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Frizzled Receptors in Development and Disease

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Abstract

Frizzled proteins are the principal receptors for the Wnt family of ligands. They mediate canonical Wnt signaling together with Lrp5 and Lrp6 coreceptors. In conjunction with Celsr, Vangl, and a small number of additional membrane and membrane-associated proteins, they also play a central role in tissue polarity/planar cell polarity (PCP) signaling. Targeted mutations in 9 of the 10 mammalian Frizzled genes have revealed their roles in an extraordinarily diverse set of developmental and homeostatic processes, including morphogenetic movements responsible for palate, ventricular septum, ocular furrow, and neural tube closure; survival of thalamic neurons; bone formation; central nervous system (CNS) angiogenesis and blood–brain barrier formation and maintenance; and a wide variety of processes that orient subcellular, cellular, and multicellular structures relative to the body axes. The last group likely reflects the mammalian equivalent of tissue polarity/PCP signaling, as defined in *Drosophila*, and it includes CNS axon guidance, hair follicle and tongue papilla orientation, and inner ear sensory hair bundle orientation. Frizzled receptors are ubiquitous among multicellular animals and, with other signaling molecules, they very likely evolved to permit the development of the complex tissue architectures that provide multicellular animals with their enormous selective advantage.

1. INTRODUCTION

Frizzled proteins are the principal receptors for the Wnt family of signaling molecules, and they are found throughout the animal kingdom, including in the most primitive metazoa, but they are not present in plants or in simpler (single cell) eukaryotes, such as yeast (Schenkelaars, Fierro-Constain, Renard, Hill, & Borchellini, 2015). Frizzled proteins share a common architecture: a conserved extracellular cysteine-rich domain (CRD) is followed by a domain with seven presumptive transmembrane segments. The genetic dissection of

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Frizzled function began in *Drosophila*, with the characterization of the *frizzled* (*fz*) phenotype by Gubb and Garcia-Bellido (1982). Loss-of-function mutations in *fz* lead to a tissue polarity phenotype, defined as a misorientation of surface structures, such as cuticular bristles and wing hairs, which normally exhibit a precise orientation relative to the body axes. This pathway is generally referred to by the not entirely accurate designation “planar cell polarity” (PCP).

The *Drosophila fz* gene was isolated by Vinson, Conover, and Adler (1989), and the first two vertebrate *Frizzled* homologues were identified several years later (Chan et al., 1992). A search for additional vertebrate *Frizzled* family members using degenerate PCR identified eight family members in mammals, as well as multiple family members in birds and fish (Wang et al., 1996). Two additional mammalian *Frizzled* genes were identified subsequently, bringing the total to 10 (Koike et al., 1999; Wang et al., 1997). The large number of vertebrate *Frizzled* genes together with their receptor-like structure led Wang et al. (1996) to suggest that “If Frizzled proteins act as receptors, that would imply the existence of a corresponding family of ligands. ... Currently the only family of ligands known to be of this size and for which no receptors have been identified are the Wnt proteins. The availability of a large number of *Frizzled* genes should facilitate a biochemical test of the possibility that these two families of proteins interact directly.” Within a year, that suggestion was confirmed with the demonstration that *Drosophila* Wingless binds to and promotes beta-catenin stabilization via Frizzled2 (Bhanot et al., 1996). These experiments also identified the Frizzled CRD as the site of Wnt binding (Bhanot et al., 1996; Hsieh, Rattner, Smallwood, & Nathans, 1999). The recently determined structure of a Wnt–Frizzled CRD complex shows an unusual ligand–receptor interaction characterized by a relatively small protein–protein interface and a relatively large interface between the CRD and a lipid that is covalently attached to the Wnt (Janda, Waghray, Levin, Thomas, & Garcia, 2012).

The identification of Frizzleds as Wnt receptors implied that Frizzleds act in at least two distinct signaling pathways: tissue polarity/PCP signaling (which may or may not involve a Wnt ligand) and canonical Wnt signaling. The former pathway appears to involve reorganization of the cytoskeleton and is characterized by a small set of asymmetrically localized plasma membrane proteins (including Frizzled) and membrane-associated cytosolic proteins (Goodrich & Strutt, 2011). The latter pathway involves Wnt–Frizzled-dependent disinhibition of a single-pass transmembrane coreceptor (Lrp5/Lrp6 in mammals; arrow in *Drosophila*) leading to inhibition of beta-catenin phosphorylation and proteolysis, with a resultant migration of beta-catenin into the nucleus to regulate transcription in combination with Lef/Tcf transcription factors (MacDonald, Tamai, & He, 2009; Nusse, 2012). A third pathway, the Wnt-calcium pathway, is less well defined and has been suggested to involve a G-protein-based transduction system (Wang & Malbon, 2003).

