Wnt signaling and cell-matrix adhesion

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ABSTRACT

Three decades after the beginning of the study of the Wnt signaling pathway, major contributions have been made to elucidate the molecular mechanisms that regulate this signaling pathway and its role in development, homeostasis and disease. However, there is still a lack of understanding about the relationships between Wnt signaling and cell-extracellular matrix (ECM) adhesion. Data gathered in the last years is helping to uncover these relationships. Several ECM proteins are able to regulate components of the Wnt pathway during development and disease, and their misregulation leads to changes in Wnt signaling. Fibronectin, a major ECM protein, regulates non-canonical Wnt signaling during embryogenesis in *Xenopus* and in muscle regeneration in mouse, whereas it modulates canonical Wnt signaling through modulation of β-catenin. Integrins, which act as Fibronectin receptors, also modulate Wnt activity, and Syndecan-4, an heparan sulphate proteoglycan, is able to regulate canonical and non-canonical Wnt pathways, notably during embryogenesis. Other secreted ECM proteins have been recently associated to the regulation of Wnt signaling, albeit molecular mechanisms are still unclear. Wnt signaling, in particular the non-canonical Wnt pathway, plays a role in the regulation of the ECM assembly, whereas Wnt/β-catenin signaling regulates the expression of genes encoding ECM proteins and modulates focal adhesion dynamics, through the direct involvement of Wnt components. This evidence indicates that Wnt signaling and cell-ECM adhesion are two closely related processes, and alterations in this cross-talk might be involved in disease.

Keywords (6-8): Adhesion, Extracellular Matrix, Fibronectin, Focal Adhesion, Integrin, Stiffness, Syndecan-4, Wnt

INTRODUCTION

Three decades since the beginning of the study of the Wnt (Wingless, Int1) signaling pathway have been celebrated, and a full account of the history behind the discoveries that led to the description of this signaling pathway has been published recently [1]. The Wnt family constitutes a large number of cysteine-rich secreted glycoproteins that regulate a variety of cellular processes such as development, homeostasis, regeneration, stem cell pluripotency, cell polarity and migration, [2,3]. The Wnt ligands bind to Frizzled receptors and activate signaling pathways in a context-dependent manner, depending on the availability of co-receptors. These co-receptors determine the specific intracellular proteins that will transduce the signal and the concomitant cellular events that will be elicited [3].

At least two main Wnt signaling pathways can be defined, depending on the ability to stabilize the β-catenin protein. The 'canonical' Wnt pathway, often referred as 'Wnt/β-catenin pathway', involves the binding of some members of Wnt ligands to Frizzled receptors and LRP5/6 co-receptors at the cell membrane, leading to the formation of multiprotein complexes known as 'signalosomes' [4]. This event leads to the mobilization of several intracellular proteins, including Axin and GSK3β [5]. Axin, APC and GSK3β, in absence of Wnt ligands, form a complex responsible of the degradation of the β-catenin protein by phosphorylation and ubiquitination; the Wnt stimuli induces the translocation of this complex to the plasma membrane, where becomes 'saturated' with phosphorylated β-catenin [6]. Newly synthesized β-catenin translocates to the nucleus and activates the transcription of target genes, together with members of the TCF/Lef family of transcription factors [7]. Several proteins regulate the Wnt/β-catenin signaling in the extracellular space, including the Dickkopf family of Wnt/β-catenin inhibitors and the R-Spondin family of Wnt/β-catenin agonists [reviewed in 8].

A second branch of the Wnt signaling pathway, commonly defined as the 'non-canonical Wnt pathway', is independent from β-catenin stabilization, and rather involves several alternating and overlapping pathways [9]. The non-canonical Wnt signaling is involved in embryonic development, specially the 'Wnt/PCP' pathway [10]. The non-canonical Wnt pathway involves the interaction of a second group of Wnt ligands to Frizzled receptors, together

with several co-receptors, including Strabismus/Vangl2, Ryk, Ror2, Ptk7 [reviewed in ref. 3]. In addition, Glypican-4 [11] and Syndecan-4 [12], two proteins belonging to the family of cell-surface heparan sulphate proteoglycans (HSPG) [13], also act as co-receptors for the non-canonical Wnt signaling [3, 11, 12]. The non-canonical Wnt pathway activates several intracellular proteins, including small GTPases Rho and Rac, JNK, CaMK and others [reviewed in 14]. Another non-canonical Wnt pathway involves the release and mobilization of intracellular calcium [9,14]. All these diverse (canonical and non-canonical) pathways, share some proteins, specially the Frizzled family of receptors, and Dishevelled, a scaffolding protein which binds to Frizzled receptors [15]. Dishevelled is involved in the scaffolding of intracelullar proteins playing functions in the Wnt pathway. For example, Dishevelled interacts with Axin, and together form large assemblies that can interact with the receptors at the cell membrane [4,16]. In turn, Axin binds to LRP5/6 [17], and this event allows LRP6 phosphorylation by GSK3β and CK1γ [18,19]. Dishevelled also interacts with the clathrin adaptor AP-2, and this interaction regulates the non-canonical Wnt signaling [20,21], and interacts with Daam-1, also regulating non-canonical Wnt signaling [22]. Hence, Dishevelled is a key component along the Wnt signaling axis. Adding another layer of complexity, Wnt ligands that activate the non-canonical pathway, most notably Wnt5a, are also able to activate the Wnt/β-catenin pathway, depending on the availability of specific proteins or receptor/co-receptor pairs [23,24].

Great effort has been made in the last years to elucidate the molecular mechanisms regulating the Wnt signaling, and also to understand how a specific branch is activated by specific Wnt ligands. However, two main challenges remain in this area: a) to understand how this pathway becomes altered during pathological conditions, and b) how the pathway is regulated *in vivo*. In this review, we will summarize and analyze recent data involving the regulation of this important signaling pathway by cell-extracellular matrix (ECM) adhesion proteins. Recent reviews have been published regarding the regulation of the Wnt pathway by cell-cell adhesion proteins [25,26]. Also, the role of some secreted ECM proteins, such as Matrix Metalloproteinases (MMPs), has been reviewed recently [27] and will not be covered in this review.

