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CONNEXIN 43 LOSS INDUCES EARLY SIGNS OF NEOPLASIA IN NORMAL MAMMARY EPITHELIUM

by

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AN ABSTRACT OF THE DISSERTATION OF

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Being one of the very few organs that continue to develop and differentiate late during the lifespan of an individual, the mammary gland is a good model to study how changes in the interaction between cells and their environment, as well as modulations in tissue architecture, might lead to tumor development. The differentiation of the mammary epithelium encompasses the attachment of epithelial cells to the basement membrane (a specialized form of extracellular matrix), thus creating the basal pole and the formation of the lumen apically by sealing cell-cell contacts with tight junctions, which overall defines the basoapical polarity axis. Perturbations in mammary epithelial cell adhesion, communication and polarity could bring about one of the most common types of cancers in women worldwide, breast cancer.

Normal differentiation at the tissue level requires establishment of a specific architecture, which is partly accomplished through cell junctions and polarity. The hallmark of breast cancer is the disruption of cellular communication between mammary epithelial cells. As such, it is essential to understand the stepwise mechanism by which cellular architecture and communication is lost prior to cancer initiation. Our laboratory has long been interested in understanding the role of connexin 43 (Cx43) as a major gap junction (GJ) protein that mediates optimal cell-cell communication and differentiation events in the mammary gland. Cx43 has been previously reported to be exclusively localized to myoepithelial cells in the mammary gland, however, we presented immunohistochemical evidence showing the localization of Cx43 at the apical membrane of luminal epithelial cells of normal breast tissue structures (acini). In order to understand the role of Cx43 in breast epithelial differentiation, a three-dimensional cell culture system that promotes the differentiation of HMT-3522 S1 (S1) non-tumorigenic breast epithelial cells was used. Our results suggested that Cx43 is the only Cx expressed in S1 cells and was abundantly found at the apical membrane of acini, consistent with our observations in human archival tissue sections. Cx43-mediated gap junction intercellular communication (GJIC) in acinar differentiation was assessed by microinjection experiments using Lucifer yellow; S1 cells have functional GJIC during and upon completion of acinar morphogenesis. Blocking GJIC with 18-α-glycyrrhetinic acid and silencing Cx43 perturbed acinar differentiation as shown by disruption of proper acinar morphology and alteration of apical polarity (i.e., the redistribution of apical polarity markers, including tight junction proteins, away from the apical cellular pole). Furthermore; blocking GJs primed cells to enter the cell cycle when exposed to nuclear architecture disruptive events or when induced with Insulin Growth Factor. In order to

understand the exact mechanism of how Cx43 regulates differentiation and apical polarity formation in the mammary epithelium, we stably silenced Cx43 using a Cx43 specific shRNA and a nonspecific (NSS) shRNA in S1 cells. We have previously demonstrated a Cx43 context dependent tumor-suppressive role mediated partially by a GJ complex assembly that sequesters β-, α-catenin and ZO-2 proteins at the cell membrane of breast epithelial tumor cell lines. shRNA S1 cells formed significantly larger acini sizes compared to control group. Moreover, silencing Cx43 disrupted epithelial polarity by down-regulating claudin-1, a TJ protein, and mislocalizing both ZO-1, a marker of apical polarity, and β-catenin, a binding partner of Cx43 and a key protein in proliferation pathways. Furthermore, Cx43 silencing induced a mislocalization of Scrib protein, a key regulator of apical polarity and a tumor suppressor, with no changes in its expression levels. In addition, we have unraveled a critical role for Cx43-mediated GJIC in the regulation of the orientation of the mitotic spindle prior to cell division, via a PI3K dependent pathway, which is essential for the maintenance of monolayered acini. We propose that the control of apical polarity-mediated quiescence and mitotic spindle orientation may contribute to the tumor suppressive role of Cx43.

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ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AGA	18α-glycyrrhetinic acid
Akt	Protein Kinase B
aPKC	Atypical protein kinase C
ATP	Adenosine Tri-Phosphate
BM	Basement membrane
BrdU	Bromodeoxyuridine
Ca^{2+}	Calcium ion
CDC42	Cell division control protein 42
CL	Cytoplasmic loop
CRB	Crumbs complex
CT	Carboxy Terminal
cAMP	Cyclic AMP
Cx	Connexin
Cx43	Connexin 43
Cx26	Connexin 26
Cx30	Connexin 30
Cx32	Connexin 32
DAPI	4’,6-diamino-2-phenylindole
DLG	Disc large homologue
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EL	Extracellular loop

EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
ER	Estrogen receptor
EV	Empty vector
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GJ	Gap junctions
GJC	Gap junction communication
GJIC	Gap junction intercellular communication
HER2	Human epidermal growth factor receptor 2
HME	Human mammary epithelial
HMT-3522	Human mammary tumor progression series 3522
IGF-1	IGF Insulin-like Growth Factor
IgG	Immunoglobulin G
IP3	Inositol 1,4,5-trisphosphate
JAM	Junctional adhesion molecule
KDA	Kilo dalton
LAMP-2	Lysosome-associated membrane protein 2
LGL	Lethal (2) giant larvae homologue
LY	Lucifer yellow CH
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MMP	Matrix metalloproteinases
MRNA	Messenger RNA
MSO	Mitotic Spindle Orientation
NB	Neurobiotin
NSS	Nonspecific sequence
NT	Amino terminal
NUMA	Nuclear organizing mitotic apparatus protein
NOG	NOD/Shi-scid/IL-2Rγnull
P-AKT	Phosphorylated Akt

PAR	Parititioning defective
PATJ	PALS1-associated tight junction protein
PBS	Phosphate buffer saline
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein Kinase C
PQ	Substituted quinolines
PR	Progesterone receptor
RD	Rhodamine-B isothiocyanate-Dextran
SA-HRP	Streptavidin-horse radish peroxidase
shRNA	Short hairpin RNA
S1	HMT-3522 S1
TBS	Tris-buffered saline
TDLU	Terminal ductal lobular unit
TJ	Tight junction
TM	Transmembrane domain
TNT	Tunneling nanotubes
Vj	Transjunctional voltage
Vm	Transmembrane voltage
WAP	Whey acidic protein
ZO	Zonula occludens
ZO-1	Zonula occluden-1

CHAPTER I

INTRODUCTION

The lifetime risk for a women to develop breast tumors is increasing globally (Ferlay et al. 2008). Based on current estimates, it is reported that one out of eight women born would be diagnosed with breast cancer at some time during her life. It has become clear that family history cannot explain the huge percentage of breast cancers that develop with no genetic predisposition (Teegarden et al. 2012). Accumulating evidence supports an immense role of tissue architecture in the regulation of normal and cancerous tissue behaviors of the mammary epithelium. As the mammary gland is responsible for milk production where it continues to develop during the lifetime of a female, it is considered a distinctive feature of mammals (Maller et al. 2010). The extensive remodeling that this organ undergoes in response to microenvironmental cues makes it a good model to study the interactions between cells and their environment. Given its dynamic development and differentiation, it illustrates the importance of cell-cell and cell-extracellular matrix (ECM) interactions that are characterized by a reciprocal exchange of mechanical and biochemical stimuli, which when altered might lead to tumor development (Hansen and Bissell 2000; Lelièvre 2009; Talhouk 2012). Importantly, the proper formation of cell junction complexes that allow adhesion and communication contributes significantly to the normalcy of the epithelium as it determines the architecture of the mammary epithelium by regulating proper cellular differentiation. Epithelial differentiation requires interaction between adjacent cells and

their microenvironment via multiprotein junction complexes. Cell junctions mediate the exchange of ions and metabolites between adjacent epithelial cells. They are asymmetrically organized creating a delicate architecture of the tissue with a basoapical axis that dictates epithelial polarity and is disrupted in early stages of tumor initiation. Epithelial cell polarity refers collectively to the asymmetrical distribution of cell junctions and polarity proteins. It is central to the coordinated behavior of epithelial cells within the tissue's two-dimensional sheet.

Polarity and cell junction proteins are in continuous interplay whereby they determine the localization of one another. Both protein complexes have been recognized to act as signaling hubs that regulate signaling pathways involved in the normal and cancer phenotypes of epithelia. The asymmetric organization of epithelial cells has been widely correlated with cell-cell junctions like tight junction, adherens junction and desmosomes (Nelson 2003); however, the role of gap junctions which mediate cell-cell communication has not been well characterized (Bazzoun et al. 2013). Therefore, research efforts to decipher the mechanisms through which gap junctions regulate essential features of mammary tissue homeostasis is the focus of this work.

In this chapter, I elaborate on the normal and cancer mammary gland development and introduce the role of cell junctions, in particular gap junctions, and polarity proteins in defining a fully differentiated architecture of the mammary epithelium. I also discuss the effects of disrupting these protein complexes on the development of a cancer phenotype in the mammary gland.

A. Normal Mammary Gland Development and Breast Cancer

The murine mammary gland shares functional and structural similarities with the human mammary gland. As such, the mouse has been considered to be the primary animal model to investigate the human mammary gland development. Although it is simple in its form, the mammary gland requires an intricate interplay between cell-cell and cell-microenvironment interactions to govern its proper development and function (Hansen and Bissell 2000). The breast undergoes extensive remodeling with the formation of ducts and glandular structures during puberty, pregnancy and lactation, and regression during involution following lactation. Branched structures in the murine mammary gland start developing during late embryonic developmental stages. This is followed by the expansion of the ductal tree within the fat pad in response to circulating ovarian hormones after three to four weeks of age, and ends around ten weeks of age (Robinson et al. 1999; Fata et al. 1999). During branching morphogenesis, the terminal end buds of the primary duct proliferate and extend towards the fat pad via a process of lateral side and terminal end bud branching. Beyond ten weeks of age, and under the influence of the estrous cycle, alveolar differentiation becomes the dominant morphological event. More lateral branching is initiated during pregnancy to form several lobules within the stroma and connect the milk-producing alveoli to the primary collecting ducts (Fig. 1A) (Ferguson et al. 1992; Robinson et al. 2000). The lobules are composed of terminal ductal lobular units (TDLUs). Each TDLU encompasses a terminal (distal) duct that leads to glandular structures called acini lined by milk producing luminal cells (Fig. 1B,C). The TDLUs expand in all directions during the development of the mammary gland, in particular during pregnancy and lactation. They are separated from the surrounding stroma by a basement membrane (BM). The stroma is composed of the ECM and an array of cell types such as fibroblasts, macrophages,

adipocytes, endothelial cells and inflammatory cells that secrete soluble factors and ECM components contributing to the homeostasis of the mammary gland. There are two main types of epithelia in the mammary gland: the luminal epithelium that forms ducts and the secretory alveoli, and the basal epithelium that consists essentially of myoepithelial cells (Runswick et al. 2001). The luminal cells are cuboidal-shaped cells that form a continuous layer lining the ducts with oval nuclei oriented perpendicularly to the lumen. On the other hand, myoepithelial cells are spindle-shaped and express α-smooth muscle actin and myosin proteins which endow these cells with their contractile capability. These cells form a continuous layer parallel to the long axis of the duct; whereas they form a discontinuous basket-like network around the acini in the lobules enabling cell-BM contacts which are crucial for normal development (Gudjonsson et al. 2005; Fata et al. 2004). During ductal elongation and lobulogenesis, myoepithelial cells produce BM components including collagen IV and laminins-111 and -332, hence providing the signaling molecules necessary to establish and maintain the newly formed epithelial structures (Toper and Freeman 1980; Gusterson et al. 1982). For example, laminin-111 and β1-integrin signaling was shown to regulate the expression of the milk protein β-casein (Streuli et al. 1991). Furthermore, myoepithelial cells secrete growth factors and matrix metalloproteinases (MMPs) like MMP2 and MMP3 necessary for tissue morphogenesis during development (Lu et al. 2012).

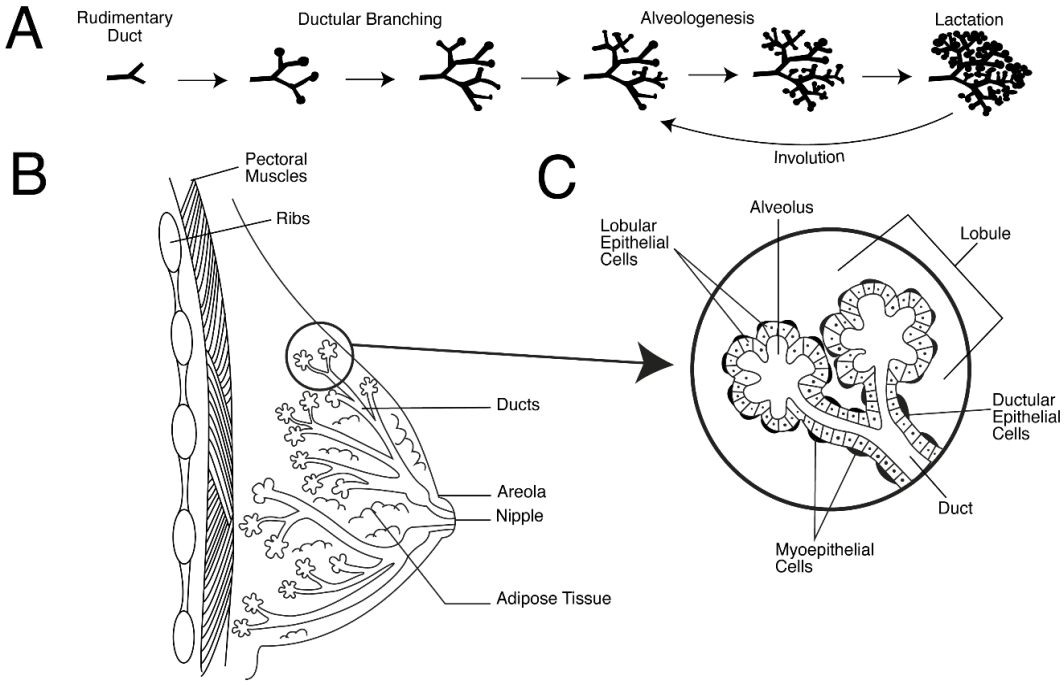

Figure 1. A schematic diagram of the different developmental stages and anatomy of the mammary gland.

A. Throughout the lifetime of a female, the breast undergoes dramatic remodeling marked by ductal growth and formation of glandular structures. During fetal development, a rudimentary structure forms and remains quiescent until puberty. Ductal elongation is initiated at puberty via continuous proliferation and migration of epithelial cells. Alveologenesis is induced upon pregnancy during which lobules and milk producing luminal epithelial cells necessary for lactation are formed. At the end of lactation, the mammary epithelium undergoes extensive apoptosis resulting in involution marked by the regression of the mammary gland back almost to the pre-pregnancy state. B. The mature female breast is composed of four main structures: lobules; milk ducts; adipose tissue and connective tissue. The external characteristic features of the breast include a nipple surrounded by a pigmented areola. Supporting the breasts are ribs that underlie the pectoral muscles. The adult mammary epithelium forms a ductal network starting at the nipple and branching into lobules. C. The lobules are composed of terminal end ducts ending in glandular structures called acini or alveoli (secreting acini). These structures are lined by luminal epithelial cells, milk secreting cells, and supported basally by myoepithelial cells that are responsible for the contractility of the epithelium (Bazzoun et al. 2014, in press).

The differentiation process that accompanies the development of the mammary epithelium incorporates complex changes in the epithelial architecture necessary to maintain the harmonious evolution of the epithelium. The disruption of this process might ultimately lead to breast cancer development. Breast cancer is a complex heterogeneous disease influenced by both genetic and environmental factors and that is tremendously variable with regards to its molecular and clinical features. It is the most common malignant cancer diagnosed among women (Jemal et al. 2011; Desantis et al. 2011). Despite being first recognized as a disease of the developed world, 50% of breast cancer cases occur in less developed countries where, according to the World Health Organization, the majority (69%) of all breast cancer deaths occurs in developing countries (Boyle and Levin 2008). Breast cancer incidence rates vary greatly worldwide, ranging from 89.7 per 100,000 women in Western Europe to 19.3 per 100,000 women in Eastern Africa (Ferlay et al. 2013). It is the second leading cause of cancer related deaths in females globally and, in the majority of the cases (more than 90%), cancer-related deaths are not due to the primary tumor itself rather the cancer metastases at distant sites. As a result of early diagnosis, improved surgical procedures and implementation of adjuvant therapies, there is decreasing trend of the prevalence of the aggressive cancers in white women compared to African American (Jones 2008).

In general, breast cancer is classified according to receptor status and molecular subtypes (Peppercorn et al. 2008). There are at least five molecular subtypes: luminal A (Estrogen receptor (ER)+ and/or progesterone receptor (PR)+, Human epidermal growth factor receptor 2 (HER2)-), luminal B (ER+ and/or PR+, HER2+), triple negative (TN) basal-like (ER-, PR-, HER2-), HER2 type (ER-, PR-, HER2+), and claudin-low (Sotiriou et al. 2003; Perou and Borresen-Dale 2011; Sorlie et al. 2011).

While the differences in the cell types at the origin of the different subtypes of breast cancer are still debated, it is thought that these cancers may be initiated from different stem/progenitor cells and possess distinct genetic and epigenetic landscapes (Holm et al. 2010; Polyak 2007). Despite this heterogeneity, pathologists have agreed on certain histological observations that mark this disease. Loss of polarity illustrated in the disruption of the bilayered epithelial architecture with piling-up of cells towards the lumen is among the earliest changes observed in breast tissues from women with increased breast cancer risk (Van de Vijver and Peterse 2003). Given the high incidence and mortality rates associated with breast cancer, a precise understanding of the molecular pathways of mammary gland differentiation is needed. Essentially, differentiation of the mammary epithelium relies on the tight regulation of cell–cell junction complexes and their link with polarity complexes that are in turn involved in the control of the asymmetrical organization of these cell-cell junction complexes.

B. Cell Junctions

The evolution of multicellular life involves the capacity of each cell to sense the state of its directly contacting neighbors. Sensing the cellular neighborhood requires the formation of junction complexes at cell–cell contacts which mediate adhesion of cells to one another and enables communication in an orderly manner. This contributes to a coordinated behavior of cells within the epithelium which mediates its normal architecture. Cell-cell junctions are classified into tight junctions (TJs), adherens junctions (AJs), desmosomes, gap junctions (GJs) and the recently described tunneling nanotubes (TNT) (Fig. 2). These junctions are often represented in text books as distinct protein entities that occupy fixed locations within the cell membrane, however; they

share protein components and cytoplasmic partners that are in continuous interaction. In this section, I will elaborate on GJs as it is the major focus of my PhD work and to a lesser extent TJs, AJs, desmosomes and TNTs.

TJs are areas of close contacts where intramembrane strands are visualized between neighboring cells (Furuse 2009). TJs act as barriers that regulate paracellular diffusion based on size and charge and restrict lipid diffusion between apical and basolateral domains (Balda and Matter 2008; Anderson and Van Itallie 2009). They are major contributors to tissue polarity as they mark the apical side of membranes. Seven TJs transmembrane proteins have been identified. They encompass transmembrane occludins, claudins, tricellulin, junctional adhesion molecules (JAMs), CRB-3, Zona Occludens (ZO) family members, and blood vessel/epicardial substance (Bves) (Chiba et al. 2008; Wang and Margolis 2007; Brennan et al. 2010). The extracellular domains of occludins regulate the paracellular diffusion of small hydrophilic molecules between cells and target occludins to TJs at the cell membrane. On the other hand, claudins dictate TJ gate function where changes in their expression were found to alter TJ conductivity (Krause et al. 2008). They also regulate occludins recruitment to TJs (Martin and Jiang 2009). The TJ cytoplasmic plaque is composed of multiple scaffold proteins among which is ZO-1. It is the first TJ protein identified, suggested to be a scaffold protein to which transmembrane and cytoplasmic proteins bind and a common binding partner of TJs, AJs and GJs proteins (Utepbergenov et al. 2006; Giepmans 2004). Several TJ proteins have dual localization at TJs and in the nucleus where they have been linked to cell proliferation, differentiation and gene expression. For example, JAM proteins act as regulators of epithelial cell morphology by enhancing β1-integrin expression through controlling Rap1 GTPase activity (Mandell et al. 2005). As such, it

contributes to cell attachment to the basement membrane. By regulating TGF-β type I receptor localization, occludins induce the TGF-β-dependent TJ disassembly during epithelial-to-mesenchymal transition, a phenomenon in which cells acquire mesenchymal features enabling them to detach from the epithelium during cancer metastasis or normal tissue development (Barrios-Rodiles et al. 2005). Interestingly, TJ proteins modulation is essential for cancer progression. The levels of expression of occludins and claudin-1, -4 and -6 proteins were reported to decrease in breast cancer cell lines while claudin-1 over-expression enhanced apoptosis in tumor nodules of MDA-MB-361 breast cancer cells produced in three-dimensional (3D) cell culture (Osanai et al. 2007; Hoevel et al. 2004).

AJs mediate intercellular adhesion. They are comprised of transmembrane proteins including the classic cadherins and nectins. Cadherins, namely E-cadherin, and nectins were reported to associate with p120-catenin and afadin, respectively (Takai et al. 2008). Classical cadherins such as E-cadherin, N-cadherin and P-cadherin permit firm cellular adhesion and anchorage of cytoskeletal intermediate filaments to the membrane (Lanigan et al. 2009). E-cadherin is a negative regulator of Wnt signaling pathway that is implicated in cell proliferation, differentiation, gene transcription and cell adhesion. By binding to E-cadherin, β-catenin is recruited to the cell membrane away from the nucleus, thus preventing its pro-proliferative potential (Wijnhoven et al. 2000). In the mammary ducts, when E-cadherin, expressed in the luminal epithelial cells, is blocked, the growth of the epithelial tissue is inhibited by interfering with downstream signaling pathways initiated at the AJ complex (Lanigan et al. 2009). Cadherins and/or their associated proteins are reported to be altered in cancer where the down-regulation of E-cadherin is correlated with the progression of breast cancer

(Cowing et al. 2005). Moreover, levels of β-catenin are usually elevated in the nuclei of tumor cells and associated with the activation of genes involved in proliferation and cell cycle progression, both features necessary for tumor development (Hatsell et al. 2003; Talhouk et al. 2013).

In addition to cadherins, desmosomal junctions contain other transmembrane proteins of the desmocollin and desmoglein subfamilies (Garrod and Chidgey 2008). Desmosomal proteins recruit intermediate filaments to the sites of their localization through binding to other proteins such as plakoglobin and plakophilins, family of catenin proteins (Nollet et al. 2000). Although desmosomes are critical for maintaining stable cell–cell adhesion, emerging evidence indicates that they are also dynamic structures that contribute to other cellular processes. It has been revealed that the altered expression of desmoglein3 in transgenic mice resulted in abnormal differentiation and epidermal hyperproliferation (Merritt et al. 2002). On the other hand, its expression in the upper layers of the epidermis induced a reduction in epidermal barrier function and early postnatal lethality due to excessive water loss (Elias et al. 2001). Furthermore; desmoplakin was significantly down-regulated in breast cancer metastases compared to the primary tumor, where it is correlated with increased proliferation and tumor size (Knudsen and Wheelock 2005). Desmosomal adhesion was enhanced in response to increased estrogen levels making it an interesting junction that requires further investigation in mammary gland differentiation and cancer development (Maynadier et al. 2012).

The transfer of information between cells in multicellular organisms seems to be essential for their evolution. The invention of direct pathways of communication in the form of communicative structures, such as the GJs, is therefore not surprising. GJs

are located beneath the TJs at cell-cell contacts and are equipped with molecular features that enable direct exchange of signaling molecules. GJs enable various functions such as rapid transmission of action potentials and diffusion of metabolites, nutrients, second messengers below 1200 Da in size such as 1,4,5-inositol-trisphosphate (IP3), calcium ions, and cyclic nucleotides, hence participating in the modulation of gene transcription, proliferation and apoptosis (Guttman and Finlay 2009). GJ channels are formed upon the pairing of two apposing connexons, which are composed of six transmembrane proteins called connexins (Cxs). Functional GJs were reported in the human non-neoplastic mammary luminal epithelial cells (Lelièvre, unpublished data) and murine mammary gland (Talhouk et al. 2005, 2008). The spatial and temporal expression of Cxs and their extensive contribution to the mammary gland development have been extensively studied (reviewed by El-Sabban et al. 2003; McLachlan et al. 2007). Cxs are no longer viewed from their structural perspective as performing a channel role rather as signaling nodes that impact both normal and cancer cell functions. Their regulation via translational and post-translational mechanisms and their interaction with cytoskeletal elements, junctional proteins and polarity proteins make them signaling nodes affecting both normal and cancer cell functions (Bazzoun et al. 2013). The role of GJs in the normal development of the mammary gland and in breast cancer will be detailed in later sections. Both GJs and TNTs mediate the transfer of intracellular molecules between the cytoplasms of neighboring cells. However, as opposed to GJs, TNTs facilitate and allow the exchange of small molecules, vesicles and organelles. They were reported to be expressed in bovine and human mammary epithelial cells, and, in particular, mediate the interaction between breast cancer cells and human mesenchymal stromal cells to promote cancer growth and progression

(Pietila et al. 2013). Given that TNTs are, relatively, recently identified cell junctions, their role in normal function and cancer development of different tissues is still poorly understood.

Cell junctions appear as separate entities involved in the maintenance of tissue morphology and homeostasis; nevertheless, they exhibit overlapping localization and interactions among each other. For example, reports have indicated that several growth factors that regulate the function of GJs are also associated with the regulation of TJs permeability. Furthermore, Cxs co-immunoprecipitate with core TJ and AJ proteins such as, claudins and occludins and N-cadherin (Nagasawa et al. 2006; Gipemans 2004). Cell junctions also share a common pool of downstream effectors and signaling partners that in turn integrate signals transduced by these junctions towards the nucleus to govern tissue differentiation. For example, GJs and AJs share a common downstream effector, β-catenin, which binds to both Cxs and cadherins (Xu et al. 2006; Ai et al. 2000). This close link between the different types of junctions reveals the close interaction required for their proper formation and function as will be discussed in later sections of this chapter. Cell junctions not only rely on interactions among each other to secure proper function, but also on their association with polarity proteins that are key players in epithelial differentiation.

C. Polarity Proteins

Epithelial cell polarity is essential for tissue function where it has been traditionally defined as “asymmetry” within the cells and tissues. Asymmetry not only refers to the differential positioning of organelles and membrane domains, also known as apico-basolateral polarity, along the basoapical axis, but also to the organization of

cells in the tissue plane (Simons and Mlodzik 2008). Apical-basolateral polarity is characterized by the distinct existence of apical and basolateral plasma domains which are differentially composed of various proteins and lipids. Most epithelia are formed of a layer of dividing cells that have their basal domain attached to the underlying BM. The apical membrane faces the luminal space and is distinguished by a set of proteins that mark the apical pole. Apico-basolateral polarity contributes to the attainment of cell shape and directional transport of molecules and vesicles (Bornens 2008). The mechanisms that support the establishment of epithelial cell polarity are not fully elucidated and variable across model organism and epithelia. Polarity formation is greatly dependent on cell junctions. It has been widely reported that two intercellular adhesion complexes, AJs and TJs, form part of the apical junctional complex and regulate the formation and the maintenance of apical and basolateral domains (Perez-Moreno et al. 2003; Tamura et al. 2008). GJs, on the other hand, have not been reported to contribute to the regulation of apico-basolateral polarity, and this regulation is a main focus of my Ph.D. project.

At the origin of the formation of polarity lies a delicately regulated interplay between AJs, TJs and cell polarity complexes. These polarity complexes have been originally identified in yeast, worms and flies where they were found to be highly evolutionarily conserved (Martin-Belmonte and Mostov 2008). The Partitioning defective (PAR) complex that comprises PAR3, PAR6, atypical protein kinase C (aPKC) and cell division control protein 42 (CDC42). This complex promotes the establishment of the apical-basolateral membrane border. The crumbs (CRB) complex which constitutes the transmembrane protein CRB and its associated cytoplasmic proteins PALS1 and PALS1-associated tight junction protein (PATJ) are collectively

required to establish the apical membrane. The third complex is the scribble complex that includes scribble homologue (SCRIB), lethal (2) giant larvae homologue (LGL) and discs large homologue (DLG) proteins which mark the basolateral plasma domain (Fig. 2). The Par and Crumbs complexes are localized apically and together regulate apical domain maintenance. They act in a mutually antagonistic fashion with the Scribble complex which is located basolaterally and is necessary for the maintenance of the basolateral membrane and basal protein restriction (Ellenbroek et al. 2012).

Initially, nectin–afadin adhesion complexes associate with PAR3 (Ooshio et al. 2007; Sakisaka et al. 2007) which is followed by E-cadherin recruitment with JAMA to the cell cortex (Adams et al. 1998; Vasioukhin et al. 2000). This association gives rise to adhesive homophilic interactions mediated by a mixture of AJ and TJs proteins (Tsukita et al. 2009; Sakisaka et al. 2007). These primordial adhesions, via their anchoring to the actin cytoskeleton and through their RHO-GTPase activity, extend the border of adhesion along the basolateral domain (Mege et al. 2006; Kovacs and Yap 2008). This extension is proceeded by the separation of AJ associated proteins from TJ associated proteins to ultimately form the AJ belt-like structures and mature TJs. For this to happen, PAR3 has to be excluded via aPKC that phosphorylates PAR3–PAR6. This phosphorylation event consequently excludes PAR3 from the subapical sites of the cell allowing the separation of the apical and subapical domains (McCaffrey and Macara 2009; Horikoshi et al. 2009). In addition, the SCRIB–LGL–DLG complex localizes with AJs and promotes basolateral membrane identity (Navarro et al. 2005). LGL phosphorylation by aPKC inhibits the interaction of LGL with PAR3 to allow the separation of the lateral domains and subapical domains (Yamanaka et al. 2003; Benton and Johnston 2003; Yamanaka and Ohno 2008). The continued expression and activity

of cell junctions and polarity complexes are vital for polarized cells to remain firmly associated with the epithelium and to synchronize signalling pathways that regulate differentiation and proliferation. As such, the loss of cell polarity complexes leads to intercellular adhesion breakage and the acquisition of cancer related behavior.

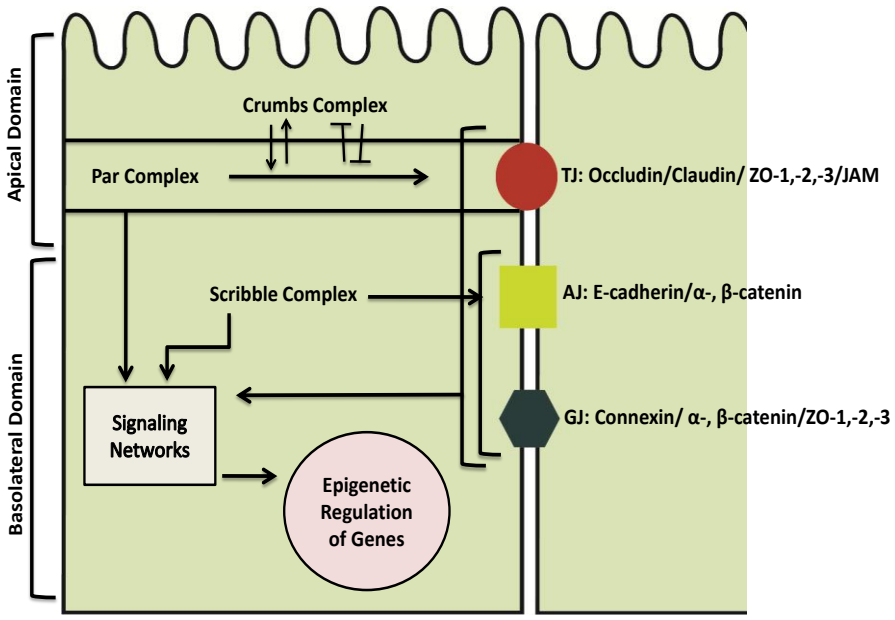

Figure 2. Epithelial cell junctions and polarity.

There is an asymmetric subcellular localization of polarity protein complexes (Crumbs, Par and Scribble) and of cell junctions (TJs, AJs and GJs) along the basoapical axis of normally polarized mammary epithelial cells. While the apical domain is specified by the Crumbs and the Par complexes, the basolateral domain is defined by Scribble proteins. In addition, cell junction complexes are made by transmembrane proteins (occludin, claudin and JAMs for TJ; cadherins for AJ; connexins for GJ) associated with various cytoplasmic partners (ZO proteins, catenins and other (Bazzoun et al. 2013).

D. Gap Junctions

1. Connexins: Gene, Protein and Biogenesis

Given the dynamic nature of the GJs throughout the developmental stages of tissues, it is worth elaborating on the nature of its building blocks, Cxs. The family of

Cxs consists of twenty-one genes in the human genome that share a similar structure consisting of a 5'-untranslated exon 1 separated from the coding exon 2 by an intron of variable length. Interestingly, Cx genes have different transcription start sites and distinct 5'-untranslated region depending on the tissue type which makes their expression tissue specific (Pfenniger et al. 2010). The Cx protein is composed of four α-helical transmembrane domains (TM1–TM4), two extracellular loops (EL1 and EL2), a cytoplasmic loop (CL) between TM2 and TM3, cytoplasmic amino-terminal (NT) and carboxy-terminal (CT) domains. The two extracellular loops, which are highly conserved, contain three cysteine residues in all but one Cx isoform (Harris and Locke 2008). In addition, the four transmembrane domains are also conserved forming α-helical sheets (Sohl and Willecke 2004). On the other hand, the intracellular loop and the C-terminal region differ in length and sequence, which brings about the diverse Cxs and connexon types (Fig. 3A) (Rhett et al. 2011; Evans et al. 2006). Some Cxs are expressed in many cell types, whereas others are restricted to one or a few cell types (Haefliger et al. 2004). Cxs orient their N-termini, cytoplasmic loops, and C-termini towards the cytosol allowing for interaction with catenins, cytoskeletal proteins, and ZO proteins among others which, in turn, contribute to Cxs' and GJs' functions (Herve et al. 2007; Dbouk et al. 2009). Cx proteins are named after their molecular weights such as Cx26, Cx32, and Cx43, corresponding to molecular weights of 26 kDa, 32 kDa and 43 kDa respectively. However, their genes are categorized into 4-5 groups (α, β, γ, δ and sometimes ε) based on sequence similarity and length of the cytoplasmic domain, thus the genes are named accordingly (for instance Cx43, which is the first connexin of the α-group, is coded by *GJA1*) (Račkauskas et al. 2007).

Biosynthesis of Cx polypeptides seems to proceed like that of most other membrane proteins. Cxs use the usual translation machinery of the ribosomes attached to the endoplasmic reticulum (ER). The nascent Cx polypeptide is released to the ER lumen through the Sec61 or translocon (Dbouk et al. 2009; Segretain and Falk 2004). It is during the integration into the ER membrane that the structural arrangement of most Cxs is established (Evans et al. 2006). It was initially thought that oligomerization of Cxs into connexons occurred in the ER, yet some reports have indicated that Cxs remain as monomers in the ER and will only oligomerize while being transported from the ER to the Golgi apparatus. The resulting connexons, formed of six Cx proteins that assemble into a homomeric (identical Cx subunits) or a heteromeric (different Cx subunits) hemichannels, are subsequently transported to the plasma membrane by vesicular carriers travelling along microtubules (Koval 2006). The docked connexons either remain uncoupled or can interact with their counterparts, via their extracellular loops, in the neighboring cell and form channels collectively termed as GJs (Fig. 3B, Fig. 4) (Evans et al. 2006; Duffy et al. 2002). GJs could be either homotypic or heterotypic when two identical or different connexons dock, respectively. Heterotypic channels have different characteristics compared to the homotypic ones due to their variable conductance and permeability (Rackauskas et al. 2007). Depending on the tissue type and the Cx expressed, connexons can either function as hemichannels, enabling transmembrane signaling, or as GJ channels that provide a direct communication pathway between the cytosols of adjacent cells (Pfenniger et al. 2007). The principle signal for Cxs assembly is located within their C-termini while the selectivity signal that determines subunit compatibility is within their N-termini. These signals, which are stretches of aminoacids, differ among Cx isoforms bringing about

different surface structures of protein/protein interfaces. As such, only compatible Cxs with similarly folded motifs will interact with one another (Segretain and Falk 2004).

Posttranslational modifications of Cxs are implicated in the regulation of hemichannel oligomerization, export of the protein to the plasma membrane, hemichannel activity, GJ assembly, GJ channel gating and Cx degradation (Solan and Lampe 2010). At least eight kinases particularly protein kinase A (PKA) and protein kinase C (PKC), and three phosphatases control the phosphorylation of various Cxs (Moreno and Lau 2007). Phosphorylation occurs on either serine/threonine or tyrosine residues, yet some Cxs (e.g. Cx36) may only be posttranslationally modified under certain conditions or others (e.g., Cx26) (Urschel et al. 2006; Hervé 2005). Phosphorylation events of Cxs are often linked to changes in the permeability, conductance and gating of the junctional channels which in turn affect the assembly, proteolytic degradation and biosynthesis of Cxs. This ultimately regulate the half-life of Cxs, which is remarkably short (3-5 h) compared to that of many other integral membrane proteins (Sohl and Willecke 2004).

