Wnt signaling: complexity at the surface

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Accepted 5 December 2005
Journal of Cell Science 119, 395-402 Published by The Company of Biologists 2006
doi:10.1242/jcs.02826

Summary

Wnts are secreted proteins that are essential for a wide array of developmental and physiological processes. They signal across the plasma membrane by interacting with serpentine receptors of the Frizzled (Fz) family and members of the low-density-lipoprotein-related protein (LRP) family. Activation of Fz-LRP promotes the stability and nuclear localization of β-catenin by compromising the ability of a multiprotein complex containing axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3 (GSK3) to target it for degradation and block its nuclear import. The Fz-LRP receptor complex probably accomplishes this by generating multiple signals in the cytoplasm. These involve activation of Dishevelled (Dsh), possibly through trimeric G proteins and LRP-mediated axin binding and/or degradation. However, individual Wnts and Fzs can activate both β-catenin-dependent and -independent pathways, and Fz co-receptors such as LRP probably provide some of this specificity. Additional, conflicting data concern the role of the atypical receptor tyrosine kinase Ryk, which might mediate Wnt signaling independently of Fz and/or function as a Fz co-receptor in some cells.

Key words: Wnt, Frizzled, LRP, Arrow, β-catenin, Ryk, Dishevelled, Dvl

Introduction

Wnts are secreted lipid-modified glycoproteins (Willert et al., 2003) found in all metozoans examined to date. During development, they are used by cells to influence the fate or behavior of neighboring cells (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Logan and Nusse, 2004). In the mature organism, Wnts are implicated in maintaining stem-cell-like fates in the intestinal epithelium (Pinto and Clevers, 2005), skin (Lowry et al., 2005) and hemopoietic cells (Reya et al., 2003; Willert et al., 2003). Inappropriate activation of Wnt signaling contributes to numerous human cancers (Polakis, 2000; van Es et al., 2003; Gregorieff and Clevers, 2005) and reduced Wnt signaling has been implicated in osteoporosis (Koay and Brown, 2005; Levasseur et al., 2005).

A combination of genetic and molecular studies has provided a biochemical model for how Wnt molecules act. They are thought to signal through several different pathways. In this Commentary, we focus mainly on signaling through β-catenin, sometimes referred to as canonical Wnt signaling. During this branch of signaling, Wnt induces stabilization and nuclear localization of β-catenin, an intracellular signal normally held in check by a complex containing axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3 (GSK3) that normally targets it for degradation (Fig. 1A,B). Reviews containing more details about the other branches of Wnt signaling can be found elsewhere (Veeman et al., 2003; Fanto and McNeill, 2004; Kohn and Moon, 2005).

Wnt/β-catenin signaling involves at least two types of receptors: Frizzled (Fz) and low-density-lipoprotein-related protein (LRP). Below, we discuss current models for how a Fz-LRP receptor complex might signal, emphasizing the possibility that it generates more than one signal. We examine these models in relation to the issue of signaling specificity of β-catenin-dependent and -independent pathways. Finally, we explore work implicating the kinase Ryk as a Wnt receptor and/or Fz co-receptor.

Fz and LRPs are essential for Wnt/β-catenin signaling

The initial connection between seven-transmembrane-span proteins of the Fz family and Wnt proteins came from studies in *Drosophila* cell culture. Transfection of *Drosophila frizzled 2* (*fz2*) confers the ability to bind Wingless (Wg; a fly Wnt) and stabilize Armadillo (Arm; the fly β-catenin) upon cells that do not express Fz2 and are unresponsive to Wg (Bhanot et al., 1996). Additional evidence implicating Fzs in Wnt signaling came from mis-expression studies in *Xenopus* (Yang-Snyder et al., 1996; He et al., 1997) and the finding that a mutation in *lin-17*, which encodes a Fz, affects T-cell polarity in *Caenorhabditis elegans* (Sawa et al., 1996), which is controlled by LIN-44, a Wnt (Herman et al., 1995).

The extracellular portion of Fz proteins contains a cysteine-rich domain (CRD), which is thought to mediate direct binding to Wnt proteins. The CRDs of Fz1 and Fz2 in flies, and Fz8 in mice, have been shown to bind to Wnt proteins with nanomolar affinity (Hsieh et al., 1999b; Rulifson et al., 2000; Wu and Nusse, 2002). Expression of truncated forms of Fz1 and Fz2 containing the extracellular domains tethered to the membrane inhibits Wg signaling, which is consistent with direct binding of Wnts to the CRD (Cadigan et al., 1998; Zhang and Carthew, 1998).

