Supplemental Data

Methods

Animals and Genotyping. Foxc1$^{lacz/+}$ mice were generated by T. Kume (Vanderbilt University). Generation and genotyping of the Foxc1-*hith* mutant allele has been described previously (Zarbalis et al., 2007). Timed-matings between *Foxc1*$^{hith/+}$ and *Foxc1*$^{lacz/+}$ to generate the three mutant genotypes were set up at ~4PM and animals were examined for a vaginal plug the following morning; the day the plug was found was E0.5. Details of genotyping are found in the Supplementary Data. The *Rdh10* mutant line was generated from an ENU mutagenesis screen for novel mutants harboring forebrain interneuron migration defects (Zarbalis et al., 2004). Further characterization of the Rdh10 mutant phenotype is forthcoming (Ashique et al., manuscript in preparation). To genotype *Foxc1*$^{l/l}$ embryos, primers that amplify a 482 bp portion of the murine *Foxc1* gene were used (5'-3': cggcgagcagagctactatc and 5'-3': ccttcactgcgtccttcttc). Genotyping *Foxc* $^{h/l}$ embryos was a two step process. First, primers that amplify the neo cassette were used to identify embryos with the *Foxc1-lacz* allele (5'-3': tgctcctgccgagaaagtatccatcatggc and 5'-3': cgccaagctcttcagcaatatcacgggtag). To identify carriers of the *Foxc1-hith* allele, we took advantage of the fact that the point mutation in the *Foxc1* gene eliminates a Bsr1 restriction enzyme site that is normally present in the wildtype *Foxc1* allele. The PCR product of *Foxc1* sequencing primers (described above) was digested with the Bsr1 restriction enzyme and run on a 2% agarose gel. Bsr1 digestion of the WT allele yielded three digestion products (256 bp, 142 bp, 86 bp) whereas the digestion of the *hith* allele results in two digestion products (396 bp and 86 bp).

For injection of atRA (Sigma), a 20 or 30 mg/ml stock solution in DMSO (Sigma) was diluted 1:1 in corn oil. The retinoic acid was given as a once-daily interperitoneal injection from E10.5 to E13.5. Alternatively, pregnant mice were given atRA-enriched food (250 mg atRA/kg

food; Harlan Teklab Custom Diets) ab libitum beginning on the afternoon of E9. On average, mice consumed 20-30 mg atRA/kg b.w.

Brain explant and meningeal cultures. On E13.5, the pregnant dams were injected with BrdU and the fetal brains collected 1 hour later. The brains, with meninges intact, were embedded in 3% SeaPlaque low-melt agarose (Cambrex) and cut into 300 μm coronal sections with a vibratome (Leica). For the transplantation experiment, a control and *Foxc1*$^{h/h}$ brains or two control brains were embedded in the same agarose block and sectioned simultaneously. This was done to ensure that the transplanted slice was being transferred to control meninges from same relative area of the brain. The transplanted explants were then cultured at 37°C, 5% CO_2 for 32 hours post-BrdU injection in 3ml Neurobasal (NB) medium (Invitrogen) supplemented with 2% B27 with vitamin A (Invitrogen), 2 mM L-glutamine, 100 mM dextrose and 100 μM penicillin/streptomycin (UCSF Cell Culture Facility San Francisco, CA) on 0.40 μm filter inserts (Millipore) in 60 mm plates (Costar). Meningeal cultures were obtained by removing the forebrain meninges from ~20, E16.5 WT brains and collected in DMEM/F12 medium containing 1mg/ml Collegenase/Dispase (Roche). Following titration with a glass pipette, the cells were pelleted via centrifugation and resuspended in DMEM/F12 (Invitrogen), 10% fetal bovine serum (FBS), 2 mM glutamine, 100 mM dextrose, 100 μM penicillin/streptomycin. Four days prior to the conditioned medium experiment, cells were plated on collagen-coated, 6-well plates (Costar) at a density of 1.0×10^5 cells/well; the cells were at confluency when the experiment began. For the meningeal conditioning experiments, DMEM/F12 media was removed 2 days prior to the explant experiment and replaced with 1.5 ml of NB media containing the B27 supplement with or without vitamin A. Thus, the media was conditioned for 48 hours prior to the explants

