Alternative Wnt Pathways and Receptors

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In addition to activating β -catenin/TCF transcriptional complexes, Wnt proteins can elicit a variety of other responses. These are often lumped together under the denominator “alternative” or “non-canonical” Wnt signaling, but they likely comprise distinct signaling events. In this article, I discuss how the use of different ligand and receptor combinations is thought to give rise to these alternative Wnt-signaling responses. Although many of the biochemical details remain to be resolved, it is evident that alternative Wnt signaling plays important roles in regulating tissue morphogenesis during embryonic development.

The very notion of an “alternative” mode of Wnt signaling implies the existence of a default response. That honor befalls the Wnt- and Frizzled/LRP-mediated stabilization of β -catenin and its concomitant association with LEF/TCF transcription factors (historically called “canonical” Wnt signaling and hereafter referred to as Wnt/ β -catenin signaling), which remains the best understood and most studied response to Wnt ligand stimulation. There are multiple instances, however, in which Wnt proteins elicit a biological response that does not appear to involve the activation of β -catenin/TCF complexes. For lack of a better description, these responses are often jointly referred to as “alternative” Wnt signaling.

An obvious first question, then, might be to ask how many alternative responses exist. Perhaps less obvious is the answer to this question: We don’t really know. The main reason for this is the fact that β -catenin-independent signaling

responses remain relatively poorly characterized at the molecular level. As a result, we lack the robust readouts that exist to probe Wnt/ β -catenin signaling. Fortunately, we are gaining an ever-increasing understanding of the molecular players involved in and the developmental events controlled by alternative Wnt signaling.

ROLES FOR β -CATENIN-INDEPENDENT WNT SIGNALING IN DEVELOPMENT

Before discussing the initiation and progression of alternative Wnt-signaling responses, I shall introduce some of the physiological processes that are controlled by β -catenin-independent Wnt signaling in vivo. In general, rather than affecting cell proliferation or cell fate specification, alternative Wnt signaling controls various aspects of cell migration and polarity, thereby having profound effects on tissue morphogenesis.

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Planar Cell Polarity

The process in which cells align themselves within the plane of a tissue is called planar cell (or tissue) polarity (PCP). Although it is observed in a wide variety of tissues, the two best-known examples are the uniform array of distally pointing hairs in the fly wing and the coordinated polarization of cochlear hair cells in the mammalian inner ear (Fig. 1A,B). However, PCP can also encompass the alignment of larger structures rather than individual cells, such as the multicellular ommatidia in the compound fly eye, and even entire hair follicles in

the mammalian skin (Montcouquiol et al. 2003; Devenport and Fuchs 2008; Jenny 2010; Maung and Jenny 2011).

Although the Wnt-pathway components frizzled (fz) and dishevelled (dsh) are core PCP proteins (Fig. 1C), it should be stressed that to date there is no evidence for the involvement of a ligand in either the establishment or maintenance of PCP in the *Drosophila* wing and ommatidia (Chen et al. 2008). This issue has been long debated, however, and recent evidence suggests that this might not hold true for all tissues (Shimizu et al. 2011). Furthermore, Wnt proteins have been implicated in PCP in vertebrates and

Figure 1. Planar cell polarity (PCP) as an example of alternative Wnt signaling. (A, top) PCP in the sensory epithelium of the mammalian cochlea manifests itself in the alignment of inner ear hair cells, which carry characteristic V-shaped stereociliary bundles of actin hairs on the apical cell surface (visualized here by phalloidin staining in red; kinocilia are stained green with an antibody against acetylated tubulin). (Bottom) PCP defects are scored based on the misorientation of these stereociliary bundles on inner (IHC) and outer (OHC) hair cells in whole-mount preparations of the organ of Corti. (B, top) In a textbook example of PCP, the surface of the fly wing is covered with a neatly organized array of hairs, all of which point toward the distal side. (Bottom) Defects in PCP are typically scored based on the misorientation of wing hairs. (C) Molecular control of PCP in the *Drosophila* wing. Complexes of the transmembrane proteins flamingo and vang, together with the cytoplasmic protein prickle, accumulate at the proximal end of the cell. At the distal border, flamingo and frizzled accumulate together with dishevelled and diego. Interactions between the extracellular domains of flamingo, frizzled, and vang are thought to play an important role in maintaining these complexes at opposite sides of the cell boundary (Chen et al. 2008; Wu and Mlodzik 2008). In addition, diego has been proposed to promote fz/dsh signaling, whereas vang/pk is thought to prevent recruitment of dsh to the cell membrane, something that is a prerequisite for PCP signaling (Tree et al. 2002; Das et al. 2004; Jenny et al. 2005).

Caenorhabditis elegans (Dabdoub et al. 2003; Dabdoub and Kelley 2005; Wu and Herman 2006; Qian et al. 2007; Yamamoto et al. 2008).

