Partial interchangeability of Fz3 and Fz6 in tissue polarity signaling for epithelial orientation and axon growth and guidance

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ABSTRACT

In mammals, a set of anatomically diverse polarity processes – including axon growth and guidance, hair follicle orientation, and stereociliary bundle orientation in inner ear sensory hair cells – appear to be mechanistically related, as judged by their dependence on vertebrate homologues of core tissue polarity/planar cell polarity (PCP) genes in Drosophila. To explore more deeply the mechanistic similarities between different polarity processes, we have determined the extent to which frizzled 3 (Fz3) can rescue the hair follicle and Merkel cell polarity defects in frizzled 6-null (Fz6−/−) mice, and, reciprocally, the extent to which Fz6 can rescue the axon growth and guidance defects in Fz3−/− mice. These experiments reveal full rescue of the Fz6−/− phenotype by Fz3 and partial rescue of the Fz3−/− phenotype by Fz6, implying that these two proteins are likely to act in a conserved manner in these two contexts. Stimulated by these observations, we searched for additional anatomical structures that exhibit macroscopic polarity and that might plausibly use Fz3 and/or Fz6 signaling. This search has revealed a hitherto unappreciated pattern of papillae on the dorsal surface of the tongue that depends, at least in part, on redundant signaling by Fz3 and Fz6. Taken together, these experiments provide compelling evidence for a close mechanistic relationship between multiple anatomically diverse polarity processes.

KEY WORDS: Planar cell polarity, Skin, Brain, Hair follicle, Tongue, Mouse

INTRODUCTION

Complex metazoan animals are replete with structures that exhibit a high degree of spatial order. One type of order is apparent in the orientation of polar structures relative to local anatomic landmarks and/or the body axes. The genetic dissection of this type of spatial order – referred to as tissue polarity or, more restrictively, planar cell polarity (PCP) – began 30 years ago with the discovery and characterization of a core set of genes in Drosophila that regulate the orientations of wing hairs and cuticular bristles (Adler, 2002; Goodrich and Strutt, 2011; Gubb and Garcia-Bellido, 1982). Subsequent work showed that these genes also control ommatidial chirality, implying a more general role in influencing vectorial processes during development (Jenny, 2010).

Homologues of Drosophila PCP genes are found in all vertebrates, with the added complexity that there are typically several homologues for each Drosophila gene. Targeted disruption of these genes in mice – including the genes coding for frizzled (Fz; ten family members), dishevelled (Dsh; three family members), Van Gogh-like (Vang; two family members) and Celsr (three family members) proteins – has revealed multiple anatomic structures that appear to require polarity signaling to attain their correct orientations (Tissir and Goffinet, 2013; Wang and Nathans, 2007; Wynshaw-Boris, 2012). These include: (1) hair follicles and their associated structures in the skin; (2) stereocilia bundles on the apical faces of inner ear sensory hair cells; and (3) motile cilia in the trachea and on the walls of the cerebral ventricles that direct the vectorial movement of mucus and cerebrospinal fluid, respectively. Two processes that involve oriented cell movements – neural tube closure in mammals and the related process of convergent extension in amphibia and fish – also require core PCP gene function (Munoz-Soriano et al., 2012; Tada and Heisenberg, 2012).

In epithelia, where PCP has been most extensively studied, current evidence suggests that PCP signaling involves the assembly of asymmetric cell-surface complexes that organize the underlying cytoskeleton (Peng and Axelrod, 2012). In these complexes, Fz proteins are localized in the plasma membrane of one cell and face Vang/Vangl proteins in the plasma membrane of the neighboring cell. Importantly, PCP protein assemblies exhibit a macroscopic asymmetry: Fz proteins assemble exclusively on one side of each cell and Vang/Vangl proteins assemble exclusively on the opposite side. The multiple cadherin-domain protein Fmi/Stan/Celsr is present on both sides of the cell and forms homophilic interactions between adjacent cells that stabilize the complex. In current models of PCP signaling, a self-assembly process in which a Fz- or Vang/Vangl-containing hemi-complex on one cell promotes the assembly of the opposite type of hemi-complex on the neighboring cell is hypothesized to be the mechanism by which polarity information is created in and propagates across the epithelial sheet (Peng and Axelrod, 2012; Simons and Mlodzik, 2008).

