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Supplemental Information

Sonic Hedgehog Is a Remotely Produced Cue that Controls Axon Guidance Trans-axonally at a Midline Choice Point

Jimmy Peng, Pierre J. Fabre, Tiphaine Dolique, Shannon M. Swikert, Laëtitia Kermasson, Tomomi Shimogori, and Frédéric Charron

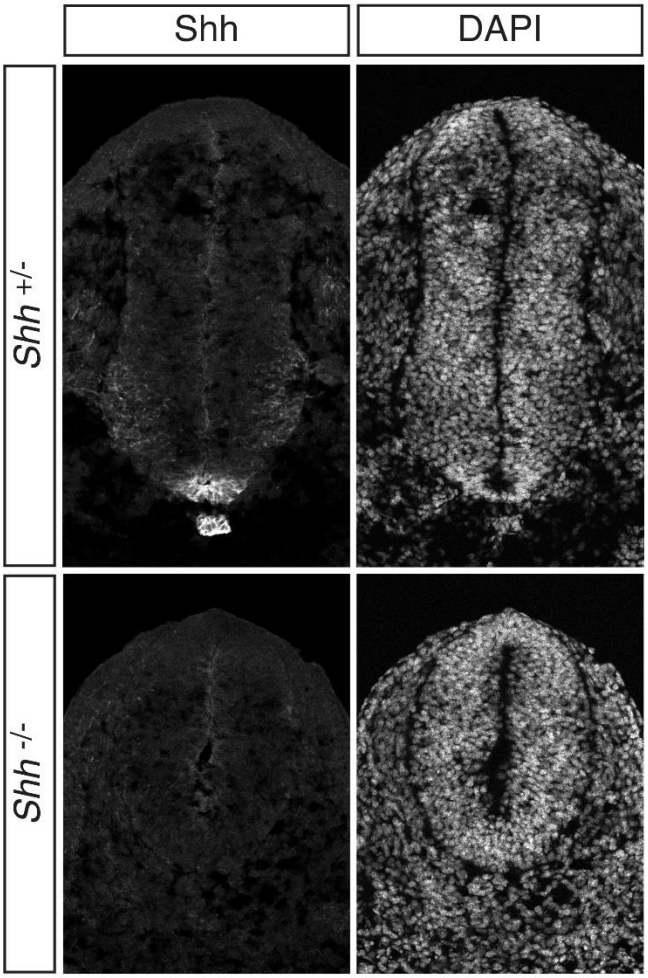

Figure S1. Related to Figure 1: Shh is detected specifically by the 95.9 rabbit anti-Shh polyclonal antibody. E10.5 mouse embryo sections were stained with the 95.9 Shh antibody, which detects Shh in the notochord and floor plate. This signal was lost when staining a *Shh*$^{-/-}$ mutant.

