RECK in Neural Precursor Cells Plays a Critical Role in Mouse Forebrain Angiogenesis

HIGHLIGHTS

- Mice lacking RECK in Foxg1-positive neural precursor cells die shortly after birth.
- These mice show vascular defects similar to those in mice lacking endothelial RECK.
- The vascular phenotype can be suppressed by LiCl, an activator of WNT signaling.
- RECK in WNT7-producing cell enhances contact-dependent WNT signaling in adjacent cells.

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RECK in Neural Precursor Cells Plays a Critical Role in Mouse Forebrain Angiogenesis

Huiping Li, Takao Miki, Glicia Maria de Almeida, Carina Hanashima, Tomoko Matsuzaki, Calvin J. Kuo, Naoki Watanabe, and Makoto Noda

SUMMARY
RECK in neural precursor cells (NPCs) was previously found to support Notch-dependent neurogenesis in mice. On the other hand, recent studies implicate RECK in endothelial cells (ECs) in WNT7-triggered canonical WNT signaling essential for brain angiogenesis. Here we report that RECK in NPCs is also critical for brain angiogenesis. When Reck is inactivated in Foxg1-positive NPCs, mice die shortly after birth with hemorrhage in the forebrain, with angiogenic sprouts stalling at the periphery and forming abnormal aggregates reminiscent of those in EC-selective Reck knockout mice and Wnt7a/b-deficient mice. The hemorrhage can be pharmacologically suppressed by lithium chloride. An effect of RECK in WNT7-producing cells to enhance canonical WNT-signaling in reporter cells is detectable in mixed culture but not with conditioned medium. Our findings suggest that NPC-expressed RECK has a non-cell-autonomous function to promote forebrain angiogenesis through contact-dependent enhancement of WNT signaling in ECs, implying possible involvement of RECK in neurovascular coupling.

INTRODUCTION
Precise bidirectional communications between the nervous and vascular systems (i.e., neurovascular coupling) is essential for proper formation and functioning of the central nervous system (CNS) in vertebrates (Paredes et al., 2018). Several neuroepithelium-derived molecules crucial for CNS-specific angiogenesis have been discovered, which include ID1/ID3 (Lyden et al., 1999), integrin alpha-V (Bader et al., 1998; McCarty et al., 2002, 2005), integrin beta-8 (Zhu et al., 2002; Proctor et al., 2005), WNT7A/B (Stenman et al., 2008; Daneman et al., 2009), and TGFBR2 (Hellbach et al., 2014). However, it remains to be elucidated exactly how these molecules contribute to the neurovascular communication.

RECK (reversion-inducing cysteine-rich protein with Kazal motifs) encodes a glycosylphosphatidylinositol-anchored glycoprotein capable of regulating several extracellular metalloproteinases (Takahashi et al., 1998). Global Reck knockout mice die around embryonic day 10.5 (E10.5) with reduced tissue integrity, abdominal hemorrhage (Oh et al., 2001), and precocious neuronal differentiation (Muraguchi et al., 2007). Around E10.5, normal mice express RECK abundantly in blood vessels (both endothelial cells [ECs] and mural cells) as well as neural precursor cells (NPCs) (Oh et al., 2001; Muraguchi et al., 2007; Chandana et al., 2010). To explore the functions of RECK in mice beyond E10.5, we generated Reck-flox mice (Chandana et al., 2010; Yamamoto et al., 2012). Our earlier study using temporally inducible Reck knockout mice revealed that inactivation of Reck around E11 results in vascular defects including forebrain hemorrhage and vascular malformation by E15.5 and embryonic death before birth (Chandana et al., 2010). The roles of RECK in different cell types, however, could not be discriminated in such system. A more recent study using cell type-selective Reck knockout mice revealed that Reck inactivation in mural cells recapitulates the E10.5 lethality of global knockout mice, whereas Reck inactivation in ECs results in perinatal death with brain hemorrhage (Almeida et al., 2015), further highlighting the importance of RECK in vascular development.

Recent studies also indicate that RECK binds and cooperates with GPR124, an orphan G-protein-coupled receptor, to facilitate the canonical WNT signaling in ECs triggered by WNT7A/B that is required for proper tip cell function, CNS angiogenesis, and blood-brain barrier maturation (Vanhollebeke et al., 2015; Ulrich et al., 2016; Cho et al., 2017; Vallon et al., 2018). Interestingly, RECK was found to directly...
bind WNT7A/B and confer ligand specificity to the FZD4-LRP5/6 receptor complex (Eubelen et al., 2018; Vallon et al., 2018).

As our earlier study using global Reck knockout mice implicated RECK in CNS development (Muraguchi et al., 2007), we attempted to confirm and extend that finding by inactivating Reck selectively in the Foxg1-positive NPCs in mice, expecting to find some neural deficiency. Characterization of these mice, however, revealed an unexpected role for NPC-expressed RECK in CNS angiogenesis.

RESULTS

Reck Knockout in Foxg1-Positive NPCs Results in Neonatal Death with Unexpected Phenotype of Forebrain Hemorrhage

To selectively inactivate Reck in NPCs, we chose to use a Foxg1-Cre transgenic line (Hebert and McConnell, 2000). When visualized with the mTmG reporter system (Muzumdar et al., 2007), Foxg1-Cre-expressed cells (i.e., green cells in mTmG;Foxg1-Cre mice) are abundant in telencephalon at E8.5 and persist in a large area of the forebrain from E9.5 onward (Figure 1A; green signals). We generated mice carrying this Foxg1-Cre allele and one or two Reck-flox allele(s). The Reck-flox heterozygotes (Reck\text{flox}/+; Foxg1-Cre) are normal in gross morphology, fertile, and hence used as a control in this study. On the other hand, the Reck-flox homozygotes (Reck\text{flox}/flox; Foxg1-Cre), which we call Reck-cKO (Foxg1), are not found among the adult littermates obtained from the mating supposed to yield such offspring at the 25% frequency (i.e., Reck\text{flox}/+; Foxg1-Cre x Reck\text{flox}/flox). Careful examination of newborn pups revealed that Reck-cKO (Foxg1) mice are viable up to the day of birth (P0) but die shortly after birth exhibiting forebrain hemorrhage (Figure 1B). When traced back, visible forebrain hemorrhage occurred in 74% (29/39) of Reck-cKO (Foxg1) mice at E12.5 (Figure 1C, red diamond) and in all Reck-cKO (Foxg1) mice at E13.5 (Figure 1D, arrow) as well as at later embryonic time points (Figure 1C, E13.5–E18.5). Histological examinations indicate that hemorrhage mainly occurs in ganglionic eminence (GE) at early stages (Figure 1E-1, arrows) but becomes prominent in cerebral cortex (Cx) at later stages (Figure 1E-3, arrows). Immunofluorescence staining of brain sections of mTmG;Foxg1-Cre reporter mice, as shown in Figure 1A, indicates that Foxg1-Cre-expressed cells are neither CD31 positive (vascular ECs) nor NG2 positive (vascular mural cells) (Figure 1F). A previous study by Hellbach et al. also indicated that Foxg1-Cre is expressed in neuronal cells, but not in vascular cells, in the forebrain in this transgenic line (Hellbach et al., 2014). These data support the idea that the phenotype of Reck-cKO (Foxg1) mice results from the lack of RECK in NPCs rather than vascular cells.