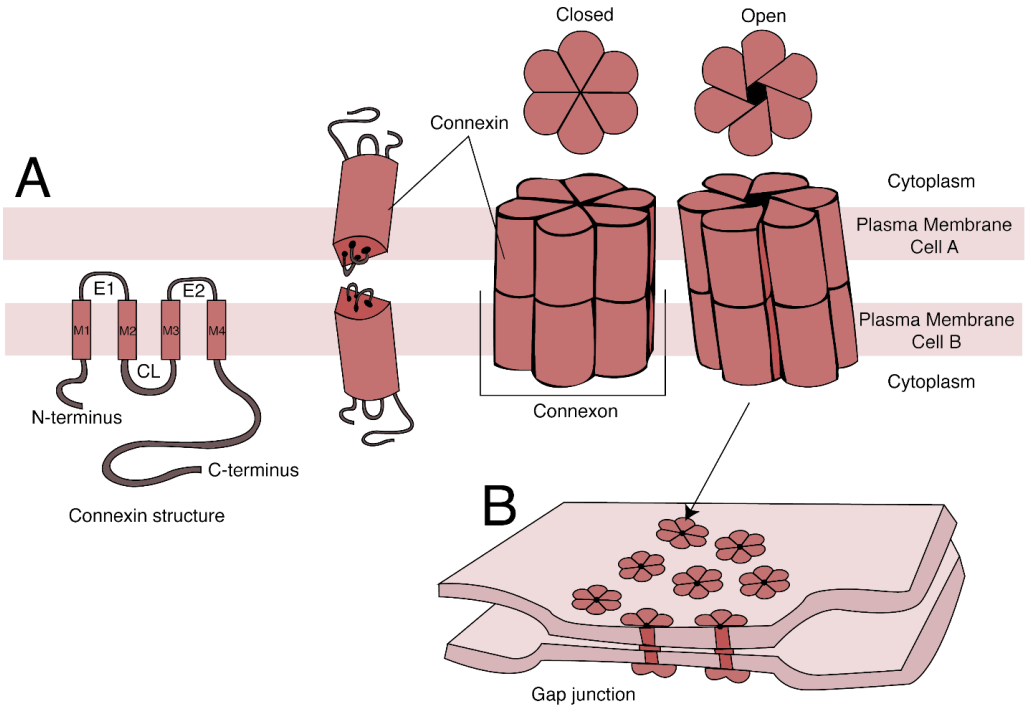

Figure 3. A schematic model illustrating Cxs assembly into GJs.

A. Cxs have their four domains (m1-m4) spanning the plasma membrane and creating two extracellular loops (E1-E2), one cytoplasmic loop (CL) with of their N- and C-termini deposited towards the cytoplasm. Upon insertion into the plasma membrane, six Cxs can position themselves into a donut-like configuration called a connexon. B. Connexons may either remain as hemichannels or dock with compatible connexons from neighboring cells to form structures collectively known as GJs (Bazzoun et al. 2014).

2. Channel: Regulation and Break Down

GJs are visualized as the close apposition of plasma membranes of adjacent cells with a uniform 2 to 3 nm intercellular space (Hervé and Derangeon 2013). Because of their mediation of the cell-to-cell diffusion of ions, metabolites and small cell-signaling molecules, GJs play fundamental roles in a wide array of physiological processes such as development, growth and proliferation, cell differentiation, neuronal signaling and hormone secretions among others (Yeager and Harris 2007; Houghton

2005; Yang et al. 2005). Junctional channel pores are indeed sufficiently wide to be permeable to a wide variety of cytoplasmic molecules, yet the channel activity of Cxs is tightly regulated mainly in response to changes in voltage, calcium concentration, and pH. GJ conductance can be modulated by transjunctional voltage (Vj), and for some Cxs, by the transmembrane voltage (Vm). The Vj gating of Cxs involve the NT, TM1, CL and CT. Each of the 21 different connexins has distinct gating properties (Verselis et al. 1994). Interestingly, studies conducted on Cx26 and Cx32 channels revealed that the cytoplasmic N-terminal domain contains charged residues that determine the response of the channel to changes in voltage. For instance, Cx26, Cx30 and Cx50 close at positive voltages, and Cx31, Cx32, Cx37, Cx40, Cx43, Cx45 and Cx57 close at negative voltages (Srinivas et al. 2006). Furthermore, Cx43 channels were reported to be relatively insensitive to changes in Vj compared to channels composed of Cx45 (Paulauskas et al. 2012). Besides voltage gating, chemical gating such as pH change has profound physiological implications on the functionality of GJs whereby the effect of alkalization or acidification on coupling differs between tissues with different Cx expression. In the case of Cx46 and Cx50, most channels will be active at pH of 7.2 with very minimal activation of Cx26 channels (Palacios-Prado et al. 2010; Eckert 2002). In contrast to Cx26 channels that close in response to an increase in pH; homotypic Cx45 and heterotypic Cx45/Cx43 GJs tend to open under such conditions (González-Nieto et al. 2008; Palacios-Prado et al. 2010). Calcium-dependent gating is another mechanism by which GJs are chemically gated. Studies have revealed that increasing intracellular Ca^{2+} uncouples GJs in a number of tissues. This is a protective mechanism that prevents cells undergoing apoptosis to damage their neighboring ones (Peracchia 2004). The proposed mechanism of the uncoupling is the interaction between

Ca^{2+} and calmodulin. Studies have reported that the expression of calmodulin mutants with increased affinity for Ca^{2+} resulted in increased Ca^{2+} sensitivity of Cx32, which has two calmodulin binding sites. When calcium levels increase, calmodulin is thought to either physically block the channel or close it through a conformational change in Cx32 (Peracchia et al. 1983; Török et al. 1997; Dodd et al. 2008). The role of Calmodulin in gating has been extended to other Cxs where it plays a role in Cx36, Cx43 and Cx45 channels gating (Burr et al. 2005; Lurtz and Louis 2002). Phosphorylation status of Cxs is another factor that regulates the function of GJ, either by modulating the efficacy of passage of certain molecules or the closing and opening the channel for the transfer of molecules (Moreno and Lau 2007). For example, the phosphorylation of Cx50 and Cx43 in lens fibers enhanced GJ permeability and stability, respectively (Liu et al. 2005; Dunn et al. 2011).

Several alterations including phosphorylation, ubiquitination, methylation SUMOylation, acetylation, glutamate γ-carboxylation and nitrosylation have been identified in Cxs (Johnstone et al. 2011). Phosphorylation and ubiquitination are well recognized mechanisms through which GJs and Cxs are degraded. Cx phosphorylation is not a standard requirement for Cxs to be targeted for degradation given that Cx26 is not phosphorylated but still appears to have a short half-life similar to that of Cx32 and Cx43 (Thomas et al. 2004). The evidence that Cxs are sensitized for degradation due to phosphorylation events is ambiguous. On the other hand, Cx ubiquitination at the GJ seems to have a major role in endocytosis and intracellular vesicle trafficking (Laird et al. 1995; Puranam et al. 1993). Among ubiquitin ligases, Nedd4, Smurf2 and TRIM21 were reported to interact with Cx43 and associated with GJ plaques (Chen et al. 2012; Fykerud et al. 2012; Leykauf et al. 2006). Cxs modification is followed by either

proteasomal or lysosomal degradation (Fig. 4). The role of the proteasome is indirect, whereby this pathway is mainly involved in degrading single unfolded polypeptides. This has been supported by a study illustrating that a short-lived protein is needed to target correctly folded Cxs for proteasomal degradation (Musil et al. 2000). In addition, treatment with proteasome inhibitors resulted in an increased activity of the Akt protein kinase, which in turn, phosphorylates Cx43 ultimately increasing it stability at the GJ (Dunn et al. 2012). On the other hand, lysosomal degradation pathway directly regulates Cx degradation before GJs are formed, during transport or after incorporation into the membrane (Laird 2006). Furthermore, degradation not only affects GJs and replenishes the plaques with newly formed channels, but also, indirectly regulates Cx stability by mediating Cx-associated proteins turnover, such as ZO-1 and others.

E. Channel-Dependent and -Independent Roles of Connexins

1. Permeability and Selectivity of Channels

Permeability of GJs is highly dependent on the Cx make-up of the channel. For example, a study has evaluated single channels permeabilities of homotypic Cx26, Cx32, Cx37, Cx40, Cx43, Cx45 and heterotypic Cx26/Cx32, Cx37/Cx43 GJ channels in *Xenopus laevis* oocytes and revealed that the permeability of heterotypic channels was determined by the permeability of the less permeable Cxs. The permeability of Cx36 channels for cationic dyes is about 10-fold higher than for anionic dyes of the same net charge and similar molecular mass (Paulauskas et al. 2012). When examining single channel permeability of GJs in cardiac cells, the ratio of single channel conductance to permeability was much higher for Cx30.2 than for Cx40, Cx43, and Cx45 channels

(Rackauskas et al. 2007). Homotypic channels composed of Cx43 have a 100-fold higher selectivity for ATP compared to channels made of Cx32; metabolites such as glutamate, glutathione, ADP and AMP displayed a 10-fold higher permeability through Cx43 channels than through Cx32 channels (Goldberg et al. 2002). These studies demonstrate that Cxs differentially regulate the intercellular transfer of natural permeants. However, the physical basis of this selectivity remains to be examined as transfer of metabolites through these channels is not simply size- or charge-dependent (Harris 2007).

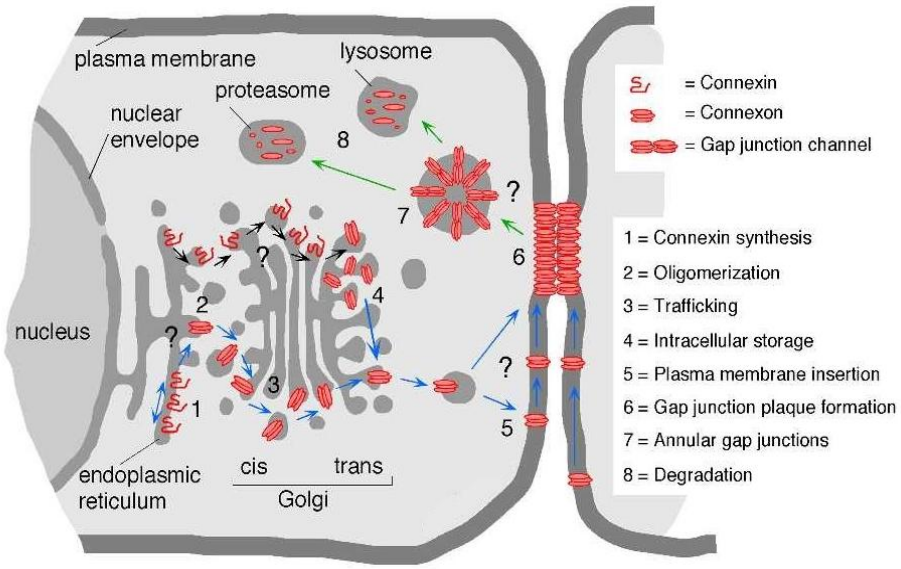

Figure 4. Schematic representation of Cx synthesis, assembly and degradation.

Cxs are synthesized on ER bound ribosomes and inserted into the ER cotranslationally. This is followed by its oligomerization that occurs between the ER and Golgi apparatus (depending on the Cx type) into connexons, which are then delivered to the membrane either directly from the ER or from the golgi apparatus. Cxs have their four transmembrane domains spanning the membrane with both its N- and C-termini deposited towards the cytoplasm. Upon insertion, connexons may remain as hemichannels or dock with compatible connexons of neighboring cells to form GJs. Collectively, GJs aggregate to form GJ plaques that enable the exchange of metabolites such as cAMP, IP3, ADP, ATP, microRNAs among others (Katakowski et al. 2010). Older gap junction fragment are degraded by internalization of a double-membrane structure called an annular junction into one of the attaching cells, where subsequent

lysosomal (not shown) or proteasomal degradation occurs (Dbouk et al. 2009; Bazzoun et al. 2014).

2. Functional Roles of the Channels

GJs provide a universal function as intercellular channels enabling the formation of communication compartments that are essential for the maintenance of tissue homeostasis. GJIC is actively involved in virtually all aspects of the cellular life starting from cell growth until cell death. They are implicated in a wide diversity of cell types where they coordinate long-distance responses initiated by mechanical, electrical or hormonal stimuli. Here we elaborate on the physiological role of GJs in major tissues.

GJs play a central role in allowing the propagation of the action potential between the cells of the myocardium. In functioning myocardium, GJs are favorably present in the region of the intercalated disks mediating an "end-to-end" coupling of the cardiomyocytes (Saffitz et al. 1995). Whereas Cx43 is the major Cx in the working myocardium of the ventricles (Verheule et al. 1997), the atria express Cx40, Cx43, and Cx45 (van der Velden et al. 1998). It has been reported that mice that are heterozygous for the Cx43 null mutation ($Cx43^{+/-}$) displayed a slow epicardial conduction in the ventricles, and a widening of the QRS complex (Thomas et al. 1998). Furthermore, mice that lack Cx43 are born with beating hearts but die shortly after birth due to obstruction of the right ventricular outflow tract (Reaume et al. 1995). In the vascular system, GJs enable the coupling of vascular smooth muscle and endothelial cells. Cx37, Cx40, Cx43 and Cx45 are the most abundant Cxs in blood vessels where they contribute to the regulation of blood pressure (Haefliger et al. 2001, 2004; Hamelin et al. 2009). Studies have shown that heterozygous Cx43 knockout mice display reduced and more

stable atherosclerotic plaques compared to homozygotes hinting on a role of Cx4 in atherosclerosis development (Kwak et al. 2003; Wong et al. 2003). On the other hand, Cx37, through regulating monocytes and macrophages recruitment, exert atheroprotective effects (Wong et al. 2006). GJs are abundantly expressed in many tissues of the female reproductive system, mainly in the smooth muscle cells of the uterus and oviduct. They are upregulated at the time of labor as it is essential for coordinating the contractile activity of the uterine smooth muscle required for fetal expulsion. GJs are also vital for the communication between the maturing oocyte and the surrounding granulosa cells for normal follicular development (Granot and Dekel 2002; Talhouk et al. 2012). Follicles from Cx43-deficient mice were found to be phenotypically abnormal; they fail to reach meiotic competency and cannot be fertilized *in vitro* (Kidder and van der Hyden 2010). In addition, mice lacking Cx37 are infertile and their follicles do not mature normally (Simon et al. 1997). GJs are also highly involved in the differentiation of the mammary gland as we will elaborate in upcoming sections of this chapter.

3. Gap Junction-Independent Roles of Connexins

In 1995, a study by Mesnil and colleagues has revealed that Cxs may have separate non-channel-related functions (Mesnil et al. 1995). Since then extensive research has been focused on the GJ-independent roles of these membrane proteins, where evidence suggests a role of Cxs in cell growth, proliferation, differentiation, injury, apoptosis, migration and inevitably tumorigenicity independent of its channel function (Jiang and Gu 2005). An accumulation of Cx-associated proteins have been

discovered, namely enzymes, adhesion molecules, cytoskeletal and signaling proteins. This association endowed Cxs with the ability to regulate a number of mechanisms involved in their channel-independent functions (Moorby and Patel 2001; Vinken et al. 2011). In this section, we provide an overview of the GJ-independent functions of Cxs in particular cell growth, differentiation, signaling and development.

Despite the evidence that GJs are involved in the regulation of cell growth, there is an explicit data showing that Cxs are able to control cell growth independent of GJs. A report by Huang et al. revealed that transfection of human glioblastoma cells with Cx43 was able to revert the transformed phenotype without changes in the activity of the GJs. In addition, Cx26 dominant negative mutants in HeLa cells enhanced their growth rate and tumorigenicity in nude mice with no effect on GJIC (Huang et al. 1998). Wild-type Cx43 inhibited the proliferation of Neuro2a cells to a similar extent when compared to Cx43 having an intact C-terminus that is unable to form functional GJs (Olbina and Eckhart 2003). These data suggest that the expression of Cxs per se, and not the functionality of GJs, correlates with a control of cell growth. Furthermore, the nonchannel forming C-terminal tail of Cx43 was found to inhibit DNA synthesis, and ultimately cell growth, via PKC phosphorylation of serine 262 (Dang et al. 2006). Not only cell growth, Cxs have demonstrated a regulatory role in cell differentiation. The overexpression of Cx45.6 but not Cx43 and Cx56 stimulated lens cell differentiation without affecting GJIC, demonstrating a GJ-independent function of Cx45.6 in lens cell differentiation. This was comparable to the effect of C-terminus of Cx45.6 which does not induce a functional GJ, further reinforcing the channel-independent role of Cx45.6 in lens cell differentiation (Gu et al. 2003; Yu and Jiang 2003). Additionally, mutants of Cx50 which are unable to form functional GJs but

retain the integrity of the protein, and in particular its C-terminus, are still able to promote lens epithelial differentiation (Jiang 2010; Shi et al. 2008).

Developmental processes such as neurogenesis is greatly influenced by Cxs. Schwann cells from Cx32 knockout mice have a mild response to mitogenic factors compared to the wild type with no change in electrical coupling between schwann cells (Freidin et al. 2009). Cx43 plays an important role in astrocytic proliferation in response to injury (Homkajorn et al. 2010), yet the inhibition of GJIC between the cells did not affect the wound closure rate. Furthermore, cardiac neural crest cells isolated from Cx43 knockout mice exhibited decreased adhesion and diminished cell directionality compared to no changes in both adhesion and migration in response to blockage of GJs. This suggests that Cx43 affects the migration of cardiac neural crest cells via its signaling functions with other proteins (Xu et al. 2006; Elias et al. 2007).

F. Crosstalk Between Gap Junctions and Protein Complexes

There is a plethora in the number and diversity in Cx-associating partners implicating tight and adherens junctional proteins, as well as protein phosphatases and kinases, in addition to cytoskeletal elements (Herve et al. 2007; El-Sabban et al. 2003; Herve et al. 2004). The C-terminus, mainly, N-terminus, cytoplasmic loop and some membrane domains of Cx protein can interact with membrane receptors, cell signaling and scaffolding proteins (Giepmans 2006).

1. Connexins and Other Junctional Proteins

The interaction of Cxs with cytoskeletal elements is necessary for the transportation of Cxs to the membrane and their rapid turnover and replenishment. Cx43

has a tubulin-binding domain through which it directly binds to α- and β-tubulin at the GJs. In addition to tubulin, Cx43 is reported to colocalize with actin in a variety of cell types and co-precipitates with α-spectrin at the gap junctional complexes (Wall et al. 2007). The interaction between GJ proteins and proteins of the AJs and TJs is a clear suggestion of the collaboration between these adhesion complexes. Both direct and indirect interactions between these junctional complexes regulate Cx and GJ lifecycle (Herve et al. 2004). A major emerging cross-talk is that between GJs and TJs. GJs are found to regulate TJs and may even regulate the expression and function of TJ proteins. Cx32 transfection in mouse hepatocytes that lack Cx32 induced TJ strands formation and enhanced occludin, claudin-1 and ZO-1 expression leading to a stronger TJ functionality (Kojima et al. 2002). In addition, occludin was reported to interact with Cx32 in immortalized mouse and cultured rat hepatocytes (Kojima et al. 2001). Both claudin-5 and occludin were shown to colocalize with Cx43 and Cx40 in porcine blood-brain barrier endothelial cells (Nagasawa et al. 2004). Moreover, Cx26 has also been reported to interact with occludin in polarized sheets of the human intestinal cell line T84 and with claudin-14 in human airway epithelial cell lines (Nusrat et al. 2000; Go et al. 2006). Cx43 was also shown to regulate the blood-testes barrier via its effects on the reassembly of TJs (Li et al. 2010). ZO-1 is highly involved in the organization and trafficking of GJs. It was shown to regulate Cx43-mediated GJ-communication in osteoblasts by altering the membrane localization of Cx43 and mediating its delivery to the GJ (Rhett et al. 2011). Furthermore, small interfering RNA knockdown of ZO-1 abolished the formation of Cx50 GJs in HeLa cells (Chai et al. 2011). Another potential role for the interaction of ZO-1 with Cx43 is to act as a scaffold for other proteins. For example, ZO-1 is essential for stabilizing GJs in myocytes as it links Cx43 at the GJs to

vinculin which anchors the actin cytoskeleton to the sarcolemma and thus preserving the myocytes integrity (Zemljic-Harpf et al. 2014).

Several studies have shown that GJ and AJ formation are intimately linked. For instance, knock-down of Cx43 in fibroblasts resulted in impaired membrane localization of AJs (Weir et al. 2006). In NIH3T3 cells, it was found that Cx43 colocalizes and co-immunoprecipitates with N-cadherin with no evidence of direct binding (Weir et al. 2006). It was also observed that blockage of Cx33 induced a mislocalization of N-cadherin (Chung et al. 1999). Cx43 interacts with β-catenin, a versatile protein with adhesive and transcriptional functions, highly involved in regulation of cell growth and proliferation. In addition, binding of E- or N-cadherins (E- or N-) to β-catenin is crucial for the inhibition of cell proliferation; thus, Cx43 and cadherins seem to regulate β-catenin signaling by sequestering it at the membrane (Gottardi et al. 2001; Kamei et al. 2003). Moreover, binding of β -catenin to Cx43 at cell–cell contact areas in cardiac myocytes was also shown to be important for down-regulation of β-catenin-dependent gene transactivation (Ai et al. 2000). Our laboratory has previously demonstrated that the functionality of GJs depends on the interaction of Cxs with their associated protein partners. We have reported that the formation of a GJ complex in heterocellular co-cultures of mouse mammary epithelial cell line SCp2, and myoepithelial like cell line SCg6, is essential for functional differentiation of the SCp2 cells and production of milk proteins. This was correlated with increased GJIC and enhanced association between Cxs and β -catenin, α-catenin, and ZO-2 (Talhouk et al. 2008). As such, the interaction of AJs and TJs proteins with Cxs greatly contributes to the functionality of GJs and consequently enables GJs to mediate their wide array of signaling roles.

2. Connexins and Polarity Proteins

There are few studies that shed light on the interaction between GJs and polarity. The interaction of Cxs with TJ core protein ZO-1 seems to contribute to the organization of GJs, either directly or indirectly, through the recruitment of partner proteins involved in various downstream signaling pathways. It has been reported that ZO-1 associates with Cx43, 36, 45, 35, 31.9, 46, 47 and 50 (Kausalya et al. 2001; Nielsen et al. 2001, 2002, 2003; Li et al. 2004a, b). Thus, it is likely to visualize that ZO-1 offers a scaffold for the localization of Cxs to GJs (Giepmans and Moolenaar 1998). In addition, GJs affect TJ proteins, markers of polarity, as shown by the up-regulation in occludin, claudin-1 and ZO-1 protein levels induced upon transfection of Cx32 into mouse hepatocytes (Kojima et al. 2002). Similarly, over-expression of Cx26 in human airway epithelial cells was associated with elevated levels of claudin-14 (Morita et al. 2004). A direct interaction between polarity proteins and Cxs was recently revealed through Drebrin, a developmentally regulated brain protein. Drebrin is an actin-binding protein involved in mediating cell polarity and maintaining the different plasma membrane domains. The interaction between Cx43 and Drebrin enhances the stability of Cx43 at the membrane since Drebrin's depletion leads to the inhibition of GJ function and Cx43 degradation in Vero cells that are derived from kidney epithelial cells (Butkevich et al. 2004). From the above, it is evident that Cxs are not solely structural proteins of the connexon at the membrane; their coordinated interactions with TJ, AJ and polarity proteins during epithelial differentiation ought to be further scrutinized.

G. Polarity in the Mammary Epithelium

In tissues where epithelial structures are arranged as one layer surrounding a lumen, such as the mammary epithelium, the establishment of polarity accompanies lumen formation. The polarized epithelium is established early in mammary development as soon as the bud emerges from the ectoderm (Jolicoeur 2005). There are two features of polarity in the mammary epithelium. The first is that of the bilayered epithelium composed of the luminal and myoepithelial (basal) layers where the two different cell types orient themselves. The second is epithelial cell polarity as in the mechanisms dictating how mammary epithelial cells (MECs) form an apical surface and consequently, lumens (Muschler and Streuli 2010). Reports have indicated that in adult tissues the spatial orientation of luminal and myoepithelial cells is greatly regulated by adhesion between both cell types. While both express desmosomal cadherins, desmoglein 2 (Dsg2) and desmocollin (Dsc2), myoepithelial cells also express Dsg3/Dsc3. The intrinsic high adhesive potential of luminal cells to one another restricts the myoepithelial cells basally. This fails to happen if Dsg3/Dsc3 is absent from the myoepithelium (Runswick et al. 2001). In addition, blocking E-cadherins selectively alter luminal cells without affecting the myoepithelium, whereas the disruption of P-cadherin disturbs the basal cell layer only (Daniel et al. 1995). Cell–ECM interactions are also central for the polarization of the mammary epithelium. The BM matrix regulates the bilayered organization because cocultures of luminal and myoepithelial cells in collagen gels form bilayers which depends on laminin-111 production by the myoepithelial cells (Gudjonsson et al. 2002).

Establishing apical–basal polarity is key for lumen formation. The apical surfaces of luminal epithelial cells are marked with transmembrane mucins, glycosylated proteins, to prevent cells to adhere at those surfaces. On the other hand, the basal cellular pole is characterized by transmembrane integrin dimers that connect cells to ECM molecules of the BM (Koukoulis et al. 1991). β1-integrins, laminin-111 and dystroglycan have central roles in establishing polarity, as known from 3D cultures of mammary and Madin-Darby canine kidney (MDCK) cells (O'Brien et al. 2001; Weir et al. 2006; Yu et al. 2005). As such, integrins through their interaction with the ECM, provide a guide within the alveoli to orient luminal cells in a way that creates fluid-filled cavities for vectorial secretion and intake of molecules, yet the signaling mechanisms through which integrins control the establishment of the apical surface remains unclear (O'Brien et al. 2002). Luminal epithelial cells are characterized by the presence of TJs at the top third of the apical cell pole opposite to the BM to seal the intercellular space and create a physical segregation between the basolateral and apical domains of the cell membrane (Plachot et al. 2009). Polarity is not only a physical feature but is also needed to direct cell movement and provide guidance for migration (Petrie et al. 2009). For example, transplantation of Par3-depleted stem cells into the mammary fat pad resulted in ductal hyperplasia, luminal filling and highly disorganized end bud structures that failed to develop normal ductal structures. Not only that, it was also found that mammary morphogenesis is highly dependent on the ability of Par3 to directly bind aPKC (McCaffrey and Macara 2009). In addition, Scrib-depleted non-neoplastic mammary epithelial MCF10A cells failed to regulate Cdc42 and attain apical polarity. MCF10A cells with no Scrib had disrupted migration due to the lack of polarization at the leading edge without affecting basal polarity (Dow et al. 2007).

Furthemore, Scrib loss from the mammary epithelium lead to the formation of ducts with diminished luminal space, typical of ductal carcinoma in situ (Zhan et al. 2008). Apico-basolateral polarity contributes to the acquisition of proper tissue architecture and spatiotemporal responses to cues that govern proper mammary epithelial functions. Thus, loss of polarity proteins is sufficient to induce a multilayered phenotype which is typical of early stages of breast cancer. It does this by prompting cells to switch to a mesenchymal phenotype and enable their migration, rearranging cells that are still within the epithelial tissue or misorienting the plane of epithelial cell division (Chatterjee and McCaffrey 2014).

H. Orientation of the Mitotic Spindle in Mammary Epithelial Differentiation

Mitosis is not only essential for the segregation of chromosomes but can also direct tissue architecture and cell fate. In general, astral microtubules position and orient the entire mitotic spindle by rotating it into the defined orientation relative to the cell axis (Giansanti et al. 2001; O'Connell and Wang 2000). As the spindle determines the plane of cytokinesis, it plays a major role in symmetric (producing identical cells) or asymmetric division (producing different cells) that is crucial for tissue morphogenesis and homeostasis (Betschinger and Knoblich 2004; Neumuller and Knoblich 2009). In most epithelia, the spindle orientation is aligned along the tissue plane, i.e., parallel to the apical and basal cell surfaces to generate daughter cells that are side by side (Fernandez-Minan et al. 2007). Alterations in this orientation could disrupt epithelial tissue architecture where daughter cells are placed on top of one another, creating vertical tissue expansion and consequently tissue hyperplasia, a premalignant change

(Pease and Tirnauer 2011) (Fig. 5A). Thus, spindle misorientation contributes to cancer development but alone is not tumorigenic (Fischer and Pontoglio 2009; Patel et al. 2008). The loss of polarity in tissues along with spindle misorientation further disrupts the orientation of cell division creating a feedback loop between spindle misorientation and altered cell polarity in premalignant tissues (Pease and Tirnauer 2011). In several epithelial systems, including the mammary epithelium, the polarity machinery directs cell division whereby the depletion of Par6, Par3, Cdc42, aPKC or Dlg resulted in tissue architecture defects due to the misorientation in cellular divisions (Hao et al. 2010; Jaffe et al. 2008; Zheng et al. 2010). The cross-talk between both cell features, polarity and direction of cell divisions, is mediated via the interaction between Dlg and adaptor protein Leu-Gly-Asn repeat-enriched (LGN) (Bergstralh et al. 2013; Johnston et al. 2011). Dlg fixes LGN to the lateral side of the plasma membrane where it, in turn, binds to the heterotrimeric G-protein; Gαi (Du and Macara 2004). For the segregation of mitotic chromosomes, LGN partners with nuclear mitotic apparatus protein (NuMA) which recognizes the astral microtubules and dynein, the force generating microtubule protein. For the lateral localization of NuMA/LGN complex, i.e. at the cell midcortex, activated aPKC is required. Par3/Cdc42-GTP complex activates aPKC at the apical surface which in turn blocks the NuMA/LGN complex from localizing at the apical surface, thereby preventing vertical alignment of the spindle (Fig. 5B) (Hao et al. 2010).

Our understanding of how spindles are oriented in the mammary epithelium is incomplete and possibly additional mechanisms are involved, however, one can imagine that defects in any step of this process may disrupt the parallel spindle orientation and induce alteration in the tissue sheets formation. Given that establishment of epithelial polarity is associated with the proper formation of cell junctions and cell-ECM

interactions, junctional proteins by generating polarity cues could also orient the orientation of the spindle. In this work, we show results that indicate that GJs, besides AJs which have been previously investigated by others, contribute to the proper orientation of the mitotic spindle in the mammary epithelium.

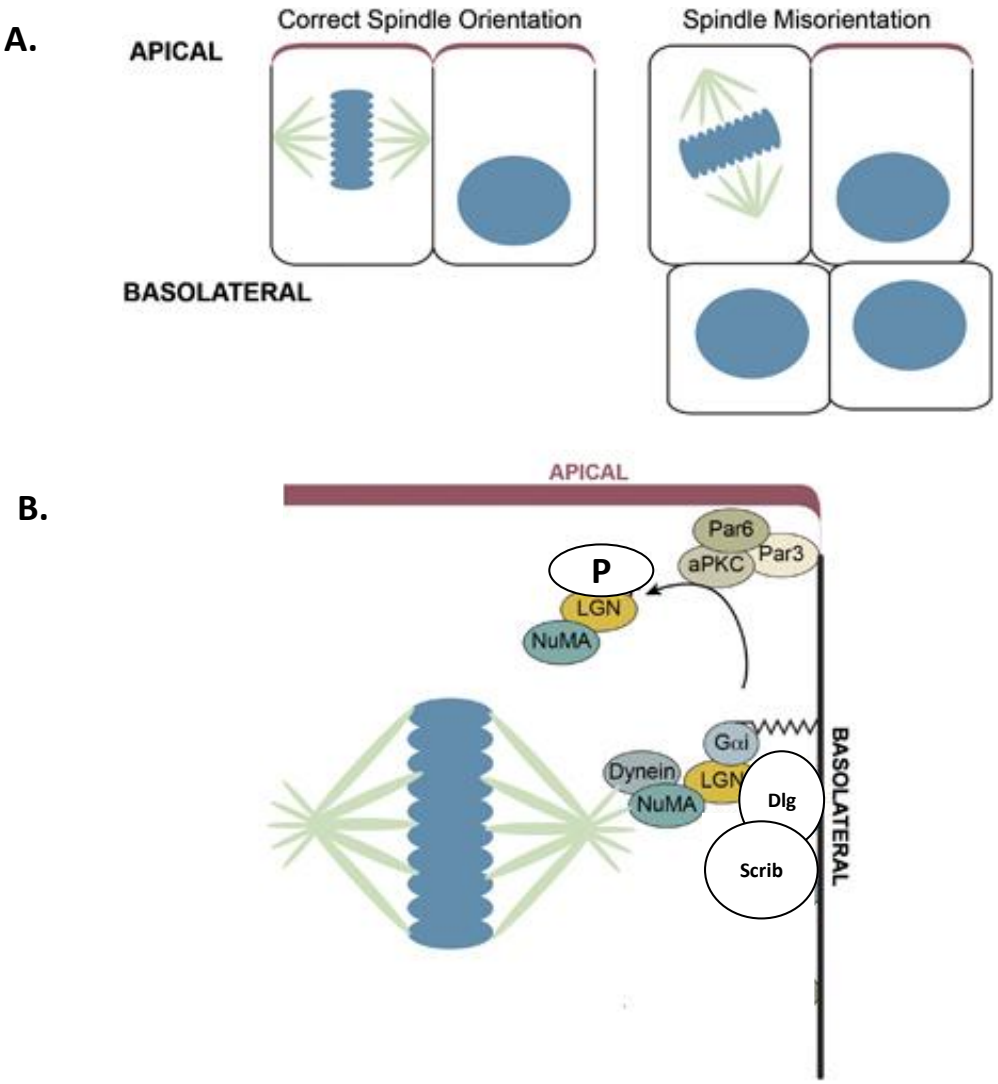

Figure 5. Planar orientation of the mitotic spindle.

A. Correctly versus incorrectly oriented mitotic spindle in most epithelia. Mitotic spindles oriented parallel to the planar cell axis leads to daughter cells dividing side by side, as opposed to on top of one another when the spindle is misoriented leading to the disorganization of the epithelial tissue. B. Apical and basolateral polarity complexes

contribute to the correct orientation of the mitotic spindle (modified from Halaoui and McCaffrey 2014).

I. Role of Connexins and Gap Junctions in the Differentiation of Mammary Epithelium

Given the dynamic development of the mammary gland, GJs provide enable the interaction between mammary epithelial cells with their neighboring cellular and acellular environment. This interaction is inevitable for the gland's differentiation and proliferation states. In fact, mounting evidence indicates that GJs are not only implicated in the normal development of the mammary gland, but also their dysregulation often occurs in, and sometimes causes, its pathophysiology. This dual role is explained by the fact that these junctions act as nodes that join adjacent cells and by complexing with associated proteins that relay downstream signaling cascades. As such, understanding GJ regulation, expression of different Cxs in a stage-specific manner and the association with binding partners is essential for the investigation of normal and cancerous processes in the mammary gland.

1. Temporal and Developmental Expression of Connexins

Among the Cxs isoforms, Cx43 and Cx26 have been identified to be the only Cxs expressed in human MECs that display functional GJIC among themselves and with human mammary fibroblasts (Lee et al. 1992; Tomasetto et al. 1993). *In vitro*, it has been revealed that Cx43 but not Cx26 is expressed in nontumorigenic human MECs, HMT-3522-S1 (Park et al. 2003, Lelièvre laboratory, unpublished data). Furthermore, Cx43 has been reported to be expressed in MCF10A (Rakib et al. 2010) where it contributes to the normal-like phenotype in 3D culture (Talhouk et al. 2013). In

normal breast samples from reduction mammoplasties, Cx26 was expressed between luminal epithelial cells while Cx43 was exclusively found at the myeopithelial cells of the ducts (McLachlan et al. 2007, Laird et al. 1999). We have revealed in the work presented in this dissertation, that Cx43, is expressed at the apical side of luminal epithelial cells in alveolar structures of normal breast tissue samples taken from reduction mammoplasties and biopsies from women with no breast cancer. These variable findings indicate that the temporal expression pattern of Cxs in the human mammary gland is not well-characterized due to the problem of obtaining normal human breast tissue samples representing different developmental stages. This has been partially solved by studying the rodent mammary gland given its high similarity to the human mammary gland and the high degree of sequence identity of their Cx genes with that of humans (El-Sabban et al. 2003; McLachlan et al. 2007; Söhl and Willecke 2004).