1. Regulation of the Wnt pathway by cell-ECM adhesion proteins

The ECM is composed by several proteins, including Fibronectin (FN), Collagen, Tenascin, Elastin and HSPGs [28,29]. Cell-ECM adhesion structures are small, discrete regions in close contact to the substratum, with actin filament bundles connecting the ECM and the cytoskeleton. A diverse array of cell-ECM structures are defined by their size, components, structure and distribution, including nascent adhesions, focal complexes, focal adhesions (FA) and fibrillar adhesions, in a process of maturation [30-32]. Although several proteins are present at these cell-matrix structures, a few of them are present at the cell membrane and directly interact with the ECM. They include members of the integrin family of ECM receptors [33]. Integrins are present as heterodimers, comprising one *alpha* (α) and one *beta* (β) subunit. Integrin heterodimers interact with Fibronectin, a secreted protein that assembles into long, fibrillar oligomers, as well as with other ECM proteins [33,34]. Several proteins regulate this process; among them Syndecan-4 (SDC4) has emerged as a key protein regulating integrin turnover [35,36]. Both integrins and SDC4 interact, via its cytoplasmic domains, with an array of intracellular components, including α-actinin, syntenin, synactin, PKCα and several others [for a complete list of interactions, see reviews 37, 38 and 39]. In this sense, cell-matrix adhesions, and specially FAs, emerge as "signaling hubs", since they comprises several proteins with diverse functions. Large-scale studies have analyzed these structures, often referred as "adhesomes", and they comprise hundreds of proteins, all of them potentially capable to regulate signaling events inside the cell [38].

A relationship between cell adhesion and Wnt signaling has been acknowledged for a long time; however, it was mainly focused in cell-cell adhesion, due to the multifunctional role of β-catenin in both biological events. β-catenin is involved in the regulation of cell-cell contacts by interacting with cadherins, proteins that strongly regulates Wnt signaling [25,26]. However, the relationship between cell-ECM adhesion and Wnt signaling is less studied, although several lines of evidence suggest that regulation of Wnt signaling by cell-ECM adhesion structures may be a highly relevant biological process. These evidences arise mainly from studies of Wnt signaling in development, notably from reports on *Xenopus laevis* embryos, and from studies in mammalian cells to address the molecular mechanisms of diseases, such as cancer and fibrosis. Importantly, independent work from several groups has shown in the last ten years that main proteins involved in FAs -Integrins, SDC4 and Fibronectin- are involved in

136 the regulation of Wnt signaling through several mechanisms.

137

138 *1.1. Syndecan-4 as an important modulator of Wnt signaling.*

139

140 SDC4 is an HSPG from the Syndecan family of type I transmembrane proteoglycans, which is involved in
141 the physiology of focal adhesions. Early studies showed that SDC4 colocalized with focal adhesions [40], and more
142 recent evidence show that SDC4 is required for β1-integrin turnover [35]; this process is dependent upon SDC4
143 phosphorylation by Src [36]. Although SDC4 is not typically found in large 'adhesome' screenings, a plausible
144 explanation argued recently relates this observation to the large size of the SDC4 extracellular domain [39].

145

146 SDC4 heparan sulphate chains bind to FN through its heparin binding domains (HBD) [41]. However, the
147 core protein of SDC4 can also possibly exert its functions in a GAG-independent fashion, due to a conserved
148 domain present on its extracellular domain, involved in the interaction with β1-integrin [42]. Engagement of SDC4
149 to FN activates Rac, regulating directional and persistent cell migration [43].

150

151 Our group showed that SDC4 regulates non-canonical Wnt signaling in *Xenopus laevis* development,
152 providing a first evidence regarding the specific involvement of this FA component on Wnt signaling [12]. During
153 *Xenopus laevis* early development, *sdc4* is present in mesoderm and neuroectoderm, two tissues that will undergo
154 convergent and extension (CE) movements, a process that is highly regulated by the non-canonical Wnt pathway
155 [10]. Manipulation of *Xenopus* SDC4 (xSDC4) levels impairs CE movements, an effect which is rescued by a form
156 of Dishevelled lacking the DIX domain involved in Wnt/β-catenin signaling, indicating that xSDC4 regulates CE
157 movements through the non-canonical Wnt pathway [12]. Another group also showed that SDC4 regulates another
158 process, neural crest migration, by a non-canonical Wnt pathway involving Rho and Rac [44].

159

160 A molecular mechanisms to link SDC4 and non-canonical Wnt signaling was provided by a recent work
161 showing that SDC4 binds R-Spondin 3. R-Spondin 3 is a member of the family of R-Spondin secreted proteins that
162 synergize with Wnt ligands to enhance Wnt/β-catenin signaling [8]; however, the binding of R-Spondin 3 to SDC4

induces activation of the non-canonical Wnt signaling in *Xenopus laevis*, by a clathrin pathway, in a Dishevelled-dependent fashion, to activate JNK [45]. There is a second mechanism involving regulation of the Wnt pathway by SDC4. Wnt5a, a prototypical non-canonical Wnt ligand, induces SDC4 ubiquitination and degradation, also in a Dishevelled-dependent manner [46]. This effect is observed in *Xenopus* embryos and in mammalian cells. Furthermore, SDC4 induces Dishevelled translocation to the cell membrane, and SDC4 itself is able to interact with Dishevelled in HEK293 cells [12]. It seems likely that SDC4 regulation of non-canonical Wnt pathway may involve interactions with several ligands and intracellular partners.

The data mentioned above indicates that SDC4 regulates non-canonical Wnt signaling in *Xenopus laevis*. Recent evidence shows that SDC4 can also regulate non-canonical Wnt signaling in mice. Bentzinger and coworkers reported that SDC4 form a complex with Frizzled-7, to transduce the signal initiated by Wnt7a and FN, through a non-canonical pathway, in satellite cells [47]. In addition, a recent work from our laboratory shows that SDC4 interacts genetically with Vangl2 to regulate neural tube closure [48]. Furthermore, SDC4 *knockout* mice, after detailed examination, also exhibit defects associated with alterations of the non-canonical Wnt pathway, including loss of orientation in the sensory hair cells from the inner ear, a hallmark of non-canonical Wnt deregulation [49]. Genetic interaction between SDC4 and Vangl2 induces an increase in occurrence of *spina bifida* in mice [48], revealing the importance of SDC4 regulation of the pathway and its possible clinical relevance. Of note, regulation of Wnt signaling by SDC4 may not be limited to the non-canonical pathway, since binding of SDC4 to Dishevelled may also influence the Wnt/β-catenin pathway. In addition, HSPG chains in SDC4 may bind Wnt ligands and modulate Wnt signaling. It has been reported that, in low serum conditions, HSPG maintain Wnt3a solubility, preventing its aggregation and stabilizing Wnt activity [50], supporting the notion that HSPG chains, like the GAG chains present in SDC4, may modulate alternative Wnt pathways.

