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Frizzled/RYK mediated signalling in axon guidance

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Successful axon navigation depends on the competence of the axon growing tip to receive and integrate information provided by multiple, spatially organised molecular cues arranged along the axon trajectory. Several recent studies have raised the intriguing possibility that 'morphogen' signalling, known to give cell-specific positional information during tissue patterning, is later used to provide part of this guidance information to the growth cone. How general this strategy is has now become apparent with new compelling evidence from the Wnt field, which shows that new ligand-receptor interactions underlie the evolutionary conserved role of the Wnt signalling cascade in the initiation, elongation and turning behaviour of the growth cone.

Introduction

The progressive specification of different tissues and organs occurring during early embryonic development is coordinated by a limited number of cell signalling molecules (known as morphogens) that belong to the hedgehog (Hh), Transforming Growth Factor-β (Tgfβ), Fibroblast Growth Factor (Fgf) and wingless (Wnt) families of secreted ligands. These ligands bind to specific receptors and activate particular intracellular cascades that ultimately control the transcription of target genes, thus influencing cell fate and cell behaviour (for a review, see Tabata and Takei, 2004). The function of these signalling pathways, however, is not limited to cell specification, as there is increasing evidence that they are also necessary to control cell proliferation, cell survival and cell movement, not only in embryogenesis, but also in postnatal life during normal and pathological conditions (Bovolenta and Marti, 2005). This is not particularly surprising as all of these processes depend heavily on changes in gene expression. Axon guidance, by contrast, has been considered for many years to depend on the competence of the growth cone to interpret information provided by different ligand-receptor signalling systems [i.e. Ephrins/Eph, Netrins/DCC/Unc5, Slits/Robo, Semaphorins/Plexin/Neuropilin (reviewed by Dickson, 2002)] and to rely also on local changes of a few intracellular mediators, like cyclic nucleotides (cAMP and cGMP) and Ca^{2+} ions, and on phosphorylation-dependent changes of certain cytoskeletal proteins (reviewed by Song and Poo, 1999). This view has been recently expanded with the discovery that local translation (reviewed by van Horck et al., 2004), particularly of mRNA of cytoskeletal proteins (Piper et al., 2006), as well as protein degradation (reviewed by van Horck et al., 2004) are important events for growth cone steering in response to guidance cues. Moreover, the local internalisation of molecules known for their activity as transcription factors also influences growth cone movement via the phosphorylation of translational regulatory proteins (Brunet et al., 2005). In this changing scenario, the demonstration that morphogen signalling is used at late stages of embryonic development to control both axon growth and directionality (reviewed by Bovolenta, 2005; Charron and Tessier-Lavigne, 2005) offers an additional exciting perspective on the mechanisms that contribute to the establishment of brain connections. Thus, in vertebrates, Shh, Bmp and Wnt signalling has been implicated in the guidance of retina ganglion cell (RGC) axons and of commissural axons of the spinal cord (Augsburger et al., 1999; Trousse et al., 2001; Butler and Dodd, 2003; Charron et al., 2003; Lyuksyutova et al., 2003; Bourikas et al., 2005).

These studies have raised an obvious question: is morphogen signalling at the growth cone a general strategy for axon guidance? Very recent work in *Drosophila*, *C. elegans* and vertebrates demonstrates that Wnt-mediated signalling at the growth cone is indeed a general and evolutionary conserved mechanism of axon guidance. Here, we review this new evidence and focus on new ligand-receptor interactions that underlie the functions of the already complex Wnt signalling pathways in axon guidance. Recent discoveries show that Wnt-induced axon guidance is mediated by at least two different families of receptors: the 'classical' Frizzled receptors (Fz) (Lyuksyutova et al., 2003; Schmitt et al., 2005; Sato et al., 2006; Pan et al., 2006; Hilliard and Bargmann, 2006; Prasad and Clark, 2006) and the newly identified atypical Tyr-kinase receptors, Derailed/Ryk (Related to Tyrosine Kinase) (Yoshikawa et al., 2003; Lu et al., 2004; Liu et al., 2005; Schmitt et al., 2005). Whereas Fz receptors appear to mediate mainly attractive responses of the growth cones to Wnts (Lyuksyutova et al., 2003; Schmitt et al., 2005; Sato et al., 2006), Derailed/Ryk mediates the repulsive ones (Yoshikawa et al., 2003; Liu et al., 2005; Schmitt et al., 2005). Furthermore, Secreted Frizzled Related Protein 1 (Sfrp1), an extracellular modulator of Wnt signalling, also provides Wnt-independent guidance information by binding to Fz2 (Rodriguez et al., 2005). Thus, the same family of ligands (Wnt) can bind to different receptor families (Fz, Ryk), and different ligands (Wnt, Sfrp) can bind to the same family of receptors (Fz), all providing information to the growing axons.

The Wnt signalling pathways

WNTs comprise a large family of lipid-modified, secreted glycoproteins that are involved in cell-to-cell communication during the embryonic development of different tissues, including the nervous system. By binding to the Fz receptors (seven-pass transmembrane proteins with characteristics of G-protein couple receptors), Wnts activate at least three different signalling pathways: the canonical or Wnt/β-catenin pathway, the planar cell polarity (PCP) pathway and the Wnt/calcium pathway. In addition, as we discuss later, Wnt can bind to Ryk/Derailed, which, in turn, can interact with Fz (Lu et al., 2004). There are many excellent and up-to-date reviews describing in detail these pathways (Logan and Nusse, 2004; Ciani and Salinas, 2005; Kohn and Moon, 2005; Cadigan and Liu, 2006; Willert and Jones, 2006), thus, we will just briefly describe their main features here.

A common step in the activation of all these three cascades is the Fz-mediated recruitment of Dishevelled (Dvl), a cytoplasmic scaffold protein with three conserved domains that mediates its interactions with different proteins (reviewed by Wallingford and

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Habas, 2005). In the canonical pathway, Dvl activation results in the inhibition of Glycogen Synthase Kinase 3β (Gsk3β), a serine/threonine kinase that has multiple substrates, including β-catenin and microtubule-associated proteins (MAPs). In the absence of a Wnt stimulus, Gsk3β forms a complex with the scaffold protein Axin and the tumor suppressor protein adenomatous polyposis coli (Apc), and recruits β-catenin, targeting it for degradation. Wnt/Fz interaction leads to the disintegration of the Gsk3β/Axin/Apc complex, with the consequent accumulation of β-catenin in the cytoplasm and its translocation to the nucleus, where, in association with the T-cell factor/lymphoid-enhancer factor (Tcf/Lef), it activates the transcription of target genes. At the cell membrane, the activation of this pathway also involves the interaction of Fz receptors with Arrow/low-density lipoprotein receptor-related protein 5 (Lrp5) and Lrp6, which function as co-receptors (Fig. 1A).

