

The Tangled Web of Non-Canonical Wnt Signalling in Neural Migration

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Key Words

Wnt • Ror • Ryk • Non-canonical Wnt signalling • Neural tube formation • Convergent extension • Axon guidance

Abstract

In all multicellular animals, successful embryogenesis is dependent on the ability of cells to detect the status of the local environment and respond appropriately. The nature of the extracellular environment is communicated to the intracellular compartment by ligand/receptor interactions at the cell surface. The Wnt canonical and non-canonical signalling pathways are found in the most primitive metazoans, and they play an essential role in the most fundamental developmental processes in all multicellular organisms. Vertebrates have expanded the number of Wnts and Frizzled receptors and have additionally evolved novel Wnt receptor families (Ryk, Ror). The multiplicity of potential interactions between Wnts, their receptors and downstream effectors has exponentially increased the complexity of the signal transduction network. Signalling through each of the Wnt pathways, as well as crosstalk between them, plays a critical role in the establishment of the complex architecture of the vertebrate central nervous system. In this review, we explore the signalling networks triggered by non-canonical Wnt/receptor interactions, focussing on the emerging roles of the non-conventional Wnt receptors Ryk and Ror. We describe the role of these pathways in neural tube formation and axon guidance where Wnt signalling controls tissue polarity, coordinated cell migration and axon guidance via remodelling of the cytoskeleton.

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A Brief History of Wnt Signalling

In all multicellular animals, complex developmental processes such as axis formation, tissue specification and polarity must be tightly coordinated in space and time if a functional organism is to be produced. Successful embryogenesis is dependent on the ability of cells within developing tissues to sense the status of their local environment and to respond to the relevant signals by initiating the appropriate cellular response. In all metazoans, including the simplest multicellular organisms, the nature of the extracellular environment is communicated to the intracellular compartment by cytoplasmic signal transduction cascades activated by ligand-receptor interactions at the cell surface. Sequencing of genomes from extant, primitive multicellular organisms such as sponges has led to the hypothesis that essential developmental signal transduction pathways, including the Wnt, sonic hedgehog and transforming growth factor β pathways, originally evolved in the earliest multicellular organisms [1].

The Wnt family of extracellular ligands, through their cell surface receptors (Frizzled, Fz; Ryk, Ror), activate several distinct, but integrated, signal transduction pathways that have diverse developmental consequences (fig. 1). The canonical (Wnt/ β -catenin) pathway regulates stem cell proliferation and self-renewal, cell fate determination and axis formation by modulating gene expression. The non-canonical planar cell polarity (PCP) pathway controls the remodelling of the cytoskeleton via a β -catenin-independent mechanism and controls tissue polarity, coordinated cell migration and axon guidance.

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The Wnt/Ca²⁺ pathway regulates cell fate determination, cell migration, axon guidance and also collaborates with the PCP pathway in regulating tissue polarity. Key components of the Wnt/ β -catenin pathway have been identified in the sponge *Amphimedon queenslandica*, a member of the oldest known extant animal phylum, thereby implicating this pathway in embryonic axis patterning at a very early evolutionary time point [2]. These components include Wnt, Fz, low-density lipoprotein receptor-related protein 5/6 (LRP5/6), Dishevelled (Dvl in mammals, Dsh in *Drosophila* and *Xenopus*), axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β) and T cell factor (TCF) (fig. 1). The non-canonical PCP and Wnt/Ca²⁺ pathways are believed to have evolved after the divergence of sponges and the eumetazoan lineage in which animals display radial or bilateral symmetry (sea anemones or jellyfish, respectively) [1, 2].

Both nematodes and insects utilise the Wnt/ β -catenin, PCP and Wnt/Ca²⁺ pathways and have additionally evolved new coreceptors and novel Wnt receptor families (Ryk and Ror) which are able to potentiate or suppress these pathways, depending on the biological context. In addition, gene duplication has expanded the number of Wnt ligands (19 in mammals) and receptors (10 Fzs in mammals) [3]. Thus, the multiplicity of potential interactions between Wnts, their receptors and downstream effectors has exponentially increased the complexity of the signal transduction network. In the context of nervous system development, the functional outcome of Wnt pathway activation has been conserved across all species. Signalling through each of the Wnt pathways, as well as crosstalk between them, plays a critical role in all facets of nervous system development, including neural induction, neuronal polarisation, cell migration, axon and dendritic growth, axon guidance and synaptogenesis [4–8].

In this review, we explore the signalling networks triggered by non-canonical Wnt/receptor interactions, focusing on the emerging roles of the non-conventional Wnt receptors Ryk and Ror. It has taken 25 years to unravel the complexities of the Fz-dependent pathways. In contrast, characterisation of the Ryk and Ror signalling cascades is in its infancy. What is becoming clear, however, is that none of these pathways operate in isolation. Instead, evidence is emerging that there is a great deal of crosstalk between them. Here, we focus on the role of Wnt signalling in the establishment of the complex architecture of the vertebrate central nervous system (CNS). In particular we concentrate on those aspects of CNS development (neural tube formation and axon guidance) where a clearer picture of the function of the non-canonical receptors

Ryk and Ror is beginning to emerge. In order to understand the relevance of Ryk and Ror signalling in the context of the entire Wnt signalling network, we start with an abridged summary of the major Wnt pathways.

Canonical and Non-Canonical Wnt Pathways

Mammalian Wnts can be subdivided into two classes, originally defined by their ability to act as oncogenes [9]. The transforming Wnts (e.g. Wnt1, Wnt3) activate the canonical Wnt/ β -catenin pathway, whereas the non-transforming Wnts (e.g. Wnt4, Wnt5a, Wnt11) act through the non-canonical (β -catenin-independent) pathways. Wnts signal via the Fz receptors [10–12] which can also be loosely classified into canonical or non-canonical receptors, depending on whether or not they prevent β -catenin degradation and trigger its translocation to the nucleus. Fzs are members of the 7-pass transmembrane domain, G-protein-coupled receptor family. The extracellular cysteine-rich domain is responsible for Wnt binding [13] and other extracellular subdomains are involved in coreceptor binding (e.g. binding to the coreceptor Van Gogh [14]). The cytoplasmic domain comprises 3 intracellular loops and a C-terminal tail that associates with a variety of downstream effectors [15]. In all Wnt pathways, Dvl is recruited to Fz at the membrane (fig. 1) and activated by phosphorylation after Wnt binding and is therefore perfectly positioned to regulate crosstalk between pathways [16]. The N-terminal and central PDZ domains of Dvl are required for Wnt/ β -catenin signalling, whereas the PDZ and C-terminal regions activate the PCP pathway [16].

The Wnt/ β -Catenin Pathway

In the absence of canonical Wnt binding, cytoplasmic β -catenin is captured by the destruction complex (containing the scaffold protein axin and the tumour suppressor gene APC) where it is phosphorylated by GSK3 β and casein kinase 1 α (CK1 α) and targeted for degradation in the proteasome (fig. 1). Upon canonical Wnt binding, Fz forms a coreceptor complex with LRP5/6, resulting in the recruitment of Dvl and components of the destruction complex to the receptor. GSK3 β - and CK1 α -mediated phosphorylation of β -catenin is subsequently inhibited, preventing degradation of β -catenin which is then free to translocate into the nucleus where it forms a transcriptional activation complex with TCF. This leads to the transcription of target genes involved in embryonic patterning and cell fate specification and proliferation [for detailed reviews, see 17, 18].

