SUPPLEMENTAL MATERIALS:

Supplemental methods:

Animals:

These studies adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Children's Hospital Boston Animal Care and Use Committee. Unless otherwise indicated, C57Bl/6J mice (stock no. 000664; Jackson Laboratory) were used for the study. The *Lrp5*$^{-/-}$ mice (stock no. 005823; Jackson Laboratory) were developed by Deltagen Inc. TOPGAL transgenic Wnt activity reporter mice (stock no. 004623; Jackson Laboratory) contain the *lacZ* gene under the control of a regulatory sequence consisting of three consensus LEF/TCF-binding motifs upstream of a minimal *c-fos* promoter, as described previously [1, 2]. Mice with macrophage marker *Csf1r*-driven GFP (stock no. 005070; JAX) were used to visualize microglia/macrophages in the retina. *Dishevelled2*$^{-/-}$ from Jackson Laboratory (stock no. 008001) were described previously [3].

Retina dissection, vessel staining and flat mount:

Mice at various ages during development were anesthetized with Avertin (Sigma-Aldrich) and sacrificed by cervical dislocation. Eyes were enucleated and fixed in 4% paraformaldehyde for 1 h at room temperature, followed by isolation and subsequent dissection of the retina. To visualize vessels, retinas were stained overnight at room temperature with fluoresceinated *Griffonia Bandeiraea Simplicifolia* Isolectin B_4 (Alexa Fluor 594 conjugated; I21413; Invitrogen; 1:100 dilution) in 1 mM $CaCl_2$ in 1x PBS. Following 2 h of washes in 1x PBS, retinas were whole-mounted onto Superfrost/Plus microscope slides (12-550-15; Fisher Scientific) with the photoreceptor side down and imbedded in SlowFade Antifade reagent (S2828; Invitrogen).

Quantification of vascular development and neovascularization:

Quantification of retinal vasculature was carried out as described previously [4-6]. Images of whole-mounted retina were taken at ×5 magnification on a Zeiss AxioObserver.Z1 microscope and merged using AxioVision 4.6.3.0 software to produce an image of the entire retina. Vascular growth during development, and neovascular tuft formation in OIR were quantified in Adobe Photoshop or Image J. The number of pixels in vascular area during development is visualized with isolectin staining and outlined in Photoshop and compared to total number of pixels in the whole retina. Retinal areas with pathologic neovascular tufts structures were visually identified by their abnormal aggregated morphology that is distinctly different from the normal finely branched vascular network. The number of pixels in pathologic neovascular area is quantified and compared with the total number of pixels in the whole retina, either by manual selection based on morphology in Photoshop [4, 5] or by the SWIFT_NV method [6] which consists of a set of macros on NIH's free ImageJ platform to isolate the neovascular structures to stand out clearly against the background fluorescence of normal vessels. Percentages of neovascularization in total retina were compared between retinas from littermate heterozygous or age-matched wild type mice with identical oxygen conditions. For quantification of endothelial sprouts during development, images were taken at ×20 magnification at the center of each quadrant of the retina. The number of vascular sprouts with extending filopodia was quantified and compared in *Lrp5*$^{-/-}$ mice and their littermate controls. Evaluation was done blind to the identity of the sample. *n* is number of eyes quantified.

Intravitreal injection:

For analysis of superficial retinal vessel development, claudin5 antibody (0.25μg/μl in 0.5μl volume) was injected intravitreally at P2 with contra-lateral eyes injected with pre-immune IgG as control. Retinas were dissected, lectin stained and flatmounted at P7. Images were taken with Zeiss AxioObserver.Z1 microscope. The experiment was repeated in 3 litters. For analysis of deep retinal vessel development, claudin5 antibody (0.25μg/μl in 0.5μl volume) was injected intravitreally at P4 with contra-lateral eyes injected with pre-immune IgG as control. Retinas were dissected at P9, permeabilized, lectin stained and

flatmounted. Images were taken with a Leica confocal microscope at 10X magnification. For each image of deep layer of retinal vessels, the focus was adjusted to capture the maximum vascular area in the outer plexiform layer. Images were merged and quantified with Adobe Photoshop.

