Prolactin Induced Molecular Mechanisms Mediating Mammary Differentiation and Tumor Suppression

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Abstract

Prolactin hormone (PRL) is a key inducer of mammary lobulo-alveolar development, terminal differentiation of the mammary epithelial cells and lactation. While the pro-differentiation role of this hormone is well established, its role in mammary tumorigenesis is still debatable and to be fully elucidated. Recently, our lab has proposed an anti-tumorigenic role for PRL in breast cancer. Here, I investigated the hypothesis that molecular mechanisms through which PRL induces its pro-differentiation function are implicated in mediating its anti-tumorigenic role in breast cancer.

Our data revealed a central role for kinesin heavy chain-5B (KIF5B), a novel PRL down-regulated target gene identified in mammary epithelial cells, as a promoter of breast tumorigenesis. Indeed, we showed that KIF5B and its partner protein kinesin light chain 1 (KLC1) play differential roles in regulating epithelial-mesenchymal plasticity (EMP) of breast cancer cells. We found KIF5B to be expressed in triple negative (TN)-basal-like/claudin-low breast cancer subtype and to be an inducer of epithelial mesenchymal transition (EMT), stemness, invasiveness and metastatic colonization. On the other hand, we found KLC1 to be expressed in epithelial/luminal breast cancer subtypes and to be a potent suppressor of EMT, invasion and stem cell marker expression as well as an inducer of epithelial/luminal phenotype.

Furthermore, we showed that PRL induces apical/basal polarity (A/B polarity) and mammary acini morphogenesis through modulating the proper localization of classical polarity protein complexes, PAR and Crumb, to the apical plasma membrane and the Scribble complex to the basal domain of plasma membrane of mammary epithelial cells. Importantly, our data also showed that PRL induced A/B polarization is

mediated through activation of the tumor suppressor Hippo pathway in mammary epithelial cells. Moreover, our data demonstrated PRL-induced mammary differentiation is linked to the regulation of centrosome duplication and genomic stability. Indeed, our results showed that CRISPR/Cas9 knockout of the PRLR in luminal A breast cancer cells resulted in increased centrosome amplification, DNA damage as well as up-regulation of the centrosome duplication kinase, Polo-like kinase 4 (PLK 4).

Collectively, our work helps in better understanding of the role of PRL and its signaling pathway in mammary tissue as well as in breast cancer. Furthermore, these findings provide molecular insights in support of the newly appreciated tumor suppressive role of PRL in breast carcinogenesis.

Résumé

La prolactine (PRL) est l'inducteur majeur du développement lobulo-alvéolaire des glandes mammaires, de la différenciation terminale des cellules épithéliales mammaires et de la lactation. Bien que son implication lors du processus de différenciation soit bien documentée, son rôle lors de la tumorigenèse mammaire est sujet à controverse. Récemment, notre laboratoire a rapporté un rôle anti-tumorigénique pour la PRL dans le cancer du sein. Dans la présente étude, j'ai traité de l'hypothèse selon laquelle les mécanismes moléculaires sous-jacents au rôle de la PRL dans la différenciation sont impliqués dans l'induction de ses effets anti-tumorigénique dans le cancer du sein.

Nous avons démontré le rôle central que joue la chaîne lourde-5B de la kinésine (*kinesin heavy chain-5B* ; KIF5B) dans la promotion de la tumorigenèse mammaire. Ce gène est une nouvelle cible régulée négativement par la PRL au niveau des cellules épithéliales mammaires. En effet, nous avons montré que KIF5B et son partenaire protéique, la chaîne légère 1 de la Kinesin (KLC1), jouent des rôles différents dans la régulation de la plasticité épithélio-mésenchymateuse (EMP) des cellules cancéreuses du sein. Nous avons trouvé que KIF5B est exprimée dans le sous-type de cancer du sein *triple negative (TN)-basal-like/claudin low*, et serait un inducteur de la transition épithélio-mésenchymateuse (EMT), du maintien de l'état souche, du caractère invasif et de la colonisation métastatique. D'autres part, nous avons trouvé que KLC1 est exprimée dans les sous-types de cancer du sein épithélial/luminal, et serait un puissant suppresseur de l'EMT, de l'invasion et de l'expression des marqueurs de cellules souches ainsi qu'un inducteur du phénotype épithélial/luminal.

Aussi, nous avons montré que la PRL induit la polarité apico-basale (polarité A/B) et la morphogenèse des acini mammaires en modulant la localisation correcte des complexes protéiques de la polarité classique ; en l'occurrence PAR et Crumb à la membrane plasmique apicale et le complexe Scribble au domaine basal de la membrane plasmique des cellules épithéliales mammaires. De plus, nous montrons que la PRL induit la polarisation A/B via l'activation de la voie de suppression de tumeur Hippo (*tumor suppressor Hippo pathway*) au niveau des cellules épithéliales mammaires. En sus, nous montrons que la différenciation mammaire induite par la PRL est liée à la régulation de la duplication des centrosomes et de la stabilité génomique. En effet, nos résultats ont montré que l'invalidation de PRLR par la stratégie CRISPR/Cas9 dans les cellules luminal A du cancer du sein entraîne une amplification accrue du centrosome, une augmentation des dommages à l'ADN ainsi qu'une régulation à la hausse de la kinase *Polo-like kinase 4* (PLK 4).

En somme, nos découvertes permettront de mieux comprendre le rôle joué par la PRL et sa voie de signalisation dans le développement des tissus mammaires et du cancer du sein. En outre, elles apportent des évidences moléculaires à l'appui du rôle de suppresseur de tumeur de la PRL dans la carcinogenèse du sein.

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Dedication

This thesis is dedicated to the most important people in my life, my parents Mr. Ibrahim Moamer and Mrs. Layla Malayo who dedicate their lives to my sisters and me. They have thought us how to be better people, stronger and different. I would not be more thankful throughout my entire life than being their daughter. I would like to dedicate this work also to my sisters Afnan, Areej and Yara who have been always behind me supporting my back. I would like to thank them for their love, care, friendship and inspiration. Finally, I dedicate this thesis to all my extended family who have granted me with the true love for whole of my life. I would not be more proud than being part of this great family.

Preface & Contribution of Authors

This thesis is presented in a manuscript-based format. This thesis is written by Alaa Moamer and revised by my supervisor, Dr. Suhad Ali. It consists of five major chapters. Chapter 1 includes the introduction, a review of related literature background. Chapter 2 contains a submitted manuscript. Chapter 3 and 4 comprise manuscripts under submission; Chapter 5 includes a general discussion and future directions.

Chapter 2 was submitted to EBioMedicine (The Lancet Group). I wrote the manuscript under my supervisor's guidance. I designed and performed all the experiments described under my supervisor directions. Dr. Ibrahim Hachim performed analyses of immunohistochemistry (IHC) experiments and analyses. Najat Binothman performed the nuclear extraction assay, immunoprecipitation assay and contributed to drafting the article. Dr. Jean Jacques Lebrun contributed design and revising the article. Dr. Suhad Ali contributed in designing the experiments, supervising the project and drafting of the article.

Chapter 3 and 4 are manuscripts under submission. I wrote the manuscripts under my supervisor's guidance. I designed and performed all the experiments described under my supervisor's directions. Dr. Suhad Ali contributed in designing the experiments, supervising the project and drafting of the article.

I have collaborated on, and published or submitted the following articles as first author or coauthor:

1. Moamer A, Hachim IY, Binothman N, Lebrun JJ, and Ali S., A Role for Kinesin-1 Subunits KIF5B/KLC1 in Regulating Epithelial Mesenchymal Plasticity in Breast Tumorigenesis (submitted manuscript).

2. Moamer A and Ali S., Prolactin Induces Mammary Acini Morphogenesis Through Regulating Apical/Basal Polarity and Hippo Pathway (under submission).
3. Moamer A and Ali S., A Novel Role of Prolactin Signaling in Maintaining Centrosome Duplication and Genomic Stability Through Regulating Polo-Like Kinase 4 (PLK4) (under submission).
4. Tian J, Raffa F, Dai M, Moamer A, Khadang B, Hachim IY, Bakdounes K, Ali S, Jean-Claude B, Lebrun JJ., Dasatinib Sensitizes Triple Negative Breast Cancer Cells to Chemotherapy by Targeting Breast Cancer Stem Cells, Br J Cancer. 2018 Dec:119(12):1495-1507.

Contribution to knowledge

In this thesis, I investigated molecular mechanisms mediating PRL-induced mammary differentiation and its tumor suppression role in mammary epithelial and breast cancer cells.

- In chapter 2, we found Kinesin-1 subunits, KIF5B and its partner protein KLC1, to play a differential role in regulating epithelial mesenchymal plasticity (EMP) of breast cancer cells.
- While KIF5B was found to be an inducer of epithelial mesenchymal transition (EMT) in triple negative (TN)-basal-like/claudin low breast cancer cells, KLC1 was found to be a potent inducer of epithelial/luminal phenotype.
- Our data showed enrichment of KIF5B within the nuclear compartment of TN-basal-like/claudin-low cells while no nuclear accumulation of KLC1 was found.
- We also highlighted a role of the KIF5B/KLC1 dynamic in TGFb and PRL regulation of EMP in breast cancer.
- In chapter 3, our data showed for the first time that PRL induces A/B polarity and mammary acini morphogenesis through modulating the proper localization of two classical polarity protein complexes, PAR and Crumb to the apical plasma membrane and Scribble complex to the basal domain of plasma membrane of mammary epithelial cells.
- We also showed in chapter 4 that PRL-induced A/B polarization is mediated through activation of the tumor suppressor Hippo pathway in mammary epithelial cells.

- In chapter3, our data demonstrated PRL induced mammary differentiation is linked to the regulation of centrosome duplication and genomic stability.
- We showed that loss of PRL signaling components, PRLR and Jak2 resulted in increased centrosome amplification, DNA damage as well as up-regulation of the centrosome duplication kinase, Polo-like kinase 4 (PLK 4).
- Our study provides better understanding of the role of PRL and its signaling pathways in mammary tissue as well as in breast cancer. Furthermore, these findings provide molecular insights in support of the newly appreciated tumor suppressive role of PRL in breast carcinogenesis.

List of Abbreviations

A/B polarity, apical/basal polarity

AJC, apical junctional complexes

aPKC, atypical protein kinase C

Amot, angiomotin

CCD, coil-coiled domain

Cdk2-E, Cyclin-dependent kinase2-cycline E

CRH, cytokine receptor homology

CIS, carcinoma *in situ*

CK5/6, cytokeratins 5/6

CK18, cytokeratin 18

Crb3, Crumbs 3

DBD, DNA binding domain

Dlg, Discs-large

ECD, extracellular domain

ECM, extracellular matrix

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

EMT, epithelial-mesenchymal transition

EMP, epithelial-mesenchymal plasticity

ER, estrogen receptor alpha

FERM, short for band 4.1 ezrin, radixin and moesin domains

FGF, fibroblast growth factor

FN1, fibronectin 1

GPCR, G protein-coupled receptor

HER2, human epidermal growth factor receptor-2

IBC, invasive breast carcinoma

ICD, intracellular domain

IHC, immunohistochemistry

Jak2, Janus kinase 2

JAMs, junction adhesion molecules

JH, Janus homology domains

KHC, kinesin heavy chain

KIFs, Kinesin superfamily proteins

KIF5B, Kinesin family member 5B

KLC, kinesin light chain

Lats1/2, Large tumor suppressor kinase 1/2

LD, linker domain

Lgl2, lethal giant larvae

LID, lipid-interacting domain

MAPK, mitogen-activated protein kinase

MMPs, metalloproteinases

Mst1/2, macrophage stimulating protein 1/2

MTOCs, microtubule-organizing centers

NF2, Neurofibromin 2

NTD, N-terminal domain

Patj, Pals1-associated tight junction protein

PCM, pericentriolar material

PCP, planar cell polarity

PLK1, Polo-like kinase 1

PLK 4, polo-like kinase 4

PR, progesterone receptor

PRL, Prolactin hormone

PRLR, Prolactin receptor

RANKL, receptor activator of nuclear factor κ-B ligand

Scrib, Scribble

SH2, Src-homology-2

Snail1, Zinc finger protein

Stat5, signal transducer and activator of transcription 5

TAD, C terminal transcriptional activation domain

TEBs, terminal end buds

TGFβ, transforming growth factor beta

TMD, transmembrane domain

TNBC, triple negative breast cancer

Twist1, Twist-related protein 1

Twist2, Twist-related protein 2

WAP, whey acidic protein

Yap1, Yes-associated protein 1

Zeb1, Zinc finger E-box-binding homeobox 1

Zeb2, Zinc finger E-box-binding homeobox 2

ZO1, zonula occluden 1

Rationale and Objectives

Prolactin hormone (PRL) is a key inducer of mammary epithelial cell differentiation, mammary gland development and lactation through activation of the Jak2/Stat5 signaling pathway. While the pro-differentiation role of this hormone is well established, its role in breast cancer development/progression and the molecular mechanisms determining its function are still controversial and need to be fully elucidated. Previous work in our lab highlighted a crucial anti-tumorigenic role for PRL in breast cancer. Indeed, we originally showed that PRL suppresses epithelial-mesenchymal-transition (EMT) and reduces the invasive properties of breast cancer cells by suppressing two invasive and pro-metastatic pathways, MAPK and TGFβ/Smad. We showed also that PRL blocks growth factor-induced mammary cell proliferation and viability of human breast cancer cells. Moreover, we have recently shown, using large cohorts of human breast cancer clinical cases, that expression of PRLR and PRL are downregulated during breast tumor progression and that their expression correlates with favorable clinicopathological parameters and better patient survival. On the other hand, our studies in mammary epithelial cells (MECs) defined a gene signature, derived from PRL upregulated target genes, that correlates with favorable patient outcomes, whereas another gene signature, derived from PRL-downregulated genes, correlates with a poor prognosis. Other work in our lab using MECs has identified PRL as a key inducer of polarized mammary acini morphogenesis and terminal differentiation of mammary epithelial cells. Together, these findings provide compelling evidence supporting the pro-differentiation role of the PRL pathway in mammary tissue and the anti-tumorigenic

function in breast carcinogenesis. Therefore, further studies are needed to investigate the role of the PRL pathway in mammary tissue and breast cancer.

The principal objective of this thesis is to detail the mechanisms by which PRL induces the mammary epithelial differentiation program, providing further insights into the anti-tumorigenic role of PRL in breast carcinogenesis. The specific objectives of this study include: (1) to elucidate the role of PRL/Jak2 signaling in regulating epithelial mesenchymal plasticity and thereby suppressing breast carcinogenesis (Chapter 2); (2) to investigate the molecular mechanisms through which PRL/Jak2 signaling induces A/B polarity and mammary acini morphogenesis (Chapter 3); and (3) to characterize the role of PRL/Jak2 signaling in maintaining genomic integrity by examining how PRL/Jak2 signaling is involved in the regulation of centrosome duplication and maintenance of genomic stability of mammary epithelial and breast cancer cells (Chapter 4).

Chapter 1: Introduction - Literature Review

1.1 Prolactin Hormone and Its Signal Transduction Pathway

1.1.1 Prolactin (PRL)

Prolactin (PRL), known as luteotropic hormone or luteotropin, is a polypeptide hormone secreted particularly from specialized cells of the anterior pituitary gland, called the lactotrophs. However, it has been revealed that synthesis and secretion of PRL is not restricted to the anterior pituitary gland, but other body organs and tissues can secrete it (Bole-Feysot et al, 1998; Chilton & Hewetson, 2005). Indeed, mammary epithelial cells are believed to be the most significant source of exta-pituitary PRL production during lactation (Steinmetz et al, 1993). Studies have shown that the myometrium and the endometrium are also other sources of PRL (Gellersen et al, 1991; Walters et al, 1983). Furthermore, other extra-pituitary sites have been identified, including the brain (Fuxe et al, 1977), the spinal cord (Harlan et al, 1989), the prostate (Nevalainen et al, 1997), the urethral gland (Tsubura et al, 1986), the gut, the adipose tissue (Brandebourg et al, 2007), lymphocytes and other immune system tissues (Wu et al, 1996).

PRL was named based on the fact that an extract of bovine pituitary gland causes crop sac growth and stimulates crop milk production in pigeons as well as its promotion of lactation in rabbits (Freeman et al, 2000). Nevertheless, it is well appreciated now that PRL has over 300 distinct biological activities not represented by its name. Indeed, not only does PRL exert various roles in reproduction other than lactation, but it also serves other roles including homeostatic, immune response in organisms as well as citrate production in the prostate. Recent studies have expanded PRL functions to regeneration of the central nervous system and pathogenesis of the cardiovascular system (Ignacak et al, 2012). Other studies have further expanded the role of PRL to regulation of

microRNAs (Yan et al, 2016) and promotion of immune cell migration into the mammary gland during lactation (Dill & Walker, 2017).

PRL is a single chain hormone produced in both endocrine and autocrine/paracrine systems thus functioning as either as a circulating hormone or as a cytokine in various physiological events (Freeman et al, 2000). Human PRL is first synthesized as a pro-peptide form. After cleavage of the signal peptide, the mature 23kDa protein, compromised of 199 amino acids with three intra-molecular disulfide bonds between cysteine residues, is released into the blood circulation (endocrine PRL) or to a cellular niche (autocrine PRL) (Sinha, 1995).

Based on genetic, structural, binding and functional properties, PRL belongs to the prolactin/ growth hormone/ placental lactogen family, which evolved from a common ancestral gene by gene duplication (Freeman et al, 2000). All three hormones belong to the large haematopoietic cytokine family/group I of the helix bundle protein family (Cooke & Liebhaber, 1995) and all of them are sharing a common tertiary structure (Forsyth & Wallis, 2002). These hormones are believed to exert their biological effects through binding to their target receptors with a non-intrinsic tyrosine kinase activity. Therefore, these ligands can substitute one for another in receptor binding and subsequently induce signal transduction. Importantly, and as consequence of ligand promiscuity, overlapped biological effects are provoked following ligand engagement, masking the discrete effect of each hormone. PRL target tissues include the mammary gland, ovary, prostate, adipocytes, liver and immune cells (Freeman et al, 2000).

PRL variants have been described in the literature as a result of alternative splicing of primary transcript, proteolytic cleavage and other post-translational

modifications including phosphorylation, N-linked glycosylation (Sinha, 1995), sulphation and deamination (Sun et al, 1996) (Figure1.1). The 23kDa PRL is proteolytically cleaved into 14 kDa, 16 kDa and 22 kD isoforms (Bernard et al, 2015). Compared to the non-phosphorylated form of PRL, phosphorylated PRL shows much lower biological activities and may act as an antagonist to PRL (Coss et al, 1999; Wang & Walker, 1993).

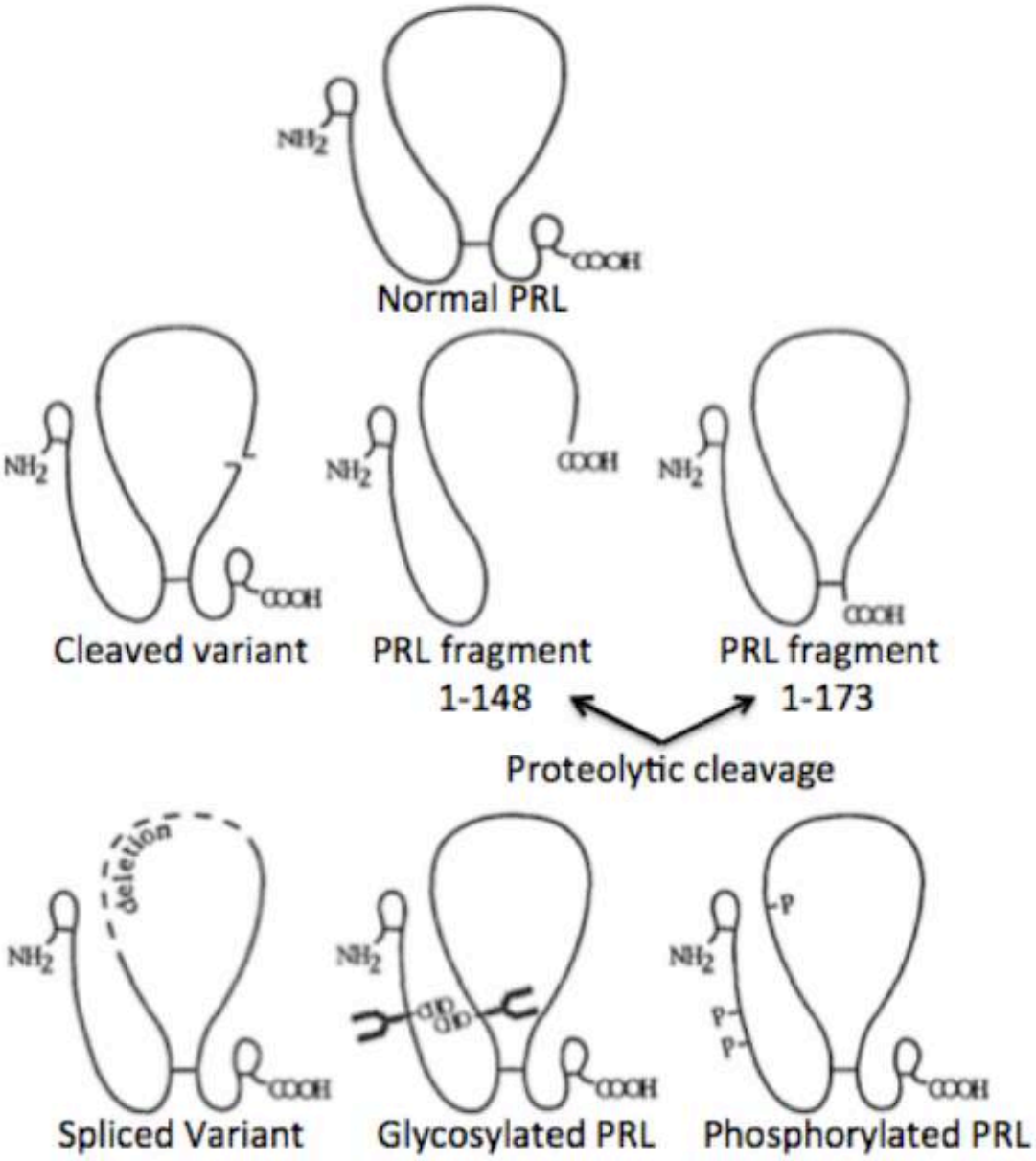

Figure 1.1 Schematic diagram of PRL structure and PRL variants

PRL variants are a result of alternative splicing, proteolytic cleavage and various post-translational modifications including glycosylation, phosphorylation, sulphation and deamination. Image adapted from (Sinha, 1995).

1.1.2 Prolactin Receptor (PRLR)

The PRLR is a transmembrane receptor that belongs to the class I cytokine receptor superfamily. This receptor exhibits three different domains: (1) the extracellular ligand binding domain (ECD) as the signal receiver, (2) the transmembrane domain (TMD) and (3) the signal transducing non-catalytic intracellular domain (ICD) (Leonard & Lin, 2000). The ECD comprises a sequence of 200 amino terminal residues, termed the cytokine receptor homology (CRH) domain. The CRH domain is further divided into D1 and D2 subunits; each consists of 100 amino acids. The D1 domain is characterized by the presence of disulfide-linked cysteines in its N-terminus, crucial for ligand binding. In contrast, D2 has a pentapeptide termed the "WS motif" in its C-terminus that undergoes conformational changes upon PRL engagement which is important for receptor activation (Bole-Feysot et al, 1998; Dagil et al, 2012). The transmembrane domain (TMD) is 24 amino acids long; however, the function of this domain is yet to be determined. The intracellular domain (ICD) is relatively conserved among different species, with a conserved proline-rich Box1 domain indispensable for docking and activation of janus kinase 2 (Jak2), and a Box 2 domain consisting of 11 amino acids that is less conserved among species (Bernard et al, 2015). Furthermore, three lipid-interacting domains (LIDs) are found in the ICD of PRLR, which might confer the specificity of signal transduction (Haxholm et al, 2015). Significant structural homology is shared between the PRLR and other members of the class I cytokine receptor family including the receptors for growth hormone, erythropoietin, leptin, and interleukins (Bernard et al, 2015). The PRLR primary transcript originates from one single gene and then undergoes alternative splicing and translates into multiple isoforms. Three isoforms were characterized in the human

including short, intermediate and long while one long and three short forms were identified in mice (Bole-Feysot et al, 1998; Davis & Linzer, 1989). These isoforms are different in length, size, and cytoplasmic tail but have identical extracellular domains (Brooks et al, 2014; Kelly et al, 1991).

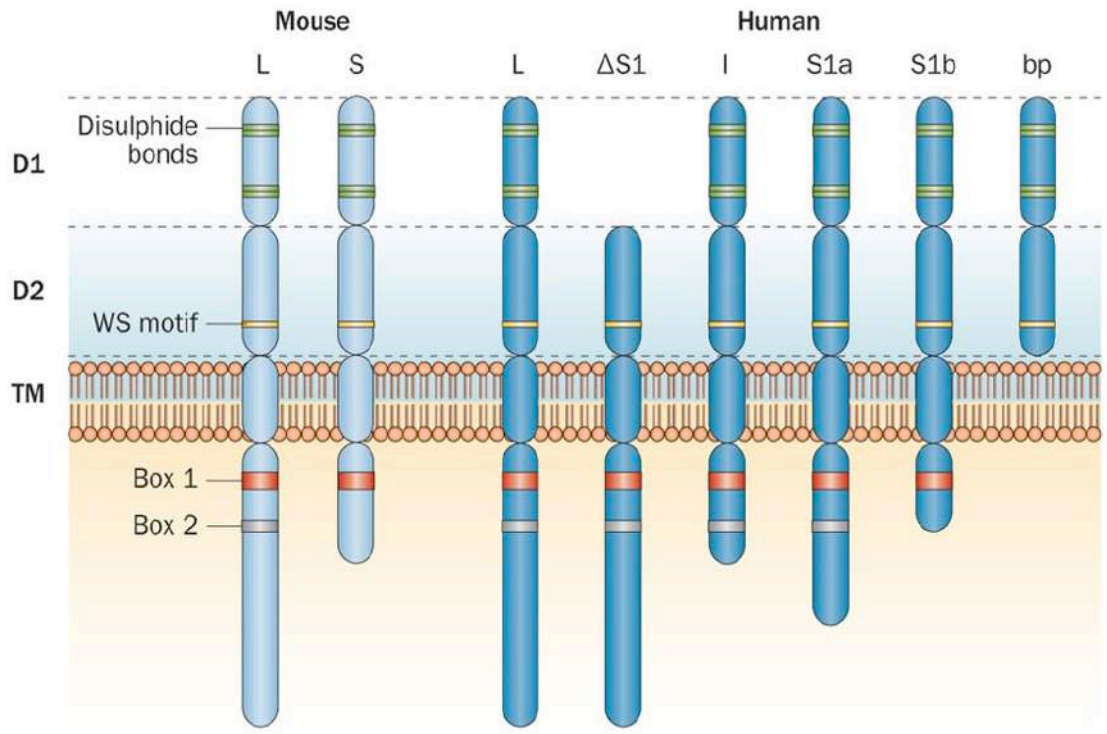

Figure 1.2 Structure of PRLR and its isoforms

The PRLR consists of an extracellular domain comprising two binding domains (D1 and D2), and a transmembrane domain that are conserved across species and a cytoplasmic domain exhibiting variable length and composition. Conserved features such as a disulphide bond and a tryptophan-serine motif, as well as Box 1 and Box 2 have been recognized. Various isoforms of PRLR resulting from alternative splicing of the primary transcript have been identified in mice and humans. Figure adapted from (Bernard et al, 2015).

1.1.3 The Janus Kinases

Four members of the mammalian Janus Kinase (Jak) family have been identified: Jak1, Jak2, Jak3 and tyrosine kinase 2 (Tyk2). Members of the Jak family vary in size from 120 to 140 kDa sharing common homology in seven distinct regions called Janus homology (JH) domains 1-7 (Figure 1.3). JH1 domain has enzymatic activity and conserved tyrosines that are fundamental for Jak stimulation, namely Y1038/Y1039 in Jak1, Y1007/Y1008 in Jak2, Y980/Y981 in Jak3, and Y1054/Y1055 in Tyk2. Phosphorylation of JH1 resulted in structural modifications within the Jak protein and enhancement of the interaction with intracellular molecules. JH2, known as the pseudokinase domain, was found to be lacking enzymatic activity and has been suggested to play a negative role in JH1 activation. Furthermore, JH3 and JH4 domains have been identified to be sharing common homology with Src-homology-2 (SH2) domains while JH4-JH7 domains share NH2 terminal ends, named FERM (short for band 4.1 ezrin, radixin and moesin) domains, implicated in binding of Jaks to cytokine receptors and/or in interacting with other kinases (Kisseleva et al, 2002). Importantly, Jak2 has been shown to be the main kinase constitutively associated with the PRLR at the Box1 consensus region and to be essential for biological activation of the PRL pathway.

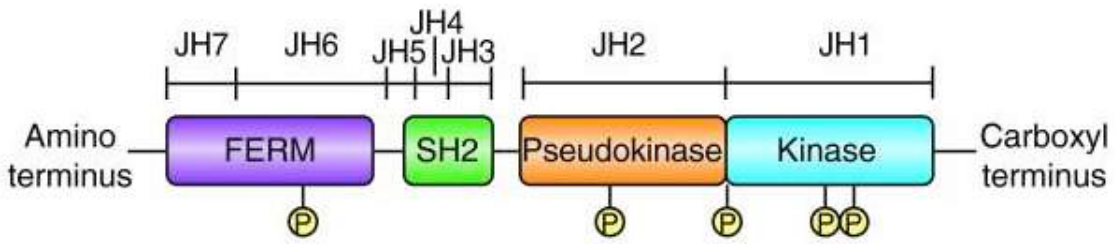

Figure 1.3 Schematic structure of Jak kinase

Jak kinase consists of a tyrosine kinase domain (JH1) localized at the C-terminus followed by a catalytically inactive pseudokinase domain (JH2). A Src homology 2 (SH2) domain localizes next to the N-terminus FERM domain. Figure adapted from (Yamaoka et al, 2004)

1.1.4 Signal Transducer and Activator of Transcription (STAT) Protein Family

STATs are a family of cytoplasmic transcription factors that respond rapidly to cytokine stimulation. Seven members of this family have been identified namely: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6. Structurally, all STATs share common features: (1) a relatively conserved N terminal domain responsible for major protein/protein interactions like homo-dimerization or interacting with other nuclear transcriptional co-activators (Shuai, 2000), (2) a DNA binding domain, (3) the SH2/tyrosine activation domain for recognition of highly specific phosphorylated tyrosine residues of cytokine receptors and subsequent recruitment to this site, and (4) the divergent C terminal transcriptional activation domain (TAD) granting the specificity of different members (O'Shea et al, 2002) (Figure 1.4).

Three Stat members have been shown to interact with the PRLR: Stat1, Stat3 and, mainly, Stat5. In 1994, Stat5 was first discovered in sheep mammary glands as mammary gland factor. Stat5 cloned from mouse (534+/-536 amino acids), rat (537 amino acids) and human (538,539 amino acids) are encoded by two genes (Stat5a and Stat5b) showing an homology of 90-95 %. The key variation between the two isoforms is in the C-terminal domain, which correlates to putative serine/threonine phosphorylation sites. Stat5a and Stat5b structurally share six common functional domains that, namely, the N-terminal domain (NTD), the coil-coiled domain (CCD), the DNA binding domain (DBD), the linker domain (LD), the SH2 domain, and the transcriptional activation domain (TAD) (Figure 1.5). The NTD maintains the interactions between the two Stat molecules. The DBD is critical in recognition of the gamma interferon activation site (GAS) element in target genes. The LD modulates the stability of Stat5a and Stat5b during DNA binding.

The SH2 domain, the most conserved domain, is responsible for Stat5a and Stat5b recruitment and dimerization. The TAD accelerates the transcription process (Bole-Feysot et al, 1998).

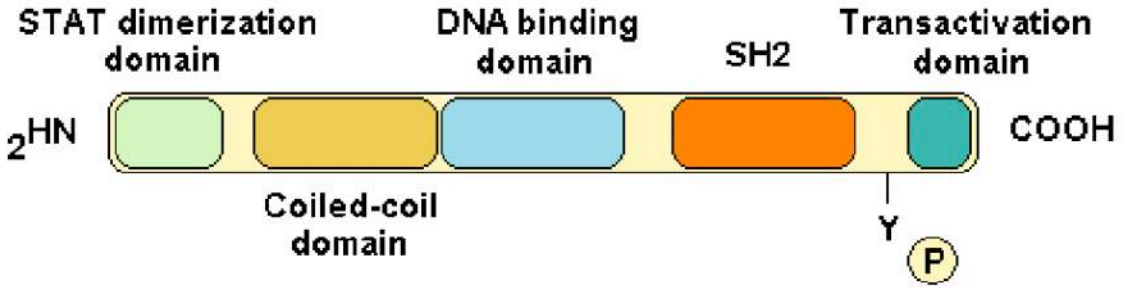

Figure 1.4 Schematic structure of STAT

The N-terminus domain is the dimerization domain upon tyrosine phosphorylation. The coiled-coil domain links a DNA binding domain and Src homology 2 (SH2) domain to the C-terminus transactivation domain, which is subject to multiple post-translational modifications. Figure adapted from (Becerra-Diaz et al, 2011).

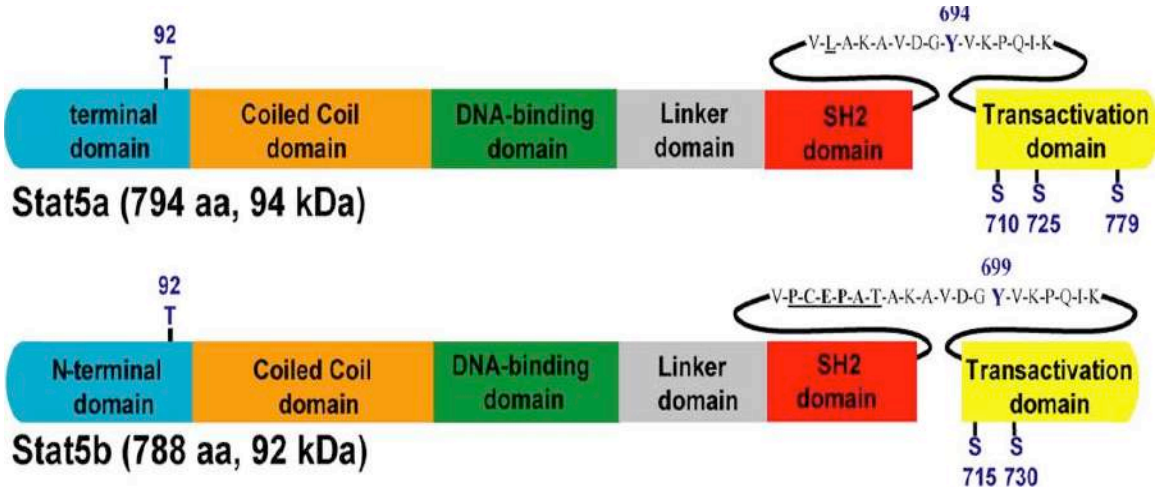

Figure 1.5 Schematic structures of Stat5a and Stat5b with domains and specific amino acids mediating their functions.

Figure adapted from (Koptyra et al, 2011)

1.1.5 Prolactin Signaling Cascade (PRL/ PRLR/ Jak2/ Stat5)

The Jak/Stat pathway is a signaling transduction cascade initiated by protein/protein interactions (ligand/receptor binding) at the cell surface and the message is transmitted directly to the nucleus (Stark & Darnell, 2012). It is well established that the Jak2/Stat5 pathway is the primary pathway activated upon PRL/PRLR engagement (Campbell et al, 1994; Rui et al, 1994). Indeed, binding of a single PRL ligand to two PRLRs triggers the dimerization of receptors, which subsequently leads to transphosphorylation and activation of constitutively associated Jak2 with PRLR. Activated Jak2 phosphorylates tyrosine residues on the intracellular domain of the PRLR, providing docking sites for Stat5 recruitment. Then, Jak2 further phosphorylates the recruited Stat5, leading to dissociation, dimerization and nuclear transportation of these transcription factors (Goffin et al, 2002) (Figure 1.6).

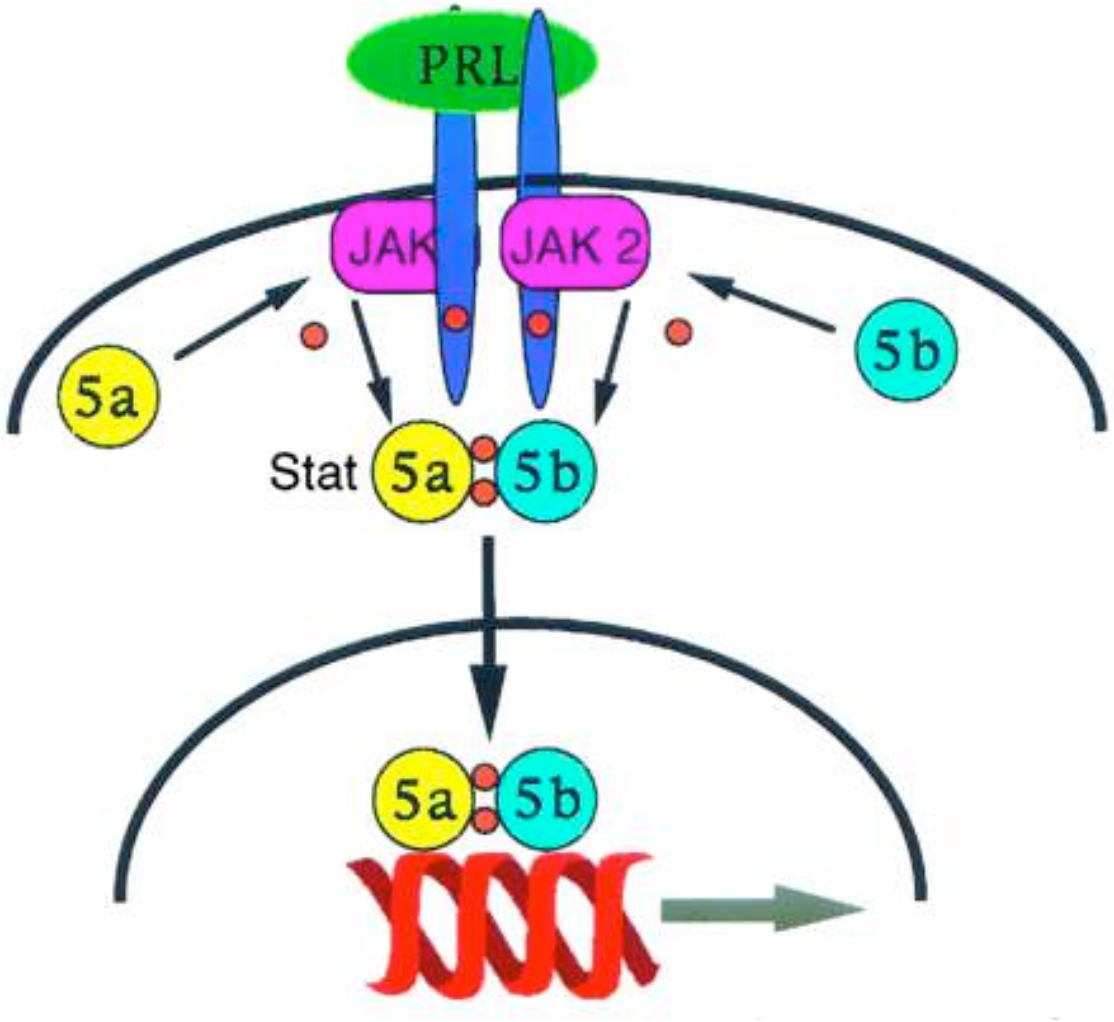

Figure 1.6 PRL/PRLR signaling pathway

Upon formation of ligand/receptor (PRL/PRLR) complex, the receptor will be activated triggering subsequent Jak2 trans-phosphorylation. This induces Jak2 activation and tyrosine phosphorylation of PRLR cytoplasmic domain, which serves as docking sites for recruitment and phosphorylation of Stat5 by Jak2. Phosphorylated Stat5 dissociates from the receptor, dimerizes to another Stat5 molecule and translocates into the nucleus, inducing the expression of various genes like β-Casein and whey acidic protein. Figure Adapted from (Hennighausen et al, 1997)

1.2 The Mammary Gland

The mammary gland distinguishes mammals from all other species with its distinctive anatomical structure that secretes milk for offspring nourishment. These milk secretory organs are epidermal appendages, evolved over 300 million years ago, most likely from apocrine sweat glands (Oftedal, 2002). They are complex organs composed of a number of various cell types: epithelial cells, growing into a fat pad that is infiltrated by immune cells, fibroblasts and vascular endothelial cells. During a female's lifetime, the mammary gland undergoes several structural and functional changes influenced by the estrous/menstrual cycle, as well as the dramatic changes that occur during pregnancy, lactation and involution. As the result of these diverse stages, mammary cells proliferate, differentiate or collapse (apoptose) in response to different cues, giving rise to significant glandular tissue architecture remodeling (Macias & Hinck, 2012).

The mammary epithelium comprises two main cell types: basal and luminal. Indeed, the basal epithelium consists mainly of myoepithelial cells, generating the outer layer of the gland, and several putative stem progenitor cell populations that supply different cell types of mammary. In contrast, the luminal epithelium forms the mammary ducts and the milk secretory alveoli; it encompasses cell populations defined by their hormone receptor status. Together with the myoepithelial structure and function, the luminal epithelium makes a bi-layered tubular structure that facilitates milk production and secretion during lactation (Capuco & Ellis, 2013).

1.2.1 Mammary Gland Development in a Glance

After embryonic development, postnatal development of the mammary gland, including ductal morphogenesis, takes place resulting in elongation and branching of the rudimentary ductal tree to fill the mammary fat pad. The process of alveologenesis then commences during the pregnancy/lactation cycle where cells at the terminal end bud proliferate and differentiate into milk-secreting units. During lactation, the mammary epithelial cells undergo further terminal differentiation and milk secretion process before they are finally eliminated by post lactation apoptosis, restoring the mammary gland pre-pregnant stage. These processes are highly controlled and regulated by multiple hormones and growth factors (Macias & Hinck, 2012) (Figure 1.7).

Studies of mammary gland development have offered deep insights into molecular mechanisms and pathways regulating normal mammary acini morphogenesis, epithelial cell polarity and cell fate specification. Furthermore, many dysregulated pathways observed in breast tumorigenesis mimic those normally observed in normal mammary gland development and breast tissue architecture remodeling. Thus, these developmental mechanisms are of interest to cancer biologists (Inman et al, 2015).

