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Gap Junctions and Wnt Signaling in the Mammary Gland: a Cross-Talk?

Sabreen F. Fostok[1] · Mirvat El-Sibai[2] · Marwan El-Sabban[3] · Rabih S. Talhouk[1]

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Abstract

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

Keywords Mammary gland · Breast Cancer · Gap junctions · Connexins · Wnt pathways

Abbreviations

GJ	Gap junction
Cx	Connexin
GJIC	Gap junctional intercellular communication
TEB	Terminal end bud
TDLU	Terminal duct lobular unit
ECM	Extracellular matrix
3-D	3-Dimensional
3'-UTR	3'-Untranslated region
STAT5	Signal transducer and activator of transcription 5
VEGF	Vascular endothelial growth factor
TSP-1	Thrombospondin 1
PCP	Planar cell polarity
APC	Adenomatous polyposis coli
CK1	Casein kinase 1
GSK-3	Glycogen synthase kinase 3
TCF	T-cell factor
LEF	Lymphoid enhancer factor
Gro	Groucho
TLE	Transducin-like enhancer
HDAC	Histone deacetylase
Fzd	Frizzled
LRP5/6	Low-density lipoprotein receptor-related protein 5 or 6
Dvl	Dishevelled
CDK1	Cyclin-dependent kinase 1
DKK1	Dikkopf 1
sFRP	Secreted frizzled-related protein
WIF1	Wnt inhibitory factor 1
Panx3	Pannexin 3
Rb	Retinoblastoma
GSC	Glioma stem cell
GFAP	Glial fibrillary acidic protein
MSC	Mesenchymal stem cell
Rho	Ras homolog
JNK	c-Jun N-terminal kinase
Daam1	Dishevelled-associated activator of morphogenesis 1
GEF	Guanine nucleotide exchange factor

✉ Rabih S. Talhouk
rtalhouk@aub.edu.lb

[1] Department of Biology, Faculty of Arts and Sciences, American University of Beirut (AUB), P.O. Box: 11-0236, Beirut, Lebanon

[2] Department of Natural Sciences, School of Arts and Sciences, Lebanese American University (LAU), Beirut, Lebanon

[3] Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut (AUB), Beirut, Lebanon

ROCK	Rho-associated kinase
ER	Endoplasmic reticulum
PKC	Protein kinase C
CaMKII	Calcium/calmodulin-dependent protein kinase II
NFAT	Nuclear factor of activated T-cells
PAK1	p21-activated kinase 1
FRET	Forster resonance energy transfer
EGF	Epidermal growth factor
MMP	Matrix metalloproteinase
MLC	Myosin light chain
TJ	Tight junction
AJ	Adherens junction
MDCK	Madin-Darby canine kidney
MTOC	Microtubule-organizing center
GDI	Guanine nucleotide dissociation inhibitor
α-MHC	α-Myosin heavy chain
BCR	B-cell receptor

Introduction

The mammary gland continues to develop postnatally and is considered a valuable model for studying epithelial physiology and pathology. In addition to the role of soluble mediators as systemic regulators of breast tissue development and differentiation, the local microenvironment has emerged as a major regulator almost two decades ago [1–7]. Disruption of the mammary epithelial microenvironment is linked to breast cancer development [8–10]. Neighboring cells with which mammary epithelial cells directly interact to establish homocellular and heterocellular junctions have gained considerable interest, both in developmental and tumorigenesis contexts [11, 12]. Gap junctions (GJs) regulate the development and differentiation of the mammary gland. Altered expression and localization of their building blocks, connexin (Cx) proteins, are reported in breast cancer, making them candidate tumor suppressors [13–28]. Indeed, a tight spatiotemporal regulation governs the expression of Cxs in the mammary gland throughout its development [29–34]. Studies addressing the role of Cxs in mammary epithelial differentiation or tumorigenesis implicate channel-independent mechanisms for Cxs beyond their classical GJ-dependent roles [24, 35, 36]. However, the downstream pathways through which Cxs act remain elusive. Both branches of Wnt signaling, the canonical or the Wnt/β-catenin pathway and the noncanonical pathway, execute key roles in mammary gland development and differentiation, and altered Wnt signaling is associated with breast cancer [37–48]. The involvement of Cxs and Wnt pathways in similar processes suggests a cross-talk in the breast tissue. Induction of Wnt1 expression in a mammary epithelial cell line enhances Cx43 expression and gap junctional intercellular communication (GJIC) [49]. Similarly, stimulation of mammary cocultures with Wnt3a upregulates the expression of Cx43 [50]. Overexpression of Wnt5a in the mammary epithelium impairs lactation in mice by altering Cx43 function [51]. Although Cxs are downstream targets of Wnt signaling in the mammary epithelium, the interplay between the two is poorly investigated and is not defined in terms of the biological context, possibly due to the scarcity of studies. In support of a cross-talk, we have demonstrated negative regulation of the Wnt/β-catenin pathway by Cxs, as a mechanism to induce differentiation [36] or to suppress tumorigenesis [24] in the mammary epithelium. Furthermore, our recent findings indicate a role for Cxs in regulating the noncanonical Wnt pathway in the breast tissue (unpublished data). In this review, we elaborate on the roles of Cxs and Wnt pathways in mammary development and breast cancer. We next discuss the cross-talk between Cxs and Wnt signaling in nonbreast tissues, and we propose a model for their interaction in the mammary gland in developmental and tumorigenic contexts.

Cxs may act as upstream negative regulators or as downstream positive effectors of Wnt/β-catenin signaling, depending on the biological context. The "positive effector" role of Cxs is linked to developmental and pathological processes, such as ovarian folliculogenesis and endometrioid adenocarcinomas [52, 53]. This role is additionally defined in the context of cardiac differentiation and function, whereby induction of Cx expression downstream of canonical Wnt signaling enhances spontaneous beat rate and improves cardiac conduction [54, 55]. Cxs act as "negative regulators" of the Wnt/β-catenin pathway as a mechanism to regulate cell cycle entry in kidney cells [56]. Furthermore, reconstitution of Cx expression reverses the malignancy of glioma and colon cancer cells by inhibiting canonical Wnt signaling [57, 58]. In light of the above findings, we propose a model in which a similar cross-talk exists between Cxs and Wnt signaling in the mammary gland. Whether Cxs play the role of an "upstream negative regulator" or a "downstream positive effector", this is likely governed by the context. During developmental stages, canonical Wnt signaling induces the expression of Cxs that execute channel-dependent and channel-independent functions to regulate the morphogenesis and differentiation of the mammary tissue, and subsequently act as inhibitors of canonical Wnt signaling to maintain homeostasis and suppress tumorigenesis. The downregulation of Cx expression in early stages of breast cancer leads to the loss of this control and induces hyperproliferation into a primary tumor. In the context of advanced-stage breast cancer, aberrant canonical Wnt signaling upregulates Cx expression to support collective migration and drive tumor metastasis.

Development of the Mammary Gland

Extensive remodeling governs the development of the mammary gland and predominates it during adulthood. The

anatomical and molecular events that accompany the development of the mammary gland from prenatal stages to weaning post lactation are well characterized [59, 60]. Murine models have been mainly used for studying the development of the mammary gland. In brief, development commences during embryogenesis, and is initiated by the formation of bilateral milk lines, or mammary ridges, which develop into mammary placodes and then into epithelial bulbs that invade the underlying mesenchyme. Bud elongation produces a mammary sprout that further invades the fat pad precursor mesenchyme. A rudimentary ductal system develops within the mammary adipose tissue upon lumen formation and branching of the sprout, and continues with the isometric growth until the neonatal phase. Subsequently, the mammary gland remains quiescent until puberty [59, 61, 62]. At puberty, estrogen mediates the formation of terminal end buds (TEBs) at the tips of the branching ducts. TEBs direct elongation and branching of the ductal tree, characterized by epithelial proliferation and migration, and regress upon reaching the edges of the fat pad [59, 63–65]. Further side branching occurs with each estrous cycle in response to progesterone [66]. During pregnancy, progesterone and prolactin stimulate the development of alveolar buds at the ends of the branching ducts. At this point, epithelial cells within alveoli undergo structural and functional differentiation [59, 67, 68]. At parturition, reduced progesterone levels and sustained production of prolactin induces milk secretion in alveoli. Upon cessation of lactation, epithelial apoptosis results in involution of the mammary gland and regression into a prepregnancy state [59, 68].

In humans, the mature female breast encompasses lobules, milk ducts, connective tissue and adipose tissue. Terminal duct lobular units (TDLUs), the functional units of the breast, consist of a terminal duct that connects to the ductal system and leads to a lobule, a cluster of glandular milk-secreting structures termed alveoli or acini. Luminal epithelial cells line alveoli (lobular epithelium) and ducts (ductal epithelium), and are surrounded by a discontinuous layer of myoepithelial cells. A basement membrane supports the mammary epithelium and forms contacts with both luminal epithelial and myoepithelial cells in TDLUs. The stroma consists of an extracellular matrix (ECM) and stromal cells (fibroblasts, adipocytes, endothelial cells and immune cells) which underlie the basement membrane [69].