For the study with claudin5 siRNA during retinal development, claudin5 siRNA (Dharmacon: Sense sequence: 5'-CGU UGG AAA UUC UGG GUC UUU dTdT-3', Antisense sequence: 5'-AGA CCC AGA AUU UCC AAC GUU dTdT-3') were injected intravitreally at P4 with contra-lateral eyes injected with control siRNA (1027310, Qiagen). At P6 retinas were dissected and RNA isolated followed by quantitative real-time PCR analysis of claudin5 gene expression. At P7, retinas were dissected and flatmounted for analysis of vascular growth. For the study of claudin5 siRNA in retinopathy, retinopathy were induced in mice by exposure to 75% oxygen from P7 to P12. At P14 claudin5 siRNA were injected intravitreally with contra-lateral eyes injected with control siRNA. Retinas were dissected and flatmounted at P17 with lectin staining to analyze pathologic neovessels.

RNA isolation and cDNA preparation:

Total RNA was extracted from the retinas of 6 mice, each from a different litter; the RNA was pooled to reduce biologic variability (n=6). Retinas from each time point were lysed with a mortar and pestle and filtered through QiaShredder columns (Qiagen, Chatsworth, MD, USA). RNA was then extracted as per manufacturer's instructions using the RNeasy Kit (Qiagen). To generate cDNA, 1 μg total RNA was treated with DNase I (Ambion Inc.) to remove any contaminating genomic DNA, and was then reverse transcribed using random hexamers, and SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA). All cDNA samples were aliquoted and stored at –80°C.

Quantitative real-time PCR analysis of gene expression:

PCR primers targeting *Lrp5* (F: 5'-AAG GGT GCT GTG TAC TGG AC-3', R: 5-AGA AGA GAA CCT TAC GGG ACG-3'), *Frizzled4* (F: 5'-TTC CTT TGT TCG GTT TAT GTG CC-3', R: 5'-CTC TCA

GGA CTG GTT CAC AGC-3'), *Norrin* (F: 5'-CGC TGC ATG AGA CAC CAT TAT-3'; R: 5'-CTC AGA GCG TGA TGC CTG G-3'), *Wnt3* (F: 5'-GGT TCG TGC GGA TGG GTG GG-3', R: 5'-GGG GCC AGA TGG GAG CTG GA-3'), *Wnt7a* (F: 5'- CAC TTG TGG TCT CAG GGG TT-3', R: 5'-GCA TCT GAG TTT CAC CAG CA-3'), *Wnt10a* (F: 5'-GCT CAA CGC CAA CAC AGT G-3', R: 5'-CGA AAA CCT CGG CTG AAG ATG-3'), *Claudin5* (F: 5'-GCA AGG TGT ATG AAT CTG TGC T-3', R: 5'-GTC AAG GTA ACA AAG AGT GCC A-3'), *Plvap* (F: 5'-GCT GGT ACT ACC TGC GCT ATT-3', R: 5'-CCT GTG AGG CAG ATA GTC CA-3') and an unchanging control gene, *Cyclophilin A* (F: 5'-AGG TGG AGA GCA CCA AGA CAG A-3', R: 5'-TGC CGG AGT CGA CAA TGA T-3'), were designed using Primer Bank and NCBI Primer Blast Software.Express software (Applied BioSystems). We used three methods to analyze primer sequences for specificity of gene detection. First, NCBI Blast modulewas used to identify primer and probe sequences that specifically detected the sequence of choice. Second, amplicons generated during a PCR reaction were analyzed using the first derivative primer melting curve software supplied by Applied BioSystems. This analysis determined the presence of amplicons on the basis of their specific melting point temperatures. Third, amplicons generated were gel purified and sequenced by the Children's Hospital Boston Core Sequencing Facility. This further confirmed the selection of the desired sequence. Quantitative analysis of gene expression was determined using an ABI Prism 7700 Sequence Detection System (TaqMan) and the SYBR Green Master mix kit. Standard curves for each gene were plotted with quantified cDNA template during each real-time PCR reaction. Each target gene mRNA copy number was normalized to 10^6 copies of the house keeping gene, *cyclophilin A.*

