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CONNEXIN 43 LOSS TRIGGERS CELL CYCLE ENTRY AND INVASION IN NON-NEOPLASTIC BREAST EPITHELIUM: A ROLE FOR NONCANONICAL WNT SIGNALING

by
SABREEN FARAJ FOSTOK

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AN ABSTRACT OF THE DISSERTATION OF

Sabreen Faraj Fostok for Doctor of Philosophy
Major: Cell and Molecular Biology

Title: Connexin 43 Loss Triggers Cell Cycle Entry and Invasion in Non-neoplastic Breast Epithelium: a Role for Noncanonical Wnt Signaling

The signaling pathways through which connexin (Cx) proteins, the building blocks of gap junctions (GJs), exert their tumor suppressive roles in the mammary gland remain elusive. The Wnt signaling pathways, including the Wnt/β-catenin or the canonical Wnt pathway and the β-catenin-independent noncanonical Wnt pathway, execute key roles in the development of the mammary gland, and their deregulation is associated with impaired development and breast tumorigenesis. Evidence suggests a cross-talk between Cxs and Wnt signaling in a multitude of nonbreast tissues and biological contexts. In the breast, Cxs act as downstream transcriptional and functional targets for Wnt signaling. However, the possible role of Cxs as upstream regulators of Wnt signaling is yet to be investigated (Fostok, El-Sibai, El-Sabban, & Talhouk, 2018). Cxs exhibit spatiotemporal expression patterns that are critical for normal development and physiology of the mammary gland. Impaired expression of Cxs leads to mammary developmental defects and predisposes the mammary gland to primary tumors and lung metastases in murine models. In the human breast, Cx43 is expressed in myoepithelial and luminal epithelial cells, which additionally express Cx26. Reduced expression and altered localization of Cx43 have been reported in human breast cancer tissues and cell lines. Furthermore, Cx43 has been proposed as an independent prognostic factor in light of the positive correlation of its levels with good prognosis in breast cancer patients. Overexpression of Cx43 in human breast cancer cell lines reduces proliferation, anchorage-independent growth, migration, invasion, xenograft tumor growth, angiogenesis and metastasis. The aim of this study is to investigate possible involvement of the Wnt pathways downstream of Cx43 signaling in the homeostasis of the mammary gland.

The non-neoplastic human mammary epithelial HMT-3522 S1 cells assemble into growth-arrested differentiated glandular structures, or acini, under 3-dimensional (3-D) culture conditions. S1 acini are characterized by an apicobasal polarity axis, a central lumen and apicolateral membrane expression of Cx43, recapitulating the normal tissue architecture in the human breast. We have previously shown that downregulation of Cx43 expression via retroviral delivery of shRNA impairs apical polarity and mitotic spindle orientation (MSO) and alters the lumen-forming ability in 3-D cultures of S1 cells (Bazzoun/Adissu et al., submitted). Here we report enhanced proliferation and cell

cycle entry, concomitantly with the upregulation of c-Myc and cyclin D1 in Cx43-shRNA S1 cells. The GJ complex, consisting of Cx43, β-catenin and ZO-2 effectively assembled in control S1 cells, whereas β-catenin and Scrib, key regulators of mammary epithelial proliferation and polarity, were mislocalized from apicolateral membrane domains in 3-D cultures of Cx43-shRNA S1 cells. While no detectable activation of Wnt/β-catenin signaling was observed, the loss of Cx43 upregulated the expression and activity of Rho GTPases (RhoA, Rac1 and Cdc42), which relay noncanonical Wnt signals, and enhanced ERK1/2 activity. This was accompanied with the formation of invadopodia-like actin-rich structures, the enhanced random 2-D migration and Matrigel-invading capacity. In 3-D cultures, Cx43-shRNA S1 cells acquired an invasive phenotype marked by the loss of the characteristic spheroidal morphology only under permissive extracellular matrix (ECM) stiffness. This observation supports the overriding impact of the microenvironment and illustrates the role of coordinated junctional and ECM signaling in dictating cellular morphology and behavior. The results of this study suggest that the loss of Cx43 activates proliferation and invasion pathways in the normal mammary epithelium in part via regulating noncanonical Wnt signaling.

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ILLUSTRATIONS

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ABBREVIATIONS

%	Percent
/	Per
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
2-D	2-Dimensional
3-D	3-Dimensional
3'-UTR	3'-Untranslated region
AJ	Adherens junction
APC	Adenomatous polyposis coli
BCR	B-cell receptor
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
Cdc42	Cell division control protein 42 homolog
CDK1	Cyclin-dependent kinase 1
circRNA	Circular RNA
CK1	Casein kinase 1
cm	Centimeter
Cx	Connexin

Cx43 KO	Cx43-shRNA S1 cells
Daam1	Dishevelled-associated activator of morphogenesis 1
DKK1	Dikkopf 1
DMBA	7,12-Dimethylbenz(a)anthracene
DMEM	Dulbecco's Modified Eagle's Medium
Dvl	Dishevelled
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
et al.	et allii (and others)
F-actin	Filamentous actin
FBS	Fetal bovine serum
FRET	Forster resonance energy transfer
Fzd	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GJ	Gap junction
GJIC	Gap junctional intercellular communication
Gro	Groucho
GSC	Glioma stem cell

GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)
HRP	Horseradish peroxidase
IP	Co-immunoprecipitation
JNK	c-Jun N-terminal kinase
LEF	Lymphoid enhancer factor
LRP5/6	Low-density lipoprotein receptor-related protein 5 or 6
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
min	Minute
miRNA	MicroRNA
ml	Milliliter
MLC	Myosin light chain
mm	Millimeter
mM	Millimolar
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MSO	Mitotic spindle orientation
MTOC	Microtubule-organizing center
NFAT	Nuclear factor of activated T-cells
p	*p*-value

PAK1	p21-activated kinase 1
Panx3	Pannexin 3
PBD	p21-binding domain
PBS	Phosphate-buffered saline
PCP	Planar cell polarity
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PKC	Protein kinase C
PMSF	Phenylmethane sulfonyl fluoride
PVDF	Polyvinylidene difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
Rb	Retinoblastoma
Rho	Ras homolog
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay
ROCK	Rho-associated kinase
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
sFRP	Secreted frizzled-related protein
shRNA	Short hairpin RNA
STAT5	Signal transducer and activator of transcription 5
TBST	Tris-buffered saline-Tween 20
TCF	T-cell factor
TDLU	Terminal duct lobular unit

TEB	Terminal end bud
Tim	Translocase of the inner membrane
TJ	Tight junction
TLE	Transducin-like enhancer
TSP-1	Thrombospondin 1
Undil	Undiluted
VEGF	Vascular endothelial growth factor
vol	Volume
WIF1	Wnt inhibitory factor 1
Wnt	Wingless/Integrated
x	Times
ZO-2	Zonula occludens 2
α-MHC	α-Myosin heavy chain

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FAMILY

CHAPTER I

GAP JUNCTIONS AND WNT SIGNALING IN THE MAMMARY GLAND: A CROSS-TALK?

Sabreen F. Fostok, Mirvat El-Sibai, Marwan El-Sabban and Rabih S. Talhouk
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A. Abstract

Connexins (Cxs), the building blocks of gap junctions (GJs), exhibit spatiotemporal patterns of expression and regulate the development and differentiation of the mammary gland, acting via channel-dependent and channel-independent mechanisms. Impaired Cx expression and localization are reported in breast cancer, suggesting a tumor suppressive role for Cxs. The signaling events that mediate the role of GJs in the development and tumorigenesis of the mammary gland remain poorly identified. The Wnt pathways, encompassing the canonical or the Wnt/β-catenin pathway and the noncanonical β-catenin-independent pathway, also play important roles in those processes. Indeed, aberrant Wnt signaling is associated with breast cancer. Despite the coincident roles of Cxs and Wnt pathways, the cross-talk in the breast tissue is poorly defined, although this is reported in a number of other tissues. Our previous studies revealed a channel-independent role for Cx43 in inducing differentiation or suppressing tumorigenesis of mammary epithelial cells by acting as a negative regulator of the Wnt/β-catenin pathway. Here, we provide a brief overview of mammary gland development, with emphasis on the role of Cxs in development and tumorigenesis of

this tissue. We also discuss the role of Wnt signaling in similar contexts, and review the literature illustrating interplay between Cxs and Wnt pathways.

B. Introduction

The mammary gland continues to develop postnatally and is considered a valuable model for studying epithelial physiology and pathology. In addition to the role of soluble mediators as systemic regulators of breast tissue development and differentiation, the local microenvironment has emerged as a major regulator almost two decades ago (Chua, Hodson, Moldenhauer, Robertson, & Ingman, 2010; Gouon-Evans, Rothenberg, & Pollard, 2000; Keely, Wu, & Santoro, 1995; Koledova & Lu, 2017; O'Brien, Martinson, Durand-Rougely, & Schedin, 2012; Taddei et al., 2008; Woodward, Mienaltowski, Modi, Bennett, & Haslam, 2001). Disruption of the mammary epithelial microenvironment is linked to breast cancer development (Insua-Rodriguez & Oskarsson, 2016; Majidinia & Yousefi, 2017; Soysal, Tzankov, & Muenst, 2015). Neighboring cells with which mammary epithelial cells directly interact to establish homocellular and heterocellular junctions have gained considerable interest, both in developmental and tumorigenesis contexts (Knudsen & Wheelock, 2005; McLachlan, Shao, & Laird, 2007). Gap junctions (GJs) regulate the development and differentiation of the mammary gland. Altered expression and localization of their building blocks, connexin (Cx) proteins, are reported in breast cancer, making them candidate tumor suppressors (Ferrati et al., 2017; Hirschi, Xu, Tsukamoto, & Sager, 1996; Kalra et al., 2006; Kańczuga-Koda et al., 2003; Laird et al., 1999; Momiyama et al., 2003; Rana

Mroue, Inman, Mott, Budunova, & Bissell, 2015; Isabelle Plante & Laird, 2008; I Plante, Stewart, Barr, Allan, & Laird, 2011; Isabelle Plante, Wallis, Shao, & Laird, 2010; Qin et al., 2002; Shao, Wang, McLachlan, Veitch, & Laird, 2005; Singal, Tu, Vanwert, Ginder, & Kiang, 2000; Stewart, Bechberger, Welch, Naus, & Laird, 2015; Stewart, Plante, Bechberger, Naus, & Laird, 2014; Talhouk et al., 2013). Indeed, a tight spatiotemporal regulation governs the expression of Cxs in the mammary gland throughout its development (Dianati, Poiraud, Weber-Ouellette, & Plante, 2016; Locke et al., 2007; Locke et al., 2000; Locke et al., 2004; Monaghan, Perusinghe, Carlile, & Evans, 1994; Talhouk et al., 2005). Studies addressing the role of Cxs in mammary epithelial differentiation or tumorigenesis implicate channel-independent mechanisms for Cxs beyond their classical GJ-dependent roles (El-Sabban et al., 2003; Talhouk et al., 2013; Talhouk et al., 2008). However, the downstream pathways through which Cxs act remain elusive. Both branches of Wnt signaling, the canonical or the Wnt/β-catenin pathway and the noncanonical pathway, execute key roles in mammary gland development and differentiation, and altered Wnt signaling is associated with breast cancer (Bagci, Laurin, Huber, Muller, & Cote, 2014; Bray et al., 2013; Ewald, Brenot, Duong, Chan, & Werb, 2008; G Fritz, Brachetti, Bahlmann, Schmidt, & Kaina, 2002; Gerhard Fritz, Just, & Kaina, 1999; Imbert, Eelkema, Jordan, Feiner, & Cowin, 2001; Lin et al., 2000; Lindvall et al., 2009; Milovanovic et al., 2004; Prasad et al., 2008; Raymond et al., 2011; van Genderen et al., 1994). The involvement of Cxs and Wnt pathways in similar processes suggests a cross-talk in the breast tissue. Induction of Wnt1 expression in a mammary epithelial cell line enhances Cx43 expression and gap junctional intercellular communication (GJIC) (Van der Heyden et al., 1998). Similarly,

stimulation of mammary cocultures with Wnt3a upregulates the expression of Cx43 (Constantinou et al., 2008). Overexpression of Wnt5a in the mammary epithelium impairs lactation in mice by altering Cx43 function (Baxley, Jiang, & Serra, 2011).

Although Cxs are downstream targets of Wnt signaling in the mammary epithelium, the interplay between the two is poorly investigated and is not defined in terms of the biological context, possibly due to the scarcity of studies. In support of a cross-talk, we have demonstrated negative regulation of the Wnt/β-catenin pathway by Cxs, as a mechanism to induce differentiation (Talhouk et al., 2008) or to suppress tumorigenesis (Talhouk et al., 2013) in the mammary epithelium. Furthermore, our recent findings indicate a role for Cxs in regulating the noncanonical Wnt pathway in the breast tissue (unpublished data). In this review, we elaborate on the roles of Cxs and Wnt pathways in mammary development and breast cancer. We next discuss the cross-talk between Cxs and Wnt signaling in nonbreast tissues, and we propose a model for their interaction in the mammary gland in developmental and tumorigenic contexts.

Cxs may act as upstream negative regulators or as downstream positive effectors of Wnt/β-catenin signaling, depending on the biological context. The "positive effector" role of Cxs is linked to developmental and pathological processes, such as ovarian folliculogenesis and endometrioid adenocarcinomas (H.-X. Wang et al., 2013; Zhai et al., 2002). This role is additionally defined in the context of cardiac differentiation and function, whereby induction of Cx expression downstream of canonical Wnt signaling enhances spontaneous beat rate and improves cardiac conduction (Z. Ai, Fischer, Spray, Brown, & Fishman, 2000; Mureli et al., 2012). Cxs act as "negative regulators" of the Wnt/β-catenin pathway as a mechanism to regulate

cell cycle entry in kidney cells (Kamei, Toyofuku, & Hori, 2003). Furthermore, reconstitution of Cx expression reverses the malignancy of glioma and colon cancer cells by inhibiting canonical Wnt signaling (Sirnes et al., 2012; S. C. Yu et al., 2012). In light of the above findings, we propose a model in which a similar cross-talk exists between Cxs and Wnt signaling in the mammary gland. Whether Cxs play the role of an "upstream negative regulator" or a "downstream positive effector", this is likely governed by the context. During developmental stages, canonical Wnt signaling induces the expression of Cxs that execute channel-dependent and channel-independent functions to regulate the morphogenesis and differentiation of the mammary tissue, and subsequently act as inhibitors of canonical Wnt signaling to maintain homeostasis and suppress tumorigenesis. The downregulation of Cx expression in early stages of breast cancer leads to the loss of this control and induces hyperproliferation into a primary tumor. In the context of advanced-stage breast cancer, aberrant canonical Wnt signaling upregulates Cx expression to support collective migration and drive tumor metastasis.

C. Development of the Mammary Gland

Extensive remodeling governs the development of the mammary gland and predominates it during adulthood. The anatomical and molecular events that accompany the development of the mammary gland from prenatal stages to weaning post lactation are well characterized (Musumeci et al., 2015; Paine & Lewis, 2017). Murine models have been mainly used for studying the development of the mammary gland. In brief, development commences during embryogenesis, and is initiated by the formation of bilateral milk lines, or mammary ridges, which develop into mammary placodes and

then into epithelial bulbs that invade the underlying mesenchyme. Bud elongation produces a mammary sprout that further invades the fat pad precursor mesenchyme. A rudimentary ductal system develops within the mammary adipose tissue upon lumen formation and branching of the sprout, and continues with the isometric growth until the neonatal phase. Subsequently, the mammary gland remains quiescent until puberty (Gjorevski & Nelson, 2011; Paine & Lewis, 2017; Veltmaat, Van Veelen, Thiery, & Bellusci, 2004). At puberty, estrogen mediates the formation of terminal end buds (TEBs) at the tips of the branching ducts. TEBs direct elongation and branching of the ductal tree, characterized by epithelial proliferation and migration, and regress upon reaching the edges of the fat pad (Brisken & O'Malley, 2010; Hinck & Silberstein, 2005; Paine & Lewis, 2017; Parsa et al., 2008). Further side branching occurs with each estrous cycle in response to progesterone (Atwood et al., 2000). During pregnancy, progesterone and prolactin stimulate the development of alveolar buds at the ends of the branching ducts. At this point, epithelial cells within alveoli undergo structural and functional differentiation (Brisken, 2002; Oakes, Rogers, Naylor, & Ormandy, 2008; Paine & Lewis, 2017). At parturition, reduced progesterone levels and sustained production of prolactin induces milk secretion in alveoli. Upon cessation of lactation, epithelial apoptosis results in involution of the mammary gland and regression into a prepregnancy state (Oakes et al., 2008; Paine & Lewis, 2017).

In humans, the mature female breast encompasses lobules, milk ducts, connective tissue and adipose tissue. Terminal duct lobular units (TDLUs), the functional units of the breast, consist of a terminal duct that connects to the ductal system and leads to a lobule, a cluster of glandular milk-secreting structures termed

alveoli or acini. Luminal epithelial cells line alveoli (lobular epithelium) and ducts (ductal epithelium), and are surrounded by a discontinuous layer of myoepithelial cells. A basement membrane supports the mammary epithelium and forms contacts with both luminal epithelial and myoepithelial cells in TDLUs. The stroma consists of an extracellular matrix (ECM) and stromal cells (fibroblasts, adipocytes, endothelial cells and immune cells) which underlie the basement membrane (Howard & Gusterson, 2000).

Development of the mammary gland is tightly regulated by systemic (endocrine) and local factors (microenvironment) that act together to ensure the proper spatiotemporal regulation of proliferation, differentiation and apoptosis, thereby preventing developmental defects and neoplastic transformation (Zhu, Xiong, Trinkle, & Xu, 2014). Stromal cells are part of the local factors that play important roles in orchestrating morphogenetic events in the developing mammary gland. Fibroblasts, for instance, trigger epithelial branching morphogenesis in a 3-dimensional (3-D) fibroblast-epithelial coculture model (Koledova & Lu, 2017). Macrophages or eosinophils are also required for mouse TEB formation and ductal branching, which are impaired in mice lacking those cells in their mammary glands (Gouon-Evans et al., 2000). Furthermore, mice dually treated with estradiol and progesterone to induce alveologenesis have reduced ability to form alveolar buds upon depletion of macrophages (Chua et al., 2010). Macrophages also regulate mammary gland involution, whereby the execution of epithelial apoptosis, alveolar regression and adipocyte repopulation fails in macrophage-devoid mice (O'Brien et al., 2012).

In addition to stromal cells, the role of ECM signaling in regulating mammary gland development is extensively documented (Keely et al., 1995; Taddei et al., 2008; Woodward et al., 2001). Interactions of the epithelial and myoepithelial compartments with the underlying ECM generate biochemical and mechanical signals that dictate normal mammary architecture and function (Xu, Boudreau, & Bissell, 2009). Thus, disruption of cell-ECM interactions is associated with developmental defects and breast tumorigenesis. Conditional deletion of β1-integrin, a major ECM receptor, from the basal compartment of mouse mammary epithelium alters ductal branching pattern and impairs lobuloalveolar development (Taddei et al., 2008). The ECM is dynamically deposited and degraded throughout the developmental stages of the mammary gland, further supporting its role in mammary morphogenesis. Indeed, ECM components and remodeling enzymes undergo spatial and temporal expression in the developing mammary glands of mice (Keely et al., 1995; Talhouk, Bissell, & Werb, 1992; Wiseman et al., 2003; Woodward et al., 2001). Therefore, normal morphogenesis of the mammary gland is not only contingent upon tight hormonal regulation, but is also dependent on the presence of a well-regulated microenvironment.

D. Connexins in Mammary Gland Development

Cxs are expressed in most cell types and exhibit evolutionary conservation among chordates (Dermietzel, Hwang, & Spray, 1990). Twenty Cx genes have been identified in mice and 21 in humans. Most Cx genes share a similar structure consisting of two exons separated by one intron. The first exon is untranslated, while the second contains the coding region and the 3'-untranslated region (3'-UTR) (Söhl & Willecke,

2004). Cx proteins consist of highly conserved cytoplasmic N-terminal domain, two extracellular loops with four transmembrane domains, and variable intracellular loop and cytoplasmic C-terminal domain that account for functional differences among Cx isoforms (Dbouk, Mroue, El-Sabban, & Talhouk, 2009; Leithe, Mesnil, & Aasen, 2017; Su & Lau, 2014). Cx43 is the most abundantly and ubiquitously expressed Cx protein, making it the most studied Cx isoform (Dbouk et al., 2009; Leithe et al., 2017; Su & Lau, 2014). Cxs oligomerize to form hexameric structures referred to as hemichannels or connexons, and docking of two connexons in adjacent cell membranes forms a GJ channel. Oligomerization of identical Cxs forms homomeric connexons, while heteromeric connexons result upon association of different Cx isoforms. In addition, homotypic or heterotypic GJ channels result from docking of identical or different connexons, respectively. Structures formed upon accumulation of thousands of GJ channels at the membrane are referred to as GJ plaques or GJs (Dbouk et al., 2009; Leithe et al., 2017; Su & Lau, 2014). GJs connect the cytoplasms of two adjacent cells, allowing intercellular exchange of ions, second messengers (Ca^{2+}, cAMP and IP3) and metabolites (sugars, amino acids and small peptides) less than 1.5 kDa in size (Dbouk et al., 2009; Leithe et al., 2017; Su & Lau, 2014). In addition to their classical channel-dependent roles, Cxs execute channel-independent functions by associating with signaling molecules, enzymes, cytoskeletal and junctional proteins, among others (Dbouk et al., 2009; Leithe et al., 2017). The expression and turnover of Cxs are tightly regulated. The loss of this regulation, whether in the form of loss of expression, mutations or altered GJIC, is associated with disease outcomes, including cancer (El-

Saghir, El-Habre, El-Sabban, & Talhouk, 2011; Kelly, Simek, & Laird, 2015; Naus & Laird, 2010).

The expression patterns of Cxs in the mammary gland are spatiotemporally defined. In mouse models, luminal epithelial cells express Cx26, Cx30 and Cx32, while the expression of Cx43 is limited to the mammary myoepithelium (Talhouk et al., 2005). In contrast, the expression of Cx43 is evident in both epithelial cell layers in reduction mammoplasties of normal women, with luminal epithelial cells expressing additionally Cx26 (Jamieson, Going, D'Arcy, & George, 1998b; Monaghan et al., 1996). Despite a well-characterized spatial expression of Cxs in the human mammary gland, temporal expression patterns remain elusive, and are linked to sampling limitations and inability to obtain normal breast tissue samples at the various developmental stages of the mammary gland. Majority of studies investigating the temporal expression of Cxs utilized mouse models (Dianati et al., 2016; Lambe, Finlay, Murphy, & Martin, 2006; Locke et al., 2007; Locke et al., 2000; Locke et al., 2004; Monaghan et al., 1994; Pozzi, Risek, Kiang, Gilula, & Kumar, 1995; Talhouk et al., 2005).

Cxs play important roles in normal development and physiology of the mammary gland. Cx26 and Cx43 knockout mice die *in utero* and at birth, respectively, making it impossible to study the role of Cx26 and Cx43 in mammary glands of these mice (Gabriel et al., 1998; Reaume et al., 1995). Autosomal dominant Cx43 mutation ($Cx43^{I130T/+}$) delays ductal elongation and reduces gland size relative to body weight in prepubertal mice. Although milk production and ejection are not affected, mutant mice have impaired mammary epithelial proliferation, leading to reduced gland size at

parturition (Stewart et al., 2013). In a similar model ($Cx43^{G60S/+}$), mammary gland development is delayed in virgin mice. Ductal elongation, branching, TEB formation and relative mammary gland weight are reduced, but the morphology of the mammary gland at parturition is not affected (Isabelle Plante & Laird, 2008). Furthermore, milk secretion and *ex vivo* oxytocin-induced milk ejection into the ducts are impaired (Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010). Indeed, knocking down Cx43 or blocking GJIC in primary mammary organoids of wild-type mice inhibits myoepithelial contractility in response to oxytocin stimulation (Rana Mroue et al., 2015). Replacement of Cx43 with Cx32 in a heterozygous knock-in mouse model ($Cx43^{Cx32/+}$) reduces postnatal growth and survival of pups. This is attributed to defects in milk ejection but not in mammary gland development or milk production (Plum et al., 2000). Heterozygous $Cx43^{Cx26/+}$ mutation has similar effects on pup survival and growth, does not affect milk production, but is associated with reduced branching of ductuli, number and size of secretory alveoli in lactating mice (Winterhager et al., 2007). In a Cx26 conditional knockdown mouse model, where the physiological surge in mammary Cx26 that accompanies pregnancy and lactation is inhibited, normal development and function of the mammary gland are retained, indicating that basal levels of Cx26 are sufficient (Stewart et al., 2014). Interestingly, transgenic mice overexpressing Cx26 under the control of keratin 5 promoter (K5-Cx26), which exhibits constitutive activity in myoepithelial cells, are unable to feed their pups despite normal mammary gland development and milk production. In fact, *ex vivo* oxytocin stimulation of mammary organoids isolated from transgenic mice fails to induce contraction, and ectopic expression of Cx26 in myoepithelial cells alters the expression of endogenous

Cx43, leading to disrupted GJIC (Rana Mroue et al., 2015). This illustrates the importance of spatial regulation of Cx expression in normal functioning of the mammary gland. Conditional inactivation of Cx26 gene in the mammary epithelial compartment ($Cx26^{fl/fl}$ x MMTV-Cre) affects mouse mammary glands in a stage-dependent manner. The loss of Cx26 before puberty does not alter ductal elongation or branching, but it impairs lobuloalveolar development and function during pregnancy and lactation, respectively. These effects are due to increased apoptosis and are not associated with reduced mammary epithelial proliferation. In contrast, the loss of Cx26 during later stages of pregnancy does not affect mammary development or function, illustrating the temporal effects of Cx expression in the mammary gland (Bry et al., 2004). Indeed, Cx26 acts downstream of prolactin signaling in the mammary epithelium during early pregnancy. Mouse mammary epithelial transplants devoid of prolactin receptor form alveolar buds that fail to undergo lobuloalveolar development during pregnancy. This is concomitant with reduced expression of Cx26, suggesting a role in prolactin-induced mammary development (Ormandy et al., 2003). The spatiotemporal expression patterns of murine mammary Cxs and the developmental defects associated with their altered expression are summarized in Table 1.1 (Bry et al., 2004; RM Mroue, El-Sabban, & Talhouk, 2011; Rana Mroue et al., 2015; Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010; Plum et al., 2000; Stewart et al., 2013; Winterhager et al., 2007).

Table 1.1. The spatiotemporal expression patterns of murine mammary Cxs and the developmental abnormalities in mouse models of altered Cx expression.

Cx Isoform	Cell Compartment	Developmental Stage	Mouse Model	Developmental Abnormality	References
Cx26	Luminal epithelium	Pregnancy Parturition Lactation	K5-Cx26: Ectopic expression of Cx26 in myoepithelial cells	Impaired milk ejection	(Rana Mroue et al., 2015)
			$Cx26^{fl/fl}$ x MMTV-Cre: Conditional deletion of Cx26 gene in mammary epithelial cells before puberty	Impaired lobuloalveolar development and lactation	(Bry et al., 2004)
			$Cx43^{Cx26/+}$: (see below)		(Winterhager et al., 2007)
Cx30	Luminal epithelium	Pregnancy Parturition Lactation			
Cx32	Luminal epithelium	Parturition Lactation	$Cx43^{Cx32/+}$: (see below)		(Plum et al., 2000)
Cx43	Myoepithelium	Pregnancy Parturition Lactation	$Cx43^{G60S/+}$: Autosomal dominant point mutation (G60S) in one Cx43 allele	Delayed ductal elongation, branching and TEB formation Reduced gland size Defective milk secretion and ejection	(Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010)
			$Cx43^{I130T/+}$: Autosomal dominant point mutation	Delayed ductal elongation Reduced gland size	(Stewart et al., 2013)

			(I130T) in one Cx43 allele		
			$Cx43^{Cx32/+}$: Replacement of one Cx43 allele with Cx32	Impaired milk ejection	(Plum et al., 2000)
			$Cx43^{Cx26/+}$: Replacement of one Cx43 allele with Cx26	Reduced ductular branching Reduced alveolar number and size	(Winterhager et al., 2007)

We have previously demonstrated channel-dependent and channel-independent roles for Cx43 in differentiation of the mammary gland (El-Sabban et al., 2003; Talhouk et al., 2008). Blocking GJIC in CID-9 mouse mammary cell strain under differentiation-permissive conditions (in the presence of exogenous basement membrane) downregulates the expression of β-casein, a milk protein and a differentiation marker. Furthermore, induction of GJIC in the absence of a basement membrane is sufficient to induce mammary epithelial differentiation (El-Sabban et al., 2003). Indeed, these effects are independent of ECM-induced signal transducer and activator of transcription 5 (STAT5) (Talhouk, Khalil, Bajjani, Rahme, & El-Sabban, 2011). Subsequently, we illustrated involvement of GJ complex assembly (Cx43, among other Cxs, α-catenin, β-catenin and ZO-2) in differentiation of mouse mammary epithelial SCp2 cells. The role of GJ complex assembly in mammary epithelial differentiation is partly mediated by the recruitment of β-catenin to the membrane, thereby preventing its nuclear translocation, which induces the expression of proliferation and cell cycle genes (Talhouk et al., 2008).

E. Connexins in Breast Tumorigenesis

Aberrant patterns of Cx expression and localization are linked to breast cancer. Reduced Cx43 expression is reported in human breast cancer tissues at various stages of tumor progression, in carcinogen-induced rat mammary tumors and in breast cancer cell lines (Laird et al., 1999). In addition to impaired expression, progressive alteration of Cx43 localization is found in human mammary dysplasia and breast cancer tissues, as compared to normal breast tissues. Cx43 exhibits intercellular punctate localization in

normal breast tissues and diffuse cytoplasmic pattern in breast cancer tissues, indicating loss of GJIC (Kańczuga-Koda et al., 2003). Indeed, a positive correlation is established between Cx43 levels and improved disease outcome in breast cancer patients, and Cx43 is proposed as an independent prognostic marker (Teleki et al., 2014). In addition to the dysregulation of Cx43, reduced or complete loss of Cx26 expression is reported in breast cancer cell lines, compared to nontumorigenic human mammary epithelial cells, conferring a potential role to Cx26 in tumor suppression (Singal et al., 2000).

The tumor suppressive roles of Cxs in the mammary gland are supported by both *in vitro* and *in vivo* studies. We have previously demonstrated a tumor suppressive role for Cx43 in the breast. Overexpression of Cx43 in MCF-7 and MDA-MB-231 cells, human breast cancer cell lines, reduces proliferation, cell cycle progression and invasiveness and reverses their characteristic malignant phenotype. These effects are independent of GJIC, given that overexpression of a C-terminus-truncated version of Cx43 fails to restore the wild-type Cx43 phenotype. Furthermore, blocking GJIC in Cx43-overexpressing cells does not reverse the effects of Cx43, corroborating the involvement of channel-independent mechanisms (Talhouk et al., 2013). Likewise, overexpression of Cx26 in MCF-7 and MDA-MB-435 cells reduces proliferation, anchorage-independent growth, migration and invasion (Kalra et al., 2006; Momiyama et al., 2003). The effects of Cx26 on MDA-MB-435 cells are channel independent, as shown by the expression of a GJIC-incompetent Cx26 form that phenocopies the effects of wild-type Cx26 (Kalra et al., 2006). Overexpression of Cx26 or Cx43 in MDA-MB-231 and MDA-MB-435 cells suppresses xenograft tumor growth in nude mice (Hirschi et al., 1996; Qin et al., 2002). Furthermore, migration of MDA-MB-231 cells is

impaired upon exposure to Cx43-rich biovesicles extracted from plasma membranes of donor cells overexpressing functional Cx43-based GJs and capable of forming GJs with cells (Ferrati et al., 2017). Conditional mammary gland-specific knockout of Cx26 in mice predisposes the mammary gland to primary tumors in DMBA-induced breast cancer model (Stewart et al., 2015). Similarly, mice with heterozygous Cx43 mutation show higher susceptibility to mammary tumor lung metastasis following DMBA treatment (I Plante et al., 2011). *In vitro*, silencing Cx43 in Hs578T cells, human breast cancer cell line, enhances proliferation and anchorage-independent growth. This is associated with the upregulation of vascular endothelial growth factor (VEGF), a proangiogenic molecule, and downregulation of thrombospondin 1 (TSP-1), an antiangiogenic molecule (Shao et al., 2005). We have recently shown that silencing Cx43 in nontumorigenic human mammary epithelial cell line, HMT-3522 S1 cells, enhances proliferation and cell cycle progression, and induces mislocalization of membranous β-catenin (unpublished data). In addition, Cx43-silenced cells display morphogenetic defects typical of breast cancer initiation. These include loss of apical polarity, misorientation of the mitotic spindle, multilayering and loss of lumen, thus indicating disruption of normal acinar morphology (Bazzoun et al.; submitted).