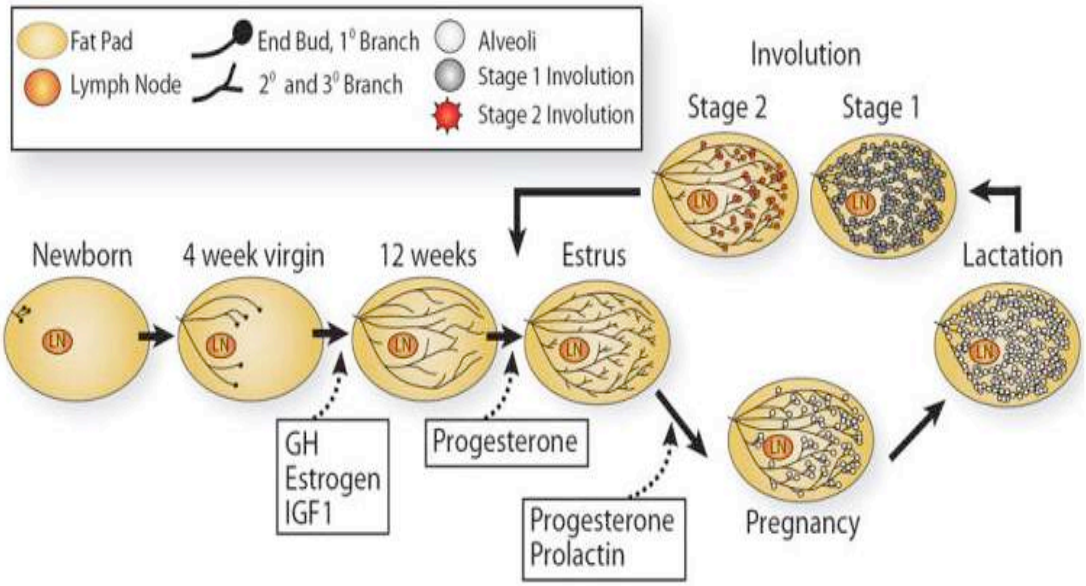

Figure 1.7 Illustration Describing Distinct Stages of Postnatal Mammary Gland Development

At birth, the mammary epithelium consists of rudiment small ducts. With the onset of puberty, ductal morphogenesis takes place influenced by growth hormone (GH), estrogen and growth factors. In the mature virgin, formation of short tertiary branches is observed under the influence of progesterone. However, alveologenesis only occurs in pregnancy with PRL induction, which together with progesterone, accelerates alveolar cell growth. PRL effects continue during the lactation stage until post-lactation involution and the mammary gland is collapsed and remodeled back to its virgin adult stage. Figure adapted from (Macias & Hinck, 2012)

1.2.1.1 Embryonic Development of the Mammary Gland

In mice, embryonic mammary gland development occurs early at embryonic day E10.5 to E18.5 (Hens & Wysolmerski, 2005; Inman et al, 2015). On E10.5, mammary lines are formed from single-layered ectoderm enlargement. These mammary lines further extend from the anterior limb bud to the posterior limb bud. At E11.5, placodes, lens-shaped multilayered ectodermal structures, are observed. The mammary placodes then become epithelial cell bulbs having distinct features from surrounding epidermis. At E12 to E13.5, the mammary buds, elevated knob-like structures, then sink into the underlying dermis at E13.5 (Watson & Khaled, 2008). The mammary mesenchyme is formed from the condensation of mesenchymal cells around the buds. In male embryos, mammary buds are degraded between E13.5 to 15.5 under the influence of androgen receptor activation (Macias & Hinck, 2012). Development of female mammary gland continues at E15.5, with the proliferation of the epithelial cells and bud elongation resulting in newly formed sprouts invading the fat pad precursor. Epidermal cells overlying the bud then form the nipple while the lumen is formed in the sprout at E16.5. The sprout then starts branching giving rise to a rudimentary ductal tree by 18.5 (Inman et al, 2015). In humans, a similar pattern is observed for mammary bud development, except (1) there is no mammary bud degradation in the male embryo, and (2) whereas there is only one single duct opening at the nipple in the mouse at birth, a ductal network merges at the nipple in humans (Howard & Gusterson, 2000).

1.2.1.2 Pubertal Development of the Mammary Gland

At birth, the mammary gland is composed of a primitive ductal system. However, exposure to maternal hormones makes this rudiment gland competent to produce milk in the infant. As these endocrine effects subside, the mammary gland grows allometrically keeping up with the overall development of the body. Branching morphogenesis is initiated during puberty creating a ductal tree that fills the fat pad through proliferation of the terminal end buds (TEBs) of the growing ducts. The TEB is described as a club-shaped, highly proliferative, hormone-dependent structure penetrating the mammary fat pad that is driven by the proliferation of a single layer of cap cells located at the tip (Figure 1.8). Bifurcation of the TEB generates the primary ductal structure and is highly regulated by the surrounding stroma. Cap cells of the TEB differentiate into myoepithelial cells that form the outer layer of the tubular duct that encircles the inner luminal cells (Paine & Lewis, 2017; Williams & Daniel, 1983). Side branching occurs besides ductal elongation through lateral sprouting from the primary ducts, which creates a tree-like pattern of ducts occupying up to 60% of the available fatty stroma (Macias & Hinck, 2012). While short tertiary branches are formed under the influence of cyclical ovarian stimulation, full blossoming of the alveolar buds into milk secretory units occurs only under the influence of pregnancy hormones. In humans, the pubertal mammary gland comprises a similarly extensive mammary tree. However, lateral branches lead to terminal ducts, which give rise to terminal ductal–lobular units comprising numerous blind-ended ducts, called acini (Howard & Gusterson, 2000). These acini are embedded in fibroblastic, intralobular stroma that is far more prominent in the human mammary

than the adipocyte-rich stroma surrounding the branches of the mouse mammary tree (Macias & Hinck, 2012; Russo et al, 1990).

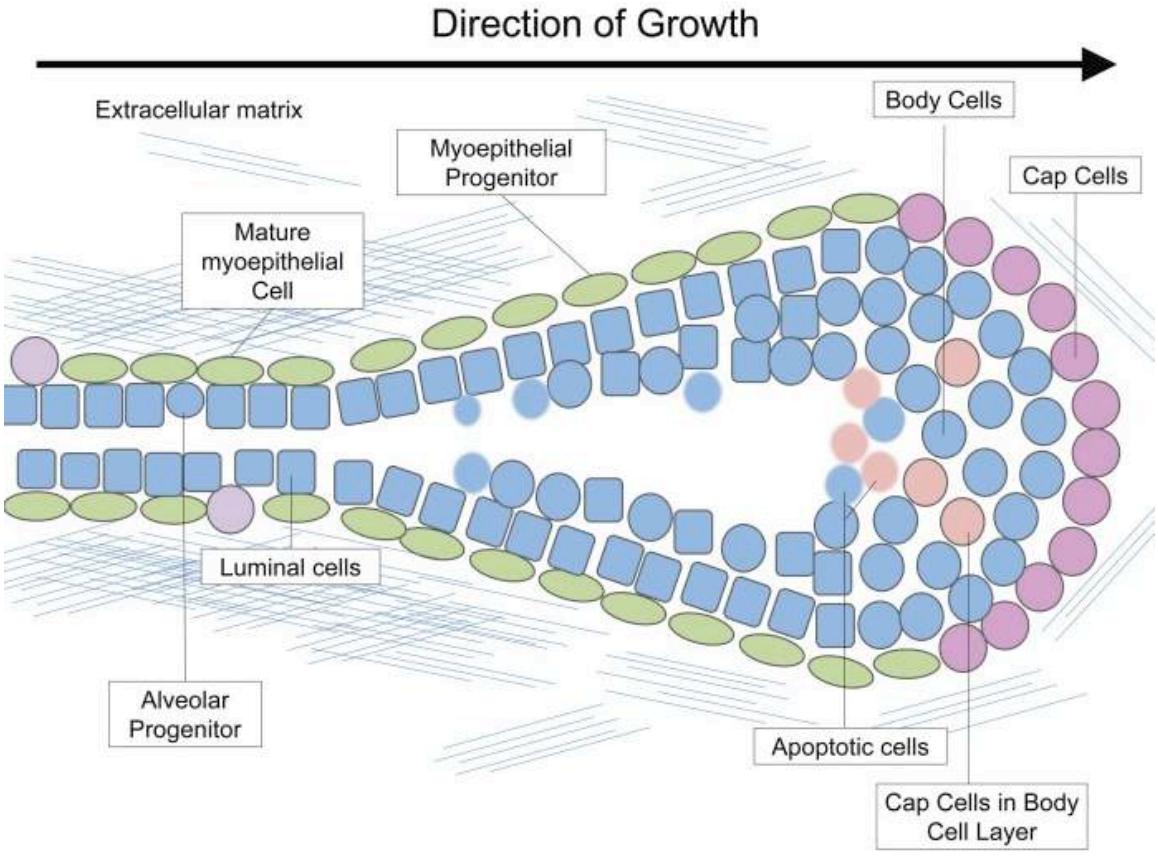

Figure 1.8 Structure of Terminal End Bud

The terminal end bud (TEB) is composed of two main compartments known as the cap and body cell layers. The cells in the front of the TEB (pink) are the least differentiated (cap cells and body cells), whereas the cells behind become more differentiated (myoepithelial progenitors-green, luminal cells- blue and mature myoepithelial cells-green). The extra-cellular matrix is light around the leading tip of the TEB while it becomes a complex meshwork in the neck of the TEB. Apoptosis in the body cell layer contributes to formation of the lumen. Figure adapted from (Paine & Lewis, 2017).

1.2.1.3 Mammary Gland Development During Pregnancy and Lactation

In adult, since the mammary epithelial cells undergo mild proliferation and differentiation under the influence of ovarian hormones during each estrous cycle, enabling limited production of milk proteins followed by structural involution (Andres & Strange, 1999). Extensive tissue remodeling occurs upon pregnancy. Indeed, the ductal tree undergoes tremendous secondary and tertiary ductal branching, providing ductal arbors for alveolar morphogenesis under the combined actions of progesterone and PRL. Luminal epithelial cells extensively proliferate, generating alveolar buds that further cleave and differentiate into milk secretory alveoli, which produce milk during lactation. The interstitial adipose tissue then disappears, giving room to proliferating epithelial cells until the alveoli encompass the majority of the mammary fat pad (McNally & Stein, 2017). During lactation, the apically oriented luminal epithelial cells synthesize and secrete milk proteins into the lumen of the mammary secretory units. Infant suckling stimulates the release of oxytocin that induces contraction of the myoepithelial cells around the alveolus, promoting the outflow of milk into the ducts (Inman et al, 2015).

1.2.1.4 The Involuting Mammary Gland

When the milk production stimuli are lost during the weaning process, the expanded epithelial compartment is collapsed by the function of apoptosis in an event referred to as involution. A number of proteases, of which metalloproteinase 3 (MMP-3) is the most prominent, remodel the mammary gland, returning it to the resting state of the pre-pregnant gland (Talhouk et al, 1991)

1.2.2 Hormonal Regulation of Mammary Gland Development

Multiple hormones are involved at different stages of mammary gland development. In the following sections, I will discuss briefly the effects of growth factors, estrogen, progesterone and focus on PRL.

1.2.2.1 Growth Factors

Members of the EGF and FGF family of growth factors signal through their respective tyrosine kinase receptors and subsequently influence mammary gland ductal morphogenesis. Indeed, it has been shown that multiple EGF ligands and ErbB receptors are expressed in mammary tissue during ductal morphogenesis, pregnancy and lactation (Schroeder & Lee, 1998). EGFR is strongly expressed in TEBs and adjacent stroma of the pubertal mammary gland (Coleman et al, 1988) and the *waved-2* mutant coupled with a kinase-impaired EGFR exhibit less outgrowth (Sebastian et al, 1998). Reciprocal transplant experimental studies between WT epithelium and EGFR mutant stroma demonstrated that EGFR signaling plays a critical role in the interaction between the stroma and the epithelium as well as it is required for mammary ductal morphogenesis (Wiesen et al, 1999).

1.2.2.2 Ovarian Hormone Estrogen

Estrogen is well known to be indispensable for the development of the pubertal mammary gland, influencing the surge of tubular morphogenesis via TEB formation and ductal branching. Initially, it was not clear whether estrogen has a direct effect on

mammary gland development, given the broad expression of estrogen receptors in both epithelial and stromal compartments of the mammary gland, or if it has indirect effect through stimulating the release of pituitary hormones like PRL (Lieberman et al, 1978). Early studies showing the direct effect of estrogen on mammary gland development revealed that local delivery of estrogen directly to the mammary tissue using the Elvax 40P implantation showed direct stimulatory effects on mammary ductal outgrowth (Silberstein & Daniel, 1982), which was blocked by local administration of the estrogen receptor antagonist, tamoxifen (Daniel et al, 1987).

In addition to the role of estrogen in stimulating cell growth during ductal morphogenesis, it is also found to function in maintaining the growth and alveolar structure during pregnancy as exhibited over fifty years ago in hormone replacement studies in ovariectomized mice (Nandi, 1958). These observations were confirmed by conditional deletion of *Esr1* in alveoli following ductal elongation, resulting in defective lobuloalveolar structures and lack of milk production (Feng et al, 2007).

1.2.2.3 Ovarian Hormone Progesterone

Progesterone is a soluble ovarian hormone that is responsible for the widespread side-branching and alveologenesis during pregnancy. In combination with PRL, progesterone induces the differentiation and maturation of mammary alveoli for milk production. Progesterone receptor knock out mice have a simple mammary ductal tree and exhibit absence of ductal proliferation and lobuloalveolar differentiation upon pregnancy. The paracrine role of the progesterone receptor in mammary gland development has further been investigated (Brisken et al, 1998; Lydon et al, 1995).

RANKL (receptor activator of nuclear factor κ-B ligand) was identified as the key mediator of progesterone induced mammary epithelial proliferation and alveologenesis. Similar to the progesterone receptor knock out, knock out of RANKL in mice exhibited no alveologenesis during pregnancy (Fata et al, 2000). Induction of RANKL expression by progesterone is essential in mediating the pro-growth response of the mammary epithelial cells (Fernandez-Valdivia et al, 2008). Furthermore, transgenic expression of RANKL in virgin mammary gland provokes side-branching of the ductal tree and alveolar budding similar to that in pregnancy (Fernandez-Valdivia et al, 2009).

1.2.2.4 Role of PRL in Mammary Gland Development

Several studies have revealed that PRL is the key driver of adult mammary gland development during pregnancy and lactation (Kelly et al, 2002). PRL and PRL receptor (PRLR) knockout mice have demonstrated that PRL indirectly regulates ductal side branching during puberty and directly controls lobuloalveolar development during pregnancy and lactating states (Naylor et al, 2003). In collaboration with progesterone, PRL is indispensable in the development of lactation-competent mammary glands through directly act on mammary epithelial cells and indirectly through modulating the systemic hormone environment, like controlling ovarian progesterone levels (Binart et al, 2000; Brisken et al, 1999). In PRL and PRLR knock out mice, normal embryonic and postnatal mammary gland development was observed, whereas ductal side-branching and alveolar budding were absent (Horseman et al, 1997; Ormandy et al, 1997). Nevertheless, grafting *PRLR-/-* mammary epithelium into pre-cleared wild type fat pads allowed *PRLR-/-* mammary epithelium to develop into a normal mammary gland,

suggesting that PRL/PRLR signaling is required in tissues other than the mammary epithelium for normal development, and endocrine PRL works in a paracrine manner to direct the development of PRLR null mammary epithelium (Brisken et al, 1999; Naylor et al, 2003). Exogenous progesterone supplementation in ovariectomized *PRL-/-* mice restored ductal side branching and partially rescued the infertility of these mice, suggesting that progesterone and PRL work in concert during lobuloalveolar outgrowth (Kelly et al, 2002).

Similarly, conditional knock out of PRL down-stream signaling components Jak2 and STAT5 in mice showed failure of alveolar development (Cui et al, 2004; Han et al, 1997; Wagner et al, 2004). It has been shown that STAT5 regulates the expression of β-casein and whey acidic protein (WAP). In the Jak2 conditional knock out mouse model, disruption of the nuclear accumulation of STAT5 and elimination of the expression of milk proteins were observed (Long et al, 2003).

1.3 Role of PRL in Breast Cancer

1.3.1 PRL Promotes Breast Cancer Progression, The Prevalent Theory

Previous epidemiological reports have indicated serum PRL as a risk marker for breast cancer development due to a positive association between high PRL serum level and increased breast cancer risk (Tworoger et al, 2004; Tworoger et al, 2007; Tworoger et al, 2013; Wang et al, 2016b). However, treatment options based on pharmacological inhibition of circulating PRL, including dopamine agonists such as bromocriptine, have not succeeded (Goffin, 2017).

Locally produced PRL in breast cancers has been proposed to mimic a growth factor function in cancer cells via an autocrine/paracrine loop. Moreover, it has been

claimed that this autocrine/paracrine loop increases in breast cancer and contributes to tumor progression (Clevenger et al, 1995; Ginsburg & Vonderhaar, 1995; Sethi et al, 2012). These findings are further supported by a number of *in vitro* studies, showing that the PRL pathway can activate mechanisms implicated in tumor progression (O'Leary et al, 2015). This comprises the activation of breast cancer cell proliferation, survival pathways and motility (Clevenger et al, 1995; Ginsburg & Vonderhaar, 1995). Other groups have highlighted the ability of the PRL pathway to activate some of the oncogenic signals, including the Ras-Raf-MAPK as well as PI3K pathways (Acosta et al, 2003; Clevenger et al, 1994; Das & Vonderhaar, 1996). A recent study has shown that PRL induces claudin-low mammary carcinomas when p53 is lost (O'Leary et al, 2014). In addition, overexpression of autocrine PRL has been shown to lead to a higher incidence of breast cancer in transgenic mouse models (Arendt et al, 2011; Rose-Hellekant et al, 2003). However, the tumor initiation effect of PRL signaling is not clear as animal models over-expressing PRL show tumor occurrence only at a late stage of the animal life span (>1year), when several cell types are prone to carcinogenesis due to aging. Moreover, it is not clear neither at which stage of cancer progression or which subpopulation of tumor initiating cells that PRL exerts its growth promoting effects on in these described studies.

As a downstream mediator of the PRL pathway, overexpression of wild-type STAT5 or constitutively active STAT5 in transgenic mice induced formation of highly differentiated micropapillary and papillary adenocarcinoma developed after a latency of 8 to 12 months (Iavnilovitch et al, 2004). A more recent study has revealed that PRL/Jak2 signaling inhibits a tumor suppressive function of BRCA1, implicating PRL/Jak2

signaling in promoting tumor progression (Chen & Walker, 2016).

1.3.2 PRL Exerts an Anti-tumorigenic Effect in Breast Cancer: A Compelling Alternative Voice

Another point of view regarding the role of PRL in breast tumorigenesis has emerged based on the previously mentioned role of PRL in mammary gland development and terminal differentiation. This normal physiologic role suggests that PRL signaling could exert an anti-tumorigenic role in breast cancer. Dekkers, Ehrenstein et al. has reported no increased risk of breast cancer among patients with hyperprolactinemia using a population-based cohort study (Dekkers et al, 2015). Another study, using qPCR analysis of autocrine PRL expression in a number of breast cancer cell lines, has demonstrated that autocrine PRL is non-detectable in the majority of cell lines tested. Furthermore, it has been found that PRL-producing cells themselves exhibited a lower proliferation rate compared to control cells. They found also that PRLR protein expression is downregulated in these cells and that they were no longer responsive to exogenous recombinant PRL. This suggests that autocrine PRL is unlikely to be a general mechanism promoting breast tumor progression (Nitze et al, 2013).

On the contrary, growing evidence has pointed to a protective effect of PRL exposure against breast cancer. An epidemiological study has shown that, despite elevated plasma PRL, breast cancer patients could benefit from pregnancy and lactation for prolonged survival (Bercovich & Goodman, 2009). Using animal models, another study has revealed that a previous pregnancy alone, and thus a PRL-driven lobuloalveolar development in the mammary gland, or in combination with lactation confers protective effects against carcinogen-induced mammary carcinomas in rodents (Yang et al, 1999).

Recently, using *in vitro* studies, activation of STAT5 in human breast cancer cells following PRL stimulation has been found to restore E-cadherin expression in these cells and to attenuate cell invasiveness (Sultan et al, 2005), accounting for the favorable prognosis associated with STAT5 activation in human breast cancer (Nevalainen et al, 2004). PRL stimulation has been shown to inhibit the expression of TGF-β and matrix metalloproteinases (MMPs) in breast cancer, subsequently regulating tumor progression (Philips & McFadden, 2004). A more recent study has shown that restoring the Jak2/STAT5 signaling cascade in mammary cancer cells reverses epithelial-mesenchymal transition (EMT) and promotes epithelial differentiation (Sultan et al, 2008). In line with the pro-differentiating role of PRL signaling components, suppressing Jak2 in mouse mammary epithelial cells is associate with de-differentiation, a hyperproliferative phenotype and constitutive activation of oncoprotein Stat3 (Xie et al, 2002). Other confirmatory evidence has come from characterization of down-stream gene products following PRL/Jak2 signaling activation. It has been shown that global profiling of PRL-modulated transcripts in xeno-transplanted T47D breast cancer cells following 48 hours of human PRL stimulation revealed PRL up-regulated genes were enriched in pathways involved in differentiation and the gene signature based on these genes was associated with prolonged relapse-free and metastasis-free survival in breast cancer patients (Sato et al, 2013). Importantly, suppression of Jak2 in T47D cells sufficiently activated ERK1/2 and SMAD2, enhancing their mesenchymal and invasive properties (Nouhi et al, 2006). Later, a negative cross talk of PRL signaling with Epidermal Growth Factor (EGF) was described, in which PRL induces tyrosine phosphorylation of growth factor receptor-bound protein 2 (Grb2) attenuating EGF-induced activation of MAPK (Haines et al,

2009). Another study, using tissue microarray analysis of PRLR protein expression in human breast cancer patients' samples, has identified PRLR as an independent predictor of favorable prognosis in human breast cancer (Hachim et al, 2016a). Recently, reconstitution of PRL/JAK2 signaling in the highly mesenchymal MDA-MB-231 breast cancer cell line was sufficient to reduce their mesenchymal phenotype and invasive properties (Lopez-Ozuna et al, 2016). Taken together, these data suggest that the role of PRL pathway in breast tumorigenesis needs to be re evaluated.

1.4 Breast Cancer Overview

According to the World Health Organization, breast cancer is the most common cancer worldwide in females and the leading cause of their deaths (World Health Organization 2015). Despite critical advances in molecular understanding of the process of carcinogenesis and improved therapies, breast cancer still represents a key health burden and it is still challenging to cure. Breast cancer is a complex heterogeneous disease that comprises several entities with different clinical, pathological and molecular profiles. Classification of breast cancer can be based on histopathological analysis or gene expression profiles (Simpson et al, 2005).

1.4.1 Histological Classification of Breast Tumors

The tumors are classified as ductal carcinoma *in situ* (DCIS) or invasive breast carcinoma (IBC). CIS is pre-malignant lesion that remains in the normal location within the mammary gland (ducts and lobes). In contrast, IBC, also known as infiltrating breast carcinoma, is the malignant lesion that infiltrates outside of the ducts or lobes and has invaded the connective and fatty tissues with the potential to metastasize (Cowell et al,

2013). IBC is the most common type of breast cancer, representing up to 80% of breast cancer diagnoses. Moreover, CIS is a non-obligate precursor of IBC, and up to 40% of these lesions can progress to invasive tumours if untreated (Figure 1.9) (Makki, 2015).

Determining the classical immunohistochemistry (IHC) markers is recommended in diagnosing invasive carcinomas. These markers, comprising estrogen receptor alpha (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) and the proliferation index (Ki67), help in guiding the clinical decision (Harris et al, 2007). Together with the traditional clinico-pathological variables including tumor size, tumor grade and lymph-node involvement, physicians can predict a patient's response to targeted therapies (Payne et al, 2008).

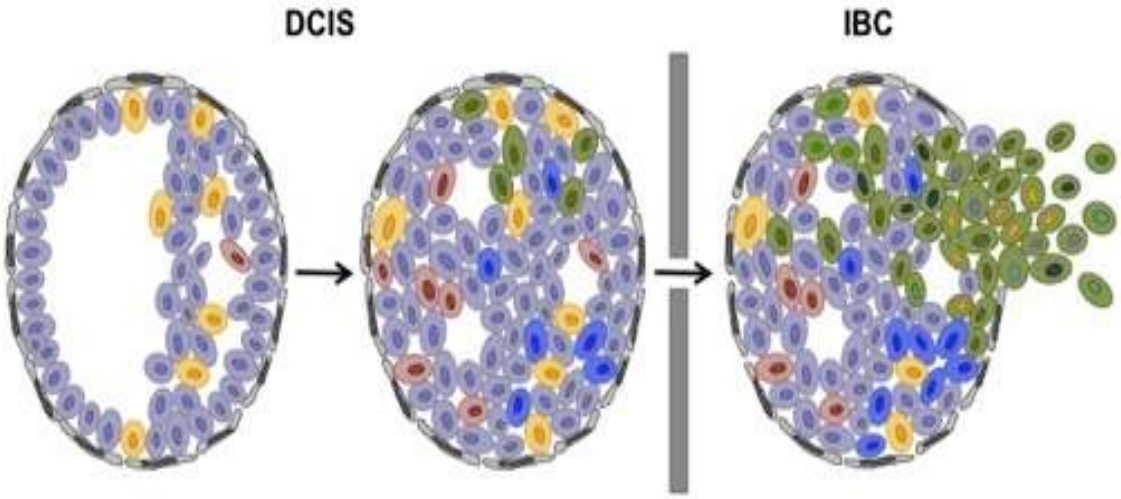

Figure 1.9 Hypothetical Schematic model of progression from *in situ* to invasive breast cancer

As DCIS develops, cancer cells accumulate somatic mutations and copy number aberrations to generate a heterogeneous lesion with distinct cell types harbouring private mutations. Only neoplastic cells that harbour a specific repertoire of genetic aberrations pass through the evolutionary bottleneck of progression to IBC. Figure adapted from (Cowell et al, 2013).

1.4.2 Molecular Classification of Breast Cancer

Breast cancer studies using differential gene expression profiling systems have identified several breast cancer subtypes beyond the traditional hormone receptor-based sub-classification. Several intrinsic molecular breast cancer subtypes have been identified using high-throughput microarray analysis (Perou et al, 2000; Sorlie et al, 2001). Together with the protein expression status of ER, PR and HER2, these gene clusters determine the breast cancer molecular intrinsic subtypes. These subtypes comprise Luminal A (ER+/PR+, HER2+), Luminal B (ER+/PR+, HER2+), HER2+ (ER-, PR-, HER2 positive) and Basal-like/triple negative (ER-,PR-, HER2-) (Figure 1.10) (Dai et al, 2015). In addition to the diversity in gene expression pattern, these breast cancer molecular subtypes differ with regard to their clinical features, patient outcome, prognosis and response to treatment (Figure 1.11).

Luminal A breast cancer subtype is defined by positive expression of ER and PR with low levels of proliferation-related genes (ki67). It represents low histological grades coupled with a good patient outcome. The luminal B subtype is determined by positivity of ER and PR expression associated with HER2 negative expression and high ki67 or ER and HER2 positive expression. In comparison to luminal A, the luminal B subgroup has a worse prognosis and a higher histological grade. The HER2+ subtype is characterized by overexpression of HER2 that is associated with high proliferation and aggressive behavior (Parker et al, 2009; Sorlie et al, 2001; Sorlie et al, 2003).

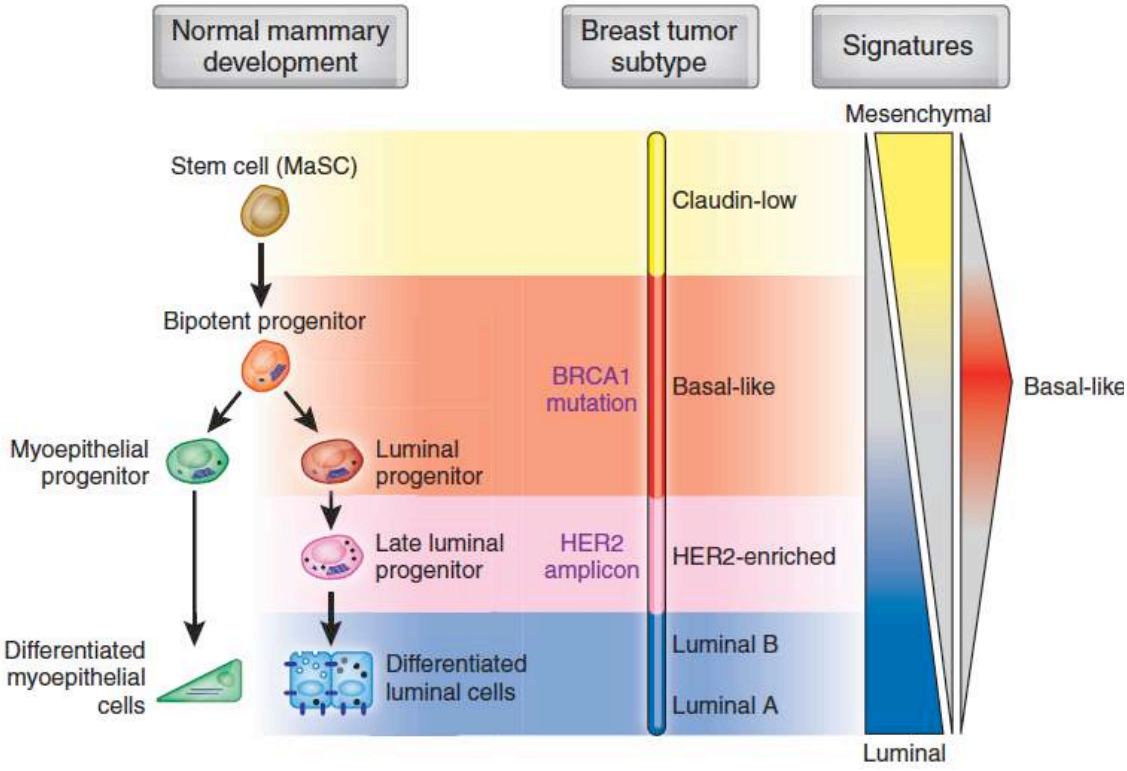

Figure 1.10 Model of human mammary epithelial hierarchy linked to various breast cancer molecular subtypes

(a) Normal subpopulations of mammary tissue and potential cells that different breast cancer intrinsic subtypes originate from. (b) The various distinct breast cancer subtypes molecularly compared to subpopulations from normal mammary tissue. (c) The defining expression patterns of luminal, claudin-low (mesenchymal), and basal-like cells. Figure adapted from (Prat & Perou, 2009).

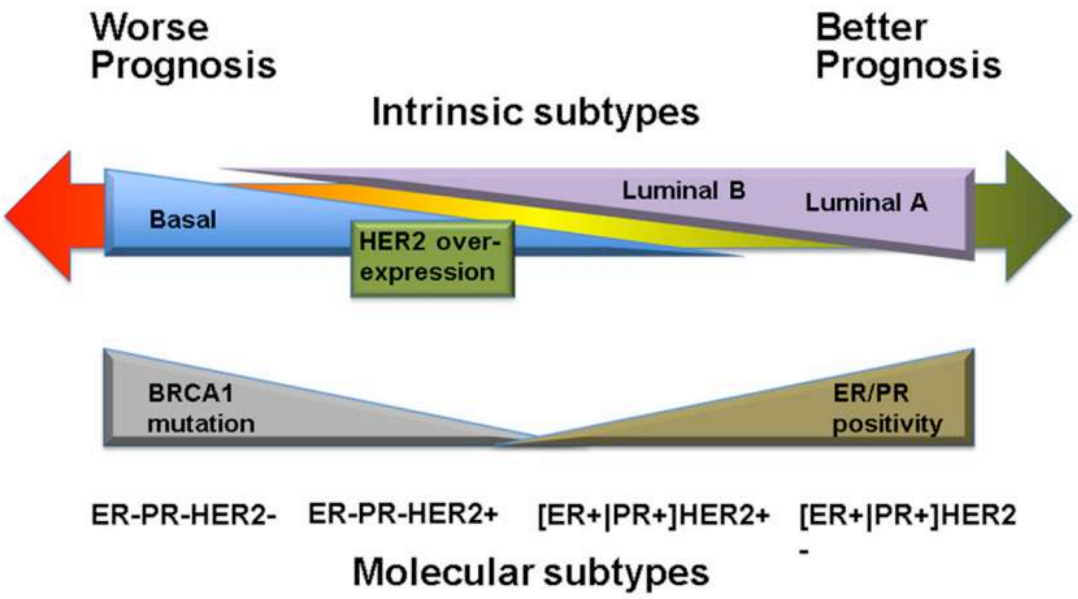

Figure 1.11 Patient outcome based on distinct breast cancer molecular subtypes. Figure adapted from (Dai et al, 2015).

The basal-like subtype is characterized by lacking the expression of ER, PR and HER2 (triple negative phenotype) coupled with gaining the expression of basal cytokeratins CK5/6 and epidermal growth factor receptor (EGFR) (Rakha et al, 2008). It is associated with high histological grade and poor patient outcome. Interestingly, breast cancer studies have linked the morphological features and molecular genetic profiles observed in basal-like subtype with BRCA1 mutation tumors (Turner & Reis-Filho, 2006).

The Claudin-Low subtype has recently been identified to be associated with genes implicated in tight junctions and intracellular adhesion formation including Claudin 3, 4 and 7, cingulin, ocludin and E-cadherin. Moreover, this subgroup is also enriched in cell proliferation and EMT genes and is highly associated with a cancer stem cell-like phenotype. This subgroup is closely related to basal-like tumors and is considered to have the least favorable prognosis (Prat et al, 2010; Turner & Reis-Filho, 2006).

1.4.3 The EMT Program

Epithelial and mesenchymal cells have been well characterised by their distinctive cellular morphology and organization within the tissue. Epithelial cells form polarized sheets connected via various cellular junctions, including adherens junctions, tight junctions and desmosomes. Moreover, epithelial cells anchor themselves to the underlying basement membrane through another cellular junction called a hemidesmosome that helps in maintaining the axis of apical-basal polarity within the epithelial sheet. Epithelial-specific cytokeratin intermediate filaments are further formed, connecting both desmosomes and hemidesmosomes. In contrast, mesenchymal cells are characterized by directly embedding themselves inside the extracellular matrix (ECM)

without establishing tight contact with neighbouring cells. During the specific stages of embryonic morphogenesis processes such as mesoderm formation and the development of the neural crest, epithelial cells exhibit massive plasticity and transit into a mesenchymal state by activating the EMT program. After acquiring EMT, these cells lose their epithelial junctions and switch to produce vimentin filaments. The main functional hallmark of the EMT is to allow stationary epithelial cells to gain the ability to migrate and invade during developmental morphogenesis (Boyer & Thiery, 1993; Hay, 1995; Tsai & Yang, 2013).

Increasing number of studies over the past decade have provided strong evidence for the reinitiation of the developmental EMT program in tumor progression and metastasis. Indeed, cancer cells exhibit many morphological and molecular features similar to those happening in developmental EMT program. Importantly, due to the heterogeneity and constant change of the tumor microenvironment, the EMT program in cancer adapts to these conditions allowing tumor cells to successfully metastasize (Tsai & Yang, 2013).

1.4.4 The Molecular Program of EMT

Introducing the EMT program to cancer cells involves the transcriptional alteration of many genes regulating several cellular programs, including cell adhesion, cell differentiation, migration, and invasion. Generally, three core groups of transcriptional regulators have been consistently demonstrated to be critical during different EMT events, thus being identified as the core EMT regulators.

The first group is the transcription factors of the Snail zinc finger family, comprising Snail1 and Snail2, both of which are capable of directly binding to the E-box

of the E cadherin promoter and repressing its transcriptional activity (Cano et al, 2000; Hajra et al, 2002). The second group is the zinc finger E-box-binding homeobox family proteins Zeb1 and Zeb2 that also have the ability to suppress E-cadherin transcription through a double-negative feedback loop controlling Zeb1/Zeb2 and the miRNA-200 family expression (Korpal et al, 2008; Park et al, 2008). Both the Snail and Zeb transcription factor families have also been shown to repress the expression of claudins and ZO-1 cellular junction proteins (Ohkubo & Ozawa, 2004; Vandewalle et al, 2005). The third group is the basic helix–loop–helix (bHLH) family of transcription factors, including Twist1, Twist2, and E12/E47. All these family members can induce EMT alone or cooperatively (Casas et al, 2011; Eckert et al, 2011; Tsai & Yang, 2013; Yang et al, 2004).

Cancer cells are believed to undergo the EMT process in response to a combination of extracellular cues in the tumor microenvironment. Major developmental signaling pathways, including TGF-β, Wnt, Notch, and growth factor receptor signaling cascades, have been shown to be implicated in some aspects of the EMT program. Most notably, the TGF-β pathway has been thought to be a primary inducer of EMT (Katsuno et al, 2013).

In summary, the EMT program comprises a large number of cellular and molecular modifications. As EMT-inducing signals are miscellaneous and context-dependent, EMT core transcription regulators are most widely used as molecular markers of EMT in cancer. Further analysis of how EMT-inducing signals impact the EMT core regulators will provide a more comprehensive knowledge of key players in the EMT (Figure

1.12).

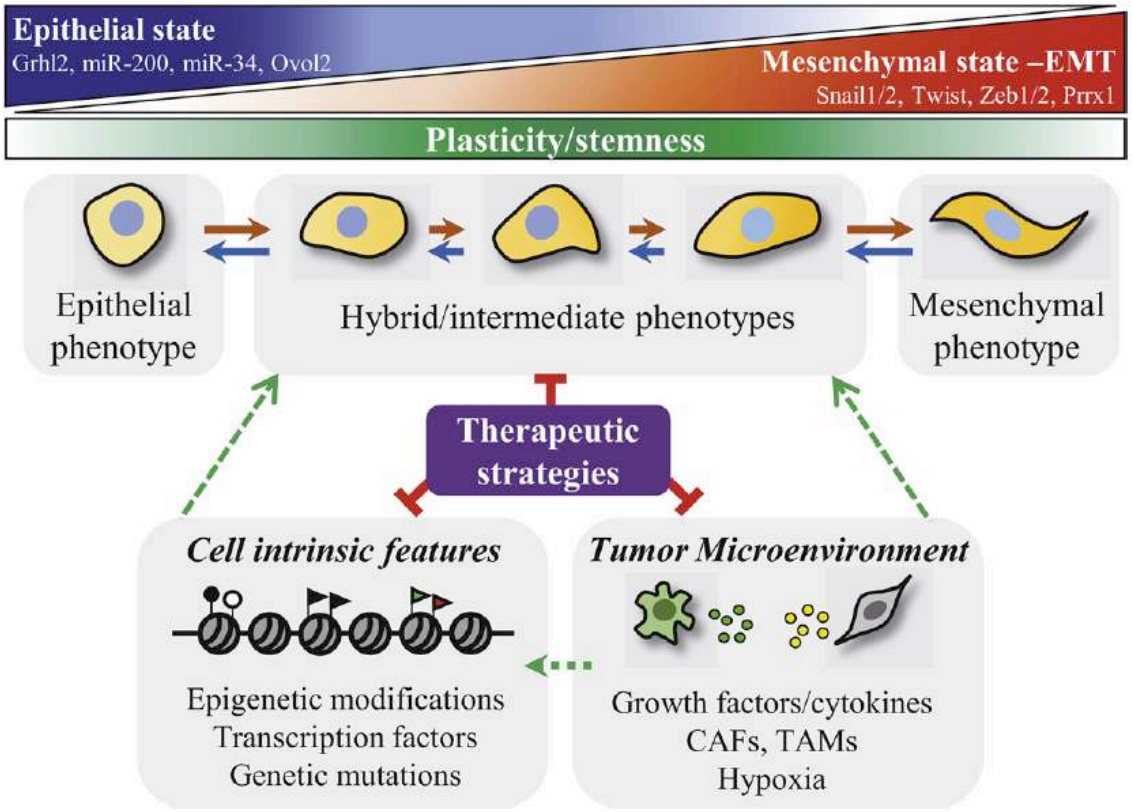

Figure 1.12 Mechanisms that regulate intratumor heterogeneity and cancer plasticity/EMT.

(A) Intratumor heterogeneity relies on genetic and epigenetic alterations as well as cancer cell plasticity. (B) This dynamic behavior is associated with the EMT induction that results into the transition from epithelial to mesenchymal-like phenotypes through plastic hybrid/ intermediate states. Targeted treatments designed to block EMT/stemness or to target mesenchymal or hybrid cancer cells, by acting on cancer cells or the tumor microenvironment of cancer cells, are promising therapeutic interventions. Figure adapted from (da Silva-Diz et al, 2018).

1.5 Kinesin Assembly and Movement in Cells

Microtubules are identified as cylindrical cytoskeletal filaments whose polymerization from α- and β-tubulin heterodimer subunits results in a structural and functional polarity of the two ends. A variety of important functions in the cells are carried out by microtubules and their associated proteins. It is well known that movement along the microtubule is driven by molecular motors proteins in the kinesin and dynein families (Figure 1.13). Indeed, molecular motors actively transport membranous organelles and macromolecules, including complex proteins as well as RNA, along the microtubule network to their appropriate destination (Hirokawa et al, 2009). Directional intracellular transport is most prominent in polarized cells, such as epithelial cells and abnormalities in motor-driven transport have been reported to be associated with several human diseases, including neurodegenerative diseases, polycystic kidney disease, and cancer (Salinas et al, 2008; Verhey et al, 2011).

Kinesin superfamily proteins (KIFs) have been identified recently as molecular motors that move along microtubules with ATP hydrolysis. Each kinesin consists of a heterodimer complex of two subunits of kinesin heavy chain (KHC), mediating the motor function along the microtubules, and kinesin light chain (KLC) recognizing cargoes and tethering them to KHC (Cyrus & Muller, 2016; Gindhart et al, 1998). Kinesin-driven transport along microtubules is mediated by the concerted effort of both KHC and KLC subunits (Hirokawa et al, 2009; Verhey & Hammond, 2009). KIFs comprise 15 kinesin families termed kinesin 1 to kinesin 14B. Our understanding of KIFs emanates mostly from studies of kinesin-1, kinesin-2 and kinesin-3 superfamilies (Lawrence et al, 2004).

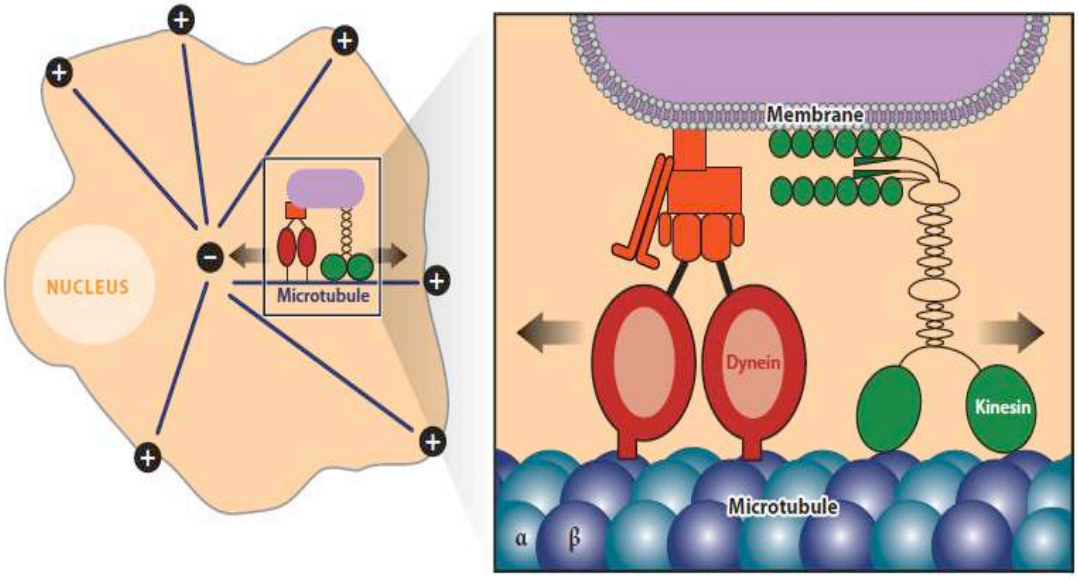

Figure 1.13 Intracellular transport in mammalian cells.

In most cells, microtubules (dark blue lines) are organized with their plus ends extending towards the cell periphery and their minus ends towards the middle of the cell. Kinesin (green) and dynein (red) motors provide the movement of cargoes (purple) such as vesicles, organelles, and protein complexes along microtubule tracks. While kinesin motors carry out long-distance transport to the plus ends of microtubules in the cell periphery, cytoplasmic dynein carries cargoes towards the minus ends of microtubules in the cell center. Figure adapted from (Verhey et al, 2011).

1.5.1 The Kinesin Family Member 5B

Kinesin-1 is the most studied family among kinesin proteins and consists of three types of KHC subunits (KIF5A, KIF5B and KIF5C) encoded by three different genes and four KLC subunits (KLC1-4) (Verhey & Hammond, 2009). Studies have linked motor-driven transport abnormalities to a wide range of diseases including cancer (Hirokawa et al, 2009; Salinas et al, 2008; Verhey et al, 2011).