experiment. Filter inserts were placed in the wells containing the meningeal cells/conditioned media and *Foxc1*$^{+/}$ (control) or *Foxc1*$^{h/h}$ explants were transferred into the inserts and cultured for 36 hours post BrdU injection. For the RA depletion experiments, meningeal cells were cultured on 10 cm culture plates in 10 ml of NB media with B27+vitamin A for 3 days prior to the explant experiment. On the day of the explant experiment, the conditioned media was removed from the cells and 5 ml was put in a foil-wrapped 15 ml conical and stored at 4º C until use and the other 5 ml was exposed to direct sunlight for 2.5 hours. For treatment with atRA, a stock solution of 100 mM atRA dissolved in DMSO was first diluted to 100 μM in NB medium then added at a final concentration of 10 μM. Following culturing, all explants were fixed for 30 min in 4% paraformaldehyde, cryoprotected and cryosectioned in 12 μm increments. Sections were immunolabeled for BrdU and Ki-67 (see *Immunostaining*) and analyzed for cell cycle exit as described above. For the meningeal transplant experiment, 13 *Foxc1*$^{+/}$ and 16 *Foxc1*$^{h/h}$ explants hemispheres from two separate transplantation experiment were analyzed (n=2). For the meningeal conditioned medium experiments using B27+vitamin A, 22 *Foxc1*$^{+/}$ and 30 *Foxc1*$^{h/h}$ explants hemispheres from 7 litters were analyzed (n=7). For the meningeal conditioned media experiments using B27-vitamin A, 9 *Foxc1*$^{+/}$ and 10 *Foxc1*$^{h/h}$ explants hemispheres from 2 litters were analyzed (n=2). For the atRA-depleted meningeal conditioned media experiments using, 5 *Foxc1*$^{+/}$ and 11 *Foxc1*$^{h/h}$ explants hemispheres from 3 litters were analyzed (n=3). For the atRA treatment, 6 control explants hemispheres and 13 *Foxc1*$^{h/h}$ slices from three litters were analyzed (n=3).

Immunohistochemistry. Tissue was processed and sectioned as described in Methods. Cells were fixed for 15 minutes in 4% paraformaldehyde and processed for immunohistochemistry as

described in Zarbalis et al., 2007. Dilutions and origins of the Zic, Foxc1, Tbr2, Tuj1, Ki-67, and Ctip2 antibodies are described in methods of the main text. The following antibodies and dilutions were used: rabbit anti-activated Caspase-3 (BDBioscience) 1:200, rabbit anti-Pax6 (Covance) 1:100, rabbit anti- pan-laminin (Sigma) 1:100, mouse anti-nestin (Millipore) 1:500.

Cell cycle length analysis. The total length of the cell cycle (T_c) and the length of S-phase (T_s) for cells in the VZ were calculated using spaced doses of the thymidine analogs idodeoxyuridine (IdU; Sigma) and bromodeoxyuridine (BrdU; Roche) (Martynoga et al., 2005; Siegenthaler et al., 2008). Briefly, the pregnant dam was injected with 50 mg/kg b.w. IdU followed by a BrdU (50 mg/kg b.w.) injection 1.5 hours later. The embryos were harvested 2 hours post IdU injection and processed for double immunohistochemistry using two BrdU antibodies and counterstained with DAPI (to label all cells in the VZ): one recognizes only BrdU (rat anti-BrdU; Novus) and a second that detects both BrdU and IdU (mouse anti-BrdU; BDBioscience). Cell counting and T_c and T_s calculations were performed as previously reported.

atRA production by meningeal fibroblasts and retinoid quantification in meninges/brain. Unconditioned media and meningeal conditioned media was collected in low light conditions and tissue samples from three separate litters (n=3) were collected under yellow lights. Tissue was homogenized in 1 mL 0.9% NaCl, extracted, and LC/MS/MS was used as described previously to measure atRA levels in both tissue and media samples (Kane et al., 2008).

References

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Figure Legends

Supplemental Figure 1.

