A Second Canon: Functions and Mechanisms of β-Catenin-Independent Wnt Signaling

Review

Michael T. Veeman,[1,2] Jeffrey D. Axelrod,[3]
and Randall T. Moon[1,*]
[1]Howard Hughes Medical Institute
Department of Pharmacology
Center for Developmental Biology and
[2]Molecular and Cellular Biology Program
University of Washington School of Medicine
Seattle, Washington 98195
[3]Department of Pathology
Stanford University School of Medicine
Stanford, California 94305

More is becoming known about so-called noncanonical Wnt pathways that signal independently of β-catenin. Here we review recent developments in both the functions and mechanisms of noncanonical Wnt signaling. We also discuss some unresolved and vexing questions. How many noncanonical Wnt pathways are there? How extensive are the parallels between *Drosophila* planar polarization and vertebrate convergence and extension? Last, we will outline some challenges and difficulties we foresee for this exciting but still very young field.

In the past 20 years, more than 2000 papers have been published about Wnt signaling. The majority of these concern the canonical Wnt pathway, which signals through the stabilization of β-catenin to regulate genes that control an impressive array of embryonic and adult processes (Huelsken and Behrens, 2002; Miller et al., 1999). An increasing body of research, however, concerns noncanonical, β-catenin-independent Wnt pathways. In vertebrates, the most robust and compelling data are for a noncanonical Wnt pathway that controls aspects of gastrulation movements through a mechanism similar to the *Drosophila* planar cell polarity (PCP) pathway (see Figures 1 and 2). There are indications that vertebrate noncanonical Wnt signaling may also be involved in processes as diverse as cochlear hair cell morphology, heart induction, dorsoventral patterning, tissue separation, neuronal migration, and cancer. Potential mechanisms of noncanonical Wnt signal transduction are also quite diverse, including signaling through calcium flux, JNK, and both small and heterotrimeric G proteins. While it is difficult to fully integrate these quite varied observations, our goal here is to provide historical and critical perspectives for this often baffling field.

Noncanonical Wnt Signaling Controls Cell Movements during Vertebrate Gastrulation

Before reviewing the experiments connecting noncanonical Wnt signaling with the control of specific morphogenetic movements during and following vertebrate gastrulation, it is necessary to discuss certain aspects of terminology. In general terms, the major cell movements affected or controlled by noncanonical Wnt signaling

*Correspondence: rtmoon@u.washington.edu

are the intercalations and directed migrations driving the mediolateral convergence and anteroposterior extension of the body axis. Such movements include, but are not limited to, the mediolaterally directed intercalations of presumptive notochord cells (Figure 2E), a process that is often referred to as convergent extension. Convergent extension is not the only movement, however, that contributes to the overall convergence and extension of the body axis. The directed migration of the prechordal plate, for example, which moves to the anterior as a coherent group without significant intercalation (Figure 2C), is also dependent on proper noncanonical Wnt signaling. We will use the term "CE movements" as a general term for the convergence and extension of the body axis, and restrict our use of "convergent extension" to those tissues that are known to converge and extend through mediolateral intercalation. It is also important to note that although these Wnt-dependent CE movements are often referred to as gastrulation movements, they represent only a subset of the cellular movements required for gastrulation and they also continue long after the germ layers have been established. For a discussion of the precise aspects of morphogenesis controlled by noncanonical Wnt signaling, there are several excellent recent reviews (Keller, 2002; Mlodzik, 2002; Myers et al., 2002b; Wallingford et al., 2002).

Overexpression of Different Wnts Causes Distinct Phenotypes

One of the first indications of functional differences between Wnt proteins came from misexpression analysis in *Xenopus* embryos. Overexpression of some Wnts, including XWnt1 and XWnt8, causes a duplication of the embryonic axis that has since been shown to depend on canonical signaling through β-catenin. See Figure 3 for a diagram of the Wnt/β-catenin pathway. Overexpression of other Wnts, however, including XWnt4, XWnt5a, and XWnt11, causes defective CE movements without affecting cell fates (Du et al., 1995; Moon et al., 1993; Figures 1E and 1F).

Dsh Domain Analysis Suggests an Overlap between Vertebrate Noncanonical Wnt Signaling and the Drosophila PCP Pathway

A series of experiments suggests that noncanonical Wnt signaling affects CE movements at least in part through a pathway similar to the *Drosophila* PCP pathway. The PCP pathway, which controls the orientation of hairs, bristles, and ommatidia, overlaps the canonical Wnt signaling pathway in that it requires Frizzled (Fz) receptors and the mysterious cytoplasmic signal transduction molecule Dishevelled (Dsh). It diverges downstream, however, in that it does not involve Axin, GSK-3, or β-catenin. A diagram of the *Drosophila* PCP pathway is given in Figure 4. For detailed reviews, see Adler (2002), Adler and Lee (2001), Axelrod and McNeill (2002), and Shulman et al. (1998).

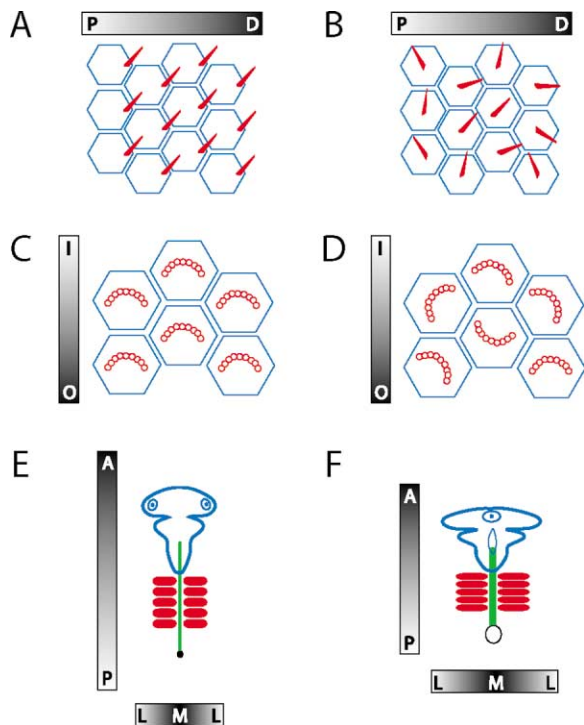Structure-function analysis of Dsh has defined distinct requirements for its three main domains in canonical

Figure 1. Some of the Main Phenotypes of PCP and Noncanonical Wnt Signaling

(A) The PCP pathway is required to restrict the growth of the actin-rich prehair (red) to the distal vertex of each pupal fly wing cell. The proximal-distal (P-D) axis is indicated.