Kinesin family member 5B (KIF5B) is the most studied KHC subunit in the kinesin-1 family and has been shown to be implicated in myogenesis and kidney development (Cui et al, 2015; Wang et al, 2013a; Wang et al, 2013b). KIF5B was also indicated to have a central role in importing of HIV-1 during early infection (Dharan et al, 2016; Malikov et al, 2015). However, the role of KIF5B itself in cancer, mainly in breast cancer is not yet fully elucidated. A recent study has shown that ectopic expression of ErbB2 in luminal-A MCF7 breast cancer cells results in an up-regulation of KIF5B that is implicated in lysosomal and mitochondrial transport. Moreover, the study revealed the involvement of KIF5B in the initial formation/ localization of autophagosomes and the transportation of components important for the autophagic process (Cardoso et al, 2009). Further study has identified a critical role of the ARF6−JIP−MT1-MMP−KIF5B axis in inducing endosomal membrane tabulation and the invasive phenotype of breast cancer (Marchesin et al, 2015). A more recent study has shown KIF5B to be implicated, with PLD2 and PA, in the vascular association of KIF5B, surface localization of MT1-MMP and invasion (Wang et al, 2017). Although KIF5B is the most studied member of the kinesin-1 family, its role in mammary tumorigenesis, the EMT process and the loss of

differentiation phenotype in aggressive breast cancer is not yet fully elucidated and still needs to be investigated.

1.6 Cell Polarity in Mammary Epithelial Cells

Solid tumor development comprises a complex progression of intrinsic and extrinsic events that prompt cells to proliferate out of control and to gain migratory and invasive capabilities (Hanahan & Weinberg, 2011). Whereas several studies have undeniably revealed that the loss of epithelial cell polarity is intricately associated with malignant progression, accumulating evidence has uncovered the critical roles of polarity regulators in the early stages of tumorigenesis.

Epithelial cell polarity is believed to be fundamental for proper tissue function, and it has been commonly defined as 'asymmetry' within cells and epithelial tissues. This denotes a differential positioning of membrane domains and organelles along the apical/basal axis, known as apical/basal polarity. Moreover, this also provides the positioning of cells within the plane of epithelial tissues, known as planar cell polarity (Bornens, 2008; Martin-Belmonte & Perez-Moreno, 2011; Simons & Mlodzik, 2008).

In the mammary gland, luminal epithelial cells exhibit apical/basal polarity that is established and maintained by asymmetric segregation of evolutionarily conserved dynamic protein complexes to distinct membrane domains. The apical plasma membrane domain faces the central lumen of the duct, into which milk is secreted during lactation. In contrast, the basolateral domain creates contact with adjacent luminal cells as well as myoepithelial cells and the basement membrane (Chatterjee & McCaffrey, 2014). Proper establishment of apical/basal cell polarity combined with planar cell polarity (PCP), the

cellular organization within the plane, is critical for normal mammary epithelium physiology and tissue homeostasis. On the other hand, loss or dysregulation of cell polarity results in misoriented cell divisions and uncontrolled cellular growth, leading to lumen filling, tissue disorganization and consequently invasive neoplastic cells, which are hallmarks of cancer (Figure 1.14).

1.6.1 Junctional Proteins in Mammary Epithelial Cells

One of the distinctive features of polarized epithelium is exhibiting adhesive structures, including adherens junctions, tight junctions, desmosomes, and gap junctions (Niessen et al, 2011). According to their relative stability and complex multi-component nature, cell–cell junctions can be considered membrane micro-domains themselves. Distinct molecular composition and structural morphology distinguish different cell–cell junctions. Generally, all cell–cell junctions consist of transmembrane proteins that bind to their counterparts on neighbouring cells, and subsequently associate with cytoplasmic proteins and the cytoskeleton through which cell–cell junctions transmit signals to the cell interior and *vice versa* (Giepmans & van Ijzendoorn, 2009).

1.6.1.1 Adherens Junctions

Adherens junctions localize at the basolateral domain of the plasma membrane at the basal side of tight junctions. They are mediated through a transmembrane calcium-dependent cell adhesion protein called E-cadherin, which forms extracellular contacts

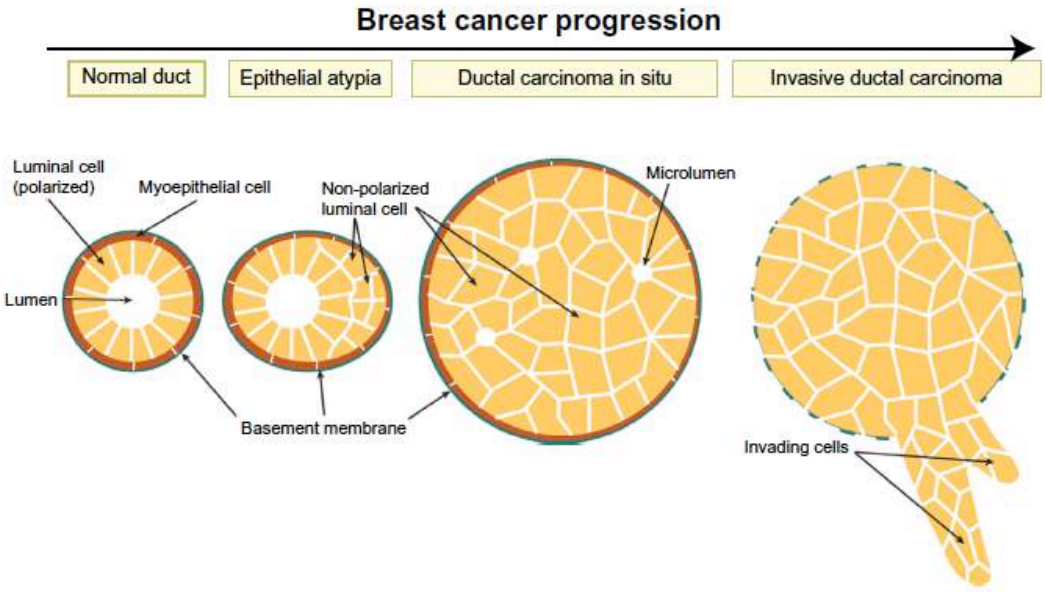

Figure 1.14 Loss of epithelial polarity and integrity—a hallmark of all advanced cancers.

Loss of epithelial cell polarity results in deterioration of cell junctions and basement membranes, which enhances the proliferative potential and migratory capacity of epithelial cells. **Note:** Cross-section of a duct that displays loss of epithelial integrity and lumen filling across the different stages of breast cancer. Figure adapted from (Chatterjee & McCaffrey, 2014)

with cadherin on neighboring cells through their N-terminal extracellular domains (Shapiro & Weis, 2009). The cytoplasmic domain of E-cadherin is connected to the intracellular actin cytoskeleton through α-catenin and β-catenin that regulate cadherin turn over. Notably, E-Cadherin is shown to regulate localization and activity of the Rho family GTPases in order to modulate actin organization and function (Fukata & Kaibuchi, 2001).

1.6.1.2 Tight Junctions

Apical tight junctions connect adjacent cells through several transmembrane proteins including claudins, occludins and junction adhesion molecules (JAMs). The adaptor proteins (zonula occludens) ZO1, ZO2 and ZO3 are highly enriched at the cytoplasmic face of tight junctions. These ZO proteins play a key role in tight junction formation as well as by establishing a connection to the actin cytoskeleton (Fanning et al, 1998; Hernandez et al, 2007). Practically, the ZO1 is usually used as a marker of tight junctions (Bazzoun et al, 2013). Tight junctions are the primary determinant of the epithelial barrier essential function that controls the exchange of nutrients, solutes and waste between biological compartments (Marchiando et al, 2010). Of note, the adherens junctions and tight junctions are sometime referred to as apical junctional complexes (AJC).

1.6.1.3 Desmosomes

Desmosomes are adherent points (localized patches) that hold two cells together and, by connecting their integral membrane proteins (desmocollin and desmoglein) to the

intermediate filaments intracellularly, providing mechanical integrity of the epithelium (Garrod & Chidgey, 2008).

1.6.1.4 Gap Junctions

Gap junctions are distinctive cell-to-cell cylinder channels composed mainly of the integral membrane proteins, connexins, which allow direct diffusion of small metabolites, second messengers, ions and other molecules (< 1kDa) between adjacent cells. This type of communication is indispensable for electrical and signaling transduction as well as nutrition diffusion. The gap junction is a highly dynamic process regulated at multiple levels (Mese et al, 2007).

1.6.1.5 Cell-Extracellular Matrix Adhesion Complexes

Besides the cell-cell adhesion, cell-ECM adhesion at the basal domain of the plasma membrane is also critical for proper function of polarized epithelium. Indeed, the ECM provides signaling cues that are important for the initiation of cell polarization. These cues are transmitted through integrin, the transmembrane ECM receptor-mediated signaling. The integrin-based cell-ECM adhesions are spot like structures that are shown to be dynamically regulated to provide proper communication between cells and the ECM. Integrins are important for proper propagation of variety of processes, including normal development, migration and proliferation (Berrier & Yamada, 2007; Giepmans & van Ijzendoorn, 2009).

1.6.2 The Cytoskeleton Network

Proper organization of the cytoskeleton network, including the actin filaments, the microtubule and the intermediate filaments is essential to maintain proper structure and

function of polarized epithelial cells. Actin filaments within polarized epithelial cells are organized in three forms: (1) as bundles within apical microvilli, (2) as filament bundles in association with the apical junctional proteins, and (3) as dense networks lining the basolateral domain of the plasma membrane (referred to as the cortical cytoskeleton). Microtubules are normally organized in bundles parallel to the lateral domain of the plasma membrane, with their minus-ends uniformly oriented toward the apical domain of the plasma membrane, or as mixed orientated networks lining the apical and basal membranes. Intermediate filaments connect desmosomes across the cell (Nelson, 2003).

1.6.3 Polarity Proteins in Epithelial Cells

Genetic studies in *Drosophila* and *C. elegans* have identified three evolutionarily conserved groups of proteins found to be key regulators for the formation and maintenance of apical/basal polarity. These protein groups assemble into dynamic protein complexes: Par, Crumbs and Scribble complexes (Bilder et al, 2000; Kemphues et al, 1988; Tepass, 2012). The Par complex, located at the apical domain of the plasma membrane, comprises multi-domain scaffolding protein, Par3, the adaptor Par6, atypical protein kinase C (aPKC), and the small GTPase cell division control protein 42 (Cdc42). Par3 is shown to bind directly with phospholipids of the plasma membrane and with the tight junction protein JAM-A19. Par3 further recruits Par6 and aPKC to the plasma membrane enabling Cdc42 to induce a conformation change in Par6 that allows aPKC activation (Ebnet et al, 2001; Suzuki & Ohno, 2006).

The apical protein complex Crumbs consists of the transmembrane protein Crumbs 3 (Crb3), which binds the multi-domain proteins Pals1, Pals1-associated tight

junction protein (Patj), and angiomotin (Amot). The Crumbs complex is required for the formation of the tight junction and to specify the apical membrane (Fogg et al, 2005).

The Scribble complex localizes at the basolateral membrane of epithelial cells and consists of the scaffolding proteins Scribble (Scrib), Discs-large (Dlg), and the adaptor lethal giant larvae (Lgl2; also known as Hugl2). This complex is required to maintain E-cadherin-mediated cell-cell adhesion and defines the basolateral membrane (Qin et al, 2005). In turn, recruitment of junctional scribble is dependent on E-cadherin engagement. Moreover, apical membrane identity is opposed by the Scrib complex and expression of Scrib mutants in *Drosophila* causes the delocalization of apical proteins to all cell surfaces (Bilder & Perrimon, 2000).

The three polarity protein complexes complement each other and act together to establish and maintain apical/basal polarity in epithelial cells. Importantly, these complexes are dynamic and exhibit interactions with each other to regulate epithelial polarity (Figure 1.15). Indeed, Lgl2 interacts with Par6 and aPKC, facilitating the trafficking of Par6/aPKC proteins. Par3 competes with Lgl2 for binding aPKC that phosphorylates and inhibits Lgl2, insuring its proper basolateral localization. Moreover, Par6 shows interactions with Pals1 and Crb3 of the Crumbs complex. Pals1 is necessary for proper localization of the Par complex (Hurd et al, 2003; Wang et al, 2004). Crb3 and Par3 compete with each other for binding Par6, which localizes Par6/aPKC to the apical domain of the cell membrane (Chatterjee & McCaffrey, 2014).

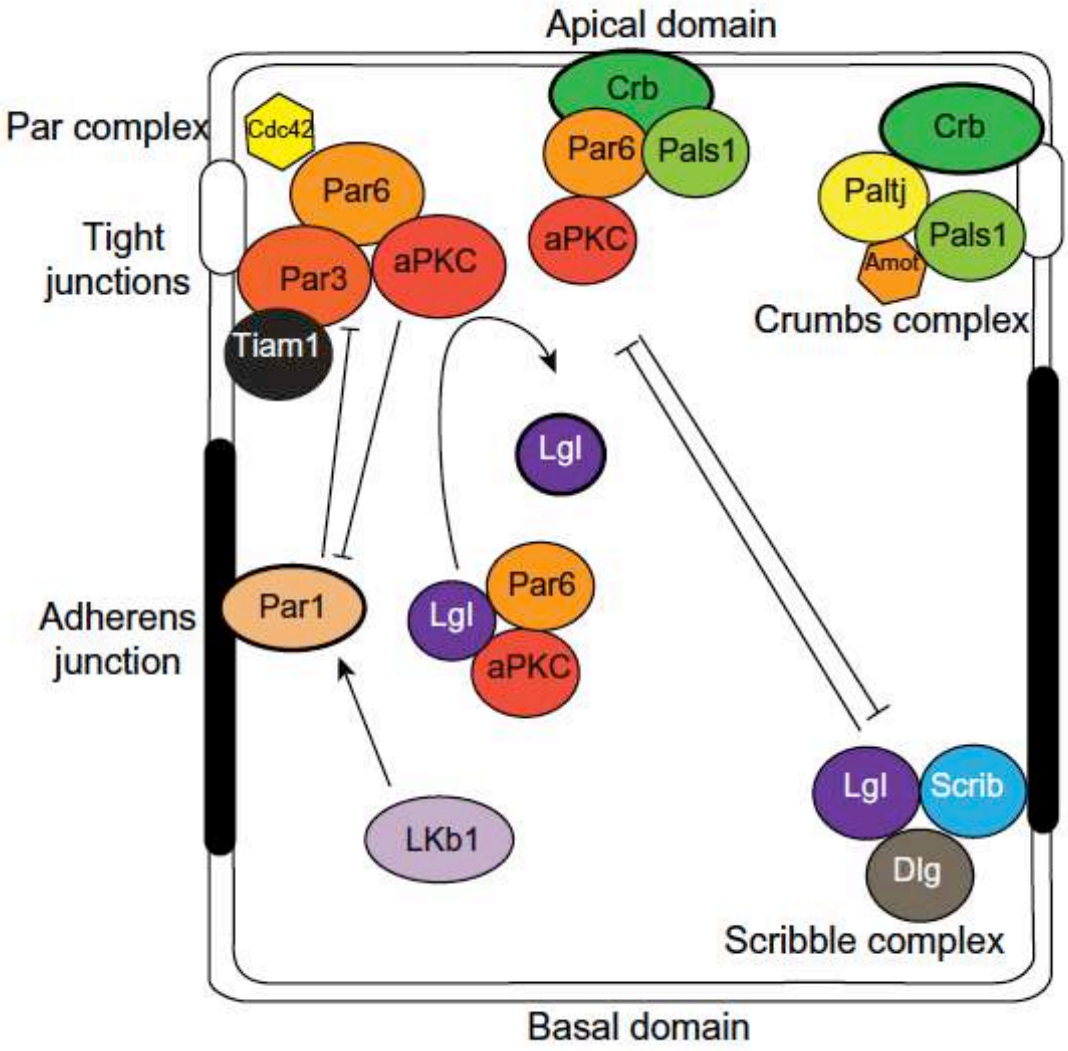

Figure 1.15 Polarity protein complexes in epithelial cells.

Figure adapted from (Chatterjee & McCaffrey, 2014)

1.6.4 Transitions in Cell Polarity States and Polarity Complexes Enable Epithelial to Mesenchymal Plasticity in Breast Tumorigenesis

Maintaining epithelial polarity is believed to be controlling cellular organization within the mammary tissue, building highly structured glandular architectures, and allowing specific cellular functions, such as the vectorial flow of milk during lactation. Several aspects of acquiring polarization states in the mammary epithelial cells appear to be inverted in breast cancer. Knowing that maintaining epithelial polarity in mammary cells is important to regulate cellular proliferation and structural morphology, it is not surprising that disturbing epithelial polarity in the mammary gland plays a key role in breast tumorigenesis and enables epithelial cells to acquire several mesenchymal characteristics defined as epithelial mesenchymal plasticity (Coradini et al, 2012; Godde et al, 2010).

Studies have revealed a number of mechanisms that can account for altered expression of apical/basal polarity proteins in breast caner and EMT. For example, TGFβ signaling regulates epithelial mesenchymal plasticity by promoting Par6 phosphorylation and consequent tight junction disassembly (Ozdamar et al, 2005). Activation of ErbB2 in breast cancer disrupts cell polarity by competing with Par3 for Par6/aPKC binding (Aranda et al, 2006). The Par6β gene, PARD6B, is shown to be frequently amplified in breast cancer (Cunliffe et al, 2012). It has been found that members of the Crumbs complex (CRB3, PATJ and PALS1) are up-regulated after the E-cadherin repressor ZEB1 is silenced in MDA-MB-231 mesenchymal breast cancer cells (Cunliffe et al, 2012).

Furthermore, loss of Par3 plays a significant role in promoting EMT in breast cancer through regulating the expression of matrix metalloproteinases (MMPs) (McCaffrey et al, 2012).

1.6.5 Epithelial Polarity and Growth Control Through Hippo Tumor Suppressor Pathway

Hippo signaling is an evolutionarily conserved pathway that controls tissue growth by regulating both cell proliferation and apoptosis. The components of this pathway are considered as significant tumor suppressors that function as key negative regulators of transcription co-factor Yes-associated protein 1 (Yap1) and the related Taz (Harvey & Tapon, 2007; Pan, 2010). It consists of a kinase cascade in which Mst1/2 phosphorylates Lats1/2, which subsequently phosphorylates Yap1/Taz, generating a binding to 14-3-3 and resulting in cytoplasmic sequestration of Yap1/Taz, and also marks them for proteasomal degradation, thus inhibiting Yap1/TAZ activity (Figure 1.16) (Huang et al, 2005; Kanai et al, 2000; Lin et al, 2013). The nuclear localization of Yap1/TAZ is associated with oncogenic transformation in mammary epithelial cells, and also correlates with metastatic breast cancer (Chan et al, 2009; Lamar et al, 2012; Vlug et al, 2013). In addition to Hippo pathway components, a number of regulators have been identified to be participating in regulating Yap1/Taz nuclear localization and activation, including cell–cell adhesion, actin cytoskeleton, G protein-coupled receptor (GPCR) signaling, and cell polarity (Boggiano & Fehon, 2012). However, the extracellular cues and receptors through which Hippo pathway is activated are yet to be fully determined.

Recent studies in both mammals and *Drosophila* have linked activation of Hippo

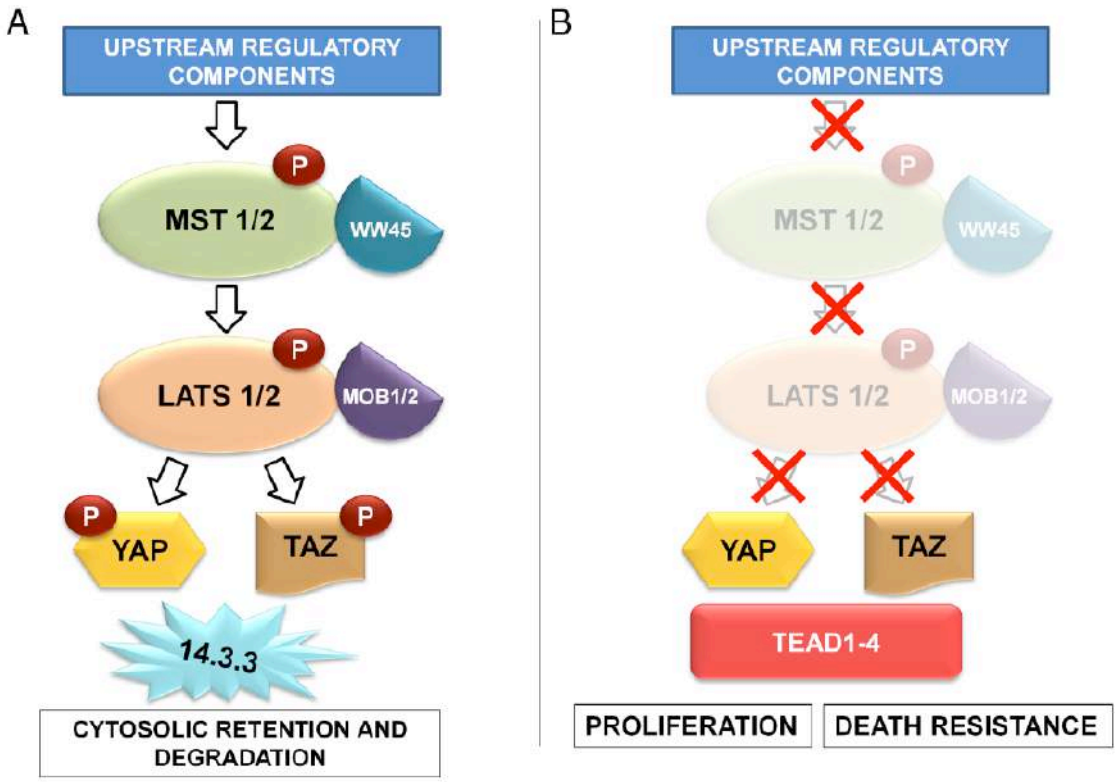

Figure 1.16 The activation of Hippo pathway.

(A) Hippo pathway activation: Yki orthologue (YAP) and TAZ are sequestered in the cytoplasm and degraded. (B) Hippo pathway inactivation: YAP and TAZ translocate to the nucleus and interact with TEAD-domain, promoting the transcription of genes involved in cell death and proliferation. P, phosphorylated protein components of the cascade. Figure adapted from (Cacemiro et al, 2017).

pathway with cell polarity proteins in determining tissue growth, limiting organ size and participating in tissue regeneration (Grzeschik et al, 2010; Zhao et al, 2011; Zhao et al, 2008). Yap1 interacts with the Crumbs complex and tight junction components, including Pals1, Patj, and Amot; deletion of either Crb or Pals1 causes Yap1/Taz to accumulate in the nucleus in mammary epithelial cells (Varelas et al, 2010). Although the Crumbs complex regulation of Yap1/Taz nuclear accumulation is dependent on upstream components of Hippo signaling (Mst1/2 and Lats 1/2), the mechanism by which the complex functions is incompletely understood. One proposed hypothesis is that the apical plasma membrane domain acts as a scaffold to regulate Hippo-mediated inhibition of Yap1/Taz activity. Neurofibromin 2 (NF2), which is apically localized in epithelial cells, recruits Lats1/2 to the apical plasma membrane (Yin et al, 2013). Another study has revealed that kidney and brain expressed protein (Kibra), which interacts apically with the Par complex, can regulate Lats1/2 activity (Moleirinho et al, 2013; Yoshihama et al, 2011). The ability of Yap1/Taz to interact with multiple members of the Crumbs complex might mean that Yap1/Taz most efficiently bind to all Crumbs complex components, ensuring that its sequestering out of the nucleus occurs in cells only when apical–basal polarity is fully established (Chatterjee & McCaffrey, 2014).

The basolateral polarity Scribbles complex has been shown also to regulate Hippo signaling. Indeed, Scrib acts as a scaffold to assemble Hippo components Mst1/2, Lats1/2 and Taz in breast cancer cells. Furthermore, either loss or mislocalization of Scrib blocks Mst1/2 induced phosphorylation and activation of Lats1/2, resulting in sustained Taz activation (Cordenonsi et al, 2011). Hence, the Hippo pathway components might have multiple interactions with apical/basal polarity complexes as a mechanism to promote

epithelial integrity. The subsequent advantage of this would be that the epithelial cell would have a great sensitivity to relatively minor disruptions in apical/basal polarity, which could enable a graduate activation of Yap1/Taz (Chatterjee & McCaffrey, 2014). Further studies should be done to enhance our understanding of the complexity by which apical/basal polarity and the Hippo pathway control each other in both normal epithelium and cancer cells.

1.7 Regulation of Centrosome Amplification and Maintenance of Genomic Stability

1.7.1 Centrosomes in Cell Division

As their name indicates, centrosomes have long been believed to have a central role in several aspects of cell organization (Azimzadeh, 2014; Bornens, 2012; Kellogg et al, 1994). A hundred years ago, it was documented that centrosomes form two poles of the bipolar mitotic spindle, separating chromosomes to daughter cells during animal cell division. In embryos from certain species, the assembly of too many centrosomes exhibited multipolar spindles that led to chromosome missegregation and usually embryonic death. However, it was reported that some embryos that survived developed into abnormal ‘monsters’, encouraging Boveri to famously speculate that chromosome imbalance might predispose cells to malignant transformation resulting in disturbance of homeostasis (Boveri, 2008).

It is recognized that centrosomes comprise a pair of special structures called centrioles. These structures display a complex behavior during the cell cycle in mammalian cells, either forming a cilium in quiescent cells or a centrosome in proliferating cells. Centrosomes are defined as subcellular organelles composed of two orthogonally positioned centrioles surrounded by pericentriolar material (PCM) (Figure

1.17). The PCM encompasses hundreds of proteins, including several cell cycle regulators, signaling components, as well as a number of proteins that help to organize and nucleate microtubules, explaining the reason why centrosomes serve as the dominant microtubule-organizing centers (MTOCs). Through the important role of centrosomes in microtubule organization and their recruitment of several important proteins, centrosomes are believed to play an important role in many cell processes (Alves-Cruzeiro et al, 2014; Andersen et al, 2003; Doxsey et al, 2005; Rieder et al, 2001) and it has been shown lately that they play key roles in cell polarity, cell division and migration (Bettencourt-Dias & Glover, 2007; Godinho & Pellman, 2014; Kim & Dynlacht, 2013).

Similar to DNA, centrosome duplication happens once, and only once, per cell cycle (Nigg & Stearns, 2011) (Figure 1.16). Centrosome duplication occurs during the S phase of the cell cycle, and by the time a cell enters mitosis, two centrosomes are formed inside the cell, which then will form the poles of the bipolar mitotic spindle (Nigg, 2007; Nigg & Stearns, 2011). Studies of cell cycle have demonstrated that cells divide halfway between the two spindle poles, therefore ensuring the inheritance of one complete set of chromosomes as well as a single centrosome to each new daughter cell (Prosser & Pelletier, 2017). According to a previous classical view of mitosis and centrosome, the occurrence of two centrosomes ensures spindle bipolarity, emphasizing the importance of strictly regulated centrosome duplication. Thus, accumulating evidence has identified a number of kinases that precisely regulate centrosome duplication, including mitotic kinase Aurora A, polo-like kinase 4 (PLK 4), Polo-like kinase 1 (PLK1), Mps-1 and Cyclin-dependent kinase2-cycline E (Cdk2-E) (Hinchcliffe, 2014).

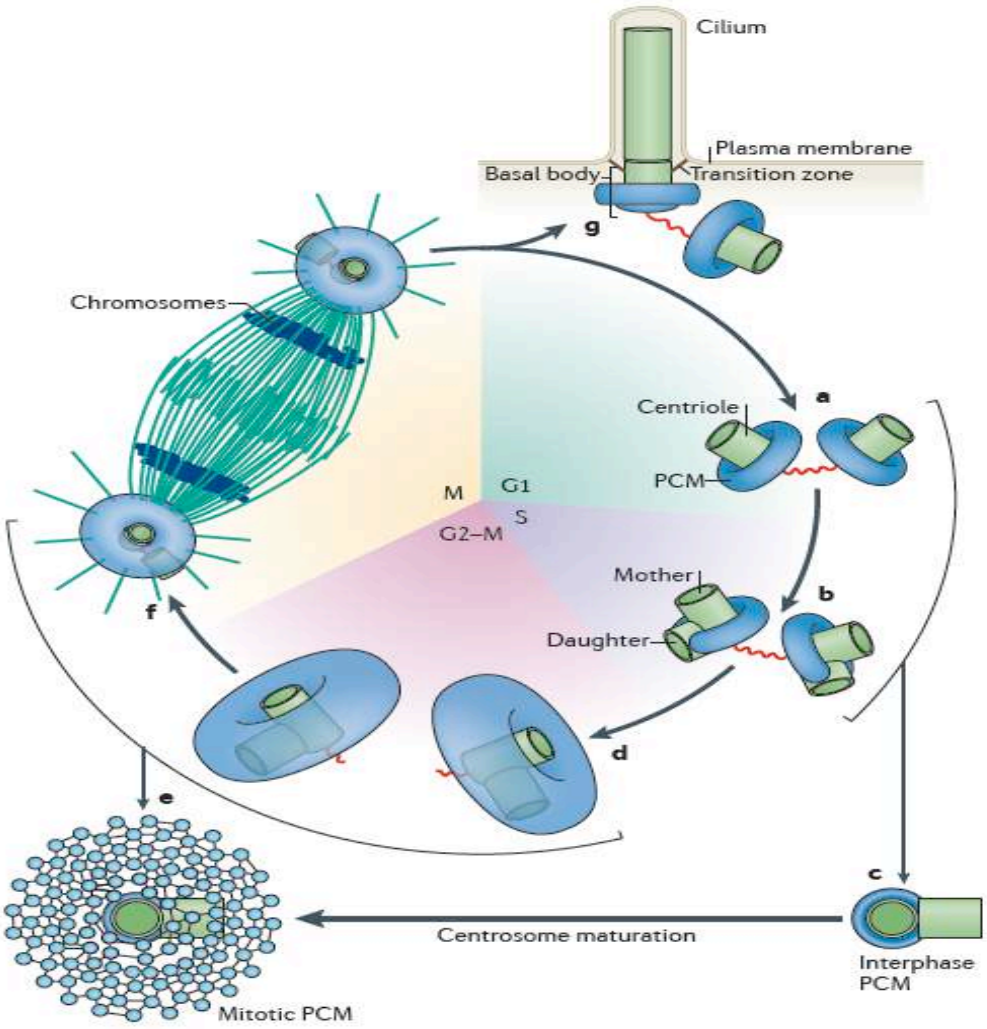

Figure 1.17 Centriole, centrosome and cilium behaviour during the cell cycle

(a) A newborn cell in the G1 phase of the cell cycle usually contains two centrioles (shown in light green). The two centrioles form centrosomes by organizing pericentriolar material (PCM; light blue) around themselves. (**b)** Centriole duplication takes place in the S phase. Although the centrioles in most cells in G1–S show very little PCM, this PCM is highly organized around the mother centriole (shown in **c**). (**d)** In mitosis (G2–M), the linkage between the two pairs of centrioles breaks, leading to the two centrioles starting to move apart. The mother centrioles start to recruit large amounts of PCM, and this is

believed to be organized by a 'scaffold' structure that assembles around the mother centrioles (shown in part **e)**. **(f)** The expanded PCM allows the centrosomes to nucleate and organize many microtubules that play a critical role in assembling and positioning the mitotic spindle (dark green). When cells exit mitosis, the chromosomes (dark blue) segregate on the mitotic spindle and the mother and daughter centrioles are completely separated. (**g)** In a number of mammalian cells that exit the cell cycle, the centriole pair migrates to the cell surface. The mother centriole forms a basal body from which a cilium extends. Figure adopted from (Conduit et al, 2015).

1.7.2 Polo-Like Kinase 4 is a Key Regulator of Centriole Biogenesis

Classical studies in both flies and humans have identified the protein kinase PLK4 as a master regulator of centriole biogenesis (Habedanck et al, 2005; Rodrigues-Martins et al, 2007). PLK4 is a distant member of the polo-like kinase family that localizes at the centrioles and plays an essential role in centriole duplication. PLK4 depletion in drosophila and human cells progressively results in impaired centriole duplication (Bettencourt-Dias et al, 2005). Embryos of $Plk4^{-/-}$ mouse do not arrest until embryonic day 7.5 and are, accordingly, apparently capable of undergoing several divisions in the absence of PLK4 (Ko et al, 2005). As PLK4 is rapidly turned over in the cells, this suggests that either there is a large supply of maternal PLK4 or that it is not essential during early embryonic divisions (Fode et al, 1996; Holland et al, 2010).

While PLK4 depletion impairs centriole duplication, overexpression of this kinase abolishes the mechanism that normally confines centriole duplication, leading to the synchronized formation of multiple daughter centrioles in a single cell cycle (Habedanck et al, 2005; Kleylein-Sohn et al, 2007; Peel et al, 2007). This demonstrates that there is no structural restriction to the formation of multiple daughter centrioles, but rather the number of centrioles generated during each cell cycle is restricted by PLK4 level (Habedanck et al, 2005).

1.7.2.1 Misregulation of PLK4 Leads to Centrosome Amplification and Subsequently Promotes Tumorigenesis

Multiple centriole assembly ultimately leads to the development of extra centrosomes. Consequently, overexpressing PLK4 in Drosophila gave rise to flies with

excessive centrosomes in ~60% of their somatic cells. These cells initially formed multipolar spindles, but these spindles eventually become bipolar. Accordingly, flies that display multiple centrosomes are normal and fertile and show only a modest increase in aneuploidy. However, transplanted larval brain tissue from animals with multiple centrosomes was able to initiate neoplastic transformation in wild type host flies. This observation offered the first direct relevant link between centrosome amplification and tumorigenesis (Basto et al, 2008).

Surprisingly, heterozygosity of PLK4 also leads to centrosome amplification and irregular spindle formation. Indeed, $PLK4^{+/-}$ murine embryonic fibroblasts display production of abnormal tetraploid daughter cells with excessive centrosome content (Rosario et al, 2010). Exactly how PLK4 acts to control centrosome duplication remains to be further investigated. Compelling evidence has suggested that the unrestrained proliferation of tetraploid cells acts as a catalyst that triggers further genetic instability and tumorigenesis (Holland & Cleveland, 2009; Macmillan et al, 2001). Consistently, it has been reported that $PLK4^{+/-}$ cells show spontaneous immortalization in culture and are capable of initiating tumors when injected into immunocompromised mice (Rosario et al, 2010). Likewise, PLK4 heterozygous mice are disposed to develop of spontaneous lung and liver cancers (Ko et al, 2005; Rosario et al, 2010). Together, these studies suggest that PLK4 has the uncommon property of acting as both a tumor suppressor and an oncogene. Importantly, regulating PLK4 level is of great importance for the cell and the organism.

1.7.3 Centrosome Amplification in Breast Cancer

Much *in vitro* evidence suggests that centrosome amplification dynamically drives aggressive disease features through promoting chromosomal instability in these cells (Funk et al, 2016). Increasing evidence has emerged linking centrosome amplification with breast tumor progression. Overexpression of Aurora Kinase A, one of the centrosome duplication regulators, results in chromosomal instability that precedes tumor initiation in mouse mammary epithelium, the incidence of which is increased by a $p53^{+/-}$ background. Therefore, centrosome amplification might drive breast tumor progression, especially in the venue of abnormal p53 (Wang et al, 2006). Similarly, overexpression of PLK4 in the mammary MCF10 cell line prompts centrosome amplification and the formation of invasive, matrix-degrading protrusions, unlike a truncated form of PLK4 that maintains kinase activity but does not induce centrosome amplification (Godinho et al, 2014). MCF10A cells treated with dihydrocytochalasin B (which causes cytokinesis failure) exhibit tetraploidy and centrosome amplification and subsequently display invasive protrusions (Ogden et al, 2017).

Centrosome amplification has also been shown to promote stem-like features. Indeed, suppression of the kinase activity of cytoplasmic Aurora Kinase A decreases the population of $CD24^{low}/CD44^{high}$ in MDA-MB-231 TNBC cells; thus, kinase-mediated activities of cytoplasmic Aurora Kinase A, which includes regulation of centrosome duplication, might be involved in imparting a stem-like phenotype in breast cancer cells (Zheng et al, 2016). Induction of centrosome amplification by overexpressing Aurora Kinase A in MCF7, T47D, and ZR-75-1 breast cancer cells increases expression of breast cancer stem cell marker ALDH1 (Liu et al, 2015b). In SUM149PT breast cancer cells,

centrosome amplification is found only in the stem-like $CD24^{low}/CD44^{high}$ subpopulation (Opyrchal et al, 2014).

In primary human cancers, several centrosome defects, including an increase in centrosome number and volume, accumulation of excessive PCM, supernumerary centrioles, as well as inappropriate accumulation of phosphocentrin during non-mitotic phases are frequently observed in specimens of high-grade metastatic breast adenocarcinoma. Furthermore, breast tumour cells exhibit functional centrosome abnormalities characterized by unusual large arrays of microtubules nucleated by an increased number of MTOCs (Lingle et al, 1998). Supporting centrosome amplification as a fundamental and early incident in breast tumourigenesis, centrosome amplification also occurs in ductal carcinoma *in situ*, and correlates with chromosomal instability in these lesions (Kronenwett et al, 2005; Lingle et al, 2002). Moreover, within lesions of ductal carcinoma *in situ*, centrosome abnormality increases with advanced histological grade, suggesting a higher propensity of progression into invasive carcinoma (Pihan et al, 2003). Greater centrosome amplification, along with abnormal mitotic spindles are observed in genomically unstable and clinically more aggressive aneuploid breast cancers, compared to genomically stable aneuploidy tumors (Kronenwett et al, 2005; Kronenwett et al, 2004). Furthermore, studies have linked centrosome amplification with high histological grade and lymph node metastasis, supporting it as a possible marker for aggressive tumors and poor breast cancer prognosis (Guo et al, 2007; Lingle et al, 2002). Some studies have reported the association of centrosome amplification with HER2 overexpression, negative estrogen receptor (ER) status, and negative progesterone receptor (PR) status in breast cancer (Montagna et al, 2002; Schneeweiss et al, 2003).

Altogether, these studies show that centrosome amplification can spearhead an array of mechanisms vital to mammary tumor initiation and progression.

1.7.4 Centrosome Amplification Promotes Genomic Instability

Given the critical role that centrosomes play in maintaining chromosomal instability, it is not surprising that their number is highly regulated during the cell cycle. If centrosome duplication happened incorrectly, coordinated cells might acquire multiple copies of centrosomes, resulting in the multi-polar spindles formation which can give rise to multi-polar divisions (Ganem et al, 2009; Nigg, 2002). Such divisions cause massive chromosome missegregation, resulting in subsequent highly aneuploid daughter cells (Figure 1.18). Therefore, mammalian cells have evolved mechanisms, suppressing multi-polar mitoses to prevent substantial chromosome missegregation and to maintain genomic integrity (Holland & Cleveland, 2009).

Accumulating studies have demonstrated the link between centrosomes and genome stability. Indeed, cellular DNA damage and incomplete DNA replication might be the intermediate events that explain the association between centrosome amplification and genomic instability. In *Drosophila* embryos, centrosomes have been shown to become inactive for nucleation of microtubules in response to ongoing mitosis with such damaged DNA; this might represent an alternative mechanism to the DNA damage checkpoint that prevents mitosis under conditions where chromosomal segregation would be catastrophic (Sibon et al, 2000). Similarly, disengagement of the centriole in mitosis has been also observed in cultured mammalian cells with compromised DNA, resulting in the formation of multipolar spindles (Hut et al, 2003). While a number of studies have reported several proteins involved in response to DNA damage to localize to

centrosomes, it is still unclear how communication between the centrosome and DNA damage sensing occurs (Nigg & Stearns, 2011).

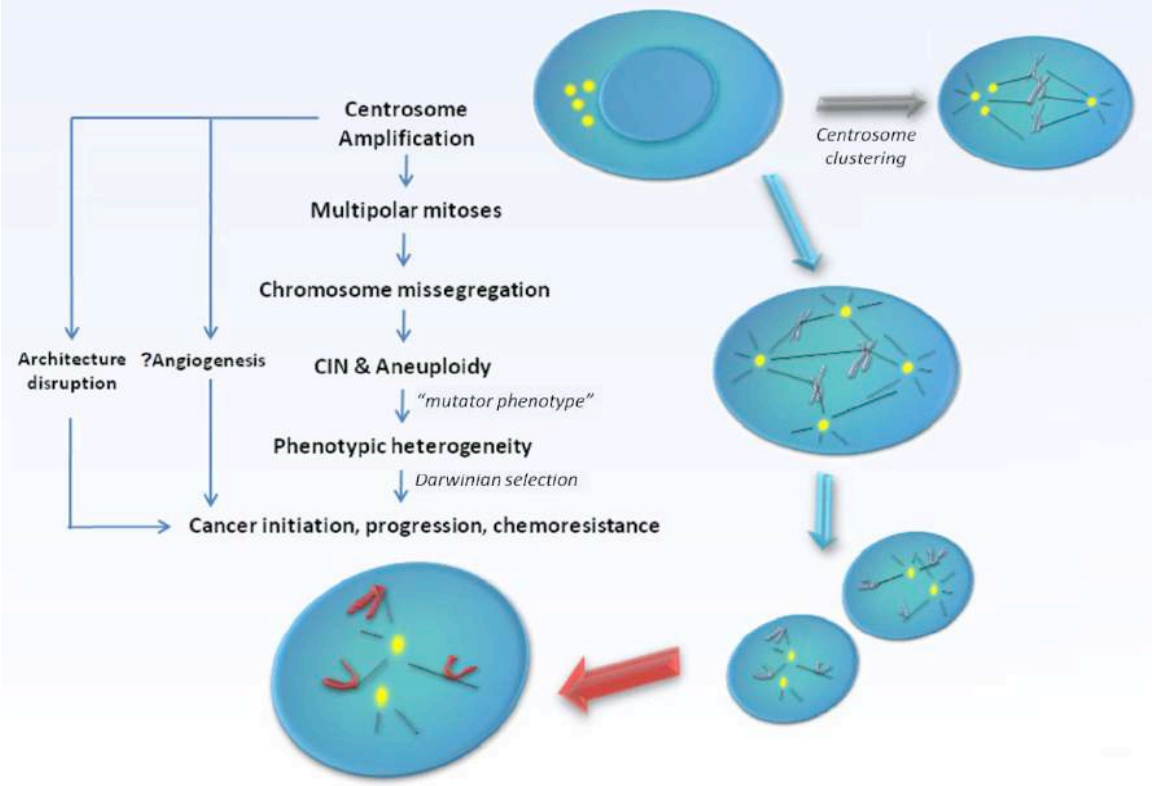

Figure 1.18 Model supporting centrosome amplification as a cause of carcinogenesis. A broad range of tumors exhibit centrosome amplification, which has been implicated in the generation of multipolar mitoses, chromosomal instability (CIN), and aneuploidy. Centrosome amplification also contributes to loss of tissue architecture, and angiogenesis in human cancers. Defective centrosomes result in abnormal nucleation of microtubules and therefore formation of disorganized mitotic spindles, leading to chromosomal missegregation and aneuploidy. Nevertheless, the presence of multiple centrosomes does not necessarily lead to major errors in cell division because extra centrosomes may undergo clustering, thereby maintaining bipolarity of the mitotic spindle. Figure adapted from (Chan, 2011)

Chapter 2: A Role for Kinesin-1 Subunits KIF5B/KLC1 in Regulating Epithelial Mesenchymal Plasticity in Breast Tumorigenesis

2.1 Preface

Prolactin hormone (PRL) is a key inducer of mammary lobulo-alveolar development, terminal differentiation of the mammary epithelial cells and lactation. While the pro-differentiation role of this hormone is well established, its role in mammary tumorigenesis is still debatable and to be fully elucidated. Recently, our lab has proposed an anti-tumorigenic role for PRL in breast cancer. Thus, investigating the molecular mechanisms through which PRL induces its pro-differentiation function and their implication in mediating its anti-tumorigenic role in breast cancer still needs to be fully elucidated. Based on microarray gene-profiling analysis of the PRL-regulated mammary epithelial cellular differentiation program, we have identified previously that kinesin family member 5B (KIF5B), a member of the kinesin-1 family, is a novel PRL down-regulated target gene. In this chapter, we investigated the role of Kinesin-1 in mammary tumorigenesis.