In this review, we focus on the roles of mammalian *Frizzled* genes *in vivo*, which have been largely defined by studying the effects of targeted mutations in mice. As seen in Fig. 1, a comparison of the amino acid sequences and intron–exon structures among the 10 mammalian *Frizzled* family members reveals branches consisting of Fz1, Fz2, and Fz7 (subfamily 1); Fz5 and Fz8 (subfamily 2); Fz9 and Fz10 (subfamily 3); Fz4 (subfamily 4); and Fz3 and Fz6 (subfamily 5, the most distant branch). Substantial genetic redundancy has

been observed between pairs of Frizzled genes within subfamilies 1, 2, and 5, but, thus far, there appears to be far less redundancy between *Frizzled* genes in different subfamilies. However, these data should be interpreted cautiously because the redundancy data are still incomplete: many of the 45 possible pairwise combinations of *Frizzled* gene knockouts—especially with *Frizzleds* from different subfamilies—have not been tested. Moreover, in many tissues, there is overlapping expression of multiple *Frizzleds*, as seen, for example, in the spiral ganglion in the inner ear (Shah, Kang, Christensen, Feng, & Kollmar, 2009), so that functional redundancy may involve more than two genes. The patterns of sequence relatedness also correlate with function: Fz3 and Fz6 appear to be devoted largely or exclusively to tissue polarity signaling, whereas Fz4 is devoted largely or exclusively to canonical Wnt signaling. Some mammalian Frizzleds could signal via more than one pathway, as shown for *Drosophila* Frizzled, which signals through both the canonical Wnt and PCP pathways (Bhanot et al., 1999; Bhat, 1998; Müller et al., 1999).

In the text that follows, we have organized the descriptions of *Frizzled* gene function by subfamily. We note that most of the phenotypic analyses in mice were performed on a mixed genetic background.

2. FRIZZLED1, FRIZZLED2, AND FRIZZLED7

Fz1^{-/-} mice show no apparent phenotype, whereas loss of *Fz2* leads to cleft palate and neonatal lethality in ~50% of mice and runting associated with reduced olfactory function in the other ~50% (Yu et al., 2010). Approximately 15% of *Fz7*^{-/-} fetuses have a ventricular septal defect (VSD), a common cardiac anomaly that results in mixing of blood between the left and the right ventricular chambers, while the remaining ~85% of *Fz7*^{-/-} mice have no apparent phenotype except for a kinked tail (Yu, Ye, Guo, & Nathans, 2012). Interestingly, a variety of additional phenotypes appear when *Fz1*, *Fz2*, and *Fz7* mutations are combined (Yu et al., 2010, 2012). 100% of *Fz1*^{-/-};*Fz2*^{-/-} fetuses have a cleft palate (Fig. 2), and approximately two-thirds of *Fz1*^{-/-};*Fz2*^{-/-} fetuses also have cardiac defects—either an isolated VSD or a combination of VSD and double outlet right ventricle, a cardiac anomaly in which the aorta is positioned partially over the right ventricular outflow tract.

Fz1^{-/-};*Fz7*^{-/-} mice have no additional defects beyond the *Fz7*^{-/-} phenotype except for a gray coat color, suggesting a defect in melanocyte migration and/or development. In contrast, *Fz2*^{-/-};*Fz7*^{-/-} embryos at embryonic day (E)8.5 show a severe loss of convergent extension, fail to close the neural tube, and fail to develop cardiac asymmetry (Fig. 3).

Given that Frizzleds control several signaling pathways (PCP, canonical Wnt, and Wnt-calcium signaling), it is of interest to explore the relationship between signaling pathways affected and developmental phenotypes observed. To this end, Yu et al. (2010, 2012) examined genetic interactions between mutations in *Fz1*, *Fz2*, and *Fz7* and in known PCP genes, such as *Vangl2* [one of the two mammalian homologues of the *Drosophila* core PCP gene *Van Gogh/Strabismus* (*Vang/Stbm*)]. When various combinations of *Fz1*, *Fz2*, or *Fz7* knockout (KO) alleles were examined in combination with one copy of the semidominant *Looptail* allele of *Vangl2* (*Vangl2*^{L^{pt}/+}), there was an enhanced frequency of neural tube closure and cleft palate defects. Similarly, the frequency of cardiac defects (mostly VSDs) was enhanced when the *Fz2*^{+/-};*Fz7*^{-/-} genotype was combined with one mutant copy of

Wnt3a (canonical signaling), *Wnt11* (noncanonical signaling), or *Dvl3* (both pathways). While these data show that *Fz1*, *Fz2*, and *Fz7* synergize with other canonical and noncanonical pathway components, they do not definitively indicate which pathway(s) is/are affected by the different *Frizzled* mutations. Palate, ventricular septum, and neural tube closures are known to involve multiple signaling pathways (Biggs, Goudy, & Dunnwald, 2015), and it is therefore plausible that defects in more than one Frizzled-based pathway combine to produce a developmental defect.

In humans, defects in tissue closure processes represent some of the most common congenital anatomic anomalies. Defects in closing the ventricular septum, palate, and neural tube occur, respectively, in ~0.4%, ~0.1%, and ~0.1% of live births, and a defect in closing the ventral urethra occurs in ~0.4% of newborn males (Au, Ashley-Koch, & Northrup, 2010; CDC, 1997; Dolk, Loane, & Garne, 2010). Congenital defects in uterine ligament, ocular furrow, and diaphragm closure are less common. All of these processes involve the directed migration and fusion of tissues, so it is not implausible that they might utilize similar or overlapping cell signaling strategies. In mice, defects in two or more canonical Wnt and/or PCP signaling components from among the *Celsr*, *Dvl*, *Frizzled*, *Lrp*, *Vangl*, and *Wnt* families have been implicated in palate, ventricular septum, neural tube, and ocular furrow closure defects (summarized in Yu et al., 2010). In humans, neural tube closure defects are associated with sequence variants in *VANGL1*, *VANGL2*, *CELSR1*, and *FZD6* in a small fraction of cases (Allache, De Marco, Merello, Capra, & Kibar, 2012; De Marco et al., 2012; Kibar et al., 2009, 2011, 2007). These data suggest that deleterious sequence variants in genes that either code for or control the expression of canonical Wnt and/or PCP signaling components may represent genetic risk factors for a wide variety of tissue closure defects in humans.