The present work focuses on Fz3 and Fz6, two mammalian Fz family members that are implicated in tissue polarity signaling. As judged by their amino acid sequences and intron-exon structures, Fz3 and Fz6 form a distinct branch within the mammalian Fz family tree (Fig. 1A,B). Fz6 is expressed in the skin and hair follicles, and Fz6−/− mice exhibit a nearly complete randomization of hair follicle orientations at early times in skin development, a phenotype that resembles the phenotypes of PCP mutants in the Drosophila cuticle (Wang et al., 2006a, 2010). By contrast, Fz3 is expressed in the developing central nervous system (CNS), and Fz3−/− mice exhibit multiple defects in axon growth and guidance, including: (1) the mis-routing of thalamocortical axons to an intra-thalamic trajectory; (2) the failure of corticothalamic axons to enter the internal capsule and reach the thalamus; (3) the absence of the corticospinal tract; and (4) the randomization of spinal cord sensory axon trajectories after
midline crossing; (5) the failure of some cranial motor axons to reach their muscle targets; and (6) the irreversible stalling of most hindlimb and some forelimb dorsal motor axons in the nerve plexus at the base of the limbs (Hua et al., 2013; Lyuksyutova et al., 2003; Wang et al., 2002, 2006c). Many of these defects are also seen in Celsr3−/− mice (Tissir et al., 2005; Zhou et al., 2008). Some of the axon guidance phenotypes observed in Fz3−/− mice—such as the failure of spinal cord sensory axons to turn rostrally—suggest a polarity signaling defect, whereas other phenotypes—such as the stalling of dorsal limb motor axons—do not. Evidence that Fz3 can engage the polarity signaling machinery in other contexts comes from the redundancy of Fz3 and Fz6 in closing the neural tube and eyelids, and in orienting inner ear sensory hair cells (Wang et al., 2006b).

The present study is aimed at determining the degree to which Fz3 and Fz6 are interchangeable, and, by inference, the degree to which polarity signaling in the skin and nervous system are mechanistically related. Our approach is to test whether it is possible to rescue Fz3−/− mice with ubiquitously expressed Fz6 or to rescue Fz6−/− mice with ubiquitously expressed Fz3. The results show a complete phenotypic rescue of Fz6−/− hair patterning by Fz3 and a partial rescue of Fz3−/− axon growth and guidance defects by Fz6. We have also searched for additional anatomic structures that exhibit macroscopic polarity to examine the contributions of Fz3 and Fz6 to that polarity. That search has revealed a hitherto unappreciated epithelial pattern that covers the dorsal surface of the mouse tongue and is partially disrupted by the combined loss of Fz3 and Fz6.

RESULTS

Alleles for ubiquitous production of Fz3 and Fz6

To test the potential of Fz6 to genetically rescue Fz3−/− mice and of Fz3 to genetically rescue Fz6−/− mice, we reasoned that the simplest experimental design would be one in which the rescuing constructs were ubiquitously expressed. To this end, triple-hemagglutinin epitope (HA)-tagged Fz3- and Fz6-coding regions were inserted into the ubiquitously expressed ubiquitin B (Ubb) locus. The Ubb

![Fig. 1. Knock-in alleles for constitutive production of Fz3 and Fz6. (A) Dendrogram showing amino acid sequence identities among the 10 mouse Fz proteins. Fz3 and Fz6 show 48% amino acid identity. (B) Schematic of coding region intron-exon structures of mouse Fz family members. Fz3 and Fz6 each have five introns, and the intron positions are precisely conserved, as seen by the alignment of encoded amino acids near each exon-exon junction. Red lettering: amino acids encoded by the 5′ exon. Blue lettering: the intron is located within that codon. Fz4 has one coding region intron; all other genes in the Fz family lack coding region introns. (C) Schematic of ROSA26-3xHA-Fz6 (top), and Z/3xHA-Fz3 and Z/3xHA-Fz6 (bottom) at the Z locus, Cre-mediated deletion of the loxp-beta-geo-stop-loxp cassette leads to constitutive expression of 3xHA-Fz3 or 3xHA-Fz6 driven by the CAG promoter. At the ROSA26 (R26) locus, Cre-mediated deletion of the loxp-stop-loxp cassette leads to constitutive expression of 3xHA-Fz6 driven by the relatively weak ROSA26 promoter. The constitutively active derivatives of these alleles are referred to as Z/Fz3C, Z/Fz6C and R26-Fz6C, respectively. (D-F) Anti-3xHA, anti-Fz6 and anti-Fz3 immunoblots of P1 brain and skin extracts from wild-type, Z/Fz3C, Z/Fz6C and R26-Fz6C mice in the presence or absence of endogenous Fz3 or Fz6 alleles, as indicated. The Fz3−/− brain was harvested at E18.5. The ubiquitously expressed (D) and endogenous (E) Fz6 proteins migrate at higher apparent molecular weights than the ubiquitously expressed (D) and endogenous (F) Fz3 proteins. Molecular weight (MW) heterogeneity may reflect heterogenous glycosylation. Black arrows, Fz6 protein; red arrows, Fz3 protein. Asterisk indicates an irrelevant cross-reacting band. MW standards are 180, 115, 82, 64, 49 and 37 kDa.]
locus is referred to hereafter as the ‘Z’ locus because it is the site into which a Cre reporter transgene, Z/AP, was found to have randomly integrated (Lobe et al., 1999; Rotolo et al., 2008). As shown in Fig. 1C, the Z3xHA-Fz3 and Z3xHA-Fz6 knock-in alleles carry a CMV enhancer/beta-actin (CAG) promoter, followed by a β-galactosidase/neo (‘beta-geo’) triple transcription stop cassette flanked by loxP sites (loxP-beta-geo-stop-loxP). In all of the experiments described here, the loxP-beta-geo-stop-loxP cassette was first removed by Cre-mediated recombination in the germline, generating derivatives in which the Fz3- or Fz6-coding regions were constitutively expressed, referred to as Z/Fz3C and Z/Fz6C, respectively. Additionally, the 3×HA-Fz6-coding region with a triple transcription stop cassette flanked by loxP sites was inserted at the ROSA26 (R26) locus to generate R26-3xHA-Fz6. Constitutive activation of this allele by germline Cre-mediated recombination generated the constitutively expressed R26-Fz6C allele. Expression of a single copy of Z/Fz3C, Z/Fz6C, or R26-Fz6C in a wild-type background produced no visible effect on viability, growth, fertility or overall health, implying that ubiquitous production of Fz3 or Fz6 is relatively innocuous.

