THE ROLE OF THE TRANSCRIPTION FACTOR ZIC2 AS A DETERMINANT OF AXONAL LATERALITY IN THE SPINAL CORD

Augusto Escalante Rodríguez

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Tesis Doctoral

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THE ROLE OF THE TRANSCRIPTION FACTOR ZIC2 AS A DETERMINANT OF AXONAL LATERALITY IN THE SPINAL CORD

Memoria para optar al grado de Doctor en Ciencias por la Universidad Miguel Hernández presentada por

AUGUSTO ESCALANTE RODRÍGUEZ

Dirigida por

Dra. Eloísa Herrera González de Molina

San Juan de Alicante, 4 de Septiembre de 2013

INSTITUTO DE NEUROCIENCIAS

D. Juan Lerma Gómez, Profesor de Investigación del Consejo Superior de Investigaciones Científicas, Director del Instituto de Neurociencias de Alicante, Centro Mixto Universidad Miguel Hernández-Consejo Superior de Investigaciones Científicas CERTIFICA que:

La Tesis Doctoral titulada: "THE ROLE OF THE TRANSCRIPTION FACTOR ZIC2 AS A DETERMINANT OF AXONAL LATERALITY IN THE SPINAL CORD", ha sido realizada por D. Augusto Escalante Rodríguez, bajo la dirección y supervisión de Dª. Eloísa Herrera González de Molina, y da su conformidad para que sea presentada ante la Comisión de Doctorado de la Universidad Miguel Hernández.

Y para que así conste a los efectos oportunos, firmo el presente Certificado,

en San Juan de Alicante, a 4 de Septiembre de 2013,

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Dª. Eloísa Herrera González de Molina, Científico Titular del Consejo Superior de Investigaciones Científicas, AUTORIZA:

La presentación de la Tesis Doctoral titulada: "THE ROLE OF THE TRANSCRIPTION FACTOR ZIC2 AS A DETERMINANT OF AXONAL LATERALITY IN THE SPINAL CORD", realizada por D. Augusto Escalante Rodríguez, bajo su inmediata dirección y supervisión en el Instituto de Neurociencias (UMH-CSIC) y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernández.

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Table of Contents

List of abbreviations

DCC: Deleted in Colorectal Cancer

DSCAM: Down Syndrome Cell Adhesion Molecule

Robo: Roundabout

BMP: Bone Morphogenetic Protein

Draxin: Dorsal repulsive axon guidance protein

Shh: Sonic hedgehog

NrCAM: Neural Cell Adhesion Molecule

GDNF: Glial cell line derived neurotrophic factor

SCF: Stem Cell Factor

Wnt: Wingless-related MMTV integration site

MDGA2: MAM domain containing glycosylphosphatidylinositol anchor 2

RGC: Retinal Ganglion Cell

VEGF: Vascular Endothelial Growth Factor

Comm: Commissureless

Lola: Longitudinals lacking

dI: Dorsal interneuron

dP: Dorsal progenitor

bHLH: basic Helix Loop Helix

Atoh1: Atonal homolog 1

LIM-HD: LIM homeodomain

Lhx2: LIM homeobox 2

Lhx9: LIM homeobox 9

Zic2: Zinc finger protein of the cerebellum 2

Isl2: Insulin related protein 2

TGF-β: Transforming Growth Factor beta

FGF: Fibroblastic Growth Factor

dIL: Dorsal interneuron late

Ngn: Neurogenin

Ascl1: Achaete-scute complex homolog 1

Tlx3: T-cell leukemia homeobox 3

Gsh2: Gs homeobox 2

Foxd3: Forkhead box D3

Olig3: Oligodendrocyte transcription factor 3

Lbx1: Ladybird homeobox 1

Ptf1a: Pancreas specific transcription factor 1a

Lmx1b: LIM homeobox transcription factor 1b

DF: Dorsal Fascicle

DLF: Dorsolateral Fascicle

Pax2: Paired box gene 2

BAC: Bacterial Artificial Chromosome

EGFP: Enhanced Green Fluorescent Protein

shRNA: short hairpin Ribonucleic Acid

Edl1: Enhancer for dorsal interneuron 1

Edl2: Enhancer for dorsal interneurons 2

CNS: Central Nervous System

RNAi: Ribonucleic Acid interference

iRGC: ipsilateral RGC

cRGC: contralateral RGC

Eph: Eph receptor

List of figures

Chapter I

Introduction

The nervous system is comprised of heterogeneous populations of cells connected to each other through an extensive network of wires. During embryonic development those wires, named axons, grow from the neural cell body location towards their final targets following stereotyped paths (Tessier-Lavigne & Goodman, 1996; Dickson, 2006). More than one century ago the Spanish anatomist Ramón y Cajal proposed the existence of special molecules that guide developing axons to their respective target tissues (Cajal, 1893). These guidance molecules, present in the environment, are detected by specific receptors expressed in the growth cone, a highly motile ameboid structure at the tip of the axon (Cajal, 1892). Later refinements of the initial hypothesis proposed by Cajal led to the four basic molecular mechanisms of axon guidance that we know today: contact-mediated or short-range

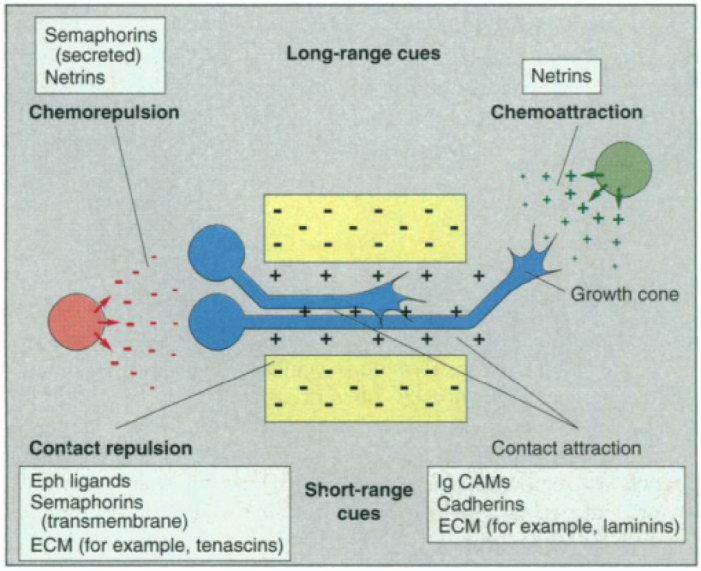

Figure 1. Guidance forces.

Four types of mechanisms contribute to the guidance of growth cones: contact attraction, chemoattraction, contact repulsion and chemorepulsion. Attraction refers here to a range of permissive and attractive effects while repulsion refers to inhibitory repulsive effects. Growth cones might be "pushed" from behind by a chemorepellent (red), "pulled" from a distance by a chemoattractant (green) and confined by attractive and repulsive local cues (yellow). From Tessier-Lavigne and Goodman, 1996.

attraction and repulsion and chemotropism or long-range attraction and repulsion (Goodman, 1996) (Figure 1).

The distances that neural axons have to cover during development to reach their final targets in the brain are, in many cases, very long and complicated. These long distances are subdivided in shorter segments, separated by the so-called intermediate targets. Intermediate targets are specialized groups of cells that provide axons the necessary information to decide which direction to follow during the next segment of their journey. The midline, a structure that separates both halves of the nervous system in species with bilateral symmetry, is one of these intermediate targets or choice-points. The binary decision of whether to cross or not to cross the midline is essential for proper circuit wiring in bilaterally symmetrical species and it has served as a model to investigate the molecular basis of axon guidance decisions.

The emergence of bilateral symmetry in evolution imposed the need for robust lines of communication between brain hemispheres (Colamarino and Tessier-Lavigne, 1995). As a consequence, the formation of contralateral (crossing) or ipsilateral (not-crossing) tracts during development became essential to distribute and integrate sensory

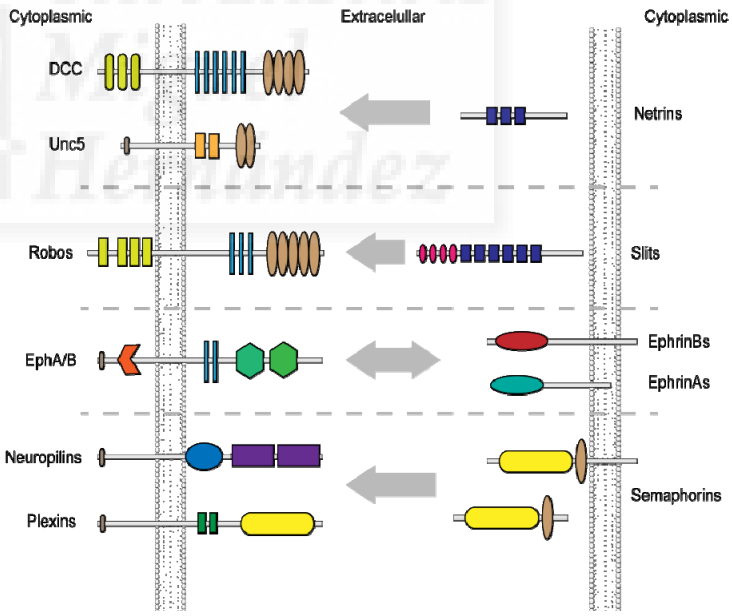

Figure 2. Major families of receptor/ligand pairs in axon guidance.

Representative members of the main families of axon guidance molecules and their receptors. Flow of the signaling cascade is indicated by a thick light grey arrow.

information from both sides of the body to subsequently generate coordinated motor responses (Engle 2010; Izzi and Charron, 2011; Nugent, 2012).