Collectively, these data suggest that SDC4 may be a relevant modulator of the Wnt signaling. Although other HSPG may also regulate Wnt signaling, the established role of SDC4 in cell-matrix adhesion opens the possibility that cell-ECM adhesion may impinge in Wnt signaling pathways (see Figure 1).

190 *1.2. Fibronectin in Wnt signaling*

191

192 Integrins and FN are among the main proteins present in cell-matrix adhesion structures. It is known that both
193 proteins are involved in regulation of non-canonical Wnt signaling, in a great extent due to studies performed in
194 *Xenopus laevis* embryos. At the onset of the gastrulation during *Xenopus* development, a group of mesodermal cells
195 from the dorsal marginal zone, at the tip of the dorsal blastopore, begin to migrate along a substrate composed by a
196 layer of FN [51,52]; this migration along the inner roof of the blastocoele is supported by the expression of
197 Integrins. The non-canonical Wnt pathway regulates *convergence and extension* (CE), the collective movements that
198 allow the massive rearrangements of cells during gastrulation [10]. FN and Integrins plays a role in regulating non-
199 canonical Wnt signaling in the tissues involved in CE movements, suggesting a cross-talk between cell adhesion and
200 Wnt signaling.

201

202 Blockage of FN fibrillogenesis in *Xenopus* by using blocking antibodies or antisense morpholinos, impairs
203 CE movements [53,54]. FN is permissive to the localization of Dishevelled at the plasma membrane [53], and also
204 regulates polarized cell division and polarized cell protrusions, processes regulated by the Wnt pathway [53-55].
205 Concomitantly, the impairment of FN function, either by knockdown using morpholinos or by blockage of FN
206 fibrillogenesis using antibodies, induces severe changes in gastrulation, slowing CE and dorsal axis extension [54].
207 It has been proposed that the main function of FN during mesodermal migration is the control of cell protrusive
208 activity [56], event that is regulated by the non-canonical Wnt pathway [57]. However, later studies showed that
209 morphogenetic movements are sensitive to the physical state of FN; fibrillar FN is required for oriented cell division
210 and protrusive activity, but dispensable for CE movements, establishing a differential role of FN, depending on its
211 assembly state [55]. Furthermore, the main approach to study FN requirement on morphogenetic movements
212 consisted in the use of blocking antibodies, usually targeting the CCBD (central cell binding domain) of FN, which
213 contains the RGD site for binding to integrin. Hence, FN seems to regulate gastrulation movements through
214 mechanisms dependent on both cell adhesion and FN assembly, and additional interactions mediate mesodermal cell
215 adhesion to the blastocoel cell roof. It must be noted, however, that a mechanistic explanation for FN regulation of
216 morphogenetic movement through regulation of non-canonical Wnt signaling is still missing in this model system.

A possible mechanism to explain the regulation of the non-canonical Wnt pathway by FN is provided by studies in mammalian cells. Recently, it was demonstrated a novel function for FN during satellite cell biology [47]. FN, together with Wnt7a, binds to a receptor complex composed by SDC4 and Frizzled-7 (FZD7); this event induces Wnt7a signaling, through a non-canonical pathway, regulating the symmetric division of satellite stem cells. Furthermore, the authors show that, after muscle injury, FN expression is induced, indicative of a transient fibrosis. *In vivo* experiments demonstrated that FN is required for satellite stem cell maintenance and expansion in muscle tissue. These results support a model whereby FN regulates the non-canonical Wnt pathway through binding of Wnt ligands to a receptor complex, activating intracellular cascades leading to changes in cell behaviour.

Whether the mechanisms described in mammals also are present during *Xenopus* gastrulation is unknown; however, it seems plausible that SDC4 and FZD7 can contribute to the effects of FN upon non-canonical Wnt signaling in *Xenopus*, considering the data previously discussed [12].

It has also been observed that FN can modulate β-catenin activity through a pathway involving Src kinase in Natural Killer (NK) cells, to promote NK survival [58]. In NK cells, FN binds to CD11b receptor, which then binds and activates Src. This event leads to the interaction between Src and β-catenin, which triggers β-catenin phosphorylation at Ser675, and nuclear translocation of β-catenin. Independent work shows that phosphorylation of β-catenin at Ser675 promotes β-catenin transcriptional activity [59]. However, whether the regulation of β-catenin by FN in NK cells results in increased Wnt/β-catenin signaling is not addressed by Zheng and coworkers, but provides a potential mechanism to regulate this pathway by the ECM.

FN has more than 50 splice variants [60]. One of these variants, the embryonic EDA (EIIIA), is expressed during wound healing. Primary dermal fibroblasts from wounds have increased active β-catenin, which correlates with increased TCF reporter activity in these cells when cultured on FN [61]. However, β-catenin activity is decreased in cells in wounds from *Fn Eda* -/- mice, and the defective phenotype observed in these animals could be rescued with pharmacological activation of β-catenin. Collectively, these data indicates that FN can regulate Wnt signaling by diverse mechanisms, depending on the cell type, availability of receptors/co-receptors, and Wnt ligands.

1.3. Integrins in Wnt signaling

Several Integrins are also able to regulate Wnt signaling, through mechanisms that are also diverse, depending on the presence of other ECM proteins and the cell type. For example, the increase of the transcriptional activity of β-catenin induced by FN in primary dermal fibroblasts is dependent on β1-integrin [61], since lack of β1-integrin abrogates the activation of β-catenin induced by FN, indicating that a β1-integrin-mediated pathway is able to regulate β-catenin activity. One mechanism is provided by a work showing that Grb2, a protein with multiple adaptor functions, regulates Wnt/β-catenin signaling by binding to Dishevelled [62]. When cells are plated upon Collagen, Grb2 and Dvl2 strongly synergize to activate the Wnt/β-catenin pathway. In line with these results, a dominant negative form of Grb2 abrogates Wnt signaling. FAK overexpression cooperates with Wnt3a to enhance Wnt signaling, suggesting a role of FAK in the regulation of the pathway by Grb2. Of note, Grb2 acts downstream β-catenin to enhance the Wnt/β-catenin signaling, and requires JNK and Rac, two cytoplasmic proteins involved in non-canonical Wnt signaling. These evidences suggest that β1-integrin may activate alternative pathways to modulate Wnt signaling at the nuclear level.