Reck-cKO (Foxg1) Embryos Exhibit Vascular Malformations

CD31 is known to be expressed in ECs and some blood cells (Privratsky et al., 2010). When forebrain sections from E12.5 embryos were stained with anti-CD31, a line of regularly spaced small loops (representing cross sections of blood vessels) was found near the ventricular edge of both GE and Cx in control mice (Figures 2B and 2C, arrows). In Reck-cKO (Foxg1) mice, however, abnormal aggregates of CD31-positive cells or loops are found in GE near the perineural vascular plexus or midway toward the ventricle (Figure 2E, arrowheads); these abnormal vessels are proliferative (Figure S1A) and reminiscent of the glomeruloid malformations found in Wnt7A/Wnt7b double-knockout mice (Stenman et al., 2008; Daneman et al., 2009) and Gpr124 knockout mice (Kuhnert et al., 2010). On the other hand, very few vessels were found in the cortex of Reck-cKO (Foxg1) mice (Figure 2F).

Less severe but similar vascular abnormalities were found in cKO (Foxg1) mice at E11.5 (Figures 2L–2P). In GE, CD31-positive (red) cells form multilayered tubes with wide opening that are associated with NG2-positive (green) cells (Figure 2O, arrowheads).

At E13.5, Reck-cKO (Foxg1) mice show more advanced vascular abnormalities, including the lack of a line of regularly spaced periventricular vessels (Figures 3G and 3H; see arrows in Figures 3C and 3D); large aggregates of CD31-positive, proliferative cells midway toward the ventricle (Figure 3G, arrowheads; Figures S1B–S1D); and a central region of apparent tissue damage (Figure 3G, asterisk) in GE. In the cortex, some vessels consisting of multilayered CD31-positive cells with round luminal space are found in the cortex (Figure 3H, arrowheads).

Taken together, these findings indicate that Reck expressed in NPCs is critical for proper angiogenesis in the forebrain, especially in GE and Cx.
Reck-cKO (Foxg1) Embryos Exhibit Precocious Neuronal Differentiation

The vascular abnormalities in the forebrains of Reck-cKO (Foxg1) mice are accompanied by the increased number and widened zone of TUJ1-positive differentiated neurons (Figure 2Q, compare green signals in panels 1 and 2) and the reduced number and narrower zone of Ki67-positive proliferative cells in both Cx and GE (Figure S2). Hence, precocious neuronal differentiation, a phenotype previously found in global Reck knockout mice (Muraguchi et al., 2007), is recapitulated in Reck-cKO (Foxg1) mice, suggesting that RECK produced by NPCs affects both neurogenesis and angiogenesis.
Deficiency in NPC and Reck Deficiency in EC Lead to Similar Vascular Phenotype

For comparison, we generated EC-selective conditional knockout mice using a Tie2-Cre transgenic line (Kisanuki et al., 2001). The Reck<sup>flex1/flex1</sup>;Tie2-Cre mice (Reck-cKO [Tie2] mice in short) show phenotypes

Figure 2. Vascular and Neuronal Phenotypes of Mice Lacking Reck Expression in NPCs

(A–F) Vascular phenotype of Reck-cKO (Foxg1) mouse at E12.5. A coronal section of the brain from control (A–C) or Reck-cKO (Foxg1) mouse (D–F) at E12.5 was stained with anti-CD31 antibodies (red) followed by nuclear counterstain (blue). Magnified views of the two areas (dotted-line box) in (A and D) are shown in (B) GE and (C) cortex and (E) GE and (F) cortex, respectively. Note that typical glomeruloid malformations are found in the GE of Reck-cKO (Foxg1) mouse (arrowheads in E) and that a line of regularly spaced small vessels along the ventricular edge in the control mouse (arrows in B and C) are absent in Reck-cKO (Foxg1) mouse (E and F).

(G–P) Vascular phenotype of Reck-cKO (Foxg1) mouse at E11.5. A coronal section of the brain from control (G–K) or Reck-cKO (Foxg1) mouse (L–P) at E11.5 was double stained with antibodies against CD31 (red) and NG2 (green) followed by nuclear counterstain (blue). Single-color images for CD31 (G and L) and NG2 (H and M) as well as three-color images (I–K and N–P) are shown. Magnified views of the two areas (dotted-line box) in (I and N) are shown in (J) GE and (K) cortex and (O) GE and P cortex, respectively. Note the abnormal vessels in GE (O) and that a line of regularly spaced small vessels, as found in the control mouse (arrows in J and K), is absent in Reck-cKO (Foxg1) mouse (O and P).

(Q) Neural phenotype of Reck-cKO (Foxg1) mouse at E12.5. A coronal section of the brain from control mouse (panel 1) or Reck-cKO (Foxg1) mouse (panel 2) at E12.5 was stained with anti-TUJ1 antibodies (green) followed by nuclear counterstain (blue). Note the expanded TUJ1-positive area in the Reck-cKO (Foxg1) mouse brain (panel 2). Scale bars, 200 μm.

See also Figures S1–S3.

Reck Deficiency in NPC and Reck Deficiency in EC Lead to Similar Vascular Phenotype

For comparison, we generated EC-selective conditional knockout mice using a Tie2-Cre transgenic line (Kisanuki et al., 2001). The Reck<sup>flex1/flex1</sup>;Tie2-Cre mice (Reck-cKO [Tie2] mice in short) show phenotypes
reminiscent of those of Reck-cKO (Foxg1) mice. For instance, at E13.5, the lack of a line of regularly spaced periventricular vessels in both the GE and cortex (Figures 3O and 3P; see arrows in Figures 3Ka and 3L), large aggregates of CD31-positive cells (Figure 3O, arrowheads), and a central tissue damage (Figure 3O, asterisk) in GE are evident. On the other hand, misexpression of TUJ1 is not observed in Reck-cKO (Tie2) mice (Figure S3), suggesting distinct effects of Reck in ECs and NPCs (see Figures 2Q and S2) on neuronal differentiation. Nevertheless, our data indicate that Reck deficiency in ECs and Reck deficiency in NPCs give rise to a vascular phenotype very similar both in timing and locations in the forebrain.

