The Wnt Coreceptor Ryk Regulates Wnt/Planar Cell Polarity by Modulating the Degradation of the Core Planar Cell Polarity Component Vangl2*\(\S\)

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Background: Wnt5a regulates planar cell polarity (PCP) in development and signals through Ryk in axon guidance.

Results: Ryk interacts with Vangl2 genetically and biochemically and mediates Wnt5a signaling in controlling PCP.

Conclusion: Ryk regulates PCP by controlling the degradation of Vangl2.

Significance: Revealing regulatory mechanisms underlying PCP is essential for understanding morphogenesis and human birth defects.

The Wnt signaling pathways control many critical developmental and adult physiological processes. In vertebrates, one fundamentally important function of Wnts is to provide directional information by regulating the evolutionarily conserved planar cell polarity (PCP) pathway during embryonic morphogenesis. However, despite the critical roles of Wnts and PCP in vertebrate development and disease, little is known about the molecular mechanisms underlying Wnt regulation of PCP. Here, we have found that the receptor-like tyrosine kinase (Ryk), a Wnt5a-binding protein required in axon guidance, regulates PCP signaling. We show that Ryk interacts with Vangl2 genetically and biochemically, and such interaction is potentiated by Wnt5a. Loss of Ryk in a Vangl2\(\S\) background results in classic PCP defects, including open neural tube, misalignment of sensory hair cells in the inner ear, and shortened long bones in the limbs. Complete loss of both Ryk and Vangl2 results in more severe phenotypes that resemble the Wnt5a\(-/-\) mutant in many aspects such as shortened anterior-posterior body axis, limb, and frontonasal process. Our data identify the Wnt5a-binding protein Ryk as a general regulator of the mammalian Wnt/PCP signaling pathway. We show that Ryk transduces Wnt5a signaling by forming a complex with Vangl2 and that Ryk regulates PCP by at least in part promoting Vangl2 stability. As human mutations in WNT5A and VANGL2 are found to cause Robinow syndrome and neural tube defects, respectively, our results further suggest that human mutations in RYK may also be involved in these diseases.

Wnt signaling pathways control many fundamentally important processes in both embryonic morphogenesis and adult physiology. Abnormal Wnt signaling leads to birth defects and other diseases, including cancer (1, 2). Among the Wnt pathways, the Wnt/planar cell polarity (PCP)\(\textsuperscript{2}\) pathway has recently emerged as an evolutionarily conserved signaling axis, which is required for embryonic morphogenesis (3–6). PCP originally refers to the polarity of epithelial cells within a plane orthogonal to their apical-basal axis and was first discovered in Drosophila melanogaster, where PCP controls polarized processes such as the directional alignment of the wing hair cells. A group of core PCP regulatory genes are identified in Drosophila, and these core PCP components are functionally conserved in vertebrates (7–9). Although a Wnt ligand has not been found to regulate PCP in Drosophila, Wnt5a and Wnt11 are required in PCP-regulated processes in vertebrates (3–6, 10, 11). In mammals, PCP signaling is essential for many critical developmental processes such as convergent extension movements, neural tube closure, inner ear hair cell polarity, and limb elongation (3, 12–22). However, despite the significantly expanded functional spectrum of PCP in vertebrate development, the mechanisms underlying Wnt-mediated regulation of PCP still remain to be elucidated.

Wnt5a, which signals mainly through β-catenin-independent pathways (23, 24), regulates the establishment of PCP by inducing the formation of a receptor complex containing the Wnt-binding receptor tyrosine kinase (RTK) Ror2 and Vangl2 (3), a core PCP component. Mouse mutants of Wnts5a, Ror2, and Vangl2 display shortened long bone cartilage (3, 14, 19, 20, 25). In humans, mutations in components of the Wnt/PCP pathway such as WNT5A and ROR2 cause Robinow syndrome and brachydactyly type B1, which are characterized by short limb dwarfism (26–29). Mutations in both VANGL1 and VANGL2 cause congenital neural tube defects (NTD) such as

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2 The abbreviations used are: PCP, planar cell polarity; NTD, neural tube defect(s); E9.5, embryonic day 9.5; MEF, mouse embryonic fibroblast.
spina bifida (30, 31). Wnt5a acts through Ror2 to induce Vangl2 phosphorylation in a Wnt5a-dose-dependent manner (3). Similar to Ror2, the receptor-like tyrosine kinase (Ryk) is a member of the RTK family (32) and its Drosophila homolog derailed transduces wnt5 signals in axon pathfinding (33). The interaction of wnt5 and derailed is conserved in mammals (34), raising the possibility that Ryk may transduce Wnt5a signals in PCP. In Xenopus and zebrafish embryos, Ryk may mediate Wnt11-regulated convergent extension (3, 35, 36). However, because the mouse Ryk<sup>−/−</sup> and Wnt11<sup>−/−</sup> mutants do not display an obvious PCP phenotype (37, 38), it is important to test whether Ryk acts as a core component transducing Wnt signal to regulate PCP.

