Supplemental Material and Methods

Atoh1/ EGFP electroporation studies

E13.5 cochleas were dissected and the ducts opened before placing on the culture inserts. Cochleas were incubated in 5% CO_2 for 24 hours at 37°C. The following day, cochleas were electroporated as previously described (Hayashi et al., 2010) with plasmids containing i) EGFP driven by the EF1 promoter or, ii) both EGFP driven by EF1 promoter and *Atoh1* driven by the EF1 promoter (Gubbels et al., 2008). The cochleas were cultured for 4 additional DIV before fixation and immunolabeling.

In situ hybridization on serial sections

cDNA templates were reverse-transcribed for *Wnt7b*, *Wnt5a* and *Wnt7a* from total RNA extracted from embryonic cochleas (Roche). *Wnt7b* has three splice variants and *Wnt5a* has two splice variants, while *Wnt7a* has only one splice variant (NCBI). Therefore, we utilized anti-sense probes that detected all isoforms. We synthesized probe templates against consensus sequences across multiple transcript variants by PCR and sub-cloned into pCR2.1-TOPO (Life Technologies). Primer sequences are as follows: *Wnt7b* 5'-*ttctttgcttctggagctg*-3' and 5'-*ctcataatataaggaccctgaagca*-3'; *Wnt5a* 5'-*gctttggccacgtttttct*-3' and 5'-*tccacaatctccgtgcact*-3'; and *Wnt7a* 5'-*atcacaggcaggatacagtttc*-3' and 5'-*ggggatggttccctcttgt*-3'. Digoxygenin-labeled anti-sense probes were transcribed with T3 or T7-specific primers (Roche). In situ hybridizations were performed on E12.5-E15.5 cryosectioned embryos and probed at the same time to gauge relative Wnt gene expression across ages, as previously described (Sienknecht and Fekete, 2008).

Imaging

High-resolution Z-stack images were acquired on a Nikon Eclipse 90i confocal with D-Eclipse C1 camera or a Zeiss LSM 710 confocal. Images were processed with NIS-Elementz and Photoshop CS6. HC counts and area measurements were analyzed using the NIS-Elementz software or Image J. All images were acquired with a 10X objective at high resolution and accompanying inset images were digitally cropped in Photoshop CS6, unless noted otherwise in the legends. In situ hybridization images were acquired on a Nikon Eclipse E800 DIC/Epifluorescence microscope with a SPOT Flex camera. Scale bars in all figures are 200 μm, unless specified. S: saccular macula

Quantification and Statistics

Cochlear lengths were measured based on Sox2 labeling across 4 conditions (control, day0 CHIR, day1 CHIR and day3 CHIR). Area measurements on day1 were quantified based on Sox2/Prox1 labeling to identify total and lateral compartment areas in the whole cochlea. The medial compartment area was calculated by subtracting the lateral area from the total area. Two-tailed t-tests were performed between control and day1 CHIR-treated cochleas to compare total areas, medial areas, lateral areas, and medial/total area ratios. Area measurements on day0 and day3 CHIR-treated cochleas were compared to controls using a one-way ANOVA test, followed by a Dunnett's multiple comparison analysis. HC counts were analyzed using a one-way ANOVA test on cochleas from Atoh1-GFP$^{+/-}$ mice that were labeled with Prox1/Sox2 to distinguish HCs of each compartment. IHCs and OHCs were further differentiated by a natural gap that forms due to the pillar cells. A Dunnett's multiple comparison analysis was used to determine significance of treatment groups to controls.