(A-B) Sections through the forebrain of WT and the three *Foxc1* mutants lines at (A) E10.5 and (B) E12.5 immunolabeled with Tuj1 (green) and Ki-67 (red).

(C) At E14.5 in the WT forebrain, Pax6 (red) and Tbr2 (green staining was restricted to the proliferative zones of the pallium. This same pattern of staining was also observed in the *Foxc1* mutant lines.

(D) Higher magnification of Pax6 in the dorsal forebrain revealed no differences in the width of the Pax6 labeled ventricular zone between wildtype and *Foxc1* mutants.

(E) Analysis of cell cycle length revealed that cells in the VZ of WT and the Foxc1 mutant lines were cycling at the approximately the same rate.

(F) Low magnification images of E13.5 WT and *Foxc1*$^{l/l}$ sections labeled with nestin (green) and laminin (red).

(G) Higher magnification images of the nestin/laminin staining highlight the radial organization of the nestin+ fibers in both genotypes. Insets in G show co-localization of nestin+ radial endfeet and laminin at the *glia limitans* (arrows) in both WT and *Foxc1*$^{l/l}$ cortices.

(H) Confocal images of nestin+ radial glial fibers in WT and *Foxc1*$^{l/l}$ cortices show individual radial glial fibers coursing from their attachment point at the *glia limitans* (identified by the dotted line) into the cortical plate (arrows).

Scale bars = (A) 250 µm (B-C, F) 500 µm; (D) 100 µm.

Supplemental Figure 2.

(A-B) The majority of cells in the meningeal cultures expressed Foxc1+ and Zic+ through there were a few cells with smaller nuclei that did not stain with either meningeal marker.

(C) The table reports the amount of atRA in pM detected in unconditioned or meningeal conditioned Neurobasal media containing B27+vitamin A (VA), meningeal conditioned media containing B27-VA, and meningeal conditioned media containing B27-VA exposed to direct sunlight for 2.5 hours.

Supplemental Figure 3.

(A-B) Low magnification of E13.5 WT (A) and *Rdh10* (B) mutants immunolabled with a pan-Zic antibody (green) and counterstained with DAPI (blue). Note that brightly labeled Zic+ cells surround the entire *Rdh10* mutant cortex.

(C-D) Higher magnification images of the cortex and meninges show Zic+ meningeal layer in the *Rdh10* mutants.

Scale bars = (A-B) 500 µm (C-D) 50 µm.

Supplemental Figure 4.

(A-C) Low magnification images of atRA-treated WT (A), *Foxc1*$^{h/l}$ (B), and *Foxc1*$^{l/l}$ (C) brains immunolabeled with anti-pan-Zic (green) and DAPI (blue). Note that Zic+ meninges are missing from part and all of the overlying mesenchyme in *Foxc1*$^{h/l}$ and *Foxc1*$^{l/l}$ brains, respectively. Higher magnification images of the boxed areas in A-C show the continuous layer of Zic+ meninges in the WT and the stop point (indicated by a dotted line in B') in the atRA *Foxc1*$^{h/l}$ despite a normal looking cortex. Despite the absence of the meninges, Zic+ positive Cajal-Retzius cells are present in the marginal zone of atRA-treated *Foxc1*$^{h/l}$, and *Foxc1*$^{l/l}$ mutant brains (arrows in B' and C').

Supplemental Figure 5.

(A) Low magnification images of three, E14.5 RA-treated *Foxc1*$^{h/l}$ mutants from two separate litters reveals the variability in forebrain elongation rescue by atRA.

(B) Activated-caspase 3, which labels cells undergoing apoptotic cell death, was prominent in the supracortical mesenchyme in RA-treated *Foxc1*$^{l/l}$ mutants though not in the brain (arrows). The cells in the cortex, however, appeared rounded and unhealthy.

