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Ankrd6 is a mammalian functional homolog of Drosophila planar cell polarity gene diego and regulates coordinated cellular orientation in the mouse inner ear

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Abstract

The coordinated polarization of neighboring cells within the plane of the tissue, known as planar cell polarity (PCP), is a recurring theme in biology. It is required for numerous developmental processes for the form and function of many tissues and organs across species. The genetic pathway regulating PCP was first discovered in Drosophila, and an analogous but distinct pathway is emerging in vertebrates. It consists of membrane protein complexes known as core PCP proteins that are conserved across species. Here we report that the over-expression of the murine Ankrd6 (mAnkrd6) gene that shares homology with Drosophila core PCP gene diego causes a typical PCP phenotype in Drosophila, and mAnkrd6 can rescue the loss of function of diego in Drosophila. In mice, mAnkrd6 protein is asymmetrically localized in cells of the inner ear sensory organs, characteristic of components of conserved core PCP complexes. The loss of mAnkrd6 causes PCP defects in the inner ear sensory organs. Moreover, canonical Wnt signaling is significantly increased in mouse embryonic fibroblasts from mAnkrd6 knockout mice in comparison to wild type controls. Together, these results indicated that mAnkrd6 is a functional homolog of the Drosophila diego gene for mammalian PCP regulation and act to suppress canonical Wnt signaling.
Keywords

PCP; Inner Ear; Ankrd6; Diversin; Inversin; Diego

Introduction

Planar cell polarity (PCP) refers to coordinated polarization of neighboring cells in reference to the plane of the tissue. PCP is exhibited by many forms across species, such as convergence of the cells along one axis of the tissue that results in concomitant extension at a perpendicular axis, a process known as convergent extension (CE), and coordinated orientation of cellular protrusions within a tissue (Bayly and Axelrod, 2011; Solnica-Krezel, 2005; Torban et al., 2004). Inner ear sensory organs consist of sensory hair cells and interdigitating non-sensory supporting cells. Each sensory hair cell is polarized, consisting of an asymmetrically positioned primary cilium, the kinocilium, abutting a bundle of microvilli-derived stereocilia of graded height with the tallest stereocilium near the kinocilium. All of the hair cells are coordinately oriented within each sensory organ of the inner ear, displaying distinctive forms of vertebrate PCP (Kelly and Chen, 2007).

In Drosophila, a set of transmembrane proteins and their associated proteins, including Van gogh (Vang), Frizzled receptor (Fz), Flamingo (Fmi), Dishevelled (Dsh), Prickle (Pk), and Diego (Dgo), form polarized membrane-associated complexes to mediate coordinated polarization of all of the cells across the entire tissue (Peng and Axelrod, 2012; Wu and Mlodzik, 2009). Known as core PCP proteins, they are required for PCP in all adult tissues with PCP features. Non-conventional cadherins Fat (Ft) and Dachsous (Ds) also regulate PCP (Adler, 2012; Lawrence and Casal, 2013; Matis and Axelrod, 2013). Several murine genes homologous to Drosophila core PCP genes, including Vang-like 2 (Vangl2) (Montcouquiol et al., 2003), Frizzled (Fz) (Wang et al., 2006b), Dishevelled (Dvl) (Wang et al., 2005), and Fmi homolog Celsr1(Curtin et al., 2003), have been shown to regulate PCP in the inner ear and several other tissues in mice (Goodrich and Strutt, 2011). Mutations in Vangl2, Fz, Dvl, and Celsr1 genes cause the loss of coordinated orientation of sensory hair cells (Curtin et al., 2003; Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b). Defective PCP regulation also results in the formation of a shortened cochlear duct with patterning defects (Montcouquiol et al., 2003; Wang et al., 2005) due to abnormal cellular boundary remodeling during CE of the cochlear duct (Chacon-Heszele et al., 2012). In addition to Drosophila PCP gene homologs, ciliary and basal body genes act in parallel to or downstream of core PCP genes and are required for the intrinsic polarization of hair cells (Jones et al., 2008; Ross et al., 2005; Sipe and Lu, 2011).