Development of the mammary gland is tightly regulated by systemic (endocrine) and local factors (microenvironment) that act together to ensure the proper spatiotemporal regulation of proliferation, differentiation and apoptosis, thereby preventing developmental defects and neoplastic transformation [70]. Stromal cells are part of the local factors that play important roles in orchestrating morphogenetic events in the developing mammary gland. Fibroblasts, for instance, trigger epithelial branching morphogenesis in a 3-dimensional (3-D) fibroblast-epithelial coculture model [4]. Macrophages or eosinophils are also required for mouse TEB formation and ductal branching, which are impaired in mice lacking those cells in their mammary glands [2]. Furthermore, mice dually treated with estradiol and progesterone to induce alveologenesis have reduced ability to form alveolar buds upon depletion of macrophages [1]. Macrophages also regulate mammary gland involution, whereby the execution of epithelial apoptosis, alveolar regression and adipocyte repopulation fails in macrophage-devoid mice [5].

In addition to stromal cells, the role of ECM signaling in regulating mammary gland development is extensively documented [3, 7, 6]. Interactions of the epithelial and myoepithelial compartments with the underlying ECM generate biochemical and mechanical signals that dictate normal mammary architecture and function [71]. Thus, disruption of cell-ECM interactions is associated with developmental defects and breast tumorigenesis. Conditional deletion of β1-integrin, a major ECM receptor, from the basal compartment of mouse mammary epithelium alters ductal branching pattern and impairs lobuloalveolar development [6]. The ECM is dynamically deposited and degraded throughout the developmental stages of the mammary gland, further supporting its role in mammary morphogenesis. Indeed, ECM components and remodeling enzymes undergo spatial and temporal expression in the developing mammary glands of mice [3, 7, 72, 73]. Therefore, normal morphogenesis of the mammary gland is not only contingent upon tight hormonal regulation, but is also dependent on the presence of a well-regulated microenvironment.

Connexins in Mammary Gland Development

Cxs are expressed in most cell types and exhibit evolutionary conservation among chordates [74]. Twenty Cx genes have been identified in mice and 21 in humans. Most Cx genes share a similar structure consisting of two exons separated by one intron. The first exon is untranslated, while the second contains the coding region and the 3′-untranslated region (3'-UTR) [75]. Cx proteins consist of highly conserved cytoplasmic N-terminal domain, two extracellular loops with four transmembrane domains, and variable intracellular loop and cytoplasmic C-terminal domain that account for functional differences among Cx isoforms [76–78]. Cx43 is the most abundantly and ubiquitously expressed Cx protein, making it the most studied Cx isoform [76–78]. Cxs oligomerize to form hexameric structures referred to as hemichannels or connexons, and docking of two connexons in adjacent cell membranes forms a GJ channel. Oligomerization of identical Cxs forms homomeric connexons, while heteromeric connexons result upon association of different Cx isoforms. In addition, homotypic or heterotypic GJ channels result from docking of identical or different connexons, respectively.

Structures formed upon accumulation of thousands of GJ channels at the membrane are referred to as GJ plaques or GJs [76–78]. GJs connect the cytoplasms of two adjacent cells, allowing intercellular exchange of ions, second messengers (Ca^{2+}, cAMP and IP3) and metabolites (sugars, amino acids and small peptides) less than 1.5 kDa in size [76–78]. In addition to their classical channel-dependent roles, Cxs execute channel-independent functions by associating with signaling molecules, enzymes, cytoskeletal and junctional proteins, among others [76, 77]. The expression and turnover of Cxs are tightly regulated. The loss of this regulation, whether in the form of loss of expression, mutations or altered GJIC, is associated with disease outcomes, including cancer [79–81].

The expression patterns of Cxs in the mammary gland are spatiotemporally defined. In mouse models, luminal epithelial cells express Cx26, Cx30 and Cx32, while the expression of Cx43 is limited to the mammary myoepithelium [33]. In contrast, the expression of Cx43 is evident in both epithelial cell layers in reduction mammoplasties of normal women, with luminal epithelial cells expressing additionally Cx26 [82, 83]. Despite a well-characterized spatial expression of Cxs in the human mammary gland, temporal expression patterns remain elusive, and are linked to sampling limitations and inability to obtain normal breast tissue samples at the various developmental stages of the mammary gland. Majority of studies investigating the temporal expression of Cxs utilized mouse models [29–34, 84, 85].

Cxs play important roles in normal development and physiology of the mammary gland. Cx26 and Cx43 knockout mice die in utero and at birth, respectively, making it impossible to study the role of Cx26 and Cx43 in mammary glands of these mice [86, 87]. Autosomal dominant Cx43 mutation ($Cx43^{I130T/+}$) delays ductal elongation and reduces gland size relative to body weight in prepubertal mice. Although milk production and ejection are not affected, mutant mice have impaired mammary epithelial proliferation, leading to reduced gland size at parturition [88]. In a similar model ($Cx43^{G60S/+}$), mammary gland development is delayed in virgin mice. Ductal elongation, branching, TEB formation and relative mammary gland weight are reduced, but the morphology of the mammary gland at parturition is not affected [21]. Furthermore, milk secretion and ex vivo oxytocin-induced milk ejection into the ducts are impaired [21, 22]. Indeed, knocking down Cx43 or blocking GJIC in primary mammary organoids of wild-type mice inhibits myoepithelial contractility in response to oxytocin stimulation [27]. Replacement of Cx43 with Cx32 in a heterozygous knock-in mouse model ($Cx43^{Cx32/+}$) reduces postnatal growth and survival of pups. This is attributed to defects in milk ejection but not in mammary gland development or milk production [89]. Heterozygous $Cx43^{Cx26/+}$ mutation has similar effects on pup survival and growth, does not affect milk production, but is associated with reduced branching of ductuli, number and size of secretory alveoli in lactating mice [90]. In a Cx26 conditional knockdown mouse model, where the physiological surge in mammary Cx26 that accompanies pregnancy and lactation is inhibited, normal development and function of the mammary gland are retained, indicating that basal levels of Cx26 are sufficient [25]. Interestingly, transgenic mice overexpressing Cx26 under the control of keratin 5 promoter (K5-Cx26), which exhibits constitutive activity in myoepithelial cells, are unable to feed their pups despite normal mammary gland development and milk production. In fact, ex vivo oxytocin stimulation of mammary organoids isolated from transgenic mice fails to induce contraction, and ectopic expression of Cx26 in myoepithelial cells alters the expression of endogenous Cx43, leading to disrupted GJIC [27]. This illustrates the importance of spatial regulation of Cx expression in normal functioning of the mammary gland. Conditional inactivation of Cx26 gene in the mammary epithelial compartment ($Cx26^{fl/fl}$ x MMTV-Cre) affects mouse mammary glands in a stage-dependent manner. The loss of Cx26 before puberty does not alter ductal elongation or branching, but it impairs lobuloalveolar development and function during pregnancy and lactation, respectively. These effects are due to increased apoptosis and are not associated with reduced mammary epithelial proliferation. In contrast, the loss of Cx26 during later stages of pregnancy does not affect mammary development or function, illustrating the temporal effects of Cx expression in the mammary gland [91]. Indeed, Cx26 acts downstream of prolactin signaling in the mammary epithelium during early pregnancy. Mouse mammary epithelial transplants devoid of prolactin receptor form alveolar buds that fail to undergo lobuloalveolar development during pregnancy. This is concomitant with reduced expression of Cx26, suggesting a role in prolactin-induced mammary development [92]. The spatiotemporal expression patterns of murine mammary Cxs and the developmental defects associated with their altered expression are summarized in Table 1 [21, 22, 27, 88–91, 93].

We have previously demonstrated channel-dependent and channel-independent roles for Cx43 in differentiation of the mammary gland [35, 36]. Blocking GJIC in CID-9 mouse mammary cell strain under differentiation-permissive conditions (in the presence of exogenous basement membrane) downregulates the expression of β-casein, a milk protein and a differentiation marker. Furthermore, induction of GJIC in the absence of a basement membrane is sufficient to induce mammary epithelial differentiation [35]. Indeed, these effects are independent of ECM-induced signal transducer and activator of transcription 5 (STAT5) [94]. Subsequently, we illustrated involvement of GJ complex assembly (Cx43, among other Cxs, α-catenin, β-catenin and ZO-2) in differentiation of mouse mammary epithelial SCp2 cells. The role of GJ complex assembly in mammary epithelial differentiation is partly mediated by the recruitment of β-catenin to the

Table 1 The spatiotemporal expression patterns of murine mammary Cxs and the developmental abnormalities in mouse models of altered Cx expression

Cx isoform	Cell compartment	Developmental stage	Mouse model	Developmental abnormality	References
Cx26	Luminal epithelium	Pregnancy Parturition Lactation	K5-Cx26: Ectopic expression of Cx26 in myoepithelial cells	Impaired milk ejection	[27]
			$Cx26^{fl/fl}$ x MMTV-Cre: Conditional deletion of Cx26 gene in mammary epithelial cells before puberty	Impaired lobuloalveolar development and lactation	[91]
			$Cx43^{Cx26/+}$: (see below)		[90]
Cx30	Luminal epithelium	Pregnancy Parturition Lactation			
Cx32	Luminal epithelium	Parturition Lactation	$Cx43^{Cx32/+}$: (see below)		[89]
Cx43	Myoepithelium	Pregnancy Parturition Lactation	$Cx43^{G60S/+}$: Autosomal dominant point mutation (G60S) in one Cx43 allele	Delayed ductal elongation, branching and TEB formation Reduced gland size Defective milk secretion and ejection	[21, 22]
			$Cx43^{I130T/+}$: Autosomal dominant point mutation (I130T) in one Cx43 allele	Delayed ductal elongation Reduced gland size	[88]
			$Cx43^{Cx32/+}$: Replacement of one Cx43 allele with Cx32	Impaired milk ejection	[89]
			$Cx43^{Cx26/+}$: Replacement of one Cx43 allele with Cx26	Reduced ductular branching Reduced alveolar number and size	[90]

membrane, thereby preventing its nuclear translocation, which induces the expression of proliferation and cell cycle genes [36].

