Testin interacts with Vangl2 genetically to regulate inner ear sensory cell orientation and the normal development of the female reproductive tract in mice

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Abstract

Background—Planar cell polarity (PCP) signaling regulates the coordinated polarization of cells and is required for the normal development and function of many tissues. Previous studies have identified conserved PCP genes, such as Van gogh-like 2 (Vangl2) and Prickle (Pk), in the regulation of coordinated orientation of inner ear hair cells and female reproductive tract development. Testin shares a PET-LIM homology with Pk. It is not clear whether Testin acts in PCP processes in mammals.

Results—We identified Testin as a Vangl2-interacting protein through a 2-hybrid screen with a cochlea cDNA library. Testin is enriched to cell-cell boundaries in the presence of Vangl2 in cultured cells. Genetic inactivation of Testin leads to abnormal hair cell orientation in the vestibule and cellular patterning defects in the cochlea. In addition, Testin genetically interacts with Vangl2 to regulate hair cell orientation in the cochlea and the opening of the vaginal tract.

Conclusions—Our findings suggested Testin as a gene involved in coordinated hair cell orientation in the inner ear and in female reproductive tract development. Furthermore, its genetic interaction with Vangl2 implicated it as a potential molecular link, responsible for mediating the role of Vangl2-containing membranous PCP complexes in directing morphologic polarization.

Keywords

Testin; Planar cell polarity; Vangl2; Inner ear; Female reproductive tract; Hair cells

Introduction

Planar cell polarity (PCP) refers to coordinated polarization of neighboring cells within the plane of a cell sheet. In vertebrates, PCP is regulated by a set of conserved genes known as core PCP genes, including Celsr, Van gogh-like (Vangl), Frizzled (Fz) receptor, and Prickle (Pk) genes (Wallingford, 2012). In vertebrates, PCP signaling is required for diverse cellular processes. Defects in PCP signaling cause abnormalities in many organs and tissues, such as the loss of coordinated orientation of inner ear sensory hair cells and a defective convergent

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extension (CE) of the cochlear duct (Curtin et al., 2003; Montcouquiol et al., 2003; Lu et al., 2004; Wang et al., 2005; Jones et al., 2008), lung branching (Ansley et al., 2003; Paudyal et al., 2010; Yates and Dean, 2011) and dermis (Devenport and Fuchs, 2008) abnormalities, kidney diseases (Kramer-Zucker et al., 2005), axonal defects (Tissir and Goffinet, 2010; Armstrong et al., 2011; Banerjee et al., 2011; Berger-Muller and Suzuki, 2011; Shimizu et al., 2011; Shafer et al., 2012), and defective closures of the palate (Topczewski et al., 2011), ventricular septum (Sheldahl et al., 2003; Phillips et al., 2005; Etheridge et al., 2008; Paudyal et al., 2010; Henderson and Chaudhry, 2011) and the neural tube (Kibar et al., 2001; Murdoch et al., 2001a; Murdoch et al., 2001b; Hamblet et al., 2002; Curtin et al., 2003; Lu et al., 2004; Ciruna et al., 2006; Paudyal et al., 2010). Individual PCP genes have been associated with additional processes, such as loss-of-function mutations in Vangl2 with cellular polarization during postnatal female reproductive tract development in mice (Vandenberg and Sassoon, 2009).

The inner ear sensory organs, i.e., the one in the cochlea and the five in the vestibule, show distinctive forms of PCP in vertebrates (Kelly and Chen, 2007). At the apical surface of each sensory hair cell, microvilli-derived stereocilia are arranged in rows of increasing length, with the tallest sterocilia positioned toward the periphery. In addition, there is a single primary cilium, known as the kinocilium, eccentrically placed near the tallest stereocilia at one edge of the cellular apex. All of the hair cells within each of the six inner ear sensory organs are coordinately polarized (Kelly and Chen, 2007). Hair cells in the cochlea and in the three vestibular cristae are uniformly oriented, while hair cells in the vestibular saccule and utricle have opposite orientations along a line of polarity reversal (Kelly and Chen, 2007). Among the conserved core PCP genes, mutations in Vangl2 (Montcouquiol et al., 2003), Celsr1 (Curtin et al., 2003), Fz (Wang et al., 2006), or Dvl genes (Wang et al., 2005) cause the disruption of coordinated hair cell orientation of in the cochlea. A vestibular PCP defect was also observed in Vangl2 and Fz mutants (Wang et al., 2006). It is thought that the membrane proteins Vangl2 and Fz form membrane-associated protein complexes with cytoplasmic PCP proteins to mediate intercellular interactions and to coordinate the polarity of neighboring cells (Wang et al., 2005; Montcouquiol et al., 2006; Wang et al., 2006; Wu and Mlodzik, 2008). Indeed, asymmetric localization of Vangl2, Fz, and Dvl proteins along the PCP axis is observed in the inner ear sensory organs (Wang et al., 2005; Wang et al., 2006), and the polarized localization of membrane PCP proteins is lost when PCP signaling is disrupted (Wang et al., 2005; Montcouquiol et al., 2006; Wang et al., 2006; Deans et al., 2007). On the other hand, the membrane-associated PCP protein complexes are normally polarized in several PCP mutants that are defective in directing the intrinsic polarization of cells (Jones et al., 2008; Grimsley-Myers et al., 2009; Sipe and Lu, 2011), which suggests that membrane PCP proteins are not sufficient to direct morphological polarization but likely recruit and interact with other cellular factors to direct morphological polarizations. However, very little is known about such cellular factors in vertebrates.

To further explore vertebrate PCP regulation, we performed a two-hybrid screen to identify proteins which interact with transmembrane PCP protein Vangl2. We used the C-terminal cytoplasmic domain of Vangl2 and a mouse embryonic cochlear cDNA library. The screen identified several proteins (Li et al., 2012) including Testin. Testin shares a PET and LIM

domain homology with a known PCP protein, Pk. In *Drosophila*, Pk functions in an intercellular feedback loop crucial to the establishment of PCP in the eye and epidermis by interacting with Vang and modulating Frizzled-Dishevelled signaling (Tree et al., 2002; Das et al., 2004; Jenny et al., 2005). While interference with Pk1 causes CE defects in zebrafish and ascidians (Carreira-Barbosa et al., 2003; Veeman et al., 2003; Jiang et al., 2005), both over-expression and depletion of Testin also inhibit activin-induced CE of animal caps and cause gastrulation CE defects in Xenopus (Dingwell and Smith, 2006). The impact of Testin modulation on these CE processes implicates a potential role for Testin in vertebrate PCP signaling. Here, we present data suggesting that Testin is a mammalian gene involved in coordinated orientation of hair cells in the inner ear and in the development of the female reproductive tract, and demonstrate a role for Testin in PCP regulated processes in mice.