Collectively, the above studies illustrate key roles of Cxs in development and tumorigenesis of the mammary gland. The involvement of channel-independent mechanisms in Cx signaling suggests a link between Cxs and cellular pathways that execute overlapping roles with those of Cxs in the mammary gland. The developmental pathways which mediate Cx signaling in the mammary gland are yet to be investigated. Evidence supports interplay between Cxs and Wnt signaling in nonbreast tissues and in

a multitude of biological contexts. In the mammary epithelium, canonical and noncanonical Wnt signaling regulate the expression and function of Cx43 (Baxley et al., 2011; Constantinou et al., 2008; Van der Heyden et al., 1998). In addition, our earlier findings indicate that the Wnt/β-catenin pathway is a modulator of Cx signaling in differentiation (Talhouk et al., 2008) and tumorigenesis (Talhouk et al., 2013) of mammary epithelial cells. This suggests that the Wnt pathways are potential candidates for relaying Cx signals within the mammary gland in development and cancer.

F. Connexins as Regulators of Wnt Signaling

1. Connexins in Canonical Wnt Signaling

a. Canonical Wnt Pathway

The Wnt/β-catenin pathway (or the canonical Wnt pathway) is one of the three best characterized Wnt pathways, which also include the planar cell polarity (PCP) and the Wnt/calcium pathways. The Wnt/β-catenin pathway is involved in β-catenin-mediated regulation of developmental gene expression, essential for embryogenesis and adult tissue homeostasis. Deregulation of this pathway is associated with developmental defects and adult diseases, including cancer (MacDonald, Tamai, & He, 2009; Rao & KUhl, 2010; Zardawi, O'Toole, Sutherland, & Musgrove, 2009).

In the absence of a Wnt ligand, two scaffolding proteins, adenomatous polyposis coli (APC) and Axin as well as casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK-3) form a complex in the cytoplasm, referred to as the β-catenin destruction complex. CK1 and GSK-3, serine/threonine protein kinases, sequentially phosphorylate β-catenin on specific N-terminal amino acid residues (on serine 45, and

subsequently on threonine 41, serine 37 and serine 33, respectively). This marks β-catenin for ubiquitination (dually phosphorylated β-catenin on serine 33 and 37 is recognized by β-TrCP, E3 ubiquitin ligase) and subsequent proteasomal degradation leading to a reduction in the cytoplasmic pool of β-catenin available for nuclear translocation. Consequently, Wnt/β-catenin target genes are repressed by the DNA-bound T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. TCF/LEF acts as transcriptional repressor by forming a complex with Groucho (Gro)/transducin-like enhancer (TLE), which interacts with histone deacetylases (HDACs) to mediate histone deacetylation and chromatin compaction (MacDonald et al., 2009; Rao & KUhl, 2010; Zardawi et al., 2009).

In the presence of Wnt, the ligand binds to its receptor and coreceptor, Frizzled (Fzd) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), respectively. This complex (Wnt-Fzd-LRP5/6) triggers Fzd-mediated recruitment of Dishevelled (Dvl), a scaffolding protein, which in turn recruits Axin along with its associated GSK-3 and CK1 to the membrane, resulting in phosphorylation of LRP5/6 by GSK-3 and CK1. Phosphorylation leads to the activation of LRP5/6, which recruits the Axin-GSK-3-CK1 complex, thereby amplifying phosphorylation of LRP5/6 and enhancing the recruitment of the Axin complex as well. As a result, the β-catenin destruction complex (APC-Axin-CK1-GSK-3) is disrupted. This stabilizes β-catenin and leads to its accumulation and translocation to the nucleus, where it acts as a transcriptional coactivator. In the nucleus, β-catenin displaces Gro/TLE to form a complex with TCF/LEF, thereby converting the latter into a transcriptional activator and inducing the expression of genes involved in cell cycle progression, including c-Myc, cyclin-dependent kinase 1 (CDK1) and cyclin

D1 (Figure 1.1). Wnt/β-catenin target genes also include components of the Wnt/β-catenin pathway itself that may act as agonists or antagonists, conferring self-regulatory properties to the pathway (MacDonald et al., 2009; Rao & KUhl, 2010; Zardawi et al., 2009).

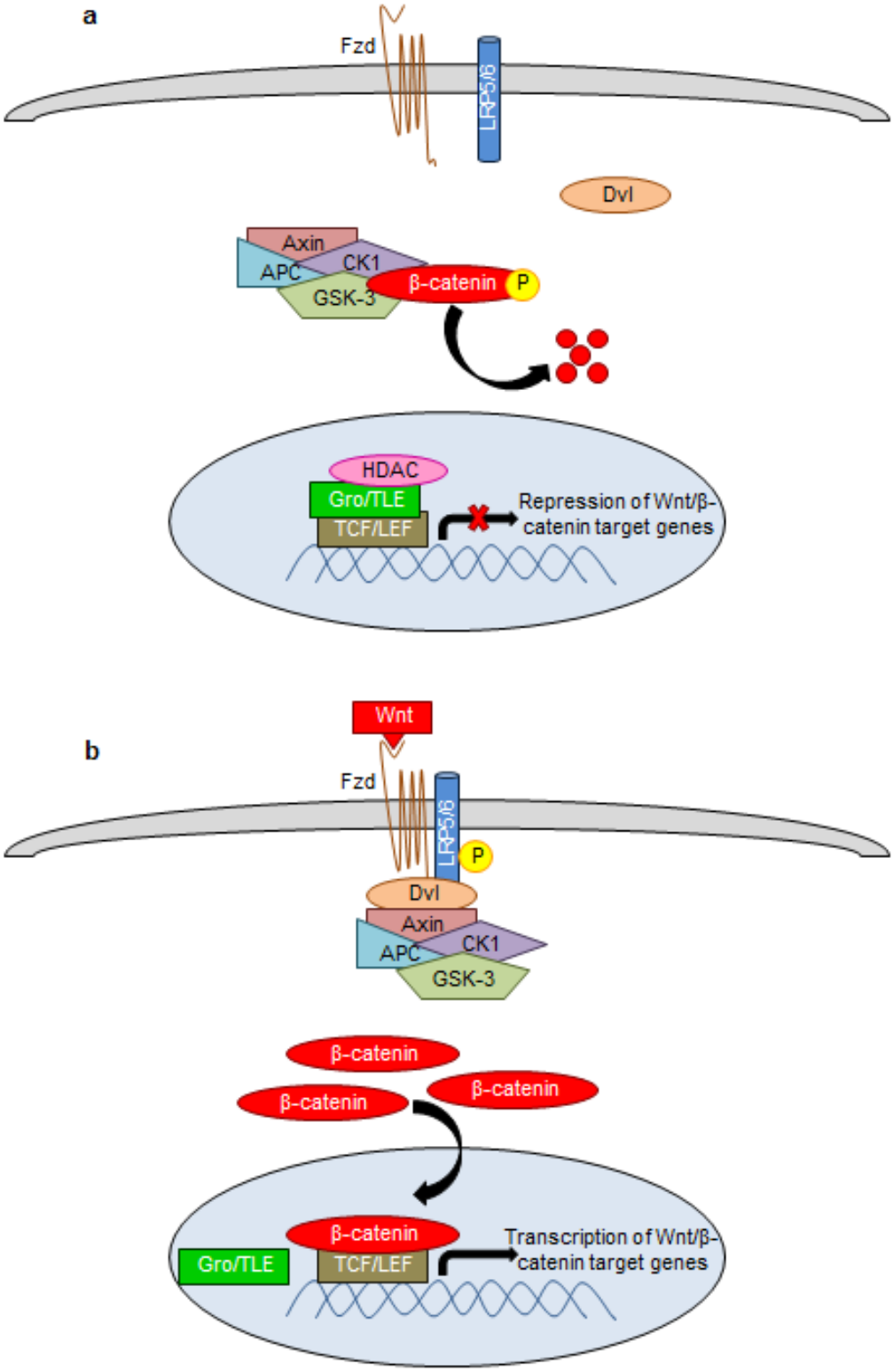

a
Fzd
LRP5/6
Dvl
Axin
APC
CK1
GSK-3
β-catenin
P
HDAC
Gro/TLE
TCF/LEF
Repression of Wnt/β-catenin target genes
b
Wnt
Fzd
LRP5/6
P
Dvl
Axin
APC
CK1
GSK-3
β-catenin
β-catenin
β-catenin
β-catenin
Gro/TLE
TCF/LEF
Transcription of Wnt/β-catenin target genes

Figure 1.1. The canonical Wnt pathway. In the absence of a Wnt ligand **(a)**, the scaffolding proteins Axin and APC form a complex with the serine/threonine protein kinases CK1 and GSK-3 in the cytoplasm, referred to as the β-catenin destruction complex. CK1 and GSK-3 sequentially phosphorylate β-catenin, marking it for ubiquitination and subsequent proteasomal degradation, thereby reducing its nuclear translocation. Consequently, the TCF/LEF family of transcription factors acts as a transcriptional repressor by forming a complex with Gro/TLE, which interacts with HDACs to mediate chromatin compaction, causing the repression of the Wnt/β-catenin target genes. In the presence of Wnt **(b)**, the ligand binds to its receptor Fzd and coreceptor LRP5/6. The resulting complex recruits the scaffolding protein Dvl, which in turn recruits the β-catenin destruction complex. CK1 and GSK-3 phosphorylate LRP5/6, causing its activation and enhancing the recruitment of the β-catenin destruction complex. This results in the stabilization and accumulation of β-catenin in the cytoplasm, and its subsequent nuclear translocation. In the nucleus, β-catenin acts as a transcriptional coactivator by displacing Gro/TLE, thereby converting TCF/LEF into a transcriptional activator to induce the expression of the Wnt/β-catenin target genes and cell cycle progression.

b. Role of Canonical Wnt Pathway in Mammary Gland Development

The role of Wnt signaling in mammary gland development and breast tumorigenesis is well documented (Incassati, Chandramouli, Eelkema, & Cowin, 2010; Turashvili, Bouchal, Burkadze, & Kolar, 2006; Q. C. Yu, Verheyen, & Zeng, 2016). The earliest detectable event marking the activation of Wnt signaling during mammary development is the expression of Wnt10b in the mammary line and Wnt6 in the surface ectoderm of mouse embryos at embryonic day E11.25 (Veltmaat et al., 2004). Wnt signaling components are expressed in a cell type-specific and stage-dependent manner in the developing mammary gland (Boras-Granic & Hamel, 2013; Jarde & Dale, 2012). The expression patterns of Wnt ligands are summarized in Table 1.2 (Cai et al., 2014; Chu et al., 2004; Ji et al., 2011; Kouros-Mehr & Werb, 2006; T. F. Lane & Leder, 1997; Roarty, Shore, Creighton, & Rosen, 2015; Veltmaat et al., 2004; Weber-Hall, Phippard, Niemeyer, & Dale, 1994).

Table 1.2. The spatiotemporal expression patterns of Wnt ligands in the murine mammary gland.

Developmental Stage	Cell Compartment	Wnt Ligand	References
Embryonic	Mammary line	Wnt5a Wnt6 Wnt10b	(Chu et al., 2004; Veltmaat et al., 2004)
	Mammary placode	Wnt1 Wnt2 Wnt3 Wnt5a Wnt6 Wnt7b Wnt10a Wnt10b Wnt11	(Chu et al., 2004; Veltmaat et al., 2004)
	Mammary bud	Wnt1 Wnt2 Wnt3 Wnt3a Wnt4 Wnt5a Wnt5b Wnt7b Wnt10b Wnt11	(Chu et al., 2004)
Pubertal	TEB	Wnt2 Wnt4	(Kouros‐Mehr & Werb, 2006)

		Wnt5a Wnt5b Wnt6 Wnt7b	
	Duct	Wnt4 Wnt5b Wnt6	(Kouros‐Mehr & Werb, 2006)
Adult	Luminal epithelium	Wnt4 Wnt5a Wnt5b Wnt7b	(Cai et al., 2014; Ji et al., 2011; Roarty et al., 2015)
	Myoepithelium	Wnt5a Wnt5b Wnt10a	(Ji et al., 2011; Roarty et al., 2015)
Pregnancy		Wnt2 (early, mid) Wnt4 (early, mid) Wnt5a (early, mid) Wnt5b (early, mid, late) Wnt6 (early, mid, late) Wnt7b (early) Wnt10b (early)	(T. F. Lane & Leder, 1997; Weber-Hall et al., 1994)

Canonical Wnt signaling initiates mammary gland morphogenesis in mouse embryos. Activation of canonical Wnt signaling in the mammary region overlaps with the onset of mammary morphogenesis and localizes to mammary placodes and buds thereafter. Forced activation of canonical Wnt signaling using Wnt3a accelerates placode formation in cultured embryos. Conversely, ectopic expression of the Wnt inhibitor Dikkopf 1 (DKK1) in the surface epithelium of transgenic embryos blocks placode development (Chu et al., 2004). Formation of rudimentary mammary buds is inhibited in mouse embryos with homozygous LEF-1 mutation (van Genderen et al., 1994), while homozygous mutation in LRP5 reduces the size of mammary placodes in mouse embryos and alters ductal elongation and TEB numbers in virgin mice (Lindvall et al., 2006). Similarly, LRP6 knockout mouse embryos have smaller mammary placodes and fat pad and lack ductal branching, whereas heterozygous LRP6 mutation alters TEB numbers and ductal branching in juvenile and adult mice, respectively (Lindvall et al., 2009). Canonical Wnt signaling also mediates progesterone-induced side branching in mammary ducts. Ectopic expression of Wnt1 rescues side branching of ducts in mammary epithelial transplants of mice with homozygous mutation in progesterone receptor, indicating that the canonical Wnt signaling acts downstream of progesterone. This latter induces the expression of Wnt4, and mammary bud implants derived from Wnt4-deficient mouse embryos show impaired ductal branching during early pregnancy (Brisken et al., 2000). Expression of a constitutively active form of β-catenin causes precocious lobuloalveolar development and differentiation in mouse mammary glands. Indeed, virgin mammary glands of these transgenic mice resemble those of wild-type pregnant mice in terms of development and functional differentiation,

show lobular hyperplasia during pregnancy and regress into a midpregnant state, characteristic of virgin transgenic mice, post lactation. The transgenic mice develop multiple aggressive adenocarcinomas early on during their lifetime (Imbert et al., 2001).

c. Role of Canonical Wnt Pathway in Breast Tumorigenesis

In addition to regulating development and differentiation of the mammary gland, aberrant Wnt/β-catenin signaling plays a role in breast cancer. Reduced levels of membranous β-catenin and enhanced nuclear activity are linked to poor disease outcome in breast cancer patients and are proposed as independent prognostic factors (Dolled-Filhart et al., 2006; Lin et al., 2000). β-catenin mutations at phosphorylation sites that target it for ubiquitination and subsequent degradation, as well as inactivating APC mutations, lead to stabilization of β-catenin and constitutive activation of the Wnt/β-catenin signaling. Although nuclear and cytoplasmic accumulation of β-catenin are reported in breast cancer, APC and β-catenin mutations, commonly associated with other cancers, are absent or rare and restricted to benign and metaplastic breast tumors (Abraham et al., 2002; Hayes, Thomas, Emmons, Giordano, & Kleer, 2008; Jönsson, Borg, Nilbert, & Andersson, 2000; Ozaki et al., 2005; Sawyer et al., 2002; Schlosshauer et al., 2000; Sørlie, Bukholm, & Børresen-Dale, 1998). This suggests that deregulated Wnt/β-catenin signaling in breast cancer is not a consequence of mutations in components of this pathway. In support of this, defective expression, localization or epigenetic patterns of canonical Wnt components are associated with breast cancer. Wnt ligands, receptors and coreceptors are overexpressed in breast cancer (Björklund, Svedlund, Olsson, Åkerström, & Westin, 2009; Huguet, McMahon, McMahon,

Bicknell, & Harris, 1994; Lejeune, Huguet, Hamby, Poulsom, & Harris, 1995; C.-C. Liu, Prior, Piwnica-Worms, & Bu, 2010; Milovanovic et al., 2004). For instance, expression of FZD1 and FZD2 receptors is upregulated in breast cancer tissues (Milovanovic et al., 2004). Similarly, LRP6 is overexpressed in breast cancer cell lines and tissues and is required for activation of canonical Wnt signaling, cell proliferation and xenograft tumor growth, while administration of LRP6 antagonist *in vivo* prevents the growth of MMTV-Wnt1 tumors (C.-C. Liu et al., 2010). Interestingly, expression of an aberrantly spliced internally truncated form of LRP5 coreceptor is found in breast cancer tissues. This form is essential for β-catenin stability and activity, cell proliferation and tumor growth in a xenograft mouse model (Björklund et al., 2009). Altered expression and epigenetic regulation of other components in the Wnt/β-catenin pathway are also common. Amplification and upregulation of Dvl1, a scaffolding protein that recruits the β-catenin destruction complex, are reported in primary breast tumors (Nagahata et al., 2003). APC promoter hypermethylation and reduced expression are detected in breast cancer tissues and correlate with active Wnt/β-catenin signaling (Prasad et al., 2008; Van der Auwera et al., 2008). Epigenetic silencing and promoter hypermethylation of Wnt antagonist genes, including secreted frizzled-related protein (sFRP) family, Wnt inhibitory factor 1 (WIF1) and DKK, are present in breast cancer cell lines and in primary breast tumors (L. Ai et al., 2006; Suzuki et al., 2008). Reduced sFRP expression accounts for activation of canonical Wnt signaling, and expression of sFRP suppresses proliferation of breast cancer cells (Shulewitz et al., 2006; Suzuki et al., 2008).

Although Cxs and Wnt/β-catenin signaling play overlapping roles in the mammary gland, scarce evidence supports a link between these pathways in the breast tissue (Constantinou et al., 2008; Van der Heyden et al., 1998). We have previously shown Cx channel-independent signaling as an upstream negative regulator of the Wnt/β-catenin pathway in the breast. Cx43 associates with β-catenin at the membrane and inhibits its nuclear translocation, as a mechanism to induce differentiation (Talhouk et al., 2008) or to suppress tumorigenesis (Talhouk et al., 2013) in mammary epithelial cells. The interplay between Cxs and canonical Wnt signaling exists in a number of other tissues, where Cxs act as upstream negative regulators or as downstream positive effectors of the Wnt/β-catenin pathway.

d. Cross-talk between Connexins and Canonical Wnt Signaling

i. Connexins as Upstream Negative Regulators of Canonical Wnt Signaling

Evidence supports negative regulation of the Wnt/β-catenin pathway by Cx signaling in cardiac, bone, kidney, nervous and colon tissues (Z. Ai et al., 2000; Bivi et al., 2013; Kamei et al., 2003; Pacheco-Costa et al., 2017; Rinaldi et al., 2015; Sirnes et al., 2012; S. C. Yu et al., 2012).

Overexpression of Cx43 in lithium-stimulated neonatal rat cardiomyocytes (lithium mimics Wnt signaling by inhibiting GSK-3β) reduces β-catenin transcriptional activity. Association and colocalization of Cx43 and β-catenin at the membrane suggests that Cx43 inhibits canonical Wnt signaling via β-catenin sequestration (Z. Ai et al., 2000).

The knockout of Cx43 or Cx37 in osteocytes results in the accumulation of β-catenin and increased expression of Wnt/β-catenin target genes. These effects are associated with enhanced Wnt/β-catenin-dependent processes, including osteogenic response to mechanical loading and resistance to fractures in bones (Bivi et al., 2013; Pacheco-Costa et al., 2017). Interestingly, pannexin 3 (Panx3), a member of a recently identified family of GJ proteins, also inhibits Wnt/β-catenin signaling in bones. Overexpression of Panx3 in osteoprogenitor cells cultured under proliferation conditions reduces proliferation and induces cell cycle arrest. Panx3 exerts its effects by enhancing the activity of GSK-3β, leading to the phosphorylation of β-catenin and the reduction of its cytoplasmic levels. This is coupled to a decrease in β-catenin nuclear localization and activity. As a result, levels of cyclin D1 and phosphorylated retinoblastoma (Rb), involved in G1 to S phase progression, are reduced (Ishikawa, Iwamoto, Fukumoto, & Yamada, 2014).

In a study on the role of adhesion molecules in cell proliferation, Cx43 synergizes the effects of N-cadherin in suppressing β-catenin/TCF transcriptional activity, as a mechanism to upregulate p21 and reduce proliferation and cell cycle progression in HEK293 human embryonic kidney cells. Notably, the effects of Cx43 are channel dependent (Kamei et al., 2003).

Reconstitution of Cx43 in glioma stem cells (GSCs) impairs tumorsphere formation and proliferation. In addition, increased expression of glial fibrillary acidic protein (GFAP), an astrocytic differentiation marker, and reduced expression of CD133, a stem cell marker, are noted, indicating differentiation and impaired self-renewal capacity. Overexpression of Cx43 is also associated with reduced invasiveness *in vitro*,

and xenografts of Cx43-transduced GSCs exhibit smaller tumor size, reduced proliferation and better differentiation, compared to their mock counterparts, suggesting that Cx43 inhibits tumorigenicity of GSCs. Notably, overexpression of Cx43 in GSCs does not restore GJIC, indicating that the observed effects of Cx43 are due to channel-independent mechanisms. Microarray analysis revealed reduced expression of Wnt/β-catenin target genes, including stemness-related genes (Nanog, Oct4 and Sox2), in Cx43-transduced GSCs. Furthermore, overexpression of Cx43 induces the expression of E-cadherin, and knocking down E-cadherin in Cx43-transduced GSCs is sufficient to restore invasiveness, indicating that Cx43 negatively regulates the Wnt/β-catenin pathway in GSCs via an E-cadherin-dependent mechanism (S. C. Yu et al., 2012). The loss of Cx43, but not GJIC, is associated with differentiation of human neural progenitor cells as a consequence of enhanced canonical Wnt signaling. Silencing Cx43 triggers neurogenesis by increasing the protein levels and transcriptional activity of β-catenin, thereby upregulating the expression of proneuronal genes (Rinaldi et al., 2015).

Ectopic expression of Cx43 in HT29 colon cancer cell line reduces anchorage-dependent, anchorage-independent and xenograft growth. Notably, ectopically expressed Cx43 localizes mainly to intracellular vesicular compartments and fails to form GJs, suggesting the implication of channel-independent mechanisms in tumor suppression. In addition, Cx43 associates with β-catenin and reduces TCF transcriptional activity in HT29 cells, indicating negative regulation of the Wnt/β-catenin signaling, a mechanism through which Cx43 could exert its tumor suppressive effects (Sirnes et al., 2012).

While the above studies described Cxs as negative regulators of canonical Wnt signaling, others reported positive regulation of Cxs downstream of the Wnt/β-catenin pathway. This illustrates possible existence of a negative feedback mechanism, whereby Cxs act as both downstream targets and inhibitors of the Wnt/β-catenin pathway.

ii. Connexins as Downstream Positive Targets of Canonical Wnt Signaling

In cardiac and skeletal muscle cells, the Wnt/β-catenin pathway upregulates the expression of Cxs, mainly Cx43, and GJIC (Z. Ai et al., 2000; Czyz, Guan, Zeng, & Wobus, 2003; Du et al., 2008; Heo & Lee, 2011; Mureli et al., 2012). GJIC and Cx43 expression are enhanced in neonatal rat cardiomyocytes and skeletal myoblasts in response to lithium-stimulated activation of canonical Wnt signaling, and are associated with increased spontaneous beat rate in cardiomyocytes (Z. Ai et al., 2000; Du et al., 2008). Indeed, activation of the Wnt/β-catenin signaling acts downstream of cyclic strain to upregulate Cx43 expression in mouse embryonic stem cells, thereby inducing cardiac differentiation (Heo & Lee, 2011). Canonical Wnt signaling also mediates the effects of β1-integrin on Cx mRNA expression (Cx40, Cx43 and Cx45) in mouse embryonic stem cell-derived cardiomyocytes at advanced stages of differentiation (Czyz et al., 2003). Furthermore, inhibition of β-catenin or GSK-3α/β in HL-1 cells, mouse cardiomyocyte cell line, prevents mesenchymal stem cell (MSC)-induced upregulation of Cx43 and improvement in cardiac conduction, suggesting that MSCs alleviate cardiac arrhythmias via activation of the canonical Wnt signaling (Mureli et al., 2012).

A similar pattern of Cx and GJ regulation is reported in Xenopus embryos, ovarian follicles and ovarian carcinomas, umbilical vein endothelial cells and retinal

pigment epithelial cells (Guger & Gumbiner, 1995; Prunskaite-Hyyryläinen et al., 2014; Samarzija, Sini, Schlange, MacDonald, & Hynes, 2009; Umazume et al., 2014; H.-X. Wang et al., 2013; Zhai et al., 2002).

Studies summarized above clearly illustrate interplay between Cxs, mainly Cx43, and the Wnt/β-catenin pathway in several tissues, with the former acting either as downstream targets (positive effectors) or as upstream negative regulators of Wnt signaling. Whether Cxs play the downstream role of a "positive effector" or are upstream "negative regulator" of Wnt signaling, the interplay between the two is context specific. Studies defining Cxs as downstream targets (positive effectors) for the Wnt/β-catenin pathway correlate tissue development and differentiation-driving events to effective GJ communication. As previously stated, induction of Cx43 expression, among other cardiac Cxs (Cx40 and Cx45), downstream of canonical Wnt signaling is associated with the acquisition of cardiac differentiation and function (Z. Ai et al., 2000; Czyz et al., 2003; Heo & Lee, 2011; Mureli et al., 2012). The "positive effector" role of Cxs is additionally associated with developmental processes, such as embryogenesis, angiogenesis and ovarian folliculogenesis (Guger & Gumbiner, 1995; Prunskaite-Hyyryläinen et al., 2014; Samarzija et al., 2009; H.-X. Wang et al., 2013). On the other hand, this role is evident in the context of disease pathogenesis, including ovarian cancer (Zhai et al., 2002) and proliferative vitreoretinopathy (Umazume et al., 2014). In contrast to acting as downstream targets in developmental contexts, the inhibitory effects of Cxs upstream (i.e. "negative regulator") of the Wnt/β-catenin pathway are associated with differentiation or tumor suppression as most studies indicate (Z. Ai et al., 2000; Kamei et al., 2003; Sirnes et al., 2012; S. C. Yu et al., 2012). Hence, Cxs

likely undergo a switch in role from a "positive effector" into a "negative regulator" of the Wnt/β-catenin pathway upon establishment of tissue development to suppress tumorigenesis. During growth and differentiation-driving events of the normal mammary gland, we speculate positive regulation of Cxs downstream of active canonical Wnt signaling to induce Cx-mediated morphogenesis and differentiation (Z. Ai et al., 2000; Czyz et al., 2003; El-Sabban et al., 2003; Guger & Gumbiner, 1995; Heo & Lee, 2011; Rana Mroue et al., 2015; Mureli et al., 2012; Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010; Prunskaite-Hyyryläinen et al., 2014; Samarzija et al., 2009; H.-X. Wang et al., 2013). Within the same context, hyperactive Wnt/β-catenin signaling impairs mammary development (Constantinou et al., 2008). In the context of a differentiated mammary tissue, however, Cxs act to suppress the Wnt/β-catenin pathway in order to maintain homeostasis and to execute tumor suppressive effects (Figure 1.2a) (Z. Ai et al., 2000; Kamei et al., 2003; Sirnes et al., 2012; Talhouk et al., 2008; S. C. Yu et al., 2012). In early stages of breast cancer, the loss of Cx expression triggers the formation of primary tumor by activating canonical Wnt signaling (Banerjee, 2016; Dbouk et al., 2009; Phillips, Williams, Zambrano, Williams, & Yeh, 2017; Talhouk et al., 2013), whereas in the context of advanced breast cancer-driving events, aberrant Wnt/β-catenin signaling induces Cx expression to support collective migration and tumor metastasis (Figure 1.2b) (Umazume et al., 2014; Zhai et al., 2002).

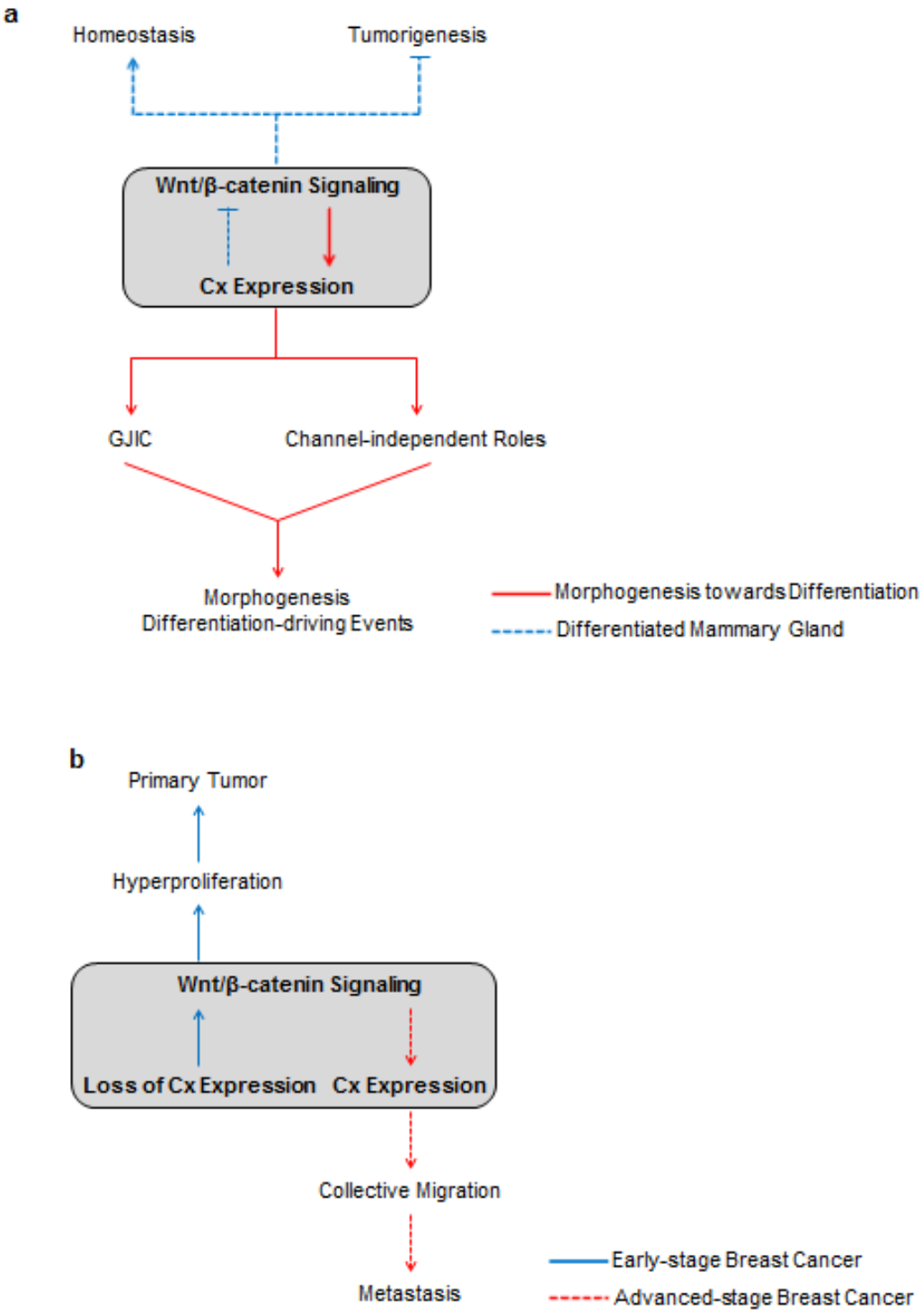

a
Homeostasis
Tumorigenesis
Wnt/β-catenin Signaling
Cx Expression
GJIC
Channel-independent Roles
Morphogenesis
Differentiation-driving Events
Morphogenesis towards Differentiation
Differentiated Mammary Gland
b
Primary Tumor
Hyperproliferation
Wnt/β-catenin Signaling
Loss of Cx Expression
Cx Expression
Collective Migration
Metastasis
Early-stage Breast Cancer
Advanced-stage Breast Cancer

Figure 1.2. A proposed model for the cross-talk between Cxs and Wnt/β-catenin signaling in the mammary gland. Depending on the context, Cxs may act as downstream "positive effectors" (red arrows) or as upstream "negative regulators" of the Wnt/β-catenin pathway (blue arrows) both in normal development and tumorigenesis of the mammary gland (grey boxes). In normal development **(a)**, active canonical Wnt signaling induces Cx expression during morphogenesis and differentiation-driving events of the mammary gland. Cxs regulate the morphogenesis and differentiation of the tissue via channel-dependent and channel-independent mechanisms (red arrows) (Z. Ai et al., 2000; Czyz et al., 2003; Guger & Gumbiner, 1995; Heo & Lee, 2011; Mureli et al., 2012; Prunskaite-Hyyryläinen et al., 2014; Samarzija et al., 2009; H.-X. Wang et al., 2013). Within a differentiated mammary gland, Cxs act as negative regulators of the Wnt/β-catenin pathway, a mechanism to sustain homeostasis and suppress tumorigenesis (blue arrows) (Z. Ai et al., 2000; Kamei et al., 2003; Sirnes et al., 2012; Talhouk et al., 2008; S. C. Yu et al., 2012). In breast cancer **(b)**, the loss of Cx expression during early stages activates canonical Wnt signaling, which mediates hyperproliferation and primary tumor formation (blue arrows) (Talhouk et al., 2013). Aberrant Wnt/β-catenin signaling induces Cx expression in advanced stages of breast cancer, supporting collective migration and tumor metastasis (red arrows) (Umazume et al., 2014; Zhai et al., 2002).

