Cell, Vol. 119, 97–108, October 1, 2004, Copyright ©2004 by Cell Press

Mammalian Ryk Is a Wnt Coreceptor Required for Stimulation of Neurite Outgrowth

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Summary

The Ryk receptor belongs to the atypical receptor tyrosine kinase family. It is a new member of the family of Wnt receptor proteins. However, the molecular mechanisms by which the Ryk receptor functions remain unknown. Here, we report that mammalian Ryk, unlike the *Drosophila* Ryk homolog Derailed, functions as a coreceptor along with Frizzled for Wnt ligands. Ryk also binds to Dishevelled, through which it activates the canonical Wnt pathway, providing a link between Wnt and Dishevelled. Transgenic mice expressing Ryk siRNA exhibit defects in axon guidance, and Ryk is required for neurite outgrowth induced by Wnt-3a and in the activation of T cell factor (TCF) induced by Wnt-1. Thus, Ryk appears to play a crucial role in Wnt-mediated signaling.

Introduction

Wnt signaling plays an essential role in multiple, diverse developmental processes, including the regulation of cell proliferation, differentiation, and migration (Cadigan and Nusse, 1997; Moon et al., 2002; Peifer and Polakis, 2000). Misregulation of the Wnt pathway is implicated in several diseases, including cancer (van Es et al., 2003). Gene targeting experiments suggest that the Wnt proteins are required for patterning of the central nervous system (Ikeya et al., 1997; McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990). Wnt is also involved in neural crest stem cell induction (Garcia-Castro et al., 2002; Lewis et al., 2004), neural precursor cell proliferation (Castelo-Branco et al., 2003; Chenn and Walsh, 2003; Ikeya et al., 1997), neurogenesis (Hari et al., 2002; Lee et al., 2004), axon guidance (Lyuksyutova et al., 2003; Yoshikawa et al., 2003), and synapse formation (Hall et al., 2000; Packard et al., 2002).

The canonical Wnt signaling cascade is mediated by β-catenin (Willert and Nusse, 1998). In the absence of Wnt signaling, β-catenin is synthesized but rapidly degraded due to phosphorylation by GSK3β (Aberle et al., 1997; Orford et al., 1997; Peifer et al., 1994; Salic et al., 2000; Yost et al., 1996). Wnt signaling inhibits the kinase activity of GSK3β, allowing β-catenin to accumulate in the cytoplasm and to translocate to the nucleus. Nuclear β-catenin binds to members of the LEF/TCF family of transcription factors to activate the Wnt target genes (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996).

The Wnt ligands are extracellullar proteins that stimulate other cells through distinct receptors such as members of the Frizzled family and the coreceptor LRP5/6 (Bhanot et al., 1996; Tamai et al., 2000; Wehrli et al., 2000). LRP5/6 and Frizzled form a receptor-ligand complex with Wnt (Tamai et al., 2000). Additionally, the intracellular downstream adaptor protein Dishevelled also plays a key role in Wnt signaling. Recently, Derailed in *Drosophila* was shown to be another receptor for Wnt (Yoshikawa et al., 2003).

Derailed and its mammalian homolog Ryk are members of the atypical receptor tyrosine kinase family (Halford and Stacker, 2001). Ryk consists of an extracellular WIF (Wnt inhibitory factor) domain, an intracellular atypical kinase domain, and a PDZ binding motif (Halford and Stacker, 2001). The kinase domain of Ryk is atypical because it contains mutations in the evolutionarily conserved tyrosine kinase residues (Hovens et al., 1992; Yee et al., 1993) and lacks protein tyrosine kinase activity. The functions of Ryk have been studied in several model organisms, including *D. melanogaster, C. elegans*, and *M. musculus*. The *Drosophila* Ryk homolog, *Linotte* or *Derailed*, was first identified as a gene involved in learning and memory (Dura et al., 1993, 1995). Furthermore, a mutation in the *derailed* gene causes defects in axon guidance (Bonkowsky et al., 1999; Callahan et al., 1995; Moreau-Fauvarque et al., 1998; Simon et al., 1998; Yoshikawa et al., 2003). Thus, the defects in learning and memory might be caused by the abnormal morphology of the central nervous system. The *C. elegans* Ryk homolog Lin-18 is required for establishing the polarity of the secondary vulval cell linage produced by a hypodermal blast cell. In vulva development, the Lin-18 mutant has a similar phenotype to the Lin-17 mutant (Sternberg and Horvitz, 1988), the *C. elegans* homolog of Frizzled, suggesting a genetic interaction of Ryk and Frizzled. Ryk knockout mice die soon after birth and exhibit a complete cleft of the secondary palate plus a distinctive craniofacial appearance (Halford et al., 2000). This phenotype is also seen in EphB2/B3 knockout mice (Orioli et al., 1996), indicating that Ryk might be genetically linked to the Eph pathway, a cascade important for the development of the nervous system.

The discovery of Ryk as another receptor for Wnt in *Drosophila* is important to the understanding of Wnt signaling in the development of the nervous system and poses several interesting questions. What is the ligand for mammalian Ryk? Does Ryk function as a separate receptor independent of Frizzled, as Derailed does? Or does it form a receptor complex with Frizzled, as LRP5/6 does? Is Dishevelled required? Can Ryk activate the TCF pathway?

Here, we report that Ryk directly binds Wnt-1 and Wnt-3a via its WIF domain and is required for the TCF activation induced by Wnt-1. The extracellular domain of Ryk forms a ternary complex with Frizzled and Wnt-1. The intracellular domain of Ryk binds Dishevelled, which is required for TCF activation in response to Wnt-3a activation. Transgenic mice expressing anti-Ryk siRNA have defects in axon guidance. Finally, Ryk allows Wnt-3a to induce neurite outgrowth in dorsal root ganglia

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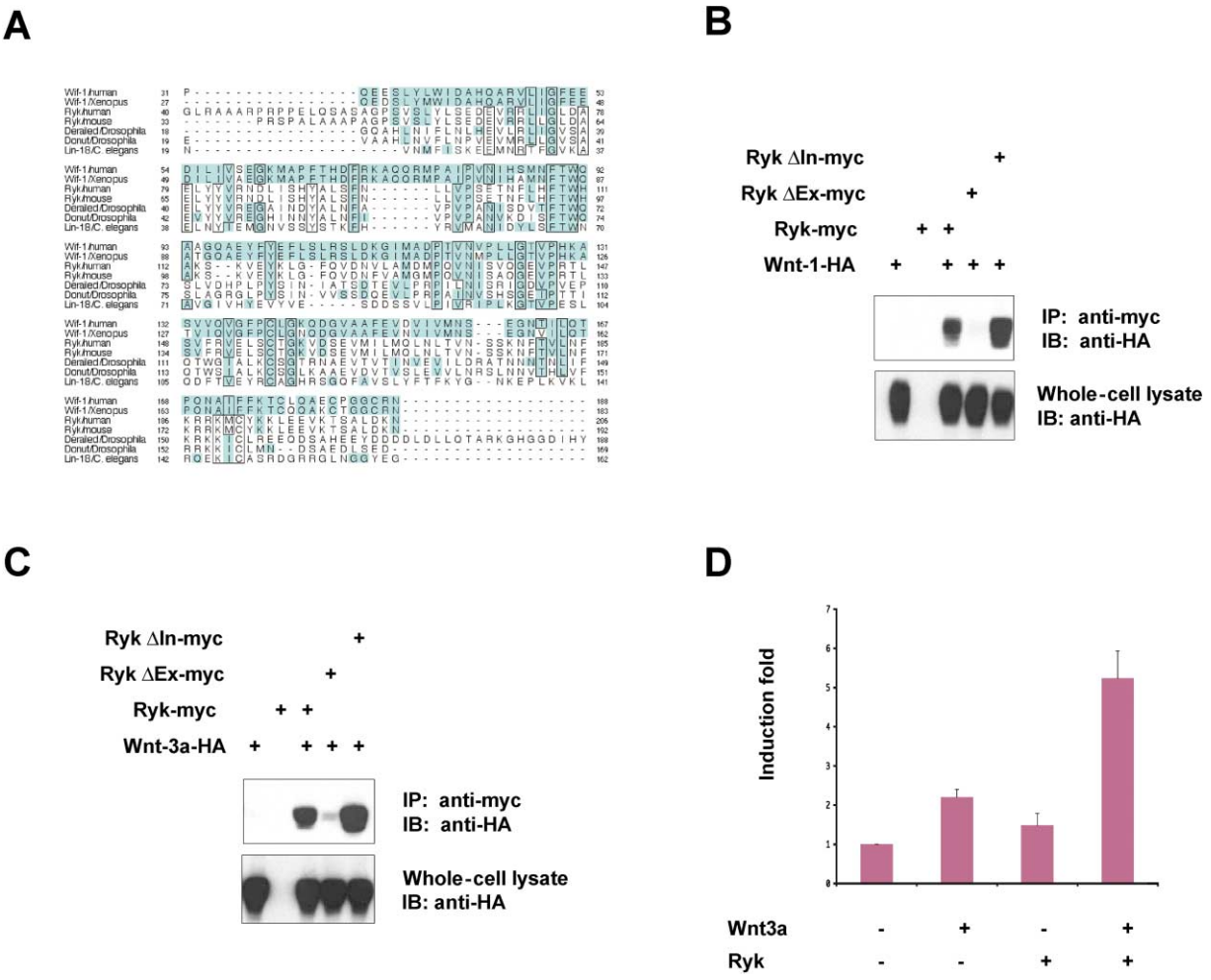