A second mechanism by which Integrins can regulate the Wnt/β-catenin pathway could involve regulation of β-catenin levels through cross-talk with other signaling pathways. For example, in epithelial cells expressing α3β1-integrin, which bind to Laminin, a complex between phosphorylated Smad2 and phosphorylated β-catenin at Tyr654 is formed in response to TGFβ1; this event depends on the turnover of α3-integrin together with the TGFβR1 and E-Cadherin [63]. A third mechanism may involve direct regulation of components of the Wnt pathway. Such a mechanism is provided by a report showing that β1-integrin engagement to Collagen Type I induces GSK3β phosphorylation and promotes nuclear translocation of β-catenin and an increase of Wnt/β-catenin signaling [64]. However, authors do not provide a molecular mechanism linking β1-integrin engagement and GSK3β inactivation.

Another mechanism, which is supported by several studies, links β1-integrin to Wnt/β-catenin signalling, through the Integrin-linked kinase (ILK) protein. Early studies showed that overexpression of ILK in epithelial cells results in translocation of β-catenin to the nucleus [65] and inhibitory GSK3β phosphorylation [66]. Furthermore,

ILK co-immunoprecipitates with APC and GSK3β, and its activity is required for the activation of the Wnt/β-catenin pathway by Wnt3a in several cell lines, including the commonly used HEK293 and L-Cells [67]. Of interest, ILK also interacts with Dishevelled [68]. However, it is not completely clear whether the kinase activity of ILK is required for the regulation of β-catenin activity, since GSK3β phosphorylation is not altered in *Ilk* -/- fibroblasts [69]. Furthermore, mutations in ILK to disrupt its kinase activity result in loss of interactions with additional partners [70]. Also, regulation of E-Cadherin levels can also explain part of the effects of ILK. It is possible that ILK may function in specific cell types, when subjected to changes in the surrounding ECM environment.

The evidence mentioned so far suggests that β1-integrin activates Wnt/β-catenin signaling through regulation of intracellular components, such as GSK3β phosphorylation via ILK activity, direct interaction between ILK and components of the pathway, and cross-talk with other intracellular signaling proteins (TGFβ1/Smad2). Less information is available regarding a role of integrins in the regulation of the non-canonical Wnt pathway. Binding of FN to β1-integrin is permissive to the translocation of Dishevelled to the membrane in explants from the dorsal marginal zone from *Xenopus* embryos [53]. Furthermore, β1-integrin regulates proper protrusive activity in cells undergoing mediolateral intercalation movements in *Xenopus*, in a process requiring ligation to FN, event that promotes polarized protrusions by suppressing random protrusions [54]. However, direct regulation of non-canonical Wnt signaling in this system is still speculative. Data gathered from studies in mammalian cells is also scarce. It is known that, during epithelial-mesenchymal transition (EMT), MDCK cells increase the expression of Wnt5a together with several integrins heterodimers; this event is also characterized by a decrease in Wnt/β-catenin activity [71]. Whether increased Integrin expression and increase of non-canonical Wnt signaling mediated by Wnt5a are two related processes, is unknown. However, considering that increased migration and invasiveness is a hallmark of EMT, it is interesting that Frizzled-2 (FZD2) localizes at adjacent puncta with β1-integrin, and that FZD2, APC and Dishevelled colocalize with focal adhesions, and stimulate focal adhesion dynamic in response to Wnt5a [72], suggesting that β1-integrin is related with focal adhesion turnover through the non-canonical Wnt pathway. Also, these results suggest that β1-integrin can regulate Wnt/β-catenin or non-canonical Wnt signaling, depending on the cell type (ephitelial versus mesenchymal). In summary, FN and Integrins regulate Wnt signaling through several mechanisms, including regulation of intracellular Wnt components, cross-talk with other signaling

pathways, and direct regulation of β-catenin activity by regulation of GSK3β or other kinases (Figure 1).

1.4. Other ECM molecules

Collagen is a family of ECM secreted proteins. More than 40 genes encode for proteins that can form at least 28 different Collagen subtypes [73]. Several integrins act as receptors for Collagen, through binding to multiple domains [73]. Two recent studies from independent groups have shown that Collagen Type I (Col1) regulates the activity of β-catenin. In pancreatic cancer cell lines, Col1 binding to β1-integrin activates Src, inducing FAK activation, association of FAK with the E-Cadherin complex, and translocation to the nucleus of β-catenin and increased TCF/Lef activity, by a mechanism independent of GSK3β [74]. These effects are not observed in cells cultured on Fibronectin, ruling out that increased adhesion, instead of features found in Col1, induce β-catenin activation. A second group observed a similar phenomenon in gastric carcinoma cells, where Col1 also promoted E-Cadherin/β-catenin disassembly, β-catenin phosphorylation and increase of cyclin-D1 (a transcriptional target of β-catenin) mRNA [75].

How Col1 exactly induces β-catenin activation through Integrins is unknown. An interesting discovery, however, is provided by two recent articles [76,77], showing that Collagen XVIII (Col18) harbors a CRD domain, with identity to the CRD domain found in Frizzled receptors, and also found in other Wnt receptors [3], providing a second mechanism by which Collagens can potentially regulate the Wnt signaling. In this case, an amino-terminal end variant of Col18, termed V3C18, is presented at the cell membrane, proteolytically processed, releasing a soluble protein containing a CRD module (FZC18), which binds Wnt3a and attenuates the Wnt/β-catenin pathway [76]. The authors find a negative correlation between the processed form of V3C18, FZC18, and Wnt/β-catenin signaling, since liver tumors, but not normal samples, were positive for FZC18. Later, the same group demonstrated that FZC18 also binds Frizzled-1 and Frizzled-8, reduces sensitivity to Wnt3a in HEK293T cells, and binds Wnt3a in a cell-free system [77].