2. Connexins in Noncanonical Wnt Signaling

a. Noncanonical Wnt Pathway

The noncanonical Wnt signaling is a branch of Wnt signaling that encompasses multiple β-catenin-independent pathways and regulates embryogenesis and adult tissue homeostasis. As such, aberrant noncanonical Wnt signaling is associated with developmental defects and adult diseases, particularly cancer (Dunn & Tolwinski, 2016; Gómez-Orte, Sáenz-Narciso, Moreno, & Cabello, 2013; Komiya & Habas, 2008; Sedgwick & D'Souza-Schorey, 2016; Segalen & Bellaïche, 2009; Sokol, 2015).

Noncanonical Wnt signaling regulates epithelial apicobasal polarity (asymmetry along the apical-basal axis within a cell), PCP (the coordinated organization of cells within a tissue plane, also referred to as tissue polarity), cell junctions, mitotic spindle orientation, actin cytoskeletal dynamics and cell migration. Noncanonical Wnt

pathways are triggered by specific family members of Wnt ligands that signal through Fzd receptors, like the canonical branch, but use alternatives to LRP5/6 where coreceptors are involved. Owing to the ligand and coreceptor differences, the noncanonical Wnt pathways regulate signaling cascades different from that underlying canonical Wnt signaling downstream of Dvl recruitment to the ligand-receptor-coreceptor complex. In addition, while the activation of the canonical Wnt pathway regulates gene expression, noncanonical Wnt signaling is also associated with nontranscriptional outcomes. The PCP and the Wnt/calcium pathways are by far the best characterized among the noncanonical Wnt pathways (De, 2011; Dunn & Tolwinski, 2016; Gómez-Orte et al., 2013; Komiya & Habas, 2008; Sedgwick & D'Souza-Schorey, 2016; Segalen & Bellaïche, 2009; Sokol, 2015).

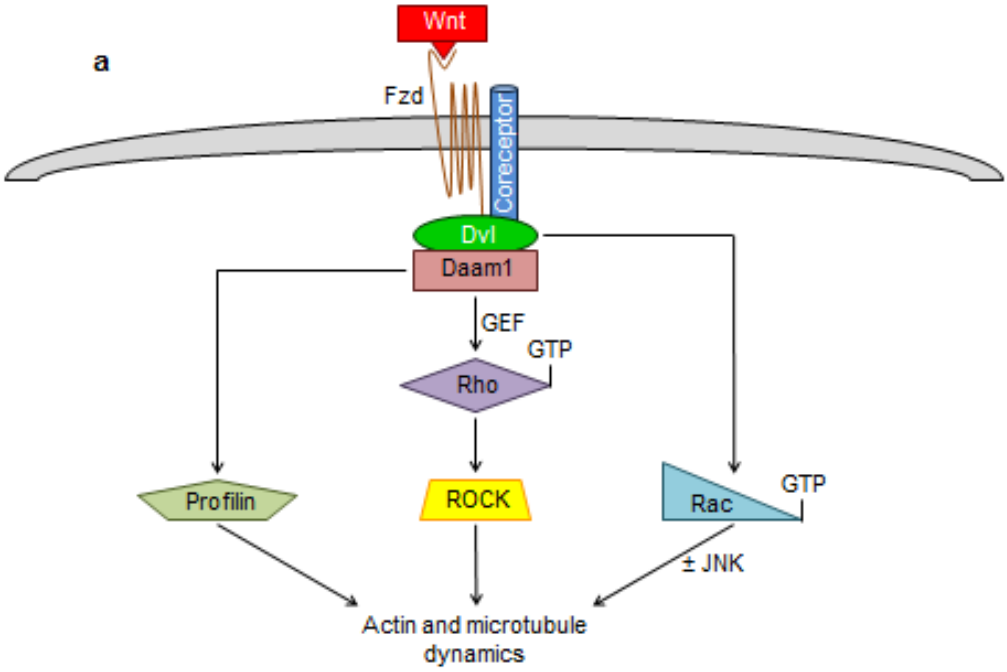
The PCP pathway activates Ras homolog (Rho) GTPases, namely Rac and Rho, and c-Jun N-terminal kinase (JNK), which induce cytoskeletal rearrangements (Gómez-Orte et al., 2013; Komiya & Habas, 2008). The PCP pathway is activated when a noncanonical Wnt ligand binds to Fzd and its coreceptor (ROR2, RYK, PTK7 or NRH1). Dvl is subsequently recruited and associates with Dishevelled-associated activator of morphogenesis 1 (Daam1), which activates Rho via a guanine nucleotide exchange factor (GEF). Rho in turn activates Rho-associated kinase (ROCK), a major regulator of the actin cytoskeleton. Daam1, on the other hand, mediates binding of profilin to actin. In addition, Dvl mediates activation of Rac, which activates JNK. Profilin, ROCK and JNK induce actin cytoskeletal reorganization (Gómez-Orte et al., 2013; Komiya & Habas, 2008; Sedgwick & D'Souza-Schorey, 2016). The PCP pathway is known to regulate actin polymerization, as a mechanism to control cell morphology

and polarized cell migration (Komiya & Habas, 2008). Microtubules constitute another cytoskeletal element regulated by the PCP pathway, which orients the mitotic spindle relative to cell-cell contacts or to an embryo symmetry axis (Segalen & Bellaïche, 2009) (Figure 1.3a). Due to its role in cell division orientation and directional cell movement, the PCP pathway regulates morphogenetic processes, such as gastrulation, neurulation and organ morphogenesis (Gómez-Orte et al., 2013; Sedgwick & D'Souza-Schorey, 2016).

The Wnt/calcium pathway, on the other hand, activates Fzd-associated heterotrimeric G proteins besides Dvl and regulates intracellular calcium levels by stimulating or inhibiting calcium release from the endoplasmic reticulum (ER). One consequence of calcium release is the activation of the Rho GTPase Cdc42 through protein kinase C (PKC). Another important outcome is the activation of calcium/calmodulin-dependent protein kinase II (CaMKII), which in turn activates nuclear factor of activated T-cells (NFAT), a transcription factor (De, 2011; Komiya & Habas, 2008) (Figure 1.3b). The Wnt/calcium pathway regulates several aspects of embryogenesis, such as ventral cell fate, tissue separation and convergent extension, and is thought of as a modulator of PCP signaling (Komiya & Habas, 2008).

Fzd-independent pathways are identified as components of noncanonical Wnt signaling, although less characterized than the PCP and the Wnt/calcium pathways (Angers & Moon, 2009; Green, Nusse, & van Amerongen, 2013). The Fzd coreceptors ROR2 and RYK harbor functional extracellular Wnt-binding domains and can act as Wnt receptors independently from Fzd activation (Green et al., 2013) (Figure 1.3c). ROR2 and RYK regulate developmental processes in several tissues and are associated

with cell polarity, migration and asymmetric cell division (Angers & Moon, 2009; Clark, Nourse, & Cooper, 2012; Debebe & Rathmell, 2015; Green et al., 2013).



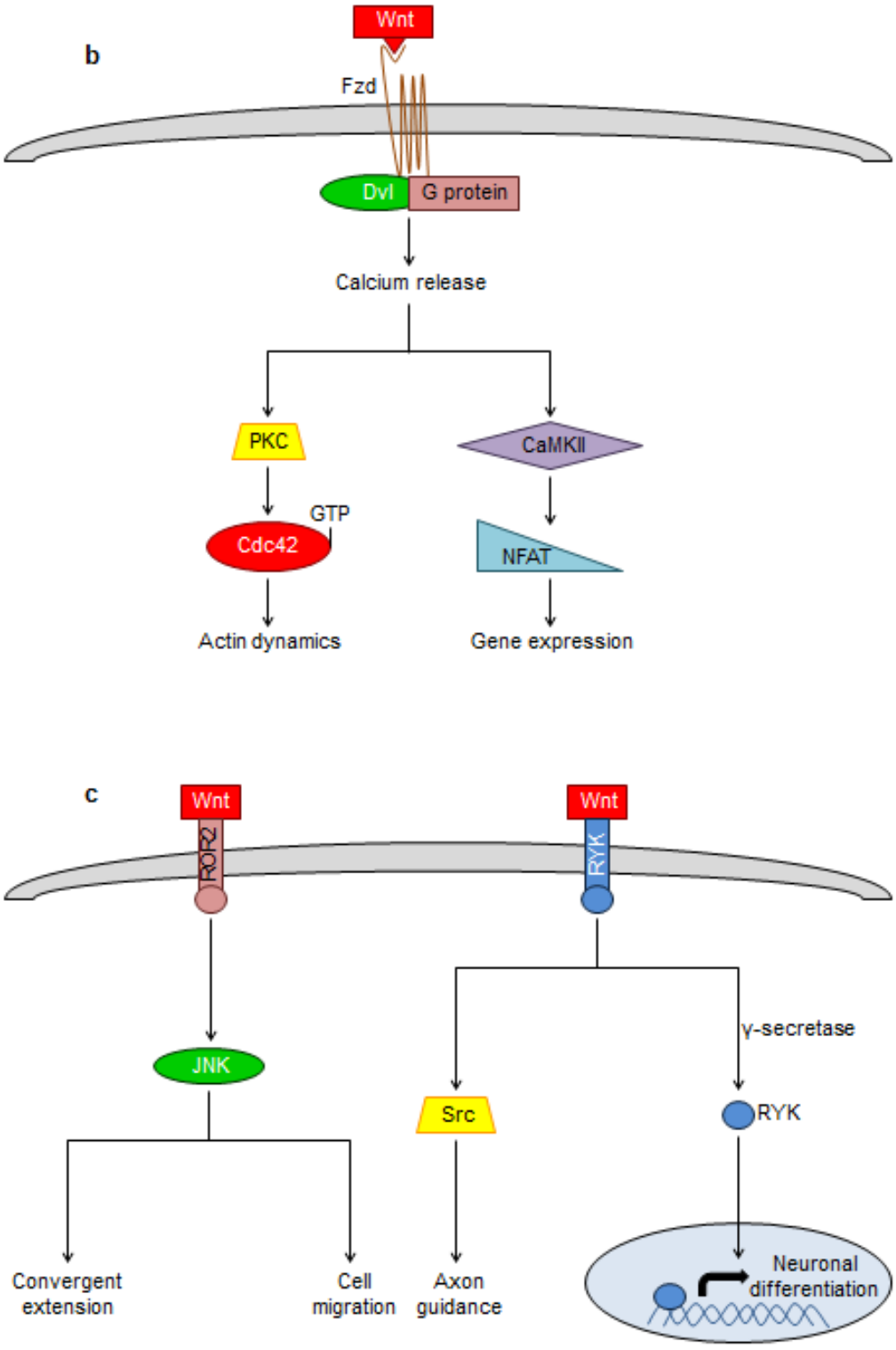


Figure 1.3. The noncanonical Wnt pathway. The PCP pathway **(a)** involves binding of a Wnt ligand to its receptor Fzd and coreceptor (ROR2, RYK, PTK7 or NRH1). The

resulting complex recruits the scaffolding protein Dvl, which in turn recruits Daam1. This leads to GEF-mediated activation of Rho, which activates ROCK. Daam1 also mediates binding of profilin to actin. On the other hand, Dvl mediates Rac activation, which acts through activating JNK or independently. Profilin, ROCK and Rac regulate the dynamics of the actin and microtubule networks, which control cellular morphology, migration and division orientation. The Wnt/calcium pathway **(b)** involves the coactivation of Dvl and Fzd-associated G protein upon binding of a Wnt ligand, leading to the intracellular release of calcium. This results in PKC-mediated activation of Cdc42, which regulates actin dynamics. Calcium release also activates CaMKII, which activates the transcription factor NFAT. The Fzd-independent pathways **(c)** are triggered upon binding of a Wnt ligand to its receptor ROR2 or RYK. ROR2 subsequently mediates JNK activation, which regulates cell migration and convergent extension, among others. RYK controls axon guidance via the Src kinase family. In addition, the intracellular domain of RYK translocates to the nucleus upon cleavage by γ-secretase, where it mediates the expression of genes required for neuronal differentiation.

Due to a cross-talk among noncanonical Wnt pathways, these pathways are alternatively considered as one signaling network with diverse biological outcomes. Studies modeling the noncanonical Wnt pathways as such highlighted the roles of Rho GTPases as important downstream effectors of noncanonical Wnt signaling. RhoA, Rac1 and Cdc42 are known to regulate cytoskeletal dynamics involving the microtubule and actin networks, thereby controlling mitotic spindle orientation, cell shape changes, motility and invasion. Rho GTPase signaling also regulates polarity, intercellular junctions and cell-ECM interactions, hence the implication of the deregulation of Rho GTPases in mammary gland tumorigenesis (Dunn & Tolwinski, 2016; Etienne-Manneville & Hall, 2002; Hanna & El-Sibai, 2013; Mack & Georgiou, 2014; Schlessinger, Hall, & Tolwinski, 2009; Sedgwick & D'Souza-Schorey, 2016).

b. <u>Role of Rho GTPases in Mammary Gland Development</u>

Rho GTPase signaling components are implicated in various stages of mammary gland development, from embryogenesis to involution, and their aberrant expression and/or activity contributes to breast tumorigenesis (Burbelo, Wellstein, & Pestell, 2004; Zuo, Oh, Ulu, & Frost, 2016).

Inhibition of Rac1 or ROCK, a downstream effector of RhoA, in an organoid culture of mammary tissue blocks duct initiation and disrupts branching pattern, respectively, indicating a role for Rac1 and RhoA in morphogenesis of the mammary gland (Ewald et al., 2008). Expression of a dominant-negative form of Rac1 or its downstream effector p21-activated kinase 1 (PAK1) enhances the contractility of mouse myoepithelial cells *in vitro*. Consistent with these observations, the expression of a constitutively active form of Rac1 or a catalytically active form of PAK1 induces myoepithelial relaxation, demonstrating a role for Rac1 signaling in controlling the contraction/relaxation cycle of myoepithelial cells and thus in lactation (Raymond et al., 2011). Conditional deletion of Rac1 in mouse mammary glands delays involution via STAT3-dependent mechanism (Bagci et al., 2014).

A study on a 3-D culture of primary mammary epithelial cells isolated from Cdc42 conditional knockout mice unveiled a role for Cdc42 in morphogenesis of the mammary gland. Cdc42 deficiency reduces cell proliferation and survival and alters the number and size of acini, concomitant with disruption of acinar morphology. Furthermore, apicobasal polarity, mitotic spindle orientation and lumen formation, which represent key morphogenetic features of normal mammary epithelium, are disrupted (Bray, Brakebusch, & Vargo-Gogola, 2011). Paradoxically, normal morphogenesis of the mammary gland is also disrupted in a tetracycline-regulatable

Cdc42 overexpression mouse model. This suggests the importance of tight regulation of Cdc42 levels for normal mammary gland development. Cdc42-overexpressing mammary glands exhibit TEB hyperbudding and trifurcation, ductal tree hyperbranching and altered epithelial-stromal interactions, which are known to regulate branching. Consistent with these observations, primary mammary epithelial cells isolated from Cdc42-overexpressing mammary glands form dysmorphic invasive acini in 3-D cultures, coupled to enhanced expression of ECM proteins and remodeling enzymes in their stromal counterparts. Interestingly, the phenotypic abnormalities observed upon Cdc42 overexpression are not a consequence of enhanced cell proliferation or survival, nor are they associated with disruptions in apicobasal polarity or mitotic spindle orientation. They are rather due to enhanced epithelial contractility and migration (Bray et al., 2013). Taken together, gain-of-function and loss-of-function studies clearly illustrate redundancy in Cdc42 effects, suggesting that its role in mammary gland morphogenesis is highly contingent upon a tight balance of its levels, and perhaps activity. In addition to regulating the morphogenesis of the mammary gland, Cdc42 plays a role in its proper functioning. Conditional knockout mice lacking Cdc42 in mammary alveolar epithelial cells during lactation inadequately nourish their pups, leading to stunted growth. This is attributed to impaired alveologenesis as a consequence of disrupted apical-basal polarity and cell-cell adhesion, which result in premature exfoliation of the alveolar epithelium (Druso et al., 2016).

c. Role of Rho GTPases in Breast Tumorigenesis

Rho GTPases are overexpressed or hyperactivated in human breast tumors (Burbelo et al., 2004; G Fritz et al., 2002; Gerhard Fritz et al., 1999). In addition, the expression of Rho GTPase regulators and effectors is altered in breast cancer tissues (Ahn et al., 2003; Burbelo et al., 2004; Hanna et al., 2014; J. Lane, Martin, Mansel, & Jiang, 2008). A link is established between Rho GTPase expression levels and cell motility and invasion *in vitro*. Cdc42 and Rac regulate the formation of filopodia and lamellipodia, respectively, at the leading edge of a motile cell, while Rho regulates the formation of stress fibers and actomyosin contractility at the rear end (Hanna & El-Sibai, 2013). The presence of a cross-talk among Rho GTPases during cell motility is also reported. For instance, Forster resonance energy transfer (FRET) biosensor imaging revealed a biphasic localization of RhoA activity at the leading edge of epidermal growth factor (EGF)-stimulated MTLn3 rat mammary adenocarcinoma cells. This spatiotemporal pattern of RhoA activity is critical for coordinating the functions of Rac1 and Cdc42 during the formation of protrusions (El-Sibai et al., 2008). Primary mammary epithelial cells from Cdc42-overexpressing mammary glands display enhanced contractility and migration. Specifically, Cdc42 overexpression upregulates ECM proteins and remodeling enzyme levels in stromal cells, and disrupts epithelial-stromal interactions, further supporting a role for Cdc42 in breast cancer invasion (Bray et al., 2013). Consistent with those findings, the knockdown of Cdc42 in MTLn3 cells impairs EGF-induced protrusion, barbed end formation and F-actin accumulation at the protruding edges, which are concomitant with reduced motility, suggesting a role for Cdc42 in breast cancer cell motility (El-Sibai et al., 2007). siRNA-mediated silencing of RhoA or RhoC impairs invasiveness of MDA-MB-231 cells (Pillé et al., 2005).

Interestingly, ROCK mediates the invasion of amoeboid breast cancer cells through matrix metalloproteinase (MMP)-independent mechanism, by regulating myosin light chain (MLC) organization and the generation of forces that cause deformation of the underlying collagen fibers, thereby allowing cells to invade the ECM (Wyckoff, Pinner, Gschmeissner, Condeelis, & Sahai, 2006). Silencing RhoC in MTLn3 cells impairs protrusion formation and directionality in response to EGF stimulation (Bravo-Cordero et al., 2013). In addition, RhoC-depleted MTLn3 cells exhibit altered morphology and function of the ECM-degrading invadopodial protrusions and reduced invasive potential (Bravo-Cordero et al., 2011). Rac1 counteracts the activity of RhoC in MTLn3 cells by inducing the disassembly of invadopodia. Considering the role of Rac1 in the formation of lamellipodia, this effect is believed to sustain the proper balance between matrix-degrading and locomotory protrusions for optimal cell invasion (Moshfegh, Bravo-Cordero, Miskolci, Condeelis, & Hodgson, 2014). In fact, knocking down Rac1 induces membrane ruffling and impairs motility in EGF-stimulated MTLn3 cells. This is due to altered formation of focal adhesions at the leading edge, rendering the protrusions unstable (Yip et al., 2007).

In addition to their role in breast cancer invasion, Rho GTPases alter the morphogenesis of mammary epithelial tissue, an event that marks breast cancer initiation, both *in vitro* and *in vivo* (Bray et al., 2013). Indeed, Rho GTPase signaling plays a role in regulating morphogenetic aspects of mammary epithelial cells, including cell-cell adhesion, cell-ECM interactions, apicobasal polarity, mitotic spindle orientation and lumen formation (Bray et al., 2011; Bray et al., 2013; Druso et al., 2016). Rho GTPases also mediate preneoplastic transformation, tumor growth,

angiogenesis and metastasis in breast cancer. Ectopic expression of RhoA leads to immortalization of primary human mammary epithelial cells (Zhao et al., 2009). In contrast, silencing RhoA reduces the proliferation of MDA-MB-231 cells and suppresses xenograft tumor growth, angiogenesis and lung metastasis in mice (Chan et al., 2010; Pillé et al., 2005). Similarly, inhibiting Rac1 in MDA-MB-435 cells impairs tumor growth, angiogenesis and metastasis in a nude mouse model (Castillo-Pichardo et al., 2014).

d. Cross-talk between Connexins and Rho GTPase Signaling

As previously mentioned, intercellular adhesion and communication, which are key aspects of a differentiated mammary epithelium, are disrupted in breast cancer. Rho GTPase activities are spatiotemporally regulated to control the establishment and maintenance of epithelial apicobasal polarity and cell-cell junctions, particularly tight junctions (TJs) and adherens junctions (AJs) (Citi, Guerrera, Spadaro, & Shah, 2014; Etienne-Manneville & Hall, 2002; Mack & Georgiou, 2014). FRET biosensor studies showed spatiotemporal localization patterns of RhoA, Rac1 and Cdc42 activities along the apical and lateral membrane domains of Madin-Darby canine kidney (MDCK) epithelial cells during cystogenesis. Specifically, Rac1 activity at the lateral membrane exceeds that at the apical membrane during late cystogenesis, and induction of Rac1 activity at the apical membrane of mature cysts disrupts apical-basolateral polarity, TJs and mitotic spindle orientation (Yagi, Matsuda, & Kiyokawa, 2012). Spatiotemporal Rac1 activity is also implicated in the establishment of AJs. FRET biosensor imaging showed that local Rac1 activation is induced upon the formation of nascent AJs, leading

to junction stabilization in endothelial cells (Timmerman et al., 2015). RhoA colocalizes with AJs in the developing mouse brain, and conditional knockout of RhoA in neural progenitor cells of the forebrain and midbrain disrupts AJs, suggesting a role for RhoA in maintenance of AJs (Katayama et al., 2011). RhoA also regulates the maintenance of both apicobasal polarity and TJ localization in retinal progenitor cells during vertebrate embryonic development (Herder et al., 2013). Similarly, Cdc42 regulates the establishment of cell polarity and junction assembly in a mammalian model of early embryonic development. Cdc42-null embryoid bodies show homogenous cortical distribution of F-actin and lack the characteristic distribution of the microtubule-organizing center (MTOC) and Golgi complex, indicating absence of cell polarity. In addition punctate cell-cell contacts containing TJ and AJ markers are formed, and continuous TJ or AJ belts fail to assemble (Wu et al., 2007).

Rho GTPase signaling is also known to regulate GJ function and assembly. Blocking the activities of Rho family proteins by overexpressing the guanine nucleotide dissociation inhibitor (GDI) Rho GDIα under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter reduces the expression levels of Cx40 in mouse hearts and is associated with conduction defects (Wei et al., 2004). In a similar study where C3-exoenzyme expression is utilized, inhibition of Rho GTPase activities in mouse lenses reduces Cx50 expression levels (Maddala et al., 2004). Consistent with those findings, calpeptin-stimulated RhoA activity in HL-1 cells, mouse cardiac myocyte cell line, upregulates the expression levels of Cx43 (L. Wang, Liu, Zhang, Hu, & Wei, 2015). In parallel, Rho GTPases also affect Cx localization. For instance, Cx43 localization is altered in response to Rac1 inhibition in neonatal rat cardiac myocytes

(Matsuda et al., 2006). Likewise, Cx26 and Cx32 are mislocalized in hepatocytes of Cdc42-deficient mouse livers (Van Hengel et al., 2008). Rho GTPases further regulate GJs at the level of assembly and permeability. Inhibiting Rho activity in primary rabbit corneal epithelial cells by C3-exoenzyme microinjection impairs the assembly of Cx43-based GJs (Anderson, Stone, Tkach, & SundarRaj, 2002). In addition, C3-exoenzyme-induced inhibition of RhoA reduces GJIC in rat cardiac myocytes (Derangeon et al., 2008). Notably, other families of GTPases, mainly the Ras family, are also implicated in the regulation of Cx expression levels, GJ formation and GJIC (BROWNELL, NARSIMHAN, et al., 1996; Brownell, Whitfield, & Raptis, 1996, 1997; Chen, Shuzo, Li, & Han, 1998; Geletu, Guy, Greer, & Raptis, 2015; Hayashi, Nomata, Chang, Ruch, & Trosko, 1998; Ito et al., 2006; Somekawa et al., 2005; Stains & Civitelli, 2005).

In contrast to above, others demonstrated that Cxs are upstream regulators of Rho GTPase signaling. Cx43 activates the RhoA-ROCK pathway, as a mechanism for bradykinin-induced vascular contraction (Zhang et al., 2015). Furthermore, a role for Cx43 in Rac1 activation and actin cytoskeletal reorganization is proposed in breast cancer cells (Sin et al., 2009). Blocking GJIC induces phosphorylation of Cdc42 in mouse ventricular zone precursors, resulting in its inactivation (X. Liu, Hashimoto-Torii, Torii, Ding, & Rakic, 2010). Unlike the aforementioned studies that reported positive regulation of Rho GTPases by Cxs, one study demonstrated enhanced Rac1 and RhoA activities in 3T3 mouse embryonic fibroblasts in response to Cx43 knockdown. This is followed by enhanced migration and actin cytoskeletal reorganization (Mendoza-Naranjo et al., 2012). The variable effect of Cxs on Rho GTPases suggests that Cxs regulate Rho GTPase signaling in a cell type-specific and/or context-dependent

manner. Cxs also regulate other GTPases, such as Rap1. In WEHI-231 cells, murine B lymphoma cell line, Cx43 mediates B-cell receptor (BCR)-, integrin (LFA-1)- and chemokine (CXCL12)-induced Rap1 activation and the subsequent spreading and adhesion of B cells to vascular endothelial cells (Machtaler et al., 2014; Machtaler et al., 2011). Cx43 further regulates BCR- and integrin-induced B cell motility, in addition to chemokine-stimulated directed and transendothelial migration downstream of Rap1 activation (Machtaler et al., 2014).

Although a cross-talk between Cxs and Rho GTPases is implied, the literature describing such a link remains scarce, and almost no evidence supports its existence in the breast tissue. In one study however, the noncanonical ligand Wnt5a is proposed to impair lactation in mice through regulating Cx functions. In contrast to wild-type mice, overexpression of Wnt5a in the mammary epithelium inhibits oxytocin-induced milk ejection and sustains the phosphorylation of Cx43 after parturition (Baxley et al., 2011). Studies summarized above suggest positive regulation of Cx expression and function downstream of Rho GTPase signaling in tissue morphogenesis, differentiation and pathology (Maddala et al., 2004; L. Wang et al., 2015; Wei et al., 2004). Considering the dual roles of Cxs and Rho GTPases in development and tumorigenesis of the mammary gland, it is conceivable that enhanced Cx expression downstream of Rho GTPase signaling drives normal morphogenesis during development while supporting metastasis during breast cancer progression. The effects of Cxs as upstream regulators of Rho GTPases, however, remain controversial, posing a challenge in defining the regulatory role of Cxs in Rho GTPase signaling within the mammary gland (X. Liu et al., 2010; Mendoza-Naranjo et al., 2012; Sin et al., 2009; Zhang et al., 2015). We have

recently delineated a role for Cx43 in regulating Rho GTPase signaling (unpublished data) and in establishing apical polarity and mitotic spindle orientation in 3-D cultures of human mammary epithelial cells (Bazzoun et al.; submitted). Given the role of Rho GTPases in establishment and maintenance of epithelial apicobasal polarity and intercellular junctions and in regulation of cytoskeletal dynamics, and considering their developmental and tumorigenic roles in the mammary gland that overlap with those of Cxs, it becomes necessary to study the involvement of Rho GTPase signaling downstream of Cxs in the mammary gland.

G. Conclusion and Future Perspectives

Understanding the molecular events associated with the development and tumorigenesis of the mammary gland is key to establishing the appropriate preventive measures and treatment strategies for breast cancer. The loss of Cx expression and GJIC characterizes early stages of breast cancer. Studies investigating Cx expression profiles in patient tissues propose Cxs as independent prognostic markers, making Cxs potential therapeutic targets in breast cancer. Considering the channel-independent roles of Cxs and the diverse cellular events they regulate, elucidating the signaling pathways that link GJs to the development and tumorigenesis of the mammary gland would ensure a better targeted therapeutic approach in breast cancer. A cross-talk between Wnt pathways on one hand and GJs on the other hand is clearly illustrated in several tissues and biological contexts. Although independent regulatory roles are established for GJs and Wnt signaling in the development and tumorigenesis of the mammary gland, the link between the two pathways in this tissue is poorly characterized. Our findings

illustrate a role for Cxs in regulating Wnt signaling as a mechanism to drive development, maintain homeostasis and to suppress tumorigenesis of the mammary gland. We speculate the involvement of both canonical and noncanonical Wnt pathways as modulators of GJ functions in development of the mammary gland, and we implicate disruption of Wnt signaling as a result of altered Cx expression and function in breast cancer.

CHAPTER II

AIMS

Connexin (Cx) expression is spatially and temporally regulated to support normal development of the mammary gland (Dianati et al., 2016; Locke et al., 2007; Locke et al., 2000; Locke et al., 2004; Monaghan et al., 1994; Rana Mroue et al., 2015; Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010; Talhouk et al., 2005). In the human breast, the expression of Cx26 is confined to luminal epithelial cells, whereas Cx43 is expressed in luminal epithelial and myoepithelial compartments (Jamieson, Going, D'Arcy, & George, 1998a; Monaghan et al., 1996). Human breast tumor tissues and cell lines harbor altered expression and localization of Cx43, suggesting a tumor suppressive role (Kańczuga-Koda et al., 2003; L Kanczuga-Koda et al., 2006; Luiza Kanczuga-Koda et al., 2005; Laird et al., 1999; Yeh et al., 2017). This has been corroborated by studies that propose Cx43 as an independent favorable prognostic factor in breast cancer patients (Chasampalioti et al., 2018; Teleki et al., 2014). Our previous studies demonstrated a role for Cx43 in inducing the differentiation of mammary epithelial cells and in partial reversion of the tumor phenotype when re-introduced into human breast cancer cell lines (Talhouk et al., 2013; Talhouk et al., 2008). We have recently unveiled a role for Cx43 in regulating acinar morphogenesis of nontumorigenic human mammary epithelial HMT-3522 S1 cells (Bazzoun/Adissu et al., submitted). Silencing Cx43 in 3-dimensional (3-D) cultures of S1 cells altered apical polarity, mitotic spindle orientation (MSO) and impaired the lumen-forming ability, which collectively represent early signs of tumor initiation. The signaling pathways

through which Cx43 exerts its tumor suppressive roles in the mammary gland remain poorly defined. The Wnt signaling pathways, encompassing the Wnt/β-catenin or the canonical Wnt pathway and the β-catenin-independent noncanonical Wnt pathway, execute key roles in the development of the mammary gland, and aberrant Wnt signaling is associated with impaired mammary development and breast tumorigenesis (Bagci et al., 2014; Bray et al., 2013; Ewald et al., 2008; G Fritz et al., 2002; Gerhard Fritz et al., 1999; Imbert et al., 2001; Lin et al., 2000; Lindvall et al., 2009; Milovanovic et al., 2004; Prasad et al., 2008; Raymond et al., 2011; van Genderen et al., 1994). Studies support a cross-talk between Cxs and Wnt signaling in a variety of nonbreast tissues and biological contexts (Fostok et al., 2018). In the breast, Cxs act as downstream transcriptional and functional targets for Wnt signaling (Baxley et al., 2011; Constantinou et al., 2008; Van der Heyden et al., 1998). However, the possible role of Cxs as upstream regulators of Wnt signaling is yet to be investigated. Our earlier studies suggest that Cx43 negatively regulates the Wnt/β-catenin pathway via assembling gap junction (GJ) complexes that recruit β-catenin to the membrane and sequester it away from the nucleus, as a mechanism to induce differentiation of mammary epithelial cells or to suppress tumorigenesis of breast cancer cells (Talhouk et al., 2013; Talhouk et al., 2008). In this study, we will extend our previous findings and further address the role of Cx43 in homeostasis of the breast tissue, using 3-D in-vitro breast culture models. We propose to elucidate the signaling pathways through which Cx43 regulates mammary epithelial homeostasis. The specific aims of this study are to:

Aim 1: determine the effects of Cx43 silencing on proliferation and migration of S1 cells.

Aim 2: assess the assembly of the GJ complex in S1 cells, and determine the effects of Cx43 silencing on the expression and localization of junctional and polarity proteins.

Aim 3: investigate the effects of Cx43 silencing on the activities of the canonical and the noncanonical Wnt pathways.

CHAPTER III

CONNEXIN 43 LOSS TRIGGERS CELL CYCLE ENTRY AND INVASION IN NON-NEOPLASTIC BREAST EPITHELIUM: A ROLE FOR NONCANONICAL WNT SIGNALING

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A. Abstract

1) Background: The expression of connexin 43 (Cx43) is disrupted in breast cancer, and re-expression of this protein in human breast cancer cell lines leads to decreased proliferation and invasiveness, suggesting a tumor suppressive role. This study aims to investigate the role of Cx43 in proliferation and invasion starting from non-neoplastic breast epithelium. 2) Methods: Nontumorigenic human mammary epithelial HMT-3522 S1 cells and Cx43 shRNA-transfected counterparts were cultured under 2-dimensional (2-D) and 3-D conditions. 3) Results: Silencing Cx43 induced mislocalization of β-catenin and Scrib from apicolateral membrane domains in glandular structures or acini formed in 3-D culture, suggesting the loss of apical polarity. Cell cycle entry and proliferation were enhanced, concomitantly with c-Myc and cyclin D1 upregulation, while no detectable activation of Wnt/β-catenin signaling was observed. Motility and invasion were also triggered and were associated with altered acinar morphology and activation of ERK1/2 and Rho GTPase signaling, which acts downstream of the noncanonical Wnt pathway. The invasion of Cx43-shRNA S1

cells was observed only under permissive stiffness of the extracellular matrix (ECM). 4) Conclusion: Our results suggest that Cx43 controls proliferation and invasion in the normal mammary epithelium in part by regulating noncanonical Wnt signaling.