1.1 Axon guidance at the midline

Genetic screens designed to recognize axon midline defects in simple animal models such as *Drosophila melanogaster* and *Caenorhabditis elegans* lead to the identification of many of the axon guidance molecules that we know today (Hedgecock, 1990; Seeger, 1993; Tear, 1996; Kidd, 1998; Kidd, 1999; Araújo and Tear, 2003) (Figure 2). In invertebrates, the Netrin family of diffusible molecules mediates attraction to the midline through its receptors DCC. On the other hand, Slits molecules are responsible for the repulsion of axons expressing Robo receptors and the large families of Semaphorin/Plexin/Neuropilins and Ephrin/Ephs usually signal repulsive responses to either keep

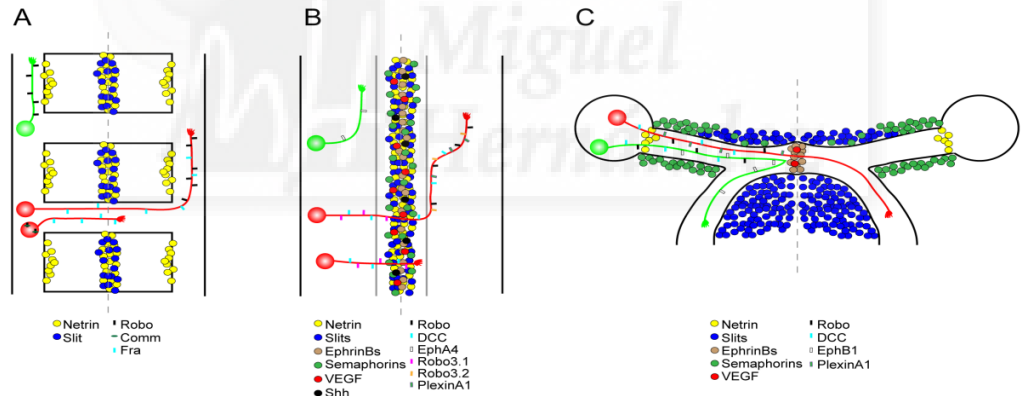

Figure 3. Three models frequently used to study axon guidance at the midline.

(A) Several segments of the ventral nerve cord of *Drosophila* are represented in a ventral view. Ipsilateral (green) neurons express Robo receptors, but not Comm, causing a repulsive response to the slits expressed at the midline. Commissural (red) neurons are first attracted to the midline by Netrin-Frazzled (the *Drosophila* ortholog of the vertebrate DCC) and allowed to overcome the repulsive Slit territory due to the lack of Robo expression in the growth cone which is kept in endosomal compartments by Comm. Following exit of the floor plate on the contralateral side Comm expression is downregulated leading to Robo upregulation, preventing axons to cross back. **(B)** The vertebrate spinal cord is depicted in an open-book configuration. Similar to the ventral nerve cord of insects, Netrin-DCC and Slit-Robo determine the attraction and later repulsion of commissural axons. Here, another member of the Robo family, Robo3, is responsible for keeping commissural axons from becoming prematurely responsive to midline repellents. In addition, several other families of guidance molecules have been shown to play a role in the guidance of commissural axons (see text). On the other hand, very few knowledge has been gathered about axon guidance of spinal ipsilateral axons in vertebrates. Till today, only the expression of EphA4 has been shown to be necessary for avoiding ventral midline crossing.**(C)** The mouse optic chiasm is shown in a horizontal section. A different arrangement of the same guidance molecules controls the guidance of RGCs. While in this model Netrin-DCC is responsible for the exit of axons from the retina and Slit-Robo for keeping them within the limits of the tract, it is the repulsive signaling mediated by ephrinB2-EphB1 binding that determines midline avoidance. Dotted lines indicate the midline. Adapted from Williams, 2004.

axons away or expel them out after midline crossing. Extensive studies in vertebrates, particularly those using the spinal cord and the optic chiasm as models, have demonstrated a high degree of conservation among these guidance molecules throughout evolution (Evans, 2010) (Figure 3).

1.1.1 Molecular mechanisms of midline crossing

In vertebrates, subsequent to the closure of the neural tube, cells from the roof plate secrete diffusible proteins such as BMPs and Draxin. These molecules act as repellents for axons coming from dorsal territories, forcing them to grow ventrally. Pioneer commissural axons from dorsal interneurons expressing DCC grow ventrally attracted by Netrin and Shh, which are secreted by floor plate cells (Keino-Masu, 1996; Ly, 2008; Liu, 2009; Charron, 2003; Okada, 2006; Yam, 2009). In pre-crossing commissural axons, the repulsive signaling mediated by Slits molecules, secreted by the ventral midline, is silenced by the expression of Robo3.1 (Chen, 2008). As commissural axons contact the floor plate, several molecular changes occur in the growth cone provoking a switch from midline attraction to repulsion. This switch involves several families of guidance molecules. DCC is inhibited by Robo1 so the attractive signaling is lost (Stein, 2001); Robo3.2 is expressed instead of Robo3.1 (Chen, 2008) and increased levels of Robo1/2 induce a repulsive response to Slits (Long, 2004). NrCAM inhibits Calpain1 and PlexinA1 accumulates in the growth cone to signal repulsion to Sema3B through Neuropilin2. The switch in response to Sema seems to be mediated by Shh and GDNF (Nawabi, 2010; Charoy, 2012). Additionally, growth promoting activity of the Stem Cell Factor through the tyrosine kinase receptor Kit, is necessary for axons to leave the midline (Gore, 2008) (Figure 4).

Another family of guidance molecules that plays an important role in the regulation of midline crossing in the spinal cord is the Eph/ephrin. EphrinB3 is expressed at the midline while EphA4 is expressed in the growth cone of several populations of spinal interneurons. Upon contact with EphrinB3, these axons will be repelled. Mice mutant for EphA4 or EphrinB3 show a great number of

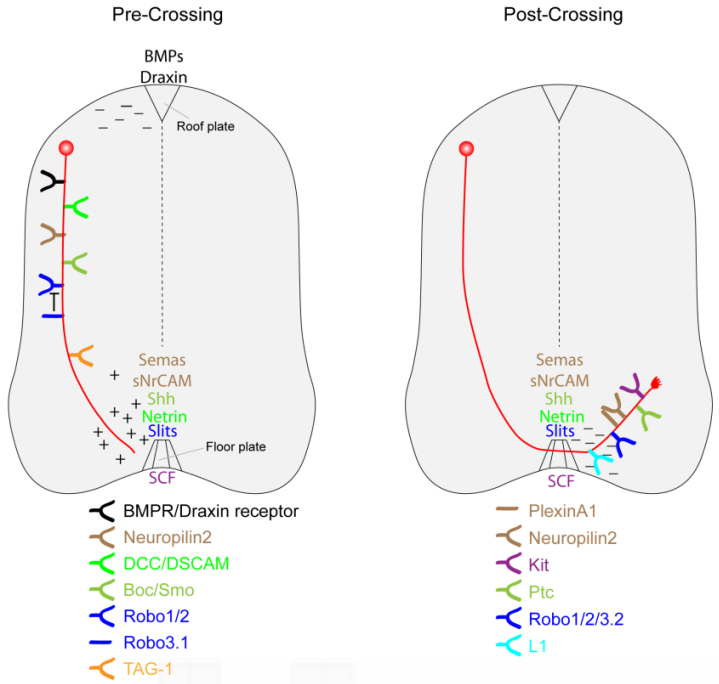

Figure 4. Molecular guidance of spinal commissural axons.

Pre-crossing axons are repelled from the dorsal part of the spinal cord by Draxin and BMPs. They are attracted to the floor plate by Netrin1 and Shh. In pre-crossing axons, Slit/Robo repulsion is inactivated by Robo3.1. At the floor plate, SCF promotes midline exit and DCC/Netrin1 attraction is silenced by Robo1 in a Slit dependent manner. The expression of the other Robo receptors is upregulated and Slit repulsion is activated. In parallel, the inhibition of Calpain1 induced by soluble NrCAM leads to an upregulation of PlexinA1 expression on post-crossing commissural axons allowing it to form a complex with Neuropilin2 that mediates Sema3B repulsion. Shh is also involved in switching Sema3B repulsion and, in chick, may also directly repel post-crossing axons.

axons crossing the dorsal midline ectopically (Kullander, 2003).

Once axons cross the midline they turn orthogonally to continue their growth longitudinally in the ventral funiculus. In vertebrates, Wnt, Shh, Nectin-like and MDGA2 signaling have been shown to play a role in the election of rostral rather than caudal growth after crossing (Lyuksyutova, 2003; Bourikas, 2005; Niederkofler, 2010; Domanitskaya, 2010; Joset, 2011). The position of the longitudinal tracts at the proper dorsoventral level in the ventro-lateral white matter has been proposed to be mediated by Slits and Robos signaling. In *Drosophila* a "Robo code" seems to control longitudinal axon sorting in the longitudinal tracts that run along the ventral nerve cord (Rajagopalan, 2000; Simpson, 2000). This "Robo code" relies preferentially on the differential gene expression of the Robo receptors rather than in structural differences among the Robo receptors (Spitzweck, 2010). In vertebrate spinal neurons, several reports have also demonstrated the conserved function of Slits and Robos in the longitudinal positioning of post-crossing axons (Long, 2004; Reeber, 2008; Jaworski, 2010; Sakai,

2012). Robo1 expels axons out of the floor plate and position them into the ventral fascicle to run longitudinally. Robo2 is necessary for axons to be expelled out of the ventral fascicle leading them into the lateral fascicle (Long, 2004; Jaworski, 2010).

The signaling mediated by the family of ephrins and Ephs has been also implicated in the establishment of the dorsal limit where post-crossing axons turn taking parallel trajectories to the rostrocaudal axis (Imondi, 2001). However the specific receptor/ligand pair controlling this decision has not been identified.

In the visual system, the guidance of contralaterally-projecting fibers relies on the same guidance cues/receptors that control commissural axons behavior in the invertebrate and the vertebrate cords. However, the patterns of expression vary and, accordingly, the outcome of the signaling cascades involved. Axons from RGCs are attracted towards the retinal exit by Netrin/DCC interaction (Deiner, 1997). Slits/Robo2 signaling channels retinal axons into the optic chiasm where Semaphorin/Plexin/NrCAM allows, and VEGF promotes, the crossing of commissural RGCs axons that express Neuropilin1 (Erskine, 2000; Fricke, 2001; Plump, 2002; Williams, 2006; Erskine, 2011; Kuwajima, 2012).

1.1.2 Molecular mechanisms that mediate axonal avoidance from the midline

The molecular mechanisms that govern axon midline avoidance in the *Drosophila* ventral nerve cord have been amply described. Ipsilateral axons express Robo but not Comm (Georgiou and Tear, 2002) and therefore are repelled by the midline, turning longitudinally without crossing to the contralateral side. Ectopic expression of Comm in ipsilateral neurons is sufficient to induce crossing (Kidd, 1998). Thus, Slit function appears to account for all the repulsive activity at the *Drosophila* midline.