Figure 1. Ryk Associates with Wnt

(A) Alignment of WIF domain of Ryk and WIF proteins among different species. Conserved residues are boxed.

(B) Coimmunoprecipitation of Ryk with Wnt-1 in 293T cells. HA-tagged Wnt-1 was cotransfected into 293T cells with myc-tagged Ryk and its deletion mutants. The expression level of Wnt was determined by Western blotting (bottom). The Ryk-associated Wnt was determined by Western blotting of the anti-Myc immunoprecipitate (top).

(C) Same as (B), except Wnt-3a was used.

(D) Activation of TCF-luciferase reporter by transfection of Ryk (lanes 3 and 4) and treatment with Wnt-3a-conditioned medium (lanes 2 and 4).

(DRG) explants, suggesting that Ryk is a functional and key receptor for Wnt in the nervous system.

Results

Ryk Binds to Wnt-1 and Wnt-3a through the Extracellular WIF Domain

The extracellular domain of Ryk is homologous to Wnt inhibitory factor (WIF) (Hsieh et al., 1999; Patthy, 2000) (Figure 1A), suggesting that Wnt may be a ligand for Ryk. To test this hypothesis, we first examined whether Ryk can bind to Wnts. Myc-tagged Ryk, myc-tagged Ryk with its extracellular domain deleted (RykΔEx), and myc-tagged Ryk with its intracellular domain deleted (RykΔIn) were cotransfected into 293T cells with either HA-tagged Wnt-1 or Wnt-3a. Cell lysates from the transfected cells were subjected to immunoprecipitation with anti-myc antibody followed by Western blotting with an anti-HA antibody. Both Wnt-1 and Wnt-3a bound Ryk (Figures 1B and 1C, lane 3). The extracellular WIF domain was required for both interactions because the RykΔEx mutant did not coimmunoprecipitate with Wnt-1 or Wnt-3a (Figures 1B and 1C, lane 4). The intracellular domain of Ryk did not contribute to the Ryk/Wnt interaction, because RykΔIn bound effectively to both Wnt-1 and Wnt-3a (Figures 1B and 1C, lane 5). Thus, Ryk had the properties of a receptor for Wnt proteins.

Ryk Protein Is Required for TCF-Driven Transcription

Activation of the canonical Wnt signaling pathway requires β-catenin stabilization and its association with TCF to activate transcriptional targets (Moon et al., 2002; Wodarz and Nusse, 1998). To determine if Ryk binding by Wnt leads to activation of the canonical Wnt pathway, a TCF-mediated luciferase reporter assay was utilized. 293T cells were cotransfected with a TCF-luciferase reporter construct and a DNA construct expressing Ryk. Forty-eight hours posttransfection, cells were treated with Wnt-3a-conditioned medium for 6 hr and lysed to

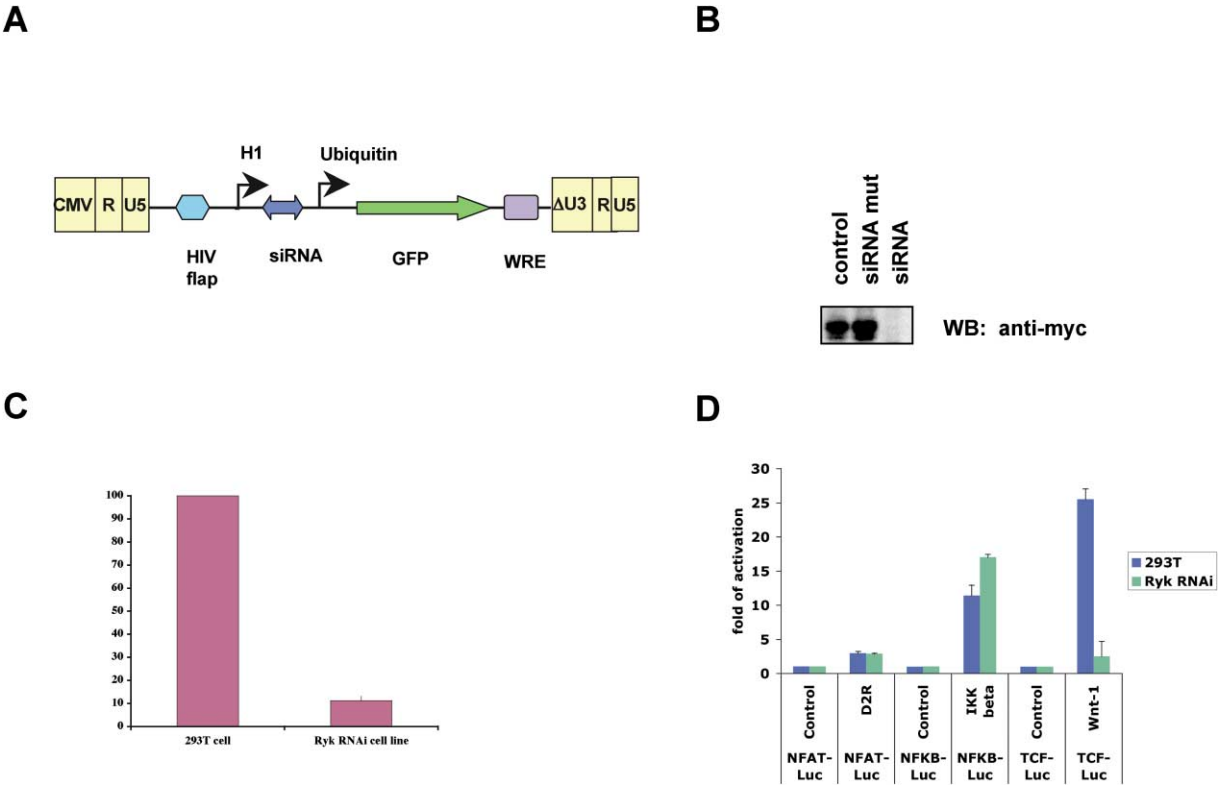

Figure 2. Ryk siRNA Blocks Tcf Activation

(A) Schematic structure of lentiviral constructs expressing Ryk siRNA. SiRNAs for Ryk were expressed under the control of the human H1 promoter. GFP under the ubiquitin promoter was used as a control for infection.

(B) Inhibition of myc-tagged Ryk expression by siRNA expressed from lentivirus. Lentivirus expressing Ryk siRNA and its mutant were used to infect L cell line expressing myc-tagged Ryk. The expression of Ryk was determined by Western blot using anti-myc antibody.

(C) The endogenous Ryk mRNA levels in 293T cells infected with lentivirus expressing Ryk siRNA. The mRNA levels were determined by real-time PCR.