Convergent Extension

The same core players that control PCP have been shown to regulate a seemingly unrelated developmental process, called convergent extension (CE) (Keller and Danilchik 1988; Warga and Kimmel 1990; Wallingford et al. 2000). This morphogenetic event refers to the simultaneous lengthening and narrowing of a tissue as the result of directional cell intercalation. As such, CE movements control extension of the primary body axis and neural tube closure (Keller et al. 1985; Davidson and Keller 1999). For experimental reasons, CE is mostly studied in *Xenopus* and zebrafish, in which gastrulation defects are relatively easy to score. In addition, CE can be monitored and manipulated in *Xenopus* animal cap assays (Wilson et al. 1989; Hertzano et al. 2004). Both Wnts and Frizzleds (Fzds), as well as Dishevelled (Dvl) and other core PCP proteins, have been implicated in CE in amphibians, fish, and mammals (Djiane et al. 2000; Heisenberg et al. 2000; Carreira-Barbosa et al. 2003; Kilian et al. 2003; Torban et al. 2004; Moeller et al. 2006; Wang et al. 2006a; Kim et al. 2008). However, despite the fact that PCP and CE are often used interchangeably when discussing the role of alternative Wnt signaling, it is important to bear in mind that they are two distinct biological phenomena that are often studied in different species. Although they share the same core components, different downstream effector mechanisms are therefore prone to exist, and PCP and CE are unlikely to be controlled by completely identical signal transduction events (Fig. 2) (see also the section on Downstream Signaling Events). Future studies will have to determine how similar (or different) their underlying machineries really are.

Other Developmental Consequences of Defects in Alternative Wnt Signaling

Because the proteins previously implicated in PCP and CE are found to control multiple aspects of mammalian development, the possibility

arises that these processes may underlie polarized cell movements in general. Indeed, the developmental consequences of deregulated alternative Wnt signaling are substantial and include deficits in left/right patterning (Zhang and Levin 2009; Antic et al. 2010; Song et al. 2010), branching morphogenesis (Shabani et al. 2008; Karner et al. 2009; Yates et al. 2010), cardiac outflow tract formation (Hamblet et al. 2002; Phillips et al. 2005; Etheridge et al. 2008), intestinal elongation (Cervantes et al. 2009; Yamada et al. 2010), limb outgrowth (Yamaguchi et al. 1999; Afzal et al. 2000; van Bokhoven et al. 2000; Schwarzer et al. 2009; Person et al. 2010; Gao et al. 2011), and neural tube closure (Kibar et al. 2001, 2011; Montcouquiol et al. 2003; Seo et al. 2011).

Of note, not all β -catenin-independent responses to Wnt-ligand stimulation have been definitively linked to PCP/CE. However, recent reports showing the hitherto unknown involvement of PCP proteins other than Fzd and Dvl in Wnt-mediated axon guidance and differentiation underscore that this may simply be a matter of time (Shafer et al. 2011; Shimizu et al. 2011).

ALTERNATIVE WNT-SIGNALING MECHANISMS

Initiation of the Wnt-signaling response is first and foremost decided at the level of ligand and receptor binding. In all animal species, however, both Wnts and Fzds belong to large, multigene families, complicating a comprehensive analysis of their signaling activities. Further diversification of the signaling response comes from the use of different (co-)receptors and cytoplasmic effectors. With this in mind, future studies have to take into account that the outcome of a given Wnt-signaling event is determined by the ligand and receptor context and the developmental history of the receiving cell.

At the tissue level, the response is ultimately controlled by a “Wnt code” or “Wnt landscape” that integrates the complex expression patterns and different signaling inputs of multiple Wnt ligands and (ant)agonists (Gleason et al. 2006; Guder et al. 2006; Green et al. 2008; Zinovyeva et al. 2008; van Amerongen and Nusse 2009; Janssen et al. 2010; Fossat et al. 2011).

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Figure 2. Knowledge gaps. In the absence of robust and quantitative readouts, it has remained difficult to dissect the biological processes (*left*) in which alternative Wnt-signaling components (*center*) play a role in distinct signal transduction events (*right*). For instance, despite the fact that multiple Wnt-pathway components show a genetic interaction with core PCP/CE proteins (boxed in the *center*), it remains unknown how Wnts and their (co-)receptors are tied to the core PCP/CE machinery at the biochemical level. As an example, the core PCP/CE protein *vangl*/Vangl2 plays a central role in tissue morphogenesis. Animals deficient for *vangl*/Vangl2 display severe defects in PCP (red ellipse; e.g., cochlear hair cell orientation) and CE (yellow ellipse; e.g., neural tube closure), as well as in developmental processes that have not (yet) been definitively linked to either PCP or CE (blue ellipse; e.g., lung branching morphogenesis). A genetic interaction with Vangl2 has been shown for both Wnt5a and Ror2, and Wnt5a/Ror2 has been reported to phosphorylate Vangl2 in the murine limb bud (Qian et al. 2007; Yamamoto et al. 2008; Gao et al. 2011). The most robust assay to monitor Wnt5a/Ror2 signaling in vitro is inhibition of the Wnt/ β -catenin-responsive TOPFLASH luciferase reporter (green ellipse). However, Wnt5a and Ror2 have also been reported to activate JNK and AP-1 in mammalian cell culture experiments (orange ellipse). It is unknown how far these two responses overlap and whether they involve Vangl2. Conversely, it is unknown whether either Wnt5a or Ror2 plays a direct role in the diverse tissue-morphogenetic events in which Vangl2 has been implicated.