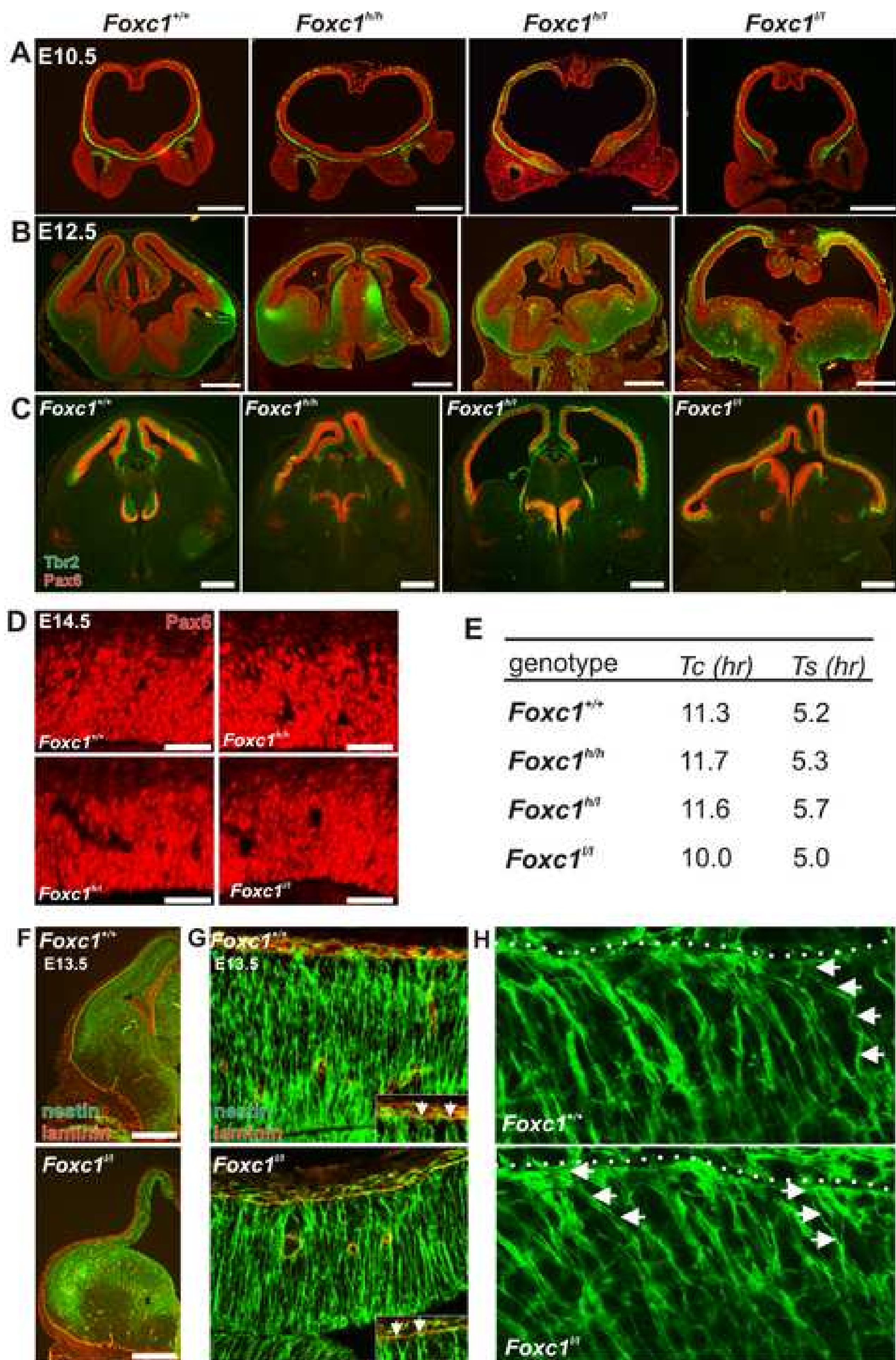
(C-D) BrdU (red) and Ki-67 (green) double immunolabeling of atRA-treated WT, *Foxc1*$^{h/h}$, and *Foxc1*$^{h/l}$. Quantification of the percent BrdU+/Ki-67- revealed that atRA-treatment significantly increased cell cycle exit in *Foxc1*$^{h/h}$ and *Foxc1*$^{h/l}$ cortices as compared to untreated mutant counterpart (indicated by *). Cell cycle exit in atRA-treated *Foxc1*$^{h/h}$ was not significantly different than untreated WT however cell cycle exit in atRA-treated *Foxc1*$^{h/l}$ was significantly lower than in untreated WT (indicated by #).