(B) When the PCP pathway is disrupted, the prehair develops near the center of the cell and hair polarity is perturbed.

(C) A strikingly similar example of planar polarity is seen in the polarized stereocilia of vertebrate cochlear hair cells, where the stereociliary bundles (red) are normally aligned toward the outer border of the cochlear duct. The axis of polarity from the inner to the outer (I-O) border of the duct is indicated.

(D) Cochlear hair cell polarity is perturbed by the application of Wnt conditioned media, or in embryos lacking the function of a homolog of the PCP gene *strabismus*.

(E) CE movements, including the movements of convergent extension, extend wild-type embryos along the anterior-posterior (A-P) axis while narrowing them along the medial-lateral (M-L) axis. The cartoon depicts a generic vertebrate embryo, showing the anterior neurectoderm (blue), the notochord (green), and the somites (red).

(F) Disruptions of noncanonical Wnt signaling perturb these movements, resulting in shorter, wider embryos. Embryos with these CE defects tend to have secondary defects that vary by species, but which can include open blastopores, open neural tubes, and cyclopia.
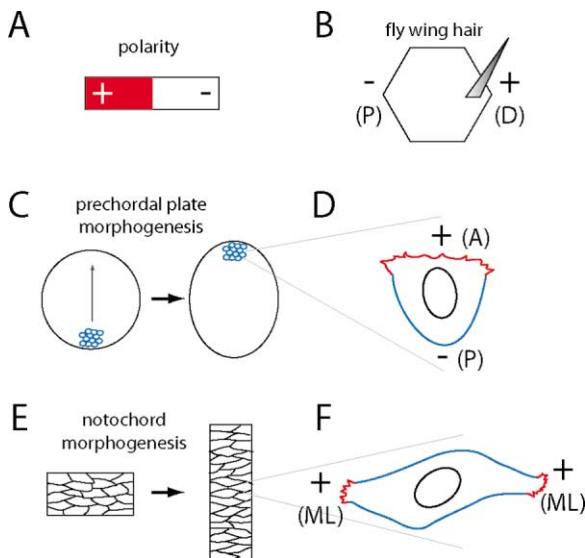

Figure 2. Manifestations of Polarity in PCP and Noncanonical Wnt Signaling

(A) A reminder that polarity, in the abstract sense, consists of having a spatial asymmetry in some quality. This is often visualized as having a plus end and a minus end.

(B) Wild-type *Drosophila* wing cells are polarized in this fashion, as they have a plus end and a minus end defined by the orientation and origin of the trichome.

(C and D) Some gastrulating vertebrate cells also show this type of polarity, such as the cells of the prechordal plate (blue), which have an axis of polarity defined by their unidirectional movement toward the anterior of the embryo (black).

(E and F) Many gastrulating vertebrate cells, however, such as presumptive notochord cells, exhibit a bipolar morphology. Such mediolateral (ML) bipolar protrusive behavior is required to drive the movements of convergent extension. (D) and (F) are close-up depictions of the cell morphologies thought to underlie the cell movements depicted in (C) and (E), with the sites of active tractive behavior indicated in red.

Wnt signaling versus PCP signaling. Dsh domain deletions have thus been used as probes for pathway specificity. There is likely some redundancy of domain function, as residual β-catenin signaling activity can be observed in the absence of any of the three conserved domains (DIX, PDZ, and DEP; Axelrod et al., 1998; Rothbacher et al., 2000; Wallingford et al., 2000). Of the three single-domain deletions, DshΔDIX is most impaired in canonical β-catenin signaling. In contrast, the DEP domain is absolutely required for *Drosophila* PCP and vertebrate CE (Axelrod et al., 1998; Boutros et al., 1998; Heisenberg et al., 2000; Tada and Smith, 2000). DshΔDIX, which is active in PCP but strongly impaired in canonical signaling, can rescue dominant-negative Wnt11 inhibition of the elongation of activin-treated *Xenopus* animal caps, a common ex vivo model of convergent extension (Tada and Smith, 2000).

Similar experiments with Dsh domain deletions have been used to examine the underlying cellular basis for CE movements in the context of noncanonical Wnt signaling. Perturbation of noncanonical Wnt function was found to disrupt the mediolateral elongation and alignment of dorsal mesodermal cells and the mediolateral stabilization of cell protrusions (Wallingford et al., 2000). While these phenotypes are not entirely comparable to *Drosophila* PCP (discussed later), aspects of cell polarity are clearly required for normal CE movements. Together, these observations with Dsh domain deletions strongly suggest that some of the morphogenetic effects of Wnt signaling in vertebrate embryos are not through the canonical β-catenin pathway, and are likely through a pathway akin to the PCP pathway.

Further Evidence from Vertebrate Loss of Function

Experiments based solely on overexpression can be difficult to interpret. The most convincing evidence, both of a role for noncanonical Wnts in CE movements and
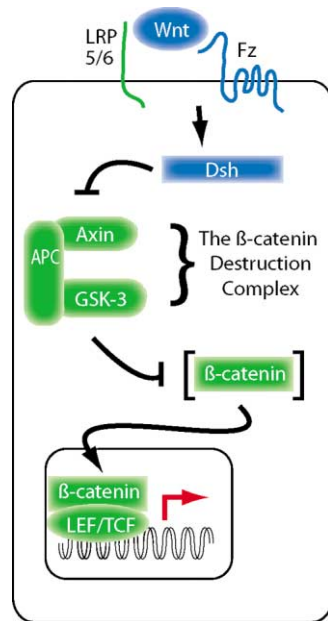
Figure 3. The Canonical Wnt/β-Catenin Pathway