Use of Alternative Wnt Ligands

From the moment it became clear that Wnt proteins were members of an evolutionarily conserved family of signaling molecules (Baker 1987; Rijsewijk et al. 1987), attempts were made to classify them into groups with distinct properties. Two assays that were regularly used for this were the morphological transformation of C57MG cells and axis duplication in *Xenopus* embryos. The former scores the ability of individual Wnt proteins to change these cells from a cuboidal to a spindle-like cell shape (Jue et al. 1992), whereas the latter makes use of the fact that ectopic Wnt/ β -catenin signaling on the ventral side of the early embryo will induce formation of a secondary body axis (McMahon and Moon 1989; Sokol et al. 1991). Although no one has ever reported an exhaustive side-by-

side comparison of the behavior of all 19 mammalian Wnt genes in both assays, it was clear that the capacity of some Wnt proteins (e.g., Wnt3a), but not others (e.g., Wnt5a), to induce transformation and cause an increase in the cellular levels of β -catenin in C57MG cells (Wong et al. 1994; Shimizu et al. 1997) corresponded to their ability to induce axis duplication in *Xenopus* (Du et al. 1995). The transforming Wnts became known as “canonical” and were assumed to activate β -catenin/TCF signaling. In contrast, the non-transforming or “non-canonical” Wnts were thought to use alternative signaling mechanisms. Since then, Wnt3a and Wnt5a have become the prototypes for either class.

Although valuable, these assays do have some drawbacks. For instance, the C57MG morphological transformation assay is not quantifiable and therefore difficult to interpret, especially for

weak transforming genes (e.g., Wnt6, Wnt7B), which might explain why they have an effect in some studies, but not others (Wong et al. 1994; Shimizu et al. 1997). More importantly, the strict subdivision into canonical and non-canonical Wnts is a vast oversimplification, and an important caveat of these early classification attempts is the fact that they overlooked the importance of receptor context.

Instead of inducing axis duplication upon injection into early *Xenopus* embryos, exogenous Wnt5a does not affect development until after the onset of gastrulation, ultimately resulting in complex head and tail malformations. In addition, Wnt5a blocks the elongation of blastula cap explants, first suggesting a role in controlling the CE movements associated with gastrulation (Moon et al. 1993). However, Wnt5a was subsequently shown to be able to induce an ectopic axis when it was coinjected with Fz5 (He et al. 1997). Likewise, Wnt5a was found to activate β -catenin/TCF signaling when it was added to 293 cells overexpressing a combination of Fzd4/LRP5 receptors (Mikels and Nusse 2006). Furthermore, when Wnt5a or Wnt11 was fused to Fzd5, both were able to induce a secondary axis in *Xenopus* embryos—something that is never observed for either Wnt alone (Holmen et al. 2002).

Importantly, in vivo studies support the idea that individual Wnt proteins can have different functions depending on the time and place of their activity. Formation of the primary body axis in *Xenopus* depends on Wnt/ β -catenin signaling on the dorsal side of the fertilized oocyte. Quite surprisingly, maternal Wnt5a and Wnt11, at the time generally considered non-canonical Wnt proteins, were shown to be required for this process (Tao et al. 2005; Cha et al. 2008). Even more remarkable was the discovery that Wnt5a and Wnt11 function together in a multimeric complex to induce Wnt/ β -catenin signaling during primary axis formation (Cha et al. 2009). Yet the same proteins clearly control β -catenin-independent gastrulation events at subsequent stages of development (Cha et al. 2008), as illustrated by the fact that both the Wnt11/*Silberblick* and Wnt5a/*Pipetail* zebrafish mutants display defects in convergent ex-

tension movements (Heisenberg et al. 2000; Kilian et al. 2003).

Thus, the same Wnt proteins can have both β -catenin-dependent and -independent functions depending on the cellular context, suggesting that any definitive conclusions regarding their signaling activity should be made on a case-by-case basis. That being said, it is a fact that some Wnts are less often associated with β -catenin/TCF signaling than others. Precisely what controls ligand and receptor affinity or intrinsic differences in signaling capacity remains to be determined.

Alternative Use of Frizzled and Dishevelled

Genetic screens in *Drosophila* provide a powerful tool for dissecting signaling pathways. Using epistasis experiments, it was shown that *wingless* (*wg*/Wnt) functioned upstream of a *frizzled* (*fz*/Fzd) transmembrane receptor and used *dishevelled* (*dsh*/Dvl) and *zeste-white* (*zw*/GSK3) to ultimately signal through *armadillo* (*arm*/ β -catenin) and *pangolin* (*pan*/TCF), thereby providing a blueprint of the now well-known Wnt/ β -catenin pathway (Siegfried et al. 1992, 1994; Noordermeer et al. 1994; Bhanot et al. 1996; van de Wetering et al. 1997). In addition to controlling segment polarity, however, both *fz* and *dsh*, but not *arm*, were found to be required for the establishment of PCP in collaboration with a completely distinct set of signaling partners (Vinson et al. 1989; Wong and Adler 1993; Axelrod et al. 1998). These include *flamingo*, *vang*, and *prickle* (*Celsr1/2/3*, *Vangl1/2*, and *Prickle1/2/3* in vertebrates) (see also Fig. 1C) (see section on Core PCP/CE Proteins).