Despite the abundant functional and physical data indicating Fz proteins are essential for Wnt signaling, this remains largely unproven for most family members, presumably owing to genetic redundancy (see Logan and Nusse, 2004). A notable exception is in flies, where loss of *fz1* and *fz2* results in a

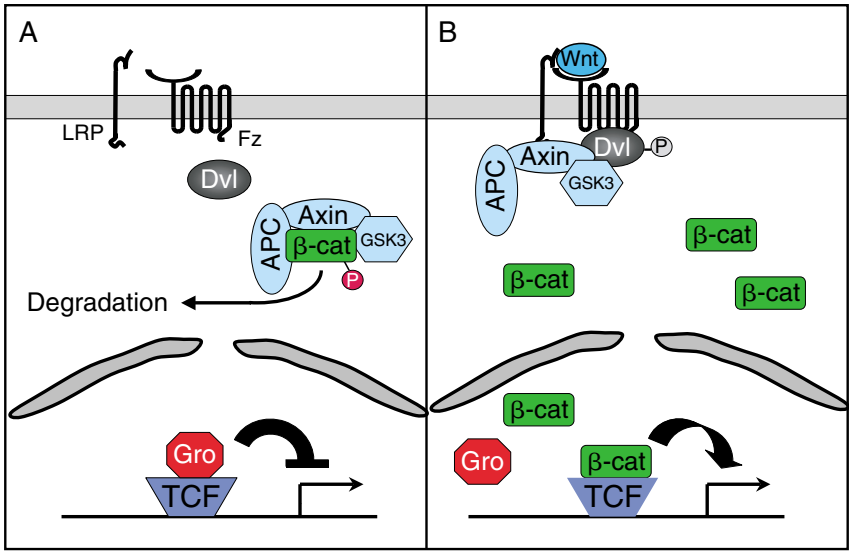

Fig. 1. Outline of Wnt/β-catenin signaling. (A) In the absence of Wnt, β-catenin (β-cat) is phosphorylated by a complex containing GSK3. This targets β-catenin for proteosomal degradation. In the nucleus, members of the T-cell factor (TCF) family of DNA-binding proteins repress Wnt targets, in concert with co-repressors such as Groucho (Gro). (B) Upon Wnt binding to Fz-LRP receptors, a combination of LRP-axin interaction and Dvl phosphorylation (P) blocks the APC-axin-GSK3 complex from phosphorylating β-catenin. The accumulated β-catenin then enters the nucleus, where it converts TCF into a transcriptional activator. See the Wnt homepage (http://www.stanford.edu/~rnusse/wntwindow.html) for a more complete description of Wnt signaling components.

phenotype that is virtually indistinguishable from *wg* mutants (Bhat, 1998; Bhanot et al., 1999; Chen and Struhl, 1999; Muller et al., 1999). Both *fz* genes must be mutated to obtain a loss of Wg signaling. The relatively simple correlation of the *wg* and *fz1*,*fz2* phenotypes makes it an ideal system for structure-function studies of these receptors.

There is also a compelling case for some LRPs transducing Wnt signals. LRPs are single-pass transmembrane proteins that have relatively large extracellular and short cytosolic domains [see He et al. (He et al., 2004), for more details concerning LRP structure]. Mutations in the fly LRP, Arrow (Arr), result in a mutant phenotype very similar to that of *wg* (Wehrli et al., 2000). Disruption of LRP6 in mice causes an embryonic phenotype that appears to be a composite of *Wnt1*, *Wnt3a* and *Wnt7a* mutants (Pinson et al., 2000). Mouse embryos lacking both *LRP6* and *LRP5* fail to form a primitive streak and lack mesoderm (Kelly et al., 2004), which is also observed in *Wnt3*- and *β-catenin*-knockouts (Liu et al., 1999; Huelsken et al., 2000). Complementing these loss-of-function data are studies demonstrating that overexpression of LRP5 or LRP6 lacking the intracellular domain can block Wnt signaling (Tamai et al., 2000). Truncations of either LRP lacking the extracellular domains but still tethered to the membrane can constitutively activate Wnt/β-catenin signaling in *Xenopus* and mammalian cell culture (Tamai et al., 2000; Mao et al., 2001b; Liu et al., 2003; Cong et al., 2004b; Gonzalez-Sancho et al., 2004; Tamai et al., 2004). These data all point towards an essential, positive role for LRP5, LRP6 and Arr in Wnt signaling.

Fz and LRPs act as Wnt co-receptors

The extracellular domains of Fz and LRPs can bind to Wnts in coprecipitation assays (Tamai et al., 2000; Kato et al., 2002; Itasaki et al., 2003; Liu et al., 2003; Cong et al., 2004b). However, the Wnt-LRP interactions appear to be weaker than the nanomolar binding observed with Wnt-Fz (He et al., 2004). Although no interaction between a native LRP and Fz has been reported, the soluble extracellular domains of LRP6 can form a complex with the murine Fz8/CRD in a Wnt1-dependent manner (Tamai et al., 2000; Semenov et al., 2001). Similar results have not been obtained for Arr, Fz2 and Wg in flies (Wu and Nusse, 2002). Nevertheless, the vertebrate results have led to the hypothesis that Wnt ligand induces association of Fz and LRP at the cell surface.