Less known is the role of other ECM components in the regulation of Wnt signaling. For example, the role of

Laminin, a key component of the ECM, is less studied. A recent work reported that Laminin-511, a major α5-containing Laminin isoform expressed in developing and adult organs, inhibits Wnt/β-catenin signaling. In the intestinal tissue lacking *lama5*, the gene encoding for the α5 subunit of Laminin, the expression of Wnt target genes was increased, and culture of cells on Laminin-511 repressed TCF activity [78]. Although a mechanism is not provided, authors suggest that Integrins may mediate the inhibitory effect of Laminin-511. In this direction, the finding that Wnt/β-catenin signaling is decreased in α3β1-integrin (a receptor for Laminin-5) null duct epithelial cells, further supports a role of Laminin in regulating the Wnt signaling [79]. Of note, in kidneys from *lama5* null embryos, there is a loss of *wnt7b* expression, as well as in kidneys from α3-integrin embryos [79]. Another family of proteins from the ECM is comprised by the Tenascins. Tenascin-C (TN-C) was the first member of the family described [31]. TN-C expression correlates with nuclear β-catenin in colorectal tumor cells, and is also expressed in these cells [80]. TN-C inhibits Wnt/β-catenin signaling in oligodendrocytes cultured on plates coated with TN-5 [81]. However, in MCF-7 cells, TN-C induced the mobilization of β-catenin from the membrane to the cytosol, as well as E-Cadherin [82], although Wnt/β-catenin signaling was not evaluated. In T98G glioblastoma cells, in addition, TN-C decreases Dkk1 (Dickkopf-1, a canonical Wnt inhibitor) levels, and Wnt/β-catenin signaling is increased [83]. Collectively, these data indicates that TN-C can have opposite effects upon Wnt/β-catenin signaling, depending on the cell type. Periostin (POSTN) is an ECM molecule involved in several biological processes, including tissue regeneration and homeostasis, and cancer invasion [84], and may also regulate Wnt/β-catenin signaling, since it has been shown that *Postn* deficiency impairs β-catenin activity [85], evaluated by the reporter TOPGAL mice. This function of POSTN likely involves the inhibition of Sclerostin (SOST), a known negative Wnt/β-catenin regulator [86], in response to parathyroid hormone (PTH), since defects in the *Postn* -/- mice are partially rescued by blocking antibodies for SOST, and *Sost* downregulation induced by PTH and mediated by POSTN, is lost in *Postn* -/- mice. Since POSTN interacts with Fibronectin and TN-C [see 84], it is a possible that the simultaneous presence of different secreted ECM proteins may regulate Wnt signaling by different mechanisms.

In summary, recent literature has expanded our view regarding the link between cell-ECM adhesion molecules and Wnt signaling. Although more attention has been devoted to cell-cell adhesion and Wnt signaling, particularly due to the role of β-catenin in both biological processes, now it is clear that cell-cell and cell-matrix

adhesion can even share molecules and mechanisms to regulate Wnt signaling. For example, most data regarding regulation of Wnt/β-catenin signaling by Integrins and Collagen-I point to the role of release of the 'membrane' pool of β-catenin through regulation via ILK; however, the function of FN and SDC4, mainly in the non-canonical Wnt pathway, seems to involve direct interaction and regulation of Wnt components, such as FZD7, Wnt ligands or Dishevelled, pointing also to a more direct effects of ECM components upon specific Wnt transducers (Figure 1).

2. Regulation of ECM proteins by Wnt signaling

2.1. ECM biosynthesis

Wnt signaling can play an important role in regulating the ECM. In particular, the Wnt/β-catenin pathway seems to directly activate ECM biosynthesis, since several Wnt target genes encode for ECM components and proteins involve in ECM regulation. Perhaps the most prominent case is FN, because it is involved in the regulation of both Wnt pathways, providing a 'feedback loop'. Two independent studies showed that the Wnt/β-catenin pathway induces *Fn* expression. In the work of Gradl and coworkers [87], it was shown that *Fn* is induced in fibroblasts, but not in kidney epithelial cells, where the *Fn* promoter is silenced, and regulatory elements in the *Fn* promoter were identified [87]. A second report showed that, in fibroblast-like synoviocytes, *Fn* expression is induced under transfection of Wnt-1 [88].

Fibrotic diseases are characterized by an excessive production of several ECM components, including FN and Collagens, including Collagen Type I [89]. The Wnt/β-catenin pathway is involved in fibrotic disorders, as several reports have demonstrated [for a recent review, see 90]. In several diseases, including idiopathic pulmonary fibrosis, liver fibrosis, systemic sclerosis and renal fibrosis, there is a correlation with increase of Wnt/β-catenin signaling or expression of canonical Wnt proteins [90]. Wnt/β-catenin activation promotes Renal Interstitial Fibrosis, ureteral obstructive injury induce Wnt/β-catenin activation in renal tubular epithelial cells, inducing *Fn* expression, and treatment with the Wnt inhibitor Dkk1 decreased FN and Col1 expression and deposition [91]. Also, in mice expressing stabilized β-catenin in fibroblasts, there is a fibrotic response characterized by accumulation of

Collagen and differentiation of fibroblast into myofibroblasts, and increased nuclear β-catenin is observed in fibroblasts from Systemic Sclerosis (SSc) skin biopsies [92].

Other reports also show similar effects of Wnt/β-catenin activation and a fibrotic response [90]. The involvement of Wnt/β-catenin signaling in fibrosis seems to be mediated by the TGFβ1 pathway. For example, in a recent report, the inactivation of β-catenin transcriptional activity impaired the TGFβ1-induced expression of several ECM genes, such as *Fn* and *Col1*, by a Smad-independent mechanism, in human kidney tubular epithelial cells [93]. At the mechanistical level, decreased Dkk1 expression is observed in biopsies from SSc patients, while Wnt1 and Wnt10b are upregulated [94]. A transgenic mouse overexpressing Dkk1 is resistant to pharmacological induction of fibrosis, and stimulation of dermal fibroblasts with TGFβ reduced Dkk1 expression via the p38 mitogen-activated kinase [94]. In addition, the inhibition of β-catenin markedly inhibited TGFβ1-induced expression of ECM genes and FN production, in airway smooth muscle cells [95]. In summary, increased Wnt/β-catenin seems to be a hallmark of several fibrotic disorders, and this pathway may mediate the profibrotic signals elicited by TGFβ through Smad-independent mechanisms, that include reduction of Wnt/β-catenin antagonist Dkk1. It must be noted, however, that the non-canonical Wnt ligand, Wnt5a, has also been related to the TGFβ-induced fibrosis in airway smooth muscle cells in a very recent report [96]. It is evident that more research is needed to reconcile all the recent data, including apparently opposite results, to understand the complex molecular mechanisms relating the Wnt signaling to the ECM synthesis and misregulation in fibrotic disorders.