Results

Testin interacts with Vangl2 in the yeast two-hybrid assay and is recruited by Vangl2 and Dvl1 to their subcellular locations

To further explore the vertebrate PCP pathway, we performed a yeast two-hybrid screen (Li et al., 2012). Vangl2 is predicted to consist of 4 transmembrane domains with a short N-terminal cytoplasmic domain and a long C-terminal cytoplasmic domain. Several mutations at the C-terminal cytoplasmic domain cause the loss-of-function for Vangl2 in PCP signaling (Torban et al., 2004a; Torban et al., 2004b), which implicates the functional importance of Vangl2 C-terminal region. The yeast two-hybrid screen was carried out using the C-terminal domain of Vangl2, Vangl2^C, as the bait against a mouse cDNA library made from embryonic day 15 (E15) cochlear tissues (Li et al., 2012). In a minimum culture medium that lacks histidine and adenine, the presence of Vangl2^C or Testin alone did not activate the expression of reporter genes which allow for cell growth independent of histidine or adenine. As a consequence, there was no cell growth on the minimum plates (Fig. 1A, B). When both Vangl2^C and Testin were present, the cells grew in the minimal medium and turned blue in the presence of galactosidase substrates (Fig. 1B). This result suggested that reporter genes are expressed in the presence of both Vangl2^C and Testin, or Vangl2^C and Testin brought the Gal4 DNA binding domain (Gal4-BD) to the Gal4 DNA activation domain (Gal4-AD) to activate reporter gene expression.

The mouse Testin protein consists of a PET domain, a ~110 amino acid motif found in the N-terminal regions of LIM domain proteins Prickle (Pk), Espinas, and Testin (PET), and three LIM domains (Bekman and Henrique, 2002; Katoh and Katoh, 2003). In mice, Testin shares homology with Pk1 and Pk2 (Katoh and Katoh, 2003). Pk1 and Pk2 are mammalian homologs of the *Drosophila* PCP protein Pk, which is known to interact with *Drosophila* core PCP proteins Van gogh and Dishevelled (Jenny et al., 2003; Jenny et al., 2005). Genetic analysis of Pk1 and Pk2, however, has not concluded essential roles of Pk in mammalian PCP processes, likely due to the overlapping functions of Pk1, Pk2, and other mammalian functional homologs. Testin shares the common PET and LIM domains with Pk1 and Pk2 (Katoh and Katoh, 2003), and has been shown to regulate gastrulation in Xenopus (Dingwell and Smith, 2006). In particular, the knocking down of Testin causes characteristic convergent extension defects in Xenopus (Dingwell and Smith, 2006). It is

thought that Testin may act similarly or redundantly with Pk1 and Pk2 in vertebrate PCP signaling (Dingwell and Smith, 2006). To test whether Testin may be a mammalian protein involved in PCP processes and to confirm its interaction with Vangl2 as indicated by the yeast two-hybrid assay, we performed coimmunoprecipitation using extracts from cultured cells co-transfected with GFP-tagged Testin (GFP-Testin) and Ha-tagged Vangl2 (HA-Vangl2), but failed to mutually pull down either of the proteins.

It is possible that the co-immunoprecipitation conditions used do not accommodate the interaction between the two proteins in vitro. To further examine the potential interaction or association of Testin and Vangl2 as implicated by the 2-hybrid assay, we transfected IMCD3 cells with Testin-GFP and HA-Vangl2 and examined their localization in the cells (Fig. 2). When transfected alone, Testin showed a general distribution in the cells, with some membrane localization (Fig. 2A), while Vangl2 accumulated at the plasma membrane (Fig. 2B). In cells co-transfected with Vangl2 and Testin (Fig. 2D-F), Testin was enriched at the cellular contact where Vangl2 was also enriched. The localization of Testin at the cellular contacts was observed in 62% of the pairs of cells co-transfected with Vangl2 (N=37 pairs), compared with 18.9% of the pairs of cells transfected with Testin alone (N=37 pairs) (Fig. 2A, D-F). Furthermore, the signal for Testin-GFP at the cellular contacts of co-transfected cell pairs was drastically enhanced (100%) in comparison to that of the localization of Testin-GFP at the contacts of cells transfected with Testin-GFP alone (Fig. 2A, D). This result suggests that the cellular distribution of Testin can be affected by Vangl2 and is recruited by Vangl2 to cell-cell contacts.

In addition to interacting with Vang proteins, Pk family members are also known to interact with Dishevelled proteins (Jenny et al., 2003; Jenny et al., 2005). We therefore also examined the localization of Testin-GFP and HA-tagged Dvl1 (HA-Dvl1) in IMCD3 cells (Fig. 2). As reported previously (Ahmad-Annuar et al., 2006; Terabayashi et al., 2009; Shafer et al., 2012), Dvl1 displayed a distinctive vesicular localization pattern in transfected cells (Fig. 2C). In cells co-transfected with GFP and HA-Dvl1 (Fig. 2G-I), GFP was uniformly distributed in the cells and was not enriched in the Dvl1-containing vesicles. In all of the cells (N>100 cells, 100%) co-transfected with Testin-GFP and Dvl1-HA, Testin-GFP was specifically re-localized to the vesicular structures consisting of Dvl1 (Fig. 2J-L), in contrast to cells transfected with only Testin-GFP (Fig. 2A) or cells co-transfected with GFP and Dvl1 (Fig. 2G-I). These data together indicate that Testin is specifically recruited by Dvl1.

Together, the data from the 2-hybrid assay and the localization experiments in cultured cells suggested that Testin could interact with Vangl2, and could be recruited to subcellular compartments by Vangl2 or Dvl1.

Testin is required for cellular patterning and interacts with Vangl2 genetically in regulating precisely coordinated orientation of hair cells in the cochlea

The findings of the recruitment of Testin to Dvl1 cellular structures and its enrichment to the same cellular contact by Vangl2 support the possibility that Testin may be involved in the same cellular processes as Dvl1 and Vangl2. To explore its potential role in PCP processes, we examined the expression of Testin in the cochlea during the establishment of PCP (Fig.

3), and accessed the loss-of-function of Testin in the inner ear and its genetic interaction with Vangl2 (Fig. 4).

We could not validate the specificity of the antibodies against Testin, and used in-situ hybridization (ISH) to assess the expression of Testin transcript in the developing cochlea (Fig. 3). The developing organ of Corti at E14-15, PCP gene Celsr1 was expressed in the entire cochlear epithelium, with higher expression levels observed in the region medial to the developing organ of Corti (Fig. 3B). Testin transcripts were detected in the cochlear epithelium, with higher levels seen in the region medial to the developing organ of Corti and in the roof of the cochlear duct (Fig. 3A). Testin transcripts were also observed in the mesenchyme cells surrounding the cochlear epithelium and in the spiral ganglion cells, in contrast to Celsr1 transcripts that were only seen in the cochlear epithelium (Fig. 3).