In contrast to demonstrated essential roles in PCP signaling for Vangl2, Fz, Dvl, and Celsr1 genes, the roles of the Dgo homologs in mammalian PCP processes have not been conclusively illustrated. Dgo interacts with the Fz-Dsh polarity complex and limits Pk to the opposite Vang polarity complex to propagate polarity signals and coordinate polarization among neighboring cells (Das et al., 2004; Jenny et al., 2005). In vertebrates, the closest Dgo homolog is Ankrd6, also known as Diversin. Morpholino studies implicated Ankrd6 in zebrafish gastrulation movement (Moeller et al., 2006; Schwarz-Romond et al., 2002).
over-expression of a truncated Ankrd6 protein lacking the ankyrin repeat domain (likely a dominant negative form). Ankrd6 has been shown to regulate gastrulation movements and is required for normal heart formation in zebrafish (Moeller et al., 2006). In addition, ectopic expression of \textit{mAnkrd6} in \textit{Drosophila} eye interferes with the establishment of PCP in \textit{Drosophila} eye (Moeller et al., 2006). Furthermore, Ankrd6 appears to localize to the basal body compartment and regulate basal body structure and the polarity of the cilia in Xenopus (Itoh et al., 2009; Itoh and Sokol, 2011; Yasunaga et al., 2011). In addition, morpholino and biochemical studies revealed a role for Ankrd6 in suppressing β-catenin-mediated canonical Wnt signaling (Schwarz-Romond et al., 2002; van Amerongen et al., 2010). Together, these data suggested that Ankrd6 could interact with \textit{Drosophila} PCP components, linked Ankrd6 to cilia polarity and CE, and indicated a biochemical role for Ankrd6 in suppressing canonical Wnt signaling. However, it is not known whether Ankrd6 functions in PCP regulation in mammals, whether mouse Ankrd6 (mA Ankrd6) is a core PCP protein that functions characteristically in association with asymmetric membrane PCP complexes, and whether it acts with primary cilia in PCP regulation. In this study, we tested the functional conservation of \textit{mAnkrd6} in \textit{Drosophila} PCP regulation and analyzed the roles of Ankrd6 in the mouse inner ear sensory epithelia. Similar to a previously reported study (Moeller et al., 2006), ectopic expression of \textit{mAnkrd6} causes PCP phenotypes in \textit{Drosophila} wing and eye. In addition, we revealed that \textit{mAnkrd6} can rescue the loss of function of \textit{diego} in \textit{Drosophila}. In the mouse inner ear sensory epithelia, Ankrd6 shows an asymmetric membrane-associated localization, characteristic of core PCP proteins, while it is not detected in the basal body or the primary cilia in the inner ear cells. The knockout of \textit{Ankrd6} gene in mice disrupts precisely coordinated cellular polarity in the cochlea and the vestibule, and leads to significantly increased canonical Wnt activity in mouse embryonic fibroblasts. These data collectively suggest that Ankrd6 is a functional homolog of Dgo in regulating epithelial PCP and are consistent with Ankrd6 playing a role in antagonizing canonical Wnt signaling.

Results and Discussions

Ankrd6 gain-of-function causes planar cell polarity defects in \textit{Drosophila}

Ankrd6 is the vertebrate ortholog of \textit{Drosophila} Dgo by Ensembl sequence homology (Schwarz-Romond et al., 2002). It shares the homologous N-terminal Ankyrin repeats and 24% identity with Dgo beyond the Ankyrin repeat region. To directly test whether Ankrd6 is a functional homolog of Dgo, we performed gain-of-function and rescue analysis for \textit{mAnkrd6} in \textit{Drosophila} (Figs. 1, 2).

The bristles of fly wing cells are normally uniformly oriented toward the distal direction. To test the gain-of-function of \textit{mAnkrd6}, the \textit{UAS: mAnkrd6} transgene expression was driven in the posterior compartments of developing flies by either \textit{en:GAL4} or \textit{hh:GAL4} (Kornberg, 1981; Ma et al., 1993). Flies that contain either the \textit{en:GAL4} driver (not shown) or the non-expressing \textit{UAS: mAnkrd6} transgene alone (not shown), or that express a \textit{UAS: GFP} transgene under the control of the \textit{en:GAL4} driver (Fig. 1A), are phenotypically wild-type with uniformly oriented bristles - the latter control experiment demonstrating that over-expression of simply any protein by \textit{en:GAL4} is not sufficient to cause bristle polarity.
defects. In flies that are mutant for diego (diego\(^{380/380}\)) (Feiguin et al., 2001), the bristles in the wing exhibit strong polarity defects and display a stereotypical whorl phenotype (Fig. 1B). We examined multiple transgenic lines of en::mAnkrd6 flies (n = 9 lines) that express the UAS:mAnkrd6 transgene in the posterior compartment of the wing under the control of the en:GAL4 driver, and found that all of the lines displayed strong bristle polarity defects (Fig. 1C), and in some cases gross defects in the overall morphology of the entire wing (not shown). In comparison, hh::mAnkrd6 flies (n = 10 lines) exhibited mild bristle polarity defects, and there were no gross defects in the wing morphology (not shown).

The photoreceptors that make up each eye facet are chiral in nature, exhibiting a characteristic trapezoid shape (Fig. 1D–F). The eye facets are polarized across the adult eye epithelium and reverse their orientation relative to a line of symmetry that runs horizontally across the midline (Fig. 1D′–F′, dashed line). Flies that contain either the hh:GAL4 driver (not shown) or the UAS:mAnkrd6 transgene alone (not shown), or that express UAS:GFP under the control of the hh:GAL4 driver (Fig. 1D, D′), are phenotypically wild-type with precisely oriented eye facets within either side of the midline and across the midline. As reported previously, flies that are mutant for diego (diego\(^{380/380}\)) exhibit strong polarity defects in the eye, which include the loss of chirality and mis-oriented facets (Fig. 1E, E′).

Flies that express the UAS:mAnkrd6 transgene under the control of the hh:GAL4 driver (or hh::mAnkrd6) contain mis-oriented facets while some facets lack the normal complement of photoreceptor cells (Fig. 1F, F′).