Vascular Phenotype of Reck-cKO (Foxg1) Mice Can Be Partially Rescued by Pharmacological Treatment with LiCl

Importance of the WNT7a/WNT7b-dependent canonical WNT signaling in brain angiogenesis (Stenman et al., 2008; Daneman et al., 2009) and the EC-autonomous function of RECK to specifically enhance such signaling (Vanhollebeke et al., 2015; Ulrich et al., 2016; Cho et al., 2017; Eubelen et al., 2018; Vallon et al., 2018) has been demonstrated. However, because RECK is a membrane-anchored protein, it is not obvious how the RECK expressed in NPCs can also affect angiogenesis. To address this question, we first tested whether canonical WNT signaling is affected in Reck-cKO (Foxg1) mice by daily administration of lithium chloride (LiCl) into the pregnant mice followed by morphological examination of Reck-cKO (Foxg1) embryos. LiCl is known to inhibit glycogen synthase kinase-3β thereby bypassing the

Figure 3. Effects of Reck Mutations in NPCs and ECs

(A–H) (A–E) Vascular phenotype of Reck-cKO (Foxg1) mice at E13.5. Serial coronal sections of the brain from control (A–D) or Reck-cKO (Foxg1) mouse (E–H) at E13.5 were prepared, and one section was H&E stained (panels A and E) and the next section was fluorescently stained with anti-CD31 antibodies (red) followed by nuclear counterstain (blue) (B–D and F–H). Magnified views of the two areas (dotted-line box) in (B and F) are shown in (C) GE and (D) cortex and (G) GE and (H) cortex, respectively. Note that large glomeruloid malformations are found in the GE of Reck-cKO (Foxg1) mouse (arrowheads in G) and that a line of regularly spaced small vessels along the ventricular edge in the control mouse (arrows in C and D) is absent in Reck-cKO (Foxg1) mouse (G and E). Instead, a few larger and irregularly located vessels are found in the cortex of mutant mice (arrowheads, H).

(I–P) Vascular phenotype of Reck-cKO (Tie2) mice at E13.5. Serial coronal sections of the brain from control (I–L) or Reck-cKO (Tie2) mouse (M–P) at E13.5 were prepared, stained, and presented as described for (A–H). Note the phenotype very similar to that of Reck-cKO (Foxg1) mice, including large glomeruloid malformations in GE (arrowheads in O) and the absence of regularly spaced small vessels along the ventricular edge (compare O and P with K and L). Scale bars, 200 μm. See also Figures S1 and S3.
ligand-receptor interactions and directly activating downstream cascade of canonical WNT signaling (Klein and Melton, 1996); the agent has been used to demonstrate the involvement of canonical WNT signaling during various developmental processes in mouse embryos (Cohen et al., 2007, 2009; Kugimiya et al., 2007; Tian et al., 2010; Griffin et al., 2011; Curtis and Griffin, 2012; Cornett et al., 2013; Cambier et al., 2014; Briggs et al., 2015; Da Silva et al., 2017).

First, untreated Reck-cKO (Foxg1) mice at E15.5 show hemorrhage in both GE and the cortex (Cx) at 100% penetrance (Figure 4A; Table 1, a; Figure S4). In contrast, daily administration of LiCl from E8.5 to E14.5 suppressed macroscopic hemorrhage completely in the cortex and partially in GE (Table 1, b). We therefore compared the effects of LiCl injected at different timings. The results indicate that the treatments from E10.5 to E14.5 (Table 1, d) are essential for this suppression. Histological examinations of the LiCl-treated mice indicated that small hemorrhagic legions were found in GE in some of the animals but not in their cortices (Figures 4B and 4C); this was in sharp contrast to the numerous large hemorrhagic legions found

Figure 4. Effects of LiCl on the Vascular Phenotype of Reck-cKO (Foxg1) Mice
(A) Vascular phenotype of Reck-cKO (Foxg1) mice at E15.5 (untreated control). Panel 1: Lateral view of an intact, typical mutant embryo exhibiting cerebral hemorrhage (red arrow). Panel 2: an H&E-stained coronal section with numerous hemorrhagic legions (black arrows). Panel 3: magnified view of the cortical area shown by dotted-line box in panel 2. Panel 4: the next section subjected to immunofluorescence staining with anti-CD31 antibodies (red, vascular endothelial cells and some blood cells) followed by nuclear counterstain (blue). Panel 5: magnified view of a cortical area indicated by dotted-line box in panel 4.
(B and C) Two Reck-cKO (Foxg1) embryos harvested at E15.5 from pregnant mice pretreated with daily injection of LiCl from E10.5 to E14.5. Images in panels 1–5 were similarly acquired as those in (A). Note that hemorrhagic legions are greatly reduced in number and size in one case (C) and almost absent in the other case (B).
(D) Control embryos harvested at E15.5 from pregnant mice pretreated with daily injection of LiCl from E10.5 to E14.5 (LiCl-treated control mouse). Scale bars, 200 μm.
See also Figure S4.
in both the GE and the cortex of untreated animals that resulted in the leakage of blood into the ventricles (Figure 4A). These findings support the idea that RECK in NPCs influences canonical WNT signaling.

Altered Gene Expression Is Found in the Embryonic Forebrain of Reck-cKO (Foxg1) Mouse

To gain some insights into the possible mechanism by which RECK in NPCs may affect canonical WNT signaling and vascular development in the forebrain, we determined the levels of mRNAs encoding some molecules relevant to brain development, angiogenesis, and WNT signaling using total RNA extracted from forebrain of embryos at three time points (E11.5, E12.5, and E13.5) by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (Figure 5A). At the first time point (E11.5; blue symbols), significant downregulation of an NPC marker, Nes, was detected (Figure 5A, group 23). At the second time point (E12.5; red symbols), upregulation of Mmp2 (protease), Id3 (transcription factor), and downregulation of Wnt7a, Itg8b (cell adhesion receptor), and two endogenous targets of canonical WNT signaling, Apcdd1 and Sox17, became significant (Figure 5A, groups 3, 6, 12, 38, and 41). At the third time point (E13.5; green symbols), significant upregulation of Mmp2, Vegfa (vascular endothelial growth factor A), downregulation of Wnt7a, Wnt7b, Apcdd1, and Sox17 were noted (Figure 5A, groups 4, 7, 10, 22, 39, and 42). Downregulation of Apcdd1 and Sox17 provides additional evidence suggesting that canonical WNT signaling is affected by the Reck deficiency. Some of the other alterations in gene expression may directly or indirectly contribute to the mutant phenotype (see Discussion).