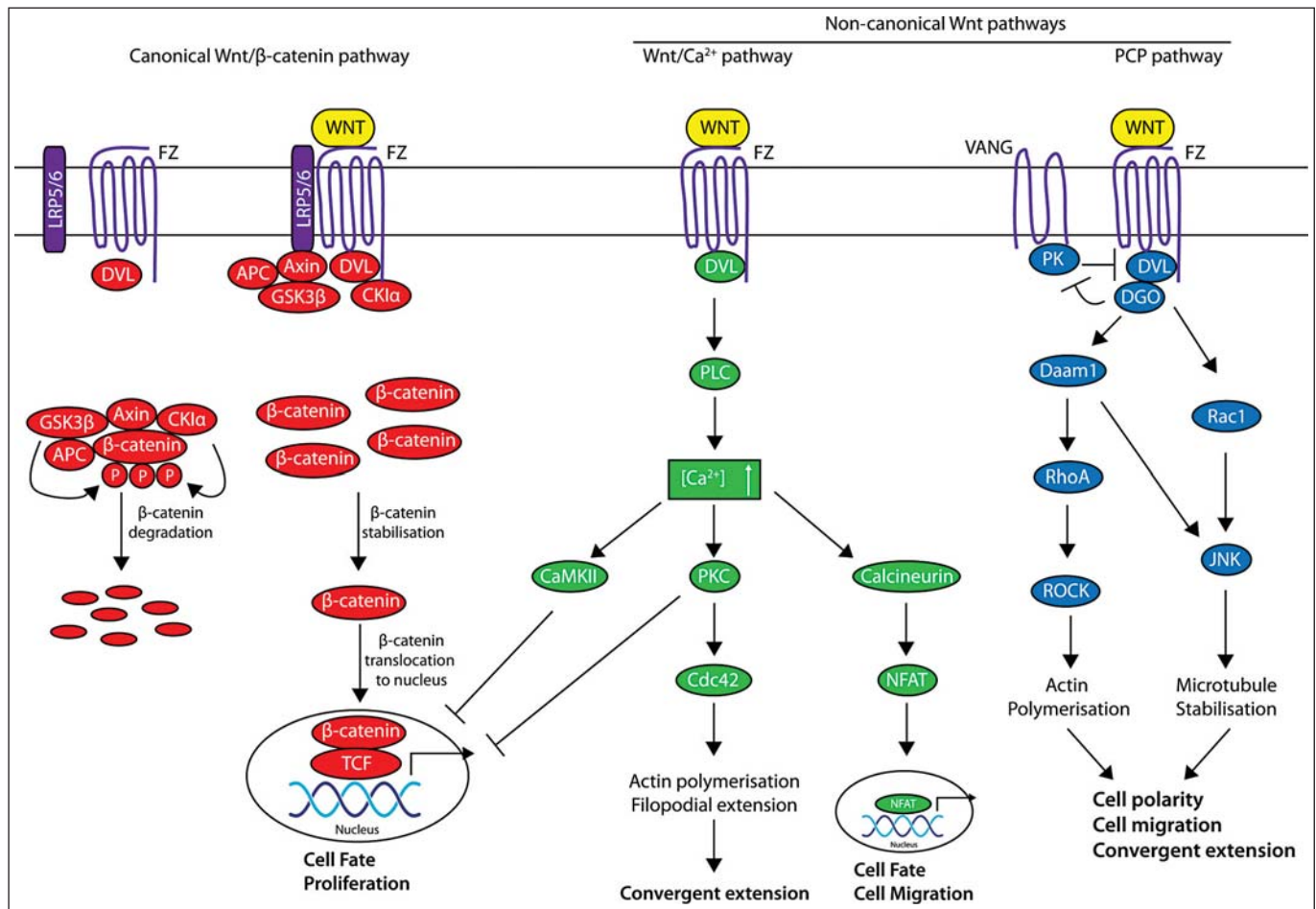


Fig. 1. Wnt signalling pathways. In the canonical Wnt/β-catenin pathway in the absence of a Wnt ligand, β-catenin is recruited by the destruction complex and phosphorylated by glycogen synthase kinase 3β (GSK3β) and casein kinase Iα (CK1α) targeting it for degradation in the proteasome; levels of cytosolic β-catenin are subsequently kept low. In the presence of a Wnt ligand, members of the destruction complex are recruited to the membrane, and β-catenin accumulates within the cytoplasm. β-Catenin then translocates to the nucleus where it affects T cell factor (TCF) transcription controlling cell fate and proliferation. The non-canonical Wnt/Ca²⁺ pathway leads to increased intracellular Ca²⁺

which activates calcium calmodulin kinase II (CaMKII), protein kinase C (PKC) and calcineurin. CaMKII and PKC inhibit canonical Wnt signalling, PKC also activates Cdc42 regulating cytoskeletal dynamics during convergent extension. Calcineurin activates NFAT which affects gene transcription controlling cell fate and cell migration. Non-canonical PCP signalling involves interactions between Fz, Vang, Pk and Dgo with activation of RhoA and ROCK via Daam1, which leads to actin polymerisation, and Rac1 and JNK, which leads to microtubule stabilisation. This regulation of cytoskeletal dynamics controls cell polarity, cell migration and convergent extension.

The Non-Canonical PCP Pathway

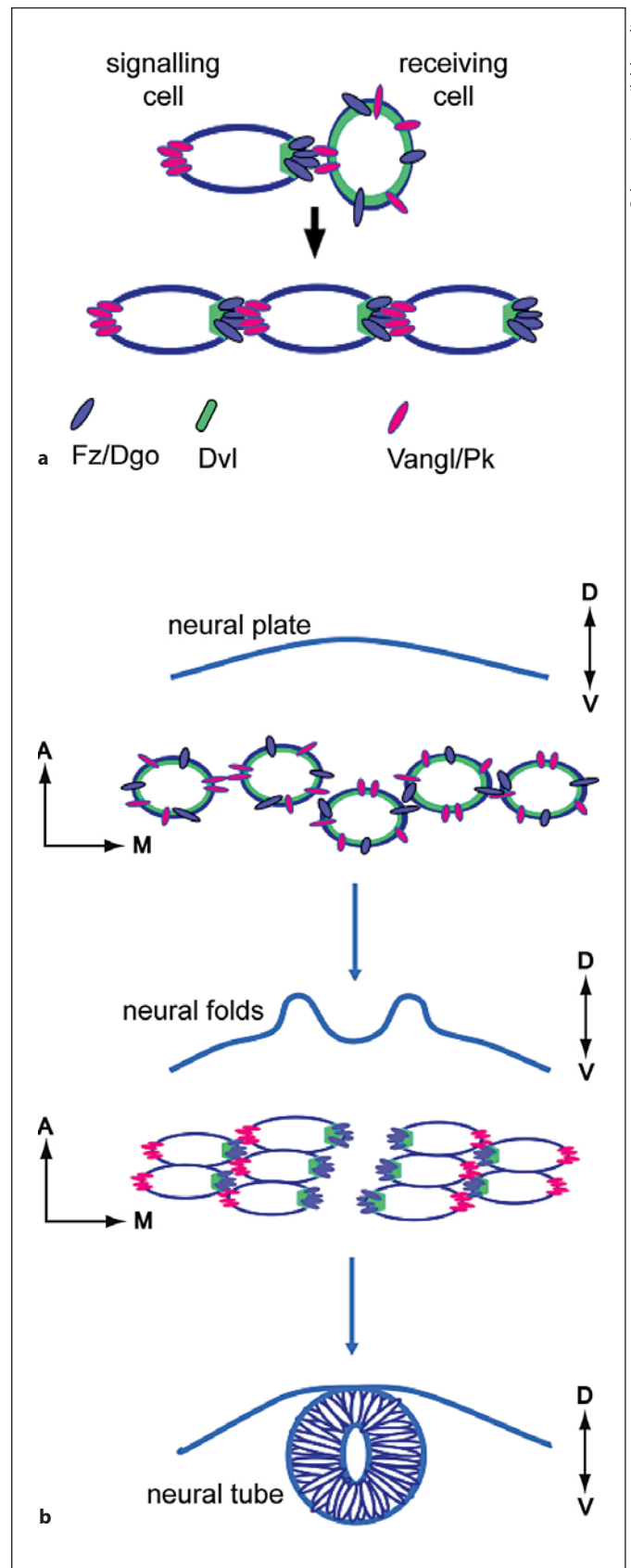
Non-canonical Wnt signalling is β-catenin independent and mediated by two distinct, but interacting pathways, the Ca²⁺-independent PCP pathway and the Ca²⁺-dependent Wnt/Ca²⁺ pathway (fig. 1). The PCP pathway is best characterised in *Drosophila* where it is responsible for establishing tissue polarity during wing and eye formation [for a detailed review, see 19]. A similar mechanism regulates tissue polarity during gastrulation and neural tube formation in the vertebrate embryo. In contrast to the cell-

autonomous function of the Wnt/β-catenin pathway, PCP signalling in the fly wing epithelium requires interaction of pathway components on neighbouring cells. Upon activation of the pathway, the polarity signal is transmitted throughout the plane of the tissue through local cell-cell interactions. Prior to polarisation, the core PCP components are distributed uniformly around the plasma membrane. In the *Drosophila* wing epithelium, the two core PCP complexes are found on opposite sides of the polarising cell (fig. 2a). The distal core complex comprises Fz,

Dsh (Dvl in mammals) and Diego (Dgo, diversin in mammals), whereas the strabismus/Van Gogh (Vang)-prickle (Pk) complex is localised on the proximal side. Both complexes also contain the cadherin-like receptor, flamingo (Fmi, Celsr in mammals), which directly links the distal and proximal complexes on adjacent cells through trans-homophilic adhesive interactions. The interaction between Fz in the signalling cell and Vang in the receiving cell triggers the asymmetric distribution of both core complexes in the receiving cell (fig. 2a). The polarised receiving cell then becomes the signalling cell and induces polarity in another neighbour. It is these reiterative local cell-cell interactions that transmit the planar polarity signal throughout the tissue. Fz-Vang interactions between cells are also required for the correct alignment of the polarised tissue relative to the body axis. Exactly how this global signal is propagated is not well understood; however, to date there is no convincing evidence in *Drosophila* that Wnts are involved [14]. In vertebrates the situation is different as Wnts are directly involved in activating Fz signalling in the context of planar polarity [20]. Moreover, a recent study has provided convincing evidence that global tissue polarity in the mouse embryo is established in response to a Wnt gradient [21].

Cell-autonomous versions of the PCP pathway drive cell migration, axon growth and pathfinding, synaptogenesis and ciliogenesis through modulation of the actin and microtubule cytoskeletons [4, 7, 20, 22–24] (fig. 1). In these situations non-canonical Wnts activate the PCP pathway through the recruitment of the cytoplasmic signalling components Dvl and Dgo to the cytoplasmic domain of Fz receptors. Here, Dgo activates Dvl by inhibiting the interaction of the Pk-Vang complex with Dvl (fig. 1). Daam1, a scaffold protein, links Dvl to RhoA, resulting in actin stress fibre formation [16, 22, 25]. Dvl also

Fig. 2. The PCP pathway establishes tissue polarity and drives convergent extension during neural tube formation. **a** Prior to polarisation in the fly wing epithelium, the core PCP components are distributed uniformly around the plasma membrane. Upon activation of PCP signalling, the interaction between Fz in the signalling cell and Vang in the receiving cell triggers the asymmetric distribution of both core complexes in the receiving cell. The polarised receiving cell then becomes the signalling cell and induces polarity in another neighbour. **b** During neurulation, the neuro-ectoderm migrates from the dorsolateral regions of the embryo towards the dorsal midline by mediolateral intercalation which drives convergent extension and neural tube formation. The PCP pathway polarises cells in the mediolateral direction, promoting mediolateral intercalation. A = Anterior; D = dorsal; M = medial; V = ventral.



activates Rac1 leading to the protrusion of actin-based lamellipodia and c-Jun-dependent transcription. Activation of Rac1, Cdc42 and JNK also leads to stabilisation of microtubule filaments [16, 22]. Thus, direct interaction of Dvl with both RhoA and Rac1 is fundamental to the regulation of cell polarity through the remodelling of the actin and microtubule cytoskeletons.

