



## Determination of the Cell Orientation Pathway and Receptor Complex Signals

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### Abstract

Vertebrate proteins activate several distinct pathways. Intrinsic differences among ligands and Frizzled receptors, and the availability of pathway-specific co receptors. A secreted glycoprotein, Cthrc1, is involved in selective activation of the planar cell polarity pathway by proteins. Cell-surface-anchored Cthrc1 bound to proteins, Frizzled proteins, and Ror2 and enhanced the interaction of proteins and Frizzled /Ror2 by forming the Cthrc1 - Frizzled /Ror2 complex. These results suggest that Cthrc1 is a Frizzled cofactor protein that selectively activates the Frizzled /PCP pathway by stabilizing ligand-receptor interaction.

**Keywords:** Vertebrate proteins; pathway-specific; Cell-surface-anchored Cthrc1; Stabilizing ligand-receptor

### Introduction

Proteins are members of a family of secreted signalling molecules that have multiple roles in the development of the organism throughout the animal kingdom. In vertebrates, proteins play central roles in regulation of body-axis specification, patterning of germ layers and tissues, differentiation and proliferation of stem/progenitor cells, morphogenetic movements during embryogenesis and organogenesis, and growth and metastasis of cancers/tumors. At the cellular level [1], such multi functionality of signalling is achieved through the activation of different intracellular signalling pathways.

The best-characterized pathway is the canonical pathway, which leads to the stabilization of  $\beta$ -catenin proteins. An increase in the  $\beta$ -catenin level leads to its translocation to the nucleus, where it interacts with TCF/LEF-family transcription factors to activate the expression of target genes. During vertebrate development, canonical signalling is involved in determining cell fate and the regulation of growth, including formation of the body axis, and amplification of neural progenitors. The non-canonical pathway is further classified into two subsidiary pathways: planar cell polarity and Ca<sup>2+</sup> [2]. The downstream components of the PCP pathway resemble those of PCP signalling in *Drosophila*. The PCP pathway promotes activation of small GTPases, including RhoA and Rac1, and their downstream protein kinases, ROCK and JNK, respectively, to regulate actin polymerization [3, 4]. In vertebrates, PCP signalling plays important roles in morphogenetic processes, including convergent-extension movements during gastrulation and the alignment of the bundle of the sensory hair cells of the organ of Corti. In contrast, the Ca<sup>2+</sup> pathway is characterized to a lesser extent. It promotes the intracellular increase in Ca<sup>2+</sup> concentration and activates Ca<sup>2+</sup>-sensitive enzymes such as PKC, CamKII, and calcineurin. Vertebrate proteins have been classified into two groups. They stimulate the canonical Wnt pathway, whereas Wnt5a and Wnt11 are classified as “noncanonical Wnt proteins [5].” Such differential activities indicate that pathway selection depends on the intrinsic nature of Wnt proteins. In contrast to these observations, the relationships between Wnt proteins and pathways are not strictly maintained in cultured cells, indicating that pathway selection by Wnt proteins depends on the cellular context. Wnt proteins use Frizzled receptors and pathway-specific co receptors for signalling. LRP5 and LRP6 are co receptors for the canonical pathway, whereas Ror2 is a co receptor for the noncanonical pathway. The availability of these

co receptors also affects pathway selection by Wnt proteins. In the Cthrc1 was originally identified as an overexpressed gene in injured rat arteries, and it encodes a secreted glycoprotein with repeats of the GXY motif, which is characteristic of collagens. The role of the Cthrc1 gene during development in the mouse mutation of a PCP gene, into this mutant background resulted in clear PCP phenotypes [6, 7]. In Cthrc1 activated the PCP pathway by promoting the formation of a ligand-receptor complex for the PCP pathway regardless of the class of protein. The results now available indicate that Cthrc1 is a Wnt signalling component that selectively activates the PCP pathway, and that its underlying mechanism is enhancement of the Wnt-receptor interaction by forming a stabilized complex.