The alternative Wnt/Ca^{2+} and PCP pathways are less well understood, but both are linked to the regulation of cell movement, including the coordinated orientation of cells within an epithelium, the orientation of stereocilia in the mammalian inner ear and the convergent extension movements that occur during gastrulation (Veeman et al., 2003). In the PCP pathway (Fig. 1B), a Wnt-Fz interaction leads to the Dvl-mediated activation of the small GTPases RhoA and Rok (Rho kinase), or of Rac and c-Jun amino (N)-terminal kinase (JNK), which in turn affects the dynamics of the cytoskeleton (Fig. 1B). The Wnt/Ca^{2+} pathway (Fig. 1C) involves G proteins, phospholipase C (PLC), phosphodiesterase (PDE) and the activation of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), calcineurin and the nuclear factor of activated T cells (NF-AT).

Biochemical and genetic studies support the existence of antagonistic crosstalk between the Wnt canonical and non-canonical pathway in different contexts (Kohn and Moon, 2005). Furthermore, the activity of the three Wnt signalling cascades is potentially modulated extracellularly by different Wnt antagonists, including Wif1 (Wnt inhibitory factor 1), Cerberus, and members of the Dickkopf and Sfrp families. Although Dickkopf proteins interfere with Wnt activity by binding to Lrp5/Lrp6, thus antagonising canonical signalling, Wif1, Cerberus and Sfrps can interact directly with Wnt proteins (reviewed by Kawano and Kypta, 2003), and, thus, can potentially interfere with all three signalling pathways. Notably, Wif1 shares structural similarity with the Ryk receptor, whereas Sfrps are modular proteins that fold into two independent domains (Chong et al., 2002), one of which is structurally related to the Cysteine Rich Domain (CRD) of Fz receptors. The other Sfrp domain, known as Netrin module (NTR), is characterised by a set of conserved disulfide bridges and by segments of hydrophobic residues that have been identified in several other proteins, including Netrin 1 (Banyai and Patthy, 1999). Possibly as a result of this modular structure, Sfrps appear to have Wnt-independent mechanisms of action (Kawano and Kypta, 2003), including a direct interaction with Fz (Bafico et al., 1999; Rodriguez et al., 2005) and being able to interfere with Bmp signalling (Lee et al., 2006b; Muraoka et al., 2006).

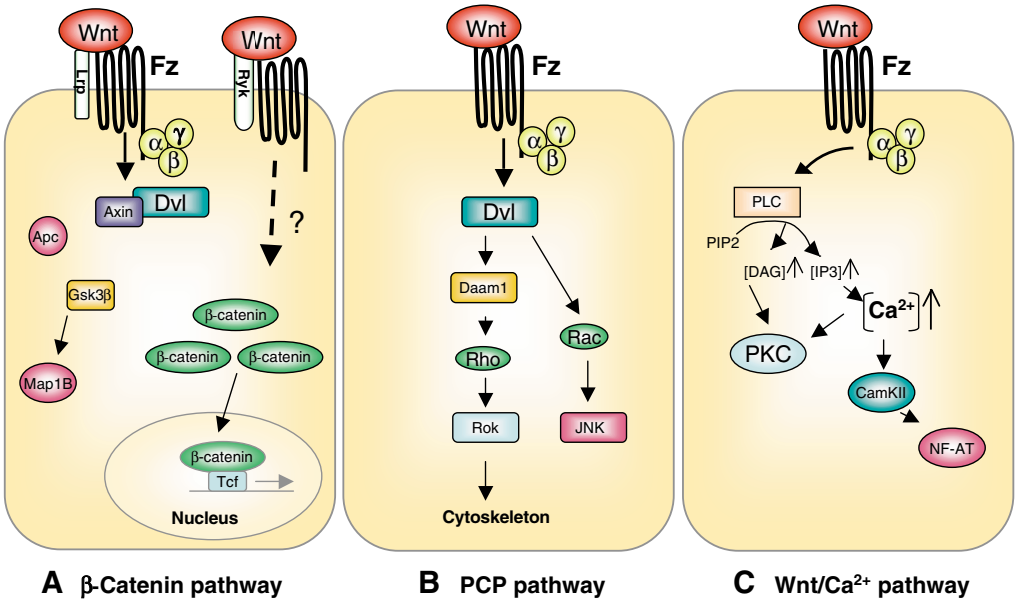

Fig. 1. The three Wnt signalling pathways and their main components. (**A**) The canonical β-catenin pathway is initiated by Wnt binding to Fz/Lrp, which activates Dvl, preventing β-catenin phosphorylation through the Apc-Axin-Gsk3β complex. β-Catenin accumulates in the cytoplasm and translocates to the nucleus, where it activates transcription in association with Tcf. Downstream of Gsk3β, a divergent pathway controls Map1B phosphorylation. The proposed Wnt-Ryk-Fz complex may function through the same pathway. (**B**) In the planar cell polarity (PCP) pathway, the binding of Wnt to Fz activates Dvl, which then signals either through Daam1, activating the small Rho GTPase, or through the small Rac GTPase, which in turn leads to JNK activation. Both GTPases then induce changes in the cytoskeleton. (**C**) In the the Wnt/calcium pathway, Wnt-Fz binding triggers PLC activation, which then hydrolyzes PIP2, generating IP3 and DAG. IP3 leads to the release of intracellular calcium, which activates the calcium/calmodulin dependent protein kinase II (CamKII) and PKC. α, β, γ, G-protein subunits; Apc, adenomatous polyposis coli; CamKII, calcium/calmodulin-dependent protein kinase II; Daam1, dishevelled associated activator of morphogenesis 1; DAG, diacylglycerol; Dvl, dishevelled; Fz, Frizzled receptor; Gsk3β, glycogen synthetase kinase 3; IP3, inositol 1,4,5-trisphosphate receptor; JNK, jun kinase; Lrp, low-density lipoprotein receptor-related protein; Map1B, microtubule-associated protein 1B; NF-AT, nuclear factor of activated T cells; PIP2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; Rok, Rho kinase; Ryk, receptor-like tyrosine kinase; Tcf, T-cell factor.

Thus far, it is unclear whether all, none, one or just part of these three pathways are used to transduce Fz- and Ryk-mediated signalling at the growth cone. However, there are clear indications that, independently of Tcf-mediated transcription, Wnt signalling can change microtubule organisation and stability through a mechanism that involves the binding of Dvl to the microtubules and the inhibition of Gsk3β (Krylova et al., 2000; Ciani et al., 2004). Furthermore, the Wnt/Ca^{2+} pathway has interesting similarities with the pathway triggered by the activation of other G-protein coupled receptors (GPCR), which has recently been shown to be involved in axon guidance (Xiang et al., 2002), suggesting that different mechanisms of action could be expected.

A conserved role for Wnt signalling in axon guidance

The first evidence of the involvement of the Wnt pathway in axon guidance came from studies of the central nerve cord of *Drosophila* (Fradkin et al., 1995). This system has also been instrumental in identifying Derailed/Ryk as the receptor that mediates Wnt-induced growth cone repulsion (Yoshikawa et al., 2003), thus defining a new ligand-receptor signalling system for the guidance of axons.