(D) Ryk is required for Wnt-1-induced TCF activation. Luciferase reporters for NFAT, NFκB, and TCF were cotransfected into 293T cells (blue bar) and Ryk siRNA cells (green bar) with dopamine receptor D2R, IKK β, and Wnt-1, respectively. The luciferase activity was examined as described.

determine luciferase activity. Ryk activated the TCF-luciferase reporter 1.5-fold, while Wnt-3a activated the TCF-luciferase reporter 2- to 3-fold (Figure 1D). However, the TCF-luciferase reporter was activated 5-fold in the cells transfected with Ryk and treated with Wnt-3a conditioned medium. A control mutant TCF-Luciferase reporter (FOPFLASH) was used, and no activation was found (data not shown). These results suggested that Wnt-3a and Ryk might function cooperatively in the activation of the TCF-luciferase reporter and supported the notion that Ryk is a receptor for Wnt. To definitively ask whether Ryk plays an essential role in the Wnt signaling pathway, siRNA technology was used to knock down the expression of the endogenous Ryk gene. Four hairpin double-strand DNAs to target Ryk were designed and tested for their ability to reduce Ryk expression (Brummelkamp et al., 2002). The strongest Ryk siRNAs, driven by an H1 promoter, were inserted into the lentiviral vector FUGW as illustrated (Figure 2A) (Lois et al., 2002). VSVG pseudotyped lentivirus was generated. A stable mouse L cell line expressing myc-tagged Ryk was infected with either siRNA or control virus. When 100% of cells were infected, myc-tagged Ryk expression was completely inhibited (Figure 2B).

To test whether Ryk plays a role in Wnt signal transduction, 293T cell lines expressing Ryk siRNA were generated. Endogenous Ryk mRNA levels were determined by real-time PCR. Messenger RNA of human GAPDH was used as an internal control. Compared with the control, Ryk mRNA from the Ryk siRNA cell line was inhibited by 88% (Figure 2C).

Signal transduction in these cells was examined using a luciferase reporter assay. In wild-type cells, the TCF-luciferase reporter was activated about 25-fold after Wnt stimulation, while, in Ryk siRNA-containing cells, TCF-driven transcription induced by Wnt-1 was greatly inhibited (Figure 2D). This suggests that Ryk is required for Wnt-induced, TCF-driven transactivation. As controls, the activation of an NFκB-luciferase reporter by IKK and an NFAT-luciferase reporter by the dopamine receptor 2 (D2R) were not inhibited in the Ryk siRNA-containing cells, demonstrating that the Ryk siRNA-mediated inhibition was specific to the TCF pathway. These results strongly support the hypothesis that Ryk is a functional receptor for Wnt.

Ryk Forms a Ternary Complex with Frizzled and Wnt

Since Frizzled is the canonical receptor for Wnt (Bhanot et al., 1996), we investigated whether Ryk acts as a

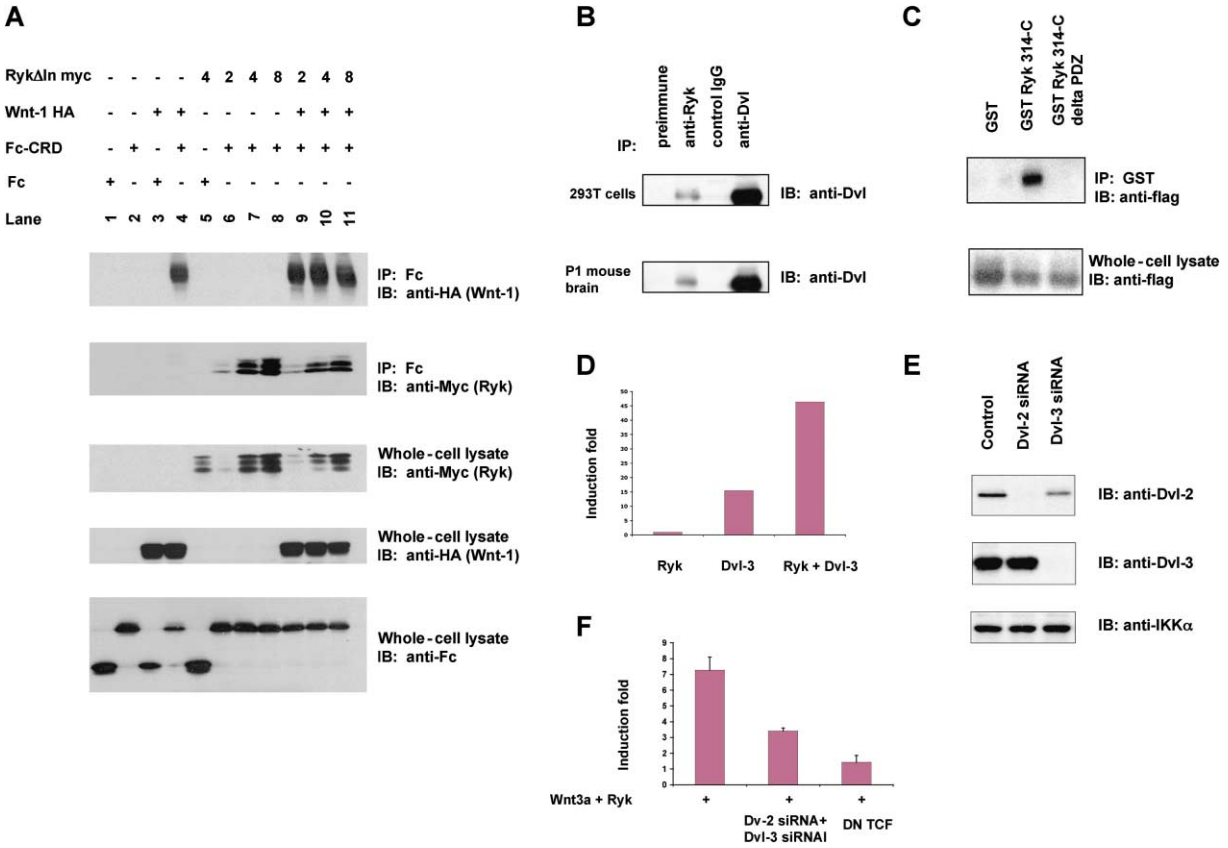

Figure 3. Interaction of Ryk with Frizzled and Dishevelled

(A) Ryk, Wnt, and Frizzled form a ternary complex. The cysteine-rich domain (CRD) of Frizzled-8 was fused with human IgG Fc fragment. 293T cells expressing Fc fusions, Wnt-1 (HA-tagged), and Ryk extracellular domain (Myc-tagged) were subjected to immunoprecipitation of Fc fragment by protein A agarose beads and then immunoblotting (top two panels). The expression of these proteins in whole-cell lysates was examined by Western blotting (bottom three panels).

(B) Interaction of endogenous Ryk and Dishevelled. 293T cell lysate and P1 mouse brain cell lysate were subject to immunoprecipitation with control IgG and anti-Ryk and anti-Dishevelled antibodies followed by Western blotting using anti-Dishevelled antibody.

(C) Coimmunoprecipitation of Ryk intracellular domain (amino acid 236 to the C terminus, GST tagged) with Dishevelled (Flag-tagged, DIX domain deletion mutant) from lysates of transfected 293T cells. Ryk 236-C ΔPDZ is a deletion mutant that lacks the PDZ binding motif.

(D) Ryk and Dishevelled synergistically activate the TCF luciferase reporter in 293T cells.

(E) siRNA specific for Dishevelled-2 and Dishevelled-3 inhibited expression of Dishevelled-2 and -3, respectively. 293T cells were transfected with siRNA of Dishevelled-2 and Dishevelled-3. The expression of Dishevelled-2 and Dishevelled-3 was determined by Western blot. IKKα level was used as a loading control.

(F) Cotransfection of Dishevelled-2 siRNA and Dishevelled-3 siRNAs blocks the activation of a Tcf-luciferase reporter, as does dominant-negative Tcf-4. Dishevelled siRNAs and dominant-negative Tcf-4 were transfected with Ryk into 293T cells. The cells were treated with Wnt-3a-conditioned medium prior to luciferase reporter assay.

coreceptor with Frizzled. The extracellular cysteine-rich domain (CRD) of Frizzled-8 was fused with the human IgG Fc fragment and was cotransfected into 293T cells with increasing amounts of myc-tagged Ryk extracellular domain with or without HA-tagged Wnt-1. As a negative control, the Fc fragment was also transfected into 293T cells. The Fc and Fc fusions were immunoprecipitated with protein A/G agarose. Associated proteins were determined by Western blotting using anti-HA and anti-myc antibodies. In the absence of Wnt-1, the CRD of Frizzled-8 binds strongly to the Ryk extracellular domain (Figure 3A, lanes 6–8), while Fc alone does not (Figure 3A, lane 5). In the absence of Ryk, the Frizzled CRD binds to Wnt as well (Figure 3A, lane 3 and 4). Furthermore, overexpression of increasing amounts of Ryk does not inhibit the Wnt/Frizzled interaction (Figure 3A, lanes 9–11), suggesting that Ryk may form a ternary coreceptor complex with Frizzled and Wnt.