2.2. ECM assembly

Regulation of ECM proteins by the Wnt pathway is not limited to fibrosis. Genes associated with the non-canonical (Wnt/PCP) Wnt pathway, including *Xpk* (*prickle*), *Xstbm* (*strabismus*) and *Xfzd7* (*frizzled-7*), regulates the matrix deposition during the development of *Xenopus laevis* [97]. As mentioned previously, the migration of mesodermal cells proceeds upon a layer of FN, which is deposited at the beginning of the gastrulation [52]. However, when *Xpk*, *Xstbm* and *Xfzd7* are overexpressed, FN disorganization and random assembly of fibrils is observed. These results unveiled a previously unknown function of these PCP genes in providing molecular cues for

406 the polarized assembly of FN fibrils along the surface of the mesoderm. FN fibrils are required for cell polarization;
407 however, the incubation of explants from embryos over-expressing *Xstbm* and *Xfzd7*, in an exogenous FN matrix,
408 does not rescue the defects in mediolateral cell polarization, evaluated by random versus polarized cell protrusive
409 activity. These results hallmark the role of FN discussed previously in regulating Wnt signaling.

410 A second report employed animal cap explants from *Xenopus* embryos to study the effect of the non-
411 canonical Wnt ligand Wnt11 on FN assembly; explants from embryos expressing a dominant negative Wnt11
412 (dnWnt11) failed to assembly FN fibrils across the surface of the explant [98]. FN fibril assembly was rescued in
413 dnWnt11-expressing embryos when a Dishevelled mutant, lacking the DIX domain involved in Wnt/β-catenin
414 signaling, was coinjected, which indicates that the effect of Wnt11 is mediated through the non-canonical Wnt
415 pathway.

416

417 *2.3. Focal adhesion dynamics*

418

419 A third layer of regulation is provided by the direct role of Wnt proteins in cell-matrix adhesion events, and
420 where the most studied is Dishevelled. Dishevelled directly participates in FA biology. Dishevelled is present in
421 focal adhesions, where colocalizes and interacts with Actin, Paxillin and ILK [68]. Dishevelled translocates to the
422 membrane in response to SDC4 overexpression, and co-immunoprecipitation with SDC4 is enhanced by FN [12].
423 Furthermore, Dishevelled, together with APC, directly contributes to focal adhesion turnover induced by Wnt5a
424 [72]. Dishevelled localizes at focal adhesions in early steps of cell adhesion, interacts with APC and, in response to
425 Wnt5a, both Dishevelled and APC localizes at the cell periphery. Furthermore, Dishevelled regulates focal adhesion
426 dynamics in response to Wn5a, and localizes in close proximity to β1-integrin. Based on co-immunoprecipitation
427 and microscopy assays, the authors propose a model for the regulation of focal adhesion turnover, where
428 Dishevelled binds to FAK, and APC binds to Paxillin; Frizzled-2 (FZD2), which interacts with α2-integrin, also
429 localizes at the focal adhesion, and the FZD2/Dishevelled/FAK complex, together with the Integrin/Paxillin/APC
430 complex, promote focal adhesion dynamics [72].

431

432

2.4. Mechanisms of ECM-Wnt cross-regulation.

How the different lines of investigation and types of ECM regulation by Wnt proteins can be reconciled in a specific model remains an open question. We may speculate that Wnt signaling differentially regulates ECM biosynthesis and cell adhesion depending on the expression of specific Wnt ligands, receptors and co-receptors at the plasma membrane and intracellular modulators. When 'canonical' Wnt proteins are present, ECM biosynthesis may be favoured, through changes in gene expression; however, failure of cells in controlling canonical Wnt activity may result in fibrotic disorders. Such a failure may be due to the lack of specific receptors mediating hypothetic inhibitory signals from ECM proteins. In this scenario, proper 'negative feedback loops' should be a key regulatory mechanism to avoid pathological responses induced by ECM biosynthesis (see Figure 1).

Since non-canonical Wnt signaling has been more linked to cell behaviour instead of gene expression, this pathway may control proper ECM assembly during development, rather than controlling ECM biosynthesis, especially for FN [97,98]. Focal adhesion dynamic at the cell membrane, induced by Wnt5a and other non-canonical components, can regulate the availability of integrins and other ECM receptors, essential for proper FN assembly. Assembled FN matrices may promote cell migration and polarized protrusive activity, which involve recycling of ECM receptors, sustaining a continuous cycle. Since focal adhesion dynamic is mediated by shared components of the Wnt pathway, such as APC, Dishevelled and Frizzled receptors, it is likely that only one Wnt pathway can operate in ECM regulation at a given time. Specific features may depend on the cell type, developmental stage, or even activating mutations of the Wnt/β-catenin pathway.

The knowledge gathered in the last years strongly suggests that Wnt signaling regulates several aspects of ECM biology, such as ECM biosynthesis, with implications in pathology and aging, focal adhesion turnover, and ECM organization during development. This also indicates that the Wnt pathway and the ECM are two biological processes in continuous interaction, and it is possible that some key steps of this mutual cross-talk may be interrupted in disease.

3. Matrix elasticity and Wnt signaling

Evidence accumulated recently shows that cells can respond to physical changes in their surrounding ECM, and mechanical cues can direct specific responses, including differentiation to specific lineages, in stem cells [reviewed in 99]. The manipulation of matrix stiffness, to mimic the elasticities found in normal tissues, regulate the fate decision of human mesenchymal stem cells (hMSC). Culture of hMSC in softer matrices (0.1-1 kPa), that mimic the elasticity found in brain tissue, induced hMSC differentiation into neurons, whereas culture in stiffer matrices, mimicking muscle (8-17 kPa) and collagenous bone (25-40 kPa) induced differentiation of hMSCs into myogenic and osteogenic phenotypes, without the addition of differentiation factors [100]. This feature of MSC may be shared with committed cells, because muscle stem cells cultured in soft substrates, with an elasticity similar to the muscle, underwent self-renewal and were more efficient in terms of muscle regeneration when were transplanted into mice [101]. Topological changes, cell shape and mechanical forces are also involved in stem cell fate decision [100]. However, molecular mechanisms linking matrix stiffness to cell fate decision and signaling events are just starting to emerge. The Wnt pathway, involved in cell fate decisions during embryogenesis, may provide a mechanism to explain the behaviour of stem cells in response to changes in matrix stiffness. A recent report related changes in matrix stiffness and Wnt signaling, via regulation of Dickkopf expression [102]. Barbolina and colleagues used endothelial ovarian cancer (EOCs), which anchor to a collagen matrix during metastasis, and matrices with different elasticities, and 2D versus 3D cultures. The authors found that *dkk1* expression was strongly down-regulated in 3D cultures. Furthermore, 3D-Col1 cultures induced β-catenin translocation to the nucleus and increased transcriptional activity. Expression of Membrane type-I matrix metalloproteinase (MT1-MMP) was upregulated in these cultures, providing a mechanism to explain increased invasiveness of EOCs. However, since *dkk1* itself is a Wnt -induced target gene [103,104], regulation of Dkk1 may also represent a secondary response that follows a first wave of Wnt regulation by the ECM matrix. Changes in matrix rigidity may also be involved in TGF-β1 induced Wnt activation in calcific aortic valve disease, since myofibroblasts exhibited increased nuclear β-catenin in response to TGF-β1, when cultured in substrates of increasing stiffness [105].

