2013) ASXL1 is frequently mutated in myeloid malignancies and hematological cancers. ASXL1 mutations are associated with disease progression and poor clinical outcome (Abdel-Wahab, Adli et al. 2012, Valletta, Dolatshad et al. 2015, Wu, Bekker-Jensen et al. 2015). ASXL1 mutations are strongly associated with poorer survival in patients with myeloid malignancies (Shih, Abdel-Wahab et al. 2012).

In our study, the examination of ASXL1 mRNA expression status in a wide spectrum of breast carcinoma datasets revealed the significant downregulation of ASXL1 mRNA expression levels in breast cancer samples compared to the control samples. We have also tested the clinical utility of ASXL1 protein expression as a prognostic biomarker of breast cancer progression through examination of the association between ASXL1 and disease outcome. In our analysis, we found that the patients with low ASXL1 expression have significantly poorer relapse-free survival than the patients with high ASXL1 expression and collectively, there is an inverse correlation of ASXL1 expression with tumor malignancy. However, in-depth studies are needed to understand the role of ASXL1 in tumorigenesis completely.

5.2 ASXL1 downregulation perturbs the global gene expression

Depletion or inactivation of tumor suppressor genes leads to the reduced function of the gene. Loss of function leads to the increase in tumor cell number by increasing cell growth or by inhibiting cell death, which is caused due to the activation of genes regulating cell cycle or by suppressing the genes involved in apoptosis (Vogelstein and Kinzler 2004). As an initial step to understand the functional role of ASXL1 in breast cancer development and progression, the transcriptional effect of ASXL1 depletion was obtained through mRNA expression profiling in normal breast epithelial cells, MCF10A. The transcriptome-wide sequencing analysis revealed that the expression of ASXL1 activates or suppresses a distinct set of genes which includes a small portion of genes involved in EMT process such as fibronectin, Vimentin, TJP-3, N-cadherin and E-cadherin and also stem cell marker, CD24. Other than EMT genes, a small subset of genes is also differentially expressed. PTEN, a tumor suppressor is one among them which is known to be mutated or lost in several

cancers, including breast cancer and involved in tumorigenesis and progression (Zhang, Liang et al. 2013). ASXL1 depletion resulted in the perturbation of genes that are involved in cell adhesion, cell proliferation and cell motility. The putative tumor suppressor role of ASXL1 was confirmed by the GSEA and the DAVID functional annotation analyses. The gene Ontology analysis reveals that ASXL1 controls cellular activities, that are exploited during cancer progression and metastasis, such as cell adhesion, cell proliferation, angiogenesis, cell death and cell motility. Furthermore, gene expression analyses of ASXL1 depleted MCF10A cells confirms that pathways related to breast cancer, epithelial differentiation, EMT and metastasis are among the top enriched pathways. This suggests that ASXL1 depletion promotes EMT.

5.3 The role of ASXL1 in cancer metastasis

EMT and MET processes play an important role in the transition between epithelial and mesenchymal phenotype and are involved in the normal development and cancer metastasis. During the transition, the epithelial cells disrupt their intracellular tight junctions and cell-cell contacts and become mesenchymal cells. These cells acquire stem cell properties and enhance their invasive and migratory properties (Thiery and Sleeman 2006, Heerboth, Housman et al. 2015). Our analysis of global gene expression suggested the role of ASXL1 in EMT regulation and thus metastasis. To assess more and test further consequences, ASXL1 is either depleted or overexpressed.

5.3.1 The role of ASXL1 in regulating EMT

One of the important clues that have been obtained in this study is the possible involvement of ASXL1 in the EMT process. EMT is known to be indispensable for the

development of the embryo and the formation of various tissues or organs. However, inappropriate activation of EMT in the normal cells leads to diseases, such as cancer by converting the epithelial cancer cells into stem-cell like mesenchymal cancer cells. In normal epithelial cells, the genes which make the cells in association with other cells in a tissue are expressed, however, the mesenchymal genes, which induce the cells to change morphology, and to breakaway for migration and invasion, are repressed. E-cadherin is involved in cell-cell adhesion and its loss is thought to be the initial step for enabling EMT and metastasis. In contrast, N-cadherin is elevated in EMT and can regulate cell-cell adhesion during the invasion. The switch of E-cadherin and N-cadherin expression induces EMT in several cancers, leading to enhanced cellular motility or invasion and decreased adhesion (Mani, Guo et al. 2008, Onder, Gupta et al. 2008, Gupta, Onder et al. 2009).

