Figures and figure supplements

Molecular determinants in Frizzled, Reck, and Wnt7a for ligand-specific signaling in neurovascular development

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Figure 1. Frizzled CRD specificity for Reck-Gpr124-Wnt7a binding and signaling. (A) Reck(CC1-5)-AP binding to live HEK293T cells transfected with Gpr124, Wnt7a, and full-length Fz (top) or Gpr124, Wnt7a, and FzCRD-Myc-GPI (bottom). (B) Reck(CC1-5)-AP binding as in (A), with Gpr124, Wnt7a, and...
the indicated FzCRD-Myc-GPI targets, together with anti-Myc and anti-1D4 controls. (C) Summary of the AP binding assay in (A) and (B). (D) Reck(CC15)-AP binding as in (A), with WT or chimeric Frizzleds. (E) Beta-catenin signaling assay using STF cells transfected with Wnt7a, Gpr124, Reck, and Lrp5 (left) or Wnt7a and Lrp5 (right), together with WT or chimeric Frizzleds. Inset: summary of AP binding (D) and STF signaling (E). In this and subsequent figures, bars represent mean ± SD. Statistical significance, determined by the unpaired t-test, is represented by * (p<0.05), ** (p<0.01), *** (p<0.001), and **** (p<0.0001). The statistical comparisons in (E), (G), and (I) are to the ‘No Fz’ control. (F) AP-3xMyc-Norrin binding assay as in (A), with WT or chimeric Frizzleds. (G) Beta-catenin signaling assay using STF cells transfected with Ts12 and Norrin (left) or Norrin (right), together with WT or chimeric Frizzleds. Inset: summary of AP binding (F) and STF signaling (G). (H) Schematic of the FzCRD-Myc-GPI competition experiment in (I). (I) The effect of FzCRD-Myc-GPI competition on beta-catenin signaling by Reck, Gpr124, and Wnt7a. Inset: summary of STF signaling.
Figure 2. Wnt7a regions that are required for Reck/Gpr124-stimulated signaling. (A) Backbone model of Wnt7a (N-terminal domain, blue; C-terminal domain, cyan) bound to Fz8 CRD (green) based on the Wnt-CRD crystal structure of Janda et al. (2012). The N-terminal domain of Wnt7a consists...
of ~270 amino acids (some of which were not resolved in the crystal structure), and the C-terminal domain consists of ~80 amino acids. (B) Beta-catenin signaling assay using STF cells transfected with Reck and Gpr124 (left) or pRK5 vector control (right), together with the indicated Wnts. Inset: summary of STF signaling. Statistical comparisons in (B), (D), and (G) are to WT Wnt7a. (C) Amino acid sequence of the N-terminal domain of mouse Wnt7a, with alanine scanning mutants indicated. (D) Beta-catenin signaling assay using STF cells transfected with Reck, Gpr124, and Fz8, together with WT or mutant Wnt7a. (E) Left, backbone model of Wnt7a bound to Fz8 CRD as in (A), with amino acids that are critical for Wnt7a signaling shown in red. Right, the boxed region is displayed at higher magnification. (F) Single alanine substitution mutants of Wnt7a, indicated in red. (G) Beta-catenin signaling assay as in (D) with WT or the indicated Wnt7a mutants. (H) Left, backbone model of Wnt7a bound to Fz8 CRD as in (A) except rotated 135 degrees, with amino acids that are critical for Wnt7a signaling shown in red. Right, the boxed region is displayed at higher magnification.

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Figure 2—figure supplement 1. Production of intact and chimeric Wnt proteins for Reck- and Gpr124-mediated signaling. (A) Detecting WT Wnt7a-1D4 proteins on the surface of HEK293T cells co-transfected with Fz8CRD-Myc-GPI and the indicated concentrations of Wnt7a-1D4 plasmid DNA. Live cells were immuno-stained with anti-1D4, fixed, and then incubated with anti-mouse antibody conjugated to AP. (B) Immunoblot of post-nuclear supernatants from HEK293T cells transfected with the indicated concentrations of Wnt7a-1D4 plasmid DNA, probed with mAb 1D4 and anti-tubulin. (C) Detecting Wnt proteins on the surface of HEK293T cells transfected with Fz8CRD-Myc-GPI, together with Wnt7a-1D4, Wnt3-1D4, Wnt3a-1D4, or their chimeric derivatives. Cell-surface AP immuno-staining was performed as described in (A). (D) Immunoblot of post-nuclear supernatants from HEK293T cells transfected with Wnt7a-1D4, Wnt3-1D4, Wnt3a-1D4, or their chimeric derivatives, probed with mAb 1D4 and anti-tubulin.

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Figure 2—figure supplement 2. Locations of alanine mutations on the Wnt7a-Fz8CRD model. Front (A) and back (B; rotated 180 degrees) views of the Wnt7a-Fz8CRD model with alanine mutations shown in red.