In each segment of the *Drosophila* ventral nerve cord, commissural axons cross the midline, selecting either the anterior (AC) or posterior (PC) commissural tract. Wnt5, also known as Dwnt3, is strongly expressed in cells positioned ventrally to the PC (Fig. 2A). If this localised production of Wnt5 is disrupted by ubiquitous overexpression, commissural tracts do not form properly (Fradkin et al., 1995). *Derailed* (*drl*), an atypical receptor tyrosine kinase initially unrelated to Wnt activity, is normally expressed in the axons of AC projecting neurons (Fig. 2A). In *drl* null mutants, AC axons abnormally cross into the PC, whereas the misexpression of *drl* in PC neurons conversely forces their axons through the AC (Fig. 2B) (Callahan et al., 1995; Bonkowsky et al., 1999). A genetic screen for mutations that suppress this last phenotype brought the observations of these two studies together, identifying Wnt5 as a ligand for Drl and as an essential cue for the proper projection of AC axons (Fig. 2C) (Yoshikawa et al., 2003). More precisely, Wnt5 repels AC growth cones that express Drl. *drl* does not show genetic interaction with *Dfz1* (*fz* – FlyBase) and *Dfz2* (*fz2* – FlyBase), suggesting that Drl-mediated, Wnt5-induced repulsion is independent of Fz. Rather, Wnt5 signalling seems to require the intracellular domain of Drl, because misexpression of its truncated version in PC cells fails to cause PC axons to cross through the AC (Yoshikawa et al., 2003). Furthermore, Wnt5 might have additional functions during the formation of the *Drosophila* nerve cord. In *wnt5* null mutants, AC and PC do not separate properly, and 'fuzzy' commissures are formed. Even more intriguingly, Wnt5 expression in AC neurons might be negatively controlled by Drl itself, as there is a twofold increase in Wnt5 protein levels in *drl* mutants (Fradkin et al., 2004).

In an interesting parallel, the interaction of Ryk, the vertebrate homologue of Drl, with Wnt ligand provides repulsive guidance information to axons as they descend in the vertebrate spinal cord (Fig. 2D) (Liu et al., 2005), a structure that is the possible vertebrate equivalent of the *Drosophila* nerve cord. Two Wnt molecules, Wnt1 and Wnt5a, are expressed in an anterior-high, posterior-low gradient

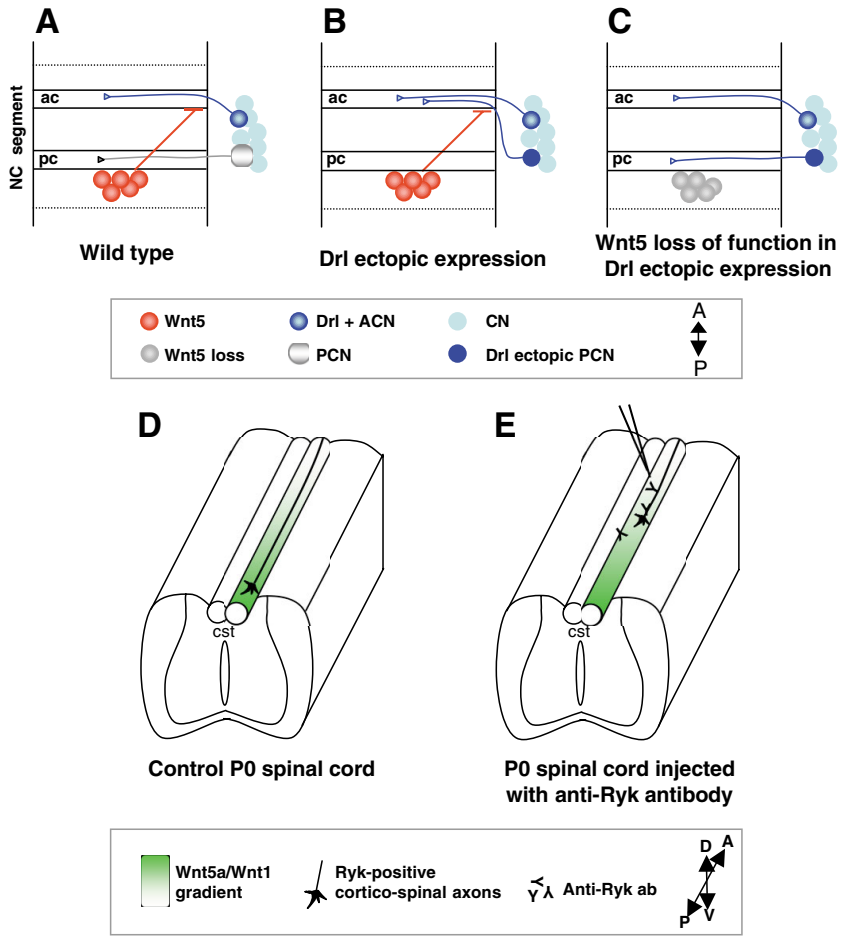

Fig. 2. Wnt-Drl/Ryk mediates axon repulsion in the *Drosophila* nerve cord and vertebrate cortico-spinal tract. (**A-C**) Schematics of a *Drosophila* nerve cord (NC) segment, illustrating anterior (ac) and posterior (pc) commissures. The axons of anterior commissural neurons (ACN) express *derailed* (*drl*; shown in blue) and are repelled by Wnt5 derived from midline cells (red circles). (A) In wild-type *Drosophila*, axons of posterior commissural neurons (PCN) do not express *drl* and enter the pc. (B) The misexpression of *drl* in PCN forces their axons through the ac. (C) *Wnt5* loss of function (grey circles) suppresses the phenotype induced by *drl* ectopic expression, demonstrating that Wnt5, through Drl, is an essential cue for proper commissural axon projection. (**D**) Schematic of the cortico-spinal tracts (cst) in the dorsal portion of the postnatal mouse spinal cord. In vertebrates, a Ryk-mediated repulsive gradient of Wnt proteins (Wnt1 or Wnt5a) guides cortico-spinal axons in the AP direction. (**E**) Injections of anti-Ryk antibodies into the cervical spinal cord stall cortico-spinal axons posterior to the site of injection, with a consequent shortening of the cst. D, dorsal; V, ventral.

along the length of the mouse dorsal spinal cord, coinciding with the trajectory of the descending motor cortico-spinal axons, which express Ryk on their surface. In collagen gel experiments, Wnt1- or Wnt5a-transfected cells (but not cells transfected with other Wnts) strongly inhibit neurite outgrowth from neonatal, but not embryonic motor cortex explants, suggesting that cortico-motor axons become receptive to Wnt activity at the time when they normally reach the spinal cord. This effect is mimicked by co-cultures with anterior, but less so with posterior, dorsal spinal cord explants, and is abolished by antibodies against Ryk, indicating that Ryk mediates the effect of a graded diffusible chemo-repellent(s) (probably Wnt1 and/or Wnt5) that forces the growth of cortico-spinal axons down the spinal cord (Liu et al., 2005). In agreement with this interpretation, intrathecal injections of anti-Ryk antibodies reduce the growth of cortico-spinal axons posterior to the site of injections (Fig. 2E). Biochemical studies have indirectly excluded Fz participation in this activity (Liu et al., 2005; Schmitt et al., 2005).