Our study reveals that depletion of ASXL1 induces a dramatic change in the morphology of MCF10A cells from epithelial to fibroblastic-like spindle-shaped phenotype. The alteration in cell morphology is one of the characteristics of EMT and is associated with changes in the expression of several genes. In our transcriptome-wide analysis, we found that ASXL1 depletion significantly upregulates or downregulates some of the EMT-associated genes, which includes a reduced expression of epithelial markers such as E-cadherin and TJP3 and an increased expression of mesenchymal markers such as Vimentin, N-cadherin and fibronectin.

To further determine the tumor suppressor role of ASXL1 in breast cancer, we evaluated the expression of ASXL1 in breast cancer cell line, MDA-MB-231 and observed that ASXL1 expression was downregulated. Furthermore, stable ASXL1 overexpression in both normal and breast cancer cells exhibited increased epithelial

marker expression and decreased mesenchymal marker expression. The epithelial marker, E-cadherin was decreased whereas the mesenchymal markers, Vimentin, and N-cadherin were increased in ASXL1 depleted cells. In contrast, there is an increase in E-cadherin expression and a decrease in N-cadherin and Vimentin expression in the cells with ASXL1 overexpression. Immunofluorescent staining confirmed increased expression of Vimentin and reduced expression of E-cadherin in ASXL1 depleted MCF10A cells compared to the control cells, suggesting that these factors might contribute to induction of the EMT phenotype. The migration and invasion of cancer cells is an important process of cancer metastasis. Our studies using *in vitro* migration assays showed an increased migration potential of the normal mammary epithelial cell lines MCF10A and MCF12A upon ASXL1 depletion, whereas ASXL1 overexpression suppressed the migratory potential of the cells (MCF10A). More importantly, ASXL1 overexpression inhibited the migration of breast cancer cells (MDA-MB-231). Enhanced cell migration is a pronounced feature of advanced tumors (Friedl, Hegerfeldt et al. 2004) and therefore decreased ASXL1 expression may potentially facilitate cancer progression.

Furthermore, due to the inverse relationship between the expression of epithelial genes and mesenchymal genes, it seems that ASXL1, with its tumor suppressor function, may suppress the mesenchymal genes. Indeed, our data highlights the same that depletion of ASXL1 results in the upregulation of mesenchymal markers whereas overexpression results in repression of mesenchymal markers. Taken together, we have shown in this study that, upon depletion of ASXL1, mammary epithelial cells undergo a series of changes through which cells lose their epithelial characteristics and acquire the mesenchymal phenotype.

5.3.2 ASXL1 expression regulates cancer stem cell pool

Several reports have shown that breast cancer stem cells (BCSCs) have been implicated as the main driver for initiation, maintenance, progression and reoccurrence of breast cancer. Cancer stem cells (CSCs) are a small portion of undifferentiated malignant cancer cells within a tumor that have the capacity to self-renew, proliferate and can differentiate/transdifferentiate into the heterogeneous population of cancer cells. (Clarke, Dick et al. 2006, Yao, Ping et al. 2011, Li, Yao et al. 2014, Liu and Fan 2015, Qiao, Liang et al. 2015). Recently, it was reported that EMT has been closely linked to the acquisition of CSCs-like characteristics such as enhanced self-renewal, CSC-gene expression and increased tumorigenicity (Mani, Guo et al. 2008, Sampieri and Fodde 2012). In our studies, we observed that the CSC marker, CD24 was downregulated in ASXL1 depleted mammary cells at both mRNA and protein level. Furthermore, ASXL1 depletion also increased the mammosphere formation which was suppressed by the overexpression of ASXL1 in MCF10A cells. Taken together, the results strongly suggest the role of ASXL1 in mammospheres formation and anchorage-independent growth by suppressing the stem cell phenotype.