B. Introduction

Connexins (Cxs), the building blocks of gap junctions (GJs), exhibit spatiotemporal expression patterns throughout development of the mammary gland (Dianati et al., 2016; Locke et al., 2007; Locke et al., 2000; Locke et al., 2004; Monaghan et al., 1994; Talhouk et al., 2005). In the human breast, Cx43 is expressed in luminal epithelial and myoepithelial compartments, while the expression of Cx26 is restricted to the luminal epithelium (Jamieson et al., 1998a; Monaghan et al., 1996). Altered expression and localization of Cxs have been reported in human breast cancer tissues and cell lines (Kańczuga-Koda et al., 2003; L Kanczuga-Koda et al., 2006; Luiza Kanczuga-Koda et al., 2005; Laird et al., 1999; Singal et al., 2000; Yeh et al., 2017) and have been associated with developmental defects in mouse models (Rana Mroue et al., 2015; Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010), suggesting that Cxs have developmental and tumor suppressive roles. Indeed, Cx43 is proposed as an independent prognostic factor in light of its positive correlation with improved disease outcome in breast cancer patients (Chasampalioti et al., 2018; Teleki et al., 2014). Exogenous expression of Cx43 in human breast cancer cell lines reduces proliferation, invasiveness, xenograft tumor growth and metastasis and restores the differentiation capacity (Hirschi et al., 1996; Z. Li, Zhou, & Donahue, 2008; Z. Li, Zhou, Welch, & Donahue, 2008; McLachlan, Shao, Wang, Langlois, & Laird, 2006; Qin et al., 2002;

Talhouk et al., 2013). Conversely, silencing Cx43 in Hs578T human breast cancer cell line enhances proliferation and anchorage-independent growth and upregulates the expression of vascular endothelial growth factor (VEGF) (Shao et al., 2005). Similarly, heterozygous Cx43 mutation enhances the susceptibility to lung metastasis in mice with 7,12-Dimethylbenz(a)anthracene (DMBA)-induced mammary tumors (I Plante et al., 2011).

The involvement of channel-independent mechanisms in the tumor suppressive functions of Cxs (Kalra et al., 2006; Qin et al., 2002; Talhouk et al., 2013) suggests a cross-talk with signaling pathways that regulate tumorigenesis of the mammary gland, such as Wnt signaling (Fostok et al., 2018). The canonical or Wnt/β-catenin and the noncanonical Wnt pathways execute key roles in the development and differentiation of the mammary gland, and altered Wnt signaling is associated with breast cancer (Bagci et al., 2014; Bray et al., 2013; Ewald et al., 2008; G Fritz et al., 2002; Gerhard Fritz et al., 1999; Imbert et al., 2001; Lin et al., 2000; Lindvall et al., 2009; Milovanovic et al., 2004; Prasad et al., 2008; Raymond et al., 2011; van Genderen et al., 1994). Although Cxs act as transcriptional and functional targets of Wnt signaling in the breast tissue (Baxley et al., 2011; Constantinou et al., 2008; Van der Heyden et al., 1998), their possible role as regulators of Wnt pathways is yet to be investigated (Fostok et al., 2018). It is widely accepted that reduced Cx expression and the subsequent loss of gap junctional intercellular communication (GJIC) promotes physical detachment of cells from the tumor microenvironment and enhances their migratory capacity in the context of a primary tumor. On the other hand, restoration of Cx expression and GJIC in advanced stages of breast cancer is believed to mediate the interaction of tumor cells

with their microenvironment, thereby enhancing tumor metastasis (Banerjee, 2016; Grek, Rhett, Bruce, Ghatnekar, & Yeh, 2016; McLachlan et al., 2007). Given that Cxs act as tumor suppressors or enhancers in a stage-specific manner (Elzarrad et al., 2008; Jamieson et al., 1998a; Luiza Kanczuga-Koda, Sulkowska, Koda, Rutkowski, & Sulkowski, 2007; L Kanczuga-Koda et al., 2006; Naoi et al., 2007; Pollmann, Shao, Laird, & Sandig, 2005; Thiagarajan et al., 2018), and considering the extracellular matrix (ECM) remodeling events in breast cancer (Insua-Rodriguez & Oskarsson, 2016; Kaushik, Pickup, & Weaver, 2016), Cxs might act in cooperation with an altered microenvironment to induce breast tumorigenesis and invasion. The signaling pathways that act downstream of Cx loss in breast cancer and the role of the mammary epithelial microenvironment in execution of Cx signaling remain poorly investigated. In this study, we assess the activity of canonical and noncanonical Wnt pathways in Cx43-silenced non-neoplastic mammary epithelium, and we describe a role for the microenvironment as a coregulator of Cx43-driven events.

In order to address the role of Cx43 loss in breast homeostasis, we utilized the nontumorigenic human mammary epithelial HMT-3522 S1 cell line (Briand, Petersen, & Van Deurs, 1987). When cultured in the presence of basement membrane components-enriched Matrigel under 3-dimensional (3-D) conditions, S1 cells assemble into growth-arrested differentiated glandular structures or acini. An apicobasal polarity axis, a central lumen and apicolateral membrane expression of Cx43 that forms functional GJs characterize S1 acini, recapitulating the normal tissue architecture in the human breast (Petersen, Rønnov-Jessen, Howlett, & Bissell, 1992; Plachot & Lelièvre, 2004). Our previous studies showed that Cx43-shRNA S1 cells in 3-D culture acquire a

perturbed apical polarity and mitotic spindle orientation (MSO), with loss of lumen formation (Bazzoun/Adissu et al., submitted). These observations are in line with our earlier findings that demonstrate a role for Cx43 in inducing differentiation of mouse mammary epithelial cells and reversing tumorigenesis of human breast cancer cells, via membrane GJ complex assembly mediated in part by ECM signaling and β-catenin/Cx43 association (El-Sabban et al., 2003; Talhouk et al., 2013; Talhouk et al., 2008).

In this study, we report enhanced proliferation and cell cycle entry, upregulation of c-Myc and cyclin D1 in Cx43-shRNA S1 cells, concomitantly with the mislocalization of β-catenin and Scrib from apicolateral membrane domains in 3-D cultures. While no detectable activation of Wnt/β-catenin signaling was observed, the loss of Cx43 upregulated the expression and activity of Rho GTPases (RhoA, Rac1 and Cdc42), thereby triggering the noncanonical Wnt pathway, and enhanced ERK1/2 activity. Migratory and invasive capacity was evident only under permissive ECM stiffness. Our results suggest that Cx43 controls proliferation and invasion pathways in the normal mammary epithelium in part via regulating noncanonical Wnt signaling.

C. Materials and Methods

1. Cell Culture

Non-neoplastic HMT-3522 S1 human mammary epithelial cells (Briand et al., 1987), between passages 52 and 60, were routinely maintained as a monolayer on plastic (2-D culture) in chemically defined serum-free H14 medium (Blaschke, Howlett,

Desprez, Petersen, & Bissell, 1994; Plachot & Lelièvre, 2004) at 37 °C and 5% CO_2 in a humidified incubator. H14 medium was changed every 2-3 days. HMT-3522 T4-2 cells, the tumorigenic counterparts of S1 cells, were maintained under similar conditions but on plastic coated with collagen I (BD Biosciences, 354236) and in the absence of epidermal growth factor (EGF). S1 and T4-2 cells were propagated as previously described (Vidi, Bissell, & Lelièvre, 2012). For 2-D cultures, cells were plated on plastic substrata at a density of $2.3x10^4$ cells/cm^2 (S1 cells) or $1.15x10^4$ cells/cm^2 (T4-2 cells). The drip method of 3-D culture was used to induce the formation of acini. Briefly, cells were plated on Matrigel™ (50 μl/cm^2; BD Biosciences, 354234) at a density of $4.2x10^4$ cells/cm^2 (S1 cells) or $2.1x10^4$ cells/cm^2 (T4-2 cells) in the presence of culture medium containing 5% Matrigel™ (Plachot & Lelièvre, 2004; Vidi et al., 2012). The EGF was omitted from the culture medium after day 7 to allow completion of acinar differentiation (usually observed on day 8 or 9) (Plachot & Lelièvre, 2004). For some experiments, the Matrigel™ was diluted at 1:5, 1:10 or 1:20 in DMEM:F-12 (Lonza, BE12-719F) and allowed to solidify by incubation for 4 hours at 37 °C.

2. Transfection and Infection Protocols

Cx43 was downregulated in S1 cells via retroviral delivery of shRNA, as previously described (Bazzoun/Adissu et al., submitted). Cx43-shRNA S1 cells were maintained, propagated and plated similarly to S1 cells, but the H14 medium was supplemented with 25 μg/ml hygromycin B (Sigma, H3274) for selection.

3. Trypan Blue Exclusion Method

S1 and Cx43-shRNA S1 cells were plated in 24-well tissue culture plates (2-D). The medium was removed, and the cells were subsequently trypsinized and collected. Cells were then diluted in trypan blue at 1:1 ratio (vol/vol) and counted using a hemocytometer. The cells were counted from triplicates on days 6 and 10. To assess proliferation in 3-D cultures, cells were plated in 35-mm tissue culture plates, and acinar diameters were measured manually on days 4, 6, 9 and 11 using an ocular micrometer calibrated against a stage micrometer. Acinar areas were then calculated and plotted as acinar size. Fifty acini were analyzed per group.

4. Cell Cycle Analysis

S1 and Cx43-shRNA S1 cells were plated in T-25 tissue culture flasks (2-D) or 35-mm tissue culture plates (3-D). Acini were isolated from 3-D cultures as described earlier (Plachot & Lelièvre, 2004). Briefly, the medium was removed and acini were released from the Matrigel^{TM} by incubation with dispase (BD Biosciences, 354235) at 37 ºC. Acini were then collected by centrifugation and washed thrice with DMEM:F-12 and once with 1x phosphate-buffered saline (PBS). Cells on days 4, 6, 9 and 11 (2-D) and acini on days 4 and 11 (3-D) were trypsinized and collected by centrifugation. They were subsequently fixed using ice-cold 70% ethanol and left at -20 ºC overnight. Cells were then centrifuged, and the pellet was washed twice with 1x PBS. The pellet was resuspended in 1x PBS containing 30 μg/ml propidium iodide (Molecular Probes,

P3566) and RNase A. Cells were transferred to flow cytometry tubes and analyzed using BD FACSAria™ III.

5. Time-lapse Imaging

S1 and Cx43-shRNA S1 cells were plated in 35-mm tissue culture plates (2-D) at a density of $9.2x10^4$ cells/cm^2, and random motility was assessed on day 5 as previously described (Khalil et al., 2014). Images of cells were collected every 60 seconds for 3 hours using a 20x objective. During imaging, the temperature was controlled using a Zeiss heating stage, which was set at 37 °C. The medium was buffered using HEPES and overlaid with mineral oil. The speed of cell migration was quantified using the ROI tracker plugin in the ImageJ software, written by Dr. David Entenberg. This software was used to calculate the total distance traveled by individual cells. The speed was then calculated by dividing this distance by the time (180 minutes) and reported in μm/min. A minimum of 15 cells were analyzed per condition. The assay was done using infinity-corrected optics on a Zeiss Observer Z1 microscope supplemented with a computer-driven Roper cooled CCD camera and operated by Zen software (Zeiss).

6. Transwell Cell Invasion Assay

Six-well format cell culture inserts (8 μm pore size) were coated with 400 μl of 1:5 diluted Matrigel™ and incubated at 37 °C for 4 hours. $3x10^5$ S1 or Cx43-shRNA S1

cells were plated in the inserts in DMEM:F-12 supplemented with 1% fetal bovine serum (FBS; Sigma, F-9665). DMEM:F-12 supplemented with 10% FBS was added below the insert. Cells were incubated for 72 hours and were then fixed using 4% formaldehyde in 1x PBS for 20 minutes at room temperature. The cells towards the inside of the insert were removed using a cotton swab, and nuclei of invading cells were stained with 1 μg/ml Hoechst 33342 (Molecular Probes, H3570) in 1x PBS for 10 minutes at room temperature. The insert was then cut, mounted on a microscope slide in ProLong® Gold antifade reagent (Invitrogen Molecular Probes, P36930), allowed to dry overnight and sealed. The inserts were examined with a fluorescence microscope, and the number of invading cells was counted and reported as fold change.

7. Immunofluorescence

S1 and Cx43-shRNA S1 cells were plated on coverslips in 12-well tissue culture plates (2-D) or 4-well chamber slides (3-D) and were stained by immunofluorescence on day 9 or 12 (2-D) or day 11 (3-D) as described earlier (Plachot & Lelièvre, 2004). Briefly, cells were washed with 1x PBS and permeabilized with 0.5% peroxide and carbonyl-free Triton X-100 in cytoskeleton buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, 5 mM MgCl2, 1 mM pefabloc, 10 μg/ml aprotinin, 250 μM NaF). Cells were washed twice with cytoskeleton buffer and fixed in 4% formaldehyde. Cells were subsequently washed thrice with 50 mM glycine in 1x PBS and blocked. Primary antibodies used were rabbit polyclonal β-catenin (4 μg/ml; Santa Cruz Biotechnology, sc-7199) and goat polyclonal Scrib (0.25 or 1 μg/ml; Santa

Cruz Biotechnology, sc-11049). Secondary antibodies conjugated to Alexa Fluor 568 (red), donkey anti-rabbit (Invitrogen, A-10042) and donkey anti-goat (Invitrogen, A-11057), were used at 1 μg/ml. Nuclei were counterstained with 1 μg/ml Hoechst 33342, and cells were mounted in ProLong® Gold antifade reagent, allowed to dry overnight and sealed. The slides were then examined and imaged with a laser scanning confocal microscope (Zeiss, LSM710). A minimum of 50 acini were analyzed per group. For F-actin staining, S1 and Cx43-shRNA S1 cells were plated on coverslips in 12-well tissue culture plates (2-D) at a density of $9.2x10^4$ cells/cm^2 and were stained on day 5. Cells were fixed using 4% formaldehyde in 1x PBS for 10 minutes at 37 °C, permeabilized with 0.5% Triton X-100 in 1x PBS for 15 minutes on ice and blocked with 1% bovine serum albumin (BSA) in 1x PBS for 1 hour at 4 °C. Cells were subsequently stained with Rhodamine-phalloidin (1:50; Molecular Probes, R415) in 1% BSA for 30 minutes at room temperature and mounted in ProLong® Gold antifade reagent.

8. Preparation of Whole Cell Protein Extracts and Western Blot Analysis

S1 and Cx43-shRNA S1 cells were plated in T-75 tissue culture flasks (2-D) or 35-mm tissue culture plates (3-D). Acini were isolated from 3-D cultures as described earlier (Plachot & Lelièvre, 2004). Cells were harvested from 2-D cultures by scraping in 1x PBS and were collected by centrifugation. Cells on days 4, 6, 9 and 11 (2-D) and acini on day 11 (3-D) were lysed, and whole cell extracts were prepared in lysis buffer (2% SDS in 1x PBS with 10 μg/ml aprotinin, 1 mM PMSF and 250 μM sodium fluoride) (Lelièvre et al., 1998) or in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1%

Nonidet P-40, 0.5% sodium deoxycholate, 4% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail). Proteins were quantified using the DC protein assay (Bio-Rad, 5000116). For Western blot analysis, equal amounts of proteins were separated on polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes that were subsequently blocked with 5% milk in tris-buffered saline-Tween 20 (TBST) and immunoblotted with the following: mouse monoclonal antibodies against c-Myc (2 μg/ml; Santa Cruz Biotechnology, sc-40), cyclin D1 (2 μg/ml; Santa Cruz Biotechnology, sc-8396), Cx43 (1 or 2 μg/ml; Santa Cruz Biotechnology, sc-271837, and 5 μg/ml; Invitrogen, 13-8300), GSK-3α/β (1 μg/ml; Santa Cruz Biotechnology, sc-7291), p-GSK-3α/β (0.5 μg/ml; Santa Cruz Biotechnology, sc-81496), p-β-catenin (2 μg/ml; Santa Cruz Biotechnology, sc-57535), ERK1/2 (1 μg/ml; Santa Cruz Biotechnology, sc-514302) and p-ERK1/2 (4 μg/ml; Santa Cruz Biotechnology, sc-7383), goat polyclonal antibodies against Scrib (1 μg/ml; Santa Cruz Biotechnology, sc-11049) and ZO-2 (2 or 4 μg/ml; Santa Cruz Biotechnology, sc-8148) and rabbit polyclonal antibodies against β-catenin (0.04 or 0.2 μg/ml; Santa Cruz Biotechnology, sc-7199) and Cx43 (2.5 μg/ml; Invitrogen, 71-0700 and 5 μg/ml; Sigma, SAB4501175). Secondary antibodies conjugated to HRP, goat anti-rabbit (Abcam, ab6721), goat anti-mouse (Abcam, ab6789) and rabbit anti-goat (Abcam, ab6741), were used at 0.13 μg/ml. Equal protein loading was verified by immunoblotting for lamin B (rabbit polyclonal, 0.05 μg/ml; Abcam, ab16048) or GAPDH (mouse monoclonal, 0.4 or 1 μg/ml; Santa Cruz Biotechnology, sc-47724). Protein levels were quantified using ImageJ software and normalized to lamin B or GAPDH.

9. Co-Immunoprecipitation

S1 and Cx43-shRNA S1 cells were plated in T-75 tissue culture flasks (2-D) or 35-mm tissue culture plates (3-D). Acini were isolated from 3-D cultures as described earlier (Plachot & Lelièvre, 2004). Cells were harvested from 2-D cultures by scraping in 1x PBS and were collected by centrifugation. Cells on day 6 (2-D) and acini on day 11 (3-D) were subjected to co-immunoprecipitation as previously described (Talhouk et al., 2008) with the following modifications: whole cell lysates were sheared using a 27-gauge needle, protein G sepharose beads (GE Healthcare, 17-0618-01) were used with 0.25 μg rabbit polyclonal Cx43 antibody (Invitrogen, 71-0700), and samples were resolved on 12% polyacrylamide gels.

10. Pulldown Assay

S1 and Cx43-shRNA S1 cells were plated in T-75 tissue culture flasks (2-D) or 35-mm tissue culture plates (3-D). Acini were isolated from 3-D cultures as previously described (Plachot & Lelièvre, 2004). The pulldown assays were performed using the RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell Biolabs, STA-405) following the manufacturer's instructions. Briefly, lysates of cells on day 9 (2-D) and acini on day 11 (3-D) were incubated with PAK PBD agarose beads (for Rac-GTP and Cdc42-GTP pulldown) for 1 hour at 4 °C with gentle agitation. The samples were then centrifuged, and the pellets were washed several times. Subsequently, the pellets were resuspended in Laemmli sample buffer and boiled for 5 minutes. GTP-Rac1 and GTP-Cdc42 were detected by Western blotting using mouse monoclonal antibodies against Rac1 or

Cdc42, respectively. Lysates were collected prior to the incubation with PAK PBD agarose beads and were immunoblotted with mouse monoclonal antibodies against RhoA, Rac1 and Cdc42 for detection of total GTPase levels.

11. Subcellular Fractionation

S1, Cx43-shRNA S1 and T4-2 cells were plated in 35-mm tissue culture plates (3-D). Acini and tumor nodules were isolated from 3-D cultures as previously described (Plachot & Lelièvre, 2004), and subcellular fractionation was performed using the Qproteome Cell Compartment Kit (Qiagen, 37502) following the manufacturer's instructions. The nuclear fractions of acini on day 7 (T4-2) or day 11 (S1 and Cx43-shRNA S1) were quantified and analyzed by Western blotting using rabbit polyclonal β-catenin antibody (0.2 μg/ml; Santa Cruz Biotechnology, sc-7199). Equal protein loading was verified by immunoblotting for lamin B (rabbit polyclonal, 0.2 μg/ml; Abcam, ab16048). The purity of nuclear fractions was assessed by immunoblotting for GAPDH (mouse monoclonal, 0.4 μg/ml; Santa Cruz Biotechnology, sc-47724) and Tim23 (mouse monoclonal, 1 μg/ml; Santa Cruz Biotechnology, sc-514463), a membrane marker.

12. Statistical Analysis

Data are presented as means ± S.D. Statistical comparisons were done using Microsoft Excel 2010 software. Unpaired t-test was used for comparison of two groups. $p < 0.05$ was considered significant.

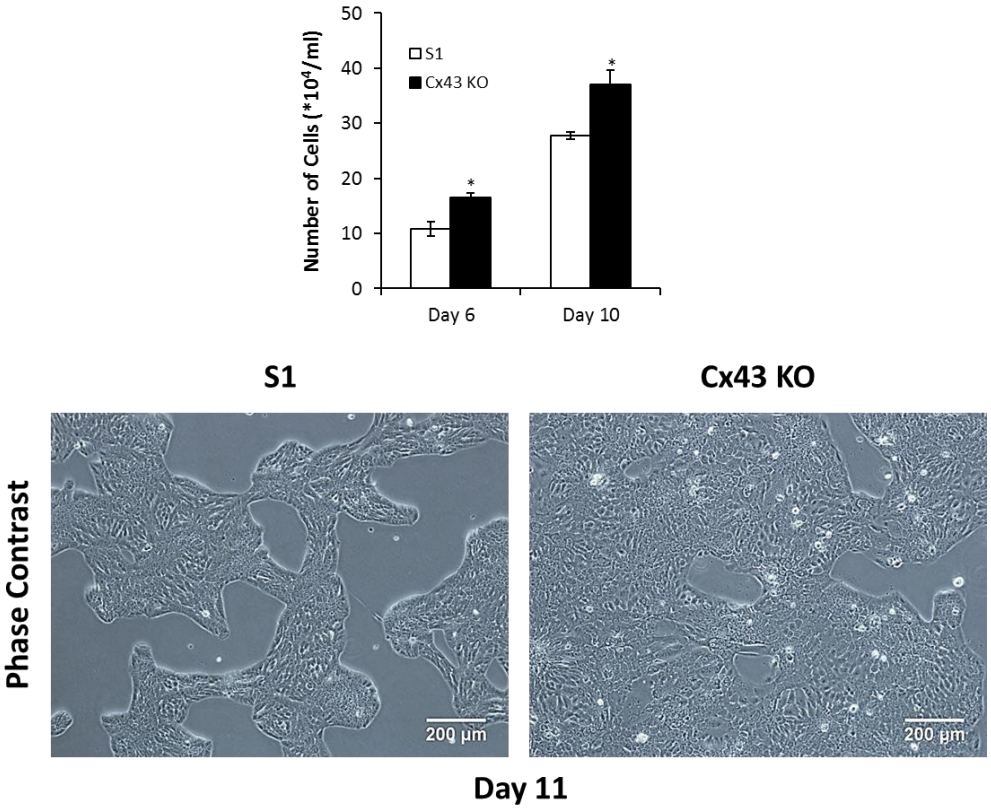
D. Results

1. Silencing Cx43 Triggers Cell Cycle Entry and Enhances the Proliferation Rate

Earlier studies demonstrated that functional GJIC and Cx43 interaction with its associated proteins observed in 3-D but not 2-D cultures of mammary epithelial cells are critical for differentiation (El-Sabban et al., 2003; Talhouk et al., 2008). We sought to investigate the effects of Cx43 silencing on the proliferation rate of nontumorigenic human mammary epithelial HMT-3522 S1 cell line (Briand et al., 1987) under 2-D and 3-D culture conditions. S1 cells form monolayers of interconnected islands with cobblestone cell morphology on plastic (2-D culture conditions), and organize into multicellular spheroidal acini, characterized by cell cycle exit, a central lumen and an apicobasal polarity axis when cultured on Matrigel, an exogenous basement membrane-rich ECM (3-D culture conditions) (Petersen et al., 1992; Plachot & Lelièvre, 2004). This differentiation potential is impaired in Cx43-shRNA S1 cells that exhibit perturbed apical polarity and lumen-forming ability (Bazzoun/Adissu et al., submitted). The 2-D cultures of Cx43-shRNA S1 cells revealed a significant increase in cell counts, by 50% and 33% on days 6 and 10, respectively, compared to control cells (Figure 3.1A). The number of dead cells was negligible and did not differ between the two groups at the different time points (data not shown). Cx43-shRNA S1 cells assembled and grew into

significantly larger acini that lacked a lumen in 3-D cultures when compared to control cells, with an increase in acinar area of 62%, 72%, 75% and 82% on days 4, 6, 9 and 11, respectively (Figure 3.1B). In addition, silencing Cx43 significantly altered the distribution of cells in the different cell cycle phases under 2-D (Figure 3.2A) and 3-D culture conditions (Figure 3.2B) at the different time points (days 4, 6, 9 and 11 in 2-D and days 4 and 11 in 3-D). Specifically, the percentage of cells in G0/G1 phase decreased in Cx43-shRNA S1 cells compared to control cells (39%-44% and 28%-34% decrease in 2-D and 3-D, respectively), suggesting enhanced cell cycle entry in the cell population. This result is supported by the concomitant increase in the percentages of cells in S (up to 723% and 344% increase in 2-D and 3-D, respectively) and G2/M phases (55%-119% and 79%-114% increase in 2-D and 3-D, respectively). Hence, the loss of Cx43 triggers cell cycle entry and enhances the proliferation rate of S1 cells under 2-D and 3-D culture conditions. Consistent with these results, Western blotting showed that c-Myc and cyclin D1 were upregulated in Cx43-shRNA S1 cells compared to control cells under 2-D and 3-D culture conditions at the different time points (days 4, 6, 9 and 11 in 2-D and day 11 in 3-D) (Figure 3.2C).

A


Number of Cells (*10⁴/ml)
50
40
30
20
10
0
S1
Cx43 KO
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Day 6
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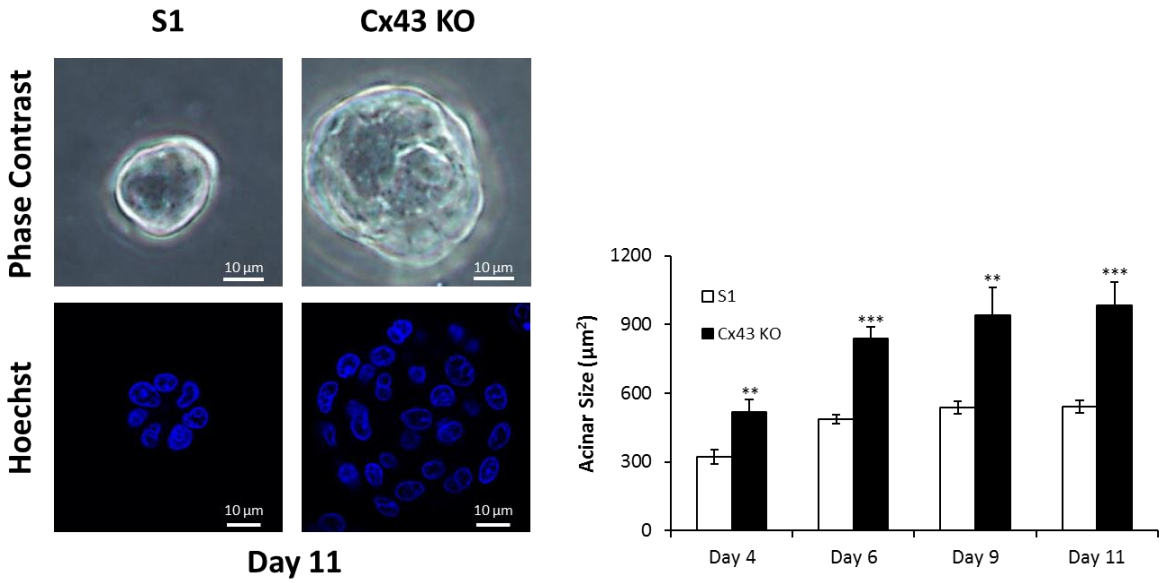
S1
Cx43 KO
Phase Contrast
200 µm
200 µm
Day 11

B


S1
Cx43 KO
Phase Contrast
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10 µm
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10 µm
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Day 11
Acinar Size (µm²)
1200
900
600
300
0
S1
Cx43 KO
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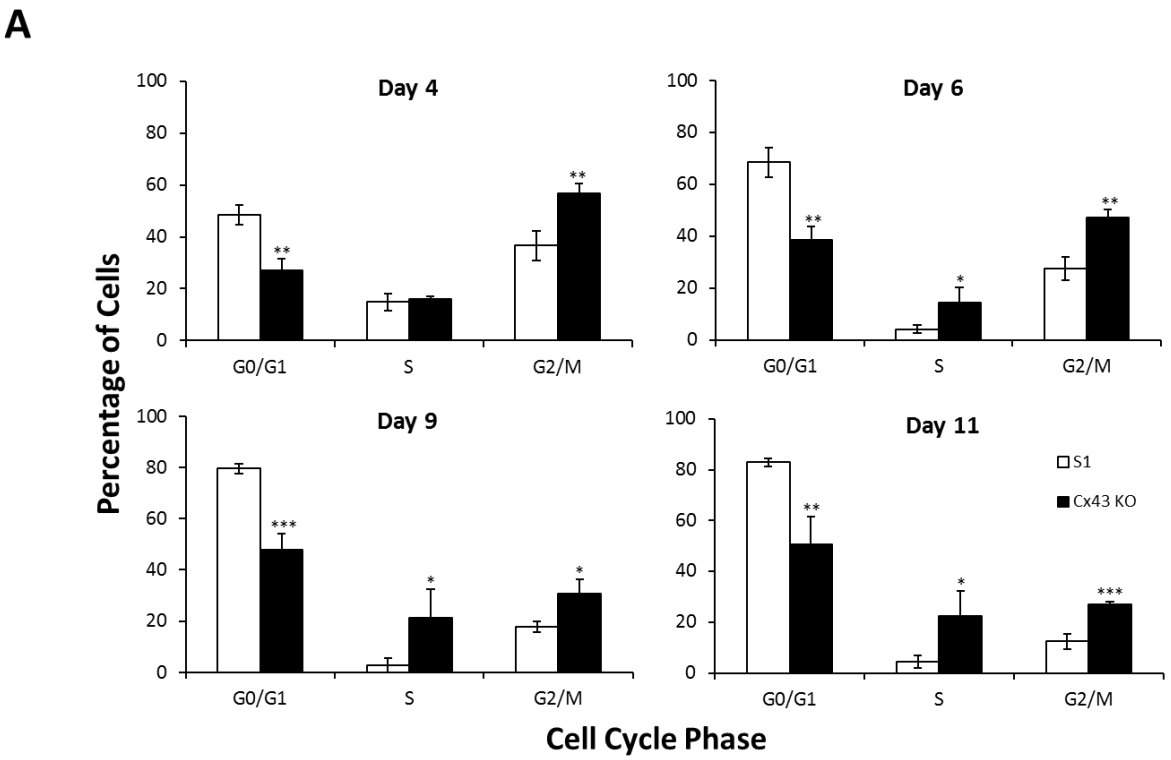
Day 4
Day 6
Day 9
Day 11

Figure 3.1. Silencing Cx43 enhances the proliferation rate of S1 cells under 2-D and 3-D culture conditions. S1 and Cx43-shRNA S1 cells (Cx43 KO) were cultured under 2-D (**A**) or 3-D conditions (**B**). Proliferation rate was assessed by cell counting on days 6 and 10 in 2-D (**A; upper panel**) and by measurement of acinar diameter on days 4, 6, 9 and 11 in 3-D (**B; right panel**). Fifty acini were analyzed per group. The values depicted in histograms are the means (± S.D.) of cell counts (**A; upper panel**) or acinar sizes (**B; right panel**) from three independent experiments. Unpaired t-test; *p < 0.05, **p < 0.01, ***p < 0.001. Representative images of cells on day 11 in 2-D (**A; lower panel**) and in 3-D (**B; left panel**) are shown. Nuclei were stained with Hoechst (blue; **B; left lower panel**). *Cell counting in 2-D: Dana Bazzoun, Thesis, AUB, 2015.*



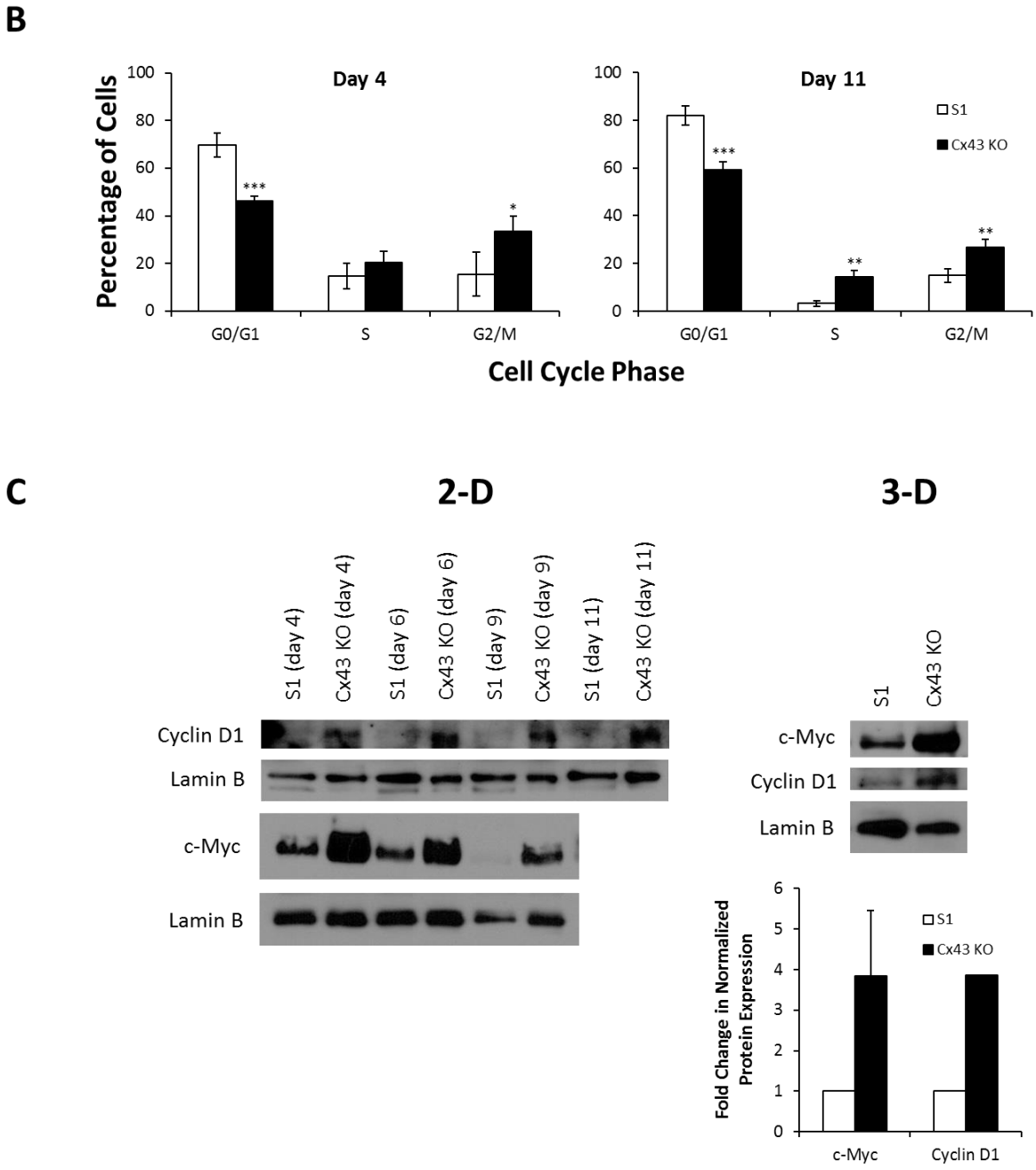


Figure 3.2. Silencing Cx43 triggers cell cycle entry and upregulates the expression of cell cycle genes in S1 cells under 2-D and 3-D culture conditions. S1 and Cx43-shRNA S1 cells (Cx43 KO) were cultured under 2-D (**A and C; left panel**) or 3-D conditions (**B and C; right panel**). **A and B.** Cell cycle analysis was performed by flow cytometry on days 4, 6, 9 and 11 in 2-D (**A**) and on days 4 and 11 in 3-D (**B**). The values depicted in histograms are the means (± S.D.) of cell percentages in the different cell cycle phases from three independent experiments. Unpaired t-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **C.** Total proteins were extracted on days 4, 6, 9 and 11 in 2-D (**left panel**) and on day 11 in 3-D (**right panel**). Expression of c-Myc and cyclin D1 was

assessed by Western blotting. Lamin B served as loading control. The values depicted in the histogram (**right lower panel**) are the means of fold change in c-Myc or cyclin D1 expression in 3-D normalized to that of Lamin B from three independent experiments. Fold change in normalized expression is set to 1 in S1 cells.