We further examined the cochleae isolated from postnatal day 6 (P6) control, *Testin* mutant (Drusco et al., 2005), and *Testin* and *Vangl2* compound mice (Fig. 4). The looptail (Lp) mutation in *Vangl2* gene causes the loss of function of Vangl2 in PCP signaling (Torban et al., 2004a; Torban et al., 2004b). The homozygous looptail mutant mice show characteristic PCP defects, including mis-orientation of hair cells and a shortened and widened sensory region with cellular patterning defects in the cochlea resulting from a CE defect (Montcouquiol et al., 2003). In the wild type control littermates at P6, hair cells are patterned into 4 rows along the entire length of the cochlear duct and oriented along the same direction, with the vertex of each hair bundle pointing from the medial (M) to the lateral (L) side of the cochlear spiral (Fig. 4A). In $Testin^{+/-}$ and $Testin^{-/-}$ animals, the orientation of the hair bundles does not show a statistically significant difference from that of the wild-type controls (Fig. 4B, C, and H). Cellular patterning in $Testin^{+/-}$ and $Testin^{-/-}$ animals from the mid-apical to apical region, however, appears abnormal in comparison to that of the wild-type controls (Fig. 4A-C). In several PCP mutants, defective CE causes shortening of the cochlear duct and consequently the widening of the tissue, with the appearance of extra rows of hair cells concentrated toward the apical region of the cochlear duct (Montcouquiol et al., 2003; Wang et al., 2005). The patterning defect of hair cells in $Testin^{-/-}$ mutants is statistically significant but only mildly so (Fig. 4I). The associated length reduction in core PCP mutants was not detected in $Testin^{-/-}$ mutants, likely due to the milder patterning defect and the fact that the sensitivity of the length measurement for a spiral structure could be influenced by variations in fixation and mounting of the tissue.

The additional loss of function of one copy of Vangl2 has a statistically significant effect on hair cell orientation in the Testin mutant background. Within the sensory region, the third row of outer hair cells in $Testin^{-/-};Vangl2^{Lp/+}$ animals showed deviations in orientation in comparison to wild-type, $Testin^{+/-}$, $Testin^{-/-}$, and $Vangl2^{Lp/+}$ littermates (Fig. 4A-H). Furthermore, a defect in hair cell orientation was observed in the extra row of hair cells in both the $Testin^{+/-};Vangl2^{Lp/+}$ and $Testin^{-/-};Vangl2^{Lp/+}$ animals (Fig. 4H).

PCP signaling in vertebrates involves a characteristically polarized enrichment of membrane associated protein complexes consisting of the core PCP proteins Fz3/6 and/or Vangl2, which presumably propagate the directional information across the tissue and coordinate polarity among neighboring cells. The membrane enrichment of the complexes is lost in

core PCP mutants (Wang et al., 2005; Montcouquiol et al., 2006; Wang et al., 2006) but is maintained in mutants of other PCP genes, including Fuzzy and Inturned genes, which act downstream of the core PCP genes (Park et al., 2006; Heydeck et al., 2009; Dai et al., 2011), and cilia genes that act parallel to the core PCP genes (Jones et al., 2008). No quantitatively significant hair cell orientation defect in $Testin^{-/-}$ mutants was detected (Fig. 4C, H), which implies that core PCP proteins are likely localized normally in $Testin^{-/-}$ mutants. Indeed, the examination of core PCP protein Fz3 in the cochlea using immunostaining showed a characteristically asymmetric localization of the Fz3 protein in both the wild type control and in $Testin^{-/-}$ cochleae at E18.5 (Fig. 4J, K).

Together, the analysis revealed that Testin is required for cellular patterning and that there exists a genetic interaction between Testin and Vangl2 in the regulation of hair cell orientation. Furthermore, the examination of Fz3 localization in $Testin^{-/-}$ cochlea implicated that Testin may act downstream of core PCP genes in these processes.

Deletion of Testin leads to characteristic PCP defects in the vestibular sensory organs

In addition to the cochlea, the vestibular sensory organs in the inner ear also show coordinated hair cell orientations within each sensory organ. In the utricle and saccule, hair cells are oriented towards or opposing to each other across a line of polarity reversal (Deans et al., 2007). In the three cristae ampullaris, all of the hair cells are oriented in the same direction (Kelly and Chen, 2007). In several known PCP mutants, hair cell orientation defects in the vestibular organs are reported (Wang et al., 2006). In particular, the $Vangl2^{Lp/+}$ animals show head bobbing, which suggests potential vestibular abnormalities. Posterior cristae isolated from $Vangl2^{Lp/+}$ animals were smaller and the hair cells were misoriented (Wang et al., 2006).

We examined the vestibular organs isolated from Testin mutant mice (Figs. 5, 6). To visualize the orientation of hair cells in the vestibular organs, we used an antibody against α-Spectrin, which is enriched in the cuticular plate of hair cells excluding the region underneath the kinocilium, known as the fonticulus (Figs. 5, 6). Characteristic PCP defects were observed in the posterior crista and saccule in $Testin^{-/-}$ mice (Figs. 5, 6). In the posterior cristae from the wild type animals, all of the hair cells were oriented in the same direction along the PCP axis perpendicular to the long axis of the crista (Fig. 5A-C, G, H). In $Testin^{-/-}$ animals, hair cells were generally orientated in opposite directions on each of the two sides of the posterior crista, and hair cells within the same side of the posterior crista were not all oriented along the same direction (Fig. 5D-F, I).

In the wild type saccule (Fig. 6), hair cells were oriented in opposite directions across the line of polarity reversal (Fig. 6A-C). In $Testin^{-/-}$ mutants (Fig. 6D-F), this line of polarity reversal was observed in most regions of the saccule but became disrupted at one region (Fig. 6E, F, n=3).

The observed hair cell mis-orientation in the vestibular organs from $Testin^{-/-}$ animals suggests that Testin is required for the regulation of the precisely coordinated hair cell orientation in the posterior crista and the saccule of the vestibule.

Testin genetically interacts with Vangl2 to regulate female reproductive tract (FRT) development

During the process of generating Testin and Vangl2 compound mutants to test the genetic interaction between Testin and Vangl2 in the cochlea, it was noted immediately that many of the females failed to breed. Upon further examination, we found that those animals did not have externally visible female reproductive tracts (FRT). The FRT in mice consists of bilateral ovaries, two oviducts (OT), two uteruses, a cervix, and a vagina that opens externally. Genotyping assays for the male-specific gene Sry were carried out and confirmed that those animals were genotypically females. We quantified this phenotype in $Vangl2^{Lp/+}$, $Testin^{-/-}$, $Testin^{+/-}$; $Vangl2^{Lp/+}$, and $Vangl2^{Lp/+};Testin^{-/-}$ female animals (Fig. 7A). The wild type (N=100) and $Testin^{-/-}$ (N=30) females do not have closed FRT (Fig. 7A, and data not shown). 7% of the $Vangl2^{Lp/+}$ females (N=100) had a closed reproductive tract while 33% of the $Testin^{+/-}$; $Vangl2^{Lp/+}$ (N=30) or $Testin^{-/-}$; $Vangl2^{Lp/+}$ (N=30) animals had the same phenotype, indicating a strong genetic interaction of the two genes in the development of FRT.