In the murine mammary gland, in addition to Cx26 and Cx43 characterized in the human mammary gland, Cx32 and Cx30 were reported to be expressed. Interestingly, there is a dynamic change in the protein and mRNA expression levels of these Cxs throughout the different developmental stages of the mammary gland. Cx26, Cx30 and Cx32 are expressed only in the luminal epithelial cells while Cx43 is expressed only in myoepithelial cells (Mroue et al. 2014). Cx26 and Cx32 were found to be expressed at the basolateral membranes of luminal cells at all developmental stages of the gland, but their expression significantly peaks around lactation and decreases during involution (Talhouk et al. 2005). In addition, Cx30 mRNA and protein levels were minimally detected in the virgin murine gland but were significantly higher throughout later stages of pregnancy and during lactation, then considerably decreased during involution. This temporal expression of Cx30 coincided with the onset of β-

casein and whey acidic protein (WAP) expression, both markers of mammary gland differentiation, suggesting the central role that Cx30 might play in inducing proper differentiation in the mammary epithelium. Cx30 expression was evident in differentiation permissive coculture conditions of SCp2 and SCg6, respectively, but not when differentiated SCp2 cells were cultured alone in the presence of Matrigel. The heterocellular interaction between SCp2 and SCg6 was characterized by membranous Cx30 forming a complex assembly with β-catenin, α-catenin and ZO-2 resulting in the production of β-casein. This suggests a complex GJ-mediated Cx30-dependent pathway for mammary epithelial differentiation (Talhouk et al. 2008, et al. 2009). Besides Cx30, Cx26 is minimally expressed at all stages of murine mammary gland development but is dramatically upregulated during pregnancy (McLachlan et al. 2007; El-Saghir et al. 2011). In an interesting report, conditional Cx26 knockdown in mouse model, meaning the absence of the physiological surge in Cx26, the mammary gland development and function were retained. It has been concluded that as long as basal levels of Cx26 expression is maintained, mammary gland development and function may be unaffected (Stewart et al. 2014). On the other hand, transgenic mice over-expressing Cx26 driven by keratin 5 promoter had an unexpected mammary phenotype whereby the mothers produced normal levels of milk proteins but were unable to feed their pups, suggesting a defect in delivery rather than milk production. Moreover, the ectopic expression of Cx26 in myoepithelial cells disrupted the endogenous expression of Cx43 resulting in impaired GJIC (Mroue et al. 2014). As for Cx43, its active phosphorylated form was up-regulated during lactation despite the significant decreased expression of its mRNA during mid-pregnancy and lactation. More so, previous studies in our laboratory suggested that Cx43 may be localized to the myoepithelial-epithelial cell contact

regions indicating that it is not only expressed by myoepithelial cells but also in secretory alveolar epithelial cells (Talhouk et al. 2005, 2008). In the *Gja1Jrt/+* mouse model, decreased Cx43 protein levels, reduced GJIC in myoepithelial cells resulting in a delay in the mammary gland development with no changes in the morphology of the gland and the expression of other Cxs compared to wild-type mice at parturition (Plante and Laird 2008). Furthermore, the knock-down of Cx43 in organoids from wild-type mice, impaired myoepithelial contraction in response to oxytocin (Mroue et al. 2014). In light of these findings, Cxs appear to be dynamically modulated to support the different stages of the mammary gland development and their expression in the different epithelial compartments of the mammary gland is critical for optimal function of the mammary epithelium. As such, the deregulation of the expression or localization of mammary Cxs might have detrimental effects on the differentiation and development of the mammary gland leading to the loss of proper function of the gland and/or tumorigenesis.

2. Gap Junctions in Normal Mammary Epithelial Differentiation

In support of the role of GJs in the mammary gland normal development, several studies reported a dynamic change in GJ channel composition and permeability at different stages of mammary gland development. In fact, initially during parturition, heteromeric Cx26/Cx32 hemichannels are formed to be later replaced with homomeric Cx32 channels after parturition, allowing the passage of larger permeants. In addition, osmolytes such as Taurine, accumulated during lactation, were shown to selectively inhibit the molecular permeability of Cx26/Cx32 channels while sparing others such as

homomeric Cx32, heteromeric Cx26/Cx30 and Cx30/Cx32 channels, suggesting a role for Cx30 and Cx32 during lactation (Locke et al. 2000, 2004, 2007). There is a scarcity in the studies that investigate the role of GJs in the differentiation of the mammary epithelium. One of these studies, however, examined the role of heterocellular GJIC in murine mammary epithelial differentiation. For this, CID-9 mouse mammary strain consisting mostly of epithelial cells in addition to myoepithelial-like and fibroblast cells was cultured on plastic and on a nonadhesive substratum. The stimulation of GJIC with cAMP was sufficient to drive β-casein expression which failed in a homogeneous SCp2 cultures in the absence of a basement membrane. Furthermore, culturing SCp2 cells with SCg6 enabled the establishment of GJIC between the two cell populations resulting in the expression of β-casein milk protein (El-Sabban et al. 2003). This GJ-dependent and β1-integrin/STAT5-independent pathway, activated β-casein expression in CID-9 cells cultured on a non-adhesive PolyHEMA substratum via OCT-1 rather than STAT5, suggesting OCT1 as downstream effector of GJ in β-casein expression (Talhouk et al. 2011). The above findings emphasize the importance of GJ-mediated epithelial/myoepithelial interactions in mammary gland development. Furthermore, evidence indicates that GJ and cell-matrix interaction-mediated signaling are essential for differentiation of mammary epithelial cells. As such, the differentiation of MECs depends on both the assembly and functionality of the GJ and the interaction of Cxs with associated partners within this complex.

J. Connexins in Mammary Tumorigenesis

Several studies have demonstrated that GJIC is a complex process capable of inhibiting or promoting breast tumor progression. This complexity exists since GJIC is

defined in a context-dependent manner as tumor cells can form either homotypic or heterotypic channels with multiple combinations of Cx protein. In addition, Cx proteins are now recognized to mediate GJ-independent roles which adds to the complexity of their contribution to tumor progression and development. Reports have clearly revealed that aberrant expression and activity of Cxs and GJs, respectively, is associated with breast cancer. This deregulation is by itself a dynamic process given that Cxs and GJs are differentially needed by tumor cells to intravasate, extravasate, migrate and invade depending on the cancer stage (Bazzoun et al. 2013).

1. GJIC-Dependent Mechanisms

The fact that GJs mediate intercellular exchange of a wide range of regulatory molecules, it has been associated with normal cellular homeostasis, yet its alteration is common in neoplastic progression. Several studies have attempted to decipher the role of GJIC in the different stages of breast tumorigenesis (Fig. 6). In breast cancer, the most frequent site of metastasis is the bone whereby RANK-RANKL axis is essential to the interaction between bone and breast cancer cells (Jones et al. 2006). In bone metastasis, GJIC was found to form between breast cancer cells and human osteoblastic cells (Kapoor et al. 2004). Interestingly, the inhibition of GJs led to a decrease in the cytoskeletal microfilaments arrangement between neighboring MDA-MB-231 cells and significantly reverted the augmented metastatic and invasion effect of silencing RANKL (Zhao et al. 2012). In addition, in MCF-7 cells, heterotypic GJIC was found to be more prevalent than homotypic GJIC between the cancer cells and hFOB, a human osteoblastic cell line which correlated with their increased metastatic and invasive

potential (Saunders et al. 2001; Kapoor et al. 2004). Another site of metastasis of breast cancer cells is the brain. Functional GJs were reported to exist between 4T-1 cells, mouse mammary cancer cells, and endothelial cells and were found to be essential for the spheroid formation and colonization of cancer cells in 3D cultures. Another metastatic breast cancer cell line, HMLE cells, was found to establish heterocellular GJs with the brain endothelium *in vivo* to mediate metastasis (Stletov et al. 2013). Similarly, GJIC-deficient HBL100 breast cancer epithelial cells exhibited increased GJIC and diapedesis through the endothelial monolayer upon Cx43 expression (Pollmann et al. 2005). In a significant number of studies examining breast cancer relapses, the bone marrow stroma, as being a continuous source of interleukins and chemokines, acts as a source of dormant breast cancer cells (Naume et al. 2007). As such, it has been revealed that an interaction coexists between breast cancer cells and the bone marrow's microenvironment and is partly mediated via GJIC (Moharita et al. 2006). In an early report, miRNAs, in particular, that specific to C-X-C motif chemokine 12 (CXCL12), have been demonstrated to be transported via GJs from the bone stroma to T47D cells. This resulted in a decrease in cell proliferation and transition of cancer cells to G0 phase of the cell cycle (Lim et al. 2011). Although cell-cell adhesion has been shown to regulate cancer cell invasion and motility, not much is known about the effect of GJs on breast cancer cell migration. Stabilizing cell adhesion and enhancing communication between cancer cells enable effective migration (Friedl et al. 2012; Friedl 2004; Carmona-Fontaine et al. 2008). In fact, a central property of collective migration of cancer cells is coordinated polarization that is established by the rearrangement of cytoskeletal and junctional proteins at the leading edge of migrating cells to facilitate synchronized retraction at the rear end of the group (Friedl and Gilmour 2009).

Interestingly, invasive tumors are characterized by an enhanced expression of E-, N-cadherin and TJ proteins that form complexes at the cell membrane (Hegerfeldt et al. 2002; Hidalgo-Carcedo et al. 2011). Furthermore, it has been reported that GJs are more concentrated in tumor cells compared to their normal counterparts and that Cx43 which forms GJs tend to accumulate in the rear half of cancer cells (Peglion et al. 2014). In addition, over-expression of Cx43 and Cx26 enhanced communication among HeLa cells and resulted in increased collective invasiveness (Friedl and Gilmour 2009). Clearly, GJs are not only essential for a differentiated mammary epithelial phenotype, they are also central coordinators of tumorigenesis as cancer cells need communication, adhesion and adequate polarization for progression and metastasis (Fig. 6).

2. GJIC-Independent Mechanisms

While Cxs have been established as essential for the functional development of the mammary gland, their role in breast cancer progression is controversial and not easily resolved. This, in part, is due to the cellular heterogeneity in cancer and the complex process of cancer development and progression. It is now realized that Cxs are differentially expressed in primary and disseminating breast cancer cells. Cx26 was found to be minimally expressed in normal breast epithelium but highly present in both primary and lymph node metastatic breast cancer cells, with higher levels in the cancer cells found in the lymph nodes (Kanczuga-Koda et al. 2006). In addition, evidence indicates that Cx43 expression is down-regulated in primary breast cancer tumors possibly to enable physical detachment of cancer cells as an early step in the metastatic process (Laird 2006; Naus and Laird 2010; Langlois et al. 2010). During later stages of metastasis, Cx43 was found to be up-regulated in established breast cancers to enable

tissue colonization and vascular penetration (Elzarrad et al. 2008; Chao et al. 2012). In parallel, conflicting data revealed that highly metastatic breast cancer cell lines exhibited reduced expression levels of Cx43 compared to the non-metastasizing ones (Li et al. 2008). Cx43 transfection in MDA-MB-231, resulted in the promotion of mesenchymal to epithelial transition and the inhibition of *in vitro* and *in vivo* angiogenesis by regulating the release of pro-angiogenic and anti-angiogenic molecules (Shao et al. 2005; McLachlan et al. 2007). This tumor reversion effects of Cx43 is GJ-independent whereby its stable transfection in breast cancer cell lines that do not express Cx43 (HBL100) or have defects in GJ assembly (MDA-MB-231) resulted in decreased tumor growth *in vivo* without the formation of GJs (Qin et al. 2002). Also, the overexpression of Cx43 in MDA-MB-231 and MCF-7, that represent different invasive stages of breast cancer, induced a partial reversion of the cancer phenotype in a culture-dependent manner. The membrane localization of Cx43 primed the recruitment of ZO-1, ZO-2 and α-catenin at the membrane. In particular, β-catenin was recruited away from the nucleus in 2D cultures of MCF-7 and 3D cultures of MCF-7 and MDA-MB-231 cells (where exogenous Cx43 was membranously localized). Thus, the Cx43 tumor suppressive effect occurs in a context-dependent manner, where the association of Cx43 with its cytoplasmic partners is involved in reducing proliferation rate, invasiveness, and, hence, tumorigenic phenotype of MCF-7 cells in 2D and 3D cultures, and of MDA-MB-231 cells only in 3D cultures (Talhouk et al. 2013). In an interesting report, mice with G60S mutation of Cx43 gene exhibited hyperplasic mammary glands, were more susceptible to lung tumors and had increased metastasis to the lung but displayed delayed onset of palpable tumors (Plante et al. 2011). This suggests that mutant Cx43 is linked to the inhibition of solid tumors formation but still induces early stages of

mammary gland tumorigenesis. The deregulation of Cxs has been implicated in breast tumorigenesis, however, their prognostic correlations have been rarely documented (Czyz 2008). In a recent study, breast cancer patients with reduced Cx26 and elevated Cx46 expression post-chemotherapy reflected improved survival rates after treatment (Teleki et al. 2013). In addition, elevated Cx43 protein and mRNA levels were linked to significantly improved breast cancer outcome as opposed to elevated Cx30 protein and mRNA levels that were associated with a reduced disease outcome. This offers both Cx43 and Cx30 as prognostic markers of breast cancer (Teleki et al. 2014).

The understanding of Cxs channel-dependent and -independent functions require the understanding of their role in a context-dependent manner. This involves examining their roles as tumor suppressors or enhancers in the light of the tumor microenvironment and the stage of cancer. Moreover, it is important to emphasize that Cxs, as part of the GJ complex, are not the sole players in the GJ complex. As described in earlier sections, Cxs are considered as signaling hubs, that when altered, in expression and/or localization, will bring about changes in their associated partners that are widely involved in the progression of breast cancers (Bazzoun et al. 2013).

K. Gap Junctions/Connexins as Potential Targets for Breast Cancer Therapy

Both the loss and gain of GJIC characterize breast tumors depending on the stage of cancer development. As such, GJs and/or Cxs are emerging as new potential targets for breast cancer therapy (Fig. 6). Restoring GJIC in tumor cells is a promising approach that enhances the spread of cytotoxic drugs consequently increasing their anticancer effects. The use of GJ enhancers has been recently assessed. A new class of substituted quinolines (PQs) was synthesized and found to possess potent inhibitory

activities against T47D breast cancer cells through the enhancement of GJIC (Shi et al. 2008; Gakhar et al. 2008). It demonstrates these anti-cancer effects by up-regulating the expression levels of Cx43 in T47D cells. Furthermore, seven intraperitoneal injections of PQ7 in nude mice with T47D xenografts resulted in a 100% decrease in tumor volume (Heiniger et al. 2010). Besides quinolones, gamma linoleic acid, a polyunsaturated fatty acid recognized for its anti-proliferative and cytotoxic effects, was shown to specifically improve the GJIC between endothelial cells and consequently reduce adhesion of breast (MDA-MB-231) and colon (HT115) cancer cells to endothelial cells (Jiang et al. 1997). Cisplatin is one of the most widely used cancer chemotherapeutic agents that induces the formation of platinum-DNA adducts to arrest the cell cycle. This damage triggers DNA-PK dependent signal which is transmitted to neighboring cells by GJIC. It has been reported that the inhibition of GJs protected mouse embryonic fibroblasts from cisplatin; whereas enhancing GJIC by transfecting MCF-7 breast cancer cells with Cx43 improved drug sensitivity (Jensen and Glazer 2004). Another study revealed that the combinational treatment of Cisplatin and PQ1 or PQ7 induced a significantly additional reduction of mammary tumor growth. This enhanced effect of Cisplatin was explained by the up-regulation of both Cx43 and Cx26 in PQ-treated tissues (Shishido and Nguyen 2012; Shishido et al. 2013). In addition to Cisplatin and PQs combinatorial therapy, tamoxifen and retinoic acid treatment of breast cancer induced a reversion in the tumor phenotype of MCF-7 cells as a result of enhanced GJIC, colocalization of Cx26 and Cx43 at cell–cell contacts, increased cell adhesion, accumulation of cells in the G0/G1 cell cycle phase and reduction in telomerase activity (Saez et al. 2003). Furthermore, it has been reported that the combinational treatment of T47D with tamoxifen and PQ1 enhanced tamoxifen efficacy

as PQ1 facilitated intercellular passage of tamoxifen and consequently, mediated its rapid action to inhibit cancer cell proliferation and colony growth (Ding et al. 2012; Gakhar et al. 2010). Despite the anti-cancer effects discussed above, introducing therapeutic agents against cell junction proteins might induce drug resistance. This is associated with the fact that some cancer cells are not proliferating or did not establish cell adhesion and communication, rendering the drug ineffective (Tsuruo et al. 2005). A better development of more targeted therapeutic agents will require a full understanding of how cell junctions and polarity proteins are modulated in relation to the genetic background of every patient.

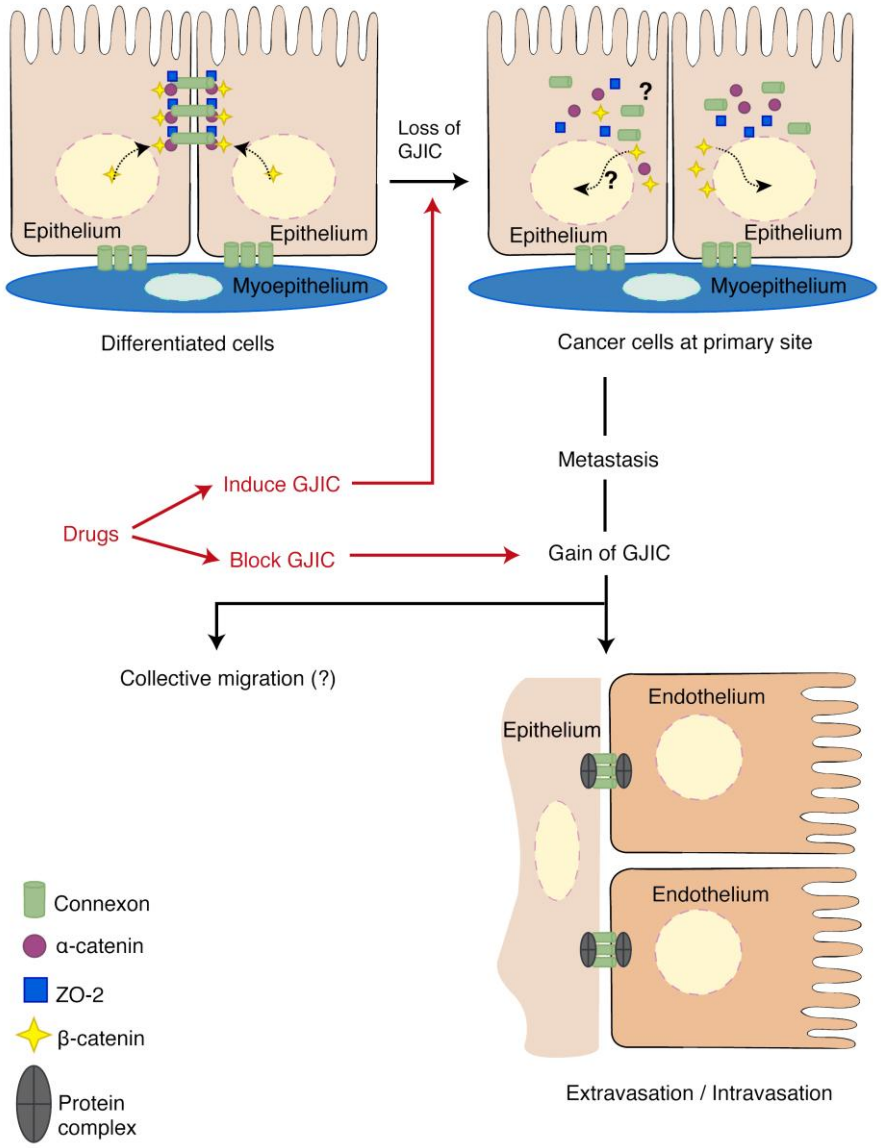

Figure 6. GJ dependent and independent role in mammary differentiation and cancer progression.

A. In a differentiated mammary epithelium, junctions such as TJs, AJs, and GJs are assembled at the membrane between two neighboring epithelial cells and their corresponding myoepithelial partners. During their life cycle, Cxs associate with different proteins that are able to transduce signals that are initiated at the gap junctional complex assembled at the membrane, towards the nucleus to bring about changes in the expression of genes involved in the differentiation of the mammary epithelium. B. Alterations in the expression and/or localization of Cxs result in an improper GJIC. GJs might dissociate thereby affecting the integrity of other junctions and releasing its associated partners which in turn can induce inadequate signaling cascades, deregulated proliferation, and detachment of epithelial cells from the basement membrane culminating in a disrupted epithelial architecture that marks tumor initiation. C. At later stages of cancer progression, invasive epithelial cells reestablish GJIC with the endothelial barrier to facilitate their intravasation/extravasation where Cxs establish interactions with other molecules to support a tumor-promoting rather than a tumor-

suppressing role of GJs that is displayed in the normal context (Modified from El-Saghir et al. 2011).

CHAPTER II

AIMS

Tumorigenesis is characterized by loss of adhesion and communication between the cells themselves and their microenvironment, inadequate cell cycle entry and improper cytokinesis among other changes that occur early on in epithelial cells. In order to understand the role of Cx43 in preventing mammary tumorigenesis we set on determining the effect of its loss on each of the mentioned early signs of tumorigenesis. Previous work at our laboratory suggests that overexpressing Cx43 in human mammary carcinoma cell lines is capable of inducing a partial reversion of tumor phenotype, in a context dependent manner, by affecting proliferation, invasiveness, cell cycle progression and proper gap junction complex assembly with its associated proteins, catenins and ZO proteins, at the membrane. Using HMT 3522-S1 (S1) cells, we aim to extend our findings to demonstrate a potential contribution of Cx43 in maintaining a normally differentiated mammary epithelium. Preliminary data revealed an apicolateral expression of Cx43 in the human breast tissue and in the S1 3D model (Hibret Adissu, Thesis, Purdue University 2007). We opted to characterize S1 cells in terms of expression and localization of GJ components, connexins, and their association with cytoplasmic partners. In addition, as part of the characterization, TJ and AJ proteins will be assessed. Since those junctional complexes are reported to be interconnected, the effect of stably silencing Cx43 on the expression and localization of TJ and AJ proteins will be examined. As being a feature of tumorigenesis, the effect of Cx43 loss on the

proliferation rate and cell cycle entry of S1 cells is to be determined in both 2D and 3D culture conditions.

In addition to junction establishment, proper cellular polarity is central to normal epithelial differentiation. Given that junctions and polarity proteins interplay, we aim to show a potential disruptive effect of GJ inhibition/Cx43 loss on apical polarity, a key feature of normal differentiation and one of the early events disrupted prior to breast tumor initiation. We will explore the signaling pathway via which Cx43 regulates apical polarity. Furthermore, a differentiated mammary epithelium is characterized by a monolayer of epithelial cells that is maintained by a proper orientation of the mitotic spindle which should result in cells dividing parallel to the substratum for the formation of a lumen. There is a gap of knowledge on the mechanism through which the mitotic spindle is oriented in the mammary epithelium; however, we have preliminary data that indicate that PI3K is required for the proper mitotic spindle orientation. As such, we aim to examine the cross talk between GJs/Cx43 and PI3K in the context of regulating cytokinesis in the mammary epithelium. By accomplishing this, we would establish, in addition to the tumor suppressive effects of Cx43 complex assembly, a novel role of Cx43 as a regulator of apical polarity and cytokinesis which are central for proper differentiation of the mammary epithelium. Our findings will contribute to the established role of Cx43 in mammary development and could serve as evidence to consider Cx43 as a biomarker for breast cancer risk assessment.

a. **AIM 1:** Characterize Cx43 protein expression, localization and GJ complex assembly in the mammary luminal epithelium and assess its role in proliferation and establishment of junctional complexes.

b. **AIM 2:** Determine the role of Cx43 in inducing and maintaining apical polarity.

c. **AIM 3:** Delineate the mechanism through which Cx43 ensures proper morphogenesis and architecture of the mammary epithelium.

CHAPTER III

CONNEXIN 43 MAINTAINS TISSUE POLARITY AND REGULATES MITOTIC SPINDLE ORIENTATION IN THE BREAST EPITHELIUM

Authors: Bazzoun, D.*, Adissu H.*; Wang L., Urazaev, A., Hodges K., Chandramouly, G., Talhouk, R. S, Lelièvre, S. A., (*first coauthors)

A. Abstract

Recent evidence suggests a regulatory role for Connexin (Cx) 43, a gap junction (GJ) protein, in the mammary epithelium where it is regarded as a tumor suppressor potentially through the promotion of phenotypically normal differentiation. Cx43 has been previously reported to be localized to myoepithelial cells in the mammary gland; we now bring immunohistochemical evidence of the localization of Cx43 at the apicolateral membrane of luminal epithelial cells of normal breast tissue structures (acini). In order to understand the role of Cx43 in breast epithelial differentiation, a three-dimensional cell culture system that promotes the differentiation of HMT-3522 S1 (S1) non-neoplastic breast epithelial cells into acini was used. Gap junction intercellular communication (GJIC) inhibition and Cx43 down-regulation perturbed acinar differentiation as shown by disruption of proper acinar morphology and alteration of apical polarity. Furthermore; blocking GJIC primed S1 cells to enter the cell cycle when induced by Insulin Growth Factor or treated with NuMA antibodies that alter nuclear organization. In addition, we have unraveled a critical role for GJIC in the regulation of the orientation of the mitotic spindle prior to cell division, which is essential for the maintenance of monolayered acini. Blocking GJIC resulted in an improper orientation of the mitotic spindle in S1 cells. We propose that the control of

apical polarity-mediated quiescence and mitotic spindle orientation contribute to the tumor suppression role of Cx43.

B. Introduction

The pathological feature corresponding to the addition of layers of epithelial cells has been recognized as an early event in cancer development (Godde et al. 2014). Cell multilayering is an illustration of the loss of epithelial architecture resulting from the disruption of the polarity axis. The recurrent demonstration of the implication of tight junction proteins in proliferation control and tumor suppression (reviewed in Bazzoun et al. 2013) and of tight junction structures in the maintenance of cell quiescence (Chandramouly et al. 2007; Yue et al. 2011) has led to the recognition that apical polarity, established by the apical location of tight junctions, is essential for the homeostasis of epithelia. The potential contribution of apical polarity in preventing cancer onset makes this architectural feature worth of consideration for cancer prevention research (Lelièvre 2010; BCJ 2013).

Another tissue structure involved in epithelial homeostasis is the gap junction (GJ), with connexins (Cxs) as building blocks. Lack of cell coupling via GJs and/or down-regulation of Cxs have been reported in liver, thyroid, stomach, lung, prostate, cervical and intestinal tumors (Kanno and Matsui 1968; Jamakosmanovic and Loewenstein 1968; Krutovskikh et al. 1994;Yamasaki et al. 1995; Ruch et al. 1998). Several studies have demonstrated the tumor suppressive role of Cxs, as the ectopic reintroduction of Cx26, Cx43 or Cx40 into tumor cells reduced tumor growth and partially re-differentiated transformed cells (Hellman et al. 1990; Eghbali et al. 1991; Zhu et al. 1991; Hirschi et al. 1996; McLachlan et al. 2006). In the mammary gland GJs

have been implicated in normal development and their dysregulation has been correlated with tumorigenesis (Wilgenbus et al. 1992; Kanczuga-Koda et al. 2003). Specifically, the down-regulation of Cx43 and Cx26 expression in primary breast tumors (Lee et al. 1992; Laird 2006; Naus and Laird 2010; Langlois et al. 2010) and the transfection of both Cxs at a time in MDA-MB-231 leading to the promotion of mesenchymal to epithelial transition and partial reversion of the tumor phenotype have earned these Cxs the attribute of proteins with tumor suppressor function (Qin et al. 2002; Shao et al. 2005; McLachlan et al. 2007, Talhouk et al. 2013).

Interestingly, Cxs 30, 40, 43 and 26 have been reported to interact with and/or regulate the expression of ZO-1, claudins and occludins in epithelia thus, hence linking elements of GJs and TJs (Nusrat et al. 2000; Nagasawa et al. 2004; Go et al. 2006; Li et al. 2010). Specifically, the co-localization of Cx43 and tight junction proteins has been reported in blood-brain barrier endothelial cells (Nagasawa et al. 2004), and Cx43 has been shown to regulate the blood-testes barrier via its effects on the reassembly of TJs (Li et al. 2010). Cx43 also binds to ZO-1 in mammary cells which, in turn, acts as a scaffold for other proteins to interact with GJs (Talhouk et al. 2013). Whether GJs and TJs, these two major mediators of tissue homeostasis work together or separately to prevent tumor development remains to be investigated.

There is a major hindrance to the investigation of the functional interaction between GJs and TJs in the breast. Indeed, so far Cx43 has been only documented in the myoepithelial cells, notoriously deprived of TJs, in human and murine mammary glands. In the myoepithelium it is strongly expressed at cell-cell contact, at constant levels throughout the cell cycle, and mediates GJ intercellular communication (GJIC) (Lee et al. 1992; Monaghan et al. 1996; Jamieson et al. 1998; Laird et al. 1999;

Kanczuga-Koda et al. 2003; El-Sabban et al. 2003; Locke et al. 2004; Talhouk et al. 2005; Plante and Laird 2008; Mroue et al. 2014). Luminal cells in the murine mammary gland express Cx32 and Cx30 that form channels with Cx26, which, in particular, is variably expressed through development, with a peak of expression during pregnancy and lactation (Locke et al. 2004; 2007) and appears to have low impact on the function of the mouse mammary gland (Stewart et al. 2014).Yet, until now the only Cx reported *in vivo* in the human luminal epithelium is Cx26. It has been detected in low amount and localized intracellularly in luminal epithelial cells when separated from the myoepithelium and cultured *in vitro*. Moreover, these luminal cells rarely displayed dye transfer and Cx26 did not form GJ plaques (Monaghan et al. 1996). The obvious lack of GJs in the luminal human breast epithelium is in contradiction with murine work showing Cx26/Cx32, homomeric Cx32, heteromeric Cx26/Cx30 and Cx30/Cx32 channels in the luminal epithelium. It is also difficult to reconcile the fact that induced expression of Cx43 in human breast cancer cells drives mesenchymal to epithelial transition (Talhouk et al. 2013) if this Cx43 is considered only present in the myoepithelium, and that Cx43 would interact with TJs proteins as we mentioned earlier.

Here we report the presence of Cx43 at the apical cell-cell contacts of the luminal breast epithelium both in 3D culture models and in resting human breast. Cx43-mediated GJs are required for the establishment and maintenance of apical polarity illustrated by the presence of core TJ marker ZO-1 at apicolateral cell-cell contacts. We further demonstrate that Cx43-dependent polarity controls glandular morphogenesis not only by regulating cell cycle entry, but also by directing the orientation of the mitotic spindle through the PI3K-aPKC-NuMA mechanism without a detectable influence of

β1-integrin. Our findings establish the novel preventive function of Cx43 in major aspects of cancer onset that are polarity loss and cell multilayering.

C. Materials and Methods

1. Cell culture

Non-neoplastic S1 HMT-3522 human mammary epithelial cells (HMECs) (Briand et al 1987), between passages 52 and 60, were routinely maintained as a monolayer (2D culture) in chemically defined serum-free H14 medium (Blaschke et al., 1994; Plachot and Lelièvre, 2004). MCF10A cells (ATCC, Manassas, VA) were cultured in DMEM/F12 supplemented with 5% donor horse serum, 20 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone and 100 ng/ml cholera toxin. The drip method of three-dimensional (3D) cell culture was used to induce the formation of acini. Briefly, S1 and MCF10A cells were plated on Matrigel^{TM} (60 µl/cm^2, BD Biosciences, Bedford, MA) at a density of 5x10^4 cells/ml in the presence of culture medium containing 5% Matrigel^{TM} Plachot and Lelièvre, 2004). EGF was omitted from the culture medium after day 7 to allow completion of acinar differentiation (usually observed on day 8 or 9; Plachot and Lelièvre 2004; Lelièvre and Bissell 2005). To induce basoapical polarity formation in S1 cells cultured as a flat monolayer, cells were seeded on coverslips coated with Laminin-Entactin Free (354239, Corning, Corning, NY) (Plachot et al. 2009).

To block gap junction intracellular communication (GJIC), cells were incubated with 10 or 50 µM of 18α-glycerrhitinic acid (AGA) (Sigma Aldrich, St. Louis, MO). Control groups were incubated with vehicle (ethanol). To inhibit aPKCζ

cells were treated with 5 μM of aPKCζ pseudosubstrate (myristoylated) inhibitor (Enzo LifeSciences, Farmingdale, NY). Cell culture medium was changed every 48 h during the culture period. To inhibit PI3-Kinase (PI3K), cells were treated with 1 μM LY294002 (Cell signaling technology, Danvers, MA). To block β1-Integrin signaling, S1 cells were incubated with 15 μg/ml rabbit polyclonal IgG anti-human β1 Integrin (AIIB2) antibody (Santa Cruz Biotechnology Inc., Dallas, TX); control cells were incubated with 15 μg/ml nonspecific IgG (Jackson ImmunoResearch, West Grove, PA) for controls.

For treatment with an antibody against NuMA, live cells in 3D culture were permeabilized with 0.01% digitonin in permeabilization buffer [25 mM Hepes (Sigma), pH 7.2; 78 mM KHOAc (Sigma), 3 mM MgHOAc (Sigma), 1 mM EGTA (Sigma), 300 mM sucrose (Sigma), 1.0% bovine serum albumin (Sigma)] as described earlier (Lelièvre et al., 1998; Abad et al., 2007). Cultures were incubated with 15 μg/ml of nonspecific mouse IgG (Zymed, San Francisco, CA) or mouse monoclonal IgG antibodies directed against NuMA (B1C11 clone, kindly provided by Jeffrey A. Nickerson; University of Massachusetts Medical School, Worcester, MA).

2. Transfection and infection protocols

The recombinant short hairpin (sh)RNA retroviral constructs against Cx43 have been described previously (Shao et al., 2005). Vectors containing shRNA, non-silencing sequence (NSS), and empty vector control (EV) were transfected into Phoenix packaging cells using Calcium Phosphate (Stratagene, La Jolla, CA). Briefly, packaging cells at 30-40% confluence were incubated with 2 μg of retroviral vectors in

transfection solution according to the supplier's protocol. Cells were maintained at 37ºC for 24 h, after which the transfection medium was replaced by DMEM/F12 containing 10% fetal bovine serum. Cells were maintained for an additional 24 h before collecting viral supernatants for the next three days. Viral supernatants were passed through 0.45 µm cellulose acetate filters and stored at -80°C. For infection, filtered retroviral supernatants were thawed at 37ºC in a water bath and applied to monolayers of S1 cells at day 3. Cells were incubated with hexadimethrine bromide (polybreen) (6 µg/ml; Sigma) for 8 h. The infection medium was removed and cells were incubated in regular H14 medium for 24 h. Infection was repeated two additional times and selection with hygromycin-B (150 µg/ml; Calbiochem, San Diego, CA) was started 72 h after the last infection.

The pCDF-MCS1 plasmid vectors containing HuCx43 and EV control were transfected into 293TN cells according to the supplier's protocol. Viral supernatants were collected 48 h after transfection, filtered and stored at -80ºC. MCF10A cells were plated in a 60 mm dish 72 h prior to viral infection to which 3.8 ml of DMEM with additives were added. Viral supernatant (2 ml) was added directly to MCF10A cells and incubated with polybreen (5 µg/ml) at 37ºC overnight. The medium was replaced with a polybreen-free medium the next day and left overnight. Infection was repeated two additional times and selection with puromycin (50 µg/ml, Sigma) in H14+EGF medium was started 48 h after the last infection.

3. GJIC assay by scrape-loading and microinjection

The scrape-loading method (El-Fouly et al., 1987) was used to determine the minimum concentration of AGA that blocks GJ in S1 cells. Cells were plated as a monolayer in 35 mm dishes and cultured for 10 days. Cells were then treated for 1 h with AGA (10, 20, 50, 75, and 100 μM) and corresponding concentrations of vehicle (DMSO). After rinsing the cells with warm Phosphate Buffer Saline (PBS) twice, a dye mixture of 2.5% Lucifer yellow CH (LY) (Sigma) and 2% Rhodamine-B isothiocyanate-Dextran (RD) (Sigma) in PBS was added. Scrapes were made through the monolayer with a surgical scalpel blade #20 (Swann-Morton, UK) and culture dishes were incubated at 37°C for 5 min. The dye mixture was removed and cultures were rinsed three times with warm PBS, and fixed with 4% buffered paraformaldehyde (Sigma). Dye spread was observed using epifluorescence microscopy and images were recorded.

GJ assay in 3D culture was performed by microinjection of a dye mixture of LY (2.5%) and RD (2%) dissolved in 0.15 M LiCl in single S1 cells of acini formed in 3D culture, in 35 mm dishes. Some of the acini populations were treated with AGA as described above. Penetration of the cell membrane was determined by monitoring the membrane potential, which ranged between -19 and -35 mV (AxoClamp 2B amplifier, Axon Instruments, Foster City, CA). Cells were injected ionophoretically with micropipettes using hyperpolarizing pulses, 4-5.5 nA/500 ms at a frequency of 0.75 Hz for 1 min, produced by a Grass S88 stimulator (Grass Instruments, West Warwick, RI). The micropipettes, when filled with 3 M KCl, had tip resistance of 15-20 MOhm and were pulled using a P-97 microelectrode puller (Sutter Instruments, Novato, CA). Single cells in nine to 12 acini in each experiment group were microinjected. During microinjections, acini were kept in culture medium at room temperature (RT).

Intercellular dye diffusion was allowed to proceed for 15 min and acini were observed using epifluorescence microscopy. Dye coupling was determined by assessing the spread of LY from the injected cell (marked by RD) to the other cells in the acinus.