In vertebrates, one of the few families of guidance molecules known to be implicated in midline avoidance are the membrane anchored proteins, ephrins, and their tyrosine kinase receptors the Ephs.

Their role in defining axonal ipsilaterality has been described in the visual system and the spinal cord. Ipsilateral but no contralateral retinal ganglion cell axons express EphB1 and respond to its ligand, ephrinB2, expressed by glial cells at the midline. EphB1/ephrinB2 binding mediates a repulsive response that provokes the turning of ipsilateral axons away from the midline (Williams, 2003; Petros, 2009). Though less-well described than in the retina, the Eph/ephrin family has been also involved in midline avoidance in the spinal cord since the disruption of EphA4/ephrinB3 interaction causes ectopic midline crossing of spinal interneurons (Kullander, 2003).

1.1.3 Transcriptional control of axon midline decisions

Despite advances in the identification of axon guidance molecules at the midline during the last few decades, little is currently known about the transcriptional control of axonal laterality in the CNS. The t-box transcription factor Midline controls the expression of two major guidance systems in *Drosophila*: frazzled, slit and robo (Liu, 2009) and the zinc finger transcription factor Lola, which controls the expression and genetically interacts with both Robo and Slit (Crowner, 2002). How the expression of guidance molecules is orchestrated at the transcriptional level is even less clear in the spinal cord where very few transcriptional determinants of axon midline decisions have been described.

One of the few examples of transcription factors directly involved in controlling midline guidance at the spinal cord are the LIM-HD proteins Lhx2 and Lhx9. These transcription factors control the axonal laterality of the dorsal population of spinal interneurons that are more dorsally generated, the dI1 interneurons. dI1 interneurons are subclassified into two populations: dI1c that cross the ventral midline and dI1i that project ipsilaterally to the lateral funiculus. dI1i expresses Lhx9 but not Lhx2 while dI1c express Lhx2 and low levels of Lhx9. Loss of function of Lhx2/9 lead to a downregulation of Robo3 expression in dI1c neurons and, as a result, dI1 axons are unable to cross the midline (Wilson, 2008).

In the visual system a member of the Zic transcription factors family, Zic2, is expressed in postmitotic retinal ganglion cells that project ipsilaterally. Mutant mice for this transcription factor lack an ipsilateral projection at the optic chiasm (Herrera, 2003) and, conversely, ectopic expression of Zic2 in contralateral RGCs generates an aberrant axonal misprojection to the ipsilateral side (García-Frigola, 2008). Zic2 induces the expression of the tyrosine kinase receptor EphB1 in ipsilateral axons, which are then repelled by ephrinB2 expressed at the midline. Conversely, the LIM homeodomain transcription factor Isl2 is specifically expressed in contralateral but not in ipsilateral RGCs, and it has been described as a repressor of Zic2 (Pak, 2004).

Because the transcriptional mechanisms controlling axonal ipsilaterality in the spinal cord have not been yet unveiled, the main aim of this thesis was to investigate whether the transcription factor Zic2, that controls axonal ipsilaterality in the visual system, plays a similar function in the spinal cord. To address this issue we focused on the dorsal horns of the vertebrate spinal cord, a structure that contains several of the major ipsilateral tracts in the CNS.

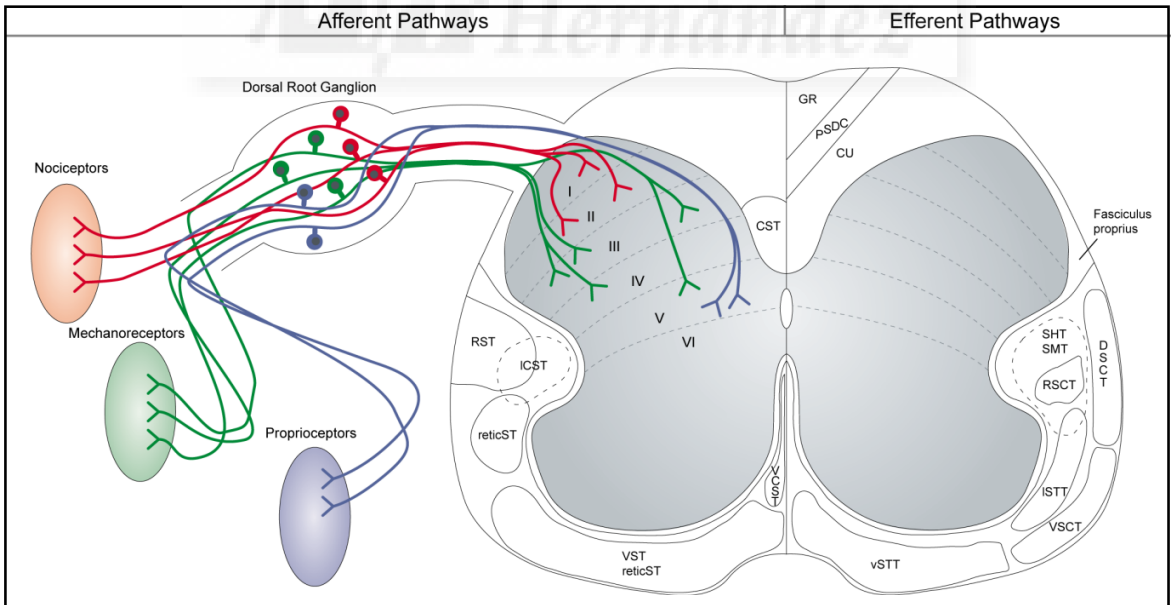

Figure 5. Spinal cord structure, afferent and efferent pathways.

Schematic representation of the neuronal circuits in the mouse spinal cord. Sensory information from dorsal root ganglion cells is delivered to specific laminae in the dorsal horns. Supraspinal centers also provide information to the spinal cord through several pathways (left side): corticospinal tract (CST), lateral corticospinal tract (ICST), ventral corticospinal tract (VSCT), rubrospinal tract (RST), reticulospinal tract (reticST) and vestibulospinal tract (VST). Spinal neurons then drive the information to supraspinal targets through different pathways (right side): gracile tract (GR), cuneate tract (CU), postsynaptic dorsal column tract (PSDC), dorsal spinocerebellar tract (DSCT), ventral spinocerebellar tract (VSCT), rostral spinocerebellar tract (RSCT), spinohypothalamic tract (SHT), spinomesencephalic tract (SMT), lateral spinothalamic tract (ISTT), ventral spinothalamic tract (vSTT). Compiled from Caspary and Anderson, 2003; Webb and Muir, 2004; Watson and Harrison, 2012.

1.2. *The dorsal horns of the spinal cord*

1.2.1. Structure and function

The dorsal horns are located in the dorsal half of the spinal cord and are subdivided into six cytoarchitectonic regions (laminae I to VI by Rexed, 1952) (Figure 5).

For the last fifty years, several authors defended a model that describes the dorsal horns as isolated homogeneous units with no significant exchange of information with other parts of the spinal cord (Szentágothai, 1964; Réthelyi and Szentágothai, 1969, 1973). In this model, the dorsal horns were simple relays for sensory information on its way to the brain. However, evidences accumulated in the last decade have challenged this view and favor the idea that the dorsal horns do not act solely as sensory relays but as processing centers in which sensory information is gathered, transmitted to different areas of the spinal grey matter and processed to generate a response. This response is transmitted to the spinal motor areas and, in case it reaches a certain threshold level, to supraspinal brain centers, where the sensory impulses reach another level of processing (Petkó, 2012) (Figure 6).

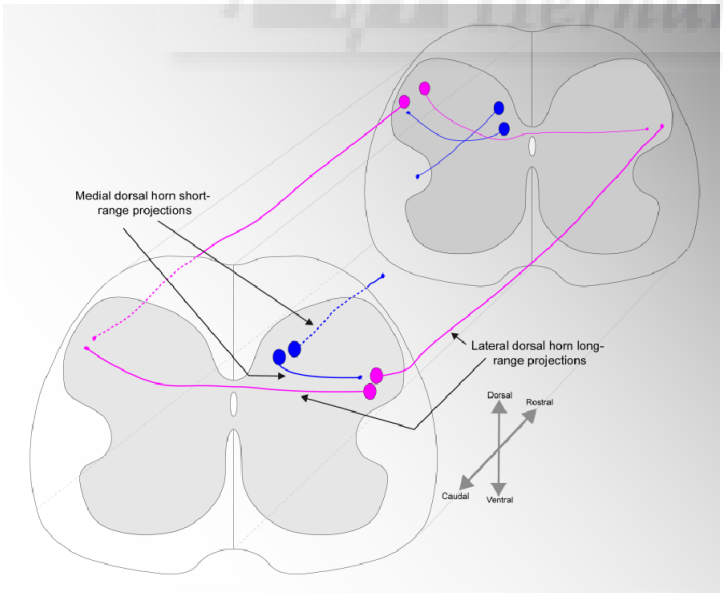

Figure 6. Propriospinal pathways in the dorsal horns

The medial and lateral aspects of the dorsal horns show differential patterns of projections. Propriospinal neurons whose somas are located in the medial dorsal horn (blue) posses short projections to the lateral aspects and to close rostrocaudal levels on the same side of the horn. However, propriospinal neurons on lateral positions of the dorsal horns (pink) show long range projections both to the same side of the spinal cord and to the contralateral dorsal horn. Based on Petkó, 2012.

The medial and lateral areas of the dorsal horns have different sets of connections. Neurons

located in the medial-dorsal horn project rostrocaudally and mediolaterally but only for short distances. Neurons in the lateral-dorsal horn, in addition to such short-range projections, possess a system of long-range projecting axons that span the whole lumbar cord. This long-range projection system is exclusively ipsilateral. In the lateral horn there are also contralateral axons that connect the lateral areas on both sides of the horns. The dorsal horns also communicate with different supraspinal areas (brainstem, cerebellum, midbrain, diencephalon and telencephalon) by means of projection neurons that give rise to several ascending spinal pathways (Willis and Coggeshall, 1991; Watson, 2012) (Figure 5).