(E-F) Tbr2 (green) immunolabeling on sections from untreated WT brain (N) and atRA treated WT (O), *Foxc1*$^{h/h}$ (P), and *Foxc1*$^{h/l}$ (Q) cortices. Quantification reveals a significantly reduced Tbr2 population in untreated *Foxc1*$^{h/h}$ and *Foxc1*$^{h/l}$ cortices as compared to untreated WT and atRA treated WT, *Foxc1*$^{h/h}$, and *Foxc1*$^{h/l}$ (indicated by *#).

(G-H) The intensity of apical aPKCλ (G) immunostaining in atRA-treated *Foxc1*$^{h/h}$ and *Foxc1*$^{h/l}$ neuroepithelium appeared similar to untreated wildtype. Apical Par3 (H) was also comparable to WT in atRA-treated *Foxc1*$^{h/h}$ and *Foxc1*$^{h/l}$ cortices. Analysis of aPKCλ (G) and Par3 (H) fluorescent intensity revealed no significant difference between WT and atRA treated *Foxc1*$^{h/h}$ and *Foxc1*$^{h/l}$ but both were significantly less than their untreated mutant counterparts (indicated by *#).

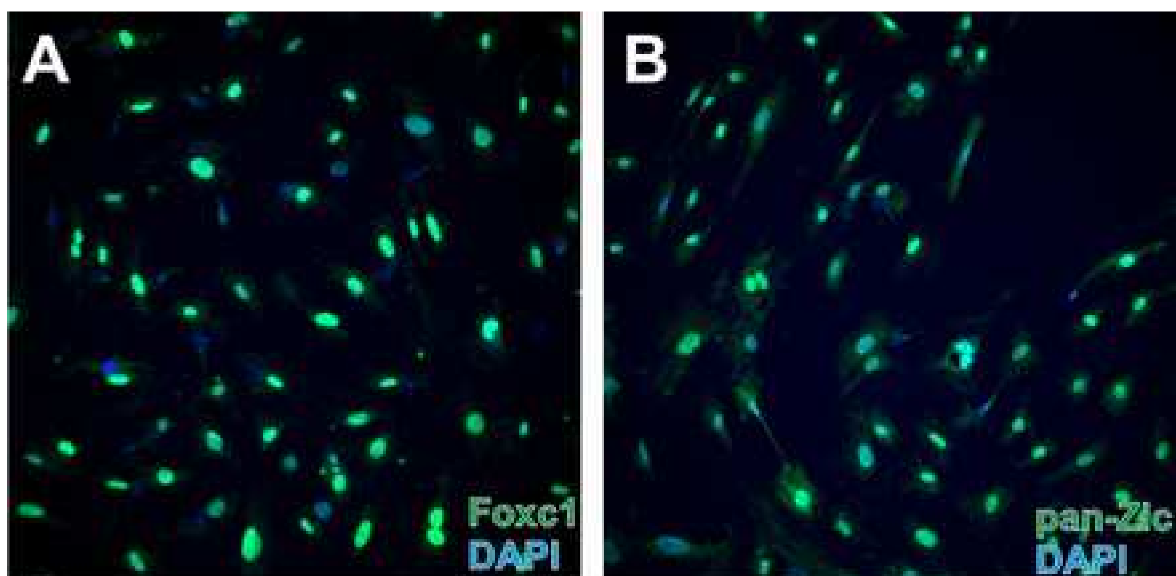
Scale bars = (A) 500 µm; (B) 200 µm (C, E) 100 µm, (G-H) 50 µm.