Canonical Wnt signaling requires the Frizzled and LRP5/6 coreceptors and leads, by a poorly understood process, to the activation of Dishevelled. Dishevelled can inhibit the activity of the so-called β-catenin destruction complex, a complex of proteins including APC, Axin, and GSK-3 that otherwise phosphorylates β-catenin, targeting it for destruction by the ubiquitin-proteasome pathway. These and other mechanisms allow Wnt ligands to both stabilize β-catenin and promote its entry into the nucleus where it recruits transactivators to HMG box DNA binding proteins of the LEF/TCF family.
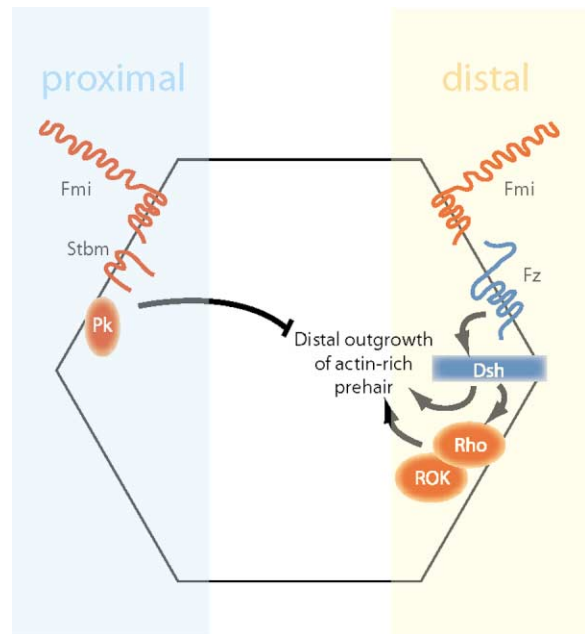

Figure 4. The *Drosophila* Planar Cell Polarity (PCP) Pathway

The PCP pathway includes Frizzled, Dishevelled, Rho, Rho Kinase, Flamingo, Strabismus, and Prickle. It is not a simple, linear pathway and is best appreciated in a spatial context. Some PCP proteins, including Flamingo, become localized to both the proximal and distal sides of the cell. Others, however, including Frizzled, Dishevelled, and Rho, become localized specifically to the distal side, whereas Prickle and Strabismus become localized to the proximal side. The function of all of these proteins is required to ensure both their correct segregation into proximal and distal domains and the subsequent development of correct planar polarity. The molecular mechanisms underlying this polarization remain unclear.

of the similarity of this pathway with the PCP pathway, comes from loss-of-function analyses. The zebrafish *silberblick* (*slb*) mutation, which has a defect in the morphogenesis of the prechordal plate, is an allele of *wnt11* (Heisenberg et al., 2000). *pipetail* (*ppt*), which is an allele of *wnt5*, has morphogenetic defects in tail extension (Rauch et al., 1997). The *slb/slb; ppt/ppt* double mutant combination is more severe, revealing considerable redundancy between Wnt5 and Wnt11 in the anterior mesendoderm (Kilian et al., 2003). Intriguingly, the relatively mild *slb* mutant phenotype can be rescued not only with Wnt11 or Wnt5 RNA but also with DshΔDIX RNA, further suggesting a similarity with the PCP pathway (Heisenberg et al., 2000). The somewhat more severe *ppt* phenotype, however, cannot be rescued with Wnt5 RNA (Kilian et al., 2003), suggesting that rescue analysis may not be the most robust means of characterizing this pathway.

Fruitful comparisons between vertebrate noncanonical Wnt signaling and *Drosophila* PCP signaling have also come from the analysis of vertebrate homologs of *Drosophila* genes thought to be specifically involved in PCP signaling. These include the membrane protein Strabismus (Stbm), the LIM domain protein Prickle (Pk), and the seven-pass transmembrane cadherin Flamingo (Fmi). Stbm is mutated in *trilobite* fish, which have a severe defect in CE (Jessen et al., 2002). This phenotype has also been seen by RNA overexpression and morpholino-mediated gene knockdown in zebrafish and *Xenopus* (Darken et al., 2002; Goto and Keller, 2002; Park

and Moon, 2002). Furthermore, Vangl2, a Stbm homolog, is mutated in *loop-tail* mice, which have failures in neural tube closure likely due to defective CE movements postgastrulation (Kibar et al., 2001). A comparable phenotype is seen in *Celsr1* mice, which are mutant for a homolog of Fmi (Curtin et al., 2003). There are no known mutations in vertebrate *pk* homologs, but morpholino knockdowns have recently been used to show a similar role for zebrafish and *Xenopus pk* genes in CE movements (Carreira-Barbosa et al., 2003; Takeuchi et al., 2003; Veeman et al., 2003). Not only are the loss-of-function phenotypes of *slb*, *ppt*, *tri*, and *pk* similar in that they all affect CE movements without obviously affecting cell fates, but also strong double-mutant, double-morpholino, or mutant/morpholino interactions have been detected between *slb* and *ppt* (Kilian et al., 2003), *pk1* and *tri* (Carreira-Barbosa et al., 2003; Veeman et al., 2003), *pk1* and *slb* (Carreira-Barbosa et al., 2003), and *pk1* and *ppt* (Carreira-Barbosa et al., 2003).

As in the *Xenopus* overexpression studies, defects in cell polarity have also been found in the zebrafish noncanonical Wnt/CE mutants and morphants. These include defects in the mediolateral elongation and alignment of dorsal ectodermal cells, and the velocity of dorsal convergence of dorsal mesodermal cells (Jessen et al., 2002; Kilian et al., 2003; Marlow et al., 2002; Topczewski et al., 2001; Veeman et al., 2003).
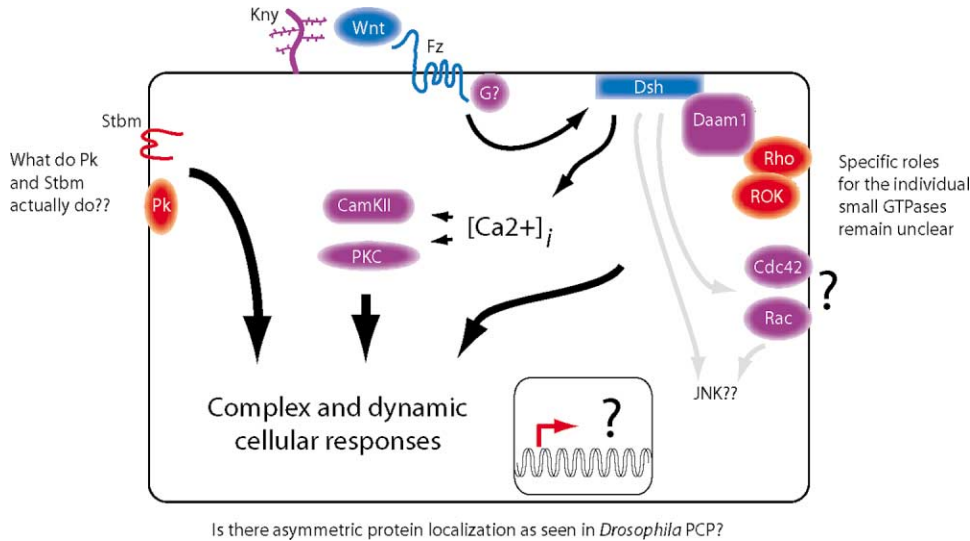
Figure 5. Vertebrate Noncanonical Wnt Signaling

