Neuron, Volume *80*

Supplemental Information

Zic2-Dependent Axon Midline Avoidance Controls the Formation of Major Ipsilateral Tracts in the CNS

Augusto Escalante, Blanca Murillo, Cruz Morenilla-Palao, Avihu Klar, and Eloísa Herrera

SUPPLEMENTAL DATA

Figures

Figure S1

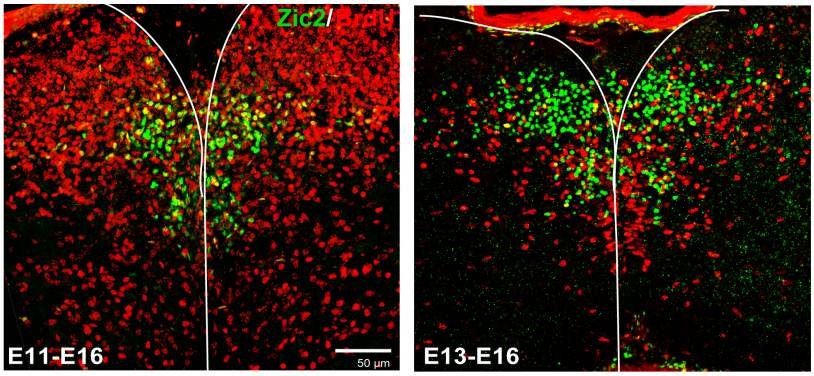

Figure S1. Transverse spinal cord sections from E16 embryos injected with BrdU at E11 and E13 and double-stained with anti-BrdU and anti-Zic2 antibodies show only few double Zic2/BrdU cells.