[G] Supplemental Figure 1



genotype	Tc (hr)	Ts (hr)
Foxc1$^{+/+}$	11.3	5.2
Foxc1$^{h/h}$	11.7	5.3
Foxc1$^{h/l}$	11.6	5.7
Foxc1$^{l/l}$	10.0	5.0

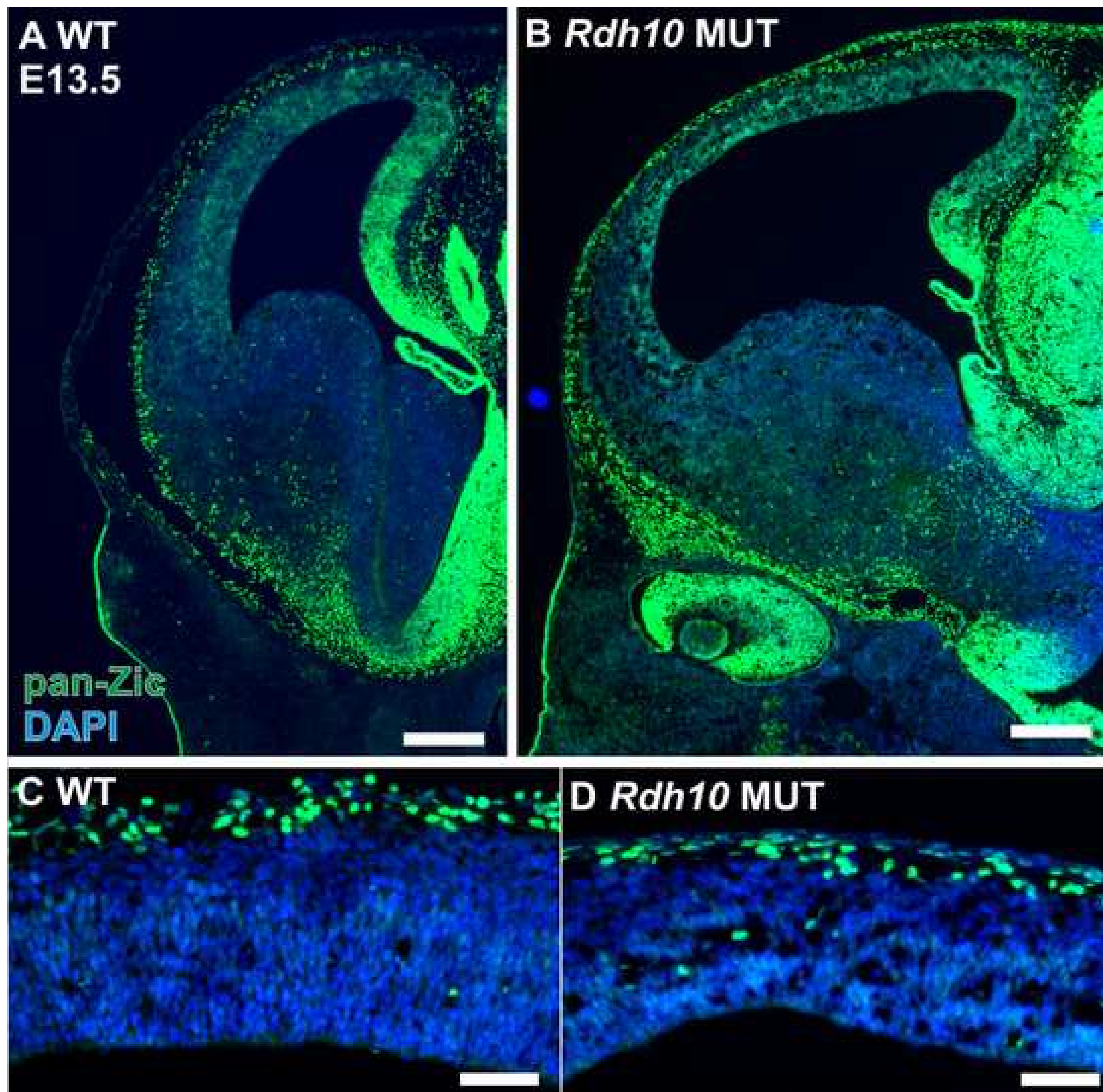
[G] Supplemental Figure 2



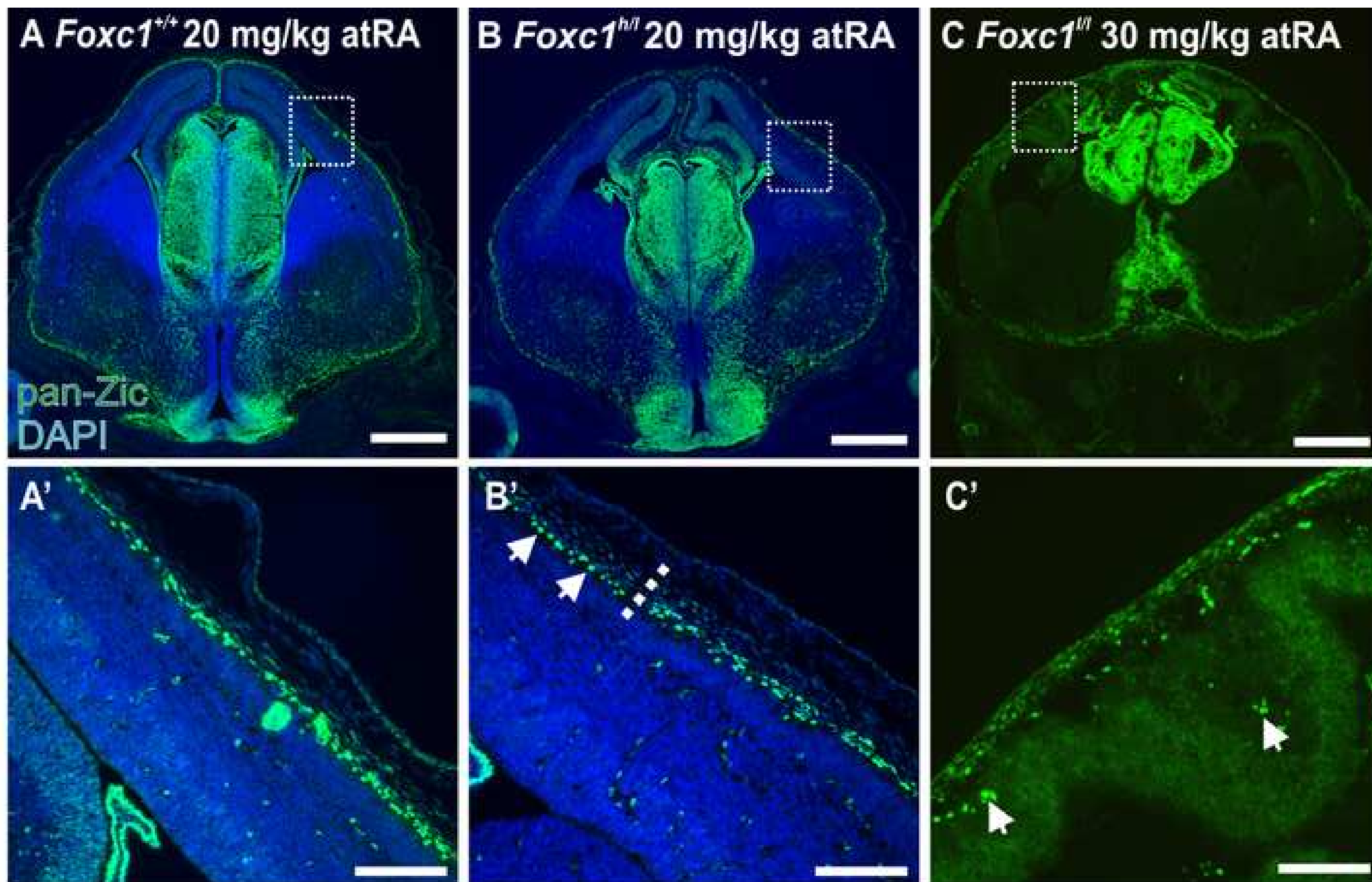
C

Treatment conditions	atRA (pM)
Neurobasal media B27+VA (no cells)	102
Conditioned Neurobasal media B27+VA	507
Conditioned Neurobasal media B27+VA exposed to 2.5 hr sunlight	118
Conditioned Neurobasal media B27-VA	72

[G] Supplemental Figure 3



[G] Supplemental Figure 4



[G] Supplemental Figure 5