Direct evidence for Wnt stimulating the Fz-LRP association is lacking; thus, neither Wnt-dependent co-immunoprecipitation of native proteins nor fluorescence resonance energy transfer (FRET) studies with tagged Fz and LRP have been reported. However, two recent studies report that forced association of Fz and LRP6 can stimulate Wnt/β-catenin signaling independently of Wnt. One approach used LRP6 fused with the neurotrophic receptor TrkN and human Fz5 tagged with NT3, a ligand of TrkN. Co-expression of these fusion proteins in cultured *Drosophila* cells resulted in >20-fold activation of a Wnt reporter gene (Cong et al., 2004b). Impressive synergy is also observed when human HEK-293T cells are co-transfected with LRP6 and a fusion of Fz5 and DKK1 (Holmen et al., 2005). DKK1 is a Wnt antagonist that is a ligand for LRP6 (Mao et al., 2001a; Semenov et al., 2001); presumably, the DKK1 moiety on Fz5 recruits LRP6 to activate Wnt/β-catenin signaling in the absence of exogenously added Wnt.

Clouding the picture painted above is the finding that the CRD, thought to be necessary and sufficient for Wnt binding (Bhanot et al., 1996; Hsieh et al., 1999b; Rulifson et al., 2000; Wu and Nusse, 2002), is dispensable for Fz function. Transgenes that produce Fz1 or Fz2 lacking the CRD are able to completely rescue fly embryos or larval tissues lacking endogenous *fz1*, *fz2* and *fz3* (Chen et al., 2004). However, another study found that similar constructs signal less efficiently than wild-type proteins (Povelones and Nusse, 2005). Fusion of the human Wnt inhibitory factor (WIF) domain, which binds to Wnts with high affinity (Hsieh et al., 1999a), to Fz lacking the CRD restores full Wnt/β-catenin signaling activity. A protein in which Wnt is fused to Fz lacking the CRD (Wnt-Fz$^{\Delta CRD}$) also had full activity (Povelones and Nusse, 2005). These data support a model in which the CRD recruits Wnt to Fz, where it can then interact with other portions of Fz or other co-receptors (see also Bejsovec, 2005).

The finding that the CRD of Fz is dispensable for Wnt/β-

catenin signaling is not incompatible with the model that Wnt stimulates assembly of a Fz-LRP oligomer but argues against the idea that Wnt acts as an adaptor between the two co-receptors. It is possible that binding of Wnt to LRP induces a conformational change that makes binding to Fz favorable. Constitutively active Wnt-Fz$^{\Delta CRD}$ fusions could bind preferentially to LRP. Indeed, the Wg-Fz$^{\Delta CRD}$ chimera requires Arr for its signaling activity (Povelones and Nusse, 2005). Clearly, more work examining the effects of Wnt binding on native Fz and LRP in the plasma membrane is needed.

A two-signal model for Fz-LRP receptor action

Activation of the Wnt/β-catenin pathway results in the stabilization and nuclear translocation of β-catenin/Arm. Wnt stimulation inhibits the ability of the axin-APC-GSK3 complex to phosphorylate β-catenin, which targets the protein for ubiquitylation and proteosomal degradation (Kitagawa et al., 1999; Farr et al., 2000; Hinoi et al., 2000; Salic et al., 2000). The PDZ-containing protein Dishevelled (Dsh in flies; Dvl1/Dvl2/Dvl3 in mammals) is required for Wnt-dependent inhibition of this complex (Yanagawa et al., 1995; Li et al., 1999; Rothbacher et al., 2000; Salic et al., 2000; Kishida et al., 2001). There is also evidence that, in *Drosophila*, axin can retain Arm in the cytoplasm, and that this form of regulation is distinct from regulation of Arm turnover (Tolwinski and Wieschaus, 2001; Tolwinski et al., 2003; Tolwinski and Wieschaus, 2004). Recent work, summarized below, suggests that activation of Fz-LRP causes two distinct events: binding of axin to the intracellular domain of LRP and Fz-induced phosphorylation of Dsh/Dvl. These branches in the signaling cascade then converge on β-catenin stability and nuclear localization (Fig. 2).