Thus, in both vertebrates and invertebrates, a Fz-independent, Wnt-Ryk/Drl interaction functions as a negative regulator of axon growth. However, in the case of the vertebrate cortico-spinal tract, the mechanism appears to be counterintuitive, as one would expect axon growth to stall when they encounter a strong repellent activity. A strictly time-regulated expression of Ryk at the axon surface, as indicated by the differential sensibility to Wnts observed in embryonic and postnatal cortical explants (Liu et al., 2005), might explain this apparent paradox. Alternatively, counteracting attractive forces emanating from the caudal spinal cord could counterbalance the repellent activity, as has been suggested for the possible cooperation of the opposite Wnt4 and Shh gradients in the rostral growth of commissural axons (Stoeckli, 2006). On commissural axons, however, Wnt exerts an attractive force, offering the first vertebrate example of a positive Wnt-induced guidance activity, probably mediated by a Fz receptor (Fig. 3A) (Luksyutova et al., 2003).

The floor plate is an essential intermediate target for spinal cord commissural axons. In the absence of the floor plate, commissural axons reach the ventral spinal cord but fail to turn rostrally, growing towards the opposite side (Bovolenta and Dodd, 1991). A search for molecules that could favour the rostral growth of commissural axons has identified several Wnts (Wnt1, Wnt4, Wnt5a, Wnt6, Wnt7b) as being the best candidates for this activity (Luksyutova et al., 2003). Among these, only Wnt4 fulfils the necessary physiological condition for this activity: an anterior-high to posterior-low graded distribution at the floor plate, a prerequisite previously determined with a series of clever in vitro tissue recombination experiments (Fig. 3B,C) (Luksyutova et al., 2003). Interestingly, commissural axons in *Fz3* null mouse embryos grow normally towards the midline but, after crossing the floor plate, project randomly along the anteroposterior (AP) axis, suggesting that Wnt4 activity might be mediated by the Fz3 receptor (Luksyutova et al., 2003).

A low concentration of Wnt, specifically Wnt3a, provides similar Fz-mediated, positive guidance information to chick dorsal (but not ventral) retina ganglion cell (RGC) axons that project to the tectum. In addition, high concentrations of Wnt3a strongly inhibit dorsal, as well as ventral, RGC axon outgrowth (Schmitt et al., 2005). The physiological significance of this observation resides in the graded distribution of Wnt3a, which decreases in a medio(dorso)-lateral(ventral) direction in the chick optic tectum and in the mouse superior colliculus. Normally retinal projections to the tectum (or superior colliculus) are spatially organised: ventral retinal axons project to the medial tectum, whereas dorsal axons terminate in the lateral tectum. This organisation is in part dictated by an attractive force provided by the EphB receptor tyrosine kinases and their EphrinB ligands, well recognised axon guidance cues with a crucial role in the establishment of topographic projections in the visual system (McLaughlin and O'Leary, 2005). However, modelling studies have suggested the need for a repellent gradient in the same direction, as a possible mechanism to achieve precise topography (Hindges et al., 2002). With a series of in vitro and in ovo experiments (Fig. 4A), Schmitt and colleagues (Schmitt et al., 2005) reached the conclusion that Wnt3a could be this additional cue, acting through two different receptors: Fz, which is uniformly distributed in RGC throughout the retina; and Ryk, which strongly localises to the ventral RGC. Thus, Ryk-positive, ventral RGC axons are repelled by Wnt3a, whereas Fz receptors mediate the attractive response of dorsal RGC axons to low concentrations of Wnt3a. In

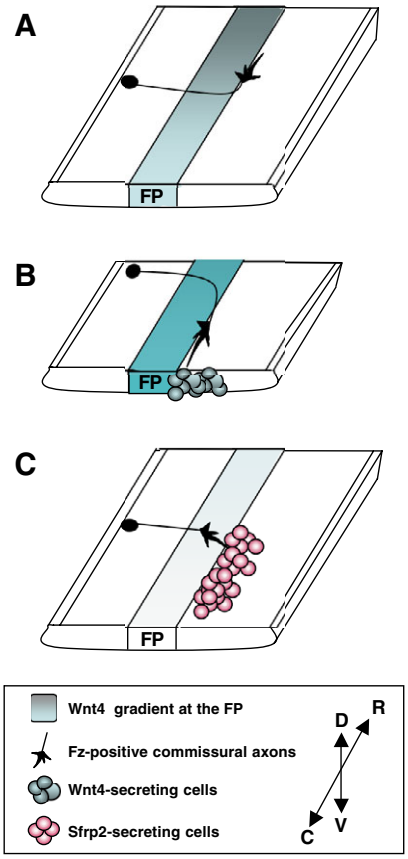

Fig. 3. Wnt-Fz interaction mediates the rostral growth of post-crossing commissural axons in the vertebrate spinal cord. (**A-C**) Schematics of the vertebrate embryonic spinal cord in the open book configuration under (A) normal or (B,C) experimental conditions. (A) Commissural growth cones turn rostrally after crossing the midline, attracted by Wnt4 expressed in the floor plate in an anterior-high to posterior-low gradient (graded grey shading). (B) The addition of Wnt4-expressing cells at the caudal end of short spinal cord explants cultured in the open book configuration disrupts the Wnt gradient at the floor plate (solid blue colour) and reorients the axons toward the posterior spinal cord. (C) The addition of cells transfected with the Wnt inhibitor Sfrp2 in the proximity of the floor plate inactivates Wnt function (pale blue), and commissural axons stall at the floor plate or turn randomly. C, caudal; D, dorsal; FP, floor plate; R, rostral; V, ventral.

this light, retinotopic organisation in the mediolateral axis, as well as growth of axon branches, would result from the competition between a repulsive Ryk-mediated signal and an attractive Fz-mediated signal derived from different concentrations of the same ligand, Wnt3a (Fig. 4A) (Schmitt et al., 2005).

Part of this signalling system is conserved in invertebrates to regulate the dorsoventral (DV) organisation of *Drosophila* visual projections to the first optic ganglion, the lamina (Sato et al., 2006). In the fly, retina R1-R6 photoreceptors project to the lamina with a precise organisation along the AP and DV axes. *Dwnt4* (Wnt4 – FlyBase) is expressed asymmetrically only in the ventral lamina, where ventral retinal axons terminate (Fig. 4B). If this asymmetry is disrupted by either *Dwnt4* loss-of-function or by ectopic expression of *Dwnt4* in the dorsal lamina, ventral axons mis-project to the dorsal lamina (Fig. 4B), indicating that Dwnt4 normally acts as a cue for ventrally projecting photoreceptors (Sato et al., 2006). The inhibition of non-canonical pathway components, including Dfz2, Dvl and hemipterous, the *Drosophila* homologue of JNK, strongly interferes with ventral photoreceptor axon projections, indicating that this pathway is likely to mediate *Dwnt4* activity. Intriguingly, the specificity of the retinal projections to the lamina appears to be reinforced by the activity of *iroquois* (*iro*), a homeobox gene that confers dorsal identity to the retina. In *iro* mutants, dorsal axons accumulate Dwnt4 and mis-project to the ventral lamina. This phenotype is suppressed in *iro;Dfz2* double mutants, suggesting that *iro* may attenuate the competence of Dfz2 to respond to Dwnt4 (Sato et al., 2006).

The *C. elegans* field has also recently added to the evidence that the Wnt-Fz signalling system directs axon outgrowth (Pan et al., 2006; Hilliard and Bargmann, 2006; Prasad and Clark, 2006). In addition, these studies have shown that this signalling also influences AP neuronal polarity and, thus, the characteristics of neuronal processes (Hilliard and Bargmann, 2006; Prasad and Clark, 2006).