Figure S2

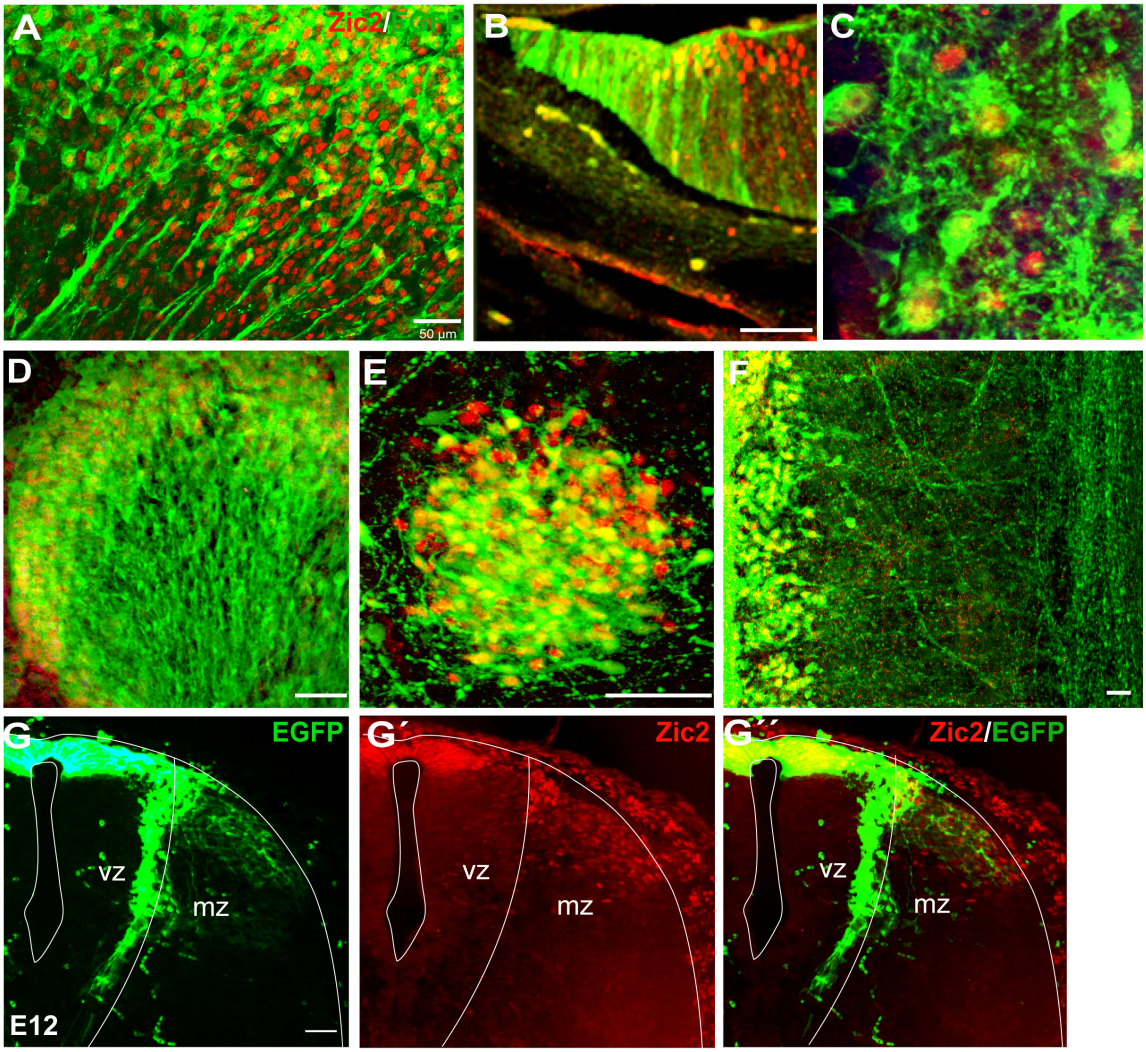

Figure S2. Zic2 reporter mice: EGFP expression recapitulates the endogenous expression of Zic2 in many tissues.

(A-F) Zic2 and EGFP co-localize in different brain areas such as the thalamus (A), ciliary margin in the retina (B), Cajal-Reltzius cells (C), hippocampus (D), amygdala (E) and spinal cord (F).

(G-G´) Transverse spinal cord sections of an E13 $Zic2^{EGFP}$ embryo

Figure S3

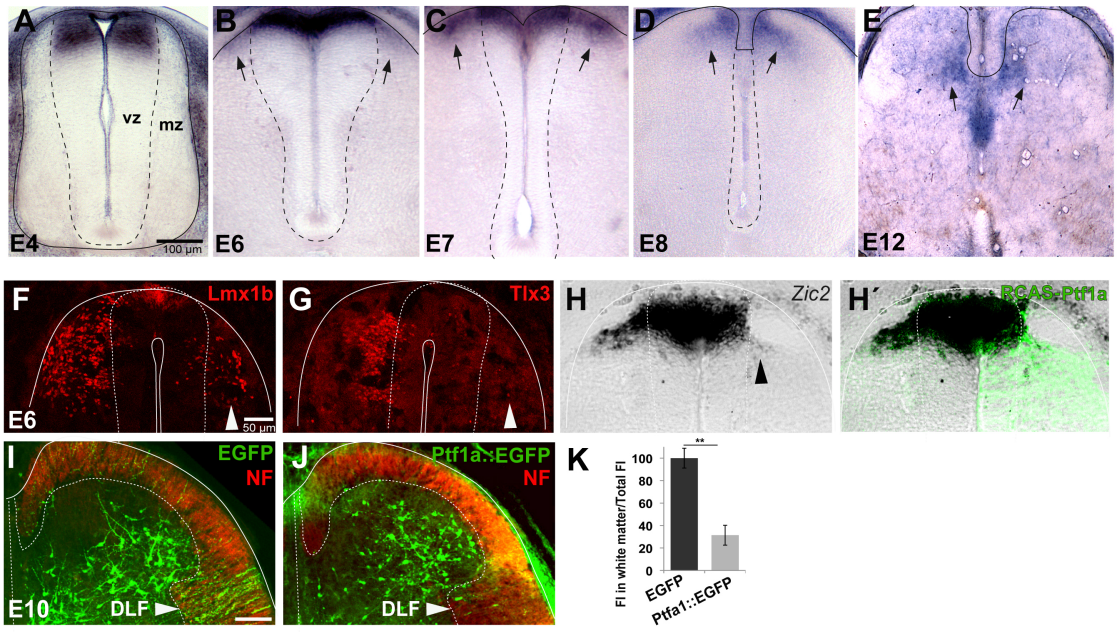

Figure S3. Prf1a-derived GABAergic neurons that project locally or contralaterally, do not express Zic2.