2. Silencing Cx43 Alters the Localization of Junctional and Polarity Proteins

We have previously shown that blocking Cx43-mediated GJIC in 3-D cultures of S1 cells is not sufficient to promote proliferation (Bazzoun/Adissu et al., submitted). Thus, we speculated the involvement of channel-independent mechanisms in the growth-regulatory functions of Cx43. Our earlier studies in breast adenocarcinoma cell lines showed that exogenously expressed Cx43 exerts its antiproliferative effects by the assembly of GJ complexes consisting of Cx43, α-catenin, β-catenin and ZO-2 at the membrane (Talhouk et al., 2013). Co-immunoprecipitation demonstrated association of Cx43 with β-catenin and ZO-2 in control S1 cells under 2-D and 3-D culture conditions (Figure 3.3). While the protein levels of Cx43 were markedly reduced by 90% in Cx43-shRNA S1 cells, Western blotting analysis did not show an effect for Cx43 loss on the levels of β-catenin or ZO-2 compared to control cells (Figure 3.4A). Similarly, immunofluorescence showed homogenous membrane distribution of β-catenin at cell-cell contacts in 2-D cultures of S1 cells and Cx43-shRNA counterparts (Figure 3.4B; left upper panel). Under 3-D conditions however, β-catenin displayed an apicolateral membrane distribution in S1 acini, consistent with the pattern previously described for Cx43 (Bazzoun/Adissu et al., submitted). Silencing Cx43 significantly altered the distribution of membranous β-catenin with 81% decrease in acini showing apicolateral localization (Figure 3.4B; left lower and right panels). The mislocalization of β-catenin

in Cx43-shRNA S1 acini was accompanied with impaired lumen formation and acinar architecture. The levels of Scrib, a key regulator of apical polarity in epithelia, were not altered in Cx43-shRNA S1 cells compared to control cells under 2-D or 3-D culture conditions, as Western blotting analysis showed (Figure 3.4A). Given the asymmetric distribution of polarity complexes along the apicobasal axis of polarized epithelial cells (Ellenbroek, Iden, & Collard, 2012), we next studied the localization of Scrib. As expected, Scrib localized at cell-cell contacts in monolayers of control and Cx43-shRNA S1 cells (Figure 3.4C; left upper panel). While 50% of S1 acini showed apicolateral Scrib distribution in 3-D cultures, this pattern was significantly altered in Cx43-shRNA acini (only 11% of acini expressed apicolateral Scrib), where a diffuse pattern was predominant (Figure 3.4C; left lower and right panels), suggesting the loss of apical polarity. Taken together, the above results indicate that silencing Cx43 alters the localization of junctional and polarity proteins in S1 cells under 3-D culture conditions, possibly through the altered assembly of GJ complexes.

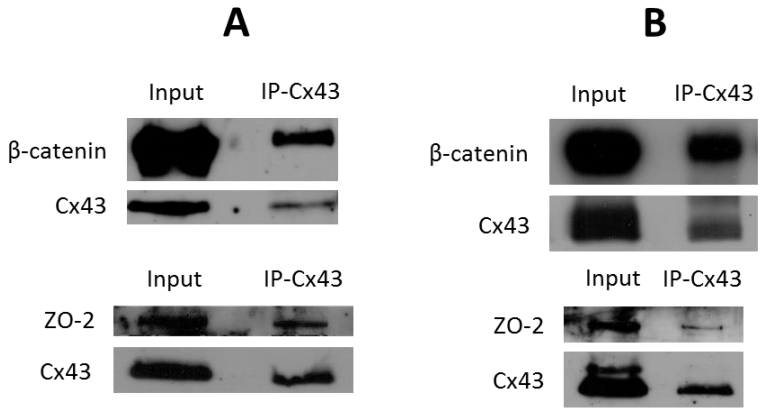
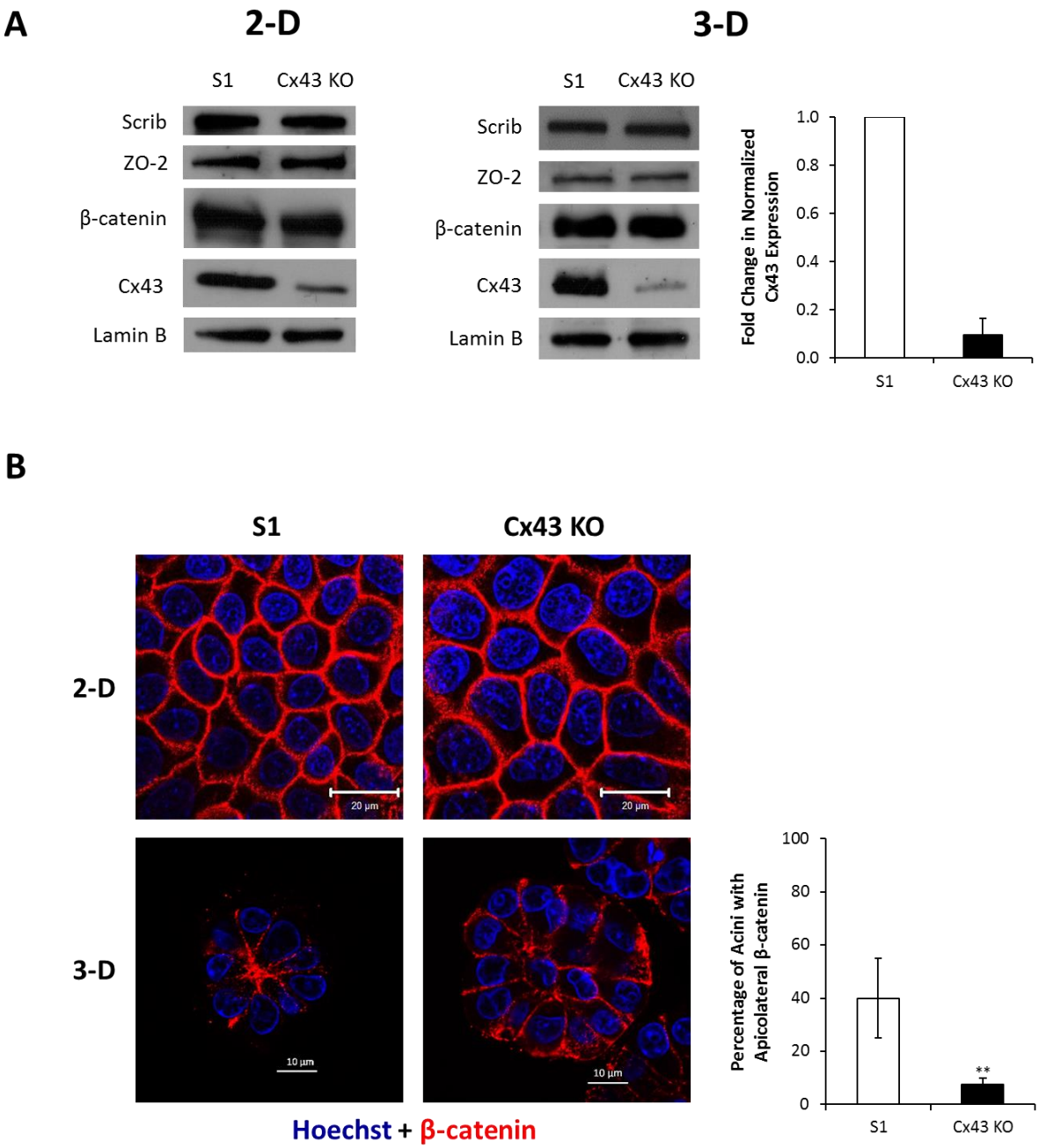


Figure 3.3. Cx43 assembles GJ complexes in S1 cells under 2-D and 3-D culture conditions. S1 cells were cultured under 2-D (**A**) or 3-D conditions (**B**). Total proteins

were extracted on day 6 in 2-D (**A**) and on day 11 in 3-D (**B**). Association of Cx43 and β-catenin (**A and B; upper panels**) or ZO-2 (**A and B; lower panels**) was assessed by co-immunoprecipitation (IP) of Cx43 followed by Western blotting for detection of Cx43, β-catenin and ZO-2. The input served as a control. Three independent experiments were performed for Cx43-β-catenin association and two for Cx43-ZO-2 association.



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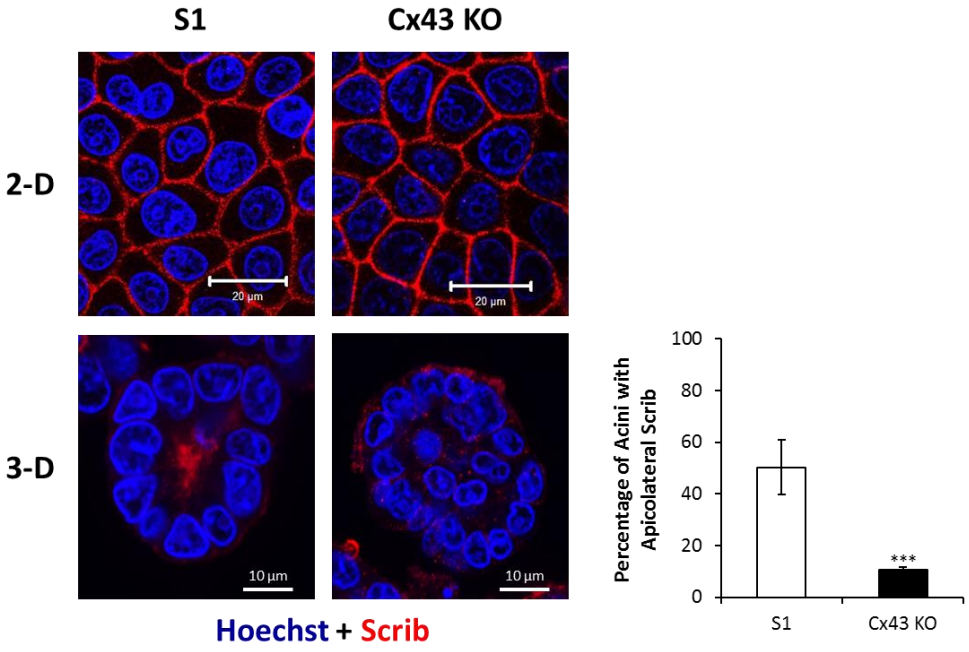


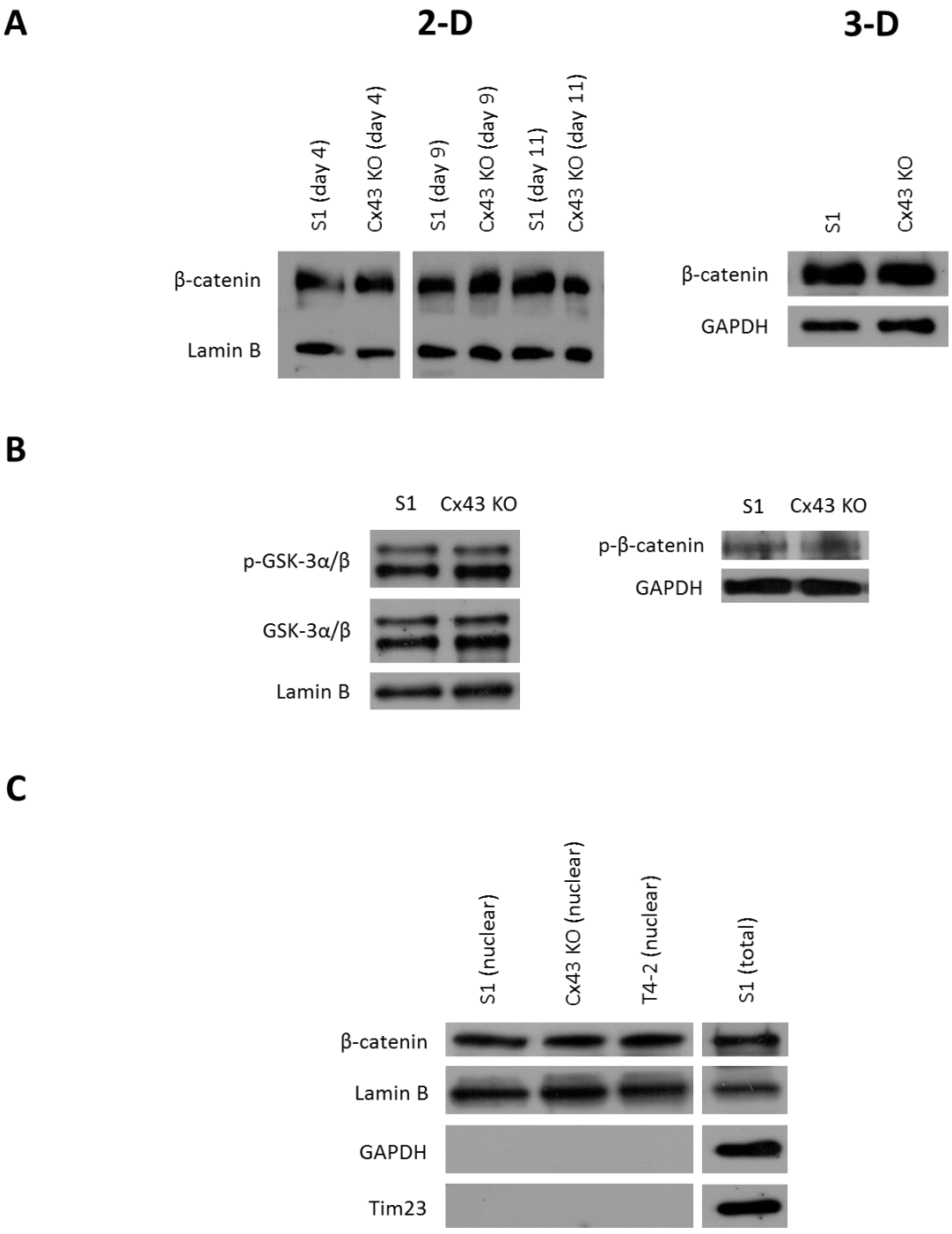
Figure 3.4. Silencing Cx43 alters the localization of junctional and polarity proteins in S1 cells under 3-D culture conditions without affecting their expression levels. S1 and Cx43-shRNA S1 cells (Cx43 KO) were cultured under 2-D or 3-D conditions. **A.** Total proteins were extracted on day 9 in 2-D (**left panel**) and on day 11 in 3-D (**right panel**). Expression of Scrib, ZO-2, β-catenin and Cx43 was assessed by Western blotting. Lamin B served as loading control. Three independent experiments were performed. The values depicted in the histogram (**right panel**) are the means of fold change in Cx43 expression in 3-D normalized to that of Lamin B from three independent experiments. Fold change in normalized expression is set to 1 in S1 cells. **B and C.** Localization of β-catenin (red; **B; left panel**) and Scrib (red; **C; left panel**) was assessed by immunofluorescence on day 9 (**B; left upper panel**) or day 12 in 2-D (**C; left upper panel**) and on day 11 in 3-D (**B and C; left lower panels**). Nuclei were counterstained with Hoechst (blue; **B and C; left panels**). Histograms show the quantification of β-catenin (**B; right panel**) and Scrib distribution in 3-D (**C; right panel**). A minimum of 100 acini were analyzed per group. The values depicted are the means (± S.D.) of acini percentages with apicolateral β-catenin or Scrib from three independent experiments. Unpaired t-test; **p < 0.01, ***p < 0.001. *Scoring of localization (β-catenin and Scrib) and images in 3-D (Scrib): Dana Bazzoun, Thesis, AUB, 2015.*

3. Silencing Cx43 Activates MAPK but not Wnt/β-catenin Signaling

Because β-catenin was mislocalized from its apicolateral membrane domains in 3-D cultures of Cx43-shRNA S1 cells (Figure 3.4B; left lower and right panels), we investigated the involvement of the Wnt/β-catenin pathway in mediating proliferation downstream of Cx43 loss. Wnt/β-catenin signaling plays important roles in development and differentiation of the mammary gland and is deregulated in breast cancer (Imbert et al., 2001; Lin et al., 2000; Lindvall et al., 2009; Milovanovic et al., 2004; Prasad et al., 2008; van Genderen et al., 1994). Our earlier studies suggest negative regulation of the Wnt/β-catenin pathway downstream of Cx43 signaling as a mechanism for tumor suppression in the mammary epithelium (Talhouk et al., 2013). A hallmark of Wnt/β-catenin signaling is the stabilization of cytoplasmic β-catenin, leading to its accumulation and nuclear translocation, where it mediates the expression of proliferation genes (MacDonald et al., 2009; Rao & KUhl, 2010; Zardawi et al., 2009). Silencing Cx43 did not alter the total levels of β-catenin in 2-D (days 4, 9 and 11) or 3-D cultures (day 11) of S1 cells, as demonstrated by Western blotting analysis (Figure 3.5A). However, since the majority of cellular β-catenin is associated with membrane complexes, total β-catenin levels might not accurately reflect those of cytosolic or nuclear β-catenin and are therefore not indicative of Wnt/β-catenin activation (Beurel, Grieco, & Jope, 2015). Thus, we studied the activity of GSK-3α/β, a serine/threonine protein kinase that regulates the stability of β-catenin within the β-catenin destruction complex (MacDonald et al., 2009; Rao & KUhl, 2010; Zardawi et al., 2009). Silencing Cx43 did not affect total GSK-3α/β or active levels (p-GSK-3α/β), as Western blotting analysis showed (Figure 3.5B). Furthermore, the levels of phosphorylated (p)-β-catenin (unstable isoform targeted for ubiquitination and

proteasomal degradation), a readout for the kinase activity of GSK-3α/β associated with the β-catenin destruction complex and an indicator of the overall status of Wnt/β-catenin signaling, did not change in Cx43-shRNA S1 cells compared to control cells, consistent with total β-catenin levels (Figure 3.5B). Although this result might rule out enhanced activation of the Wnt/β-catenin pathway downstream of Cx43 silencing, we reasoned that since Cx43 associates with β-catenin at the membrane, the loss of Cx43 expression might indirectly activate Wnt/β-catenin signaling. Given that the activity of GSK-3α/β associated with the β-catenin destruction complex did not change (Figure 3.5B), the release of β-catenin sequestered by Cx43 at the membrane could lead to its cytoplasmic accumulation and nuclear translocation in Cx43-shRNA S1 cells. Western blotting analysis of nuclear fractions did not show an increase in β-catenin levels in Cx43-shRNA S1 cells compared to control cells (Figure 3.5C). Interestingly, co-analysis of T4-2 cells, the tumorigenic counterparts of S1 cells, did not show an altered level of nuclear β-catenin either (Figure 3.5C). These data suggest the involvement of proliferation pathways other than the Wnt/β-catenin downstream of Cx43 signaling in S1 cells. One possible candidate is the ERK1/2 pathway, since we measured alterations in the levels of c-Myc and cyclin D1 that are among the downstream targets of ERK1/2 signaling (Chambard, Lefloch, Pouysségur, & Lenormand, 2007; Grill et al., 2004). The ERK1/2 pathway is the best characterized among three other MAPK signaling cascades (ERK5, p38 and JNK) involved in mammary gland development and breast cancer progression (Whyte, Bergin, Bianchi, McNally, & Martin, 2009). While total levels of ERK1/2 remained unchanged, active levels (p-ERK1/2) were upregulated in Cx43-shRNA S1 cells compared to control cells, as Western blotting analysis showed (Figure

3.5D). Thus, the loss of Cx43 activates MAPK signaling, a mechanism that could induce cell cycle entry and proliferation in S1 cells.



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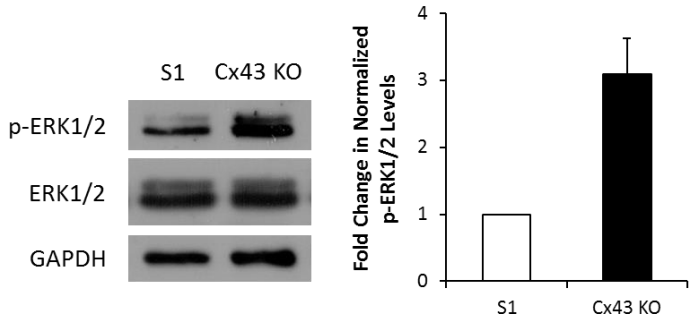


Figure 3.5. Silencing Cx43 activates MAPK but not Wnt/β-catenin signaling in S1 cells under 3-D culture conditions. S1 and Cx43-shRNA S1 cells (Cx43 KO) were cultured under 2-D (**A**) or 3-D conditions (**A-D**). **A, B and D.** Total proteins were extracted on days 4, 9 and 11 in 2-D (**A; left panel**) and on day 11 in 3-D (**A; right panel, B and D**). Expression of β-catenin, p-β-catenin, GSK-3α/β, p-GSK-3α/β, ERK1/2 and p-ERK1/2 was assessed by Western blotting. Lamin B and GAPDH served as loading controls. Three independent experiments were performed. The values depicted in the histogram (**D; right panel**) are the means of fold change in p-ERK1/2 levels in 3-D normalized to that of GAPDH from three independent experiments. Fold change in normalized levels is set to 1 in S1 cells. **C.** Nuclear proteins were extracted on day 11 (S1 and Cx43 KO cells) or on day 7 in 3-D (T4-2 cells). β-catenin levels were assessed by Western blotting. Lamin B served as loading control. Purity of nuclear fractions was determined by analyzing cytosolic (GAPDH) and membrane markers (Tim23). Total proteins extracted on day 11 in 3-D (S1 cells) were co-analyzed as a control for the detection of GAPDH and Tim23. Three independent experiments were performed.

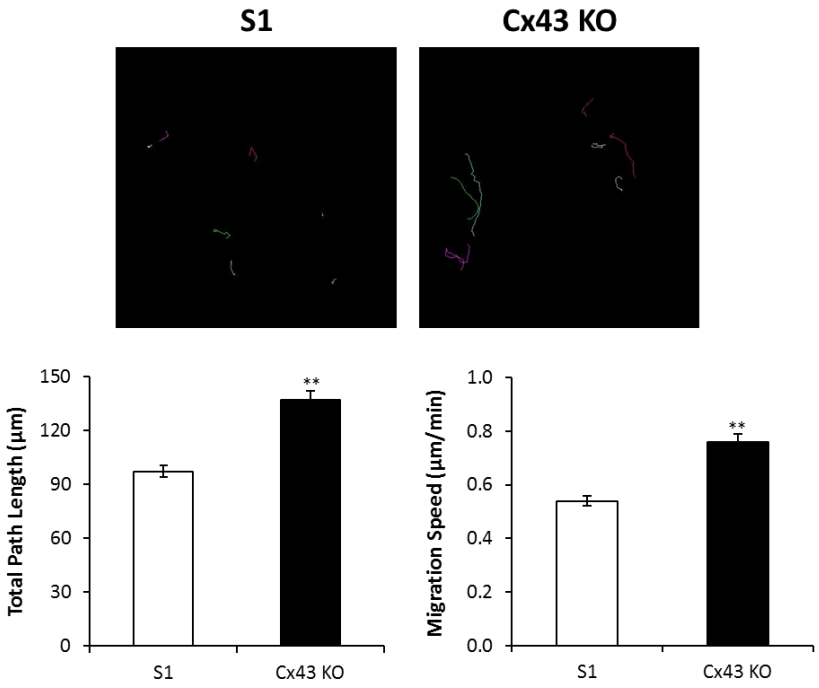
4. Silencing Cx43 Induces Motility and Invasion

ERK1/2 signaling is known to regulate cell motility and invasion in addition to proliferation (Reddy, Nabha, & Atanaskova, 2003; Santamaria & Nebreda, 2010; Stupack, Cho, & Klemke, 2000). Random motility was significantly enhanced in cultures of Cx43-shRNA S1 cells compared to control cells, as time-lapse imaging of random 2-D migration revealed (Movies 1 and 2). Specifically, quantitative analysis showed 41% increase in the total distance traveled by Cx43-shRNA S1 cells compared to control cells over time (Figure 3.6A). In transwell cell invasion assay, Cx43-shRNA

S1 cells showed a significant increase of four-fold in the number of Matrigel-invading cells compared to control cells (Figure 3.6B; upper panel). In addition, invadopodia-like actin-rich dots were evident in 2-D cultures of Cx43-shRNA S1 cells (Figure 3.6B; lower panel). Both control and Cx43-shRNA S1 cells formed spheroid acini when cultured on undiluted Matrigel and did not display an apparent invasive behavior. The phenotype and invasive ability of normal, oncogene-initiated and malignant mammary epithelial cells are largely determined by the stiffness of the ECM (Cassereau, Miroshnikova, Ou, Lakins, & Weaver, 2015; Levental et al., 2009; Miroshnikova et al., 2011). Compared to normal tissues, breast tumors are associated with increased ECM stiffness, which correlates with aggressive subtypes (Acerbi et al., 2015). *In vitro* studies revealed that ECM stiffening promotes the invasion of premalignant and malignant mammary epithelial cells (Cassereau et al., 2015; Levental et al., 2009). However, matrix stiffness was found to enhance matrix metalloproteinase (MMP) activity in a pancreatic cancer cell line (Haage & Schneider, 2014), suggesting that motility and invasion of cancer cells require permissive ECM stiffness. This is in line with a recent demonstration of enhanced motility in breast cancer cells on matrices with moderate or low stiffness (Ansardamavandi, Tafazzoli-Shadpour, & Shokrgozar, 2018). Thus, we found it reasonable to study the effects of Cx43 loss on the behavior of S1 cells in the context of variable ECM stiffness. For this purpose, we utilized 3-D cultures with different Matrigel concentrations (undiluted, 1:5, 1:10 and 1:20 dilutions). Matrix stiffness and network density are altered in response to the concentration of the Matrigel (Zaman et al., 2006). While S1 cells maintained their spheroid acinar morphology, Cx43-shRNA S1 cells displayed loss of this characteristic morphology under conditions

of diluted Matrigel (1:5, 1:10 and 1:20). Indeed, Cx43-shRNA S1 acini showed progressive morphological alterations over the culture period, with an increase in size and acquisition of granular edges marked by the presence of migrating cells (Figure 3.6C; lower panel). We used the term "nonspheroid" to describe all observed patterns of dysmorphic structures. Such structures represented 53% and 58% of Cx43-shRNA S1 cultures on 1:5 and 1:10 diluted Matrigel, respectively, and predominated in cultures on 1:20 diluted Matrigel by day 11, forming 76% of the population of multicellular structures. The percentages of nonspheroid structures in control S1 cultures were negligible under all conditions and did not exceed 4% (Figure 3.6C; upper panel). These results indicate that the loss of Cx43 induces the acquisition of an invasive phenotype in S1 cells in a context-dependent manner and highlight the integration of Cx and ECM signals in determining mammary epithelial morphology and behavior. Importantly, the lumen-forming ability of control S1 cells was recapitulated under conditions of 1:5 diluted Matrigel, while a significant loss of lumen formation was noted in cultures on 1:10 and 1:20 diluted Matrigel (Figure 3.7A). In addition, the percentages of S1 acini with apicolateral Scrib localization did not differ between cultures on undiluted and 1:5 diluted Matrigel (Figure 3.7B). Since the 3-D conditions of 1:5 diluted Matrigel favored an invasive phenotype in Cx43-shRNA S1 cells while maintaining normal morphology in control cells, it becomes possible to further assess the role of Cx43 loss in invasion of S1 cells under permissive ECM stiffness.