**Ankrd6 functionally complements for the loss of the Drosophila diego gene**

The PCP phenotypes associated with over-expression of mAnkrd6 in Drosophila (Fig. 1) support a conserved role for mAnkrd6 in PCP regulation. We further tested whether mAnkrd6 could rescue for the loss of diego in Drosophila (Fig. 2). Because we could distinguish between the mild bristle polarity defects in hh::mAnkrd6 wings from the whorl phenotype of diego wings, and because the anterior compartment could serve as an internal control in the same wing, we used the hh:GAL4 driver to express mAnkrd6 in the posterior compartments of wings of diego flies for the rescue experiments in the wing. Control flies that express either the hh:GAL4 driver (Fig. 2A) or the non-expressing UAS-mAnkrd6 transgene (Fig. 2B) in the diego background display whorls that are indistinguishable from those of the diego mutants (Fig. 1B). Flies that express the UAS-mAnkrd6 transgene under the control of the hh:GAL4 driver in the diego background (Fig. 2C, D) show a complete suppression of the diego mutant whorl phenotype in the posterior wing compartments (n=25, Fig 2C–D), while whorls were observed in the anterior compartment of the wings (n=25, Fig. 2E), demonstrating the specificity of the functional rescue by mAnkrd6. Residual mild polarity defects were attributed to hh::mAnkrd6 gain-of-function.

A previous study examined the functional rescue of diego by mAnkrd6 expression using the sevenless:GAL4 driver in the Drosophila eye and reported the failure to functionally rescue the diego phenotype (Moeller et al., 2006). Because sevenless:GAL4 expresses specifically only in the R3 and R4 photoreceptor cells, we turned to the hh:GAL4 driver that expresses in the entire eye disc, and therefore all of the photoreceptor cells, for the rescue experiments. The diego PCP phenotype in Drosophila eye is very strong (Fig. 1) (Feiguin et al., 2001).
We examined for a functional rescue of the prevalent diego phenotype by hh::mAnkrd6 in the Drosophila eye and observed the rescue of apparent mis-rotated or achiral ommatidia (n=163 ommatidia) that are characteristically present in the diego mutants.

Together, mAnkrd6 over-expression and rescue experiments in Drosophila indicate that mAnkrd6 is homologous to Dgo and able to interact with components of the Drosophila PCP pathway to influence cellular polarity in Drosophila.

**Ankrd6 protein is asymmetrically localized in the inner ear sensory organs**

The inner ear has six sensory organs, one in the cochlea for hearing and five in the vestibule for positional sensations. The sensory hair cells in each of the inner ear sensory organs are polarized coordinately, displaying distinctive forms of epithelial PCP (Kelly and Chen, 2007). A hallmark of the core PCP proteins in Drosophila and vertebrates is their asymmetric and polarized membrane localization that is parallel to the axis of PCP (Rida and Chen, 2009; Wu and Mlodzik, 2009), which is thought to coordinate the polarity of neighbor cells along the PCP axis. To evaluate the cellular role for Ankrd6, we generated an antibody against mAnkrd6, and examined the subcellular localization of Ankrd6 in the cochlea and vestibule (Fig. 3).

In the cochlea, the microvilli-derived stereocilia of graded height are arranged into a V-shaped bundle on the apical surface of each hair cell and all of the hair cells in the cochlea are oriented uniformly with the vertex of the stereociliary bundle pointing to the periphery of the cochlear spiral or in the medial-to-lateral direction (Fig. 3A, B). Ankrd6 is enriched to distinct membrane regions in the organ of Corti (Fig. 3A, B, B′, B″), at the boundaries between a hair cell and a supporting cell and between supporting cells (Fig. 3A, B, B′, B″). Moreover, this enrichment of Ankrd6 at the boundaries between a hair cell and a supporting cell appears on the medial side of the hair cells and is polarized along the medial-to-lateral PCP axis (Fig. 3B′), characteristic of a core PCP protein.

In the macula of the utricle, the hair cells are also polarized in a coordinated manner (Rida and Chen, 2009). The hair bundle protruding from the apical surface of each vestibular hair cell consists of numerous stereocilia arranged in a bundle with graded height from the center to one edge of the apical cortex and a single eccentrically positioned kinocilium near the tallest stereocilia. The orientation of hair bundles in the vestibule can be visualized by the position of the kinocilium, recognized as a region devoid of α-spectrin staining in the apical cortex of each hair cell that is known as the foniculus (Fig. 3C–H, H′). The hair cells at the medial region of the utricle are oriented with their kinocilium positioned toward the periphery or lateral edge of the utricle while the hair cells at the periphery region of the utricle are oriented in the opposite direction with their kinocilia positioned toward the medial edge, forming an imaginative line of polarity reversal where the two populations of hair cells with opposite orientations meet (Fig. 3C–E). Ankrd6 localization in the utricle is also polarized along the PCP axis of the sensory epithelium (Fig. 3C–H, H′). Ankrd6 is enriched at the cellular boundaries between a hair cell and a supporting cell and cellular boundaries between two supporting cells (Fig. 3C–H, H′). It is noted that the localization of Ankrd6 at the hair-supporting cell boundaries appears at the medial side of hair cells across the entire sensory epithelium, regardless of the opposite polarity of hair cells across the line.
of polarity reversal (Fig. 3E, E′, H, H′). For instance, Ankrd6 localization is near the
kinocilium or away from the kinocilium in hair cells that are at the lateral side or the medial
side, respectively, of the line of polarity reversal in the utricle (Fig. 3C–H, H′).