RECK in NPCs or Ligand-Producing Cells Can Enhance Contact-Dependent Activation of Canonical WNT Signaling in Reporter Cells

Neuroepithelium-derived WNT7A/B (leading to canonical Wnt signaling in CNS ECs) is known to be specifically required for CNS vascularization (Stenman et al., 2008; Daneman et al., 2009). To test whether RECK in NPCs may enhance canonical WNT signaling in a non-cell-autonomous fashion, we prepared NPCs from Reck-cKO (Foxg1) mice and control mice at E11.5. These NPCs (Figures 5B and 5C, groups 1 and 2; Figure S5, group 11) or their conditioned media (Figure 5C, groups 3 and 4) were overlaid onto the TOP-Flash reporter cells expressing FZD4, LRP5, GPR124, and RECK, and the cultures were incubated for 24 h followed by luciferase assay to detect canonical WNT signaling. NPCs stimulated WNT signaling when mixed with the reporter cells (Figure S5, groups 9 versus 11; Figures 5B and 5C, group 1), but their conditioned media failed to do so (Figure S5C, group 3), indicating contact-dependent stimulation. Furthermore, NPCs prepared from Reck-cKO (Foxg1) mice were less effective than the control NPCs in this assay (Figures 5B and 5C, groups 2 versus 1). These results suggest that NPCs display ligand(s) (on their surface) stimulating canonical WNT signaling in adjacent reporter cells and that RECK somehow enhances this stimulation.

We also performed a model experiment using HEK293 cells in which the effects of RECK in both ligand-producing cells and TOP-Flash reporter cells were assessed. To prepare the assay system, we first established a series of derivatives of RECK-deficient HEK293 cells producing WNT7A or WNT7B (Producers) or

| Stage (E) | 8.5 | 9.5 | 10.5 | 11.5 | 12.5 | 13.5 | 14.5 |
|-----------|-----|-----|------|------|------|------|------|
| Ganglionic Eminence | | | | | | | |
| Cerebral Cortex | | | | | | | |
| a | 45/45 | 45/45 |
| b | x | x | x | x | x | x |
| c | x | x | x | x | x |
| d | x | x | x | x | x |
| e | x | x | x | x |
| f | x | x |

Table 1. Suppression of Forebrain Hemorrhage by LiCl-Administration

i.p., intraperitoneal injection; cKO, conditional knockout (Reckflex1/flex1;Foxg1-Cre); x, LiCl injected. See also Figure S4.

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harboring the TOP-Flash reporter plasmid (Reporter). The Producer cells were then transfected with either a vacant vector (V) or RECK expression vector (R); the Reporter cells were transfected with expression vectors encoding the WNT7 receptor components: FZD4, LRPS, GPR124, and RECK (R) (or control vector [V]).

Figure 5. The Effects of RECK on Gene Expression in the Forebrain and Canonical WNT Signaling in Adjacent Cells

(A) Changes in the levels of mRNAs encoding 15 proteins considered to be relevant to the vascular or neural phenotypes of Reck-cKO (Foxg1) mice and WNT/β-catenin signaling. Total RNA extracted from the forebrains of control mice and Reck-cKO (Foxg1) mice at E11.5, E12.5, and E13.5 were subjected to qRT-PCR using primer sets specifically amplifying indicated mRNAs and internal control (Gapdh or Mars). The level of mRNA relative to the control in each sample was calculated, and then the ratio of the values for Reck-cKO (Foxg1) mice and control mice is presented in dot plots with open bar representing mean ± SEM. Vertical axis is in the log2 scale. *p < 0.05, **p < 0.01.

(B) The ability of NPCs to activate canonical WNT signaling in the reporter cells in mixed culture. NPCs prepared from the forebrains of control embryos (n = 4, group 1) or Reck-cKO (Foxg1) embryos (n = 6, group 2) were co-cultured for 24 h with TOP-Flash reporter cells followed by dual-luciferase assay. Horizontal lines in each group represent mean (brown) and SEM (blue). *p < 0.05. Note that NPCs from Reck-cKO (Foxg1) mice show significantly lower activity to stimulate WNT signaling in the Reporter cells (group 2).

(C) The ability of NPCs and their conditioned media to activate canonical WNT signaling in the reporter cells. NPCs (groups 1 and 2) and their conditioned media (groups 3 and 4) were subjected to TOP-Flash assay as described in (B). Note that the activity of conditioned media (groups 3 and 4) were very low in this assay, indicating that the activity of NPCs to stimulate canonical WNT signaling in the reporter cells is contact dependent.

(D) The effect of RECK in the reporter cells on, and contact-dependence of, the WNT7-triggered canonical WNT signaling. HEK293 cells producing WNT7A (groups 1 and 2) or WNT7B (groups 3 and 4) or their conditioned media (groups 5 and 6) were added to the RECK-deficient TOP-Flash reporter cells transfected with either vacant vector (groups 1 and 3) or RECK expression vector (groups 2 and 4–6). After 24-h incubation, dual luciferase assay was performed. ***p < 0.003. Note that RECK in the reporter cells greatly enhances WNT signaling in this co-culture assay (groups 2 and 4) and that the stimulation could not be achieved with conditioned media (groups 5 and 6), demonstrating a cell-autonomous effect of RECK to enhance the contact-dependent, WNT7-stimulated WNT signaling.

(E) The effect of RECK in WNT7-producing HEK293 cells to stimulate WNT signaling in the reporter cells in mixed culture. RECK-deficient HEK293 cells stably expressing WNT7A (groups 1 and 2) or WNT7B (groups 3 and 4) were transfected with either control vector (V) or RECK expression vector (R), incubated for 48 h, and then co-cultured for 24 h with TOP-Flash reporter cells lacking RECK, followed by dual-luciferase assay (in triplicate). *p < 0.05. Note that RECK in WNT7-producing cells has the ability to enhance (1.6–1.7 fold) WNT signaling in the RECK-null reporter cells (i.e., non-cell-autonomous effect). Experiments were repeated three times with similar results. See also Figure S5.
Organic compounds have been postulated. Known properties and biological functions of these proteins, however, are intriguing in
multiple tissues (including the nervous and vascular system as well as mesenchyme of several developing
organisms); it interacts with WNT3A and LRP5 and possibly prevents WNT-FZD binding (Shimomura et al.,
2010; Cruciat and Niehrs, 2012). Hence, APCDD1 shares many similarities with RECK except that it inhibits
WNT transport (Routledge and Scholpp, 2019).

**DISCUSSION**

Our previous study indicated that RECK in ECs is required for proper vascular development in mouse CNS (Almeida et al., 2015). It was also demonstrated that RECK facilitates the WNT7A/B-mediated canonical
WNT-β-catenin signaling in ECs (Cho et al., 2017; Eubelen et al., 2018; Ulrich et al., 2016; Vallon et al.,
2018; Vanhollebeke et al., 2015) that is required for CNS angiogenesis (Liebner et al., 2008; Stenman
et al., 2008; Daneman et al., 2009). Our findings reported here indicate that RECK in neuroepithelial cells
is also required for proper angiogenesis in the forebrain. Several lines of evidence, including (1) phenotypic
resemblance among Reck-cKO (Foxg1) mice (Figures 1, 2, and 3), Wnt7a/Wnt7b double-knockout mice,
and EC-specific β-catenin knockout mice (Liebner et al., 2008; Stenman et al., 2008; Daneman et al.,
2009); (2) the effect of LiCl to suppress the vascular phenotype of Reck-cKO (Foxg1) mice (Figure 4); (3)
reduced expression of Apccdf1 and Sox17 in the mutant forebrain (Figure 5A); and (4) the TOP-Flash
data in mixed culture (Figures 5B–5E), point to the model that RECK in NPCs somehow facilitates con-
tact-dependent, WNT7A/B-stimulated WNT-β-catenin signaling in ECs.