Ryk Links Wnt to Dishevelled

We have established that Ryk may be involved in the canonical pathway of Wnt signaling, but it is not clear whether it involves Dishevelled, one of the key components of the Wnt pathway. We first tested if there was any interaction of endogenous Ryk and Dishevelled. Both 293T cell lysate and mouse brain cell lysate were subjected to immunoprecipitation with mice polyclonal anti-Ryk serum followed by immunoblotting with anti-Dishevelled antibody. Dishevelled was found to coimmunoprecipitate with Ryk in both 293T cells and brain cells (Figure 3B), suggesting that endogenous Ryk and Dishevelled associated with each other in vivo. We rea-

soned that the binding was most likely mediated by the PDZ domain of Dishevelled and the C-terminal PDZ binding motif of Ryk. To test this hypothesis, the GST fusion protein of the Ryk intracellular domain and its PDZ binding domain deletion mutant were transfected with Dishevelled (ΔDIX) into 293T cells. The Dishevelled (ΔDIX) mutant was used because the DIX domain is associated with lipids and actin and caused a high background in coimmunoprecipitation experiments. Western blotting of the GST Ryk immunoprecipitate showed that the Flag-tagged Dishevelled mutant associated with Ryk (Figure 3C). This association was mediated by the PDZ binding motif of Ryk because its deletion abolished binding.

We tested whether Ryk and Dishevelled can synergize in activating the TCF pathway. Overexpression of Dishevelled led to the activation of the TCF-luciferase reporter about 15-fold. Coexpression of Ryk further enhanced the activation to about 25-fold, supporting the hypothesis of a functional interaction between Ryk and Dishevelled (Figure 3D). We then asked whether the activation of TCF-luciferase reporter induced by Wnt-3a and Ryk is mediated by Dishevelled. This was done by knocking down the Dishevelled expression by RNAi. In 293T cells, Dishevelled-2 and Dishevelled-3 are expressed, while Dishevelled-1 is not (data not shown). Therefore, siRNA against Dishevelled-2 and -3 were used to inhibit the expression of endogenous Dishevelled in 293T cells. SiRNA for Dishevelled-2 blocked the expression of Dishevelled-2 specifically and had no effect on Dishevelled-3, while Dishevelled-3 siRNA only knocked down the expression of Dishevelled-3 (Figure 3E). Overexpression of siRNA against Dishevelled-2 and siRNA against Dishevelled-3 together blocked the TCF-luciferase reporter activation in the cells transfected with Ryk gene and treated with Wnt-3a-conditioned medium, as did the dominant-negative TCF-4 (Figure 3F). These results demonstrate that Ryk regulates the canonical TCF pathway by acting with Dishevelled protein. The interaction of Dishevelled and Ryk provides a link between Wnt and Dishevelled.

Generation of Ryk siRNA Mice

To address the roles of Ryk in in vivo neural development, transgenic mice expressing Ryk siRNA were generated by lentiviral infection of mouse one-cell-stage embryos. These embryos were transferred to pseudopregnant recipient mice, and those that came to term were examined further. Transgenesis was determined by fluorescent microscopy of mouse tails and FACS analysis of tail blood. Among 18 offspring, eight mice were GFP positive. Three of them were runted (Figure 4A, denoted by an asterisk). The copy number of each transgene was determined by Southern blotting (Figure 4A). Most transgenic lines had three to four copies of integrated lentiviral transgenes. The relative radiographic density of some transgene bands was weak, suggesting these transgenic lines might be mosaic. FACS analysis of tail blood further confirmed that the transgenic mice were mosaic, as less than 30% of white blood cells were GFP positive. Mice with multiple copies of the transgenes were mated to wild-type C57BL6 mice to segregate nonmosaic, single transgene-containing lines. Offspring were analyzed for Ryk RNA levels using real-time PCR (data not shown) in combination with Northern blotting. Northern blotting showed that the Ryk mRNA level in the brain from one line of Ryk siRNA mice was reduced 5- to 10-fold (Figure 4B).

Many Ryk siRNA transgenic mice died after birth, as has been observed with Ryk knockout mice (Halford et al., 2000). Some of the surviving mice were runted. These were about half the size of their control siblings on days 3 and 8 (Figure 4C). The difference in weight became less dramatic over time. These mice also displayed developmental defects, such as an unsteady gait.

Ryk siRNA Mice Have Defects in Axon Guidance

Drosophila derailed is involved in axon guidance as well as learning and memory (Callahan et al., 1995; Dura et al., 1995; Moreau-Fauvarque et al., 1998; Simon et al., 1998). Therefore, we also examined the role of Ryk in axon guidance using Ryk siRNA transgenic mice. Neurafilament staining of E10 embryos showed that the majority of axons projected correctly in Ryk siRNA mice, compared to wild-type mice (Figure 5A). However, glossopharyngeal nerves and vagus nerves prematurely connected (Figure 5B), and craniofacial motor neuron axons were less fasciculated in Ryk siRNA mice (Figure 5C). In the E10.5 embryos of Ryk siRNA mice, the ophthalmic axons, instead of projecting to the anterior, wandered posterior and were less fasciculated (Figure 5D). The projection and fasciculation of the DRG axons was normal.

Wnt-3a Induces Neurite Outgrowth in DRG Neurons

In *Drosophila*, the Ryk homolog Derailed is involved in the regulation of the guidance of anterior commissural axons (Bonkowsky et al., 1999; Yoshikawa et al., 2003). Moreover, Wnt has been shown to be involved in axon arborization in rats (Hall et al., 2000; Krylova et al., 2002; Lucas and Salinas, 1997). To establish a system for examining the role of Ryk in in vitro neuronal development, dorsal root ganglion (DRG) explants from rat E14 embryos were cocultured with a mouse L cell line overexpressing Wnt. Sixteen hours afterward, the explants were fixed and immunostained for synapsin expression, a marker for presynaptic terminals. Compared with the control cell line, Wnt-3a, Wnt-4, and Wnt-7b all induced a significantly greater expression of synapsin (Figure 6A).

Not only was the synapsin signal significantly increased in the Wnt-3a-treated DRG explants, neurite numbers were also visibly increased, as demonstrated by staining for GAP43 protein (data not shown), suggesting that Wnt-3a can induce neurite outgrowth. To further confirm the role of Wnt-3a in neurite outgrowth, DRG explants from E13 mouse embryos were harvested, placed in a collagen gel, and incubated in DMEM/F12 growth medium supplemented with concentrated Wnt-3a conditioned medium. The control explants were cultured in the same medium plus an addition of concentrated conditioned medium from normal L cells. Neurites were visualized using neurafilament antibody (2H3) after 24–48 hr of culture. The explants in the normal L cell-conditioned medium had few neurites, while the number and length of neurites in Wnt-3a-conditioned medium