In the *C. elegans* embryo, *egl-20(wnt)* is expressed in the tail (Fig. 5A), behind the anterior (AVM) and posterior (PVM) ventral mechanosensory neurons. These neurons grow a single process that extends ventrally, enters the nerve cord and then turns anteriorly (Fig. 5B). In embryos mutants for two Wnt genes (*cwn-1;egl-20)* or two frizzled receptors (*mig-1;mom-5)*, AVM and PVM axons join the ventral nerve cord as normal but then present variable pathfinding defects: they stall, turn in the posterior direction or bifurcate, sending anterior and posterior processes (Fig. 5C), indicating that Wnt/Fz are required to direct growth cones in the anterior direction (Pan et al., 2006; Prasad and Clark, 2006). Ectopic overexpression of *egl-20(wnt)* in the posterior region of *cwn-1;egl-20* double-mutant animals significantly rescues the mutant phenotype, whereas a similar ectopic expression in the anterior position enhances the pathfinding defects. The phenotype induced

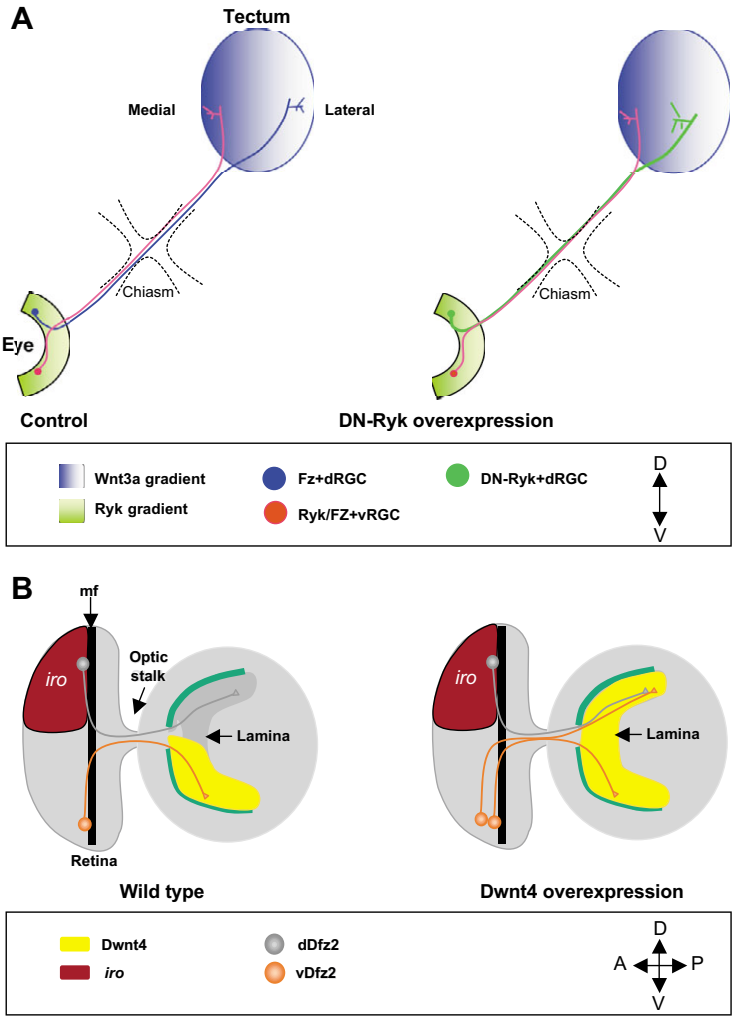

Fig. 4. The retinotopic organisation of retinal projections requires Wnt activity in both vertebrates and invertebrates. (**A**) Schematic of the chick retinotectal projections under normal (left) or experimental conditions (right). In the chick, ventral retinal axons expressing both Ryk and Fz (red) project to the medial tectum, whereas dorsal axons expressing only Fz (blue) terminate in the lateral tectum. This organisation is achieved in part through the graded medial-high distribution (graded blue staining) of Wnt3a. Fz-positive dorsal retinal cell axons are attracted by low doses of Wnt3a. Misexpression of a dominant-negative (DN)-Ryk form in the dorsal RGCs (green staining) induces a medial shift of their terminal arborisations. (**B**) Schematic of the *Drosophila* visual system. Photoreceptor cells differentiate behind the morphogenetic furrow (mf) and project in an organised manner to the lamina after crossing though the optic stalk: ventral axons expressing Dfz2 (orange) are attracted by Dwnt4 (yellow) expressed in the ventral lamina. *Iro* expression (red) in the dorsal retina attenuates the competence of Dfz2-positive dorsal photoreceptors (grey) to respond to Dwnt4. Ectopic expression of *Dwnt4* in the dorsal lamina attracts ventral axons to the dorsal lamina.

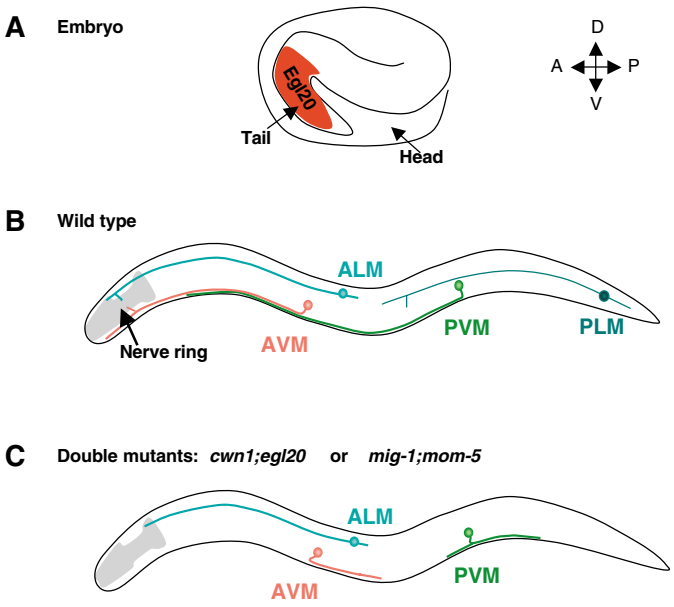

Fig. 5. Wnt-Fz signalling controls anterior-directed projections of mechanosensory neurons in *C. elegans*. (**A**) In the 2-fold stage *C. elegans* embryo, *egl-20(wnt)* is expressed (red shading) in the tail behind anterior (AVM) and posterior (PVM) ventral mechanosensory neurons. (**B**) The position and projections of the different mechanosensory neurons of the worm (only one of the ALM and PLM bilateral pairs is shown; PVM and AVM are positioned on opposite hemi-sides of the embryo). (**C**) In double Wnt (*cwn-1;egl-20*) mutants or double Fz (*mig-1; mom-5*) mutants, the anterior projection of the AVM and PVM axons is impaired. Similarly, ALM axons do not branch in the nerve ring.

by the anterior misexpression of *egl-20(wnt)* is reduced in Fz double mutants (*mig-1*;*mom-5*), whereas posterior ectopic expression of *egl-20(wnt)* does not rescue the Fz double mutant phenotype.