GJ assay also encompassed microinjection of neurobiotin (NB) (Invitrogen Molecular Probes, Eugene, OR). Cells were plated in 3D on Matrigel™-covered 13 mm diameter cell culture Thermanox™ Coverslips (Nunc, Rochester, NY). The coverslips were then transferred into 35 mm cell culture dishes and maintained in H14 medium. On day 10, 10 to 12 acini were microinjected with 3% NB in 0.15 M LiCl. A single cell in an acinus was injected ionophoretically with depolarizing pulses of 2 nA/600 ms at a frequency of 1 Hz for 2 min. Intercellular dye diffusion was allowed to proceed for 15 min. Acini were dual-immunostained for Cx43 using rabbit antibody (Sigma) and streptavidin-FITC (against NB) (1:40 dilution, Vector Laboratories, Burlingame, CA) according to the protocol described in the immunostaining section.

4. Immunohistochemistry

Archival formalin-fixed normal adult breast tissues were obtained from the Department of Surgical Pathology at the Indiana School of Medicine, Indianapolis, IN. Tissue samples were used according to Institutional Review Board approval # 03-135E. For HRP immunostaining, tissue sections (4 μm) were sequentially deparaffinized, rehydrated and antigens were revealed by boiling in Antigen Unmasking Solution (Vector). Following washes with Tris-Buffered Saline (TBS) [10 mM Tris base; 150 mM NaCl], endogenous peroxidase was blocked by incubating in 0.03% hydrogen peroxide in TBS for 15 min. Samples were then washed with TBS and TBST (TBS with

0.05% Tween-20) and then incubated for 1 h with blocking reagents (Avidin/Biotin Blocking Kit, Vector) followed by overnight incubation with rabbit anti-Cx43 primary antibody. Samples were washed with TBS and TBST and incubated with biotynyl-conjugated anti-rabbit polyclonal antibody (DakoCytomation, Carpinteria, CA) for 1h at RT. Following washes with TBS and TBST, specimens were incubated for 30 min at RT in streptavidin-horse radish peroxidase (SA-HRP) (PerkinElmer Life Sciences, Waltham, MA) diluted to 1:100 in blocking reagent. Following washes with TBS and TBST, specimens were incubated for 15 min at RT with biotinyl-tyramide signal amplification solution (PerkinElmer Life Sciences). Specimens were then washed with TBS and TBST and incubated with streptavidin-HRP for 30 min at RT. After washes with TBS and TBST, specimens were incubated with 3, 3'-diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector) for 5 min. Nuclear counter-staining was performed for 45 s with Gill III Hematoxylin (Leica Biosystems, Buffalo Grove, IL), and coverslips were fixed to the tissue sections on slides with Permount mounting reagent (Fisher Scientific, Fair Lawn, NJ).

5. Immunofluorescence labeling

Fresh 3D cultures or cryosections from frozen embedded cultures were stained for markers of differentiation as described earlier (Lelièvre et al. 1998; Plachot and Lelièvre 2004; Abad et al. 2007). Briefly, cells were either permeabilized with 0.5% peroxide and carbonyl-free Triton X-100 (Sigma) in cytoskeleton buffer [100 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, 5 mM $MgCl_2$, 1 mM pefabloc, 10 µg/ml aprotinin, 250 µM NaF] prior to fixation in 4% paraformaldehyde (Sigma), or directly fixed with 4% paraformaldehyde. Antibodies used were rabbit polyclonal against Cx43

(C6219, 1:400, Sigma-Aldrich), NuMA (B1C11, 1:2, a gift from Dr Jeffrey Nickerson, UMass, Worcester, USA), Ki-67 (VP-K451, 1:1000, Vector), and mouse monoclonal against ZO-1 (33-9100, 1:200, Life Technologies; Grand Island, NY), α-tubulin (T5168, 1:500, Sigma), β-catenin (Clone CD14, 1:800, BD Biosciences), E-cadherin (610181, 1:50, BD biosciences), and rat α-6 integrin (clone NKI-GoH3, 1:200, EMD Millipore, Billerica, MA). For immunostaining for Lysosomal Associated Membrane 2 (LAMP-2) (mouse, A15464, Life Technologies), cells were fixed with methanol acetone solution (1:1 ratio) without permeabilization. Secondary antibodies conjugated with Alexafluor 488 (green) or Alexafluor 594 (red) (Invitrogen Molecular Probes) were used at the manufacturer's proposed dilutions. Nuclei were counterstained with 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) and specimens were mounted in ProLong® Gold antifade reagent (Invitrogen Molecular Probes).

6. BrdU labeling

Active cell proliferation was determined by immunofluorescence detection of 5-Bromo-2'-deoxy-Uridine (BrdU) incorporated into DNA during the S phase of the cell cycle. The BrdU assay was performed on day 4 of cell culture using a BrdU labeling kit according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN).

7. Preparation of whole cell protein extracts and Western blot analysis

Cells were harvested from 3D cultures as described earlier (Plachot and Lelièvre, 2004). Briefly, acini were released from the Matrigel^{TM} by incubation with

Dispase (BD Biosciences) and whole cell extracts for the immunoblotting of all proteins were prepared in Laemmli buffer (2% SDS in PBS) including PPI cocktail (10 mg/ml Aprotonin, 100 mM Pefabloc, 250 mM Sodium fluoride) (Lelièvre et al., 1998) except for p-Akt which was prepared in RIPA buffer (25 mM Tris HCL, 150 mM NaCl, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS). For western blot analysis equal amounts of proteins were separated and immunoblotted with rabbit polyclonal antibodies against Cx43 (C6219, 1:1200, Sigma), p-Akt (#9271, 1:100, Cell signaling) and Akt (#9272, 1:1000, Cell signaling), monoclonal antibodies against Cx26 (1-2 μg/ml, Zymed Laboratories San Francisco, CA), and mouse ZO-1 (33-9100, 1:50, Life Technologies). Equal protein loading was verified by immunoblotting for lamin B (Rabbit, Ab16048, 1:14000, Abcam) and β-actin (Rabbit, A8227, 1:1000, Abcam). Protein levels were quantified using Scion NIH Image software (Scion Image, Scion Corporation, NIH) or *ImageJ* (http://imagej.nih.gov/ij/) and normalized to Lamin B or β-actin.

8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (1 μg) was reversed transcribed to cDNA using Revertaid 1st strand cDNA synthesis kit (Fermentas, Grand Island, NY). qRT-PCR was performed using iQSYBR Green Supermix in a CFX96system (Bio-Rad Laboratories, Hercules, CA). Products were amplified using primers for Cx43 5′CAA TCA CTT GGC GTG ACT TC 3′ (forward) and 5′ GTT TGG GCA ACC TTG AGT TC 3′ (reverse), Cx30 5′ TCA ACA AAC ACT CCA CCA GC 3′ (forward) and 5′ CAA TCC CAC ATT TCA ACA CC 3′ (reverse), Cx32 5′ GAC AGG TTT GTA CAC CTT GC 3′ (forward) and 5′ CGT CGC ACT TGA CCA GCC GC 3′

(reverse), Cx26 5′ TCT TTT CCA GAG CAA ACC GC 3′ (forward) and 5′ GAC ACG AAG ATC AGC TGC AG 3′ (reverse), Cx45 5′ GGA GCT TTC TGA CTC GCC TG 3′ (forward) and 5′ CGG CCA TCA TGC TTA GGT TT 3′ (reverse) and GAPDH 5′ AAG GTG AAG GTC GGA GTC AAC 3′ (forward) and 5′ GGG GTC ATT GAT GGC AAC AAT A 3′ (reverse). To quantify changes in gene expression, the ΔCt method was used to calculate the relative-fold changes normalized to GAPDH.

9. Image processing

Images of immunofluorescence labeling were recorded using a laser scanning MRC-1024 UV (Bio-Rad Laboratories, Hemel, Hampstead, England) linked to a Diaphot 300 (Nikon, Tokyo, Japan) inverted microscope, and oil immersion 60X, numerical aperture (NA) 1.4 apochromatic and 40X, NA 1.4 fluor lenses. Images were processed using Confocal Assistant™ 4.02 (Bio-Rad Laboratories) and assembled using Adobe Photoshop® 6.0 (Adobe Systems, San Jose, CA).

10. Statistical analysis

Data are presented as means ± SEM and statistical comparisons were done using GraphPad Prism 3.0 software (GraphPad Software Inc, San Diego, CA). Non-paired and paired t-test were used for comparison of two groups or paired samples, respectively; whereas one-way ANOVA was employed for three or more groups of treatments. $P < 0.05$ was considered significant.

D. Results

1. Cx43 is located apically in breast luminal epithelial cells.

We used the non-neoplastic human mammary epithelial HMT-3522 S1 cell line (Briand et al 1987) known to form well-differentiated acini, as characterized by the establishment of a basoapical polarity axis and cell cycle exit (Petersen et al 1992; Plachot and Lelièvre 2004) to examine whether Cxs 26 and 43 are expressed via western blot analysis; only Cx43 was detectable (Fig. 7A). Analysis of mRNA expression for *GJB6*, *GJB1*, *GJB2*, *GJA7* and *GJA1* coding for Cx30, 32, 26, 30, 45 and 43, respectively, confirmed transcription of the *GJA1* gene only (Fig. S1). Immunostaining for Cx43 in S1 cells cultured as 2D monolayer revealed a pattern compatible with cell-cell localization; surprisingly, labeling performed in S1 acini obtained in 3D culture demonstrated a concentration of Cx43 towards the center of the acini thus, within the apical cellular poles (Fig. 7B). Immunohistochemistry for Cx43 performed on archival biopsy sections of normal appearing breast tissue confirmed the apicolateral concentration of the protein in the luminal epithelium (Fig. 7C), but also reaffirmed the presence of Cx43 in the myoepithelial cell layer as previously reported (Monaghan et al. 1996; Laird et al. 1999). Dual immunofluorescence labeling of Cx43 and α-smooth muscle actin protein (α-SMA), a marker of myoepithelial cells, confirmed that apicolateral Cx43 did not overlap with possible myoepithelial cell expansions in real tissue and thus, was strictly confined to luminal cells (Fig. 7D). Dual immunostaining for Cx43 and lysosomal marker LAMP-2 showed only minimal colocalization in acini formed by S1 cells (Fig. 7D), enabling us to rule out the possibility that the signal observed for Cx43 is linked to lysosomal degradation. Indeed, earlier work established that such degradation is the fate of Cxs in cells that are defective for Cx trafficking and GJ assembly (Qin et al. 2001). Dual immunostaining

for Cx43 and ZO-1 revealed extensive colocalization at the apical side of luminal cells (Fig. 7E), suggesting a close association of Cx43 with tight junction proteins as described in the polarized thyroid tissue (Guerrier et al. 1995). Moreover, Cx43 was primarily localized along lines marked by cell-cell adhesion marker β-catenin, indicating its presence at cell-cell junctions and consequently, its possible involvement in GJIC (Fig. 7D).

2. Cx43-mediated GJIC controls epithelial homeostasis

Communication among S1 cells via Cx43-enabled GJ was initially determined by scrape loading of a mixture of Lucifer yellow (LY) and rhodamine-B isothiocyanate-Dextran (RD) in 2D culture. LY diffused through a longer distance inside the cell layer compared to RD that remained at the wound site (Fig. S2A). For the assessment of GJIC in the differentiated glandular epithelium a mixture of LY and RD was microinjected into a single cell of an acinus and repeated for at least 10 acini. LY diffused throughout each of the acini treated in this manner (Fig 8A). A concentration of AGA that effectively blocked GJs without toxicity, based on TUNEL and Trypan blue exclusion assays, was first determined in 2D culture (Fig. S2B). Treatment of cells with AGA at day 4 (during the proliferation stage of acinar morphogenesis) (Fig S2C) or at day 10 (upon completion of acinar morphogenesis) of 3D culture indeed confirmed blockage of GJ communication, as shown by the strict localization of LY in the injected cells (Fig. 8A).

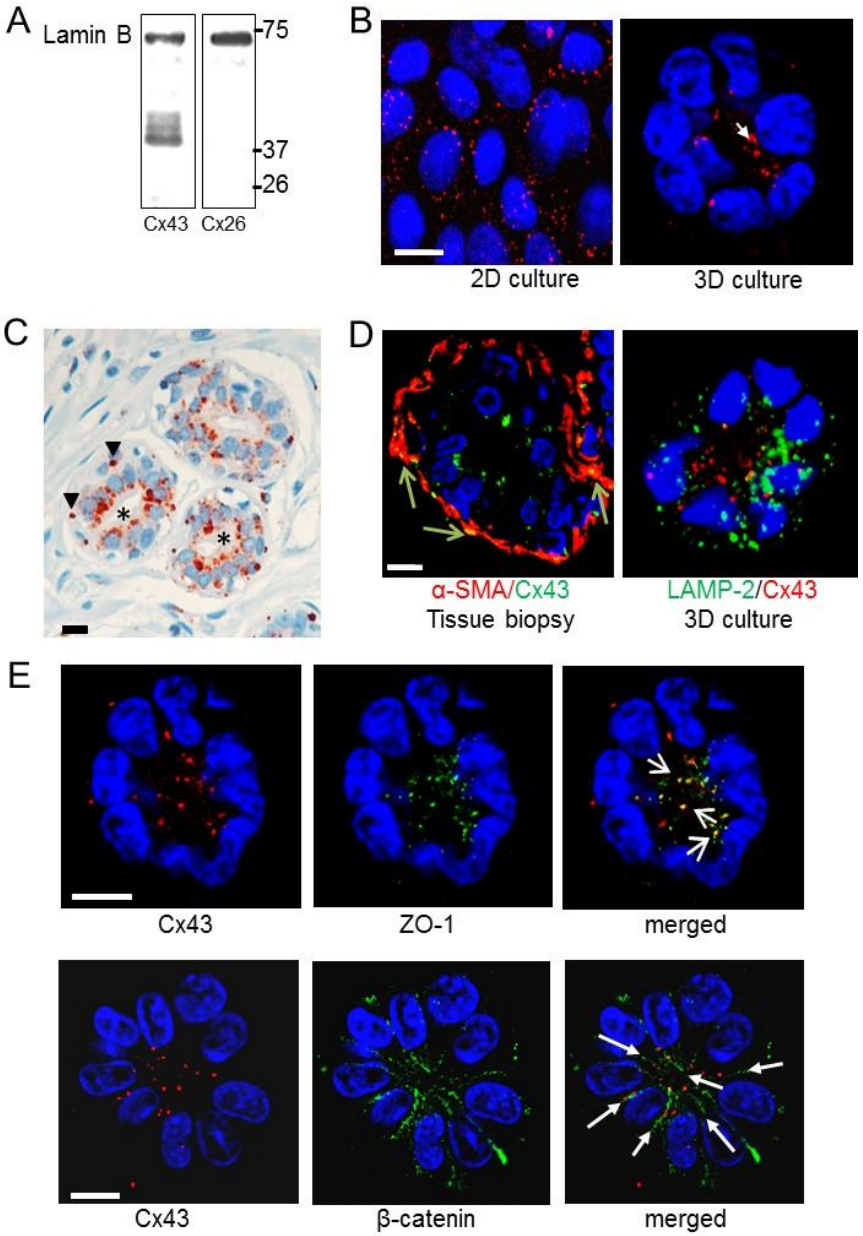

Figure 7. Characterization of Cx43 in breast luminal epithelial cells in vivo and in vitro.

S1 non-neoplastic mammary epithelial cells were culture in 2D culture (B) or in 3D culture (B; D [right image; E) for 10 days. A. Western blot for Cx43 (left panel) and Cx26 (right panel) from whole protein extracts; lamin B is used as loading control. B. Fluorescence immunostaining for Cx43 (red); the apical localization of Cx43 in the acinus is indicated by the arrow. Nuclei are counterstained with DAPI (blue). C. Immunohistochemistry for Cx43 (redish-brown) on a paraformaldehyde-fixed section of normal-appearing breast glandular tissue; examples of basal localization in

myoepithelial cells (arrowheads) and apical localization in luminal cells (asterisk) are shown. Nuclei are counterstained with hematoxylin. D. Left image: Dual fluorescence staining for Cx43 (green) and a myoepithelial cell marker (α-smooth muscle actin protein/α-SMA) (red) on a paraformaldehyde-fixed section of normal-appearing breast glandular tissue. Cx43 staining overlap with α-SMA staining in myoepithelial cells appears in yellow (arrow). Right image: Dual immunostaining for Cx43 (red) and a lysosomal marker (Lysosomal-Associated-Membrane-Protein-2/LAMP-2) (green) in an acinus formed by S1 cells. Nuclei are counterstained with DAPI (blue). E. Dual staining for Cx43 (red) and ZO-1 (green) or Cx43 (red) and Beta-catenin (green). Colocalization of Cx43 (red) and ZO-1 (green) staining appears yellow (arrows); cell-cell contacts with Cx43 aligned with beta-catenin are indicated (arrows). Nuclei are counterstained with DAPI (blue). Size bar = 10 μm. "Hibret Adissu, Thesis, Purdue University 2007".

The effect of blocking GJIC on acinar differentiation was examined by assessing proliferation, acinar morphology, and basoapical polarity following treatment of S1 cells with AGA during the 10-day morphogenesis process. A marked defect in acinar morphology was visualized under phase contrast microscopy. Acini appeared bumpy and lacked the usual pie-like organization of cells in ~70% of the structures in the AGA-treated group compared to ~45% in the control group (Fig. 8B). Noticeably, immunostaining for β-catenin revealed the aberrant presence of cells in the center of the acini of the AGA-treated group (Fig. 8C). Altered cell arrangement was not accompanied by a change in proliferation rate as measured by BrdU labeling on day 4 of 3D culture (Fig. 8D). Treatment of cells with AGA during acinar differentiation (days 1 to 10) or following acinar differentiation (days 9 to 12) resulted in no apparent alteration in the localization of the basal polarity marker α-6-integrin compared to untreated acini (Fig. 8E). Whereas, the distribution of polarity marker ZO-1, typically apical in differentiated breast acini *in vivo* (Martin et al., 2004) and *in vitro* (Plachot and Lelièvre, 2004), was significantly altered by AGA treatment under both treatment timelines, with a diffused and/or punctual basal staining pattern (Fig. 8E). Such alteration was not accompanied by a drastic change in ZO-1 expression, as shown by western blot analysis (Fig. 8F).

These results collectively suggest that GJIC is required for the proper organization of tight junctions, hence for the establishment of apical polarity in acini. We have shown that acini lacking apical polarity can be pushed into the cell cycle upon altering the distribution the nuclear protein NuMA with antibody B1C11 (Chandramouly et al. 2007). To determine whether apical polarity loss induced by GJ blockage also primed acinar cells for cell cycle entry, we treated S1 acini in which GJs were blocked by AGA treatment from days 1 to 10 or from days 9 to 12 with B1C11 from days 9 to 12. The percentage of cells positive for Ki-67 was only significantly increased (nearly four-fold) in the group of acini with combined AGA and B1C11 treatments compared to groups of acini treated with a nonspecific immunoglobulin (IgG), regardless of AGA treatment, or with B1C11 alone (Fig. S3). We confirmed the impact of GJIC blockage on cell cycle entry using a potent mitogenic factor implicated in normal (Dickson and Lippman 1995) and cancerous mammary development (Yu and Rohan 2000). Insulin growth factor 1 (IGF-1) added to the cell culture medium from days 9 to 12 of 3D culture significantly increased the percentage of Ki67 positive cells in only the AGA-treated group of acini (from days 1 to 10) (Fig. 9A-B), Thus, both gap and tight junctions control quiescence, a major aspect of tumor onset.

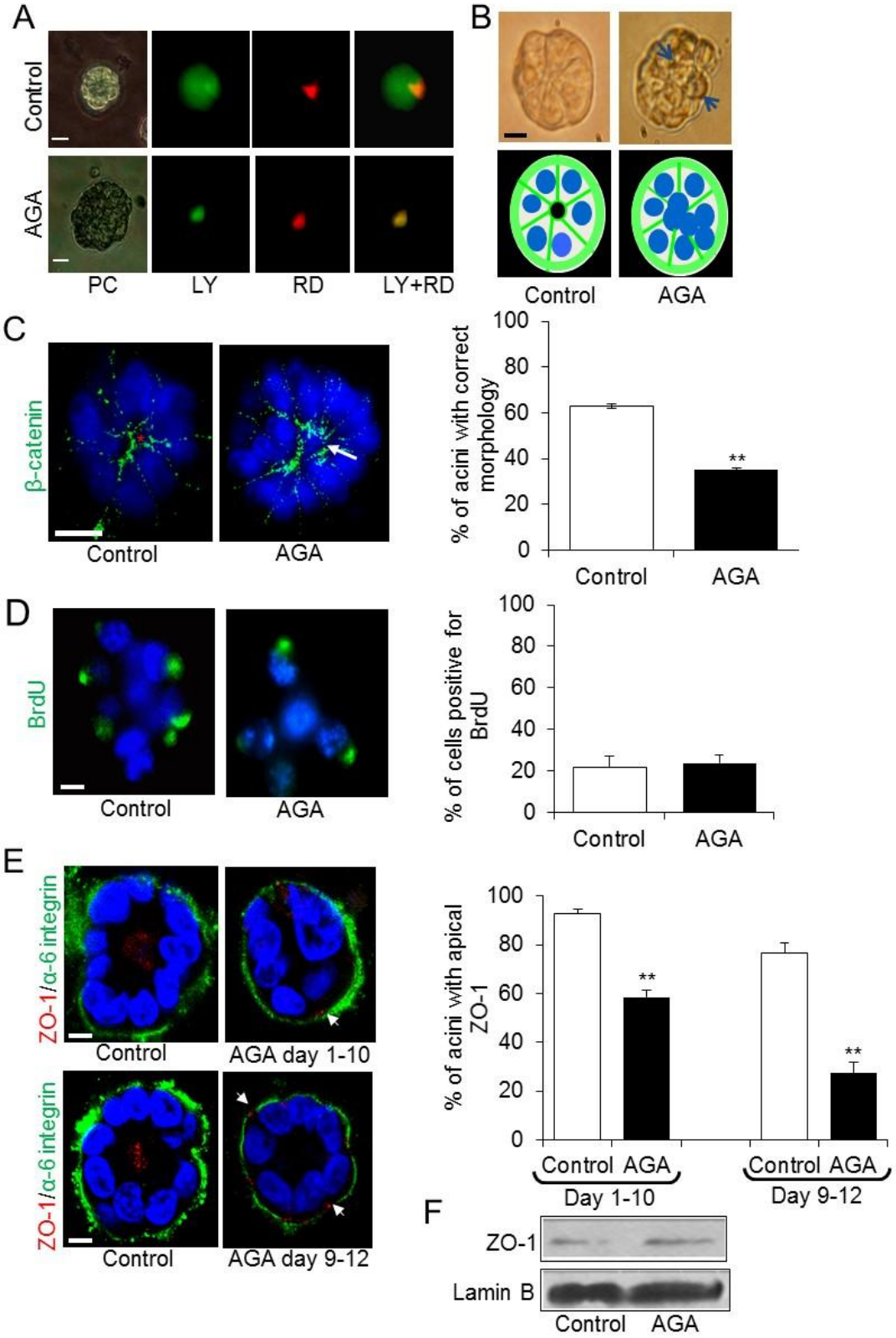

Figure 8. Loss of GJIC disrupts acinar morphogenesis and differentiation of breast luminal epithelial cells.

S1 cells were cultured in 3D for 10 days and treated with AGA (50 μM) or vehicle. A. One cell per acinus was ionophoretically microinjected with a mixture of Lucifer yellow (LY, 2.5%, w/v) and rhodamine B isothiocyanate-Dextran/RD (2%, w/v) in 0.1 mM LiCl. Cells were observed with epifluorescence microscopy 15 min following injection. Representative acini are shown. The gap junction impermeable dye, RD (red), marks the injected cells. LY diffuses throughout the acinus when gap junctions are functional. B. Phase contrast (PC) mages with accompanying drawings of a normal-looking acinus (left panel) and an acinus with cells positioned abnormally (right panel; arrows). C. Immunostaining for β-catenin (green) and DAPI (blue) to better visualize the arrangement of cells. The correct acinar morphology is defined as one layer of cells; the arrow indicates an aberrantly localized (central) cell observed on the optical section through the middle of an acinus. Histogram of the percentage of acini with correct morphology; at least 100 acini were analyzed; n = 3. D. BrdU labeling on day 4 of 3D culture. A minimum of 500 cells were analyzed per condition; n = 3. E, Dual immunostaining for apical polarity marker ZO-1 (red) and basal polarity marker α-6 integrin (green); arrows indicate the aberrant basal localization of ZO-1. Shown is the histogram of the percentages of S1 acini with apical location of ZO-1 presented; at least 100 acini were analyzed per condition; n = 3. F. Western blot for ZO-1 with Lamin B was used as loading control. Size bar= 10 μm; ** $p < 0.01$, nonpaired *t*-test. "Hibret Adissu, Thesis, Purdue University 2007".

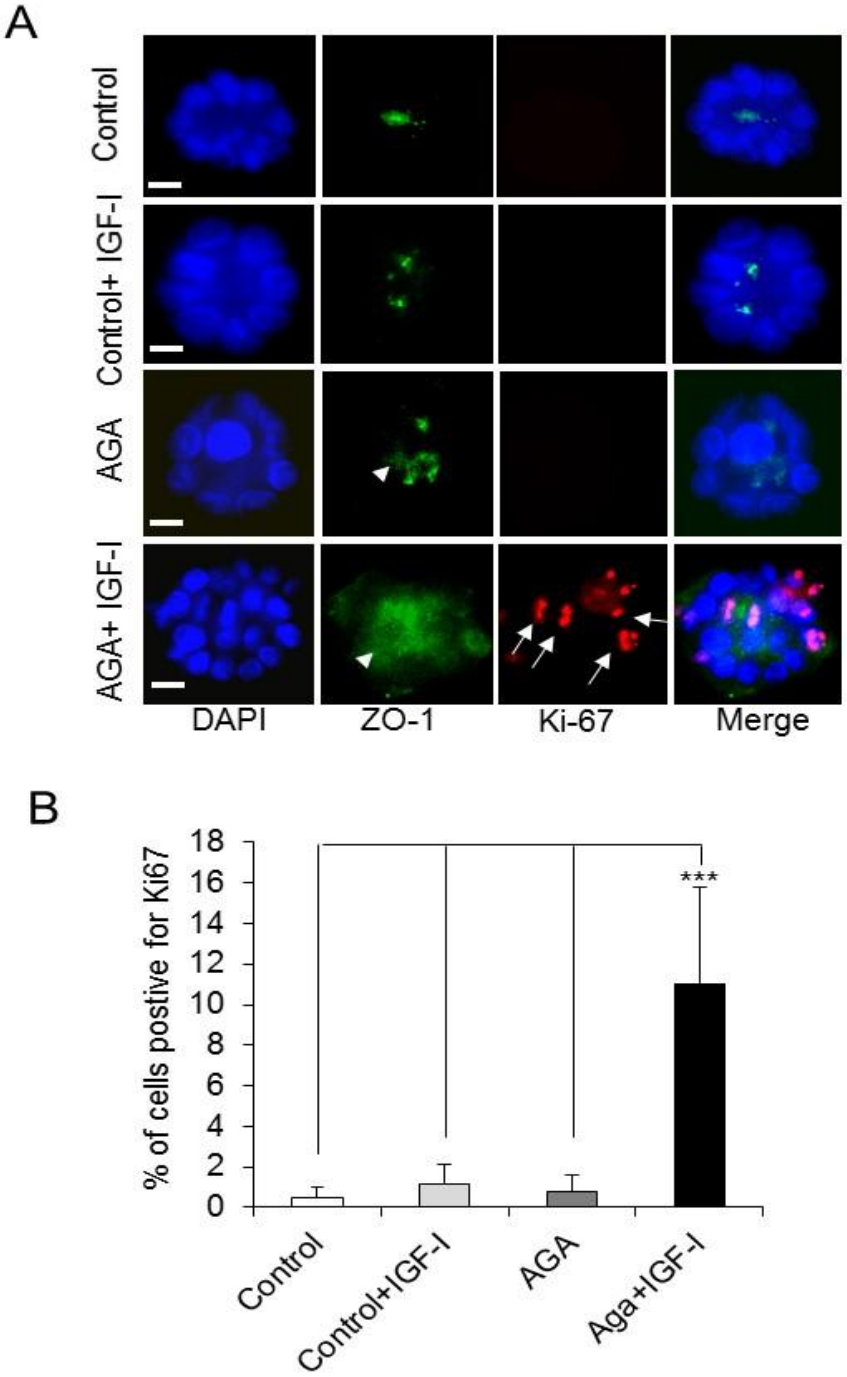

Figure 9. Acini that lack apical polarity as a result GJ blockage are primed to enter the cell cycle.

S1 cells were cultured for nine days in 3D with or without AGA followed by incubation with 100 ng/ml of insulin-like growth factor-I (IGF-I) of vehicle for 36 h. A. Dual fluorescence immunostaining for ZO-1 (green) or Ki67 (red). Nuclei are counterstained with DAPI (blue). Diffused ZO-1 localization is indicated by arrow-heads; positive intranuclear Ki67 localization is shown by arrows. B. Histogram of percentages of cells positive for Ki-67. A minimum of 1000 cells were analyzed for each condition, n = 3. *** $p < 0.001$; one-way ANOVA with Dunn's comparison. Size bar= 10 μm. "Hibret Adissu, Thesis, Purdue University 2007".

3. Cx43 controls the establishment of apical polarity

The phenotypic alterations observed upon blockage of GJIC in mammary acini are likely attributable to the disruption of Cx43 function because it was the only usual Cx found to be expressed in the luminal epithelium, and this protein has been previously involved in the maintenance of intestinal and testis epithelial homeostasis (Ey et al. 2009; Li et al. 2010). To confirm the direct implication of Cx43 in regulating mammary acinar differentiation, Cx43 was down-regulated in S1 cells via retroviral delivery of shRNA (Shao et al. 2005). The expression of Cx43 was markedly reduced (by ~70% for the mRNA and ~85% for the protein) in Cx43-shRNA cells compared to cells infected with the nonsilencing sequence (NSS) or the empty vector (EV) (Fig. 10A). Effective silencing of Cx43 was also evident based on immunostaining for the protein in cells in 2D and 3D cultures. Notably, ~65% of the acini in the shRNA group did not express Cx43; whereas, ~90% of the acini in the control groups exhibited apical location of Cx43 (Fig. 10B). The effect of silencing Cx43 on GJIC in differentiated acini was determined by microinjection of neurobiotin (NB), a fixable GJ permeable dye. Communication was inhibited in the majority (~75%) of the structures analyzed in the shRNA group as revealed by lack of NB transfer; in contrast, acini in the control group had an effective dye coupling, as demonstrated by the diffusion of NB throughout the

acini (Fig. 10C). All the acini that displayed a lack of GJIC were deficient in Cx43, as shown by immunostaining.

Similar to the inhibition of GJIC, the down-regulation of Cx43 expression was accompanied with a marked effect on acinar morphogenesis. The pie-like organization of luminal epithelial cells was lost in a significant portion of the Cx43-shRNA acini population as revealed by immunostaining for β-catenin, with ~85% of properly organized acini in the control group compared to ~40% in the Cx43-shRNA group (Fig. 10D). Silencing *GJA1* did not have any observable effect on basal polarity, as revealed by α-6 integrin distribution (Fig. S4), which was comparable to results obtained upon blockage of GJs during acinar morphogenesis; yet, apical polarity was generally compromised as measured by ZO-1 distribution. Apical localization of ZO-1 was evident in ~47% of the acini in the Cx43-shRNA group compared to ~85% of the acini in the NSS group (Fig. 10E). ZO-1 mislocalization was not accompanied by a detectable change in expression level (Fig. 10F). Thus, Cx43 influences the apical localization, but not the expression of ZO-1 in S1 cells. These data are similar to those obtained upon blockage of GJs, confirming that Cx43-mediated GJIC plays a critical role in the glandular differentiation of S1 cells.

We reasoned that if Cx43 is essential to mediate apical polarity formation in the mammary epithelium, its re-expression in non-neoplastic mammary epithelial MCF10A cells that normally harbor negligible endogenous Cx43, might improve their notorious inability to form apical polarity (Plachot et al. 2009). Following infection with a lentiviral construct coding for Cx43, MCF10A/HuCx43 cells displayed a significant amount of Cx43 protein compared to uninfected cells and cells infected with the empty vector control in both 2D and 3D culture conditions (Fig. 11A). Accordingly, there was

a significantly higher percentage of MCF10A acini (~75%) expressing Cx43 in comparison to the EV group (~15%) (Fig. 11B). Importantly, *de novo* expression of Cx43 was accompanied with an apical localization of the protein in MCF10A acini, as shown by immunostaining. Specifically, there was a higher percentage of acini with apical (~64%) compared to nonapical (~36±5%) Cx43 in MCF10A/HuCx43 (Fig. 11B). Moreover, immunostaining for ZO-1 revealed an apical localization in MCF10A/HuCx43 acini that also displayed an apically localized Cx43 as opposed to MCF10A/EV structures that showed infrequent apical localization for ZO-1. Fifty percent of MCF10A/HuCx43 acini displayed apical ZO-1 in the group with apically localized Cx43, which was significantly higher than in the group with nonapically localized Cx43 (~15%). On average 14.3% of the MCF10A/EV structures positive for Cx43 displayed ZO-1 apically localized, which represents only ~2% of the whole MCF-10A/EV population (Fig. 11C). The change in ZO-1 distribution associated with Cx43 *de novo* expression was not accompanied by striking alterations in the level of expression of ZO-1 (Fig. 11A). These data indicate that Cx43 drives the establishment of apical polarity in the mammary epithelium.

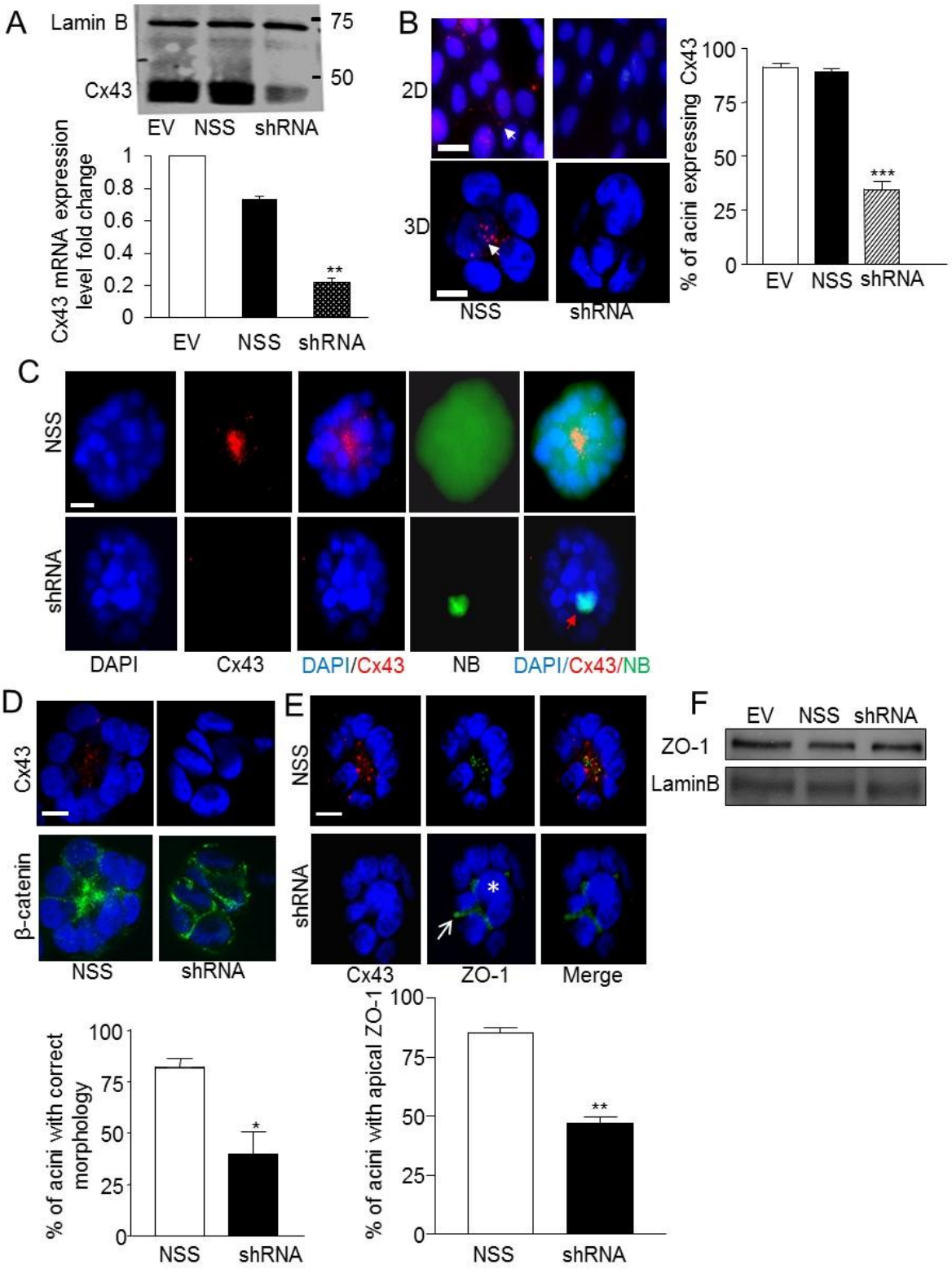

Figure 10. Down-regulation of Cx43 expression disrupts GJIC and apical polarity in S1 cells.