We further compared the relative localizations of Ankrd6 with core PCP protein Vangl2
(Fig. 3I–J, J′). Vangl2 is essential for all the known PCP processes in vertebrates and is
asymmetrically localized in the inner ear sensory epithelia (Kibar et al., 2001; Montcouquiol
et al., 2006; Qian et al., 2007). It appears that Ankrd6 and Vangl2 are localized to the same
cellular boundaries between a hair cell and a supporting cell in the cochlea (Fig. 3I, I′).
However, the localizations of Ankrd6 and Vangl2 are not overlapped at the same cellular
boundaries, but appear to be on the opposing cellular sides that form the boundaries (Fig. 3I
′). Moreover, examination of the localization of Ankrd6 at the cellular boundaries between
supporting cells 1 and 3, and between supporting cells 2 and 3 (Fig. 3B, B′) suggests that
Ankrd6 is located to the medial side of supporting cells 1 and 2, rather than to the lateral
side of the supporting cell 3, since the lateral side of the supporting cell 3 is continuous but
the line of Ankrd6 protein signal at the boundary is broken at the line of the separation
between supporting cells 1 and 2 (Fig. 3B, B′). In the vestibule, Ankrd6 and Vangl2 show a
partially overlapping localization to some of the boundaries while only Vangl2 is detected in
other cellular boundaries (Fig. 3I, J′).

In vertebrates, Inversin also shares the homology of N-terminal Ankyrin-repeat domains
with Dgo (Fig. S1). Inversin has been shown to be a ciliary protein in some tissues and
functions as a switch between Wnt signaling pathways (Simons et al., 2005), sharing similar
functions to reported roles of Ankrd6. Ankrd6 was reported to be localized to the cilia in
Xenopus dermis (Itoh et al., 2009; Simons et al., 2005; Yasunaga et al., 2011). In the
cochlea, due to the background staining, we could not determine whether Ankrd6 has above
background levels in the basal body or kinocilia of hair cells (Figs. 3, S3). Using a
transgenic mouse line that carries a functional Inversin-GFP fusion at the Inversin locus
(Watanabe et al., 2003), we found that Inversin does not show a membrane enrichment in the
cochlear epithelium (Fig. S4), but a distinct localization to the primary cilia in the cochlea
(Fig. S4).

These localization data show that, Ankrd6 is asymmetrically localized along the PCP axis
characteristic of a core PCP protein, implicating a potential role in PCP regulation in the
inner ear. Furthermore, the non-overlapping localization of Ankrd6 and Vangl2 at the same
cellular boundaries suggests that the two proteins may be localized to the opposing sides
from two different cells that form the boundary. Finally, the localization of Ankrd6
regardless of the polarity of individual hair cells in the vestibule is similar to what was
observed for Pk2, supporting the hypothesis that additional regulatory mechanisms for
intrinsic polarity of hair cells are involved (Deans et al., 2007; Ezan and Montcouquiol,
2013). The apparently distinctive localization of Ankrd6 and Inversin in the inner ear cells
suggests unique functions and functional compartments for the two proteins.
Ankrd6 interacts with Vangl2 to regulate hair cell polarity in the cochlea

The asymmetric and polarized subcellular localization of Ankrd6 in the cochlear and vestibular epithelia is highly suggestive of its potential role in PCP regulation in the inner ear sensory organs. We generated a mouse knockout line to examine the role of Ankrd6.

The mAnkrd6 gene has 17 exons. The start codon ATG is located within the 4th exon and the next ATG codon codes for amino acid 405 of the Ankrd6 protein. We created a construct using sequences flanking exon 4 and generated ES cells and mice carrying the Ankrd6 knockout allele with exon 4 deleted (Fig. S2). Despite the loss of Ankrd6, the homozygous Ankrd6 null mutants survive with no apparent behavior or other noticeable morphologic abnormalities. In particular, examination and quantification of the polarity of hair cells in the cochlea from Ankrd6 mutants showed that there is no statistically significant deviation for hair cell orientation in comparison to controls at from E17 to postnatal day 10 (P10) (Fig. 4A–H, Figs. S5, S6). Lgn is a component of the apical compartmentation complexes and localized to the lateral region of the apical cortex of the hair cells to regulate the positioning of the basal body and the polarity of hair bundle (Tarchini et al., 2013). Its localization in the hair cells at E18 is not affected in the absence of Ankrd6 (Fig. S5). PCP in the organ of Corti could also be revealed by the orientation of phalangeal processes of supporting cells in the mature organ of Corti (Copley et al., 2013). The examination of supporting cell phalangeal processes did not reveal any abnormality in P10 Ankrd6−/− animals (Fig. S6). Together, these data provided an additional support that PCP is mostly not affected in cochleae from Ankrd6−/− animals during development and at P10.