Supplemental Figures

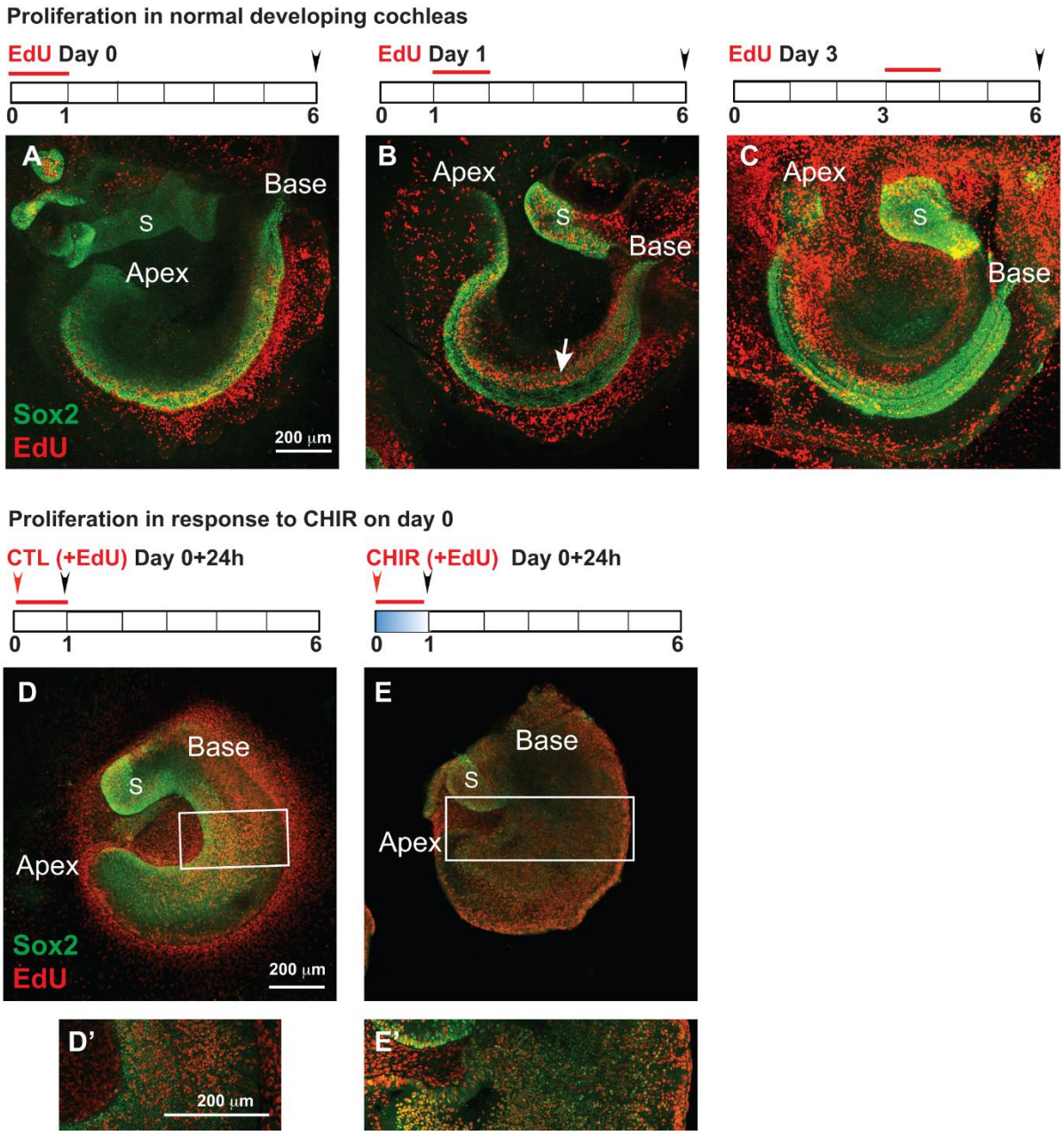

Figure S1: Proliferation was assessed in normally developing cochleas *in vitro* (A-C) (n=3 per condition). EdU for 24 hours was pulsed during day0, day1 and day3. EdU was washed out, chased until 6DIV and immunolabeled for Sox2 and EdU. A) After a 24-hour pulse of EdU on day0, proliferating cells were present across the OC. B) An EdU pulse on day1 shows that the zone of non-proliferation was already established. The future GER fell outside this zone and was actively proliferating (arrow). C) On day3, there was relatively minimal

proliferation within the sensory domain. Cells that appear to be proliferating in the sensory domain are localized to a different focal place in the overlying and underlying mesenchyme surrounding the cochlea. D,E) CHIR treatment of cochleas on day0 stimulated proliferation after 24 hours. D) Control cochlea treated with EdU for 24 hours on day0, fixed and labeled for EdU/Sox2 (n=8). D') Region across the radial axis in the mid-cochlea with a 60X objective. E) CHIR-treated cochlea treated with EdU for 24 hours on day0, fixed and labeled for EdU/Sox2 (n=8). E') Region across the radial axis in the mid-cochlea with a 60X objective.