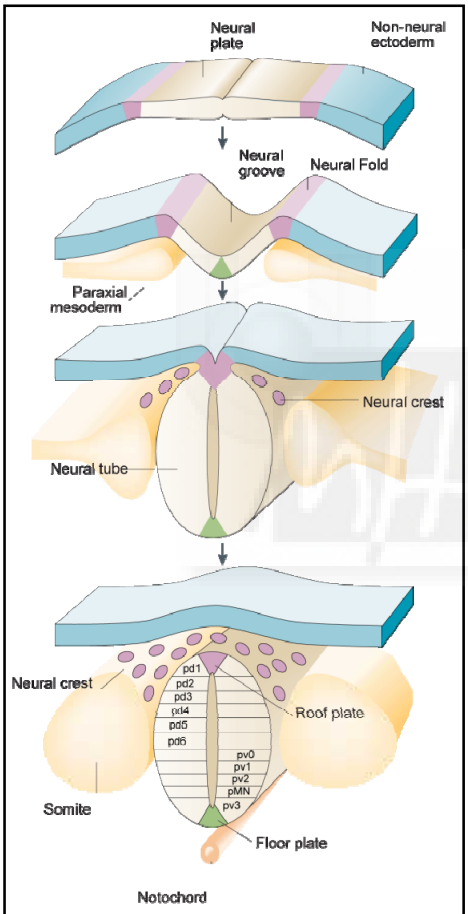

Figure 7. The formation of the neural tube.

The neural tube arises from invagination of the neuroectoderm induced by signals coming from the non-neural ectoderm (blue) and the underlying paraxial mesoderm (yellow). The neural plate borders fold and elevate causing the neural plate to roll into a neural tube. Neural crest cells delaminate from the dorsal neural tube and the remaining cells become the roof plate which constitutes a new signalling center. Adapted from Gammill and Bronner-Fraser, 2003 and Liu and Niswander, 2005.

1.2.2. Development of the dorsal spinal cord

The spinal cord is the most caudal part of the central nervous system and arises from the closure of the neural tube (or neurulation) during early embryonic development. After neurulation three different cell populations differentiate: i) dorsal midline cells that will give rise to the roof plate, which constitutes a signaling center essential for the proper patterning of dorsal interneurons; ii) neural crest cells that delaminate and migrate out of the neural tube to form diverse structures, and iii) the rest of the cells that become neural progenitors (Figure 7). As development proceeds, roof plate cells start to secrete different families of dorsalizing molecules (TGF-β, Wnt, FGF). This complex molecular system regulates the expression of several cross-inhibitory transcription factors of the bHLH family and causes a subdivision of the precursor

area in six dorsal domains of interneuron progenitors (dP1-6) organized dorsoventrally.

Subsequent activation of LIM-HD factors further specifies these domains of progenitors to give rise to six postmitotic populations of dorsal interneurons (dI1-6). Following differentiation of the dI1-6, two more populations of late dorsal interneurons (dIL_A and dIL_B) originate from the dorsal subventricular zone (Helms and Johnson, 2003; Caspary and Anderson, 2003; Lewis, 2006) (Figure 8). dP1 cells are determined by the expression of Atoh1 (Ben-Arie, 1996; Helms and Johnson, 1998), which in turn drives the expression of the postmitotic BarHl transcription factors BarHl1 and BarHl2, necessary to determine dI1 identity. Barhl2 seems to be responsible for the specification of dl1 neurons in dl1i and dl1c because in the Barhl2 knockout mice dl1i neurons are respecified to dl1c that ectopically express Lhx2 and Robo3 and aberrantly cross the midline (Liem, 1997; Saba, 2003; Saba, 2005; Wilson, 2008; Ding, 2012). Cross-inhibitory activities between Atoh1 and Ngn1/2 in the ventricular zone allow the segregation of the next ventral subpopulation of dorsal interneurons, the dP2 interneurons (Gowan, 2001). dP2 neurons are characterized by the postmitotic expression of Foxd3.

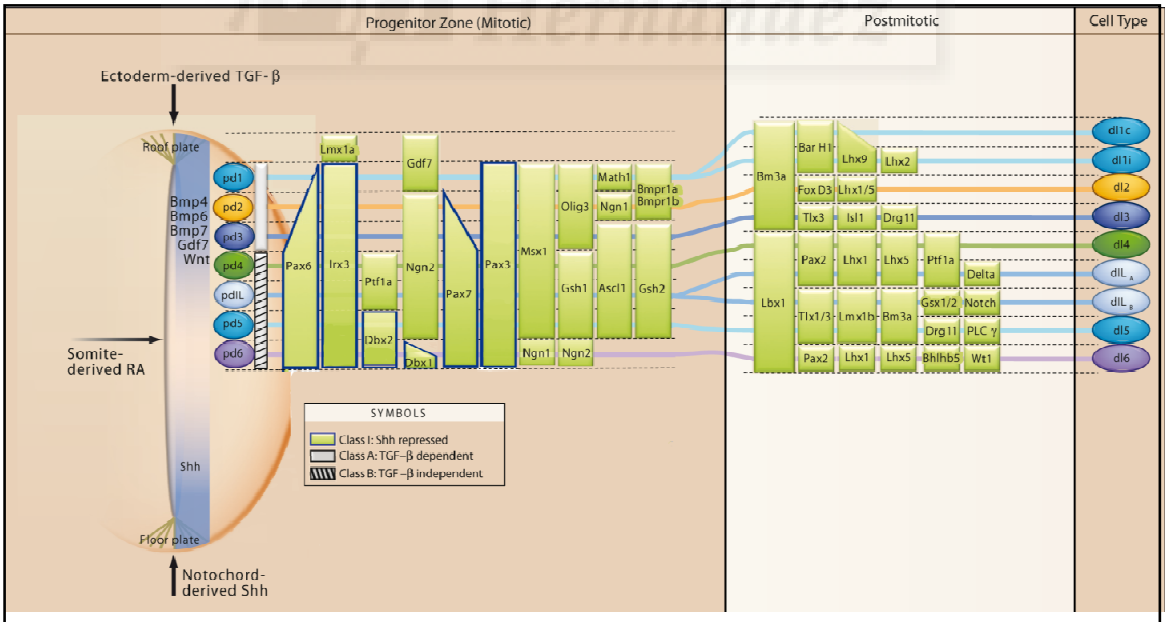

Figure 8. Genetic specification of dorsal interneuron populations.

Cellular identities are defined by the influences of a two-dimensional system of morphogen gradients acting on the neuroepithelia of the ventricular zone. Floor plate and notochord derived Shh repress the expression of dorsal identities while ectoderm and roof plate derived BMPs and Wnts induce their expression. Crossinhibitory mechanisms between transcription factors of neighboring progenitor domains prevents cells from developing hybrid identities. Adapted from Alaynick, 2011.

Cells expressing Ascl1 give rise to the dI3 and dI5 populations (Helms, 2005), Gsh2 represses Ngn1 to specify dI3 neurons (Kriks, 2005) and Tlx3 has been implicated in the proper development of both dI3 and dI5 neurons (Qian, 2002). Absence of Olig3 causes misspecification of dI1-dI3 switching their identity to dI4-6 cells. Conversely, loss of the transcription factor Lbx1 switches dI4-6 identity to dI1-3, pointing to this transcription factor as essential for the specification of dI4-6 neurons (Gross, 2002; Müller, 2002).

Late-born dIL neurons are initially specified by the transcription factors Ascl1 and Lbx1 (Gross, 2002, Müller, 2002). Later, dIL neurons generate the two subpopulations that form the dorsal horns: dIL_A and dIL_B. Ptf1a determines the dIL_A GABAergic phenotype (Glasgow, 2005), while Tlx3 acts as a postmitotic selector gene that specifies the glutamatergic fate of dIL_B (Cheng, 2004; Cheng, 2005). In addition, the Lmx1b transcription factor controls the differentiation and migration of dIL_B (Ding, 2004).

The laminar organization of the mature dorsal horns observed in the adult spinal cord is the result of complex migratory pathways followed by the distinct domains of dI1-6 interneurons (Figure 9).

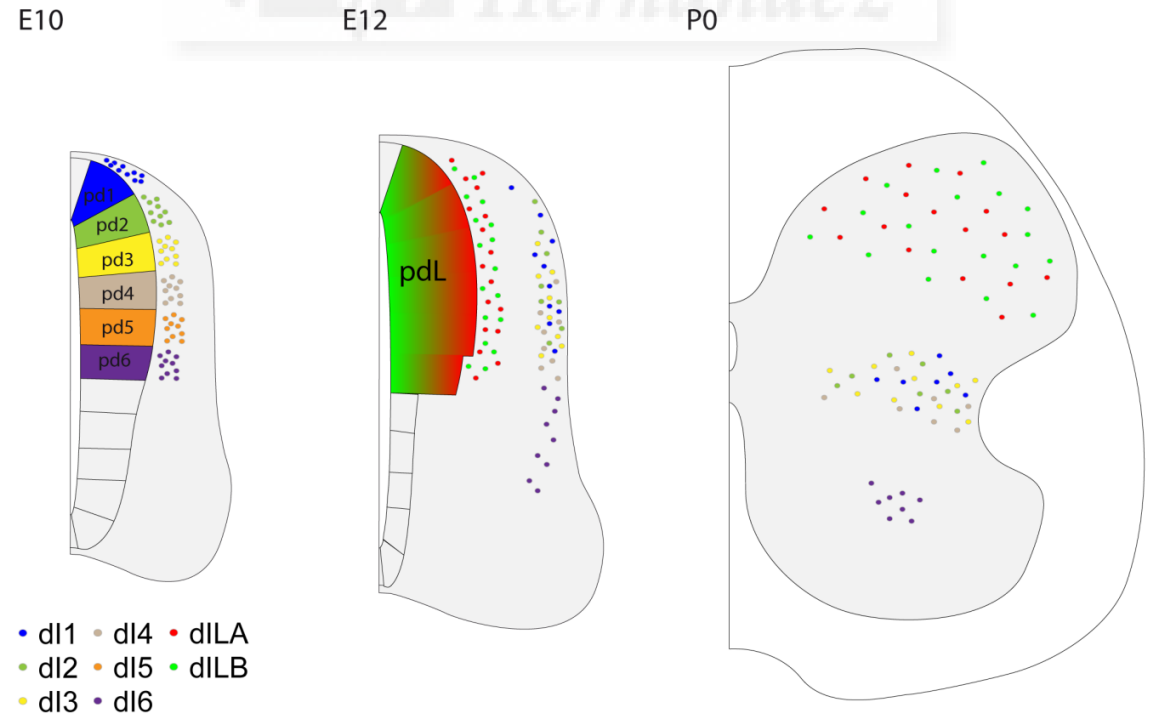

Figure 9. Migratory paths and final location of dorsal spinal interneurons.