We further examined the expression of Vangl2 and Testin transcripts in the FRTs of wild type controls, Vangl2 mutants, and Vangl2 and Testin compound mutants (Fig. 7B-F). Both Vangl2 and Testin RNA transcripts were detected in FRT from postnatal day 1 (P1) to P30 and the expression of both genes is higher at birth (Fig. 7B). Furthermore, Vangl2 protein is located to the lateral side of the FRT epithelial cells (Fig. 7C, D). The dissected FRTs from the control and mutant female animals appeared to be similar at postnatal day 12 (Fig. 7E). However, in contrast to the control females (N=100) in which FRT opens by P30, 1/3 of the Testin and Vangl2 compound mutant females (N=30 each for $Testin^{+/-};Vangl2^{Lp/+}$ and $Testin^{-/-};Vangl2^{Lp/+}$) and 7% of the $Vangl2^{Lp/+}$ females (N=100) had a closed FRT at P30 (Fig. 7F). The FRTs in these females were greatly enlarged and filled with fluid (Fig. 7F).

Together, the data suggested that Testin and Vangl2 interact genetically to regulate the maturation or opening of the FRT in mice.

Conclusions and Discussion

Studies in Drosophila have identified a conserved genetic pathway that is required for various forms of PCP. In vertebrates, a similar set of genes, including Fz, Dvl, Vangl2, and Celsr1, regulate many PCP processes (Barrow, 2006). It appears that the vertebrate PCP pathway uses a similar mechanism of polarized sorting of membrane-associated complexes to propagate the polarity signal and to recruit intracellular effectors for morphological polarization (Jones and Chen, 2008). In particular, Vangl2 has emerged as a central player essential for all of the known PCP processes. However, the molecular mechanisms underlying the roles of Vangl2 and other conserved PCP genes in PCP signaling remain elusive. In this study, we identified Testin as an interacting protein with Vangl2 by a two-hybrid screen, and demonstrated that Testin is required in several processes involving known PCP genes.

The requirement for Testin in inner ear development

Testin is a structural homolog of Drosophila PCP protein Pk, consisting of a characteristic PET domain and three LIM domains (Bekman and Henrique, 2002; Katoh and Katoh, 2003). Moreover, a functional study in Xenopus implicated that Testin plays a role in gastrulation involving several PCP processes (Dingwell and Smith, 2006). We sought to determine whether Testin functions in mammalian PCP regulation after we identified Testin by its interaction with Vangl2 in the two-hybrid screen we carried out with Vangl2 C-terminal domain (Fig. 1).

The sensory organs in the inner ear display distinct forms of epithelial PCP. In addition, CE occurs during terminal differentiation of the cochlear epithelium (Wang et al., 2005). As a consequence, defective PCP signaling also causes hair cell patterning defects in a shortened cochlear duct (Montcouquiol et al., 2003; Wang et al., 2005). We examined the inner ears of Testin mutant mice to test whether Testin is essential for any PCP processes in the inner ear (Figs. 4-6), and to determine the genetic interaction between Vangl2 and Testin during inner ear PCP processes (Fig. 4).

Deletion of the third exon of the Testin gene leads to the elimination of the protein in homozygous knockout animals (Drusco et al., 2005). Depletion of Testin in the mouse inner ear causes two inner ear phenotypes: a patterning defect of cochlear hair cells and the mis-orientation of vestibular hair cells (Figs. 4-6). In Vangl2, Fz, or Dvl core PCP mutants, defective CE causes the shortening of the cochlear duct and widening of the sensory organ, or the appearance of extra rows of hair cells in a shortened cochlear duct (Montcouquiol et al., 2003; Wang et al., 2005). In cochleae from $Testin^{-/-}$ animals, the precise pattern of 4 rows of sensory hair cells was disrupted and extra hair cells were present (Fig. 4). This phenotype, albeit statistically significant in comparison to control animals, is still mild. The associated shortening of the cochlear duct in PCP mutants was not detected in $Testin^{-/-}$ animals. It is possible that the patterning defect is due to mild deregulation of CE in $Testin^{-/-}$ animals, and the quantification of extra hair cells is more sensitive than the measurement of minor changes in cochlear length, which could be affected by several factors in sample preparation. Alternatively, other potential roles of Testin may account for the patterning abnormalities observed in $Testin^{-/-}$ animals. It is noted that the patterning defect is limited to the mid-apical to apical region of the cochlear duct; similar to what has been observed in core PCP mutants (Montcouquiol et al., 2003; Wang et al., 2005). This may be the consequence of the differentiation gradient of the organ of Corti (Chen and Segil, 1999) and the extension of the cochlear duct from the base to the apex (Wang et al., 2005).

The mis-orientation of hair cells in the vestibular organs was distinct and conclusive of the role of Testin in regulating precisely coordinated hair cell orientation (Figs. 5, 6). In the posterior crista from $Testin^{-/-}$ animals, the uniform orientation of hair cells was disrupted (Fig. 5), while the organ was normal in appearance. In the saccules isolated from $Testin^{-/-}$ animals, the line of polarity reversal was consistently disrupted in one area (Fig. 6). These data together indicated that Testin plays a role in regulating hair cell orientation in the vestibule and implicated Testin's potential role in cochlear CE.

Notably, despite the patterning defect in the cochleae from Testin mutants, hair cell orientation appears to be normal in Testin mutants (Fig. 4B, C, and H). The lack of apparent cochlear hair cell orientation defects may be due to the overlapping roles of Pk homologs, and the different sensitivities of individual PCP processes to the loss of Testin in the cochlea. We crossed Testin mutant mice with *Vangl2* looptail mutants to test whether the inactivation of another gene in the same pathway would reveal a masked requirement for Testin in regulating cochlear hair cell orientation (Fig. 4E, F). Indeed, cochlear hair cells showed orientation defects in $Testin^{+/-};Vangl2^{Lp/+}$ and $Testin^{-/-};Vangl2^{Lp/+}$ animals (Fig. 4).

Together, the data suggested that Testin is a mammalian gene involved in PCP processes in the inner ear, including coordinated hair cell orientation and CE.

Testin in female reproductive tract development

An important feature of mammalian development is the generation of sexually dimorphic reproductive tracts from the Müllerian and Wolffian ducts. In females, Müllerian ducts develop into the oviduct, uterus, cervix and vagina, whereas Wolffian ducts regress. A number of genes have been identified to be essential for FRT development, such as Lim1, Pax2, Emx2, Wnt4, Alk3, Hoxa10/11/13, P63, Wnt7a, Wnt5a (Mericskay et al., 2004), and Vangl2 (Vandenberg and Sassoon, 2009).