Laser capture microdissection of retinal vessels:

Eyes were embedded in OCT and flash frozen immediately following enucleation. Eyes were cyrosectioned under RNase free conditions into 8-μm sections, and collected on RNase-free polyethylene naphthalate glass slides (11505189, Leica). Sections were dehydrated with 50%, 70%, and 100% ethanol washes and stained with isolectin (1:50 in 1mM $CaCl_2$) and counterstained with H&E. Retinal vessels

were microdissected with a Leica LMD 6000 system (Leica Microsystems) and collected directly into RNA stabilizing buffer from the RNeasy Micro kit (Qiagen, Chatsworth, CA). RNA was extracted from microdissected tissues using the RNeasy Kit as described above (Qiagen), and real-time PCR was performed with the generated cDNA.

Permeability assay:

For visualizing retinal vascular permeability, P8 *Lrp5*$^{-/-}$ mice or WT control were injected with fluoresceinated dextran 70kD (10mg/ml) with Isolectin B_4 (Alexa Fluor 594 conjugated). Mice were sacrificed after 5 min, followed by eye fixation, retinal dissection and flatmounting. In addition, retinal cross-section from adult *Lrp5*$^{-/-}$ mice or WT control were stained immunohistochemically for mouse IgG.

Endothelial cell culture and spheroid sprouting assay:

Human retinal microvascular endothelial cells (HRMECs, Cell Systems, Kirkland, USA) were culture in Complete Medium and used from passage 3–7 for experiments. Small interference siRNA-mediated knockdown was performed using the RNA interference technique. siRNA duplexes used were as follows; human Lrp5: 5'-GCCCUACAUCAUUCGAGGAAU-3', human Sox18: 5'-GGGUUACAUUUUUGAAGCA-3', Ambion, ID: 109098). Cells were transfected with 10nM of siRNA duplexes using Silentfect (BioRad, Hercules, CA) according to the manufacturer's instructions and cells were harvested after incubation for 48 hours. As a control, siRNA duplex with irrelevant sequence (Ambion) was used. The silencing efficiency and gene expression were assessed with RT-qPCR. For spheroid sprouting assay, cells were cultured as monolayers at 37°C, 5% CO2 in a humidified atmosphere in Complete Media (Cell Systems, Kirkland, USA). The preparation of EC spheroids was performed as described previously [7, 8]. Briefly, cells were harvested from subconfluent monolayers by trypsinization and suspended in Complete Media containing 10% FBS and 0.25% (w/v) carboxymethylcellulose (Sigma). 500 cells were seeded together in one hanging drop to assemble into a single spheroid within 24 h at 37°C, 5% CO2. After 24 h spheroids were harvested and used for sprouting analysis in a matrix of

type I collagen. Briefly, 30 EC spheroids per group were seeded into 0.5 ml collagen solution in non-adherent 24-well plates, with a final concentration of rat type I collagen of 1.5 mg/ml. Freshly prepared gels were transferred rapidly into a humidified incubator (37°C, 5% CO2) and after the gels had solidified, 0.1 ml Serum-Free Media (Cell Systems, Kirkland, USA) was added per well containing Wnt3a (100ng/ml), Wnt7a (100ng/ml), VEGF (25ng/ml), and Cln5 antibody (10μg/ml) as indicated. After 24 h, gels were photographed and spheroid sprouting was assessed quantitatively using Adobe Photoshop®. Results are expressed as mean ± SEM. n is number of spheroids quantified.

Immunohistochemical staining.

For retina cross-sections immunostaining, eyes were fixed in 4% paraformaldehyde for 1 h and incubated in 30% sucrose at 4°*C*, followed by embedding in OCT. 10-μm thick sections were blocked in PBS with 0.1% Triton X-100 and 5% goat serum. Sections were stained with Isolectin to visualize vessels and/or primary antibodies followed by secondary antibodies. For whole-mount immunohistochemical staining, retinas fixed in 4% paraformaldehyde for 1 h were rinsed in 1x PBS, permeabilized overnight at 4°C with 0.5% Triton X-100 in PBS, stained with Isolectin B_4, followed by primary and secondary antibody staining. Retinal whole-mounts were prepared and imaged as described previously. Retinal microglia/macrophages were visualized in retinas from mice with *Csf1r*-driven GFP, with fluorescence enhanced with FITC labeled rabbit antibodies against GFP.