How could RECK in NPCs exert such non-cell-autonomous effects on WNT signaling in ECs? Recent studies
have revealed the ability of RECK to specifically bind WNT7A/B, to keep them in a bioactive form (i.e., hy-
drophobic monomer) (Vallon et al., 2018), and to confer ligand specificity to the classical FZD-LRP5/6 re-
ceptor complex (Eubelen et al., 2018). One feasible model is that RECK expressed by WNT7-producing
cells may help presenting bioactive ligands on their surface, thereby facilitating the contact-dependent
cell-cell communication via these short-range ligands (Figure 6). We also speculate that the divergent nature
of RECK dimer (Omura et al., 2009) may facilitate this task further, for instance, by promoting signalosome
assembly. Such mechanism may also provide a solution to the “hand-over problem” in the intercellular
WNT transport (Routledge and Scholpp, 2019).

Our gene expression data indicate upregulation of Mmp2 in the forebrains of Reck-cKO (Foxg1) embryos
(Figure 5A, groups 3 and 4). Although the relevance of this phenomenon to the mutant phenotype is
unclear, upregulation of Mmp2 mRNA was also observed in Gpr124 knockout mice (Cullen et al., 2011).
Downregulation of the NPC marker Nes at E11.5 (Figure 5A, group 23) may reflect the precocious neuronal
differentiation (Figures 2Q-2 and S2), and yet upregulation of Tubb3/Tuj1 was not so prominent. With re-
gard to the neuronal differentiation, the ID family is also of interest, because Id1/Id3 double-knockout mice
die by E13.5 with brain hemorrhage and precocious neuronal differentiation (Lyden et al., 1999), which is
similar in nature to, but different in timing of death from, the Reck mutant mice (Oh et al., 2001; Muraguchi
et al., 2007; Chandana et al., 2010; Almeida et al., 2015). However, it is unclear whether the observed
upregulation (rather than downregulation) of Id3 at E12.5 (Figure 5A, group 33) contributes to the pheno-
type of Reck-cKO (Foxg1) mice.

Decreased expression of Apccdf1 and Sox17 (Figure 5A, groups 37–42) are indicative of attenuated
WNT-β-catenin signaling in the forebrain of Reck-cKO (Foxg1) mice. As the products of these WNT-induc-
ible genes are known to inhibit WNT-β-catenin signaling, their roles as negative feedback regulators have
been postulated. Known properties and biological functions of these proteins, however, are intriguing in
the present context. For instance, APCDD1 is a membrane-bound, cysteine-rich glycoprotein expressed
in multiple tissues (including the nervous and vascular system as well as mesenchyme of several developing
organs); it interacts with WNT3A and LRP5 and possibly prevents WNT-FZD binding (Shimomura et al.,
2010; Cruciat and Niehrs, 2012). Hence, APCDD1 shares many similarities with RECK except that it inhibits
rather than promotes) Wnt/β-catenin signaling. Importantly, APCDD1 was found to coordinate vascular remodeling and barrier maturation of retinal blood vessels (Mazzoni et al., 2017). On the other hand, SOX17 is a transcription factor that forms a complex with β-catenin and several TCF/LEF family transcription factors and promotes their degradation (Zorn et al., 1999; Sinner et al., 2007). Of note, Sox17 bridges Wnt and NOTCH signaling to promote arterial specification (Corada et al., 2013; Morini and Dejana, 2014) and blood-brain barrier maturation (Corada et al., 2019). Contribution of the reduction of these proteins to the vascular and/or neural phenotype of Reck-cKO (Foxg1) mice is an interesting possibility to be tested in future studies.

Wnt7a, Wnt7b, and Itgb8 are known to be critical for CNS angiogenesis (Zhu et al., 2002; Proctor et al., 2005; Stenman et al., 2008; Daneman et al., 2009). Their downregulation from E12.5 or E13.5 in Reck-cKO
(Foxg1) mice (Figure 5A, groups 6, 7, 10, and 12) may also contribute to the progression of vascular pheno-
type in these mice, although the mechanisms by which these genes get downregulated remain unclear. The 
Vegfa upregulation found at E13.5 could be a consequence of hypoxia due to the halted vascularization, 
especially in the bulky tissues such as GE. The tissue damage at the center of GE (Figure 3G, asterisk) seems 
to support this idea. As vascular endothelial growth factor overexpression has been implicated in glomer-
uloid malformation (Sundberg et al., 2001) and intracranial hemorrhage (Yang et al., 2013), Vegfa upregu-
lation may also contribute to the progressive nature of vascular defects in these mice.

In summary, we found that RECK on both endothelial and neuroepithelial components is essential for 
proper forebrain angiogenesis in mice. Our findings raise an interesting possibility that RECK mediates 
neurovascular communication by facilitating contact-dependent WNT signaling triggered by the short-
range ligands WNT7A/B.

Limitations of the Study
Although our qRT-PCR experiments for Apcdd1 and Sox17 indicated attenuated WNT/β-catenin signaling 
in the forebrain of Reck-cKO (Foxg1) mice, it was not determined in which cell type(s) this attenuation oc-
curs. We may address this issue more directly by cell-type-specific activation of canonical WNT signaling, 
for instance, using a dominant stable mutant of β-catenin (Harada et al., 1999). Our TOP-Flash assay in vitro 
supported the model that RECK in WNT7A/B-producing cells enhances contact-dependent activation of 
canonical WNT signaling in adjacent cells. However, the detailed molecular mechanism of this enhance-
ment and whether such mechanism may account for all the vascular phenotype of Reck-cKO (Foxg1) 
mice remain to be elucidated. Previous studies indicate that WNT7A stimulates neuronal differentiation 
during brain development (Hirabayashi et al., 2004), that RECK promotes neurogenesis (NPC proliferation) 
by inhibiting Notch-ligand shedding (Muraguchi et al., 2007), and that GPR124 is not expressed in NPCs 
(Kuhnert et al., 2010; Anderson et al., 2011; Cullen et al., 2011). Whether RECK supports WNT7 signaling 
in NPCs (in the absence of GPR124) and whether defects in this mechanism contribute to the neural pheno-
type of Reck-cKO (Foxg1) mice remain to be addressed in future studies.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.08.009.