3. FRIZZLED5 AND FRIZZLED8

Homozygous deletion of *Fz5* leads to embryonic lethality at midgestation secondary to placental insufficiency (Ishikawa et al., 2001). When the placental phenotype is bypassed by conditionally ablating *Fz5* in embryonic but not extraembryonic tissues (*Fz5^{CKO/CKO};Sox2-Cre*), the resulting mice survive to adulthood and exhibit interesting phenotypes in the eye and the thalamus (*CKO*, conditional knockout). The ocular phenotype is ~50% penetrant and is characterized by increased cell death in the ventral retina and failure to close the ventral furrow (coloboma), an excess of mesenchymal cells in the vitreous cavity, an excess of astrocytes on the vitreal face of the retina, and persistence of the vascular network within the vitreous cavity (the hyaloid vasculature) into adulthood (Liu & Nathans, 2008). The same ocular phenotype is seen when *Fz5* is selectively ablated in the retina and in retinal astrocytes but not in vascular endothelial cells (ECs; Zhang, Fuhrmann, & Vetter, 2008). In the normal course of ocular development, the hyaloid vasculature regresses as the retinal vasculature matures, leaving an optically clear path between the lens and the retina. In humans, persistence of a hyaloid vascular remnant is a relatively common ocular anomaly (Duke-Elder, 1964; Goldberg, 1997).

In the brain, *Fz5* is expressed selectively in neurons in the parafascicular nucleus (PFN) of the thalamus, and *Fz5^{CKO/CKO};Sox2-Cre* mice show a progressive loss of PFN neurons over

the first several months of postnatal life (Liu, Wang, Smallwood, & Nathans, 2008). Genetic mosaic experiments in which *Fz5*^{-/-} cells are marked by expression of a reporter show that this loss is cell autonomous. Numerous changes in gene expression in PFN neurons precede neuronal loss, including downregulation of the genes coding for beta-catenin, Wnt4, and Wnt9b, suggesting that a Fz5-dependent positive feedback loop maintains canonical Wnt signaling in the PFN. The physiological role of the PFN is not well understood, but the work of Liu et al. (2008) implicates the PFN in sensorimotor function because loss of PFN neurons in *Fz5*^{CKO/CKO}; *Sox2-Cre* mice is accompanied by impaired motor coordination.

Fz8^{-/-} mice have no apparent phenotype. However, loss of both *Fz8* and *Fz5* in embryonic tissues leads to mid-gestational lethality, and loss of one *Fz8* allele in combination with loss of *Fz5* in embryonic tissues leads to 100% penetrance and greater severity of the *Fz5* ocular phenotypes and to a reduction in retinal progenitor proliferation (Liu, Bakeri, Li, & Swaroop, 2012). *Fz8* also exhibits a genetic interaction *Fz4* in the with context of kidney development (Ye, Wang, Rattner, & Nathans, 2011). *Fz4*^{-/-}; *Fz8*^{-/-} fetuses show reduced growth of ureteric buds and smaller kidneys, a phenotype similar to that described earlier for *Wnt11*^{-/-} mice (Chi et al., 2004; Majumdar, Vainio, Kispert, McMahon, & McMahon, 2003). The genetic mosaic experiments of Ye, Wang, et al. (2011) revealed strong competition between cells within the ureteric bud, which leads to a dramatic underrepresentation of *Fz4*^{-/-} or *Fz4*^{-/-}; *Fz8*^{-/-} cells relative to wild-type cells when both were initially present in roughly equal numbers. Intriguingly, in *Fz4*^{-/-}; *Fz8*^{-/-} embryos, in which all cells share the same genotype, the reduction in kidney size is more modest than would be predicted from the almost complete elimination of mutant cells in the mosaic kidney. This last observation suggests the possibility of (1) a feedback signal that enhances cell proliferation and/or survival when overall kidney growth is reduced and/or (2) an interaction between WT and mutant cells that selectively eliminates mutant cells.

4. FRIZZLED9 AND FRIZZLED10

Fz9 has long intrigued human geneticists because it is one of ~20 genes in the chromosome 7 region that is present in a hemizygous state in Williams–Beuren syndrome, a complex multisystem disorder (OMIM 194050). Although *Fz9*^{-/-} mice are viable and fertile, in-depth studies by three research groups have identified: (1) a subtle abnormality in B-cell development (Ranheim et al., 2005), (2) an increase in cell death in the dentate gyrus and a defect in visuo-spatial learning (Zhao et al., 2005), and (3) reduced bone mass and a reduction in newly formed bone in response to fractures, due to impaired osteoblast function (Fig. 4; Albers et al., 2011; Heilmann et al., 2013). Curiously, the bone phenotypes do not appear to be associated with alterations in canonical Wnt signaling, as judged by a comparison of the transcriptomes of *Fz9*^{-/-} and *Fz9*^{+/+} osteoblasts, a cell type in which transcriptome changes in response to canonical Wnt signaling are well defined. This finding contrasts with the well-established role of Lrp5-dependent canonical Wnt signaling in promoting bone formation in mice and humans (Baron & Kneissel, 2013; Joeng, Schumacher, Zylstra-Diegel, Long, & Williams, 2011). Albers et al. (2011) have provided evidence that, in osteoblasts, *Fz9* responds to Wnt5a and acts through a noncanonical pathway to control a distinct set of transcripts, including several coding for chemokines.