Here, we present evidence to show that Ryk is a core PCP regulatory component that controls PCP in multiple developmental processes. Ryk interacts with Vangl2 genetically and biochemically, and this interaction is enhanced by Wnt5a. Mechanistically, Ryk may regulate PCP by binding to Vangl2 and increasing the stability of Vangl2 protein. Our findings suggest that human mutations in Ryk may also be involved in NTD, Robinow syndrome, and brachydactyly.

EXPERIMENTAL PROCEDURES

Mouse Lines and Genotyping—Vangl2, Ryk, and Wnt5a mouse strains have been described previously (14, 20, 37).

Skeletal Preparation—Embryos were skinned, eviscerated, and fixed in ethanol for 24 h and then transferred to acetone for 24 h. Embryos were stained in Alizarin red and Alcian blue for 3 days and subsequently cleared in 1% KOH and stored in 80% glycerol.

Immunostaining and Confocal Microscopy—Cochleae were dissected in PBS and fixed in 4% paraformaldehyde overnight at 4 °C and incubated according to standard protocol of fluorescent immunohistochemistry. Confocal images were acquired using a LSM 510 NLO Meta system (Carl Zeiss). Projected z-stack images were acquired at 0.5-μm intervals for 5–10 μm and combined by Photoshop Elements (Adobe) software.

Immunoprecipitation and Immunoblotting—For co-IP experiment, HEK 293T cells were transfected with Ryk (c-terminal FLAG tag), Vangl2 (N-terminal HA tag) and Wnt5a expression constructs using Lipofectamine 2000 (Invitrogen). Cells were lysed in lysis buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40) with Halt protease inhibitor mixture (Thermo Scientific) and Halt phosphatase inhibitor mixture (Thermo Scientific) and sonicated. Mouse embryonic fibroblasts were isolated according to standard protocols.

RESULTS

Ryk and Vangl2 Interact Genetically—Wnt5a interacts with Ryk during axon guidance (34). To address whether Wnt5a also signals through Ryk to regulate other developmental processes, we generated compound mutants of Wnt5a and Ryk. By phenotypic comparison, we found that loss of Ryk did not enhance the phenotypes of the Wnt5a+/− or Wnt5a<sup>−/−</sup> mutants. The Wnt5a+/−;Ryk<sup>−/−</sup> embryos displayed the same phenotypes as the Wnt5a<sup>−/−</sup> embryos (supplemental Fig. S1) (20). These results suggest that during development, Ryk may act in the same pathway of Wnt5a and may only transduce a subset of Wnt5a signals.

As Wnt5a has been identified to control PCP in mammals (3, 5), we reasoned that Ryk may also regulate PCP if it acts in the Wnt5a signaling pathway. To test this, we crossed the Ryk<sup>−/−</sup> mice with the Vangl2<sup>+/−</sup> mice. The Ryk<sup>−/−</sup>;Vangl2<sup>+/−</sup> mice were viable and fertile and did not display any obvious defects (Fig. 1). However, the Ryk<sup>−/−</sup>;Vangl2<sup>+/−</sup> embryos exhibited open neural tubes; 61% (<i>n</i> = 17/28) of these embryos displayed a completely open neural tube (craniorachischisis), similar to that shown in the Vangl2<sup>−/−</sup> embryos. The Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryos appeared to be similar to the Vangl2<sup>−/−</sup> mutant. However, the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryo exhibited more severe phenotypes than the Vangl2<sup>−/−</sup> mutant. The anterior-posterior body axis and the limbs of these embryos were markedly shortened. In addition, the ventral body wall in the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryos failed to close, similar to what has been observed in the Vangl1<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryo (14). Importantly, the phenotypes of the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryos, including shortened limbs, anterior-posterior body axis and frontonasal processes, were similar to those of the Wnt5a<sup>−/−</sup> embryo (20). Furthermore, we found, that the closure of the eyelid, which is controlled by PCP (39), was affected in the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryos. This defect was not observed in the single mutants of Ryk or Vangl2 (supplemental Fig. S2). The long bones in the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> forelimb were significantly shortened, but their widths were slightly increased, leading to a reduced length-to-width ratio (Fig. 2, A–F). A similar defect was observed in the Wnt5a<sup>−/−</sup> mutants (19, 20). However, the digits of the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryo were less affected than those in the Wnt5a<sup>−/−</sup> embryo (Fig. 2, F and E'). Only one of the three phalanges was absent in the
FIGURE 1. Genetic interaction of Ryk and Vangl2. Lateral views of E18.5 mouse embryos (top row) and their skeletal preparations (bottom row). The Vangl2−/− embryo showed a complete open neural tube and shortened frontonasal process, limbs, and anterior-posterior body axis. The Vangl2+/− phenotype was not enhanced by heterozygous loss of Ryk. However, the Ryk+/−;Vangl2+/− embryo phenocopied the Vangl2−/− embryos. The Ryk+/−;Vangl2+/− embryo resembled the Wnt5a−/− embryo and was more severe phenotypically compared with the Vangl2−/− embryo.