Early born (E10-E12) neurons in the dorsal neural tube (dI1-6) differentiate laterally from the ventricular zone into the marginal zone and follow a ventral migration to their finallocation. Meanwhile, late born (E12-E14) neurons (dIL) give rise to the dorsal horns.

Contrary to what might be expected, the most dorsal lamina in the adult spinal cord, the Rexed's lamina I, does not correspond to interneurons generated in the dI1 domain during development. Indeed, dI1-3 neurons migrate ventrally and end up at the base of the dorsal horn. The final location of dI4-5 neurons is not clear but apparently they migrate even more ventrally than dI1-3. dI6 neurons migrate ventromedially to the ventral horn. Finally, the late-born interneurons $dIL_{A/B}$, are the only ones that will populate the dorsal horns (Lewis, 2006).

1.2.3. The wiring of the dorsal spinal cord

Each population of dorsal interneurons is characterized by specific patterns of axonal projections. Experimental evidences based on the existence of enhancers that are specific for dorsal, dI1-3, neurons have allowed the description of the main axonal trajectories of these three types of dorsal interneurons. However data for dI4-6 and $dL_{A/B}$ interneurons are sparse (Figure 10). dI1 neurons are a mixed population comprised by both ipsi- and contralaterally projecting neurons (Wilson, 2008; Avraham, 2009; Ding, 2012) while dI2 neurons are mostly contralateral (Avraham, 2009). dI3 neurons are exclusively ipsilateral but they differ in the choice point taken by their axons to turn longitudinally. Some dI3 neurons project ventrally towards the ventro-lateral fascicle while some others project dorsally towards the dorsal root entry zone (Avraham, 2010). dI4 neurons seem to be ipsilaterally-projecting association neurons that target proprioceptive afferents on their course to motoneurons (Alaynick, 2011), and dI6 cells are premotor neurons with both ipsi- and contralateral axons (Vallstedt and Kullander, 2013). The projection pattern of dI5 neurons remains unknown.

Regarding the late-born interneurons, it has been shown that at least part of the Lbx1-derived cells become association interneurons with ipsilateral projections into the DLF (Gross, 2002). Together with Petkó's data on propriospinal projections these observations indicate that in the dorsal horns, dIL_A inhibitory interneurons are locally or contralateral projecting neurons, while dIL_B excitatory neurons are

long-range ipsilateral projection neurons (Petkó, 2012).

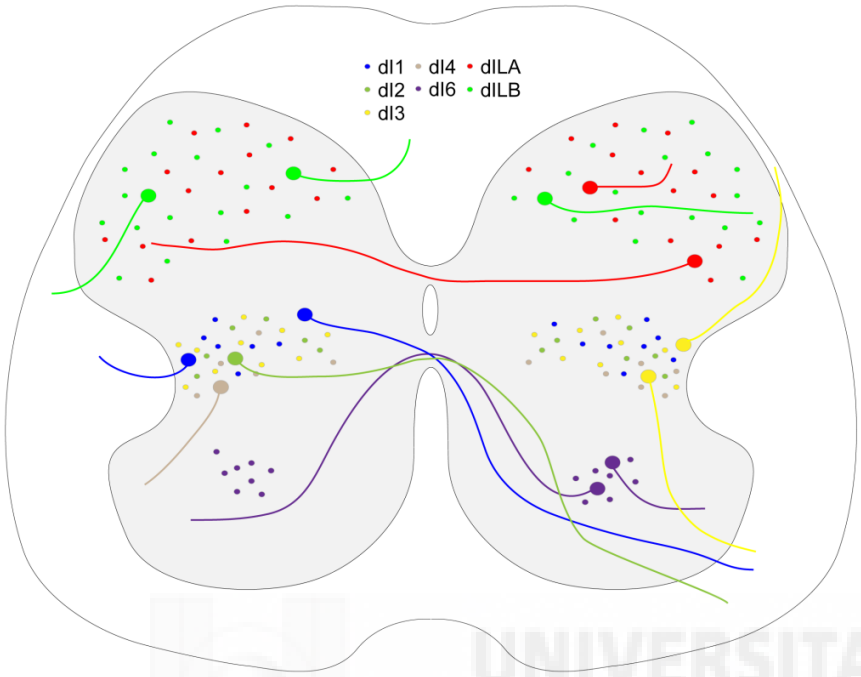

Figure 10. Projection patterns of dorsally-generated spinal interneurons.

Representation of the known axonal trajectories with respect to the midline of dorsal interneurons populations. dI1 and dI6 form both ipsi- and contralateral projections, dI2 is considered to be almost exclusively contralateral while dI3 and dI4 have been shown to project only ipsilaterally. dILA neurons are local and contralateral neurons. dILB are ipsilateral neurons which project to different fascicles into the white matter.

Chapter II

Aims

The main objective of this thesis was to investigate whether the transcription factor Zic2 determines axonal ipsilaterality in the spinal cord as it does in the visual system. To this end we focused on the following objectives:

1. To define the spatiotemporal expression of Zic2 in the developing spinal cord
2. To characterize the spinal neurons expressing Zic2
3. To perform Zic2 loss-of-function experiments in the spinal cord
4. To perform Zic2 gain-of-function experiments in the spinal cord
5. To identify Zic2 effector molecules in the spinal cord

Chapter III

Material and Methods

3.1 Mice

The Tg(Zic2^{EGFP})HT146Gsat/Mmcd line (identification number RP23-158G6) was generated by GENSAT (Gong, 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. TgZic2^{EGFP} mice were crossed with Zic2$^{kd/+}$ mice, and the resulting F1 progeny were crossed to generate double Zic2^{EGFP} Zic2$^{kd/kd}$ and Zic2^{EGFP} Zic2$^{+/+}$ mice. 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.

3.2 Expression constructs

The following plasmid constructs were described previously: RCAS-Ptf1a (Huang, 2008); CAG-DsRed2 (Borrell, 2010); CAG-GFP (García-Frigola, 2007); Edl1-Cre, CAG-loxP-STOP-loxP-IRES-EGFP, Ngn1-Cre, Foxd3-GAL4 and UAS-loxP-STOP-loxP-IRES-EGFP (Avraham, 2009); CAG-Robo3.1myc (Chen, 2008). CAG-Zic2-IRES-EGFP was constructed by inserting a human Zic2 cDNA into pIRES2EGFP (Clontech) and subcloning the Zic2-IRES-EGFP cassette into the multicloning site of pCAGSE (provided by Dr. Oscar Marín). CAG-IRES-EGFP was generated by removing Zic2 from CAG-Zic2-IRES-EGFP. CAG-loxP-STOP-loxP-Zic2-IRES-EGFP and UAS-loxP-STOP-loxP-Zic2-IRES-

EGFP were derived from CAG-loxP-STOP-loxP-IRES-EGFP and UAS-loxP-STOP-loxP-IRES-EGFP respectively.

3.3 Immunohistochemistry

Embryos fixed overnight at 4ºC in 4% paraformaldehyde/PBS, were washed in PBS and sectioned in a vibratome (70 µm sections) or in a cryostat (20 µm sections). Sections were blocked in 0.02% porcine gelatin/PBT (0.25% TritonX/PBS) and incubated with the proper primary antibodies in the same blocking solution. Appropriate Alexa (Invitrogen) secondary antibodies were used. The primary antibodies used in this thesis were: PCNA (Santa Cruz), HuC/D (Molecular Probes), NeuN (Millipore), BrdU (Abcam), Lbx1, Lmx1b and Tlx3 (Dr. Carmen Birchmeier), Pax2 (Zymed), GFP (Aves Labs), RFP (Clontech), Parvalbumin (Sigma), Lhx2/9 (Thomas Jessell), Robo3 (R&D), Brn3a (Chemicon) and Chick neurofilament (8D9), Mouse neurofilament (2H3), Lhx1/5 (4F2), Isl1/2 (39.4D5) from the Developmental Studies Hybridoma Bank. Zic2 antibody was generated in our laboratory from rabbits immunized with a GST-tagged recombinant protein corresponding to residues in the N-terminus of mouse Zic2.as in (Brown, 2003). 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.

3.4 In situ hybridization

Whole mount in situ hybridization in chick embryos was performed as previously described in (Acloque, 2008). In situ hybridization in spinal cord cryosections were performed as in (Marcos-Mondejar et al 2012). In situ hybridization in spinal cord vibrosections were performed as in (Borrell, 2012). At least 5

sections/embryo and 5 embryos/probe were used for in situ hybridization analysis in functional experiments. The following cDNA probes have been previously described: mouse Zic2 (Herrera, 2003), mouse Lhx2 (Marcos-Mondejar, 2012), chick Lhx2 and chick Lhx9 (obtained from Addgene), mouse ephrinB2, mouse EphB1 and mouse EphB2 (Williams, 2003), mouse Robo1 and mouse Robo2 (gift from Katja Brose), mouse PlexinA2 and mouse Unc5c (gift from Salvador Martínez), mouse EphA5, mouse EphA6 and mouse ephrinA5 (gift from Robert Hindges). Mouse Robo3 probe was obtained from E13.5 mouse spinal cord cDNA using primers according to (Kuwako, 2010). Chick Zic2 probe was cloned from chick E14 cerebellum cDNA using primers CTCAGAGCCACCTCCTGTTC and ATACCGTTTCCTCTGTAGCAAG. Chick Robo3 was generated from chick E3 spinal cord cDNA using the primers CAGCATGCTGCGGTACCTTCTGA and CTCACCGTGATGCGCTCATCCT. Mouse EphA4, mouse ephrinB3, mouse PlexinA1 and mouse Neuropilin1 probes were isolated from mouse E13.5 spinal cord cDNA with the following primers: EphA4 (GGGCCACTGAGCAAGAAA and GCCTGGACCAAAGCAATG), ephrinB3 (GTTAGGTTTTGCGGGGCT and TTCCTAGCTCCCCAGGCT), PlexinA1 (ACCCAATCCCATGATGCTCG and AGCTGCAGGGTGAGGTAGTA), Neuropilin1 (CTCCGGAACCCTACCAGAGA and ATCCAGTCCTCTCCGTTGGA). Probes were cloned into the pGEMT-Easy system (Clontech) and sequenced for verification.