One puzzle that arises when comparing the non-cell-autonomous PCP pathway responsible for planar polarity, to the cell-autonomous PCP signalling governing processes such as axon guidance, has been the role of Vang. In the *Drosophila* wing, Vang (proximal core complex) and Fz (distal core complex) are localised on apposing cell membranes. The polarity signal initiated by intracellular adhesion between the core complexes is then transmitted intracellularly (from the proximal side) via Vang to the distal side of the same cell, where it induces polarised proximal localisation of the Fz complex (fig. 2a). Therefore, the initiation of cell polarity requires that Fz and Vang operate in apposing cells. In contrast, in axon growth cones Fz and Vang are always present on the same membrane, indicating a fundamental difference in the interaction between core PCP components. A recent study from the Zou laboratory may have, at least partially, solved this puzzle [23]. Previous studies had shown that Wnt activation of the PCP pathway requires that Fz be internalised through clathrin-mediated endocytosis [26]. Shafer et al. [23] now show that Vangl2 is highly enriched in the tips of growth cone filopodia and that, upon Wnt activation, it selectively promotes the internalisation of the non-phosphorylated, active form of Fz3, thereby focussing PCP pathway activation to a localised membrane domain. These very different modes of PCP signalling demonstrate the versatility of the core components and provide an explanation for why non-canonical signalling plays such a prominent role in many fundamental morphological processes during embryonic development.

The Non-Canonical Wnt/Ca²⁺ Pathway

The Ca²⁺-dependent branch of non-canonical Wnt signalling is important in cell fate determination, cell migration, convergent extension (CE) and axon guidance. During gastrulation and neural tube formation, it works in parallel with the PCP pathway through its ability to activate a distinct set of actin modulators. Receptor binding activates phospholipase C which in turn stimulates diacylglycerol and inositol triphosphate (IP3) production, leading to Ca²⁺ release from intracellular stores and the activation of the Ca²⁺-sensing enzymes, calcium

calmodulin kinase II (CaMKII), calcineurin and protein kinase C (PKC) [27–30] (fig. 1). Localised increases in intracellular Ca²⁺ stimulate the formation of new filopodial protrusions via regulation of actin dynamics [31]. Dvl is also required for pathway activation as blocking Dvl function leads to decreased activation of PKC by Fz7 [29].

Dvl is a critical component of the Wnt/ β -catenin, PCP and Wnt/Ca²⁺ pathways, placing it in an ideal position to regulate crosstalk between pathways. A Dvl mutant lacking the N-terminal DIX domain (Dsh Δ DIX) is sufficient to activate the PCP pathway but not the Wnt/ β -catenin pathway, which requires the DIX domain [32, 33]. Dsh Δ DIX is also sufficient to activate the Wnt/Ca²⁺ pathway via stimulation of Ca²⁺ fluxes and activation of PKC and CaMKII [29]. This modular functionality of Dvl provides a mechanism by which individual Wnt pathways can directly influence each other's activity. For example, Wnt/Ca²⁺ signalling is able to antagonise Wnt/ β -catenin signalling during CE [27, 34–36]. The Wnt/ β -catenin pathway is required indirectly for CE movements of mesodermal cells in the early *Xenopus* embryo through TCF-mediated transcriptional activation of Xnr1, a modulator of cell migration [34]. However, Wnt5- or Wnt11-mediated Wnt/Ca²⁺ pathway activation suppresses the Wnt/ β -catenin pathway at two levels (fig. 1). Dvl-dependent activation of PKC blocks Dvl-induced β -catenin function and Dvl-dependent activation of CaMKII inhibits TCF-mediated transcription (fig. 1). Thus, Dvl activates the Wnt/Ca²⁺ pathway while simultaneously inhibiting the Wnt/ β -catenin pathway [34].

The Non-Canonical Ror Pathway

The non-canonical, β -catenin-independent Wnt receptors Ror1 and Ror2 are receptor tyrosine kinases required for the establishment of cell polarity, asymmetric cell division and cell migration during key morphological processes, including neural tube formation, testes development, and heart and bone formation [for a detailed review, see 37]. Vertebrate Rors possess an extracellular cysteine-rich domain (similar to that of Fz receptors) and a membrane proximal kringle domain. Within the cytoplasmic region there is an active tyrosine kinase domain and 2 serine/threonine-rich domains flanking a proline-rich domain. The extracellular cysteine-rich domain is responsible for the interaction of Ror receptors with Wnt ligands [38–40] and mediates the formation of a coreceptor complex with Fz2 [40]. The immunoglobulin-like domains may also be involved in receptor-ligand and receptor-coreceptor interactions [37]. The function of the krin-

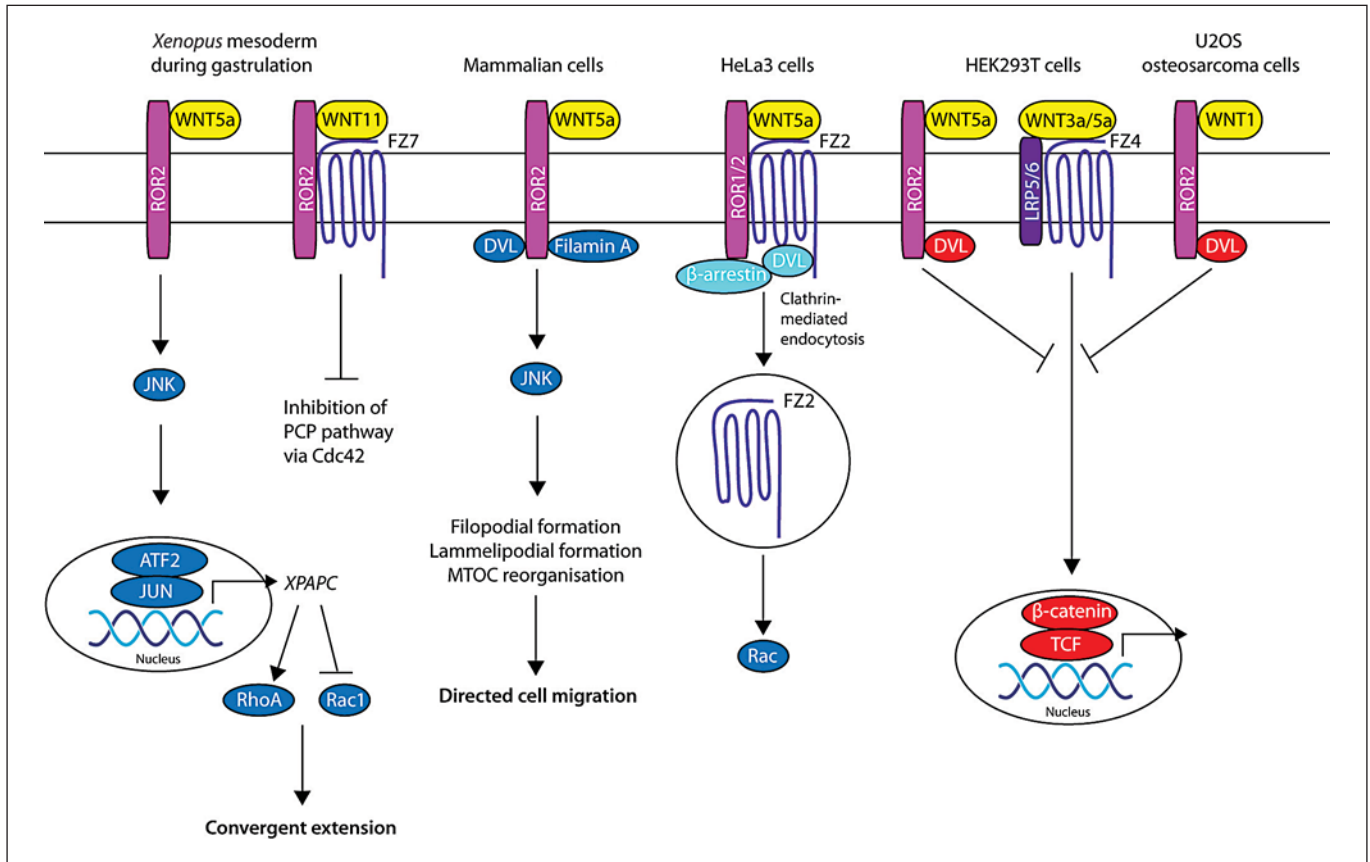


Fig. 3. Non-canonical Ror signalling. In *Xenopus* mesoderm during gastrulation, Wnt5a/Ror2 binding leads to activation of the PCP pathway affecting convergent extension. Conversely, Wnt11 activation of Ror2-Fz7 leads to inhibition of the PCP pathway. In mammalian cells [for specific cell types, see 113], Wnt5a activation of Ror2 leads to PCP pathway activation via recruitment of filamin A to the membrane controlling directed cell migration.

In HeLa3 cells, Wnt5a/Ror2-Fz2 binding leads to recruitment of β-arrestin and Dvl and clathrin-mediated endocytosis of Fz2 which activates the PCP pathway. In HEK293 cells, Wnt3a/5a can activate TCF transcription via Fz4-LRP5/6. Wnt5a/Ror2 binding can inhibit this pathway, and in U2OS osteosarcoma cells Wnt1/Ror2 binding can inhibit TCF transcription.

gle domain has not been determined but it is predicted to associate with intracellular signalling molecules. The serine/threonine-rich and proline-rich domains also enable binding to downstream effectors, including the scaffold protein filamin A, and serine/threonine and tyrosine kinases [37, 41]. The tyrosine kinase domain is responsible for autophosphorylation and the phosphorylation of other intracellular effectors upon Wnt5a binding [42–44].