Our data revealed a central role for the Kinesin-1 Yin/ Yang in PRL regulation of epithelial mesenchymal plasticity (EMP) in breast cancer. Indeed, we showed that KIF5B and its partner protein kinesin light chain 1 (KLC1) play differential roles in regulating EMP in breast cancer. Furthermore, we demonstrated that while KIF5B is required for the pro-invasive activity of TGF-β, we found the EMT suppressor, PRL hormone, to induce KIF5B/KLC1interaction and relocalization of KIF5B to the cytoplasm in basal/claudin-low TNBC cells similar to what is observed in less aggressive breast cancer subtypes. Together, these results highlight for the first time a KIF5B/KLC1 switch regulating EMP in breast cancer that may prove useful for EMP-targeted therapies.

Chapter 2:

A Role for Kinesin-1 Subunits KIF5B/KLC1 in Regulating Epithelial Mesenchymal Plasticity in Breast Tumorigenesis

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2.2 Abstract

Epithelial mesenchymal plasticity (EMP) is deemed vital in breast cancer progression, metastasis, stemness and resistance to therapy. Therefore, characterizing molecular mechanisms contributing to EMP are in need enabling the development of more advanced therapeutics against breast cancer. While kinesin superfamily proteins (KIFs) are well known for their role in intracellular cargo movement, our knowledge of their function in breast tumorigenesis is still limited. In breast cancer, we show that kinesin family member 5B (KIF5B) and its partner protein kinesin light chain 1 (KLC1), subunits of kinesin-1, to play differential roles in regulating EMP. Indeed, we found KIF5B to be expressed in triple negative (TN)-basal-like/claudin low breast cancer subtype and to be an inducer of epithelial-Mesenchymal transition (EMT), stemness, invasiveness and metastatic colonization. On the other hand, we found KLC1 to be expressed in epithelial/luminal breast cancer subtypes and to be a potent suppressor of EMT, invasion and stem cell markers expression as well as to be an inducer of epithelial/luminal phenotype. Interestingly, in TN-basal-like/claudin low cells we found a novel nuclear accumulation of KIF5B and its interaction with the EMT transcriptional regulator Snail1 independent of KLC1. In addition, TGF-β mediated pro-invasive activity was found to be dependent on KIF5B expression. In contrast, the epithelial differentiation factor and EMT suppressor prolactin (PRL) was found to repress KIF5B gene expression and KIF5B-Snail1 nuclear accumulation, but enhanced KLC1 gene expression and KIF5B-KLC1 interaction. Together, these results highlight a new paradigm for kinesin-1 function in breast tumorigenesis by regulating EMP programing and aggressiveness.

2.3 Introduction

Despite improvements in early detection and advances in treatment options, breast cancer progression to a metastatic disease remains a major clinical challenge. Epithelial mesenchymal plasticity (EMP) is now well recognized cellular process contributing to cancer cell diversity and intra-tumor heterogeneity associated with disease progression and impaired response to therapy (Chaffer et al, 2016; Skibinski & Kuperwasser, 2015). The role of EMP in promoting aggressive breast cancer phenotype is further emphasized in recent molecular subclassification of breast cancer. Indeed the mesenchymal-basal-like (claudin low) TNBC subtype, frequently characterized by high histological grade and poor differentiation, to be associated with unfavorable pathological features and poor patient outcome in comparison to the epithelial/luminal subtypes (Sorlie et al, 2001; Viale, 2012). Furthermore, pro-oncogenic and pro-metastatic growth factors such as TGF-β are known to potently induce EMT and promote the transition of breast cancer cells from non-invasive epithelial to invasive mesenchymal with stem-like phenotype (Katsuno et al, 2013; Massague & Obenauf, 2016). Conversely, EMT suppressors such as prolactin hormone (PRL), is shown to supress the mesenchymal properties and induce an epithelial phenotype in breast cancer cells and subsequently supress their invasive and tumorigenic behaviour (Lopez-Ozuna et al, 2016; Nouhi et al, 2006). Therefore, regulators of EMP represent important targets for the development of novel therapeutics in breast cancer. Clinically, whereas current targeted breast cancer treatments are directed toward the epithelial/luminal subtypes, there are no targeted treatments for the mesenchymal aggressive breast cancer subtypes represented by TNBC. Therefore, better understanding of molecular mechanisms that regulate EMP, and subsequently the critical

switch/conversion of breast cancer cells from epithelial to acquiring mesenchymal and stem-like properties leading to tumor heterogeneity may offer much needed new targets for prognosis and therapy in breast cancer.

Kinesin superfamily proteins (KIFs) are well known to be involved in intracellular movement and cytoplasmic transport of membranous organelles and macromolecules including complex proteins as well as RNA along the microtubules network. These superfamily proteins comprise 15 kinesin families termed kinesin-1 to kinesin-14B (Hirokawa et al, 2009; Lawrence et al, 2004). Each kinesin consists of a heterodimer complex of two subunits the kinesin heavy chain (KHC), mediating the motor function along the microtubules, and the kinesin light chain (KLC), recognizing cargoes and tethering them to KHC (Cyrus & Muller, 2016; Gindhart et al, 1998). Kinesin-driven transport along microtubules is mediated by the concert function of both KHC and KLC subunits (Hirokawa et al, 2009). Kinesin-1 is the most studied family among kinesin proteins consisting of three types of KHC subunits (KIF5A, KIF5B and KIF5C) encoded by three different genes and four KLC subunits (KLC1-4) (Verhey & Hammond, 2009). Studies have linked abnormalities in motor-driven transport to a wide range of diseases including cancer (Hirokawa et al, 2009; Salinas et al, 2008; Verhey et al, 2011). Kinesin family member 5B (KIF5B) is the most studied KHC subunit in kinesin-1 family and has been shown to be implicated in myogenesis, nuclear infusion and kidney development (Cui et al, 2015; Wang et al, 2013a; Wang et al, 2013b). To date the role of kinesin-1 in cancer including breast cancer has not been extensively studied. Indeed, KIF5B has been shown to play a role in breast tumorigenesis through regulating transport of lysosomes, mitochondria and membrane-type 1 matrix metalloproteinase

(MT1-MMP) contributing to cell migration (Cardoso et al, 2009; Marchesin et al, 2015; Wang et al, 2017). Still however it is not known whether KIF5B role in breast tumorigenesis is related to its function as a motor protein. As well, the role of its partner protein KLC in breast tumorigenesis is not yet elucidated.

In this study, we found KIF5B and KLC1, subunits of kinesin-1, to be critical regulators of EMP in breast cancer showing opposite roles. We found that KIF5B to be highly expressed in the most aggressive basal/claudin low TNBC subtype and is essential for cell viability, migration, invasion, stemness as well as metastatic potential in this breast cancer subtype. Interestingly, we found atypical nuclear accumulation and interaction of KIF5B with the EMT inducer zinc finger transcription factor Snail 1 in basal/claudin low TNBC cells. In contrast, we found its classical partner protein KLC1 to be expressed in luminal/epithelial breast cancer cells and to possess anti-invasive activity. Importantly, we found that loss of KLC1 expression in luminal breast cancer subtypes resulted in nuclear accumulation of KIF5B and acquisition of mesenchymal, invasive and stem-like phenotype with loss of epithelial properties. Finally, we demonstrated that while KIF5B is required for the pro-invasive activity of TGF-β, we found the EMT suppressor PRL hormone to induce KIF5B/KLC1interaction and relocalization of KIF5B to the cytoplasm in basal/claudin low TNBC cells. Together, these results highlight for the first time a KIF5B/KLC1 switch regulating EMP in breast cancer that may prove useful for EMP-targeted therapies.

2.4 Results

2.4.1 Differential Expression of KIF5B and its Partner Protein KLC1 in Breast Cancer Molecular Subtypes

Based on microarray gene-profiling analysis of PRL-regulated mammary epithelial cellular differentiation program we have identified KIF5B to be a novel PRL down-regulated target gene (Hachim et al, 2016c). As shown in Fig S2.1, while PRL treatment of the mammary luminal/ epithelial HC11cells resulted in a significant decrease in KIF5B m-RNA levels, on the other hand, blocking PRLR signaling by suppressing expression of Jak2, a major PRL downstream kinase, resulted in increased KIF5B expression both at the mRNA and protein levels. Thus, demonstrating that the PRL/Jak2 signaling pathway leads to downregulation of KIF5B gene expression in mammary epithelial cells prompting us to investigate the role of KIF5B in breast tumorigenesis. To address the molecular role of KIF5B in breast cancer we examined the mRNA as well as the protein expression levels of KIF5B in cell lines representative of the different breast cancer molecular subtypes, including luminal A (T47D and MCF7 cells), luminal B (BT474 cells), Her-2E (SKBR3 cells), triple negative (TN)-luminal-androgen receptor (LAR) (MDA-MB-453) and TN-basal-like/claudin low (MDA-MB-231, SCP2 and SUM159 cells) (Jiang et al, 2016; Lehmann et al, 2011; Prat et al, 2010). Interestingly, as shown in Fig 2.1A, left panel, KIF5B m-RNA levels were significantly higher in all breast cancer cells representative of the aggressive TN-basal-like/claudin low subtype, in comparison to the breast cancer cells representative of the TN-LAR (MDA-MB-453) which is characterized by a luminal-epithelial phenotype (Lopez-Ozuna et al, 2016) as well as in breast cancer cells representative of the luminal A, luminal B and Her-2E

subtypes. Similarly, as shown in Fig 2.1A, right panel, KIF5B protein was readily detectable in all TN-basal-like/claudin low cells but not in breast cancer cells of epithelial/luminal subtypes. This data suggests a potential role for KIF5B in TN-basal-like/claudin low tumorigenesis.

The kinesin-1 proteins KIF5B and KLC1 work in concert to mediate intracellular cargo transport (Adio et al, 2006; Rahman et al, 1999). Having shown that KIF5B is upregulated in TN-basal-like/claudin low cells, we next examined the expression level of its partner protein KLC1. Interestingly, examining mRNA expression levels of KLC1 in the different breast cancer molecular subtypes showed a complete opposite pattern to that observed for KIF5B. Indeed, as shown in Fig 2.1B, KLC1 is least expressed in the TN-basal-like/claudin low cells (MDA-MB-231, SCP2 and SUM159) in comparison to luminal A (T47D), luminal B (BT474), Her-2E (SKBR3) and TN-LAR (MDA-MB-453) cells both at the m-RNA and protein levels.

To further evaluate the role of Kinesin-1 components, KIF5B and KLC1 in breast cancer, we next examined their clinical significance using Curtis dataset, (ONCOMINE database) containing gene profiling data of 1700 breast cancer cases (Rhodes et al, 2004). Interestingly, we found KIF5B mRNA levels to be significantly higher in invasive breast cancer carcinoma (1556 cases) compared to normal breast tissue (144 cases) (P=5.47E-5) (Fig 2.1C, left panel). In contrast, KLC1 mRNA levels were found to be significantly lower in invasive breast cancer carcinoma (1556 cases) compared to normal breast tissue (144 cases) (P=1.83E-24) (Fig 2.1C, right panel). We next examined the mRNA levels of KIF5B using a cohort of 1411 breast cancer cases in GOBO database. We found higher KIF5B mRNA levels in the poorly differentiated grade III tumors compared to grades II

and I tumors while we found KLC1 mRNA levels trend to be higher in well differentiated tumor grade 1 tumors compared to grade II and III with a P value of (P=0.03047) and (0.08243) respectively (Fig 2.1D). Moreover, examining KIF5B mRNA levels in GOBO database (containing 1881 human breast cancer cases) using HU and PAM50 sub-classifications (Ringner et al, 2011). We found KIF5B mRNA levels to be highest in the basal-like subtype and lowest in the luminal A subtype (P=<0.00001) (Fig 2.1E, left panel). In contrast to what we found for KIF5B, we found that KLC1 mRNA levels to be highest in luminal subtypes and lowest in basal-like subtype, (Hu; P=<0.00001; PAM50; P= 0.0084) (Fig 2.1E, right panel). Lastly, we analyzed the association between KIF5B mRNA levels and patient outcome represented as distant metastasis free survival (DMFS) and relapse free survival (RFS). For this we used Kaplan Meier plotter database which allow monitoring of survival of a large number of breast cancer patients for > 10 years (Gyorffy et al, 2010). Interestingly, patients with higher KIF5B mRNA levels showed worse outcome presented as reduced DMFS and RFS respectively (Fig 2.1F). On the other hand, when analyzing the association between KLC1 mRNA levels and patient RFS outcome, we found that patients with higher KLC1 mRNA levels show significantly better RFS outcome (Fig 2.1G). Taken together, these findings demonstrate that KIF5B and KLC1 are differentially expressed in the different breast cancer molecular subtypes and suggest that these two components of the kinesin-1 complex have independent and potentially opposite functions in breast cancer.

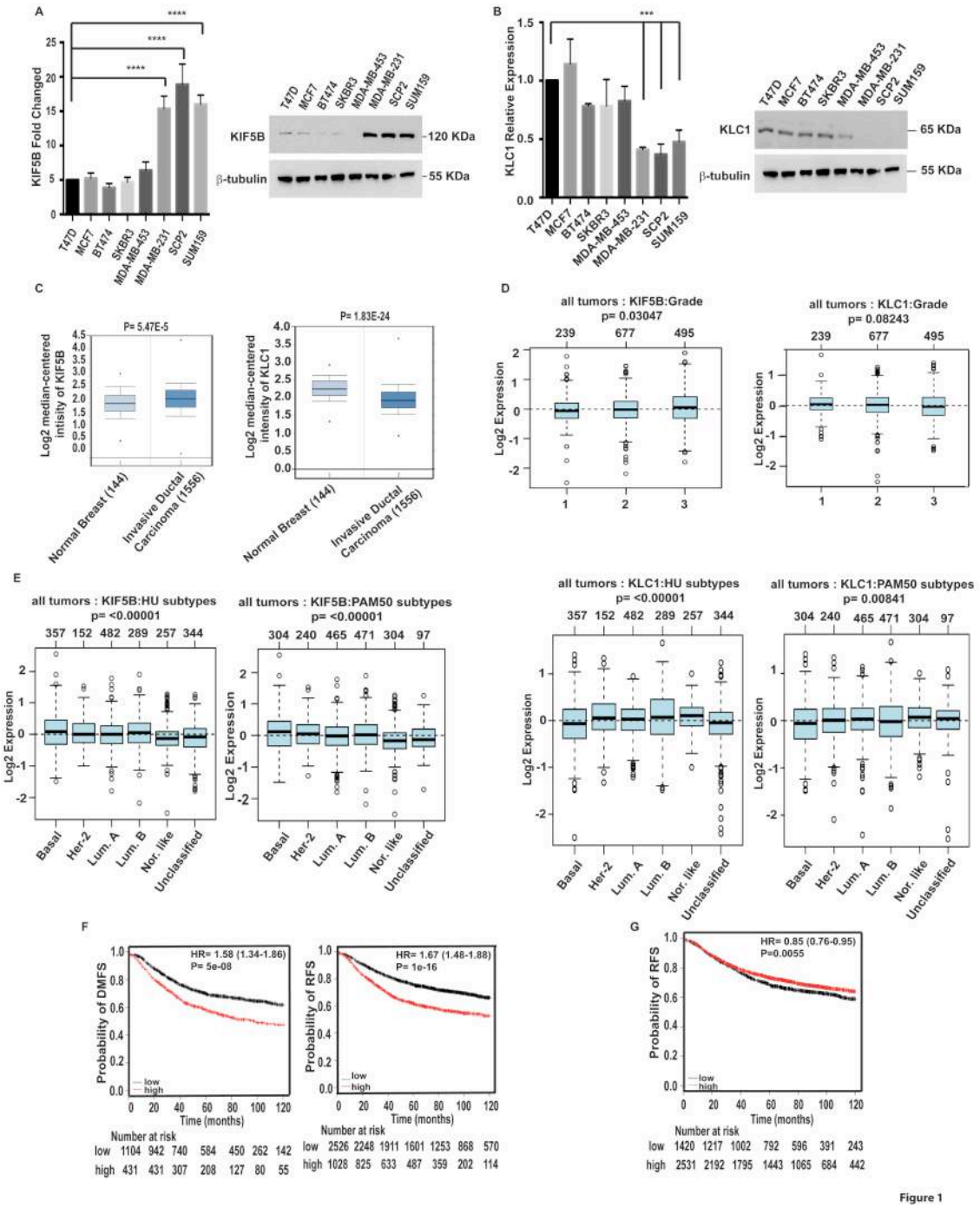


Figure 2.1 KIF5B and KLC1 expression in relation to breast cancer molecular subtypes and patient outcome

A. Left panel, expression of KIF5B was examined using q-RT-PCR in T47D (control), MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 cells. Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$. Right panel, T47D, MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 cells were lysed and western blotting was carried out using monoclonal antibodies against KIF5B and β-tubulin.

B. Left panel, m-RNA levels of KLC1 using q-RT-PCR in T47D (control), MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159. Results are expressed as relative expression of triplicates of three independent experiments ***$p \leq 0.001$.

Right panel, immunoblot analysis of total cell lysates of breast cancer cells using monoclonal antibodies against KLC1 and β-tubulin.

C. KIF5B (left panel) and KLC1 (right panel) mRNA expression levels in 144 normal and 1556 invasive breast cancer cases using Curtis dataset of ONCOMINE database.

D. KIF5B (left panel) and KLC1 (right panel) mRNA expression levels stratified according to tumor grade in a cohort of 1411 cases using GOBO database.

E. KIF5B (left panel) and KLC1 (right panel) mRNA expression levels in association with breast cancer molecular subtypes stratified according to Hu et al as well as PAM50 sub-classification methods in 1881 human breast cancer samples using GOBO database.

F. Kaplan-Meier survival curves of KIF5B gene expression in association with patient outcome (1535 patients, KM-plotter database) using DMFS as an end point (left panel). Kaplan-Meier survival curves of KIF5B gene expression in association with patient outcome (3554 patients, KM-plotter database) using RFS as an end point (middle panel).

G. Kaplan-Meier survival curves of KLC1 gene expression in association with patient outcome (3951 patients, KM-plotter database) using RFS as an end point.

2.4.2 TN-Basal-Like/Claudin Low Breast Cancer Cells Show Dependency on KIF5B for Viability and Invasion Activity

We next investigated the functional role of KIF5B in breast tumorigenesis by means of RNA interference, using two independent KIF5B-specific shRNAs in the TN-basal-like/claudin low MDA-MB-231 cells. Efficacy of KIF5B knockdown was verified at both m-RNA and protein levels for each ShRNA (Fig 2.2A). As can be seen in Fig 2B, blocking KIF5B expression resulted in a significant loss of cell viability, most evident at 96h time point. Our data also showed that KIF5B knockdown significantly decreased the migratory property of the MDA-MB-231 cells (Fig S2.2A). Moreover, KIF5B knockdown also resulted in a significant loss in the invasive capacity of the MDA-MB-231 cells (Fig 2.2C). To avoid the limitation of a single cell line, these findings were reproduced in the other aggressive TN-basal-like/claudin low cell model system SUM159, in which KIF5B was also found to be highly expressed and showed the same effects as that seen in MDA-MB-231 cells (Fig 2.2D). Moreover, upon knockdown of KIF5B, we observed a change in cell morphology of the TN-basal-like/claudin low cells MDA-MB-231 and SUM159 from mesenchymal to more cuboidal shape (Fig 2.2E). To further elaborate on the role of KIF5B in breast cancer, we suppressed KIF5B expression in the luminal A T47D cells normally expressing low levels of KIF5B. Interestingly, as shown in Fig 2.2F, no change in cell viability or morphology was observed following KIF5B knockdown. Altogether these results highlight a pro-oncogenic role for KIF5B in mediating cell viability, migration and invasion capacities in TN-basal-like/claudin low cells.

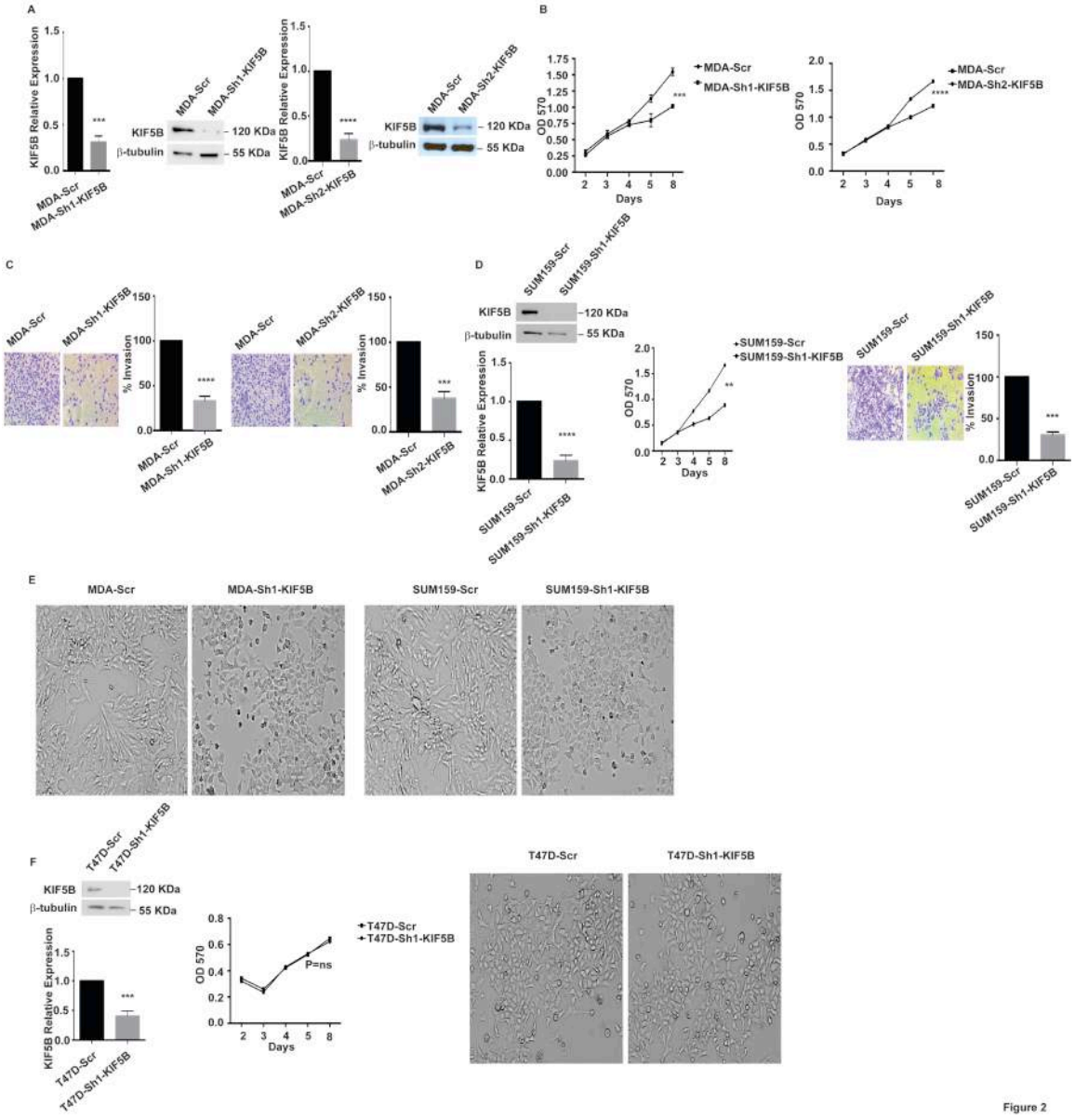


Figure 2

Figure 2.2 KIF5B is required for TN-basal-like/claudin low cell viability and invasion capacity

A. Left panel, m-RNA levels of KIF5B were assessed using q-RT-PCR in MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells. Results are expressed as relative expression of triplicates of three independent experiments ***p <

0.001. Immunoblot analysis of total cell lysates of MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells using antibodies against KIF5B and β-tubulin. Right panel, m-RNA levels of KIF5B were assessed using q-RT-PCR in MDA-MB-231-Scr (control) & MDA-MB-231-Sh2-KIF5B cells. Results are expressed as relative expression of triplicates of three independent experiments ****$p < 0.0001$. Immunoblot analysis of total cell lysates of MDA-MB-231-Scr (control) & MDA-MB-231-Sh2-KIF5B cells using antibodies against KIF5B and β-tubulin.

B. MTT assays were performed in MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B (left panel) and in MDA-MB-231-Scr & MDA-MB-231-Sh2-KIF5B (right panel) for 2, 3, 4, 5 and 8 days. Results are expressed as mean ± SEM of triplicates of three independent experiments. ***$p \leq 0.001$, ****$p < 0.0001$

C. Quantitative invasion assays of MDA-MB-231-Scr and MDA-MB-231-Sh1-KIF5B (left panel) and MDA-MB-231-Scr and MDA-MB-231-Sh2-KIF5B (right panel). Results presented are of triplicates of three independent experiments ****$p < 0.0001$. ***$p \leq 0.001$.

D. Left panel, immunoblot analysis of total cell lysates of SUM159-Scr (control) & SUM159-Sh-KIF5B cells using antibodies against KIF5B and β-tubulin (upper panel). m-RNA levels of KIF5B were assessed using q-RT-PCR in SUM159-Scr (control) & SUM159-Sh1-KIF5B cells (lower panel). Middle panel, MTT assays were performed in SUM159-Scr & SUM159-Sh1-KIF5B for 2, 3, 4, 5 and 8 days. Right panel, quantitative invasion assays of SUM159-Scr and SUM159-Sh1-KIF5B. Results are expressed as mean ± SEM of triplicates of three independent experiments ****$p < 0.0001$, ***$p \leq 0.0001$ and **$p \leq 0.01$

E. Representative images of MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B and SUM159-Scr & SUM159-Sh-KIF5B

F. Upper left panel, immunoblot analysis of total cell lysates of T47D-Scr (control) & T47D-Sh1-KIF5B cells using antibodies against KIF5B and β-tubulin. Lower left panel, m-RNA levels of KIF5B were assessed using q-RT-PCR in T47D-Scr (control) & T47D-Sh1-KIF5B cells. Results are expressed as relative expression of triplicates of three independent experiments ***$p < 0.001$.

Middle panel, MTT assays were performed in T47D-Scr & T47D-Sh1-KIF5B for 2, 3, 4, 5 and 8 days. Results are expressed as mean ± SEM of triplicates of three independent experiments. ns: non-significant

Right panel, representative images of T47D-Scr & T47D-Sh-KIF5B.

2.4.3 KIF5B Plays a Central Role in Inducing the EMT Program and Stemness in TN Basal-Like/Claudin Low Cells

It is well known that the invasive capacity and distant metastasis properties of TN-basal-like/claudin low cells are mediated through acquisition of molecular signals that activate the function and/or expression of various EMT transcription factors and markers while suppressing the expression of epithelial markers (Wu et al, 2016; Ye et al, 2017). To further decipher the role of KIF5B in breast cancer we then examined the role of KIF5B in regulating the EMT process. For this we examined the expression levels of EMT markers in MDA-MB-231 cells following knock-down of KIF5B. As shown in Fig 2.3A, our data revealed that blocking KIF5B expression significantly decreased the m-RNA levels for several EMT transcription factors (ZEB1, ZEB2, Slug and Snail1) and mesenchymal markers (vimentin and FN1). On the other hand, loss of KIF5B resulted in a significant up-regulation in the expression levels of the epithelial markers E-cadherin, CK18 as well as of the PRLR (Fig 2.3B). Together, these data strongly underscore the critical role of KIF5B in mediating induction of EMT reprograming and suppression of epithelial differentiation pathways in TN-basal-like/claudin low tumors.

Breast cancer stem-like cells (BCSCs) are largely responsible for the aggressive phenotype, high invasive capacity and high rate of recurrence of TN-basal-like/claudin low tumors. In particular, the $CD44^{high}/CD24^{low}$ stem-like cell sub-population has been identified as a mesenchymal-tumorigenic subpopulation with high metastatic activity (Dontu et al, 2003; Fillmore & Kuperwasser, 2007; Jin et al, 2016). We thus examined the role of KIF5B in regulating the stem cell phenotype of TN-basal-like/claudin low cells. Interestingly, loss of KIF5B expression in MDA-MB-231 cells resulted in a

significant reduction in the stem-like cell sub-population $CD44^{+}/CD24^{-}$ with a significant increase in the number of the non-tumorigenic $CD44^{-}/CD24^{-}$ cell subpopulation (Fig 2.3C). Furthermore, examining individual stem cell marker expression levels, we found that loss of KIF5B led to a significant decrease in CD44 but not CD24 m-RNA levels (Fig 2.3D). This was followed by a significant decrease in gene expression levels of the self-renewal transcriptional factors OCT4, NANOG and SOX2 (Fig 2.3E). Finally, we assessed the overall effect of loss of KIF5B expression on the tumorigenic capacity of MDA-MB-231 cells using colony formation assay. As shown Fig 2.3F, loss of KIF5B expression resulted in a significant decrease of the clonogenic capacity of MDA-MB-231 cells. On the other hand, there was no effect of loss of KIF5B on the clonogenic capacity of the luminal A T47D cells (Fig S2.2B). Together, these results clearly demonstrate a critical role for KIF5B in generating the mesenchymal and stem-like phenotype characteristics of EMP resulting in aggressive TN-basal-like/claudin low phenotype.

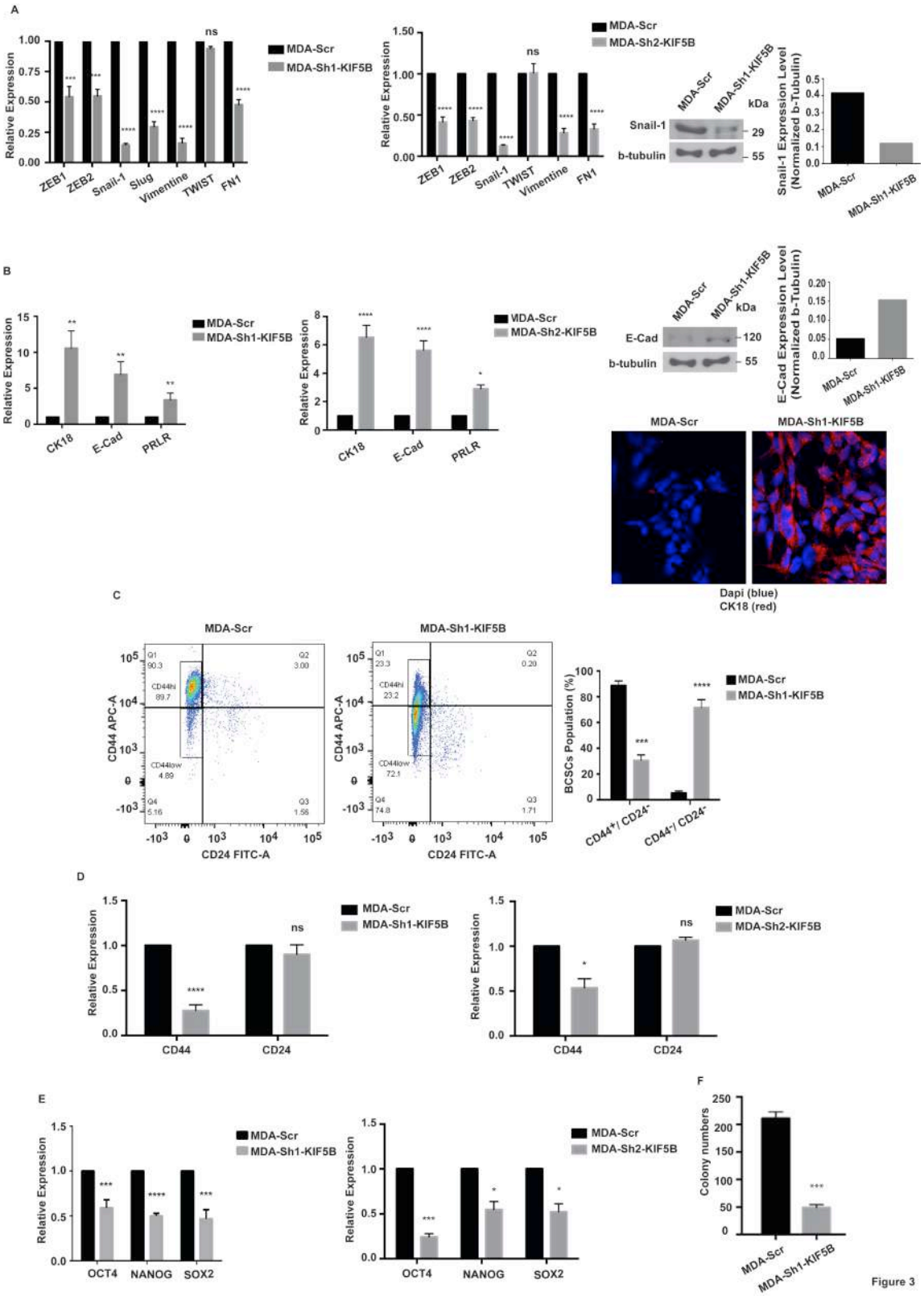

A
Relative Expression
MDA-Scr
MDA-Sh1-KIF5B
ZEB1
ZEB2
Snail-1
Slug
Vimentine
TWIST
FN1
MDA-Sh2-KIF5B
kDa
Snail-1
b-tubulin
29
55
Snail-1 Expression Level (Normalized b-Tubulin)
B
CK18
E-Cad
PRLR
E-Cad Expression Level (Normalized b-Tubulin)
Dapi (blue)
CK18 (red)
C
CD44 APC-A
CD24 FITC-A
BCSCs Population (%)
CD44+/ CD24-
CD44-/ CD24-
D
CD44
CD24
E
OCT4
NANOG
SOX2
F
Colony numbers

Figure 3

Figure 2.3 KIF5B is required for EMT programing and stemness in TN-basal-like/claudin low cells

A. Left and middle panels, m-RNA expression levels of EMT transcription factors and markers using q-RT-PCR in both MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B and MDA-MB-231-Scr & MDA-MB-231-Sh2-KIF5B. Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$, ***$p \leq 0.001$ and ns: not significant.

   Right panel, immunoblot analysis of total cell lysates of MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells using antibodies against Snail1 and β-tubulin.

B. Left and middle panels, m-RNA expressions levels of epithelial markers CK18, E-cadherin and PRLR using q-RT-PCR in MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B and MDA-MB-231-Scr & MDA-MB-231-Sh2-KIF5B. Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$, **$p \leq 0.01$ and *$p \leq 0.05$.

   Upper right panel, immunoblot analysis of total cell lysates of MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells using antibodies against E-cadherin and β-tubulin.

   Lower right panel, confocal immunofluorescence images CK18 (red) and nucleus (Dapi) (blue) of MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells. Scale bar, 10 μm.

C. The percentage content of breast CSCs ($CD44^{+}$ /$CD24^{-/low}$) in MDA-MB-231-Scr and MDA-MB-231-Sh1-KIF5B were determined by flow cytometry

(representative image of dot plot). Quantification analysis of three independent experiments expressed as mean ± SEM of triplicates of three independent experiments ****$p \leq 0.0001$ and ***$p \leq 0.001$.

D. m-RNA levels of breast stem cell markers CD44 and CD24 were examined using q-RT-PCR in MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B (left panel) and MDA-MB-231-Scr & MDA-MB-231-Sh2-KIF5B (right panel). Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$, *$p \leq 0.05$ and ns: not significant.

E. m-RNA levels of breast cancer stem cell self-renewal markers OCT4, NANOG and SOX2 were examined using q-RT-PCR in MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B and MDA-MB-231-Scr & MDA-MB-231-Sh2-KIF5B. Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$, ***$p \leq 0.001$ and *$p \leq 0.05$.

F. Colony formation assays were performed using MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B for a period of three weeks. Results are expressed as mean ± SEM of triplicates of three independent experiments ***$p \leq 0.001$

2.4.4 KLC1 is Required to Maintain an Epithelial and Non-Invasive Breast Cancer Phenotype

To address the role of KIF5B partner protein, KLC1, in breast cancer, we next suppressed KLC1 expression in the luminal A (T47D) and Her-2E (SKBR3) cells, using specific KLC1 siRNA (Fig 2.4A). While knocking down KLC1 expression did not affect T47D and SKBR3 cell viability (Fig 2.4B), importantly, we found that loss of KLC1 in T47D and SKBR3 cells reprogramed the normally non-invasive cells to an invasive phenotype (Fig 2.4C). Furthermore, suppression of KLC1 resulted in a pronounced increase in expression of the EMT markers (ZEB1, ZEB2, Snail1, Slug, Vimentin and FN1) in the two epithelial/luminal breast cancer subtypes (Fig. 2.4D). On the other hand, loss of KLC1 resulted in significant up-regulation of m-RNA levels of the epithelial markers E-cadherin and the PRLR (Fig 2.4E). Next, examining individual stem cell marker expression levels, we found that loss of KLC1 led to a significant increase in CD44 m-RNA levels while CD24 m-RNA level was significantly decreased (Fig 2.4F). Altogether, these findings highlight a clear association between KLC1 expression and maintenance of an epithelial and non-invasive breast cancer cellular phenotype emphasizing an EMP suppressor role for KLC1 in breast cancer opposite to KIF5B.

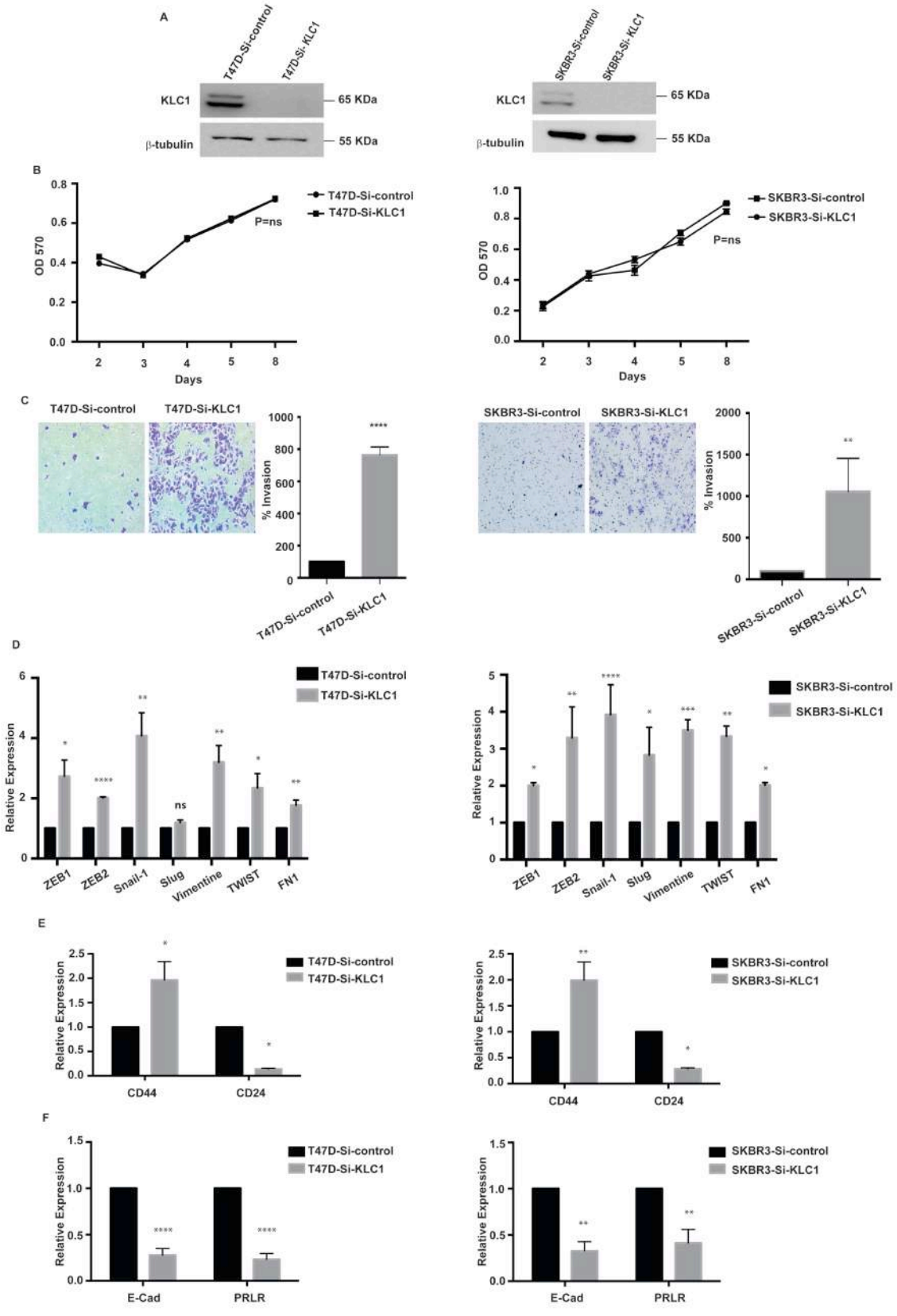

A
T47D-Si-control
T47D-Si-KLC1
KLC1
65 KDa
β-tubulin
55 KDa
SKBR3-Si-control
SKBR3-Si-KLC1
B
T47D-Si-control
T47D-Si-KLC1
P=ns
OD 570
Days
SKBR3-Si-control
SKBR3-Si-KLC1
C
T47D-Si-control
T47D-Si-KLC1
% Invasion
SKBR3-Si-control
SKBR3-Si-KLC1
D
Relative Expression
ZEB1
ZEB2
Snail-1
Slug
Vimentine
TWIST
FN1
E
CD44
CD24
F
E-Cad
PRLR

Figure 4

Figure 2.4 Loss of KLC1 expression in breast cancer cells induces aggressive phenotype

A. Immunoblot analysis of total cell lysates of T47D-Si-control and T47D-Si-KLC1 (left panel) and of SKBR-Si-control and SKBR-Si-KLC1 (right panel) using monoclonal antibodies against KLC1 and β-tubulin.
B. MTT assays of T47D- Si-control and T47D-Si-KLC1 (left panel) and of SKBR-Si-control and SKBR-Si-KLC1 (right panel) for 2, 3, 4, 5 and 8 days. Results are expressed as mean ± SEM of triplicates of three independent experiments. ns: non significant
C. Quantitative invasion assay of T47D- Si-control and T47D-Si-KLC1 (left panel) and SKBR3-Si-control & SKBR3-Si-KLC1 (right panel). Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$ and **$p \leq 0.01$
D. m-RNA levels of EMT transcription factors and markers ZEB1, ZEB2, Snail1, Slug, Vimentin, TWIST and FN1 using q-RT-PCR in T47D- Si-control & T47D-Si-KLC1 (left panel) and SKBR3-Si-control & SKBR3-Si-KLC1 (right panel). Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ and ns: non significant
E. m-RNA expressions levels of epithelial markers E-cadherin and PRLR using q-RT-PCR in T47D- Si-control & T47D-Si-KLC1 (left panel) and SKBR3-Si-control & SKBR3-Si-KLC1 (right panel). Results are expressed as relative

expression of triplicates of three independent experiments ****$p \leq 0.0001$ and **$p \leq 0.01$.

F. m-RNA levels of breast stem cell markers CD44 and CD24 were examined using q-RT-PCR in T47D- Si-control & T47D-Si-KLC1 (left panel) and SKBR3-Si-control & SKBR3-Si-KLC1 (right panel). Results are expressed as relative expression of triplicates of three independent experiments **$p \leq 0.01$ and *$p \leq 0.05$.