Two additional types of mechanosensory neurons, the anterior lateral (ALM) and posterior lateral (PLM), are polarised cells that have a short unbranched posterior process and a long anterior one that branches near its distal end (Fig. 5B). In ALM neurons, the branches that terminate in the anterior nerve ring are either not formed or re-routed in a posterior direction in different Wnt double mutants (*cwn-1*;*egl-20* and *cwn-1*;*cwn-2*) (Hilliard and Bargmann, 2006; Prasad and Clark, 2006), or after ectopic expression of *egl-20* anterior to the ring (Pan et al., 2006). Furthermore, simultaneous inactivation of either *cwn-1;egl-20* or *cwn-1;cwn-2* interferes with the proper establishment of ALM anteroposterior polarity. Strong polarisation defects are also observed in the PLM processes of *Fz(Lin-44)* mutants alone, or combined with the *cwn-1* or *egl-20* Wnt mutants (Prasad and Clark, 2006).

In summary, in *C. elegans*, Wnt-Fz interaction is required to establish the proper polarisation of specific neurons and, in contrast to the attractive information observed in the fly and vertebrates, provides a repellent signal that forces mechanosensory axons to grow in the anterior direction.

Additional functions of Fz or Ryk in axon guidance

The work described above strongly supports that Wnt-Ryk and Wnt-Fz are ligand-receptor pairs with a conserved role in neuronal process development (Table 1). However, there are additional examples in vertebrates that illustrate the importance of Ryk or Fz as mediators of axon guidance cues. In *Ryk* null mice, cortical axons grow through the corpus callosum in a defasciculated manner and stall at the contralateral side without reaching their targets. Wnt5a, which is expressed in the surrounding of the corpus callosum, inhibits the extension of embryonic cortical axons in explant cultures and interacts with Ryk in vitro, indicating that additional chemorepulsive information is provided by the Wnt/Ryk interaction (Keeble et al., 2006). Furthermore, inactivation of *Fz3* in mice causes the absence of, or a great reduction in, several axon tracts, including the anterior commissure, cortico-spinal tract, corpus callosum, fornix, thalamo-cortical and cortico-thalamic tracts, stria medullaris, stria terminalis and hippocampal commissure (Wang et al., 2002; Wang et al., 2006). Interestingly, *Fz3* is co-expressed in striatal and thalamic cells with *Celsr3* (Tissir et al., 2005), an ortholog of the *Drosophila flamingo*, a protocadherin involved in PCP. Mice defective in *Celsr3* show defects in thalamo-cortical connectivity that are similar to those observed in $Fz3^{-/-}$ mice (Wang et al., 2002; Tissir et al., 2005), further associating genes involved in PCP with axon guidance.

The general trend emerging from what we have discussed so far is that Wnts are bi-fuctional axon guidance cues. However, the growth cone response seems to be mainly dictated by the receptor. For example, Wnt1 or Wnt5a can provide both repulsive and attractive information depending on whether they interact with Fz, as in commissural neurons (Luksyutova et al., 2003), or with Ryk, as in cortico-spinal and callosal axons (Liu et al., 2005; Keeble et al., 2006). Similar considerations apply to Wnt3a in the chick visual system (Schmitt et al., 2005). This is not a peculiarity of Wnt proteins, as analogous situations exist for Shh and Netrins. Shh-induced attraction of pre-crossing commissural axons is presumably mediated by the Smoothened receptor (Charron et al., 2003), whereas Hip (Hedgehog Interacting Protein) is involved in Shh-induced repulsion of post-crossing commissural axons (Bourikas et al., 2005). Unc5 conveys Netrin1-induced repulsion in both vertebrates and invertebrates, whereas Dcc (Deleted in Colorectal Cancer) can mediate both positive and negative Netrin1 signalling, but, in the latter case, by forming a complex with Unc5 (reviewed by Chilton, 2006). An interaction between Ryk and Fz has been also reported. In co-immunoprecipitation assays, Ryk interacts with Dvl, and with both Wnt and Fz; furthermore, interfering with Ryk expression in vitro abolishes Wnt3a-induced neurite outgrowth from DRG explants, suggesting that when interacting with Fz, Ryk can enhance axon growth (Lu et al., 2004).

With this one exception, most studies, however, suggest that Wnt-Ryk and Wnt-Fz signalling at the growth cone are independent. This seems to be the case in the *Drosophila* nerve cord (Yoshikawa et al., 2003) and is particularly evident in the vertebrate visual system. Indeed, binding assays demonstrate that anti-Ryk antibody can block Wnt-Ryk, but not Wnt-Fz, binding; by contrast, Sfrp2, an extracellular modulator of Wnts, can partially block Wnt-Fz, but not Wnt-Ryk, binding. Consistent with this, Sfrp2, but not Ryk antibodies, block Wnt3a-induced axon outgrowth from the dorsal retina. Conversely, anti-Ryk antibodies, but not Sfrp2, interfere with the axon outgrowth inhibition caused by Wnt3a on the ventral retina (Schmitt et al., 2005). Similarly, Sfrp2 does not prevent Wnt-Ryk-mediated repulsion on cortico-spinal axons (Liu et al., 2005).

Besides indicating that Ryk and Fz function independently, these studies also indicate that different Wnt domains are involved in the binding of Wnts to Ryk or Fz. In the most common model, Wnts bind to either Fz or Sfrps through their respective CRD domains. In this way, Sfrps sequester Wnts in the extracellular space by

Table 1. Evolutionary conserved roles of Wnt signalling in axon guidance mediated by Fz or Ryk

Organism	Ligand	Receptor	Function	Reference
D. melanogaster	Wnt5	Drl	Repulsion of anterior commissural axons	Yoshikawa et al., 2003
D. melanogaster	Dwnt4	Dfz2	Attraction of ventral photoreceptor axons	Sato et al., 2006
C. elegans	*cwn1;egl-20* (Wnt)	*mig-1;mom-5* (Fz)	Repulsion of AVM and PVM axons	Pan et al., 2006; Prasad and Clark, 2006
C. elegans	*cwn1;egl 20;cwn-2* (Wnt)	?	Absence of ALM branches in the nerve ring	Hilliard and Bargmann, 2006
Rat, mouse	Wnt4	Fz3	Attraction of post-crossing commissural axons	Lyuksyotova et al., 2003
Mouse	Wnt1; Wnt5a	Ryk	Repulsion of cortico-spinal axons	Liu et al., 2005
Chick, mouse	Wnt3	Ryk	Repulsion of RGC axons	Schmitt et al., 2005
Chick, mouse	Wnt3	Fz	Attraction of RGC axons	Schmitt et al., 2005
Mouse	Wnt5a	Ryk	Repulsion of callosal axons	Keeble et al., 2006

competing with Fz (Kawano and Kypta, 2003). The domain of Wnt that interacts with Fz or Sfrps is undefined but clearly has to be different from that mediating Wnt binding to Ryk, given the null effect of Sfrp2 on Ryk-mediated Wnt3a activity (Schmitt et al., 2005). Alternatively, Sfrp2 could block Wnt-mediated axon outgrowth by binding to the Fz receptor. This possibility is supported by in vitro studies that demonstrate a direct binding of Sfrps to Fz (Bafico et al., 1999; Rodriguez et al., 2005), and by the observation that interference with *Fz2* expression abolishes the Wnt-independent activity of Sfrp1 on RGC growth cone movement, thus pointing to a new ligand for Fz in axon guidance (Rodriguez et al., 2005).