Connexins in Breast Tumorigenesis

Aberrant patterns of Cx expression and localization are linked to breast cancer. Reduced Cx43 expression is reported in human breast cancer tissues at various stages of tumor progression, in carcinogen-induced rat mammary tumors and in breast cancer cell lines [14]. In addition to impaired expression, progressive alteration of Cx43 localization is found in human mammary dysplasia and breast cancer tissues, as compared to normal breast tissues. Cx43 exhibits intercellular punctate localization in normal breast tissues and diffuse cytoplasmic pattern in breast cancer tissues, indicating loss of GJIC [17]. Indeed, a positive correlation is established between Cx43 levels and improved disease outcome in breast cancer patients, and Cx43 is proposed as an independent prognostic marker [95]. In addition to the dysregulation of Cx43, reduced or complete loss of Cx26 expression is reported in breast cancer cell lines, compared to nontumorigenic human mammary epithelial cells, conferring a potential role to Cx26 in tumor suppression [15].

The tumor suppressive roles of Cxs in the mammary gland are supported by both in vitro and in vivo studies. We have previously demonstrated a tumor suppressive role for Cx43 in the breast. Overexpression of Cx43 in MCF-7 and MDA-MB-231 cells, human breast cancer cell lines, reduces proliferation, cell cycle progression and invasiveness and reverses their characteristic malignant phenotype. These effects are independent of GJIC, given that overexpression of a C-terminus-truncated version of Cx43 fails to restore the wild-type Cx43 phenotype. Furthermore, blocking GJIC in Cx43-overexpressing cells does not reverse the effects of Cx43, corroborating the involvement of channel-independent mechanisms [24]. Likewise, overexpression of Cx26 in MCF-7 and MDA-MB-435 cells reduces proliferation, anchorage-independent growth, migration and invasion [18, 20]. The effects of Cx26 on MDA-MB-435 cells are channel independent, as shown by the expression of a GJIC-incompetent Cx26 form that phenocopies the effects of wild-type Cx26 [20]. Overexpression of Cx26 or Cx43 in MDA-MB-231 and MDA-MB-435 cells suppresses xenograft tumor growth in nude mice [13, 16]. Furthermore, migration of MDA-MB-231 cells is impaired upon exposure to Cx43-rich biovesicles extracted from plasma membranes of donor cells overexpressing functional Cx43-based GJs and capable of forming GJs with cells [28]. Conditional mammary gland-specific knockout of Cx26 in mice predisposes the mammary gland to

primary tumors in DMBA-induced breast cancer model [26]. Similarly, mice with heterozygous Cx43 mutation show higher susceptibility to mammary tumor lung metastasis following DMBA treatment [23]. In vitro, silencing Cx43 in Hs578T cells, human breast cancer cell line, enhances proliferation and anchorage-independent growth. This is associated with the upregulation of vascular endothelial growth factor (VEGF), a proangiogenic molecule, and downregulation of thrombospondin 1 (TSP-1), an antiangiogenic molecule [19]. We have recently shown that silencing Cx43 in nontumorigenic human mammary epithelial cell line, HMT-3522 S1 cells, enhances proliferation and cell cycle progression, and induces mislocalization of membranous β-catenin (unpublished data). In addition, Cx43-silenced cells display morphogenetic defects typical of breast cancer initiation. These include loss of apical polarity, misorientation of the mitotic spindle, multilayering and loss of lumen, thus indicating disruption of normal acinar morphology (Bazzoun et al.; submitted).

Collectively, the above studies illustrate key roles of Cxs in development and tumorigenesis of the mammary gland. The involvement of channel-independent mechanisms in Cx signaling suggests a link between Cxs and cellular pathways that execute overlapping roles with those of Cxs in the mammary gland. The developmental pathways which mediate Cx signaling in the mammary gland are yet to be investigated. Evidence supports interplay between Cxs and Wnt signaling in nonbreast tissues and in a multitude of biological contexts. In the mammary epithelium, canonical and noncanonical Wnt signaling regulate the expression and function of Cx43 [49–51]. In addition, our earlier findings indicate that the Wnt/β-catenin pathway is a modulator of Cx signaling in differentiation [36] and tumorigenesis [24] of mammary epithelial cells. This suggests that the Wnt pathways are potential candidates for relaying Cx signals within the mammary gland in development and cancer.

Connexins as Regulators of Wnt Signaling

Connexins in Canonical Wnt Signaling

Canonical Wnt Pathway

The Wnt/β-catenin pathway (or the canonical Wnt pathway) is one of the three best characterized Wnt pathways, which also include the planar cell polarity (PCP) and the Wnt/calcium pathways. The Wnt/β-catenin pathway is involved in β-catenin-mediated regulation of developmental gene expression, essential for embryogenesis and adult tissue homeostasis. Deregulation of this pathway is associated with developmental defects and adult diseases, including cancer [96–98].

In the absence of a Wnt ligand, two scaffolding proteins, adenomatous polyposis coli (APC) and Axin as well as casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK-3) form a complex in the cytoplasm, referred to as the β-catenin destruction complex. CK1 and GSK-3, serine/threonine protein kinases, sequentially phosphorylate β-catenin on specific N-terminal amino acid residues (on serine 45, and subsequently on threonine 41, serine 37 and serine 33, respectively). This marks β-catenin for ubiquitination (dually phosphorylated β-catenin on serine 33 and 37 is recognized by β-TrCP, E3 ubiquitin ligase) and subsequent proteasomal degradation leading to a reduction in the cytoplasmic pool of β-catenin available for nuclear translocation. Consequently, Wnt/β-catenin target genes are repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. TCF/LEF acts as transcriptional repressor by forming a complex with Groucho (Gro)/transducin-like enhancer (TLE), which interacts with histone deacetylases (HDACs) to mediate histone deacetylation and chromatin compaction [96–98].

In the presence of Wnt, the ligand binds to its receptor and coreceptor, Frizzled (Fzd) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), respectively. This complex (Wnt-Fzd-LRP5/6) triggers Fzd-mediated recruitment of Dishevelled (Dvl), a scaffolding protein, which in turn recruits Axin along with its associated GSK-3 and CK1 to the membrane, resulting in phosphorylation of LRP5/6 by GSK-3 and CK1. Phosphorylation leads to the activation of LRP5/6, which recruits the Axin-GSK-3-CK1 complex, thereby amplifying phosphorylation of LRP5/6 and enhancing the recruitment of the Axin complex as well. As a result, the β-catenin destruction complex (APC-Axin-CK1-GSK-3) is disrupted. This stabilizes β-catenin and leads to its accumulation and translocation to the nucleus, where it acts as a transcriptional coactivator. In the nucleus, β-catenin displaces Gro/TLE to form a complex with TCF/LEF, thereby converting the latter into a transcriptional activator and inducing the expression of genes involved in cell cycle progression, including c-Myc, cyclin-dependent kinase 1 (CDK1) and cyclin D1 (Fig. 1). Wnt/β-catenin target genes also include components of the Wnt/β-catenin pathway itself that may act as agonists or antagonists, conferring self-regulatory properties to the pathway [96–98].