Vertebrate noncanonical Wnt signaling requires Frizzled receptors and the proteoglycan coreceptor Knypek. Like Wnt/β-catenin signaling, this pathway involves the cytoplasmic signal transduction protein Dishevelled. The pathway differs, however, in requiring Dishevelled to be localized to the cell membrane via its DEP domain. A main branch downstream of Dishevelled involves the small GTPases of the Rho family. Dishevelled activation of Rho requires the bridging molecule Daam1. The precise roles of Rho versus other Rho family small GTPases such as Rac and Cdc42 remain unclear, as is the potential role of the JNK pathway. Dsh can also stimulate calcium flux and the activation of the calcium-sensitive kinases PKC and CamKII, suggesting that the Wnt/calcium pathway, which had previously been thought of as distinct from PCP-like signaling, may be a joint part of a common noncanonical Wnt pathway. As in the *Drosophila* PCP pathway, Prickle and Strabismus appear to have some role in this process, but not in a linear or well-understood way. While we have depicted a single, unified noncanonical Wnt pathway, it is possible that certain components are only used in distinct contexts.

Mechanisms of Noncanonical Wnt Signaling
Calcium Signaling

A separate line of research suggests that some noncanonical Wnt signaling may involve intracellular calcium release (see Figure 5 for a consensus view of vertebrate noncanonical Wnt signaling). In zebrafish blastulae, overexpression of Wnt5a or rat Fz2 stimulates the frequency of calcium fluxes in the enveloping layer (EVL) cells as directly measured with a calcium-sensitive dye (Slusarski et al., 1997a, 1997b). In *Xenopus* embryos, overexpression of Wnt5a or Wnt11 can activate the calcium-sensitive kinase protein kinase C (PKC; Sheldahl et al., 1999) and calcium/calmodulin-dependent kinase II (CamKII; Kühl et al., 2000a). PKC is also upregulated in response to Wnt5a overexpression in UACC 1273 melanoma cells (Weeraratna et al., 2002). The calcium-responsive transcription factor NF-AT has been suggested as a potential target in certain contexts (Murphy and Hughes, 2002; Saneyoshi et al., 2002). This pathway has been called the Wnt/calcium pathway, to distinguish it from the canonical Wnt/β-catenin pathway (Kühl et al., 2000b).

A potential role for Dsh in Wnt/calcium signaling has only recently been tested (Sheldahl et al., 2003). Full-length Dsh was found to have a modest ability to activate calcium signaling in the calcium flux, PKC, and CamKII assays. A Dsh construct lacking the DIX domain, and therefore likely active in PCP signaling but not canonical Wnt/β-catenin signaling, is a strong activator in these assays. Overexpression of zebrafish Prickle1 also stimulates calcium flux, albeit not as quickly (Veeman et al., 2003). That these reagents, which are thought to specifically affect PCP signaling, can also stimulate calcium

release, suggests a potential overlap between the PCP and Wnt/calcium pathways.

Interestingly, PKCδ has recently been shown to form a complex with Dishevelled and to be required to translocate Dishevelled to the cell membrane in response to *Xenopus* Frizzled7 (Kinoshita et al., 2003). Dishevelled and PKC may thus be acting together as part of a protein complex, as opposed to being in a linear pathway as suggested by the stimulation of PKC activity by Dishevelled. PKCδ activity was also found to be required for proper CE movements (Kinoshita et al., 2003).

Rho Family GTPases

Other potential mediators of noncanonical Wnt signaling include the small GTPases of the Rho family. Rho acts downstream of Dishevelled in *Drosophila* PCP signaling (Strutt et al., 1997), with Rho-associated kinase (ROK) acting as one effector (Winter et al., 2001). A function for Cdc42 in PCP signaling has also been suggested, through expression of a dominant-negative construct (Eaton et al., 1996). Rho, Rac, and Cdc42 have all been implicated in vertebrate noncanonical Wnt signaling (Choi and Han, 2002; Habas et al., 2001, 2003; Penzo-Mendez et al., 2003). The Formin homology protein Daam1 binds to both Dsh and Rho, and is required for both Wnt activation of Rho and correct CE movements (Habas et al., 2001). A zebrafish homolog of the Rho effector Rho kinase (ROK2) has been shown to act downstream of noncanonical Wnt signaling in the regulation of CE movements (Marlow et al., 2002). One report shows that, in addition to activating Rho, Wnt signaling can also activate Rac but not Cdc42 (Habas et al., 2003). In contrast, other groups have used different functional assays to argue that noncanonical Wnt signaling does

involve Cdc42 (Choi and Han, 2002; Penzo-Mendez et al., 2003). These latter works are still somewhat preliminary, as they used overexpressed G proteins instead of assaying the activities of the endogenous proteins. Studies of the Rho family GTPases in other systems have also been rife with confusion, both from crosstalk and overexpression artifacts. Despite these complications, the Rho family GTPases and their effectors remain the most obvious connection between noncanonical Wnt signaling and the cytoskeleton.

Heterotrimeric G Proteins

While small Rho family G proteins likely act downstream of Dsh in noncanonical Wnt signaling, there is also evidence that heterotrimeric G proteins may be involved upstream of Dsh. Stimulation of calcium flux in zebrafish embryos by noncanonical Wnts and Frizzleds is sensitive to pertussis toxin (Slusarski et al., 1997a), as is PKC and CamKII activation in *Xenopus* (Kühl et al., 2000a; Sheldahl et al., 1999). DshΔDIX stimulates calcium activity in a pertussis toxin-insensitive manner, consistent with it acting downstream of the relevant heterotrimeric G proteins (Sheldahl et al., 2003). A recent report suggests that $G_{\beta\gamma}$ subunits may be the relevant signaling moiety in this context (Penzo-Mendez et al., 2003). Experiments in cultured cells have implicated both $G_{i/o}$ and transducin family G proteins (Dejmek et al., 2003; Murphy and Hughes, 2002; Wang and Malbon, 2003). Specific heterotrimeric G protein subunits, however, remain to be tested by rigorous loss-of-function methods in embryos for roles in noncanonical Wnt signaling.