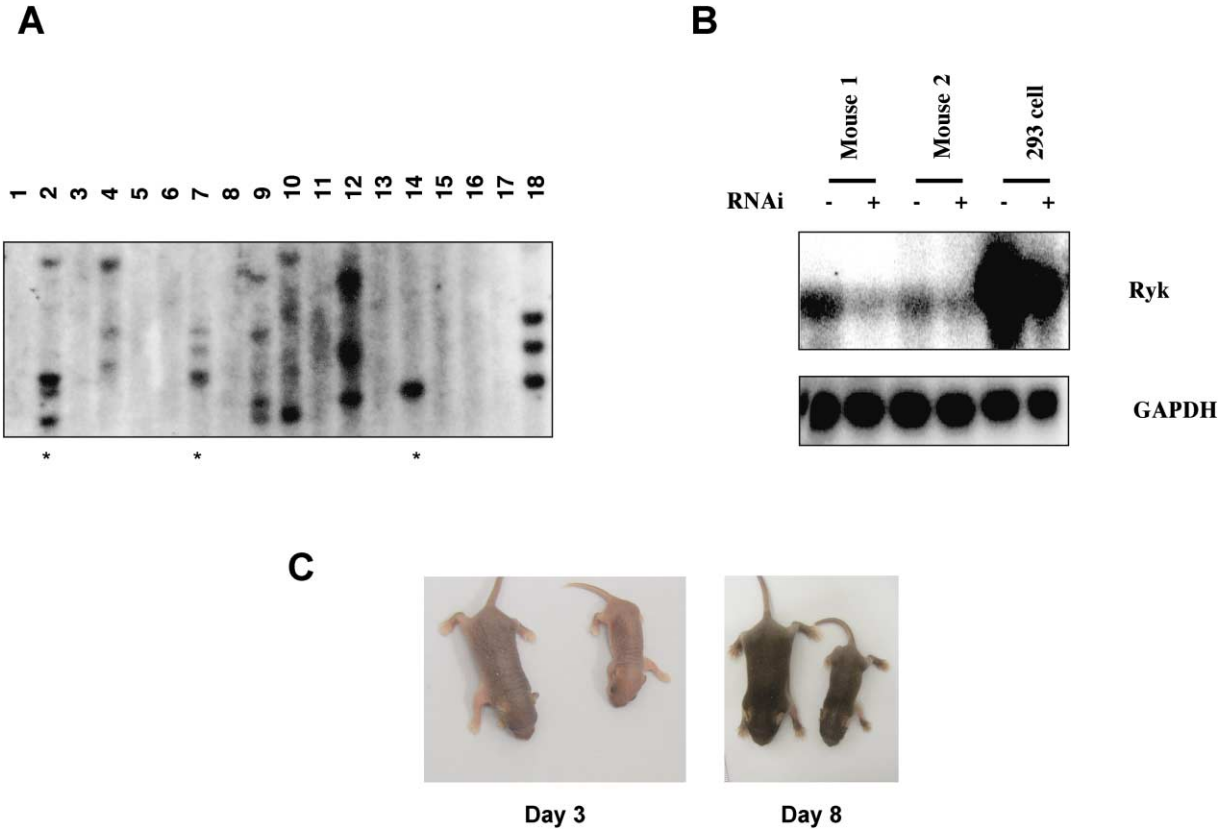

Figure 4. Generation of Ryk siRNA Mice

(A) Southern blot of genomic DNA of transgenic mice. The GFP-WRE fragment was used as a probe. Three lines of transgenic mice labeled "*" were runted after birth.

(B) Northern blot of total RNA from brains of F1 generation transgenic mice. A Ryk DNA fragment corresponding to 236-C was used as a probe. GAPDH was used as a control.

(C) Phenotype of Ryk siRNA transgenic mice.

was dramatically increased (Figures 6B and 6C). These results support the role of Wnt in inducing neurite outgrowth. The unconcentrated Wnt-3a-conditioned medium had a similar effect (Figures 6D and 6E).

It has been reported that Ryk is expressed in DRGs (Kamitori et al., 1999). However, the localization of Ryk in DRG neurons was not known. The anti-Ryk antiserum we generated in mice was not sufficient; therefore, to detect the localization of Ryk in DRG neurons, dissociated DRG neurons were infected with lentivirus expressing a Ryk/GFP fusion (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/1/97/DC1/). The expression of Ryk/GFP and GAP 43 was determined by immunohistochemistry. The overlay of Ryk/GFP and GAP 43 suggested that Ryk was localized not only in the cell body but also in growth cones, consistent with its roles in neurite outgrowth and axon guidance.

Ryk siRNA Mice Have Defects in Neurite Outgrowth in Response to Wnt-3a Induction

Although our results showed that Wnt-3a induced neurite outgrowth in DRG explants, the DRG axon outgrowth looked normal in Ryk siRNA mice (Figure 5A). The Ryk mRNA level in the DRG explants isolated from Ryk siRNA mice was inhibited to 14% of the wild-type control (data not shown). To assess whether the DRG neurons in Ryk siRNA mouse had defects in neurite outgrowth in response to Wnt stimulation, DRG explants were isolated from E13 embryos of both wild-type and Ryk siRNA mice and cultured in a collagen gel with DMEM/F12 supplemented with unconcentrated Wnt-3a-conditioned medium. While numerous neurites projected from the wild-type DRG explants, DRG explants from Ryk siRNA mice had fewer and shorter neurites emanating from them (Figure 7A). The number of neurites decreased 4-fold (Figure 7B), while the average length of neurites was reduced by 2-fold (Figure 7C). As a control, neurite outgrowth from wild-type and Ryk siRNA DRG was similar when the medium was supplemented with nerve growth factor (NGF) (Figures 7D and 7E), suggesting that Ryk is specifically involved in Wnt-induced neurite outgrowth. This may also explain why the neurite outgrowth of DRG is normal in Ryk siRNA mice, since NGF and other growth factors might be also involved in inducing neurite outgrowth in vivo. Based on the results that Ryk is required for the Wnt-3a-induced neurite outgrowth and the binding of Ryk and Wnt-3a, we conclude that Ryk is a biological receptor of Wnt in vivo.

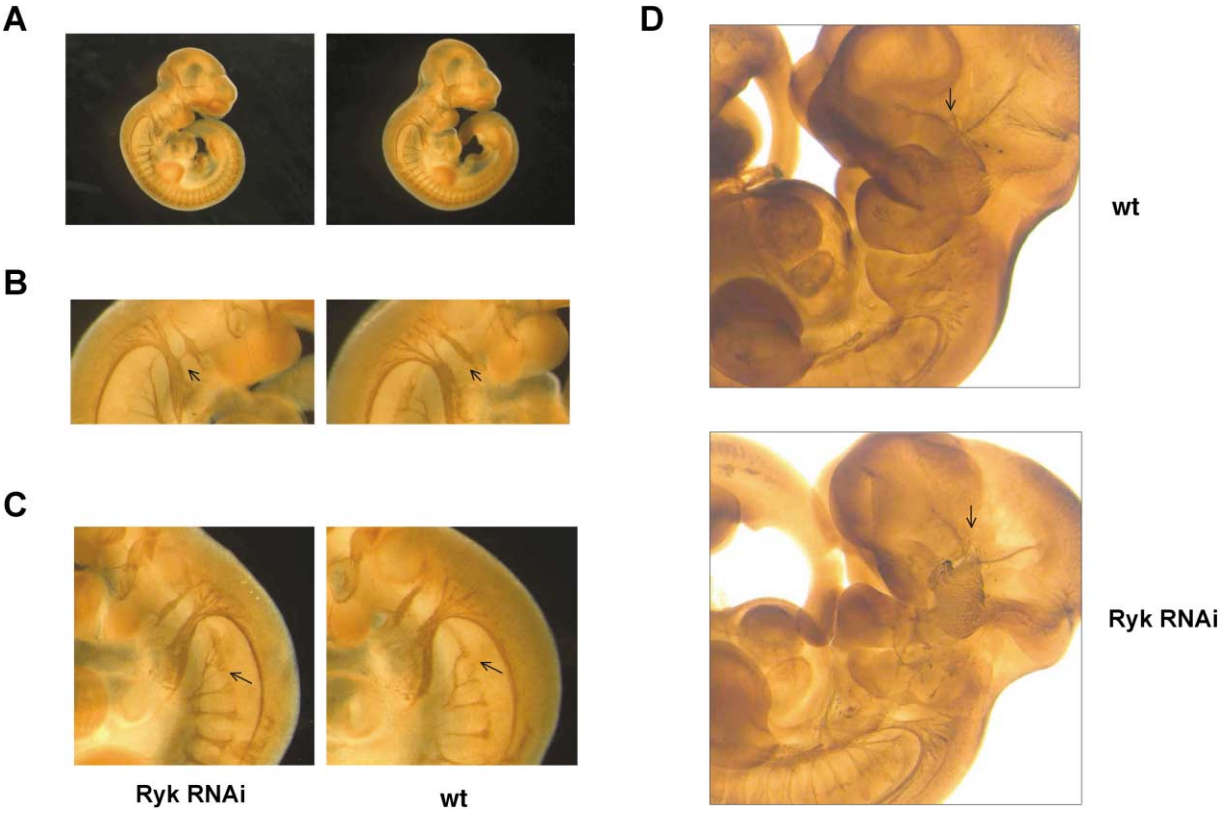

Figure 5. Comparison of Axonal Projection in Ryk siRNA Transgenic Mice and Wild-Type Mice

All figures are lateral views of mouse E10.5 embryos after whole-mount immunostaining of neurafilament (2H3). (A) Overall structure of axonal projections in Ryk siRNA mouse was similar to that of wild-type mouse. (B) Vagus nerves and glassopharyngeal nerves were prematurely connected in Ryk siRNA transgenic mice. (C) Craniofacial motor neuron axons in Ryk siRNA transgenic mice were less fasciculated. (D) Ophthalmic nerves in Ryk siRNA transgenic mice were less fasciculated, and some nerves projected posteriorly.