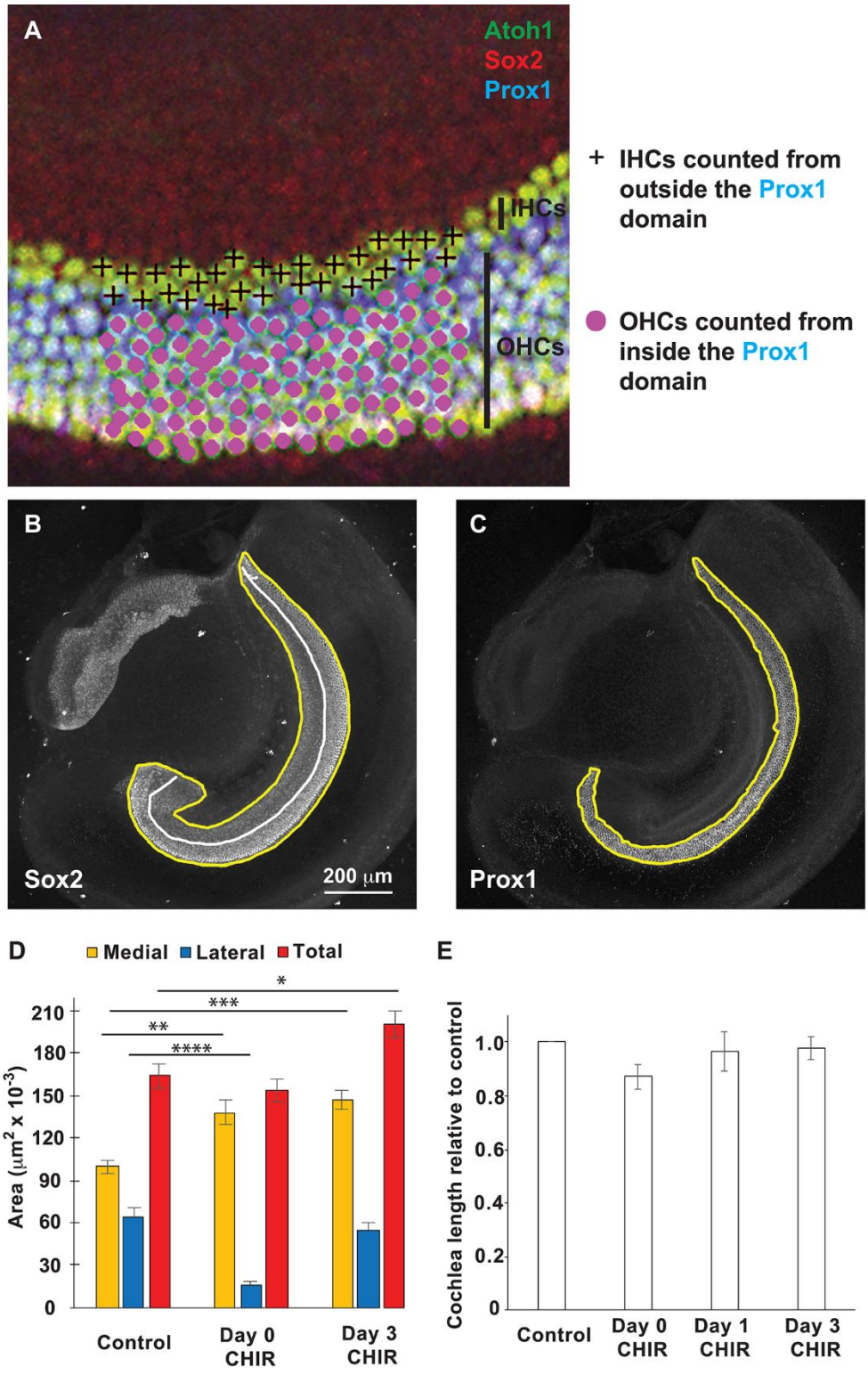

Figure S2: Quantification of compartments. A) IHC (+) and OHC (purple dot) counts were performed on Atoh1-GFP cochleas co-labeled for Sox2 and Prox1. IHCs reside in the Sox2-positive/Prox1-negative domain, while OHCs reside in the Sox2-postive/Prox1-positive

domain. B) The total area of the sensory domain was measured by outlining the Sox2-positive sensory domain (yellow). The length of the cochlea was measured down the midline of the sensory domain along the longitudinal axis (white line). C) The lateral compartment of the sensory domain was measured by outlining the Prox1-positive domain (yellow). D) Day0 CHIR did not affect the total size of the sensory domain, but increased the size of the medial compartment, while abolishing the lateral compartment. Day3 CHIR increased the total size of the sensory domain and the medial compartment, while the lateral compartment was unaffected. Control (n=9), day1 CHIR (n=10) and day3 CHIR (n=8). One-way ANOVA test was performed and statistical significance was determined post-hoc by Dunnett's multiple comparison analysis. *$p<0.05$; **$p<0.01$; ***$p<0.0005$; ****$p<0.0001$. E) Cochlear lengths for CHIR-treated samples were normalized to controls. N=6 experiments containing all four conditions: control (n=21), day 0 (n=19), day 1 (n=19) and day 3 (n=19). One-way ANOVA test shows no statistical differences in cochlear lengths. Abbreviation: S, saccular macula.