Fz10 is the most enigmatic of the Frizzled family members. The *Fz10* gene is expressed widely in the CNS, including in multiple thalamic nuclei (Zhao, Guan, & Pleasure, 2006), but no *Fz10* loss-of-function phenotypes have been reported thus far.

5. FRIZZLED4

Among the Frizzleds that are known or suspected to activate the canonical Wnt pathway, Fz4 is the best understood in terms of its biological function, its interactions with ligands and other receptor components, and its role in human disease. *Fz4*^{-/-} mice exhibit a severe defect in retinal vascularization, progressive hearing loss, and a slowly progressive cerebellar degeneration that is associated with loss of the blood–brain barrier (BBB) in the cerebellum (Wang, Huso, Cahill, Ryugo, & Nathans, 2001; Wang et al., 2012; Xu et al., 2004; Ye et al., 2009). In humans, heterozygosity for loss-of-function mutations in *FZD4* leads to a variable degree of retinal hypovascularization, a syndrome referred to as familial exudative vitreoretinopathy (FEVR; Nikopoulos, Venselaar, et al., 2010; Qin et al., 2005; Robitaille et al., 2002, 2011; Salvo et al., 2015; Toomes et al., 2004).

In WT mice during the first week of postnatal life, ECs emerge from the optic disc and migrate radially outward across the vitreal face of the retina to form a superficial vascular plexus. During the second postnatal week, ECs migrate vertically into the retina and form two additional tiers of capillaries, one immediately proximal and the other immediately distal to the central layer of neuronal cell bodies (the inner nuclear layer; Fruttiger, 2007). The mature retinal vasculature in mice, as in humans, consists of these three tiers of vessels. In *Fz4*^{-/-} mice, EC invasion into the retina is aborted after only a short distance, resulting in a complete absence of the two intraretinal capillary beds.

A critical clue to the biochemical mechanism of Fz4 action came from studies of Norrie disease, an X-linked disorder characterized by congenital blindness and slowly progressive hearing loss (Berger & Ropers, 2001). The gene responsible for Norrie disease, *NDP*, codes for a distant relative of the transforming growth factor beta (TGF-beta) family, a dimeric cysteine knot protein with no sequence similarity to Wnts (Berger et al., 1992; Chen et al., 1992). Strikingly, *Ndp*^{KO} mice (either *Ndp*^{-/-} females or *Ndp*^{-Y} males) exhibit exactly the same retinal phenotype as *Fz4*^{-/-} mice (Fig. 5), providing strong evidence that the congenital blindness of Norrie disease patients most likely reflects a severe retinal hypovascularization (Luhmann et al., 2005; Rehm et al., 2002). This identity in retinal vascular phenotypes suggested that Norrin (the protein product of the *Ndp* gene) might be a ligand for Fz4. Indeed, this is the case: dimeric Norrin binds a pair of Fz4 CRDs with several nanomolar affinity, it activates canonical Wnt signaling in concert with Fz4 and either Lrp5 or Lrp6 coreceptors, and the *Ndp*^{KO} retinal vascular phenotype can be fully rescued by artificially stabilizing beta-catenin in developing ECs, thereby circumventing the signaling defect at the level of ligand and receptor (Xu et al., 2004; Zhou et al., 2014).

Recent structural and biochemical experiments have revealed the precise three-dimensional architecture of the complex between dimeric Norrin and a pair of symmetrically bound Fz4 CRDs and defined the sequence determinants of ligand–receptor recognition (Chang et al., 2015; Ke et al., 2013; Smallwood, Williams, Xu, Leahy, & Nathans, 2007). The dimeric

nature of the Norrin–Fz4 CRD complex suggests that Fz4 may normally signal as a dimer, perhaps interacting symmetrically with dimeric Lrp5 or Lrp6 (Liu, Bafico, Harris, & Aaronson, 2003). If a pair of Wnt–Fz complexes is required for signaling, this would create the potential for a nonlinear dependence of signal amplitude on Wnt concentration and for a sharpening of the spatio-temporal boundaries of Wnt action.

Fz4 is expressed in all ECs throughout the body from the earliest times in vascular development through adulthood, and *Ndp* is expressed in selective anatomic regions of the prenatal CNS and in glia throughout the postnatal CNS: Muller glia in the retina, Bergman glia in the cerebellum, and astroglia elsewhere in the brain and spinal cord (Ye, Smallwood, et al., 2011; Ye et al., 2009). Experiments with conditional *Fz4* knockout alleles show that eliminating *Fz4* in ECs produces the same vascular phenotype as global elimination of *Fz4*.

An additional component of the Norrin/Fz4/Lrp signaling complex, Tspan12, was identified in a screen of knockout mice for ocular phenotypes (Junge et al., 2009). Tspan12 has four putative transmembrane segments and is a member of the large and diverse tetraspanin family. In cell culture, Tspan12 acts in conjunction with Fz4 and Lrp5 to specifically enhance Norrin signaling. In humans, *TSPAN12* and *LRP5* mutations are found in a subset of FEVR patients (Nikopoulos, Gilissen, et al., 2010; Poulter et al., 2010, 2012; Salvo et al., 2015). In summary, retinal angiogenesis is controlled by a glial ligand (Norrin) that is sensed by an EC receptor (Fz4), coreceptor (Lrp5), and coactivator (Tspan12).