3.5 In ovo electroporation

Fertilized White Leghorn chicken eggs were incubated at 38°C until desired developmental stage according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) was reached. Plasmidic DNA solution was injected into the lumen of the neural tube. Platinum electrodes were used with 5 x 10 ms pulses at 25 V generated by a TSS20 Ovodyne electroporator. Embryos were then incubated for further 2-4 days before analysis. Chick RNA interference experiments were performed using the

pRFPRNAiC plasmid (Das, 2006). Chick Zic2 RNAi target sequence was: GATCCACAAACGGACGCAC.

3.6 In utero electroporation

Electroporation experiments were carried out in BDL1 (DBA2 × C57/B6) mice. Spinal cord in utero electroporation was performed as described in (Garcia-Frigola, 2007) but injecting the DNA solution through the base of the tail and using the CUY567-0.5 electrodes placed in the lumbar spinal cord. Zic2 shRNAs 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) following manufacturer recommendations. Mouse Zic2 RNAi target sequence: GGCCAAATACAAACTGGTC.

3.7 Quantifications

Unless othersiwe stated, all images used for quantification were obtained using a Leica DM IRE2 Confocal microscope.

Downregulation of Zic2 by in utero electroporation of Zic2RNAi was quantified measuring the levels of Zic2 protein expression detected by immunohistochemistry. Zic2 intensity levels were determined in sections of E16 mouse embryos that were electroporated at E12 both in the electroporated side and the non electroporated side.

For the chick experiments, ImageJ software was used to generate two mirror ROIs (Ipsilateral (iROI) and contralateral (cROI)) per image taken from the white matter where axons project longitudinally (marked with L1 antibodies). Ipsilateral Index (IpI) was obtained as a measure of GFP levels in iROI and cROI and expressed as (iROI /iROI+cROI) x100. For the mouse experiments, Adobe Photoshop CS5 was used to select two different ROIs per section in the non-electroporated side adjacent to the

midline (Figure 19. M,N): one from the central canal to the dorsal fascicle (Dorsal Crossing ROI, dcROI) and another one from the central canal to the floor plate (Ventral Crossing ROI, vcROI). dcROI and vcROI-GFP signals were normalized to the GFP levels in the grey matter of the electroporated side ($_{EGFP}$ROI). Contralateral Index (CoI) in dorsal and ventral cord was obtained as (dcROI/$_{EGFPROI}$)x1000 and (vcROI/$_{EGFPROI}$)x1000 respectively. Quantifications were performed in, at least, four sections per embryo and three embryos per condition.

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.

Chapter IV

Results

4.1 Zic2 is transiently expressed in postmitotic late-born spinal neurons

To investigate a possible role of Zic2 in the guidance of spinal axons, we first analyzed its spatio-temporal expression pattern during embryonic development in the mouse spinal cord. We detected high expression of Zic2 protein in the ventricular zone of the dorsal neural tube from embryonic day 10 (E10) to E12 (Figure 11. A-B) as it has been previously reported (Nagai, 1997), In addition, we found

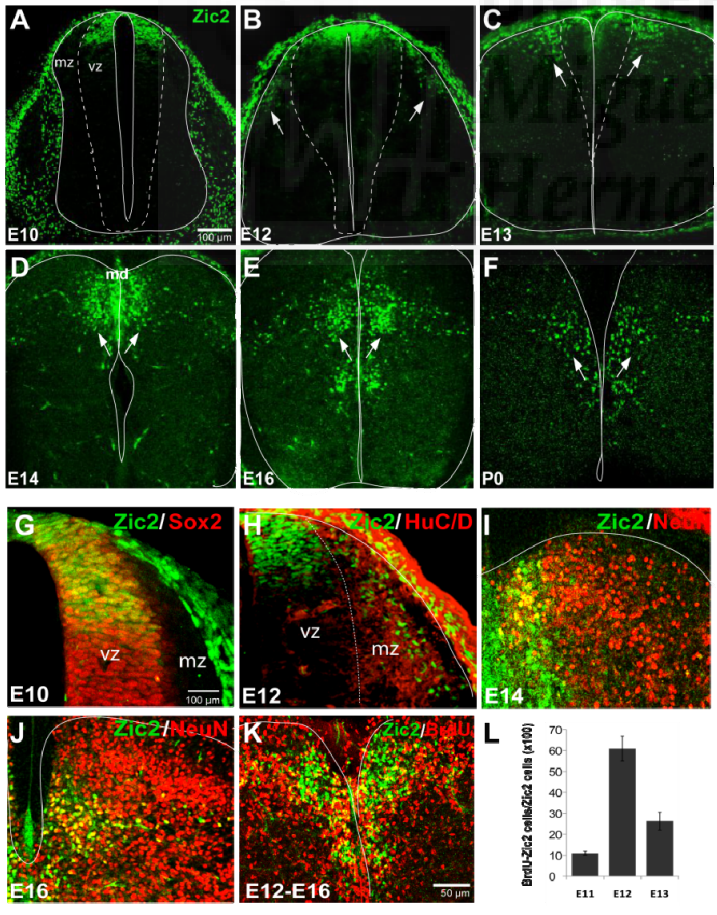

Figure 11. Spatiotemporal expression of Zic2 in the developing spinal cord.

(A-F) Transverse spinal cord sections from wildtype mouse embryos at the indicated stages stained with antiZic2 antibodies show the spatiotemporal exprssion pattern of Zic2 throughout spinal cord development.
(G-J) Transverse spinal cord sections double-stained with Sox2, HuC/D, NeuN and Zic2 show that Zic2 is expressed in progenitors at early stages and in postmitotic neurons at later stages. Arrows points to the region of double-labeled cells
(K) Transverse spinal cord sections from E16 embryos injected with BrdU at E11, E12 and E13 and double stained with anti-BrdU and anti-Zic2 antibodies.
(L) Quantification of the number of double-labelled $Zic2^{+}/BrdU^{+}$ cells in E16 embryos injected at E11, E12 and E13 indicates that most of the $Zic2^{+}$ cells are born at E12. At least five sections/embryo and four embryos/stage were used to quantify. Scale bar: mm. Error bars indicate ±SEM (**p<0.01, Student´s unpaired t-test).

that at later stages, Zic2 is expressed in a population of dorsal cells that by E16 forms two symmetric patches at both sides of the midline (Figure 11. C-F), a pattern maintained until early postnatal stages.

To further characterize Zic2 expression we used different markers for progenitors (Sox2), postmitotic cells (HuC/D) and differentiated neurons (NeuN). We observed that Zic2 is first expressed in dorsal progenitors (positive for Sox2) then become restricted to a dorsal subpopulation of NeuN- postmitotic neurons (Figure 11. G-J). Neurogenesis in the dorsal spinal cord takes place in two phases. In the first phase, from E10 to E12, six populations of interneurons organized dorsoventrally (dI1-6) are born. In the second phase from E12 to E14, the so-called late-born interneurons (dIL) are generated (Helms and Johnson, 2003; Caspary and Anderson, 2003; Lewis, 2006). To determine the birth-date of Zic2 postmitotic neurons we performed BrdU uptake experiments by injecting pregnant females at different developmental stages during neurogenesis and analyzed the expression of BrdU in Zic2 cells at E16, two days after neurogenesis finalization in the spinal cord (Nornes and Carry, 1978). Even though some Zic2 neurons were born at E11 and at E13, the results showed that the vast majority of Zic2 positive postmitotic spinal cord cells are born at E12, suggesting that they are late-born dIL neurons (Figure 11. K, L).

4.2 Zic2 expressing spinal cells are dIL$_B$ neurons

While early born dI1-6 interneurons predominantly settle in the deep dorsal horn, late born dIL neurons migrate dorsally giving rise to the dorsal horns (Lewis, 2006). dIL neurons is comprised of inhibitory (dIL$_A$) and excitatory (dIL$_B$) cell types. dIL$_A$ are GABAergic interneurons determined by the expression of the transcription factor Ptf1a and characterized by expression of the transcription factor Pax2, while dIL$_B$ are glutamatergic neurons defined by the expression of Tlx3 and Lmx1b (Muller, 2002; Gross, 2002; Cheng, 2004 and 2005; Glasgow, 2005; Mizuguchi, 2006). Glutamatergic dorsal horn neurons

extend axons ipsilaterally within the dorsal and dorsolateral fasciculi (DF and DLF, respectively) to ascend longitudinally along the cord white matter and connect interspinal segments (propriospinal neurons) or form the major tracts to the brain (projection neurons). GABAergic interneurons are thought to be part of a local circuit mainly formed by contralateral neurons with short-projections (Szentagothai, 1964; Brown, 1981; Gross, 2002; Bannatyne, 2006; Petkó, 2012).

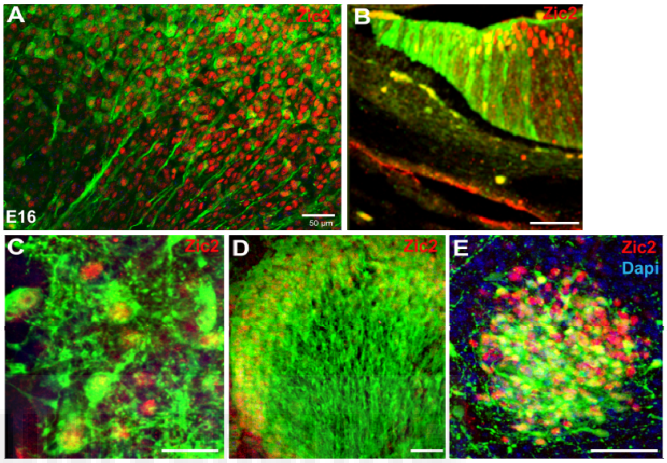

Figure 12. Zic2 reporter mice: EGFP expression recapitulates the endogenous expression of Zic2.