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AUTHOR CONTRIBUTIONS
H.L. designed and performed a large part of the experiments and analyzed data. T. Miki, N.W., C.H., and 
C.J.K. provided useful materials and advices. G.M.A. and T. Matsuzaki helped some part of the experi-
ments. M.N. organized and supervised the study, designed and performed a part of the experiments, 
and analyzed data. H.L. and M.N. co-wrote the draft, which was improved by opinions and ideas provided 
by other authors.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

RECK in Neural Precursor Cells

Plays a Critical Role in Mouse Forebrain Angiogenesis

Huiping Li, Takao Miki, Glícia Maria de Almeida, Carina Hanashima, Tomoko Matsuzaki, Calvin J. Kuo, Naoki Watanabe, and Makoto Noda
| Gene | 5’-3’ Primers | Related to Figures 1-5 |
|------|---------------|-----------------------|
| Cre  | Forward: GCCTGCATTACC GGTCGATGCAAGG | 65 | 700 |
|      | Reverse: AAATCCATCGCTCGAGTTTAGTTACC |          |      |
| Reck | Forward: TGGCGGAAGGGAACGTAGTACG | 57–61 | 480 |
|      | Reverse: CATCAGCAGCTGAGCAGCAA |          |      |
| Mars | Forward: GAGGGTGTGTGTCCTTCTGT | 59 | 202 |
|      | Reverse: CTGCTTCTCCCAACCAGTCC |          |      |
| Gapdh| Forward: TCAACGGCAACAGTCAAGG | 59 | 126 |
|      | Reverse: ACTCCAGCACATGTCACG |          |      |
| Reck | Forward: AGGTCTCACAGTCGATTGTCG | 59 | 137 |
|      | Reverse: GCAGTTCTCGAGTTTGTG |          |      |
| Mmp2 | Forward: CAAGTTCCCGGCGATGTC | 59 | 171 |
|      | Reverse: TTCTGGTCAAGGTCACCTG |          |      |
| Wnt7a| Forward: GCCTTCTTTTGTGTTAGTC | 59 | 155 |
|      | Reverse: TGAAGACTGACACTCTGAG |          |      |
| Wnt7b| Forward: TTTGGCTCTCTCTACGTGAAG | 59 | 145 |
|      | Reverse: CCCGGATGACATGAGC |          |      |
| Itgb8| Forward: AGTGAACACAATAGATGTCGAAC | 59 | 115 |
|      | Reverse: TTTGGTCAAGGTCACCTG |          |      |
| Itgb1| Forward: AGGCCCAATCTTGCAGGAAT | 59 | 209 |
|      | Reverse: TTTGGTCAAGGTCACCTG |          |      |
| Glut1| Forward: CAGTTCCGGCTCTGAGTTAGT | 59 | 156 |
|      | Reverse: GCCGATGACGAACTGCAG |          |      |
| Vegfa| Forward: GCACATAGGAGAGTACGAGTAAGCT | 59 | 105 |
|      | Reverse: CTCCGCTTGAAACAGCT |          |      |
| Nes  | Forward: CCCTGAACTCGAGGAGC | 59 | 166 |
|      | Reverse: CTCCGCTTGAAACAGCT |          |      |
| Tubb3| Forward: TAGACCAGCAGGAACTAT | 59 | 127 |
|      | Reverse: GTCCGCTTGAAACAGCT |          |      |
| Id1  | Forward: CCTAGCTTCTTGCCTAAGAC | 59 | 60 |
|      | Reverse: GCCGACAGAAGTACCAC |          |      |
| Id3  | Forward: CTGGCAAGAGAAGGAGC | 59 | 90 |
|      | Reverse: GGTGGCTGTCCTCAAAGA |          |      |
| Axin2| Forward: TGACTCTCCTCCACATC | 59 | 105 |
|      | Reverse: TGCCGACACTGAGCTGAC |          |      |
| Apcc | Forward: ATGAACCACCTCCCCATCA | 59 | 161 |
|      | Reverse: GTCAATGCCTGCCGCTAG |          |      |
| Sox7 | Forward: GTGCCGGAAGGAGGAACTG | 59 | 136 |
|      | Reverse: CACCACACCTCGCCTAC |          |      |
Figure S1. Characterization of abnormal vessels, Related to Figures 2 and 3.

(A) Proliferating cells as detected by Ki67-immunofluorescence staining. Coronal sections of the brain from control (panels 1-4) and Reck-cKO (Foxg1) mice (panels 5-8) at E12.5 were doubly stained with anti-CD31 (endothelial marker, green; panels 2 and 6) and anti-Ki67 (proliferation marker, red; panels 3 and 7) antibodies followed by nuclear counterstaining (blue; panels 1 and 5). Merged images of the red, green, and blue fluorescence are also included (panels 4 and 8). Typical images focusing on vessels in ganglionic eminence are shown. Asterisk indicates the ventricular side. Note that CD31-positive vascular endothelial cells are largely Ki67-negative in control mice (arrowheads in panels 3 and 4) while clusters of CD31-positive cells found in Reck-cKO (Foxg1) mice...
are often Ki67-positive (arrow in panels 7 and 8), indicating proliferative nature of these cells.

(B, C) Distribution of mesenchymal antigens in vascular malformations found in Reck-cKO (Foxg1) mice. Coronal sections of the brain from Reck-cKO (Foxg1) mice at E13.5 were stained for endothelial marker (CD31, red; panel 2) and mesenchymal marker [SM22a (B, panel 3) or α-smooth muscle actin (αSMA; C, panel 3)], without (B) or with Ki67 (C, magenta, panel 4), followed by nuclear counterstaining (blue; panel 1). Note that the endothelial and mesenchymal signals are largely non-overlapped in vascular malformations in merged images (B4 and C5), which does not support the involvement of endothelial-to-mesenchymal transition (EndMT) (Dejana and Lampugnani, 2018). Ki67-signals are associated with many CD31-positive cells (C6 and C7), again indicating the proliferative nature of these endothelial cell clusters.