Comparisons of the levels of protein production from the three ubiquitously expressed knock-in loci and from the endogenous Fz3 and Fz6 loci by immunoblotting show that: (1) the CAG promoter at the Z locus produces many fold higher levels of Fz3 and Fz6 than the ROSA26 locus in both brain and skin (Fig. 1D); (2) the levels of Fz3 and Fz6 produced from the Z locus are higher in skin than in brain, but the levels of Fz6 produced from the ROSA26 locus in skin and brain are more nearly equivalent (Fig. 1D); (3) the level of endogenous Fz6 in skin is barely detectable (compare Fz6+/+ and Fz6−/− lanes in Fig. 1E), is several fold below the level of Fz6 produced from the ROSA26 locus, and is many fold below the level of Fz6 produced from the Z locus (Fig. 1E); and (4) the level of endogenous Fz3 in brain is readily detectable and is close to the level of Fz3 produced from the Z locus (Fig. 1F). We note that in both brain and skin, the comparison between endogenous and ubiquitously expressed proteins does not correct for the more limited anatomic distribution of the endogenous protein, an effect that acts to minimize endogenous versus ubiquitous protein concentration differences in the relevant cell types compared with the ratios observed in the whole tissue immunoblots. For example, in the skin, endogenous Fz6 is expressed only in the epidermis and hair follicles, whereas the Z and ROSA26 loci are active in the epidermis, hair follicles, dermis and vasculature, all of which were included in the tissue homogenate.

Ubiquitous production of Fz3 rescues the Fz6−/− hair polarity phenotype
In the skin, Fz6 expression – as measured with a Fz6lacZ knock-in allele (Guo et al., 2004) – is readily detected in the epidermis and in hair follicles, whereas Fz3 expression – as measured with a Fz3lacZ knock-in allele (Wang et al., 2002) – is undetectable (Fig. 2A). Crossing Z/Fz3C into Fz6−/− background showed, as expected, a complete rescue of the Fz6−/− hair orientation phenotype. More interestingly, crossing Z/Fz3C into the Fz6−/− background also showed a complete rescue of the Fz6−/− hair orientation phenotype, as assessed on both the back and the paws.
Table 1. Rescue of the Fz6+/− hair follicle orientation phenotype by Z/Fz3C and Z/Fz6C.

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<td><strong>Cross: Fz6+/−;Z/Fz3C × Fz6+/−</strong></td>
<td>Progeny genotype</td>
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(Table 1, Fig. 2B). At postnatal day (P) 3, hair follicles on the back are well oriented in an anterior-to-posterior direction in Fz6+/− mice but are severely disordered in Fz6−/− mice. In Fz6+/−;Z/Fz3C and Fz6−/−;Z/Fz6C mice, the normal anterior-to-posterior follicle orientation in back skin is restored. Similarly, at P5, hair follicles on the dorsal surface of the paws are aligned in a proximal-to-distal direction in Fz6−/− mice, whereas they form a macroscopic whorl in the center of the paws in Fz6+/− mice. In Fz6−/−;Z/Fz3C and Fz6+/−;Z/Fz6C mice, the normal proximal-to-distal follicle orientation on the paws is restored.