Indeed, in different vertebrates, *Sfrp1* is strongly expressed in crucial regions of the initial visual pathway, including the optic nerve head, the chiasm and the initial portion of the optic tract. Consistent with this distribution, interfering with normal Sfrp1 levels disrupts the growth of *Xenopus* RGC axons along the optic tract. Notably, Sfrp1 causes RGC growth cones to undergo a bi-functional chemotropic turning response, when tested in stripe and turning assays, depending on its interaction with extracellular matrix (ECM) molecules (Rodriguez et al., 2005). Similar to what is observed with Netrin1 (Hopker et al., 1999), Sfrp1-laminin combinations result in RGC axons showing a strong repulsion behaviour, whereas Sfrp1-fibronectin pairs induce growth cone attraction (Rodriguez et al., 2005). Fz2 is required for the Sfrp1-laminin response (Rodriguez et al., 2005), but whether it mediates Sfrp1-fibronectin induced attraction still needs to be determined.

In conclusion, it seems that the Wnt-Ryk interaction has a conserved function in mediating only repulsive information at the growth cone. By contrast, Fz receptors appear to be less 'strict': they mediate both positive and negative axon guidance information and can bind different ligands, including Wnts, Sfrps and norrin, a protein involved in an X-linked congenital retinal dysplasia that is structurally unrelated to Wnts (Xu et al., 2004). Whether different Fz subtypes (i.e. Fz3 versus Fz2) have specific preferences for different ligands or whether additional components (i.e. ECM molecules or Ryk interaction) can modulate Fz-mediated responses needs to be established.

Cascades downstream of Fz- and Ryk-mediated axon guidance

Even though it is clear that Fz and Ryk have important functions in the initiation, elongation and turning of the axon of different neuron types, it is still unclear how they transduce information from the surface to the other growth cone components, particularly the cytoskeleton, to modify their behaviour. This is especially true for Ryk, which is an atypical tyrosine kinase receptor that lacks intrinsic catalytic activity; it is therefore unclear what mechanism is used for signal transduction. One interesting possibility is its reported association with the EphB receptors (Fig. 6) (Trivier and Ganesan, 2002), the loss of which causes defects similar to those described for the Ryk null mice (Halford et al., 2000). A connection between the two systems may be Dvl, which seems to interact with both Ryk and EphrinB1 (Lu et al., 2004; Lee et al., 2006a). In addition, interaction between Ryk and Fz has been reported, suggesting that the two proteins may form a multi-receptor complex signalling through the canonical pathway (Lu et al., 2004). Although this may occur, the evidence derives mostly from in vitro overexpression studies that need to be confirmed in different experimental contexts.

The Wnt-Fz signalling cascade has been extensively studied and, thus, there are many more data pointing to the mechanisms that may mediate Fz functions in axon guidance, but only one so far has provided direct evidence (Sato et al., 2006). Most of the other studies link Wnt signalling to cytoskeletal dynamics in different contexts.

Of the three Wnt signalling cascades, the PCP and Wnt/calcium pathways are the best candidates. Indeed, both pathways have been strongly involved in the control of Wnt-induced cell migration, a process that has many similarities with axon guidance. As mentioned earlier, the Wnt/calcium pathway has striking similarities with that described for the activation of other GPCRs in axon guidance, both involving pertussis toxin-sensitive G proteins, PKC, CamKII, and the modulation of cytosolic levels of cGMP and Ca^{2+} (Slusarski et al., 1997; Xiang et al., 2002). Furthermore, components of both pathways, like the small GTPases RhoA and Rac, or intracellular levels of Ca^{2+} and cyclic nucleotides, are considered to be necessary pieces of the network that controls the response of a growth cone to many different guidance cues (Wen and Zheng, 2006). In agreement with this idea, proteins involved in the PCP pathway, including JNK, are required for Dwnt4-mediated retinotopic organisation in the fly (Sato et al., 2006), and Rac and JNK have been implicated in Wnt-induced dendrite morphogenesis acting downstream of Dvl (Rosso et al., 2005). A link between Dlv and Rho GTPase could be Daam1, a member of the mammalian diaphanous-related formin family, which binds to both proteins and mediates convergent extension movements during vertebrate gastrulation (Habas et al., 2001). An association among Wnt and Rho and Rok (Veeman et al., 2003), a kinase that indirectly regulates ADF/cofilin, a depolymerising actin factor, has been also reported to control microfilament dynamics in other developmental contexts