(D) Distribution of basement membrane markers around the abnormal vessels found in Reck-cKO (Foxg1) mice. The brain sections were stained for endothelial marker (CD31, red; panel 3) and two basement membrane markers, laminin (LN, green; panel 2) and type IV collagen (Col4, magenta; panel 4). Scale bar: 50 µm.
### [1] E11.5

| Cortex | GE |
|--------|----|
| **A**  | **B** |
| SOX2   | SOX2 |
| TUJ1   | TUJ1 |
| Nuc/SOX2/TUJ1 | Nuc/SOX2/TUJ1 |

- **Cont**
- cKO (Fragl1)

| C | D |
|---|---|
| KI67 | CD31 |
| Nuc/KI67/CD31 | Nuc/KI67/CD31 |

### [2] E12.5

| Cortex | GE |
|--------|----|
| **A**  | **B** |
| SOX2   | SOX2 |
| TUJ1   | TUJ1 |
| Nuc/SOX2/TUJ1 | Nuc/SOX2/TUJ1 |

- **Cont**
- cKO (Fragl1)

| C | D |
|---|---|
| KI67 | CD31 |
| Nuc/KI67/CD31 | Nuc/KI67/CD31 |

### [3] E13.5

| Cortex | GE |
|--------|----|
| **A**  | **B** |
| SOX2   | SOX2 |
| TUJ1   | TUJ1 |
| Nuc/SOX2/TUJ1 | Nuc/SOX2/TUJ1 |

- **Cont**
- cKO (Fragl1)

| C | D |
|---|---|
| KI67 | CD31 |
| Nuc/KI67/CD31 | Nuc/KI67/CD31 |
Figure S2. Neural phenotype of Reck-cKO (Foxg1) mouse at three embryonic stages, Related to Figure 2q.

Coronal sections of the brain from a control (panels 1-3) or a Reck-cKO (Foxg1) mouse (panels 4-6) at E11.5 (set [1]), E12.5 (set [2]), or E13.5 (set [3]) were doubly stained with anti-Sox2 (neural precursor marker, red; A, B, panels 1, 4) and anti-TUJ1 (neuronal marker, green; A, B, panels 2, 5) antibodies or with anti-Ki67 (proliferation marker, red; C, D, panels 1, 4) and anti-CD31 (endothelial marker, green; C, D, panels 2, 5) antibodies followed by nuclear counterstain (blue). Merged images of the red, green, and blue fluorescence are also included (panels 3 and 6). Typical images focusing on the cerebral cortex (A, C) or ganglionic eminence (B, D) are shown. Symbols in [1]-[3]: asterisk, ventricle; arrowhead, signals of interest; bracket and double-pointed arrow, layer of positive cells, with some semi-quantitative information such as thickness of the layer (length), intensity (line thickness), and sparseness (dotted line). Scale bar: 50 µm. The images in set [3] A and B are at a lower magnification (x10 objective lens) than others (x20 objective lens). [4] Fluorescence density per unit area in
images as shown in [1]-[3] was determined using ImageJ (see Methods for detail). Dot represents the density in one area, and brown horizontal bar the mean of 5 areas. Significance of difference is indicated by asterisk(s): * P<0.05, ** P<0.01. Note that in Reck-cKO (Foxg1) mice, TUJ1-positive mature neurons tend to be more abundant and widely distributed while Ki67-positive cells are less abundant than the control at all three stages. At E13.5, decreased abundance of Sox2-positive cells becomes also apparent (bottom row, group 2 in A and B).
Figure S3. Neural phenotype of Reck-cKO (Tie2) mouse at E13.5, Related to Figures 2q and Figure 3I-P.

A coronal section of the brain from control (A, B) or Reck-cKO (Tie2) mouse (C, D) at E13.5 were stained with anti-TUJ1 antibodies (neuronal marker, red) followed by nuclear counterstain (blue). Magnified views of the cortical area (dotted-line box) in panels A and C are shown in B and D, respectively. Note the less prominent TUJ1 signals (red) in the Reck-cKO (Tie2) mouse brain (panels C, D) than the control (panels A, B). Scale bars: 200 µm.
Figure S4. Vascular phenotype of Reck-cKO (Foxg1) mice at E15.5 after LiCl-treatment, Related to Table 1 and Figure 4. Lateral (A, B) and dorsal (C, D) views, focusing on the head region, of Reck-cKO (Foxg1) embryos left untreated (A, C) or treated with LiCl daily from E10.5 to E14.5 (B, D) and harvested at E15.5. GE, hemorrhage in ganglionic eminence. Cx, hemorrhage in cerebral cortex. Scale bar: 1 mm.
Figure S5. Effects of RECK and GPR124 in WNT7-producing HEK293 cells on WNT signaling in the reporter cells in mixed culture, Related to Figure 5, D and E.

RECK-deficient HEK293 cells stably expressing WNT7A (groups 1-4) or WNT7B (groups 5-8) were transfected with either control vector (V), RECK-expression vector (R), GPR124-expression vector (G), or both expression vectors (R+G), incubated for 48 h, and then co-cultured for 24 h with TOP-Flash reporter cells lacking RECK, followed by dual-luciferase assay (in triplicate). The cells without WNT7-expression (groups 9, 10) and wild type NPCs (group 11) were also included for comparison. *P<0.05, **P<0.01. Note that GPR124 suppressed, rather than enhanced, the activity of RECK to enhance WNT signaling in adjacent reporter cells. Experiments were repeated three times with similar results.
TRANSPARENT METHODS

Mice
All animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University and conducted according to its regulation. The engineered Reck allele Reck\textsuperscript{flex1}, carrying two lox-P sites franking the exon 1 (“flex1” stands for “floxed at exon 1”; tm2.1Noda, RRID:IMSR_RBRC10232), has been reported (Yamamoto et al., 2012). To generate Reck-cKO (Foxg1) mice, Reck\textsuperscript{flex1/flex1} mice were crossed with mice carrying the Foxg1-Cre allele (Hebert and McConnell, 2000) to obtain the heterozygous Reck\textsuperscript{flex1/+}; Foxg1-Cre mice. These mice were crossed again with Reck\textsuperscript{flex1/flex1} mice to obtain Reck\textsuperscript{flex1/flex1}; Foxg1-Cre [Reck-cKO (Foxg1)] and Reck\textsuperscript{flex1/+}; Foxg1-Cre [Cont] mice among others. To generate the EC-selective Reck knockout mice [Reck-cKO (Tie2)], Reck\textsuperscript{flex1/flex1} mice were crossed with Tie2-Cre mice (Kisanuki et al., 2001) to obtain the heterozygous Reck\textsuperscript{flex1/+}; Tie2-Cre mice. These mice were crossed again with Reck\textsuperscript{flex1/flex1} mice to obtain Reck\textsuperscript{flex1/flex1}; Tie2-Cre [Reck-cKO (Tie2)] and Reck\textsuperscript{flex1/+}; Tie2-Cre [Cont] mice among others. To visualize Foxg1-Cre-expressed cells, the Foxg1-Cre mice were crossed with homozygous mTmG/mTmG mice (Muzumdar et al., 2007). Mice were mated between 16:00 and 9:00, and the day on which vaginal plug was found was designated as 0.5 dpc (E0.5). Genotype was determined by PCR using the primers listed in Supplemental Table S1.