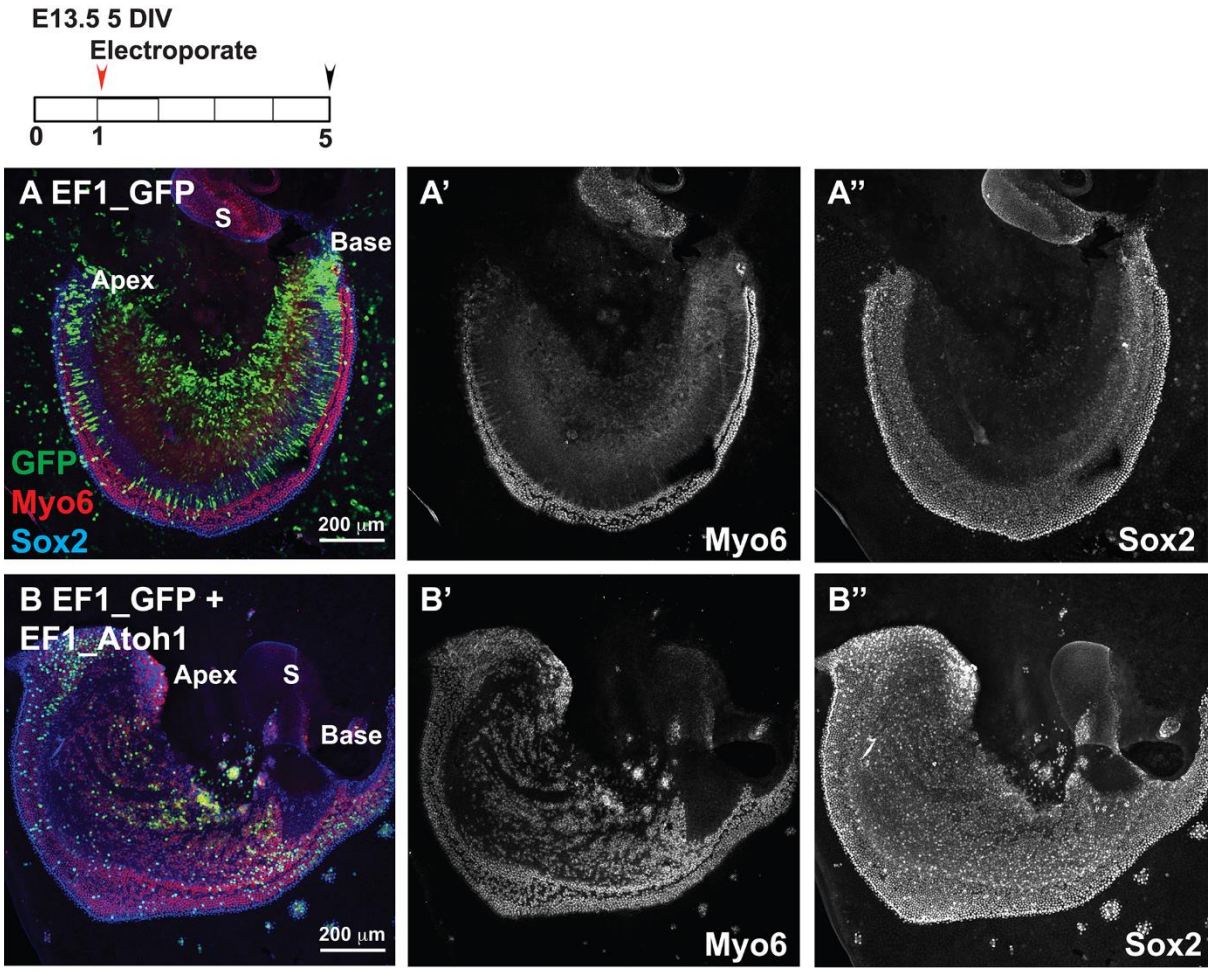

Figure S3: *Atoh1* over-expression induces medial sensory specification. Cochleas (n=5 per group) were immunolabeled for Myo6 and Sox2. A-A") E13.5 cochleas were cultured for 24 hours, electroporated with control EGFP expression plasmid and cultured for an additional 4 DIV. B-B") Cochleas were electroporated with expression plasmids for EGFP and *Atoh1*.