Non-canonical Wnt5a has been identified as the principal ligand for Ror1 and Ror2 on the basis that *Wnt5a* loss-of-function mice exhibit an array of phenotypes that closely parallel those seen in *Ror* knockout mice [45–48]. Upon Wnt5a binding, Ror2 promotes cell migration in a Dvl-dependent manner [41] (fig. 3). In a parallel pathway, the actin-binding protein filamin A is recruited to the Ror2 cytoplasmic domain, resulting in reorganisation of

the actin cytoskeleton via the JNK pathway and filopodial extension [41, 49] (fig. 3). Activation of the filamin A-JNK axis also promotes lamellipodial formation [49] (fig. 3), perhaps by activating paxillin and focal adhesion kinase [50, 51] and reorientation of the microtubule-organising centre [49] (fig. 3). Thus, the Ror and PCP pathways cooperate at the level of the cytoskeleton to establish cell polarity, an essential requirement for cell division and directed cell and axon migration.

Wnt5a/Ror2 interactions also impinge on the Wnt/β-catenin pathway. However, this activity appears to be dependent on the stimulating canonical Wnt and cell type. Wnt3a stimulation of TCF-mediated transcription is blocked by Wnt5a-Ror2 activation in HEK293 cells [39, 52, 53] (fig. 3), whereas Ror2 augments Wnt1-induced transcription but inhibits Wnt3a-induced transcription

in U2OS osteosarcoma cells [38] (fig. 3). Thus, there is a complex regulatory crosstalk between these pathways. Ror2 is known to interact with several Fzs and forms a receptor complex under specific conditions [40, 54–56]. One explanation for these opposing activities may lie in the identity of the Fz receptor. However, there is also evidence that Ror can act independently of Fz. A recent study has shown that the C terminus of Dvl can bind Ror2, resulting in the inhibition of the Wnt/ β -catenin pathway [57]. Therefore, Dvl-mediated activation of the Wnt/ β -catenin pathway appears to be overruled by the Ror/Dvl negative feedback loop, at least in some contexts. Another point of intersection between Ror and the Wnt/ β -catenin pathway is CK1, a key component of the β -catenin destruction complex [58]. CK1 phosphorylates the Fz coreceptor, LRP5/6, reinforcing the interaction with axin, leading to stabilisation of β -catenin (reviewed in Angers and Moon [17]; fig. 1). CK1 ϵ also binds Ror2 and activates Ror2 autophosphorylation [58]. In addition, Wnt5a binding initiates Ror2 tyrosine autophosphorylation and the activation of Src family kinases [52]. However, the kinase domain is not required for filopodial extension and migration [55]. How these different phosphorylation states are differentially regulated is still not well understood.

The Non-Canonical Ryk Pathway

The Ryk orthologue *derailed* (Drl) was initially identified as a chemorepulsive axon guidance receptor in the *Drosophila* nerve cord where its cognate ligand was identified as Wnt5 [59, 60]. It has since been shown to be required for synaptogenesis and memory and learning in the adult fly [61, 62]. In vertebrates, more diverse roles are emerging for Ryk in a range of neurodevelopmental processes. In addition to chemorepulsive axon guidance [63–66], Ryk is important for establishing planar polarity during neural tube development in the *Xenopus* embryo [67], differentiation of mouse neural progenitor cells [68, 69], specification of GABAergic neurons during mouse cortical development [70] and neuronal migration into the expanding cortical plate [71]. Although the function of Ryk is currently best understood with respect to its role in nervous system development, its widespread expression, both in developing tissues and in the adult, suggests that Ryk regulates many biological processes. This hypothesis is supported by the multiple developmental phenotypes apparent in *Ryk* loss-of-function mice, including skeletal, craniofacial and cardiac abnormalities [72].

Ryk is an atypical receptor tyrosine kinase due to its inactive tyrosine kinase domain [73–75]. Although it is

clearly a member of the receptor tyrosine kinase superfamily, there are numerous amino acid substitutions in its kinase domain that render it ‘kinase dead’. Interestingly, although human Ryk is kinase dead, it is able to activate the mitogen-activated protein kinase pathway [75], possibly via its interaction with the Src kinase family [76]. The Ryk extracellular domain is also unusual as it is considerably shorter than that found in other receptor tyrosine kinases [74]. The predominant features in the extracellular domain are the 2 leucine-rich motifs, which bear significant homology to the N-terminal domain of Wnt-inhibitory factor 1 (WIF-1) [77]. It has been shown that Ryk binds Wnts via these WIF-like domains [63].

Both the canonical (Wnt1, Wnt3) and non-canonical Wnts (Wnt5a, Wnt11) have been identified as Ryk ligands [63, 66, 67], and as for the Ror receptors, Wnt5a/Ryk interactions have been most intensely studied [63, 65, 78]. Our knowledge of the Ryk signalling pathway comes largely from analysis of its axon guidance function. As guidance is dependent on establishing the correct polarity within the growth cone by the assembly and disassembly of the actin cytoskeleton, one would predict that Ryk signals through the non-canonical pathway. Indeed, Wnt5a/Ryk-mediated axon outgrowth and chemorepulsion have been shown to utilise the Wnt/ Ca^{2+} pathway [64] (fig. 5). There is also evidence that Ryk may activate the Wnt/ β -catenin pathway by forming a coreceptor complex with Fz8 [66] (fig. 4).

Further light has been shed on the Ryk signal transduction pathway in *Drosophila* where chemorepulsion was found to be dependent on the Src non-receptor tyrosine kinase family. Src64B and Src42A are necessary for commissure formation, and loss of either kinase leads to disorganised commissures and longitudinal tract defects, a phenotype comparable to that seen in *Wnt5* and *derailed* mutants [76]. It has been shown that Ryk and Src form a complex whose stability is dependent on Src activation and Drl phosphorylation [76] (fig. 5). Furthermore, the Wnt5/Drl/Src pathway does not affect TCF-dependent transcription. Together these data suggest that Drl does not signal via the Wnt/ β -catenin pathway but uses a novel Src-mediated non-canonical pathway. Whether mammalian Ryk also works through Src is unknown; however, the mammalian orthologues of Ryk and Src do form complexes [76], suggesting that Src signalling may be a universal requirement for Ryk-dependent chemorepulsion.

Recently, a novel mechanism for Ryk signal transduction has been uncovered in the early embryonic mouse cortex. The intracellular domain (ICD) of Ryk was shown to be cleaved by γ -secretase and then translocated to the

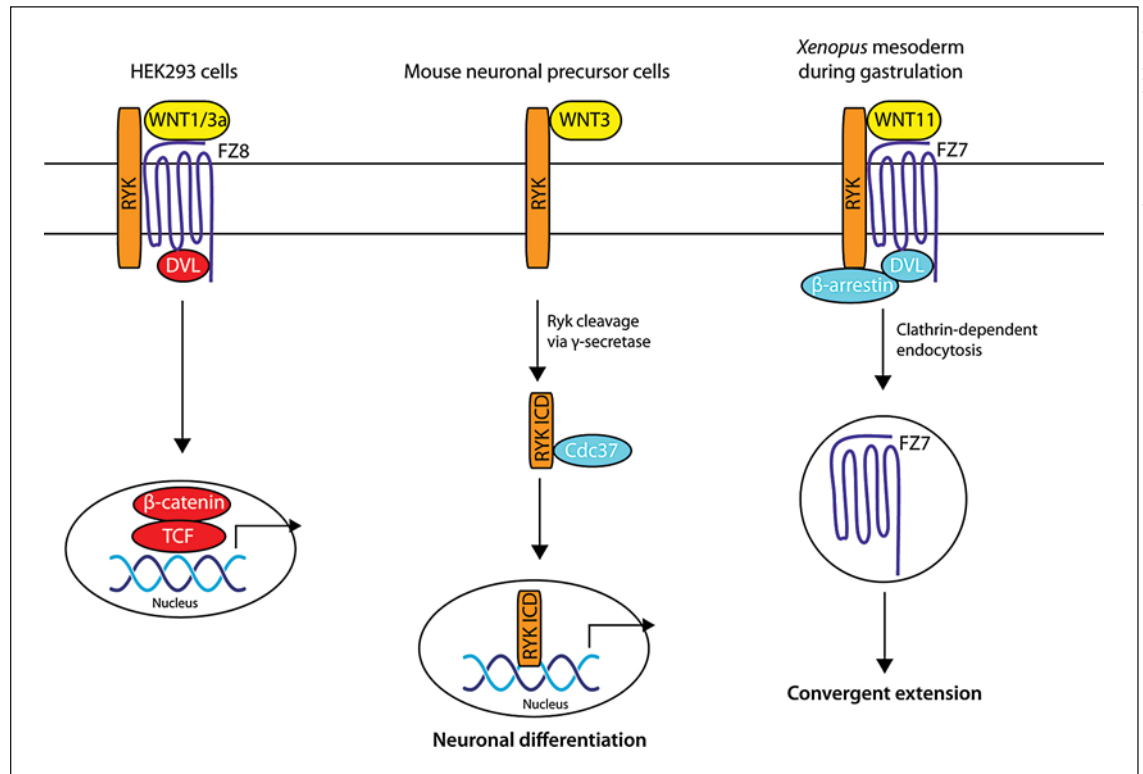


Fig. 4. Non-canonical Ryk signalling. In HEK293 cells, Ryk forms a complex with Fz8 and Dvl, and Wnt1 or Wnt3a stimulation leads to TCF transcription. In mouse neuronal precursor cells, Wnt3 binds Ryk, and the Ryk intracellular domain is cleaved by γ -secretase. The Ryk intracellular domain is chaperoned to the nucleus

by Cdc37 where it affects gene transcription regulating neuronal differentiation. In *Xenopus* mesoderm during gastrulation, Ryk forms a complex with Fz7, Dvl and β -arrestin. Wnt11 activation of this complex leads to clathrin-dependent endocytosis of Fz which regulates convergent extension.

nucleus of newborn pyramidal projection neurons where it was postulated to promote the transcription of factors required for neuronal differentiation [68] (fig. 4). Wnt3 is necessary for Ryk ICD translocation but not cleavage, and the cochaperone protein Cdc37 is required for stabilisation of the Ryk ICD [68, 69] (fig. 4). Neither β -catenin stabilisation nor TCF-mediated transcription was observed after ICD translocation, indicating that Ryk signalling does not occur via the Wnt/ β -catenin pathway [68]. Conversely, Wnt3-mediated neuronal differentiation was shown to be dependent on the nuclear localisation of the Ryk ICD [68]. Clearly, there are multiple facets to Ryk signalling, and its interplay with the Wnt/ β -catenin pathway is complex.