5.4 Genome-wide distribution of ASXL1

From our transcriptome-wide data, we found the potential role of ASXL1 in regulating EMT. In order to gain mechanistic insight into the function of ASXL1 in EMT regulation, we need to understand ASXL1 mediated regulation of gene transcription. From our genome-wide analyses, we observed that the occupancy of ASXL1 was mostly confined to the distal intergenic regions. However, a significant portion of ASXL1 binding sites was confined to the promoter regions throughout the genome.

Furthermore, aggregate plot analyses also revealed that the highest enrichment of ASXL1 was observed near the TSS region of the genes. Possible mechanistic explanations for the presumed tumor-suppressor activity of ASXL1 can be studied by identifying the ASXL1 regulated genes. In our IGV data significant ASXL1 peaks were found around the promoter regions of a small subset of ASXL1 regulated genes such as PTEN, TJP3 and ARID1B. Furthermore, the qRT-PCR analysis showed that there was a significant decrease of ASXL1 binding on the TSS of PTEN, ARID1B and TJP3 upon ASXL1 knockdown. Moreover, there was a significant increase in ASXL1 occupancy upon ASXL1 overexpression which proves the specificity of ASXL1.

A key issue that remains to be elucidated is whether ASXL1 binding has a direct function in gene transcription. Indeed, we observed that these genes were significantly downregulated upon ASXL1 depletion and upregulated upon ASXL1 overexpression. These results demonstrate that ASXL1 regulates gene transcription.

5.5 ASXL1 regulates EMT by targeting PTEN

PTEN, a tumor suppressor gene, is a negative regulator of (PI3K)-Akt pathway and participates in the regulation of cell cycle, proliferation, apoptosis, cell adhesion and EMT during embryonic development and cancer progression. PTEN is known to dephosphorylate Akt and inactivate PI3K/Akt pathway and its depletion leads to accumulation of an active form of Akt (Cantley and Neel 1999, Nicholson, Streuli et al. 2003). PTEN mutations were reported in several cancers and its loss leads to induction of tumor invasion and metastasis (Wang, Quah et al. 2007, Keniry and Parsons 2008, Bao, Yan et al. 2013). In our ChIP-seq analysis, we found that ASXL1 occupancy on the PTEN promoter and PTEN expression was regulated

depending upon ASXL1 expression. Thus, loss of PTEN perturbs the EMT genes. As a result of depletion of PTEN in MCF10A and MDA-MB-231 cells, the expression of epithelial markers, CDH1 and TJP3 were downregulated and mesenchymal markers, CDH2, VIM and FN1 were upregulated without any effect on the expression of ASXL1. Thus, this indicates that PTEN may be one of the main downstream regulators of ASXL1. However, the ectopic expression of ASXL1 was able to suppress the expression of mesenchymal markers and retain the expression of epithelial markers. Furthermore, p-Akt was increased upon ASXL1 depletion whereas decreased upon ASXL1 overexpression. As PTEN is a downstream regulator of ASXL1, knockdown of PTEN in ASXL1 overexpressing MDA-MB-231 cells rescued the phosphorylation of Akt. Thus, our results suggest that ASXL1 inhibits Akt signaling through positive regulation of PTEN expression.

5.6 ASXL1 and post-translational modifications

It was reported that ASXL1 regulates epigenetic marks and transcription through interaction with polycomb complex proteins, thus ASXL1 loss results in the reduction of K3K27me3, the repressive mark regulated by the PRC2 complex (Cho, Kim et al. 2006, Boultwood, Perry et al. 2010, Wang, Li et al. 2014). It was also reported that ASXL1 depletion disrupts the PR-DUB complex which in turn results in an increase in H2Aub1 levels. In our study, we show that depletion of ASXL1 is associated with global changes in histone modifications such as decreased levels of global H3K4me3 levels and increased levels of global H2Aub1 levels. We also observed that there is a slight reduction of H2Bub1 levels. However, H3K27me3 levels were not affected. Global loss of H2Bub1 has been reported in breast cancer (Prenzel, Begus-Nahrmann et al. 2011) and H2Bub1 is known to recruit the methyltransferase

complex, which is involved in H3K4 methylation (Wood, Schneider et al. 2005, Kim, Guermah et al. 2009). H3K4me3 is associated with transcription activation and enrichment of ASXL1 at promoter regions genome-wide suggesting the potential role of that ASXL1 in the regulation of gene transcription.