(A-E) *In situ* hybridization against Zic2 in transverse chick spinal cord sections shows a similar spatiotemporal expression pattern of Zic2 in both the chick and mouse.

(F-H´) Transverse spinal sections of E6 chick embryos that were unilaterally electroporated *in ovo* at E4 with Ptf1a-encoding plasmids (RCAS-Ptf1a) show a reduction in the expression of glutamatergic markers (Lmx1b and Tlx3; white arrows) and in the postmitotic expression of Zic2 mRNA (black arrow).

(I-J) Transverse spinal cord sections from E10 chick embryos electroporated *in ovo* at E6 with EGFP-encoding plasmids (CAG-EGFP) or together with plasmids encoding Ptf1a (RCAS-Ptf1a) show that GABAergic neurons do not extend long axons into the dorsolateral fascicle (DLF) or into the rest of the white matter (labeled with neurofilament, NF, red); on the contrary, these axons project locally.

(K) Quantification of the axons in the white matter after electroporation of RCAS-Ptf1a/CAG-EGFP or CAG-EGFP plasmids. Quantification was performed in at least four sections per embryo and three embryos per condition.

Figure S4

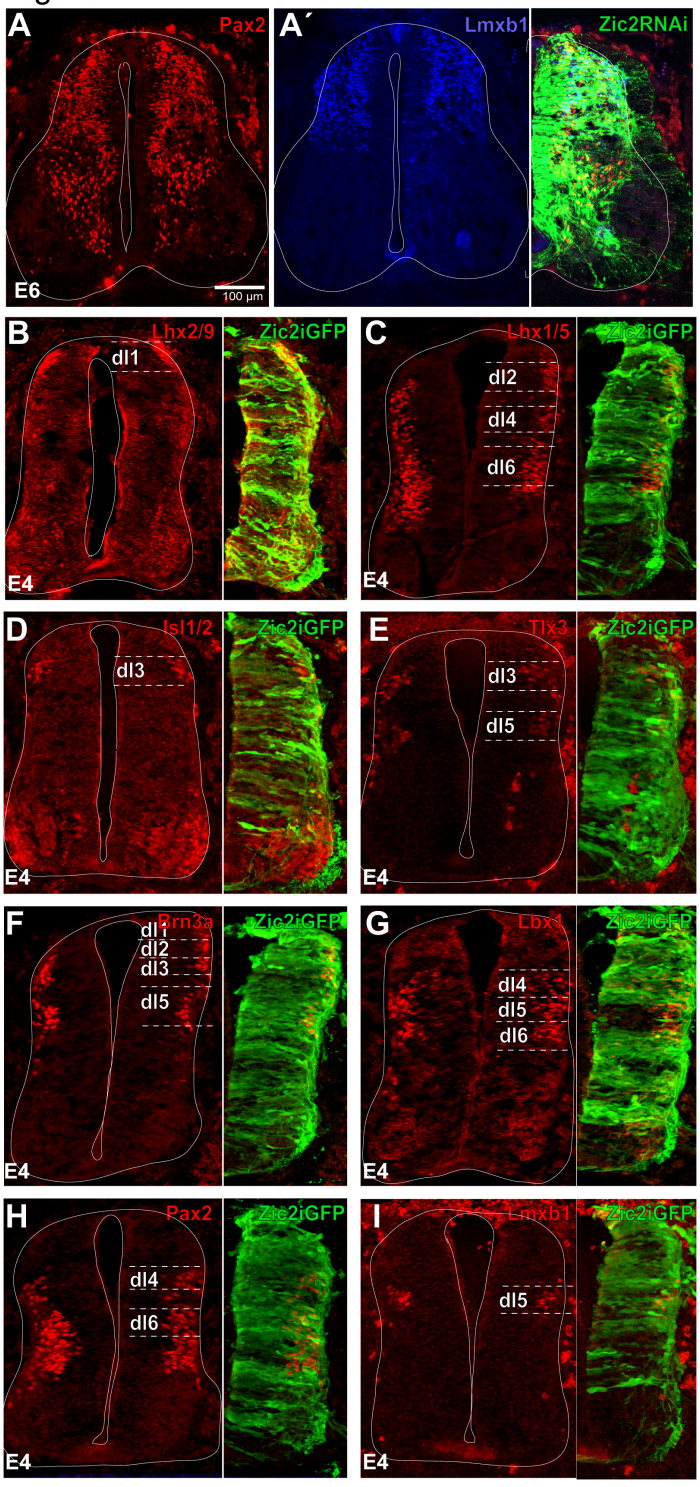

Figure S4. Alterations in the expression of Zic2 do not affect cell fate.

(A) Immunolabeling of Pax2 and **(A´)** Lmxb1 in transverse spinal cord sections of an E6 chick embryo electroporated at E4 with Zic2RNAi shows no alterations in the cell number or localization of dIL neurons in the electroporated compared to the non-electroporated side. The electroporated side is shown on the right.

(B-I) Transverse spinal cord sections of E4 chick embryos electroporated with Zic2iEGFP plasmids at E2 and immunostained with the indicated markers for the different domains of the dorsal spinal cord do not show alterations in cell fate in the electroporated compared to the non-electroporated side. The electroporated sides are shown to the right of the corresponding images.

Figure S5

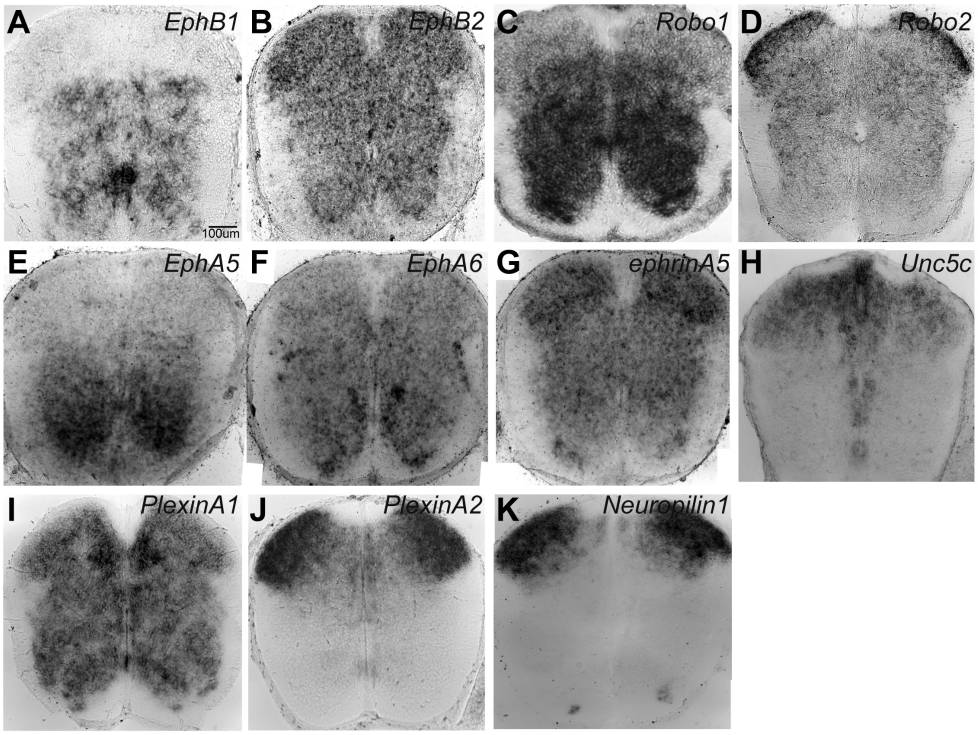

Figure S5. Expression pattern of different guidance receptors in the late developing spinal cord.