In the mature retinal and cerebellar vasculatures, Norrin/Fz4 signaling is required for maintenance of the blood–retina barrier and the BBB, respectively (Wang et al., 2012; Ye et al., 2009). This barrier consists of a series of cellular and molecular specializations that distinguishes the CNS and peripheral vasculatures. In CNS ECs, these specializations include the presence of tight junctions, suppression of transcytosis, an absence of fenestrations, and expression of a variety of extrusion pumps and transporters (Daneman & Prat, 2015). The gene expression program that leads to CNS barrier competence is induced and maintained by canonical Wnt signaling (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008; Wang et al., 2012; Zhou et al., 2014).

Experiments in which beta-catenin, Fz4, or Norrin production is either activated or extinguished in the mature vasculature show that CNS barrier integrity is plastic (Liebner et al., 2008; Wang et al., 2012; Zhou et al., 2014). As demonstrated both by expression of molecular markers and by directly assaying the leakiness of CNS vessels, loss of canonical Wnt signaling leads within days to a loss of barrier competence. Conversely, a congenital deficiency in barrier function due to reduced canonical Wnt signaling can be reversed by restoring signaling later in life. Interestingly, the choroid plexus, a highly permeable CNS vascular bed that is the site of cerebrospinal fluid production, shows a cell-autonomous conversion to the barrier competent state (as judged by molecular markers) following EC-specific expression of a stabilized beta-catenin (Zhou et al., 2014). Loss-of-function experiments reveal CNS region-specific variations in ligands (Norrin, Wnt7a, Wnt7b, and possibly other Wnts), receptors (Fz4 and other Frizzleds), and coreceptors (Lrp5 and Lrp6), and the extent of redundancy among these components (Zhou et al., 2014). Taken together,

the data reveal a system in which multiple canonical Wnt ligands derived from the CNS parenchyma control vascular barrier integrity.

Recent work on Fz4 signaling has led to the discovery of two additional proteins, Gpr124 and Reck, that function in ECs as Wnt7a- and Wnt7b-specific coactivators of canonical Wnt signaling. Based on its predicted trans-membrane topography, Gpr124 had been categorized as a member of the adhesion class of orphan G-protein-coupled receptors. The first link between Gpr124 and vascular biology came with the discovery that *GPR124* transcripts are abundant in human tumor vasculature but rare in quiescent adult vasculature (Carson-Walter et al., 2001; St Croix et al., 2000). The second link came with the discovery that *Gpr124*^{-/-} embryos exhibit anatomically localized defects in CNS angiogenesis that are virtually identical to the defects observed in *Wnt7a* and *Wnt7b* double mutant embryos (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010; Stenman et al., 2008). This second observation led Zhou and Nathans (2014) and Posokhova et al. (2015) to test whether Gpr124 promotes signaling by Wnt7a and Wnt7b. Remarkably, in transfected cells Gpr124 stimulated signaling by these two Wnts but had little or no effect on signaling by Norrin or any of the other 17 mammalian Wnts. *In vivo*, various double mutant combinations show that the Wnt7a/Wnt7b/Gpr124/Fz4 system (which likely signals through Fz4 as well as other EC-expressed Frizzleds) and the Norrin/Tspan12/Fz4 system function redundantly to promote angiogenesis in the embryonic spinal cord and hindbrain and to maintain BBB integrity in many brain regions, including the cerebral cortex (Fig. 6).

The second coactivator, Reck, is a GPI-anchored protein that synergizes with Gpr124 to specifically stimulate Wnt7a/Wnt7b-mediated canonical Wnt signaling in transfected cells (Vanhollebeke et al., 2015). In zebrafish, *Reck* morphants phenocopy *Gpr124* mutants and morphants, and eliminating either Reck or Gpr124 blocks CNS angiogenesis and eliminates EC-specific expression of a reporter for canonical Wnt signaling.

These experiments reveal a general role for canonical Wnt signaling in ECs during embryonic and retinal angiogenesis and BBB development and maintenance. They also reveal the existence of auxiliary receptor proteins that enhance ligand specificity (Fig. 7). The high level of *GPR124* expression in tumor ECs suggests that canonical Wnt signaling in ECs may play a role in tumor angiogenesis.

6. FRIZZLED3 AND FRIZZLED6

Among mammalian Frizzled genes, *Fz6* is the family member that most closely resembles *Drosophila fz*, as determined by the macroscopic appearance of its loss-of-function phenotype (Guo, Hawkins, & Nathans, 2004).

Fz6 is expressed in the epidermis and in hair follicles starting at ~E13. *Fz6*^{-/-} mice are healthy and fertile, with skin and hair follicles that appear microscopically normal. However, at the earliest stages of their development, the orientations of *Fz6*^{-/-} hair follicles are aberrant and in many regions nearly randomized. By contrast, in WT mice, immature follicle orientations generally differ by less than 30° from their final orientations, which are precisely coordinated with the body and limb axes. During the first postnatal week, *Fz6*^{-/-}

follicles gradually reorient to create progressively expanding zones of local order, such as whorls or cruciforms (Fig. 8; Wang, Badea, & Nathans, 2006; Wang et al., 2010). As the number of follicles encompassed by the individual patterns increases and the follicle orientations conform more perfectly to the local geometry of each pattern, the number of patterning centers decreases, a progression that appears to represent a competition between neighboring patterns. In WT mice, a more subtle shift in follicle orientations occurs during the same time period, leading to the nearly parallel alignment that characterizes the mature coat.