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Figure 2—figure supplement 3. Cell-surface CRD binding by Wnt7a alanine mutants. Detecting WT and mutant Wnt7a-1D4 proteins on the surface of HEK293T cells co-transfected with Fz8CRD-Myc-GPI and the indicated WT or mutant Wnt7a-1D4 plasmids. Live cells were immuno-stained with anti-1D4, fixed, and then incubated with anti-mouse antibody conjugated to AP.

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Figure 3. Reck CC4 is necessary for multi-protein complex formation and signaling with Gpr124, Wnt7a, and Fz. (A) Reck(CC1)-, (CC1-2)-, (CC1-3)-, (CC1-4)-, or (CC1-5)-AP binding to live HEK293T cells transfected as indicated at right. (B) Schematic of the AP binding assay in (A). (C) Amino acid Conservation of P256 and W261 in Reck CC4

Mus musculus  CT-KPLPQPLWQC
Homo sapiens  CT-KPLPQPLWQC
Rattus norvegicus  CT-KPLPQPLWQC
Gallus gallus  CT-KPLPQPLWQC
Taeniopygia guttata  CT-KPLPQPLWQC
Xenopus tropicalis  CT-KPLPQPLWQC
Callorhinchus milii  CT-KPLPQPLWQC
Danio rerio  CGSQPLPQWQC

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Figure 3 continued on next page
sequence of mouse Reck CC4, with alanine scanning mutants indicated. (D) Beta-catenin signaling assay using STF cells transfected with Gpr124 and WT Reck or the indicated Reck CC4 mutant, in combination with WT Wnt7a (left), Wnt7a Ala #16 (middle), or Wnt7a Ala #17 (right). Red arrows, Reck CC4 Ala #8 and Ala #10 transfections. Statistical comparisons in (D) and (G) are to WT Reck. (E) Sequence of mouse Reck CC4 in the region of Ala#8 and Ala#10, with additional alanine substitution mutants indicated in red. (F) HEK293T cells were transfected with WT Reck or the indicated Reck mutants. Post-nuclear supernatants (input) and surface biotinylated proteins (captured with NeutrAvidin agarose) were immunoblotted for Reck and actin. (G) Beta-catenin signaling assay using STF cells transfected with Gpr124, Wnt7a, and WT Reck or the indicated Reck CC4 mutant. Red arrows, Reck CC4 mutants that eliminate signaling. (H) Alignment and conservation of the Reck CC4 region shown in (E) across vertebrates, generated by Clustal Omega. (*) denotes fully conserved residues. P256 and W261 are highlighted in red. (I) Reck(CC1-5)-AP and Reck(CC1-5 Ala #21)-AP binding to live HEK293T cells transfected as indicated at right. (J) Schematic of the AP binding assay in (I).

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Figure 3—figure supplement 1. Tests of Reck ΔCC2-3 for Wnt7a/Fz/Gpr124 signaling and complex formation. (A) HEK293T cells were transfected with WT Reck or the indicated Reck deletion mutants. Post-nuclear supernatants (input) and surface biotinylated proteins (captured with NeutrAvidin agarose) were immunoblotted for Reck and actin. (B) Beta-catenin signaling assay using STF cells transfected with Gpr124, Wnt7a, and WT Reck or the indicated Reck deletion mutants. Inset: summary of STF signaling. (C) Reck(CC1-5)-AP and Reck(CC1-5, Δ2-3)-AP binding to live HEK293T cells transfected as indicated at right. (D) Schematic of the AP binding assay in (C).

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**Figure 4.** Embryos with Reck<sup>P256A/W261A</sup> have severe defects in CNS angiogenesis that match the defects in Gpr124 null embryos. (A) CRISPR/Cas9 strategy for introducing P256A and W261A into Reck exon 9 in the mouse germline and sequencing chromatograms from cloned genomic PCR products from WT (top) vs. Reck<sup>P256A/W261A</sup> alleles (bottom). (B) Immunoblot of proteins from E11.5 embryos of the indicated genotypes, probed with anti-Reck and anti-actin antibodies. (C) Gross appearance of WT vs. Reck<sup>P256A/W261A/Δex2</sup> E13.5 embryos. The arrow points to intracranial bleeding in the Reck<sup>P256A/W261A/Δex2</sup> embryo. (D,E) Coronal sections of WT vs. Reck<sup>P256A/W261A/Δex2</sup> E13.5 embryos immunostained for PECAM and GLUT1, and stained for GS-lectin. Sections are at the levels of (a) the ganglionic eminences, (b) the thalamus (center) flanked by the cerebral cortices, and (c) the hindbrain. In (E), arrows point to the avascular and hypoplastic cerebral cortex, and arrowheads point to the avascular and hypoplastic ganglionic eminences. Scale bar, 500 μm.

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