The Ryk and Ror pathways share some intriguing commonalities. Both receptors bind canonical and non-canonical Wnts. Like Ror receptors, Ryk's favourite ligand is the non-canonical Wnt5a; however, other Wnts may come into focus as a deeper understanding of the biological roles of these receptors is revealed. Both path-

ways cooperate with the PCP pathway to establish cell polarity, a fundamental requirement for directed cell and axon migration and asymmetric progenitor division. They accomplish these tasks by influencing the stability and orientation of the cytoskeletal network. Both pathways also modulate the Wnt/ β -catenin pathway, although the evidence in the case of Ryk needs to be bolstered. To date there is no evidence that the Ryk and Ror pathways interact, but given that they share ligands and some downstream effectors, it is unlikely that they operate independently. The above studies further suggest that the biological outcomes of Ryk and Ror signalling are likely to be context dependent.

Wnt Signalling in Neural Tube Formation

In vertebrate embryos, the neural tube, the first 3-dimensional structure of the CNS, comprises a pseudostratified neuro-epithelium surrounding a central lumen.

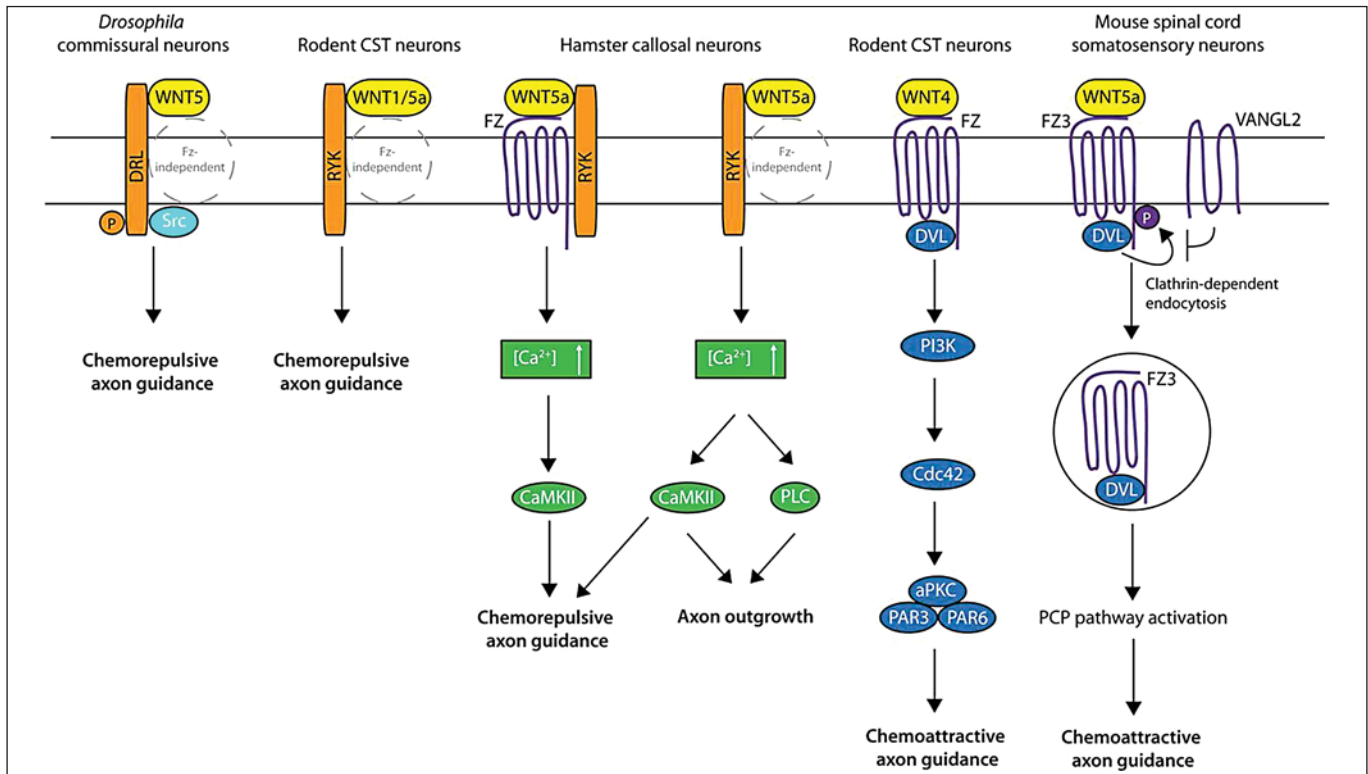


Fig. 5. Non-canonical Wnt signalling in axon guidance. In *Drosophila* commissural neurons, Wnt5/Drl signalling via Src controls chemorepulsive axon guidance. Similarly, in rodent corticospinal tract (CST) neurons Wnt5a and Wnt1 control chemorepulsive axon guidance. These functions occur independently of Fz receptors. In contrast, in hamster callosal neurons, Wnt5a/Fz/Ryk signalling via the Wnt/ Ca^{2+} pathway controls chemorepulsive axon guidance whereas Wnt/Ryk signalling alone, also via the Ca^{2+} pathway, controls axon outgrowth. Chemo-attractive axon guid-

ance is mediated by Wnt/Fz interactions in rodent CST and spinal cord neurons. In rodent CST axons, Wnt4/Fz signalling via Cdc42 and the aPKC/Par3/Par6 complex regulates chemo-attraction. In mouse spinal cord commissural somatosensory neurons, Wnt5a/Fz3 signalling stimulates Fz3-Dvl endocytosis to activate the PCP pathway and control chemo-attractive axon guidance. This process is regulated via Dvl-mediated Fz3 phosphorylation which is antagonised by Vangl2.

This neuro-epithelium ultimately gives rise to all neural cell types in the CNS. The neural tube derives from a flat sheet of neuro-ectoderm (the neural plate) arising on the dorsal surface of the blastoderm at the end of gastrulation [79, 80]. During *Xenopus* neurulation, the neuro-ectoderm and the underlying mesoderm migrate from the dorsolateral regions of the embryo towards the dorsal midline by mediolateral intercalation, a specialised form of migration where cells shuffle between each other while remaining in close contact [81] (fig. 2b). These synchronised cell movements contribute to the morphological process known as CE in which coordinated tissue movements shape the body axis by narrowing the embryo in the mediolateral direction and lengthening the antero-posterior axis [82, 83]. As the neural plate approaches the midline, it elevates dorsally and the dorsal tips then fuse, leading to neural tube closure. The PCP pathway drives

the medially directed movement of the neural folds [80, 84–87] (fig. 2b), but not the elevation of the folds, which is regulated by the repulsive guidance molecule A receptor neogenin [88]. Mediolateral intercalation requires that the neuro-ectodermal cells polarise in a mediolateral orientation, which is established by the PCP pathway [80, 84, 87, 89, 90]. Failure of the PCP pathway abolishes mediolateral polarity, preventing migration towards the dorsal midline and neural fold fusion, resulting in devastating congenital malformations in which the brain and spinal cord are fully or partially open (anencephaly, craniorachischisis, spina bifida) [79].

The PCP Pathway and CE

In *Xenopus* and zebrafish, the interaction between non-canonical Wnt5a/Wnt11 and Fz3/Fz7 triggers a signalling cascade that induces mediolateral polarity in neu-

ro-ectodermal cells during neural tube formation as well as in the gastrulating mesoderm [22, 32, 91–93]. The plasma membrane proteins Vang, Fmi (Celsr1) and the heparan sulphate proteoglycans knypek and syndecan 4 as well as the intracellular proteins Dvl and Pk are required for PCP-induced polarised cell intercalation [20, 22, 94]. Binding of Wnt concentrates Fz and Dvl to localised membrane subdomains [22]. Knypek and syndecan 4 are able to bind Wnts, and syndecan 4 can also bind Fz and Dvl. Therefore, these molecules may act to increase the local Wnt concentration at the receptor and/or to stabilise Fz-Dvl complexes at specific membrane subdomains. Vang interacts with Pk to negatively regulate Dvl activity (fig. 1). Pk antagonises the Dvl-Rho-Rac axis by preventing Dvl translocation to the membrane and can also promote its degradation. Thus, Vang-Pk activity dampens PCP-mediated GTPase activity, a function that would allow tight regulation over filopodial extension, thereby fine-tuning the response to the Wnt gradient. Vang and Pk are also able to activate JNK, a downstream effector of PCP signalling that triggers transcriptional activation. In *Drosophila* planar polarity, Fmi is responsible for homophilic cell-cell adhesion between the signalling and receiving cell (fig. 2) and interacts directly with the Fz cytoplasmic domain. In vertebrates, Fmi (Celsr) also has a cell adhesion function but does not appear to bind Fz [95]. Nonetheless, Fmi is required for efficient PCP signalling during vertebrate CE [96].