In wild-type mice, a semicircle of ~30 Merkel cells partially surrounds each hair guard on the back skin, with the opening of the semicircle facing anteriorly. In Fz6−/− mice, the anterior-posterior polarity of the Merkel cell cluster is lost and the Merkel cells are arranged in a complete circle (Chang and Nathans, 2013; Fig. 2B). In Fz6+/−;Z/Fz3C and Fz6−/−;Z/Fz6C mice, Merkel cell polarity is restored (Fig. 2B).

To assess the specificity of Z/Fz3C function, we asked whether it could rescue the palate closure defect that occurs in Fz1−/−;Fz2−/− embryos (Yu et al., 2010). In 9/9 Fz1−/−;Fz2−/−;Z/Fz3C embryos examined, there was a failure of palate closure indistinguishable from the palate closure defect seen in Fz1−/−;Fz2−/− embryos (supplementary material Fig. S1). Thus, Z/Fz3C is capable of rescuing some Fz mutations but not others.

**Ubiquitous production of Fz6 partially rescues Fz3−/− axon growth and guidance phenotypes**

In the embryonic brain, Fz3 is widely expressed (Tissier and Goffinet, 2006; Wang et al., 2002) but Fz6 expression is largely confined to the vasculature (Z.L.H., H.C., Y.W., P.M.S. and J.N., unpublished; Daneman et al., 2009; Stenman et al., 2008). Crossing Z/Fz3C into the Fz3−/− background showed that the neonatal lethality exhibited by Fz3−/− mice is completely rescued by ubiquitous production of Fz3 (Yu et al., 2010). In 9/9 Fz1−/−;Fz2−/−;Z/Fz3C embryos examined, there was a failure of palate closure indistinguishable from the palate closure defect seen in Fz1−/−;Fz2−/− embryos (supplementary material Fig. S1). Thus, Z/Fz3C is capable of rescuing some Fz mutations but not others.

The severe defect in thalamocortical and corticothalamic tract formation in Fz3−/− embryos was completely rescued in Fz3−/−;Z/Fz3C embryos (n=5), but only partially rescued in Fz3−/−;Z/Fz6C embryos (n=6; Fig. 4A-D'). In Fz3−/− embryos, thalamocortical and corticothalamic axons do not enter the internal capsule. In Fz3−/−;Z/Fz6C embryos, many of these axons enter the internal capsule, but the resulting tracts appear narrower (arrow in Fig. 4D) and some of the axons exhibit aberrant trajectories around the globus pallidus (arrowhead in Fig. 4D').

Fz3−/−;Z/Fz6C embryos showed variable rescue of the migratory defects of Vth cranial nerve cell bodies. In the Fz3−/− brainstem, these cells fail to migrate tangentially and caudally from rhombomere 4 to rhombomere 6, and, as a consequence, their axons fail to loop around the VIth cranial nerve nucleus (compare Fig. 4E with Fig. 4F). In Fz3+/−;Z/Fz6C embryos this defect was fully rescued (Fig. 4G). Among three Fz3−/−;Z/Fz6C embryos examined, one was completely rescued (Fig. 4H) and two showed no rescue (Fig. 4I).

In earlier work, we characterized the stalling of dorsal motor axons at the base of both fore- and hindlimbs in midgestation Fz3−/− embryos by determining the width of the dorsal nerve at different locations along its trajectory (Hua et al., 2013). Stalling is more...
We conclude that axon development nor an effect that is redundant with active in promoting axon growth and guidance than Fz3 or that Fz6 functional Fz6. The data suggest either that Fz6 is quantitatively less complete rescue of the axon stalling defect (Table 3; Fig. 4J-L). NF immunostaining, corresponding motor neurons in the spinal cord. By whole-limb complete in the hindlimb and leads to rapid death of the

Polarization of lingual papillae involves Fz3 and Fz6

The observation that Fz3 can replace Fz6 in skin PCP signaling prompted us to examine whether Fz3 might normally play a role in patterning epithelial structures. In keeping with the absence of detectable Fz3 expression in the skin over most of the body (Fig. 2A), loss of Fz3 did not perturb hair follicle orientation at E18.5 (supplementary material Fig. S3A,B), and the combined loss of Fz3 and Fz6 in the epidermis (Fz3K0/−:Fz6K0/−;K14-Cre;K17-GFP) produced a phenotype of hair follicle mis-orientation at P0 that was indistinguishable from the phenotype produced by global loss of Fz6 (supplementary material Fig. S3C). These data imply that Fz3 plays little or no role in hair follicle orientation. [In these experiments and the ones described below, we have bypassed the CNS defects that lead to neonatal lethality in Fz3−/− mice by using a Keratin14-Cre (K14-Cre) transgene to selectively delete Fz3 in the epidermis starting at ~E12.5-E13.5 (Beronja et al., 2010). In unpublished work we observed that Fz6K0/−:K14-Cre and Fz6−/− mice exhibit identical hair patterning phenotypes, implying that K14-Cre acts sufficiently early to eliminate PCP in the developing epidermis.]