Among the genes known for FRT development, Vangl2 appears to be required for the polarization of epithelial cells in the FRT (Vandenberg and Sassoon, 2009), and Wnt5a (Mericskay et al., 2004) is considered to be a Wnt molecule for PCP signaling. The requirement of Vangl2 and Wnt5a in FRT development implicates the involvement of PCP signaling or a signaling cascade consisting of PCP genes. In this study, we found a genetic interaction between Vangl2 and Testin which enhances the vaginal blockage phenotype originally observed in $Vangl^{Lp/+}$ females.

The potential role of Testin in vertebrate PCP signaling

The genetic interactions of Testin with Vangl2 and its role in regulating hair cell orientation and FRT development suggested Testin could be a mammalian gene involved in PCP processes in multiple tissues, including regulating the establishment of polarity features at the apical surface of the epithelial cells and cellular morphogenesis in epithelial tissues to create an opening in the tubular tissues.

Notably, Testin has been well known to be associated with actin cytoskeleton structures (Coutts et al., 2003; Garvalov et al., 2003; Griffith et al., 2005; Rotter et al., 2005; Boeda et al., 2007). Its biochemical properties and cellular functions suggest that Testin may be directly involved in cellular polarization during PCP signaling. Testin appears to have different conformations corresponding to its various localizations and associations (Zhong et al., 2009). It can bind to a variety of cytoskeleton proteins, including paxillin (Garvalov et al., 2003), talin, zyxin, VASP, Mena (Coutts et al., 2003; Griffith et al., 2005), EVH, and alpha-II–Spectrin (Rotter et al., 2005; Boeda et al., 2007), and also regulates a plethora of cell behaviors. In rat and chicken fibroblasts, Testin is associated with actin stress fibers and promotes cell spreading on fibronectin (Coutts et al., 2003). In Hela cells, Testin is localized

to focal adhesions and regulates the actin cytoskeleton (Garvalov et al., 2003; Griffith et al., 2005). Our cellular localization data (Fig. 2) showed that Testin has a general cytoplasmic localization, and its membrane enrichment is greatly enhanced when Vangl2 is over-expressed and can be recruited to Dvl1 domains (Fig. 2). It is possible that Testin shares an overlapping role with Pk in establishing and propagating the polarity signal across a tissue. Given the extensive evidence of Testin association with actin structures, Testin may concurrently act as a link between membranous PCP complexes and the actin cytoskeleton, allowing it to regulate cellular morphogenesis. Conversely, Pk genes may also play a role in directly linking membranous PCP complexes with the actin cytoskeleton. Growing evidence has indicated a tumor suppressor role for Testin for loss of cell polarity when Testin levels are abolished or reduced (Drusco et al., 2005; Zhu et al., 2012), consistent with its role in directly regulating cytoskeleton polarization.

In summary, our current study showed that Testin is involved in the coordinated orientation of hair cells in the inner ear and regulates FRT development. The interaction of Testin with known PCP protein Vangl2 along with its well-established association with actin cytoskeleton structures suggested a role for Testin in linking the membranous PCP complexes with actin cytoskeletons in regulating cellular morphological polarization.

Experimental Procedures

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. LPT/Le mice or Vangl2 looptail mutant mice were obtained from the Jackson Laboratories (Torban et al., 2004a; Torban et al., 2004b). Vangl2–green fluorescent protein (GFP) transgenic mice were generated as reported (Qian et al., 2007). Testin mutant mice were gifts from Drusco and colleagues (Drusco et al., 2005). Vangl2 looptail mutant and Testin mutant strains were maintained on B6/129 background.

Yeast two-hybrid analysis

We utilized the Matchmaker Gal4 yeast two-hybrid system (Clontech, USA) and a cDNA library from embryonic day 15 mouse cochlear epithelia constructed in Gal4-AD (Li et al., 2011). A 283-aa C-terminal cytoplasmic tail of Vangl2 served as the bait. The plasmid pGal4-BD/Vangl2^C alone with the empty Gal4-AD vector does not activate reporter expression. Yeast two-hybrid assays were performed as described in the Clontech protocol. We co-transfected pGal4-BD/Vangl2^C and the cDNA library DNA into yeast reporter cells and screened for colonies that grew on histidine- and adenine-deficient plates and turned blue under the conditions for α-galactosidase (α-gal) and β-gal assays.

Plasmid construction, cell culture and plasmid transfection

HA-tagged Vangl2 (HA-Vangl2) and GFP-tagged Testin (Testin-GFP) were used for cell cultures. Dvl1-HA was a gift from Dr. Gerd Walz (Germany). IMCD3 cells were used for transfection and co-localization experiments.

Inner ear dissection, immunostaining, and imaging

Inner ear dissection, staining and imaging were performed as described previously (Jones et al., 2008). Briefly, the inner ears were isolated from mice at various developmental stages, fixed in 4% paraformaldehyde overnight at 4°C. The sensory organs were further dissected and subjected to staining. Rhodamine or Alexa-Fluor-488 conjugated phalloidin dyes (Invitrogen, 1:1000) were used to stain actin-rich structures such as stereocilia bundles and the cuticular plate of hair cells. An anti-α-Spectrin antibody (1:200, Chemicon, MAB1622) was used to depict the polarity of vestibular hair cells by revealing the eccentric position of the kinocilium. An antibody against Fz3 (a gift from J. Nathans, Johns Hopkins University, Maryland, USA) was used to visualize the subcellular localization of membrane associated PCP complexes. The following microscopes were used for image acquisition: Olympus SZX12 upright microscope and Zeiss LSM510 confocal microscope.

Quantification of hair cell orientation and statistical analysis

The number of inner or outer hair cells in each cochlea was counted by scoring phalloidin[+] hair bundles. To determine stereocilia bundle orientation in the cochlea, we drew a line from the vertex of the "V"-shaped stereocilia bundle through the middle of the "V"-shaped stereocilia (bisecting line). The angle formed between this line and the line parallel to the diagonal or the mediolateral axis of the cochlea spiral was measured and used for quantifications. In wild type animals, this angle is close to 0°. For histogram plots of distribution of the angles, the angles form at the right side or the left side of the line representing mediolateral axis are designated to be positive (+) or negative (−), respectively. For quantification of the average deviation from the normal orientation (angle=0°), the angles were quantified without positive (+) or negative (−) designations to avoid cancellation of the angle deviations. At least 50 hair cells in each row at the medial-apical regions were quantified for each sample. For average deviations, the absolute rotation away from the 0° orientation was determined regardless of direction. The measurement of hair cell angels in the crista was performed as diagramed in figure 5.

Data collected from each experimental group with at least 3 samples per genotype are expressed as mean ± S.E.M. SPSS (13.0) was used for statistic analyses.

Histology of female reproductive tracts

Female reproductive tracts (FRT) were isolated from postnatal day 1 (P1), P12, and P30 animals, fixed in 4% paraformaldehyde in PBS at 4°C overnight for imaging.