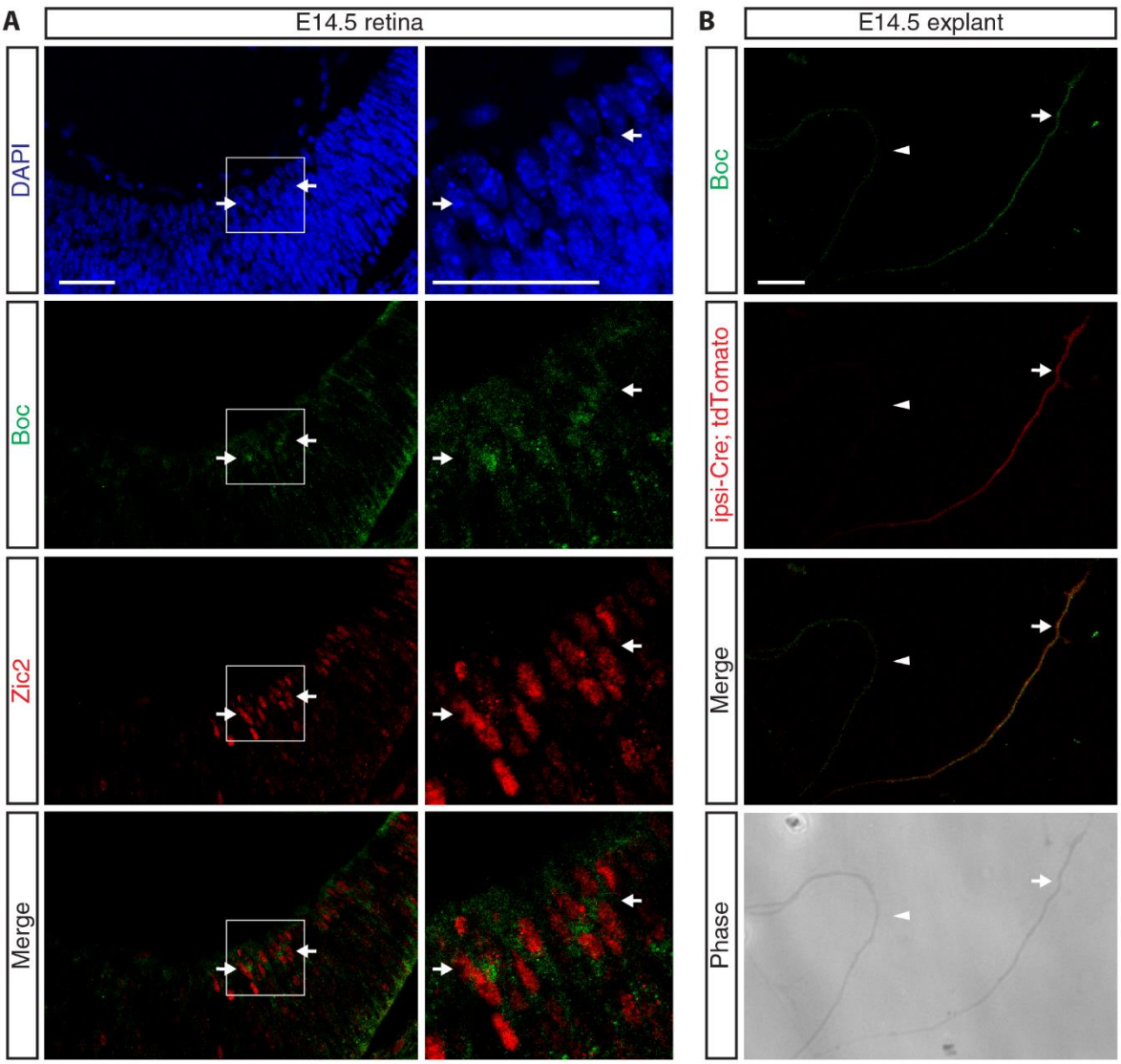

Figure S2. Related to Figure 3 and Figure 5: Boc is expressed by Zic2-positive cells and ipsi-Cre; tdTomato positive axons during midline crossing at E14.5. A) Immunofluorescence images of retina sections of wild-type E14.5 embryos stained with DAPI, Boc, and Zic2 antibodies. Areas of high expression of Boc, a membrane-bound receptor, can be detected surrounding RGC cell bodies positive for the Zic2 nuclear transcription factor (arrows). White boxes indicate the enlarged areas shown in the right column. B) Immunofluorescent and phase contrast images of RGC axons from an explant culture dissected from ipsi-Cre; tdTomato E14.5 embryos. The right RGC axon (arrow) is tdTomato-positive,

indicating it is an ipsilateral axon, and expresses Boc strongly. The left RGC axons (arrowhead) are tdTomato-negative and express residual levels of Boc. Scale bar = 20 μm.