Discussion

We have presented evidence that implicates cell-surface Ryk as an important element of the Wnt-mediated signaling pathway. We show that Ryk binds to Wnt-1 and Wnt-3A through its extracellular WIF domain and likely forms a coreceptor with Frizzled by binding the CRD of Frizzled. Ryk is required for Wnt-1-mediated TCF activation and Wnt-3A-mediated neurite outgrowth, confirming that Ryk is a functional receptor for Wnt. Ryk binds Dishevelled, providing a link between Wnt and the downstream scaffold protein Dishevelled. Transgenic mice expressing Ryk siRNA exhibit defects in axon guidance, a phenotype also observed in Derailed mutant flies. This work provides strong evidence that Ryk is a crucial part of the Wnt signaling pathway.

Ryk Is a Functional Receptor for Wnt

Since Ryk siRNA blocks Wnt-1-induced TCF activation and Wnt-3a-induced neurite outgrowth, in certain settings, Ryk appears to be a required mediator of Wnt function. The requirement of Ryk for Wnt signaling during development might be context dependent. There are 19 Wnt genes and only one Ryk gene in mammals. Gene deletion experiments demonstrate that Wnt genes are required for a variety of developmental processes (Peifer and Polakis, 2000; Veeman et al., 2003; Wodarz and Nusse, 1998), while the Ryk gene is only involved in a few specific developmental procedures (Halford et al., 2000). This implies that Ryk is only involved in Wnt signaling in some specific cell types and maybe in response to specific Wnt ligands. The expression pattern of Ryk and the different binding affinity of Ryk with various Wnt ligands can contribute to the specificity of Ryk function.

Mammalian Ryk forms a complex with Wnt ligand and Frizzled. Ryk can bind Frizzled in a ligand-independent manner, suggesting that Ryk likely functions as a coreceptor with Frizzled. While there is only one Ryk gene in mammals, there are three Ryk homolog genes in *Drosophila*. They are Derailed (Drl), Doughnut (Dnt), and Derailed-2 (Drl-2). The molecular mechanism of each protein is different since Dnt can only partially rescue the muscle attachment defects in Drl mutant (Oates et al., 1998). Although Derailed functions independently of Frizzled for commissural axon guidance (Lyuksyutova et al., 2003), it will not be surprising if Dnt or Drl-2 interacts with Frizzled.

Ryk Provides a Link between Wnt and Dishevelled

This study demonstrates that Ryk associates with Dishevelled and that this association requires the PDZ binding motif of Ryk. It was suggested previously that

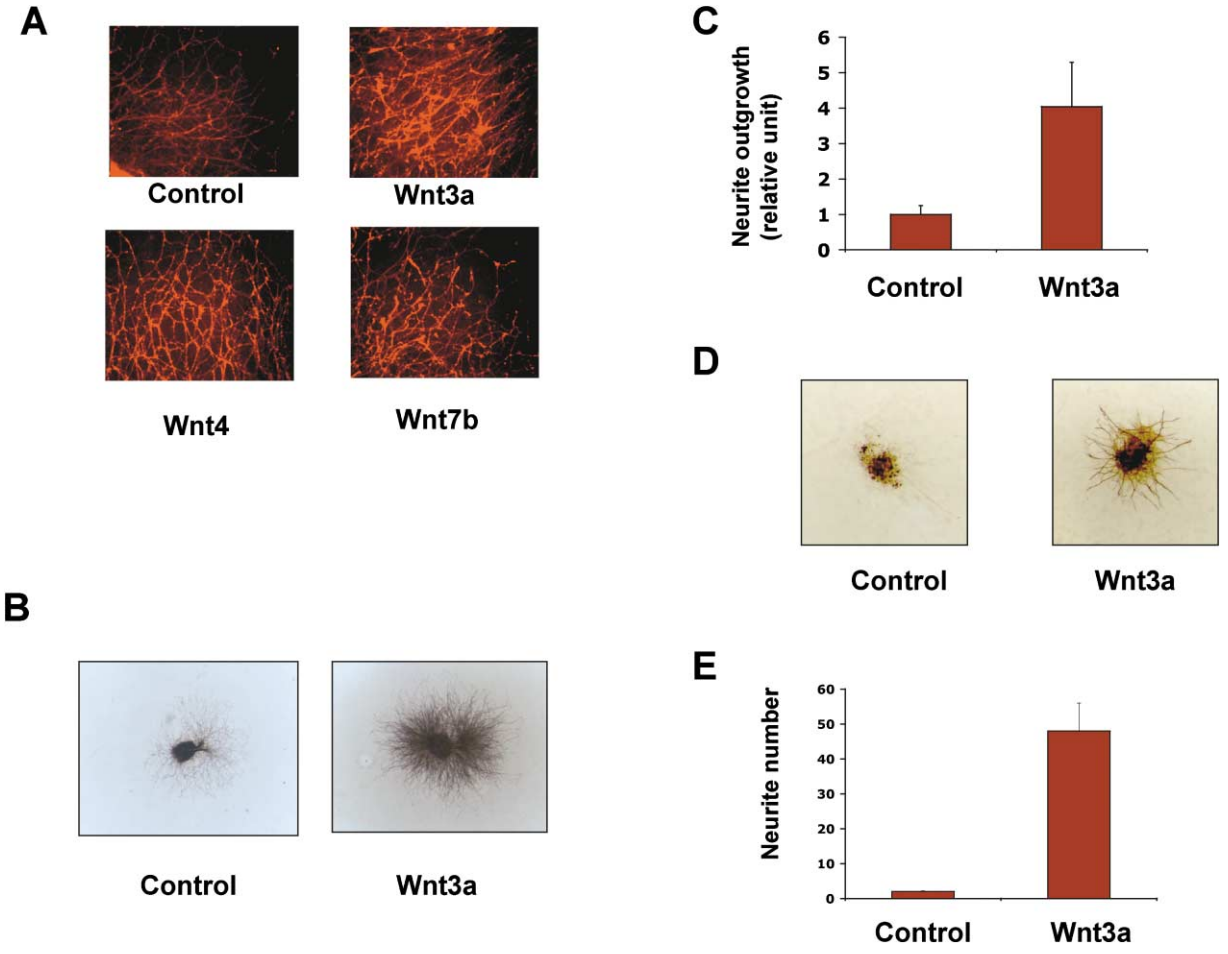

Figure 6. Wnt Induces Synapse Formation and Neurite Outgrowth in DRG Neurons

(A) Wnt-induced synapse formation. DRG explants from E14 rat embryos were harvested and cultured on the fibroblast cell line expressing Wnt as labeled. Presynaptic specification was demonstrated by immunostaining of synapsin.

(B) Wnt-3a-induced neurite outgrowth of DRG explants. DRG explants from E13 mice embryos were cultured in collagen gel in DMEM/F12 supplemented with concentrated Wnt-3a-conditioned medium. The control is the conditioned medium from L cells.

(C) Quantification of neurite outgrowth in (B).

(D) Same as (B), except unconcentrated conditioned medium was used.

(E) Quantification of neurite outgrowth in (D). The quantitation in (C) and (E) is different. Details are described in Experimental Procedures.

the PDZ domain of Dishevelled binds to a sequence of Frizzled at the C terminus (Wong et al., 2003). However, this interaction is relatively weak. The interaction of Ryk with Wnt extracellularly and Dishevelled intracellularly as reported here provides a link between Wnt and Dishevelled.

Ryk Is Required for TCF Activation

An RNAi directed at the Ryk gene in 293T cells inhibited the TCF activation induced by Wnt-1, suggesting that Ryk is required for the TCF pathway in this situation. While anti-Ryk siRNA blocks the activation of the TCF-luciferase reporter, overexpression of Ryk only modestly activates it, suggesting that endogenous Ryk levels might be near saturating level for activation of the TCF pathway. There are two pathways that regulate the TCF-driven target gene expression. One is through the accumulation and nuclear translocation of β-catenin, which then binds to TCF and changes TCF from a repressor to an activator (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). The second pathway is less well-characterized but is mediated by a nemo-like kinase (NLK), which inhibits the TCF transactivation (Ishitani et al., 1999, 2003; Smit et al., 2004). The fact that Dishevelled is required in TCF activation induced by Ryk and Wnt-3a clearly suggests that Ryk is involved in the canonical Wnt pathway leading to TCF activation.