Acknowledgments

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Salient features of the manuscript

1. Identified Testin as a Vangl2-interacting protein in a 2-hybrid screen;

2. Enrichment of Testin in PCP protein-containing compartments in culture cells;

3. Genetic inactivation of Testin in mice leads to misorientation of hair cells in the vestibule;

4. Testin interacting with Vangl2 genetically to coordinate hair cell orientation in the cochlea;

5. Testin is required for precise patterning of the organ of Corti;

6. Testin interacting with Vangl2 genetically during female reproductive tract development in mice
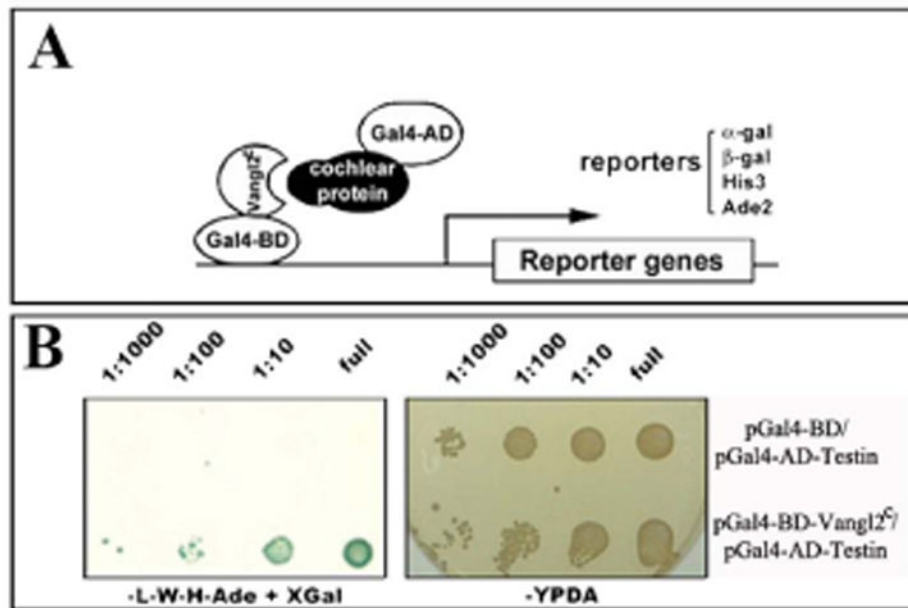
Fig.1.
Testin interacts with Vangl2 in the yeast two-hybrid assay.

(**A**) Schematic diagram of the two hybrid screen in which the C-terminal cytoplasmic tail of Vangl2, Vangl2^C, was fused in-frame with Gal4 DNA binding domain (gal4-BD) and a cochlear cDNA was fused to Gal4 DNA activation domain (Gal4-AD). (**B**) Yeast cells were serially diluted and replicated on rich media YPDA (yeast media containing yeast extract, peptone, dextrose plus adenine) plates and minimal media plates lacking Leucine (L), Tryptophan (W), Histidine (H), Adenosine (Ade). The survival of cells on the minimum media depends on the presence of the two plasmids (Gal4-BD and Gal4-AD) and the expression of reporter genes. pGBTK7 plasmid contains Gal4-DB only while pVangl2-C contains Vangl2^C fused to Gal4-BD.
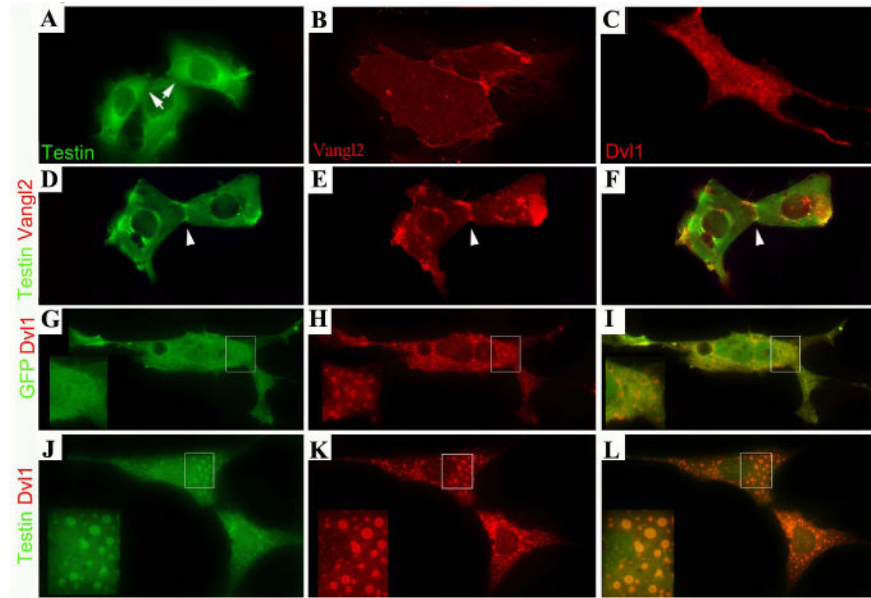
Fig. 2.
Testin is localized to the same subcellular compartments as Vangl2 and Dvl1.
(**A-C**) IMCD3 cells transfected with Testin-GFP (**A**), HA-Vangl2 (**B**), or HA-Dvl1 (**C**). The arrows (**A**) indicate the cellular boundaries of Testin-transfected cells. (**D-F**) IMCD3 cells transfected with Testin-GFP (green) and HA-Vangl2 (red). The arrowhead marks the cellular boundary with significantly enriched Testin signals in the presence of Vangl2. (**G-I**) IMCD3 cells transfected with GFP (green) and HA-Dvl1 (red). The inserts are the larger images of the boxed areas in each panel to better visualize the localization of Dvl1 to the vesicular structures and the uniform distribution of GFP in the cells. (**J-L**) IMCD3 cells transfected with Testin-GFP (green) and HA-Dvl1 (red). The inserts are the larger images of the boxed areas in each panel to visualize the enrichment of Testin-GFP to Dvl1-positive vesicles.
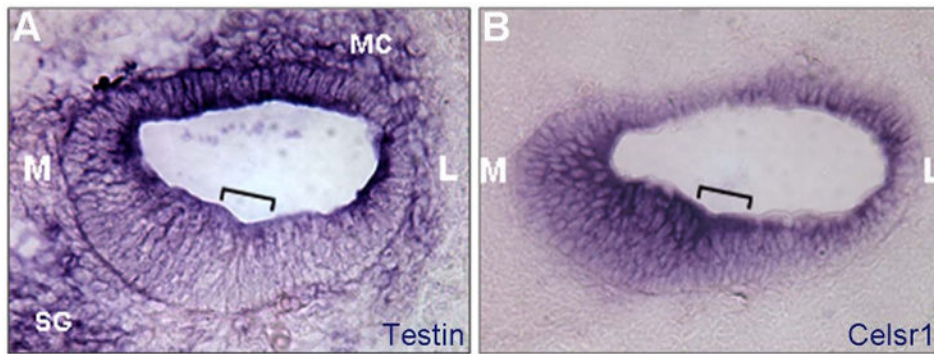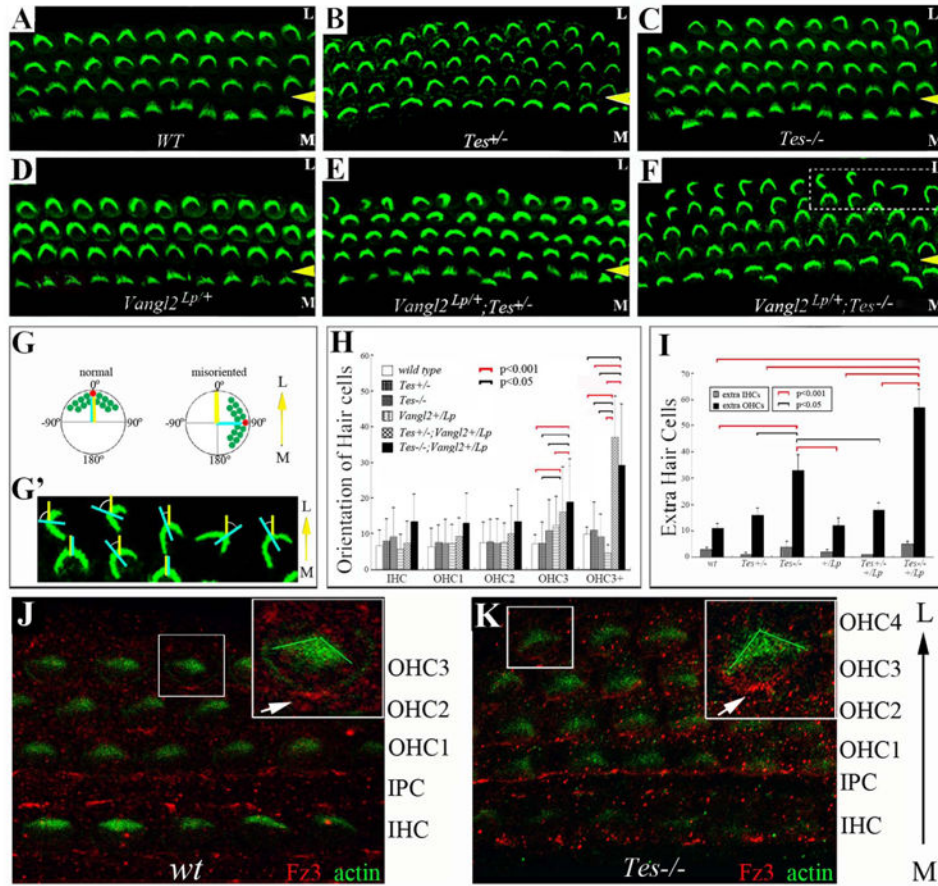
Fig. 3.
Testin is expressed in the inner ear during development.
(**A-B**) E14.5 cochlear sections probed for Testin (**A**) or Celsr1 (**B**). The bracket in each panel marks the developing organ of Corti. M: medial or center of the cochlear spiral; L: Lateral or periphery of the cochlear spiral; MC: mesenchyme cells; SG: spiral ganglion neurons.