A. Western blot (upper image) and real-time quantitative PCR (bottom graph) for Cx43 expression in S1 cells following retroviral delivery of shRNA empty vector (EV) or non-silencing sequence (NSS) used as negative controls, or Cx43-specific shRNA (shRNA). Lamin B serves as loading control, n=3. B. Fluorescence immunostaining for Cx43 (red) in S1 cells cultured for 10 days in 2D or 3D. Nuclei are counterstained with DAPI (blue). Arrows indicate Cx43 foci. Bar graph of the percentages of acini that express Cx43. At least 200 acini were analyzed per condition, n=3. C. S1 cells expressing NSS (upper panel) or shRNA (lower panel) were cultured in 3D for ten days and microinjected with 3% NB in 0.15 M LiCl following by dual fluorescence staining with streptavidin-FITC and against Cx43 (red). Merged images show the extent of NB spread within the acini. At least 10 acini were analyzed. D. Dual immunostaining of acini for Cx43 (red) and cell-cell contact marker β-catenin. The peripheral organization of cells around a hollow center (left lower panel) is considered morphologically correct. Bar graph: percentages of acini with correct morphology. At least 100 acini were analyzed per condition, n=3. E. Dual immunostaining for Cx43 (red) and ZO-1 (green) in acini formed by S1 cells. The arrow points to the peripheral location of ZO-1 and the asterisk indicates the central location of a cell (optical section through the middle of the acinus), illustrating abnormal morphogenesis. Bar graph: percentages of acini with apical ZO-1 staining. At least 100 acini were analyzed per condition, n=3. F. Western blot for ZO-1 in 10-day old S1 acini. Lamin B was used as loading control. *** $p < 0.001$, ** $p < 0.01$, *$p<0.05$; one way ANONA, with Dunn's comparison (A,B), nonpaired *t*-test (C-E). Nuclei are counterstained with DAPI (blue). Size bar = 10 μm.

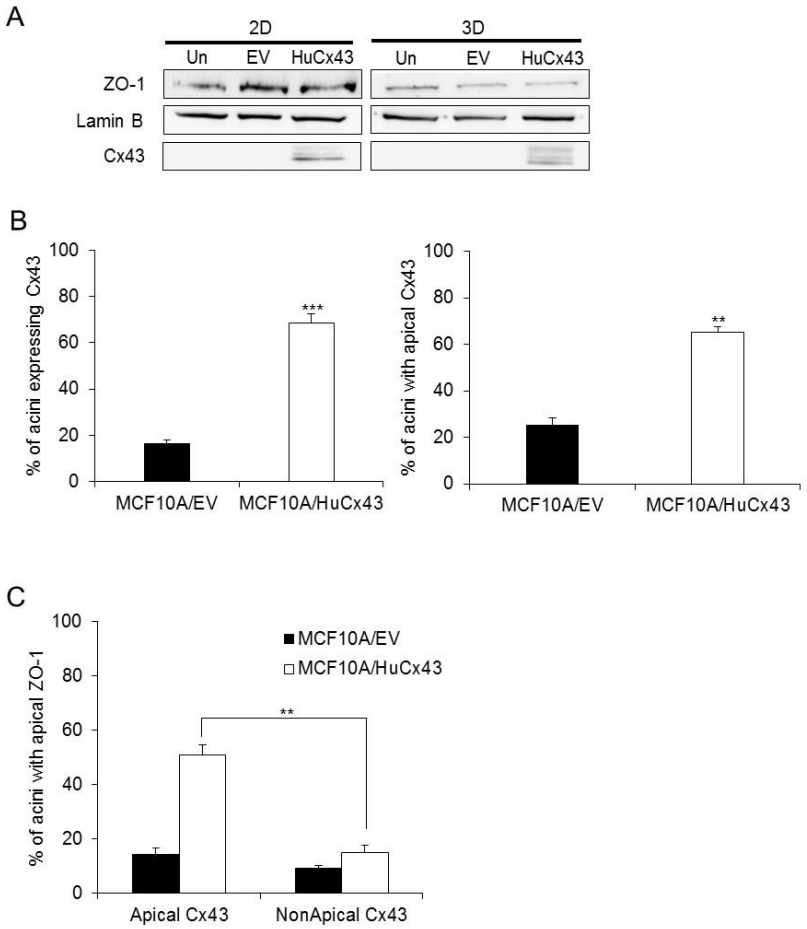

Figure 11. Cx43 expression induces the formation of apical polarity in MCF-10A Acini.

(EV) as controls, or infected with HuCx43 vector (HuCx43). Lamin B is used as loading control. B. MCF10A cells stably infected with EV (MCF10A/EV) or with HuCx43 (MCF10A/HuCx43) were cultured in 3D for 10 days to induce acinar differentiation. Bar graphs: percentages of acinar structures expressing Cx43 (left) and percentages of acinar structures with Cx43 apically localized among those expressing Cx43 (right). At least 100 acini were analyzed per condition; n = 3; nonpaired-test. C. Representative images of dual immunostaining for Cx43 (green) and ZO-1 (red) in acinar structures formed by MCF10A/EV and MCF10A/HuCx43. Nuclei are counterstained with DAPI (blue). Bar graph: percentages of acinar structures with apically localized ZO-1 among those with apical Cx-43 and nonapical Cx43. A minimum of 100 structures were scored in each condition although the number of acini analyzed was less in EV population due to the paucity of structures expressing ZO-1, n =3; one-way ANOVA with Dunn's comparison. ** $p < 0.01$, *** $p < 0.001$. Size bar= 10 µm

4. Cx43-mediated GJIC influences acinar morphology via the control of mitotic spindle orientation (MSO)

The loss of apical polarity has been linked to multilayering of cells in pathological specimens (Jones and Young 1994; Fitzgibbons et al. 1998; Tobi 1999). A recurrent observation following blockage of GJIC and silencing Cx43 in the experiments presented earlier is the disruption of acinar morphogenesis with loss of the single layering of the luminal epithelium, as exemplified by the presence of cells in the center of the acinus (see Figs. 8B-C; 9A, 10D-E). We further investigated the link between Cx43 localization and epithelial multilayering in the human breast. Immunohistochemical analysis of Cx43 in archival biopsy sections from breast cancer free women revealed that the majority of the acini (~62%) displayed apically localized Cx43 compared to basally localized Cx43 (~38%) (Fig. 12A). Furthermore, the apical localization of Cx43 significantly correlated with a monolayered luminal epithelium (Fig. 12B-C), and multilayered acini were more frequently associated with basally localized Cx43 than apically localized Cx43. We also confirmed that the loss of Cx43 expression was accompanied with a significant increase in the number of acini displaying cell multilayering upon 3D culture of S1 cells in which Cx43 was constitutively silenced (Fig. 12D).

Factors such as cell adhesion, polarity and the extracellular matrix that contribute to the formation of monolayered epithelia have been considerably investigated (Martin-Belmonte and Perez-Moreno 2012; Akhtar and Streuli 2013); however the mechanisms underlying abnormal cell multilayering remain poorly understood. We sought to investigate how Cx43 might participate in cellular organization using the 3D cell culture model of acinar differentiation. One possible explanation for multilayering is an impaired localization of cells due to compromised

adhesion. We considered this possibility since studies have revealed a significant correlation between the expression of Cx43 and E-cadherin, a central adhesion marker (Jin et al. 2010; Tang et al. 2011). We observed no significant difference, as revealed by immunostaining, in the percentage of acini displaying E-cadherin at cell-cell junctions *vs*. a cytoplasmic diffused distribution when comparing AGA-treated acini (in which the apical location of Cx43 is greatly compromised) and control acini formed by S1 cells (Fig. S5A-B) thus, ruling out loss of cell-cell adhesion as a likely cause of acini malformation.

Another possibility is that cell multilayering in the acini results from improper directional division, a known phenomenon for improper epithelial morphogenesis and disorganization of the neuroepithelium (Zheng et al. 2010; Peyre et al. 2011). In the case of the mammary gland an incorrect cytokinesis axis would be any direction that is not parallel to the substratum of the acinus. In such a situation, one or more of the daughter cells would remain in the center of the acinar structure upon completion of cytokinesis. On the contrary, division of cells tangential to the circumference of the acinus would only allow positioning of daughter cells within the layer of luminal cells and thus, increase the size of the acinus, like what occurs in lactation for instance (Fig. 13A). The orientation of the mitotic spindle was assessed in cells treated with AGA and with vehicle until day 4 of 3D culture by immunostaining for α-tubulin. Only ~35% of the mitotic cells in the AGA-treated cultures displayed proper mitotic spindle orientation (MSO), which was significantly lower than in the control group (~63% of acini with proper MSO) (Fig. 13B-C). Dual immunostaining for α-tubulin and Cx43 revealed that ~73% of acini containing mitotic cells with proper MSO (there is usually at most one mitotic cell per acinus) displayed apical Cx43, whereas only ~27% of acini

containing mitotic cells with proper MSO had Cx43 nonapically localized. Findings were similar in S1 acini treated with AGA whereby a higher percentage of acini containing mitotic cells with proper MSO displayed apically localized Cx43 (Fig. 13D). To confirm that expression of Cx43 is required for proper MSO, we also analyzed MCF10A/EV cells, that barely express Cx43, and MCF10A/HuCx43 cells in which we have shown that Cx43 expression is associated mainly with apical localization (see Fig. 11B). The MSO tangential to the circumference of the acinar structure was significantly less frequently observed (~38% of cells) in MCF10A/EV group than in the group of MCF10A/HuCx43 cells (~61% of cells) (Fig. 13E). Like in the acini formed by S1 cells there was a higher percentage of MCF10A/HuCx43 acini with mitotic cells displaying proper MSO when Cx43 was apically localized (~71%) compared to structures with nonapical Cx43 (Fig. 13F).

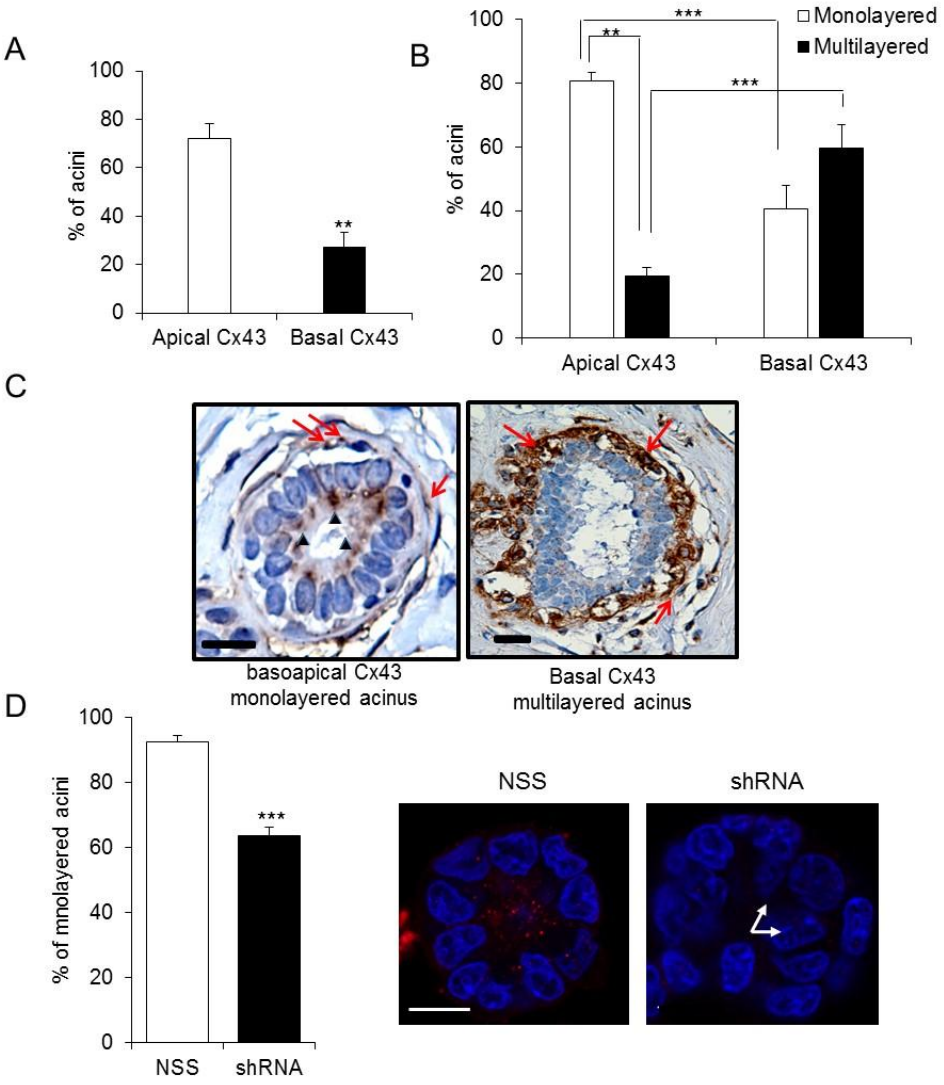

Figure 12. Absence of luminal expression of Cx43 is associated with cell multilayering.

A-C. Immunohistochemical staining for Cx43 was performed on archival breast tissue biopsy sections from twenty two women with no history of breast of cancer. A. The bar graphs show the percentages of acini with apical localization of Cx43 in the luminal epithelium. B. and the percentages of acini displaying a monolayer or a multilayer of luminal cells depending on the location of Cx43, n=22; paired *t*-test. C. Representative images of acini with basal (arrows) and apical (arrowheads) Cx43 in a normal-appearing structure and only basal Cx43 (arrows) in a multilayered structure are shown. Nuclei are stained with hematoxylin (blue). D. S1 cells stably silenced for Cx43 were cultured in 3D for 10 days and immunostained for Cx43. The bar graph indicates the percentages of monolayered acini in nonspecific sequence (NSS) control acini and shRNA-Cx43 acini. Shown are representative images of a monolayered acinus with Cx43 (red) apically localized and a multilayered (arrows) acinus lacking Cx43 staining. Nuclei are stained with DAPI. At least 100 acini were scored; n=3, nonpaired *t*-test. ** $p < 0.01$, *** $p < 0.001$. Size bar = 10 µm

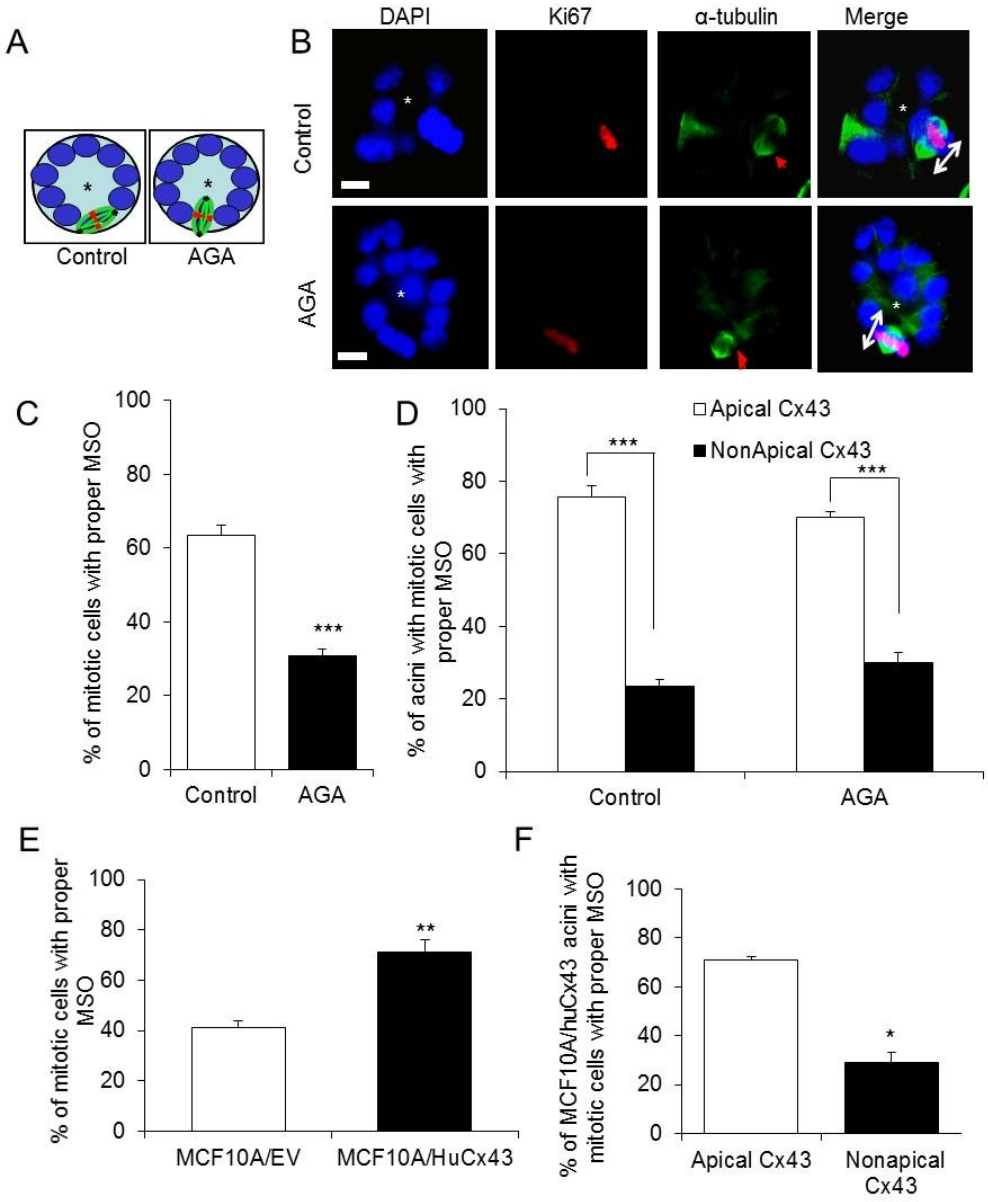

Figure 13. Cx43-mediated GJs regulate mitotic spindle orientation (MSO).

A-D. S1 cells were cultured in 3D with vehicle or with AGA up to day 4. Resulting multicellular structures were dual-immunostained for Ki-67 (red) and α-tubulin (green). MSO was analyzed based on the directionality of the α-tubulin poles, either parallel to the substratum (proper MSO to maintain a monolayered epithelium) or nonparallel to the substratum (conducive to cell multilayering) as drawn in A, and shown on representative structures in B, red arrows point to mitotic spindles and double head arrows indicate MSO; the asterisk indicates the center of the acinar structure; nuclei are counterstained with DAPI (blue). C. The bar graphs indicate the percentages of S1 cells that show 'correct' MSO; minimum of 60 cells was analyzed; n=3; nonpaired t-test. D. The percentages of S1 cells that show 'correct' MSO depending on the location of

Cx43. A minimum of 60 cells in each treatment group was analyzed; n=3; One way ANOVA, with Dunn's comparison E-F. MCF10A/HuCx43 and MCF10A/EV cells were cultured in 3D for four days and dual-immunostained for Cx43 and α-tubulin. E. The bar graphs show the percentages of cells that display 'correct' MSO; n= 3; F, and the percentages of MCF10A/HuCx43 cells that show 'correct' MSO depending on the location of Cx43. A minimum of 60 cells was analyzed; n = 3. ** $p < 0.01$, *** $p < 0.001$; nonpaired *t*-test. Size bar = 10 µm

It has been established that β1-integrins regulate MSO in many cell types including epithelial cells (Thery et al. 2005; Reverte et al. 2006, Toyoshima and Nishida 2007). A PI3-kinase (PI3K) dependent pathway through which β1-integrins regulate MSO was uncovered in normal and cancer cells. Specifically, β1-integrins activated PI3K which, in turn, lead to the accumulation of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the midcortex of the cell; consequently, orienting the mitotic spindle parallel to the substratum (Toyoshima and Nishida 2007; Toyoshima et al. 2012). Since β1-integrin is part of the making of mammary acini (Berdichevsky et al. 1994), we investigated the potential role of this adhesion molecule in regulating MSO in the breast epithelium. Upon incubation of S1 cells with 15 µM of AIIB2, a function blocking antibody against β1-integrin (Damsky et al. 1992) from days 4 till 7 of 3D culture, there was no significant alteration of MSO compared to acini treated with 15 µM of nonspecific IgG. In addition, inhibition of both GJs and β1-integrin (AGA+AIIB2 treatment) resulted in a significant loss of tangential orientation of the mitotic spindle similar to when only GJs were blocked (Fig. S6), suggesting a prominent role for Cx43-mediated GJ in MSO in the breast epithelium. We also investigated the involvement of PI3K, not as part of a β1-integrin-dependent MSO controlling pathway anymore, but potentially as part of the Cx43-dependent MSO controlling pathway. Indeed, it is well established that PI3K is involved in the MSO

parallel to the cell layer. Integrins localize PI3K in the equatorial part of the plasma membrane to enrich PIP3 which, in turn, recruits dynactin allowing a cortical dynein-dynactin motor complex to pull on the astral microtubules and orient the spindle correctly (Toyoshima et al., 2007). Interestingly, Cx43 can indirectly activate PI3K in cardiomyocytes to protect them against necrosis (Ishikawa et al. 2012). Inhibition of PI3K in S1 cells up to day 4 of 3D culture with 1 μM of LY294002 induced a significant decrease in the percentage of cells with proper MSO (i.e., parallel to the substratum) (~32% of cells with LY treatment compared to ~68% of cells in the control group) as shown with α-tubulin immunostaining (Fig. 14A), suggesting that PI3K is an important player in MSO also in the mammary epithelium. The percentage of cells with proper MSO in the group treated with both LY294002 and AGA (~34%) was comparable to the groups treated only with AGA (~32%) or with LY294002 (~32%) potentially, suggesting epistasis between the two pathways. This hypothesis was confirmed by measuring the expression levels of p-Akt (a downstream target of PI3K) under the different treatment conditions. Western blot analysis revealed that p-Akt was significantly down-regulated and to the same extent in the AGA-treated group and in the LY294002-treated group compared to control; combined AGA and LY294002 treatments further down-regulated p-AKT (Fig. 14B). Thus, blocking GJs inhibits PI3K, consequently disrupting the PI3K-dependent pathway regulating proper MSO.

Among the top candidates that might act as effector proteins downstream of the PI3K to control MSO is aPKCζ. This kinase is part of the par6/aPKC polarity complex; once activated by cdc42 it excludes NuMA/LGN, a major protein complex controlling MSO, from the apical domain of epithelial cells during mitosis (Zheng et al. 2010; Durgan et al. 2011). S1 acini were treated from days 1 to 4 with 5 μM aPKC

pseudosubstrate inhibitor to inhibit aPKCζ activity. As revealed by immunostaining for α-tubulin, the treated group of acini had only 36% of cells with proper orientation of the mitotic spindle as opposed to 75% in the control group (Fig. 14C). Dual immunostaining for Cx43 and NuMA proteins in AGA-treated acini showed that GJ inhibition resulted in a significant decrease in the number of cells with proper MSO measured by the presence of NuMA at the mitotic spindle pole (~24% of the cells with MSO parallel to the substratum in AGA treated group compared to ~78% in the untreated group) (Fig. 14D). Furthermore, in the control group, a majority of cells with properly oriented mitotic spindle were associated with apical Cx43 in the acini. If Cx43 helps orient MSO via an impact on NuMA we reasoned that their respective location in mitotic cells might reveal such relationship. To get a clearer indication of this potential spatial relationship, we cultured S1 cells as a monolayer on a flat surface with induction of polarity via laminin 111 coating of the culture surface (Plachot 2009; Grafton 2011). Cx43 staining at cell-cell contacts was observed predominantly 'against' NuMA staining of the spindle poles when MSO was parallel to the substratum (~91% of mitotic cells). On the other hand, when Cx43 staining appeared diffuse, the majority of the cells displayed a random MSO based on NuMA staining (Fig. 14E).

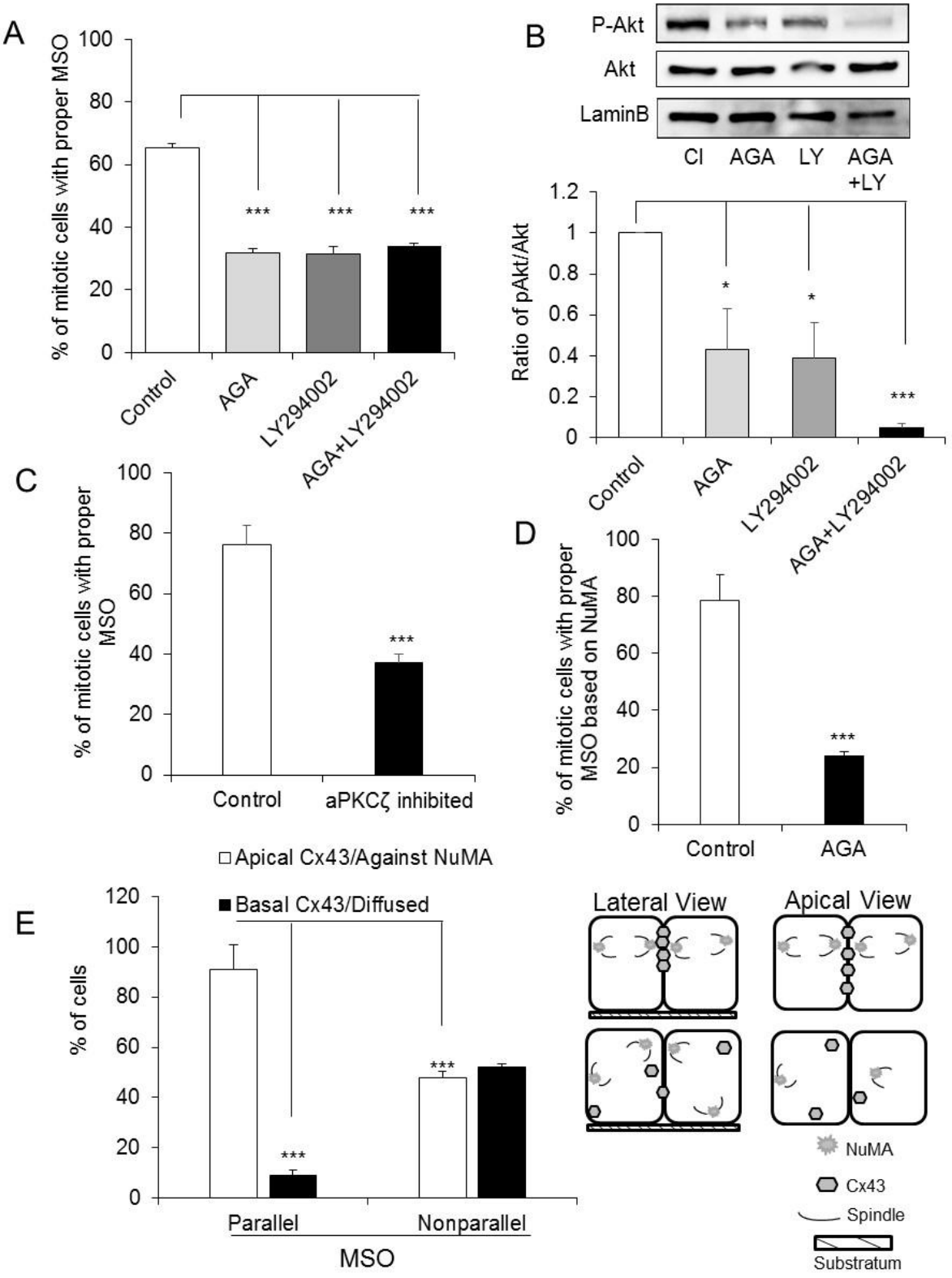

Figure 14. Regulation of MSO by Cx43 is via the PI3K-pAKT-NuMA pathway.

A-B. S1 cells were treated with vehicle (control) or with AGA, or with 1 μM of PI3K inhibitor LY294002, or with LY294002+AGA from day 0 till 4 in 3D culture. A. The

bar graphs represent the percentage of cells with proper MSO (i.e., parallel to the substratum) based on immunostaining for α-tubulin, a minimum of 60 cells was analyzed. B. The ratios of downstream effector of PI3K, p-Akt, over Akt relative to the control group based on quantification of western blot bands; n=3, one-way ANOVA, with Dunn's comparison. C. S1 cells were treated with vehicle (control) or with 5 μM of aPKCζ pseudosubstrate inhibitor, up to day 4 in 3D culture. The bar graph represents the percentage of acini with proper MSO based on α-tubulin immunostaining and analysis of a minimum of 60 cells; n=3; nonpaired *t*-test. D. S1 cells were cultured in 3D conditions with vehicle (control) or with AGA. On day 4, structures were dual immunostained for NuMA and Cx43. The bar graph shows the percentage of cells with proper MSO based on NuMA location at the spindle pole; n=3; nonpaired *t*-test. E. S1 cells were cultured on laminin 111, a substratum conducive to polarity induction while cells organize as a flat monolayer, until day 4 when they were dual-stained for NuMA and Cx43. For each dividing cell, the relative localization of Cx43 and NuMA was assessed. For each NuMA localization pattern (parallel versus nonparallel) the corresponding Cx43 localization, within the same cell, was determined; either being apicolateral or diffused throughout the membrane (schematic). The bar graph shows the percentages of cells with NuMA-stained mitotic spindle pole against Cx43 foci depending on MSO; n=3; paired *t*-test. * $p < 0.05$, *** $p < 0.001$. Size bar= 10 μm

E. Discussion

The contribution of apical Cx43 to various aspects of mammary epithelial differentiation suggests a tumor initiation preventive role for this protein. Here, we showed for the first time that Cx43 is present in the luminal breast epithelium where it is apically localized. We demonstrated that Cx43-mediated GJIC is vital for the establishment of apical polarity and, consequently participates in the control of the cell cycle as a mechanism to prevent cancer onset. We have also revealed that the localization of Cx43 is associated with cell multilayering, as we identified its novel role in regulating the proper mitotic spindle orientation via a PI3K-dependent pathway.

Thus far, Cx43, as part of the GJ, has been widely established to be a junctional protein found at cell-cell contact and mainly expressed in the myoepithelial compartment of the mammary gland (Monaghan et al. 1996). In addition to Cx26 which is the only Cx reported in the human luminal mammary epithelium *in vivo*, we

introduced Cx43 as a major mediator of luminal epithelial homeostasis. Our immunohisto- and cytochemical analysis in tissue samples of normal human breasts and in our S1 3D *in vitro* model, revealed a distinct apical localization of Cx43 at cell-cell contact. Cx43 has been previously reported to be apical in two major epithelia, the intestine and the lens (Gao and Spray 1998; Ey et al. 2009). In the lens, Cx43 and Cx50 are abundantly expressed at cell-cell contact and apical in epithelial cells where they form functional GJs and hemichannels (Donaldson et al. 1994; White et al. 2001). While the *in vivo* deletion of Cx43 did not affect the development of the lens and has not been linked to pathologies and lens abnormalities, Cx50 demonstrated a major role in epithelial development (White et al. 2007; Calera et al. 2009). In addition, in Caco-2 cells, intestinal epithelial cell line, Cx43 forms functional GJs at the lateral domains of the plasma membrane. Interestingly; however, only upon bacterial infection was Cx43 found to localize apically where it formed functional hemichannels, along with the lateral GJs to contribute to the observed release of ions and electrolytes associated with diarrhea during infection (Ey et al. 2009; Guttman et al. 2009). Unlike its minor role and redundancy with other Cxs in the lens epithelium and its infection-induced apical location in the intestinal epithelium, we showed that Cx43 is found apicolateral in the human breast tissue under normal conditions; whereby its deletion or loss of function disrupted mammary epithelial differentiation. This finding raised an intriguing possibility that the formation and/or maintenance of differentiation characteristics of the breast tissue could be influenced by the location of Cx43. A critical aspect of epithelial differentiation is the establishment and maintenance of basoapical polarity (McCaffrey and Macara 2011). The epithelial polarity program has long been introduced in the literature in the context of the formation and maturation of cell junctions such as, TJs

and AJs that in turn maintain polarity, with no emphasis on GJs in such a process. Apical polarity is marked by the presence of TJs that separate the apical and basolateral domains (Rodriguez-Boulan and Nelson 1989; Cereijido et al. 1998), and is regulated by a conserved group of core polarity proteins, Crumbs, Par and Scribble complexes that define the cell domains through distinct localization and complex interactions (reviewed in Bazzoun et al. 2013). Polarity starts upon the establishment of cell–cell contacts, a required step for the subsequent formation of TJ (Miyoshi and Takai 2005; Yeaman et al. 2004). Then, JAM and Nectin, adhesion molecules, recruit Par3 that binds Cdc42 and aPKC and leads to TJ formation (Joberty et al. 2000; Etienne-Manneville and Hall 2003). Our observation that Cx43 is apical in the proximity of TJs in the mammary epithelium, that only properly functions when polarized, suggested a possible role for this protein in regulating polarity and in particular, apical polarity. We have revealed that whether Cx43-mediated GJIC is inhibited or *GJA1* is silenced, there is an evident loss of apical polarity as indicated by the mislocalization of ZO-1. In fact, our data revealed that Cx43-mediated GJIC controls not only the maintenance of apical polarity, but also its *de novo* formation regardless of the developmental and differentiation stage of the epithelium. To the best of our knowledge, there are no reports that link GJs to apical polarity, however there is an emerging cross-talk between GJs and TJs where Cxs interact with and induce the formation and expression of TJ proteins such as, occludins, claudins and ZO proteins (Kojima et al. 2002; Morita et al. 2004; Nagasawa et al. 2006; Talhouk et al. 2013; Zemljic-Harpf et al. 2014). The regulation of apical polarity by Cx43-mediated GJIC implied a cell cycle regulatory role of apical Cx43 in the mammary epithelium since apical polarity is recognized as a major restraint on epithelial cell proliferation and a modulator of cell quiescence (Balda et al.

2003; Chandramouly et al. 2007; Cong et al. 2010). Cell proliferation continues in many epithelial tissues even after growth is complete, which opens a window for events leading to cancer initiation to occur. In fact, our data indicated that the inhibition of Cx43-mediated GJIC, in the presence of mitogenic factors or nuclear architecture disruptive events, predisposed the cells to readily enter the cell cycle, a phenomenon considered necessary for tumorigenesis.

Apical polarity disruption not only promotes cell cycle entry, but also influences the direction of cell division. Reports have shown that the depletion of apical polarity proteins such as, Par3 and aPKC resulted in improper mitotic spindle orientation and the formation of multilayered mammary ducts with no luminal space (McCaffrey et al. 2012; Xue et al. 2013; Kojima et al. 2008). In this study, we unprecedentedly identified that Cx43-mediated GJIC regulates the direction of cell division as revealed by a random orientation of the mitotic spindle in GJ blocked S1 acini compared to being parallel to the substratum in the control ones. The mechanism of mitotic spindle orientation in the mammary gland has not been well characterized, yet it is demonstrated that luminal cells divide within the plane of the epithelium (Villegas et al. 2014). Several studies showed that cell-ECM interactions and in particular, those mediated by β1-integrins, play an important role in the organization and orientation of the mitotic spindle (Fernández-Miñán et al. 2007). In β1-integrin mutant mammary epithelium, the orientation of the division plane was random with numerous cells dividing perpendicular to the basement membrane. Thus, a lack of β1-integrins leads to disruptions in the orientation of the division axis in basal cells (Taddei et al. 2008; Akhtar et al. 2013), but not in the luminal layer of the mammary epithelium as our data demonstrated. Here, we delineated a PI3K dependent pathway through which Cx43-

mediated GJIC regulates directionality of cell division. It is known that NuMA/LGN complex forms a belt at the lateral cortex of epithelial cells where it binds to the astral microtubules and orient the spindle parallel to the basement membrane (Johnston et al. 2009). This complex is excluded from the apical cortex by aPKC that is activated by Cdc42-GTP (Jaffe et al. 2008). Our findings illustrated that PI3K which activates Cdc42, had decreased kinase activity as a result of GJ inhibition, with aPKCζ being downstream in this pathway. Interestingly, we revealed that in dividing cells, NuMA was found located parallel to the substratum when Cx43 was apicolateral, in other words, lies perpendicular to the plane of the spindle containing NuMA at the poles. However; when Cx43 was diffused, NuMA displayed a random orientation, further illustrating the tight link between proper orientation of the mitotic spindle and the apical location of Cx43. This finding suggested that besides guiding the orientation of the mitotic spindle via PI3K, Cx43 might also be involved, during later stages of mitosis, in the maintenance of the spindle orientation by possibly contributing to the proper location of NuMA. Moreover, immunohistochemical analysis of normal human breast tissues supported the correlation between apical Cx43 and the maintenance of monolayered luminal epithelium *in vivo*. The direction of cell division i.e., the orientation of the mitotic spindle, is a critical determinant of the epithelial tissue architecture and consequently its normalcy. For example, the earliest stages of breast tumorigenesis involve the loss of lumen formation and multilayering that characterize premalignant breast lesions such as breast ductal hyperplasia (Godde et al. 2014). Indeed, mitotic spindle misorientation has been suggested to contribute to several aspects of cancer progression be it increasing aneuploidy, facilitating tissue disorganization and expanding the cancer stem cell pool (Caldwell et al. 2007; Qin et al.