Role of Canonical Wnt Pathway in Mammary Gland Development

The role of Wnt signaling in mammary gland development and breast tumorigenesis is well documented [99–101]. The earliest detectable event marking the activation of Wnt signaling during mammary development is the expression of Wnt10b in the mammary line and Wnt6 in the surface ectoderm of mouse embryos at embryonic day E11.25 [61]. Wnt

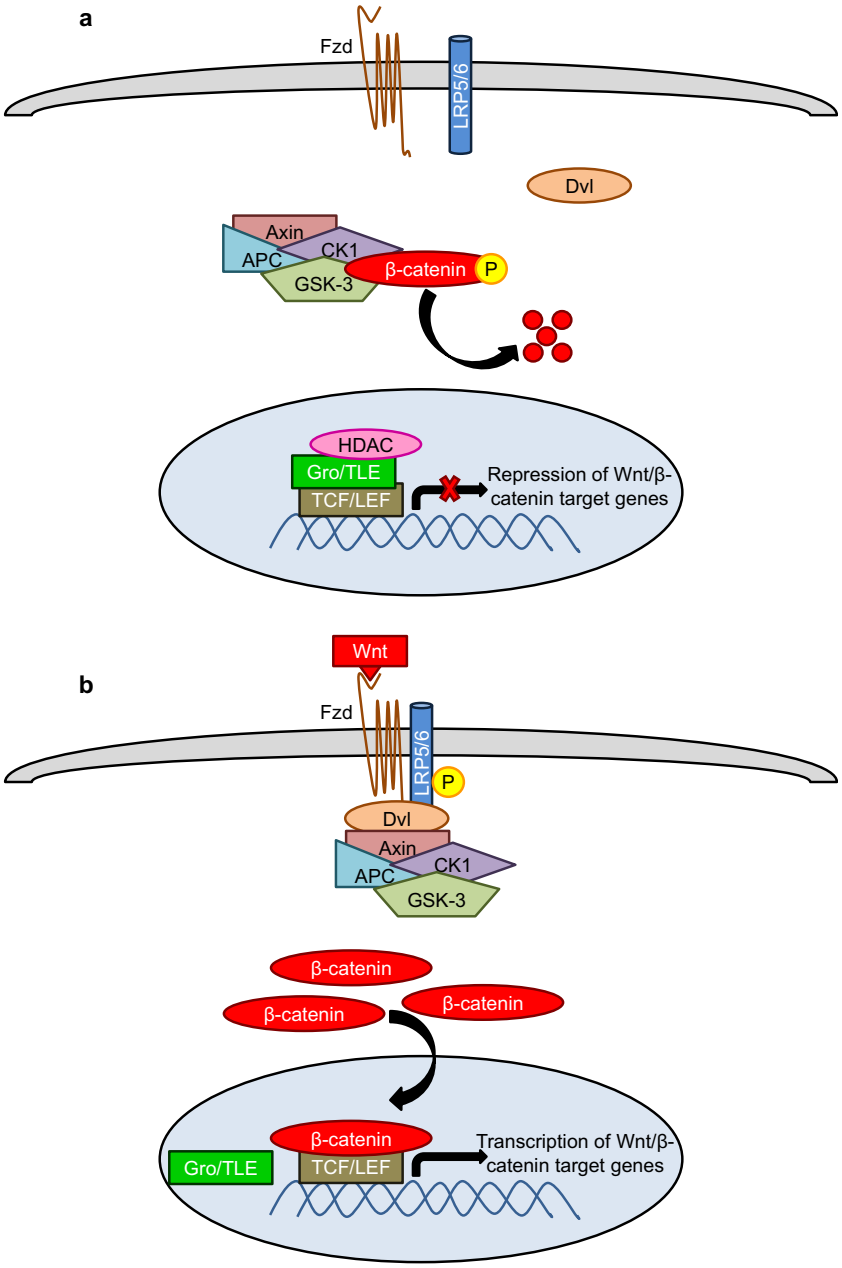


Fig. 1 The canonical Wnt pathway. In the absence of a Wnt ligand (**a**), the scaffolding proteins Axin and APC form a complex with the serine/threonine protein kinases CK1 and GSK-3 in the cytoplasm, referred to as the β-catenin destruction complex. CK1 and GSK-3 sequentially phosphorylate β-catenin, marking it for ubiquitination and subsequent proteasomal degradation, thereby reducing its nuclear translocation. Consequently, the TCF/LEF family of transcription factors acts as a transcriptional repressor by forming a complex with Gro/TLE, which interacts with HDACs to mediate chromatin compaction, causing the repression of the Wnt/β-catenin target genes. In the presence of Wnt (**b**), the ligand binds to its receptor Fzd and coreceptor LRP5/6. The resulting complex recruits the scaffolding protein Dvl, which in turn recruits the β-catenin destruction complex. CK1 and GSK-3 phosphorylate LRP5/6, causing its activation and enhancing the recruitment of the β-catenin destruction complex. This results in the stabilization and accumulation of β-catenin in the cytoplasm, and its subsequent nuclear translocation. In the nucleus, β-catenin acts as a transcriptional coactivator by displacing Gro/TLE, thereby converting TCF/LEF into a transcriptional activator to induce the expression of the Wnt/β-catenin target genes and cell cycle progression

signaling components are expressed in a cell type-specific and stage-dependent manner in the developing mammary gland [102, 103]. The expression patterns of Wnt ligands are summarized in Table 2 [61, 104–110].

Canonical Wnt signaling initiates mammary gland morphogenesis in mouse embryos. Activation of canonical Wnt signaling in the mammary region overlaps with the onset of mammary morphogenesis and localizes to mammary placodes

Table 2 The spatiotemporal expression patterns of Wnt ligands in the murine mammary gland

Developmental stage	Cell compartment	Wnt ligand	References
Embryonic	Mammary line	Wnt5a	[61, 105]
		Wnt6	
		Wnt10b	
	Mammary placode	Wnt1	[61, 105]
		Wnt2	
		Wnt3	
		Wnt5a	
		Wnt6	
		Wnt7b	
		Wnt10a	
		Wnt10b	
		Wnt11	
	Mammary bud	Wnt1	[105]
		Wnt2	
		Wnt3	
		Wnt3a	
		Wnt4	
		Wnt5a	
		Wnt5b	
		Wnt7b	
		Wnt10b	
		Wnt11	
Pubertal	TEB	Wnt2	[106]
		Wnt4	
		Wnt5a	
		Wnt5b	
		Wnt6	
		Wnt7b	
	Duct	Wnt4	[106]
		Wnt5b	
		Wnt6	
Adult	Luminal epithelium	Wnt4	[104, 109, 110]
		Wnt5a	
		Wnt5b	
		Wnt7b	
	Myoepithelium	Wnt5a	[109, 110]
		Wnt5b	
		Wnt10a	
Pregnancy		Wnt2 (early, mid)	[107, 108]
		Wnt4 (early, mid)	
		Wnt5a (early, mid)	
		Wnt5b (early, mid, late)	
		Wnt6 (early, mid, late)	
		Wnt7b (early)	
		Wnt10b (early)	

and buds thereafter. Forced activation of canonical Wnt signaling using Wnt3a accelerates placode formation in cultured embryos. Conversely, ectopic expression of the Wnt inhibitor Dikkopf 1 (DKK1) in the surface epithelium of transgenic embryos blocks placode development [105]. Formation of rudimentary mammary buds is inhibited in mouse embryos with homozygous LEF-1 mutation [37], while homozygous mutation in LRP5 reduces the size of mammary placodes in

mouse embryos and alters ductal elongation and TEB numbers in virgin mice [111]. Similarly, LRP6 knockout mouse embryos have smaller mammary placodes and fat pad and lack ductal branching, whereas heterozygous LRP6 mutation alters TEB numbers and ductal branching in juvenile and adult mice, respectively [45]. Canonical Wnt signaling also mediates progesterone-induced side branching in mammary ducts. Ectopic expression of Wnt1 rescues side branching of ducts in mammary epithelial transplants of mice with homozygous mutation in progesterone receptor, indicating that the canonical Wnt signaling acts downstream of progesterone. This latter induces the expression of Wnt4, and mammary bud implants derived from Wnt4-deficient mouse embryos show impaired ductal branching during early pregnancy [112]. Expression of a constitutively active form of β-catenin causes precocious lobuloalveolar development and differentiation in mouse mammary glands. Indeed, virgin mammary glands of these transgenic mice resemble those of wild-type pregnant mice in terms of development and functional differentiation, show lobular hyperplasia during pregnancy and regress into a midpregnant state, characteristic of virgin transgenic mice, post lactation. The transgenic mice develop multiple aggressive adenocarcinomas early on during their lifetime [41].

Role of Canonical Wnt Pathway in Breast Tumorigenesis

In addition to regulating development and differentiation of the mammary gland, aberrant Wnt/β-catenin signaling plays a role in breast cancer. Reduced levels of membranous β-catenin and enhanced nuclear activity are linked to poor disease outcome in breast cancer patients and are proposed as independent prognostic factors [40, 113]. β-catenin mutations at phosphorylation sites that target it for ubiquitination and subsequent degradation, as well as inactivating APC mutations, lead to stabilization of β-catenin and constitutive activation of the Wnt/β-catenin signaling. Although nuclear and cytoplasmic accumulation of β-catenin are reported in breast cancer, APC and β-catenin mutations, commonly associated with other cancers, are absent or rare and restricted to benign and metaplastic breast tumors [114–120]. This suggests that deregulated Wnt/β-catenin signaling in breast cancer is not a consequence of mutations in components of this pathway. In support of this, defective expression, localization or epigenetic patterns of canonical Wnt components are associated with breast cancer. Wnt ligands, receptors and coreceptors are overexpressed in breast cancer [42, 121–124]. For instance, expression of FZD1 and FZD2 receptors is upregulated in breast cancer tissues [42]. Similarly, LRP6 is overexpressed in breast cancer cell lines and tissues and is required for activation of canonical Wnt signaling, cell proliferation and xenograft tumor growth, while administration of LRP6 antagonist in vivo prevents the growth of MMTV-Wnt1 tumors [124]. Interestingly, expression of an aberrantly spliced internally truncated form of LRP5 coreceptor is found in breast cancer tissues. This form is essential for β-catenin stability and activity, cell proliferation and tumor growth in a xenograft mouse model [123]. Altered expression and epigenetic regulation of other components in the Wnt/β-catenin pathway are also common. Amplification and upregulation of Dvl1, a scaffolding protein that recruits the β-catenin destruction complex, are reported in primary breast tumors [125]. APC promoter hypermethylation and reduced expression are detected in breast cancer tissues and correlate with active Wnt/β-catenin signaling [44, 126]. Epigenetic silencing and promoter hypermethylation of Wnt antagonist genes, including secreted frizzled-related protein (sFRP) family, Wnt inhibitory factor 1 (WIF1) and DKK, are present in breast cancer cell lines and in primary breast tumors [127, 128]. Reduced sFRP expression accounts for activation of canonical Wnt signaling, and expression of sFRP suppresses proliferation of breast cancer cells [128, 129].