FIGURE 2. Severe length reduction in the Ryk+/−;Vangl2+/− humerus. A–F, Skeletal preparation of the forelimbs of the indicated genotypes at E18.5. Bone length was only mildly reduced in the Vangl2−/− (C) and Ryk+/− (B) mutant, and a more severe reduction in limb elongation was found in the Ryk−/−;Vangl2+/− limb (D). Reduction in humerus length in Ryk−/−;Vangl2−/− embryos (E) was very severe and similar to that in the Wnt5a−/− limb (F). A′–F′, enlarged views of the anterior paws shown in A–F. One phalange in the Ryk−/−;Vangl2−/− embryo was missing (E′). Distal elements were completely missing in the Wnt5a−/− limb (F). G–J, boxed rib regions of E18.5 embryos are shown at higher magnification. G, normal development of the rib cage in the Ryk−/−;Vangl2+/− embryo. H, single bifurcation in the Vangl2−/− rib cage. I, reduced number of ribs accompanied by severe bifurcation in the Ryk−/−;Vangl2−/− embryo. J, bifurcation of a subset of the ribs in the Wnt5a−/− embryo. G′–J′, ventral views of sternum in embryos shown in G–H. The sternum in the Ryk−/−;Vangl2−/− (I′) and the Wnt5a−/− (J′) embryos were closed incompletely (arrows).
Ryk regulates Vangl2 degradation to control PCP.

Ryk regulates PCP signaling in the inner ear—To further investigate the role of Ryk as a PCP regulator, we examined the polarity of sensory hair cells in the inner ear. Expression of Ryk was recently reported by Macheda et al. (36). The hair cell polarity was mostly normal in the Ryk$^{-/-}$/Vangl2$^{+/−}$ and Ryk$^{-/-}$ embryos (Fig. 3, A and B). However, they were misaligned in the Ryk$^{−/−}$/Vangl2$^{+/-}$ inner ears at E18.5 (Fig. 3D). This phenotype was most severe in the outer hair cell row 3 (Fig. 3I). Furthermore, we found an additional row of hair cells in the middle cochlea (arrow in Fig. 3D), and a similar defect was found in the inner ear of the Wnt5a$^{−/-}$ mutant (5). These results further support the notion that Ryk mediates Wnt5a signaling and regulates PCP in the mouse inner ear.

Ryk promotes Vangl2 stability but not Vangl2 phosphorylation—To understand the mechanism underlying the regulation of PCP by Ryk, we first tested whether Ryk and Vangl2 form a Wnt5a receptor complex. Indeed, we were able to detect Ryk and Vangl2 in the same immunoprecipitated complex (Fig. 4, A and B). As a negative control, we could not detect Ryk and another membrane protein Smoothened (Smo) in the same complex (supplemental Fig. S4). Co-expression of Wnt5a enhanced the formation of the Ryk-Vangl2 complex (Fig. 4, A and B). Previously, we have shown that Wnt5a acts through Ror2 to induce phosphorylation of Vangl2 (3). To test whether Ryk regulates PCP in a similar fashion, we investigated Vangl2 phosphorylation. However, unlike Ror2, Ryk did not induce any obvious protein mobility shift of Vangl2, suggesting that Vangl2 phosphorylation was not altered by Ryk (Fig. 5, A and B). Instead, Vangl2 protein levels were increased by Ryk expression suggesting a reduction of Vangl2 degradation in the presence of Ryk. We then tested whether Ryk regulates Vangl2 protein levels in vivo and whether such regulation contributes to the observed genetic interaction between Ryk and Vangl2 by examining Vangl2 protein levels in the E9.5 whole embryo lysisates. Indeed, a decrease in Vangl2 protein levels was detected in the Ryk$^{−/−}$/Vangl2$^{+/-}$ embryos compared with their Ryk$^{+/-}$/Vangl2$^{+/-}$ littermate (Fig. 6A). Furthermore, we observed a similar regulation of Vangl2 protein levels by Ryk in the mouse embryonic fibroblasts, as Ryk$^{−/−}$ cells showed less Vangl2 protein compared with cells isolated from a wild type littermate (Fig. 6B).