Frizzled

Similar to the situation observed for Wnts, not all Frizzled proteins are equal. The mammalian genome encodes ten Frizzled proteins. Of these only Fzd3 and Fzd6 have been implicated in PCP signaling so far (Guo et al. 2004; Montcouquiol et al. 2006; Wang et al. 2006b, 2010). Of the four *frizzled* receptors present in *Drosophila*, *fz* and *fz2* function redundantly in transmitting the *wg/arm* signal (Bhat 1998; Kennerdell and

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Carthew 1998; Bhanot et al. 1999). However, *fz2* has a higher affinity for *wg*, and only *fz* is required for PCP signaling (Rulifson et al. 2000) or capable of recruiting *dsh* to the cell membrane (Axelrod et al. 1998).

An intrinsic difference between the two receptors was shown to reside in their cytoplasmic tails, which affected both their subcellular localization and their capacity to activate either the Wnt/ β -catenin or the PCP pathway (Boutros et al. 2000; Wu et al. 2004). Despite the fact that no Wnt protein appears to be required for PCP signaling in the *Drosophila* wing, the *fz* cysteine-rich domain (CRD), which constitutes the *wg* binding site, is indispensable and might interact with *flamingo/vang* complexes on adjacent cells (Wu and Herman 2006; Wu and Mlodzik 2008).

Again, however, the involvement in either Wnt/ β -catenin or alternative signaling responses is not black and white. Even though certain Fzds may be more prone to participate in one pathway than the other, a single Fzd receptor can have multiple signaling activities. For instance, Fzd7 has been well documented to control β -catenin-dependent as well as -independent developmental events in *Xenopus* (Medina et al. 2000; Sumanas et al. 2000; Abu-Elmagd et al. 2006). In addition, overexpression of Fzd7 was shown to inhibit convergent extension movements through Dvl (Djiane et al. 2000), whereas loss of Fzd7 was shown to disrupt the separation of mesodermal and ectodermal tissues in a Dvl-independent manner (Medina et al. 2000; Winklbauer et al. 2001). Similarly, Fzd2 has been linked to both β -catenin-dependent as well as -independent responses in mammalian cells (Liu et al. 1999; Ma and Wang 2007; Verkaar et al. 2009; Sato et al. 2010). This shows that, similar to Wnts, individual Fzds can signal through multiple independent branches (see below), only some of which may require Dvl.

Dishevelled

The first indication for a dual signaling role for Dishevelled (Dvl) came from the identification of the *Drosophila dsh¹* allele, which causes defects in PCP, but not in Wnt/ β -catenin signal-

ing (Adler 1992). Since then, much effort has been made to map the domains that are required for the different signaling activities of Dvl. The protein contains three well-conserved domains: an amino-terminal DIX domain, a central PDZ domain, and a carboxy-terminal DEP domain. Of these, the DIX domain is critically required for inducing Wnt/ β -catenin signaling. It has been shown to mediate dynamic polymerization, resulting in the recruitment of Fzd/LRP complexes into large signalosomes (Bilic et al. 2007; Schwarz-Romond et al. 2007a,b; Fiedler et al. 2011). Deletion of the DIX domain renders Dvl functionally inactive in a Wnt/ β -catenin-responsive TOPFLASH reporter assay as well as in *Xenopus* axis duplication experiments. Conversely, the PDZ and DEP domains are largely dispensable for axis duplication (Rothbacher et al. 2000). Instead, deletion mutants lacking the DEP domain are defective in disrupting CE movements (Wallingford et al. 2000).

The observation that wild-type *dsh* was recruited to the plasma membrane by *fz* (the receptor involved in PCP signaling), but not by *fz2* and *wg* (the ligand/receptor combination that controls *wg/arm* signaling), in *Xenopus* animal cap cells suggested that membrane localization was critical for the role of *dsh* in establishing PCP/CE (Axelrod et al. 1998). In support of this, the mutant *dsh* protein encoded by the *dsh¹* allele was found to contain a K $\rightarrow$ M transition mutation in the DEP domain and had lost its capacity to be recruited to the cell membrane.

These observations were elegantly confirmed in the mouse, using an allelic series of BAC transgenes (Wang et al. 2006a). The mammalian genome encodes three *Dvl* homologs, and mice deficient for both *Dvl1* and *Dvl2* display severe neural tube closure defects as well as cochlear hair cell orientation defects, similar to mice that carry a mutated version of the core PCP gene *Vangl2* (Kibar et al. 2001; Hamblet et al. 2002; Wang et al. 2005). The neural tube closure defects observed in the *Dvl1^{-/-}*; *Dvl2^{-/-}* mutants could be rescued by either a *Dvl2* or a *Dvl2 Δ DIX*, but not by a *Dvl2 Δ DEP* BAC transgene or by a *Dvl2* BAC transgene carrying a single point mutation identical to that observed in the *Drosophila dsh¹* allele (Wang et al. 2006a).

Summarizing, the amino-terminal DIX domain of Dvl is dispensable, whereas its carboxy-terminal DEP domain is required for PCP/CE signaling. The opposite holds true for Wnt/ β -catenin signaling. In addition to differences in subcellular localization, the different pools of Dvl also appear to use different downstream effectors to activate the different branches of the Wnt pathway (see below).