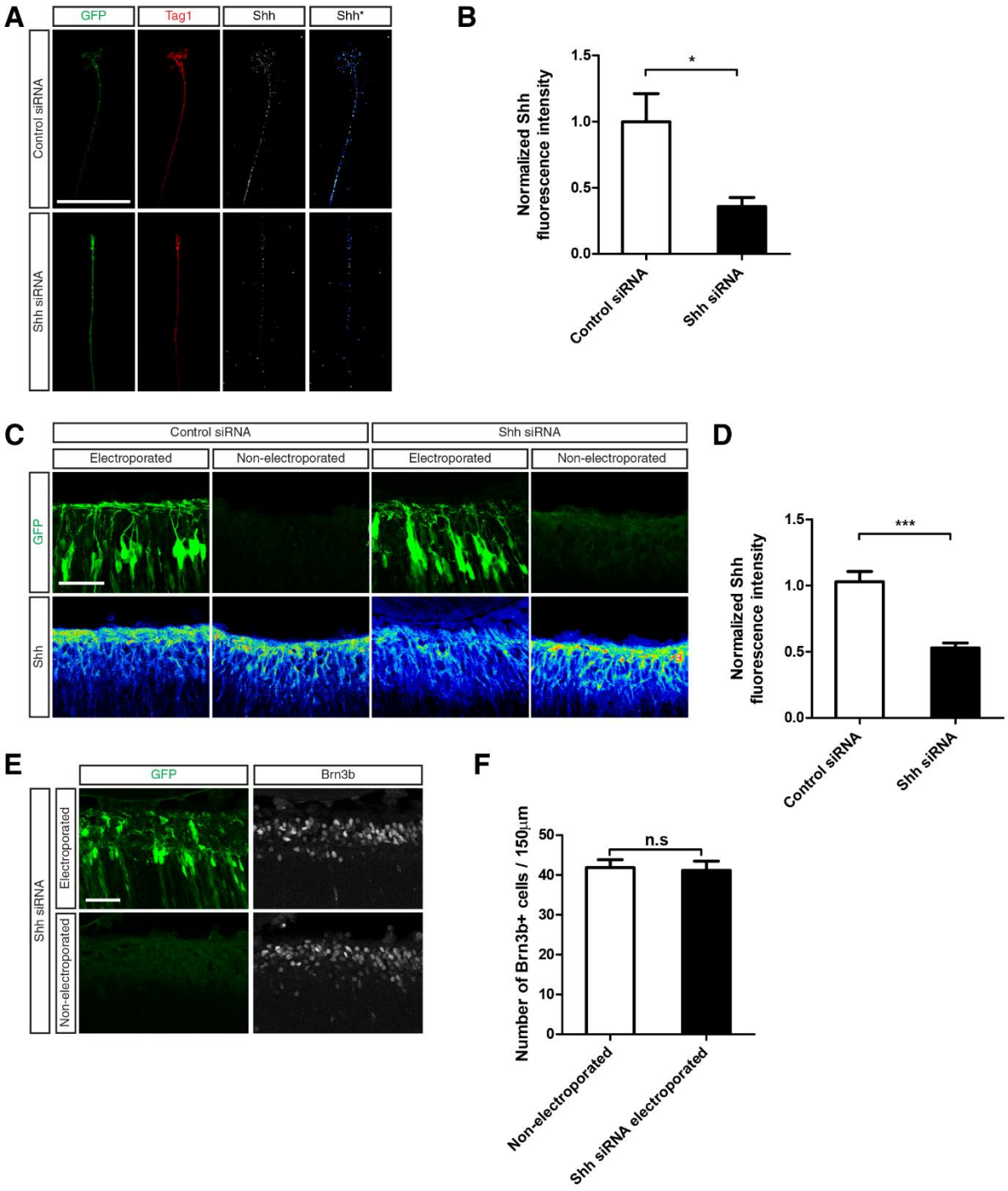

Figure S3. Related to Figure 6: *Shh* siRNA is effective at reducing endogenous Shh levels in RGCs and does not lead to perturbation in RGC specification when electroporated *in utero* at E13.5. A) Non-permeabilized immunofluorescent images of RGC axons from retina explants dissected from wild-type