The LRP/axin branch

Expression of constructs comprising the intracellular domain of LRP5 or LRP6 tethered to the membrane can constitutively stabilize β-catenin and activate Wnt reporter genes (Mao et al., 2001b; Li et al., 2002; Brennan et al., 2004; Cong et al., 2004b; Tamai et al., 2004). This appears to be independent of Fz, since it is observed in S2 cells in which all four *Drosophila fzs* are expressed at extremely low levels, i.e. <1 transcript/cell (Brennan et al., 2004). A similar truncated form of Arr does not activate Wg signaling in flies (Tolwinski et al., 2003). However, a chimera containing the intracellular domain of Arr fused to the C-terminus of Fz2 activates Wg targets independently of endogenous *wg*, *fz1*/*fz2* or *arr* (Tolwinski et al., 2003). Perhaps mis-expression of the truncated Arr from a single-copy transgene is not sufficient to bypass the other (Fz-dependent) branch of Wnt signaling. Fusion of this domain to a Fz presumably activates both branches of the pathway, leading to Arm stabilization and nuclear translocation.

The ability of intracellular LRP to activate β-catenin strongly correlates with its ability to bind axin (Mao et al., 2001b; Cong et al., 2004b; Tamai et al., 2004). This depends upon the phosphorylation of several motifs containing Ser/Thr residues in the cytoplasmic domain of LRP6, which act redundantly to activate Wnt/β-catenin signaling (Tamai et al., 2004; Davidson et al., 2005). Each motif contains two clusters containing casein kinase I (CKI) consensus sites and a PPPSP site (Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005). CKIγ has been shown to phosphorylate the CKI sites and is required for Wnt/β-catenin signaling (Davidson et al., 2005). The PPPSP sites are phosphorylated by GSK3, and this is required for the adjacent phosphorylation of the CKI sites (Zeng et al., 2005). Importantly, Wnt stimulation promotes rapid (10-15 minutes) phosphorylation at both GSK3 and CKI sites (Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005) and FRET has shown Wnt-dependent binding of axin to LRP5 with similar kinetics (Mao et al., 2001b). These data support a model in which Wnt stimulation promotes phosphorylation of LRP by GSK3 and CKIγ, which recruits axin to the plasma membrane, where it is inactivated and/or targeted for degradation. Wnt stimulation can promote axin degradation in mammalian cell culture (Willert et al., 1999; Yamamoto et al., 1999; Mao et al., 2001b) and fly embryos (Tolwinski et al., 2003). This should promote β-catenin/Arm stability because axin is an essential component of the degradation complex (Lee et al., 2003). It should also promote

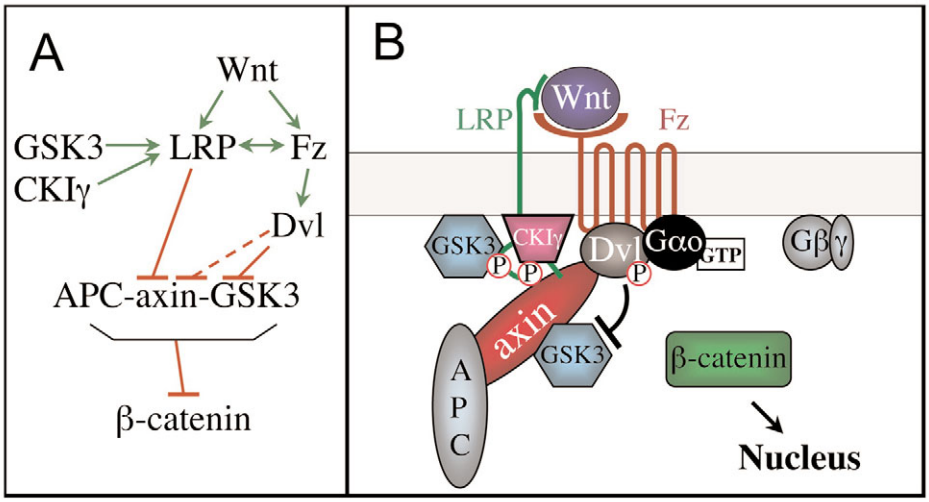

Fig. 2. The two-signal model of Fz-LRP signaling to β-catenin showing the regulatory relationships between each component (A) and summarizing the probable physical interactions (B). In this model, Wnt stimulation promotes Fz-LRP oligomerization, which transduces two separate signals to the cytoplasm. The first signal is LRP phosphorylation, mediated by membrane-localized GSK3 and LRP-bound CKIγ. We speculate that neither kinase is activated by Wnt. Rather, the LPR-Wnt-Fz interaction allows LRP to become phosphorylatable, which then promotes the recruitment of axin to the plasma membrane, leading to its inactivation and/or degradation. The second signal is Fz-dependent Dsh/Dvl phosphorylation, which we tentatively propose involves trimeric G proteins. Activated Dvl then inhibits the APC-axin-GSK3 complex by a poorly understood mechanism. The dashed line in panel A reflects the fact that Dsh/Dvl also participates in recruitment of axin to the plasma membrane. Although hyperactivation of either branch by overexpression can stabilize β-catenin, both signals are required under physiological conditions.