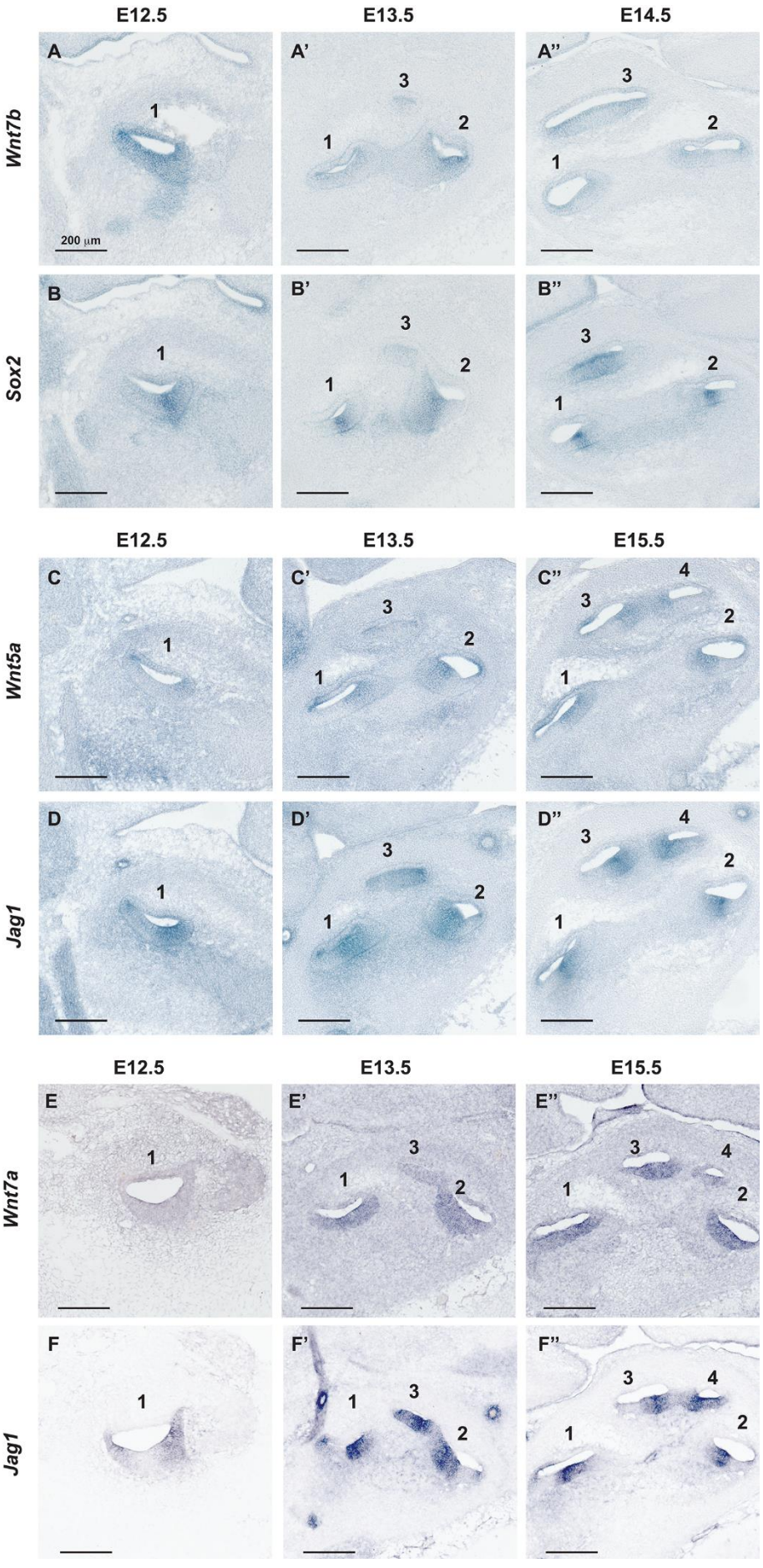

Figure S4: Dynamic expression patterns of Wnt ligand in the mouse during mid-gestation stages. A-A") *Wnt7b* expression series at E12.5, E13.5 and E14.5. B-B") Alternate sections for (A-A") were probed for *Sox2* transcripts. C-C") *Wnt5a* expression series at E12.5, E13.5 and E15.5. D-D"). Alternate sections for (C-C") were probed for *Jag1* transcripts. E-E"). *Wnt7a* expression series at E12.5, E13.5 and E15.5. F-F") Alternate sections for (E-E") were probed for *Jag1* transcripts. At least 2 embryos were analyzed for each time point.