2.4.5 Nuclear Accumulation of KIF5B and KIF5B/SNAIL Interaction in TN-Basal-Like/Claudin Low Cells Independent of KLC1

Having found a new role for KIF5B in promoting EMP, independent of KLC1, this prompted us to investigate whether KIF5B plays a nuclear role in breast cancer. Therefore, next we examined the subcellular localization of KIF5B in the different breast cancer molecular subtypes, using immunoblotting of nuclear fractions. Interestingly, as shown in Fig 2.5A, we found high nuclear accumulation of KIF5B in the TN-basal-like/claudin low cells (MDA-MB-231, SCP2 and SUM159) while no nuclear signal for KIF5B could be detected in the epithelial/luminal breast cancer cells. In contrast, we could not detect any KLC1 nuclear accumulation in all breast cancer cells types. Moreover, immunofluorescence analysis of KIF5B showed strong nuclear localization of KIF5B in TNBC MDA-MB-231cells, while remaining unfocalized in luminal A T47D cells (Fig 2.5B). In contrast, KLC1 showed cytoplasmic localization in both T47D and MDA-MB-231 cells (Fig 2.5C). To investigate whether the nuclear accumulation of KIF5B is determined by KLC1 expression levels, we examined KIF5B nuclear levels following suppression of KLC1 expression in the luminal breast cancer cells T47D and Her-2E SKBR3 cells. Interestingly, decreasing KLC1 levels in the two luminal molecular subtypes led to KIF5B nuclear accumulation (Fig 2.5D), suggesting that KLC1 expression levels influence and control KIF5B nuclear localization. These results suggest a potential nuclear function for KIF5B in TN-basal-like/claudin low cells deriving EMP independent of KLC1.

Next, we examined whether this nuclear accumulation of KIF5B in TN-basal-like/claudin low cells permits new function for KIF5B in the nucleus. Therefore, we

examined whether there is a physical interaction between KIF5B and the transcriptional regulators of EMT in TN-basal-like/claudin low cells. Indeed, as can be seen in Fig 2.5E, all TN-basal-like/claudin low cells, MDA-MB-231, SCP2 and SUM159, showed KIF5B interaction with Snail1. Screening the possible interaction between KIF5B and transcription factors ZEB1, ZEB2 as well as Slug, our data did not detect any interaction between KIF5B and these proteins (data not shown). To address whether KIF5B regulates Snail1 function, we examined Snail1 nuclear localization in MDA-MB-231 cells following loss of KIF5B expression. Interestingly, as can be seen in Fig 2.5F, MDA-MB-231-Sh1-KIF5B cells showed loss of Snail1 nuclear accumulation and its re-localization to the cytoplasm, highlighting an important function for KIF5B in regulating Snail1 nuclear accumulation highlighting a functional role for KIF5B as a regulator of Snail1 function in TN-basal-like/claudin low cells. Altogether, these findings indicate that KIF5B nuclear accumulation in breast cancer is context dependent and is regulated by the expression of KLC1 promoting EMT transcriptional programing.

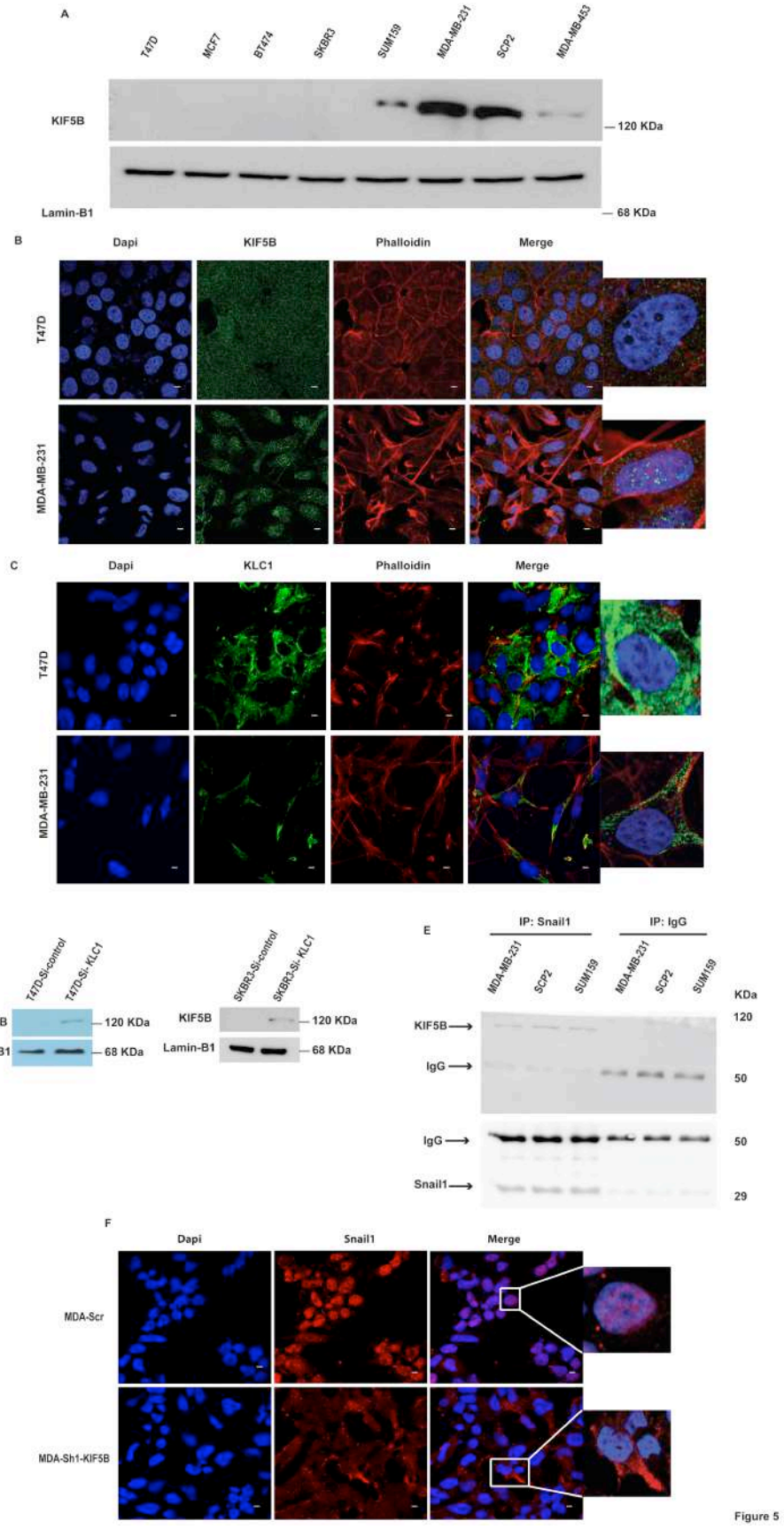

A
T47D
MCF7
BT474
SKBR3
SUM159
MDA-MB-231
SCP2
MDA-MB-453
KIF5B
120 KDa
Lamin-B1
68 KDa
B
Dapi
KIF5B
Phalloidin
Merge
T47D
MDA-MB-231
C
Dapi
KLC1
Phalloidin
Merge
T47D
MDA-MB-231
D
T47D-Si-control
T47D-Si- KLC1
KIF5B
120 KDa
Lamin-B1
68 KDa
SKBR3-Si-control
SKBR3-Si- KLC1
KIF5B
120 KDa
Lamin-B1
68 KDa
E
IP: Snail1
IP: IgG
MDA-MB-231
SCP2
SUM159
MDA-MB-231
SCP2
SUM159
KDa
KIF5B
120
IgG
50
IgG
50
Snail1
29
F
Dapi
Snail1
Merge
MDA-Scr
MDA-Sh1-KIF5B

Figure 5

Figure 2.5 KIF5B shows high nuclear accumulation and interaction with Snail1 in TN-basal-like/claudin low cells

A. Immunoblot analysis of nuclear extracts of breast cancer cells using monoclonal antibodies against KIF5B and Lamin-B1.

B. Confocal immunofluorescence images of KIF5B (green), phalloidin (red) and nucleus (Dapi) (blue) of T47D (upper panel) & MDA-MB-231 (lower panel) cells. Scale bar, 10 μm.

C. Confocal immunofluorescence images of KLC1 (green), phalloidin (red) and nucleus (Dapi) (blue) of T47D (upper panel) & MDA-MB-231 (lower panel) cells. Scale bar, 10 μm.

D. Immunoblot analysis of nuclear extracts of T47D-Si-control (control) and T47D-Si-KLC1 (left panel) and in SKBR-Si-control and SKBR-Si-KLC1 (right panel) using monoclonal antibodies against KIF5B and Lamin-B1.

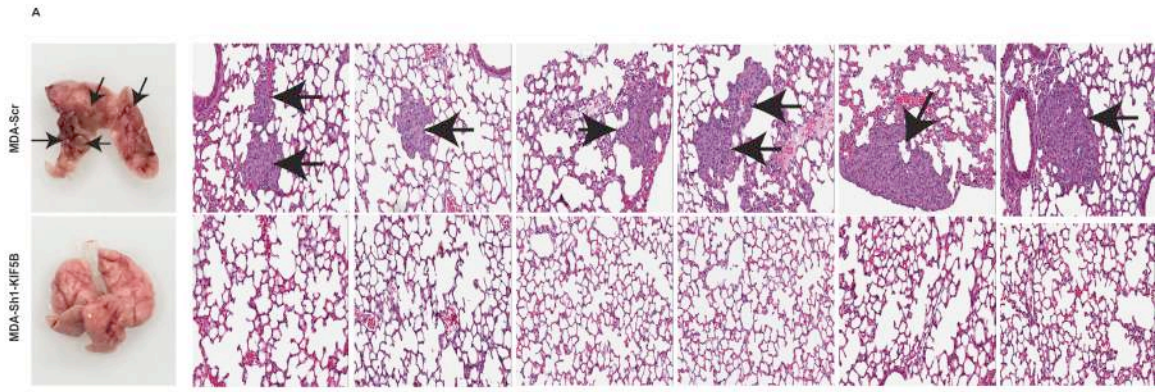
E. MDA-MB-231, SCP2 and SUM159 cells were lysed and immunoprecipitations were performed using a goat polyclonal antibody against Snail1 or control normal goat IgG. Western blotting was carried out using a rabbit monoclonal antibody against KIF5B.

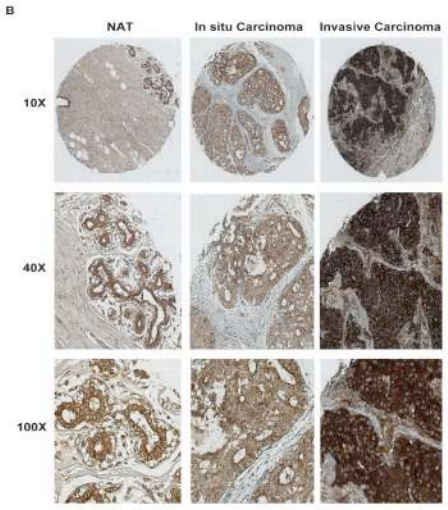
F. Confocal immunofluorescence images of Snail1 (red) and nucleus (Dapi) (blue) of MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells. Scale bar, 10 μm.

2.4.6 KIF5B is Essential for Metastatic Colonization Propensity and is a Clinically Relevant Marker of High-Grade Invasive Breast Cancer

The above data implicated high expression of KIF5B as a critical inducer of EMT, stemness and invasion in breast cancer cells. Therefore, next we examined the role of KIF5B in driving organ metastasis *in vivo*. For this, we monitored the effects of loss of KIF5B gene expression on lung metastasis using tail vein injection preclinical xenograft mouse model. Remarkably, while 7 out of the 8 mice injected with the control MDA-MB-231 cells expressing the scrambled shRNA developed lung macrometastases, none of the 8 mice injected with MDA-MB-231-Sh1KIF5B cells developed lung metastasis (Fig 2.6A). These results demonstrate that KIF5B is a driver of metastatic colonization, the functional endpoint of EMP.

As cancer cells adopt the EMP programing and invasive/metastatic behavior, they acquire cellular features clinically associated with high-grade invasive malignancy (Nieto, 2011; Thiery et al, 2009). Next we investigated the clinical features of breast cancer cases expressing KIF5B protein. For this, we used a TMA of 102 cases including 97 breast cancer cases and 5 normal/benign breast tissues. Importantly, we found KIF5B protein expression to be significantly higher in invasive ductal carcinoma in comparison to *in situ* carcinoma ($p= 0.04$). Moreover, KIF5B protein expression was associated with poorly differentiated tumors (grade III) (81.25%) in comparison to the moderately differentiated, grade II (60.38%) and grade I (31.58%) tumors ($P=0.01$) (Fig 2.6B, right panel). This data implicates KIF5B as a novel clinically relevant biomarker of high-grade invasive breast cancer.





Pathology	Negative	Positive	Total	%	P-value
Normal/Benign	4	1	5	20.00	
In situ	5	1	6	16.67	0.04529766
Invasive	38	52	90	57.78	
Grade	Negative	Positive	Total	%	P-value
I	13	6	19	31.58	
II	21	32	53	60.38	0.01
III	3	13	16	81.25	

Figure 6

Figure 2.6 Breast cancer shows dependency on KIF5B for invasive capacity and metastasis in preclinical mouse model

A. Representative gross photo of lungs as well as H& E histological images of NOD-SCID tail vein mouse xenografts of MDA-MB-231-Scr MDA-MB-231-Sh-KIF5B. Black arrow heads indicate macro and micrometastasis.

B. Left panel, positive immunohistochemical staining of KIF5B in normal adjacent tissue, in situ and invasive breast cancer lesions (10X and 40X). Right panel, associations between KIF5B protein expression and different clinicopathological parameters.

2.4.7 KIF5B/KLC1 function downstream of TGF-β and PRL in TN-Basal-Like/Claudin Low Cells

Our results so far emphasized a key role for kinesin-1 components KIF5B/KLC1 in regulating EMP in breast cancer. Extensive research has identified the growth factor TGFb as a critical inducer of EMT, stemness, invasion and tumorigenesis in TN-basal-like/claudin low cells, whereas, we have previously shown PRL to block TGFb function and to suppress EMT, invasion and tumorigenesis of TN-basal-like/claudin low cells (Haines et al, 2009; Kim et al, 2015; Mani et al, 2008). Therefore, next we examined whether KIF5B regulates TGFb-mediated pro-invasive function. Interestingly, we found that loss of KIF5B expression in MDA-MB-231 cells significantly blocked TGFβ-mediated cell invasion (Fig 2.7A). Next, we investigated PRL regulation of KIF5B/KLC1 function. Interestingly, as seen in Fig 2.7B, PRL treatment of MDA-MB-453 cells, TN-LAR subgroup has been shown previously to express PRLR endogenously (Lopez-Ozuna et al, 2016), resulted in a significant suppression of KIF5B m-RNA levels concomitant with a significant increase in KLC1 m-RNA expression levels. These results were further confirmed using the TN-basal-like/claudin low cell line MDA-MB-231 cells engineered to overexpress the PRLR upon doxycycline treatment, designated as MDA-MB-231/PRLR cells (Lopez-Ozuna et al, 2016). As can be seen in Fig 2.7C, upon doxycycline treatment to induce PRLR expression, whereas PRL treatment suppressed KIFB expression, it resulted in increased expression of KLC1. We then evaluated the role of PRL in regulating KIF5B nuclear accumulation, using the MDA-MB-231/PRLR cell model system. As shown in Fig 2.7D, our data show that PRL treatment of MDA-MB-231 cells induced to express the PRLR, resulted in the re-localization of KIF5B to the

cytoplasm, while no change was observed in control cells. In parallel, PRL was found to induce the relocalization of Snail1 from the nuclear compartment to the cytoplasm (Fig 2.7E). Finally, we also found that PRL promotes the physical interaction between KIF5B and KLC1 in MDA-MB-231/PRLR cells (Fig 2.7F). Together, these results emphasize a role for KIF5B/KLC1 in TGFb and PRL regulation of EMP, highlighting a central role for KIF5B in mediating TGFb pro-invasive activities and showing the ability of PRL to regulate and suppress KIF5B function in TN-basal-like/claudin low cells.

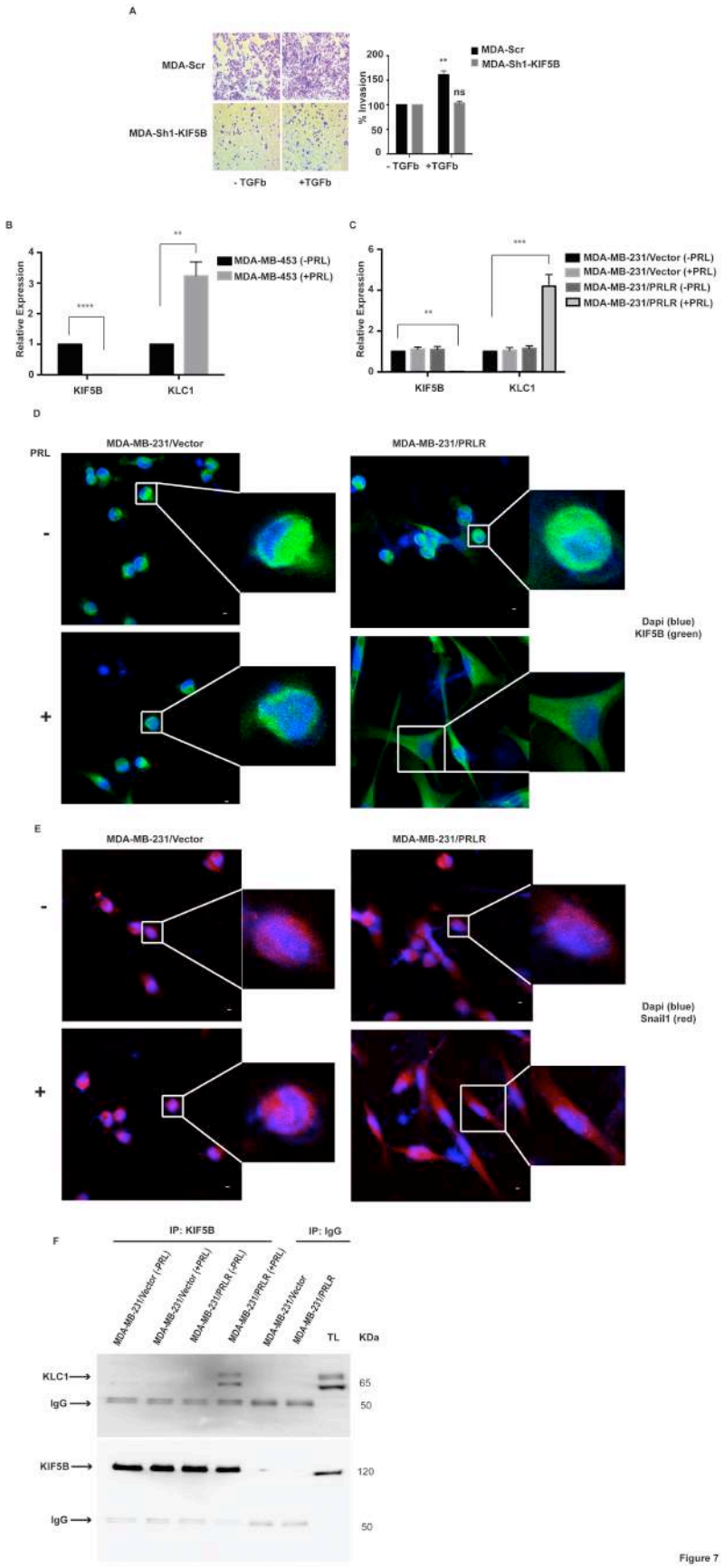

A
MDA-Scr
MDA-Sh1-KIF5B
- TGFb
+TGFb
% Invasion
MDA-Scr
MDA-Sh1-KIF5B
ns
B
Relative Expression
MDA-MB-453 (-PRL)
MDA-MB-453 (+PRL)
KIF5B
KLC1
C
Relative Expression
MDA-MB-231/Vector (-PRL)
MDA-MB-231/Vector (+PRL)
MDA-MB-231/PRLR (-PRL)
MDA-MB-231/PRLR (+PRL)
KIF5B
KLC1
D
PRL
MDA-MB-231/Vector
MDA-MB-231/PRLR
Dapi (blue)
KIF5B (green)
E
MDA-MB-231/Vector
MDA-MB-231/PRLR
Dapi (blue)
Snail1 (red)
F
IP: KIF5B
IP: IgG
MDA-MB-231/Vector (-PRL)
MDA-MB-231/Vector (+PRL)
MDA-MB-231/PRLR (-PRL)
MDA-MB-231/PRLR (+PRL)
MDA-MB-231/Vector
MDA-MB-231/PRLR
TL
KDa
KLC1
IgG
KIF5B
IgG
65
50
120
50

Figure 7

Figure 2.7 KIF5B/KLC1 loop downstream of TGFβ and PRL in TNBC

A. Quantitative invasion assays of MDA-MB-231-Scr and MDA-MB-231-Sh1-KIF5B following TGFβ stimulation. Results presented are of triplicates of three independent experiments ***$p \leq 0.001$.

B. MDA-MB-453 cells were treated with rhPRL (150 ng/ml) for 24 h and the expression of KIF5B and KLC1 mRNA were examined using q-RT-PCR. Results are expressed as relative expression of triplicates of three independent experiments ****$p \leq 0.0001$ and **$p \leq 0.01$.

C. Control MDA-MB-231/Vector and MDA-MB-231/PRLR cells were treated or not with dox (100 ng/ml) and rhPRL (250 ng/ml) for 24 hrs. KIF5B and KLC1 mRNA expression were assessed using q-RT-PCR.

D. Confocal immunofluorescence images of KIF5B (green) and nucleus (Dapi) (blue) of MDA-MB-231/Vector and MDA-MB-231/PRLR cells following treatment or not with rhPRL (250 ng/ml) for 72h. Scale bar, 10 μm.

E. Confocal immunofluorescence images of Snail1 (red) and nucleus (Dapi) (blue) of MDA-MB-231/Vector and MDA-MB-231/PRLR cells following treatment or not with rhPRL (250 ng/ml) for 72h. Scale bar, 10 μm.

F. MDA-MB-231/Vector and MDA-MB-231/PRLR treated or not with rhPRL for 72h were lysed and immunoprecipitations were performed using a rabbit monoclonal antibody against KIF5B or control normal rabbit IgG. Western blotting was carried out using a rabbit monoclonal antibody against KLC1. TL: total lysates

2.5 Discussion

Kinesins encompass a large family of proteins that are well-known to mediate intracellular movement and cytoplasmic transport of membranous organelles and macromolecules along the microtubules network. Kinesin-driven transport is mediated by the concert function of two subunits forming a complex of the motor protein (KHC) and the adaptor protein (KLC) (Gindhart et al, 1998; Hirokawa et al, 2009). Here we present evidence highlighting for the first time that kinesin-1 subunits, KIF5B and KLC1, as distinct regulators of EMP thereby contributing to breast cancer heterogeneity and aggressiveness (Fig 2.8).

2.5.1 The Differential Expression of KIF5B vs. KLC1 in Breast Cancer

The roles of Kinesin-1subunits KIF5B and KLC1 in breast tumorigenesis are still to be fully determined. Here using IHC analysis of breast cancer clinical cases we found KIF5B to be highly expressed in invasive ductal carcinoma and to be associated with poorly differentiated tumors. Moreover, and in agreement with a previous report (Marchesin et al, 2015), our IHC data also showed that TNBC clinical cases to exhibit high expression levels of KIF5B in comparison to other breast cancer subtypes. This finding was further confirmed using large bioinformatics dataset showing `KIF5B to be enriched in the basal subtype based on PAM50 and Hu et al, subclassifications. These results together implicate KIF5B as a novel biomarker of high-grade invasive breast cancer. To further examine the expression of KIF5B in relation to breast cancer subtypes, we made use of breast cancer cell lines representative of the various breast cancer molecular subtypes (Jiang et al, 2016; Lehmann et al, 2011; Prat et al, 2010). Importantly, our data showed that KIF5B to be overexpressed in breast cancer cell lines

characterized as TN-basal-like/claudin low subtype and least expressed in cell lines representative of the luminal/epithelial subtype. Importantly, cell fractionation experiments showed enrichment of KIF5B within the nuclear compartment of only TN-basal-like/claudin low cells. On the other hand, expression of KLC1, was found to correlate with favorable patient outcome and was found to exhibit different expression pattern than KIF5B. Interestingly, breast cancer cell lines as well as bioinformatics data of clinical breast cancer cases, showed KLC1 to be most expressed in luminal breast cancer subtypes including luminal A, luminal B and Her2-E and least expressed in basal-like subtype. Off note, no nuclear accumulation of KLC1 was observed in all breast cancer cells examined. Together our data emphasizes the differential expression and highlight possible independent functions of these two proteins in breast cancer.

2.5.2 Role of Kinesin-1 Subunits (KIF5B/KLC1) in Determining EMP

EMP is believed to be a critical regulator of cancer heterogeneity, disease progression and metastasis. When fully implemented cancer cells will acquire stem-like mesenchymal features exhibiting invasive/metastatic behavior resulting in high grade malignancy and resistance to available therapies. EMP may also contribute to molecular subtype conversion. Indeed, it has been shown that metastatic breast tumors of luminal but not basal-like subtype may undergo interconversion to more aggressive subtype (Cejalvo et al, 2017). These considerations underscore the interest in identifying further markers and molecular players driving the transition and switch from epithelial to mesenchymal states providing closer insights into understanding breast cancer progression and opening new avenues to more advanced therapies. TN-basal-like/claudin low breast cancer cells are known to be enriched for genes associated with EMT and to

exhibit full EMT (Dias et al, 2017; Prat et al, 2010). Our data showed that loss of KIF5B expression in these basal-like/claudin low breast cancer cells resulted in suppression of cell viability, EMT, migration, invasion, stemness and metastatic colonization of the lung. This result highlight KIF5B as a critical regulator of the EMP programming associated with the TN-basal-like/claudin low breast cancer subtype. On the other hand, KLC1 was found to be required to maintain an epithelial phenotype and to suppress EMT as well as stem cell markers endowing the cells with less invasive and less aggressive features. How kinesin1 regulates EMP is still to be fully discovered and it may involve various mechanisms. A previous report did show KIF5B to contribute to cell migration as part of the formation of invadopodia within the cytoplasm in the context of NT-basal-like/claudin low breast cancer cells (Marchesin et al, 2015). Importantly, our data point to a new mechanism. Indeed, we found KIF5B to localize in the nucleus and to interact with the EMT inducer Snail 1 transcription factor in basal/claudin low breast cancer cells. Additionally, we found that loss of KLC1 to be a determinant in the nuclear accumulation of KIF5B. These data suggest that KIF5B/KLC1 determine the transition between epithelial and mesenchymal phenotypes thereby defining the EMP status and aggressiveness of breast cancer.

2.5.3 Regulation of EMP Inducer (TGFβ) and EMP Suppressor (PRL) of Kinesin-1 Subunits KIF5B/KLC1 in Breast Cancer

Extensive studies have placed TGFβ ligands "center-stage" in regulating EMP leading to breast cancer cell invasion, metastasis and stemness (Lv et al, 2013). On the other hand, PRL is known to play an essential role in regulating mammary alveologenesis during pregnancy lactation cycle (Cui et al, 2004; Lv et al, 2013; Wagner et al, 2004).

Recently, PRL was shown to have direct effects in inducing apical/basal polarity and mammary luminal/epithelial stem cell terminal differentiation (Liu et al, 2015a). Interestingly, PRL was also found to have negative cross-talk with TGFb-Smad pathway and to suppress EMT, invasion and the tumorigenic phenotype of TNBC cells highlighting PRL as a critical suppressor of EMP (Haines et al, 2009; Lopez-Ozuna et al, 2016; Nouhi et al, 2006). Interestingly, our data implicates a central role for KIF5B/KLC1 loop in TGFβ and PRL regulation of EMP in breast cancer. Whereas TGFβ-pro-invasive activity requires KIF5B, PRL blocks KIF5B function through stable KIF5B/KLC1 complex formation there by suppressing EMP.

This manuscript highlights the role of kinesin-1 subunits KIF5B/KLC1 in breast cancer. While kinesin-1 is a large superfamily compromising various protein members, further studies are needed to investigate the role of other KIFs and KLCs in relation to each other as well as their role in breast cancer. Collectively, our study revealed a new understanding of the role of kinesin-1 in breast cancer mediating EMP programing. We propose here that the expression pattern of the two components of kinesin-1, KIF5B and KLC1, play an important role in determining breast cancer phenotype and aggressiveness.

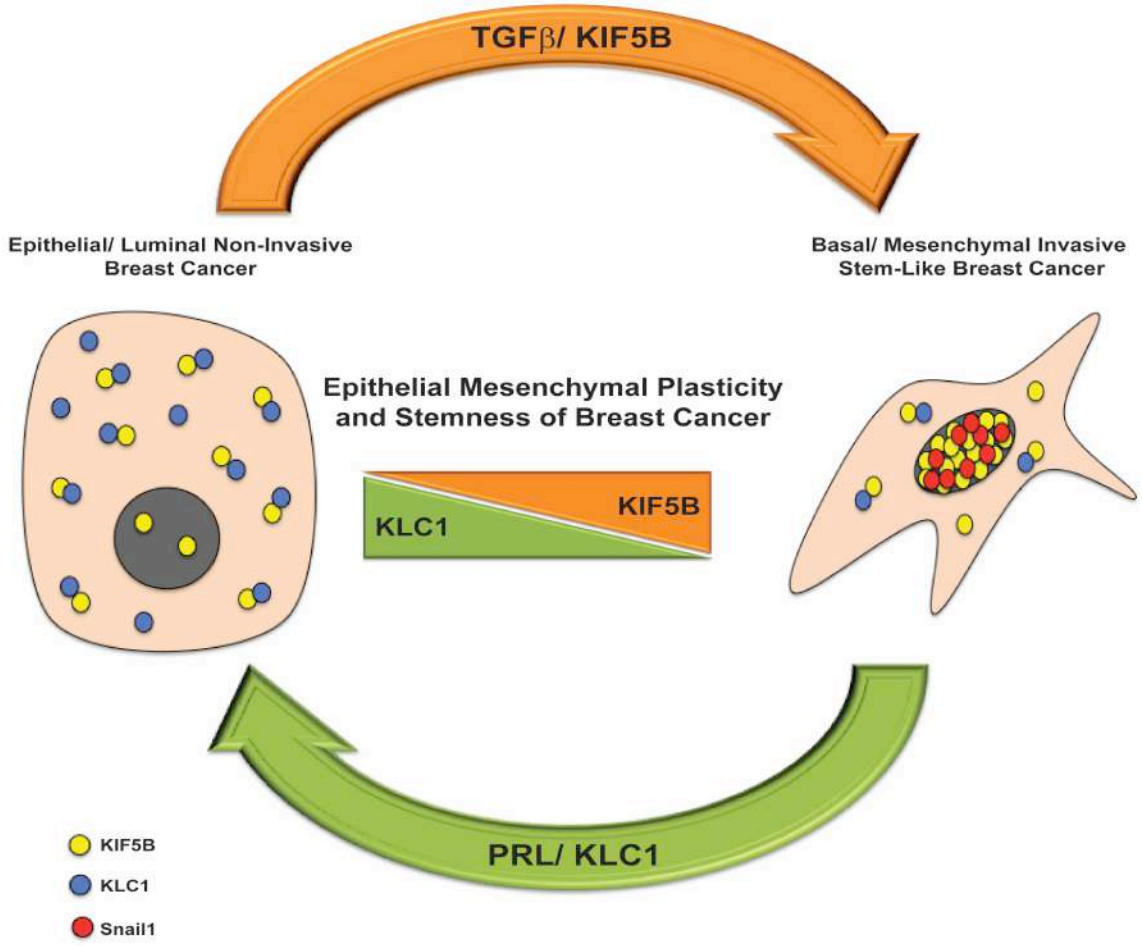


Figure 2.8 KIF5B/KLC1 complex regulates epithelial mesenchymal plasticity programing in breast cancer determining breast cancer phenotype, stemness and aggressiveness

Expression levels of KIF5B and KLC1 varies according to the molecular subtypes of breast cancer. Whereas KLC1 is expressed in the luminal/epithelial subtypes, KIF5B is expressed in the mesenchymal basal subtype showing novel nuclear accumulation and interaction with Snail1 transcription factor. Moreover, KIF5B/KLC1 loop plays a central role in TGFβ and PRL regulation of EMP in breast cancer.

2.6 Material and Methods

2.6.1 Antibodies, plasmids and reagents

Antibodies: anti-KIF5B rabbit monoclonal antibody (abcam #ab167429), anti-UKHC rabbit polyclonal antibody (Santa-Cruz #sc-28538), anti-KLC1 rabbit monoclonal antibody (abcam #ab174273), anti-PRLR rabbit polyclonal antibody (Santa-Cruz #sc-20992), anti-SNAIL1 goat polyclonal antibody (Santa-Cruz #sc-10433), anti-Cytokeratin 18 mouse monoclonal antibody (Santa-Cruz #sc-32329) and anti-E-Cadherin mouse monoclonal antibody (BD Biosciences #610182)

Antibodies used for FACS analysis were FITC mouse anti-human CD24 (BD Biosciences #555427) and APC mouse anti-human CD44 (BD Biosciences #559942)

Secondary antibodies used were goat anti-rabbit IgG HRP (Santa-Cruz #sc-2004), rabbit anti-goat IgG-HRP (Santa-Cruz #sc-2922) as well as goat anti-mouse IgG-HRP (Santa-Cruz #sc-2005). Secondary antibodies for confocal immunofluorescence studies were: donkey anti-rabbit IgG (H+L) Fluor 546 (Invitrogen), donkey anti-mouse Fluor 488 (Invitrogen), donkey anti-goat IgG-R Rhodamine conjugated (Santa-Cruz #sc-2094) and Alexa Fluor 568 phalloidin (Invitrogen #A12380).

The dilutions of antibodies for western blotting analysis are as indicated: 1: 1000 for all primary antibodies. The dilutions for secondary antibodies for western blotting analysis are 1:5000. For immunofluorescence staining: 1:100 for primary antibodies and 1: 200 for secondary antibodies. The dilution for antibodies for FACS analysis is 20:100 as recommended.

Other reagents used include: Recombinant human prolactin (rhPRL) (150 ng/ml and 250ng/ml) used for human cell stimulation was purchased from Feldan Therapeutics (1F-02-008), Recombinant ovine prolactin (oPRL) (2 μg/ml) used for HC11 cell stimulation was purchased from Sigma-Aldrich (L6520-SIGMA), SosoFast EvaGreen Supermix (Bio-Rad # 172-5201), protein A-Sepharose beads (Amersham Biosciences and GE Healthcare), 12-well plates HTS multi-well insert system format (BD Falcon) and 96-well plates (Corning #3753 and Fisher #7201216).

2.6.2 Cell culture

Normal mammary epithelial cells: mouse HC11 cells were obtained from N. Hynes (Friedrich Miescher Institute, Basel, Switzerland) and were maintained in RPMI–1640 containing 10% fetal bovine serum (FBS) (Multicell Invitrogen). Human breast cancer cells: MDA-MB-231 obtained from Dr. Shafaat Rabbani (McGill University), MDA-MB-453, SKBR3 and BT474 were obtained from Dr. Morag Park (McGill University), SCP2 and SUM159 were obtained from Dr. Jean Jacques Lebrun (McGill University). MDA-MB-231, MDA-MB-453, SKBR3 MCF7 and SCP2 cells were maintained in DMEM media (Multicell Invitrogen) containing 10% fetal bovine serum (FBS) (Multicell Invitrogen). SUM159 were maintained in Ham F-12 media (Gibco by Life Technologies) containing 5% fetal bovine serum (FBS) (Multicell Invitrogen).

2.6.3 KIF5B stable knock-down in human breast cancer cells

Lentiviral particles expressing human shRNA against KIF5B was obtained from Sigma and scramble shRNA were obtained from Addgene. The scramble shRNA is in pLKO.1

(Addgene plasmid 338651) and human KIF5B MISSION shRNA in bacterial GlycerolStock (TRCN#0000338651) (CCGGTCGGCAACTTTAGCGAGTATACTCGAGTATACTCGCTAAAGTTGCCGATTTTTG) and (TRCN#0000338580) (CCGGTTACAACTGTGGCCCTATTTACTCGAGTAAATAGGGCCACAGTTGTAATTTTTG). MDA-MB-231, SUM159 and T47D cells were infected with lentiviral particles. Stable cell lines were then generated using puromycin selection (InvivoGen) 1 μg/ml puromycin for MDA-MB-231 and 2 μg/ml for SU159 and T47D cells

2.6.4 KLC1 transient knock-down in human breast cancer cells

Silencer pre designed SiRNA against human KLC1 and Negative control SiRNA were obtained from Thermo Fisher Scientific. T47D and SKBR3 cells were infected with 28 nM SiRNA using lipofectamine 2000 protocol obtained from Thermo Fisher Scientific.

2.6.5 Western blotting analysis

Nuclear extraction lysates were collected by hypotonic buffer (10mM HEPES-KOH pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DDT, 1mM Na_3VO_4, 20mM NaF and Protease inhibitors cocktail). Then, the pellet was washed with 1X PBS 3 times. After wash, High salt buffer (20mM HEPES-KOH pH7.9,25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA pH8.0, 1mM Na_3VO_4, 20mM NaF and Protease inhibitors cocktail) were used to get the nuclear extract from the pellet. 20 μg protein was loaded in the SDS-PAGE gel.

Total protein lysates were obtained using RIPA lysis buffer (50 mM Tris pH 8, 150 mM

sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM Na_3VO_4 and Protease inhibitors cocktail). 30 µg proteins were loaded in the gel. Cell lysates were separated by electrophoresis in 8–12% sodiumdodecyl sulphate-polyacrylamide gradient minigel (SDS-PAGE) and electrophoretically transferred to anitrocellulose membrane. Western blots were probed with the relevant primary antibodies and secondary antibodies.

2.6.6 Immunoprecipitation

RIPA lysis buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM Na3VO4 and Protease inhibitors cocktail) was used to obtain the total protein lysates. Mixer of 1 µg of anti-KIF5B antibody, protein A/G beads (20 µl) and 500 µg of cell lysates were incubated for 3hrs at 4 °C. After washing the beads, western blotting with SDS-PAGE gel was performed with specific antibodies.

2.6.7 Immunofluorescence

Cells were grown on coverslips with 80% confluency. Fixation process were performed of coverslips coated with cells in 4% Paraformaldehyde for 15 minutes at room temperature, followed by permeabilization process with 0.1% Triton X-100 (Fisher). Cells were subsequently immunostained with primary antibody for an overnight period at 4 °C and followed by secondary antibody and Dapi for 1h at room temperature. Mounting media (Lerner # 13800) was used to mount the coverslips and stored at 4 °C. Confocal

microscopy was performed using Zeiss LSM 780 confocal microscope equipped with a Plan- Apochromat x63-1.4 oil immersion objective.

2.6.8 RNA isolation and RT-qPCR

HC11 cells were grown to confluence then allowed to undergo differentiation for 24 h in media containing 10% FBS, insulin and hydrocortisone. Then, cells were starved or treated with ovine PRL (sigma) for 24 h. Breast cancer cells T47D, MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 were also grown to confluence before RNA extraction was performed. MDA-MB-453 cells, MDA-MB-231/Vector and MDA-MB-231/PRLR were grown to confluence then were starved or treated with recombinant human PRL for 48h. All cells were lysed in 1 ml of trizol. Total RNA was isolated following RNA extraction protocol (Abcam, United States).

Nanodrop was used to quantify RNA concentrations at 260 nm. Total RNA 1mg was used for reverse transcription by using (iScript Reverse Transcription supermix kit # 170-8841). RT-qPCR of KIF5B, Slug, Snail, Twist, FN1, Vimentin, ZEB1, ZEB2, PRLR E-cadherin, CK18, CD44, CD24, OCT4, NANOG and SOX2) was performed.

2.6.8 Scratch assay

5×10^3 cells were seeded on 6 well plate and grown until reached confluency. A straight scratch was obtained by yellow pipette tip and scratch or wound was monitoring by taking picture at 0, 24 and 48h.

2.6.9 Invasion assay

80×10^3 cells were seeded in 24-well plates HTS multi-well insert system coated with Matrigel. Invasion assays were performed for 24 hours migrated cells were counted using five fields of triplicates for each experimental point.

2.6.10 Soft Agar Transformation Assay

30×10^3 cells were seeded into 24-well plate coated with 1% agar gel and grown in growth media with 0.6 % agar for 3 weeks. Colonies were stained by 0.05 % crystal blue. Number of colonies was counted using low power lens microscopy.

2.6.11 MTT Assay

5×10^3 cells were seeded into 96-well plate and grown for a period of 2 to 8 days. Then, cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT) at 37 °C for 2 h.

2.6.12 Gene Expression Analysis

Publically available (ONCOMINE) and (GOBO) databases were used to determine associations between KIF5B and KLC1 m-RNA expression levels and different

clinicopathological parameters in large human breast cancer cohorts. KM plotter was used to determine associations of gene expression in relation to patient outcome.

2.6.13 Tissue microarray

Tissue microarrays (TMA) (BIOMAX, BC081120) including102 human breast cancer patient's samples were used. TMA include information containing; age, grade, stage and TNM. The virtual H & E slides for those cases were available and were reviewed by a pathologist to confirm the diagnosis and that they are representative of the tumor.

2.6.14 Immunohistochemistry

Immunohistochemical staining was performed to paraffin embedded slides. After deparaffinization and rehydration slides were immersed in retrieval solution (sodium citrate 10mM, pH 6.0 buffer). The slides were incubated in hydrogen peroxide block s, followed by Ultra V Block. Slides were incubated with a rabbit anti-KIF5B Antibody. UltraVision LP Detection System HRP Polymer & DAP Plus Chromogen (Thermo Fisher Scientific, Fremont CA) was used for detection. The TMA slides were scanned using Aperio XT slide scanner (Leica Biosystems).

2.6.15 Animal models

All experimental animal work was performed in a specific-pathogen-free animal facility according to the guidelines and ethical regulations of the Research Institute

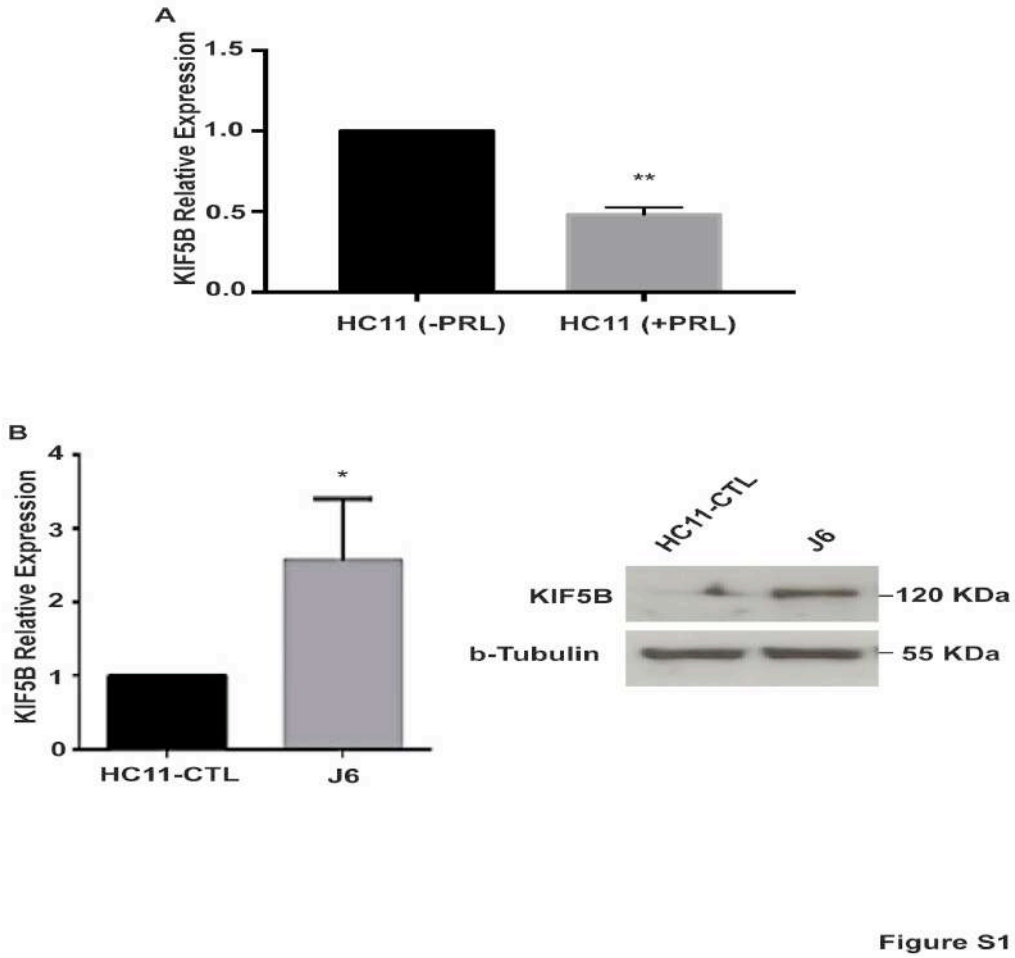
McGill University Health Centre approved animal used protocol (#2014-7492) in accordance with Canadian Council of animal care guidelines.