Western blot:

Retinas were dissected as described above. 25 μg protein of retinal lysate from *Lrp5*$^{-/-}$ or WT mice was loaded on an SDS-PAGE gel and transferred onto a PVDF membrane. After blocking, the membranes were incubated overnight with rabbit anti-mouse Cln5 antibody followed by anti-rabbit secondary antibody conjugated with horseradish peroxidase for one hour at room temperature. *Chemiluminescence* signals were generated with ECL plus substrate and captured with KODAK film.

Antibodies:

The following primary antibodies were used in this study: anti-Lrp5 (36-5400, Invitrogen), anti-Frizzled4 (MAB194, R&D systems), anti-Norrin (AF3014, R&D systems), rabbit antibody against GFP (A11122; Invitrogen); anti-β-GAL (ab616, Abcam), anti-Claudin5 (34-1600 and 352588, Invitrogen) and anti-Mouse IgG (A-21200, Invitrogen). Secondary antibodies: Chicken anti-rabbit Alexa 488 (A-21441, Invitrogen); Goat anti-Rabbit conjugated with horse-radish peroxidase (NA934V, Amersham Pharmacia).

Supplemental References:

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7. Stahl A, Paschek L, Martin G, et al. Rapamycin reduces VEGF expression in retinal pigment epithelium (RPE) and inhibits RPE-induced sprouting angiogenesis in vitro. *FEBS Lett.* 2008;582:3097-3102.

8. Korff T, Augustin HG. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol.* 1998;143:1341-1352.

Supplemental Figure 1. Time course of *Lrp5* mRNA expression. **A.** Quantification of retinal *Lrp5* mRNA with RT-qPCR during normal retinal development. **B.** Specificity of Lrp5 antibody is verified with absence of Lrp5 antibody staining in *Lrp5*$^{-/-}$ eyes. Scale bar: 100μm.

Supplemental Figure 2. Normal retinal vasculature in *Dvl2*$^{-/-}$ eyes. **A.** Retinal flat mount of *Dvl2*$^{-/-}$ mice (1 month old) shows normal retinal vascular structure. Retinal vessels are visualized with isolectin B_4 staining (red). **B.** Retinal cross section of *Dvl2*$^{-/-}$ mice (1 month old) stained with isolectin (red, vessels) and DAPI (blue, nucleus), showing normal retinal vessels in all three layers. Scale bars: A: 1000μm. B: 100μm.

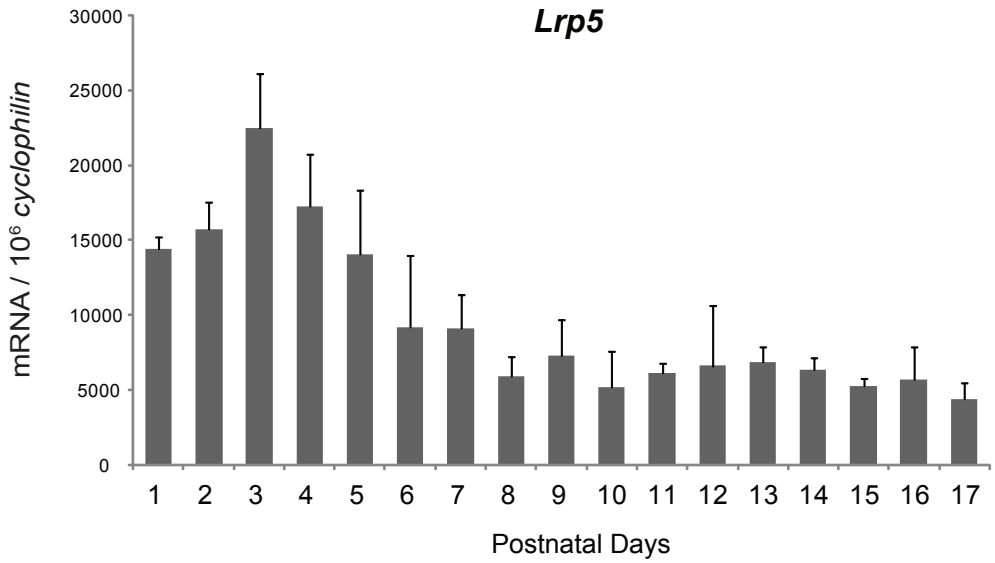
Supplemental Figure 3. Pathways not regulated at mRNA level in *Lrp5*$^{-/-}$ eyes. **A.** Quantification of *Angiopoietin* (Ang) 1 and *Ang2* in WT and *Lrp5*$^{-/-}$ retina at P5 and P8. **B.** Quantification of *Dll4*, *Notch1* and *Notch2* in WT and *Lrp5*$^{-/-}$ retina at P5 and P8.