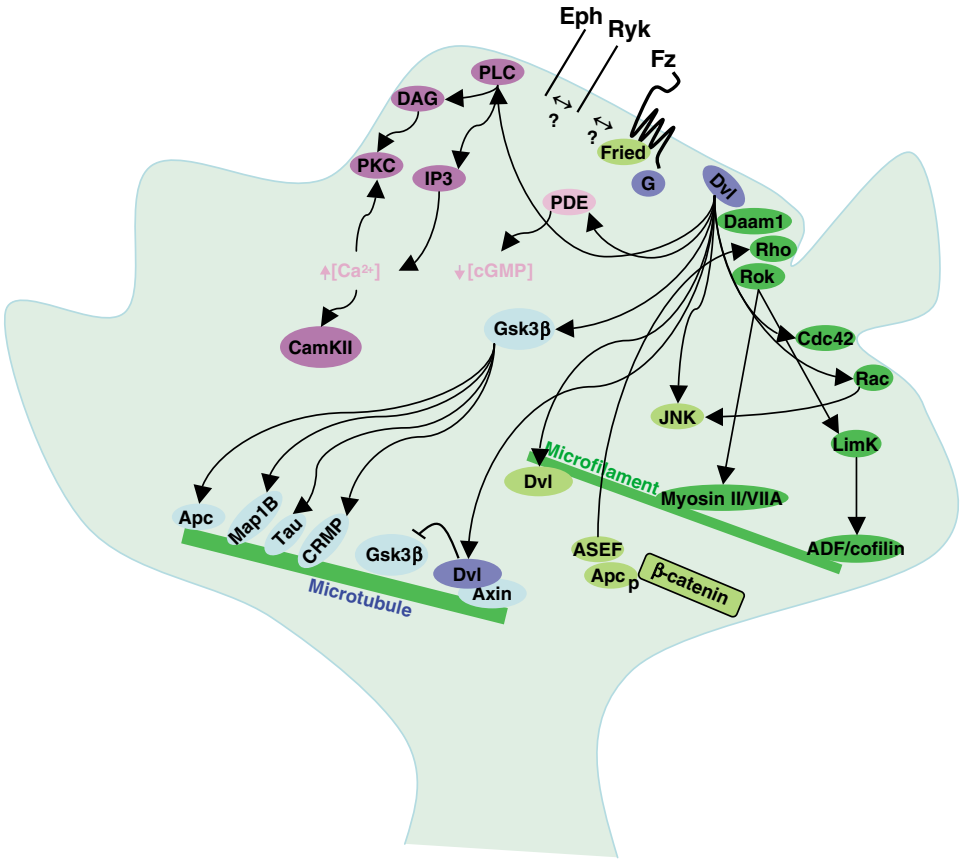

Fig. 6. Hypothetical pathways that might link Wnt signalling to changes in the cytoskeletal organisation of the growth cone. Components typical of the non-canonical PCP pathway are represented in dark green, the classical or divergent canonical pathway in light blue, and the Ca^{2+} pathway in purple. General modulators of 'classical' guidance cues are represented in pink. This hypothetical link suggests that components of the PCP pathway could mainly regulate actin dynamics (green), whereas, independently of transcriptional activity, the canonical pathway could regulate microtubule dynamics through Gsk3β. Apc may function to link microtubule and microfilament dynamics. Apc, adenomatous polyposis coli; ASEF, rac-specific guanine nucleotide exchange factor; Axin, axis inhibition protein; CamKII, calcium/calmodulin-dependent protein kinase II; Cdc42, cell division cycle 42; cGMP, cyclic guanosine monophosphate; CRMP, collapsin response mediator protein; Daam1, dishevelled associated activator of morphogenesis 1; DAG, diacylglycerol; Dvl, dishevelled; Eph, ephrin receptor; Fried, frizzled-8 associated multidomain protein; Fz, Frizzled receptor; G, G-protein; Gsk3β, glycogen synthetase kinase 3; IP3, inositol 1,4,5-trisphosphate receptor; JNK, jun kinase; LIMK, LIM domain kinase; Map1B, microtubule-associated protein 1B; PDE, phosphodiesterase; PKC, protein kinase C; PLC, phosphatidylinositol-specific phospholipase C; Rac, GTPase activator; Rho, ras homolog; Rok, Rho kinase; Ryk, receptor-like tyrosine kinase; Tau, neurofibrillary tangle protein.

(Maekawa et al., 1999). Furthermore, overexpression of Fried, a protein tyrosine phosphatase that interacts with the C-terminal PDZ domain of Fz8, reorganises cortical actin in *Xenopus* ectoderm (Itoh et al., 2005).

Altogether, these data suggest that similar mechanisms, downstream of Wnt-Fz signalling, could regulate actin polymerisation in the growth cone (Fig. 6). Evidence for a related crosstalk between Wnt signalling components and microtubule organisation does also exist. In this case, independently of β-catenin transcription activity (Ciani et al., 2004; Orme et al., 2003; Rosso et al., 2005; Veeman et al., 2003), Dvl, Apc and Gsk3β, which are involved in the initial steps of the canonical pathway, are the main players.

During synaptogenesis, Wnt-Fz interaction leads to axonal remodelling via the inhibition of Gsk3β (Ciani and Salinas, 2005), a kinase that phosphorylates microtubule-associated proteins, including Tau (Wagner et al., 1996), Map1B (Microtubule Associated Protein 1B) (Lucas et al., 1998), Apc (Zumbrunn et al., 2001) and Crmp2 (Collapsin Response Mediator Protein 2) (Yoshimura et al., 2005). The phosphorylation state of these proteins is crucial for their binding to the microtubules, and, therefore, Gsk3β indirectly controls microtubule dynamics. This activity also requires Dvl, which, through its PDZ domain inhibits a Gsk3β pool locally bound to microtubules (Ciani et al., 2004; Krylova et al., 2000). Interestingly, a different Dvl domain, DIX, appears necessary for both actin binding (Capelluto et al., 2002) and neurite outgrowth (Fan et al., 2004). Finally, Apc may function as a link between microtubule and actin components, as its binding affinity to either microtubules or the actin cytoskeleton changes depending on its phosphorylation state (Zhou et al., 2004; Zumbrunn et al., 2001).

Thus, there is a strong case for a local activity of Wnt on the growth cone (Fig. 6). By modifying cytoskeleton dynamics, the Wnt-Fz signalling system could orient axons by using mechanisms that are comparable to those adopted by other more 'traditional' guidance cues (Wen and Zheng, 2006). This does not exclude that Fz and Ryk may also act on the growth cone via gene transcription, local protein synthesis regulation or the control of endocytic recycling (Piper et al., 2005). In this respect, retromer, a protein complex that mediates endosome-to-Golgi protein trafficking, has been associated with the Wnt-induced establishment of neuronal polarity in *C. elegans* (Prasad and Clark, 2006).

Conclusion and perspectives

In conclusion, Fz and Ryk serve as receptors for a conserved class of axon guidance cues: the morphogen Wnts. Generally, Fz receptors mediate attractive responses, whereas Drl/Ryk is responsible for conveying repulsive information to the growth cone. In addition, Fz can mediate the activity of Sfrp1, a Wnt signalling modulator that, independently of Wnt, controls RGC growth cone movements. It is possible that this last ligand-receptor pair might only operate in vertebrates, as no clear

homologues of Sfrps have been found among invertebrates (Kawano and Kypta, 2003), although their modular structure may have complicated the search.

In mammals, there are 19 Wnt genes, 10 Fz receptors, one Ryk, five Sfrps and several possible signalling cascades, which are in constant expansion, as recently indicated by the finding that Wnt can also operate through the receptor tyrosine kinase-like orphan receptors (Ror), repressing Wnt-β-catenin signalling (Mikels and Nusse, 2006). At the moment, there are perhaps more questions than answers as to how this crowded scenario may fit into the control of axon guidance. For example, a point to be resolved is the binding specificity of all the receptor-ligand pairs. Wnts seems to have a greater affinity for Ryk than for Fz (Liu et al., 2005). Is this the case for all Wnt proteins? An answer to this question should help to define relevant physiological situations, as is the case for the ventral RGC, where Fz and Ryk compete for Wnt3a binding (Liu et al., 2005). The generation of appropriately engineered Wnt mouse mutants should also give important information, as the mutants currently available have not been reported to show major alterations in axon tract formation, suggesting that Ryk and Fz in vivo may be activated by multiple ligands.

Many other, and possibly more general, questions come to mind. Where are Wnt proteins really localised? As most of our information on Wnt distribution comes from in situ hybridisation studies, this question remains open. How far do Wnts (or other morphogens involved in axon guidance) diffuse? Is there a differential distribution of receptors along the axon or the growth cone? How is the timing of receptor expression regulated? This is a crucial point to understand; for instance, why pre-crossing commissural axons are insensitive to the Wnt4-attractive gradient at the ipsilateral side of the floor plate. The example of the Eph2A receptor, which is locally translated in the commissural growth cones as they cross the midline (Brittis et al., 2002), offers an attractive example of how sensitivity to Wnt4 could be regulated. Alternatively, as in RGC, Sfrp1, strongly expressed in the ventral midline (Esteve et al., 2000), may provide guidance information to commissural axons at the ipsilateral side by binding to Fz, thus temporarily excluding it from Wnt binding.

Specific experiments are needed to answer these hypotheses. Because Wnt signalling pathways are involved in many different biological processes (from development to disease), advances in this field may provide useful information in other contexts where Wnt-Fz, Fz-Sfrp or Ryk-Wnt interactions might also be relevant.

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