We further bred mice carrying the Ankrd6 knockout allele with mice carrying the looptail loss-of-function allele of Vangl2 (Kibar et al., 2001), and examined hair cell polarity in Ankrd6 and Vangl2 compound mutants in comparison with Ankrd6 and Vangl2 single mutants (Fig. 4). In contrast to Andrd6−/− mutants, Ankrd6−/−;Vangl2Lp/+ mice showed both patterning defects and statistically significant hair cell polarity abnormality (Fig. 4G–M). In PCP mutants, such as Vangl2 looptail mutants, there is the loss of coordinated hair cell polarity (Montcouquiol et al., 2003). Furthermore, PCP mutants show loss of precise cellular patterning with the appearance of two rows of outer hair cells in the base and sporadic inner and outer hair cells additional to the normal one row of inner and three rows of outer hair cells, indicating defective CE of the cochlea that is also regulated by PCP genes (Montcouquiol et al., 2003; Wang et al., 2006a; Wang et al., 2005). There are regions in the cochlea where the precise patterning of four rows of hair cells is disrupted in Ankrd6−/−;Vangl2Lp/+ samples (Fig. 4K, L). In addition, the coordinated orientation in the outer-most row of hair cells toward the periphery or lateral side of the cochlear spiral is significantly disrupted in Ankrd6−/−;Vangl2Lp/+ samples (Fig. 4). 29.2% of the 4th row of hair cells in Ankrd6−/−;Vangl2Lp/+ animals have an orientation deviation of 30° or larger from the PCP axis, in comparison to 0.9% in wild-type control, 0.3% in Ankrd6−/−;Vangl2Lp/+ or 1.7% in Ankrd6−/− samples (Fig. 4K–M). The coordinated orientation of the outer-most row of outer hair cells is often affected most severely in PCP mutants (Wang et al., 2005; Wang et al., 2006b). Interestingly, in-situ hybridization showed that the level of Ankrd6 transcripts is higher in the lateral region of the cochlear spiral during development (Fig. S7), while PCP genes, such as Celsr1, show higher expression
levels at the medial region of the cochlear epithelium (Ren et al., 2013). The stronger mis-orientation phenotype in the outer-most row of hair cells in Vangl2 and Ankrd6 compound mutants may represent the sensitivity of this row of hair cells to the alteration of general PCP regulation and the sensitivity to the loss of Ankrd6 in the region.

In addition to the patterning and orientation abnormalities, 100% of Ankrd6−/−;Vangl2Lp/+ females (N=20) are sterile due to a blocked vaginal track, in contrast to <10% of Vangl2Lp/+ females examined to date (N>100). The female reproductive track phenotype is observed in other PCP compound mutants (Ren et al., 2013). The observed genetic interaction between Ankrd6 and Vangl2 in the female reproductive track development further supports that Ankrd6 and Vangl2 act in the same genetic pathway(s).

Ankrd6 is required for precise orientation of hair cells in the utricle

The vestibular sensory organs show distinct PCP. In the three cristae at the end of three semi-circular channels, hair cells are oriented uniformly. In the saccule and utricle, hair cells are oriented away from or toward, respectively, the line of polarity reversal. We examined the loss-of-function of Ankrd6 on the polarity of hair cells in the vestibular sensory organs.

In the three cristae and saccule of the vestibule, no hair cell polarity defect was observed in Ankrd6−/− animals. In the wild-type control utricles, hair cells across the line of polarity reversal are oriented toward each other and the hair cells on the same side of the line of polarity reversal is oriented uniformly (Fig. 5A, A'). In the Ankrd6−/− utricles, the line of polarity reversal is recognizable (Fig. 5B, B'). However, the uniform orientation of neighboring hair cells within the same side of the line of polarity reversal is disrupted in sporadic areas (Fig. 5B, B').

Ankrd6 suppresses canonical Wnt signaling

Ankrd6 has distinct functional domains. The N-terminal Ankyrin repeats domain of Ankrd6 is homologous to Dgo and mediates Ankrd6 interaction with the PCP-specific domain of Dvl (Moeller et al., 2006; Schwarz-Romond et al., 2002). Biochemical and *in vitro* cell culture analysis revealed that Ankrd6 also consists of a central casein kinase-binding domain and a C-terminal conductin-binding domain, which are essential for regulating or suppressing β-catenin-mediated canonical Wnt signaling in cultured cells (Moeller et al., 2006; Schwarz-Romond et al., 2002). In Ankrd6−/− animals, no gross morphological abnormalities associated with abnormal canonical Wnt signaling in the development of lung, heart, brain and limbs that impact the survival or size of the animals were observed. The absence of system developmental defects associated with deregulation of canonical Wnt signaling in Ankrd6−/− animals suggests that there may be compensatory or redundant mechanisms for Ankrd6 in regulating canonical Wnt signaling in certain tissues *in vivo*.

To test whether Ankrd6 could participate in regulating elicited activation of canonical Wnt signaling in mouse cells as observed in cultured cells (Moeller et al., 2006; Schwarz-Romond et al., 2002), we isolated embryonic fibroblasts (MEFs) from E12.5 wild-type and Ankrd6−/− animals, co-transfected MEFs with β-galactosidase and TOPFLASH luciferase reporter (TOP) constructs (Fig. 6). The TOPFLASH reporter construct consists of luciferase reporter cassette under the control of tandem repeats of TCF binding sites, which is activated
when canonical Wnt signaling is augmented. A mutated TCF binding site TOPFLASH luciferase reporter construct, or FOP construct, was included as a control to determine the specificity of the reporter gene expression response. Transfected cells were treated with canonical Wnt ligand Wnt3a-containing conditioned medium or control medium for eight hours and harvested for standard luciferase assays to measure activated and base line canonical Wnt activities, respectively (Li et al., 2012). All of the measurements were normalized against β-galactosidase activity for transfection efficiency, and all data were subjected to a Student’s t-test. Ankrd6−/− MEFs have a higher base level of canonical Wnt activity in comparison to wild type MEFs, and the exposure of Ankrd6−/− MEFs to canonical Wnt molecule Wnt3a elicited a significantly higher canonical Wnt activity (Fig. 6). The canonical Wnt activity in wild type MEFs in response to Wnt3a conditional medium is diminutive in comparison (Fig. 6). The data supports that removal of Ankrd6 enhances the canonical Wnt response and Ankrd6 could act as a suppressor for canonical Wnt signaling under certain conditions.