2010; Quyn et al. 2010). Although misoriented spindles have been seen in several tumor models, not all tumors show spindle misorientation and not all spindle misorientation events are tumorigenic (Fleming et al. 2009; Fischer et al. 2006; Patel et al. 2008). Yet, it is conceivable that it might play a considerable synergistic role with other changes that occur at various stages of tumorigenesis.

It is evident, from our data, that Cx43 apical localization contributes to the 3D organization machinery that the cell uses to establish polarity, yet how it does that remains largely unknown, especially with the lack of reports on the impact of GJs on polarity. We can speculate that GJs might mediate this role early on when cells contact one another by enabling the exchange of signals involved in polarity initiation between neighboring cells. This is exemplified in adult epithelial tissue of *Drosophila* that is characterized by the formation of tissue polarity which is a second polarity axis in the plane of the epithelium. Tissue polarity is known to be locally mediated across the tissue by the passage of polarity signals through direct cell-cell interactions (Park et al. 1994). This observation could be extrapolated to a potential role of GJs, mediators of cell-cell communication, in mammalian epithelia, as it could be essential for the transfer of polarity cues from one epithelial cell to the other to initiate the formation of the epithelial polarity axis. On the other hand, GJs might be involved in later stages of basoapical axis formation, possibly by regulating core polarity and signaling molecules such as polarity lipids. The polarized segregation of proteins and their confinement in distinct membrane domains is partly mediated by polarity lipids such as, PtdIns(3,4,5)P3 (PIP3) and PtdIns(4,5)P2 (PIP2). Gain and loss of function experiments showed that PIP3 confers its identity on the lateral membranes in the vicinity of TJs, whereas PIP2 identifies the membrane domains facing the lumen (Gassama-Diagne et

al. 2006; Martin-Belmonte et al. 2007). The polarization of PIPs is regulated by the recruitment and activation of PI3K (synthesizes PIP3) to the lateral domains and the enrichment of phosphatase PTEN (degrades PIP3 to PIP2) at the apical membrane facing the lumen, implying the importance of both PTEN and PI3K regulation in maintaining lipid segregation. Having said that and given our finding that Cx43-mediated GJIC regulates PI3K activity, one would expect that Cx43 contributes to the maintenance and formation of the pool of PIPs at the different membrane domains, consequently regulating polarity. It is also possible by being apical, Cx43 could physically interact with polarity proteins such as members of Par and Crumbs group and influence their localization; similar to JAM and Nectin's (as being apical membranous proteins) role in recruiting Par3 at the apical domains early on during polarity initiation (Joberty et al. 2000). Both apical polarity and Cx43 are lost early in cancer development, it may be proposed that the down-regulation or mislocalization of Cx43 sets the stage for hyperplastic changes in the presence of permissive microenvironmental cues.

F. Supplementary Figures

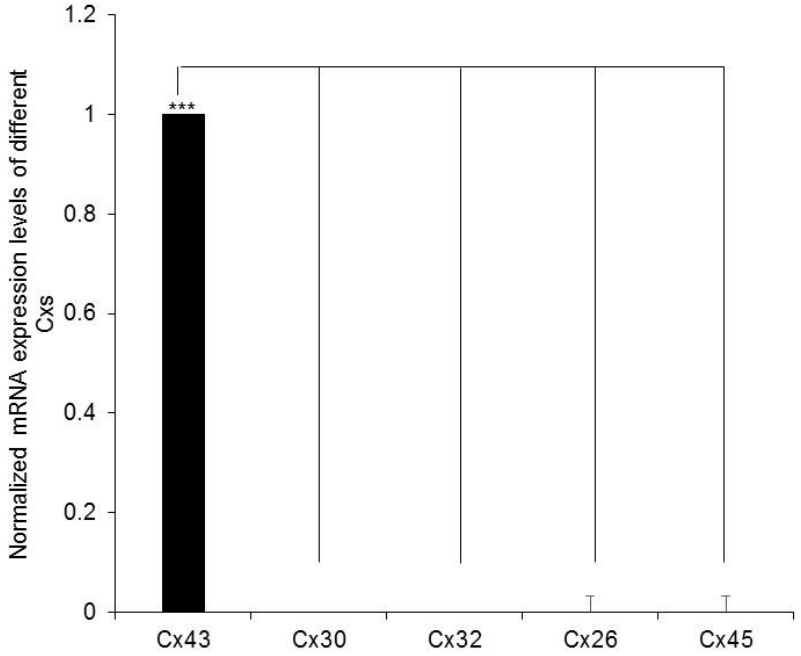

Figure S 1. Characterization of Cxs in S1 cells.

Real-time PCR analysis for the expression of Cx43, 30, 32, 26 and 45 using their corresponding forward and reverse primers in S1 acini (day 10). Histogram represents the normalized expression levels of the different Cxs against GAPDH. n=3 one-way ANOVA with Dunn's comparison. *** $p < 0.001$.

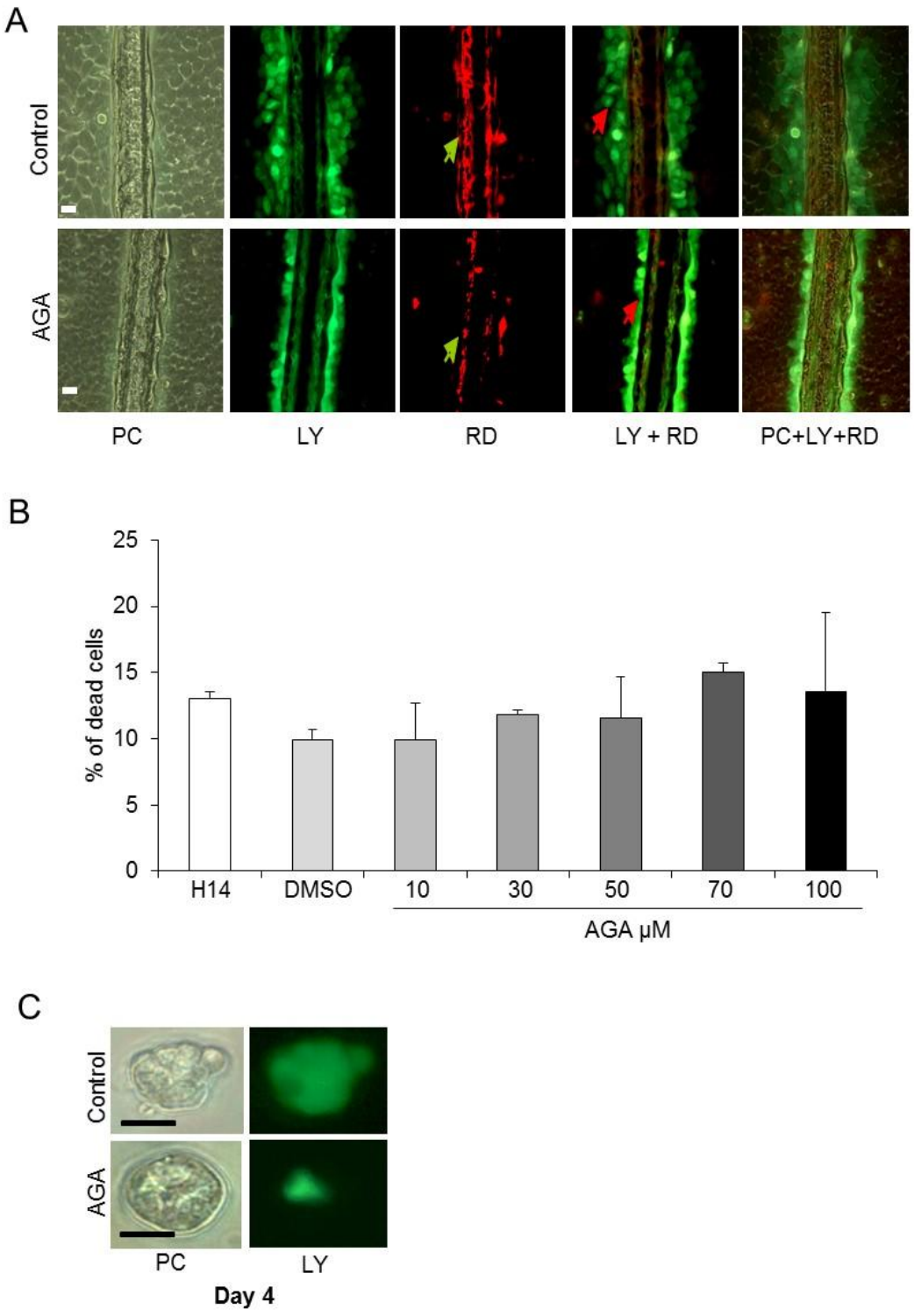

Figure S 2. Assessment of GJ.

A. S1 cells were plated as monolayer in presence of gap junction blocker 18-alpha-glycyrrhetinic acid (AGA), with medium replaced every 48 h. On day 10, scrape loading of a dye mixture of LY (green) + RD (red) was performed, with dye coupling for 5 min followed by incubation in paraformaldehyde and epifluorescence microscopy

analysis within 5 min following dye-loading. Restriction of LY to a single line of scraped cells in the AGA treated group is indicated by a red arrow (bottom fourth panel, AGA treatment). Spreading of LY (red arrow) to adjacent cells beyond the scraped cells marked by RD is indicated by a red arrow (upper fourth panel, control). B. Histogram of the percentage of dead cells (trypan blue staining) upon treatment with different concentrations of AGA in 2D culture of S1 cells. H14 and DMSO groups are controls, n=3, one-way ANOVA with Dunn's comparison. C. One cell per acinus was ionophoretically microinjected with a mixture of Lucifer yellow (LY, 2.5%, w/v) and rhodamine B isothiocyanate-Dextran/RD (2%, w/v) in 0.1 mM LiCl. Cells were observed with epifluorescence microscopy 15 min following injection. Representative acini are shown. The gap junction impermeable dye, RD (red), marks the injected cells. LY diffuses throughout the acinus when gap junctions are functional. PC = phase contrast. Size bar = 10 µM. "Hibret Adissu, Thesis, Purdue University 2007".

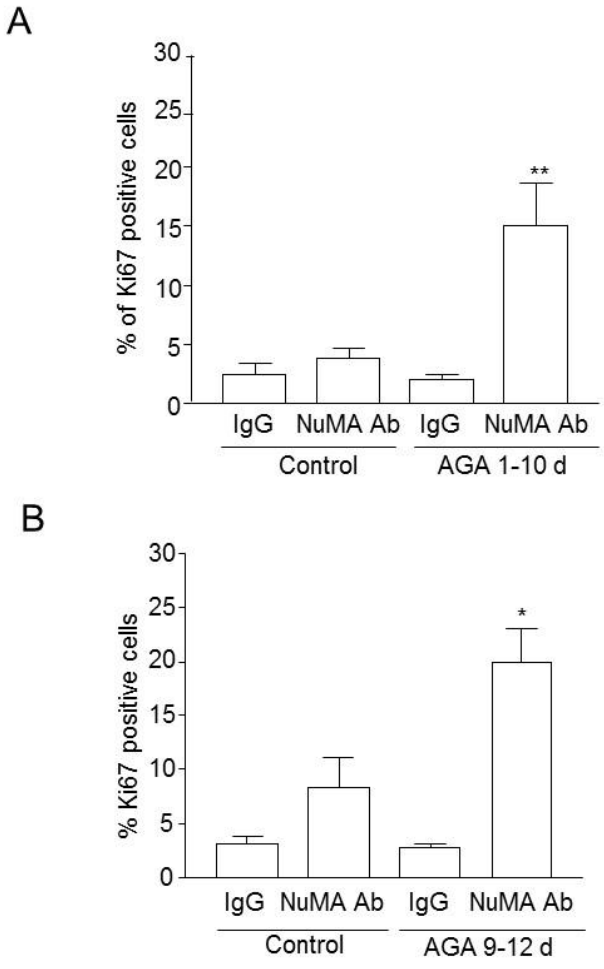

Figure S 3. Effect of NuMA alteration on acini with compromised GJ.

A. S1 cells were cultured in 3D with vehicle or AGA for 9 days followed by permeabilization with digitonin and incubation with NuMA antibodies (NuMA Ab) or a nonspecific immunoglobulin (IgG) from days 9 to 12. B. S1 cells were cultured in 3D for nine days to produce basoapically polarized acini (control). From days 9 to 12, acini were treated for three days with NuMA Ab and AGA, IgG and AGA, NuMA Ab only, or IgG only. Under all conditions cell cycle activity was assessed by immunostaining for Ki-67 on day 12. Bar graph: percentage of cells positive for Ki67. A minimum of 500 cells were scored in each condition. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Dunn's comparison. "Hibret Adissu, Thesis, Purdue University 2007".

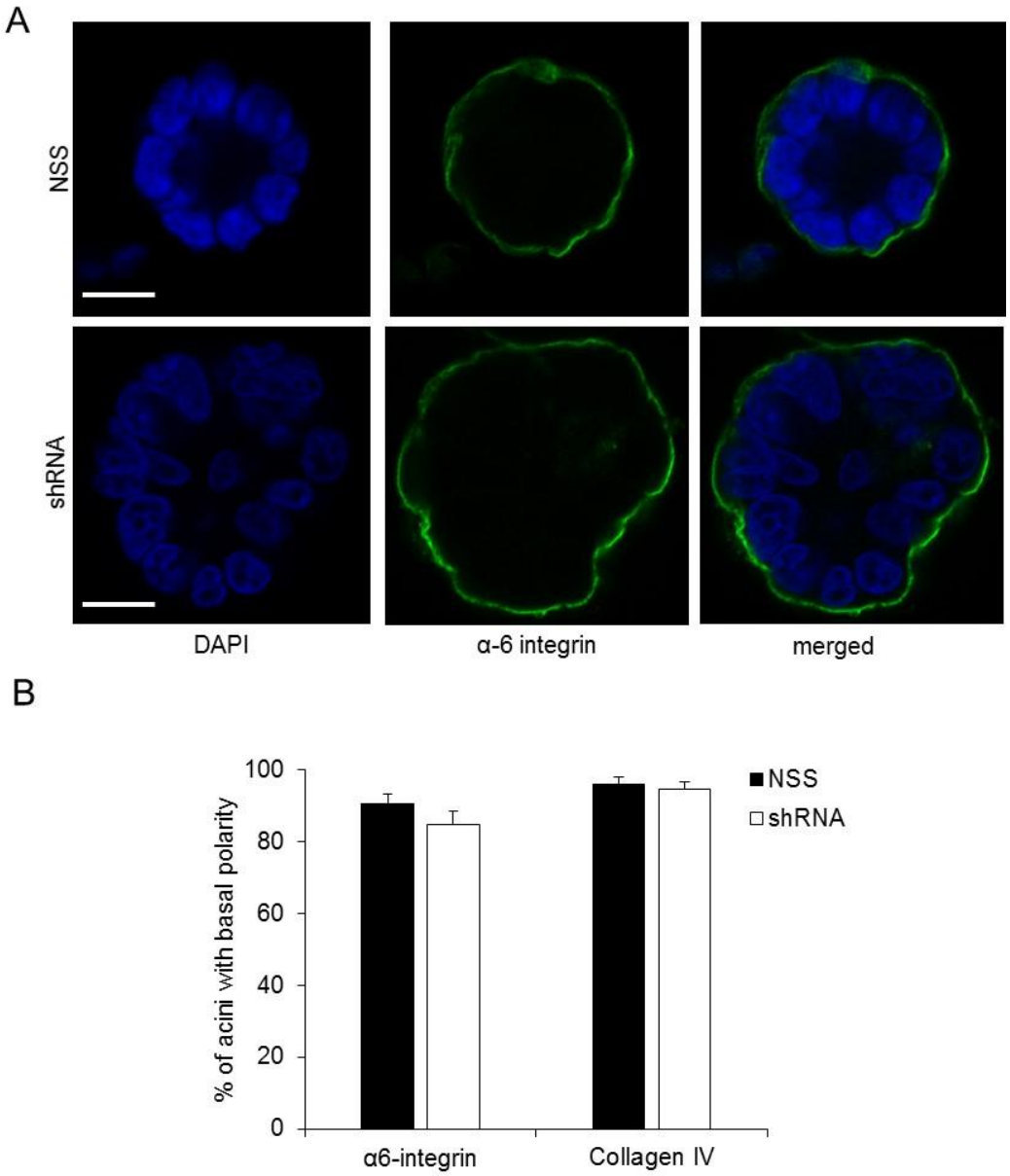

Figure S 4. Impact of Cx43 silencing on basal polarity.

A. Representative acini of both Cx43-shRNA and nonspecific sequence (NSS) groups immustained for α6-integrin (green) with nuclei stained with DAPI (blue). B. Bar graphs of the percentages of acini with basal polarity based on α6-integrin localization or on collagen IV localization. Size bar= 10 μm.

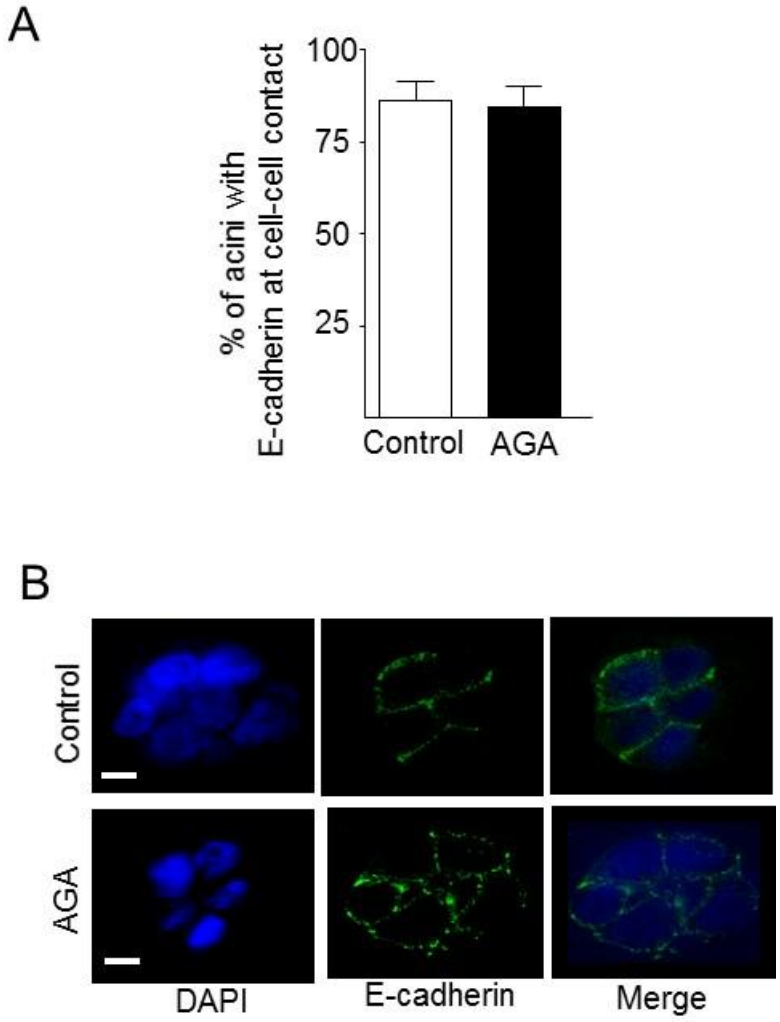

Figure S 5. Impact of GJ blockage on cell-cell adhesion.

S1 cells were treated with AGA in 3D culture for ten days and immunostained for cell-cell contact marker E-cadherin (green). **A.** The bar graph indicates the percentages of acini with E-cadherin strictly at cell-cell contacts (i.e., in-line staining pattern around each cell without diffuse staining). **B.** Representative images are shown. Nuclei were counterstained with DAPI (blue). A minimum of 100 acini were scored; n=3; Size bar= 10 μm. "Hibret Adissu, Thesis, Purdue University 2007".

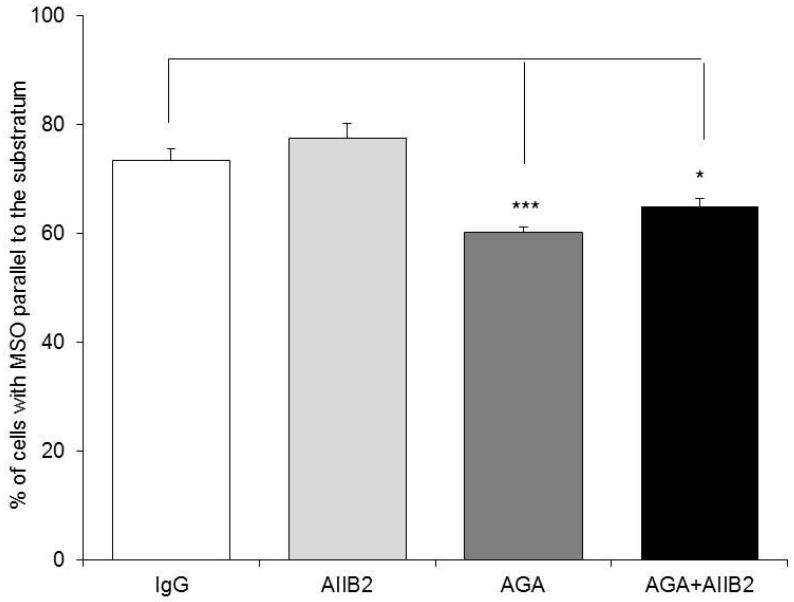

Figure S 6. Impact of β1-integrin on MSO in S1 cells.

S1 acini were treated with non-specific IgG (15 μg/ml), AIIB2 (15 μg/ml), AGA, and a combination of AGA and AIIB2 from days 4 to 7 followed by immunostaining for α-tubulin to assess MSO. The bar graph shows the percentages of cells with proper MSO (i.e., parallel to the substratum) in a minimum of 60 cells; n=3; * $p < 0.05$, *** $p < 0.001$, one-way ANOVA with Dunn's comparison test. "Hibret Adissu, Thesis, Purdue University 2007".

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CHAPTER IV

CONNEXIN 43 LOSS DISRUPTS POLARITY AND INITIATES TUMORIGENIC PATHWAYS IN PHENOTYPICALLY NORMAL BREAST EPITHELIUM

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A. Abstract

Studies suggest that Connexin (Cx) 43, a gap junction (GJ) protein, acts as a tumor suppressor in mammary epithelium, potentially through the promotion of functional and phenotypic differentiation. However, the role of Cx43 in tumor suppression is not fully elucidated. In this study, HMT-3522 S1 (S1) nontumorigenic breast epithelial cell line was used to decipher the mechanism through which the loss of Cx43 contributes to tumor initiation and the disruption of homeostasis of the mammary epithelium. Cx43 was found to be apically localized in three dimensional (3D) structures of S1 cells (S1 acini) (Hibret Adissu, Thesis, Purdue University 2007). Its stable shRNA mediated silencing, was marked by the inhibition of GJ intercellular communication in 3D culture of S1 cells. Moreover, apical polarity was disrupted in ~60% of acini upon Cx43 silencing, which correlated with mislocalization of Scrib, regulator of apical polarity. This was associated with a mislocalization of β-catenin, a Cx43-associated protein, away from the GJ complex towards basolateral sites of the membrane. In addition, there was ~50% increase in the proliferation rate, ~50% decrease in the percentage of cells in G0/G1 phase of the cell cycle and around two-fold increase in matrigel-invading potential of Cx43 silenced S1 cells compared to the

control. Loss of apical polarity was accompanied by improper acinar morphogenesis and a defect in the ability of S1 cells to form monolayered acini. Mitotic spindle orientation in multilayered acini of Cx43 silenced cells was disrupted along with a significant decrease in PI3K activity. Furthermore, subcutaneous injection of Cx43 silenced S1 cells resulted in significant tumor growth compared to control cells in NSG immunodeficient mice. We propose that Cx43 loss can initiate early signs of tumorigenesis by altering pathways involved in proper architecture and differentiation of the mammary epithelium.

B. Introduction

Gap junctions (GJs) are located at cell-cell contacts and equipped with molecular features that enable direct exchange of signaling molecules. They mediate various functions such as transmission of action potential and diffusion of metabolites, nutrients and second messengers below 1500 Da in size, such as 1,4,5-inositol-trisphosphate, calcium ions, and cyclic nucleotides, hence participating in the modulation of gene transcription, proliferation and apoptosis (Guttman and Finlay 2009). Their building blocks, connexins (Cxs), are no longer viewed as solely performing a channel role, but rather as signaling nodes that impact both normal and cancer cell functions, as they interact with cytoskeletal elements, junctional and polarity proteins (Dbouk et al. 2009; Bazzoun et al. 2013). While GJs have been established as essential for the functional development of the mammary gland, their role in breast cancer initiation remains poorly explored.

GJs were originally described between epithelial cells in the mammary gland of virgin, pregnant and lactating mice (Pitelka et al. 1973). Cx26, Cx32 and Cx30 are

present in the luminal epithelial cells while Cx43 is primarily expressed between myoepithelial cells in the murine mammary gland (El-Sabban et al. 2003; Talhouk et al. 2005). In the human breast, myoepithelial cells express Cx43 at cell-cell contact, and very recently, we reported the expression of Cx43 in luminal epithelial cells both apically and at cell-cell contact (Monaghan et al. 1996; submitted Bazzoun et al. 2015). In addition, luminal cells minimally express non-membranous diffused Cx26, which seems to form nonfunctional GJs and, when lost, does not significantly tone down developmental and functional roles of the mammary gland (Laird et al. 1999; McLachlan et al., 2007; Stewart et al. 2014). On the other hand, Cx43 is highly expressed during all stages of mammary gland development suggesting that it may have important roles in differentiation. Interestingly, in human breast cancer tissues, Cx43 is commonly down-regulated compared to the nearby nonneoplastic tissue (Laird et al. 1999). It is also reported to be down-regulated in many mammary carcinoma cell lines, suggesting that Cx43 plays a role in maintaining cell differentiation and preventing transformation (Lee et al. 1992; Tomasseto et al. 1993; Hirschi et al. 1996). Conversely, when Cx43 was overexpressed in mammary carcinoma cells, tumor growth is slowed, and the cells regain the capacity to form at least partially differentiated structures (Talhouk et al. 2013). Cxs rely on their interactions among each other and association with key effector proteins involved in proliferation and polarity (Bazzoun et al. 2013), two basic features of epithelial differentiation. We propose that this tumor-suppressive role of Cxs is accomplished by regulating signaling pathways involved in cell proliferation, differentiation and architecture.

Several studies have illustrated the critical role of the GJ assembly in development, differentiation and homeostasis of different tissues, including the

mammary gland (El-Sabban et al. 2003; Talhouk et al. 2008). In fact, Cx43 forms its complex assembly by sequestering β-catenin at the membrane, which is a versatile protein with adhesive and transcriptional roles, consequently regulating cell growth and proliferation (Gottardi et al. 2001; Talhouk et al. 2013). On the other hand, basoapical polarity contributes to the acquisition of proper tissue architecture and spatiotemporal responses to cues that govern proper mammary epithelial functions (Bornens 2008; Bazzoun et al. 2013). Thus, not only enhanced proliferation but also loss of polarity proteins are typical of early stages of breast tumorigenesis. As such, understanding the contribution of Cxs to attain polarity and proper tissue architecture is essential to further clarify pathways controlling normal differentiation of the mammary epithelium.

To explore the unique contributions of Cx43 to proliferation, proper morphogenesis and polarity of the mammary epithelium, we silenced *GJA1* (gene coding for Cx43) in a nontumorigenic human mammary epithelial cell line HMT3522 S1 (S1) cells. S1 cells were characterized for their expression pattern of Cx43, Cx32, Cx30 and Cx26. The results indicated that Cx43 is the predominant Cx expressed in this cell line with negligible expression of other Cxs even when Cx43 was silenced. Cx43 loss induced a disruption in the GJ complex assembly, which it forms with β-catenin at the membrane. This has been accompanied by an alteration in apical polarity and mislocalization of Scrib protein, an apical polarity regulator. More so, Cx43 silencing brought about improper morphogenesis of S1 acini, whereby cells lost their proper direction of division and formed more than one luminal epithelial layer within an acinus. Our data suggested that Cx43 regulates PI3K, which plays a major role in determining the orientation of the mitotic spindle that dictates the number of layers of which an acinus is formed. This culminated in changes in the nontumorigenic

phenotype of S1 cells towards a tumor-initiation one marked by the enhanced cell cycle entry, proliferation rate and potential to invade the matrigel. These findings clearly propose that Cx43 loss induces early signs of tumor initiation as supported by our *in vivo* data, where subcutaneous injection of Cx43 silenced S1 cells induced tumor formation in NSG immunodeficient mice compared to the control group.

C. Materials and Methods

1. Cell culture and control of acinar differentiation

Non-neoplastic S1 HMT-3522 human mammary epithelial cells (HMECs), between passages 52 and 60, were routinely maintained as a monolayer (2D culture) in chemically defined serum-free H14 medium (Plachot and Lelièvre 2004). Three-dimensional (3D) cell culture was used to induce the formation of acini. Briefly, S1 cells were plated on Matrigel^{TM} (60 µl/cm2, BD Biosciences, Bedford, MA) at a density of $5x10^4$ (BD Biosciences, Bedford, MA) in the presence of culture medium containing 5% Matrigel^{TM} (Plachot and Lelièvre, 2004). EGF was omitted from the culture medium after day 7 to allow completion of acinar differentiation (usually observed on day 8 or 9; Plachot and Lelièvre, 2004; Lelièvre and Bissell, 2005).

2. Transfection and infection with short hairpin RNAs

The recombinant short hairpin (sh)RNA retroviral constructs against Cx43 have been described previously (Shao et al., 2005). Vectors containing shRNA, non-silencing sequence (NSS), and empty vector control (EV) were transfected into Phoenix packaging cells using Calcium Phosphate (Stratagene, La Jolla, CA). Briefly, packaging

cells at 30-40% confluence were incubated with 2 µg of retroviral vectors in transfection solution according to the supplier's protocol. Cells were maintained at 37°C for 24 h, after which the transfection medium was replaced by DMEM/F12 containing 10% fetal bovine serum. Cells were maintained for an additional 24 h before collecting viral supernatants for the next three days. Viral supernatants were passed through 0.45 µm cellulose acetate filters and stored at -80°C. For infection, filtered retroviral supernatants were thawed at 37°C in a water bath and applied to monolayers of S1 cells at day 3 of 2D culture. Cells were incubated with hexadimethrine bromide (polybreen) (6 µg/ml; (Sigma Aldrich, St. Louis, MO) for 8 h. The infection medium was removed and cells were incubated in regular H14 medium for 24 h. Infection was repeated two additional times and selection with hygromycin-B (150 µg/ml; Calbiochem, San Diego, CA) was started 72 h after the last infection.

3. Immunofluorescence

Fresh 3D cultures were stained for various markers of differentiation as described (Plachot and Lelièvre, 2004; Abad et al., 2007). Cells were either permeabilized with 0.5% peroxide and carbonyl-free Triton X-100 (Sigma-Aldrich) 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) prior to fixation in 4% paraformaldehyde (Sigma-Aldrich), or directly fixed with 4% paraformaldehyde (Lelièvre et al., 1998). Antibodies used were rabbit polyclonal against Cx43 (C6219, 1:400, Sigma-Aldrich), and mouse monoclonal against ZO-1 (33-9100, 1:200, Life Technologies; Grand Island, NY), α-tubulin (T5168, 1:500, Sigma-Aldrich), β-catenin (Clone CD14, 1:800, BD Biosciences), E-cadherin (610181, 1:50, BD biosciences), and

rat α-6 integrin (clone NKI-GoH3, 1:200, EMD Millipore, Billerica, MA). Secondary antibodies conjugated with Alexafluor 488 (green) or Alexafluor 594 (red) (Invitrogen Molecular Probes, Eugene, OR) were used at the manufacturer's proposed dilutions. Nuclei were counterstained with 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and specimens were mounted in ProLong® Gold antifade reagent (Invitrogen Molecular Probes). A minimum of one hundred acini were analyzed for each immunostaining using epifluorescence microscopy.

4. Preparation of whole cell protein extracts and western blot analysis

Cells were harvested from 3D cultures as described earlier (Plachot and Lelièvre, 2004). Briefly, S1-acini were released from the MatrigelTM by incubation with Dispase (BD Biosciences) and whole cell extracts for the immunoblotting of all proteins were prepared in Laemmli buffer (2% SDS in PBS) including PPI cocktail (10 mg/ml Aprotonin, 100 mM Pefabloc, 250 mM Sodium fluoride) (Lelièvre et al., 1998) for except for p-Akt which was prepared in RIPA buffer (25 mM Tris HCL, 150 mM NaCl, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS). For western blot analysis equal amounts of proteins were separated and immunoblotted with rabbit polyclonal antibodies against Cx43 (C6219, 1:1200, Sigma), claudin-1 (71-7800, 1:1000, Invitrogen) β-catenin (sc7199, 1:400, Santa Cruz), LaminA/C (sc20681, 1:400, Santa Cruz), p-Akt (#9271, 1:100, Cell signaling) and Akt (#9272, 1:1000, Cell signaling), monoclonal antibodies against Cx26 (1-2 µg/ml, Zymed Laboratories San Francisco, CA), and mouse ZO-1 (33-9100, 1:50, Life Technologies), E-cadherin (13-1700, 1:200, Invitrogen), γ-tubulin (sc17787, 1:400, Santa Cruz). Equal protein loading was verified by immunoblotting for lamin B (Rabbit, Ab16048, 1:14000, Abcam) and GAPDH

(Goat, sc20357, 1:10000). Protein levels were quantified using Scion NIH Image software (Scion Image, Scion Corporation, NIH) or ImageJ (http://imagej.nih.gov/ij/) and normalized to Lamin B or β-actin.

5. Extraction of nuclear proteins

Cells were gently scraped with 1 ml 1x PBS (Lonza, Belgium) into a microcentrifuge tube and centrifuged at 1000x g for 10 min at 4ºC to obtain a cell pellet. The pellet was lysed by rapid freezing and thawing and then resuspended into 70 µl of hypotonic Buffer A (10 mM Hepes PH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM Dithiothreitol), incubated for 10 min at 4ºC, and then vortexed for 10 s. The mixture was centrifuged at 4,500x g for 11 min, and the pellet (representing nuclei) was resuspended in 15 µl of hypertonic Buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1.5 mM MgCl2, 25% (v/v) Glycerol, 0.2 mM EDTA, 1 mM Dithiothreitol, 0.5 mM PMSF), and placed on a shaker for 30 min at 4ºC, and then centrifuged at 14,000x g for 20 min. The supernatant was diluted with 30 µl of Diluting Buffer D (20 mM Hepes, 50 mM KCl, 20% (v/v) Glycerol, 0.2 mM EDTA, 1 mM Dithiothreitol, and 0.5 mM PMSF) and stored at -80ºC.

6. Co-Immunoprecipitation

Cell pellets resulting from 2D or 3D cultures were suspended in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% Sodium deoxycholate and 0.1% SDS) supplemented with Protease inhibitors (Complete^{TM}) at a concentration of 40 µl/ml. Cell extracts were centrifuged at 14,000x g for 30 min. The supernatants

were collected and precleared with protein A agarose beads (Roche Applied Science, Indianapolis, IN) and incubated at 4ºC for 1 hr. Protein A agarose beads were collected by centrifugation at 14,000x g at 4ºC for 10 min, supernatants were removed and incubated with 1µg of primary antibody at 4ºC for 2 h. A volume of 20 µl of protein A agarose was added to each 1ml of lysate and incubated at 4ºC over two nights. The agarose beads bound to the antibody-protein complex were collected by centrifugation at 14,000x g at 4ºC for 10 min. The supernatants were discarded and the beads washed with PBS and centrifuged at 14000x g at 4ºC three times. Finally the beads were re-suspended in 40 µl 2X sample buffer, boiled for 1-2 min and stored at -20ºC.

7. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions.1 µg of total RNA was reversed transcribed to cDNA using Revertaid 1st strand cDNA synthesis kit (Fermentas, Grand Island, NY). RT-PCR was performed using iQSYBR Green Supermix in a CFX96system (Bio-Rad Laboratories, Hercules, CA). Products were amplified using primers for the below genes. To quantify changes in gene expression, the comparative Ct method was used to calculate the relative-fold changes normalized to GAPDH.

Primer	Sense	Antisense
Cx43	CAA TCA CTT GGC GTG ACT TC	GTT TGG GCA ACC TTG AGT TC
Cx30	TCA ACA AAC ACT CCA CCA GC	CAA TCC CAC ATT TCA ACA CC
Cx32	GAC AGG TTT GTA CAC CTT GC	CGT CGC ACT TGA CCA GCC GC
Cx26	TCT TTT CCA GAG CAA ACC GC	GAC ACG AAG ATC AGC TGC AG
Claudin 1	GGCAGATCCAGTGCAAAGTC	TCTTCTGCACCTCATCGTCTT
Claudin 3	AGAAGTACACGGCCACCAAG	CCTGCGTCTGTCCCTTAGAC
Claudin 4	CTCCATGGGGCTACAGGTAA	CGTACACCTTGCACTGCATC
Claudin 5	GCCCTTAACAGACGGAATGA	AAGTAAGGCAGCAGCCAAGA
Claudin 7	GGGGAGACGACAAAGTGAAG	GCCATACCAGGAGCAAGCTA
GAPDH	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA

Table 1. Forward and Reverse primers of Cxs and claudins.