A


S1
Cx43 KO
Total Path Length (µm)
150
120
90
60
30
0
**
S1
Cx43 KO
Migration Speed (µm/min)
1.0
0.8
0.6
0.4
0.2
0.0
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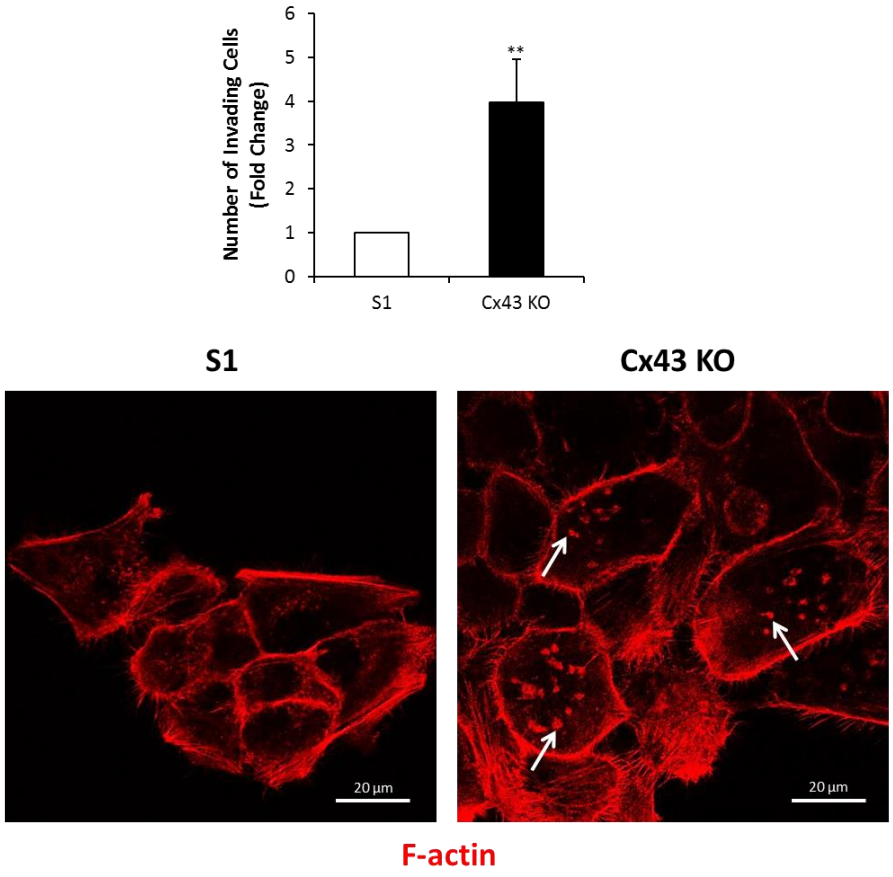
S1
Cx43 KO

B


Number of Invading Cells
(Fold Change)
6
5
4
3
2
1
0
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S1
Cx43 KO
S1
Cx43 KO
20 µm
20 µm
F-actin

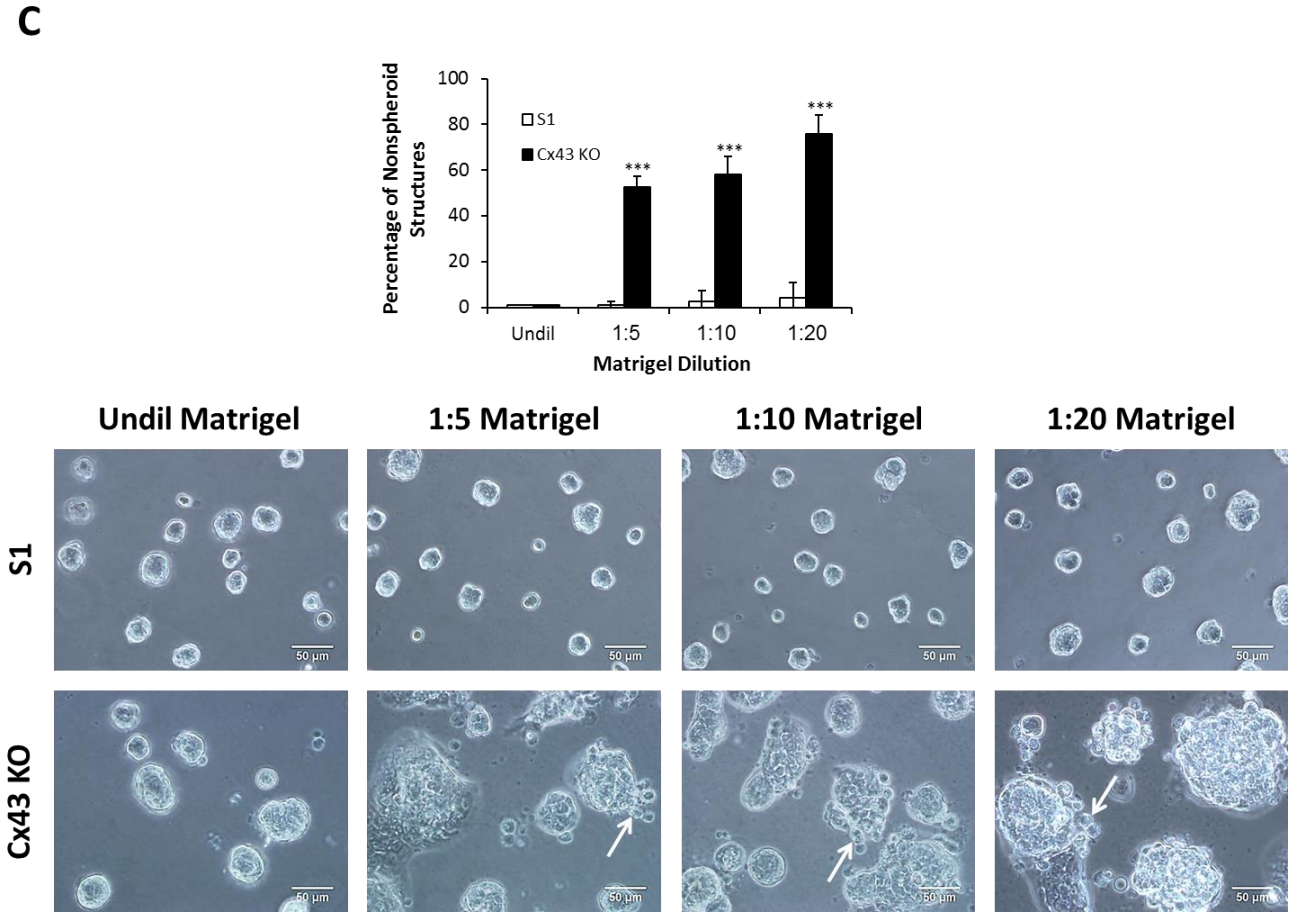

Figure 3.6. Silencing Cx43 induces motility and invasion in S1 cells. A. S1 and Cx43-shRNA S1 cells (Cx43 KO) were cultured under 2-D conditions. Motility was assessed by time-lapse imaging on day 5. The total paths of representative S1 and Cx43 KO cells from time-lapse movies (**upper panel**) are shown (different colors represent different cells). Histograms show the quantification of cell motility. A total of 50 cells were analyzed per group. The values depicted are the means (± S.D.) of total path lengths (**left lower panel**) or migration speeds (**right lower panel**) from two independent experiments. Unpaired t-test; **p < 0.01. **B.** Invasion of S1 and Cx43 KO cells across diluted Matrigel (1:5) was assessed by transwell cell invasion assay (**upper panel**). The values depicted in the histogram are the means (± S.D.) of fold change in number of Matrigel-invading cells from three independent experiments. Unpaired t-test; **p < 0.01. Representative images of cells cultured under 2-D conditions and stained for F-actin on day 5 (**lower panel**) are shown. Arrows indicate invadopodia-like actin-rich dots. **C.** S1 and Cx43 KO cells were cultured under 3-D conditions atop of different Matrigel dilutions (undiluted, 1:5, 1:10 and 1:20 dilutions). Invasion was assessed by counting nonspheroid structures on day 11. One hundred structures were analyzed per group. The values depicted in the histogram (**upper panel**) are the means (± S.D.) of nonspheroid structures from three independent experiments. Unpaired t-test; ***p < 0.001. Representative images of cells (**lower panel**) are shown. Arrows indicate migrating Cx43 KO cells. Undil; Undiluted

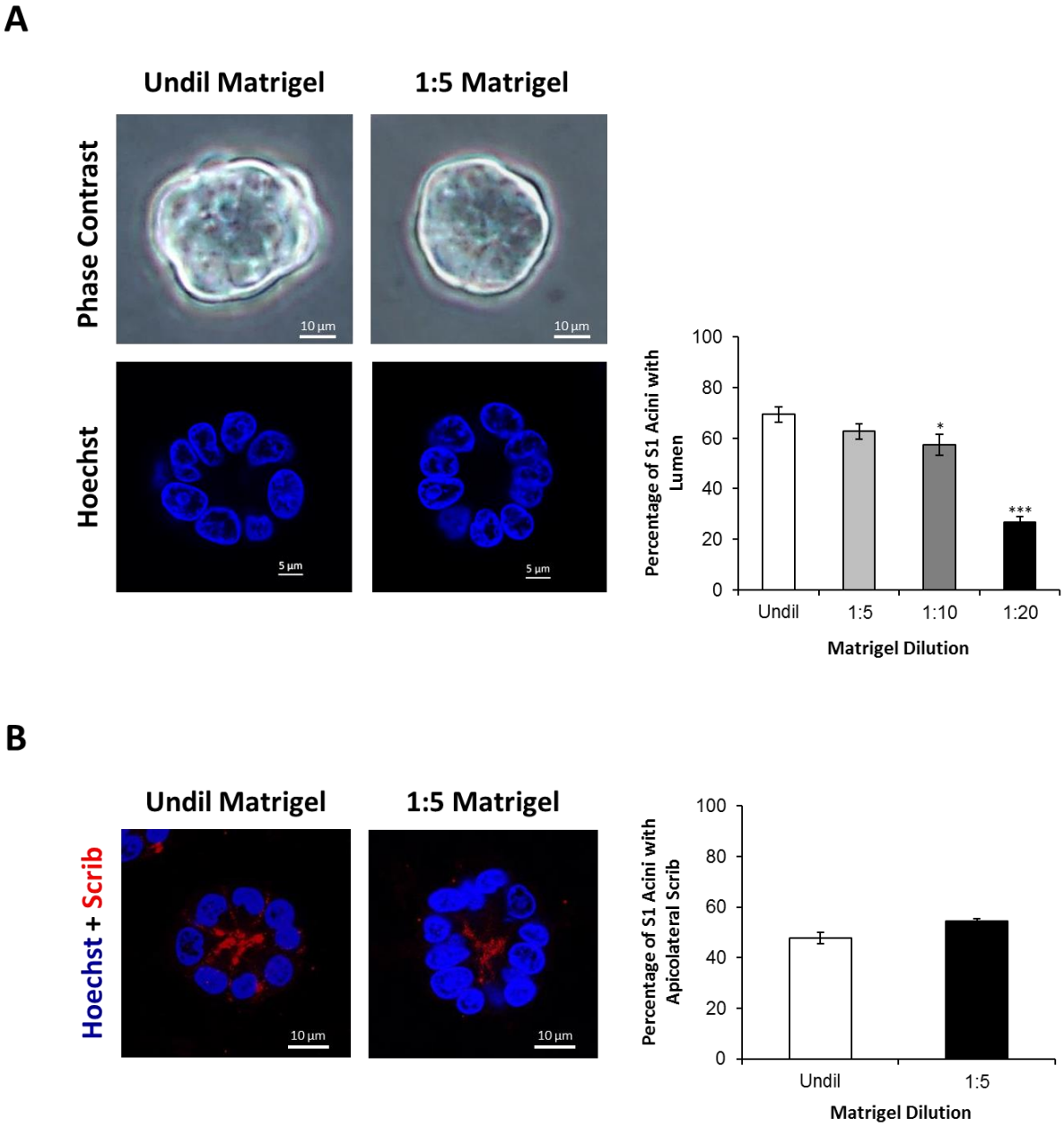

Figure 3.7. S1 cells maintain their characteristic spheroid morphology, lumen-forming ability and polarity under 3-D culture conditions of reduced ECM stiffness. S1 cells were cultured under 3-D conditions atop of different Matrigel dilutions (undiluted, 1:5, 1:10 and 1:20 dilutions). Lumen formation (**A**) and polarity (**B**), demonstrated by apicolateral Scrib localization (red; **B; left panel**), were assessed by immunofluorescence on day 11. **A.** Fifty acini were analyzed per condition. The values depicted in the histogram (**right panel**) are the means (± S.D.) of acini percentages with lumen from three independent experiments. Unpaired t-test; *$p < 0.05$, ***$p < 0.001$. Representative images of acini (**left panel**) are shown. Nuclei were stained with Hoechst (blue; **left lower panel**). **B.** Sixty seven acini were analyzed per

condition. The values depicted in the histogram (**right panel**) are the means (± S.D.) of acini percentages with apicolateral Scrib from two independent experiments. Unpaired t-test. Representative images of acini (**left panel**) are shown. Nuclei were counterstained with Hoechst (blue). Undil; Undiluted

5. Silencing Cx43 Activates Rho GTPase Signaling

Rho GTPase signaling regulates the development of the mammary gland, and its aberrant activation contributes to breast tumorigenesis (Burbelo et al., 2004; Zuo et al., 2016). The role of Rho GTPase signaling in motility and invasion of breast cancer cells is well established (Bravo-Cordero et al., 2011; Bravo-Cordero et al., 2013; Castillo-Pichardo et al., 2014; Chan et al., 2010; El-Sibai et al., 2007; El-Sibai et al., 2008; Moshfegh et al., 2014; Pillé et al., 2005; Wyckoff et al., 2006; Yip et al., 2007). Thus, we next investigated the expression and activity of RhoA, Rac1 and Cdc42. Western blotting analysis demonstrated upregulation of all Rho GTPases in Cx43-shRNA S1 cultures under 2-D (Figure 3.8A) and 3-D conditions of undiluted (Figure 3.8B) and 1:5 diluted Matrigel (Figure 3.8C). Furthermore, the levels of active Rho GTPases (GTP-bound) were enhanced, as pulldown assay showed (Figure 3.8). These data suggest that under permissive ECM stiffness, the loss of Cx43 induces invasion in S1 cells by altering Rho GTPase signaling.

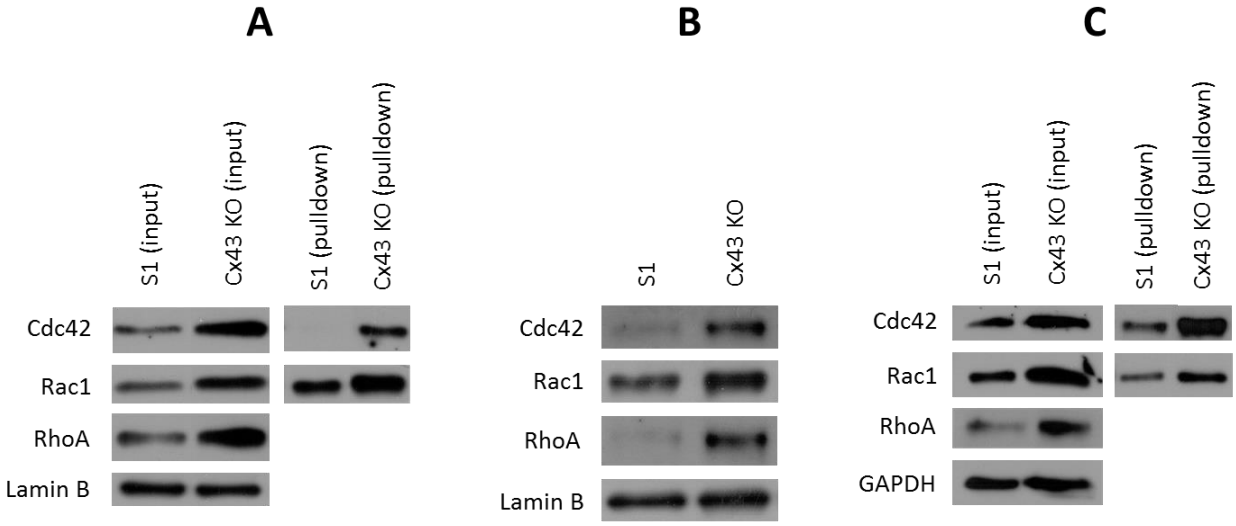

Figure 3.8. Silencing Cx43 upregulates the expression and activity of Rho GTPases in S1 cells under 2-D and 3-D culture conditions. S1 and Cx43-shRNA S1 cells (Cx43 KO) were cultured under 2-D (**A**), 3-D conditions of undiluted (**B**) or diluted Matrigel (1:5; **C**). Total proteins were extracted on day 9 in 2-D (**A**) and on day 11 in 3-D (**B and C**). Expression of RhoA, Cdc42 and Rac1 was assessed by Western blotting. Lamin B and GAPDH served as loading controls. Three independent experiments were performed. Activity of Rho GTPases was assessed by pulldown assay followed by Western blotting for detection of active Rho GTPases. Three independent experiments were performed.

E. Discussion

In this study, we report a role for the GJ protein Cx43 in controlling proliferation and migration capabilities of the mammary epithelium. Cell proliferation and migration are fundamental for tissue development and homeostasis (Demirkan, 2013; Micalizzi, Farabaugh, & Ford, 2010; Nakaya & Sheng, 2013). Deregulation of the signaling pathways that underlie these processes is associated with developmental defects and disease outcomes, such as cancer (Komiya & Habas, 2008; MacDonald et al., 2009).

GJs execute key roles in development and tumorigenesis of the mammary gland (McLachlan et al., 2007). Impaired Cx expression, localization and function alter the development of the mammary gland (Rana Mroue et al., 2015; Isabelle Plante & Laird, 2008; Isabelle Plante et al., 2010) and are linked to breast cancer (Kańczuga-Koda et al., 2003; Luiza Kanczuga-Koda et al., 2005; Laird et al., 1999; Singal et al., 2000; Yeh et al., 2017), suggesting that Cxs have both developmental and tumor suppressive roles in the mammary gland. In the human breast, Cx43 is expressed apically in luminal epithelial cells and in the myoepithelial cell layer (Bazzoun/Adissu et al., submitted), while the expression of Cx26 is confined to the luminal epithelium (Monaghan et al., 1996). Reduced expression or altered localization of Cx43 is reported in human breast cancer tissues and cell lines (Kańczuga-Koda et al., 2003; Luiza Kanczuga-Koda et al., 2005; Laird et al., 1999). Furthermore, the levels of Cx43 exhibit a positive correlation with good prognosis in breast cancer patients (Chasampalioti et al., 2018; Teleki et al., 2014). Overexpression of Cx43 in human breast cancer cell lines reduces proliferation, anchorage-independent growth, migration, invasion, xenograft tumor growth, angiogenesis and metastasis and restores the differentiation capacity, indicating a role for Cx43 in maintaining homeostasis and suppressing tumorigenesis in the mammary gland (Hirschi et al., 1996; Z. Li, Zhou, & Donahue, 2008; Z. Li, Zhou, Welch, et al., 2008; McLachlan et al., 2006; Qin et al., 2002; Talhouk et al., 2013). In order to dissect out the role of Cx43 in mammary epithelial homeostasis, we used the nontumorigenic human mammary epithelial HMT-3522 S1 cell line (Briand et al., 1987). When cultured under 3-D conditions, S1 cells form growth-arrested differentiated acini, characterized by an apicobasal polarity axis and a central lumen,

recapitulating features of their *in vivo* counterparts (Petersen et al., 1992; Plachot & Lelièvre, 2004). In addition, S1 cells express Cx43 at apicolateral membrane domains and form functional GJs, further resembling luminal epithelial cells in their natural context (Bazzoun/Adissu et al., submitted). Interestingly, of all major mammary Cxs, Cx43 is the only isoform expressed in S1 cells, making it possible to specifically study its participation in mammary homeostasis. Our earlier findings demonstrated perturbation of apical polarity and loss of lumen formation in 3-D cultures of Cx43-shRNA S1 cells (Bazzoun/Adissu et al., submitted). In this study, we show that silencing Cx43 in S1 cells triggered cell cycle entry and enhanced proliferation. This phenotype was concomitant with the upregulation of c-Myc and cyclin D1 expression. In support of our findings, silencing Cx43 was shown to enhance proliferation and anchorage-independent growth in Hs578T human breast cancer cell line (Shao et al., 2005), suggesting that the decrease in Cx43 levels has a long-lasting impact throughout cancer development. The effects of silencing Cx43 on proliferation and cell cycle entry in S1 cells were reproducible under 2-D and 3-D culture conditions, suggesting that the growth-regulatory role of Cx43 is not impeded by the substratum. This finding is in line with reports demonstrating reduced proliferation of human breast cancer cell lines overexpressing Cx genes in 2-D, 3-D cultures and xenografts (Hirschi et al., 1996; Kalra et al., 2006; Z. Li, Zhou, Welch, et al., 2008; Momiyama et al., 2003; Qin et al., 2002; Talhouk et al., 2013). Moreover, Scrib, an apical polarity regulator localized at apicolateral membrane domains in S1 acini, was mislocalized into a diffuse pattern in Cx43-shRNA counterparts, in line with Scrib redistribution in human breast cancer tissues and cell lines (Feigin et al., 2014; Zhan et al., 2008). Apicobasal polarity

regulates epithelial proliferation, migration, apoptosis, morphology and differentiation (Chatterjee & McCaffrey, 2014). The recapitulation of the effects of Cx43 silencing on proliferation and cell cycle entry of S1 cells in 2-D cultures that lack apicobasal polarization might rule out the direct involvement of apical polarity. However, this observation does not exclude the possibility that the loss of apical polarity measured in 3-D cultures sensitizes Cx43-shRNA S1 cells to other polarity-independent mechanisms downstream of Cx43 loss. Indeed, it was previously demonstrated that the loss of apical polarity primes S1 acini into cell cycle entry, but it is not sufficient to enhance proliferation (Chandramouly, Abad, Knowles, & Lelièvre, 2007). In addition, depletion of Scrib in the breast, prostate and liver accelerates tumor progression in the presence of other tumor-driving events but is not sufficient to drive tumorigenesis (Kapil et al., 2017; Pearson et al., 2011; Zhan et al., 2008). Blocking GJIC in 3-D cultures of S1 cells is not sufficient to enhance proliferation, although it acts in cooperation with extrinsic proliferation signals (Bazzoun/Adissu et al., submitted). Furthermore, 2-D cultures of mammary epithelial cells have compromised GJIC (El-Sabban et al., 2003). This suggests that Cx43 loss induces proliferation and cell cycle entry in S1 cells by mechanisms that are independent of GJIC. Indeed, overexpression of Cx43 in human breast cancer cell lines reduces proliferation and anchorage-independent growth via a channel-independent mechanism (Z. Li, Zhou, Welch, et al., 2008; McLachlan et al., 2006; Talhouk et al., 2013). Our earlier findings demonstrated that this effect is partly mediated by the membrane assembly of GJ complexes that recruit β-catenin and sequester it away from the nucleus (Talhouk et al., 2013), suggesting that the loss of Cx43 in our model triggers cell cycle entry as a consequence of altered assembly of GJ

complexes. While the GJ complex (Cx43, β-catenin and ZO-2) effectively assembled in 2-D and 3-D cultures of S1 cells, only 3-D cultures of Cx43-shRNA S1 cells exhibited evident mislocalization of β-catenin from apicolateral membrane domains, at which it associated with Cx43. In light of the dynamic cross-talk among epithelial junctions and their common associated partners (Giepmans, 2004; Giepmans & van IJzendoorn, 2009), β-catenin that is not incorporated into GJ complexes possibly relocalizes to other membrane domains where it associates with different junctional complexes in Cx43-shRNA S1 cells. In endothelial cells, the altered distribution of β-catenin within the membrane is associated with impaired integrity and stability of cell-cell contacts, the presence of intercellular gaps and the enhanced endothelial permeability (Martin Scholz et al., 2004; M Scholz et al., 2003). Similarly, the relocalization of membranous β-catenin in endometrial epithelial cells during the implantation window of the menstrual cycle is linked to altered intercellular adhesion necessary for trophoblast invasion (Buck, Windoffer, Leube, & Classen-Linke, 2012). Thus, the mislocalization of β-catenin downstream of Cx43 silencing in S1 cells might trigger proliferation and migration as a result of weakened cell-cell contacts. This enhanced proliferation downstream of Cx43 silencing may hence be mediated by the Wnt/β-catenin pathway, which regulates the development of the mammary gland and is deregulated in breast cancer (Imbert et al., 2001; Lin et al., 2000; Lindvall et al., 2009; Milovanovic et al., 2004; Prasad et al., 2008; van Genderen et al., 1994). While the membrane fraction of β-catenin associates with junctional complexes, such as adherens junctions (AJs) and GJs (Behrens, 1999; Talhouk et al., 2013; Talhouk et al., 2008), the stability of cytoplasmic β-catenin is regulated by the activity of the Wnt/β-catenin pathway. In the "on" state, β-

catenin accumulates in the cytoplasm, translocates to the nucleus and acts as a transcriptional coactivator for cell cycle genes (MacDonald et al., 2009). Breast cancers harbor reduced levels of membranous β-catenin and increased cytoplasmic and nuclear levels. The mislocalization of β-catenin correlates with poor clinical outcome and overall survival in breast cancer patients, and β-catenin is proposed as an independent prognostic factor (Dolled-Filhart et al., 2006; S. Li, Li, Sun, & Li, 2014; Lin et al., 2000). The enhanced activity of the Wnt/β-catenin pathway in breast cancer is not associated with mutations in β-catenin or other components of the pathway, but is rather linked to altered expression, localization or epigenetic patterns (S. Li et al., 2014; C.-C. Liu et al., 2010; Milovanovic et al., 2004; Ozaki et al., 2005; Prasad et al., 2008; Sørlie et al., 1998; Van der Auwera et al., 2008). We have shown previously that Cx43 acts as a negative regulator of the Wnt/β-catenin pathway by reducing the nuclear localization of β-catenin as a mechanism to induce differentiation in mammary epithelial cells or to suppress tumorigenesis in breast cancer lines (Talhouk et al., 2013; Talhouk et al., 2008). In the present studies with S1 cells, we did not detect any direct or indirect effects for Cx43 silencing on the activity of Wnt/β-catenin signaling. Specifically, the mislocalization of membranous β-catenin in Cx43-shRNA S1 cells was not accompanied with evident nuclear translocation of β-catenin. Interestingly, T4-2 cells, the tumorigenic counterparts of S1 cells, did not show increased Wnt/β-catenin activity compared to S1 cells, suggesting the involvement of other signaling pathways. Moreover, studies investigating the status of Wnt/β-catenin signaling in breast tumor tissues reported enhanced activity only in a subset of patient samples, and the correlation between Wnt/β-catenin activity and c-Myc or cyclin D1 expression was not

consistent among reports (Jönsson et al., 2000; Khan, Arafah, Shaik, Mahale, & Alanazi, 2018; Lim & Lee, 2002; Lin et al., 2000; Ozaki et al., 2005). In support of the activation of other signaling pathways downstream of Cx43 silencing in S1 cells to induce proliferation, we measured enhanced MAPK signaling, particularly ERK1/2, in Cx43-shRNA S1 cells. ERK1/2 signaling regulates mammary epithelial morphology, proliferation, motility and invasion, among others, and is involved in mammary gland development and breast cancer progression (Grill et al., 2004; Whyte et al., 2009). In line with our findings, other studies established a link between Cx32 deficiency and ERK1/2 activity in liver, lung and adrenal tumors (King et al., 2005; King & Lampe, 2004). Transfection of a glioma cell line with Cx30 abolishes ERK1/2 activity and thereby reduces proliferation, migration and invasion (Arun, Vanisree, & Ravisankar, 2016). In contrast, blocking GJs or Cx43 expression in tubular proximal epithelial cells activates ERK1/2 signaling (Gao et al., 2017). Our results showed that silencing Cx43 in S1 cells could be associated with enhanced motility and invasion, consistent with the reduced migration, invasion and xenograft tumor metastasis that we and others previously observed in breast cancer cell lines overexpressing Cx43 (Z. Li, Zhou, & Donahue, 2008; Z. Li, Zhou, Welch, et al., 2008; McLachlan et al., 2006; Talhouk et al., 2013). The enhanced motility and invasion were concomitant with upregulated expression and active levels of Rho GTPases (RhoA, Rac1 and Cdc42) and the formation of invadopodia-like actin-rich dots. Rho GTPases, which relay noncanonical Wnt signals, regulate growth, motility and invasion, and are implicated in mammary gland development and breast cancer progression (Bray et al., 2013; Burbelo et al., 2004; El-Sibai et al., 2007; Pillé et al., 2005; Schlessinger et al., 2009; Welsh, 2004; Yip

et al., 2007; Zuo et al., 2016). Consistent with our data, the knockdown of Cx43 in mouse embryonic fibroblasts leads to an increase in Rac1and RhoA activities and enhances migration (Mendoza-Naranjo et al., 2012). Overexpression of an active form of RhoA upregulates ERK1/2 in breast cancer cells and enhances motility (Cáceres, Guerrero, & Martínez, 2005). Thus, ERK1/2 signaling could be activated downstream of Rho GTPases in Cx43-shRNA S1 cells, where both pathways act in synergy to induce proliferation, motility and invasion. Importantly, migration and invasion were clearly apparent upon silencing Cx43 in 2-D cultures of S1 cells that represent nonphysiologically relevant conditions. Moreover, in 3-D cultures of S1 cells, silencing Cx43 triggered invasion only under conditions of diluted Matrigel, emphasizing the role of ECM stiffness in determining the phenotype and the invasive ability of mammary epithelial cells (Ansardamavandi et al., 2018; Cassereau et al., 2015; Levental et al., 2009; Miroshnikova et al., 2011). In line with our findings, a study on the effect of matrix stiffness on the behavior of mammary epithelial cells showed that stiff substrates restrict the motility of both normal and breast cancer cell lines. While the motility of breast cancer cells is enhanced on substrates with moderate or low stiffness, the migratory behavior of normal cells is less sensitive to changes in substrate stiffness (Ansardamavandi et al., 2018). Compared to normal tissues, breast cancer tissues are usually characterized by increased stiffness (Acerbi et al., 2015; Lopez, Kang, You, McDonald, & Weaver, 2011), but lower stiffness locally is necessary for invasion by virtue of MMP activity (Haage & Schneider, 2014; Lopez et al., 2011; Radisky & Radisky, 2010) and correlates with tumor progression and metastasis (Cross, Jin, Rao, & Gimzewski, 2007; Fenner et al., 2014; Plodinec et al., 2012). Therefore, although the

invasive phenotype linked to a decrease in Cx43 expression is not observed on normal matrix stiffness mimicked by undiluted Matrigel, once cells have become cancerous and activate mechanisms to decrease matrix stiffness locally, the Cx43 loss-mediated invasive phenotype is effective. This possibility supports the overriding impact of the ECM on intracellular changes illustrated initially two decades ago (Weaver et al., 1997) and explains the absence of an invasive phenotype in Cx43-shRNA S1 cells despite the enhanced Rho GTPase signaling observed on undiluted Matrigel. Interestingly, while S1 cells maintained their characteristic polarity and lumen-forming ability in 1:5 diluted Matrigel, these conditions unveiled an invasive phenotype in Cx43-shRNA S1 cells. This observation indicates that the resulting phenotype due to loss of Cx43 is partially mediated by coordinated signaling between cell-cell junctions and the mammary epithelial microenvironment, since neither the loss of Cx43 nor the permissiveness of the matrix was sufficient to induce invasion.

In summary, our results demonstrate that the loss of Cx43 induces the noncanonical Wnt pathway, which acts through Rho GTPase signaling to trigger cell cycle entry, motility and invasion in the phenotypically normal mammary epithelium. We propose that while the loss of Cx43 expression contributes to breast cancer initiation by perturbing apical polarity and normal morphology (Bazzoun/Adissu et al., submitted), it activates invasion pathways that become effective under permissive mechano-transducing cues from the matrix, suggesting a long-lasting impact for Cx43 loss on breast cancer development.

CHAPTER IV

CONCLUSION

Altered expression of Cxs impairs the normal development and functioning of the mammary gland and is associated with susceptibility to primary tumors and lung metastases in mouse models (Bry et al., 2004; Rana Mroue et al., 2015; Isabelle Plante & Laird, 2008; I Plante et al., 2011; Plum et al., 2000; Stewart et al., 2015; Stewart et al., 2013; Winterhager et al., 2007). In the human breast, Cx43 is expressed in myoepithelial cells and apically in luminal epithelial cells (Bazzoun/Adissu et al., submitted), which additionally express Cx26 (Monaghan et al., 1996). The expression and localization of Cx43 are altered in human breast cancer tissues and cell lines (Kańczuga-Koda et al., 2003; Luiza Kanczuga-Koda et al., 2005; Laird et al., 1999), suggesting that Cx43 exerts a tumor suppressive role in the mammary gland. Furthermore, Cx43 has been proposed as an independent prognostic factor in breast cancer (Chasampalioti et al., 2018; Teleki et al., 2014), making it critical to study its role in homeostasis and tumorigenesis of the breast tissue. The current consensus indicates that reduced Cx expression in early stages of breast cancer enhances the physical detachment of cells from the tumor microenvironment, an event required for cell migration. In contrast, restored Cx expression in advanced stages of breast cancer is essential for the interaction of tumor cells with their microenvironment and thus facilitates tumor metastasis (Banerjee, 2016; Grek et al., 2016; McLachlan et al., 2007). To address the role of Cx43 in homeostasis of the mammary gland and breast cancer

initiation, we used the non-neoplastic human mammary epithelial HMT-3522 S1 cells (Briand et al., 1987) that mimic luminal epithelial cells within their natural context. When cultured under 3-D conditions, S1 cells assemble into growth-arrested differentiated glandular structures, or acini, characterized by a central lumen and an apicobasal polarity axis (Petersen et al., 1992; Plachot & Lelièvre, 2004). In addition, S1 cells express Cx43 at apicolateral membrane domains, form functional GJs (Bazzoun/Adissu et al., submitted) and assemble GJ complexes that play a role in mammary epithelial differentiation and homeostasis (Talhouk et al., 2013; Talhouk et al., 2008). Those features collectively make S1 cells a relevant model for studying the role of Cx43 in mammary epithelial homeostasis and tumorigenesis, given that Cx43 is the only Cx isoform expressed in S1 cells (Bazzoun/Adissu et al., submitted). We have previously shown that silencing Cx43 in 3-D cultures of S1 cells impairs apical polarity, MSO and lumen-forming ability, events that mark the initiation of breast cancer (Bazzoun/Adissu et al., submitted). In this study, we show that the loss of Cx43 expression further contributes to breast cancer initiation by triggering cell cycle entry and enhancing proliferation with the upregulation of c-Myc and cyclin D1 expression. This is in line with the reduced proliferation and xenograft tumor growth demonstrated in human breast cancer cell lines overexpressing Cx43 (Hirschi et al., 1996; Qin et al., 2002; Talhouk et al., 2013). Scrib, a principal apical polarity regulator, was mislocalized from apicolateral membrane domains in Cx43-shRNA cells, consistent with its redistribution in human breast cancer tissues and cell lines (Feigin et al., 2014; Zhan et al., 2008). Although apical polarity is known to regulate epithelial proliferation (Chatterjee & McCaffrey, 2014), our results ruled out direct involvement of its loss in

the enhanced proliferation observed in Cx43-shRNA cells. The loss of apical polarity could instead act as a priming event for cell cycle entry in S1 cells downstream of Cx43 silencing (Chandramouly et al., 2007). The impaired GJIC that accompanies the loss of Cx43 in S1 cells acts in a similar manner but is not sufficient to enhance proliferation (Bazzoun/Adissu et al., submitted). Our results suggest that the role of Cx43 in controlling the proliferation of S1 cells is mediated by a channel-independent mechanism. We have reported a similar mechanism for Cx43 in inducing the differentiation of mammary epithelial cells and reversing the tumorigenesis of breast cancer cells via the assembly of GJ complexes that sequester β-catenin at the membrane and negatively regulate Wnt/β-catenin signaling (Talhouk et al., 2013; Talhouk et al., 2008). While the GJ complex effectively assembled in S1 cells, silencing Cx43 altered the apicolateral membrane distribution of β-catenin without evident activation of the Wnt/β-catenin pathway that regulates proliferation, an event that is associated with breast cancer (Jönsson et al., 2000; Khan et al., 2018; Lim & Lee, 2002; Lin et al., 2000; Ozaki et al., 2005). The mislocalization of β-catenin membrane distribution could lead to enhanced proliferation and migration by disrupting intercellular adhesion (Buck et al., 2012; Martin Scholz et al., 2004; M Scholz et al., 2003). In addition to the increased proliferation, random 2-D migration and invasion through Matrigel were enhanced in Cx43-shRNA S1 cells, consistent with the reduced migration, invasion and xenograft tumor metastasis that we and others previously observed in breast cancer cell lines overexpressing Cx43 (Z. Li, Zhou, & Donahue, 2008; Z. Li, Zhou, Welch, et al., 2008; McLachlan et al., 2006; Talhouk et al., 2013). Interestingly, silencing Cx43 in S1 cells activated ERK1/2 and Rho GTPase signaling, which integrates noncanonical Wnt

signals. Rho GTPase and ERK1/2 signaling regulate proliferation, motility and invasion, and are activated in breast cancer (Bray et al., 2013; Burbelo et al., 2004; El-Sibai et al., 2007; Grill et al., 2004; Pillé et al., 2005; Welsh, 2004; Whyte et al., 2009; Yip et al., 2007; Zuo et al., 2016). To the best of our knowledge, this is the first demonstration of a negative regulatory role for Cxs upstream ERK1/2 and Rho GTPase signaling in the mammary gland. In 3-D cultures, only permissive ECM stiffness unveiled an invasive phenotype in Cx43-shRNA S1 cells, emphasizing the upstream role of the microenvironment in determining cellular phenotype and behavior. This also suggests that the loss of Cx43 contributes to breast cancer initiation and promotes invasion at later stages of breast cancer.