5.7 Loss of ASXL1 is associated with occupancy of H3K4me3

As it was observed in this study, depletion of ASXL1 leads to a reduction in global H3K4me3 levels. Therefore, the H3K4me3 binding on various direct ASXL1 target genes (PTEN, TJP3, and ARID1B) following ASXL1 knockdown and overexpression was analyzed. Our ChIP-qRT-PCR in ASXL1 depleted cells revealed a significant reduction of H3K4me3 enrichment on ASXL1 target genes whereas ASXL1 overexpression showed increased occupancy of H3K4me3. Furthermore, aggregate plot analyses of publicly available ChIP-seq data for H3K4me3 showed that the highest enrichment of H3K4me3 was observed near the TSS region of the ASXL1 bound genes. Our data suggests that ASXL1 target genes expression was altered through direct binding and also by altering the chromatin state of promoters.

5.8 ASXL1 physically interacts with BAP1 and SET1 methyltransferase complex members

Recent studies have identified several interacting partners for ASXL family members, such as BAP1, PRC2 complex members (EZH2 and EED) and nuclear receptors (Cho, Kim et al. 2006, Scheuermann, de Ayala Alonso et al. 2010, Park, Yoon et al. 2011, Lai and Wang 2013). In this study, we showed that ASXL1 depletion leads to the reduction in both global and promoter-associated H3K4me3. Our results clearly demonstrate that ASXL1 interacts with BAP1, Cfp1 and Wdr82, whereas BAP1 interacts with ASXL1 and Wdr82. BAP1 is the catalytic subunit of PR-DUB, which

deubiquitinates the H2Aub1. Furthermore, our studies demonstrated for the first time that ASXL1 physically interact with SET1 complex members. Taken together, our results indicate that ASXL1 may form a complex with SET1 complex members and co-regulate target gene expression in part through H3K4me3.

5.9 ASXL1 regulates expression of SET1 complex members

It was reported that BAP1 not only interacts with HCF1 but also deubiquitinates HCF1, which is a member of SET1 complex and is involved in the recruitment of Setd1A and MLL1, which leads to transcription activation through H3K4me3 (Lee, Tate et al. 2007, Narayanan, Ruyechan et al. 2007, Tyagi, Chabes et al. 2007, Machida, Machida et al. 2009). Our results show that SET1 complex members were interacting with ASXL1 and BAP1. Interestingly, we also observed the expression of Wdr82 was significantly decreased whereas Cfp1 expression was partially decreased upon ASXL1 depletion. Furthermore, we also observed increased expression of Wdr82, but there was no change in Cfp1 expression upon ASXL1 overexpression. It was reported that depletion of Wdr82 leads to decreased expression of Setd1A, which in turn leads to the decreased H3K4me3 at the TSS as a result of decreased occupancy of Setd1A. It was also reported that depletion of Wdr82 downregulates Set1A expression and effects the stability of SET1 complex (Lee and Skalnik 2008, Wu, Wang et al. 2008). Our data suggests that ASXL1 mediates the expression of SET1 complex members and regulates H3K4me3 through SET1 complex.

6. Conclusion and Future Perspectives

Altogether, in this study, we have elucidated the tumor suppressor role of ASXL1 in breast cancer, which inhibits EMT and regulates stem cell properties in mammary cells. RNA-seq analysis revealed that the loss of ASXL1 results in upregulation of EMT gene signatures and also induces migratory properties. Furthermore, based on the genome-wide and transcriptome-wide analysis, we found PTEN as a direct target gene of ASXL1 and depletion of PTEN leads to EMT phenotype. Interestingly, we also found that ASXL1 interacts with a methyltransferase, SET1 complex members and their expression were also regulated by ASXL1. More studies are required to further validate the mechanism and regulatory function of ASXL1 that is coupled with transcription and EMT. Furthermore, ChIP analysis or ChIP-seq against SET1 complex members to determine genome-wide co-occupancy of ASXL1 and SET1 complex members on ASXL1 target genes to promote transcription activation.

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