(A-K) *In situ* hybridization against the indicated guidance receptors performed on transverse spinal cord sections of E14 mouse embryos shows that the expression patterns of these molecules do not match Zic2 expression at this stage.

Supplemental Experimental Procedures

Mouse Lines. The Tg(Zic2^{EGFP})HT146Gsat/Mmcd line (identification number RP23-158G6) was generated by GENSAT (Gong et al., 2003) and obtained from the MMRRC (http://www.mmrrc.org/strains/17260/017260.html). Zic2 knockdown mice (Zic2^{kd} mice) were obtained from the RIKEN Repository. All mouse lines were congenic on a C57BL/6 background and were kept in a timed pregnancy breeding colony at the Instituto de Neurociencias (IN). The animal protocols were approved by the IN Animal Care and Use Committee and met European and Spanish regulations.

In situ hybridization, immunohistochemistry and BrdU experiments. Whole-mount *in situ* hybridization of chick embryos was performed as previously described (Acloque et al., 2008). *In situ* hybridization of spinal cords and thalamic cryosections (Marcos-Mondéjar et al., 2012) and of the spinal cord vibrosections (Borrell et al., 2005) were performed as previously described. At least 5 sections/embryo and 5 embryos/probe were used for the *in situ* hybridization analyses during the functional experiments. For quantification of EphA4 mRNA, *in situ* hybridization levels were quantified in Photoshop both after electroporation of Zic2 and electroporation of Zic2RNAi by selecting a ROI on the non-electroporated side covering the wildtype pattern of expression in the dorsal horn and using the same ROI on the electroporated side. Levels were normalized to the non-electroporated side. For BrdU experiments five injections of BrdU (50mg BrdU/Kg of body weight) were performed intraperitoneally every 2 hours to label all the dividing cells in E11, E12 or E13 pregnant females. Embryos were analyzed at E16. Cryostat sections were pretreated with HCl and borate to perform the BrdU staining. The antibodies used in this study are indicated in Table S2. The information for the different probes used in this article is indicated in Table S3.

ChIP assays. For ChIP-qPCR analysis, chromatin was obtained from 7 E16.5 mouse spinal cord as previously described (Lopez-Atalaya et al., 2011). Diluted chromatin was then incubated overnight at 4ºC with Zic2 antibodies or rabbit IgG ChIP grade. Immunocomplexes were pulled-down with protein G-Sepharose (Sigma), washed, eluted and processed for qPCR. qPCR was performed using the following primers: F-5´-GGTCTCCTTCACAACCCCACCG3´ and R-5´-AGGCCCCACCTCATCAGCGG3´ for

the negative control sequence and F-5′-GGGCCAGATTTTCCTCCTCC-3′ and R-5′-GGCTGAGGTCTTTGTCCGTT-3′for the EphA4 promoter region. qPCR samples were analyzed by two dilutions and duplicated. ChIP enrichment was calculated as percentage of input recovery.

In ovo and in utero electroporation. Plasmidic DNA solution was injected in the lumen of the neural tube. A TSS20 Ovodyne electroporator was used for *in ovo* electroporation (5 x 10 ms pulses of 25 V). Chick RNA interference experiments were performed using the pRFPRNAiC plasmid (Das et al., 2006). The chick Zic2 RNAi target sequence was GATCCACAAACGGACGCAC. *In utero* electroporation experiments were carried out in BDL1 (DBA2×C57BL/6) mice as described previously (Garcia-Frigola et al., 2007), with the exception that the DNA solution was injected through the base of the tail into the spinal cord, and electrodes were placed in the lumbar spinal cord. Zic2 shRNA target sequences were designed using the GenScript siRNA Target Finder tool located at https://www.genscript.com/ssl-bin/app/rnai and cloned into the pSilencer2.1 plasmid using the pSilencer Kit (Life Technologies) in accordance with the manufacturer's recommendations. Mouse Zic2RNAi target sequence was GGCCAAATACAAACTGGTC.