8. Cell Counting by Trypan Blue

S1 cells were plated in 24-well tissue culture plates in each well. The cells were counted from triplicates on days 6 and 10. First, media was removed, and the cells subsequently trypsinized and collected. Cells were then diluted in Trypan Blue (1:1) ratio (vol/vol) and counted using a hematocytometer. Experiments were repeated at least three times. Acinar sizes measurements were performed manually on day 10 by recording the diameter of each acinus with respect to the stage micrometer. One hundred acini were included in the scoring.

9. Cell cycle analysis

Cells were trypsinized on day 10 in both 2D and 3D culture conditions and collected by centrifugation at 300 g for 5 min at 4ºC. They were then fixed using ice-

cold 70% ethanol and left at -20ºC overnight. Cells were then centrifuged (300 g, 5 min, 4ºC) and the pellet was washed twice with 1X PBS. RNase A and 30 µl of 2 mg/ml propidium iodide in 1X PBS were then added to each flow tube and cells were analyzed using Flowcytometer FACSAria SORP.

10. Invasion Assay

Six-well tissue-culture plates were fitted with inserts (8 µm pore size). The inserts were coated with 300µl of EHS-Growth media solution of 1:20 ratio and incubated at 37°C for 4 hrs. $3x10^5$ S1 cells were seeded in the inserts. After 24 hours, the cells were fixed using 4% paraformaldehyde in PBS for 20 minutes at room temperature (or kept at 4ºC for a maximum of 2 weeks). The cells towards the inside of the insert were removed by using a cotton swab, and nuclei of migrated cells were counterstained with Hoechst (DAPI; 4,6-diamino-2-phenylindole) (Molecular Probes, Eugene, OR, USA) at a concentration of 0.5 µg/ml, for 10 minutes at RT. The insert was then cut and mounted on a microscopic slide using Vector labs hardmount fluorescence media (Vector Laboratories, Burlingame, CA). The inserts were then examined by fluorescence microscopy.

11. In vivo injections

This study was approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut. Five weeks aged female and male NOD-SCID (NSG) mice were injected sub-dermally with 7×10^6 EV and shRNA S1 cells into the subcutaneous area of the neck region, in 50% Matrigel. Mice were

monitored for tumor onset. Tumor volumes were assessed weekly, for seven weeks, by measurement of tumor size (length, width, height) with a caliper device. The data were plotted as the average tumor size of 6 mice/treatment group and percentage of mice that developed tumors over the seven weeks.

12. Image processing

Images of immunofluorescence labeling were recorded using LSCM fluorescent confocal microscope (LSM 410, Zeiss, Germany). Images were processed using ZEN lite software and *ImageJ* (http://imagej.nih.gov/ij/) and assembled using Adobe Photoshop® 6.0 (Adobe Systems, San Jose, CA).

13. Statistical analysis

Data were presented as means±SEM and statistical comparisons were done using Microsoft Excel and GraphPad Prism 3.0 software (GraphPad Software Inc, San Diego, CA). Non-paired and paired t-test was used for comparison of two groups whereas one-way ANOVA was employed for three or more groups of treatments. Significance levels was at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

D. Results

1. Morphology of S1 cells in 2D and 3D Culture Conditions

The nontumorigenic human mammary epithelial S1 cells were either cultured on plastic representing the 2D culture conditions or in a matrigel based 3D culture to induce the formation of alveolar-like structures also known as acini (singular, acinus). As already established, in 2D, S1 cells were left for ten to twelve days to form connected islands of cobble-stone-like cells (Fig. 15A). In the presence of matrigel, acinar morphogenesis was marked by cellular proliferation from day 1 to 7 to form multicellular spherical structures with a central lumen (30 μm in diameter containing 25-35 cells). Proliferation was followed by differentiation on day 7, as a result of epidermal growth factor (EGF) withdrawal (Fig. 15A), and establishment of basoapical polarity (Petersen et al. 1992; Plachot and Lelièvre 2004). On day 12, the acini were fixed and immunostained using fluorescent antibodies directed against markers for basal (α-6 integrin, Fig. 15B, upper panel) and apical (ZO-1, Fig. 15B, lower panel) polarity. Notably, acinar structures expressed ZO-1, a tight junction protein, at the apical membrane of S1 cells while they expressed α-6 integrin at the basal membrane (Fig. 15B; Plachot et al. 2009). In vivo, such a polarized 3D organization is required for the differentiation of the mammary epithelium, indicating that the 3D model of S1 cells recapitulated the polarized real tissue architecture of the mammary gland.

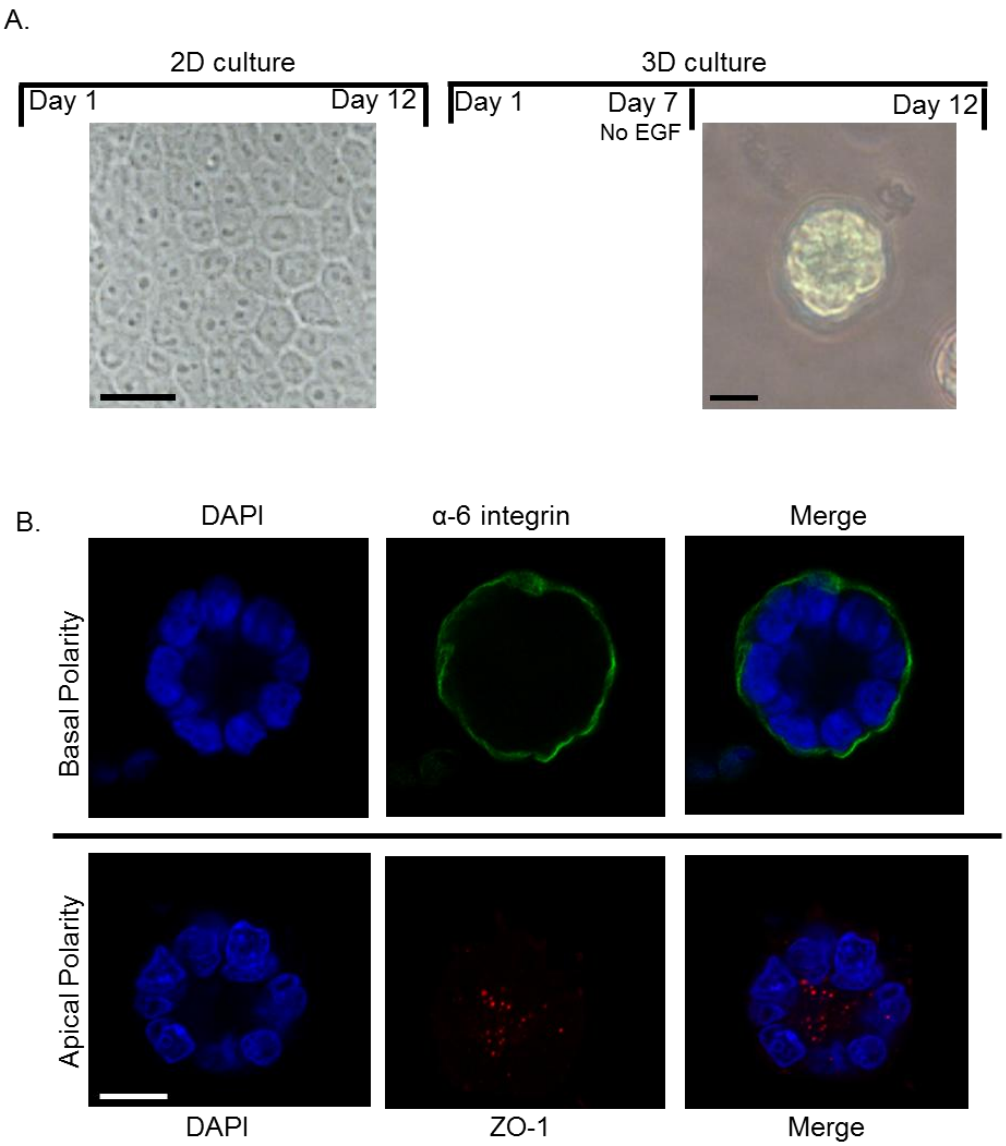

Figure 15. Morphological growth and differentiation features of S1 cells in 2D and 3D culture conditions on day 12.

A. S1 cells organize in 2D monolayers when cultured on plastic (left panel) but form 3D spherical clusters known as acini in the presence of reconstituted extracellular matrix (rECM) components (right panel). B. Confocal fluorescence images of S1 acini immunostained for basal (α-6 integrin, upper panel) and apical (ZO-1, lower panel). Nuclei were counterstained with DAPI (blue). The merged images show the apical and basal localization of both polarity markers in S1 acini. Size bar = 10 µm.

Besides recapitulating the in vivo architecture, we opted to check whether this 3D model adequately enabled the study of Cx43 loss on differentiation and tumor

initiation in the mammary gland. Our current knowledge of Cxs expression in the mammary gland proposes that Cx43 is expressed at cell-cell contact in the human and murine myoepithelia and, very recently, apical in human luminal epithelial tissue (Monaghan et al. 1996; submitted, Bazzoun et al. 2015). In addition to Cx43, the mouse mammary gland expresses Cx32, Cx30 (that are not reported in the human mammary gland) and Cx26, that was also found to be occasionally localized at luminal interfaces in the human breast (Monaghan et al. 1996; Locke et al. 2004; Talhouk et al. 2005; Locke et al. 2007). Given this difference in Cx expression between the murine and the human mammary glands, and the lack of studies that assess Cxs expression in the human breast tissue at different developmental stages, we opted to screen for the endogenous expression of Cxs in our human cell model. As such, we examined the mRNA and protein levels of Cx43, Cx30, Cx32 and Cx26 in S1 acini. Real-time PCR analysis revealed that *GJA1* was the most abundantly transcribed Cx gene in S1 cells with negligible transcription levels for *GJB6, GJB1* and *GJB2* (coding for Cx30, 32 and 26 respectively) (Fig. 16A, left panel). This was further confirmed by western blot analysis of total protein extracts, no bands were present at the 30, 32 and 26 kDa sizes and only two bands around the 43 kDa size corresponding to Cx43 were observed (Fig. 16A, right panel). It is therefore likely that Cx43 is the only Cx protein expressed in S1 acini.

To further characterize Cx43 expression pattern, immunofluorescence was performed in both 2D and 3D cultures of S1 cells. On day 12, Cx43 showed membranous localization at cell-cell contact in S1 monolayers and acini, and it accumulated mainly at the apical side of the acini (Fig. 16B). Given that S1 acini mimic the alveolar structure of the human breast and its Cx43 expression and localization

patterns, this model could be considered to be physiologically relevant and suitable to study the role of Cx43 in glandular differentiation.

2. Effect of Cx43 Silencing on Junctional Proteins

GJs, adherens (AJs) and tight junctions (TJs) play overlapping roles in the development and differentiation of many tissues, and the disruption of any of them has been associated with various diseases, including cancer (Goodenough et al. 1996; Liu et al. 2000). Given this interaction, we aimed to determine whether loss of Cx43 modulated the expression of other mammary Cxs, TJ and AJ proteins, such as claudin-1 and E-cadherin, respectively. As we previously reported, Cx43 stably silenced S1 cells, significantly displayed down-regulated Cx43 mRNA and protein levels in 3D culture without exhibiting possible compensatory expression of any of the other Cxs (Fig. 16C; Bolon et al. 2007). mRNA expression levels of Cx30, 32, and 26 were quantified using real-time PCR in shRNA acini (Cx43 shRNA vector carrying cells) compared to the control ones (Empty vector and non-specific sequence vectors carrying cells are referred to as EV and NSS, respectively). The data indicated that there was no significant change in the expression levels of any of the mentioned Cxs in the shRNA group compared to the EV and NSS control groups (Fig. 16C). This suggested that S1 model enables us to strictly assess the effect of Cx43 loss since no other mammary gland Cx is being expressed or up-regulated in response to its silencing.

The levels of claudin-1 and E-cadherin determined by western blots indicated that Cx43 silencing in S1 acini induced a significant down-regulation in the protein levels of claudin-1 compared to the control groups (Fig. 16D). Claudin 1 was the only

claudin expressed among the breast claudins that we assessed (claudins 1, 3, 4, 5 and 7) (data not shown). On the other hand, the expression levels of E-cadherin did not seem to change upon Cx43 silencing (Fig. 16E). Although Cx43 loss did not affect E-cadherin, the downregulation of claudin-1 sheds some light on the interplay between GJs and TJs in our model.

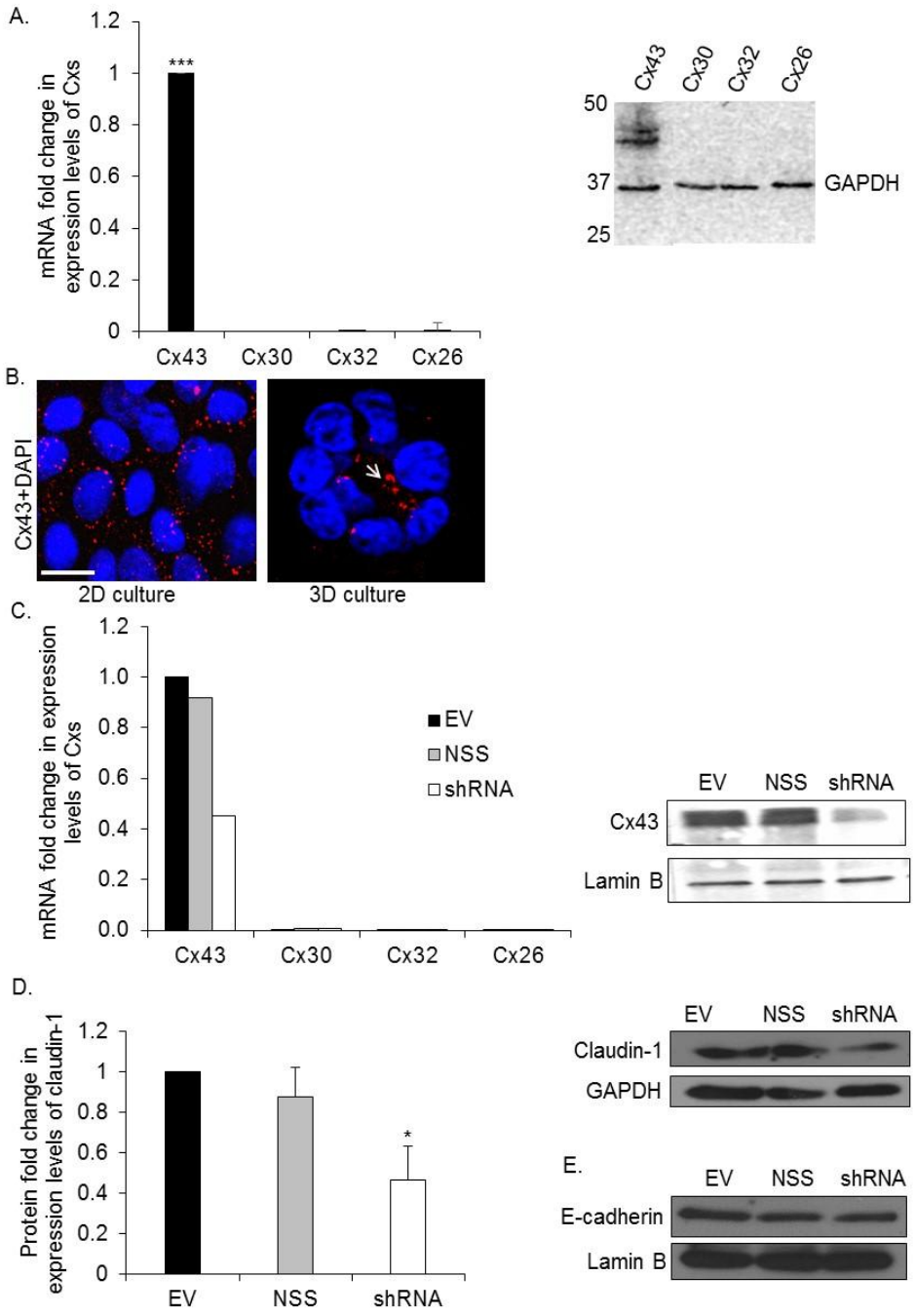

Figure 16. Effect of Cx43 silencing on junctional proteins in S1 cells.

A. Real-time PCR using Cx43, Cx30, Cx32 and Cx26 forward and reverse primers was performed. Histogram represents the normalized expression levels of the different Cxs against GAPDH. In addition, western blot analysis for the expression of Cx43, 30, 32 and 26 was performed on whole protein extracts from S1 acini (day 10) where GAPDH was used as a loading control. B. Immunostaining of Cx43 in 2D and 3D cultures of S1 cells revealed the expression of Cx43 at cell-cell contact (left panel) and apical (white arrow), respectively. Nuclei were counterstained with DAPI (blue). Size bar = 10 µm.

C. Cx43 was stably silenced using shRNA as revealed in the histogram (left panel) and the western blot (right panel). Cx43, Cx30, 32 and 26 mRNA expression levels were assessed using real-time PCR. The histogram shows the relative expression level of each Cx normalized to EV group (left panel). ***$p<0.001$; Non-paired t-test. D. Western blot reveals the protein expression level of claudin-1 in EV, NSS and shRNA S1 acini. The quantification is plotted in the histogram for the the shRNA group compared to EV and NSS groups. GAPDH was used as a loading control *$p<0.05$; nonpaired t-test. E. Western blot reveals the protein expression level of E-cadherin in EV, NSS and shRNA acini. Lamin B was used as a loading control. n=3 for all.

3. Cx43 Loss Alters Scrib Distribution

In order to reveal the mechanism through which Cx43 regulates apical polarity, we examined the effect of Cx43 silencing on the expression and localization of Scrib, a key polarity regulator and a member of Scribble polarity group of proteins. For this purpose, we characterized the expression pattern of Scrib in both 2D and 3D cultures of S1 cells. Scrib localized at cell-cell contacts in S1 monolayers and exhibited an apicolateral localization in S1 acini (Fig. 17A), similar to that of Cx43 and to what has been shown before (Yue et al. 2012). We showed that Cx43 loss induced mislocalization of Scrib protein from the apicolateral side of S1 acini to a diffused localization pattern (Fig. 17A). As indicated in Fig. 17B, apicolateral localization of Scrib was evident in only ~11% of the acini in the shRNA group compared to ~50% of the acini in the NSS group (Fig. 17B). On the other hand, western blot analysis indicated that Scrib protein levels did not change upon Cx43 silencing (Fig. 17C). This observation, in addition to claudin-1 down-regulation, mislocalization of ZO-1 indicated a possibility that Scib distribution could be affected by Cx43 as a mechanism to regulate apical polarity.

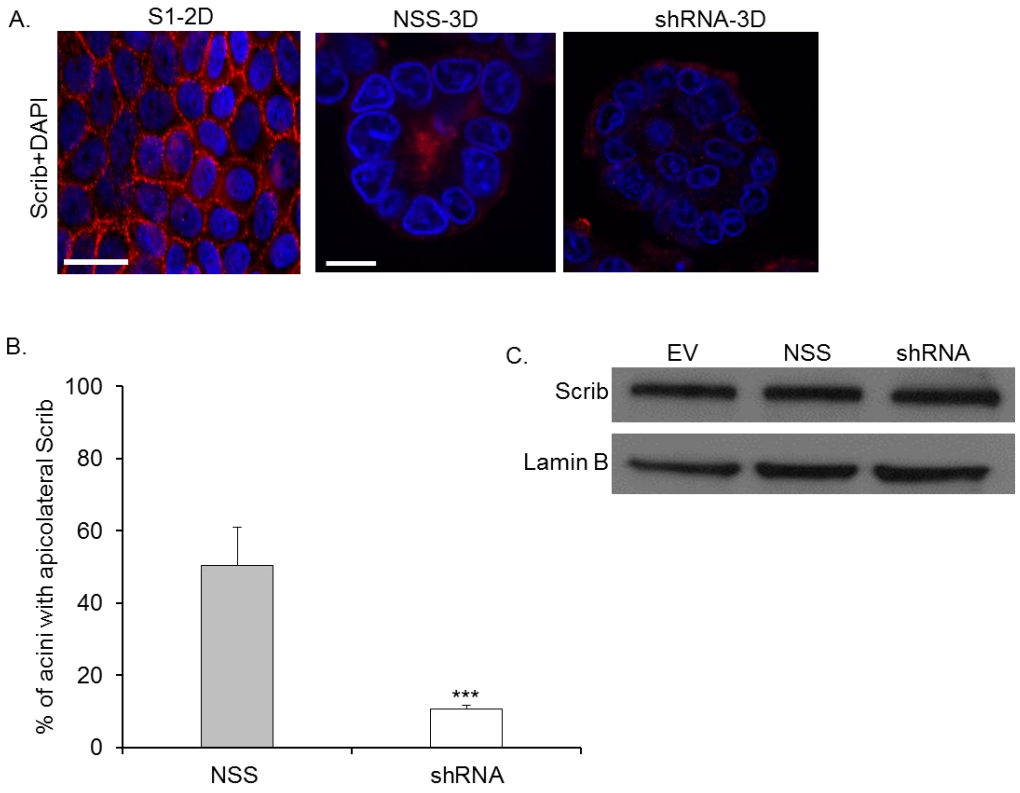

Figure 17. Cx43 impacts Scrib distribution.

A. Scrib was localized at cell-cell junctions in 2D cultures of S1 cells. S1-acini were immunostained for Scrib (red). Nuclei were counterstained using DAPI (blue). B. Western blots were performed to assess alterations in Scrib expression as a result of loss of Cx43. Lamin-B was used as loading control. C. Immunostaining of Scrib (red) in S1 acini revealed the mislocalization of Scrib from an apicolateral localization to a diffused signal in the shRNA group compared to NSS group. Nuclei were counterstained with DAPI (blue). Histogram shows the quantification data of Scrib localization and ****p<0.001*; nonpaired t-test. n=3 for all. Size bar = 10 µm.

4. Cx43 Loss Disrupted β-Catenin Localization and Assembly without Altering its Total Levels

Given the intimate link between cell junctions and polarity proteins, it is likely that the loss of apical polarity is characterized by the dissolution of junctional complexes associated with it, which further enhances apical polarity disruption. One of

the markers of the junctional disassembly is the deregulation of β-catenin which acts as a signaling protein downstream of GJs and AJs. As such, we pursued to determine whether Cx43 loss induced β-catenin mislocalization. S1 acini revealed an apicolateral and laterolateral localization of β-catenin in line with Cx43 (Fig. 18A). In fact, Co-IP analysis revealed that β-catenin binds to Cx43 in S1 acini (Fig. 18B). In order to check whether Cx43 loss triggered redistribution of β-catenin's intercellular localization, immunostaining for β-catenin was performed in shRNA and control acini. The quantification of the localization pattern demonstrated a significant relocalization of β-catenin. There was no change in the percentage of acini exhibiting a laterolateral expression pattern of β-catenin, but a significant decrease in the percentage of acini with an apicolateral localization from ~40% in the NSS group to ~10% in the shRNA group. This was associated with an increase in the percentage of acini with a basolateral β-catenin localization from ~7% in the NSS group to ~25% in the shRNA group (Fig. 18B).On the other hand, western blot analysis of total cell extracts showed that the protein levels of β-catenin were not altered in shRNA acini compared to the control (Fig. 18C).

5. Cx43 Silencing Increased Proliferation Rate, Invasive potential and Induced Cell Cycle Progression of S1 Cells

Numerous reports have revealed that Cx over-expression in cell lines of primary mammary tumor origin decreases their growth rates and reduces their invasive capacity (Qin et al. 2002; Mamiyama et al. 2003; Talhouk et al. 2013), hence mediating a tumor suppressive role. Moreover, when Cx43 was overexpressed in breast cancer cell lines, it recruited β-catenin away from the nucleus, consequently, minimizing its

proproliferative effects (Talhouk et al. 2013). We pursued to determine whether the mislocalization of β-catenin as a result of Cx43 loss could enhance proliferation and modulate the cell cycle activity of S1 cells. As such, we assessed the effect of Cx43 silencing on the proliferation rate of S1 cells in both 2D and 3D culture conditions. The numbers of NSS and shRNA S1 cells were counted using trypan blue dye exclusion assay. shRNA S1 cells showed a significant increase in cell counts by ~50% and 37% on days 6 and 10, respectively, when compared to NSS control group (Fig. 19A). The number of dead cells was negligible and did not differ between the groups at the different time points (data not shown). Moreover, shRNA acini displayed ~57% increase in their acini areas compared to NSS group on day 10 of 3D culture, suggesting an increase in the proliferation rate of shRNA cells (Fig. 19B). In order to examine whether Cx43 silencing affected cell cycle progression of S1 cells, given that it enhanced their proliferation rate in both 2D and 3D culture conditions, PI cell cycle analysis was performed on NSS and shRNA acini. Notably, the shRNA group displayed around two-fold the percentage of cells in both the S- and G2/M phases, with a concomitant ~30% decrease in the cells distribution in the G0/G1 phase of their cell cycle compared to the control (Fig. 19C). Similarly in 2D, there was ~30±2% decrease in the percentage of S1 cells in the G0/G1 phase and was two-fold the percentage of cells in both the S- and G2/M phases in shRNA group compared to the control (data not shown). This finding was in line with studies reporting the critical role that Cx43 plays in regulating proliferation and cell cycle entry in epithelial cells. To determine whether Cx43 loss induced cells to invade matrigel, trans-well invasion assay was performed by seeding equal numbers of EV, NSS and shRNA S1 cells over trans-well filters (having pores of 8 μm in diameter) coated with matrigel components. After 24 hr, there was

~two-fold increase in the capacity of shRNA S1 cells to invade the matrigel compared to minimally invading EV and NSS cells (Fig. 19D).

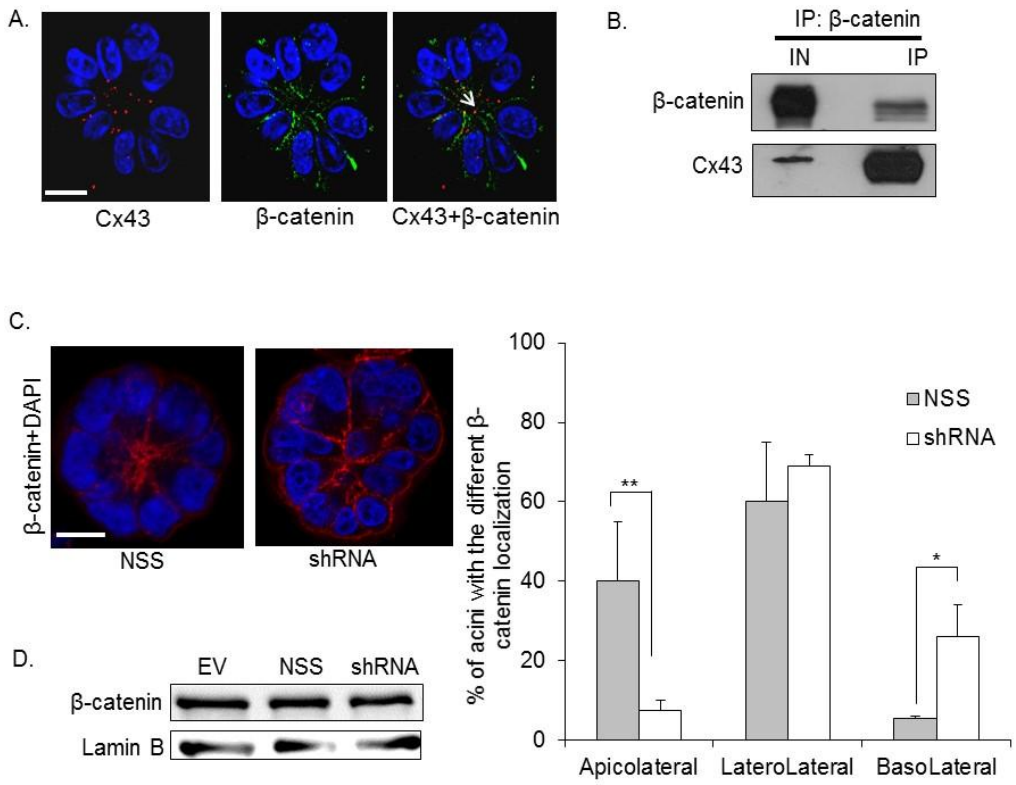

Figure 18. Cx43 is localized at cell-cell junctions and forms the complex assembly with β-catenin.

A. S1-acini were coimmunostained for Cx43 (red) and β-catenin (green). Cx43 showed predominantly apical localization (left panel). β-catenin staining represented cell-cell contact localization (middle panel). The merged image shows Cx43 at cell-cell contacts overlaps with lateral β-catenin (yellow) (arrow). B. Immunoprecipitation using anti-β-catenin, followed by western blots for Cx43 in S1 control acini. C. Immunostaining of β-catenin (red) in 3D cultures of S1 cells revealed the mislocalization of β-catenin from the apicolateral side to laterolateral and basolateral side in the NSS compared to shRNA S1 cells. Nuclei were counterstained with DAPI (blue). Histogram shows the quantification data of β-catenin localization and $**p<0.05$; nonpaired t-test. D. Western blots were performed to assess alterations in of β-catenin expression as a result of loss of Cx43. Lamin-B was used as loading control. n=3 for all. Size bar = 10 μm.

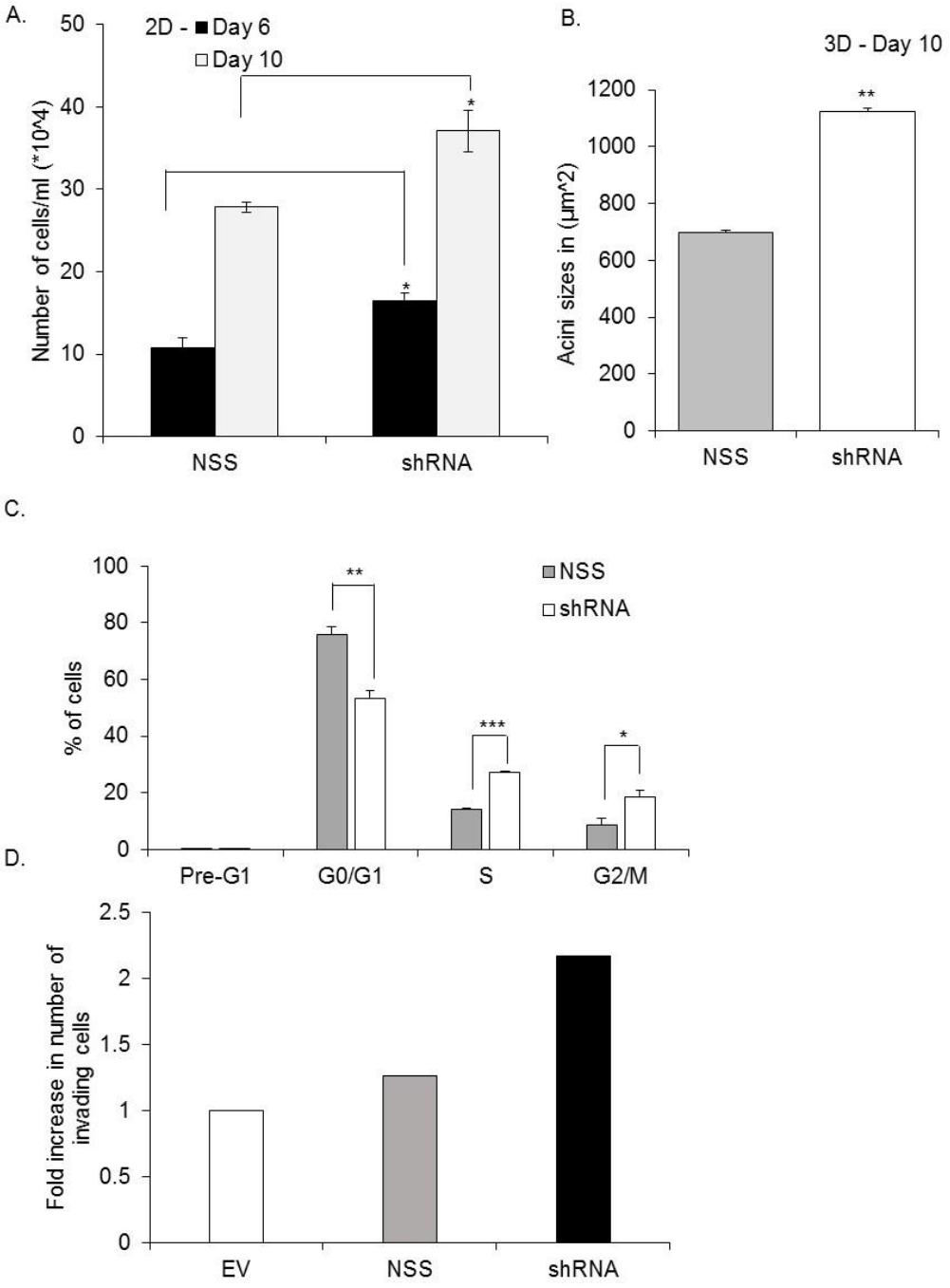

Figure 19. Effect of Cx43 loss on the proliferation rate, cell cycle activity and matrigel-invading potential of S1 cells.

A. Histogram of cell number of NSS and shRNA group represents their proliferation rates in 2D on days 6 and 10. (B) Histogram represents the acinar sizes in control versus shRNA acini on day 10, *$p<0.05$, **$<p0.01$, nonpaired t-test. C. Histogram shows the percentage of NSS and shRNA cells grown in 3D culture in the different cell cycle phases, *$p<0.05$, **$p<0.01$, ***$p<0.001$; nonpaired t-test. (n=3 for A,B and C) D. Effect of Cx43 silencing on the potential of S1 cells to invade matrigel. Histogram shows the fold increase in the number of invading cells in the shRNA and NSS groups compared to EV, two independent experiments.

6. Cx43 Loss Disrupted Lumen Formation by Misorienting the Mitotic Spindle

Loss of polarity could disrupt epithelial tissue architecture by leading to improper cell cytokinesis. This results in luminal epithelial cells dividing towards the center of an acinus and ultimately changing a monolayered acinus into a multilayered one, consequently resulting in tissue hyperplasia, a premalignant change (Pease and Tirnauer 2011). Previously, we showed that blocking GJs induced the formation of multilayered S1 acini as a result of mitotic spindle misorientation (submitted, Bazzoun et al. 2015). Furthermore, silencing Cx43 resulted in a marked defect in acinar morphogenesis. Acinar malformation was evident from the gross morphology of acini that had cells filling their lumen as depicted by the DAPI images of the control and shRNA acini. Detailed analysis of this observation showed that only ~62% of the acini in the shRNA group had normal acinar morphology compared to ~92% in the NSS group (Fig. 20A). These results clearly suggested that Cx43 is required for proper mammary acinar morphogenesis and maintenance of the monolayered acinar phenotype. Given this, we aimed to elucidate the mechanism through which Cx43 could mediate this effect. For this purpose, shRNA and NSS control acini were immunostained for α-tubulin, mitotic spindle marker, on day 4 of culture (optimal observation of mitotic figures). The results revealed that only ~37% of S1 acini in the shRNA group exhibited

mitotic spindle orientation that is parallel to the surrounding substratum, compared to ~70% in the control group (Fig. 20B). This indicated that when Cx43 is apically present, there is a higher tendency that the mitotic spindle is aligned with single layer of luminal epithelial cells, thus preventing lumen disruption and multilayering.

A recent study described that integrins were found to activate PI3K, which in turn orients the mitotic spindle parallel to the substratum in HeLa cells (Toyoshima et al. 2007). In addition, Cx43 was found to indirectly activate PI3K in cardiomyocytes (Ishikawa et al. 2012). In order to illustrate the pathway through which Cx43 contributes to the regulation of mitotic spindle orientation in our model, we asked whether PI3K could be a downstream effector in this pathway. While PI3K expression levels were constant in shRNA acini and in controls, as demonstrated by the western blot analysis (Fig. 20C), Cx43 loss induced a significant decrease in its enzymatic activity. The expression levels of p-Akt (downstream target of PI3K) in shRNA acini were measured and the quantification results of the ratios p-Akt/Akt indicated ~five-fold decrease in the expression levels of p-Akt in shRNA acini group compared to controls (Fig. 20D). As such, it is conceivable that Cx43 loss down-regulates PI3K activity and alters the downstream pathway which blocks proper mitotic spindle orientation, ultimately leading to multilayering.