### Method

To identify the novel genes expressed in the node and the notochord, we generated a single-cell cDNA library of the node/notochord cell of embryonic day of mouse embryos and performed in situ hybridization screening. This screening identified Cthrc1 as a gene specifically expressed in the node and the notochord. No signal was observed. Concomitant with node formation, Cthrc1 expression was initiated and was extended anteriorly to the notochord. Cthrc1 was detected in the ventral midline of the midbrain, dorsal hindbrain [8], and optic vesicle, as well as the posterior notochord. The first exon with a LacZ gene by using homologous recombination in embryonic stem cells. Cthrc1LacZ/+ mice were apparently normal and the intercross. Northern blot analysis of embryos confirmed the absence of Cthrc1 transcripts in Cthrc1LacZ/LacZ embryos indicating the null mutation of Cthrc1 [9]. Expression of the adjacent gene, Fzd6, was not affected. The absence of clear abnormalities in Cthrc1 mutants prompted us to further test our hypothesis by introducing a heterozygous mutation of the PCP gene Vangl2. Vangl2 is a homolog of the *Drosophila* PCP

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gene Van Gogh/Strabismus and encodes a four-transmembrane protein with a PDZ-domain-binding motif. *Vangl2Lp/Lp* homozygous mutants display typical PCP phenotypes, including a shortened body axis, an open neural tube [10], and misorientation of sensory hair cells of the cochlea, whereas *Vangl2Lp/+* heterozygous mutant mice show a weak PCP-related phenotype, that is, a looped tail. Mice with a heterozygous mutation of *Vangl2* display a weak PCP-related phenotype; additional introduction of a PCP mutation would exacerbate the mutant phenotype. The *Cthrc1LacZ/+;Vangl2Lp/+* embryos were indistinguishable from those of *Vangl2Lp/+* mice. *Cthrc1LacZ/LacZ;Vangl2Lp/+* embryos displayed a neural tube closure defect in the midbrain region reminiscent of the PCP mutation [11].

To confirm the genetic interactions of *Cthrc1* and *Vangl2*, we examined another characteristic feature of the PCP mutants of the sensory hair cells in the cochlea. The normal cochlea has four rows of sensory hair cells one row of inner hair cells and three of outer hair cells. Each cell contains an asymmetrically located at the outer side accompanied by a bundle of stereocilia [12]. *Cthrc1LacZ/+;Vangl2Lp/+* embryos showed a normal arrangement of four rows of hair cells and a uniform hair bundle orientation. In *Cthrc1LacZ/LacZ;Vangl2Lp/+* embryos. The orientation of the hair bundles was disrupted significantly; Scanning electron microscopy confirmed the normal organization of bundles in the mutants, indicating that the defect was restricted to cell polarity. Among the four rows of auditory hair cells, the IHCs were the most severely affected. Severe defects were also present in the outermost row of OHCs and modest effects were observed in the inner rows. This defect was not affected by the genetic background of the mutants. In addition to the defects in the orientation of individual hair cells, we observed that some hair cells deviated from the rows, and, in an extreme case, additional sensory hair cells outside of the four rows were observed. The results demonstrate the genetic interaction of *Cthrc1* with *Vangl2* for PCP signalling and suggest that *Cthrc1* is involved in the regulation of PCP signalling interaction of *Cthrc1* with Extracellular Components of PCP Signalling.

## Results

Involvement of *Cthrc1* in PCP signalling led us to investigate how *Cthrc1* controls PCP signalling. Because *Cthrc1* is a secreted protein and because vertebrate PCP signalling is regulated by Wnt proteins, The interaction of *Cthrc1* with various extracellular components of Wnt signalling. We developed an interaction assay that immunoprecipitates the proteins from the lysates of co cultured. The conditioned medium of *Cthrc1*-expressing cells had no activity. We first verified the specificity of the protein interactions detected with this assay by using established interactions. The co cultured IP to interactions between *Cthrc1* and Wnt proteins. The cell lysates co precipitated both canonical Wnt and no canonical Wnt proteins [13]. Because Wnt proteins were not co precipitated with *Cthrc1-ΔC*, the observed *Cthrc1*-Wnt interactions are specific. The interaction of *Cthrc1* with a trans membrane PCP signalling component, *Vangl2*. In summary, *Cthrc1* interacts with multiple extracellular components of Wnt signalling, which include both canonical and non-canonical Wnt proteins, proteins, and the PCP co receptor *Ror2*, but not with the canonical Wnt co receptor *LRP6* or the PCP component *Vangl2*. These results suggest that *Cthrc1* regulates PCP signalling by modulating Wnt signalling in the extracellular space.