Supplemental Tables

Table 1. Sources of the plasmids used in this study

Plasmid	Reference
CAG-IRES-EGFP	This study
RCAS-Ptf1a	Huang et al., 2008
CAG-DsRed2	Borrell, 2010
CAG-GFP	Garcia-Frigola et al., 2007
pSilencer2.1	Ambion
EdI1-Cre	Avraham et al., 2009
CAG-RAGE-IRES-GFP	Avraham et al., 2009
CAG-RAGE-Zic2-IRES-GFP	This study
Ngn1-Cre	Avraham et al., 2009
Foxd3-GAL4	Avraham et al., 2009
UAS-RAGE-IRES-GFP	Avraham et al., 2009
UAS-RAGE-Zic2-IRES-GFP	This study
CAG-Zic2-IRES-EGFP	This sutdy
CAG-Zic2	Garcia-Frigola et al., 2008
CAG-Robo3.1myc	Chen et al., 2008
pRFPRNAiC	Das et al., 2006

Table 2. Sources of the antibodies used in this study

Antibody	Reference
Zic2	Brown et al., 2003
PCNA	Santa Cruz Biotechnologies
HuC/D	Molecular Probes
NeuN	Millipore
BrdU	Abcam
Lbx1	Carmen Birchmeier, Max-Delbrück-Centrum for Molecular Medicine
Pax2	Zymed
Lmx1b	Carmen Birchmeier, Max-Delbrück-Centrum for Molecular Medicine
Tlx3	Carmen Birchmeier, Max-Delbrück-Centrum for Molecular Medicine
Chick NF (8D9)	Developmental Studies Hybridoma Bank
GFP	Aves Labs
RFP	Clontech
Parvalbumin	Sigma
Mouse NF (2H3)	Developmental Studies Hybridoma Bank
Lhx2/9	Thomas Jessell, Columbia University
Robo3	R&D
Rabbit IgG (ChIP grade)	Abcam
L1	Millipore
Calretinin	Swant
Lhx1/5 (4F2)	Developmental Studies Hybridoma Bank
Isl1/2 (39.4D5)	Developmental Studies Hybridoma Bank
Brn3a	Chemicon

Table 3. Sources of the probes used in this study

Probe	Reference	Primers
Chick Zic2	This study	F-CTCAGAGCCACCTCCTGTTC and R-ATACCGTTTCCTCTGTAGCAAG
Mouse Zic2	Herrera et al., 2003	
Mouse Lhx2	Marcos-Mondejar et al., 2012	
Chick Lhx2	T. Jessell, Columbia U.	
Chick Lhx9	T. Jessell, Columbia U.	
Chick Robo3	This study	F-CAGCATGCTGCGGTACCTTCTGA and R-CTCACCGTGATGCGCTCATCCT
Mouse Robo3	This study	Kuwako et al, 2010
Mouse EphA4	This study	F-GGGCCACTGAGCAAGAAA and R-GCCTGGACCAAAGCAATG
Mouse EphrinB2	Williams et al., 2003	
Mouse EphrinB3	This study	F-GTTAGGTTTTGCGGGGCT and R-TTCCTAGCTCCCCAGGCT
Mouse EphB1	Williams et al., 2003	
Mouse EphB2	Williams et al., 2003	
Mouse Robo1	K. Brose	
Mouse Robo2	K. Brose	
Mouse PlexinA1	This study	F-ACCCAATCCCATGATGCTCG and R-AGCTGCAGGGTGAGGTAGTA
Mouse PlexinA2	S. Martínez, I.Neurociencias	
Mouse Neuropilin1	This study	F-CTCCGGAACCCTACCAGAGA and R-ATCCAGTCCTCTCCGTTGGA
Mouse Unc5c	S. Martínez, I. Neurociencias	

Supplemental References

Aruga, J. (2004). The role of Zic genes in neural development. Mol. Cell. Neurosci. 26, 205–221.