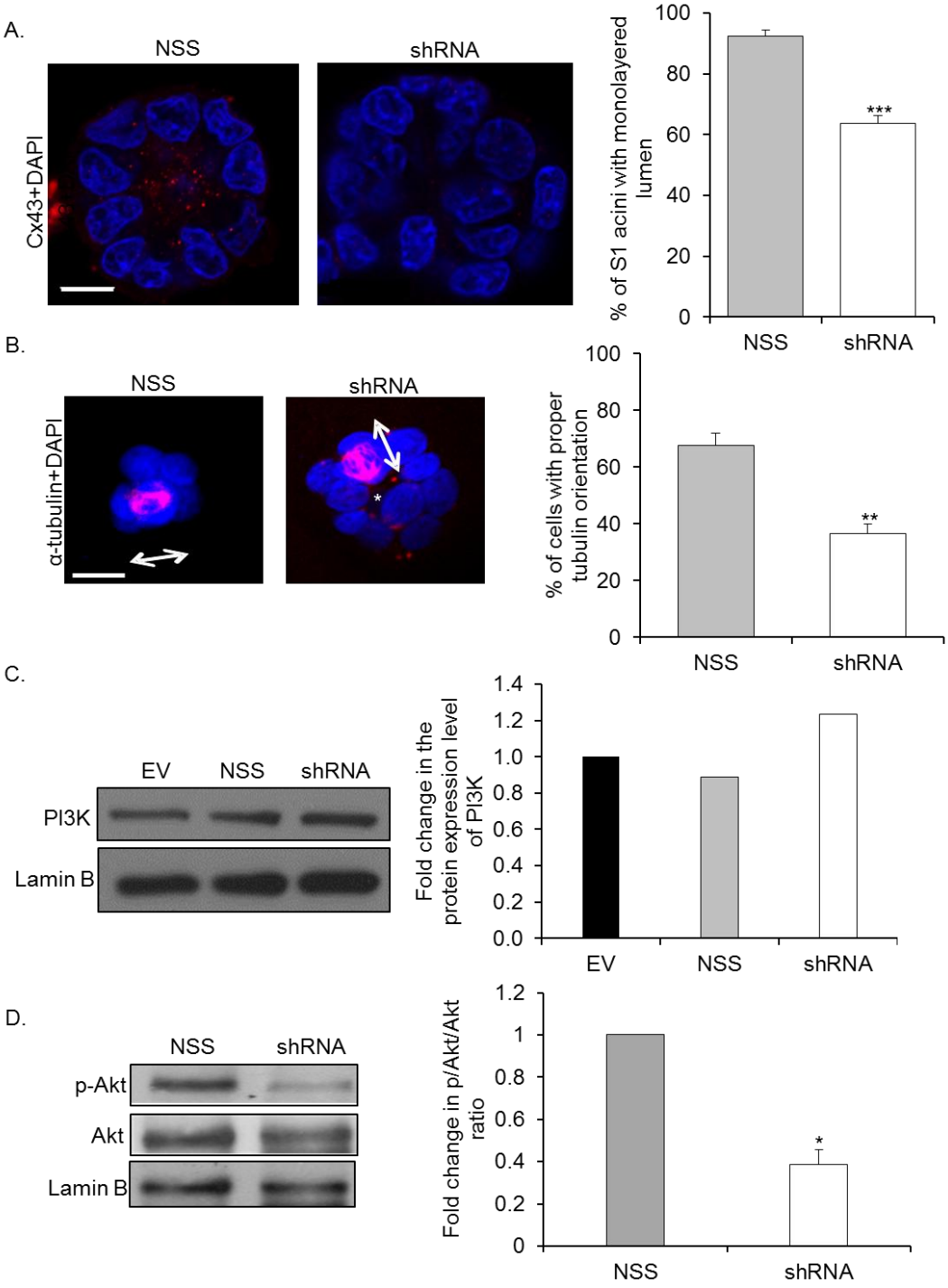

Figure 20. Cx43 regulates proper acinar morphogenesis and controls mitotic spindle orientation in a PI3K dependent pathway.

(A) S1 cells with NSS and Cx43 specific shRNA were grown in 3D culture conditions for twelve days. Acini were stained for Cx43 (red) and nuclei were counterstained with DAPI (blue), (monolayer, left panel and multilayering, right panel). Size bar = 10 µm ****p < 0.001*; nonpaired t-test. (B) On day 4, S1 acini from shRNA and control groups

were immunostained for and α-tubulin (used to mark mitotic spindle) (red). Nuclei were counterstained with DAPI (blue). Histogram shows the percentage of S1 acini in both groups that show 'correct' cytokinesis axis. The orientation of mitotic spindle was analyzed on a minimum of sixty structures in each group and the percentage of structures with predicted 'correct' cytokinesis axis is presented in the histogram. ***p < 0.01*; nonpaired *t*-test. Size bar = 10 µm. (C) Western blots were performed to assess changes in PI3K protein expression levels as a result of Cx43 loss. Lamin B was used as loading control. Histogram indicates no change in expression levels of PI3K in both groups. (D) Western blot analysis of p-Akt and Akt expression levels in total cell extracts was performed and the histogram reveals p-Akt/Akt ratio shRNA compared to control NSS group. **p<0.05*; nonpaired t-test.

7. Cx43 silenced S1 cells Initiated Tumors in NSG Mice

To validate the effect of Cx43 loss on inducing early signs of tumor initiation, $7x10^5$ EV and shRNA S1 cells were sub-dermally injected into the subcutaneous area of the neck region of NSG mice. Tumor onset and volume were monitored weekly for seven weeks. By week 2, only 16.6% of the mice in the control group developed tumors compared to 60% in the shRNA group. All mice injected with shRNA cells developed tumors by week 4 compared to only 66% in the control group (Fig. 21A). Tumor volumes in the shRNA group were significantly more than that of the control group starting week 5 and towards the end of week 7, where shRNA cells induced larger tumors (~0.3 cm^3) compared to EV S1 cells (~0.05 cm^3) (Fig. 21B).

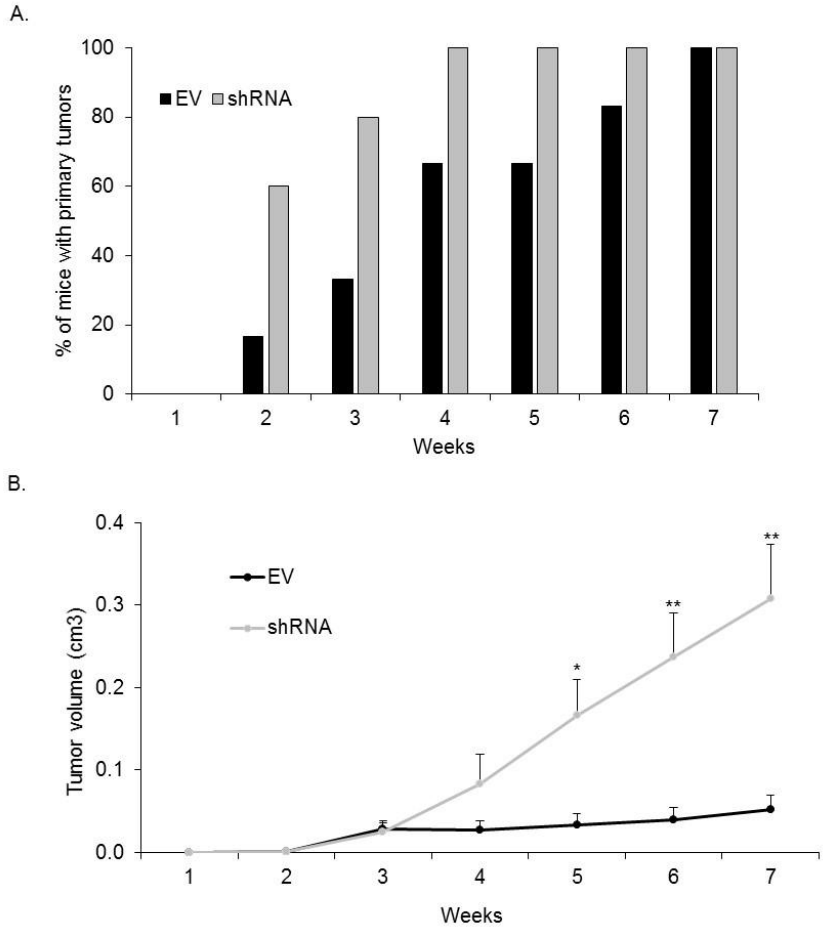

Figure 21. Cx43 silenced S1 cells initiate tumors in NOG-SCID mice.

7x 10^6 EV and shRNA S1 cells were sub-dermally injected under the dorsal neck region of NSG mice. (A) Percent of mice that developed primary tumors sustained for the duration of the experiment. The growth of the primary tumor was monitored by measuring its dimensions on a weekly basis. (B) Tumor volume (V, cm^3) was determined by the equation: V = length × width × height). Data were plotted as the average tumor size of one experiment (six mice/group). **p<0.05, **p<0.01*; nonpaired *t*-test.

E. Discussion

The establishment of cell junctions is essential for the maintenance of cellular architecture and tissue physiology. This is mediated by an array of junctional proteins that enable attachment and communication between neighboring epithelial cells. Among the cell junctions, GJs mediate intercellular communication and allow the transfer of

molecules and metabolites that are essential for the coherent development and function of the tissue. However, GJs are not mere channels that mediate communication; they have transcended their structural role and are now recognized as signaling hubs that relay signals from the external and internal cellular environment towards the nucleus and, consequently, regulate development and differentiation of tissues (Jiang and Gu 2004; Dbouk et al. 2009). The Cx family of GJ proteins has been widely reported to contribute to cell proliferation, differentiation and tissue homeostasis (Herve et al. 2007; Talhouk et al. 2008). These variable roles are mediated via the interaction of Cxs with an array of cytoplasmic and membrane associated partners to form the GJ complex assembly. In cancer, Cxs are usually mutated, down-regulated or mislocalized, and, are studied as tumor suppressors (Hanahan 2000; Naus and Laird 2010). In fact, there is accumulating evidence that sheds light on the effect of Cxs' loss in promoting breast tumorigenesis (Lee et al.1991,1992; Laird et al. 1999; Kanczuga-Koda et al. 2006; McLachlan et al. 2007), yet the current understanding of how these proteins are linked to cancer initiation and onset is relatively limited. We had previously shown that Cx43 overexpression in mammary adenocarcinoma cell lines induced a partial reversion of the tumor phenotype of the cancer cells as illustrated by the change in their invasive potential, proliferation and growth morphology. These phenotypic modulations involved the assembly of the GJ complex comprising Cx43, α-catenin, β-catenin and ZO-2 at the membrane (Talhouk et al. 2013). In this study, we aimed to unravel the mechanism mediating the tumor promoting effect of Cx43 loss in the nontumorigenic human mammary epithelial cell line, HMT-3522 S1 cells. We showed that Cx43 silencing altered the cellular architecture by altering apical polarity and disrupting the GJ

complex assembly at the membrane, ultimately leading to increased proliferation and misorientation of the direction of cell division.

As an initial step, we characterized the growth and differentiation features of our cell model in different culture conditions. S1 cells formed alveolar-like structures also known as acini in 3D. These cells have been previously used by Plachot et al. (2009) to study mammary gland function and polarity. The S1 cell model recapitulates the formation of hemidesmosomes with α-6 integrins in 3D that are observed *in vivo* and central for the establishment of basal polarity (Koukoulis et al. 1991; Weaver et al. 1997). Our results are in line with these observations where the immunostaining of α-6 integrin revealed a basal localization in S1 acini. Furthermore, S1 cells were found to exhibit apical polarity as assessed by immunostaining for the core tight junction protein, ZO-1, which similar to what we detected, was compartmentalized to the apical side of S1 cells, against the lumen (Plachot et al. 2009; submitted Bazzoun et al. 2015). This further supports that the S1 model is indeed an adequate system to study the differentiation of the mammary epithelium, as it closely recapitulates the *in vivo* characteristics.

As part of the characterization, we also identified that S1 cells do not endogenously express Cx30, 32, and 26, commonly reported Cxs in the mammary gland of mice. However, Cx43 displayed abundant protein expression levels in S1 cells similar to its pattern identified *in vivo*. Interestingly, in addition to the commonly described cell-cell contact expression in the myoepithelium (Monaghan et al. 1996), we observed a distinct apicolateral localization of Cx43 in S1 acini comparable to that we reported in the human breast tissue (submitted, Bazzoun et al. 2015). It has been found to colocalize with ZO-1 at the apical and lateral membranes of the human intestinal

epithelial cell model Caco2 cells, where it plays an essential role in maintaining mucosal homeostasis (Ey et al. 2009). In addition, Cx43 was also found to be apically localized in lens epithelial cells of mice, contributing to the proper function of the lens (Gao and Spray 1998). Given the contribution of apical Cx43 to the homeostasis of the mentioned epithelia, we suggest that it possibly has a role in the normalcy and differentiation of the mammary epithelium.

We have previously shown that the tumor suppressive effects of Cx43 overexpression in breast cancer cells exhibited a context-dependent manner. The phenotypic tumor reversion was evident when MDA-MB-231 cells were grown in 3D culture conditions that, unlike 2D, permitted the membranous localization of Cx43 and, consequently the formation of the GJ complex assembly (Talhouk et al. 2013). We sought to assess the effect of Cx43 shRNA stable silencing on the nontumorigenic differentiated status of S1 cells in 3D. Our results revealed that Cx43 silencing abolished GJIC among the cells within acini, marking the disruption of one of the fundamental components of mammary cellular differentiation. Communication is central for tissue homeostasis, as evidence indicated that its loss is associated with tumorigenesis. For example, CID-9 mouse mammary cell strain were reported to differentiate and express β-casein when GJs are formed and coupling is attained (El-Sabban et al. 2003). In addition, the human cytomegalovirus induced gliomagenesis by targeting Cx43 for proteasomal degradation, consequently disrupting functional GJIC (Khan et al. 2013). On the other hand, the ectopic expression of Cx43 significantly reduced melanoma growth by enhancing GJIC among keratinocytes (Ableser et al. 2013). Moreover, treatment with inorganic arsenic trioxide displayed tumor promotion

changes in nontumorigenic liver epithelial cells as a result of GJ inhibition (Hsiao et al. 2014).

Loss of communication was also accompanied by changes in the cellular architecture, in particular polarity. In epithelial structures that are organized as one layer surrounding a lumen, the establishment of apical polarity is characterized by the formation of TJs (Lubarsky and Krasnow 2003). Apical polarity loss, as shown by the redistribution of TJ markers away from apicolateral sites, has been used as a parameter for the characterization of early lesions in certain cancerous diseases (Konska et al. 1998). In the mammary epithelium, when apical polarity is altered, ZO-1 has been observed to be mislocalized from the apical membranes (Chandramouly et al. 2007). This goes in line with our results where we found that shRNA acini had disrupted apical polarity, as marked by ZO-1 mislocalization, when compared to control. Besides the mislocalization of ZO-1 in shRNA S1 acini, the expression level of TJ protein claudin-1 was decreased, further demonstrating disruption of apical polarity proteins as a consequence of Cx43 loss. Knowing that claudin-1 down-regulation was previously reported in primary breast tumors (Kramer et al. 2000), it is suggested that the decrease in its expression levels in S1 cells, as a result of Cx43 loss, might be indicative of early tumorigenic changes. Given the above, Cx43 regulates apical polarity which is a major barrier against tumor initiation, and therefore, its loss results in the disruption of cell architecture rendering the mammary epithelium prone to tumorigenic phenotypic changes.

The basoapical polarity of epithelial cells is controlled in part by the Par, Crumbs, Scribble, polarity proteins first identified in *Drosophila* (reviewed in Bazzoun et al. 2013). Loss of such proteins, or their deregulation, might therefore be expected to

play a significant role in tumorigenesis. Scrib, a key apical polarity regulator, is required for the homeostasis of the mammary ductal luminal epithelium where its mislocalization or down-regulation is commonly reported in human mammary tumors (Zhan et al. 2008). As such, we focused on Scrib as a potential downstream effector of Cx43 in regulating apical polarity. Scrib was expressed in S1 cells at cell-cell contact in 2D and apically in 3D. Interestingly, silencing of Cx43 resulted in the mislocalization of Scrib protein from the apicolateral side of S1 acini to display a heterogeneous localization. This suggested that Cx43 regulates apical polarity in S1 cells by maintaining proper localization of Scrib at the apicolateral side of the acini. Notably, Scrib was found to be essential for mammary duct morphogenesis and has a specific role in the control of the early steps of breast cancer. Its deletion significantly induced ductal hyperplasia and expansion of atypical luminal cells as a result of disrupted basoapical polarity (Godde et al. 2014). Furthermore, silencing of Scrib in murine mammary epithelial cells induced tumor growth, driven by c-*myc* (Zhan et al. 2008). To the best of our knowledge, the only reported interaction between Cxs and polarity proteins was that between Cx43 and Drebrin, an actin-binding protein involved in mediating epithelial cell polarity (Butkevich et al. 2004). Yet it is well known that GJs affect TJ proteins as shown by the up-regulation of claudin-1 and ZO-1 protein levels upon transfection of Cx32 into mouse hepatocytes (Kojima et al. 2002). As such, our finding that Cx43 regulates apical polarity in the mammary epithelium possibly *via* ensuring proper localization of Scrib warrants further investigation to reveal the role of Cx43 in regulating differentiation-related signaling pathways.

To elucidate the effects of Cx43 loss on its downstream signaling pathways that might potentially mediate the observed functional and phenotypic changes, we

examined the consequences of Cx43 loss on its binding partner β-catenin. Interestingly, whereas β-catenin was localized at the apical and lateral sides of the membrane in control S1 acini, it redistributed from the apical domain towards the lateral and basal ones in shRNA acini. Previous studies in our laboratory showed that Cx43 over-expression in breast cancer cell lines resulted in the recruitment of β-catenin away from the nucleus to the membrane to form the complex assembly with Cx43, consequently preventing it from displaying its proproliferative effects (Talhouk et al. 2013). In fact β-catenin/Wnt signaling pathway has been implicated in mammary gland tumorigenesis. The genetic ablation of β-catenin decreases breast cancer cell proliferation *in vitro*, as well as mammary tumor growth and metastasis in the mammary fat pad (Wagh et al. 2012), whereas increased nuclear β-catenin was associated with poor prognosis and was observed in about 40% of primary breast tumors (Geyer et al. 2011). Given that β-catenin is known to regulate the expression of various proteins involved in cell proliferation, invasiveness and cell cycle (Kota et al. 2009), and that Cx43 regulates cell proliferation (Ogawa et al. 2012), we noticed an increase in the proliferation rate and an evident exit from the G0/G1 phase towards cell cycle progression in shRNA S1 cells in both 2D and 3D. Enhanced proliferation as a result of Cx43 loss has been also reported by Gangozo et al. (2012), where its down-regulation in astrocytes induced higher proliferation rate. On the other hand, Cx43 over-expression in breast cancer cells significantly inhibited cell proliferation whereby cells were arrested in their G0/G1 phase (Talhouk et al. 2013). Moreover, control S1 cells only minimally invaded matrigel compared to shRNA S1 cells which displayed a two-fold increase in their potential to invade matrigel. This is consistent with a study by Rizki et al. (2008) whereby S1 cells had a minimal potential to invade matrigel compared to T4-2, their

tumorigenic counterparts that exhibited a threefold enhanced invasiveness. These findings suggested that the phenotypic changes observed in S1 cells as a result of Cx43 silencing were indicative of loss of differentiation and early signs of tumor initiation, and were possibly related to the disruptive effect of the GJ complex disassembly on its downstream signaling pathways.

Cx43 silencing was marked by changes in the 3D growth morphology of S1 cells. shRNA acini showed disrupted structures with cells inside the lumen, in contrast to control acini that displayed typical lumen structures enclosed within a single layer of cells. To reveal the mechanism behind this disrupted morphology, we examined changes in the orientation of the mitotic spindle. In most epithelia, the orientation of the mitotic spindle is aligned parallel to the apical and basal cell surfaces in order to generate daughter cells that are next to one another (Fernandez-Minan et al. 2007). Shifts in this orientation could alter epithelial architecture and lead to tissue hyperplasia where daughter cells are instead placed on top of one another (Pease and Tirnauer 2011). Interestingly, there was a higher percentage of acini with misoriented mitotic spindles in shRNA acini group compared to a majority of properly oriented ones in the control group. Since this is the first report indicating a role for Cx43 in regulating the orientation of the mitotic spindle in epithelia, we asked whether PI3K could be its downstream effector, given that Cx43 was found to indirectly activate PI3K in cardiomyocytes (Ishikawa et al. 2012). Moreover, it has been well established that PI3K is involved in various signaling pathways implicated in cellular differentiation, growth and proper mitotic spindle orientation (Toyoshima et al. 2007). In fact, it is established that PI3K activates Cdc42, which in turn activates aPKCζ. Subsequently, the activated par6/aPKCζ complex excludes the NuMA/LGN, a major spindle pole protein complex,

from the apical domain of epithelial cells towards the midcortex during mitosis to establish the parallel mitotic spindle orientation (Zheng et al. 2010; Durgan et al. 2011). Interestingly, Cx43 loss did not affect the expression levels of PI3K, but induced a decrease in its activity, possibly leading to the disruption in the downstream pathway required for the proper orientation of the mitotic spindle. The contribution of Cx43 to the mitotic spindle parallel orientation in the mammary epithelium was corroborated by our earlier report where the inhibition of GJs using 18α-glycyrrhetinic acid induced a significant misorientation of the mitotic spindle (submitted Bazzoun et al. 2015).

To verify the effect of Cx43 loss on tumor initiation signs obtained from our *in vitro* studies, NSG immunodeficient mice were injected with shRNA and control S1 cells. A significant difference in both tumor onset and volume between both groups was noted. However; this growth *in vivo* might also be not more than an inflammatory reaction initiated in response to the subcutaneous injection of cells, yet, if so, the enhanced reaction in response to shRNA cells injection is worth examining. The loss of Cx43 in mouse models was associated with improper regulation of epithelial morphogenesis and stromal development in the virgin gland (Plante and Laird 2008). In addition, mice with Cx43 deletion in their mammary glands exhibited defects in milk ejection (Stewart *et al.* 2013; Mroue et al. 2015). Besides its *in vivo* developmental role, our data imply a tumor prevention effect of Cx43 in the mammary epithelium.

In conclusion, we propose that Cx43 tumor suppressive role is mediated by regulating several downstream effector signaling pathways involved in mammary differentiation. This work revealed a unique role for Cx43 where it regulates apical polarity, possibly through a Scrib-dependent pathway. In addition, it promoted the formation of the GJ complex assembly with β-catenin at the membrane, consequently

preventing uncontrolled proliferation, cell cycle entry and invasion of nontumorigenic mammary epithelial cells. Our study further demonstrated a novel contribution of Cx43 in directing proper cell division, *via* a PI3K-dependent pathway, ultimately warranting the establishment of a normal epithelial cell architecture to act as a barrier against tumor initiation.

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CHAPTER V

CONCLUSION

Intercellular communication is essential for the complex biological functions of multicellular organisms including regulation of growth, differentiation and development. These functions are in part mediated by cell-cell interactions. Among the intercellular complexes, gap junctions (GJs) are unique in that they allow communication by connecting the cytoplasms of two neighboring cells. Yet, connexins (Cxs), the building blocks of GJs, are also recognized beyond their channel roles and are now considered as signaling hubs that relay cues from the microenvironment all the way to the nucleus. As such any alteration in their expression and/or localization may cause a variety of disorders including cancer (reviewed in Dbouk et al. 2009; Bazzoun et al. 2014). Given the dynamic development of the mammary gland, Cxs provide a fundamental link between mammary epithelial cells and their neighboring cellular and acellular environments which is inevitable for their differentiation (Talhouk et al. 2008). Here, we examined the effect of Cx43 loss on the differentiation status of the mammary epithelium. We used a phenotypically normal cell model (HMT3522-S1) that recapitulated the complete basoapical polarity axis required for mammary epithelial differentiation (Petersen et al. 1992; Plachot and Lelièvre 2004; Lelièvre and Bissell 2005). S1 cells, which are phenotypically normal breast luminal epithelial cells, mimicked the polarized glandular morphogenesis as well as relevant Cx expression, observed in epithelial tissue, when grown in a Matrigel-based 3D culture system. We reported that S1 acinar structures expressed Cx43 at their apical membranes similar to

what we found in the epithelial breast tissue. This resemblance in both the growth morphology and Cx43 expression pattern renders the S1 3D culture system an appropriate model to study the effect of Cx43 loss on glandular differentiation of the breast.

In differentiated epithelia, such as the glandular mammary epithelium, a key feature in tissue architecture, homeostasis and function is the polarity axis which constitutes a basal pole against the basement membrane, and an apical pole facing the lumen (Bilder et al. 2000; O'Brien et al. 2001; Plachot and Lelièvre 2004). Junctional and polarity proteins are intertwined and regulate key signaling pathways for the control of tissue homeostasis (reviewed in Bazzoun et al. 2013), consequently their disruption has been associated with the development of epithelial cancers (Hoover et al. 1998; Lee et al. 2005). Given that both cell junctions and polarity should be coordinately regulated for normal differentiation, understanding how they affect one another is essential. In this study, we hypothesized that the loss of Cx43 expression and function altered the differentiated status in mammary epithelial cells.

Both GJ blockage and loss of Cx43 were marked by the disruption of the apical localization of ZO-1 without altering its expression levels. This observation suggested a unique role for Cx43 in the establishment of apical polarity in mammary epithelial cells. Loss of apical polarity, in Cx43 silenced cells, was associated with the mislocalization of Scrib, a regulator of apical polarity formation and maintenance. In human breast and other epithelial cancers, expression of Scrib is often mislocalized and deregulated, suggesting that polarity proteins play a role in maintaining homeostasis of epithelial tissues and preventing tumorigenesis (Nakagawa et al. 2004; Gardiol et al. 2006; Zhan et al. 2008; Pearson et al. 2011). As a result, Scrib-mediated apical polarity

maintenance could potentially demonstrate a mechanism *via* which Cx43 regulates apical polarity.

The loss of apical polarity has been shown to contribute to tumorigenesis by deregulating normal proliferation of epithelial cells (Balda et al. 2003). For example, ZO-1 has been shown to bind and sequester the transcription factor ZONAB away from its proliferative role in the nucleus. In addition, Par3 binds to ASPP2, a regulator of p53, to prevent inadequate proliferation events (Cong et al. 2010; Sttocornola et al. 2010). Similarly, it has been suggested that Cx43 mediates its tumor suppressive effects by controlling cell proliferation. In fact, it forms a complex assembly with β-catenin, a versatile transcription factor, and consequently prevents its nuclear translocation that would otherwise promote proliferation (Talhouk et al. 2013). Whereas GJ blockage only induced cells to enter the cell cycle when exposed to nuclear architecture disruptive events or in the presence of mitogenic factor IGF, the loss of Cx43 was sufficient to induce cell cycle entry, promote matrigel invasion and enhance proliferation of the nontumorigenic epithelial cells. This could be explained by the mislocalization of β-catenin upon Cx43 loss, which was not observed when GJs were blocked. Such an observation suggested a role for β-catenin downstream of Cx43 in enhancing proliferation and supporting tumor initiation phenotypic changes resulting from Cx43 loss-mediated apical polarity disruption (Fig. 22A).

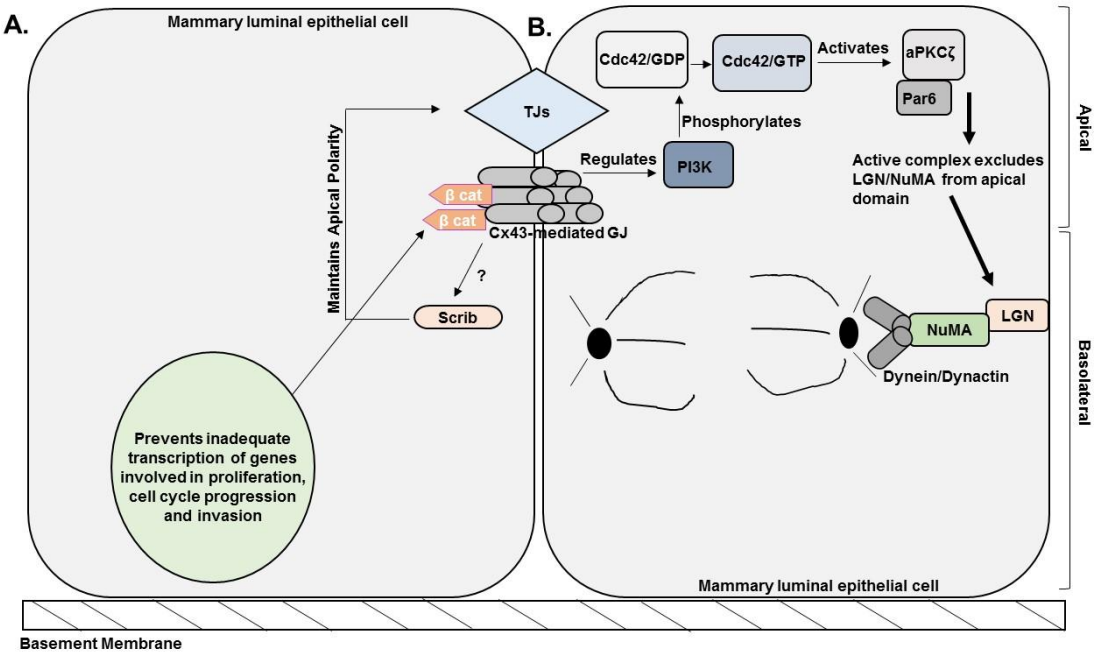

Figure 22. Intersection of tumor suppressive pathways through gap junctions.

A. Cx43-mediated GJIC regulates polarity and signaling pathways involved in tumor initiation in mammary luminal epithelial cells. Cx43 controls the localization of Scrib which in turn contribute to apical polarity establishment. In addition, via forming a complex assembly with β-catenin at the membrane, Cx43 inhibits inadequate transcription of genes involved in proliferation, cell cycle progression and invasion. B. Cx43-mediated GJIC regulates the orientation of the mitotic spindle by modulating the activity of PI3K in luminal mammary epithelial cells. Epithelial cells divide within the plane of the epithelium parallel to the basement membrane by orienting the spindle poles towards the basolateral membranes. Spindle poles are anchored to the cell cortex through the interaction of LGN/NuMA and the dynein/dynactin complex. Anchoring of spindle poles to the apical membrane is inhibited by apically localized aPKCζ which through the active complex it forms with Par6 excludes the LGN/NuMA from the apical domains. aPKCζ/Par6 complex is activated by Cdc42-GTP that is phosphorylated by PI3K.

Cx43 loss was marked by the disruption of acinar morphogenesis. In this study, we revealed a critical role for Cx43 in regulating proper glandular morphogenesis of human breast epithelial cells. Alterations in Cx43 by either silencing its gene or blocking its function during acinar morphogenesis resulted in marked altered acinar growth as evidenced by an unusual presence of cells filling the lumen. This finding

raised an intriguing possibility that, within a 3D context of an acinus, the specific positioning of cells could be influenced by Cxs. As a result, we proposed that the direction of cell division during mitosis might ultimately determine their position within an acinus. Indeed, GJ-compromised and Cx43-lacking acinar structures showed more random orientation of the mitotic spindle axis compared to controls. Furthermore, we characterized the mechanism through which Cx43-mediated GJIC accomplished this role. Our data suggested a PI3K-dependent pathway where Cx43 regulates the enzymatic activity of PI3K that phosphorylates Cdc42. In turn Cdc42 activates aPKCζ to exclude the NuMA complex away from the apical sides of the membrane towards the basolateral domains (Fig. 22B). Moreover, immunohistochemical analysis of normal human breast tissues supported the correlation between apical Cx43 and the maintenance of monolayered luminal epithelium *in vivo*. The direction of cell division i.e., the orientation of the mitotic spindle, is a critical determinant of the epithelial tissue architecture and consequently its normalcy (Fig. 23). Although not all tumors, such as colon tumors, show spindle misorientation and not all spindle misorientation events are tumorigenic, improper direction of cell division is recognized as a contributor to cancer progression (Fleming et al. 2009; Qin et al. 2010; Quyn et al. 2010). In the breast, multilayering and luminal filling characterize premalignant breast lesions observed in breast ductal hyperplasia (Godde et al. 2014). As such, apical Cx43 by regulating proper spindle orientation could potentially prevent multilayering, and ultimately inhibit the development of an early sign of tumor initiation in the mammary epithelium.

Curiously, as both apical polarity and Cx43 are lost early in cancer development, it may be proposed that the down-regulation or mislocalization of Cx43 and the subsequent disruption of apical polarity in breast epithelial cells set the stage for

hyperplastic changes in the presence of permissive microenvironmental cues. In this work, we have revealed an apical polarity regulation role of Cx43 that could potentially be mediated by Scrib. A cause to effect relationship between Cx43 loss and Scrib mislocalization is yet to be determined to better delineate the mechanism through which Cx43 controls apical polarity. As an indication of polarity disruption, Cx43 complex assembly at the membrane was compromised, culminating in the execution of β-catenin tumor promoting effects such as enhanced proliferation and sensitivity to enter the cell cycle, which both characterize tumor initiation. However, further experiments, in Cx43 silenced cells, are needed to determine the interplay between membranous Cx43 and the Wnt-dependent regulation of β-catenin. In addition, we aim to confirm the involvement of tumor promoting genes under direct Wnt/β-catenin regulation including c-myc, and cyclin D1, in the tumor initiation phenotypic changes observed upon Cx43 loss. The influence of Cx43 on the enzymatic activity of PI3K to regulate the direction of cell division requires further elucidation. Whether it is a direct binding or an indirect interaction *via* intermediate effector proteins is a question that is to be answered in upcoming studies. In addition, the *in vivo* tumor initiating potential of Cx43 lacking mammary epithelial cells requires further investigation to characterize the expression of cancer-associated markers, and potentially identify other tumor-initiating affected pathways as a result of Cx43 loss. Our findings being, loss of apical polarity, disruption of tumor suppressive signaling pathways downstream of junctional complexes, enhanced proliferation and misorientations in cytokinesis are all features of tumor initiation that could result from Cx43 loss.

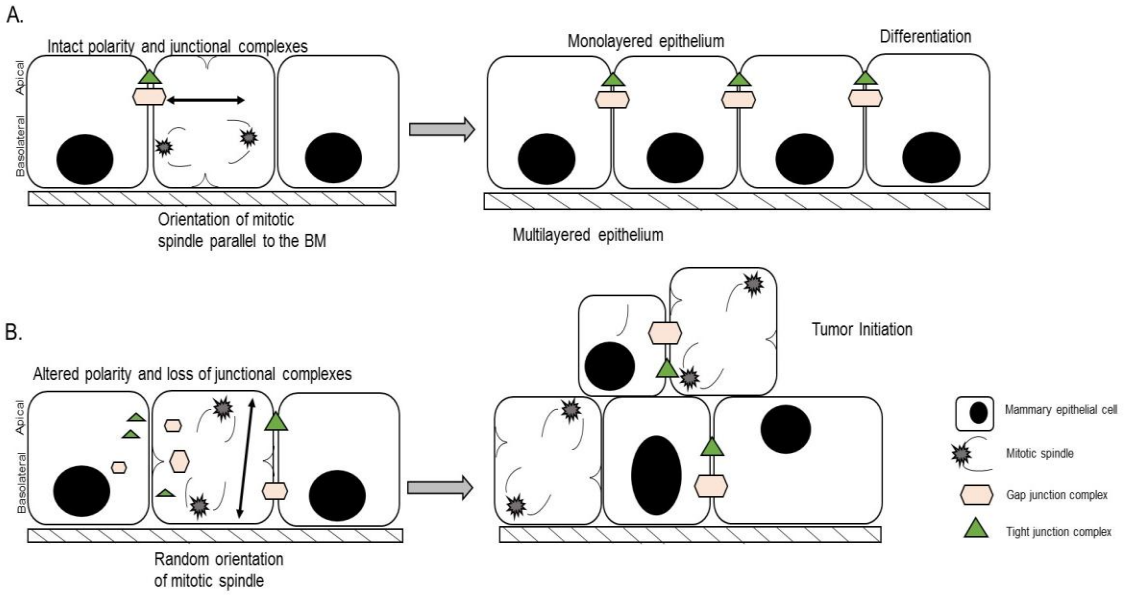

Figure 23. Junctional integrity regulates epithelial cell polarity to prevent tumor initiation events.

A. Intact basoapical polarity that includes the proper formation and maturation of junctional complexes induces a parallel orientation (parallel to the basement membrane) of the mitotic spindle in the mammary epithelium resulting in cells to divide side by side and form a monolayer of epithelial cells. B. Decreased expression of core junctional proteins is linked to alterations in the polarity axis and activation of oncogenic pathways downstream of junctions which might lead to the manifestation of tumorigenic phenotypic changes. Of these changes is the improper direction of cell division, whereby cells will divide on top of one another as a result of a random orientation of the mitotic spindle leading to a multilayered epithelium. Such a detrimental effect can initiate other events like improper cellular and nuclear architecture and further misorientations of the mitotic spindles in daughter cells which in turn would predispose the epithelium to tumor initiation (Qin et al. 2010).

There is an immense body of knowledge illustrating that cues from the cellular microenvironment including hormones, growth factors, cell-cell and cell-ECM interactions are essential for proper function of the mammary gland. These signals modulate the establishment and maintenance of both cell junctions and polarity axis proteins during epithelial morphogenesis. However, deciphering the dominant player in this interaction is not evident as both sets of proteins are interlinked and converge to

common key signaling pathways for the control of tissue homeostasis. Filling the gap in understanding the molecular mechanisms through which gap junctions and polarity proteins execute their effects in the normal and the oncogenic contexts is paramount as both types of proteins could serve as effective targets for drug therapy in a number of carcinomas, including breast cancer.

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