The JNK Pathway

Another potential, albeit controversial, mediator of noncanonical Wnt signaling is the JNK pathway. Loss of JNK cascade components can dominantly suppress the *Drosophila* eye PCP phenotype resulting from Dsh overexpression (Boutros et al., 1998). The importance of this observation is open to question, however, because null alleles of Jun cause polarity defects in only 1.6%–3% of ommatidia in the fly eye (Weber et al., 2000), and mutations in JNK pathway components have no PCP phenotype in the otherwise unperturbed fly. Dsh can induce JNK activity in vertebrate cells (Boutros et al., 1998; Li et al., 1999; Moriguchi et al., 1999), as can certain Wnts (Habas et al., 2003; Yamanaka et al., 2002), but the mechanisms for this remain quite unclear. The DEP domain is necessary for JNK induction, suggesting a connection with the PCP pathway (Boutros et al., 1998; Li et al., 1999; Moriguchi et al., 1999). The DEP domain alone, meanwhile, which is generally antimorphic with respect to PCP signaling, is also able to induce JNK activity (Li et al., 1999; Moriguchi et al., 1999). There are contradictions in the literature as to whether the Rho family GTPases are involved in JNK activation by Dsh, with one report that JNK activation by Dsh is via Rac but not Rho or Cdc42 (Habas et al., 2003), another that it requires Rac and Cdc42 (Moriguchi et al., 1999), and a third report that the small GTPases are not required (Li et al., 1999). Pk and Stbm also have modest abilities to activate JNK signaling in vertebrate cells (Park and Moon, 2002; Takeuchi et al., 2003; Veeman et al., 2003), and Pk has even been reported to form a complex with JNK (Takeuchi et al., 2003). Adding a further layer of confusion, Axin, which has no known role in PCP or

noncanonical Wnt signaling, can also induce JNK activity and can form a complex with the Jun kinase kinase kinase MEKK1 (Zhang et al., 1999). Given that these experiments used a variety of different assays for JNK induction and that the activations observed were usually quite small, it is currently difficult to draw any firm conclusions about the JNK pathway in Wnt signaling. This is especially true given that JNK activity is known to be induced by a wide range of cellular stresses.

Other Functions of Noncanonical Wnt Signaling
Cochlear Hair Cell Polarity

While the majority of research in vertebrates has focused on functions during gastrulation, there is growing evidence that noncanonical Wnt signaling may also have other functions. Given the potential connection with *Drosophila* PCP signaling, it has long been a matter of speculation whether a similar pathway might control the orientation of comparable vertebrate structures with strong planar polarity. The most dramatic of these, the polarized hair cells of the vertebrate cochlea, have recently been shown to depend on Wnt and PCP signaling (Figures 1C and 1D). Application of conditioned media containing either Wnt7a or Wnt-sequestering proteins can block normal hair cell polarization in cochlear explants (Dabdoub et al., 2003). Even more compelling, strong defects in hair cell planar polarity are seen in *loop-tail* mice, which are mutant for a homolog of *stbm* (Montcouquiol et al., 2003), and *Celsr1* mice, mutant for a homolog of *fmi* (Curtin et al., 2003). Similar but milder defects were also seen in mice mutant for *scribble1* (Montcouquiol et al., 2003). *Drosophila scribble* is known to be involved in the apical-basal polarity of epithelia (Bilder and Perrimon, 2000), but had not been previously implicated as a potential PCP gene. It will be interesting to see whether similar mechanisms control other vertebrate structures with strong PCP, such as the polarized cilia of the respiratory and reproductive tracts.

Tissue Separation

While numerous studies have shown a role for noncanonical Wnt signaling in CE movements, it has also been suggested that such signaling might play a quite different role during gastrulation in maintaining proper separation between the germ layers (Winklbauer et al., 2001). A morpholino targeting zygotic *Xenopus frizzled7* causes severe gastrulation defects, including an open neural tube and a shortened body axis. On closer examination, however, convergent extension was found to be unaffected in these embryos, but a defect was observed in the separation of the involuting mesoderm from the overlying ectoderm. In an explant assay for this tissue separation behavior, the Fz7 morphant phenotype could be rescued with PKC but not with Dsh, DshΔDIX, Cdc42, or β-catenin. These results can be interpreted as indicating that the Fz7 tissue separation behavior requires the action of a Wnt/calcium pathway distinct from Wnt/PCP or canonical Wnt signaling. We would suggest, however, that the absence of rescue in a given assay is not necessarily an informative result. While the mechanisms of Fz7 signaling remain uncertain in this context, the tissue separation phenotype remains of considerable interest given the known influences of noncanonical Wnt signaling on cell adhesion (Torres et al., 1996).

Cardiogenesis and Myogenesis

The majority of research on noncanonical Wnt signaling has emphasized phenotypes involving cell behavior but not cell fates. There are indications, though, that noncanonical Wnts may also be involved in more traditional inductive events. Wnt11, for example, has been implicated in cardiogenesis in both quail (Eisenberg and Eisenberg, 1999) and *Xenopus* (Pandur et al., 2002). An XWnt11 morpholino causes defects in heart patterning, and XWnt11 overexpression is sufficient to induce early cardiac markers in *Xenopus* animal caps. The analysis of noncanonical Wnt signaling in heart development is difficult, however, as the inhibition of canonical Wnt/β-catenin signaling is known to be a crucial step in heart induction (Schneider and Mercola, 2001) and there is thought to be crosstalk (discussed later) between canonical and noncanonical Wnt signaling. It is also difficult to exclude that cardiac phenotypes in whole embryos might be secondary to earlier morphogenetic defects. Despite these challenges, some evidence from *Xenopus* explant assays suggests that XWnt11 may have effects on cardiac gene expression that are not explained by either the inhibition of Wnt/β-catenin signaling or the perturbation of morphogenetic movements (Pandur et al., 2002).

Canonical and noncanonical Wnt signaling have also recently been shown to have intertwined roles in the development of skeletal muscle in the chick wing bud. Wnt/β-catenin signaling controls the numbers of terminally differentiated myoblasts, whereas Wnt5a and Wnt11 affect the relative proportions of slow and fast twitch muscle fibers (Anakwe et al., 2003). Curiously, Wnt5a and Wnt11 have opposite effects in this context, which is a rare example of these proteins not being equivalent in effect. The mechanism for these distinct functions is unknown, but the Wnt5a effect could be phenocopied with activated CamKII.