Alternative Receptors

The interaction with Wnt ligands is mediated by the Fzd CRD domain. This same domain, which is also used by the extracellular antagonists of the secreted Frizzled-related protein (SFRP) family, was found to be present in the otherwise completely unrelated tyrosine kinase receptors Ror1 and Ror2 (Saldanha et al. 1998). A different Wnt binding domain, used by the extracellular Wnt-inhibitory factor (WIF), resides in the extracellular domain of the tyrosine kinase-related receptor Ryk (Yoshikawa et al. 2003; Inoue et al. 2004; Lu et al. 2004). Since then, both Ryk and Ror have been shown to be bona fide Wnt-pathway components that have been linked to both β -catenin-dependent and -independent Wnt-signaling events.

Ror

The single-pass transmembrane proteins of the Ror family are evolutionarily conserved across vertebrate and invertebrate species, including *Drosophila*, *C. elegans*, *Xenopus*, mice, and humans. The genomes of most species harbor two homologs. Studies performed in mammals to date have focused on the role of Ror2, but both Ror1 and Ror2 have been shown to mediate alternative Wnt signaling (Paganoni et al. 2010), although at present the experimental data support a role for Ror1 as a pseudokinase (Gentile et al. 2011). The fact that Ror1 as well as Ror2 can bind Wnt ligands directly (Oishi et al. 2003; Mikels and Nusse 2006; Fukuda et al. 2008; Liu et al. 2008) suggests that both can function as bona fide Wnt receptors. Indeed, Ror2 has been shown to interact with multiple Wnt proteins (Billiard et al. 2005; Mikels and Nusse 2006; Winkel et al. 2008).

Alternative Wnt Pathways and Receptors

Ror2-deficient mice present, among others, with craniofacial abnormalities and shortened limbs. Their external appearance somewhat resembles that of Wnt5a-null mice, suggesting that Wnt5a and Ror2 might have a similar function during embryonic development (Oishi et al. 1999; Yamaguchi et al. 1999; Schwabe et al. 2004). Indeed, Ror2 has since been shown to transmit a Wnt5a signal that results in inhibition of the Wnt/ β -catenin signaling response (Mikels and Nusse 2006; Mikels et al. 2009). One of the earliest indications of the existence of such an inhibitory branch of the pathway was the observation that *Xenopus* axis duplication induced by *XWnt1* could be inhibited by *XWnt5a* (Torres et al. 1996). Since then, Wnt5a has been shown to counteract Wnt/ β -catenin signaling in a broad range of cells and tissues (MacLeod et al. 2007; Nemeth et al. 2007; Roarty et al. 2009).

One possible mechanism by means of which Ror (or Fzd proteins for that matter) can affect Wnt signaling is by the competition for and sequestration of other Wnt ligands. This mechanism has been shown to exist for the Ror homolog *cam-1* in *C. elegans* (Green et al. 2007). However, the Wnt5a/Ror2-mediated inhibition of Wnt/ β -catenin signaling was shown to require an intact tyrosine kinase domain (Liu et al. 2008; Mikels et al. 2009), suggesting an active signaling function. In contrast, Ror2 has also been reported to enhance Wnt/ β -catenin signaling as a coreceptor together with Fzd, independent from its intracellular, carboxy-terminal domain (Li et al. 2008), suggesting that Ror may have both kinase-dependent and -independent functions. The final verdict is also still out on whether Ror2 mainly functions alone or in concert with other receptors (Mikels and Nusse 2006; Li et al. 2008; Yamamoto et al. 2008; Nishita et al. 2010; Sato et al. 2010). Studies that have analyzed ligand/receptor complex formation have mostly depended on protein overexpression, and as such, it is unknown to what extent these complexes exist at the physiological level. Furthermore, despite the binding partners and potential effectors that have been reported for Ror2, the precise molecular makeup of the Ror2 signaling cascade remains unknown (Matsuda et al. 2003; Sammar et al. 2004, 2009; Liu

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et al. 2007; Winkel et al. 2008; van Wijk et al. 2009; Feike et al. 2010; Witte et al. 2010). Wnt5a/Ror2-mediated inhibition of Wnt/ β -catenin signaling does not appear to involve NFAT, CamKII, or calcium fluxes (Topol et al. 2003; Mikels and Nusse 2006). Although Wnt5a has been shown to induce the expression of the E3 ubiquitin ligase *Siah2* in a human colon adenocarcinoma cell line, resulting in the degradation of β -catenin (Topol et al. 2003), other data suggest that the Wnt5a/Ror2-mediated inhibition of Wnt/ β -catenin signaling occurs downstream from β -catenin nuclear entry (Verkaar et al. 2010). In agreement with the latter, we found no evidence of a transcriptional response following stimulation of Ror2-expressing 293 cells with purified Wnt5a, even though the Wnt3a-mediated induction of *Axin2* expression was prevented (R van Amerongen and R Nusse, unpubl.). Of note, Wnt5a/Ror2 signaling has also been reported to inhibit convergent extension movements through the activation of JNK/AP-1 (Oishi et al. 2003). Similar to Fzd, therefore, Ror proteins might elicit multiple, independent signaling responses.