Conclusions

PCP emerges as a common feature for many tissues in multi-cellular organisms. The regulation of PCP employs conserved genes from Drosophila to mammals. In this study, we show that Ankrd6, a mammalian gene homologous to a core PCP gene diego in Drosophila, could interact with Drosophila PCP components to regulate PCP in Drosophila wing and compound eyes. We further show that Ankrd6 is asymmetrically localized in the inner ear sensory organs, characteristic of core PCP proteins. While Ankrd6 appears to be dispensable for mouse development, it interacts with core PCP gene Vangl2 to regulate PCP in the inner ear and in the female reproductive track. Finally, we confirmed that Ankrd6 could act to suppress canonical Wnt signaling. The study demonstrated that Ankrd6 is a mammalian core PCP gene with a potential regulatory role in canonical Wnt signaling. Ankrd6 could act to suppress canonical Wnt signaling.

Materials and Methods

Drosophila stocks and constructs

Flies used for the wild type phenotypes were w1118. Transgenic stocks were: UAS-mAnkrd6, the Ankrd6 cDNA cloned in the pUAST vector and germ line transformations performed as described previously (Rubin and Spradling, 1982); hh:GAL4 UAS:GFP/TM6B, a GAL4-expressing enhancer trap allele of hedgehog driving UAS-GFP expression (referred as hh::GFP, Tanimoto, et al. Molecular Cell, 2000); en:GAL4 UAS:GFP/TM6B, a GAL4-expressing enhancer trap allele of engrailed driving UAS-GFP expression (referred as en::GFP, Marenda, et al. Development, 2005); diego380/CyO, a strong loss-of-function allele of diego (gift from Marek Mlodzik).

Drosophila adult eye and wing preparations

Sectioning and microscopic analysis of adult eyes were performed as previously described (Tomlinson and Ready, 1987). Adult wings were dissected, dehydrated in ethanol, mounted in DPX (Zeiss) and examined under an Olympus SZX12 upright microscope.
Mouse strains and animal care

Animal care and use was in accordance with US National Institutes of Health (NIH) guidelines and was approved by the Animal Care and Use Committee of Emory University. The following mouse strain was obtained from the Jackson Laboratories: LPT/Le (Vangl2<sup>Lp</sup>) carrying a single nucleotide G to A mutation, resulting in the change from serine to asparagine, at the C terminal cytoplasm domain (Kibar et al., 2001).

Ankrd6 antibody generation

cDNA for <i>mAnkrd6</i> was cloned from a cDNA library prepared from embryonic day 15.5 (E15.5) cochlear tissues and inserted into the plasmid pET-28a (+) to fuse <i>mAnkrd6</i> cDNA in frame with the His6 tag. The Ankrd6-His6 fusion protein was purified and used to generate antibodies against Ankrd6 in rabbit according to standard protocols (Spring Valley Laboratories, Inc. Maryland, USA). Sera from injected rabbits were tested by Western blot and immunoassaying. Western blot analysis indicated cross-reactivity with non-specific proteins while immunostaining with Ankrd6 wild-type and knockout tissues indicated specific signals at the plasma membrane.

Generation of Ankrd6 knockout mice

An 1851bp of DNA fragment and a 4243bp of DNA fragment upstream and downstream of Ankrd6 exon 4, respectively, were amplified and cloned to flank the LacZ and PGK-Neo cassette in the target vector (supp Fig. 2). The verified targeting vector was electroporated into 129 ES cells. Southern blots with probes outside the homologous arms and on the right homologous arm were carried out to identify ES cell clones that carry an Ankrd6 knockout allele. The identified ES cells were used to generate chimera mice. Southern blots were carried out to identify the founders and the germ line transmitted animals.

Inner ear sensory epithelia preparation and antibody immunostaining

Standard procedures were used to isolate and prepare whole mount inner ear sensory epithelia (Wang et al., 2005). The primary antibodies used in this study were raised against Ankrd6 (1:800), α-Spectrin (Chemicon, MAB1622, 1:200), p27<sup>Kip1</sup> (BD Transduction Laboratories, K25020, 1:200), and Myosin VI (Proteus Biosciences, 25-6791, 1:400). In addition, Rhodamine- or Alexa-Fluor-488 conjugated phalloidin (Invitrogen, 1:1000) were used for staining the actin-rich structures such as stereocilia, the cuticular plate of the hair cell, and the cortex of cells.

For image acquisition the following microscopes were used: Olympus SZX12 upright microscope, Olympus Fluoroview FV-1000 confocal microscope, and Zeiss LSM510 confocal microscope.