As a membrane protein, Vangl2 can be degraded by lysosomes. Indeed, treating cells with bafilomycin A1, a vacuolar-type H$^{+}$-ATPases inhibitor which prevents acidification of endosomes and lysosomes that degrade membrane proteins (40), led to increased Vangl2 protein levels (Fig. 6C). Ryk overexpression, similar to bafilomycin A1 treatment, resulted in
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Increased Vangl2 protein levels (Fig. 6C). Furthermore, the effect of Ryk on Vangl2 protein levels was dose-dependent; higher doses of Ryk in transfection resulted in higher levels of Vangl2 protein (Fig. 6C). As Ryk did not seem to affect Vangl2 phosphorylation, we hypothesized that regulation of Vangl2 levels by Ryk may be independent of Vangl2 phosphorylation. To test this, we employed a Vangl2 construct in which all Wnt5a-dependent phosphorylation sites (Ser and Thr residues) have been mutated (3). Ryk also increased the levels of this phospho-deficient Vangl2, indicating that this activity of Ryk is independent of Vangl2 phosphorylation (Fig. 6D).

To further investigate whether Ryk regulates Vangl2 protein levels by controlling its stability, we tested whether Ryk regulates the half-life of Vangl2. CHO expressing Vangl2 were treated with cycloheximide to block protein synthesis. After 2 h, we observed a reduction of Vangl2 protein in control cells, whereas Vangl2 behaved very stable in the presence of Ryk (Fig. 6E). To test whether Wnt5a also regulates Vangl2 stability through Ryk, we asked whether loss of Wnt5a affected Vangl2 levels. In the limbs of E13.5 embryos, we observed a reduction of Vangl2 protein in the Wnt5a−/− tissue compared with that from the control littermates (Fig. 7A). Next, we asked whether Wnt5a is able to enhance the stability of Vangl2. Therefore, we cultured CHO cells stably expressing Vangl2 in the presence of Wnt5a conditioned medium. Wnt5a increased Vangl2 protein levels (Fig. 7B). We then tested whether Wnt5a affects Vangl2 stability through Ryk in mouse embryonic fibroblast (MEF) cells. Wnt5a treatment increased Vangl2 protein levels in the wild type MEF cells, but not in the Ryk−/− MEF cells (Fig. 7C). To further substantiate this finding, we expressed Ryk and Wnt5a in the Vangl2-expressing CHO cells. Wnt5a or Ryk expression increased the levels of Vangl2, and the effects were further increased when both Ryk and Wnt5a were co-expressed (Fig. 7D). Taken together, our data show that Wnt5a enhances Ryk and Vangl2 complex formation and that Wnt5a may signal through Ryk to regulate Vangl2 activity by increasing its stability.

**DISCUSSION**

In this study, we demonstrate that Ryk is a regulator of the Wnt/PCP pathway in mice. We found that loss of Ryk in a Vangl2+/− background resulted in typical PCP phenotypes in multiple developmental processes, although the Ryk mutant itself had no obvious PCP defects. We further showed biochemically that Ryk and Vangl2 formed a complex, which was enhanced by Wnt5a, and that Ryk was necessary to maintain Vangl2 protein levels. Our results suggest that there is considerable redundancy at the level of Wnt5a signaling reception on the plasma membrane in controlling PCP. Multiple Wnt5a-binding proteins can receive Wnt5a signals at the plasma membrane and regulate Vangl2 in different ways. Collectively, all of these Wnt5a-binding proteins contribute to the regulation of PCP.