Daam1 is a downstream scaffold protein linking Dvl to RhoA (responsible for the formation of actin stress fibres) and Rac1 (responsible for the protrusion of lamellipodia), both of which are activated by directly binding to Dvl (fig. 1). The direct interaction of Dvl with RhoA and Rac is essential for Fz7-induced medially directed protrusion of lamellipodia. Furthermore, RhoA and Rac1 directly link the detection of Wnt at the cell surface to actin cytoskeleton remodelling and lamellipodial formation. Disruption of the PCP pathway in *Xenopus* embryos by expression of mutated forms of *Wnt11*, *Fz7*, *Vang*, *Fmi*, *Pk* or *Dvl* produces defective CE movements leading to the failure of neural tube closure. In *Dvl* mutants, neuro-ectodermal cells do not undergo mediolateral polarisation, which impedes intercalation-driven migration to the dorsal midline [97, 98]. The result is a broader floorplate, which prevents apposition of the neural folds.

Mutations in the PCP components are also responsible for neural tube defects in mammals. The neural tube of mice homozygous for the *looptail* mutation in *Vangl2* is completely open at the hindbrain and spinal cord levels (i.e. craniorachischisis) [99, 100] and mutations in *Vangl1*

and *Vangl2* have been linked to neural tube defects in humans [101, 102]. Craniorachischisis-like phenotypes are also observed in the naturally occurring *Celsr1* mutant mouse and in knockout mice lacking *Fz3*, *Fz6* or both *Dvl1* and *Dvl2* [97, 103–106]. Therefore, these phenotypes provide persuasive evidence that the core PCP pathway drives CE in the neuro-ectoderm in mammals. The ability of the PCP pathway to induce tissue polarity is reliant on the asymmetrical distribution of the core PCP complexes at the cell surface. Elegant studies in the cochlea have demonstrated that *Vangl2*, *Fz3*, *Fz6* and *Dvl2* are asymmetrically distributed in hair cells and that this distribution is lost in the *Vangl2* mutant *looptail* [106–109]. Polarised localisation of Fz and Dvl has also been demonstrated in the converging neuro-ectoderm of the mouse [109]. Surprisingly this polarised localisation in the neuro-ectoderm was not perturbed in the absence of *Vangl2* function as seen in the hair cells of the cochlea [108]. Why there should be a differential requirement for *Vangl2* in these two converging tissues is unclear.

The Wnt/Ca²⁺ Pathway and CE

The Wnt/Ca²⁺ pathway participates in CE through PKC-mediated activation of the small GTPase Cdc42 [110], which promotes actin polymerisation and filopodial extension. In converging neuro-ectoderm and mesoderm Wnt5a-PKC stimulation of Cdc42 occurs independently of Dvl [110]. In parallel, Wnt5a-triggered Ca²⁺ release controls the ability of cells to adhere to each other during CE. Wnt/Ca²⁺ signalling also antagonises Wnt/ β -catenin signalling through inhibition of both Dvl and TCF [27, 34–36]. A similar mechanism occurs in zebrafish *pipetail* (*wnt5*) mutants where decreased Ca²⁺ leads to CE defects due to a lack of inhibition of Wnt/ β -catenin signalling [36].

A Role for Ryk in CE

A recent *Xenopus* study has uncovered a novel Ryk-dependent endocytosis mechanism regulating the levels of Fz7 on the cell surface, thereby modulating PCP pathway activity during neurulation [67]. Previous studies had shown that Wnt5a binding to Fz4 triggers receptor endocytosis through recruitment of the scaffold protein β -arrestin to Dvl [111]. The clathrin adaptor protein AP2 binds β -arrestin and also directly associates with Dvl and is required for Wnt5a-induced endocytosis via the clathrin-mediated pathway [26, 111]. In *Xenopus* the Dvl-AP2 interaction is required for Wnt4-triggered Fz4-Dvl internalisation, which leads to rapid degradation of the receptor in the lysosome [26]. Moreover, blocking the inter-

nalisation of the Fz-Dvl complex prevents CE of the mesoderm during gastrulation. Kim et al. [67] have now shown that Fz endocytosis also requires Ryk. In parallel with the Wnt11/Fz7 interaction, binding of Wnt11 to Ryk recruits β -arrestin to Dvl, which associates with the Ryk cytoplasmic domain, leading to β -arrestin-mediated clathrin-dependent endocytosis of Fz7 [67] (fig. 4). Both receptors colocalise at the cell surface and in early endosomes, and removal of either receptor from the complex prevents internalisation. Therefore, endocytosis is codependent on Fz and Ryk activation by Wnt11 and provides a mechanism by which cell surface levels of the receptor complexes can be tightly regulated. Such a mechanism would ensure a finely tuned spatiotemporal response to the concentration of Wnt in the local environment, allowing appropriate cell polarisation within the mesoderm and neuro-ectoderm during CE.

Ror Regulation of CE

A role for Ror2 in *Xenopus* CE and neurulation was first revealed by Hikasa et al. [112] who demonstrated that overexpression of Ror2 inhibited CE by blocking PCP pathway activity. Ror-dependent inhibition required the interaction between Wnt11 and Ror2 and the presence of Fz7, suggesting that a trimeric complex was formed (fig. 3). Co-expression of dominant-negative Cdc42 partially rescued the Ror overexpression phenotype, indicating that Cdc42 may integrate incoming signals from both the PCP and Ror pathways (fig. 3). More recently, Ror has also been shown to activate Rac [113], another downstream target of PCP signalling. Thus, the small GTPases may lie at the centre of a complex network of non-canonical signalling cascades that work together to establish mediolateral polarity driving CE.

Given that PCP pathway activity is dependent on Wnt5a/Ryk-mediated Fz endocytosis, one might predict that Wnt5a/Ror interactions might also trigger endocytosis. To date this has not been shown in converging mesoderm or neuro-ectoderm. However, a recent study in HeLa3 cells has provided evidence to support this contention [113]. Wnt5a/Fz2 activation of Rac was found to be dependent on Fz2 internalisation via clathrin-mediated endocytosis (fig. 3). Moreover, knockdown of *Ror1* and *Ror2* inhibited Fz2 endocytosis, suggesting that these receptors are required for internalisation. Due to the inability to demonstrate Ror colocalisation with Fz2 in early endosomes, it is not possible to conclude that Ror acts as a coreceptor to promote Fz2 capture in endocytosis. Instead, Ror may be playing an indirect role where it is not required to enter the endosomal compartment. Intrigu-

ingly, the requirement for Dvl and β -arrestin in this process (fig. 3) points to the involvement of Ryk in promoting Fz2 endocytosis.

A second pathway by which Ror regulates CE in the *Xenopus* mesoderm during gastrulation has been uncovered by Schambony and Wedlich [114]. In the absence of the paraxial protocadherin XPAPC or Wnt5a, cells are unable to align in the mediolateral direction in a coordinated fashion. Instead individual cells move randomly within the tissue leading to an inability to form an intercalating sheet of cells – that is they fail to converge. Pathway mapping revealed that Wnt5a/Ror2 interactions activated JNK through Cdc42 and phosphatidylinositol-3-kinase (PI3K), resulting in c-jun- and ATF2-mediated upregulation of XPAPC (fig. 3). XPAPC then activates RhoA while inhibiting Rac1, thereby modulating the PCP pathway [115]. Thus, Wnt5a/Ror2 promote the transcription of a key polarity gene. Moreover, Wnt11, the PCP pathway activator, was not able to replace Wnt5a in this signalling cascade, indicating that the Wnt5a/Ror2 pathway is distinct from the Wnt11/PCP pathway [114]. Whereas Wnt5a/Ror2 controls convergence by establishing coordinated mediolateral polarity throughout the sheet of cells, Wnt11/Fz establishes polarity at the level of the individual cell by promoting efficient lamellipodial protrusion. Inhibition of this pathway leads to loss of migration, preventing the tissue from extending perpendicularly to the mediolateral axis. Together these studies show that both pathways act in parallel to coordinate the complex set of cell-cell interactions required for effective CE of the mesoderm. Whether a similar pathway is important for CE in the neuro-ectoderm remains to be determined.

Wnt5a, a Master Regulator of Non-Canonical Wnt Signalling?

While the non-canonical Wnts, i.e. Wnt4 and Wnt11, drive the establishment of mediolateral polarity through the PCP pathway, Wnt5a is emerging as a central modulator of CE via its ability to activate the other non-canonical pathways. Downstream effectors of the Wnt/Ca²⁺ pathway activated upon Wnt5a/Fz interactions work in parallel with the PCP pathway to achieve efficient CE. Wnt5a/Ryk and Wnt5a/Ror signalling are also required for neural tube closure and mesodermal CE in *Xenopus* embryos. These receptors directly modulate PCP signalling by forming complexes with Fz receptors at the cell surface or by sharing or influencing the activity of downstream effectors. Thus, Wnt5a may act as a master regulator to coordinate this complex network of non-canonical effectors. Given the high conservation in non-canonical

signalling components across species, one would predict that these pathways would also be important drivers of neurulation in mammals. However, loss of Wnt5a, Ryk or Ror2 does not result in an open neural tube in the mouse, whereas CE defects are seen in the cochlea of *Wnt5a*^{-/-} and *Ror2*^{-/-} mice [116, 117]. Why this should be so is unclear. It should be noted, however, that in the mouse the neural plate consists of a single sheet of neuro-ectoderm cells. In contrast, the *Xenopus* neural plate comprises a superficial epithelium and a deeper mesenchyme-like layer, both of which need to become mediolaterally polarised. In addition, as the neural folds rise and fuse, these 2 layers must intercalate to form a single-layered neuro-epithelium. Therefore, neurulation in *Xenopus* is a more complex process and may require additional Wnt signalling pathways to regulate the central PCP pathway.