Functions of Wnt and Ryk in Neurite Outgrowth and Axon Guidance

Ryk siRNA mice have defects in axon guidance of craniofacial motor nerves, ophthalmic nerves, and other nerves, suggesting an essential role of Ryk in axon guidance. Although there is no obvious deficiency in DRG neurite outgrowth in Ryk siRNA transgenic mice, DRG explants isolated from Ryk siRNA mice exhibit defects

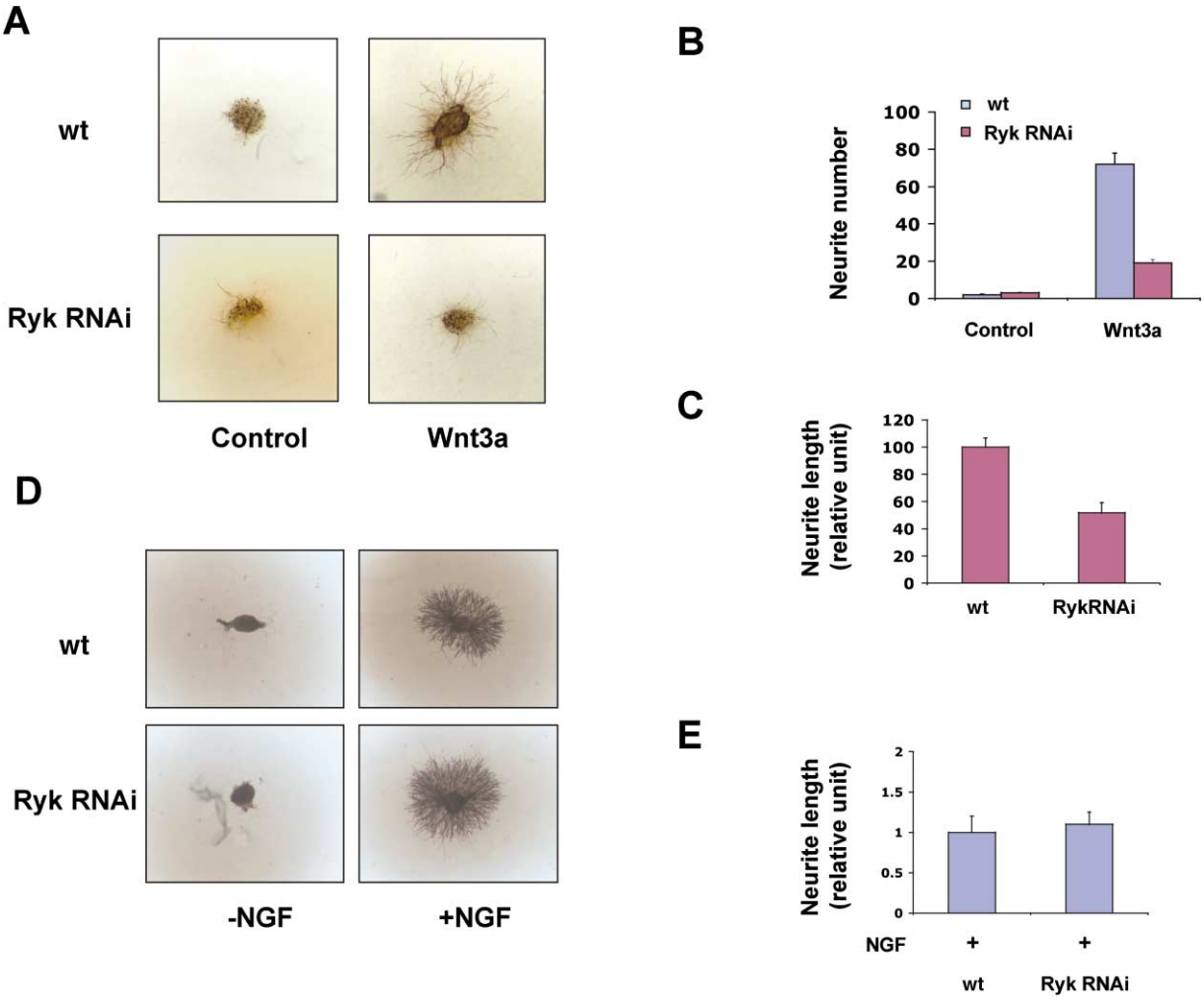

Figure 7. Ryk Is Required for Wnt-3a-Induced Neurite Outgrowth

(A) Wnt-3a-induced neurite outgrowth in DRG explants from wild-type and Ryk siRNA mice.

(B) Quantification of neurite number in (A).

(C) Quantification of neurite length in (A).

(D) Neurite outgrowth of wild-type and Ryk siRNA DRG in response to NGF.

(E) Quantification of neurite outgrowth in (D).

in neurite outgrowth in response to Wnt-3a stimulation. As mentioned before, the lack of deficiency in DRG neurite outgrowth in Ryk siRNA mice is probably because NGF and other growth factors are also involved in inducing neurite outgrowth in vivo. The fact that the Wnt-3a-induced neurite outgrowth of DRG explants is inhibited in Ryk siRNA mice provides strong evidence that there is a functional interaction between Wnt and Ryk in neurite outgrowth.

Taken together, our studies demonstrate that Ryk functions as a receptor for Wnt and is required for Wnt-1-induced TCF activation and Wnt-3a-induced neurite outgrowth. The interaction of Ryk with Dishevelled provides a link between Wnt ligand and downstream scaffold protein Dishevelled. Elucidation of more downstream components of Ryk will provide a detailed mechanism of Wnt/Ryk signal transduction.

Experimental Procedures

Transient Transfection, Coimmunoprecipitation, and Western Blotting

293T cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml of penicillin and streptomycin, and 2 mM glutamine in a 37°C incubator with 5% humidified CO_2. Twenty-four hours before transfection, 4 million 293T cells were seeded in 10 cm dishes. The cells were transfected with plasmid DNA using the calcium phosphate precipitation method. For Wnt/Ryk interaction, a total of 16 μg DNA was transfected, including 8 μg of HA-tagged Wnt-1 or Wnt-3A and 8 μg myc-tagged Ryk or its mutants. For interaction of Ryk and Dishevelled, 8 μg of EBG-Ryk 314-C or EBG-Ryk ΔPDZ was transfected with 8 μg of plasmid for Flag-tagged Dishevelled (ΔPDZ). Forty-eight hours posttransfection, cells were lysed in 1 ml ice-cold kinase lysis buffer (25 ml Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, and an appropriate amount of protease inhibitor mix [Roche]). Monoclonal antibodies or affinity-purified polyclonal antibodies (1 μg) were incu-

bated with 200 μl cell lysate for 2 hr at 4°C and precipitated with 10 μl protein G agarose (Pierce). GST-Ryk was pulled down directly by glutathione-agarose beads (Amersham Biosciences). The immunoprecipitates were washed extensively four to five times before SDS-PAGE analysis and immunoblotting. The primary antibodies used were anti-HA (1:200, Santa Cruz), anti-myc (1:200, Santa Cruz), and anti-Flag (1:2000, Sigma). Anti-Dishevelled antibodies are a mixture of Dishevelled-1, -2, and -3 from Santa Cruz. Mouse polyclonal antiserum was generated using GST fusion protein of Ryk amino acid 236 to the C terminus. The secondary antibodies were HRP-conjugated goat anti-mouse and goat anti-rabbit (1:10,000, Pierce).