Noncanonical Wnt Signaling Antagonizes Wnt/β-Catenin Signaling

One of the earliest observations about noncanonical Wnt signaling was that it has an ability to inhibit canonical Wnt signaling. Overexpression of Wnt5a, for example, can block secondary axis induction by Wnt8 in *Xenopus* embryos (Torres et al., 1996). A plethora of nonexclusive mechanisms has been proposed to explain this antagonism. It could be an indirect effect of reduced cell adhesion (Torres et al., 1996). It might involve the activation of Nemo-like kinase (NLK), which can phosphorylate TCF/LEF transcription factors and thereby interfere with their DNA binding ability (Ishitani et al., 1999). Wnt5a has been reported to activate NLK through CamKII and TAK1 (Ishitani et al., 2003). Another potential mechanism for noncanonical Wnt-mediated inhibition of canonical signaling would involve competition for molecules such as Dishevelled that are shared between the two pathways. This latter mechanism is a likely cause of the relatively small inhibitory effects of Stbm and Pk overexpression on Wnt/β-catenin signaling (Park and Moon, 2002; Veeman et al., 2003). Yet another proposed mechanism involves the Wnt5a-induced transcriptional upregulation of Siah2, which can stimulate β-catenin degradation (Topol et al., 2003).

Given these multiple potential interactions between canonical and noncanonical Wnt signaling, it has been a matter of some debate as to whether noncanonical Wnt signaling might inhibit Wnt/β-catenin signaling in developmental contexts. Relatively subtle effects on β-catenin-dependent dorsoventral patterning can be seen upon overexpression of dominant-negative Wnt11 or mutant forms of the noncanonical Wnt effector CamKII in *Xenopus* embryos (Kühl et al., 2000a). It has also been suggested that Wnt5a might signal through the calcium effector NF-AT to regulate ventral fates (Saneyoshi et al., 2002). Zebrafish mutations in *wnt5*, *wnt11*, and *stbm* have no obvious effect on dorsoventral patterning, initially suggesting that the proposed interactions of noncanonical Wnt signaling with the Wnt/β-catenin pathway are not of physiological relevance. It has recently been reported, however, that MZ*ppt* zebrafish embryos lacking maternal and zygotic Wnt5 function are somewhat dorsalized (Westfall et al., 2003). A similar phenotype was not seen with a different, slightly less severe allele of *ppt* (Kilian et al., 2003; Rauch et al., 1997), so it will be important to confirm that the dorsalized phenotype results from the null *ppt* allele and not some other genetic difference between these strains. Another recent report has documented expanded domains of Wnt/β-catenin signaling in the limb buds of *wnt5a* knockout mice (Topol et al., 2003), further demonstrating a potential in vivo context for the long-hypothesized negative interaction between noncanonical and canonical Wnt signaling.

Noncanonical Wnt Signaling and Cancer

Given the observed antagonisms between noncanonical Wnt signaling and Wnt/β-catenin signaling, and the extremely well-established link between hyperactivated β-catenin signaling and cancer (reviewed by Miller et al., 1999), it is possible that noncanonical Wnt signaling might suppress tumor formation. Indeed, antisense depletion of *wnt5a* in C57MG mammary epithelial cells can mimic the transforming effects of Wnt-1 overexpression (Olson and Gibo, 1998). Wnt5a expression can also inhibit the constitutively high Wnt/β-catenin signaling activity of SW48 colon cancer cells (Topol et al., 2003). Wnt5a does not, however, always act as a simple tumor suppressor. Wnt5a expression levels have been found to positively correlate with the severity of certain melanomas; and activation or inhibition of Wnt5a signaling can, respectively, enhance or inhibit the motility and invasiveness of UACC 1273 melanoma cells (Weeraratna et al., 2002). It is intriguing that Wnts known to affect cell adhesion and movements in embryos can also affect metastatic behaviors in some cancers. While these preliminary results are exciting, the breadth and depth of noncanonical Wnt functions in cancer remain to be explored.

New Complexities from *Drosophila*
Dwnt4 and Ovarian Morphogenesis

There is recent evidence from *Drosophila* that noncanonical Wnt signaling may be more diverse than previously appreciated. Mutation of *Drosophila wnt4* causes a defect in ovariolar morphogenesis whereby the ovariolar sheath cells fail to properly migrate over the developing ovarioles (Cohen et al., 2002). This effect cannot be phenocopied by perturbing TCF function, so it appears to not involve the Wnt/β-catenin pathway. The phenotype is seen in flies mutant for *dsh[1]*, an allele that disturbs

planar cell polarity without affecting Wnt/β-catenin signaling, suggesting some connection with PCP signaling. It is not seen, however, in mutants for *stbm*, *pk*, *fmi*, *diego* (*dgo*), or other PCP genes, nor are any of these proteins found to be polarized in the ovariolar sheath cells as they are in cells acquiring planar polarity. The ovariolar defect can also be phenocopied by misexpressing a pseudosubstrate inhibitor of PKC. We speculate that this pathway may represent a core noncanonical Wnt pathway that transduces signals by a mechanism similar to the PCP pathway but without a cassette of proteins, including Pk and Stbm, required for intracellular polarity.

Derailed and Neuronal Morphogenesis

A more dramatically heterodox example of *Drosophila* noncanonical Wnt signaling comes from a recent study of the *derailed* mutation, in which contralaterally projecting axons that should cross the midline at the anterior commissure of each segment instead cross at the posterior commissure (Yoshikawa et al., 2003). Derailed is a RYK family atypical receptor tyrosine kinase. Its extracellular region contains a Wnt inhibitory factor domain, which suggested that its ligand might be a Wnt molecule. A screen for modifiers of a Derailed misexpression phenotype identified *wnt5* (orthologous to vertebrate *wnt5* genes) as a dominant suppressor. *wg*, *wnt4*, *fz fz2* double heterozygotes, and *dsh* all failed to suppress the phenotype. Wnt5 was also found to form a complex with Derailed. Further genetic tests will be necessary to fully exclude the possibility that canonical or PCP signaling might be involved in this pathway, but it appears likely that Derailed is a receptor for an entirely novel Wnt pathway.

It is currently unknown whether Derailed homologs act downstream of any vertebrate Wnts to control axonal pathfinding or any other processes. We note, however, that aspects of noncanonical Wnt signaling have been implicated in neuronal morphogenesis. Wnt7a and Wnt3a, acting independently of β-catenin, are involved in synaptic remodeling and dendritic arborization (Hall et al., 2000; Krylova et al., 2002). While this pathway is noncanonical in that it does not involve β-catenin, it may not be homologous to PCP-like signaling as it is thought to involve GSK-3. Also, zebrafish Stbm (Jessen et al., 2002) and Prickle1 (Carreira-Barbosa et al., 2003) are required for the correct migration of hindbrain facial motor neurons from rhombomere 4 to rhombomeres 6 and 7. This may represent a novel function for Stbm and Prickle1, as it cannot be phenocopied by overexpressing dominant-negative Dishevelled (Jessen et al., 2002). The intricacies of all of these proteins in neuronal patterning will require much further study.