Although Cxs and Wnt/β-catenin signaling play overlapping roles in the mammary gland, scarce evidence supports a link between these pathways in the breast tissue [49, 50]. We have previously shown Cx channel-independent signaling as an upstream negative regulator of the Wnt/β-catenin pathway in the breast. Cx43 associates with β-catenin at the membrane and inhibits its nuclear translocation, as a mechanism to induce differentiation [36] or to suppress tumorigenesis [24] in mammary epithelial cells. The interplay between Cxs and canonical Wnt signaling exists in a number of other tissues, where Cxs act as upstream negative regulators or as downstream positive effectors of the Wnt/β-catenin pathway.

Cross-Talk between Connexins and Canonical Wnt Signaling

Connexins As Upstream Negative Regulators of Canonical Wnt Signaling Evidence supports negative regulation of the Wnt/β-catenin pathway by Cx signaling in cardiac, bone, kidney, nervous and colon tissues [54, 56–58, 130–132].

Overexpression of Cx43 in lithium-stimulated neonatal rat cardiomyocytes (lithium mimics Wnt signaling by inhibiting GSK-3β) reduces β-catenin transcriptional activity. Association and colocalization of Cx43 and β-catenin at the membrane suggests that Cx43 inhibits canonical Wnt signaling via β-catenin sequestration [54].

The knockout of Cx43 or Cx37 in osteocytes results in the accumulation of β-catenin and increased expression of Wnt/β-catenin target genes. These effects are associated with enhanced Wnt/β-catenin-dependent processes, including osteogenic response to mechanical loading and resistance to fractures in bones [130, 132]. Interestingly, pannexin 3 (Panx3), a member of a recently identified family of GJ proteins, also inhibits Wnt/β-catenin signaling in bones. Overexpression of Panx3 in osteoprogenitor cells cultured under proliferation conditions reduces proliferation and induces cell cycle arrest.

Panx3 exerts its effects by enhancing the activity of GSK-3β, leading to the phosphorylation of β-catenin and the reduction of its cytoplasmic levels. This is coupled to a decrease in β-catenin nuclear localization and activity. As a result, levels of cyclin D1 and phosphorylated retinoblastoma (Rb), involved in G1 to S phase progression, are reduced [133].

In a study on the role of adhesion molecules in cell proliferation, Cx43 synergizes the effects of N-cadherin in suppressing β-catenin/TCF transcriptional activity, as a mechanism to upregulate p21 and reduce proliferation and cell cycle progression in HEK293 human embryonic kidney cells. Notably, the effects of Cx43 are channel dependent [56].

Reconstitution of Cx43 in glioma stem cells (GSCs) impairs tumorsphere formation and proliferation. In addition, increased expression of glial fibrillary acidic protein (GFAP), an astrocytic differentiation marker, and reduced expression of CD133, a stem cell marker, are noted, indicating differentiation and impaired self-renewal capacity. Overexpression of Cx43 is also associated with reduced invasiveness in vitro, and xenografts of Cx43-transduced GSCs exhibit smaller tumor size, reduced proliferation and better differentiation, compared to their mock counterparts, suggesting that Cx43 inhibits tumorigenicity of GSCs. Notably, overexpression of Cx43 in GSCs does not restore GJIC, indicating that the observed effects of Cx43 are due to channel-independent mechanisms. Microarray analysis revealed reduced expression of Wnt/β-catenin target genes, including stemness-related genes (Nanog, Oct4 and Sox2), in Cx43-transduced GSCs. Furthermore, overexpression of Cx43 induces the expression of E-cadherin, and knocking down E-cadherin in Cx43-transduced GSCs is sufficient to restore invasiveness, indicating that Cx43 negatively regulates the Wnt/β-catenin pathway in GSCs via an E-cadherin-dependent mechanism [58]. The loss of Cx43, but not GJIC, is associated with differentiation of human neural progenitor cells as a consequence of enhanced canonical Wnt signaling. Silencing Cx43 triggers neurogenesis by increasing the protein levels and transcriptional activity of β-catenin, thereby upregulating the expression of proneuronal genes [131].

Ectopic expression of Cx43 in HT29 colon cancer cell line reduces anchorage-dependent, anchorage-independent and xenograft growth. Notably, ectopically expressed Cx43 localizes mainly to intracellular vesicular compartments and fails to form GJs, suggesting the implication of channel-independent mechanisms in tumor suppression. In addition, Cx43 associates with β-catenin and reduces TCF transcriptional activity in HT29 cells, indicating negative regulation of the Wnt/β-catenin signaling, a mechanism through which Cx43 could exert its tumor suppressive effects [57].

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

Connexins as Downstream Positive Targets of Canonical Wnt Signaling In cardiac and skeletal muscle cells, the Wnt/β-catenin pathway upregulates the expression of Cxs, mainly Cx43, and GJIC [54, 55, 134–136]. GJIC and Cx43 expression are enhanced in neonatal rat cardiomyocytes and skeletal myoblasts in response to lithium-stimulated activation of canonical Wnt signaling, and are associated with increased spontaneous beat rate in cardiomyocytes [54, 135]. Indeed, activation of the Wnt/β-catenin signaling acts downstream of cyclic strain to upregulate Cx43 expression in mouse embryonic stem cells, thereby inducing cardiac differentiation [136]. Canonical Wnt signaling also mediates the effects of β1-integrin on Cx mRNA expression (Cx40, Cx43 and Cx45) in mouse embryonic stem cell-derived cardiomyocytes at advanced stages of differentiation [134]. Furthermore, inhibition of β-catenin or GSK-3α/β in HL-1 cells, mouse cardiomyocyte cell line, prevents mesenchymal stem cell (MSC)-induced upregulation of Cx43 and improvement in cardiac conduction, suggesting that MSCs alleviate cardiac arrhythmias via activation of the canonical Wnt signaling [55].

A similar pattern of Cx and GJ regulation is reported in Xenopus embryos, ovarian follicles and ovarian carcinomas, umbilical vein endothelial cells and retinal pigment epithelial cells [52, 53, 137–140].

Studies summarized above clearly illustrate interplay between Cxs, mainly Cx43, and the Wnt/β-catenin pathway in several tissues, with the former acting either as downstream targets (positive effectors) or as upstream negative regulators of Wnt signaling. Whether Cxs play the downstream role of a "positive effector" or are upstream "negative regulator" of Wnt signaling, the interplay between the two is context specific. Studies defining Cxs as downstream targets (positive effectors) for the Wnt/β-catenin pathway correlate tissue development and differentiation-driving events to effective GJ communication. As previously stated, induction of Cx43 expression, among other cardiac Cxs (Cx40 and Cx45), downstream of canonical Wnt signaling is associated with the acquisition of cardiac differentiation and function [54, 55, 134, 136]. The "positive effector" role of Cxs is additionally associated with developmental processes, such as embryogenesis, angiogenesis and ovarian folliculogenesis [52, 137–139]. On the other hand, this role is evident in the context of disease pathogenesis, including ovarian cancer [53] and proliferative vitreoretinopathy [140]. In contrast to acting as downstream targets in developmental contexts, the inhibitory effects of Cxs upstream (i.e. "negative regulator") of the Wnt/β-catenin pathway are associated with differentiation or tumor suppression as most studies indicate [54, 56–58]. Hence, Cxs likely undergo a switch in role from a "positive effector" into a "negative regulator" of the Wnt/β-catenin pathway upon establishment of tissue