2.6.16 NOD- SCID Xenograft

16 Female NOD-SCID mice were purchased from Charles River Laboratories (Sain-Constant, QC, Canada), housed and maintained under specific pathogen-free conditions (RI-MUHC animal facility). The mice were randomly divided into two groups (n = 8 mice per group). At 7 to 9 weeks of age, the first group was injected in the tail vein with 5 X 10^5 MDA-MB-231-Scr and the second group was injected with MDA-MB-231-Sh-KIF5B. Mice were monitored up to 5 weeks after injection. Mice were sacrificed by CO2 asphyxiation and lungs were collected.

2.6.17 Statistical Analysis

Statistical analysis were performed using GraphPad prism 6 software using Student's *t*-test or one-way ANOVA analysis accordingly. Results were shown as mean ± SEM and $P < 0.05$ was considered as cut-off for significant association.



Supplementary Figure 2.1

A. HC11 cells were treated with oPRL (2 μg/ml) for 24 hrs and the expression of KIF5B was examined using q-RT-PCR. Results are expressed as relative expression of triplicates of three independent experiments **$p \leq 0.01$.

B. Left panel, the expression of KIF5B was examined using q-RT-PCR in both HC11-CTL and Jack2 KD HC11 (J6). Results are expressed as relative expression of triplicates of three independent experiments *$p \leq 0.05$. Right panel, immunoblot analysis of total cell lysates of HC11-CTL (control) & J6 cells using antibodies against KIF5B and β-tubulin.

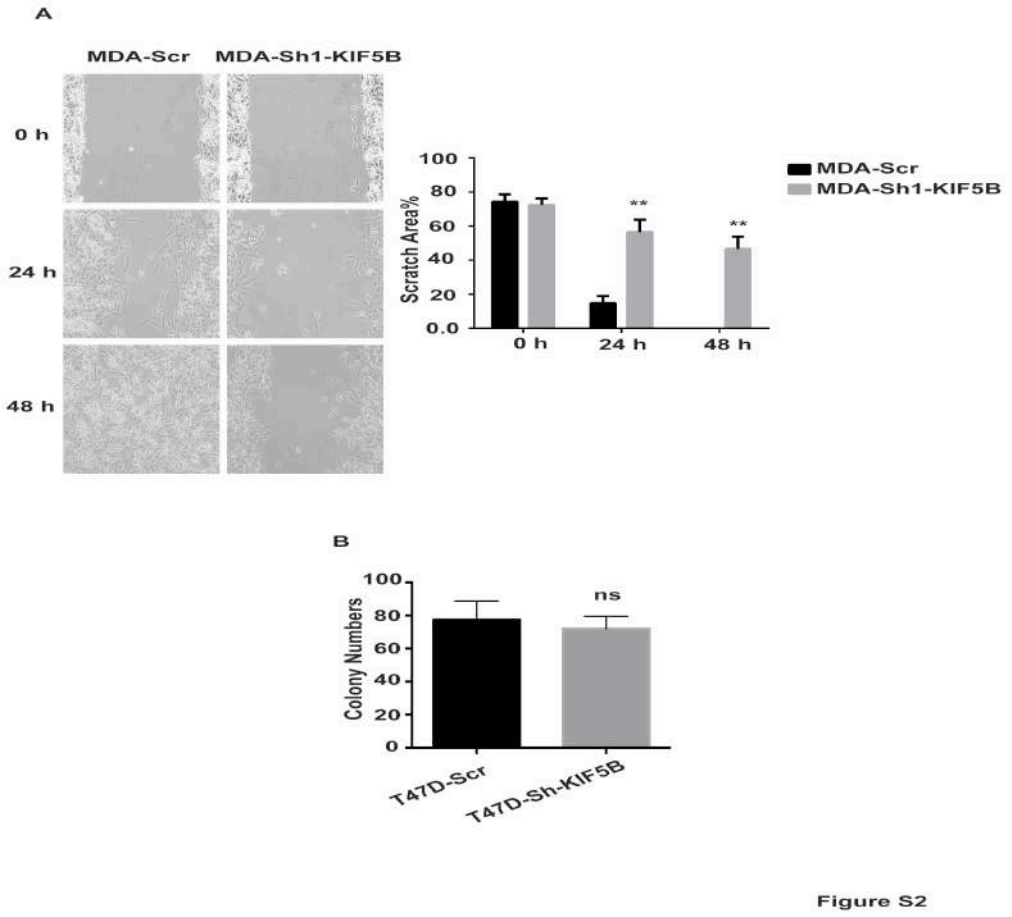


Figure S2

Supplementary Figure 2.2

A. MDA-MB-231-Scr & MDA-MB-231-Sh1-KIF5B cells were subjected to scratch wound assay (cell migration). Cells were analyzed at 24 and 48 hours. Graph shows mean ± SEM of triplicates of three independent experiments **$p \leq 0.01$.

B. Colony formation assays were performed using T47D-Scr & T47D-Sh-KIF5B for a period of three weeks (lower panel). Results are expressed as mean ± SEM of triplicates of three independent experiments. ns: non-significant

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Chapter 3: Prolactin Induces Mammary Acinar Morphogenesis Through Regulating Apical/Basal Polarity and Hippo Pathway

3.1 Preface

In our previous study, we identified the biological role of PRL in regulating Kinesin-1-induced epithelial mesenchymal plasticity (EMP). Significantly, we demonstrated that this PRL/Kinesin-1 dynamic provides a mechanism by which PRL induces differentiation and reverts/suppresses tumorigenesis. In this chapter, we investigate another mechanism through which PRL induces differentiation in mammary epithelial cells and, thus, suppresses tumorigenesis.

We have previously identified PRL as a polarity cue in mediating A/B polarity, acini morphogenesis as well as the terminal differentiation in mammary epithelial cells. Here we show that PRL induces A/B polarity and functional acini morphogenesis through modulating the proper localization of three classical polarity protein complexes, Par, Crumb and Scribble, in mammary epithelial cells. Importantly, our data also show that PRL-induced A/B polarization is mediated through activation of the tumor suppressor Hippo pathway in mammary epithelial cells.

Collectively, our findings reveal novel mechanisms through which PRL induces A/B polarity and acini morphogenesis in the mammary gland. Furthermore, our data highlight the critical pro-differentition role of prolactin within the mammary gland, thereby emphasizing the newly appreciated role of PRL as an antitumorigenic hormone.

Chapter 3:

Prolactin Induces Mammary Acini Morphogenesis Through Regulating Apical/Basal Polarity and Hippo Pathway

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3.2 Abstract

The mammary gland is a highly complex structure composed of networks of branching ducts comprised of a bilayer of ductal and myoepithelial cells. The mammary gland undergoes dramatic changes in both cellular architecture and phenotype in response to pregnancy and lactation. These changes include the establishment of apical/basal (A/B) polarity and spatially organized intercellular adhesion. When these tightly controlled events are skewed, mammary cells will acquire capabilities to initiate neoplastic transformation. The actual cellular mechanisms and hormonal changes regulating these events are not yet fully elucidated. Our previous data strongly underscored the novel role of PRL hormone in inducing the establishment of A/B polarity and acinar morphogenesis in mammary epithelial cells obtained from mid-pregnant mice. Here we show that the PRL/Jak2 pathway is a potent inducer of A/B polarity and functional acini morphogenesis in virgin state mammary cells, as determined by the proper localization of junctional proteins ZO-1 and E-cadherin as well as directional secretion of milk protein β-casein into the luminal space. Our results also highlight a new novel role for PRL in modulating the proper localization of polarity protein complexes in mammary epithelial cells. Furthermore, we interestingly show that PRL can override epidermal growth factor (EGF)-induced cellular proliferation and induce A/B polarization in the presence of EGF. Importantly, our data also show that PRL-induced A/B polarization is mediated through the activation of the tumor suppressor Hippo pathway in mammary epithelial cells. Collectively, our findings underline novel mechanisms by which PRL induces A/B polarity and acini morphogenesis in mammary gland. Moreover, our data highlight the

critical pro-differentiation role of prolactin within the mammary gland, thereby emphasizing the newly appreciated role of PRL as an antitumorigenic hormone.

3.3 Introduction

The development and remodeling of epithelial tissue compel epithelial cells to acquire specific modifications at the level of their architecture and phenotype. During the pregnancy/lactation cycle, mammary cells undergo extensive morphological and phenotypic alterations, forming a complex network of alveolar structure composed. This cellular structure is composed of differentiated epithelial cells with well-established apical/basal (A/B) polarity, functionally capable of synthesis and directional secretion of milk components into the luminal space of the mammary gland during lactation (Macias & Hinck, 2012; Rosen et al, 1993). A/B polarity is defined as asymmetric segregation of the cellular membrane where the apical domain faces the lumen while the basal domain adheres to the basement membrane. Furthermore, specialized junctional complexes including tight junctions, adherens junctions, desmosomes and GAP junctions, are formed at the lateral plasma membrane domains to maintain the intercellular adhesions, to create diffusion barriers for solutes and to define the boundary between apical and basolateral membrane domains (Brennan et al, 2010; Gumbiner, 2005). Three distinct polarity complexes (apical PAR (Par3/ Par6/ aPKC/ Cdc42) and CRB (Crumbs/ PALS/ PATJ) complexes, and basolateral SCRIB (Scribble/ Dlg/ Lgl) complex) serve as A/B polarity regulators in the mammary gland. These core polarity modules are often mutually reliant for their proper positioning in establishing cell asymmetry (Dow & Humbert, 2007; Godde et al, 2010).

Several epithelial-mesenchymal-transformation (EMT) features emerge during breast cancer progression and as A/B polarity is engaged at the interface between the morphology and proliferation control of normal mammary cells, it undeniably plays a

fundamental role in governing the EMT process in breast cancer (Godde et al, 2010). Indeed, cumulative studies have shown that loss of A/B polarity during the EMT process, excessive growth and tissue disorganization are the hallmark of aggressive breast cancer. Our knowledge of hormonal regulations and cellular mechanisms regulating these morphogenic and phenotypic events are still limited. Elucidating these mechanisms will not only expand our comprehension of the normal physiology of the mammary gland but also will enhance our understanding of breast carcinogenesis as these mechanisms are intimately involved in cancer development and progression.

The Hippo pathway is well known to mediate cell proliferation in epithelial tissues and cancers. This pathway is composed of a kinase cascade that supresses the nuclear localization and the activity of transcriptional co-activators YAP and TAZ. Indeed, activation of the Hippo pathway results in TAZ/YAP phosphorylation mediating their cytoplasmic retention and proteasomal degradation. Yet, how YAP and TAZ are physiologically regulated needs to be more fully investigated. Intriguingly, a large number of proteins, including cellular junction components (α-catenin and E-cadherin) and A/B polarity protein complexes (Crb3 and Par3), have been identified as upstream intracellular regulators of Hippo pathway activation. However, the extracellular signals that regulate this pathway are still largely unknown. Moreover, the cellular events by which the Hippo pathway and A/B polarity regulate each other are yet to be fully elucidated.

The hormone prolactin (PRL) is recognized to be indispensable in regulating mammary gland development. PRL is known to mediate its effects through activation of the Jak2/Stat5 pathway. Most importantly we have recently demonstrated that PRL

hormone is a key regulator of the establishment of A/B polarity as well as cell fate determination in mammary epithelial cells (Liu et al, 2015a). These results are in agreement with our findings showing that PRL/Jak2 signaling induces re-epithelialization of mesenchymal breast cancer cells and suppresses their invasive capacity by suppressing the process of EMT, suggesting a role for PRL in directing mammary epithelial cellular phenotype (Lopez-Ozuna et al, 2016; Nouhi et al, 2006).

Here we continue to explore the role of PRL as an inducer of A/B polarity establishment in mammary epithelial cells. Using 3D-cellular culture systems of mammary epithelial cells, we report that PRL specifically is a key factor in regulating A/B cell polarization, determined by the proper localization of polarity protein complexes. Moreover, we also show that PRL induces functional acini morphogenesis capable to synthesize and secrete milk protein within the lumen. Interestingly this complex function of PRL hormone was linked to the ability of this signaling cascade to activate the tumor suppressor Hippo pathway signaling in mammary epithelial cells, thereby activating A/B polarity. Together our data further emphasize the novel functions for PRL as an A/B polarity inducer. Furthermore, our study demonstrates a new novel role of PRL in activating Hippo pathway providing a new paradigm in PRL-induced A/B polarization in mammary gland.

3.4 Results

3.4.1 PRLR and Jak2 Localize at the Basal Domain of Mammary Acini

Studies of cell biology have shown that the use of 3D mammary cell culture *in vitro* systems offers better recapitulation of the spatial and architectural nature of the mammary gland development *in vivo*. Accordingly, using 3D cellular model systems of mammary organoids, primary mammary epithelial cells (MECs) as well as the mammary cell line HC11, we demonstrated that PRL hormone uniquely is indispensible in promoting mammary acini morphogenesis (Liu et al, 2015a). For better understanding of PRL signaling transmission in mammary acini, we first studied the localization of both PRLR and Jak2, one of the main components of PRL signaling, in mammary acini using a previously described 3D culture system. As can be seen in Fig 3.1A, PRLR as well as Jak2 interestingly localize at the basal domain of PRL-induced polarized mammary acini. These data can give better insight of how PRL signaling is transmitted in these mammary acini. Moreover, it suggests that events involving in the acquisition of A/B polarization in mammary epithelial cells begin from the basal domain of epithelial cells.

3.4.2 PRL Induces A/B Polarity in Virgin State Mammary Epithelial Cells

Our previous work revealed PRL as a crucial cue that induces the establishment of A/B polarity in mid-pregnant mammary epithelial cells (Liu et al, 2015a). As the mid pregnant mammary glands are already exposed and sensitized to various pregnancy hormones and growth factors, which make them in specific maturation stage, in the present study we studied the ability of PRL to induce A/B polarity in virgin mammary epithelial cells. We performed similar 3D experiments using MECs dissociated from

virgin C57/BL6 mice. We studied cell behavior in response to different treatments of 2% FBS (CTL), PRL and lactogenic hormone combinations of hydrocortisone (H), insulin (I) and PRL (HIP) or HI. Interestingly, we observed that colonies treated with either PRL or HIP formed mammospheres in which the cells are polarized and organized around a single lumen resembling the inner lobular core of alveoli in lactating mammary glands. These mammary acini exhibited A/B polarity determined by apical localization of ZO-1 and basal/lateral localization of E-cad. In contrast, cells grown in the presence of serum or HI formed disorganized mammospheres that are characterized by the lack of lumen and A/B polarization (Fig 3.1B). We quantified the polarized acini within different treatments and observed development of polarized acini significantly increased only in cells treated with PRL and HIP compared to cells treated with serum or HI (Fig 3.1C). Thus, our data suggest that PRL is a determinant cue of A/B polarity and a master regulator of mammary morphogenesis in spite of the differentiation state of mammary epithelial cells.

3.4.3 PRL Induces a Functional Mammary Acinar Morphogenesis Determined by Directional Secretion of β-Casein

Directional secretion and trafficking events that take place during lactation are substantial mammary alveolar properties determined by the establishment of A/B polarity. Indeed, we showed previously that PRL is required for lipid droplet apical trafficking/accumulation, signature of alveolar cell secretory differentiation (Liu et al, 2015a). To further confirm the functionality of PRL-induced acinar morphogenesis, we immunostained the mammospheres for β-casein, one of the major proteins in mammalian milk. Remarkably, only PRL and HIP treated acini were found to secrete and accumulate

β-casein in the luminal space of mammary acini (Fig 3.1D). No β-casein secretion was observed in all other conditions tested. These data together indicate PRL as a main inducer of a functional mammary acini determined by directional secretion of milk protein into the luminal space.

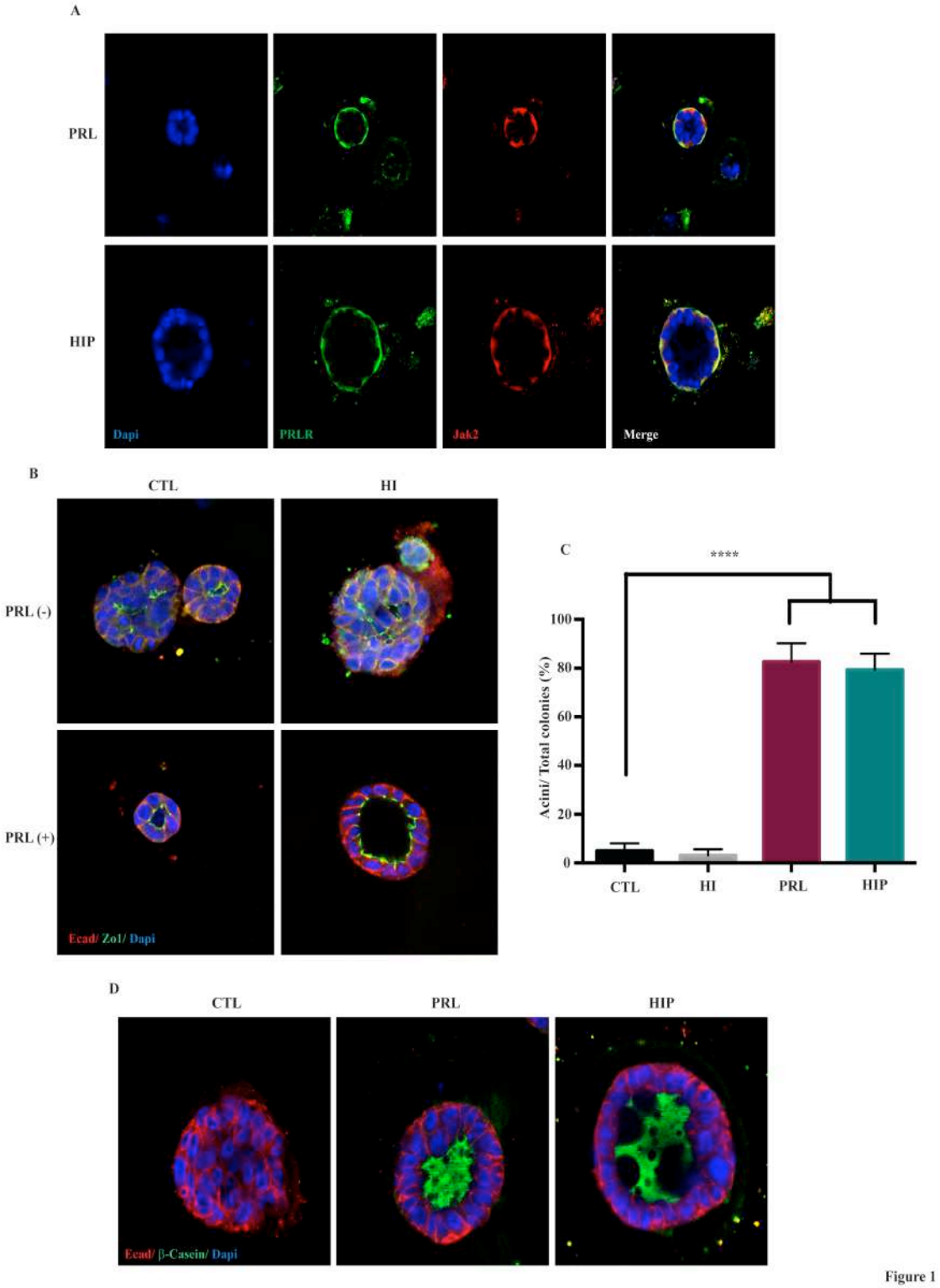
A
PRL
HIP
Dapi
PRLR
Jak2
Merge
B
CTL
HI
PRL (-)
PRL (+)
Ecad/ Zo1/ Dapi
C
****
Acini/ Total colonies (%)
100
80
60
40
20
0
CTL
HI
PRL
HIP
D
CTL
PRL
HIP
Ecad/ β-Casein/ Dapi

Figure 1

Figure 3.1 PRL induces functional mammary acini morphogenesis in virgin state mammary epithelial cells

A. MECs grown in 3D culture (Materials and Methods) were stained with antibody to PRLR (green) and Jak2 (red). Nuclei were counter stained with DAPI (blue).

B. MECs obtained from virgin state mice were grown in 3D culture and stained with ZO-1 (green) and E-cad (red) while nucleus was counter stained with DAPI (blue).

C. Percentage of mammary acini/ total colonies (> 100 colonies in triplicates) in MECs. Graph shows mean ± SEM of triplicates of three independent experiments ****$p \leq 0.0001$. P values derived from ANOVA test.

D. MECs grown in 3D culture were stained with antibody to β-casein (green) and E-cad (red). Nuclei were counter stained with DAPI (blue).

3.4.4 PRL Overrides EGF-Induced Mammary Cell Proliferation and Induces Acini Morphogenesis

We showed previously that PRL blocks EGF-induced MAPK and cell proliferation (Haines et al, 2009). However, the crosstalk of PRL with EGF in terms of regulating mammary acini morphogenesis and terminal differentiation still needs to be investigated. In order to decipher whether PRL modifies EGF signaling in mammary acini morphogenesis and terminal differentiation, we first studied mammary cell behavior using the mammosphere system in response to EGF or combined treatment of PRL and EGF (PRL/EGF). Surprisingly, cells grown in the presence of PRL/EGF combined treatment formed mammary acini in which the cells are polarized and organized around a single lumen compared to cells treated with EGF alone (Fig 3.2A). As can be seen in (Fig 3.2B), cells treated with PRL/EGF treatment showed a significant increase in the development of organized acini in comparison to cells treated with EGF alone. Importantly, PRL/EGF-induced mmammary acini were remarkably bigger in size than those induced by either PRL or HIP. Therefore, we examined the ability of PRL to attenuate the EGF-induced proliferation effect. As can be seen in Fig 3.2C, PRL pre-exposed cells were significantly less responsive to the EGF proliferative effect. As we had shown in previous work that EGF has a potent inhibitory effect on PRL-Stat5a-mediated gene transcription (Haines et al, 2009), we next tested the functionality of PRL/EGF induced mammary acini by examining the ability of these acini to produce and secrete milk into there lumens. Intriguingly, PRL/EGF treated acini failed to form and secrete β-casein into their lumens despite their fully polarized structure (Fig 3.2D). Thus, these results reveal the powerful role of PRL in overriding EGF-induced mammary cell

proliferation and promoting acinar morphogenesis independent of the functional state of these acini.

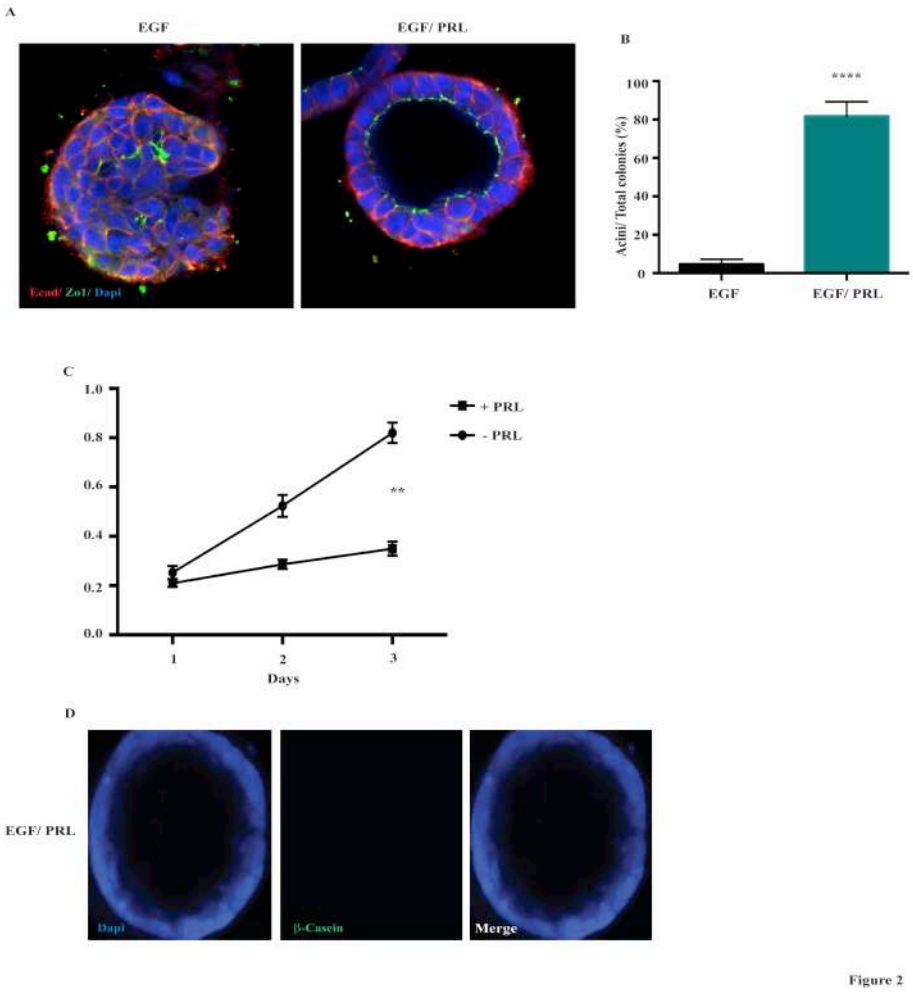

Figure 2

Figure 3.2 PRL attenuates EGF-induced cell proliferation and induces mammary acinar morphogenesis independent of their functional state and β-casein secretion

A. MECs grown in 3D culture in presence of either EGF or PRL/EGF were stained with antibody to ZO-1 (green) and E-cad (red). Nuclei were counter stained with DAPI (blue).

B. Percentage of mammary acini/ total colonies (> 100 colonies in triplicates) in MECs. Graph shows mean ± SEM of triplicates of three independent experiments ****$p \leq 0.0001$. P values derived from unpaired two-tailed Student's *t*-test.

C. MECs were grown in presence of PRL before they were treated with EGF. MTT assays were performed for 1, 2 and 8 days. Results are expressed as mean ± SD of triplicates of three independent experiments. **$p \leq 0.01$

D. MECs grown in 3D culture were stained with antibody to β-casein (green). Nuclei were counter stained with DAPI (blue).

3.4.5 PRL Impacts the Establishment of A/B Polarity by Regulating the Localization of Polarity Protein Complexes

It has been well established that A/B polarity in mammary epithelial cells is determined by evolutionarily conserved polarity protein complexes, comprising Par, Crumbs and Scribble (Bryant & Mostov, 2008b). In polarized epithelial cells, members of the Par and Crumbs complexes are known to be localized at the apical domain of the plasma membrane while Scribble complex's members localize basolaterally. Accordingly, we next examined whether PRL can also induce the proper localization of core polarity protein complexes in mammary epithelial cells. First, we examined the localization of PKCζ and Par3, members of Par complex, in PRL-induced acini. As can be seen in Fig 3.3A, PRL induces the proper localization of PKCζ and Par3 to the apical domain of the PRL-and HIP-induced acini while these proteins where found to be mislocalized in serum-or EGF-treated colonies. Examination of the Crumb complex member, Crb3, demonstrated that PRL also induces the proper apical localization of Crb3 compared to serum-or EGF-treated colonies (Fig 3.3B). Our data also showed that Llgl, a member of Scribble complex, localized properly at the basolateral domain of PRL-induced acini while it is found to be mislocalized in serum-or EGF-treated colonies (Fig 3.3C). Together, our data interestingly point out that PRL impacts A/B polarity in mammary epithelial cells by regulating the proper localization of polarity protein complexes.

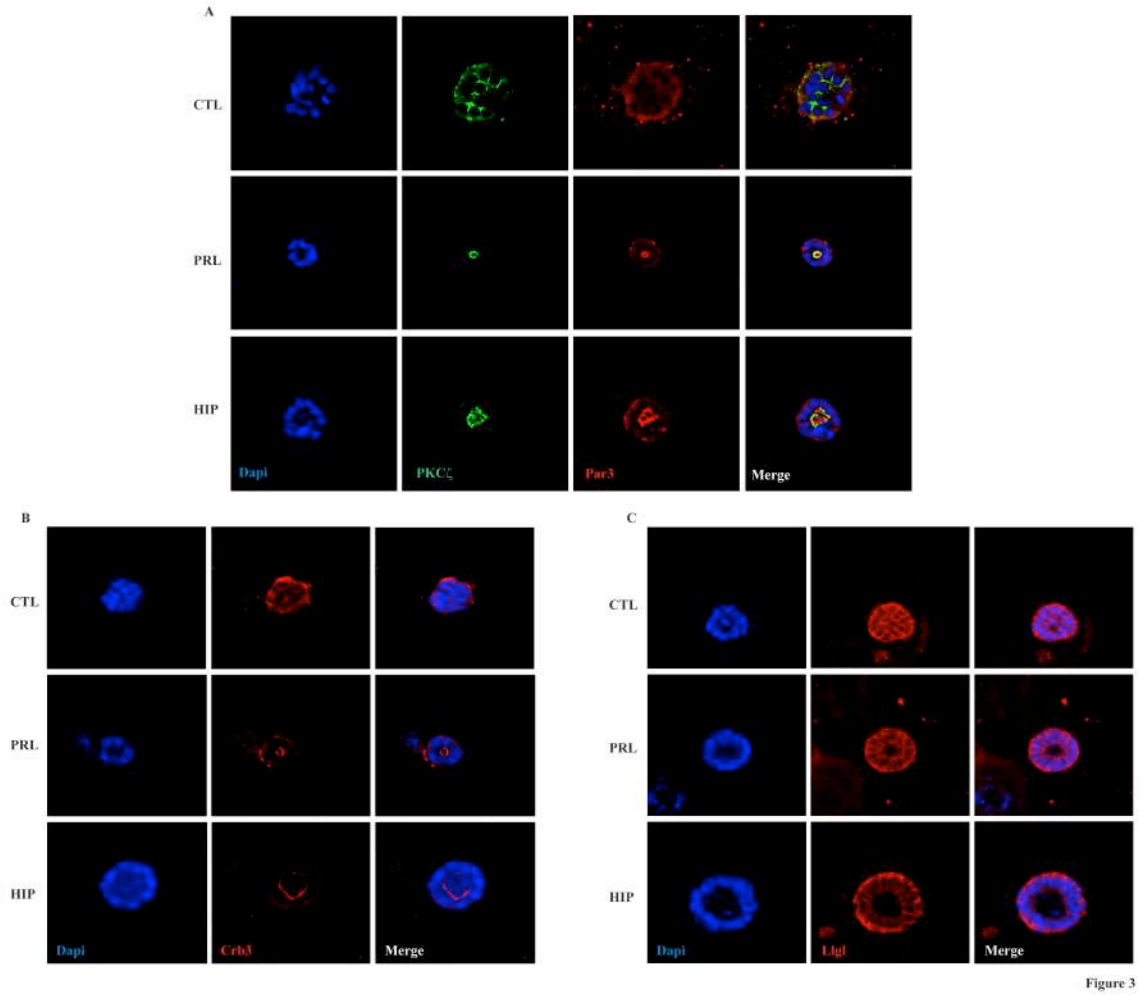

Figure 3.3 PRL induces the proper localization of polarity protein complexes in mammary epithelial calls

A. MECs grown in 3D culture were stained with antibody to PKCζ (green) and Par3 (red). Nuclei were counter stained with DAPI (blue).

B. MECs grown in 3D culture were stained with antibody to Crb3 (red). Nuclei were counter stained with DAPI (blue).

C. MECs grown in 3D culture were stained with antibody to Llgl (red). Nuclei were counter stained with DAPI (blue).

3.4.6 Prolactin/Jak2 Signaling Mediates Polarized Mammary Acinar Morphogenesis

The Jak2 kinase is well recognized to be the major kinase that mediates PRL signaling in mammary gland development and lactation. In a previous study, we identified Jak2 as a critical mediator of PRL signaling in acinar morphogenesis, A/B polarization and junctional organization in the mammary epithelial cell HC11 cell line (Liu et al, 2015a). To further confirm these findings, we next suppressed Jak2 expression in primary MECs obtained from virgin C57/BL6 mice, using specific Jak2 siRNA. Efficacy of Jak2 knockdown was verified at both mRNA and protein levels (Fig 3.4A). To further evaluate the loss of Jak2 in primary MECs, we used Stat5 phosphorylation to screen for PRL/Jak2 signaling. In contrast to Si-control cells, PRL was unable to induce Stat5 phosphorylation in Si-Jak2 cells (Fig 3.4B). We then examined the ability of Jak2 suppressed primary MECs to form acini in 3D culture. Importantly, our data showed that Si-Jak2 cells failed to form organized polarized acini in the presence of PRL or HIP compared to Si-control cells (Fig 3.4C). Therefore, our data further confirm the critical role of Jak2 in mediating PRL induced acini morphogenesis in mammary gland.

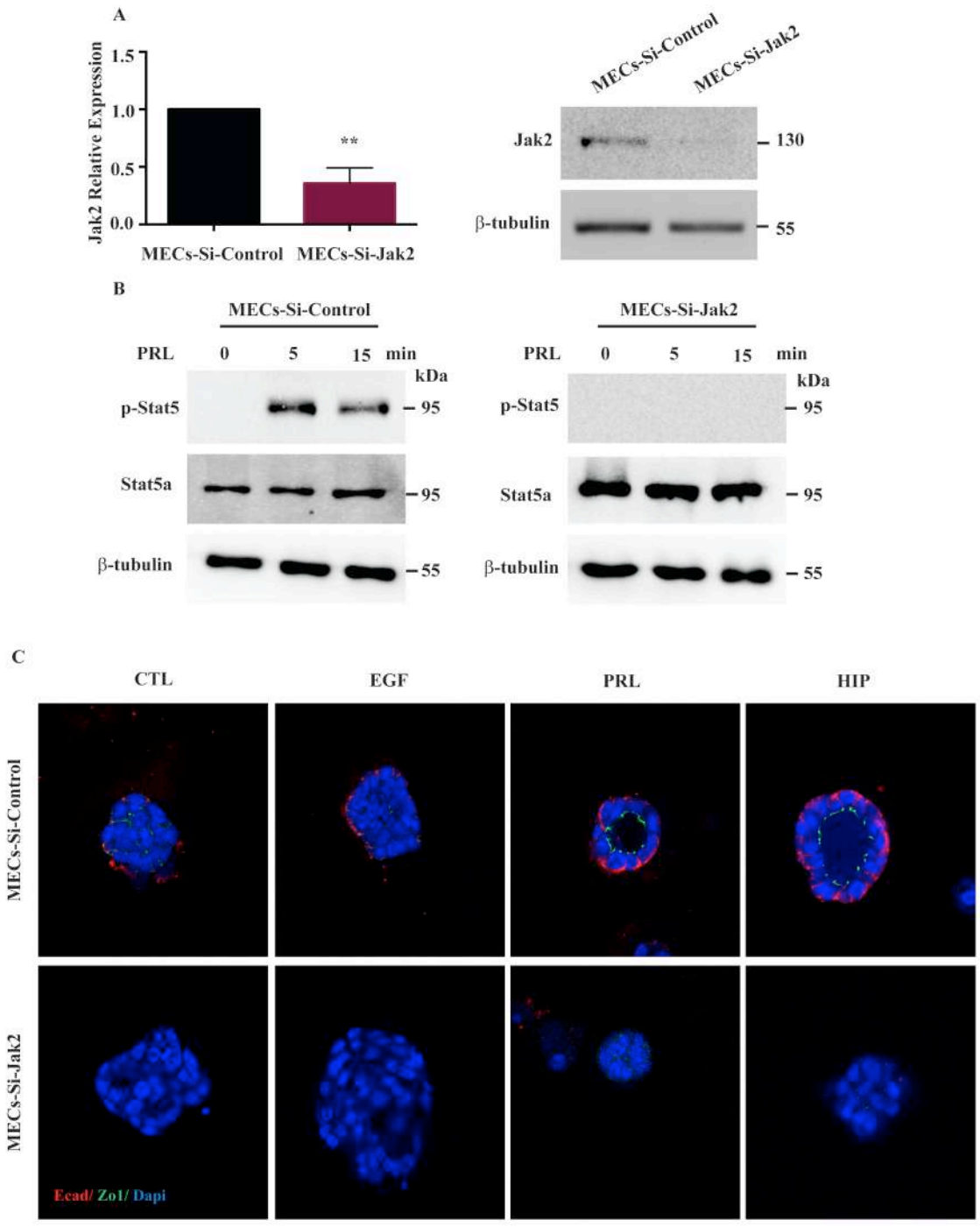
A
Jak2 Relative Expression
1.5
1.0
0.5
0.0
**
MECs-Si-Control
MECs-Si-Jak2
MECs-Si-Control
MECs-Si-Jak2
Jak2
130
β-tubulin
55
B
MECs-Si-Control
MECs-Si-Jak2
PRL
0
5
15
min
kDa
p-Stat5
95
Stat5a
95
β-tubulin
55
C
CTL
EGF
PRL
HIP
MECs-Si-Control
MECs-Si-Jak2
Ecad/ Zo1/ Dapi

Figure 4

Figure 3.4 Prolactin/Jak2 signaling mediates mammary epithelial polarity establishment

A. Left panel, m-RNA levels of Jak2 were assessed using q-RT-PCR in MECs-Si-Control & MECs-Si-Jak2 cells. Results are expressed as relative expression of triplicates of three independent experiments $^{**}p < 0.01$. Immunoblot analysis of total cell lysates of MECs-Si-Control & MECs-Si-Jak2 cells using antibodies against Jak2 and β-tubulin.

B. Western blot showing Stat5 phosphorylation in MECs-Si-Control (left panel) & MECs-Si-Jak2 (right panel) cells following PRL stimulation for 5 and 15 min. Membranes were reprobed for total Stat5 and β-tubulin.

E. MECs-Si-Control (upper panel) & MECs-Si-Jak2 (lower panel) cells grown in 3D culture were stained with antibody to ZO-1 (green) and E-cad (red). Nuclei were counter stained with DAPI (blue).

3.4.7 Prolactin/Jak2 Signaling Activates Hippo Pathway Mediated Mammary Acinar Morphogenesis

To further decipher the mechanisms by which PRL/Jak2 signaling mediates polarized mammary alveologenesis, we studied the role of PRL/Jak2 signaling in activating the Hippo pathway and thereby inhibiting YAP nuclear translocation. First, we examined YAP phosphorylation in response to PRL treatment using primary MECs. Surprisingly, YAP phosphorylation was observed in MECs following 1 hr of PRL treatment suggesting that PRL could be a potential activator of the Hippo pathway (Fig 3.5A). In order to investigate whether PRL is able to activate MST, the first kinase component of Hippo pathway, we examined MST phosphorylation following PRL stimulation. As can be seen in Fig 3.5B, MST phosphorylation was detected following 30 min of PRL stimulation. To further investigate the consequence of Jak2 loss in YAP nuclear accumulation, we used previously generated stable populations of mammary epithelial HC11 cells with Jak2 suppression (J6) (Liu et al, 2015a). Interestingly, immunofluorescence analysis of YAP showed strong nuclear accumulation of YAP in J6 cells in contrast to HC11 (Fig 3.5C). Together, our data suggest a potential novel role of PRL/Jak2 signaling in mediating Hippo pathway activation. To further evaluate the role of Hippo pathway in PRL induced mammary acinar morphogenesis and A/B polarization, we next suppressed MST1 expression in primary MECs, using specific MST1 siRNA. Efficacy of MST knockdown was verified at protein levels (Fig 3.55D). We then examined the impact of loss of MST1 in PRL induced mammary acini formation and A/B polarity using the 3D culture system. Importantly, our data showed that more that 50% of Si-MST1 colonies were unable to form organized polarized acini in response to PRL or

HIP stimulation compared to Si-control colonies where around 80% of them were found to be forming polarized mammary acini determined by the proper localization of ZO-1 and E-cad (Fig 3.5E). Altogether, our data suggest that PRL induces mammary acini morphogenesis through activating the Hippo pathway.

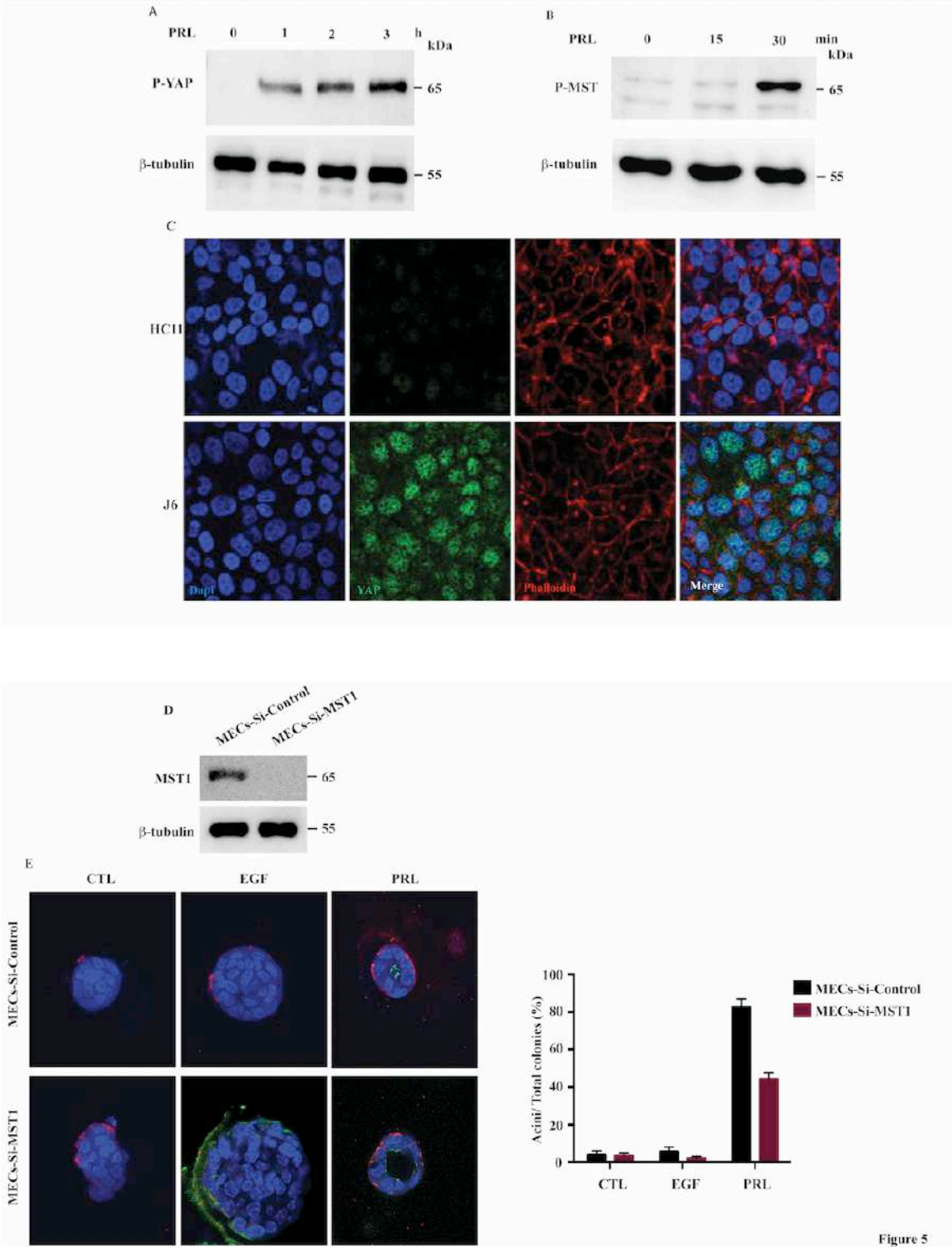
A
PRL 0 1 2 3 h
kDa
P-YAP
65
β-tubulin
55
B
PRL 0 15 30 min
kDa
P-MST
65
β-tubulin
55
C
HC11
J6
Dapi
YAP
Phalloidin
Merge
D
MECs-Si-Control
MECs-Si-MST1
MST1
65
β-tubulin
55
E
CTL
EGF
PRL
MECs-Si-Control
MECs-Si-MST1
Acini/ Total colonies (%)
100
80
60
40
20
0
CTL
EGF
PRL
MECs-Si-Control
MECs-Si-MST1

Figure 5

Figure 3.5 Prolactin induced mammary acinar morphogenesis is mediated via activation of Hippo pathway

A. Western blot showing YAP phosphorylation in MECs cells following PRL stimulation for 1, 2 and 3 h. Membranes were reprobed for β-tubulin.
B. Western blot showing MST phoshorylation in MECs cells following PRL stimulation for 15 and 30 min. Membranes were reprobed for β-tubulin.
C. Confocal immunofluorescence images of YAP (green), phalloidin (red) and nucleus (Dapi) (blue) of HC11 (control) & J6 cells. Scale bar, 10 μm.
D. Immunoblot analysis of total cell lysates of MECs-Si-Control & MECs-Si-MST1 cells using antibodies against MST1 and β-tubulin.
E. Left panel, MECs-Si-Control (upper panel) & MECs-Si-MST1 (lower panel) cells grown in 3D culture were stained with antibody to ZO-1 (green) and E-cad (red). Nuclei were counter stained with DAPI (blue). Right panel, percentage of mammary acini/ total colonies (> 100 colonies in triplicates) in MECs.