Analyses of stereociliary bundle orientation and morphology

The V-shaped hair bundle orientation was determined by drawing a line from the position of the kinocilium through the middle of the V-shaped stereocilia (bisection line). We defined the angle of orientation as the angle formed between the bisection line and the line parallel to the medial to lateral axis of the cochlear duct. In wild-type animals, this angle is close to
0°. Each row of hair cells was divided into three groups according to its position along the longitudinal axis of the cochlea: base, middle, and apex. Due to the differentiation gradient within the single cochlea during development, hair cells in the apex region are less developed. Only hair cells from the base and middle regions of the cochlea were included for polarity quantification. At least 25 hair cells in each row in each region were quantified for each sample, and at least three animals per genotype were analyzed. The distribution of angles along the length of the cochlear duct was plotted using Oriana3. Cells that had a central foniculus and/or circular stereocilia were classified as having the maximum deviation from the normal distribution, namely 180°. Data are presented as means. Statistical significance was analyzed by Chi-square analysis and Mardia Watson Wheeler tests using Oriana3.

Lef/Tcf canonical Wnt reporter assays

TOPFLASH luciferase reporter construct (Dr. Randall T. Moon, University of Washington, provided by Dr. Xing Dai, University of California, Irvine) and a pSV-β-galactosidase expression vector (a gift from Dr. Harish Joshi, Emory University) were cotransfected into primary cultured fibroblast cells isolated from Ankrd6 mutant mice and control wild type mice using Lipofectamine 2000. Transfected cells were treated with Wnt3a conditioned medium (isolated from Wnt3A-expressing cell line, ATCC# CRL-2647) or control medium (isolated from L2648, ATCC#CRL-2648) for 8 hours and harvested for standard luciferase assays to detect the canonical Wnt activity, all of which were normalized using β-galactosidase activity. All data were subjected to the Student’s t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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### Highlights

1. Over-expression of mAnkrd6 leads to planar cell polarity defects in *Drosophila*.
2. mAnkrd6 rescues the loss of planar cell polarity gene *diego* in *Drosophila*.
3. Ankrd6 shows asymmetric sub-cellular localization in the inner ear cells.
4. The loss of mAnkrd6 causes planar cell polarity defects in the inner ear.
5. Ankrd6−/− MEFs have significantly higher canonical Wnt activity.
Fig. 1. Ankrd6 gain-of-function causes planar cell polarity defects in the Drosophila eye and wing. (A–F) UAS:mAnkrd6 transgene expression was driven in the posterior compartments of developing flies by either en:GAL4 or hh:GAL4. Shown at high magnification are images of wings (A–C) and cross-sections of eyes (D–F) taken from adult flies of the indicated genotypes. Schematics in D′–F′ depict the orientations of eye facets in D–F. Control flies that contain either the en:GAL4 driver or the non-expressing UAS:mAnkrd6 transgene alone, or that express UAS:GFP under the control of the en:GAL4 driver (A) are phenotypically wild-type with uniformly oriented bristles. Flies that are mutant for diego (B, diego380/380), the fly homolog of Ankrd6, exhibit strong defects in bristle polarity in the wing displaying stereotypical whorls (B, yellow arrowheads). Flies that express the UAS:mAnkrd6 transgene in the posterior compartment of the wing under the control of the en:GAL4 driver (C, en::Ankrd6) exhibit strong defects in bristle polarity. The eye facets are polarized across the adult eye epithelium in control flies expressing either the en:GAL4, hh:GAL4 or UAS:mAnkrd6 transgene alone (D, D′) and reverse their orientation relative to
a line of symmetry (D', dotted line) that runs horizontally across the midline. The photoreceptors that make up each eye facet are chiral in nature, exhibiting a characteristic trapezoid shape. As reported elsewhere, flies that are mutant for diego (E, E', diego\textsuperscript{380/380}) exhibit strong polarity defects in the eye, which include the loss of chirality and mis-oriented facets. Flies that express the \textit{UAS:mAnkrd6} transgene under the control of the \textit{hh:GAL4} driver (F, F', \textit{hh::Ankrd6}) contain mis-oriented facets (F, F', yellow arrowheads), while some facets lack the normal complement of photoreceptor cells (F, F', red arrowheads).
Fig. 2.
Ankrd6 functionally complements for the loss of the Drosophila diego gene. (A–E) Shown at high magnification are the wings taken from adult flies of the indicated genotypes, diego<sup>380/380</sup>, hh:GAL4 control (A, posterior compartment), diego<sup>380/380</sup>, UAS:mAnkrd6 control (B, posterior compartment), and diego<sup>380/380</sup>, hh::Ankrd6 (C, D, posterior compartment; E, anterior compartment). Control flies that express either the hh:GAL4 driver (A) or carry the non-expressing UAS-mAnkrd6 transgene (B) in the diego background contain mis-oriented bristles displaying characteristic whorls (A, B, red arrowheads) that are indistinguishable from those of the diego mutants by themselves. Flies
that express the *UAS-Ankr6* transgene under the control of the *hh:GAL4* driver in the *diego* background (C, D) show a complete suppression of the *diego*-specific whorl mutant phenotype in the posterior wing compartments (n=25), while whorls were observed in the anterior compartment of the wings (E, red arrowhead).