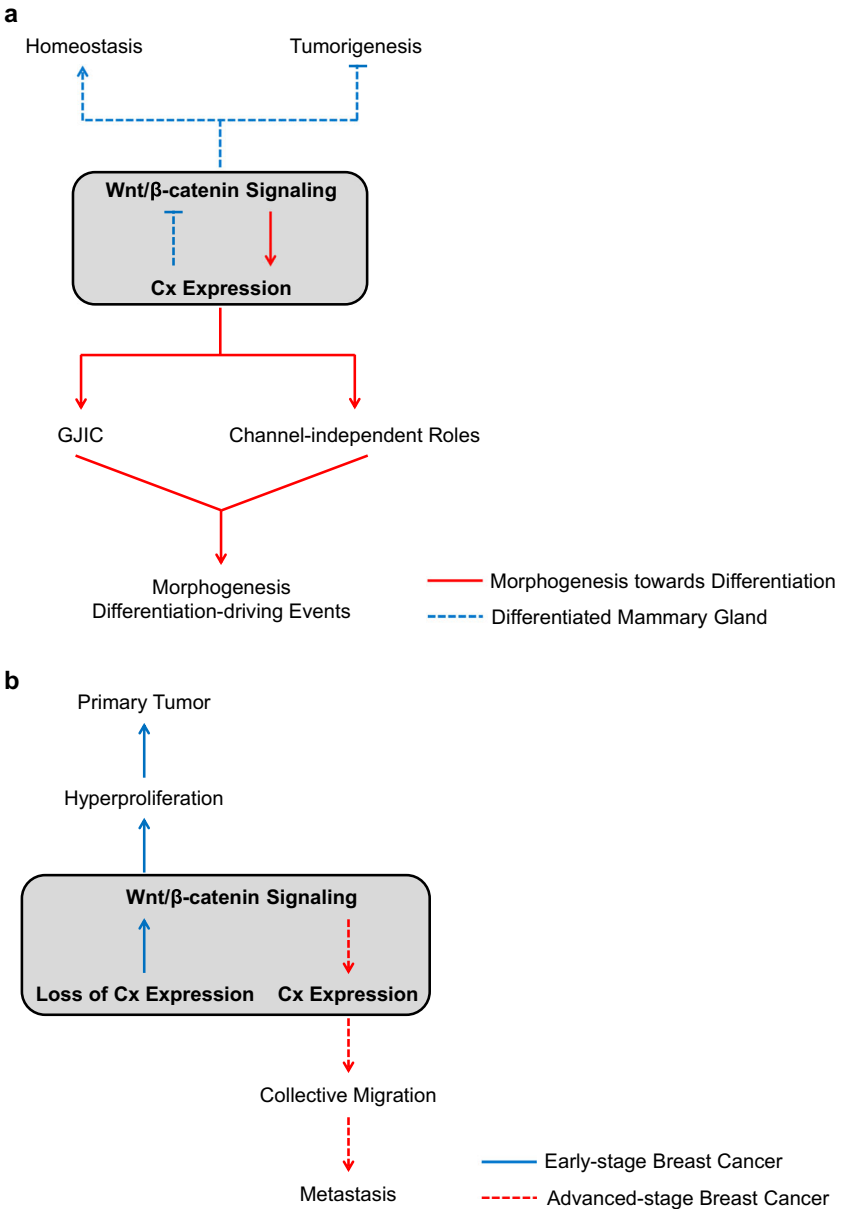
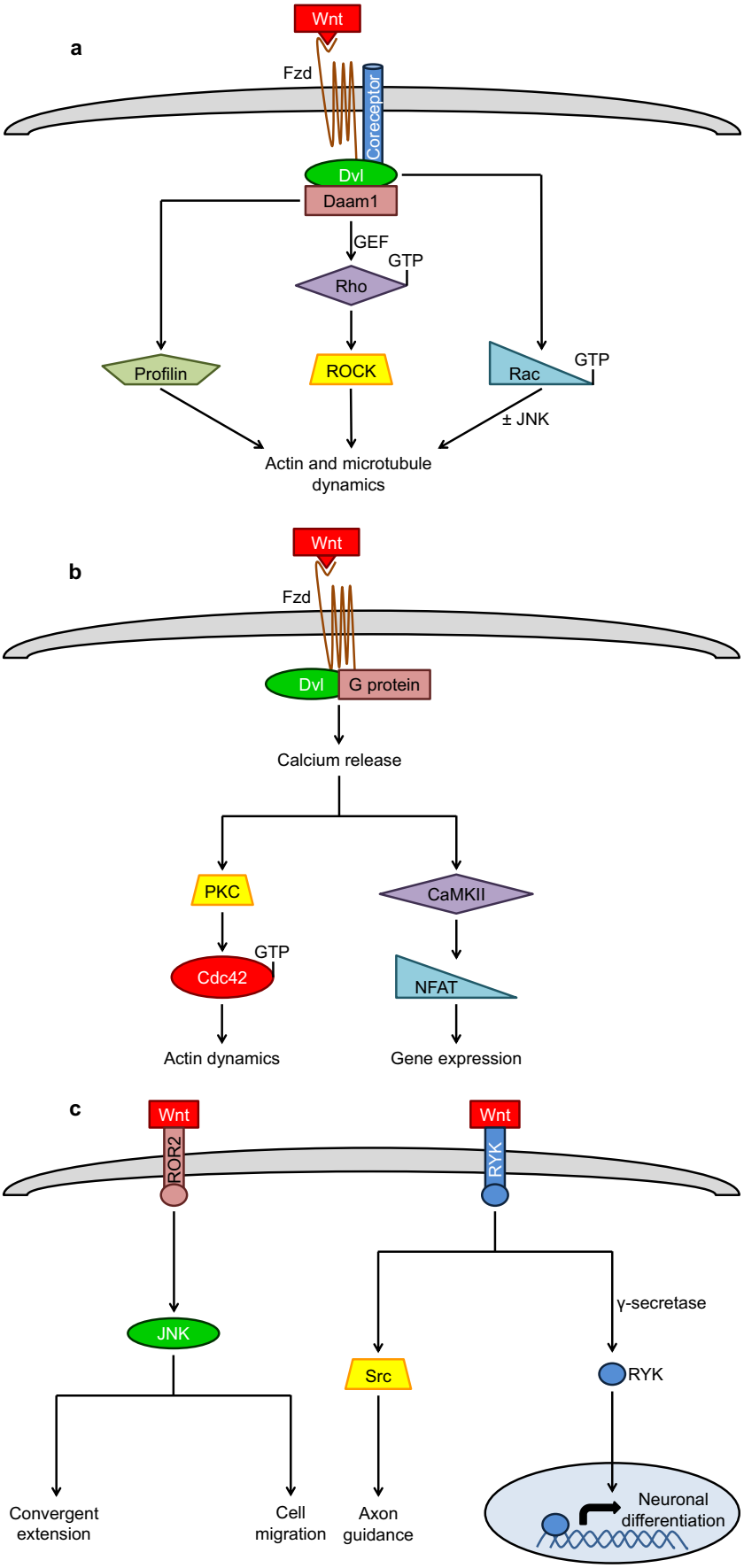


Fig. 2 A proposed model for the cross-talk between Cxs and Wnt/β-catenin signaling in the mammary gland. Depending on the context, Cxs may act as downstream "positive effectors" (red arrows) or as upstream "negative regulators" of the Wnt/β-catenin pathway (blue arrows) both in normal development and tumorigenesis of the mammary gland (grey boxes). In normal development (**a**), active canonical Wnt signaling induces Cx expression during morphogenesis and differentiation-driving events of the mammary gland. Cxs regulate the morphogenesis and differentiation of the tissue via channel-dependent and channel-independent mechanisms (red arrows) [52, 54, 55, 134, 136–139]. Within a differentiated mammary gland, Cxs act as negative regulators of the Wnt/β-catenin pathway, a mechanism to sustain homeostasis and suppress tumorigenesis (blue arrows) [36, 54, 56–58]. In breast cancer **(b)**, the loss of Cx expression during early stages activates canonical Wnt signaling, which mediates hyperproliferation and primary tumor formation (blue arrows) [24]. Aberrant Wnt/β-catenin signaling induces Cx expression in advanced stages of breast cancer, supporting collective migration and tumor metastasis (red arrows) [53, 140]

development to suppress tumorigenesis. During growth and differentiation-driving events of the normal mammary gland, we speculate positive regulation of Cxs downstream of active canonical Wnt signaling to induce Cx-mediated morphogenesis and differentiation [21, 22, 27, 35, 52, 54, 55, 134, 136–139]. Within the same context, hyperactive Wnt/β-catenin signaling impairs mammary development [50]. In the context of a differentiated mammary tissue, however, Cxs act to suppress the Wnt/β-catenin pathway in order to maintain homeostasis and to execute tumor suppressive effects (Fig. 2a) [36, 54, 56–58]. In early stages of breast cancer, the loss of Cx expression triggers the formation of primary tumor by activating canonical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RYK
JNK
γ-secretase
Src
RYK
Convergent extension
Cell migration
Axon guidance
Neuronal differentiation

◀ **Fig. 3 The noncanonical Wnt pathway.** The PCP pathway (**a**) involves binding of a Wnt ligand to its receptor Fzd and coreceptor (ROR2, RYK, PTK7 or NRH1). The resulting complex recruits the scaffolding protein Dvl, which in turn recruits Daam1. This leads to GEF-mediated activation of Rho, which activates ROCK. Daam1 also mediates binding of profilin to actin. On the other hand, Dvl mediates Rac activation, which acts through activating JNK or independently. Profilin, ROCK and Rac regulate the dynamics of the actin and microtubule networks, which control cellular morphology, migration and division orientation. The Wnt/calcium pathway (**b**) involves the coactivation of Dvl and Fzd-associated G protein upon binding of a Wnt ligand, leading to the intracellular release of calcium. This results in PKC-mediated activation of Cdc42, which regulates actin dynamics. Calcium release also activates CaMKII, which activates the transcription factor NFAT. The Fzd-independent pathways **(c)** are triggered upon binding of a Wnt ligand to its receptor ROR2 or RYK. ROR2 subsequently mediates JNK activation, which regulates cell migration and convergent extension, among others. RYK controls axon guidance via the Src kinase family. In addition, the intracellular domain of RYK translocates to the nucleus upon cleavage by γ-secretase, where it mediates the expression of genes required for neuronal differentiation

Wnt signaling [24, 76, 141, 142], whereas in the context of advanced breast cancer-driving events, aberrant Wnt/β-catenin signaling induces Cx expression to support collective migration and tumor metastasis (Fig. 2b) [53, 140].