Supplemental Figure 4. Blocking cln5 with a second antibody suppresses spheroid sprouting. **A.** Human retinal microvascular endothelial cells (HRMEC) were cultured as multicellular spheroids in the presence of Wnt 3a, Wnt7a and VEGF in combination with a second Cln5 antibody to confirm the results shown in Figure 4c. Quantification of HRMEC spheroid sprouting shows that blocking Cln5 with this second antibody also suppresses HRMEC spheroid sprouting. n=10-20 per group, n.s.: not significant, *$p \leq 0.05$, ***$p \leq 0.001$. **B.** Quantification of HRMEC spheroid sprouting in the presence of VEGF in combination with NaN_3 shows lack of toxic effects of the antibody solution containing NaN_3. n=10-20 per group, n.s.: not significant.

Supplemental Figure 5. Expression levels of Frizzled(Fzd) receptors in *Lrp5*$^{-/-}$ eyes. Quantification of *Fzd2, Fzd3 Fzd4 Fzd7 and Fzd8* mRNA in WT and *Lrp5*$^{-/-}$ retina at P5 and P8.

Supplemental Figure 1

A



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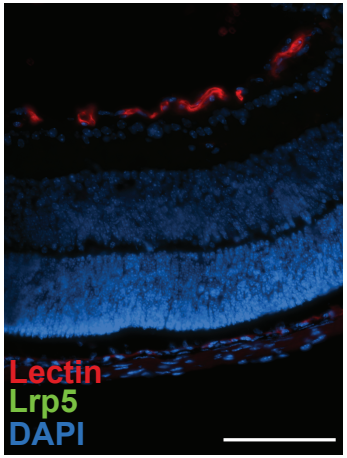
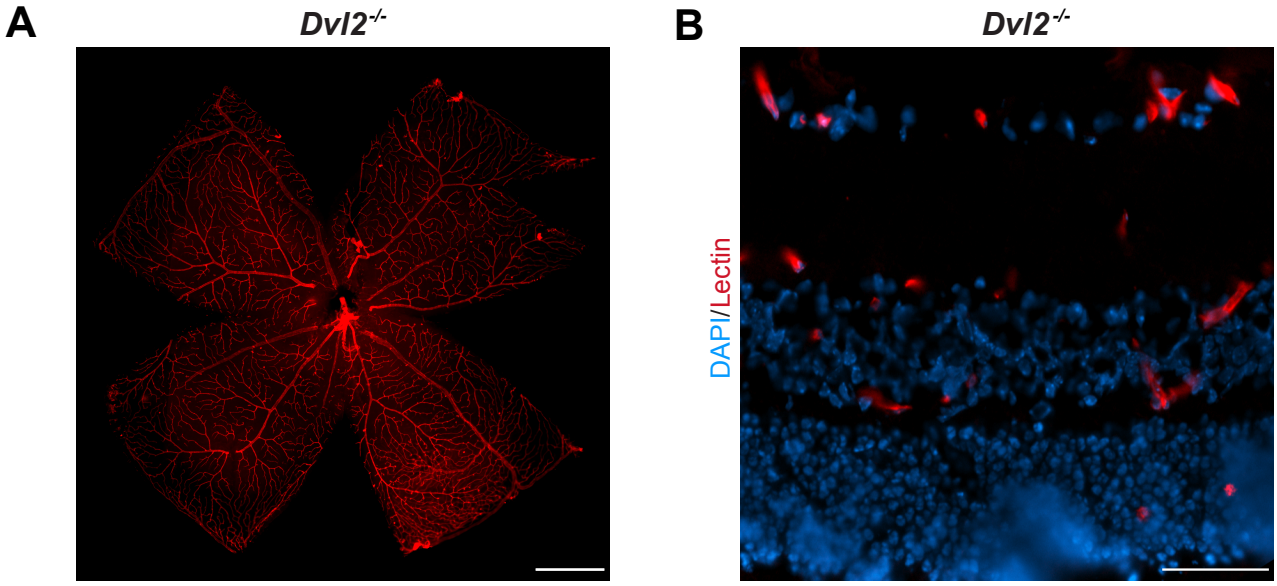
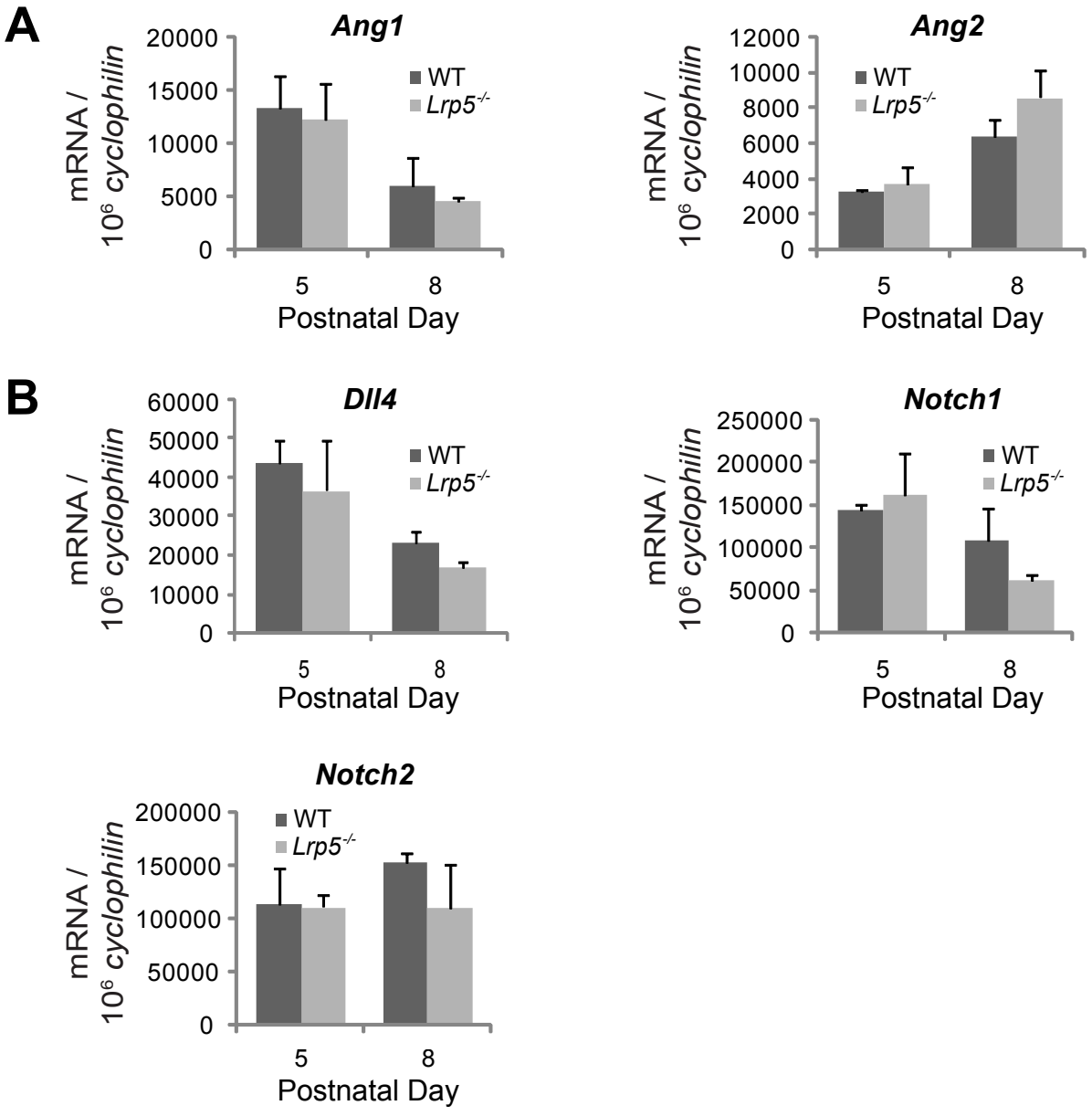