Zic2 and EGFP co-localize in different brain areas such the thalamus **(A),** ciliary margin and ipsilateral ganglion cells in the retina **(B),** Cajal-Reltzius cells **(C),** hippocampus **(D)** or amygdala **(E)**.

To determine the identity of Zic2 positive neurons, we used a Zic2 reporter mouse line ($Zic2^{EGFP}$ mice) generated from a BAC in which EGFP replaced the coding sequence of Zic2. We first confirmed that these Zic2 EGFP reporter mice faithfully reproduce the expression pattern of Zic2 in differrent tissues including the spinal cord (Figure 12 and Figure 13. A-C) and noticed that EGFP expression could be used as a transient fate-map reporter for Zic2 expression. Zic2/EGFP double-labeled neurons are located near the dorsal midline around E14. Later, these neurons turn off Zic2 but the expression of EGFP is maintained and thus allows to determine the final location of Zic2-derived neurons in the dorsal horns. Zic2-derived dorsal horn neurons maintained EGFP expression until postnatal stages (Figure 13. A-D), an expression that was similar all along the rostrocaudal extent of the spinal cord.

dIL neurons are specified by the expression of Lbx1 (Muller, 2002; Gross, 2002). Immunostaining for Lbx1 in transverse spinal cord sections of $Zic2^{EGFP}$ embryos confirmed that Zic2/EGFP neurons are dIL neurons (Figure 13. E). In all the analyzed stages (from E13 to P0) most

Zic2/EGFP cells were positive for Tlx3 and Lmx1b but negative for Pax2 (Figure 13. F-H), indicating that glutamatergic, but not GABAergic dIL neurons expressed Zic2. In fact, 91.15±0.85% of the Lmx1b neurons were also positive for EGFP, suggesting that almost the entire population of dIL_B neurons

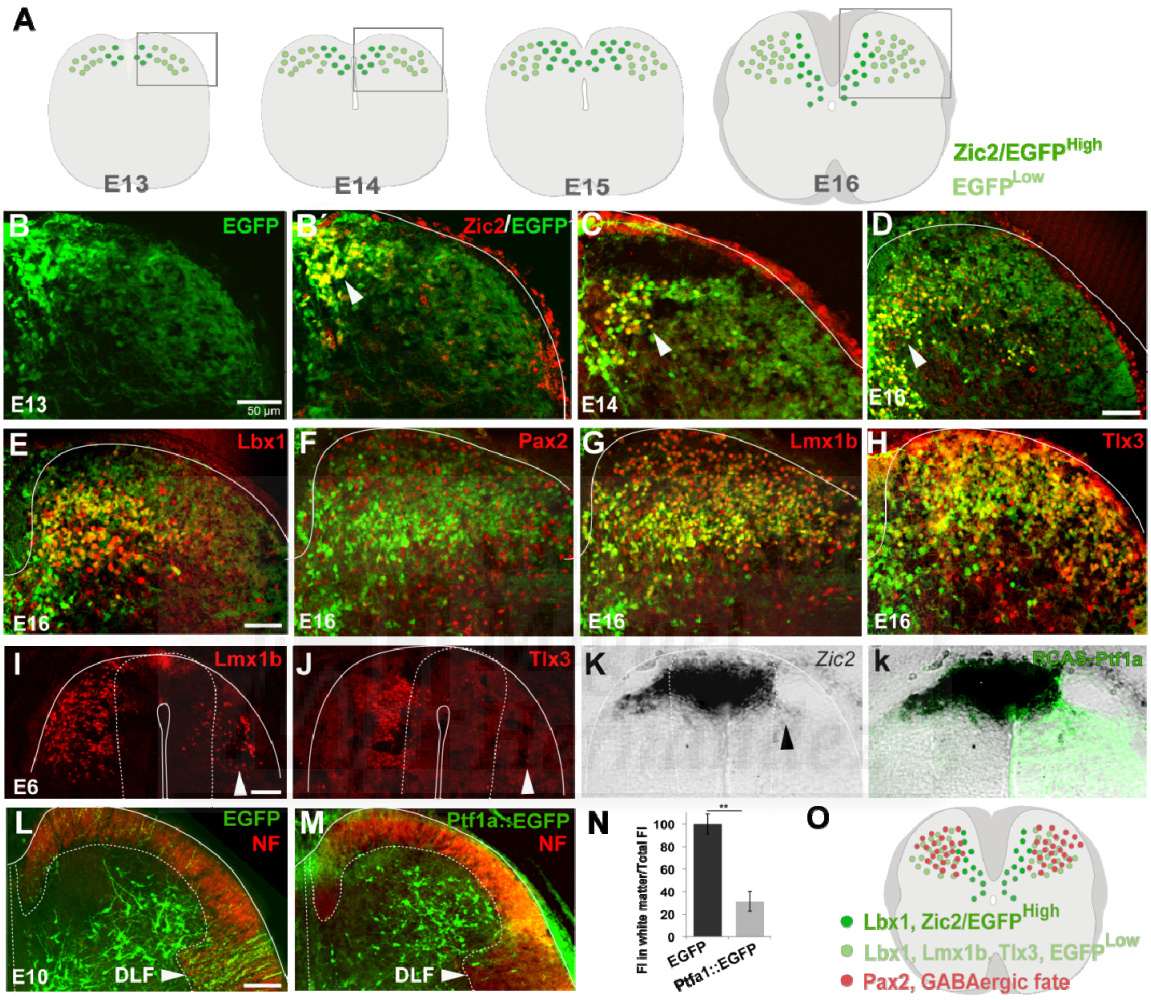

Figure 13. Spinal neurons that transiently express Zic2 are dIL_B projection neurons.

(A-C) Dorsal quadrant of transverse spinal cord sections of $Zic2^{EGFP}$ embryos stained with Zic2 antibodies shows that Zic2 cells are also positive for EGFP. Arrows point to the region with a larger concentration of double-labeled Zic2/EGFP cells. **(D)** Scheme summarizing the spatiotemporal expression of Zic2 and EGFP in $Zic2^{EGFP}$ embryos. At early stages $Zic2^{+}$/$EGFP^{+}$ cells (yellow) are located in the dorsocentral region. Through development, cells expressing high levels of EGFP (dark green) separate from the midline and go to more dorsal and lateral locations. Cells expressing low levels of EGFP (light green) are in the superficial dorsal horn layers. **(E-H)** Dorsal quadrant of transverse spinal cord sections from $Zic2^{EGFP}$ E16 embryos stained with the indicated markers. Bar scale: 100 µm. **(I-K)** Transverse spinal sections of E6 chick embryos that were electroporated *in ovo* at E4 with plasmids driving the coding sequence of Ptf1a (RCAS-Ptf1a) reduces the expression of glutamatergic markers such as Lmx1b and Tlx3 (red, white arrows) as well as postmitotic expression of *Zic2* mRNA in the dorsal cord (black arrow). Bar scale: 100 µm. **(L-M)** Dorsal quadrant of transversal spinal cord sections from E10 chick embryos electroporated *in ovo* at E6 with plasmids driving EGFP (CAG-EGFP) alone or together with Ptf1a expressing plasmids (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) but rather project locally. Bar scale: 100 µm. **(N)** Quantification of the amount of axons into the white matter after electroporation of RCAS-Ptf1a/CAG-EGFP or CAG-EGFP plasmids.

expressed Zic2 at some point. This result led us to classify Zic2 dIL neurons among the B-subtype. To further assess the relationship between Zic2 and glutamatergic fate in the dorsal horns, we reasoned that if we artificially increase the number of GABAergic neurons at the expense of the glutamatergic subtype, a decrease in the expression of Zic2 should be observed. To achieve such fate-switch from dIL_B to dIL_A we took advantage of previous work demonstrating that overexpression of the transcription factor Ptf1a causes an increase of dIL_A with a concomitant decrease of dIL_B neurons (Mizuguchi, 2006). We performed chick spinal cord electroporations with Ptf1a at the stage of development when dIL neurons are differentiating. Chick embryos were electroporated unilaterally at E4 with plasmids encoding Ptf1a plus reporter plasmids expressing EGFP (RCAS-Ptf1a and CAG-IRES-EGFP). Electroporated chick embryos were analyzed at E6 and a clear reduction in the number of cells expressing Tlx3 and Lmx1b was found, which confirmed the expected switch of fate in dIL subtype identity from dIL_B to dIL_A. We confirmed first that expression of Zic2 in the chick spinal cord is similar to the mouse Zic2 expression (Figure 14) and then analyzed Zic2 expression in Ptf1a-electroporated embryos. As expected the loss of dIL_B neurons led to a concomitant decrease in the expression of Zic2 mRNA in the electroporated side (Figure 13. I-K). In addition we analyzed the projection pattern of Ptf1a electroporarted neurons. While E10 embryos electroporated with EGFP plasmids showed axons distributed among the DF and DLF with few contralateral axons in the ventral funiculus, Ptf1a-electroporated embryos showed very few axons in the white matter revealing that GABAergic interneurons (that are negative for Zic2) are locally-projecting interneurons that do not project to the

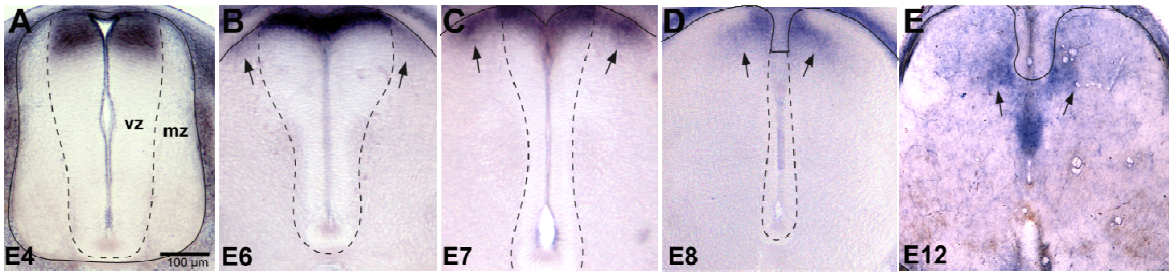

Figure 14. Spatiotemporal expression pattern of Zic2 in the chick spinal cord.

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

DLF nor to the DF (Figure 13. L-N).