The Wnt/PCP signal is transduced to two parallel signalling cascades, a process that starts with the activation of the small GTPases *Rac1* and *RhoA*, downstream of *dishevelled*. *Rac1* and *RhoA* in HEK293T cells, which have been successfully used for the analysis of Wnt/PCP signalling in mammals. To detect the activation of *Rac1*

and *RhoA*, we used an established biochemical assay that employs a fusion protein of glutathione S-transferase and the p21-binding domain of human PAK-1 and GST-RBD that recognizes GTP-bound form of *Rac* and *Rho*, respectively [14]. Transfection of the expression plasmids for *Wnt3a*, *Wnt5a*, or *Dvl2* activated endogenous *Rac1* and a similar activation was also observed by transfection of the *Cthrc1* expression plasmid. Co expression of *Cthrc1* with *Wnt3a*, *Wnt5a*, or *Dvl2* further enhanced the activation of *Rac1*. *Cthrc1* also activated *RhoA* and enhanced activation of *RhoA* by *Wnt3a*, *Wnt5a*, or *Dvl2*. These results suggest that *Cthrc1* activates both cascades of the Wnt/PCP pathway and this effect is synergistic with pathway activation by other signalling components.

Co culture of *Cthrc1*-expressing cells suppressed canonical Wnt signalling, unlike its conditioned medium. To reveal the cause of such differences, we compared *Cthrc1* proteins in the cell lysate and in the conditioned medium. *Cthrc1*-CM showed slower migration than *Cthrc1*-L on SDS-PAGE indicating that only the modified protein is released into the medium. In a co culture IP, immunoprecipitation of *Fzd6*, *Ror2*, or *Wnt5a* co precipitated *Cthrc1*-L from the lysate, but not *Cthrc1*-CM, although *Cthrc1*-CM was also present during the co culture period. In addition to the 75 kDa band, which was previously characterized as a trimmer of *Cthrc1* peptides, we observed strong bands at 150 kDa, 250 kDa, and a higher molecular mass.

## Discussion

The differences of Wnt and Fzd proteins and the availability of co receptor proteins *Ror2* and *LRP5/6*. This finding suggests the existence of a regulatory mechanism for pathway selection by Wnt proteins, which we would like to, summarize in the following model. There are some intrinsic preferences for interactions among Wnt, Fzd, and co receptor proteins. In the conditions in which appropriate receptors are available, these Wnt proteins may although Wnt-Fzd and Wnt-Ror2 have been implicated in noncanonical Wnt signalling, whether Fzd and *Ror2* act within a single receptor complex, or whether they act in two parallel pathways [15], is currently not known. and because the cell migratory activity of *Wnt5a/Ror2* signalling requires *Dvl*, a downstream effector of Fzd signalling. that of *Wnt5a* with *Ror2*, and it also establishes new interactions of *Wnt3a* with *Ror2*. The normal active form of *Cthrc1* is an N-glycosylated trimmer anchored on the cell surface. Because *Cthrc1* interacts with Wnt proteins, Fzd proteins, and *Ror2*, *Cthrc1* may bridge these proteins through trimer formation. Suppression of the canonical pathway should be a secondary effect as a result of strong activation of the PCP pathway, because interaction.

The model of PCP activation by the *Cthrc1*-Wnt-Fzd/*Ror2* complex is best supported by inner ear development. Such morphogenesis is disrupted in mouse mutants of PCP signalling components; it is conceivable that the *Cthrc1*-*Wnt5a*-*Fzd3*/*Fzd6*/*Ror2* complex activates PCP signalling in this tissue. Although *Wnt7a* is also expressed in the cochlea, no activation of canonical Wnt signalling is observed. Because the PCP pathway regulates actin polymerization through activation of the small GTPases, *JNK*, and *ROCK*, During cancer development and wound healing, activation of canonical Wnt signalling by transcriptional activation of canonical Wnt proteins and/or epigenetic silencing of secreted Wnt antagonists is observed. Therefore, additional expression of *Cthrc1* to these cells likely results in the activation of the PCP pathway and promotion of cellular motility [16]. Consistent with this notion, Wnt/PCP signalling-related genes are overexpressed in some malignant human cancers. It is conceivable that *Cthrc1* is a multifunctional protein and promotes cell migration through activation of Wnt/PCP signalling and reduction in collagen matrix

deposition. Multi functionality of Cthrc1 is also reported for TGF- $\beta$  signalling. activity (this study), inhibits TGF- $\beta$  signalling, suggesting that modifications control the activity and localization of Cthrc1.

## Conclusion

We identified a Wnt cofactor protein, Cthrc1, that promotes selective activation of the PCP pathway by enhancing the Wnt-receptor interaction. This mechanism may be used widely in the regulation of morphogenesis during development and cell motility in wound healing and cancer metastasis. In an evolutionary context, it is of interest to note that Cthrc1 is present only in chordates, which use Wnt proteins for PCP signalling. Identification of such genes in the future should facilitate understanding of the mechanism of pathway selection by Wnt proteins.

## Acknowledgement

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## Conflict of Interest

None

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