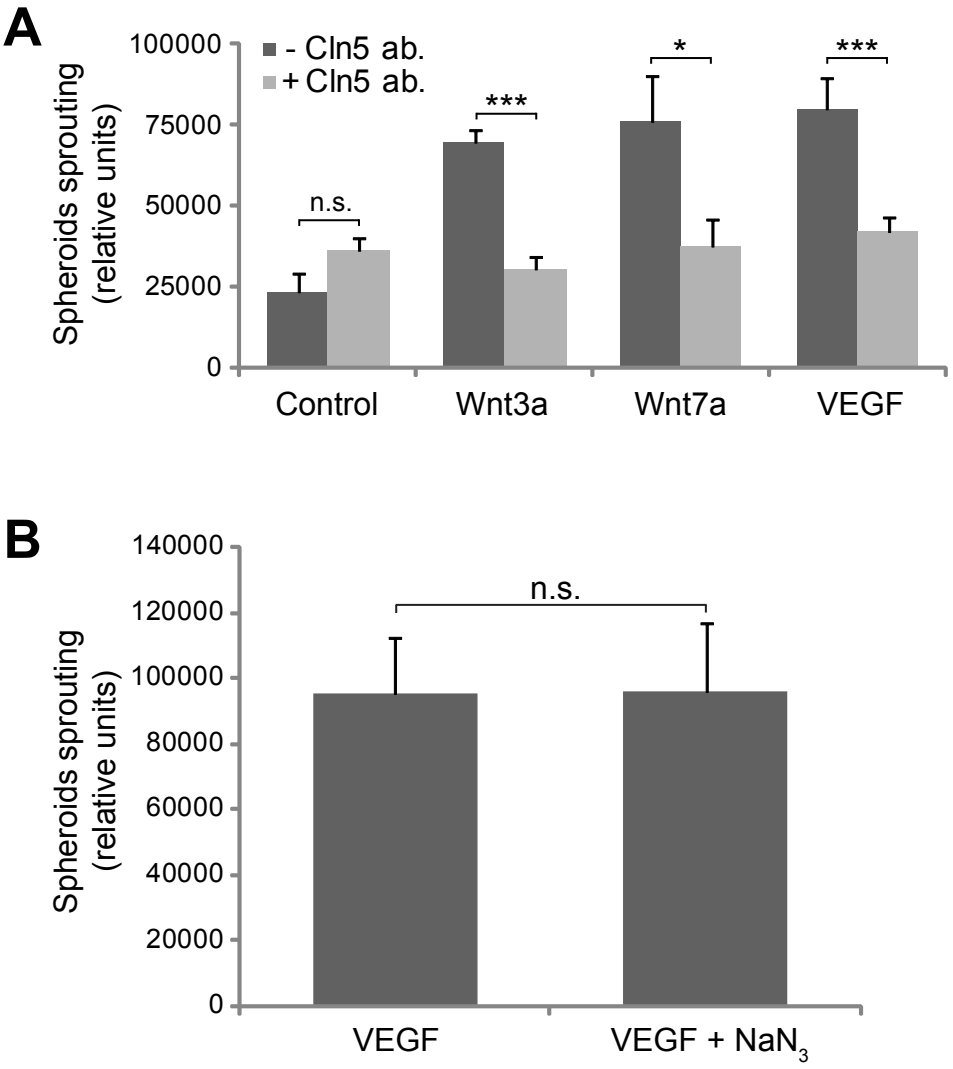
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5