Interestingly, a novel Ror1/Ror2 double-knockout mouse model was recently reported to resemble the Wnt5a-null phenotype to a larger extent than existing models. Yet, in mouse embryo fibroblasts, the combined loss of Ror1 and Ror2 was shown to solely affect the phosphorylation of Dvl, but not the phosphorylation of c-Jun or the Wnt5a-mediated inhibition of Wnt/ β -catenin signaling (Ho et al. 2012). Thus, to what extent all of the aforementioned signaling activities of Wnt5a and Ror2 play a role under physiological conditions remains to be determined. In either case, it appears that Wnt5a/Ror signaling controls complex cell movements during embryonic development.

Ryk

Ryk transmembrane receptors are also conserved across vertebrate and invertebrate species. Mammalian Ryk is critically required for axon guidance and neurite outgrowth in response to multiple Wnt ligands (Lu et al. 2004; Keeble et al. 2006). It was shown to bind both

Fzd and Dvl and to be essential for Wnt/ β -catenin signaling (Lu et al. 2004; Berndt et al. 2011). Similar to Fzd and Ror, Ryk can have different functions depending on the cellular context. For instance, the fly homologs *derailed1* and *derailed2* play opposing roles in Wnt5a signaling during development of the *Drosophila* olfactory system (Sakurai et al. 2009).

Mammalian Ryk, as well as its *C. elegans* homolog *lin-18*, are catalytically inactive, suggesting a coreceptor function. Indeed, although the extracellular WIF domain of *lin-18* and *derailed* was required, the intracellular atypical kinase domain was found to be largely dispensable for their function (Inoue et al. 2004; Taillebourg et al. 2005). Although Ryk has mostly been studied in a neurobiological context, it was also shown to regulate gastrulation movements. In *Xenopus*, it was found to cooperate with Fzd7 and Wnt11 during CE (Kim et al. 2008), whereas in zebrafish, it was shown to control directional cell movements in response to Wnt5b (Lin et al. 2010). Similar to Ror1 and Ror2, at present the signaling mechanism downstream from Ryk remains incompletely understood. Interestingly, Ryk was shown to undergo cleavage, after which the carboxy-terminal fragment translocated to the nucleus in response to Wnt3 stimulation. This processing appears to be essential for the function of Ryk in controlling the differentiation of neuronal progenitor cells (Lyu et al. 2008, 2009). Ryk signaling during axon guidance in *Drosophila* was shown to require Src kinases (Wouda et al. 2008). However, at present there is no evidence that the Src-mediated signaling events involve Ryk cleavage.

Taken together, multiple independent responses are likely to exist downstream from ligand and receptor binding for both Frizzled and non-Frizzled receptors. Future studies will have to be aimed at understanding how specificity is achieved in light of such dynamic interactions.

Downstream Signaling Events

Most alternative Wnt-signaling responses remain ill characterized at the molecular level. The main reason for this stems from the fact

that it has proven difficult to find a reliable experimental system in which to study alternative Wnt-signal transduction events to begin with, because complex developmental and tissue morphogenetic processes such as PCP and CE are not easily translated to a tissue culture dish (Endo et al. 2012). Molecular analyses have been further complicated by the fact that alternative Wnt signaling often leads to cytoskeletal and migratory changes, rather than a transcriptional response, resulting in the lack of specific and easily quantifiable in vitro readouts, analogous to the TOPFLASH reporter assay for Wnt/ β -catenin signaling.

Unlike β -catenin/TCE, many of the implicated downstream components (e.g., small Rho GTPases, JNK/AP-1, Ca^{2+} /NFAT) are not exclusive to alternative Wnt signaling and instead play a role in a vast array of cellular processes. Furthermore, in contrast to later-generation TOPFLASH constructs, existing luciferase reporters for monitoring AP-1 and NFAT signaling, for instance, show low-amplitude responses following stimulation with Wnt ligands, and it is probably safe to say that they have cost many a researcher a headache. The fact that many of these readouts are not as robust as the typical TOPFLASH reporter assay has surely contributed to some disagreement as to whether the measured responses are “real,” and at present there is no consensus in the field that these reporters truly reflect alternative Wnt signaling.

Core PCP/CE Proteins

PCP is established by the asymmetric distribution of the so-called core PCP proteins at the cell membrane (Fig. 1C) (for review, see Bayly and Axelrod 2011; Goodrich and Strutt 2011; Gray et al. 2011). In cells of the fly wing, flamingo/vang/prickle complexes accumulate on the proximal side of the cell, whereas complexes of flamingo/fz/dsh/diego localize to the distal side. The asymmetric localization of Fzd, Vangl, and Prickle is also observed in cochlear hair and support cells, suggesting that a comparable, although not identical, mechanism operates in mammals (Montcouquiol et al. 2006; Deans et al. 2007).

These same proteins have also been shown to mediate CE movements in frogs and fish (Djiane et al. 2000; Wallingford and Harland 2001; Darken et al. 2002; Veeman et al. 2003) and/or to control neural tube closure in mice (Curtin et al. 2003; Wang et al. 2006a; Torban et al. 2008).

One interesting characteristic is that both gain- and loss-of-function mutants for core PCP/CE proteins display typical (albeit not identical) PCP/CE phenotypes, suggesting that it is the correct gradient and not the absence or presence of signaling per se that is required for proper PCP/CE (Krasnow and Adler 1994; Djiane et al. 2000; Darken et al. 2002).