Matrix elasticity influences cell-matrix adhesion distribution, structure and dynamics [106]. Hence, ECM

proteins are bona-fide proteins to explain changes in cellular events inducing different transcriptional programs leading to differentiation. Future studies may help to unveil molecular mechanisms linking ECM proteins to Wnt signaling in response to changes in matrix elasticity. Some recent results shed light into possible mechanisms. Murikipudi and colleagues reported that endothelial cells (ECs) cultured in stiff 3D matrices, expressed less *perlecan* and *biglycan* (two HSPG), *Fn*, *itga5* (α5-integrin), *itgb1* (β1-integrin) and *collagen-IV*, and increased expression of elastin and tissue inhibitors of matrix metalloproteinases (*timp1* and *timp2*), compared with softer 3D matrices, affecting ECs growth [107]. A second mechanism is provided by the recent discovery of YAP and TAZ as mediators of mechanic cues, regulating signaling pathways [108]. Although the role of YAP and TAZ is beyond the scope of this review (for a recent review see ref. 109), it must be highlighted that recent evidence shows that these transcription factors exerts their effects as mechanotransducers in a Hippo-independent fashion [108]. Furthermore, YAP/TAZ mediate Wnt/β-catenin signaling by Hippo-independent mechanisms [110,111] and involving direct interaction with Wnt components, such as the β-TrCP ubiquitin ligase present at the β-catenin destruction complex [110], and β-catenin itself [112]. Also, a cross-talk between the Hippo and Wnt pathways is reported [113, 114]. These evidences support the interesting possibility that matrix elasticity and ECM proteins may induce signaling events through a novel Wnt/YAP/TAZ pathway, and current efforts of several groups are helping to uncover this new relationship between mechanical cues, signaling pathways and cell behaviour.

Therefore, the study of matrix stiffness and its relationship with Wnt signaling may help to understand pathological processes and to develop new therapeutic strategies. Since changes in matrix elasticity are commonly observed in cancer [114], further research on matrix-ECM cross-talk will help to increase the array of tools to address clinical challenges. Of note, some recent articles shed light into the possible mechanisms linking ECM proteins, Wnt and cancer. Two relevant articles show that cancer cells produce ECM proteins, and these proteins play a role in the tumorigenic process. Oskarsson and colleagues [115] showed that breast cancer cells produce TN-C, which behaves as a metastatic molecule to facilitate the colonization of the lung by these cells, and that TN-C induces the expression of LGR5, a receptor for the R-Spondin family of agonists of the Wnt/β-catenin pathway [116]. Although the study by Oskarsson and colleagues does not fully address the regulation of the Wnt pathway by TN-C in this scenario, it seems likely that TN-C may sensitize breast cancer cells to low levels of R-Spondin

ligands. In fact, R-Spondin gene fusions have been associated with colon cancer [117], and R-Spondin2 is required for lung morphogenesis [118], suggesting that circulating R-Spondin may activate Wnt/β-catenin signaling in breast cancer cells with increased LGR5 expression induced by secreted TN-C. A second study shows that POSTN is required by cancer stem cells to initiate lung colonization [119]. POSTN is a stromal niche component, and its expression by fibroblasts is induced by infiltrating cancer cells, and is required for the cancer stem cell maintenance. At the mechanistic level, POSTN binds Wnt ligands (Wnt3a and Wnt1), increasing Wnt/β-catenin signaling. It must be noted that the studies by Oskarsson [115] and Malanchi [119] does not address the relevance of matrix stiffness, it seems reasonable that changes in matrix stiffness may increase local concentrations of ECM proteins or sensitize cells to mechanical stimulus, by regulating Integrin activity. Nonetheless, these works show how ECM proteins may be involved in the establishment of tumours through regulation of Wnt signaling, and future studies relating these and other ECM proteins to matrix stiffness will be of great value to unveil new therapeutic avenues for cancer treatment.

4. Concluding remarks

A new picture is emerging in the last years, with the increasing number of studies linking cell-ECM adhesion and Wnt signaling. Several molecules that regulate Wnt signaling are also ECM components and Wnt signaling regulates the expression of several ECM genes. At the same time, the attachment of cells to the matrix induces changes in intracellular proteins that play functions in Wnt signaling. Subtle differences in matrix stiffness or composition may regulate Wnt signaling. Considering the complexity of the processes involved, it is not surprising this level of cross-regulation between seemingly separate biological events, and impairment of any of the steps involved may underlie the establishment of pathological conditions. Therefore, appropriate understanding of the molecular mechanisms relating cell-matrix adhesion and Wnt signaling is required. Careful consideration must be given to focal adhesion components, since some of them participate in Wnt signaling and cell adhesion. Turnover of focal adhesion proteins may be the critical point relating both biological processes. Internalization and recycling of adhesion receptors proceed through the same pathways employed by Wnt signaling, providing another crossroad.

Future work on this topic will allow to understand the molecular basis of Wnt signaling, cell-matrix adhesion,

and the processes impaired in fibrotic and carcinogenic disorders, providing alternatives for therapeutic strategies that will be valuable in the near future.

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FIGURE LEGENDS

Figure 1. Cross-talk between cell-matrix adhesion proteins and Wnt signaling.