These and other observations indicate that hair follicle orientation is determined by two fundamentally distinct systems. An embryonic system that requires Fz6 sets up the global pattern of hair follicles orientations, and a postnatal system that is independent of Fz6 refines the initial orientations to minimize differences between neighboring follicles. The refinement system can be modeled with a two-dimensional Ising lattice and a local consensus algorithm of the type that is commonly used to study the behavior of interacting vectors in condensed matter physics (Wang, Badea, et al., 2006). These models are agnostic with regard to molecular and cellular mechanisms, but they capture the global behavior of the system and its responses to various perturbations, such as the juxtaposition of WT and mutant tissue. It is interesting that the wing hairs of *Drosophila* PCP mutants also exhibit large-scale patterns, such as whorls, that are characterized by local order (Taylor, Abramova, Charlton, & Adler, 1998). The similarity in the large-scale patterns formed by PCP-deficient mammalian and insect epidermal structures is especially striking in light of the very different cellular structures involved: a mammalian hair follicle is an elongated cylinder formed by dozens to hundreds of epidermal cells, whereas a *Drosophila* wing hair is an actin-based protrusion that emerges from one side of an epithelial cell (Wong & Adler, 1993).

Hair follicles are associated with auxiliary structures that are polarized in a direction that matches the central follicle: a pair of follicle-derived sebaceous glands, arrector pili muscles, sensory nerve endings, and Merkel cell clusters (epithelial derivatives that are involved in mechanosensation). The largest follicles, which give rise to guard or tylotrich hairs, have all of these structures; the smaller follicles lack Merkel cells. The role of the central follicle in directing the polarized assembly of these different structures was analyzed in *Fz6*^{-/-} mice (Chang & Nathans, 2013). Sebaceous glands, arrector pili muscles, and sensory nerve endings were observed to track the orientation of their associated follicle. Merkel cell clusters on the back skin are normally arranged in a semicircle around the base of the guard hair follicles with the opening of the semicircle pointing toward the anterior. In *Fz6*^{-/-} mice, the Merkel cell clusters are arranged in a closed circle (Fig. 9). These data are consistent with the idea that *Fz6*^{-/-} skin is missing global polarity information and that the circularly symmetric Merkel cell arrangement represents a default response to a nonpolarized environment.

In humans, *FZD6* loss-of-function mutations have been identified in several families with autosomal recessive nail dysplasia (Frojmark et al., 2011; Naz et al., 2012; Wilson et al., 2013). A follow-up histologic and molecular analysis of claw development in *Fz6*^{-/-} mice shows dysplastic growth and downregulation of multiple claw-specific keratin genes in

addition to Wnt, bone morphogenetic protein, and Hedgehog genes (Cui et al., 2013). The data imply that Fz6 affects epidermal derivatives in a manner that extends beyond spatial patterning: the simplest explanation is that Fz6 couples directly to the canonical Wnt pathway in nail bed epithelial cells.

Like Fz6, Fz3 appears to signal through the PCP/tissue polarity system, but the *Fz3*^{-/-} phenotype is limited to the nervous system where it controls axon growth and guidance in the forebrain, in cranial and spinal motor neurons, in spinal sensory neurons, and in the sympathetic nervous system (Armstrong, Ryu, Chieco, & Kuruvilla, 2011; Fenstermaker et al., 2010; Hua, Jeon, Caterina, & Nathans, 2014; Hua, Smallwood, & Nathans, 2013; Lyuksyutova et al., 2003; Wang, Thekdi, Smallwood, Macke, & Nathans, 2002). In the *Fz3*^{-/-} forebrain, thalamocortical axons grow extensively but never enter the internal capsule, instead projecting inferiorly to innervate the contralateral thalamus (Fig. 10). Similarly, nigrostriatal dopaminergic neurons and brainstem serotonergic axons show extensive misrouting, with many axons growing caudally instead of rostrally. In the WT spinal cord, sensory axons that originate in the dorsal horn cross the midline before turning rostrally toward the brain. In the *Fz3*^{-/-} spinal cord, these sensory axons cross the midline appropriately but appear to lack rostrocaudal cues once their growth cones arrive on the contralateral side. *Fz3*^{-/-} spinal sensory axons appear to be incapable of finding their appropriate targets in the brainstem and thalamus as determined by behavioral testing of mice that lack Fz3 in all tissues caudal to the neck (*Fz3*^{CKO/-}; *Cdx1-Cre*). As adults, these mice show no evidence that thermal or mechanical information from the feet can be transmitted to the brain (Hua, Jeon, et al., 2014). Finally, *Fz3*^{-/-} motor neurons in the dorsal fore- and hindlimbs exhibit a distinctive stalling phenotype in the nerve plexus at the base of the limbs, suggesting that Fz3-dependent signals are required to sense or respond to molecular cues from intermediate targets (Hua et al., 2013).

The developmental roles of Fz3 and Fz6 extend substantially beyond the processes revealed by single gene mutations. *Fz3*^{-/-}; *Fz6*^{-/-} embryos reveal redundant functions in neural tube closure and in rotationally orientating the kinocilium/stereociliary bundle complex on the apical face of inner ear sensory hair cells (Wang, Guo, & Nathans, 2006). Additionally, epidermis-specific double knockout of *Fz3* and *Fz6* (in *K14-Cre*; *Fz3*^{CKO/CKO}; *Fz6*^{-/-} mice) reveals a redundant role for these genes in orienting the filiform papillae on the dorsal surface of the tongue (Hua, Chang, et al., 2014). Stimulated by the partial redundancy of *Fz3* and *Fz6*, Hua, Chang, et al. (2014) asked whether this redundancy can be extended to other tissues by enlarging the domains of *Fz3* and *Fz6* gene expression. These experiments demonstrated that ubiquitous expression of *Fz3* fully rescues the *Fz6*^{-/-} hair follicle orientation phenotype and ubiquitous expression of *Fz6* partially rescues the *Fz3*^{-/-} axon guidance phenotype. Thus, the many diverse contexts in which Fz3 and Fz6 act are likely based on a conserved signaling role for these two proteins.