In a search for other epithelial structures that exhibit large-scale polarity and that might reveal effects of Fz3 and/or cooperative effects of Fz3 and Fz6, we investigated the dorsal surface of the tongue, which in most mammals, including rodents, is covered with asymmetric epithelial protrusions (papillae). The development and micro-anatomy of lingual (i.e. tongue) papillae have been extensively studied, but their large-scale spatial organization has received little attention (Hume and Potten, 1976; Iwasaki et al., 1996). Both Fz3 and Fz6 are expressed in the tongue epithelium at E13.5, as judged by expression of the corresponding lacZ knock-in alleles (Fig. 5A). (We note that the X-gal staining intensities of these two alleles are not directly comparable: the lacZ knock-in at the Fz3 locus includes an intron in the 3′ UTR and lacks a nuclear localization signal, and it is therefore likely to be a weaker reporter than the nuclear localized lacZ knock-in at the Fz6 locus.)

differs in some qualitative manner so that it cannot fully recapitulate Fz3 function.
To obtain a global assessment of the polarity of lingual papillae, we viewed the tongue as a flat-mount, using a combination of GFP expression from a Keratin17-GFP (K17-GFP) transgene and in vivo labeling with AM4-65 to assist in visualizing papillae. The wild-type mouse tongue shows a highly stereotyped pattern of papillae orientations (Fig. 5B-G). Along the front and sides, papillae make an angle of ∼45° to the midline, with their raised edges pointing toward the center and posterior of the tongue. Moving more medially from each side, the papillae progressively rotate until they point posteriorly at the midline. The most distinctive feature of the papillae pattern is the presence of a single circularly symmetric rosette located at the midline ∼70% of the distance from the anterior tip to the base of the tongue. The orientations of papillae at progressively greater distances from the rosette change smoothly to accommodate the vector fields at the sides and front of the tongue (Fig. 5B,F; supplementary material Fig. S4). The result is that the tongue surface exhibits two ‘singularities’, i.e. locations where there are large differences in the orientations of neighboring papillae. One singularity is at the center of the rosette and the second is ∼1 mm anterior to the rosette, where posteriorly directed papillae from the anterior half of the tongue encounter anteriorly directed papillae from the rosette (supplementary material Fig. S4). As the entire papillae pattern is almost perfectly left-right symmetric, both singularities are located at the midline.

To determine whether Fz3 and/or Fz6 controls the patterning of lingual papillae, we examined tongues from mice missing either or both genes in the tongue epithelium at ages ranging from P3 to 9 months. As noted above in the context of supplementary material Fig. S3C, the Fz3CKO allele was recombined with K14-Cre, which is expressed uniformly in the dorsal epithelium of the mature tongue, as assessed with a Hprt-LSL-tdTomato reporter (Wu et al., 2014; data not shown). The genotype/phenotype relationships are summarized in Table 4. In the absence of both Fz3 and Fz6 (Fz3CKO−/−;Fz6−/−;K14-Cre;K17-GFP), the dorsal tongue surface at P20 shows no changes in the number and locations of taste buds or in the density of lingual papillae. However, papillae in the anterior half of the tongue show a general disorganization in their orientations within the plane of the tongue epithelium that is strongly reminiscent of the bristle and wing hair patterning defects observed in PCP mutants in Drosophila (Fig. 6C,D; Adler, 2002). In cross-sections near the midline, Fz3CKO−/−;Fz6−/−;K14-Cre;K17-GFP tongues reveal numerous papillae that lack the regular anterior-to-posterior pattern.
orientation observed in control tongues (Fig. 6E). By contrast, the presence of single Fz3 allele in the absence of Fz6 (Fz3CKO/+, Fz6−/−;K14-Cre;K17-GFP) or a single Fz6 allele in the absence of Fz3 (Fz3CKO/−;Fz6+/−;K14-Cre;K17-GFP) produces a papillae pattern that is indistinguishable or nearly indistinguishable from the wild type (Fig. 6A,B; supplementary material Figs S4, S5; Table 4). These data imply that Fz3 and Fz6 function redundantly in tongue epithelial patterning.