Questions and Concerns for the Future
How Similar Are the Drosophila PCP and Vertebrate Noncanonical Wnt Pathways?

Despite the many advances in understanding noncanonical Wnt signaling, many questions and controversies remain. While it is increasingly well established that a β-catenin-independent Wnt pathway similar to the *Drosophila* PCP pathway is required for vertebrate CE movements, it is unclear how far this similarity extends. The most obvious, though easily overlooked, difference is that the vertebrate noncanonical pathway clearly involves Wnt ligands, such as Silberblick (Wnt11) and Pipetail (Wnt5), whereas no Wnt ligand is known to be involved in *Drosophila* PCP signaling. While it is difficult to prove a negative result, this disparity suggests a dramatic difference in how the two pathways function. That said, there is no evidence that the Wnt proteins involved in vertebrate CE act instructively as directional cues. Indeed, apparently uniform Wnt expression appears to suffice in at least one instance (Heisenberg et al., 2000). Another distinction is that the *Drosophila* pathway functions solely in epithelia, and the proteins involved are first recruited to apical epithelial junctions, whereas the vertebrate pathway also acts in mesenchymal tissues where such junctions are not thought to exist (Keller, 2002).

Unipolarity versus Bipolarity

A further potential difference between noncanonical Wnt signaling during vertebrate gastrulation and *Drosophila* planar polarity concerns the nature of the cell polarity involved (Figure 2). The *Drosophila* PCP pathway controls the polarity of several distinct structures: the trichomes of the wing, bristles on the body, and the ommatidia of the compound eye. In each case, however, the structure is unipolar: trichomes point distally (Figure 2B), bristles point to the posterior, and ommatidia are oriented toward the eye's equator. It is somewhat more difficult to measure the polarity of gastrulating cells, as they do not have obvious markers of polarity. Cell polarity can be inferred, however, as a function of cell shape and cell movement. Unlike *Drosophila* PCP signaling, noncanonical Wnt-dependent polarity during vertebrate gastrulation involves both unipolar and bipolar cell morphologies (reviewed in Keller, 2002; Myers et al., 2002b; Wallingford et al., 2002). Prechordal plate cells, for example, migrate in a coherent, unipolar fashion toward the animal pole (Figures 2C and 2D). *Xenopus* dorsal ectodermal cells intercalate via unipolar medial protrusions (Elul and Keller, 2000). Presumptive notochord cells, in contrast, are elongated and aligned along the mediolateral axis in a bipolar fashion that is known to be required for the intercalatory movements of convergent extension (Figures 2E and 2F). While it is possible that the key aspect of polarity in each case is that cells are aligned with each other and with the axes of the embryo, the specification of bipolar structures is still a significant difference with respect to the *Drosophila* PCP pathway.

Asymmetric Protein Localization in Vertebrates?

Concerns about unipolarity versus bipolarity are especially valid given what is known about asymmetric protein localization during *Drosophila* PCP signaling. While the molecular mechanisms for generating polarity remain mysterious, it is now known that many PCP proteins become asymmetrically localized during planar polarization (Figure 4). Fz (Strutt, 2001) and Dsh (Axelrod, 2001) become localized to the distal side of the pupal wing cell, whereas Stbm (Bastock et al., 2003) and Pk (Tree et al., 2002) become localized to the proximal side. Fmi (Usui et al., 1999) appears to be localized in a bipolar manner to both proximal and distal sides, and Dgo (Feiguin et al., 2001) also localizes to the boundaries but on which side is not known. It is a matter of great interest as to whether there is a comparable asymmetric localization of the homologous proteins during vertebrate

gastrulation. Given that both unipolar and bipolar morphologies seem to be involved, are there corresponding unipolar and bipolar arrangements of the relevant proteins? If so, how can bipolar arrangements be reconciled with proposed models for generating asymmetry?

Dsh tagged with GFP (DshGFP) is recruited from cytoplasmic vesicles to the cell membrane in *Xenopus* dorsal explant cells undergoing active convergent extension (Wallingford et al., 2000). On at least a superficial level, it appears that asymmetric PCP protein localization may not be essential in this assay, as the DshGFP can be expressed at levels that do not perturb convergent extension without it displaying any obvious mediolateral asymmetry (Wallingford et al., 2000). A recent report, however, describes bipolar DshGFP localization in similar explants (Kinoshita et al., 2003). A thorough examination of this issue will require better antibodies to visualize the endogenous proteins.

What Do Prickle and Strabismus Do?
Another important question concerns the functions of Prickle and Strabismus. While these proteins are clearly essential for correct *Drosophila* PCP and normal vertebrate gastrulation movements, it is still unclear what these molecules actually do. In *Drosophila*, they do not fit in a simple linear pathway downstream of Fz or Dsh. The activity of all of the main PCP genes is required to generate the reciprocal localization of Pk and Stbm to the proximal side and Fz and Dsh to the distal side of the pupal wing cell. Various physical interactions have been proposed: between Stbm and Dsh (Bastock et al., 2003; Park and Moon, 2002), between Pk and Dsh (Takeuchi et al., 2003; Tree et al., 2002), and between Pk and Stbm (Bastock et al., 2003). That said, knowledge of these potential protein complexes has not, to date, illuminated a molecular mechanism for the polarization of cell structures. Clonal analyses and protein localization studies have led to the proposal that Pk might act in an intercellular feedback loop to amplify some initial bias by excluding Dsh from the proximal half of the cell while encouraging Fz and Dsh to localize to the distal side of the adjacent cell (Tree et al., 2002). This model remains preliminary, however, pending biochemical and mechanistic evidence. It remains controversial whether Pk can affect Dsh membrane localization in other systems. One group has reported that Pk can block Dsh translocation to the membrane in response to Fz in cultured cells (Tree et al., 2002), but another has not observed any such inhibition (Bastock et al., 2003). Two separate groups have not detected any inhibition of Dsh membrane translocation by Pk in *Xenopus* animal cap explants (Takeuchi et al., 2003; Veeman et al., 2003), but a third has reported such an effect in zebrafish blastulae (Carreira-Barbosa et al., 2003). One might predict quite distinct effects of PCP protein overexpression in unipolar, bipolar, and unpolarized cell types. Further studies are clearly required to reconcile the specificity and universality of these observations, and it will likely prove important to test these proteins in their natural contexts.