Design of siRNA Constructs in pSUPER and Lentiviral Vectors and Preparation of Lentivirus

Ryk siRNAs were designed and cloned into pSUPER as described (Brummelkamp et al., 2002). SiRNAi-1 targets human Ryk 341–360. The two oligos used are GATCCCC**GTCCAGGTTGAATATAAG**TTCAAGAGA**CTTATATTCAACCTTGGAC**TTTTTGGAAA and AGCTTTTCCAAAAAA**GTCCAAGGTTGAATATAAG**TCTCTTGAA**CTTATATTCAACCTTGGAC**GGG. SiRNA-2 targets human Ryk1659–1678. The sequences of the two oligos are GATCCCC**GATGGTTACCGAATAGCCC**TTCAAGAGA**GGGCTATTCGGTAACCATC**TTTTTGGAAA and AGCTTTTCCAAAAAA**GATGGTTACCGAATAGCCC**TCTCTTGAA**GGGCTATTCGGTAACCATC**GGG. The siRNA oligos targeting Dishevelled-2 are GATCCCC**CATGGAGAAGTACAACTTC**TTCAAGAGA**GAAGTTGTACTTCTCCATG**TTTTTGGAAA and AGCTTTTCCAAAAAA**CATGGAGAAGTACAACTTC**TCTCTTGAA**GAAGTTGTACTTCTCCATG**GGG. The siRNA oligos targeting Dishevelled-3 are GATCCCC**GTTCTTCTTCAAGTCTATG**TTCAAGAGA**CATAGACTTGAAGAAGAAC**TTTTTGGAA and AGCTTTTCCAAAAA**GTTCTTCTTCAAGTCTATG**TCTCTTGAA**CATAGACTTGAAGAAGAAC**GGG. In bold are regions identical to both human and mouse Ryk genes. Therefore, these siRNAs can be used in both human and mouse cells to target endogenous Ryk mRNA. Each pair of oligos was annealed at 20 μM in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), 2 mM magnesium acetate) at 95°C for 4 min, followed by incubation at 70°C for 10 min and slow cooling to room temperature. Forty picomoles of annealed oligos were phosphorylated by T4 polynucleotide kinase before they were ligated into pSUPER vector digested by BglII and HindIII. To put siRNA constructs into lentiviral vectors, siRNA together with human H1 promoter was digested with SmaI and HincII and ligated into pFUGW digested with PacI, followed by blunting using T4 DNA polymerase. The orientations of the fragments were confirmed by ClaI and EcoRI digestion. Lentivirus-expressing siRNA were generated using retroviral vectors and a previously described packaging system (Lois et al., 2002). Concentrated lentivirus was titered using 293T cells to test GFP expression.

Real-Time PCR

RNAs from 293T cells, Ryk siRNA cells, and mouse brains were extracted using trireagents (Molecular Research Center). First-strand cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems). The final concentration of the reaction was 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM of dNTPs, 2.5 μM of Random Hexamer, 0.4 u/μl of RNase inhibitor, 1.25 u/μl of Multoscribe Reverse Transcriptase, and 10–100 ng of total RNA. The thermal cycling parameter of the RT reaction was 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The real-time PCR was performed using the ABI 5700 Real-Time PCR Instrument. The reaction included 1× SYBR Green PCR Master Mix (Applied Biosystems), forward and reverse primer (0.5 μM each), and the appropriate amount of cDNA. The thermal cycling parameter was 50°C for 2 min; 95°C for 10 min; and 40 cycles of melting, annealing, and extension. The melting condition was 95°C for 15 s, and annealing and extension was at 60°C for 1 min. Results are analyzed according to the manufacturer's instructions.

The primers for real-time PCR were designed using Primer Express 1.5 software. The primers were designed to be around 100 bp with a Tm of 58°C–60°C. Oligos used for amplification of human Ryk genes were the following: hryk-F2, AGGTGACAATGATGCT CACTGAA; hryk-R2, TGTGATGAAGACCTCGCAGCT; hryk-F3, CAG GTGACAATGATGCTCACTGA; and hryk-R3, GTGATGAAGACCTC GCAGCTTA. Oligos used for human GAPDH were the following: GAPDH-F, GGTGGTCTCCTCTGACTTCAACA; and GAPDH-R, GCG TCAAAGGTGGAGGAGTG.

Northern Blot

The Northern blotting was performed as described (Tanaka et al., 1997). Radioactive-labeled antisense probes were used for hybridization. Antisense RNA probes were synthesized from pBS KSII Ryk236-C and pTri-GAPDG-mouse (Ambion) using T7 RNA polymerase (Promega).

Luciferase Reporter Assay

293T cells were plated at 10^5 cells per well in 24-well dishes 24 hr before transfection. The plasmids used in the transfection included 2 ng of pCSK-lacZ, 20 ng of TOPFLASH TCF-luc (a gift from Bert Vogelstein), and 350 ng of other DNA. FOPFLASH, which has a mutation in the TCF binding site, was used as a control in some cases. The medium was changed 24 hr following transfection. Post-transfection (48 hr), the cells were lysed in 100 μl of reporter lysis buffer (Promega). Cells were collected and spun at 13,000 rpm for 5 min. Twenty microliters of supernatant was used to measure luciferase activity using the luciferase assay system (Promega) and a luminometer (Optocomp I, MGM Instruments). Thirty microliters of supernatant were used to measure the β-galactosidase (β-gal) activity using the chemiluminescent β-gal reporter gene assay (Roche) according to the manufacturer's instructions. β-gal activity was used to normalize the amount of cell lysate.

Generation of Ryk siRNA Transgenic Mice using Lentivirus

Transgenic mice expressing Ryk siRNA were generated as described (Lois et al., 2002). Approximately 10–100 pl of concentrated virus at 10^6 IU/μl were injected into the perivitelline space of single-cell mouse embryos. Around 40 embryos were implanted into two-timed pseudopregnant female mice and carried to term. Genomic DNA from tails of transgenic mice was subjected to Southern blotting using a GFP-WRE DNA fragment as a probe. The mice were also tested for GFP expression by fluorescent microscopy of their tails. Transgenic mice were crossed with C57BL6, and mice carrying single-copy transgenes were selected for experiments.

DRG Explants Collagen Gel Assay

E13 embryos from both wild-type and Ryk siRNA mice were collected and washed with ice-cold PBS. Dorsal root ganglia were isolated and incubated in L15 medium on ice. Ten microliters of 10× DMEM/F12 was mixed with 90 μl of collagen gel (BD Biosciences) and put on ice. Ten microliters of collagen gel mix was put on the surface of a small culture dish and placed at room temperature until the gel solidified. DRG explants were placed on top of this surface, and another 20 μl of gel mix was added and incubated at 37°C for 10 min. DMEM/F12 medium (2 ml) supplemented with Wnt-3a-conditioned medium or control-conditioned medium was added. The explants were cultured between 24 and 72 hr before they were fixed for immunostaining.

Neurite outgrowth induced by concentrated Wnt-3a or NGF is semiquantified using ImageQuant software. Briefly, the picture of neurite was first converted to grayscale. The background and explant's core signal were subtracted so that only signals for neurite would be quantified and compared. With unconcentrated Wnt-3a, when the neurite number is low, the length of neurite is compared in arbitrary units.

Whole-Mount Immunostaining of Mouse Embryos and DRG Explants in Collagen Gel

The mouse embryos and DRG explants from the appropriate stage were fixed in 4% paraformaldhyde overnight. The tissues were then washed with PBS twice and dehydrated serially in 25%, 50%, 75%, and 100% methanol and stored in methanol overnight or longer. Prior to antibody staining, tissues were first treated with 0.3% H_2O_2 for 0.5 hr followed by rehydration serially with 75%, 50%, and 25% methanol, PBS, and PBT (PBS + 1% Tween) twice for 5 min each. The tissues were blocked in PBS with 10% heat-inactivated goat serum for 2 hr. Primary antibody 2H3 (hybridoma cell bank) at 1:50 dilution in PBS was added for incubation overnight followed by six washes, 30 min each, with PBS/2% goat serum. The HRP-conju-

gated goat anti-mouse IgG1 antibody (Southland Biotechnology, 1:300) was incubated with the tissues for 2 hr followed by six washes of PBS/2% goat heat-inactivated serum for 30 min each. The color reaction was developed using DAB staining.

Acknowledgments

We are especially grateful to Thea Lu for her technical assistance. We are in debt to Xi He, Raymond Habas, Martin Garcia Castro, Leonard Zon and Bert Vogelstein for reagents, to Susan Ou for generation of the mouse anti-Ryk serum, to Shirley Pease, Jennifer Alex and Bruce Kennedy for generation and maintenance of transgenic mice. We thank Kai Zinn, Chunming Liu, Raad Nashmi, Qiang Lu, Yosuke Mukoyama, Michiline Laurant and members of the Baltimore lab for reading this manuscript. This work was supported by NIH grant 2R01CA51462-13 (to D.B.) and Baxter Foundation (to W.L.).

Received: April 1, 2004
Revised: September 16, 2004
Accepted: September 16, 2004
Published: September 30, 2004

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