Avraham, O., Hadas, Y., Vald, L., Zisman, S., Schejter, A., Visel, A., and Klar, A. (2009). Transcriptional control of axonal guidance and sorting in dorsal interneurons by the Lim-HD proteins Lhx9 and Lhx1. Neural Dev *4*, 21.

Borrell, V. (2010). In vivo gene delivery to the postnatal ferret cerebral cortex by DNA electroporation. J Neurosci Methods *186*, 186–195.

Borrell, V., Cárdenas, A., Ciceri, G., Galcerán, J., Flames, N., Pla, R., Nóbrega-Pereira, S., Garcia-Frigola, C., Peregrín, S., Zhao, Z., et al. (2012). Slit/Robo signaling modulates the proliferation of central nervous system progenitors. Neuron *76*, 338–352.

Brown, L.Y., Kottman, A.H., and Brown, S. (2003). Immunolocalization of zic2 expression in the developing forebrain. Gene Expression Patterns *3*, 361–367.

Chen, Z., Gore, B.B., Long, H., Ma, L., and Tessier-Lavigne, M. (2008). Alternative splicing of the Robo3 axon guidance receptor governs the midline switch from attraction to repulsion. Neuron *58*, 325–332.

Das, R.M., Van Hateren, N.J., Howell, G.R., Farrell, E.R., Bangs, F.K., Porteous, V.C., Manning, E.M., McGrew, M.J., Ohyama, K., Sacco, M.A., et al. (2006). A robust system for RNA interference in the chicken using a modified microRNA operon. Dev. Biol. *294*, 554–563.

Garcia-Frigola, C., Carreres, M.I., Vegar, C., and Herrera, E. (2007). Gene delivery into mouse retinal ganglion cells by in utero electroporation. BMC Dev. Biol. *7*, 103.

Garcia-Frigola, C., Carreres, M.I., Vegar, C., Mason, C., and Herrera, E. (2008). Zic2 promotes axonal divergence at the optic chiasm midline by EphB1-dependent and -independent mechanisms. Development *135*, 1833–1841.

Gong, S., Zheng, C., Doughty, M.L., Losos, K., Didkovsky, N., Schambra, U.B., Nowak, N.J., Joyner, A., Leblanc, G., Hatten, M.E., and Heintz, N. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature 425, 917–925.

Herrera, E., Brown, L., Aruga, J., Rachel, R.A., Dolen, G., Mikoshiba, K., Brown, S., and Mason, C.A. (2003). Zic2 patterns binocular vision by specifying the uncrossed retinal projection. Cell *114*, 545–557.

Huang, M., Huang, T., Xiang, Y., Xie, Z., Chen, Y., Yan, R., Xu, J., and Cheng, L. (2008). Ptf1a, Lbx1 and Pax2 coordinate glycinergic and peptidergic transmitter phenotypes in dorsal spinal inhibitory neurons. Dev. Biol. *322*, 12–12.

Lopez-Atalaya, J.P., Ciccarelli, A., Viosca, J., Valor, L.M., Jimenez-Minchan, M., Canals, S., Giustetto, M., and Barco, A. (2011). CBP is required for environmental enrichment-induced neurogenesis and cognitive enhancement. EMBO J. 30, 4287–4298.

Marcos-Mondéjar, P., Peregrín, S., Li, J.Y., Carlsson, L., Tole, S., and López-Bendito, G. (2012). The lhx2 transcription factor controls thalamocortical axonal guidance by specific regulation of robo1 and robo2 receptors. J. Neurosci. *32*, 4372–4385.

Merzdorf, C.S. (2007). Emerging roles for zic genes in early development. Dev. Dyn. 236, 922–940.

Williams, S.E., Mann, F., Erskine, L., Sakurai, T., Wei, S., Rossi, D.J., Gale, N.W., Holt, C.E., Mason, C.A., and Henkemeyer, M. (2003). Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. Neuron *39*, 919–935.