Connexins in Noncanonical Wnt Signaling

Noncanonical Wnt Pathway

The noncanonical Wnt signaling is a branch of Wnt signaling that encompasses multiple β-catenin-independent pathways and regulates embryogenesis and adult tissue homeostasis. As such, aberrant noncanonical Wnt signaling is associated with developmental defects and adult diseases, particularly cancer [143–148].

Noncanonical Wnt signaling regulates epithelial apicobasal polarity (asymmetry along the apical-basal axis within a cell), PCP (the coordinated organization of cells within a tissue plane, also referred to as tissue polarity), cell junctions, mitotic spindle orientation, actin cytoskeletal dynamics and cell migration. Noncanonical Wnt pathways are triggered by specific family members of Wnt ligands that signal through Fzd receptors, like the canonical branch, but use alternatives to LRP5/6 where coreceptors are involved. Owing to the ligand and coreceptor differences, the noncanonical Wnt pathways regulate signaling cascades different from that underlying canonical Wnt signaling downstream of Dvl recruitment to the ligand-receptor-coreceptor complex. In addition, while the activation of the canonical Wnt pathway regulates gene expression, noncanonical Wnt signaling is also associated with nontranscriptional outcomes. The PCP and the Wnt/calcium pathways are by far the best characterized among the noncanonical Wnt pathways [143–149].

The PCP pathway activates Ras homolog (Rho) GTPases, namely Rac and Rho, and c-Jun N-terminal kinase (JNK), which induce cytoskeletal rearrangements [143, 145]. The PCP pathway is activated when a noncanonical Wnt ligand binds to Fzd and its coreceptor (ROR2, RYK, PTK7 or NRH1). Dvl is subsequently recruited and associates with Dishevelled-associated activator of morphogenesis 1 (Daam1), which activates Rho via a guanine nucleotide exchange factor (GEF). Rho in turn activates Rho-associated kinase (ROCK), a major regulator of the actin cytoskeleton. Daam1, on the other hand, mediates binding of profilin to actin. In addition, Dvl mediates activation of Rac, which activates JNK. Profilin, ROCK and JNK induce actin cytoskeletal reorganization [143, 145, 147]. The PCP pathway is known to regulate actin polymerization, as a mechanism to control cell morphology and polarized cell migration [143]. Microtubules constitute another cytoskeletal element regulated by the PCP pathway, which orients the mitotic spindle relative to cell-cell contacts or to an embryo symmetry axis [144] (Fig. 3a). Due to its role in cell division orientation and directional cell movement, the PCP pathway regulates morphogenetic processes, such as gastrulation, neurulation and organ morphogenesis [145, 147].

The Wnt/calcium pathway, on the other hand, activates Fzd-associated heterotrimeric G proteins besides Dvl and regulates intracellular calcium levels by stimulating or inhibiting calcium release from the endoplasmic reticulum (ER). One consequence of calcium release is the activation of the Rho GTPase Cdc42 through protein kinase C (PKC). Another important outcome is the activation of calcium/calmodulin-dependent protein kinase II (CaMKII), which in turn activates nuclear factor of activated T-cells (NFAT), a transcription factor [143, 149] (Fig. 3b). The Wnt/calcium pathway regulates several aspects of embryogenesis, such as ventral cell fate, tissue separation and convergent extension, and is thought of as a modulator of PCP signaling [143].

Fzd-independent pathways are identified as components of noncanonical Wnt signaling, although less characterized than the PCP and the Wnt/calcium pathways [150, 151]. The Fzd coreceptors ROR2 and RYK harbor functional extracellular Wnt-binding domains and can act as Wnt receptors independently from Fzd activation [150] (Fig. 3c). ROR2 and RYK regulate developmental processes in several tissues and are associated with cell polarity, migration and asymmetric cell division [150–153].

Due to a cross-talk among noncanonical Wnt pathways, these pathways are alternatively considered as one signaling network with diverse biological outcomes. Studies modeling the noncanonical Wnt pathways as such highlighted the roles of Rho GTPases as important downstream effectors of noncanonical Wnt signaling. RhoA, Rac1 and Cdc42 are known to regulate cytoskeletal dynamics involving the microtubule and actin networks, thereby controlling mitotic spindle

orientation, cell shape changes, motility and invasion. Rho GTPase signaling also regulates polarity, intercellular junctions and cell-ECM interactions, hence the implication of the deregulation of Rho GTPases in mammary gland tumorigenesis [147, 148, 154–157].

Role of Rho GTPases in Mammary Gland Development

Rho GTPase signaling components are implicated in various stages of mammary gland development, from embryogenesis to involution, and their aberrant expression and/or activity contributes to breast tumorigenesis [158, 159].

Inhibition of Rac1 or ROCK, a downstream effector of RhoA, in an organoid culture of mammary tissue blocks duct initiation and disrupts branching pattern, respectively, indicating a role for Rac1 and RhoA in morphogenesis of the mammary gland [43]. Expression of a dominant-negative form of Rac1 or its downstream effector p21-activated kinase 1 (PAK1) enhances the contractility of mouse myoepithelial cells in vitro. Consistent with these observations, the expression of a constitutively active form of Rac1 or a catalytically active form of PAK1 induces myoepithelial relaxation, demonstrating a role for Rac1 signaling in controlling the contraction/relaxation cycle of myoepithelial cells and thus in lactation [46]. Conditional deletion of Rac1 in mouse mammary glands delays involution via STAT3-dependent mechanism [48].

A study on a 3-D culture of primary mammary epithelial cells isolated from Cdc42 conditional knockout mice unveiled a role for Cdc42 in morphogenesis of the mammary gland. Cdc42 deficiency reduces cell proliferation and survival and alters the number and size of acini, concomitant with disruption of acinar morphology. Furthermore, apicobasal polarity, mitotic spindle orientation and lumen formation, which represent key morphogenetic features of normal mammary epithelium, are disrupted [160]. Paradoxically, normal morphogenesis of the mammary gland is also disrupted in a tetracycline-regulatable Cdc42 overexpression mouse model. This suggests the importance of tight regulation of Cdc42 levels for normal mammary gland development. Cdc42-overexpressing mammary glands exhibit TEB hyperbudding and trifurcation, ductal tree hyperbranching and altered epithelial-stromal interactions, which are known to regulate branching. Consistent with these observations, primary mammary epithelial cells isolated from Cdc42-overexpressing mammary glands form dysmorphic invasive acini in 3-D cultures, coupled to enhanced expression of ECM proteins and remodeling enzymes in their stromal counterparts. Interestingly, the phenotypic abnormalities observed upon Cdc42 overexpression are not a consequence of enhanced cell proliferation or survival, nor are they associated with disruptions in apicobasal polarity or mitotic spindle orientation. They are rather due to enhanced epithelial contractility and migration [47]. Taken together, gain-of-function and loss-of-function studies clearly illustrate redundancy in Cdc42 effects, suggesting that its role in mammary gland morphogenesis is highly contingent upon a tight balance of its levels, and perhaps activity. In addition to regulating the morphogenesis of the mammary gland, Cdc42 plays a role in its proper functioning. Conditional knockout mice lacking Cdc42 in mammary alveolar epithelial cells during lactation inadequately nourish their pups, leading to stunted growth. This is attributed to impaired alveologenesis as a consequence of disrupted apical-basal polarity and cell-cell adhesion, which result in premature exfoliation of the alveolar epithelium [161].