β-catenin/Arm nuclear localization, owing to loss of cytoplasmic tethering (Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004).

Several research groups have demonstrated that truncated LRP5/LRP6 activates β-catenin/Arm independently of Dsh/Dvl (Li et al., 2002; Schweizer and Varmus, 2003; Cong et al., 2004b; Liu, G. et al., 2005). This suggests that it can recruit axin to the plasma membrane independently of Dsh/Dvl. Although this might be true under some conditions, axin-GFP is recruited to the plasma membrane in a *dsh*-dependent manner in the fly embryo (Cliffe et al., 2003). In addition, overexpression of Dsh can recruit axin to the plasma membrane (Cliffe et al., 2003) and downregulate its levels (N. Tolwinski, personal communication). These results can be reconciled if one proposes that, under physiological conditions (i.e. Wnt stimulation of receptors), both LRP and Dsh/Dvl participate in recruitment of axin to the membrane and its degradation (Fig. 2).

There are several important gaps in our understanding of the regulation of axin by LRPs. The mechanism of axin degradation is not understood and it is also not clear whether axin degradation is a secondary consequence of its inhibition (see He et al., 2004).

The Fz/Dsh branch

Dsh/Dvl is essential for Wnt signaling in flies (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994) and mammalian cells (Li et al., 2002; Liu, G. et al., 2005). Wg can promote membrane association of Dsh (Yanagawa et al., 1995; Cliffe et al., 2003), and expression of several Fz proteins causes Dsh/Dvl to be recruited to the plasma membrane (Axelrod et al., 1998; Boutros et al., 2000; Rothbacher et al., 2000; Umbhauer et al., 2000). Direct binding of the C-terminus of murine Fz7 to Dvl1 has been reported (Wong et al., 2003) and point mutations in the cytoplasmic loops or the C-terminus of human Fz5 and *Xenopus* Fz3 abolish binding to Dsh/Dvl (Umbhauer et al., 2000; Cong et al., 2004b). These Fz mutants cannot activate β-catenin, even though they reside on the cell surface, which suggests they fold properly and are trafficked correctly (Umbhauer et al., 2000; Cong et al., 2004b).

Fz can also promote Dsh/Dvl phosphorylation (Willert et al., 1997; Rothbacher et al., 2000; Umbhauer et al., 2000; Takada et al., 2005). This is thought to be important for Wnt/β-catenin signaling since Wnt causes hyperphosphorylation of Dsh/Dvl in vivo (Yanagawa et al., 1995; Gonzalez-Sancho et al., 2004). Although the phosphoacceptor sites on Dsh/Dvl required for activation have not been identified (these proteins are notoriously Ser/Thr rich), the strong correlation between Dsh/Dvl phosphorylation and its ability to activate β-catenin/Arm (Yanagawa et al., 1995; Capelluto et al., 2002) suggests that this is an important part of Wnt/β-catenin signal transduction.

Does Dsh/Dvl phosphorylation form the second branch of Fz-LRP signaling? A recent study found that dominant-negative LRP6 or Arr does not block Wnt-induced Dvl phosphorylation, despite blocking β-catenin stabilization (Gonzalez-Sancho et al., 2004). Likewise, the LRP antagonist Dkk1 blocks β-catenin stabilization without affecting Dvl phosphorylation. In addition, LRP or Arr lacking the extracellular domain activates β-catenin/Arm without apparent phosphorylation of Dsh/Dvl (Gonzalez-Sancho et al., 2004). These data argue that LRP and axin act independently of Dsh/Dvl phosphorylation.

How does Fz activate Dsh/Dvl phosphorylation? Several kinases can phosphorylate Dsh/Dvl, including CKIε and Par1, which also appear to be required for Wnt/β-catenin signaling (Peters et al., 1999; Sun et al., 2001; Gao et al., 2002; Hino et al., 2003; Cong et al., 2004a; Ossipova et al., 2005). CKIε has been reported to be activated by Wnt stimulation, although the mechanism is not clear (Swiatek et al., 2004).

Intriguing candidates for a link between the serpentine Fz receptors and Dsh/Dvl activation are trimeric G proteins. Pertussis toxin, which inactivates some Gα subunits, and antisense oligonucleotides have been used to show that Gαo and Gαq are required for Wnt/β-catenin signaling in cultured cells and *Xenopus* embryos (Liu et al., 2001; Liu et al., 2002; Malbon, 2004). Moreover, expression of GTPase-defective (i.e. constitutively active) forms of these Gα subunits activates a Wnt/β-catenin reporter gene (Liu et al., 2001). These results are consistent with Gαo/Gαq mediating Wnt/β-catenin signaling. Similarly, reduction of Gαo gene activity compromises Wg signaling in the developing fly wing (Katanaev et al., 2005). Overexpression of wild-type or constitutively active Gαo activates Wg targets, and epitasis experiments demonstrate that Gαo activates the pathway downstream of *fz1*/*fz2* and upstream of *dsh*. These data are consistent with a model where Gαo is required for Fz to activate Dsh in a Wnt-dependent manner (Fig. 2). However, there is no biochemical confirmation of this relationship at present and the direct effectors of GTP-bound Gαo/Gαq remain to be identified.