Non-Canonical Wnt Signalling in Axon Guidance

The correct navigation of axonal growth cones to their specific targets is essential for the establishment of the intricate network of axon projections found in the mature CNS. Directionality in axonal migration is determined by the response of the growth cone to the local environment through which it travels. Long-range guidance cues, secreted from intermediate or final targets, establish concentration gradients along the pathway of the exploring growth cone; in addition, membrane-bound or secreted short-range guidance cues are also employed to effect changes in the direction of growth cone migration along axon pathways and at specific choice points [118]. The intracellular signalling cascade initiated upon detection of the guidance cue by membrane-bound guidance receptors triggers dynamic rearrangements of the actin cytoskeleton within the growth cone. These rearrangements promote cycles of extension and retraction of filopodia and lamellipodia [119, 120], allowing the continual and spontaneous reassessment of the immediate environment by the growth cone. Given that non-canonical Wnt pathways promote polarised filopodial and lamellipodial activity by impinging directly on cytoskeletal dynamics, it is not surprising that they are major players in directing axonal navigation. Exploration of Wnt signalling in axon guidance has revealed essential roles for Fzs and Ryk in the formation of major axon tracts in vertebrates. To date there is no evidence that the Ror receptors participate in axon pathfinding in vertebrates but there are reports that the *Caenorhabditis elegans* orthologue CAM1 does have this function [121].

Here, we focus on the interplay between Fz- and Ryk-mediated non-canonical pathways in axon navigation along the major axon tracts in the mammalian nervous system, the corpus callosum in the telencephalon, and the corticospinal tract (CST) and ascending somatosensory tract within the spinal cord. Guidance of axons within these tracts involves numerous molecular guidance systems [122, 123] including chemo-attractive cues such as netrin 1 and chemorepulsive cues such as Slit. Non-canonical Wnt signalling via the PCP and Ryk-Wnt/Ca²⁺ pathways acts in parallel with these guidance systems to ensure error-free axon pathfinding. These examples emphasise the absolute requirement for the correct interpretation of Wnt gradients by the migrating growth cones.

Navigation in the Developing Spinal Cord – The PCP Pathway and Ascending Somatosensory Axons

In the embryonic rodent spinal cord dorsally situated commissural somatosensory neurons project axons ventrally following chemo-attractive guidance cues, including netrin 1 and sonic hedgehog and then cross the floor-plate [124–126]. On the contralateral side, these axons turn anteriorly and migrate to their targets in the brain. Anterior turning into the spinal cord is driven by a chemo-attractive Wnt4 anterior-high to posterior-low gradient detected by Fz3 on the postcrossing axons [127]. Wnt4/Fz3-mediated guidance involves the PI3K/atypical PKC (aPKC ζ) signalling pathway [128] (fig. 5). In vitro explant assays have shown that precrossing axons are unable to respond to Wnt4 [128]. However, upon overexpression of the catalytic subunit of PI3K, p110Y, these axons are attracted to Wnt4, demonstrating that PI3K is a key determinant of axonal responsiveness to the Wnt4 gradient. Upon Wnt/Fz binding, activation of PI3K leads to formation of the aPKC ζ /Par3/Par6 polarity complex, known to be downstream of Cdc42 and essential for the establishment of axodendritic polarity in neurons and apicobasal polarity in epithelia [for reviews, see 129–131] (fig. 5). The interrelationship between Wnt signalling (both canonical and non-canonical) and the apicobasal polarity machinery is complex, with many points of intersection, including Dvl and GSK3 β [132]. Apicobasal signalling components operate upstream to suppress the non-canonical pathway and augment canonical Wnt signalling [133, 134]. Conversely, Wnt/Fz can influence the localisation or transcription of polarity pathway components [135–137]. Interestingly, activation of the aPKC ζ /Par3/Par6 polarity complex has also been shown to lie downstream of Wnt5a in the induction of axonal differ-

entiation in cultured hippocampal neurons [138]. Thus, the PI3K/aPKC ζ pathway is emerging as a divergent branch of non-canonical Wnt signalling able to coordinate distinct Wnt signalling cascades governing neuronal polarity, axon growth and pathfinding.

The anterior turning of commissural somatosensory axons in the spinal cord also requires Wnt5a, which is also expressed in an anterior-high to posterior-low gradient. In this system Wnt5a acts as a chemo-attractive guidance cue activating the PCP pathway by triggering internalisation of Fz3/Dvl complexes followed by JNK activation [23] (fig. 5). The ability of Fz3 to enter the endocytic pathway is strictly regulated by its level of phosphorylation. A negative feedback loop is initiated whereby activated Dvl prevents endocytosis by increasing Fz3 phosphorylation (fig. 5). Vangl2, at the cell surface, opposes this activity by reducing Fz3 phosphorylation (fig. 5), promoting another round of endocytosis. Vangl2 protein is enriched at the tips of filopodia and in patches of membrane where filopodia emerge. Therefore, Vangl2 is perfectly positioned to cell-autonomously restrict Fz3-mediated actin dynamics to the membrane of the gradient detectors (i.e. the filopodia) of the growth cone [23].

Ryk, a Chemorepulsive Guidance Receptor

The initial evidence that Ryk was an axon guidance receptor came from the *Drosophila* orthologue Drl. The *Drosophila* embryonic CNS comprises a brain and a ventral nerve cord, a structure that has served as a useful model for the study of axon fasciculation and axon guidance. The ventral nerve cord consists of two longitudinal axon tracts that contact the brain anteriorly and extend approximately halfway down the embryo's longitudinal axis. Communication between the two longitudinal tracts occurs via the anterior and posterior commissures within each body segment. *Drl* is expressed selectively in neurons whose axons cross the ventral nerve cord via the anterior commissure. In wild-type animals, axons from these neurons never cross through the posterior commissure, indicating the presence of a chemorepulsive cue surrounding this tract. However, in *derailed* mutants, anterior axons frequently project through this region [59]. *Wnt5* is expressed in the posterior commissure and is the chemorepulsive ligand that prevents Drl-positive axons from entering [60] (fig. 5).

Ryk and the Descending CST

The CST is made up of axons originating from the pyramidal corticospinal projection neurons in layer 5 of the neocortex and projects along the length of the spinal cord.

CST motor axons project subcortically via the internal capsule and then posteriorly within the midbrain, cross at the pyramidal decussation in the ventral midbrain before projecting through the hindbrain and along the dorsal funiculus of the spinal cord [139]. Guidance of CST axons begins embryonically with axons first entering the spinal cord at postnatal day 0 (P0) in the mouse, with the remainder of CST axon guidance occurring postnatally.

Ryk-dependent chemorepulsion is required for the posteriorly directed migration of CST axons down the postnatal spinal cord in response to Wnt1 and Wnt5a gradients [65]. *Ryk* is expressed on layer 5/6 neurons at P0 and on CST axons at P5 in the mouse, while *Wnt1* and *Wnt5a* mRNAs are expressed along the trajectory of the descending CST axons in an anterior-high to posterior-low gradient [65]. In vitro explant assays demonstrated that both Wnt1 and Wnt5a were able to repel P0 CST axons and that anti-Ryk antibodies blocked this effect. Similar results were observed in vivo where anti-Ryk antibodies blocked the posterior pathfinding of CST axons [65]. These data demonstrate a role for Ryk-mediated guidance of CST axons via Wnt1 and Wnt5a chemorepulsive gradients (fig. 5). This effect was shown to be specific to Wnt1/Wnt5a/Ryk-mediated chemorepulsive guidance as Wnt4/Fz3 chemo-attraction was not affected by anti-Ryk antibodies [65].

In their study, Liu et al. [65] also found that secreted Fz-related protein 2 did not block Wnt/Ryk-dependent posteriorly directed repulsion down the spinal cord, providing further evidence that Ryk functions independently of Fzs. In the *Drosophila* embryo, Drl was also found to signal independently of the Fz receptors and did not require Dvl to promote Wnt5-dependent chemorepulsion [60] (fig. 5), providing an early example of an Fz-independent non-canonical Wnt signalling pathway. Conversely, Ryk has been shown to bind Fz8 and Dvl, suggesting that it can act as an Fz8 coreceptor [66] (fig. 4). Unfortunately, no direct evidence for binding between Fz8 and Ryk in the presence of Wnt1 was provided in this study. However, evidence was presented that Wnt/Ryk interactions result in the activation of the downstream target of the canonical Wnt pathway, the TCF transcription factor, suggesting that the Ryk signalling pathway may intersect with the canonical Wnt pathway upstream of TCF [66] (fig. 4). As this study was carried out in HEK293T cells, its relevance to Ryk-dependent chemorepulsive axon guidance is not clear.

Ryk and Navigation across the Corpus Callosum

Within the mammalian forebrain, the corpus callosum is the largest interhemispheric commissure, respon-

sible for information transfer between the two cerebral hemispheres. This facilitates the coordinated brain activity present in mammals allowing for the mastery of skills including the performance of complex motor and behavioural tasks and language and communication skills. More than 50 different human congenital syndromes, often associated with mental retardation and epilepsy, have been described in which dysgenesis or agenesis of the corpus callosum occurs (reviewed in Paul et al. [140]). The corpus callosum is made up of axons from pyramidal callosal projection neurons situated in neocortical layers 2/3 and 5. From mouse embryonic day 15 (E15), callosal axons project ventrally, turn medially within the intermediate zone, and then approach the callosal midline. The majority of axons cross the midline between E16 and E17 and from E18 onwards, project dorsally, making homotypic connections in the contralateral hemisphere [140, 141].

Ryk is expressed on callosal projection neurons within the cortex and their axons as they project across the corpus callosum and into the contralateral hemisphere [63]. In the absence of *Ryk*, callosal axons successfully cross the midline but fail to project into the contralateral hemisphere [63]. Thus, *Ryk*-mediated axon guidance is required specifically for postcrossing callosal axon guidance. *Wnt5a* is expressed in glial cell populations that surround the callosal midline and is responsible for *Ryk*-mediated chemorepulsion of postcrossing (E18) axons. Conversely, *Ryk* is not required for the guidance of pre-crossing (E16–E17) axons, but does promote their fasciculation [63].