Pathway Specificity
There is also considerable uncertainty as to how specificity is determined between canonical and noncanonical Wnt signaling. Overexpression of Wnt5a or Wnt11 clearly causes entirely different embryonic phenotypes in *Xenopus* than overexpression of Wnt1 or Wnt8 (Du et al., 1995; Moon et al., 1993). Coexpression of human Frizzled5 with Wnt5a has been reported to cause duplicated axes (He et al., 1997), suggesting that specificity is not absolutely determined by the ligand. Zebrafish Frizzled2 (Sumanas et al., 2001) and *Xenopus* Frizzled7 (Djiane et al., 2000; Medina et al., 2000) have been implicated as putative receptors for noncanonical Wnt signaling during gastrulation. In the *Xenopus* PKC and CamKII assays, however, Fz2, Fz3, Fz4, and Fz6 predominantly activate noncanonical signaling, whereas Fz1 and Fz7 predominantly activate β-catenin signaling (Kühl et al., 2000a; Sheldahl et al., 1999). Specificity may also be determined by the presence of putative coreceptors such as LRP5/6 for Wnt/β-catenin signaling (Tamai et al., 2000; Wehrli et al., 2000) versus Knypek (Topczewski et al., 2001) or Ror2 (Oishi et al., 2003) for noncanonical Wnt signaling. In *Drosophila*, Fz can couple to both the canonical and PCP pathways, while Fz2 is dedicated to the canonical pathway (Bhanot et al., 1999). As pathway specificity is apt to be context dependent, it may prove challenging to decipher.

How Many Noncanonical Wnt Pathways Are There?
Another unresolved issue concerns the classification of β-catenin-independent Wnt signaling phenomena into discrete pathways. Different authors have, depending on their own predilections, referred to Wnt/calcium signaling, Wnt/PCP signaling, Wnt/JNK signaling, and Wnt/Rho signaling. Do these represent a single, unified pathway, or are there a multitude of noncanonical Wnt pathways? While the issue is not yet fully resolved, the evidence that the Wnt/PCP and Wnt/calcium pathways are, if not fully congruent, at least significantly overlapping, is certainly provocative. The same Wnts, Wnt5a and Wnt11, that are proposed to be involved in PCP-like signaling also activate calcium signaling (Heisenberg et al., 2000; Kilian et al., 2003; Sheldahl et al., 1999; Slusarski et al., 1997b). The ostensibly PCP-specific DshΔDIX can also activate calcium signaling (Sheldahl et al., 2003), as can zebrafish Pk1 (Veeman et al., 2003). The Wnt/calcium pathway, however, has been defined in overexpression assays at blastula stages. While it is known that there are dramatic calcium waves during gastrulation that are required for normal cell movements (Wallingford et al., 2001), it will be important to determine whether these waves are affected in *ppt*, *slb*, or *tri* embryos.

If the Wnt/calcium and Wnt/PCP pathways represent different views of the same underlying pathway, this would not imply that all β-catenin-independent Wnt signaling is the same. It has not been fully examined, but there is as yet no evidence that the PCP-specific proteins Strabismus and Prickle are involved in cardiogenesis, tissue separation, dorsoventral patterning, or other potential noncanonical Wnt functions. One possibility is that there is a core noncanonical Wnt signaling module that signals through Fz and the DEP domain of Dsh, and that PCP proteins such as Pk and Stbm are only used when intracellular polarity is required. It is also possible that the calcium response is only one of several distinct effector branches available for noncanonical Wnt signaling. There is also considerable evidence for a β-catenin-independent yet GSK-3-dependent Wnt pathway in neuronal morphogenesis (Hall et al., 2000;

Krylova et al., 2002). The recent characterization of Drl as a putative receptor for dWnt5 in axonal pathfinding (Yoshikawa et al., 2003) suggests yet another potential type of noncanonical Wnt signaling.

The Need for Better Assays

One of the most pressing needs is for better assays for noncanonical Wnt signaling. The analysis of canonical Wnt/β-catenin signaling has been greatly aided by the excellent tools available to measure pathway activity. These include known direct target genes, the β-catenin-responsive luciferase reporter TOPFlash, and assays for β-catenin stability and nuclear localization. There is a lack of correspondingly robust and facile assays for noncanonical signaling. While calcium, CamKII, PKC, Rho GTPases, heterotrimeric G proteins, and the JNK pathway have all been implicated, the assays used tend to be technically difficult, are subject to confounding effects, and/or show rather modest fold changes of activation. While the development of better assays will require a better understanding of the biological processes involved, it is important to note that a simple JNK activity assay is likely not an adequate test of a protein's potential role in noncanonical Wnt signaling. An important goal for the future will be to determine whether different effector pathways are used in distinct contexts, using loss-of-function as well as overexpression-based methods.

Some Hazards of Studying
Gastrulation Movements

Another important concern is that, despite the abundance of information connecting noncanonical Wnt signaling with gastrulation and CE movements, these movements remain quite difficult to study. One aspect of this is that gastrulation movements are easy to perturb in a nonspecific manner. It is relatively easy to generate cyclopic zebrafish embryos or *Xenopus* embryos with open blastopores by overexpressing LacZ or GFP RNAs. Appropriate and numerous injection controls are essential to show that an overexpression phenotype is specific. Another complication is the sheer number of factors and pathways that have been implicated in such movements, including JAK/STAT (Yamashita et al., 2002), PKA (Song et al., 2003), nodal (Smith et al., 1995), BMP (Myers et al., 2002a), and FGF (Slack et al., 1996) signaling. If the only readout is perturbed gastrulation, it can be difficult to tell whether two molecules act in parallel or in series. Pseudoepistatic analysis can be particularly difficult to interpret given that under- and overactivation of noncanonical Wnt signaling both perturb gastrulation movements.

Outlook

Despite these concerns, we are optimistic about the insights to be gained from studying noncanonical Wnt signaling pathways. While there is a multitude of signaling pathways that modulate transcription factors to control cell fate, there are relatively few known to impinge directly on cell movement, shape, and polarity. Noncanonical Wnt signaling during vertebrate gastrulation is thus handily positioned at the intersection of cell and developmental biology, as is *Drosophila* PCP signaling. It will be interesting to see the full range of developmental and adult processes controlled by noncanonical Wnt signaling. We hope that the canon for β-catenin-independent Wnt signaling will soon be as impressive as that for canonical Wnt/β-catenin signaling.

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