Wnt signaling specificity through Fz-LRP

Wnt proteins are known to activate several pathways through Fz receptors in addition to the β-catenin pathway (Fig. 3). Some Wnt and some Fz proteins tend to activate β-catenin signaling; others preferentially activate β-catenin-independent pathways. For example, Wnt1, Wnt3a and Wnt8 are often referred to as 'canonical/β-catenin Wnts', whereas Wnt 5a and Wnt11 are considered 'non-canonical Wnts' (Du

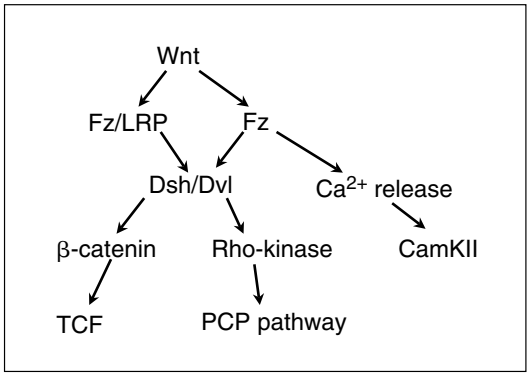

Fig. 3. Outline of several Wnt signaling pathways. The Wnt/β-catenin, PCP and Wnt/Ca^{2+} pathways are shown. All three require Fz-family members. Dsh/Dvl is required for both the β-catenin and PCP pathways; its role in Wnt/Ca^{2+} signaling is less clear (see Veeman et al., 2003). LRP is thought to be specific for Wnt/β-catenin signaling.

et al., 1995; Shimizu et al., 1997; Kuhl et al., 2000). Although these preferences have largely been determined by mis-expression studies in *Xenopus* embryos, they are also observed in other systems (e.g. Heisenberg et al., 2000; Gonzalez-Sancho et al., 2004; Liu, G. et al., 2005; Takada et al., 2005).

Many studies blur the distinction between these two Wnt subclasses. The planar cell polarity (PCP) pathway is a non-canonical Wnt pathway that acts through Dsh/Dvl, Rho and Rho-associated kinase (Rho-kinase) (Strutt et al., 1997; Boutros and Mlodzik, 1999; Habas et al., 2001; Winter et al., 2001; Fanto and McNeill, 2004). However, stimulation with the canonical Wnt1 and Wnt3a leads to rapid (10 minutes) activation of Rho and Rho-kinase in several mammalian cell lines (Habas et al., 2001; Kishida et al., 2004). Conversely, the non-canonical Wnt5a can activate β-catenin in mammary epithelial cells (Civenni et al., 2003). Wnt11, which is required for convergent extension (a β-catenin-independent pathway) in frogs and fish (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000), is also required for the β-catenin-dependent specification of dorsal cell fate in the early *Xenopus* embryo (Tao et al., 2005).

Fz proteins also refuse to be pigeonholed. Fz7 acts in both β-catenin-dependent and non-canonical Wnt signaling (Djiane et al., 2000; Medina et al., 2000; Sumanas et al., 2000; Habas et al., 2003). In flies, Fz1 and Fz2 act redundantly in Wnt/β-catenin signaling (Bhat, 1998; Bhanot et al., 1999; Chen and Struhl, 1999a; Muller et al., 1999), whereas Fz1 is also required in PCP signaling (Adler, 2002; Vinson et al., 1989). Attempts to map the area of Fz1 required for PCP signaling through the use of Fz1-Fz2 chimeras reveal sequences in both the N- and C-termini of Fz1 (Strapps and Tomlinson, 2001; Wu et al., 2004).

One explanation for Fz signaling diversity is that Fz co-receptors provide specificity. Wnt11 signaling in dorsal specification in *Xenopus* embryos requires the EGF-CFC protein FRL1, and FRL1 and Wnt11 can be co-immunoprecipitated when overexpressed (Tao et al., 2005). Perhaps FRL1 enables the non-canonical Wnt11 to signal through the β-catenin pathway.