The localization of the patterning defect to the anterior of the tongue in Fz3CKO/−;Fz6+/−;K14-Cre;K17-GFP mice is puzzling. As lingual papillae are part of a larger integrated pattern, one might predict that any PCP gene mutations that affect lingual patterning would affect the entire pattern. Although we do not have a resolution to this apparent paradox, we speculate that if the patterning of lingual papillae roughly coincides with the time when expression of the K14-Cre transgene begins, and if the anterior tongue pattern develops 1-2 days later than the posterior tongue pattern or if Cre-mediated recombination occurs 1-2 days earlier in the anterior tongue, then the patterning defect in Fz3CKO/−;Fz6+/−;K14-Cre;K17-GFP mice could be limited to the anterior tongue.

Sequence divergence and evolutionary history of Fz3 and Fz6

Which domains of Fz3 and Fz6 might account for the subtly different activities of these two proteins? Alignment of mouse Fz3 and Fz6 sequences reveals a pattern of amino acid conservation that differs between domains, with 50% identity in the extracellular N-terminal cysteine-rich domain and 68% amino acid identity in the transmembrane domain, but only 23-29% identity in the cytoplasmic C-terminal tail (the range reflecting the different lengths of Fz3 and Fz6 C-terminal tails; supplementary material Fig. S6). Similar results are obtained in comparisons across other vertebrate species. Additionally, the C-terminal tails of Fz3 (169 amino acids) and Fz6 (212 amino acids) are substantially longer than the C-terminal tails of other Fz family members. The high degree of divergence between Fz3 and Fz6 in the C-terminal tail suggests that part of the activity difference between these two proteins lies in the C-terminal tails.
proteins in the context of CNS development may reside in this region, an idea that could be tested by swapping this domain.

Alignments of Fz amino acid sequences deduced from genomic and cDNA sequences of diverse vertebrates and invertebrates show that the Fz3/Fz6 branch within the Fz family arose in the vertebrate lineage (Fig. 7). Moreover, the division of that branch into Fz3 and Fz6 sub-branches appears to have occurred early in the vertebrate lineage, as the two sub-branches are found among all major vertebrate divisions. By contrast, the genome of C. intestinalis, a primitive chordate, codes for only one Fz3/Fz6-like sequence. [The identification of a Fz3 homologue but not a Fz6 homologue among P. marinus (lamprey) sequences should be interpreted cautiously, as genomic sequences from this species are substantially incomplete.] In sum, current evidence indicates that all jawed vertebrates (Gnathostomata) use distinct Fz3 and Fz6 proteins, suggesting that the division of function between these two family members occurred at least 400 million years ago.

**DISCUSSION**

The experiments reported here provide strong evidence that Fz3 and Fz6 have broadly similar functional properties. They also imply that the molecular mechanisms of transmembrane signaling in Fz3-mediated axon growth and guidance and in Fz6-mediated hair follicle polarity involve at least some homologous protein–protein interactions. As Fz3 and Fz6 homologues exist in amphibians, birds, fish and mammals, the signaling mechanisms that use their shared structural and functional motifs likely pre-date the ancestral duplication event that created separate Fz3 and Fz6 genes at the beginning of the vertebrate radiation. This general view is further supported by the observation that loss-of-function mutation of the
core PCP gene \textit{Celsr1} produces a hair follicle orientation phenotype closely resembling the \textit{Fz6}^{-/-} phenotype (Ravni et al., 2009), and, as noted in the Introduction, loss-of-function mutation of the homologous \textit{Celsr3} gene produces an axon guidance phenotype closely resembling the \textit{Fz3}^{-/-} phenotype (Tissir et al., 2005). The emerging picture is one in which Celsr and Fz represent the core components of an ancient and versatile polarity signaling complex. It is not clear whether other core PCP genes, which were initially defined in the context of epithelial polarity, also play a role in axon growth and guidance.