Mutations of *Celsr1*, *Celsr2*, and *Celsr3*, mammalian homologues of the *Drosophila* core PCP gene *Flamingo/Starry night* (*Fmi/Stan*), produce neural tube closure, axon guidance, neuronal migration, inner ear sensory hair cell, and hair follicle phenotypes that closely resemble the phenotypes in *Fz3*^{-/-}, *Fz6*^{-/-}, and *Fz3*^{-/-}; *Fz6*^{-/-} mice (Curtin et al., 2003; Qu et al., 2010; Ravni, Qu, Goffinet, & Tissir, 2009; Tissir, Bar, Jossin, De Backer, & Goffinet,

2005; Zhou et al., 2008). Similarly, *Vangl2^{Lp/Lp}* fetuses exhibit defects in inner ear sensory hair cell orientation and neural tube closure that closely resemble the defects observed in *Fz3^{-/-}*; *Fz6^{-/-}* fetuses (Kibar et al., 2001; Montcouquiol et al., 2003; Murdoch, Doudney, Paternotte, Copp, & Stanier, 2001; Strong & Hollander, 1949). *Vangl2^{Lp/Lp}* fetuses also exhibit a hair follicle orientation defect, but, interestingly, the *Vangl2^{Lp/Lp}* follicles are initially oriented perpendicular to the plane of the skin whereas WT and *Fz6^{-/-}* follicles are oriented obliquely (Devenport & Fuchs, 2008; Wang et al., 2010). Despite such minor differences, the overall similarities in affected tissues and in polarity defects among *Celsr*, *Vangl*, and *Fz3/Fz6* mutants support the idea that the diverse *Fz3^{-/-}*, *Fz6^{-/-}*, and *Fz3^{-/-}*; *Fz6^{-/-}* phenotypes reflect the activity of a mammalian version of *Drosophila* PCP.

7. CONCLUSION

The past three decades represent the first phase of exploration of the Frizzled family. At least some of the functions of most mammalian Frizzled family members are now known at a descriptive level, and for three family members the signaling pathways in which they participate have been identified. Despite the enormous progress made during this time, our current level of understanding remains rudimentary. To cite three examples: (1) the determinants of Wnt–Frizzled specificity are still unknown, and there is a strong suspicion that additional modulatory/specificity proteins remain to be discovered, (2) the regulation of Frizzled expression, trafficking, and stability is just starting to be explored, with the discovery of R-spondin regulation of Frizzled degradation via the ubiquitin ligase Znf3 representing a tantalizing appetizer (Hao et al., 2012; Koo et al., 2012), and (3) the role of Frizzled receptors in human disease—both inherited and acquired—is still in its infancy.

Perhaps the greatest challenge is to define the biochemical role of Frizzled proteins in polarity signaling. Tissue polarity has been recognized as an essential attribute of animal development for more than 75 years, with the early microsurgical investigations of polarity in the insect cuticle preparing the intellectual foundations for today's molecular genetic experiments (Lawrence, 1966; Locke, 1959; Wigglesworth, 1940). Tissue polarity signaling also has a connection to the larger world of complex systems, of which biological pattern formation represents one part (Ball, 1999). The local nature of cell–cell signaling suggests that tissue polarity uses conceptually simple “rules” of information transfer that can be modified in various ways to create anatomic diversity. In keeping with this idea, the study of cellular automata demonstrates that large-scale iterative applications of simple rules can generate remarkably complex patterns (Wolfram, 2002). As new mechanistic insights emerge in the field of tissue polarity signaling, it will be of great interest to try to capture the fundamental characteristics of this system in mathematical models, an effort that is already underway (Amonlirdviman et al., 2005; Axelrod & Tomlin, 2011; Wang, Badea, et al., 2006).

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Figure 1.

Evolutionary relationships among mammalian Frizzled proteins and genes. (A) Dendrogram showing the relatedness of the 10 mammalian Frizzled amino acid sequences. (B) Intron–exon structures of mammalian *Frizzled* genes, with coding regions depicted schematically as the same length. Only *Fz3*, *Fz4*, and *Fz6* have introns within the coding region. The five coding introns in the *Fz3* and *Fz6* genes are located at identical positions. *From* Hua, Chang, Wang, Smallwood, and Nathans (2014).

Figure 2.

Palate closure defect in *Fz1*^{-/-};*Fz2*^{-/-} fetuses. (A and B) Scanning electron microscopy of the palate at E14. PS, palatal shelf. (C and D) Coronal section through the head at E17, stained with hematoxylin and eosin. N, nasal septum; T, tongue; Yellow asterisks, palatal shelves. Scale bars: B, 200 μ m; D, 500 μ m. From Yu et al. (2010).

Figure 3.