LRP might also regulate the signaling readout of Wnt-Fz interaction. Aaronson and colleagues (Liu, G. et al., 2005) converted Wnt5a into a canonical Wnt by fusing it to the C-terminus of Dkk2 (Dkk2C), a ligand for LRP5/LRP6. In HEK-293T cells, Wnt5a cannot activate β-catenin, even when co-transfected with ten different Fz isoforms or Dkk2C (Liu, G. et al., 2005). However, a Wnt5a-Dkk2C fusion protein efficiently activates β-catenin (Liu, G. et al., 2005). The implication is that Wnt5a is less efficient at promoting Fz-LRP oligomerization than are other Wnt molecules. The ability of particular Wnt molecules to trigger Fz-LRP interaction might determine the likelihood of their activating β-catenin signaling.

Is Ryk a Wnt receptor or co-receptor?

Adding to the complicated relationship between Wnt and its receptors is the recent finding that the atypical receptor tyrosine kinase Ryk and its fly (Derailed; DRL) and worm (LIN-18) orthologs play important roles in Wnt signaling. These single-transmembrane-span proteins contain an extracellular domain similar to the secreted Wnt inhibitor WIF (Patthy, 2000), which can bind to Wnts with nanomolar affinity (Hsieh et al., 1999a). On the cytoplasmic side, Ryk contains a PDZ-binding domain and a tyrosine kinase domain lacking some residues thought to be important for catalysis (see Halford and Stacker, 2001).

Ryk-family members have been shown to be required for Wnt signaling in several contexts. In the fly embryonic nervous system, *drl* mutants display improper axonal guidance across the midline similar to that observed in *Wnt5* mutants (Bonkowsky et al., 1999; Yoshikawa et al., 2003; Fradkin et al., 2004). In the mouse, the repulsion of corticospinal tract axons by Wnt1 and Wnt5a, and the inhibition of retinal ganglion cell axon outgrowth, is blocked by incubation with an antibody directed against the WIF domain of Ryk (Liu, Y. et al., 2005; Schmitt et al., 2005). Small interfering (si)RNA-mediated knockdown of mouse *Ryk* also inhibits Wnt3a-induced neurite outgrowth in dorsal root ganglion (DRG) explants (Lu et al., 2004). In *C. elegans*, loss of *lin-18* alters the P7 vulval cell fate in a fashion similar to that seen in Wnt mutants (Inoue et al., 2004). Finally, knocking down *Ryk* dramatically reduces the ability of Wnt1 to activate a Wnt/β-catenin reporter gene in human HEK-293T cells (Lu et al., 2004).

There are several reports of physical interaction between Ryk and Wnt molecules. Ryk can co-immunoprecipitate with Wnt1 and Wnt3a when co-expressed (Lu et al., 2004) and an exogenously added extracellular domain of Drl (containing the WIF domain) precipitates endogenous Dwnt5 from fly embryonic extracts (Yoshikawa et al., 2003). The affinity of the Wnt1-Ryk interaction is in the nanomolar range (Liu, Y. et al., 2005). The loss-of-function data combined with the observed protein interaction argue strongly for Ryk acting as a Wnt receptor.

Does Ryk mediate Wnt signaling in concert with Fz-LRP or independently? The picture is not clear. The WIF domain of Ryk interacts with the CRD of Fz8, and the two proteins may form a ternary complex with Wnt1 (Lu et al., 2004). Several other reports argue that Ryk, LIN-18 or Drl act independently of Fz, but this idea is based largely on negative evidence. For example, a twofold reduction in *fz1* and *fz2* gene activity in flies has no effect on a *Drl* mis-expression phenotype (Yoshikawa et al., 2003). In the worm vulva, genetic studies argue that LIN-18 acts in parallel with the LIN-17 Fz receptor (Inoue et al., 2004), although it is not clear whether another Fz is involved. Ryk might thus act by Fz-dependent and -independent mechanisms (Fig. 4).

Downstream, Ryk and Dvl have been shown to interact in HEK-293T cells, in which Ryk is clearly required for β-catenin signaling (Lu et al., 2004). For the other examples mentioned, it is not clear what signaling pathway Ryk uses. In flies, Drl mis-expression is not affected by the loss of zygotic *dsh* (Yoshikawa et al., 2003), but this could easily be explained by the presence of maternally provided *dsh*. Although no requirement for *β-catenin* has been shown to be necessary for proper P7 cell fate (Eisenmann et al., 1998), there are at least four *β-catenin* genes in *C. elegans* (Kidd et al., 2005). This opens the possibility that several β-catenins act redundantly in this process downstream of Wnt/LIN-18.

Whatever the mechanism(s) by which Ryk mediates Wnt signaling, it cannot be obligatory for Wnt signaling in all cell types, since knockouts of the only family member in mice or worms do not display widespread defects in Wnt signaling (Halford et al., 2000; Inoue et al., 2004).