Ror2 is required for Wnt5a signaling. However, the phenotypes of Ror2−/− and even Ror2+/−;Vangl2−/− embryos were not as severe as those of the Wnt5a−/− mutant in many developmental processes, suggesting the existence of functional redundancy in Wnt5a signal transduction. This notion is supported by the recently identified redundancy between Ror1 and Ror2 in receiving Wnt5a signaling (41). Our results uncover that Ryk is one of the other Wnt5a-binding proteins on the plasma membrane that regulates PCP. Ryk has been found to mediate Wnt5a activity in regulating axon guidance (34). However, Ryk−/− mouse mutants or derailed fly mutants failed to show obvious PCP defects morphologically (37, 42), raising the question whether Ryk is mediating Wnt5a activity in controlling PCP in general. Here, we found that Ryk regulates Vangl2 activity by modulating its protein stability. As Vangl2 regulates PCP in a dose-dependent manner, it would not be surprising that in a wild type background, the Ryk−/− mutant has no obvious PCP defects because the Vangl2 protein is not reduced to below threshold levels. When Vangl2 protein levels are reduced in the Vangl2+/− mutant, further loss of Ryk decreases the Vangl2 protein levels below a critical threshold, resulting in PCP defects. As axon guidance is a process that can be regulated by PCP (43), the axon guidance defects, shown in Ryk knockdown mice (44), may also be a result of PCP deficiency. It is likely that Vangl2 protein stability is differential regulated in distinct developmental processes. It is also possible that axon guidance may be more sensitive to reduced Vangl2 protein levels than other morphogenetic processes.

It is interesting to note that the relative contribution of Ror2 and Ryk in regulating Vangl2 is tissue-dependent. In the limb, Ror2 appears to be a major coreceptor of Vangl2 as the Ror2−/−;Vangl2−/− limb phenotype is more severe than that of the Ryk−/−;Vangl2−/− mutant. However, in the neural tube, Ryk appears to be more important than Ror2 as 61% of the Ryk−/−;Vangl2−/− mutants showed NTD, whereas only 5% of the Ror2−/−;Vangl2−/− had similar defects (3). This may reflect tissue-specific expression of Ryk and Ror2 or other tissue-specific components involved in PCP signaling.

The occurrence of additional hair cell rows has previously been reported in the Wnt5a−/− inner ears, but in contrast to the Ryk−/−;Vangl2+/− or Ryk−/−;Vangl2−/− mutants, the Wnt5a−/− mutant does not have sensory hair cell polarity.
defects (5). These findings suggest that Wnt5a signaling is impaired in the absence of Ryk and Vangl2 and that loss of Wnt5a may be compensated for by other Wnts in regulating hair cell polarity (45). These results may also imply that in the inner ear, normal convergent extension requires higher Wnt doses than maintaining normal hair cell polarity. Consistent with this, the absence of additional phenotypes in the Ryk<sup>−/−</sup>;Vangl2<sup>−/−</sup> embryo suggests that Wnt5a and/or other Wnt ligands are expressed adequately such that reduction of one Wnt ligand is not enough to perturb the PCP signaling in morphogenesis even in the absence of Ryk. Given the fundamentally important function of PCP in morphogenesis, it is not surprising that multiple Wnts and multiple Wnt-binding receptors have evolved to ensure proper PCP signaling under different circumstances. Similar to our studies, Ryk has been found to interact with Vangl2 and together they might mediate the function of Wnt11 in the zebrafish (36). However, the Wnt11<sup>−/−</sup> mice do not display PCP defects, and Ryk interaction with Wnt5a has been well established in axon guidance in mice; our genetic and biochemical analysis including characterization of the skeletal phenotypes of the Wnt5a and Ryk;Vangl2 double mutant embryos indicate that Wnt5a is the major Wnt ligand that regulates PCP through Ryk.

The PCP pathway is highly conserved in animals and mutations in human VANGL1 and VANGL2 have been identified to cause NTD (30, 31). Furthermore, human mutations in
WNT5A can result in Robinow syndrome (29). As human and mouse Ryk are highly conserved, it is very likely that the role of Ryk in regulating PCP, identified in this work, is also conserved in human. The striking similarity between the phenotype of the long bones in the Ryk;Vangl2 mutants and the defects observed in patients diagnosed with Robinow syndrome and brachydactyly indicate that Ryk may play a role in these malformations. Therefore, our results suggest that mutations in human RYK might be involved in NTD and Robinow syndrome and provide new insight into the etiology of these diseases.

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