Convergent extension, neural tube closure, and cardiac asymmetry defects in $Fz2^{-/-};Fz7^{-/-}$ embryos. E8.5 embryos are shown intact in a dorsal view (left) and in paraffin sections stained with hematoxylin and eosin (right). Left panels, the $Fz2^{-/-};Fz7^{-/-}$ embryo is shorter and wider than the $Fz2^{+/+};Fz7^{-/-}$ control due to a failure of convergent extension. Right panels, in cross section, the $Fz2^{-/-};Fz7^{-/-}$ embryo shows an open neural tube (red arrows) and symmetric cardiac chambers (green arrowheads). The $Fz2^{+/+};Fz7^{-/-}$ embryo is phenotypically WT. Scale bars: left panels, 1 mm; right panels, 200 μ m. From Yu et al. (2012).

Figure 4. Reduced bone density in $Fz9^{-/-}$ mice. The vertebral body shows reduced bone density in a 52-week-old $Fz9^{-/-}$ mouse (right) compared to an age- and sex-matched control (left). Scale bar, 250 μ m. From Albers et al. (2011).

Figure 5.

Defective retinal vascularization in *Ndp*^{KO} mice. Z-stacked confocal images of flat mount adult WT and *Ndp*^{KO} retinas, with ECs visualized with GS-lectin. The depth of the different vascular structures has been color coded as indicated at left. The *Ndp*^{KO} retina has only a superficial vascular plexus from which numerous EC clusters invade a short distance into the retina. IPL, inner plexiform layer; OPL, outer plexiform layer. Scale bar, 100 μm. *From* Wang et al. (2012).

Figure 6.

Redundant actions of Gpr124 and Norrin in maintaining BBB integrity. Coronal sections through the cortex and thalamus of postnatal day (P)6 mice with the indicated genotypes. *Pdgfb-CreER* recombines specifically in ECs. 4-Hydroxytamoxifen (4HT) was delivered by intraperitoneal injection at P3–P4. Intravascular Sulfo-NHS-biotin leaks from the choroid plexus in the lateral ventricles in all brain samples (upper panels), but it leaks extensively into the brain parenchyma only in the sample that is deleted for both *Gpr124* and *Ndp* (upper right panel). Intracerebral capillaries in the WT brain (lower left) and in the brains from mice with either one functional copy of *Gpr124* or one functional copy of *Ndp* (lower middle two panels) express claudin5 (a hallmark of CNS vasculature) and suppress plasmalemma vesicle-associated protein (PLVAP; a hallmark of peripheral vasculature). Deletion of both *Gpr124* and *Ndp* reverses this expression pattern (lower right panel). Scale bar, 200 μ m. From Zhou and Nathans (2014).

Figure 7.

Schematic of ligand and receptor components involved in canonical Wnt signaling in CNS ECs. Horizontal black lines represent the plasma membrane; extracellular space is above and cytoplasm is below. Additional components, such as Lrp6, other Frizzleds, and other Wnts, are also involved, but their roles are not yet well defined. *Modified from Zhou and Nathans (2014).*

Figure 8.

Hair patterns in a *Fz6*^{-/-}; *Vangl2*^{Lp/+} back skin at P8. (A) Vector map showing hair follicle orientations over the head and back in a flat-mount skin from a P8 *Fz6*^{-/-}; *Vangl2*^{Lp/+} mouse. Anterior is down; posterior is up. The small ovals are the eye positions; the larger ovals are the ear positions. Four patterning centers are present along the midline. (B–I) The central regions of the two most posterior patterning centers are enlarged in panels B, C, F, and G, and further enlarged in panels D, E, H, and I, as indicated by the rectangles in panels A, B, C, F, and G. Spatial order among follicles is evident at long range (~1 cm) and short range (e.g., between neighboring follicles; tens of microns). Scale bars: A, 1 cm; B and F, 2 mm; D and H, 300 μ m. From Wang, Chang, and Nathans (2010).

Figure 9.

Loss of polarity in the arrangement of Merkel cells in *Fz6*^{-/-} mice. (A) Vertical section of P1 back skin from a phenotypically WT *Fz6*^{+/-} mouse. Merkel cells (MC) are visualized with anticytokeratin8 (CK8) immunostaining and afferent sensory nerve fibers (AF) are visualized with antineurofilament (NFL) immunostaining. Ep, epidermis; Der, dermis. (B–D) Flat mounts of P1 skin in which Merkel cells are visualized with CK8 and the central guard hair follicle is visualized with a *Keratin (K)17-GFP* transgene. Anterior is to the left. The phenotypically WT *Fz6*^{+/-} Merkel cells form a semicircle that opens toward the anterior. The *Fz6*^{-/-} Merkel cells form a closed circle. Scale bar, 50 μm. *From* Chang and Nathans (2013).

Figure 10.

Thalamocortical axon guidance defects in the *Fz3*^{-/-} forebrain at E18.5. Serial coronal sections through the forebrains of E18.5 *Fz3*^{-/-} and *Fz3*^{+/+} fetuses stained with anti-neurofilament antibodies. The midline of each hemibrain image is at the right border of each panel. The sections are arranged from rostral (A and A') to caudal (E and E'). In the *Fz3*^{+/+} brain, arrows in (A–D) trace the thalamocortical projections originating in the thalamus (D) and projecting via the internal capsule (A–C) to the cortex (A–D). In the *Fz3*^{-/-} brain, thalamic axons track inferiorly with most crossing the midline to the contralateral thalamus (D') and a minority projecting to the inferior border of the cortex (C'). Additional axon guidance defects are indicated by arrowheads in (A'–E'); see Hua, Jeon, et al. (2014) for details. GP, globus pallidus. Scale bar, 1 mm. *From* Hua, Jeon, et al. (2014).