Partial redundancy and partial interchangeability of Fz3 and Fz6

The \textit{Fz3} and \textit{Fz6} rescue experiments described here can be conceptualized as a genetically engineered extension of the ‘experiment of nature’ in which \textit{Fz3} and \textit{Fz6} function redundantly in the context of neural tube and eyelid closure, inner ear sensory hair cell orientation and (as shown here) patterning of lingual papillae. Because functional redundancy is predicated on spatiotemporal overlap in expression, a failure to observe redundancy in any particular context could simply reflect the absence of expression of one of the genes, as appears to be the case for \textit{Fz3} in skin and hair follicles. In the experiments described here, we engineered ubiquitous expression of the rescuing constructs to maximize the chances of observing functional rescue, essentially creating a synthetic form of redundancy. Conveniently, it appears that ubiquitous production of \textit{Fz3} or \textit{Fz6} is not deleterious, although we cannot rule out the possibility of subtle effects. It also appears that ubiquitous production of \textit{Fz3} or \textit{Fz6} does not elicit the induction of aberrantly oriented epithelial structures of the type observed in the \textit{Drosophila} wing and abdomen at the boundaries between clones that differ in the level of \textit{Fz} gene expression (Adler, 2002; Struhl et al., 2012).

The contrast between the ability of ubiquitous \textit{Fz3} to fully rescue the \textit{Fz6}^{-/-} hair follicle orientation/epithelial polarity phenotype and the failure of ubiquitous \textit{Fz6} to fully rescue the \textit{Fz3}^{-/-} axon growth and guidance phenotype suggests that epithelial polarity may be the more fundamental of the two processes, whereas axon growth/guidance may represent a process for which \textit{Fz3} has evolved subtle alterations relative to \textit{Fz6}. The conserved nature of epithelial PCP signaling can be seen in the morphological and molecular similarities between PCP in mammalian epithelia and in the \textit{Drosophila} cuticle and wing, both of which require \textit{Frizzled}, \textit{Stan/Fmi/Celsr} and \textit{Vang/Vanl} genes, and both of which feature asymmetric PCP protein complexes (Devenport and Fuchs, 2008; Goodrich and Strutt, 2011; Wang et al., 2006b). In \textit{Drosophila} and \textit{C. elegans}, \textit{Stan/Fmi/Celsr} and \textit{Frizzled} genes have been implicated in axon guidance, branching and target selection, and \textit{Drosophila} \textit{Stan/Fmi} has been implicated in self-avoidance in sensory dendrite tiling (Huarca Najarro and Ackley, 2013; Lee et al., 2003; Matsubara et al., 2011; Ng, 2012; Senti et al., 2003; Steinel and Whittington, 2009). In view of the subtly different biological activities of \textit{Fz3} and \textit{Fz6} observed here, it would be interesting to investigate whether specific classes of mutations in \textit{Drosophila} \textit{Stan/Fmi} might differentially affect epidermal polarity versus axonal/dendritic pathfinding/target selection/tiling.

Evolution of PCP genes

A general issue in the study of genome evolution relates to the biochemical and evolutionary forces that determine the sizes of gene/protein families in different species and the extent of sequence divergence among family members. In some instances, the functional properties of a gene/protein family are sufficiently well understood to provide some insights into this issue; this is especially true when the relevant biochemical properties of the proteins can be defined \textit{in vitro}. For example, the diversification of the mammalian globin family was likely driven by the advantages associated with the different oxygen affinities of embryonic, fetal and adult hemoglobins, which promote oxygen transfer from the mother to the embryo or fetus; the diversification of the vertebrate immunoglobin family was likely driven by the advantages associated with distinct binding specificities imparted by different variable regions, which increase the diversity of the immune repertoire; and the diversification of the visual pigment family was likely driven by the advantages associated with different absorbance spectra of visual pigment receptors, which determine the wavelengths of light that can be detected, and by the advantages associated with increasing numbers of receptors, which permits a higher dimensionality in the resulting color vision. However, for many gene families, defining the relationships between sequence, function and evolutionary pressure is more challenging, especially if the encoded proteins function in multiple biological processes. Proteins in this category include, for example, myosin (over 40 family members in mammals), small GTPases (over 100 family members in mammals) and matrix metalloproteinases (over 25 family members in mammals).
For the gene families encoding PCP signaling proteins, the evolutionary pressures and relationships are rendered more complex by the additional involvement of some members of these protein families in canonical Wnt signaling and/or Wnt/calcium signaling. For example, Drosophila Fz, one of four Drosophila Frizzled proteins, functions in both canonical and PCP signaling (Strutt et al., 2012), as do Drosophila Dsh and mammalian Dvl proteins (Gao and Chen, 2010; Wynshaw-Boris, 2012). In mammals, the extent to which each of the ten Fz proteins participate in more than one signaling pathway is not clear, although current in vivo data are consistent with a division of labor in which some Fz proteins, such as Fz4, signal predominantly or exclusively via the canonical Wnt pathway, and others, such as Fz3 and Fz6, signal predominantly or exclusively via the PCP pathway (Wang and Nathans, 2007; Xu et al., 2004). One evolutionary force for PCP gene diversification is likely to act at the level of gene expression, as the requirements for spatially and temporally distinctive cell type-specific patterns of expression may exert selective pressures that can only be satisfied by evolving multiple family members with distinct promoter and enhancer sequences. The success of our genetic rescue experiments based on ubiquitous production of Fz3 and Fz6 serves as a reminder that we are still largely ignorant of the role that spatiotemporal control of gene expression plays in polarity signaling.