Histology
Mouse embryos at indicated time points were harvested on ice, and the forebrain were harvested and fixed in 4% PFA solution overnight. After immersed in 10%/20%/30% sucrose/PBS solution, the forebrains were embedded in OCT and frozen. For Hematoxylin and Eosin (HE) staining, 10-µm thick cryosections were used. For immunofluorescence staining, 5-µm thick cryosections were incubated with primary antibodies overnight, washed, incubate with appropriate secondary antibodies (conjugated with either Alexa Fluor-488, -555, or -647) for 2 h at room temperature, and then washed and mounted using DAPI Fluoromount-G (0100-20, Southern Biotechnology Associates). Micrographs were acquired using Zeiss AxioPlan or Keyence.
BZ-9000. Primary antibodies used: rat monoclonal anti-PECAM/CD31 (BD Bioscience, 553370), rabbit polyclonal anti-NG2 (Merck Millipore, AB5320), Mouse monoclonal anti-Laminin(clone LAM-89) (Sigma-Aldrich, L8271), Rabbit polyclonal anti-Type IV Collagen (PROGEN Biotechnik,10760), Goat polyclonal anti-Sox2 (Santa Cruz Biotechnology, sc-17320), Rabbit polyclonal anti-Neuronal Class III beta-Tubulin/TUJ1 (COVANCE, PRB-435P), and Rabbit polyclonal anti-Ki67 (Leica Biosystems, NCL-Ki67p). Secondary antibodies used: Goat anti-rat IgG(H+L) highly cross-adsorbed secondary antibodies (Alexa-555, Alexa-594, or Alexa-647 conjugates), and goat anti-rabbit IgG(H+L) highly cross-adsorbed secondary antibodies (Alexa-488 or Alexa-647 conjugates), Donkey anti-goat IgG(H+L) cross-adsorbed secondary antibodies (Alexa-647 conjugates), Donkey anti-rabbit IgG(H+L) cross-adsorbed secondary antibodies (Alexa-488 conjugates) (Thermo Fisher Scientific). For densitometry, mean density of fluorescence signals per pixel in a scare ROI (90 x 90 pixels) was determined using ImageJ. Five ROIs were randomly selected in the region adjacent to the ventricle (in the cases of Sox2 and Ki67) or outer surface of the brain (in the case of TuJ1) in microscopic images acquired under comparable conditions.

**Lithium Chloride Treatment**

Reckflex1/+;Foxg1-Cre male mice and Reckflex1/flex1 female mice were timed-mated, and aqueous LiCl solution was injected (200 mg/kg, i.p.) into the female mice on several consecutive days as described in Table 1. Embryos were harvested at E15.5, and their cerebral hemorrhage was visually inspected (see Supplemental Figure S4), while their genotypes determined using yolk sac DNA.

**qRT-PCR**

Total RNA was extracted from forebrains of embryos at E11.5, E12.5, and E13.5 using Nucleo Spin RNA kit (Macherey-Nagel). cDNA was prepared using PrimeScript II 1st Strand cDNA synthesis kit (Takara), and quantitative PCR was performed with the Stratagene Mx3005P (Agilent) using KAPA SYBR Fast Universal qPCR kit (Kapa Biosystems) and the primers listed in Supplemental
Table S1. Ratio of mRNA in Reck-cKO (Foxg1) and Cont samples was calculated using the delta delta Ct method.

**NPCs**

NPCs were prepared according to the method of Kitani et al. (Kitani et al., 1991). Briefly, the forebrains of E11.5 embryos were cut into several pieces in DME/F12 medium. Eye cups, nasal plates, and other non neuroepithelial tissues were removed under a dissection microscope. The tissue pieces were treated with PBS containing 0.5% trypsin for 1 h at 37°C and then with DNase I to reduce viscosity. The cells were transferred to DME/F12 medium supplemented with 10% FBS on 12 well plates. After incubation for 7 h at 37°C in a CO₂ incubator, cells in the NPC-enriched fraction (floating cells) were collected, counted, and sedimented by centrifugation. The cells and the supernatant were separated and used in TOP-Flash assays.

**TOP-Flash Assay**

A RECK-deficient HEK293 sub-line, ADA99-25 (Matsuzaki et al. unpublished), was generated using the CRISPR/Cas9 double nickase method (Ran et al., 2013). To establish the RECK-deficient TOP-Flash reporter, ADA99-25 cells were transfected with pGL4.49 (Clontech) using CalPhos Mammalian Transfection Kit (Clontech), selected in growth medium (DMEM supplemented with 10% fetal bovine serum and Pen Strep) containing 100 µg/ml Hygromycin B Gold (Invitrogen), and a clone (named HNM1) whose firefly luciferase activity shows robust response to 20 µM LiCl was selected. To establish a WNT7-selective TOP-Flash reporter (named LC20b), HNM1 cells were co-transfected with four expression vectors (expressing Fzd4, Lrp5, GPR124, and RECK) (Takahashi et al., 1998; Vallon et al., 2018) and two marker plasmids [pRL (Promega) and pUCSV-BSD (Kimura et al., 1994)], selected in growth medium containing 8 µg/ml Blasticidin-S, and a clone was isolated whose firefly luciferase activity showed a robust response when co-cultured with HEK293 cells producing WNT7A. For TOP-Flash assay with NPCs, LC20b cells (3 x 10⁴/well) were plated onto a white 96-well plate (BD 353296) at day 0. NPCs (3 x 10⁴/well) or their culture supernatant were overlaid at day 1, and luciferase
assay was performed at day 2 using Dual-Glo Luciferase Assay System (Promega) or twinlite Firefly & Renilla Luciferase Reporter Gene Assay System (PerkinElmer). For TOP-Flash assay with HEK293 cells, we first established RECK-deficient HEK293 cells expressing either WNT7A or WNT7B by stably transfecting ADA99-25 cells with an expression vector (carrying the neo marker) expressing WNT7A or WNT7B (Vallon et al., 2018) followed by selection in growth medium containing 1 mg/ml G418. These WNT7 producer cells were transfected with either vacant vector (V) or RECK-expression vector (R) using FuGENE HD (Promega) and plated onto a white 96-well plate (1.5 x 10⁴/well) at day 0 (Producer). In parallel, HNM1 cells were co-transfected with five expression vectors (pRL, Fzd4, Lrp5, GPR124, plus control or RECK-expressing vector) using FuGENE HD and plated onto regular dishes (Reporter). At day 1, media in all cultures were replaced to fresh growth medium. At day 2, culture supernatant on white plate (Producer) were transferred to new wells (Conditioned media), and then the Reporter cells, suspended using trypsin and washed with growth medium, were plated (3 x 10⁴/well) onto the wells containing either the Producer cells or the Conditioned media. After 1-day incubation, luciferase assay was performed using the twinlite system.

Statistics
Quantitative data are presented in the form of dot plots with either open bar (Figure 5A) or horizontal lines (brown and blue) representing mean and the standard error of mean (s.e.m.) (Figure 5B-E). Significance of difference between two groups was assessed using Student’s t-test.
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