PCP/CE Effectors

Precisely how the core PCP/CE machinery connects to its downstream effectors remains unknown (Fig. 2). The first insight into the PCP signaling cascade came from *Drosophila*. Flies deficient for *rhoA* (a small Rho GTPase) and *jun* (an AP-1 transcription factor) presented with typical PCP defects in the eye and the wing (Kockel et al. 1997; Strutt et al. 1997; Weber et al. 2000). These and other genes, namely, *hep* (MKK7), *bsk* (JNK), and the small Rho GTPase *rac*, but not *cdc42*, were also mapped into the PCP pathway in genetic epistasis experiments, based on the ability of their loss-of-function mutants to rescue the PCP defects induced by overexpression of either *fz* or *dsh* (Strutt et al. 1997; Boutros et al. 1998).

Other genes shown to be involved in *Drosophila* PCP signaling downstream from *fz* and *dsh* are *inturned*, *fuzzy*, and *multiple wing hairs*, which together appear to be responsible for restricting wing hair formation to the distal side of the cell (Adler 1992; Lee and Adler 2002). However, these effectors only control PCP in the wing and not in the ommatidia. Conversely, *nemo* was found to be required for PCP in the fly eye, but not the wing (Choi and Benzer 1994).

The vertebrate orthologs of *inturned* and *fuzzy* are required for ciliogenesis. As a result of aberrant cilia formation, both mice and frogs deficient for *Inturned* or *Fuzzy* have defects in Hedgehog signaling (Park et al. 2006; Gray et al. 2009; Zeng et al. 2010). However, unlike the loss

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of core PCP proteins such as Vangl2, loss of Inturned or Fuzzy does not result in clear CE defects. Moreover, unlike Wnt5a or Ror2, neither Inturned nor Fuzzy shows a genetic interaction with Vangl2 in neural tube closure (Heydeck and Liu 2011). In contrast, the vertebrate ortholog of *nemo*, Nemo like kinase (NLK), genetically interacts with Wnt11 in CE during zebrafish development (Thorpe and Moon 2004). Together, these findings not only underscore that important differences may exist between the signal transduction events that control PCP and those that regulate CE, but also that even within the same species, the effectors that function downstream from the core PCP/CE protein complexes are likely to be tissue specific.

Rho, Rac, and Cdc42 in Vertebrates

The small Rho GTPases Rho, Rac, and Cdc42 have all been implicated in alternative Wnt-signal transduction in vertebrates, but their functions appear to be divergent. Evidence for the involvement of Cdc42 comes from the observation that exogenous Cdc42 affects *Xenopus* CE movements and the demonstration that Cdc42 is activated downstream of Wnt, Fzd, and PKC (Djiane et al. 2000; Choi and Han 2002; Penzo-Mendez et al. 2003). Both endogenous Rho and Rac were shown to be required for CE movements during gastrulation in the frog. In addition, the overexpression of Dvl1 and Dvl2, as well as of Fzd1 and Fzd7 but not Fzd2 or Fzd5, resulted in the activation of Rho and Rac, but not Cdc42, in 293T cells (Habas et al. 2001, 2003).

Although both Rho and Rac can activate JNK (Li et al. 1999; Moriguchi et al. 1999; Habas et al. 2001, 2003; Marinissen et al. 2004; Kim and Han 2005; Rosso et al. 2005), it has been suggested that Dvl activates two separate branches in mammalian cells. The first results in the activation of Rho, but not JNK, and requires full-length Dvl, whereas the second results in the activation of Rac and JNK and requires only the Dvl DEP domain (Habas et al. 2003). The Dvl-mediated activation of Rho was, in fact, shown to actively repress the activation of Rac during the directional migration of neural crest cells in developing zebrafish embryos (Matthews et al. 2008).

JNK and AP-1 in Vertebrates

Many experiments in amphibians, mice, and mammalian cell lines have placed JNK and AP-1 signaling downstream from a Wnt-signaling cascade. For instance, in *Xenopus* animal cap explants, the expression of dominant-negative JNK or its activator MKK7 rescued CE movements that had been blocked by exogenous Wnt5a (Yamanaka et al. 2002). Likewise, in mice deficient for Wnt9B, which develop cystic kidneys, the levels of activated JNK2 were found to be significantly decreased (Karner et al. 2009).

Wnt as well as Dvl proteins have also been shown to activate JNK in mammalian NIH3T3 and 293T cells (Boutros et al. 1998; Li et al. 1999; Habas et al. 2003). In addition, both Wnt and Dvl can activate AP-1 transcriptional complexes, causing AP-1 luciferase reporters to be used as a surrogate readout to monitor alternative Wnt-signaling responses (Li et al. 1999; Tufan et al. 2002; Yamanaka et al. 2002; Le Floch et al. 2005). A more specific and robust assay was recently described in the form of a luciferase reporter based on an ATF2 response element (Ohkawara and Niehrs 2011). This reporter was used in *Xenopus* embryos to monitor the effects of both gains and losses of Wnts, their receptors, and various intracellular mediators, including JNK. At present it remains unknown, however, whether this reporter can be used to monitor alternative Wnt-signaling responses in other species as well.