We propose that the loss of Cx43 expression in the mammary epithelium contributes to breast cancer initiation by altering normal tissue architecture and enhancing proliferation while activating invasion pathways that become effective at advanced stages when the local ECM stiffness becomes permissive. The altered apical polarity and GJIC downstream of Cx43 loss prime cells into the channel-independent cell cycle entry through mechanisms that remain to be investigated. In addition, the loss of Cx43 activates Rho GTPases that relay proliferation and migration signals from the noncanonical Wnt pathway. The implication of channel-dependent or channel-independent mechanisms for Cx43 loss in invasion should be addressed in future studies. The activation of ERK1/2 could occur downstream of Rho GTPase signaling (Cáceres et al., 2005), where both pathways act synergistically to induce proliferation, motility and invasion. Given the spatiotemporal patterns that govern the normal activities of Rho GTPases, the effect of Cx43 loss on the localization of Rho GTPase

activities warrants further investigation. Finally, the loss of Cx43 alters the distribution of membranous β-catenin, an event that disrupts intercellular contacts to facilitate proliferation and migration. Although 3-D cultures of S1 cells represent a physiologically relevant *in vitro* model that recapitulates features of a differentiated mammary epithelium, it remains essential to study the effects of Cx43 loss under *in vivo* conditions, where a full complement of the normal microenvironment and macroenvironment exists. Future studies should be directed towards investigating the proliferation and invasive potential of Cx43-shRNA S1 mammary xenografts in mouse models. Elucidating the morphological events and the signaling pathways that are altered downstream of Cx43 loss in the mammary epithelium would fill the gap in knowledge and provide a platform for the development of the appropriate preventive measures and treatment strategies for breast cancer. Furthermore, the identification of biomarkers associated with breast cancer initiation events observed downstream of Cx43 loss, such as the impaired polarity and the enhanced proliferation, is key for early diagnosis and effective treatment. Circular RNAs (circRNAs) and microRNAs (miRNAs) have been proposed as non-invasive biomarkers for breast cancer by virtue of their stability and abundance in body fluids. Current research in our lab aims to identify alterations in miRNAs and their upstream sponging circRNAs that could drive the transition of normal into tumor-initiated mammary epithelium.

BIBLIOGRAPHY

Abraham, S. C., Reynolds, C., Lee, J.-H., Montgomery, E. A., Baisden, B. L., Krasinskas, A. M., et al. (2002). Fibromatosis of the breast and mutations involving the APC/β-catenin pathway. *Human pathology, 33*(1), 39-46.

Acerbi, I., Cassereau, L., Dean, I., Shi, Q., Au, A., Park, C., et al. (2015). Human breast cancer invasion and aggression correlates with ECM stiffening and immune cell infiltration. *Integrative Biology, 7*(10), 1120-1134.

Ahn, S.-J., Chung, K.-W., Lee, R.-A., Park, I.-A., Lee, S.-H., Park, D. E., et al. (2003). Overexpression of βPix-a in human breast cancer tissues. *Cancer letters, 193*(1), 99-107.

Ai, L., Tao, Q., Zhong, S., Fields, C. R., Kim, W.-J., Lee, M. W., et al. (2006). Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis, 27*(7), 1341-1348.

Ai, Z., Fischer, A., Spray, D. C., Brown, A. M., & Fishman, G. I. (2000). Wnt-1 regulation of connexin43 in cardiac myocytes. *The Journal of clinical investigation, 105*(2), 161-171.

Anderson, S. C., Stone, C., Tkach, L., & SundarRaj, N. (2002). Rho and Rho-kinase (ROCK) signaling in adherens and gap junction assembly in corneal epithelium. *Investigative ophthalmology & visual science, 43*(4), 978-986.

Angers, S., & Moon, R. T. (2009). Proximal events in Wnt signal transduction. *Nature reviews Molecular cell biology, 10*(7), 468.

Ansardamavandi, A., Tafazzoli-Shadpour, M., & Shokrgozar, M. A. (2018). Behavioral remodeling of normal and cancerous epithelial cell lines with differing invasion potential induced by substrate elastic modulus. *Cell adhesion & migration*, 1-17.

Arun, S., Vanisree, A. J., & Ravisankar, S. (2016). Connexin 30 downregulates Insulin-like growth factor receptor-1, abolishes Erk and potentiates effects of an IGF-R inhibitor in a glioma cell line. *Brain research, 1643*, 80-90.

Atwood, C., Hovey, R., Glover, J., Chepko, G., Ginsburg, E., Robison, W., et al. (2000). Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. *Journal of Endocrinology, 167*(1), 39-52.

Bagci, H., Laurin, M., Huber, J., Muller, W., & Cote, J. (2014). Impaired cell death and mammary gland involution in the absence of Dock1 and Rac1 signaling. *Cell death & disease, 5*(8), e1375.

Banerjee, D. (2016). Connexin's connection in breast cancer growth and progression. *International journal of cell biology, 2016*.

Baxley, S. E., Jiang, W., & Serra, R. (2011). Misexpression of wingless-related MMTV integration site 5A in mouse mammary gland inhibits the milk ejection response and regulates connexin43 phosphorylation. *Biology of reproduction, 85*(5), 907-915.

Behrens, J. (1999). Cadherins and catenins: role in signal transduction and tumor progression. *Cancer and Metastasis Reviews, 18*(1), 15-30.

Beurel, E., Grieco, S. F., & Jope, R. S. (2015). Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacology & therapeutics, 148*, 114-131.

Bivi, N., Pacheco‐Costa, R., Brun, L. R., Murphy, T. R., Farlow, N. R., Robling, A. G., et al. (2013). Absence of Cx43 selectively from osteocytes enhances responsiveness to mechanical force in mice. *Journal of Orthopaedic Research, 31*(7), 1075-1081.

Björklund, P., Svedlund, J., Olsson, A.-K., Åkerström, G., & Westin, G. (2009). The internally truncated LRP5 receptor presents a therapeutic target in breast cancer. *PloS one, 4*(1), e4243.

Blaschke, R. J., Howlett, A. R., Desprez, P.-Y., Petersen, O. W., & Bissell, M. J. (1994). [25] Cell differentiation by extracellular matrix components *Methods in enzymology* (Vol. 245, pp. 535-556): Elsevier.

Boras-Granic, K., & Hamel, P. A. (2013). Wnt-signalling in the embryonic mammary gland. *Journal of mammary gland biology and neoplasia, 18*(2), 155-163.

Bravo-Cordero, J. J., Oser, M., Chen, X., Eddy, R., Hodgson, L., & Condeelis, J. (2011). A novel spatiotemporal RhoC activation pathway locally regulates cofilin activity at invadopodia. *Current Biology, 21*(8), 635-644.

Bravo-Cordero, J. J., Sharma, V. P., Roh-Johnson, M., Chen, X., Eddy, R., Condeelis, J., et al. (2013). Spatial regulation of RhoC activity defines protrusion formation in migrating cells. *J Cell Sci*, jcs. 123547.

Bray, K., Brakebusch, C., & Vargo-Gogola, T. (2011). The Rho GTPase Cdc42 is required for primary mammary epithelial cell morphogenesis in vitro. *Small GTPases, 2*(5), 247-258.

Bray, K., Gillette, M., Young, J., Loughran, E., Hwang, M., Sears, J. C., et al. (2013). Cdc42 overexpression induces hyperbranching in the developing mammary gland by enhancing cell migration. *Breast Cancer Research, 15*(5), R91.

Briand, P., Petersen, O., & Van Deurs, B. (1987). A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In vitro cellular & developmental biology, 23*(3), 181-188.

Brisken, C. (2002). Hormonal control of alveolar development and its implications for breast carcinogenesis. *Journal of mammary gland biology and neoplasia, 7*(1), 39-48.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S. K., et al. (2000). Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes & development, 14*(6), 650-654.

Brisken, C., & O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harbor perspectives in biology*, a003178.

BROWNELL, H. L., NARSIMHAN, R. P., CORBLEY, M. J., MANN, V. M., WHITFIELD, J. F., & RAPTIS, L. (1996). Ras is involved in gap junction closure in proliferating fibroblasts or preadipocytes but not in differentiated adipocytes. *DNA and cell biology, 15*(6), 443-451.

Brownell, H. L., Whitfield, J. F., & Raptis, L. (1996). Cellular Ras partly mediates gap junction closure by the polyoma virus middle Tumor antigen. *Cancer letters, 103*(1), 99-106.

Brownell, H. L., Whitfield, J. F., & Raptis, L. (1997). Elimination of intercellular junctional communication requires lower Ras (leu61) levels than stimulation of

anchorage-independent proliferation. *Cancer detection and prevention, 21*(4), 289-294.

Bry, C., Maass, K., Miyoshi, K., Willecke, K., Ott, T., Robinson, G. W., et al. (2004). Loss of connexin 26 in mammary epithelium during early but not during late pregnancy results in unscheduled apoptosis and impaired development. *Developmental biology, 267*(2), 418-429.

Buck, V. U., Windoffer, R., Leube, R. E., & Classen-Linke, I. (2012). Redistribution of adhering junctions in human endometrial epithelial cells during the implantation window of the menstrual cycle. *Histochemistry and cell biology, 137*(6), 777-790.

Burbelo, P., Wellstein, A., & Pestell, R. G. (2004). Altered Rho GTPase signaling pathways in breast cancer cells. *Breast cancer research and treatment, 84*(1), 43-48.

Cáceres, M., Guerrero, J., & Martínez, J. (2005). Overexpression of RhoA-GTP induces activation of the Epidermal Growth Factor Receptor, dephosphorylation of focal adhesion kinase and increased motility in breast cancer cells. *Experimental cell research, 309*(1), 229-238.

Cai, C., Yu, Q. C., Jiang, W., Liu, W., Song, W., Yu, H., et al. (2014). R-spondin1 is a novel hormone mediator for mammary stem cell self-renewal. *Genes & development,* gad. 245142.245114.

Cassereau, L., Miroshnikova, Y. A., Ou, G., Lakins, J., & Weaver, V. M. (2015). A 3D tension bioreactor platform to study the interplay between ECM stiffness and tumor phenotype. *Journal of biotechnology, 193*, 66-69.

Castillo-Pichardo, L., Humphries-Bickley, T., De La Parra, C., Forestier-Roman, I., Martinez-Ferrer, M., Hernandez, E., et al. (2014). The Rac inhibitor EHop-016 inhibits mammary tumor growth and metastasis in a nude mouse model. *Translational oncology, 7*(5), 546-555.

Chambard, J.-C., Lefloch, R., Pouysségur, J., & Lenormand, P. (2007). ERK implication in cell cycle regulation. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1773*(8), 1299-1310.

Chan, C.-H., Lee, S.-W., Li, C.-F., Wang, J., Yang, W.-L., Wu, C.-Y., et al. (2010). Deciphering the transcriptional complex critical for RhoA gene expression and cancer metastasis. *Nature cell biology, 12*(5), 457.

Chandramouly, G., Abad, P. C., Knowles, D. W., & Lelièvre, S. A. (2007). The control of tissue architecture over nuclear organization is crucial for epithelial cell fate. *Journal of cell science, 120*(9), 1596-1606.

Chasampalioti, M., Green, A. R., Ellis, I. O., Rakha, E. A., Jackson, A. M., Spendlove, I., et al. (2018). Connexin 43 is an independent predictor of patient outcome in breast cancer patients. *Breast Cancer Research and Treatment*, 1-10.

Chatterjee, S. J., & McCaffrey, L. (2014). Emerging role of cell polarity proteins in breast cancer progression and metastasis. *Breast Cancer: Targets and Therapy, 6*, 15.

Chen, X., Shuzo, O., Li, Y., & Han, R. (1998). Effect of d-limonene, Salvia miltiorrhiza and turmeric derivatives on membrane association of Ras gene product and gap junction intercellular communication. *Yao xue xue bao= Acta pharmaceutica Sinica, 33*(11), 821-827.

Chu, E. Y., Hens, J., Andl, T., Kairo, A., Yamaguchi, T. P., Brisken, C., et al. (2004). Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development, 131*(19), 4819-4829.

Chua, A. C., Hodson, L. J., Moldenhauer, L. M., Robertson, S. A., & Ingman, W. V. (2010). Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium. *Development, 137*(24), 4229-4238.

Citi, S., Guerrera, D., Spadaro, D., & Shah, J. (2014). Epithelial junctions and Rho family GTPases: the zonular signalosome. *Small GTPases, 5*(4), e973760.

Clark, C. E., Nourse, C. C., & Cooper, H. M. (2012). The tangled web of non-canonical Wnt signalling in neural migration. *Neurosignals, 20*(3), 202-220.

Constantinou, T., Baumann, F., Lacher, M. D., Saurer, S., Friis, R., & Dharmarajan, A. (2008). SFRP-4 abrogates Wnt-3a-induced β-catenin and Akt/PKB signalling and reverses a Wnt-3a-imposed inhibition of in vitro mammary differentiation. *Journal of Molecular Signaling, 3*(1), 10.

Cross, S. E., Jin, Y.-S., Rao, J., & Gimzewski, J. K. (2007). Nanomechanical analysis of cells from cancer patients. *Nature nanotechnology, 2*(12), 780.

Czyz, J., Guan, K., Zeng, Q., & Wobus, A. M. (2003). Loss of beta1 integrin function results in upregulation of connexin expression in embryonic stem cell-derived cardiomyocytes. *International Journal of Developmental Biology, 49*(1), 33-41.

Dbouk, H. A., Mroue, R. M., El-Sabban, M. E., & Talhouk, R. S. (2009). Connexins: a myriad of functions extending beyond assembly of gap junction channels. *Cell Communication and signaling, 7*(1), 4.

De, A. (2011). Wnt/Ca2+ signaling pathway: a brief overview. *Acta biochimica et biophysica Sinica, 43*(10), 745-756.

Debebe, Z., & Rathmell, W. K. (2015). Ror2 as a therapeutic target in cancer. *Pharmacology & therapeutics, 150*, 143-148.

Demirkan, B. (2013). The roles of epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) in breast cancer bone metastasis: potential targets for prevention and treatment. *Journal of clinical medicine, 2*(4), 264-282.

Derangeon, M., Bourmeyster, N., Plaisance, I., Pinet-Charvet, C., Chen, Q., Duthe, F., et al. (2008). RhoA GTPase and F-actin dynamically regulate the permeability of Cx43-made channels in rat cardiac myocytes. *Journal of Biological Chemistry, 283*(45), 30754-30765.

Dermietzel, R., Hwang, T., & Spray, D. (1990). The gap junction family: structure, function and chemistry. *Anatomy and embryology, 182*(6), 517-528.

Dianati, E., Poiraud, J., Weber-Ouellette, A., & Plante, I. (2016). Connexins, E-cadherin, Claudin-7 and β-catenin transiently form junctional nexuses during the post-natal mammary gland development. *Developmental biology, 416*(1), 52-68.

Dolled-Filhart, M., McCabe, A., Giltnane, J., Cregger, M., Camp, R. L., & Rimm, D. L. (2006). Quantitative in situ analysis of β-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Cancer research, 66*(10), 5487-5494.

Druso, J. E., Endo, M., Lin, M.-c. J., Peng, X., Antonyak, M. A., Meller, S., et al. (2016). An essential role for Cdc42 in the functioning of the adult mammary gland. *Journal of Biological Chemistry, 291*(17), 8886-8895.

Du, W., Li, J., Du, W., Li, J., Wang, Q., Hou, J., et al. (2008). Lithium Chloride Regulates Connexin43 in Skeletal Myoblasts In Vitro: Possible Involvement in Wnt/β-Catenin Signaling. *Cell communication & adhesion, 15*(3), 261-271.

Dunn, N. R., & Tolwinski, N. S. (2016). Ptk7 and Mcc, unfancied components in non-canonical wnt signaling and cancer. *Cancers, 8*(7), 68.

El-Sabban, M. E., Sfeir, A. J., Daher, M. H., Kalaany, N. Y., Bassam, R. A., & Talhouk, R. S. (2003). ECM-induced gap junctional communication enhances mammary epithelial cell differentiation. *Journal of cell science, 116*(17), 3531-3541.

El-Saghir, J. A., El-Habre, E. T., El-Sabban, M. E., & Talhouk, R. S. (2011). Connexins: a junctional crossroad to breast cancer. *International Journal of Developmental Biology, 55*(7-8-9), 773-780.

El-Sibai, M., Nalbant, P., Pang, H., Flinn, R. J., Sarmiento, C., Macaluso, F., et al. (2007). Cdc42 is required for EGF-stimulated protrusion and motility in MTLn3 carcinoma cells. *Journal of cell science, 120*(19), 3465-3474.

El-Sibai, M., Pertz, O., Pang, H., Yip, S.-C., Lorenz, M., Symons, M., et al. (2008). RhoA/ROCK-mediated switching between Cdc42-and Rac1-dependent protrusion in MTLn3 carcinoma cells. *Experimental cell research, 314*(7), 1540-1552.

Ellenbroek, S. I., Iden, S., & Collard, J. G. (2012). *Cell polarity proteins and cancer.* Paper presented at the Seminars in cancer biology.

Elzarrad, M. K., Haroon, A., Willecke, K., Dobrowolski, R., Gillespie, M. N., & Al-Mehdi, A.-B. (2008). Connexin-43 upregulation in micrometastases and tumor vasculature and its role in tumor cell attachment to pulmonary endothelium. *BMC medicine, 6*(1), 20.

Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature, 420*(6916), 629.

Ewald, A. J., Brenot, A., Duong, M., Chan, B. S., & Werb, Z. (2008). Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Developmental cell, 14*(4), 570-581.

Feigin, M. E., Akshinthala, S. D., Araki, K., Rosenberg, A. Z., Muthuswamy, L. B., Martin, B., et al. (2014). Mislocalization of the cell polarity protein scribble promotes mammary tumorigenesis and is associated with basal breast cancer. *Cancer research*, canres. 3415.2013.

Fenner, J., Stacer, A. C., Winterroth, F., Johnson, T. D., Luker, K. E., & Luker, G. D. (2014). Macroscopic stiffness of breast tumors predicts metastasis. *Scientific reports, 4*, 5512.

Ferrati, S., Gadok, A. K., Brunaugh, A. D., Zhao, C., Heersema, L. A., Smyth, H. D., et al. (2017). Connexin membrane materials as potent inhibitors of breast cancer cell migration. *Journal of The Royal Society Interface, 14*(133), 20170313.

Fostok, S. F., El-Sibai, M., El-Sabban, M., & Talhouk, R. S. (2018). Gap Junctions and Wnt Signaling in the Mammary Gland: a Cross-Talk? *Journal of mammary gland biology and neoplasia*, 1-22.

Fritz, G., Brachetti, C., Bahlmann, F., Schmidt, M., & Kaina, B. (2002). Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *British journal of cancer, 87*(6), 635.

Fritz, G., Just, I., & Kaina, B. (1999). Rho GTPases are over‐expressed in human tumors. *International journal of cancer, 81*(5), 682-687.

Gabriel, H.-D., Jung, D., Bützler, C., Temme, A., Traub, O., Winterhager, E., et al. (1998). Transplacental uptake of glucose is decreased in embryonic lethal connexin26-deficient mice. *The Journal of cell biology, 140*(6), 1453-1461.

Gao, S., Zhang, X., Gao, K., Zhang, Z., Huang, Y., Yoda, R., et al. (2017). The pivotal role of extracellular signal-regulated kinase in gap junction-mediated regulation of TXNIP. *Cellular signalling, 38*, 116-126.

Geletu, M., Guy, S., Greer, S., & Raptis, L. (2015). Differential effects of polyoma virus middle tumor antigen mutants upon gap junctional, intercellular communication. *Experimental cell research, 336*(2), 223-231.

Giepmans, B. N. (2004). Gap junctions and connexin-interacting proteins. *Cardiovascular research, 62*(2), 233-245.

Giepmans, B. N., & van IJzendoorn, S. C. (2009). Epithelial cell–cell junctions and plasma membrane domains. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1788*(4), 820-831.

Gjorevski, N., & Nelson, C. M. (2011). Integrated morphodynamic signalling of the mammary gland. *Nature reviews Molecular cell biology, 12*(9), 581.

Gómez-Orte, E., Sáenz-Narciso, B., Moreno, S., & Cabello, J. (2013). Multiple functions of the noncanonical Wnt pathway. *Trends in genetics, 29*(9), 545-553.

Gouon-Evans, V., Rothenberg, M. E., & Pollard, J. W. (2000). Postnatal mammary gland development requires macrophages and eosinophils. *Development, 127*(11), 2269-2282.

Green, J., Nusse, R., & van Amerongen, R. (2013). The role of Ryk and Ror receptor tyrosine kinases in Wnt signal transduction. *Cold Spring Harbor perspectives in biology*, a009175.

Grek, C. L., Rhett, J. M., Bruce, J. S., Ghatnekar, G. S., & Yeh, E. S. (2016). Connexin 43, breast cancer tumor suppressor: missed connections? *Cancer letters, 374*(1), 117-126.

Grill, C., Gheyas, F., Dayananth, P., Weihong, J., Wei, D., Ping, Q., et al. (2004). Analysis of the ERK1, 2 transcriptome in mammary epithelial cells. *Biochemical Journal, 381*(3), 635-644.

Guger, K. A., & Gumbiner, B. M. (1995). β-Catenin Has Wnt-like Activity and Mimics the Nieuwkoop Signaling Center inXenopusDorsal–Ventral Patterning. *Developmental biology, 172*(1), 115-125.

Haage, A., & Schneider, I. C. (2014). Cellular contractility and extracellular matrix stiffness regulate matrix metalloproteinase activity in pancreatic cancer cells. *The FASEB Journal, 28*(8), 3589-3599.

Hanna, S., & El-Sibai, M. (2013). Signaling networks of Rho GTPases in cell motility. *Cellular signalling, 25*(10), 1955-1961.

Hanna, S., Khalil, B., Nasrallah, A., Saykali, B. A., Sobh, R., Nasser, S., et al. (2014). StarD13 is a tumor suppressor in breast cancer that regulates cell motility and invasion. *International journal of oncology, 44*(5), 1499-1511.

Hayashi, T., Nomata, K., Chang, C.-C., Ruch, R. J., & Trosko, J. E. (1998). Cooperative effects of v-myc and c-Ha-ras oncogenes on gap junctional intercellular communication and tumorigenicity in rat liver epithelial cells. *Cancer letters, 128*(2), 145-154.

Hayes, M. J., Thomas, D., Emmons, A., Giordano, T. J., & Kleer, C. G. (2008). Genetic changes of Wnt pathway genes are common events in metaplastic carcinomas of the breast. *Clinical Cancer Research, 14*(13), 4038-4044.

Heo, J. S., & Lee, J. C. (2011). β‐catenin mediates cyclic strain‐stimulated cardiomyogenesis in mouse embryonic stem cells through ROS‐dependent and integrin‐mediated PI3K/Akt pathways. *Journal of cellular biochemistry, 112*(7), 1880-1889.

Herder, C., Swiercz, J. M., Müller, C., Peravali, R., Quiring, R., Offermanns, S., et al. (2013). ArhGEF18 regulates RhoA-Rock2 signaling to maintain neuro-epithelial apico-basal polarity and proliferation. *Development, 140*(13), 2787-2797.

Hinck, L., & Silberstein, G. B. (2005). Key stages in mammary gland development: the mammary end bud as a motile organ. *Breast Cancer Research, 7*(6), 245.

Hirschi, K. K., Xu, C., Tsukamoto, T., & Sager, R. (1996). Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell Growth and Differentiation-Publication American Association for Cancer Research, 7*(7), 861-870.

Howard, B. A., & Gusterson, B. A. (2000). Human breast development. *Journal of mammary gland biology and neoplasia, 5*(2), 119-137.

Huguet, E. L., McMahon, J. A., McMahon, A. P., Bicknell, R., & Harris, A. L. (1994). Differential expression of human Wnt genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. *Cancer research, 54*(10), 2615-2621.

Imbert, A., Eelkema, R., Jordan, S., Feiner, H., & Cowin, P. (2001). ΔN89β-catenin induces precocious development, differentiation, and neoplasia in mammary gland. *The Journal of cell biology, 153*(3), 555-568.

Incassati, A., Chandramouli, A., Eelkema, R., & Cowin, P. (2010). Key signaling nodes in mammary gland development and cancer: β-catenin. *Breast Cancer Research, 12*(6), 213.

Insua-Rodriguez, J., & Oskarsson, T. (2016). The extracellular matrix in breast cancer. *Advanced Drug Delivery Reviews, 97*, 41-55.

Ishikawa, M., Iwamoto, T., Fukumoto, S., & Yamada, Y. (2014). Pannexin 3 inhibits proliferation of osteoprogenitor cells by regulating Wnt and p21 signaling. *Journal of Biological Chemistry, 289*(5), 2839-2851.

Ito, S., Ito, Y., Senga, T., Hattori, S., Matsuo, S., & Hamaguchi, M. (2006). v-Src requires Ras signaling for the suppression of gap junctional intercellular communication. *Oncogene, 25*(16), 2420.

Jamieson, S., Going, J. J., D'Arcy, R., & George, W. D. (1998a). Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 184*(1), 37-43.

Jamieson, S., Going, J. J., D'Arcy, R., & George, W. D. (1998b). Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. *The Journal of pathology, 184*(1), 37-43.

Jarde, T., & Dale, T. (2012). Wnt signalling in murine postnatal mammary gland development. *Acta physiologica, 204*(1), 118-127.

Ji, H., Goode, R. J., Vaillant, F., Mathivanan, S., Kapp, E. A., Mathias, R. A., et al. (2011). Proteomic profiling of secretome and adherent plasma membranes from distinct mammary epithelial cell subpopulations. *Proteomics, 11*(20), 4029-4039.

Jönsson, M., Borg, Å., Nilbert, M., & Andersson, T. (2000). Involvement of adenomatous polyposis coli (APC)/β-catenin signalling in human breast cancer. *European journal of cancer, 36*(2), 242-248.

Kalra, J., Shao, Q., Qin, H., Thomas, T., Alaoui-Jamali, M. A., & Laird, D. W. (2006). Cx26 inhibits breast MDA-MB-435 cell tumorigenic properties by a gap junctional intercellular communication-independent mechanism. *Carcinogenesis, 27*(12), 2528-2537.

Kamei, J., Toyofuku, T., & Hori, M. (2003). Negative regulation of p21 by β-catenin/TCF signaling: a novel mechanism by which cell adhesion molecules regulate cell proliferation. *Biochemical and biophysical research communications, 312*(2), 380-387.

Kańczuga-Koda, L., Sulkowska, M., Koda, M., Reszeć, J., Famulski, W., Baltaziak, M., et al. (2003). Expression of connexin 43 in breast cancer in comparison with mammary dysplasia and the normal mammary gland. *Folia morphologica, 62*(4), 439-442.

Kanczuga-Koda, L., Sulkowska, M., Koda, M., Rutkowski, R., & Sulkowski, S. (2007). Increased expression of gap junction protein--connexin 32 in lymph node metastases of human ductal breast cancer. *Folia histochemica et cytobiologica, 45*(I), 175-180.

Kanczuga-Koda, L., Sulkowski, S., Lenczewski, A., Koda, M., Wincewicz, A., Baltaziak, M., et al. (2006). Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. *Journal of clinical pathology, 59*(4), 429-433.

Kanczuga-Koda, L., Sulkowski, S., Tomaszewski, J., Koda, M., Sulkowska, M., Przystupa, W., et al. (2005). Connexins 26 and 43 correlate with Bak, but not with Bcl-2 protein in breast cancer. *Oncology reports, 14*(2), 325-329.

Kapil, S., Sharma, B. K., Patil, M., Elattar, S., Yuan, J., Hou, S. X., et al. (2017). The cell polarity protein Scrib functions as a tumor suppressor in liver cancer. *Oncotarget, 8*(16), 26515.

Katayama, K.-i., Melendez, J., Baumann, J. M., Leslie, J. R., Chauhan, B. K., Nemkul, N., et al. (2011). Loss of RhoA in neural progenitor cells causes the disruption of adherens junctions and hyperproliferation. *Proceedings of the National Academy of Sciences, 108*(18), 7607-7612.

Kaushik, S., Pickup, M. W., & Weaver, V. M. (2016). From transformation to metastasis: deconstructing the extracellular matrix in breast cancer. *Cancer and Metastasis Reviews, 35*(4), 655-667.

Keely, P. J., Wu, J. E., & Santoro, S. A. (1995). The spatial and temporal expression of the α2β1 integrin and its ligands, collagen I, collagen IV, and laminin, suggest important roles in mouse mammary morphogenesis. *Differentiation, 59*(1), 1-13.

Kelly, J. J., Simek, J., & Laird, D. W. (2015). Mechanisms linking connexin mutations to human diseases. *Cell and tissue research, 360*(3), 701-721.

Khalil, B. D., Hanna, S., Saykali, B. A., El-Sitt, S., Nasrallah, A., Marston, D., et al. (2014). The regulation of RhoA at focal adhesions by StarD13 is important for astrocytoma cell motility. *Experimental cell research, 321*(2), 109-122.

Khan, Z., Arafah, M., Shaik, J. P., Mahale, A., & Alanazi, M. S. (2018). High-frequency deregulated expression of Wnt signaling pathway members in breast carcinomas. *OncoTargets and therapy, 11*, 323.

King, T. J., Gurley, K. E., Prunty, J., Shin, J.-L., Kemp, C. J., & Lampe, P. D. (2005). Deficiency in the gap junction protein connexin32 alters p27 Kip1 tumor suppression and MAPK activation in a tissue-specific manner. *Oncogene, 24*(10), 1718.

King, T. J., & Lampe, P. D. (2004). Mice deficient for the gap junction protein Connexin32 exhibit increased radiation-induced tumorigenesis associated with elevated mitogen-activated protein kinase (p44/Erk1, p42/Erk2) activation. *Carcinogenesis, 25*(5), 669-680.

Knudsen, K. A., & Wheelock, M. J. (2005). Cadherins and the mammary gland. *Journal of cellular biochemistry, 95*(3), 488-496.

Koledova, Z., & Lu, P. (2017). A 3D Fibroblast-Epithelium Co-culture Model for Understanding Microenvironmental Role in Branching Morphogenesis of the Mammary Gland *Mammary Gland Development* (pp. 217-231): Springer.

Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis, 4*(2), 68-75.

Kouros‐Mehr, H., & Werb, Z. (2006). Candidate regulators of mammary branching morphogenesis identified by genome‐wide transcript analysis. *Developmental dynamics: an official publication of the American Association of Anatomists, 235*(12), 3404-3412.