(1) Secreted proteins present in the Extracellular Matrix bind to receptors at the cell surface. **(2)** Fibronectin binds to Syndecan-4 and regulates non-canonical Wnt signaling, although the exact mechanisms are unknown and likely involve other secreted proteins, such as R-Spondin 3, and non-canonical Wnt ligands such as Wnt5a or Wnt7, in a Dishevelled-dependent manner. FN and Collagen also binds to α5β1-integrin, which regulates ILK function. **(3)** ILK, in turns, is able to directly inhibit GSK3β, inducing its phosphorylation, and also interacts with components from the Wnt/β-catenin pathway, possibly titrating such proteins from the signalosomes. **(4)** Collagen and Laminin are also able to bind to other integrins, such as α3β1 in epithelial cells, to activate intracellular cascades leading to disruption of E-Cadherin/β-catenin complexes, increasing β-catenin levels. **(5)** β-catenin can also interact with Smad2 phosphorylated after activation by TGFβ1. **(6)** Nuclear β-catenin can then activate the transcription of Wnt target genes. **(7)** Some of the Wnt target genes can also play a role in fine-tuning the cell-matrix adhesion and Wnt crosstalk. These genes include *fibronectin* and *dickkopf-1*. FN and Dkk1 can regulate Wnt signaling, providing a feedback loop mechanism. **(8)** Secreted ECM proteins may also be able to bind to Wnt proteins; for example, the FZC18 protein, derived from a cryptic CRD domain found in Collagen-18, can bind Wnt proteins and regulate Wnt signaling. **(9)** Focal adhesions (FA) formed by Syndecan-4 and α5β1-integrin, may potentially sequester proteins from the Wnt pathway, such as Dishevelled, that regulates FA dynamics, and Frizzled-7, regulating Wnt signaling. SDC4 also regulate the non-canonical Wnt pathway, through specific mechanisms [12, 45, 47, 48]. **(10)** Members of the Glypican family of HSPG, bind Wnt proteins through cystein-rich domains, and may regulate Wnt signaling depending on its localization in specific plasma membrane (PM) microdomains, as reported recently for Glypican-4 [120]. For simplicity, other components and regulatory pathways, such as calcium [121], are omitted. Also, some interaction partners are omitted, and some interactions are depicted as direct. COL, Collagen; DVL, Dishevelled; FZD7, Frizzled-7; FN, Fibronectin; GPC4, Glypican-4; ILK, Integrin-linked kinase; LAM, Laminin; SDC4, Syndecan-4.

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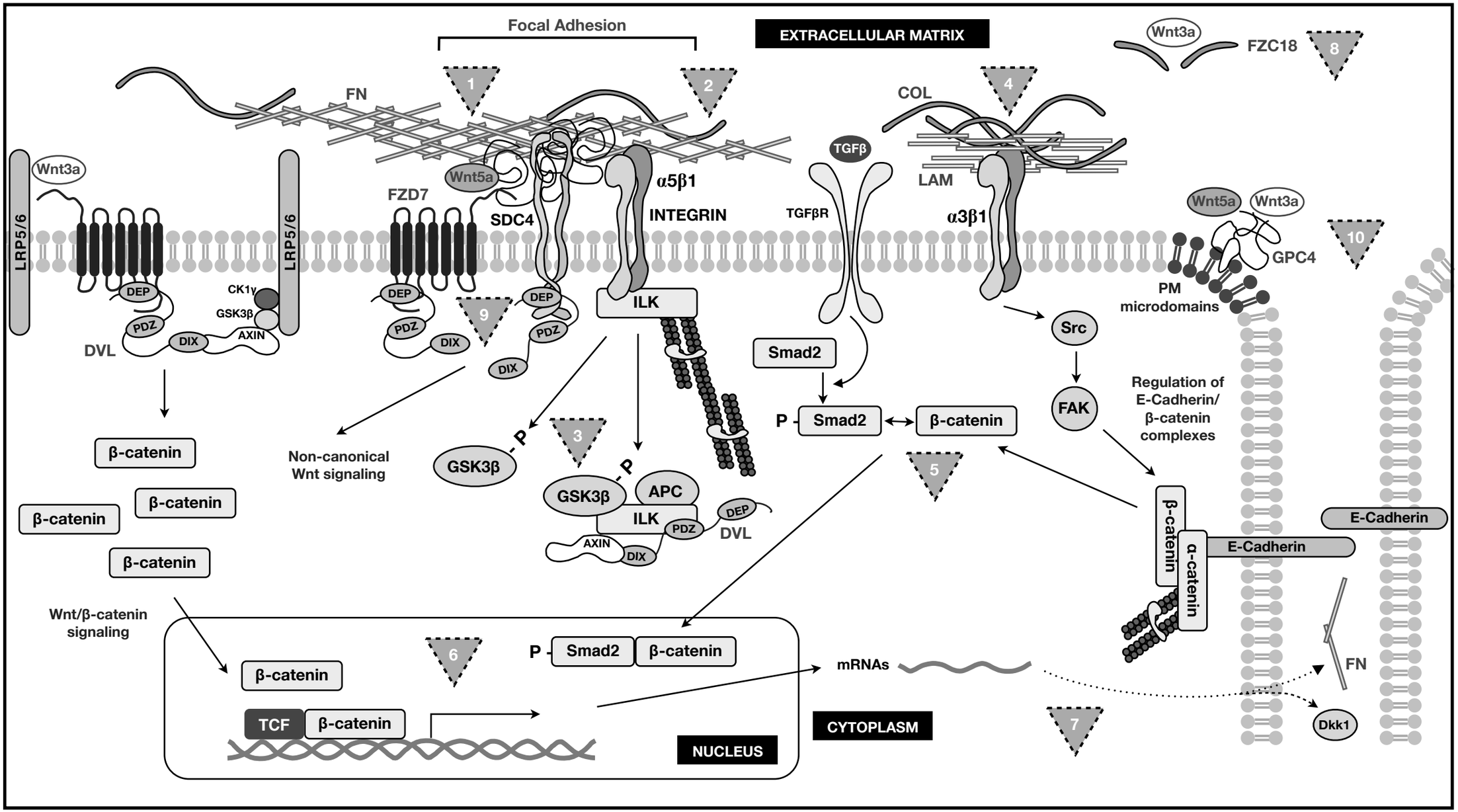
Figure 1


Focal Adhesion
EXTRACELLULAR MATRIX
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COL
LAM
Wnt3a
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LRP5/6
LRP5/6
Wnt3a
DEP
PDZ
DIX
CK1γ
GSK3β
AXIN
DVL
FZD7
Wnt5a
SDC4
α5β1
INTEGRIN
ILK
TGFβ
TGFβR
α3β1
Wnt5a
Wnt3a
GPC4
10
PM
microdomains
9
Smad2
Src
FAK
Regulation of
E-Cadherin/
β-catenin
complexes
β-catenin
Non-canonical
Wnt signaling
GSK3β
P
3
APC
AXIN
5
P
Smad2
β-catenin
E-Cadherin
α-catenin
Wnt/β-catenin
signaling
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mRNAs
FN
Dkk1
TCF
β-catenin
NUCLEUS
CYTOPLASM
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