Other Effectors: G-Proteins, Ca²⁺, and NFAT

Not all alternative Wnt-signaling events have been linked to PCP and/or CE processes, and this article would not be complete without discussing them.

Frizzled proteins have officially been classified as a distinct class within the superfamily of G-protein-coupled receptors (GPCRs), although they lack certain features that are well conserved in other, conventional GPCRs (Schulte 2010).

It should be noted that, to date, controversy remains in the field regarding the requirement of G-proteins for Wnt-signal transduction,

despite the fact that they have been implicated in both Wnt/ β -catenin and alternative signaling responses. For instance, RNAi-mediated depletion of $G\alpha_o$ and $G\alpha_q$ or treatment with the G-protein inhibitor pertussis toxin (PTX) was reported to prevent the Wnt3a-mediated increase in cytosolic β -catenin levels in mammalian cells (Liu et al. 2005), whereas in *Drosophila*, G_o hypomorphic clones showed compromised expression of *wg* target genes (Katanaev et al. 2005). Until G-proteins are shown to be directly required for transmitting Wnt signals by using a genetic knockout approach in mice, the GPCR nature of Fzds will likely remain contested.

Signaling through GPCRs generally results in the activation of heterotrimeric G-proteins, phospholipase C, and Ca^{2+} fluxes. The first evidence for the existence of a Wnt/ Ca^{2+} pathway came from experiments in zebrafish, where the injection of ectopic Wnt5a and Fzd2, but not Wnt8 or Fzd1, was shown to cause an increase in the frequency of intracellular Ca^{2+} transients (Slusarski et al. 1997a,b). Calcium fluxes control the activity of multiple intracellular proteins, including PKC, CamKII, and calcineurin, and, indeed, all of these have been implicated in alternative Wnt signaling. For instance, Wnt5a and Fzd2 were also shown to promote the translocation of PKC from the cytoplasm to the cell membrane (Sheldahl et al. 1999), whereas Wnt5a and Wnt11 were shown to activate CamKII (Kuhl et al. 2000). All of these activities could be inhibited by pertussis toxin (PTX), as could the aforementioned effect of Fzd7 on tissue separation in *Xenopus* (Winklbauer et al. 2001), suggesting the involvement of G-proteins.

In *Xenopus* animal cap explants, Wnt5a and Fzd2 were shown to promote the nuclear translocation of NFAT (Saneyoshi et al. 2002). This process is controlled by the calcium-dependent phosphatase calcineurin, thereby indicating NFAT as a downstream effector of the Wnt/ Ca^{2+} pathway. Indeed, Wnt5a can induce slight activation of an NFAT luciferase reporter in a Dvl-dependent manner in mouse F9 teratocarcinoma cells overexpressing Fzd2 (Ma et al. 2010). However, the precise requirement for Dvl in Wnt/ Ca^{2+} signaling remains to be deter-

mined (Winklbauer et al. 2001; Sheldahl et al. 2003).

CONCLUDING REMARKS

Probing any of the alternative Wnt-signaling responses discussed above will remain a challenge until their precise molecular makeup is unraveled. For this, more specific players need to be identified, and the exact temporal order of signaling events needs to be revealed. One of the major challenges for future research will be to grasp how the different Wnt-signaling responses interact during development. For instance, Wnt/ β -catenin and Wnt/ Ca^{2+} signaling were shown to have opposing activities in convergent extension, with PKC and CamKII impacting on the signaling activities of β -catenin (Kuhl et al. 2001). Likewise, distinct Wnt-signaling pathways were shown to have opposing roles in tail fin regeneration (Stoick-Cooper et al. 2007). In *Drosophila*, an attractive *wnt4/fz* and a repulsive *wnt5/ryk* signal together control salivary gland migration (Harris and Beckendorf 2007). As such, multiple Wnt pathways might collaborate in controlling complex morphogenetic events, by each regulating different aspects of cell polarization and directional migration. For instance, development of the organ of Corti in the cochlea, which has become the mammalian paradigm for studying PCP, displays aspects of both PCP and CE. Whereas disruption of Vangl2 function causes the typical misorientation of inner ear hair cells associated with the former, loss of Wnt5a results in a shorter and wider cochlear duct, with an extra row of hair cells, more reminiscent of the latter (Montcouquiol et al. 2003; Qian et al. 2007). It is evident, however, that few if any of the extracellular and transmembrane components are exclusively dedicated to a single branch of the pathway. This includes proteins that were previously thought to be solely required for Wnt/ β -catenin signaling, such as Lrp6, showing that cross talk between the different branches might be more common than previously anticipated (Tahinci et al. 2007; Andersson et al. 2010).

A second aspect worthy of attention concerns the initiation of the different signaling

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events. At present, our understanding of how the cellular context determines the assembly of Wnt/(co-)receptor complexes, and how these complexes elicit specific responses remains ill understood. Recent data suggest that part of the answer may lie in the localization of receptor complexes to distinct microdomains within the cell membrane and corresponding different mechanisms of receptor internalization (Sato et al. 2010). As the literature on this topic is expanding, it is important to remember that at the same time many of the ligand and receptor combinations remain unexplored. As a result, we have only begun to uncover the tip of the iceberg when it comes to understanding the role of alternative Wnt signaling in development and disease.

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