Laird, D. W., Fistouris, P., Batist, G., Alpert, L., Huynh, H. T., Carystinos, G. D., et al. (1999). Deficiency of connexin43 gap junctions is an independent marker for breast tumors. *Cancer research, 59*(16), 4104-4110.

Lambe, T., Finlay, D., Murphy, M., & Martin, F. (2006). Differential expression of connexin 43 in mouse mammary cells. *Cell biology international, 30*(5), 472-479.

Lane, J., Martin, T. A., Mansel, R. E., & Jiang, W. G. (2008). *The expression and prognostic value of the guanine nucleotide exchange factors (GEFs) Trio, Vav1 and TIAM-1 in human breast cancer.* Paper presented at the International Seminars in Surgical Oncology.

Lane, T. F., & Leder, P. (1997). Wnt-10b directs hypermorphic development and transformation in mammary glands of male and female mice. *Oncogene, 15*(18), 2133.

Leithe, E., Mesnil, M., & Aasen, T. (2017). The connexin 43 C-terminus: A tail of many tales. *Biochimica et Biophysica Acta (BBA)-Biomembranes*.

Lejeune, S., Huguet, E. L., Hamby, A., Poulsom, R., & Harris, A. L. (1995). Wnt5a cloning, expression, and up-regulation in human primary breast cancers. *Clinical Cancer Research, 1*(2), 215-222.

Lelièvre, S. A., Weaver, V. M., Nickerson, J. A., Larabell, C. A., Bhaumik, A., Petersen, O. W., et al. (1998). Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus. *Proceedings of the National Academy of Sciences, 95*(25), 14711-14716.

Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., et al. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell, 139*(5), 891-906.

Li, S., Li, S., Sun, Y., & Li, L. (2014). The expression of β-catenin in different subtypes of breast cancer and its clinical significance. *Tumor Biology, 35*(8), 7693-7698.

Li, Z., Zhou, Z., & Donahue, H. J. (2008). Alterations in Cx43 and OB-cadherin affect breast cancer cell metastatic potential. *Clinical & experimental metastasis, 25*(3), 265-272.

Li, Z., Zhou, Z., Welch, D. R., & Donahue, H. J. (2008). Expressing connexin 43 in breast cancer cells reduces their metastasis to lungs. *Clinical & experimental metastasis, 25*(8), 893-901.

Lim, S.-C., & Lee, M.-S. (2002). Significance of E-cadherin/β-catenin complex and cyclin D1 in breast cancer. *Oncology reports, 9*(5), 915-928.

Lin, S.-Y., Xia, W., Wang, J. C., Kwong, K. Y., Spohn, B., Wen, Y., et al. (2000). β-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proceedings of the National Academy of Sciences, 97*(8), 4262-4266.

Lindvall, C., Evans, N. C., Zylstra, C. R., Li, Y., Alexander, C. M., & Williams, B. O. (2006). The Wnt signaling receptor Lrp5 is required for mammary ductal stem cell activity and Wnt1-induced tumorigenesis. *Journal of Biological Chemistry, 281*(46), 35081-35087.

Lindvall, C., Zylstra, C. R., Evans, N., West, R. A., Dykema, K., Furge, K. A., et al. (2009). The Wnt co-receptor Lrp6 is required for normal mouse mammary gland development. *PloS one, 4*(6), e5813.

Liu, C.-C., Prior, J., Piwnica-Worms, D., & Bu, G. (2010). LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy. *Proceedings of the National Academy of Sciences, 107*(11), 5136-5141.

Liu, X., Hashimoto-Torii, K., Torii, M., Ding, C., & Rakic, P. (2010). Gap junctions/hemichannels modulate interkinetic nuclear migration in the forebrain precursors. *Journal of Neuroscience, 30*(12), 4197-4209.

Locke, D., Jamieson, S., Stein, T., Liu, J., Hodgins, M. B., Harris, A. L., et al. (2007). Nature of Cx30-containing channels in the adult mouse mammary gland. *Cell and tissue research, 328*(1), 97-107.

Locke, D., Perusinghe, N., Newman, T., Jayatilake, H., Evans, W. H., & Monaghan, P. (2000). Developmental expression and assembly of connexins into homomeric and heteromeric gap junction hemichannels in the mouse mammary gland. *Journal of cellular physiology, 183*(2), 228-237.

Locke, D., Stein, T., Davies, C., Morris, J., Harris, A. L., Evans, W. H., et al. (2004). Altered permeability and modulatory character of connexin channels during mammary gland development. *Experimental cell research, 298*(2), 643-660.

Lopez, J. I., Kang, I., You, W.-K., McDonald, D. M., & Weaver, V. M. (2011). In situ force mapping of mammary gland transformation. *Integrative Biology, 3*(9), 910-921.

MacDonald, B. T., Tamai, K., & He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Developmental cell, 17*(1), 9-26.

Machtaler, S., Choi, K., Dang-Lawson, M., Falk, L., Pournia, F., Naus, C. C., et al. (2014). The role of the gap junction protein connexin43 in B lymphocyte motility and migration. *FEBS letters, 588*(8), 1249-1258.

Machtaler, S., Dang-Lawson, M., Choi, K., Jang, C., Naus, C. C., & Matsuuchi, L. (2011). The gap junction protein Cx43 regulates B-lymphocyte spreading and adhesion. *J Cell Sci, 124*(15), 2611-2621.

Mack, N. A., & Georgiou, M. (2014). The interdependence of the Rho GTPases and apicobasal cell polarity. *Small GTPases, 5*(2), e973768.

Maddala, R., Deng, P.-F., Costello, J. M., Wawrousek, E. F., Zigler, J. S., & Rao, V. P. (2004). Impaired cytoskeletal organization and membrane integrity in lens fibers of a Rho GTPase functional knockout transgenic mouse. *Laboratory investigation, 84*(6), 679.

Majidinia, M., & Yousefi, B. (2017). Breast tumor stroma: A driving force in the development of resistance to therapies. *Chemical biology & drug design, 89*(3), 309-318.

Matsuda, T., Fujio, Y., Nariai, T., Ito, T., Yamane, M., Takatani, T., et al. (2006). N-cadherin signals through Rac1 determine the localization of connexin 43 in cardiac myocytes. *Journal of molecular and cellular cardiology, 40*(4), 495-502.

McLachlan, E., Shao, Q., & Laird, D. W. (2007). Connexins and gap junctions in mammary gland development and breast cancer progression. *Journal of Membrane Biology, 218*(1-3), 107-121.

McLachlan, E., Shao, Q., Wang, H.-l., Langlois, S., & Laird, D. W. (2006). Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis. *Cancer research, 66*(20), 9886-9894.

Mendoza-Naranjo, A., Cormie, P., Serrano, A. E., Hu, R., O'Neill, S., Wang, C. M., et al. (2012). Targeting Cx43 and N-cadherin, which are abnormally upregulated in venous leg ulcers, influences migration, adhesion and activation of Rho GTPases. *PloS one, 7*(5), e37374.

Micalizzi, D. S., Farabaugh, S. M., & Ford, H. L. (2010). Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *Journal of mammary gland biology and neoplasia, 15*(2), 117-134.

Milovanovic, T., Planutis, K., Nguyen, A., Marsh, J. L., Lin, F., Hope, C., et al. (2004). Expression of Wnt genes and frizzled 1 and 2 receptors in normal breast epithelium and infiltrating breast carcinoma. *International journal of oncology, 25*(5), 1337-1342.

Miroshnikova, Y., Jorgens, D., Spirio, L., Auer, M., Sarang-Sieminski, A., & Weaver, V. (2011). Engineering strategies to recapitulate epithelial morphogenesis within

synthetic three-dimensional extracellular matrix with tunable mechanical properties. *Physical biology, 8*(2), 026013.

Momiyama, M., Omori, Y., Ishizaki, Y., Nishikawa, Y., Tokairin, T., Ogawa, J., et al. (2003). Connexin26 ‐ mediated gap junctional communication reverses the malignant phenotype of MCF ‐ 7 breast cancer cells. *Cancer science, 94*(6), 501-507.

Monaghan, P., Clarke, C., Perusinghe, N. P., Moss, D. W., Chen, X.-Y., & Evans, W. H. (1996). Gap junction distribution and connexin expression in human breast. *Experimental cell research, 223*(1), 29-38.

Monaghan, P., Perusinghe, N., Carlile, G., & Evans, W. H. (1994). Rapid modulation of gap junction expression in mouse mammary gland during pregnancy, lactation, and involution. *Journal of Histochemistry & Cytochemistry, 42*(7), 931-938.

Moshfegh, Y., Bravo-Cordero, J. J., Miskolci, V., Condeelis, J., & Hodgson, L. (2014). A Trio–Rac1–Pak1 signalling axis drives invadopodia disassembly. *Nature cell biology, 16*(6), 571.

Mroue, R., El-Sabban, M., & Talhouk, R. (2011). Connexins and the gap in context. *Integrative Biology, 3*(4), 255-266.

Mroue, R., Inman, J., Mott, J., Budunova, I., & Bissell, M. J. (2015). Asymmetric expression of connexins between luminal epithelial-and myoepithelial-cells is essential for contractile function of the mammary gland. *Developmental biology, 399*(1), 15-26.

Mureli, S., Gans, C. P., Bare, D. J., Geenen, D. L., Kumar, N. M., & Banach, K. (2012). Mesenchymal stem cells improve cardiac conduction by upregulation of connexin 43 through paracrine signaling. *American Journal of Physiology-Heart and Circulatory Physiology, 304*(4), H600-H609.

Musumeci, G., Castrogiovanni, P., Szychlinska, M. A., Aiello, F. C., Vecchio, G. M., Salvatorelli, L., et al. (2015). Mammary gland: From embryogenesis to adult life. *Acta histochemica, 117*(4-5), 379-385.

Nagahata, T., Shimada, T., Harada, A., Nagai, H., Onda, M., Yokoyama, S., et al. (2003). Amplification, up ‐ regulation and over ‐ expression of DVL ‐ 1, the human counterpart of the Drosophila disheveled gene, in primary breast cancers. *Cancer science, 94*(6), 515-518.

Nakaya, Y., & Sheng, G. (2013). EMT in developmental morphogenesis. *Cancer letters, 341*(1), 9-15.

Naoi, Y., Miyoshi, Y., Taguchi, T., Kim, S. J., Arai, T., Tamaki, Y., et al. (2007). Connexin26 expression is associated with lymphatic vessel invasion and poor prognosis in human breast cancer. *Breast cancer research and treatment, 106*(1), 11-17.

Naus, C. C., & Laird, D. W. (2010). Implications and challenges of connexin connections to cancer. *Nature Reviews Cancer, 10*(6), 435.

O'Brien, J., Martinson, H., Durand-Rougely, C., & Schedin, P. (2012). Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development, 139*(2), 269-275.

Oakes, S. R., Rogers, R. L., Naylor, M. J., & Ormandy, C. J. (2008). Prolactin regulation of mammary gland development. *Journal of mammary gland biology and neoplasia, 13*(1), 13-28.

Ormandy, C. J., Naylor, M., Harris, J., Robertson, F., Horseman, N. D., Lindeman, G. J., et al. (2003). Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice. *Recent Progress in Hormone Research, 58*, 297-324.

Ozaki, S., Ikeda, S., Ishizaki, Y., Kurihara, T., Tokumoto, N., Iseki, M., et al. (2005). Alterations and correlations of the components in the Wnt signaling pathway and its target genes in breast cancer. *Oncology reports, 14*(6), 1437-1443.

Pacheco-Costa, R., Kadakia, J. R., Atkinson, E. G., Wallace, J. M., Plotkin, L. I., & Reginato, R. D. (2017). Connexin37 deficiency alters organic bone matrix, cortical bone geometry, and increases Wnt/β-catenin signaling. *Bone, 97*, 105-113.

Paine, I. S., & Lewis, M. T. (2017). The terminal end bud: The little engine that could. *Journal of mammary gland biology and neoplasia, 22*(2), 93-108.

Parsa, S., Ramasamy, S. K., De Langhe, S., Gupte, V. V., Haigh, J. J., Medina, D., et al. (2008). Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Developmental biology, 317*(1), 121-131.

Pearson, H. B., Perez-Mancera, P. A., Dow, L. E., Ryan, A., Tennstedt, P., Bogani, D., et al. (2011). SCRIB expression is deregulated in human prostate cancer, and its deficiency in mice promotes prostate neoplasia. *The Journal of clinical investigation, 121*(11), 4257-4267.

Petersen, O. W., Rønnov-Jessen, L., Howlett, A. R., & Bissell, M. J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proceedings of the National Academy of Sciences, 89*(19), 9064-9068.

Phillips, S. L., Williams, C. B., Zambrano, J. N., Williams, C. J., & Yeh, E. S. (2017). Connexin 43 in the development and progression of breast cancer: What's the connection? *International journal of oncology, 51*(4), 1005-1013.

Pillé, J.-Y., Denoyelle, C., Varet, J., Bertrand, J.-R., Soria, J., Opolon, P., et al. (2005). Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Molecular therapy, 11*(2), 267-274.

Plachot, C., & Lelièvre, S. A. (2004). DNA methylation control of tissue polarity and cellular differentiation in the mammary epithelium. *Experimental cell research, 298*(1), 122-132.

Plante, I., & Laird, D. W. (2008). Decreased levels of connexin43 result in impaired development of the mammary gland in a mouse model of oculodentodigital dysplasia. *Developmental biology, 318*(2), 312-322.

Plante, I., Stewart, M., Barr, K., Allan, A., & Laird, D. (2011). Cx43 suppresses mammary tumor metastasis to the lung in a Cx43 mutant mouse model of human disease. *Oncogene, 30*(14), 1681.

Plante, I., Wallis, A., Shao, Q., & Laird, D. W. (2010). Milk secretion and ejection are impaired in the mammary gland of mice harboring a Cx43 mutant while expression and localization of tight and adherens junction proteins remain unchanged. *Biology of reproduction, 82*(5), 837-847.

Plodinec, M., Loparic, M., Monnier, C. A., Obermann, E. C., Zanetti-Dallenbach, R., Oertle, P., et al. (2012). The nanomechanical signature of breast cancer. *Nature nanotechnology, 7*(11), 757.

Plum, A., Hallas, G., Magin, T., Dombrowski, F., Hagendorff, A., Schumacher, B., et al. (2000). Unique and shared functions of different connexins in mice. *Current biology, 10*(18), 1083-1091.

Pollmann, M.-A., Shao, Q., Laird, D. W., & Sandig, M. (2005). Connexin 43 mediated gap junctional communication enhances breast tumor cell diapedesis in culture. *Breast Cancer Research, 7*(4), R522.

Pozzi, A., Risek, B., Kiang, D. T., Gilula, N. B., & Kumar, N. M. (1995). Analysis of multiple gap junction gene products in the rodent and human mammary gland. *Experimental cell research, 220*(1), 212-219.

Prasad, C. P., Mirza, S., Sharma, G., Prashad, R., DattaGupta, S., Rath, G., et al. (2008). Epigenetic alterations of CDH1 and APC genes: relationship with activation of Wnt/β-catenin pathway in invasive ductal carcinoma of breast. *Life sciences, 83*(9-10), 318-325.

Prunskaite-Hyyryläinen, R., Shan, J., Railo, A., Heinonen, K. M., Miinalainen, I., Yan, W., et al. (2014). Wnt4, a pleiotropic signal for controlling cell polarity, basement membrane integrity, and antimüllerian hormone expression during oocyte maturation in the female follicle. *The FASEB Journal, 28*(4), 1568-1581.

Qin, H., Shao, Q., Curtis, H., Galipeau, J., Belliveau, D. J., Wang, T., et al. (2002). Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. *Journal of Biological Chemistry, 277*(32), 29132-29138.

Radisky, E. S., & Radisky, D. C. (2010). Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. *Journal of mammary gland biology and neoplasia, 15*(2), 201-212.

Rao, T. P., & KUhl, M. (2010). An updated overview on Wnt signaling pathways: a prelude for more. *Circulation research, 106*(12), 1798-1806.

Raymond, K., Cagnet, S., Kreft, M., Janssen, H., Sonnenberg, A., & Glukhova, M. A. (2011). Control of mammary myoepithelial cell contractile function by α3β1 integrin signalling. *The EMBO journal, 30*(10), 1896-1906.

Reaume, A. G., de Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., et al. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science, 267*(5205), 1831-1834.

Reddy, K. B., Nabha, S. M., & Atanaskova, N. (2003). Role of MAP kinase in tumor progression and invasion. *Cancer and Metastasis reviews, 22*(4), 395-403.

Rinaldi, F., Hartfield, E., Crompton, L., Badger, J., Glover, C., Kelly, C., et al. (2015). Cross-regulation of Connexin43 and β-catenin influences differentiation of human neural progenitor cells. *Cell death & disease, 5*(1), e1017.

Roarty, K., Shore, A. N., Creighton, C. J., & Rosen, J. M. (2015). Ror2 regulates branching, differentiation, and actin-cytoskeletal dynamics within the mammary epithelium. *J Cell Biol, 208*(3), 351-366.

Samarzija, I., Sini, P., Schlange, T., MacDonald, G., & Hynes, N. E. (2009). Wnt3a regulates proliferation and migration of HUVEC via canonical and non-

canonical Wnt signaling pathways. *Biochemical and biophysical research communications, 386*(3), 449-454.
Santamaria, P. G., & Nebreda, A. R. (2010). Deconstructing ERK signaling in tumorigenesis. *Molecular cell, 38*(1), 3-5.
Sawyer, E. J., Hanby, A. M., Rowan, A. J., Gillett, C. E., Thomas, R. E., Poulsom, R., et al. (2002). The Wnt pathway, epithelial–stromal interactions, and malignant progression in phyllodes tumours. *The Journal of pathology, 196*(4), 437-444.
Schlessinger, K., Hall, A., & Tolwinski, N. (2009). Wnt signaling pathways meet Rho GTPases. *Genes & development, 23*(3), 265-277.
Schlosshauer, P. W., Brown, S. A., Eisinger, K., Yan, Q., Guglielminetti, E. R., Parsons, R., et al. (2000). APC truncation and increased β-catenin levels in a human breast cancer cell line. *Carcinogenesis, 21*(7), 1453-1456.
Scholz, M., Nowak, P., Blaheta, R., Schuller, A., Menon, S., Cinatl, J., et al. (2004). Relocalization of endothelial cell β-catenin after coculture with activated neutrophils from patients undergoing cardiac surgery with cardiopulmonary bypass. *Journal of Investigative Surgery, 17*(3), 143-149.
Scholz, M., Wimmer-Greinecker, G., Simon, A., Dzemali, O., Chang, H.-Y., Kleine, P., et al. (2003). Perioperative elastase activity in cardiac surgery and its role in endothelial leakage. *Inflammation Research, 52*(10), 433-438.
Sedgwick, A. E., & D'Souza-Schorey, C. (2016). Wnt signaling in cell motility and invasion: drawing parallels between development and cancer. *Cancers, 8*(9), 80.
Segalen, M., & Bellaïche, Y. (2009). *Cell division orientation and planar cell polarity pathways.* Paper presented at the Seminars in cell & developmental biology.
Shao, Q., Wang, H., McLachlan, E., Veitch, G. I., & Laird, D. W. (2005). Down-regulation of Cx43 by retroviral delivery of small interfering RNA promotes an aggressive breast cancer cell phenotype. *Cancer research, 65*(7), 2705-2711.
Shulewitz, M., Soloviev, I., Wu, T., Koeppen, H., Polakis, P., & Sakanaka, C. (2006). Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer. *Oncogene, 25*(31), 4361.
Sin, W.-C., Tse, M., Planque, N., Perbal, B., Lampe, P. D., & Naus, C. C. (2009). Matricellular protein CCN3 (NOV) regulates actin cytoskeleton reorganization. *Journal of Biological Chemistry, 284*(43), 29935-29944.
Singal, R., Tu, Z., Vanwert, J., Ginder, G., & Kiang, D. (2000). Modulation of the connexin26 tumor suppressor gene expression through methylation in human mammary epithelial cell lines. *Anticancer research, 20*(1A), 59-64.
Sirnes, S., Bruun, J., Kolberg, M., Kjenseth, A., Lind, G. E., Svindland, A., et al. (2012). Connexin43 acts as a colorectal cancer tumor suppressor and predicts disease outcome. *International journal of cancer, 131*(3), 570-581.
Söhl, G., & Willecke, K. (2004). Gap junctions and the connexin protein family. *Cardiovascular research, 62*(2), 228-232.
Sokol, S. Y. (2015). *Spatial and temporal aspects of Wnt signaling and planar cell polarity during vertebrate embryonic development.* Paper presented at the Seminars in cell & developmental biology.
Somekawa, S., Fukuhara, S., Nakaoka, Y., Fujita, H., Saito, Y., & Mochizuki, N. (2005). Enhanced functional gap junction neoformation by protein kinase A–

dependent and Epac-dependent signals downstream of cAMP in cardiac myocytes. *Circulation research, 97*(7), 655-662.

Sørlie, T., Bukholm, I., & Børresen-Dale, A. (1998). Truncating somatic mutation in exon 15 of the APC gene is a rare event in human breast carcinomas. Mutations in brief no. 179. Online. *Human mutation, 12*(3), 215-215.

Soysal, S. D., Tzankov, A., & Muenst, S. E. (2015). Role of the tumor microenvironment in breast cancer. *Pathobiology, 82*(3-4), 142-152.

Stains, J. P., & Civitelli, R. (2005). Gap junctions regulate extracellular signal-regulated kinase signaling to affect gene transcription. *Molecular biology of the cell, 16*(1), 64-72.

Stewart, M. K., Bechberger, J. F., Welch, I., Naus, C. C., & Laird, D. W. (2015). Cx26 knockout predisposes the mammary gland to primary mammary tumors in a DMBA-induced mouse model of breast cancer. *Oncotarget, 6*(35), 37185.

Stewart, M. K., Gong, X.-Q., Barr, K. J., Bai, D., Fishman, G. I., & Laird, D. W. (2013). The severity of mammary gland developmental defects is linked to the overall functional status of Cx43 as revealed by genetically modified mice. *Biochemical Journal, 449*(2), 401-413.

Stewart, M. K., Plante, I., Bechberger, J. F., Naus, C. C., & Laird, D. W. (2014). Mammary gland specific knockdown of the physiological surge in Cx26 during lactation retains normal mammary gland development and function. *PloS one, 9*(7), e101546.

Stupack, D. G., Cho, S. Y., & Klemke, R. L. (2000). Molecular signaling mechanisms of cell migration and invasion. *Immunologic research, 21*(2-3), 83-88.

Su, V., & Lau, A. F. (2014). Connexins: mechanisms regulating protein levels and intercellular communication. *FEBS letters, 588*(8), 1212-1220.

Suzuki, H., Toyota, M., Caraway, H., Gabrielson, E., Ohmura, T., Fujikane, T., et al. (2008). Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. *British journal of cancer, 98*(6), 1147.

Taddei, I., Deugnier, M.-A., Faraldo, M. M., Petit, V., Bouvard, D., Medina, D., et al. (2008). β1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. *Nature cell biology, 10*(6), 716.

Talhouk, R. S., Bissell, M. J., & Werb, Z. (1992). Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *The Journal of Cell Biology, 118*(5), 1271-1282.

Talhouk, R. S., Elble, R. C., Bassam, R., Daher, M., Sfeir, A., Mosleh, L. A., et al. (2005). Developmental expression patterns and regulation of connexins in the mouse mammary gland: expression of connexin30 in lactogenesis. *Cell and tissue research, 319*(1), 49-59.

Talhouk, R. S., Fares, M.-B., Rahme, G. J., Hariri, H. H., Rayess, T., Dbouk, H. A., et al. (2013). Context dependent reversion of tumor phenotype by connexin-43 expression in MDA-MB231 cells and MCF-7 cells: Role of β-catenin/connexin43 association. *Experimental cell research, 319*(20), 3065-3080.

Talhouk, R. S., Khalil, A. A., Bajjani, R., Rahme, G. J., & El-Sabban, M. E. (2011). Gap junctions mediate STAT5-independent β-casein expression in CID-9 mammary epithelial cells. *Cell communication & adhesion, 18*(5), 104-116.

Talhouk, R. S., Mroue, R., Mokalled, M., Abi-Mosleh, L., Nehme, R., Ismail, A., et al. (2008). Heterocellular interaction enhances recruitment of α and β-catenins and ZO-2 into functional gap-junction complexes and induces gap junction-dependant differentiation of mammary epithelial cells. *Experimental cell research, 314*(18), 3275-3291.

Teleki, I., Szasz, A. M., Maros, M. E., Gyorffy, B., Kulka, J., Meggyeshazi, N., et al. (2014). Correlations of differentially expressed gap junction connexins Cx26, Cx30, Cx32, Cx43 and Cx46 with breast cancer progression and prognosis. *PloS one, 9*(11), e112541.

Thiagarajan, P. S., Sinyuk, M., Turaga, S. M., Mulkearns-Hubert, E. E., Hale, J. S., Rao, V., et al. (2018). Cx26 drives self-renewal in triple-negative breast cancer via interaction with NANOG and focal adhesion kinase. *Nature communications, 9*(1), 578.

Timmerman, I., Heemskerk, N., Kroon, J., Schaefer, A., van Rijssel, J., Hoogenboezem, M., et al. (2015). A local VE-cadherin and Trio-based signaling complex stabilizes endothelial junctions through Rac1. *J Cell Sci, 128*(16), 3041-3054.

Turashvili, G., Bouchal, J., Burkadze, G., & Kolar, Z. (2006). Wnt signaling pathway in mammary gland development and carcinogenesis. *Pathobiology, 73*(5), 213-223.

Umazume, K., Tsukahara, R., Liu, L., de Castro, J. P. F., McDonald, K., Kaplan, H. J., et al. (2014). Role of retinal pigment epithelial cell β-catenin signaling in experimental proliferative vitreoretinopathy. *The American Journal of Pathology, 184*(5), 1419-1428.

Van der Auwera, I., Van Laere, S. J., Van den Bosch, S., Van den Eynden, G., Trinh, B., Van Dam, P., et al. (2008). Aberrant methylation of the Adenomatous Polyposis Coli (APC) gene promoter is associated with the inflammatory breast cancer phenotype. *British journal of cancer, 99*(10), 1735.

Van der Heyden, M., Rook, M. B., Hermans, M., Rijksen, G., Boonstra, J., Defize, L., et al. (1998). Identification of connexin43 as a functional target for Wnt signalling. *Journal of Cell Science, 111*(12), 1741-1749.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R.-G., Parslow, T. G., Bruhn, L., et al. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes & development, 8*(22), 2691-2703.

Van Hengel, J., D'Hooge, P., Hooghe, B., Wu, X., Libbrecht, L., De Vos, R., et al. (2008). Continuous cell injury promotes hepatic tumorigenesis in cdc42-deficient mouse liver. *Gastroenterology, 134*(3), 781-792.

Veltmaat, J. M., Van Veelen, W., Thiery, J. P., & Bellusci, S. (2004). Identification of the mammary line in mouse by Wnt10b expression. *Developmental dynamics: an official publication of the American Association of Anatomists, 229*(2), 349-356.

Vidi, P.-A., Bissell, M. J., & Lelièvre, S. A. (2012). Three-dimensional culture of human breast epithelial cells: the how and the why *Epithelial Cell Culture Protocols* (pp. 193-219): Springer.

Wang, H.-X., Gillio-Meina, C., Chen, S., Gong, X.-Q., Li, T. Y., Bai, D., et al. (2013). The canonical WNT2 pathway and FSH interact to regulate gap junction assembly in mouse granulosa cells. *Biology of reproduction, 89*(2), 39, 31-37.

Wang, L., Liu, S., Zhang, H., Hu, S., & Wei, Y. (2015). RhoA activity increased in myocardium of arrhythmogenic cardiomyopathy patients and affected connexin 43 protein expression in HL-1 cells. *International journal of clinical and experimental medicine, 8*(8), 12906.

Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C., Briand, P., Damsky, C., et al. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *The Journal of cell biology, 137*(1), 231-245.

Weber-Hall, S. J., Phippard, D. J., Niemeyer, C. C., & Dale, T. C. (1994). Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. *Differentiation, 57*(3), 205-214.

Wei, L., Taffet, G. E., Khoury, D. S., Bo, J., Li, Y., Yatani, A., et al. (2004). Disruption of Rho signaling results in progressive atrioventricular conduction defects while ventricular function remains preserved. *The FASEB journal, 18*(7), 857-859.

Welsh, C. F. (2004). Rho GTPases as key transducers of proliferative signals in G 1 cell cycle regulation. *Breast cancer research and treatment, 84*(1), 33-42.

Whyte, J., Bergin, O., Bianchi, A., McNally, S., & Martin, F. (2009). Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. *Breast Cancer Research, 11*(5), 209.

Winterhager, E., Pielensticker, N., Freyer, J., Ghanem, A., Schrickel, J. W., Kim, J.-S., et al. (2007). Replacement of connexin43 by connexin26 in transgenic mice leads to dysfunctional reproductive organs and slowed ventricular conduction in the heart. *BMC developmental biology, 7*(1), 26.

Wiseman, B. S., Sternlicht, M. D., Lund, L. R., Alexander, C. M., Mott, J., Bissell, M. J., et al. (2003). Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *The Journal of cell biology, 162*(6), 1123-1133.

Woodward, T., Mienaltowski, A., Modi, R., Bennett, J., & Haslam, S. (2001). Fibronectin and the α5β1 integrin are under developmental and ovarian steroid regulation in the normal mouse mammary gland. *Endocrinology, 142*(7), 3214-3222.

Wu, X., Li, S., Chrostek‐Grashoff, A., Czuchra, A., Meyer, H., Yurchenco, P. D., et al. (2007). Cdc42 is crucial for the establishment of epithelial polarity during early mammalian development. *Developmental Dynamics, 236*(10), 2767-2778.

Wyckoff, J. B., Pinner, S. E., Gschmeissner, S., Condeelis, J. S., & Sahai, E. (2006). ROCK-and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. *Current Biology, 16*(15), 1515-1523.

Xu, R., Boudreau, A., & Bissell, M. J. (2009). Tissue architecture and function: dynamic reciprocity via extra-and intra-cellular matrices. *Cancer and metastasis reviews, 28*(1-2), 167-176.

Yagi, S., Matsuda, M., & Kiyokawa, E. (2012). Suppression of Rac1 activity at the apical membrane of MDCK cells is essential for cyst structure maintenance. *EMBO reports, 13*(3), 237-243.

Yeh, E. S., Williams, C. J., Williams, C. B., Bonilla, I. V., Klauber-DeMore, N., & Phillips, S. L. (2017). Dysregulated connexin 43 in HER2-positive drug resistant breast cancer cells enhances proliferation and migration. *Oncotarget, 8*(65), 109358.

Yip, S.-C., El-Sibai, M., Coniglio, S. J., Mouneimne, G., Eddy, R. J., Drees, B. E., et al. (2007). The distinct roles of Ras and Rac in PI 3-kinase-dependent protrusion during EGF-stimulated cell migration. *Journal of cell science, 120*(17), 3138-3146.

Yu, Q. C., Verheyen, E. M., & Zeng, Y. A. (2016). Mammary development and breast cancer: a Wnt perspective. *Cancers, 8*(7), 65.

Yu, S. C., Xiao, H. L., Jiang, X. F., Wang, Q. L., Li, Y., Yang, X. J., et al. (2012). Connexin 43 Reverses Malignant Phenotypes of Glioma Stem Cells by Modulating E‐Cadherin. *Stem Cells, 30*(2), 108-120.

Zaman, M. H., Trapani, L. M., Sieminski, A. L., MacKellar, D., Gong, H., Kamm, R. D., et al. (2006). Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *Proceedings of the National Academy of Sciences, 103*(29), 10889-10894.

Zardawi, S. J., O'Toole, S. A., Sutherland, R. L., & Musgrove, E. A. (2009). Dysregulation of Hedgehog, Wnt and Notch signalling pathways in breast cancer. *Histology and histopathology, 24*(3), 385-398.

Zhai, Y., Wu, R., Schwartz, D. R., Darrah, D., Reed, H., Kolligs, F. T., et al. (2002). Role of β-catenin/T-cell factor-regulated genes in ovarian endometrioid adenocarcinomas. *The American journal of pathology, 160*(4), 1229-1238.

Zhan, L., Rosenberg, A., Bergami, K. C., Yu, M., Xuan, Z., Jaffe, A. B., et al. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell, 135*(5), 865-878.

Zhang, J., Yang, G.-m., Zhu, Y., Peng, X.-y., Liu, L.-m., & Li, T. (2015). Bradykinin induces vascular contraction after hemorrhagic shock in rats. *journal of surgical research, 193*(1), 334-343.

Zhao, X., Lu, L., Pokhriyal, N., Ma, H., Duan, L., Lin, S., et al. (2009). Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. *Cancer research, 69*(2), 483-491.

Zhu, J., Xiong, G., Trinkle, C., & Xu, R. (2014). Integrated extracellular matrix signaling in mammary gland development and breast cancer progression. *Histology and histopathology, 29*(9), 1083.

Zuo, Y., Oh, W., Ulu, A., & Frost, J. A. (2016). Minireview: Mouse Models of Rho GTPase Function in Mammary Gland Development, Tumorigenesis, and Metastasis. *Molecular Endocrinology, 30*(3), 278-289.