3.5 Discussion

Understanding the biology of the mammary gland is of critical significance that provides deeper insight into the breast tumorigenesis. Extensive cell biology studies have used *ex vivo* 3D culture models of mammary epithelial cells grown in extracellular matrices in the presence of hormonal and growth factors to identify molecular mechanisms involved in regulating mammary alveologenesis. Information obtained from these 3D culture model studies have emphasized the critical function of ECM and extracellular cues in the induction of mammary acinar morphogenesis (Lee & Streuli, 2014; Lo et al, 2012). However, these studies have lacked information of the PRL hormone role in governing various aspects of mammary acinar morphogenesis. Indeed, we have identified recently for the first time a novel regulatory PRL-dependent mechanism harmonizing mammary acini organization, regulating A/B polarization as well as inducing luminal cell fate determination (Liu et al, 2015a). Here we further describe the role of PRL as a critical regulator of A/B polarization, polarity protein complex localization and Hippo pathway activation in mammary epithelial cells.

Most of the current studies of A/B polarization have been obtained from drosophila and MDCK model systems (Chen et al, 2018; Halbsgut et al, 2011; Wang et al, 2016a). Moreover, limited information regarding the biology of mammary acinar morphogenesis as well as the molecular events by which mammary epithelial cells acquire and maintain A/B polarization state is available and still to be expanded. Although the fibrocystic human cell line MCF10A is arguably the most widely used breast cell model to study A/B polarity and alveologenesis, emerging studies have argued that this cell model does not fully resemble the characterization of epithelial cells and

needs to be further evaluated (Imbalzano et al, 2009; Qu et al, 2015). Therefore, using primary MECs gives better insight into normal physiology of mammary epithelial cells and molecular events regulating their morphogenesis. Using the 3D cell model system obtained from virgin state mice, we confirm here our original findings that identified PRL/Jak2 as a critical regulator of mammary acini morphogenesis and A/B polarity. Indeed, we found that PRL signaling through Jak2 kinase is indispensible in the induction of mammary acini morphogenesis and A/B polarity. This finding was supported by another original work revealing that a conditional knock out of PRL down-stream signaling components, Jak2 and STAT5, in mice resulted in failure of alveolar development (Cui et al, 2004; Han et al, 1997; Wagner et al, 2004).

Members of the EGF family have been shown to be influencing mammary gland ductal morphogenesis in pregnancy and lactation (Schroeder & Lee, 1998; Sebastian et al, 1998). On the other hand, we showed previously that PRL blocks EGF-induced MAPK and cell proliferation (Haines et al, 2009). In this study, we highlight the role of PRL in overriding EGF-induced mammary cell proliferation and promoting acini morphogenesis. These data give a better understanding of the crosstalk between PRL and EGF in regulating mammary alveologenesis.

The current view of mammary acini organization and lumen formation still lacks the information of molecular mechanisms regulating these events. Our results suggest two potential mechanisms regulating lumen formation. Indeed, we show that PRL induces the proper localization of polarity protein complexes, thereby inducing the A/B polarization and acinar morphogenesis in mammary gland. Further studies are needed to investigate the dynamics between PRL signaling and the members of the polarity

complexes that are implicated in the establishment of A/B polarity in mammary epithelial cells. Furthermore, while the link between the Hippo pathway and A/B polarity still needs to be fully determined, our data show that PRL mediates the A/B polarization induction effect through activating the Hippo pathway and blocking the nuclear accumulation of transcription factor YAP. These data identify PRL as a novel extracellular ligand regulating the activation of Hippo pathway in mammary epithelial cells through which it induces the establishment of A/B polarity.

Finally the results described here have significant implications in expanding our knowledge of the role of PRL in inducing mammary acinar morphogenesis and thereby in preventing breast tumorigenesis. Importantly, in another study we found PRL to supress the mesenchymal properties, induce an epithelial phenotype in breast cancer cells and subsequently suppress their invasive and tumorigenic behaviour (Lopez-Ozuna et al, 2016; Nouhi et al, 2006). Therefore, we believe that restoration of PRL/Jak2 signaling in breast cancer cells can induce A/B polarization in these cells and suppress breast tumorigenesis. Moreover, our study has another important implication in providing a new insight into the role of PRL in activating the tumour suppressor Hippo pathway and the how activation of this kinase cascade is involved in inducing A/B polarity in mammary epithelial cells. These results, combined with our findings that PRL, PRLR and Jak2 are markers of favorable prognosis and their expression correlate with good patient outcome, strongly implicate PRL as an anti-tumorigeneic agent and underline this pathway as an important therapeutic target against breast cancer.

3.6 Material and Methods

3.6.1 Antibodies, plasmids and other reagents

Antibodies: anti-Jak2 rabbit monoclonal antibody (Cell Signaling #3230), anti-Jak2 mouse monoclonal antibody (Santa-Cruz #sc-390539), anti-PRLR rabbit polyclonal antibody (Santa-Cruz #sc-20992), anti-phospho-Stat5 rabbit polyclonal antibody (Invitrogen #71-6900), anti-Stat5a mouse monoclonal antibody (ThermoFisher #13-3600), anti-β-casein mouse monoclonal antibody (Santa-Cruz #sc-166520), anti-β-Tubulin mouse monoclonal antibody (Santa-Cruz #sc-53140), anti-E-Cadherin rat monoclonal antibody (Sigma # U3254), anti-aPKCζ rabbit polyclonal antibody (abcam #ab59364), anti-CRB3 rat monoclonal antibody (abcam #ab180835), anti-ZO-1 mouse monoclonal antibody Alexa Fluor® 488 (Invitrogen #339188), anti-Hugl-1 goat polyclonal antibody (Santa-Cruz #sc-49990), anti-PAR3 rabbit polyclonal antibody (Millipore #07-330), anti-phospho-YAP rabbit monoclonal antibody (Cell Signaling #13008), anti-YAP rabbit polyclonal antibody (Cell Signaling #4912), anti-phospho-MST1 rabbit monoclonal antibody (Cell Signaling #49332) and anti-MST1 rabbit polyclonal antibody (Cell Signaling #3682).

Secondary antibodies used were goat anti-rabbit IgG HRP (Santa-Cruz #sc-2004), rabbit anti-goat IgG-HRP (Santa-Cruz #sc-2922) as well as goat anti-mouse IgG-HRP (Santa-Cruz #sc-2005). Secondary antibodies for confocal immunofluorescence studies were: donkey anti-rabbit IgG (H+L) Fluor 546 (Invitrogen #A10040), donkey anti-mouse Fluor 488 (Invitrogen #A21202), goat anti-rat IgG (H+L) Fluor 555 (Invitrogen #A21434), donkey anti-goat IgG-R Rhodamine conjugated (Santa-Cruz #sc-2094) and Alexa Fluor 568 phalloidin (Invitrogen #A12380).

The dilutions of antibodies for western blotting analysis are as indicated: 1: 1000 for all primary antibodies. The dilutions for secondary antibodies for western blotting analysis are 1:5000. For immunofluorescence staining: 1:100 for primary antibodies and 1: 100 for secondary antibodies.

Other reagents used include: Recombinant ovine prolactin (oPRL) (2 μg/ml) used for primary mammary epithelial cell stimulation was purchased from Sigma-Aldrich (L6520-SIGMA) and SosoFast EvaGreen Supermix (Bio-Rad # 172-5201).

3.6.2 Jak2 transient knock-down in primary mouse mammary epithelial cells

Silencer pre-designed SiRNA against human Jak2 and negative control SiRNA were obtained from Thermo Fisher Scientific. Primary mouse mammary epithelial cells were infected with 50 nM SiRNA using the lipofectamine 2000 protocol obtained from Thermo Fisher Scientific.

3.6.3 Western blotting analysis

Total protein lysates were obtained using RIPA lysis buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM Na_3VO_4 and Protease inhibitors cocktail). 30 μg proteins were loaded in the gel. Cell lysates were separated by electrophoresis in 8% sodium dodecyl sulphate-polyacrylam]=======ide gradient minigels (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane. Western blots were probed with the relevant primary antibodies and secondary antibodies.

3.6.4 3D cell culture

The Poly-D-Lysine coated 8-well culture slides (BD Biosciences) were utilized for 3D culture. Briefly, each well of the culture slide was first coated with 100 µL growth factor reduced Matrigel® (BD Biosciences), after polymerization, 5,000 cells in 100 µL growth medium were plated and allowed 1.5 hours for cells to attach. 100µL growth media containing 10% Matrigel® was added on top, creating a final concentration of 5% Matrigel® in full growth medium. Cells were maintained in growth medium with 5% Matrigel® for two days for mammosphere outgrowth. The morphology of the mammospheres was evaluated after 3 days of different treatments: (1) control (CTL): 2% FBS, (2) EGF: EGF 10ng/ml and 2% FBS or (3) HIP, 1µM hydrocortisone, 5µg/ml insulin, 2µg/ml ovine PRL and 2% FBS, (4) PRL, 2µg/ml ovine PRL and 2% FBS or (5) EGF+P: EGF 10ng/ml, 2µg/ml ovine PRL and 2% FBS. Mouse primary mammary epithelial cells (MECs) were prepared from virgin C57BL/6 (Jackson Mice) females in DMEM/F12 media with 5% FBS using a kit, STEMCELL Technologies Inc. (Canada). Isolated MECs (5,000 cells/well) were plated for 3D culture.

3.6.5 RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from confluent primary MECs with the Trizol (Invitrogen) kit according to the manufacturer's instructions. Total RNA was reverse-transcribed. The resulting cDNA was diluted 1:10 and subject to qRT-PCR analysis in a 20µl final reaction volume. Each sample was run in triplicate for Jak2 and normalized to GAPDH.

3.6.6 Immunofluorescence staining and confocal microscopy

For all 2D immunostainings, cells were plated to confluence on poly-D-Lysine (Sigma Aldrich) coated coverslips and were fixed in 4% PFA for 15 minutes at room temperature. Samples were permeabilized in 0.1% Triton X-100/PBS (PBST) and blocked with 5% normal donkey serum in PBST for 1 hour; incubations with primary and secondary antibodies were done in the same buffer. Samples were mounted in FluorSave™ (Calbiochem) supplemented with 10 µg/ml DAPI to stain nuclei. Cells in 3D culture were fixed in 4% PFA with for 1 hour at room temperature and stained as described above. Samples were imaged on a Zeiss 510 or 780 LSM confocal microscope with an Axiovert 200M microscope and a C-Apochromat 63x/1.2W Core lens.

3.6.7 MTT Assay

5 x 10^3 cells were seeded into 96-well plate and grown for 1 day. Cells then were treated with PRL for 2 days before they were exposed to EGF for 3 days. Then, cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT) at 37 °C for 2 h.

3.6.8 Statistical Analysis

Statistical significance was determined by a paired t test and ANOVA. P values less than 0.05 were considered significant.

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Chapter 4: A Novel Role of Prolactin Signaling in Maintaining Centrosome Duplication and Genomic Stability Through Regulating Polo-Like Kinase 4 (PLK4)

4.1 Preface

In chapter 3, we demonstrated the molecular mechanism through which PRL induces differentiation and thereby suppresses tumorigenesis in mammary tissue. We showed that PRL induces A/B polarity through modulating classical polarity protein complexes. Moreover, we found that PRL activates the tumor suppressor Hippo pathway in order to induce A/B polarity in mammary epithelial cells.

Here we found that PRL induced mammary differentiation is linked to the regulation of centrosome duplication and genomic stability. Indeed, our results showed that CRISPR/Cas9 knockout of the PRLR in luminal A breast cancer cells resulted in increased centrosome amplification, DNA damage as well as up-regulation of the centrosome duplication kinase, Polo-like kinase 4 (PLK 4). These data expand our knowledge of mammary cell division and add to the pool of known regulators of controlled cell division. Moreover, these newly described roles of PRL signaling in regulating centrosome duplication and genomic stability enhance our understanding of PRL functions in mammary tissue. Most importantly, these findings provide molecular insights into the protective role of PRL against mammary tumorigenesis, suggesting PRL as a valuable target for therapeutic strategies against breast cancers.

Chapter 4:

A Novel Role of Prolactin Signaling in Maintaining Centrosome Duplication and Genomic Stability Through Regulating Polo-Like Kinase 4 (PLK4)

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Keywords: Prolactin/Jak2 signaling, centrosome, genomic stability.

4.2 Abstract

Proper cell division in mammary epithelial cells is key critical for normal aciniar morphogenesis and apical/basal polarization (A/B polarization). It involves a number of events that are tightly controlled including the centrosome duplication cycle that ensures the presence of two centrosomes with optimal microtubule organization during mitosis and allows proper segregation of chromosomes to the daughter cells. Aberrant cell division and/or centrosome duplication results in DNA damage and genomic instability that contribute to tumor development and progression. Our previous data strongly revealed the novel role of prolactin hormone (PRL) in inducing the establishment of A/B polarity and acini morphogenesis in mammary epithelial cells suggesting its role in directing the axis of mammary epithelial cell division. Here we demonstrate that PRL is a key regulator of centrosome duplication and genomic stability. Our data show that loss of the PRL signaling kinase, Jak2, in primary mammary epithelial cells is sufficient to induce centrosome amplification and DNA damage in these cells. This abnormal centrosome/genomic phenotype was also observed in luminal A breast cancer MCF7 cells following CRISPR/Cas9 knockout of the prolactin receptor (PRLR). Moreover, our data demonstrate PRL as a key regulator of the centrosome duplication kinase, Polo-like kinase 4 (PLK 4), through which PRL maintains centrosome duplication and genomic stability. Lastly, our data show that mRNA expression of PLK4, pericentrin and phosphorylated histone H2A (γH2AX) is negatively associated with PRLR expression in breast cancer cases. Together, these results identify a novel role of PRL signaling in maintaining centrosome duplication and genomic stability through regulating PLK4 expression.

4.3 Introduction

The centrosome is defined as the main microtubule (MT)-organizing centre in animal cells that plays a central role in A/B polarity, migration and cell division of the cells. This sophisticated structure consists of a pair of orthogonally positioned centrioles, embedded in a complex proteinaceous structure called the pericentriolar material (PCM) (Bettencourt-Dias & Glover, 2007; Godinho & Pellman, 2014; Kim & Dynlacht, 2013). Similar to DNA, centrosome duplication happens once, and only once, per cell cycle insuring the formation of two centrosomes, which then will form the poles of the bipolar mitotic spindle (Nigg, 2007; Nigg & Stearns, 2011). Studies of the cell cycle have demonstrated that cells divide halfway between the two spindle poles, therefore ensuring the inheritance of one complete set of chromosomes as well as a single centrosome to each new daughter cell (Prosser & Pelletier, 2017). Aberrationa in centrosome duplication are sufficient to exert a profound impact on disturbing epithelial architecture and epithelial A/B polarity that favor cell expansion in response to proliferative stimuli and overproliferation, two phenotypes typically associated with human carcinomas (Godinho & Pellman, 2014; Srsen & Merdes, 2006).

Accumulating evidence has identified a number of kinases that precisely regulate centrosome duplication, including mitotic kinase Aurora A, polo-like kinase 4 (PLK 4), Polo-like kinase 1 (PLK1), Mps-1 and Cyclin-dependent kinase2-cycline E (Cdk2-E) (Hinchcliffe, 2014). PLK4 has been identified as a fundemental centriole duplication kinase that leads to centrosome amplification. PLK4 overexpression has been linked to carcinogenesis, including breast cancer (Brownlee & Rogers, 2013; Coelho et al, 2015; Levine et al, 2017). Therefore, regulating PLK4 level is of great importance for

maintaining proper centrosome duplication thereby maintaining proper orientation of cell division.

Accumulating evidence has demonstrated a link between proper centrosome duplication and maintaining genome stability. Indeed, cellular DNA damage and incomplete DNA replication may be the intermediate events that explain the association between centrosome amplification and genomic instability (Sibon et al, 2000). While a number of studies have reported several proteins involved in response to DNA damage to localize to centrosomes, it is still unclear how communication between the centrosome and DNA damage sensing occurs (Nigg & Stearns, 2011).

We have identified the differentiation factor, prolactin hormone (PRL), as a key inducer of acinar morphogenesis and A/B polarization in mammary epithelial cells (Liu et al, 2015a). These data suggest that PRL endows mammary epithelial cells to undergo symmetric cell division determined by the axis of cell division within the plane of the epithelial sheet, thereby maintaining acinar organization. In this study, we show that PRL is key regulator of centrosome duplication and genomic stability in mammary epithelial and breast cancer cells. Indeed, our data demonstrate that PRL signaling components, PRLR and Jak2, are indispensable to maintain proper centrosome number and genomic stability. Moreover, we identify PRL as a critical suppressor of PLK4 expression, resulting in maintenance of centrosome duplication and genomic stability. Lastly, we show that the mRNA expressions of PLK4, pericentrin and phosphorylated histone H2A (γH2AX) correlate with poorly differentiated tumors and poor patient outcome and are associated negatively with PRLR expression in breast cancer cases. Together, these results highlight for the first time PRL as a master regulator of centrosome duplication

and genomic stability in mammary epithelial and breast cancer cells. These findings expand our understanding of the role of PRL in mammary acinar morphogenesis as an inducer of polarity in mammary epithelial cells, and as an antitumorigenic hormone in breast cancer.

4.4 Results

4.4.1 Loss of PRLR/Jak2 Leads to Centrosome Amplification and Mitotic Spindle Aberration in Mammary Epithelial and Breast Cancer Cells

Mammary epithelial cells require A/B polarization as well as proper orientation of cell division in order to undergo appropriate mammary acinar morphogenesis. We have previously demonstrated that PRL induces mammary acinar morphogenesis and A/B polarization in mammary epithelial cells (Liu et al, 2015a). Studies have shown that centrosome aberrations are sufficient to exert a profound impact on epithelial architecture causing not only a disturbance of epithelial polarity but also favoring overproliferation of epithelial cells typically associated with human carcinomas (Schnerch & Nigg, 2016). For a better understanding whether of PRL signaling induces proper cell division of mammary epithelial cells, we first checked the consequences of suppression of Jak2, the major kinase that mediates PRL signaling, on centrosome number in mammary epithelial cells. We suppressed Jak2 expression in primary MECs obtained from virgin C57/BL6 mice, using specific Jak2 siRNA. Efficacy of Jak2 knockdown was verified at both m-RNA and protein levels (Fig 4.1A). To further evaluate the loss of Jak2 in primary MECs, we used Stat5 phosphorylation to screen PRL/Jak2 signaling. In contrast to Si-control cells, PRL was unable to induce Stat5 phosphorylation in Si-Jak2 cells (Fig 4.1B). These cells were then examined by confocal immunofluorescence using antibodies to pericentrin and γ-tubulin (major components of the centrosomes). Interestingly, our data revealed that a significant number of Si-Jak2 cells exhibit multiple centrosomes, similar to that observed in aggressive breast cancer, in comparison to Si-control cells which show a normal centrosome number (one or two per cell). Quantification of centrosome

aberrations in Si-Jak2 cells showed around 30% of the total cell population harbor multiple centrosomes (Figure 4.1C, right panel). These data demonstrate that Jak2 is a critical regulator of centrosome duplication in mammary epithelial cells.

To further confirm the role of PRL signaling in regulating centrosome amplification, we used a previously generated luminal A MCF7 human breast cancer cell line in which we used CRISPR/Cas9 technology to knockout PRLR expression (unpublished data). We synchronized both MCF7/ Scramble and MCF7/ PRLR-KO cells in order to examine the mitotic spindle assembly in these cells. As can be seen in Fig 4.1D, left panel, PRLR K/O cells exhibit multipolar centrosome organization and mitotic spindles while MCF7/Scramble cells show normal bipolar centrosome organization and mitotic spindles suggesting the crucial role of the PRL pathway in regulating centrosome number in breast cancer as well as normal cells. Centrosome and mitotic spindle aberration in MCF7/ PRLR-KO cells was quantified showing that more than 25% of the total cell population exhibit multiple centrosomes and multipolar mitotic spindles (Figure 4.1D, right panel). Together, these data implicate PRL signaling components as critical regulators of centrosome duplication and mitotic spindle orientation, thereby regulating the proper orientation of cell division in mammary epithelial and breast cancer cells.

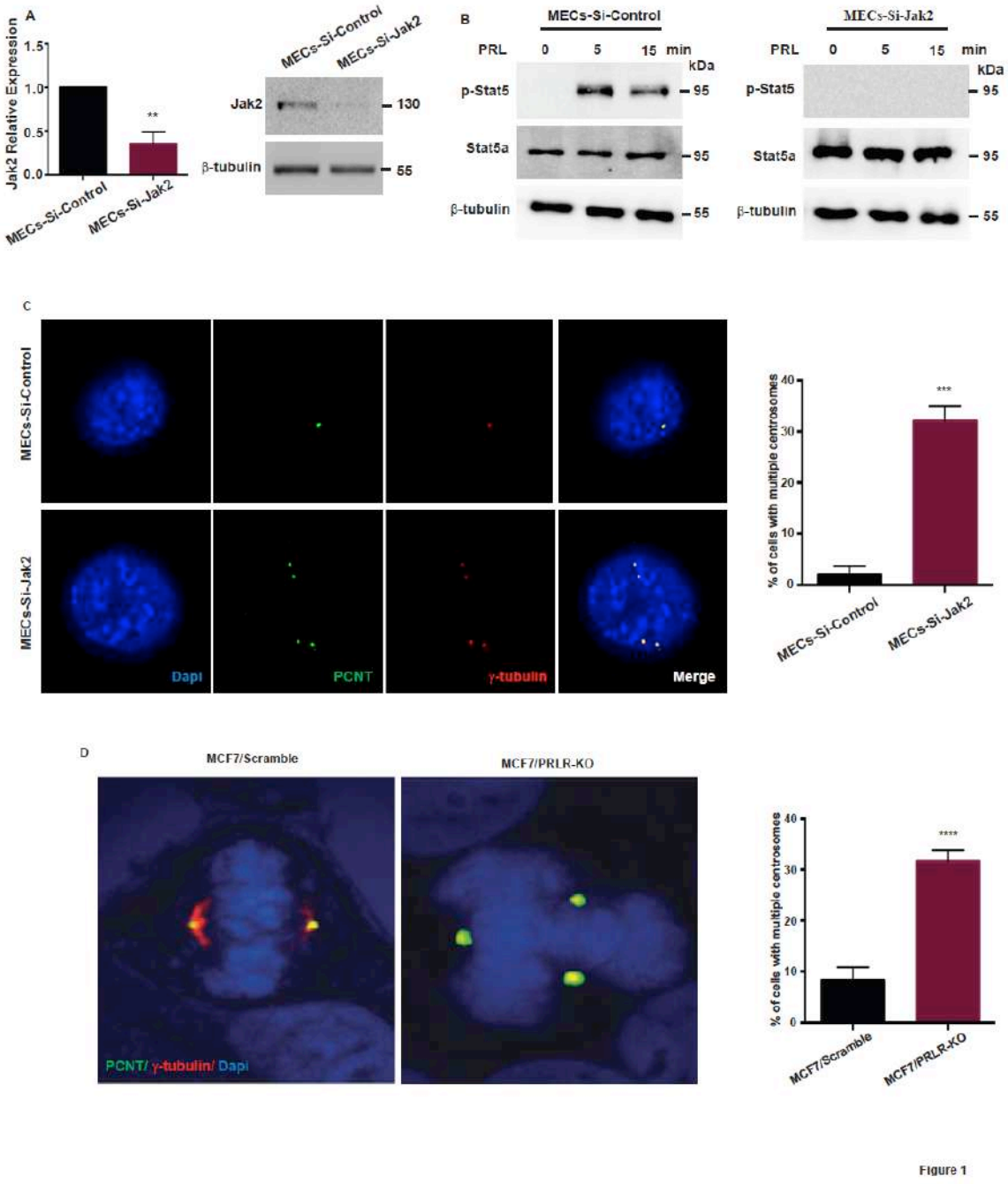

Figure 4.1 Loss of PRLR/Jak2 leads to multiple centrosomes

A. Left panel, m-RNA levels of Jak2 were assessed using q-RT-PCR in MECs-Si-Control & MECs-Si-Jak2 cells. Results are expressed as relative expression of

triplicates of three independent experiments **p < 0.01. Immunoblot analysis of total cell lysates of MECs-Si-Control & MECs-Si-Jak2 cells using antibodies against Jak2 and β-tubulin.

B. Western blot showing Stat5 phosphorylation in MECs-Si-Control (left panel) & MECs-Si-Jak2 (right panel) cells following PRL stimulation for 5 and 15 min. Membranes were reprobed for total Stat5 and β-tubulin.

C. Left panel, confocal immunofluorescence images of pericentrin (PCNT) (green), γ-tubulin (red) and nucleus (Dapi) (blue) of MECs-Si-Control (upper panel) & MECs-Si-Jak2 (lower panel) cells. Scale bar, 10 μm. Right panel, percentage of cells with multiple centrosomes/ total cells. Graph shows mean ± SD of triplicates of three independent experiments ***p ≤ 0.001. P values derived from unpaired two-tailed Student's *t*-test.

D. Left panel, confocal immunofluorescence images of pericentrin (PCNT) (green), γ-tubulin (red) and nucleus (Dapi) (blue) of MCF7/Scramble (left panel) & MCF7/PRLR-KO (right panel) cells in metaphase. Scale bar, 10 μm. Right panel, percentage of cells with multiple centrosomes/ total cells. Graph shows mean ± SEM of triplicates of three independent experiments ****p ≤ 0.0001. P values derived from unpaired two-tailed Student's *t*-test.

4.4.2 Loss of PRLR/Jak2 Leads to Genomic Instability in Mammary Epithelial and Breast Cancer Cells

Studies have shown that dysregulation in the axis of cell division and centrosome duplication results in DNA damage and genomic instability, contributing to tumor development and progression (Cosenza & Kramer, 2016). Therefore, we next examined DNA double strand breaks following loss of Jak2 in MECs using antibody to phosphorylated histone H2A designated as γH2AX (a marker of DNA damage). Interestingly, we observed increased DNA double strand breaks in Si-Jak2 primary MECs in contrast to Si-control cells (Fig 4.2A, left panel). We quantified the percentage of DNA damage in Si-Jak2 MECs compared to Si-control cells and then we observed as shown in Fig 4.2A right panel, exhibited more than 30% of Si-Jak2 MECs DNA double strand breaks in contrast to Si-control cells. To confirm the implication of PRL signaling components in maintaining genomic stability, we next examined the consequence of PRLR knockout on inducing DNA double strand breaks in the luminal A breast cancer cell line MCF7. As can be seen in Fig 4.2B, left panel, interestingly MCF7/ PRLR-KO cells exhibited increased DNA double strand breaks compared to MCF7/ Scramble cells. DNA double strand breaks occurred in more than 40% of the MCF7/ PRLR-KO cells compared to < 5% of the MCF7/ Scramble cells (Fig 4.2B, right panel). Together, our data suggested a novel role of PRL signaling in maintaining genomic stability in both mammary epithelial and breast cancer cells.

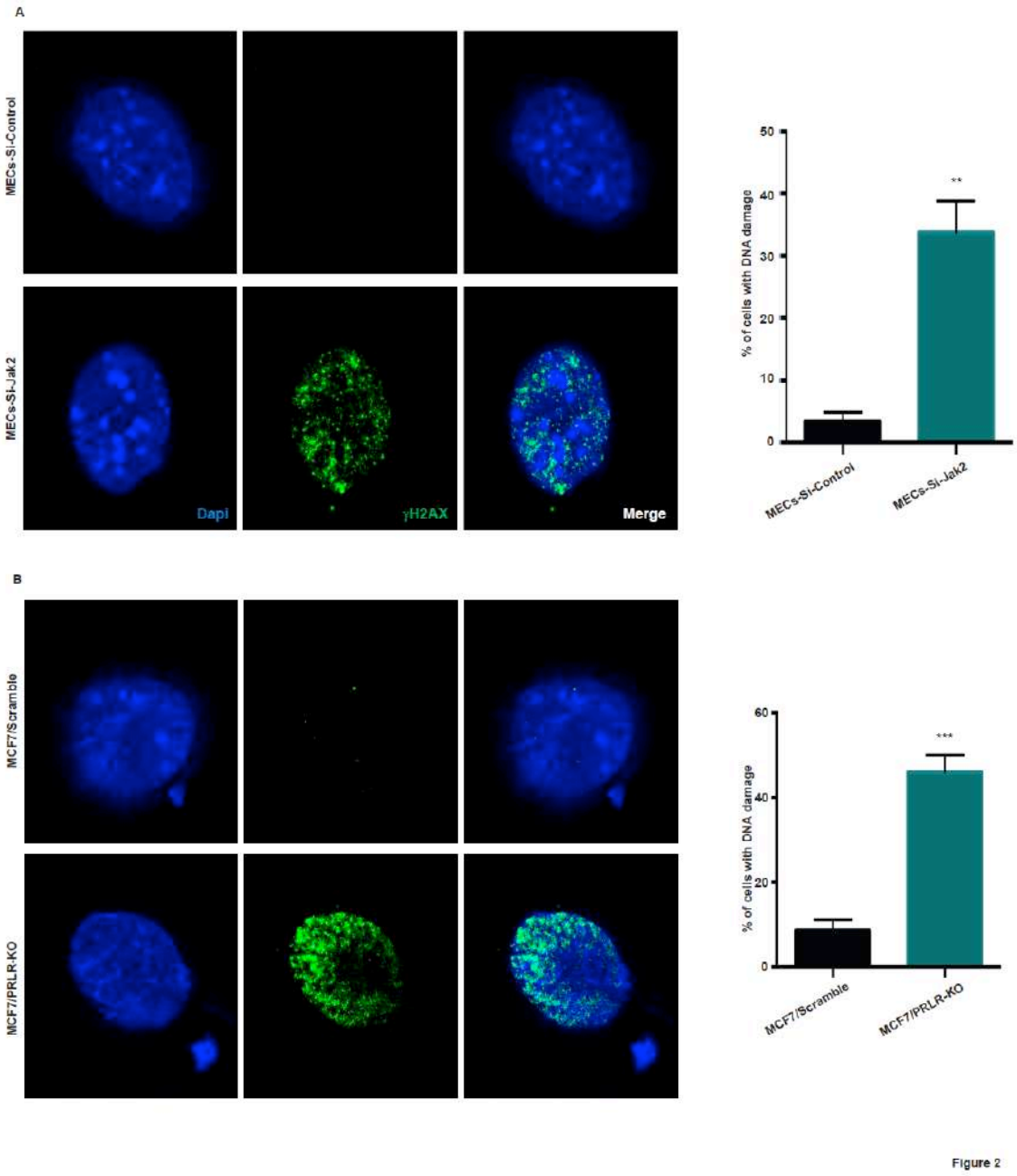

Figure 4.2 Loss of PRLR/Jak2 results in accumulation of DNA damage in nucleus

A. Left panel, confocal immunofluorescence images of γH2AX (green) and nucleus (Dapi) (blue) of MECs-Si-Control (upper panel) & MECs-Si-Jak2 (lower panel) cells. Scale bar, 10 μm. Right panel, percentage of cells with DNA damage/ total

cells. Graph shows mean ± SEM of triplicates of three independent experiments **p ≤ 0.01. P values derived from unpaired two-tailed Student's *t*-test.

B. Left panel, confocal immunofluorescence images of γH2AX (green) and nucleus (Dapi) (blue) of MCF7/Scramble (upper panel) & MCF7/PRLR-KO (lower panel) cells in metaphase. Scale bar, 10 μm. Right panel, percentage of cells with DNA damage/total cells. Graph shows mean ± SEM of triplicates of three independent experiments ***p ≤ 0.001. P values derived from unpaired two-tailed Student's *t*-test.

4.4.3 PRL Signaling Maintains Proper Centrosome Duplication and Genomic Stability Through Regulating PLK4 Expression

While centrosome duplication is a complex cellular process, polo-like kinase-4 (PLK4) has emerged as the main centriole duplication kinase; its overexpression leads to centrosome amplification and has been linked to carcinogenesis, including breast cancer (Brownlee & Rogers, 2013; Coelho et al, 2015; Levine et al, 2017). For a better understanding of the mechanism through which PRL signaling regulates centrosome duplication and thereby maintains genomic stability, we first studied PLK4 expression following loss of PRLR in the MCF7 luminal A breast cancer cell line. As can be seen in Fig 4.3A, loss of PRLR resulted in a pronounced increase in mRNA expression of PLK4 in the MCF7/PRLR-KO cells compared to MCF7/Scramble cells, suggesting the role of PRL signaling as a master regulator of PLK4 expression. This result was further confirmed using the TN-basal-like/claudin low cell line MDA-MB-231 cells engineered to overexpress the PRLR upon doxycycline treatment and designated as MDA-MB-231/PRLR cells. As can be seen in Fig 4.3B, upon doxycycline treatment to induce PRLR expression, PRL treatment suppressed PLK4 expression in MDA-MB-231/PRLR cells in contrast to MDA-MB-231/Vector cells. These data demonstrate a novel role of PRL in regulating PLK4 expression. To further assess the ability of PRL to maintain centrosome duplication and reduce the genomic instability induced in aggressive breast cancer cells, we used MDA-MB-231/PRLR breast cancer cells where we treated them with 2% FBS (control), PLK4 inhibitor (PLK4 In), PRL or a combination treatment of PLK4 inhibitor and PRL (PLK4 In/PRL). Interestingly, we observed that cells treated with PRL, PLK4 In or PLK4 In/PRL exhibited the proper number of centrosomes (one or two) compared to

untreated cells (Fig 4.3C, left panel). Of note, quantifying cells exhibiting centrosome amplification interestingly revealed that cells treated with PRL as well as PLK4 In/PRL show a significant reduction in centrosome amplification compared to PLK4 In alone (Fig 4.3C, right panel). Moreover, our data showed that while untreated MDA-MB-231/PRLR cells exhibit high DNA damage, cells treated with PLK4 In, PRL or PLK4 In/PRL showed less DNA damage, suggesting restoration of genomic stability in these cells. Interestingly, this observation was more prominant in both PRL alone and PLK4 In/PRL-treated cells compared to PLK4 inhibitor-treated cells (Fig 4.3D, left panel). Quantification of the percentage of cells exhibiting DNA damage is shown in Fig 4.3D, right panel. Collectively, our results demonstrate the novel role of PRL signaling in regulating the expression of PLK4 and thereby maintaining centrosome duplication and genomic stability.

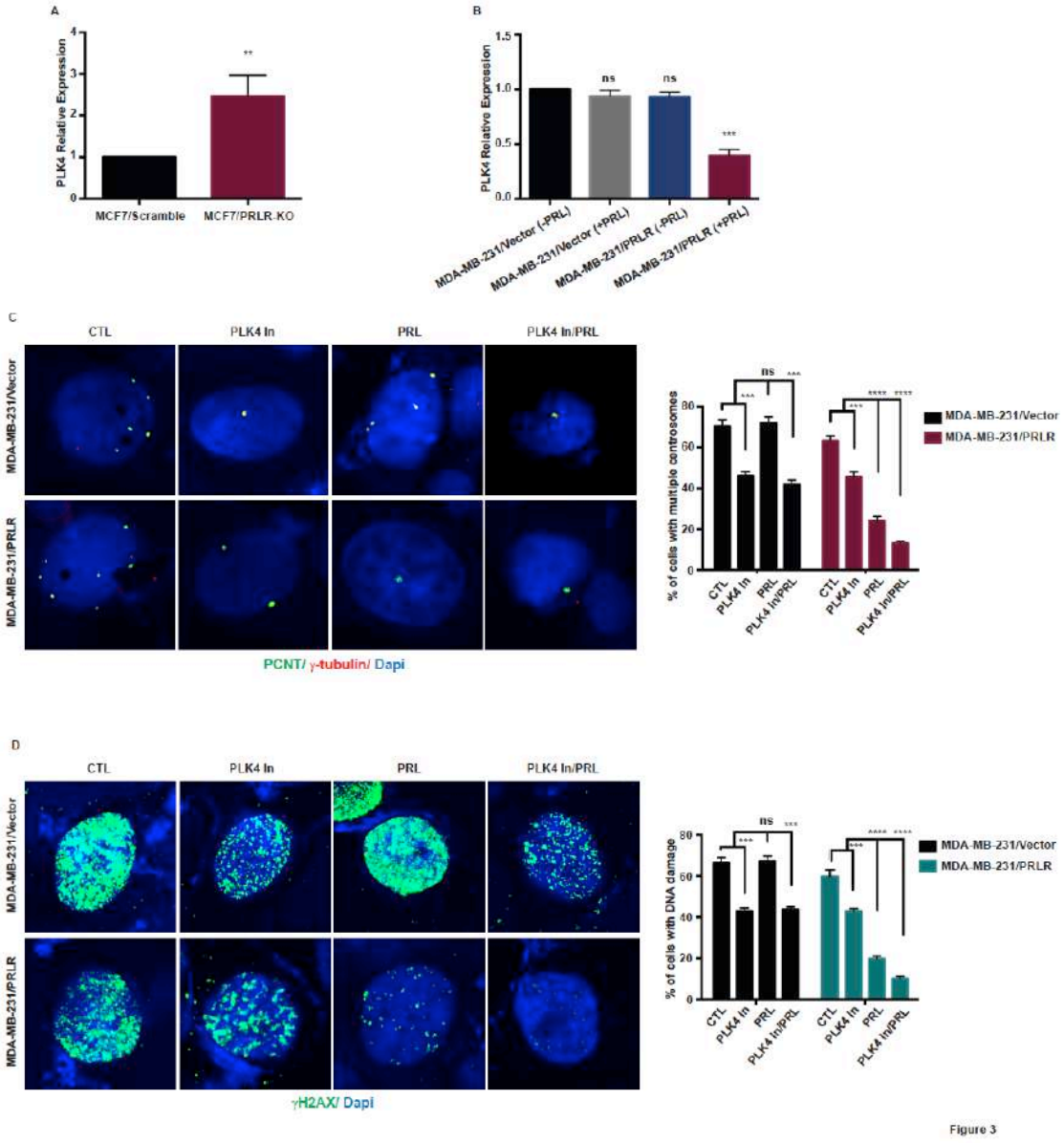

Figure 4.3 PRLR/ Jak2 regulation of PLK4

A) mRNA levels of PLK4 were assessed using q-RT-PCR in MCF7/Scramble (control) & MCF7/PRLR-KO cells. Results are expressed as relative expression of triplicates of three independent experiments; **p < 0.01.

B) Control MDA-MB-231/Vector and MDA-MB-231/PRLR cells were treated or not with dox (100 ng/ml) and rhPRL (250 ng/ml) for 72 hrs. PLK4 mRNA expression was assessed using q-RT-PCR.

C) Left panel, confocal immunofluorescence images of pericentrin (PCNT) (green), γ-tubulin (red) and nucleus (Dapi) (blue) of MDA-MB-231/Vector (upper panel) and MDA-MB-231/PRLR (lower panel) cells treated with 2% FBS, PLK4 In, PRL or PLK4 In/PRL. Scale bar, 10 μm. Right panel, percentage of cells with multiple centrosomes/total cells. Graph shows mean ± SEM of triplicates of three independent experiments ****≤ 0.0001, ***p ≤ 0.001, ns= non significant

D) Left panel, confocal immunofluorescence images of γH2AX (green) and nucleus (Dapi) (blue) of MDA-MB-231/Vector (upper panel) and MDA-MB-231/PRLR (lower panel) cells treated with 2% FBS, PLK4 In, PRL or PLK4 In/PRL. Scale bar, 10 μm. Right panel, percentage of cells with multiple centrosomes/total cells. Graph shows mean ± SEM of triplicates of three independent experiments ****≤ 0.0001, ***p ≤ 0.001, ns= non significant

4.4.4 PLK4 Induced Centrosome Amplification and DNA Damage Correlates Negatively with PRLR Expression and Positively with Poor Patient Outcome

To further evaluate the role of the previously described centrosome components, pericentrin and PLK4, as well as the DNA damage marker γH2AX in human breast cancer, we next examined their clinical relevance using publicly available databases: ONCOMINE, GOBO, Kaplan Meier plotter and bc-GenExMiner 3.0 (Gyorffy et al, 2010; Rhodes et al, 2004; Ringner et al, 2011). We first checked the clinical significance of pericentrin (PCNT) in breast cancer using the Curtis dataset (ONCOMINE) containing gene profiling data of 1700 breast cancer cases. Interestingly, we found PCNT mRNA levels to be significantly higher in invasive ductal breast cancer carcinoma (1556 cases) compared to ductal breast carcinoma *in situ* (10 cases) (P=1.78E-5) (Fig 4.4A, left panel). We next examined the mRNA levels of PCNT in the GOBO database (containing a cohort of 1411 breast cancer cases) using HU and PAM50 sub-classifications. As can be seen in Fig 4.4A, middle panel, we found PCNT mRNA levels to be highest in the basal-like subtype and lowest in the luminal A subtype (P=<0.00001). Then, we analyzed the association between PCNT mRNA levels and patient outcome represented as relapse free survival (RFS). For this we used Kaplan Meier plotter database which allow monitoring of survival of 1764 breast cancer patients for > 10 years. Interestingly, patients with higher PCNT mRNA levels showed worse outcome presented as reduced RFS (Fig 4.4A, right panel).

We then examined the clinical significance of DNA damage marker γH2AX in breast cancer using the ONCOMINE database. Our data showed that γH2AX mRNA

levels to be significantly higher in invasive ductal breast cancer carcinoma (1556 cases) compared to normal breast tissue (144 cases) (P=5.17E-77) (Fig 4.4B, left panel). Next, examining the mRNA levels of γH2AX in the GOBO database revealed that γH2AX mRNA levels were highest in the basal-like subtype in contrast to other molecular subtypes (P=<0.00001) (Fig 4.4B, upper middle panel). Using the same cohort, we found higher γH2AX mRNA levels in the poorly differentiated grade III tumors compared to grade II and I tumors with a P value of (P=<0.00001) (Fig. 4.4B, lower middle panel). Analyzing the association between γH2AX mRNA levels and patient outcome, represented as distant metastasis free survival (DMFS) and relapse free survival (RFS) using Kaplan Meier plotter database, showed that patients with higher γH2AX mRNA levels showed worse outcome presented as reduced DMFS and RFS (Fig. 4.4B, right panel).