It has generally been accepted that chemorepulsive guidance receptors (e.g. the semaphorin receptors, neuropilins/plexins) achieve their effect by causing the growth cone to stall once the chemorepellent gradient has been detected [142]. One intriguing puzzle highlighted by the chemorepulsive activity of *Ryk* in both the CST and corpus callosum is that the axons continue to grow through high levels of the Wnt chemorepellent. Studies from the Kalil laboratory have uncovered a dual activity for *Ryk* in callosal axon migration that provides an explanation for these apparently conflicting observations. Investigation of *Ryk* signalling in cortical neurons from P2 hamster sensorimotor cortex when cortical axons are entering the corpus callosum [143] revealed that 2 distinct but interconnected arms of the Wnt/ Ca^{2+} pathway are activated by *Wnt5a* (fig. 5). Upon bath application of recombinant *Wnt5a*, *Ryk* induced an increase in intracellular Ca^{2+} through the activation of cell surface transient receptor potential channels and from intracellular stores by

activation of IP3 receptors on the endoplasmic reticulum [64]. This led to the activation of CaMKII and phospholipase C and a subsequent increase in the rate of axon outgrowth [64] (fig. 5). In contrast, using an in vitro guidance assay, *Wnt5a*-dependent chemorepulsive guidance was shown to require *Ryk* and an unidentified Fz, extracellular Ca^{2+} entry through transient receptor potential channels and activation of CaMKII [64], but IP3-mediated Ca^{2+} release was not involved (fig. 5). A later study using cortical slice cultures confirmed these findings in callosal axons actively navigating across the cortical midline [78]. Taken together these results invoke a model in which *Ryk*-dependent outgrowth and *Ryk*/Fz-dependent guidance act through distinct arms of the Wnt/ Ca^{2+} pathway to coordinate axon growth and navigation at key decision points in developing axon tracts. As both guidance and growth require rearrangement of the cytoskeleton, it is not surprising that both arms intersect at CaMKII. However, pathway specificity is determined upstream by the involvement of IP3-mediated release of intracellular Ca^{2+} stores. It should be noted that no direct interaction between *Ryk* and Fz was demonstrated in this study. These findings lead to the novel hypotheses that Wnt/*Ryk* signalling is primarily responsible for axon outgrowth, whereas *Ryk* and Fz work together to promote chemorepulsive axon guidance.

Another intriguing observation from the study of Keeble et al. [63] was that despite the bilateral *Wnt5a* expression surrounding the callosal midline, wild-type callosal axons were not sensitive to the chemorepulsive activity of *Wnt5a* prior to crossing the midline. Lack of repulsive activity on the ipsilateral side, however, cannot be attributed to the absence of *Ryk* on ipsilateral axons as the *Ryk*^{-/-} axons exhibit a defasciculation phenotype as they approach the midline, indicating that *Ryk* is present on these axons. Therefore, the ability of cortical axons to respond to *Wnt5a* must be temporally regulated. Using an siRNA knockdown approach in hamster cortical slice cultures, Hutchins et al. [78] have shown that loss of *Ryk* decreases the growth rate of postcrossing callosal axons but has no effect on pre-crossing growth. Moreover, there was a concomitant decrease in the frequency of Ca^{2+} transients in postcrossing growth cones. In addition, transfection of a CaMKII inhibitor also disrupted growth and guidance in postcrossing but not pre-crossing axons. Together these observations implicate Ca^{2+} signalling, specifically dynamic Ca^{2+} fluxes, in the temporal regulation of growth cone responsiveness to *Wnt5a*. Since *Ryk* is present on both pre- and postcrossing axons, another regulatory mechanism must be in-

volved in modulating Ryk/Ca²⁺ activity. The obvious candidate is Fz3, which is known to be important for callosal axon growth and guidance [105, 106]. Fz3 is crucial for the formation of numerous axon tracts within the developing forebrain implicating Fz3 as a key cortical axon guidance receptor [105, 106]. In *Fz3*^{-/-} mice there is a reduction or complete absence of the corpus callosum, internal capsule (thalamocortical and corticothalamic projections), anterior commissure and cerebral peduncles (CST) [105, 106]. Therefore, one hypothesis would be that Fz3 suppresses Wnt5a/Ryk induction of Ca²⁺ fluxes in precrossing axons.

A complex interplay between Ryk and Fz5 has been revealed during the formation of retinotectal topographic maps in the chick [144]. Retinal ganglion cell (RGC) axons express *Ryk* in a ventral-high to dorsal-low gradient across the retina. In contrast, *Fz5* expression is equivalent on ventral and dorsal RGC axons. In the tectum *Wnt3* is expressed in a medial-high to lateral-low gradient. On reaching the tectum, interstitial axon branches projecting from ventral RGC axons are repelled in a Ryk-dependent manner from the medial tectal domain expressing high levels of *Wnt3*, whereas Ryk activity is absent on dorsal RGC axonal branches in the lateral domain. Concomitantly, branches from dorsal RGC axons are guided via Wnt3/Fz5 chemo-attractive activity towards the lateral tectum. Therefore, a single Wnt gradient (Wnt3) directs Fz-dependent attraction of dorsal branches and Ryk-dependent repulsion of ventral branches.

Conclusions

In this review we have focussed on the interplay between the non-canonical Wnt receptors in two fundamental morphological processes essential to the viability of the embryo – CE and axon pathfinding in the embryonic CNS. However, the basic principles of Wnt signalling emphasised here are also relevant to the morphogenesis of most embryonic tissues. From the many studies discussed in this review, it is clear that the non-canonical pathways (Fz-, Ror-, Ryk-dependent pathways) do not operate in isolation. Instead, each pathway cooperates in the remodelling of the cytoskeleton, leading to the establishment of cell polarity, an essential requirement for cell division and directed cell and axon migration. All non-canonical pathways also interact with the β -catenin pathway, depending on the biological context. Throughout this review we have highlighted the complex network of pathway crosstalk be-

tween Wnt signalling pathways. Dvl and Vangl have emerged as key nodes in the network. Dvl interacts with all the Wnt receptors (Fz, Ror, Ryk) at the membrane and is therefore perfectly positioned to regulate crosstalk between pathways. Vangl2 is also a central player in Fz- and Ror-dependent polarity pathways, although as yet there is no evidence that it is involved in Ryk signalling. Wnt5a is also emerging as a master regulator of non-canonical Wnt signalling due to its ability to bind all receptor families.

However, many basic questions remain to be answered. It is clear that we do not understand the nature of the interactions between Fzs, Ryk and Ror. In some experimental paradigms, especially in *in vivo* situations, these receptors have been shown to signal independently, whereas in biochemical studies, often in generic cell lines, coreceptor complexes have been identified. One explanation may be that receptor interactions are very dynamic *in vivo*, making it difficult to capture receptor complexes. Moreover, we have no insight into the rules governing receptor complex formation and disassembly. In addition, interactions with Dvl, Vang and other PCP core proteins have yet to be fully explored in the context of Ryk and Ror. These potential interactions may determine the make-up of the receptor complexes at the cell surface. A further puzzle is how the responses triggered by different Wnt receptor interactions are integrated at the network level, especially when the local environment contains multiple Wnts. The answer may lie in an emerging paradigm in which the most effective receptor signalling occurs within endocytic vesicles. Evidence is now accumulating that Wnt signalling requires Fz endocytosis. This process is dependent on other Wnt receptors such as Ryk, which recruits components of the endocytic machinery to Fz [67]. Wnt-driven endocytosis ensures that cell surface levels of receptor complexes are tightly regulated and provides a mechanism with which to finely tune spatiotemporal responses to the concentration of Wnt in the local environment. It is also possible that different Wnts or receptor complexes trigger internalisation through distinct endocytic compartments. This would allow divergent signalling cascades to occur in parallel.

One of the most intriguing questions, especially with respect to CNS development, is how the extracellular Wnt gradient is translated into the establishment of correct cellular or growth cone polarity and directed migration. We have only recently started to comprehend the behaviour of pathway components at the spatiotemporal level. Clues to this fundamental question in the context of planar cell polarity have recently been uncovered in the developing limb [21]. In this system, the local Wnt concen-

tration at a given point in the gradient determines the extent of Vangl2 phosphorylation within the Wnt/Ror/Vangl2 complex. The gradient of Vangl2 phosphorylation then translates into a corresponding gradient of Vangl2 activity, asymmetric distribution and polarity induction. This study therefore provides insight into the cell-autonomous mechanism by which the current position of the cell within the Wnt gradient modulates the intensity of Wnt signal transduction. Vangl2 also appears to be a key player in the detection of Wnt gradients in the navigating growth cone. The observation that Vangl2 restricts Fz3 signal transduction to the tips of mature and nascent filopodia in the growth cone [23] provides an important insight into how the spatial response to Wnt in the local environment is generated. Again we have no idea how the interplay between Fz, Ryk, Ror and other core PCP proteins influences the interpretation of Wnt gradients. Nor do we understand how responses to differ-

ent Wnt gradients, or gradients of other guidance or polarity cues (e.g. netrins, sonic hedgehog), are integrated. Intriguingly, distinct endocytic compartments may again be the key to sorting out these multiple polarising inputs.

Despite 25 years of intensive study, there remains much to discover about the intricacies of Wnt signalling. However, recent advances highlighted in this review have set the scene for a new wave of investigations that should shed light onto the most fundamental and enduring questions in Wnt signal transduction.

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Note Added in Proof

Further evidence of Ryk-mediated activation of the Wnt/ β -catenin pathway has recently been shown. This is dependent on interaction of Ryk with the E3 ubiquitin ligase, Mindbomb 1, downstream of Wnt3a activation of Ryk.

Reference

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