E15.5 embryos, electroporated with control siRNA+GFP or *Shh* siRNA+GFP, labelled with the RGC marker Tag1 and Shh. Heatmap images of Shh intensity are shown in the Shh* column. B) Quantification of the mean Shh fluorescence intensity of the axon and growth cones of RGCs electroporated with *Shh* siRNA and control siRNA, normalized to the average intensity for control siRNA. The mean Shh intensity is significantly lower in *Shh* siRNA electroporated RGCs compared to control siRNA (control: 1.0±0.21, *Shh*: 0.36±0.07, Student's two-tailed t-test, n=7 & 6, p=0.02). Data points from 7 control siRNA and 6 *Shh* siRNA-electroporated RGCs are included. C) Coronal sections of wild-type E15.5 retina co-electroporated with GFP along with control or *Shh* siRNA, immunostained with Shh antibody. Heatmap images of Shh intensity are shown in the lower panels. The Shh fluorescence intensity is reduced only in the portion of the retina electroporated with *Shh* siRNA. D) Quantification of the mean Shh fluorescence intensity in the RGC axon layer in GFP-positive areas of the retina electroporated with control versus *Shh* siRNA. Each mean Shh intensity value was normalized to an adjacent GFP-negative area of the same retina. The Shh fluorescence intensity was significantly reduced in the *Shh* siRNA electroporated retina compared to control siRNA (control: 1.03±0.07, Shh: 0.53±0.04, Student's two-tailed t-test, n=5 & 6, p=0.0002). Data points from 5 control siRNA retina and 6 *Shh* siRNA-electroporated retina are included, with each retina value being the average of at least 2 sections. E) Coronal sections of wild-type E17.5 retina co-electroporated with *Shh* siRNA and GFP, immunostained with the RGC transcription factor marker Brn3b. RGC cell numbers are unchanged in GFP-positive retina electroporated with *Shh* siRNA compared to non-electroporated retina. F) Quantification of Brn3b-positive cells in a 150 µm length of central retina in non-electroporated versus *Shh* siRNA-electroporated retina. The average number of Brn3b-positive cells is unchanged in non-electroporated versus *Shh* siRNA-electroporated retina (unelectroporated: 41.8±2.0, *Shh* siRNA electroporated: 41.1±2.4, Student's two-tailed t-test, n=4, p=0.83). Data points are from 4 single-eye *Shh* siRNA electroporated embryos. All error bars indicate SEM. n represents number of embryos. *$p<0.05$, ***$p<0.001$, n.s. $p>0.05$. Scale bar = 50 µm.

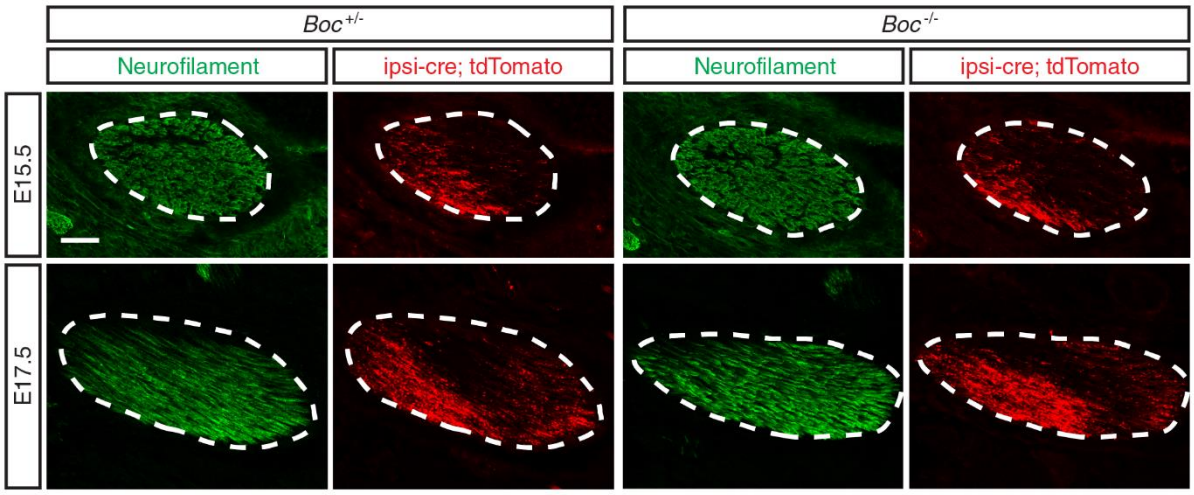

Figure S4. Related to Figure 6: *Boc* does not play a role in ipsilateral RGC segregation along the optic nerve. Coronal sections from E15.5 and E17.5 *ipsi-Cre; tdTomato* embryos were immunostained with the RGC marker neurofilament (NF-M) to visualize the optic nerve (white dashed outline) and anti-dsRed to visualize tdTomato-positive ipsilateral RGC axons. In both $Boc^{+/-}$ and $Boc^{-/-}$ animals, the ipsilateral RGC axons remain in the latero-ventral zone (bottom-left in all images) of the optic nerve. Scale bar = 50 µm.