Role of Rho GTPases in Breast Tumorigenesis

Rho GTPases are overexpressed or hyperactivated in human breast tumors [38, 39, 158]. In addition, the expression of Rho GTPase regulators and effectors is altered in breast cancer tissues [158, 162–164]. A link is established between Rho GTPase expression levels and cell motility and invasion in vitro. Cdc42 and Rac regulate the formation of filopodia and lamellipodia, respectively, at the leading edge of a motile cell, while Rho regulates the formation of stress fibers and actomyosin contractility at the rear end [156]. The presence of a cross-talk among Rho GTPases during cell motility is also reported. For instance, Forster resonance energy transfer (FRET) biosensor imaging revealed a biphasic localization of RhoA activity at the leading edge of epidermal growth factor (EGF)-stimulated MTLn3 rat mammary adenocarcinoma cells. This spatiotemporal pattern of RhoA activity is critical for coordinating the functions of Rac1 and Cdc42 during the formation of protrusions [165]. Primary mammary epithelial cells from Cdc42-overexpressing mammary glands display enhanced contractility and migration. Specifically, Cdc42 overexpression upregulates ECM proteins and remodeling enzyme levels in stromal cells, and disrupts epithelial-stromal interactions, further supporting a role for Cdc42 in breast cancer invasion [47]. Consistent with those findings, the knockdown of Cdc42 in MTLn3 cells impairs EGF-induced protrusion, barbed end formation and F-actin accumulation at the protruding edges, which are concomitant with reduced motility, suggesting a role for Cdc42 in breast cancer cell motility [166]. siRNA-mediated silencing of RhoA or RhoC impairs invasiveness of MDA-MB-231 cells [167]. Interestingly, ROCK mediates the invasion of amoeboid breast cancer cells through matrix metalloproteinase (MMP)-independent mechanism, by regulating myosin light chain (MLC) organization and the generation of forces that cause deformation of the underlying collagen fibers, thereby allowing cells to invade the ECM [168]. Silencing RhoC in MTLn3 cells impairs protrusion formation and directionality in response to EGF stimulation [169]. In addition, RhoC-depleted MTLn3 cells exhibit altered morphology and

function of the ECM-degrading invadopodial protrusions and reduced invasive potential [170]. Rac1 counteracts the activity of RhoC in MTLn3 cells by inducing the disassembly of invadopodia. Considering the role of Rac1 in the formation of lamellipodia, this effect is believed to sustain the proper balance between matrix-degrading and locomotory protrusions for optimal cell invasion [171]. In fact, knocking down Rac1 induces membrane ruffling and impairs motility in EGF-stimulated MTLn3 cells. This is due to altered formation of focal adhesions at the leading edge, rendering the protrusions unstable [172].

In addition to their role in breast cancer invasion, Rho GTPases alter the morphogenesis of mammary epithelial tissue, an event that marks breast cancer initiation, both in vitro and in vivo [47]. Indeed, Rho GTPase signaling plays a role in regulating morphogenetic aspects of mammary epithelial cells, including cell-cell adhesion, cell-ECM interactions, apicobasal polarity, mitotic spindle orientation and lumen formation [47, 160, 161]. Rho GTPases also mediate preneoplastic transformation, tumor growth, angiogenesis and metastasis in breast cancer. Ectopic expression of RhoA leads to immortalization of primary human mammary epithelial cells [173]. In contrast, silencing RhoA reduces the proliferation of MDA-MB-231 cells and suppresses xenograft tumor growth, angiogenesis and lung metastasis in mice [167, 174]. Similarly, inhibiting Rac1 in MDA-MB-435 cells impairs tumor growth, angiogenesis and metastasis in a nude mouse model [175].

Cross-Talk between Connexins and Rho GTPase Signaling

As previously mentioned, intercellular adhesion and communication, which are key aspects of a differentiated mammary epithelium, are disrupted in breast cancer. Rho GTPase activities are spatiotemporally regulated to control the establishment and maintenance of epithelial apicobasal polarity and cell-cell junctions, particularly tight junctions (TJs) and adherens junctions (AJs) [154, 157, 176]. FRET biosensor studies showed spatiotemporal localization patterns of RhoA, Rac1 and Cdc42 activities along the apical and lateral membrane domains of Madin-Darby canine kidney (MDCK) epithelial cells during cystogenesis. Specifically, Rac1 activity at the lateral membrane exceeds that at the apical membrane during late cystogenesis, and induction of Rac1 activity at the apical membrane of mature cysts disrupts apical-basolateral polarity, TJs and mitotic spindle orientation [177]. Spatiotemporal Rac1 activity is also implicated in the establishment of AJs. FRET biosensor imaging showed that local Rac1 activation is induced upon the formation of nascent AJs, leading to junction stabilization in endothelial cells [178]. RhoA colocalizes with AJs in the developing mouse brain, and conditional knockout of RhoA in neural progenitor cells of the forebrain and midbrain disrupts AJs, suggesting a role for RhoA in maintenance of AJs [179]. RhoA also regulates the maintenance of both apicobasal polarity and TJ localization in retinal progenitor cells during vertebrate embryonic development [180]. Similarly, Cdc42 regulates the establishment of cell polarity and junction assembly in a mammalian model of early embryonic development. Cdc42-null embryoid bodies show homogenous cortical distribution of F-actin and lack the characteristic distribution of the microtubule-organizing center (MTOC) and Golgi complex, indicating absence of cell polarity. In addition punctate cell-cell contacts containing TJ and AJ markers are formed, and continuous TJ or AJ belts fail to assemble [181].

Rho GTPase signaling is also known to regulate GJ function and assembly. Blocking the activities of Rho family proteins by overexpressing the guanine nucleotide dissociation inhibitor (GDI) Rho GDIα under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter reduces the expression levels of Cx40 in mouse hearts and is associated with conduction defects [182]. In a similar study where C3-exoenzyme expression is utilized, inhibition of Rho GTPase activities in mouse lenses reduces Cx50 expression levels [183]. Consistent with those findings, calpeptin-stimulated RhoA activity in HL-1 cells, mouse cardiac myocyte cell line, upregulates the expression levels of Cx43 [184]. In parallel, Rho GTPases also affect Cx localization. For instance, Cx43 localization is altered in response to Rac1 inhibition in neonatal rat cardiac myocytes [185]. Likewise, Cx26 and Cx32 are mislocalized in hepatocytes of Cdc42-deficient mouse livers [186]. Rho GTPases further regulate GJs at the level of assembly and permeability. Inhibiting Rho activity in primary rabbit corneal epithelial cells by C3-exoenzyme microinjection impairs the assembly of Cx43-based GJs [187]. In addition, C3-exoenzyme-induced inhibition of RhoA reduces GJIC in rat cardiac myocytes [188]. Notably, other families of GTPases, mainly the Ras family, are also implicated in the regulation of Cx expression levels, GJ formation and GJIC [189–197].

In contrast to above, others demonstrated that Cxs are upstream regulators of Rho GTPase signaling. Cx43 activates the RhoA-ROCK pathway, as a mechanism for bradykinin-induced vascular contraction [198]. Furthermore, a role for Cx43 in Rac1 activation and actin cytoskeletal reorganization is proposed in breast cancer cells [199]. Blocking GJIC induces phosphorylation of Cdc42 in mouse ventricular zone precursors, resulting in its inactivation [200]. Unlike the aforementioned studies that reported positive regulation of Rho GTPases by Cxs, one study demonstrated enhanced Rac1 and RhoA activities in 3 T3 mouse embryonic fibroblasts in response to Cx43 knockdown. This is followed by enhanced migration and actin cytoskeletal reorganization [201]. The variable effect of Cxs on Rho GTPases suggests that Cxs regulate Rho GTPase signaling in a cell type-specific and/or context-dependent manner. Cxs also regulate other GTPases,

such as Rap1. In WEHI-231 cells, murine B lymphoma cell line, Cx43 mediates B cell receptor (BCR)-, integrin (LFA-1)- and chemokine (CXCL12)-induced Rap1 activation and the subsequent spreading and adhesion of B cells to vascular endothelial cells [202, 203]. Cx43 further regulates BCR- and integrin-induced B cell motility, in addition to chemokine-stimulated directed and transendothelial migration downstream of Rap1 activation [202].

Although a cross-talk between Cxs and Rho GTPases is implied, the literature describing such a link remains scarce, and almost no evidence supports its existence in the breast tissue. In one study however, the noncanonical ligand Wnt5a is proposed to impair lactation in mice through regulating Cx functions. In contrast to wild-type mice, overexpression of Wnt5a in the mammary epithelium inhibits oxytocin-induced milk ejection and sustains the phosphorylation of Cx43 after parturition [51]. Studies summarized above suggest positive regulation of Cx expression and function downstream of Rho GTPase signaling in tissue morphogenesis, differentiation and pathology [182–184]. Considering the dual roles of Cxs and Rho GTPases in development and tumorigenesis of the mammary gland, it is conceivable that enhanced Cx expression downstream of Rho GTPase signaling drives normal morphogenesis during development while supporting metastasis during breast cancer progression. The effects of Cxs as upstream regulators of Rho GTPases, however, remain controversial, posing a challenge in defining the regulatory role of Cxs in Rho GTPase signaling within the mammary gland [198–201]. We have recently delineated a role for Cx43 in regulating Rho GTPase signaling (unpublished data) and in establishing apical polarity and mitotic spindle orientation in 3-D cultures of human mammary epithelial cells (Bazzoun et al.; submitted). Given the role of Rho GTPases in establishment and maintenance of epithelial apicobasal polarity and intercellular junctions and in regulation of cytoskeletal dynamics, and considering their developmental and tumorigenic roles in the mammary gland that overlap with those of Cxs, it becomes necessary to study the involvement of Rho GTPase signaling downstream of Cxs in the mammary gland.

Conclusion and Future Perspectives

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

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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