Fig. 4.

Testin is involved in cellular patterning and hair cell orientation in the cochlea.

(**A-F**) Cochlear whole mounts were prepared from wild type (*WT*) (**A**), *Testin$^{+/-}$* (*Tes$^{+/-}$*) (**B**), *Testin$^{-/-}$* (*Tes$^{-/-}$*) (**C**), *Vangl2$^{Lp/+}$* (**D**), *Vangl2$^{Lp/+}$*;*Tes$^{+/-}$* (**E**), and *Vangl2$^{Lp/+}$*;*Tes$^{-/-}$* (**F**) animals at P6. The samples were stained for phalloidin to visualize actin-rich stereocilia (green) of hair cells. The yellow arrowheads mark the outer pillar cell region that separates the inner (IHCs) from outer hair cells (OHCs). M: medial region of the cochlea duct; L: lateral region of the cochlear duct. The control organ of Corti consists of one row of inner and three rows of outer hair cells (**A**). Extra rows of outer hair cells were seen in the heterozygous and homozygous Testin mutants (**B**, **C**), as well as in Testin and Vangl2 compound mutants (**E**, **F**). (**G-G′**) Schematic diagrams illustrating the measurement of hair cell orientation. The yellow line in (**G,G′**) represents the medial-to-lateral radius of the cochlear spiral, and the light blue line in (**G,G′**) is the bisecting line of the V-shaped stereocilia bundle of hair cells. The angle formed between the yellow and light blue lines is the angle measured to represent the orientation of each hair cell. The measurement of the hair bundle orientation is illustrated in (**G′**) using hair cell images from the boxed area in (**F**). (**H**) The orientation of each row of hair cells from comparable mid-apical region of the cochlea was measured, quantified, and plotted. (**I**) Extra hair cells were counted and plotted for the genotypes included in (**A-F**). Pairs of groups with p value <0.005 for their difference were indicated by brackets in (**H, I**). The red and black brackets indicate the p values are less than 0.001 or 0.005, respectively (**H,I**). (**J-K**) Fz3 localization in wild type (**J**) and *Testin$^{-/-}$* (*Tes$^{-/-}$*) (**K**) cochleae at P0 was visualized using an antibody against Fz3.
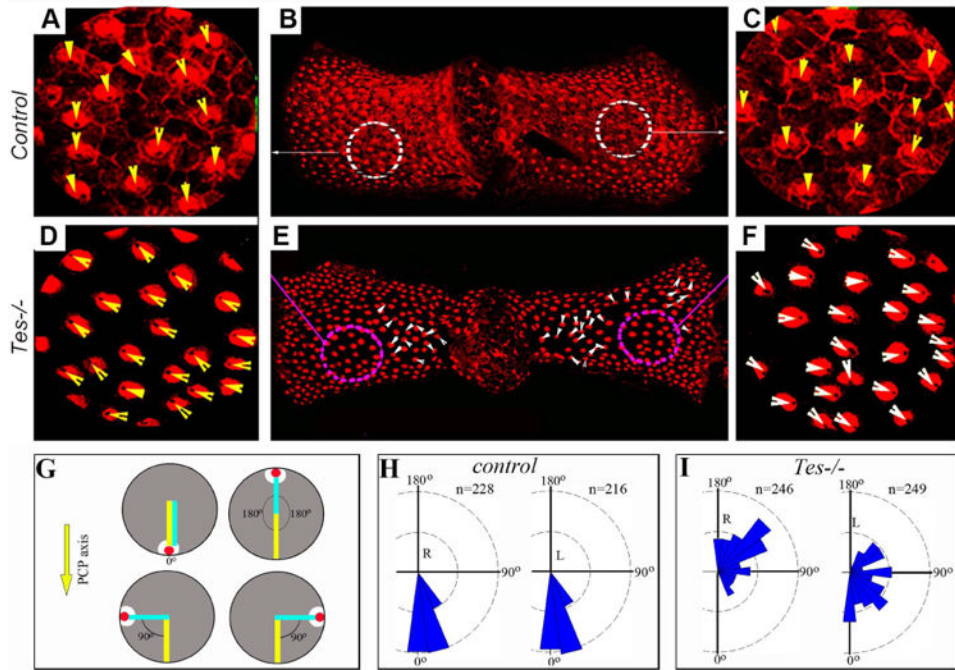
Fig. 5.
Testin is required for hair cell orientation in the posterior crista.