(F) The anterior-posterior compartment boundary (red line) and the relative wing regions shown at high magnification in panels A–E (black boxes) are indicated in a control wing.
Fig. 3.
Ankrd6 protein shows asymmetric distribution along the PCP axis in the inner ear sensory organs characteristic of core PCP proteins.

(A–I, I′) Shown are confocal projections of cochlear whole mounts isolated from wild-type mice at P1 (A–B, B′) and of utricule whole mounts isolated from wild-type mice at E18.5 (C–H, H′). Actin staining (A, white; B–B″, green) highlights the microvilli-derived stereocilia and illustrates the orientation of hair cells in the cochlea. Spectrin staining (C, F, white; E–E′ and H–H′, green) visualizes the apical cortex of individual hair cells in which the position of the kinocilium of each hair cell, known as the fonticulus, is devoid of Spectin staining and illustrates the orientation of hair cells in the utricle. Boxes in B, E and H outline regions presented in B′, B″, E′, and H′, respectively, at a higher magnification. Black
arrowheads indicate the supporting cell region separating the IHCs from the OHCs (A, B). m: medial; l: lateral. The dotted magenta line (C–E) marks the line of polarity reversal across which hair cells have opposite orientations in the utricle. Note that the asymmetric localization of Ankrd6 is observed at the cellular boundaries between hair cells and supporting cells (B′, E′, H′, white arrowheads) and at the cellular boundaries between supporting cells (B, B′, E′, H′, blue arrowheads); that the boundaries between supporting cells 1 and 3, and between supporting cells 2 and 3 are indicated by blue arrows (B, B″); and that hair cells on either side of the line of polarity reversal (C–E, dotted purple line) are oriented toward each other, and that the localization of Ankrd6 is not changed across the line of polarity reversal (E).

(I–J, J′) Shown are confocal images of P2 cochlear whole mounts (I, I′) and E18.5 vestibular whole mounts (J, J′) isolated from mice carrying a Vangl2-GFP transgene (green). The whole mounts were stained with the antibody against Ankrd6 (red). Note that, in the cochlea, Ankrd6 is asymmetrically localized to the same cellular boundaries as Vangl2-GFP protein, but its localization at the cellular boundaries does not completely overlap with that of the Vangl2-GFP fusion protein (I, I′). In the vestibule, both Ankrd6 and Vangl2 are localized to some of the cellular boundaries while only Vangl2-GFP is detectable in others (J, J′). The localization of Vangl2 and Ankrd6 does not completely overlap in the cellular boundaries consisting of both Ankrd6 and Vangl2 (J, J′). Scale bars: 10μm.
Fig. 4. 
Ankrd6 interacts with PCP gene Vangl2 to regulate the precise orientation and patterning of hair cells in the cochlea.

(A–L) Shown are confocal projections of cochlear whole mounts isolated from wild-type (A, B), Vangl2<sup>Lp/+</sup> (C, D), Ankrd6<sup>+/−</sup> (E, F), Ankrd6<sup>−/−</sup> (G, H), Vangl2<sup>Lp/+; Ankrd6<sup>−/−</sup></sup> (I, J), and Vangl2<sup>Lp/+; Ankrd6<sup>−/−</sup></sup> (K, L) mutant mice at the newborn stage or postnatal day 0 (P0). The samples were stained for phalloidin to visualize the stereociliary bundles and the cortical outline of hair cells. Yellow arrowheads (A–L) mark the supporting cell region that separates the first row of hair cells (inner hair cells) to the other three rows of hair cells (outer hair cells). Red arrowheads indicate the patterning abnormality (K, L). The images were taken from the base and 30% from the base of each cochlea. m: medial side of the cochlea; l: lateral side of the cochlea.

(M) The orientation of the last row of hair cells within the region 30% from the base was measured and plotted for the genotypes indicated.
Fig. 5.
Ankrd6 regulates coordinated orientation of hair cells in the utricle.
(A–B, B′) Shown are confocal images of utricle whole mounts isolated from P5 wild-type (A) and Ankrd6−/− mutants (B) and the corresponding drawings of hair cell orientation (A′, B′). The samples were stained with an antibody against α-Spectrin (A, B, white) to visualize the position of the kinocilium, or the orientation, of each hair cell. The dotted magenta lines denote the line of polarity reversal of the utricle. The red ovals (B) mark hair cells with deviated orientation indicated by the red arrow in Ankrd6−/− mutants.
Fig. 6. Canonical Wnt signaling is enhanced in *Ankrd6<sup>−/−</sup>* mouse embryonic fibroblast cells. Mouse embryonic fibroblasts (MEFs) were isolated from E12.5 wild-type and *Ankrd6<sup>−/−</sup>* animals and co-transfected with β-galactosidase and TOPFLASH luciferase reporter construct (TOP) or mutated TCF binding site TOPFLASH luciferase reporter construct (FOP). Transfected cells were treated with Wnt3a conditioned medium (Wnt3a) or control medium (control) for eight hours and harvested for standard luciferase assays to measure activated and base line canonical Wnt activities, respectively. All of the measurements were normalized against β-galactosidase activity for transfection efficiency, and all data were subjected to the Student’s t-test.