Similarly, we checked the clinical significance of PLK4 in breast cancer using the same databases. As can be seen in Fig 4.4C, left panel, we found`q1` PLK4 mRNA levels to be significantly higher in invasive ductal breast cancer carcinoma (1556 cases) in contrast to normal breast tissue (144 cases) (P=3.82E-85). PLK4 mRNA levels were also found to be highest in the basal-like subtype compared to other molecular subtypes (P=<0.00001) (Fig 4.4C, upper middle panel). Moreover, PLK4 mRNA expression was found to be highly associated with the poorly differentiated grade III tumors compared to grade II and I tumors with a P value of (P=<0.00001) (Fig 4.4C, lower middle panel). Lastly, high PLK4 mRNA expression was shown to be associated with worse DMFS and RFS (Fig 4.4C, right panel).

To further confirm the positive association between centrosome amplification and genomic instability and their association with PLK4, we then analyzed the correlations between PCNT, γH2AX and PLK4 mRNA expression using the Breast Cancer Gene-Expression Miner v3.2 database that includes data of 5861 breast cancer patients. In agreement with our observations, we found PCNT to be positively associated with γH2AX mRNA expression (Fig 4.4D, left panel). Our data also found that PCNT and γH2AX mRNA levels are both associated with PLK4 mRNA expression (Fig 4.4D, middle and right panels). These data confirmed the positive correlation between PLK4 expression and centrosome amplification and subsequent genomic instability in breast cancer.

Next we assessed the association between PRLR and PCNT as well as PLK4 in breast cancer. As can be seen in Fig 4.4E and in agreement with our previously described data, both PCNT and PLK4 mRNA levels were found to be negatively correlated with PRLR expression, suggesting the possible role of PRL signaling in regulating centrosome duplication through regulating PLK4 activity. Collectively, these results highlight the novel role of PRL signaling in regulating PLK4 and therefore centrosome duplication and genomic stability in mammary and breast cancer cells.

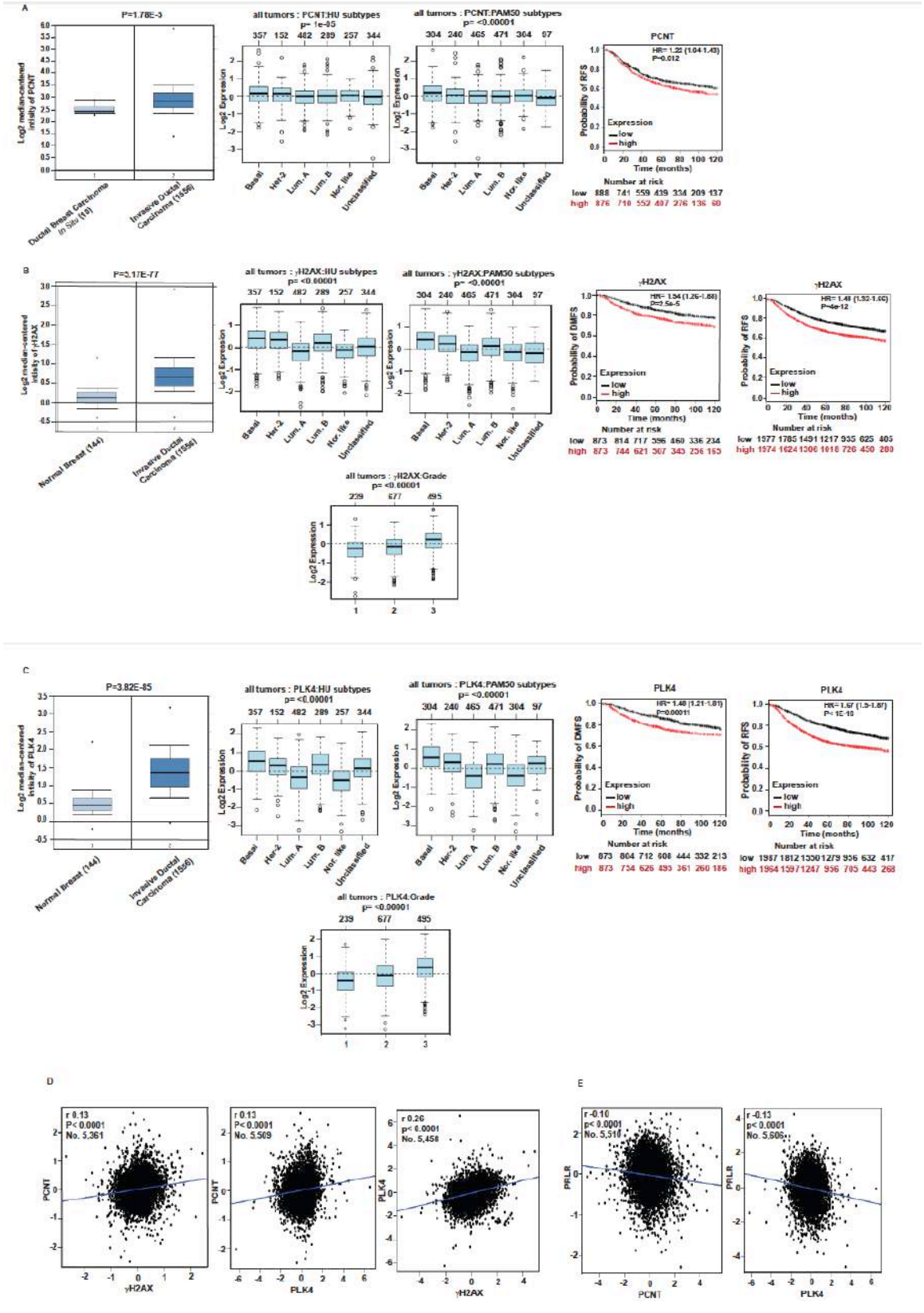
A
P=1.78E-3
Log2 median-centered intisity of PCNT
Ductal Breast Carcinoma in Situ (19)
Invasive Ductal Carcinoma (1556)
all tumors : PCNT:HU subtypes
p= 1e-05
357 152 482 289 257 344
Log2 Expression
Basal Her-2 Lum. A Lum. B Nor. like Unclassified
all tumors : PCNT:PAM50 subtypes
p= <0.00001
304 240 465 471 304 97
PCNT
HR= 1.25 (1.04-1.49)
P=0.012
Probability of RFS
Expression
low
high
Time (months)
Number at risk
low 888 741 559 439 334 209 137
high 876 710 552 497 276 136 60
B
P=3.17E-77
Log2 median-centered intisity of γH2AX
Normal Breast (144)
Invasive Ductal Carcinoma (1556)
all tumors : γH2AX:HU subtypes
p= <0.00001
357 152 482 289 257 344
all tumors : γH2AX:PAM50 subtypes
p= <0.00001
304 240 465 471 304 97
γH2AX
HR= 1.34 (1.26-1.88)
P=2.5e-5
Probability of DMFS
HR= 1.48 (1.32-1.66)
P=4e-12
Probability of RFS
low 873 814 717 596 460 336 234
high 873 744 621 507 343 256 155
low 1977 1785 1491 1217 935 625 405
high 1974 1624 1306 1018 726 450 280
all tumors : γH2AX:Grade
p= <0.00001
239 677 495
C
P=3.82E-85
Log2 median-centered intisity of PLK4
Normal Breast (144)
Invasive Ductal Carcinoma (1556)
all tumors : PLK4:HU subtypes
p= <0.00001
357 152 482 289 257 344
all tumors : PLK4:PAM50 subtypes
p= <0.00001
304 240 465 471 304 97
PLK4
HR= 1.48 (1.21-1.81)
P=0.00011
HR= 1.67 (1.5-1.87)
P< 1E-16
low 873 804 712 608 444 332 213
high 873 734 625 493 361 260 186
low 1987 1812 1330 1279 936 632 417
high 1964 1597 1247 956 705 443 268
all tumors : PLK4:Grade
p= <0.00001
239 677 495
D
r 0.13
P< 0.0001
No. 5,361
PCNT
γH2AX
r 0.13
P< 0.0001
No. 5,509
PLK4
r 0.26
p< 0.0001
No. 5,458
γH2AX
E
r -0.10
p< 0.0001
No. 5,510
PRLR
PCNT
r -0.13
p< 0.0001
No. 5,606
PRLR
PLK4

Figure 4.4 PLK4, pericentrin (PCNT) and γH2AX expression in relation to breast cancer molecular subtypes, patient outcome and PRLR expression

A. Left panel, PCNT mRNA expression levels in 10 ductal breast carcinoma *in situ* and 1556 invasive breast cancer cases using the Curtis dataset (ONCOMINE). Middle panel, PCNT mRNA expression levels in association with breast cancer molecular subtypes stratified according to Hu et al as well as PAM50 sub-classification methods in 1881 human breast cancer samples using the GOBO database. Right panel, Kaplan-Meier survival curves of PCNT gene expression in association with patient outcome (1764 patients, KM-plotter database) using RFS as an end point.

B. Left panel, γH2AX mRNA expression levels in 144 normal breast and 1556 invasive breast cancer cases using the Curtis dataset (ONCOMINE). Middle panel, γH2AX mRNA expression levels in association with breast cancer molecular subtypes stratified according to Hu et al as well as PAM50 sub-classification methods in 1881 human breast cancer samples using the GOBO database. Right panel, Kaplan-Meier survival curves of γH2AX gene expression in association with patient outcome (1746 patients, KM-plotter database) using DMFS as an end point (left panel) and Kaplan-Meier survival curves of γH2AX gene expression in association with patient outcome (3951 patients, KM-plotter database) using RFS as an end point (right panel).

C. Left panel, PLK4 mRNA expression levels in 144 normal breast and 1556 invasive breast cancer cases using the Curtis dataset of (ONCOMINE). Middle panel, PLK4 mRNA expression levels in association with breast cancer molecular

subtypes stratified according to Hu et al as well as PAM50 sub-classification methods in 1881 human breast cancer samples using the GOBO database. Right panel, Kaplan-Meier survival curves of PLK4 gene expression in association with patient outcome (1746 patients, KM-plotter database) using DMFS as an end point (left panel) and Kaplan-Meier survival curves of PLK4 gene expression in association with patient outcome (3951 patients, KM-plotter database) using RFS as an end point (right panel).

D. Correlation between PCNT and γH2AX (left panel), PCNT and PLK4 (middle panel) and between γH2AX and PLK4 (right panel) mRNA expression using thr bc-GenExMiner 3.0 database.

E. Correlation between PCNT and PRLR (left panel) and between PLK4 and PRLR (right panel) mRNA expression using the bc-GenExMiner 3.0 database.

4.5 Discussion

Supernumerary centrosomes have frequently been observed in different aggressive cancers including breast cancer (Srsen & Merdes, 2006). Here we show that PRL/Jak2 signaling is a critical regulator of centrosome duplication and maintenance of genomic stability in both mammary epithelial and breast cancer cells.

We have previously shown that PRL/Jak2 signaling induces A/B polarity and mammary acinar morphogenesis in primary mammary epithelial cells, the major phenomenon that prevents lumen filling and breast tumor progression (Liu et al, 2015a). The present data suggest that PRL/Jak2 signaling induces symmetric cell division determined by the axis of cell division within the plane of the epithelial sheet, thereby maintaining acinar organization. In agreement with this hypothesis another study has demonstrated that structural centrosome aberration disrupts the integrity of the mammary epithelial mammospheres and A/B polarity, favoring sustained proliferation in response to growth factor stimuli (Schnerch & Nigg, 2016). Our data reveal that loss of the PRL signaling components, Jak2 and PRLR, is sufficient to trigger centrosome amplification in mammary epithelial cells. These, in turn, confer properties to mammary epithelial cells that closely resemble typical features of breast carcinoma cells.

Alteration in the axis of cell division and dysregulation of centrosome duplication results in DNA damage and genomic instability, such as aneuploidy, that contribute to tumor development and progression (Cosenza & Kramer, 2016). Loss of either Jak2 or PRLR led to accumulated DNA damage in the nucleus of mammary epithelial cells, resembling cancerous cells with aggressive features. Our findings suggest that PRL signaling maintains genomic stability in mammary epithelial cells providing a protective

mechanism against breast tumorigenesis. Bcause these descriptive data do not elucidate the underlying signaling cross-talk that regulates centrosome duplication and subsequent genomic stability, further studies are needed, to provide a better understanding of PRL regulation of these two interconnected mechanisms.

While regulation of centrosome duplication is a complex cellular process, PLK4 has emerged as the master centriole duplication kinase and its overexpression has been linked to tumorigenesis, including that of mammary tissue (Brownlee & Rogers, 2013; Coelho et al, 2015; Levine et al, 2017). Indeed, inhibitor of PLK4 is currently in clinical trials as anti-cancer therapeutics (Schoffski, 2009). Our data surprisingly demonstrate PRL signaling as a critical regulator of PLK4 expression through which it regulates both centrosome duplication and genomic stability. In contrast to PLK4, this study shows PRL as a powerful supressor of centrosome amplification and DNA damage in breast cancer cells. Furthermore, our data show a negative correlation between PRLR and PLK4/ pericentrin emphasizing the potentiali role of PRL signaling components in maintaining centrosome duplication and genomic stability.

This manuscript highlights PRL regulation of proper cell division in mammary epithelial and breast cancer cells. We propose here that PRL maintains proper centrosome duplication and thereby preventing DNA damage via PLK4 regulation. These data collectively provide a new avenue in our understanding of the role of PRL in inducing mammary acini morphogenesis and of the antitumorigenic role of PRL in breast tumorigenesis.

4.6 Material and Methods

4.6.1 Antibodies, plasmids and other reagents

Antibodies: anti-Jak2 mouse monoclonal antibody (Santa-Cruz #sc-390539), anti-phospho-Stat5 rabbit polyclonal antibody (Invitrogen #71-6900), anti-Stat5a mouse monoclonal antibody (ThermoFisher #13-3600), anti-β-Tubulin mouse monoclonal antibody (Santa-Cruz #sc-53140), anti-pericentrin rabbit polyclonal antibody (abcam #ab4448), anti-γ-Tubulin mouse monoclonal antibody (Sigma #T6557), anti-γH2AX rabbit polyclonal antibody (abcam #ab11174).

Secondary antibodies used were goat anti-rabbit IgG HRP (Santa-Cruz #sc-2004) as well as goat anti-mouse IgG-HRP (Santa-Cruz #sc-2005). Secondary antibodies for confocal immunofluorescence studies were: donkey anti-rabbit IgG (H+L) Fluor 488 (Invitrogen #A21206), donkey anti-mouse Fluor 546 (Invitrogen #A10036) and Dapi (Sigma #D9542).

The dilutions of antibodies for western blotting analysis are as indicated: 1: 1000 for all primary antibodies. The dilutions for secondary antibodies for western blotting analysis are 1:5000. For immunofluorescence staining: 1:200 for primary antibodies and 1: 200 for secondary antibodies.

Other reagents used include: Recombinant human prolactin (rhPRL) (250ng/ml) used for human cell stimulation was purchased from Feldan Therapeutics (1F-02-008)-Aldrich (L6520-SIGMA) and SosoFast EvaGreen Supermix (Bio-Rad # 172-5201).

4.6.2 Isolation of primary mouse mammary epithelial cells.

Mouse primary mammary epithelial cells (MECs) were dissociated from mid-pregnant (E14-16) C57BL/6 (Jackson Mice) females using a kit from Stemcell Technologies following the manufacture's instruction.

4.6.3 Jak2 transient knock-down in primary mouse mammary epithelial cells

Silencer pre-designed SiRNA against human Jak2 and Negative control SiRNA were obtained from Thermo Fisher Scientific. Primary mouse mammary epithelial cells were infected with 50 nM SiRNA using the lipofectamine 2000 protocol obtained from Thermo Fisher Scientific.

4.6.4 Western blotting analysis

Total protein lysates were obtained using RIPA lysis buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM Na_3VO_4 and Protease inhibitors cocktail). 30 µg proteins were loaded in the gel. Cell lysates were separated by electrophoresis in 8% sodium dodecyl sulphate-polyacrylamide gradient minigels (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane. Western blots were probed with the relevant primary antibodies and secondary antibodies.

4.6.5 RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from confluent primary MECs with the Trizol (Invitrogen) kit according to the manufacturer's instructions. Total RNA was reverse-transcribed using the iScript Reverse Transcription supermix kit # 170-8841. The resulting cDNA was

diluted 1:10 and subject to qRT-PCR analysis in a 20µl final reaction volume. Each sample was run in triplicate for Jak2 and normalized to GAPDH.

MDA-MB-231/Vector and MDA-MB-231/PRLR cells were grown to confluence and then were starved or treated with recombinant human PRL for 72h. All cells were lysed in 1 ml of Trizol. Total RNA was isolated following the Abcam RNA extraction protocol (Abcam, United States). The nanodrop was used to quantify RNA concentrations at 260 nm. Total RNA 1mg was used for reverse transcription using the iScript Reverse Transcription supermix kit # 170-8841. RT-qPCR of PLK4 was then performed.

4.6.6 Immunofluorescence staining and confocal microscopy

For all 2D immunostainings, cells were grown to confluence on poly-D-Lysine (Sigma Aldrich) coated coverslips and were fixed in ice-cold 100% Methanol for 15 minutes at -20°C. Samples were blocked with 5% normal donkey serum in PBST for 1 hour; incubations with primary and secondary antibodies were done in the same buffer. Samples were mounted in FluorSave™ (Calbiochem) supplemented with 10 µg/ml DAPI to stain nuclei. Samples were imaged on a Zeiss 510 or 780 LSM confocal microscope with an Axiovert 200M microscope and a C-Apochromat 63x/1.2W Core lens.

4.6.7 Gene Expression Analysis

Publically available (ONCOMINE and GOBO) databases were used to determine associations between PCNT, γH2AX and PLK4 m-RNA expression levels and different clinicopathological parameters in large human breast cancer cohorts. The KM plotter was used to determine associations of gene expression in relation to patient outcome.

4.6.8 Statistical Analysis

Statistical significance was determined by a paired t test and ANOVA. P values less than 0.05 were considered significant.

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Chapter 5: General Discussion and Conclusions

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General Discussion and Conclusions

5.1 Overview

The role of the lactogenic PRL hormone in mammary tumorigenesis is still controversial. A number of reports support a prooncogenic role of PRL and have provided evidence of the implication of PRL/Jak2 signaling pathway in breast cancer in comparison to normal mammary tissue (Chen et al, 2012; Chen et al, 2002; Clevenger et al, 1995; Clevenger et al, 2003; Rose-Hellekant et al, 2003; Sutherland et al, 2016). In contrast, PRL/Jak2 signaling has been proposed recently as a pro-differentiation pathway in breast cancer that correlates with favorable prognosis and better patient outcome. This has led to a different hypothesis that PRL exerts an antitumorigenic role in breast carcinogenesis (Hachim et al, 2016a; Lopez-Ozuna et al, 2016; Nouhi et al, 2006; Sultan et al, 2005). The opposing evidence and points of view could be attributed to various factors related to the specificity of the antibody used, technical issues and the proposed animal models. In the presentstudies, I investigated the hypothesis that molecular mechanisms through which PRL induces its pro-differentiation function are implicated in mediating its anti-tumorigenic role in breast cancer. The specific objectives of this thesis include: (1) to elucidate the role of PRL signaling in the regulation of Kinesin-1-mediated epithelial mesenchymal plasticity and thereby suppressing breast carcinogenesis; (2) to decipher molecular mechanisms through which PRL/Jak2 signaling induces A/B polarity and mammary acinar morphogenesis; and (3) to investigate the role of PRL signaling in the regulation of centrosome duplication and genomic stability of mammary epithelial and breast cancer cells.

5.2 PRL regulates epithelial mesenchymal plasticity in breast tumorigenesis through regulating Kinesin-1 subunits KIF5B/KLC1

It is well known that the intracellular movement and the cytoplasmic transport of membranous organelles are facilitated by a concerted effort of a complex of two kinesin subunits, the motor protein (KHC) and the adaptor protein (KLC) (Gindhart et al, 1998; Hirokawa et al, 2009). **In chapter 2,** we underscored for the first time the regulatory role of kinesin-1 subunits, KIF5B and KLC1, of EMP process and their consequent contribution to breast cancer heterogeneity and aggressiveness.

KIF5B mRNA has been reported to be up-regulated in several types of cancer tissues including breast cancer indicating its possible role in tumor pathogenesis (Yu & Feng, 2010). It has been shown to be implicated in motility, migration and invasion activity in breast cancer through its involvement in the regulation of the plasma membrane targeting of MT1-MMP and its associated invadopodia (Cardoso et al, 2009; Marchesin et al, 2015; Wang et al, 2017). In agreement with this, using IHC analysis of breast cancer clinical cases, we found KIF5B to be upregulated in invasive ductal carcinoma (IDC) compared to *in situ* carcinoma and its expression correlates with aggressive phenotype, such as high grade and poor patient survival outcomes. These results indicate that KIF5B can stratify breast cancer patients with poor prognosis and poor patient outcome.

In this study, our data also demonstrated that TNBC clinical cases show high expression levels of KIF5B in comparison to other breast cancer subtypes using IHC data. Using a large bioinformatics dataset, we further confirmed this finding showing KIF5B to be enriched in the basal subtype based on PAM50 and Hu et al,

subclassifications. These results together implicate KIF5B as a novel biomarker of high-grade invasive breast cancer. This observation was in agreement with our data in breast cancer cell lines representative of the various breast cancer molecular subtypes (Jiang et al, 2016; Lehmann et al, 2011; Prat et al, 2010). Significantly, our data showed KIF5B to be overexpressed in breast cancer cell lines characterized as TN-basal-like/claudin-low subtype and least expressed in cell lines representative of the luminal/epithelial subtype. Importantly, our results also showed enrichment of KIF5B within the nuclear compartment of only TN-basal-like/claudin-low cells. On the other hand, we found KLC1 to correlate with favorable patient outcome and to exhibit different expression patterns than KIF5B. Interestingly, using bioinformatics data of clinical breast cancer cases we found KLC1 to be most expressed in luminal breast cancer subtypes including luminal A, luminal B and Her2-E and least expressed in basal-like subtype. These data were confirmed using breast cancer cell lines representative of different molecular subtypes. Of note, no nuclear accumulation of KLC1 was observed in any of the breast cancer cells examined. Together our data emphasizes the differential expression of these two proteins and highlight possible independent functions in breast cancer.

EMP is known to be a key regulator of cancer heterogeneity, tumor progression and metastasis. This allows cancer cells to acquire stem-like mesenchymal characteristics and invasive/metastatic behavior, resulting in high grade malignancy and resistance to different available remedies. A recent study has shown that the interconversion between distinct breast cancer molecular subtypes is mediated through the EMP process (Cejalvo et al, 2017). These considerations emphasize the importance of identifying further markers and molecular players driving the transition between epithelial to mesenchymal

states. This will provide a better understanding of the biology of breast cancer and will open new avenues to more advanced therapies. TN-basal-like/claudin-low breast cancer cells are known to be enriched for genes associated with EMT and to exhibit full EMT (Dias et al, 2017; Prat et al, 2010). Our data demonstrated that KIF5B expression in basal-like/claudin-low breast cancer cells is indispensable for cell viability, EMT, migration, invasion, stemness and metastatic colonization of the lung, suggesting that KIF5B is a critical regulator of EMP programming in TN-basal-like/claudin-low breast cancer cells. In contrast, our results showed that KLC1 expression is required to maintain an epithelial phenotype and to suppress EMT as well as stem cell markers resulting in cells with less invasive features. To date, molecular mechanisms through which kinesin1 regulates EMP is still to be fully determined. The Marchesin group demonstrated the contribution of KIF5B in cell migration and the formation of invadopodia within the cytoplasm of NT-basal-like/claudin-low breast cancer cells (Marchesin et al, 2015). However, this study pointed to a new mechanism by which Kinesin-1 regulated EMP in NT-basal-like/claudin-low breast cancer cells. Importantly, we found KIF5B to be enriched in the nucleus and to interact with the EMT inducer Snail 1 transcription factor in basal/claudin low breast cancer cells. Moreover, our data showed that loss of KLC1 expression to be a determinant of the nuclear accumulation of KIF5B. Therefore, our observations suggest that KIF5B/KLC1 define the transition between epithelial and mesenchymal phenotypes and consequently determine the EMP status and aggressiveness of breast cancer.

Our data interestingly implicated a central role for the KIF5B/KLC1 dynamic in the regulation of TGFβ, the EMT inducer, and PRL, EMT suppressor, of EMP in breast

cancer. Whereas TGFβ-pro-invasive activity requires KIF5B, PRL blocks KIF5B function through stable KIF5B/KLC1 complex formation thereby suppressing EMP.

In chapter 2, we demonstrated the role of kinesin-1 subunits KIF5B/KLC1 in breast cancer. Moreover, our study revealed a new understanding of the role of kinesin-1 in breast cancer mediating EMP programing. We propose here that the expression pattern of the two components of kinesin-1, KIF5B and KLC1, play an important role in determining breast cancer phenotype and aggressiveness. These findings provide a better understanding of molecular mechanisms implicated in EMP programing and therefore can be exploited for the development of new therapies targeting EMP in breast cancer.

Further studies to be done, While kinesin-1 is a large superfamily comprised of several protein members, further studies are needed to investigate the role of other KIFs and KLCs in relation to each other as well as their role in breast cancer. This will provide a better view of the role of these family members in breast tumorigenesis. Moreover, the interaction between KIF5B and the EMT inducer Snail1 in mediating EMP in breast tumorigenesis needs to be further investigated. Further studies examining possible interactions between KIF5B and other EMT transcription factors (e.g. Zeb1, Zeb2, Twist and Slug) mediating EMP in TN-basal-like/claudin-low breast cancer cells are also needed to expand our knowledge of the role of KIF5B in promoting EMP in breast carcinogenesis. While this study provided an important data of the role of the KIFB/KLC1 dynamic promoting EMP in breast cancer, still we need to investigate the molecular mechanisms through which KIF5B and KLC1 mediate their function and localization within breast cancer cells.

5.3 Prolactin/Jak2 signaling activates Hippo pathway mediated mammary acini morphogenesis

Epithelial tissues are widely distributed, lining the surfaces of internal and external organs of human bodies and playing several fundamental roles. Epithelial function is achieved by the distinctive structural organization of epithelial cells within epithelial sheet. Thus, the integrity of epithelial architecture is important in maintaining proper function of the tissue. The majority of human cancers are derived from epithelial tissue as a result of tissue disorganization. Indeed, studies have demonstrated that most of human carcinomas show loss of A/B polarity during cancer progression from benign to invasive. Therefore, A/B polarity is often considered as a gatekeeper against the carcinogenesis and metastasis (Royer & Lu, 2011). It is well known that the developing mammary gland is a highly proliferative and invasive tissue. Aberrant activation of developmental programs in mature mammary gland could be involved in breast cancer progression. Mammary luminal epithelial cells exhibit A/B polarity, and the failure to maintain this structural organization is implicated in promoting hyperplasia and breast tumorigenesis through various mechanisms, including the loss of tissue organization and function but also due to mitotic defects leading to genomic instability. (Rejon et al, 2016). Thus, investigating molecular mechanisms and extracellular cues underlying the establishment and maintenance of A/B polarity will enhance our knowledge of the early stages leading to progression of breast cancer. We previously demonstrated that PRL induces A/B polarity in the in mammary epithelial cells as well as terminal differentiation of mammary stem/progenitor cells (Liu et al, 2015a). Moreover, we have also shown that PRL hormone exerts anti-tumorigenic function in breast cancer and serves to identify

patients with better survival outcomes (Hachim et al, 2016a; Hachim et al, 2016b; Lopez-Ozuna et al, 2016).

In chapter 3, we further investigated the role of PRL in inducing A/B polarization in mammary epithelial cells. We found that PRL is a potent inducer of A/B polarity and mammary acinar morphogenesis in virgin state mammary epithelial cells. This finding was important in showing that PRL works as a polarity cue not only in mid-pregnant state mammary epithelial cells but also in that cells not exposed yet to pregnancy hormones. We also found that PRL regulates the directional secretion of milk protein β-casein into the lumen of PRL-induced mammary acini. This data was in agreement with previous report showed that PRL is required for apical trafficking/accumulation of lipid droplets, signature of alveolar cell secretory differentiation, within mammary epithelial cells (Liu et al, 2015a). Moreover, we found that PRL signaling pathway through Jak2 kinase is indispensible in the induction of mammary acini morphogenesis and A/B polarity. This finding was supported by another original work demonstrated that conditional knock out of PRL down-stream signaling components Jak2 and STAT5 resulted in failure of alveolar development in these mice (Cui et al, 2004; Han et al, 1997; Wagner et al, 2004).

A number of studies using different developmental systems have revealed that the establishment of A/B polarity requires integration of multiple external cues such as the level of diffusible morphogen, cell-cell contact and the adjacent ECM composition (Gumbiner, 2005; Gurdon & Bourillot, 2001; Kass et al, 2007). In contrast to Madin-Darby canine kidney (MDCK) cells, the most widely studied, which polarize in response to type I collagen and deposit laminin that used to establish the basement membrane,

culturing mammary epithelial cells in collagen I reverse polarization and require myoepithelial cells for laminin-1 synthesis thereby enhance basement membrane formation and direct the establishment of cell polarization (Gudjonsson et al, 2002). This suggests that the establishment of A/B polarity is separable responses and also underlines the cooperative role of myoepithelial cells during mammary acini morphogenesis. Studies of epithelial cell polarity and lumen morphogenesis have identified two main events involved in the commencement of polarization and acinar morphogenesis of epithelial cells: an primary cue that determine the axis of polarity, mediated by the interaction of epithelial cells with ECM; and then lumen formation that regulated by transport and exocytosis of membrane vesicles enclosing apical surface components (Mostov & Martin-Belmonte, 2006). Interestingly, our work reveals PRL hormone as a key extracellular cue that regulates the establishment of A/B polarity in mammary epithelial cells.

A/B polarity is maintained by three major conserved polarity complexes: the PAR, Crumbs and Scribble complexes. These protein complexes are highly dynamic and are regulated by protein-protein interactions and phosphorylation events that ultimately affect their subcellular localization and function (Bryant & Mostov, 2008a; Martin-Belmonte & Perez-Moreno, 2012). Loss of cellular polarity and mislocalization and/or downregulation of polarity protein complexes, including scribble and par3, have been reported in different type of cancers, including breast cancer (McCaffrey et al, 2012; Rothenberg et al, 2010; Xue et al, 2013; Zhan et al, 2008). The mechanisms by which polarity proteins regulate A/B polarity and thereby suppress carcinogenesis are diverse and still need to be fully characterized. In chapter 3, we found that PRL modulates the

proper localization of polarity protein complexes in PRL-induced mammary acini. Indeed, our data showed that PRL treatment regulates the proper apical localization of PAR and Crumbs complex while it regulates the Scribble complex basolateral localization. These findings suggest that PRL may regulate the establishment of A/B polarity in mammary epithelial cells through modulating the proper localization of polarity protein complexes. Further studies need to be done to determine molecular mechanisms through which PRL induces proper localization of polarity protein complexes. Indeed, we observed a preliminary data of a rapid interaction between Crb3/PKCζ in primary mouse MECs following PRL stimulation (data not shown). Moreover, we found that PRL stimulation of primary mouse MECs resulted in tyrosine phosphorylation of PKCζ (data not shown). These optimistic data could raise further questions regarding PRL regulation of A/B polarity and mammary acinar morphogenesis.

PAR complex member Cdc42 has been shown to bind annexin that is localized to PIP2-rich plasma membranes then Cdc42 guanine nucleotide exchange factor (Cdc42 GEF) protein locally activates Cdc42 (Bryant et al, 2010; Martin-Belmonte et al, 2007). This endorses the accumulation of Par6-aPKCζ complex at the apical plasma membrane (Hutterer et al, 2004). Microtubules motor proteins Dynein and Kinesins have might mediate the apical transport of polarity proteins. Indeed, Kinesins have been shown driving the apical delivery of Crumbs proteins in adult eyes, but it is not yet known this is also happening in other epithelial systems including mammary epithelium (League & Nam, 2011; Li et al, 2008). Once apical membrane established, mutual antagonism and negative feedback regulation take place between the apical and basolateral protein complexes to maintain A/B polarization axis within the epithelial sheet (Bilder et al,

2003; Tanentzapf & Tepass, 2003). For instance, the phosphorylation of Lgl and Par1 that mediated through aPKC prevents the accumulation of these basolateral proteins at the apical domain of plasma membrane. Par1 and Lgl then inhibit further basolateral localization of Baz and Par6 (Hutterer et al, 2004). The detailed mechanisms of Lgl-Par6 antagonism still need to be further studied. Moreover, a feedback loop between Rac1 and PI3K has been shown to antagonize basolateral diffusion of Crumbs (Chartier et al, 2011). These interactions, together with other antagonistic interactions maintain the establishment of distinct apical and basal domains of plasma membrane of epithelial cells, as well as they stimulate domain-specific signaling and scaffolding signaling molecules, including regulators of Rho GTPases. Examining previously described observations using our primary MECs model system in response to PRL would expand our knowledge of the molecular events underlying PRL regulation of A/B polarity and mammary acinar morphogenesis.

Most the current studies of A/B polarization have been obtained from drosophila and MDCK model systems (Chen et al, 2018; Halbsgut et al, 2011; Wang et al, 2016a). Moreover, limited information regarding the biology of mammary acinar morphogenesis as well as the molecular events by which mammary epithelial cells acquire and maintain A/B polarization state is available and still to be expanded. Although the fibrocystic human cell line MCF10A has arguably the most widely used breast cell model to study A/B polarity and alveologenesis, emerging studies have argued that this cell model does not fully resemble the characterization of epithelial cells and needs to be further evaluated (Imbalzano et al, 2009; Qu et al, 2015). Therefore, using primary MECs gives better insight into normal physiology of mammary epithelial cells and molecular events

regulating their morphogenesis. In our work, we found that using mouse MECs 3D model provides an ideal system for studying cell polarization and cell behavior in response to different physiological ligands and extracellular cues.

The current view of mammary acini organization and lumen formation still lack the information of molecular mechanisms regulating these events. However, defective in polarity protein complexes has been linked to uncontrolled tissue growth through regulating the Hippo pathway via different and context-dependent mechanisms. Indeed, Crb3 has been shown to regulate Hippo pathway via regulating the localization and stability of Expanded (Ex), the Hippo pathway regulator, while Lgl–aPKC and Scrib-Dlg act in opposite manner (Grzeschik et al, 2010; Ling et al, 2010). Cdc42 activates the non-receptor tyrosine protein kinase, Ack that phosphorylates Ex to induce Yki-dependent proliferation and organ growth (Hu et al, 2016). Whether Ack regulation of Ex is linked to A/B polarity regulation, via Cdc42 and the PAR complex, remains to be fully investigated. The regulation of Hippo signalling pathway by Lgl-aPKC is context dependent that rely on the degree of cell polarity disruption. In eye epithelium where Lgl knockdown or activation of aPKC does not disrupt cell polarity, alteration in Hpo localization is observed correlated with activation of Yki (Parsons et al, 2014a). In contrast, when the cell polarity is disrupted in the wing epithelium, deregulation of Lgl and aPKC increases Yki activity promoting cell polarity loss (Sun & Irvine, 2011). The mechanism through which the deregulation of Lgl and aPKC affects Hpo localization in the eye epithelium remains unclear. Studies have suggested that Lgl and aPKC might regulate the apical trafficking of Hpo since Lgl knockdown affects endocytic trafficking (Parsons et al, 2014b; Sun et al, 2015).

While all the previously described studies have been done in drosophila, the actual role of Hippo pathway in mammary gland development and A/B polarity still unclear. Studies have shown that nuclear YAP accumulation is dramatically observed in proliferating alveolar cells. YAP expression is significantly decreased during lactation. Furthermore, YAP-KO mice show a considerable reduction in alveolar structure. Interestingly, YAP-deficient alveoli were positive to P-Stat5, one of the downstream components of PRL pathway (Li & Gumbiner, 2016). In chapter 3, we show that PRL mediates A/B polarization induction effect through activating Hippo pathway and blocking the nuclear accumulation of transcription factor YAP. This data identifies PRL as a novel extracellular ligand regulating the activation of Hippo pathway in mammary epithelial cells through which it induces the establishment of A/B polarity.

Finally the results described in chapter 3 have significant implications in expanding our knowledge of the role of PRL in inducing mammary acini morphogenesis and thereby in preventing breast tumorigenesis. Importantly, in another study we found that PRL to supress the mesenchymal properties and induce an epithelial phenotype in breast cancer cells and subsequently supress their invasive and tumorigenic behaviour (Lopez-Ozuna et al, 2016; Nouhi et al, 2006). Therefore, we believe that restoration of PRL/Jak2 signaling in breast cancer cells can induce A/B polarization in these cells and supress breast tumorigenesis. Moreover, our study has another important implication in providing a new insight in the role of PRL in activating the tumour suppressor Hippo pathway and the how activation of this kinase cascade involved in inducing A/B polarity in mammary epithelial cells. These results combined with our findings that PRL, PRLR and Jak2 to be markers of favorable prognosis and their expression correlate with good

patient outcome strongly implicate PRL as an anti-tumorigenic agent and underline this pathway as an important therapeutic target against breast cancer.

Further studies to be done, while our data showed that PRL-induced A/B polarity in mammary epithelial cells is mediated through regulation of classical polarity protein complexes Par, Crumb and Scribble, further studies are needed to investigate how PRL modulates their localization in mammary epithelial cells. Indeed, we need to investigate if PRL directly regulates the interactions between these polarity protein complexes or it regulates other molecular pathways that indirectly modulate the proper localization of the complexes. Moreover, further studies are also needed to investigate how PRL induces activation of the Hippo pathway. Possible interactions between Hippo pathway components, MST1/2, Lats1/2, YAP and TAZ, and PRL signalling components, PRLR and Jak2 are needed to be studied. Also we need to further investigate the role of Hippo pathway components in mediating PRL induction of A/B polarity and acinar morphogenesis in mammary epithelial cells. These studies will provide better molecular insight into the role of PRL in inducing A/B polarity and mammary acini morphogenesis.

5.4 The role of prolactin signaling in maintaining centrosome duplication and genomic stability

Supernumerary centrosomes have frequently been observed in different aggressive cancer including breast cancer (Srsen & Merdes, 2006). **In chapter 4,** we showed that PRL signaling is a critical regulator of centrosome duplication thereby maintains genomic stability in mammary epithelial and breast cancer cells.

A/B polarity is intimately linked to cell division through axis of cell division positioning (orientation of the centrosomes and the microtubule network) (Ahringer, 2003). Proper cell division is also ensured by tight regulation of the centrosome duplication cycle, ensuring the presence of two centrosomes with optimal microtubule arrays at mitosis allowing proper segregation of chromosomes to the daughter cells. Aberrant regulation in the axis of cell division and centrosome duplication results in DNA damage and genomic instability contributing to carcinogenesis and tumor progression (Cosenza & Kramer, 2016). The centrosome is a non-membrane associated organelle that is a high order protein complex. Studies have identified hundreds of centrosome-interacting proteins (centrosome interactome) (Andersen et al, 2003; Galletta et al, 2016; Jakobsen et al, 2011).

We have previously showed that MECs grown in 3D culture in the presence of PRL form organized acini with well defined A/B polarization (Liu et al, 2015a). However, in the absence of PRL signaling these colonies show loss of acinar organization characterized by abnormal cell division and the lack of lumen formation. These data emphasize that, in the presence of PRL, MECs undergo symmetric cell division determined by the axis of cell division within the plane of the epithelial sheet, thereby maintaining acini organization. In agreement with this hypothesis another study has demonstrated that structural centrosome aberration disrupts the integrity of the mammary epithelial mammospheres and A/B polarity, favoring sustained proliferation in response to growth factor stimuli (Schnerch & Nigg, 2016). Our data revealed that loss of PRL signaling components, Jak2 and PRLR, is sufficient to trigger centrosome amplification

in mammary epithelial cells. These in turn confer properties to mammary epithelial cells that closely resemble typical features of breast carcinoma cells.

Alteration in the axis of cell division and dysregulation of centrosome duplication results in DNA damage and genomic instability, such as aneuploidy that contribute to tumor development and progression (Cosenza & Kramer, 2016). Loss of either Jak2 or PRLR led to accumulated DNA damages in the nucleus of mammary epithelial cells resembling cancerous cells with aggressive features. Our findings suggested that PRL signaling maintains genomic stability in mammary epithelial cells providing a protective mechanisms against breast tumorigenesis. Although these descriptive data are far from elucidating the underlying signaling cross-talks that regulates centrosome duplication and subsequent genomic stability, further extensive studies are needed to investigate these molecular signaling and provide better understanding of PRL regulation of these two interconnected mechanisms.

While regulation of centrosome duplication is a sophesticated cellular process, polo-like kinase-4 (PLK4) has emerged as the master centriole duplication kinase and its overexpression has been linked to tumorigenesis, including that of the mammary tissue (Brownlee & Rogers, 2013; Coelho et al, 2015; Levine et al, 2017). Indeed, inhibitors of PLK4 are currently in clinical trials as anti-cancer therapeutics (Schoffski, 2009). Our data surprisingly demonstrated PRL signaling as a critical regulator of PLK4 expression level throgh which it regulates centrosome duplication and genomic stability. In contrast to PLK4, this study showed PRL as a powerful supressor of centrosome amplification and DNA damage in breast cancer cells. Furthermore, our data showed the negative

correlation between PRLR and PLK4/ pericentrin emphasizing the potentiali role of PRL signaling components in maintaining centrosome duplication and genomic stability.

Chapter 4 highlighted PRL regulation of proper cell division in mammary epithelial and brreast cancer cells. We proposed that PRL maintains proper centrosome duplication and therefore preventing DNA damage via PLK4 regulation. These data collectively provide a new avenue in understading of the role of PRL in inducing mammary acini morphogenesis and of the antitumorigenic role of PRL aginst breast tumorigenesis.

Further studies to be done, while we identified the role of PRL in regulating PLK4 expression in mammary epithelial and breast cancer cells, further studies are needed to investigate the role of PRL signalling in regulating the expression of other centrosome duplication regulators including AURKA, PLK1 and Cdk2-E. We need to investigate how loss of PRL signaling components PRLR, Jak2 and Stat5 affects centrosome duplication regulators in mammary epithelial as well as in breast cancer cells. Moreover, further studies are needed to demonstrate the link between PRL induced mammary differentiation and acini morphogenesis and the regulation of centrosome duplication and genomic stability.

5.5 Conclusions

The results described in this thesis have important implications in expanding our knowledge of the role of PRL in inducing epithelial differentiation as well as in exerting antitumorigenic function in mammary epithelial and breast cancer cells. Indeed, we elucidate different molecular mechanisms mediating the tumor supressive role of PRL in mammary tissue.

Our data revealed a central role for Kinesin-1 in PRL regulation of epithelial mesenchymal plasticity in breast cancer. Moreover, we show that KIF5B and its partner protein kinesin light chain 1 (KLC1) play differential roles in regulating EMP in breast cancer.

Also, we found that PRL induced A/B polarization is mediated through the regulation of the localization classical polarity protein complexes as well as through activation of tumor suppressor Hippo pathway in mammary epithelial cells.

In addition, we proposed here that PRL-induced mammary acinar morphogenesis is linked to the regulation of centrosome duplication and genomic stability. Our data also showed that PRL to be a key regulator of the centrosome duplication kinase, Polo-like kinase 4 (PLK 4), through which PRL maintains centrosome duplication and genomic stability. All together, our work provides better molecular insight into the role of PRL and its signaling pathway in mammary tissue as well as in breast cancer. Furthermore, these findings are in support of the newly appreciated tumor suppressive role of PRL in mammary tumorigenesis and they expand our knowledge of the protective effect of PRL against breast cancer.

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