MATERIALS AND METHODS

Mouse lines

The Z/3xHA-Fz3, Z/3xHA-Fz6 and R26-3xHA-Fz6 alleles were generated by homologous recombination in mouse embryonic stem (ES) cells using standard techniques. Targeting constructs (Fig. 1C) were electroporated into R1 mouse ES cells. Colonies were grown in medium containing G418 and ganclovir, and were screened by karyotyping and Southern blot hybridization. Positive clones were injected into C57BL/6 blastocysts to generate chimeric founders, and germline transmission was confirmed by Southern blot hybridization and PCR.

The following mouse alleles were also used: Fz1<sup>−/−</sup> and Fz2<sup>−/−</sup> (Yu et al., 2010), Fz3<sup>−/−</sup> (Wang et al., 2002), Fz3<sup>5/5<sup>0</sup></sup> (Hua et al., 2012), Fz6<sup>−/−</sup> (Gao et al., 2004), Hprt-LSL-tdTomato (Wu et al., 2014), K14-Cre (Dessaud et al., 2007), K17-GFP (Bianchi et al., 2005), Osig<sup>−/−</sup> (Dessaud et al., 2007) and Sos2-Cre (Hayashi et al., 2002). Mice were handled and housed according to the approved Institutional Animal Care and Use Committee (IACUC) protocol MO13M469 of the Johns Hopkins Medical Institutions.

Reagents and immunohistochemistry

The following primary antibodies were used for immunohistochemistry or western blotting: mouse monoclonal anti-neuromelanin (165 kDa; 2H3, Developmental Studies Hybridoma Bank; 1:1000), rabbit anti-3×HA (T. Rotolo and J.N., unpublished; 1:500), rabbit anti-Fz3 and anti-Fz6 antibodies (Wang et al., 2002), rabbit anti-Fz3CKO (Hua et al., 2013), anti-K17 (a gift from Dr Pierre Coulombe, Johns Hopkins University, Baltimore, MD, USA; Developmental Studies Hybridoma Bank; 1:1000), rat mAb anti-cytokeratin8 (CK8; TROMA-1; Developmental Studies Hybridoma Bank; 1:500), rabbit anti-Fz3 and anti-Fz6 antibodies (Wang et al., 2006b; 1:1000-1:5000), and rabbit anti-3×HA (T. Rotolo and J.N., unpublished; 1:10,000). Secondary antibodies were Alexa Fluor 488 or 594 conjugated at room temperature for 2 days with gentle end-over-end rotation, or color-coded based on depth. BBBA-cleared embryos were positioned in custom-built metal embryo holders consisting of a shallow triangular trough (sides, 2 cm×2 cm×1 cm; depths, 1, 2, 3, or 4 mm). The trough was filled with BBBA and coverslipped during imaging.

Microscopy and image analysis

Immunostained samples were imaged using a Zeiss LSM700 confocal microscope with Zen software. Images of whole-mount samples were acquired with a 10× air objective at 10 µm intervals in the z dimension, and the entire z stack was either collapsed using a maximum intensity projection or color-coded based on depth. BBBA-cleared embryos were positioned in custom-built metal embryo holders consisting of a shallow triangular trough (sides, 2 cm×2 cm×1 cm; depths, 1, 2, 3, or 4 mm). The trough was filled with BBBA and coverslipped during imaging.

Sequence alignments

Amino acid sequences were aligned with ClustalW (Vector NTI software). The dendrogram branch lengths are proportional to percent amino acid non-identity.

Acknowledgements

This work was supported by the Howard Hughes Medical Institute. The authors thank John Williams for assistance with genotyping, Terry Stromsky for assistance with artwork, and Alisa Mo and Amir Rattner for helpful comments on the manuscript.
Competing interests
The authors declare no competing financial interests.

Author contributions
Z.L.H., H.C., Y.W. and J.N. designed experiments; P.M.S. constructed knock-in mice; Z.L.H., H.C. and Y.W. conducted experiments; Z.L.H., H.C., Y.W. and J.N. analyzed data and wrote the paper.

Funding
This work was supported by the Howard Hughes Medical Institute. Deposited in PMC for release after 6 months.

Supplemental material
Supplemental material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110189/-/DC1

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