(**A-F**) Posterior cristae were prepared from control (**A-C**) and *Testin*^{-/-} (*Tes*^{-/-}) (**D-F**) animals and stained for an antibody against α-Spectrin. At the apical surface of the vestibular hair cells, the basal body region beneath the kinocilium is devoid of α-Spectrin staining. The arrowheads mark the orientation of hair cells. (**G-I**) A diagram for hair cell orientation measurement in the posterior crista (**G**) and plots of hair cell orientation in wild type control (**H**) and *Testin*^{-/-} (**I**) posterior cristae. The red dot and the white area represent the position of the kinocilium and the fonticulus devoid of α-Spectrin staining in a hair cell, respectively (**G**). The orientation of hair cells from each side of the crista was plotted separately (**H**, **I**). 3 cristae for each genotype were used for quantification. R: right side; L: left side.
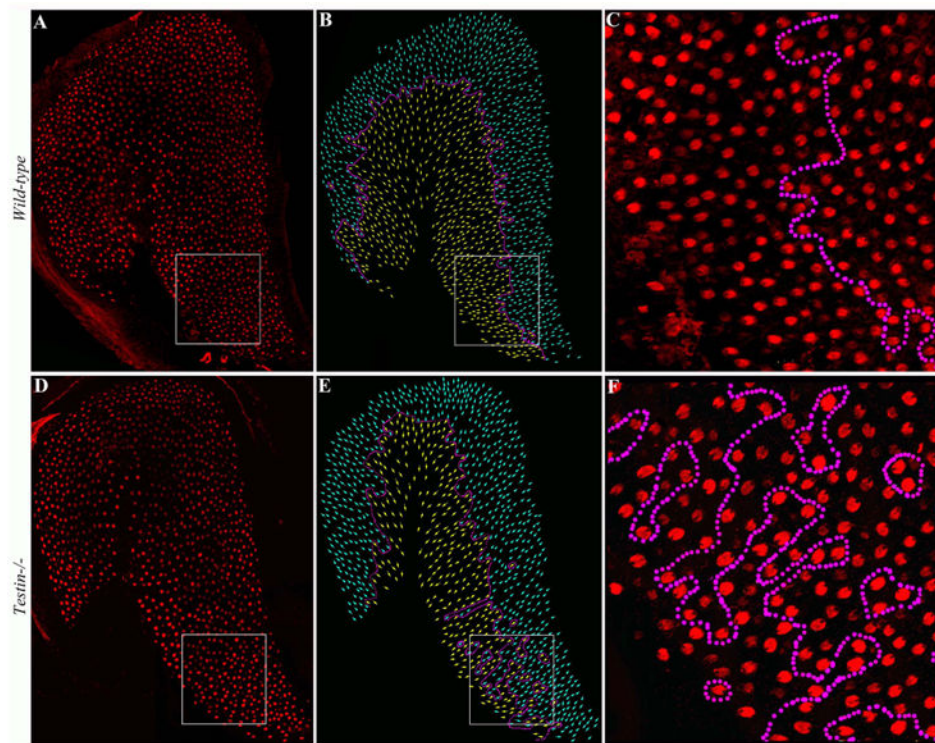
Fig. 6.
Testin is required for precisely coordinated orientation of hair cells in the saccule.
(**A-F**) Saccule whole mounts prepared from wild type control (**A-C**) and *Testin*⁻/⁻ (*Tes*⁻/⁻) E18.5 mice (**D-F**) stained for α-Spectrin to visualize the orientation of hair cells. In the wild type saccule, the orientations of hair cells on either side of the line of polarity reversal are coordinated (**A-C,** the dotted magenta line). In the *Testin*⁻/⁻ saccule (**D-F**), the orientation of hair cells is mostly coordinated along a distinct line of polarity reversal excluding one area (D, E, boxed region) where the line of polarity reversal is lost (**F**).
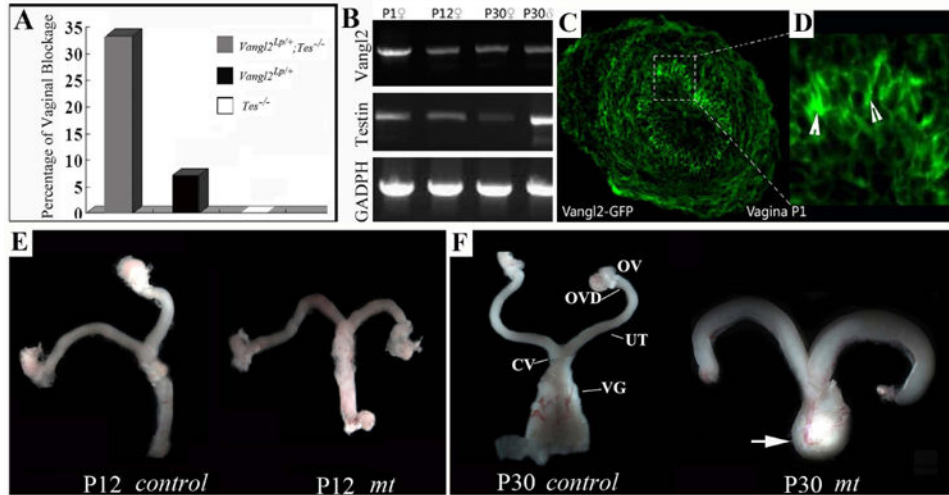
Fig. 7.
Testin genetically interacts with Vangl2 to regulate the normal development of the female reproductive tract in mice.
(**A**) Female mice without an externally visible female reproductive tract (FRT) were quantified and plotted. (**B**) The analysis of Testin and Vangl2 transcripts in FRTs from P1 to P30 and in Testis at P30. RT-PCR reactions were carried out to detect the transcripts. GADPH was used as a house-keeping gene control. (**C,D**) A cross section of the distal FRT from a postnatal day 1 (P1) animal shows the cellular localization of Vangl2-GFP. Vangl2 (green) was observed on the lateral side of the FRT epithelial cells (**C,D**). (**E,F**) Dissected female reproductive tracts from control and *Vangl2$^{Lp/+}$;Testin$^{-/-}$* mutant animals at P12 (**E**) and P30 (**F**). OV: ovary; OVD: Oviduct; UT: uterus; CV: cervix; VG: vagina.