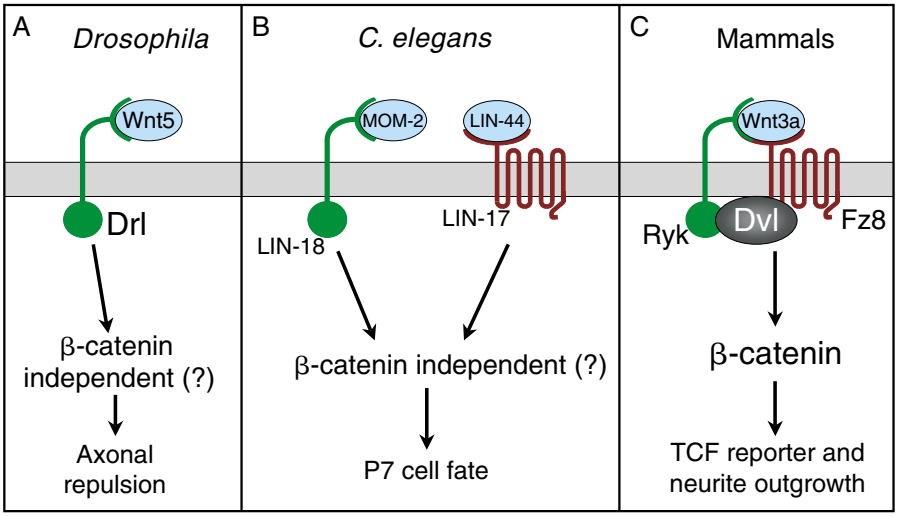

Fig. 4. Three models for Ryk in Wnt signaling. (A) In *Drosophila*, Drl acts as a receptor for Wnt5 to mediate axonal guidance signaling (Yoshikawa et al., 2003). Whether Drl acts with a Fz and through β-catenin is not clear. (B) In *C. elegans* vulva development, a Ryk (LIN-18) and Wnt (MOM-2) are thought to act in parallel to a Wnt-Fz pair (LIN-44–LIN17) to specify P7 cell fate (Inoue et al., 2004). The downstream signaling mechanism is not clear. (C) In human HEK-293T cells, Ryk is thought to act as a co-receptor for Fz8 to mediate Wnt/β-catenin signaling (Lu et al., 2004).

Conclusions and perspectives

Additional components of Wnt signaling that interact with Wnt molecules or their receptors remain to be identified, and others must be more fully understood. Indeed, we have not discussed several secreted factors (e.g. Norrin) that can activate or repress Wnt/β-catenin signaling (Kawano and Kypta, 2003; Xu et al., 2004). Other surface proteins containing CRD-like domains – e.g. Ror (Oishi et al., 2003) and heparin sulfate proteoglycans (Lin, 2004) – have also been implicated in Wnt signaling. There is also evidence that intracellular trafficking of Wnt signaling components can regulate the sensitivity of cells to Wnt molecules. Maturation and trafficking of Fz are inhibited by an endoplasmic reticulum protein called Shisa (Yamamoto et al., 2005). Protein kinase C has been shown to act with Wnt5A to stimulate internalization of Fz4 through Dvl2 and β-arrestin2-dependent endocytosis, which could regulate Wnt signaling (Chen et al., 2003). Endocytosis has also been suggested to be important for Wnt transport between cells and perhaps for signaling activity (Seto and Bellen, 2004). Understanding how these various factors and processes modulate the various Wnt pathways and influence signal specificity will continue to occupy researchers in this field.

Much of our understanding about Wnts and their receptors comes from overexpression studies, often using proteins not found in nature. These are complemented by loss-of-function analyses (based on mutants or RNA interference) to ensure that the proteins being manipulated are physiologically relevant. Although this has been a successful formula for understanding the outlines of Wnt signaling, future research should increasingly focus on what happens to the endogenous receptors immediately after Wnt binding. Does Wnt stimulate the interaction or modification of endogenous Fz, LRP or Ryk? These experiments could be complemented by FRET-based assays, as has been done for LRP5 and axin (Mao et al., 2001b).

If we aim to understand the exact series of biochemical events involved in Wnt reception, can a functional receptor complex be reconstituted in artificial membranes? To date, several Wnts have been purified in biologically active form (Willert et al., 2003; Kishida et al., 2004; Povelones and Nusse, 2005). Whether Wnt receptors can be purified in a useful way remains to be seen. Since the ultimate goal is to learn what happens when Wnt signaling is activated by Wnt molecules (as opposed to overexpression of receptor components), the field will benefit from an increased focus on membrane protein biochemistry.

We thank Nick Tolwinski, Takao Inoue and Wange Lu for helpful discussions concerning their data. Y.I.L. was supported by a pre-doctoral fellowship from the American Heart Association (0315219Z) and by NIH grants RO1 GM59846 and CA95869 to K.M.C.

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