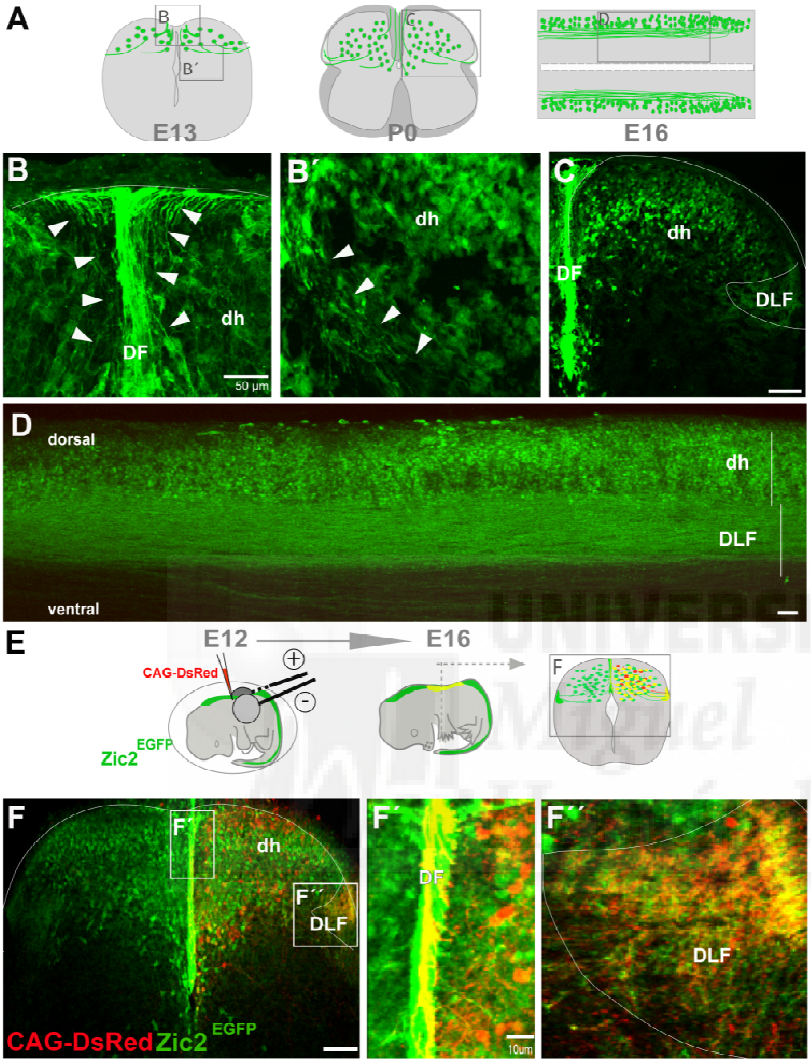

Figure 15. Zic2 spinal neurons project ipsilaterally.

(A-C) Schematic representation that illustrates the expression of Zic2 (green) at E14, P0 in transversal sections and at E15 in open-book preparations. **(a)** Dorsal quadrant of a transverse section from an E14 $Zic2^{EGFP}$ embryo shows that axons from Zic2 neurons are located outside the spinal cord (white arrows) in regions that correspond to the future DF. **(a´)** In the same transverse section, another population of EGFP axons grows to the DLF. **(b)** Dorsal quadrant of a transverse spinal cord section from a $Zic2^{EGFP}$ newborn mouse shows that many $Zic2/EGFP^{+}$ axons are in the DLF while some others are in the DF. **(c)** Open-book preparation from an E15 $Zic2^{EGFP}$ embryo shows that Zic2/EGFP axons run ipsilaterally into the DLF. **(D)** Schematic representation of the experimental procedures. CAG-DsRed plasmids were unilaterally injected in the spinal cord of E12 $Zic2^{EGFP}$ embryos by *in utero* electroporation and transverse spinal cord sections from electroporated embryos were analyzed four days later. The trajectory of axons double labelled with DsRed and EGFP (yellow) was analyzed. **(E)** Transverse spinal cord section of an E16 $Zic2^{EGFP}$ embryo unilaterally electroporated with CAG-DsRed plasmids shows that double-labeled DsRed/$Zic2^{EGFP}$ axons project ipsilaterally into the DF as well as into the DLF. **(e, e´)** Higher magnifications of squared areas in E. **(F)** An open-book preparation of an E15 $Zic2^{EGFP}$ embryo electroporated with CAG-DsRed plasmid show that DsRed/EGFP axons run ipsilaterally along the DLF. **(G)** A transverse spinal cord section from a P0 mouse stained with Zic2 (green) and Parvalbumin (red) antibodies show that the arrival of PV afferents coincide with the location of Zic2 neurons in the dorsal cord. DLF, dorsolateral fascicle; DF, dorsal fascicle; dh, dorsal horns.

4.3 Zic2 dIL$_B$ neurons project ipsilaterally

We have demonstrated that GABAergic neurons are locally projecting neurons that do not project to

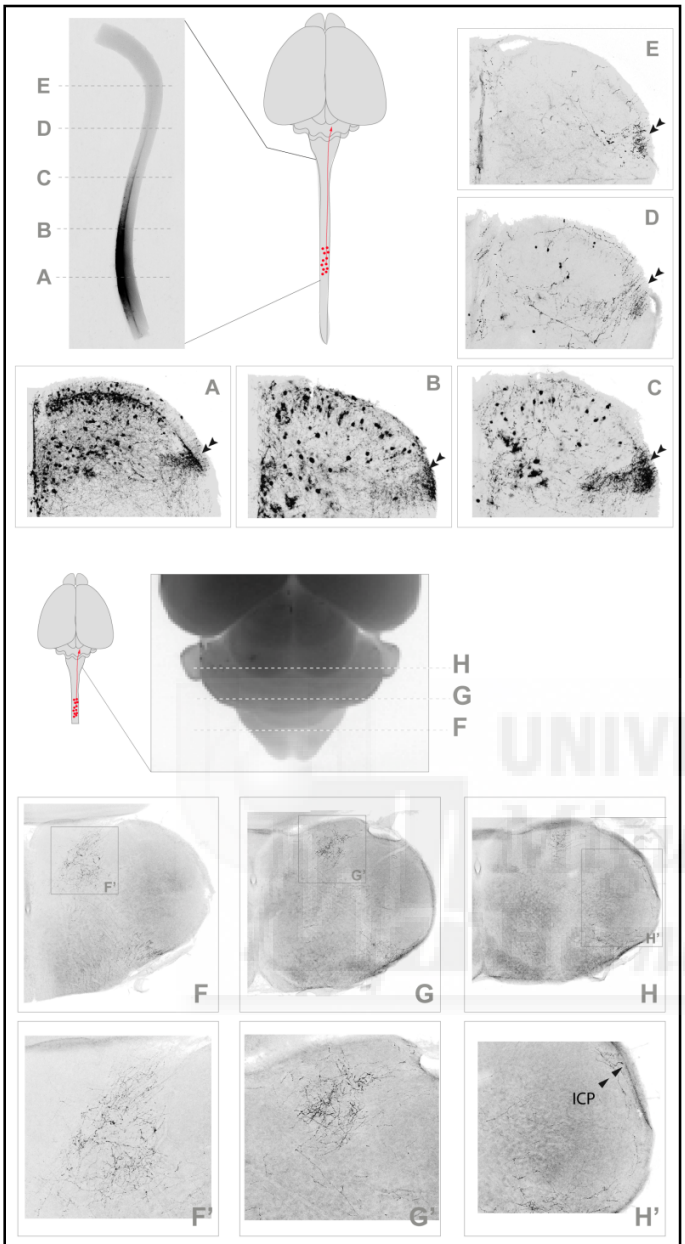

Figure 16. Late-born dorsal spinal neurons project propriospinally and supraspinally.

(A-E) E12 embryos were focally electroporated with DsRed2 encoding plasmids in the lumbar region. Coronal sections at the indicated levels from P7 electroporated spinal cords were analyzed. **(A-C)** Transverse sections of the same spinal cord at different rostrocaudal levels in the electroporated region where targeted cell bodies reside. DLF is intensively labeled (arrowheads). **(D-E)** Transverse sections rostral to the targeted area show a reduction in the number of axons projecting in the DLF (arrowheads). **(F-H)** A P7 mouse brain electroporated locally at E12 in the spinal thoracic region. Coronal sections taken at the indicated levels. **(F-G)** Tranverse sections show axons projecting to the cuneate nucleus. **(H)** Axons could be followed to the inferior cerebellar peduncle (ICP).

the DF or the DLF. Now, to define the projection phenotype of Zic2 glutamatergic interneurons we have used a Zic2^{EGFP} reporter mouse line. In these mice the expression of EGFP allows the visualization of axons from Zic2 neurons. At E13/E14 two different populations of EGFP axons were detected. A set of axons extended laterally towards the DLF while another group of fibers grew into the DF (Figure 15. A). By birth, many EGFP axons were seen in both the DLF and the DF (Figure 15. B). In open-book preparations from the spinal cord of E15 Zic2^{EGFP} embryos, EGFP axons were observed ascending ipsilaterally into the DLF (Figure 15. C). In the case of the DF, EGFP labeled axons came from both sides and therefore it was difficult to distinguish whether they run ipsilaterally or not. To determine whether Zic2 axons exit the spinal cord through the DF running ipsilaterally, we unilaterally

electroporated a plasmid encoding the red fluorescence protein (pCAG-DsRed2) in the dorsal spinal cord of E12 Zic2^{EGFP} embryos. Most DsRed-axons were positive for EGFP (Figure 15. D, E) and yellow axons never crossed the mildine (Figure 15. F) demonstrating that Zic2 dIL$_B$ neurons that project into the DF also run ipsilaterally.

We next asked whether Zic2 neurons project to supraspinal regions or if they are part of the propriospinal system. Since the expression of EGFP in Zic2^{EGFP} mice is transient we could not use these mice to define the targets of the Zic2 neurons in the brain, therefore we performed electroporations in E12 wildtype embryos and analyzed only those P7 mice with electroporations restricted to very few segments of the spinal cord (Figure 16). Near the electroporated region, we found a significant number of axons travelling through the ipsilateral DF and DLF. However few axons were found in supraspinal regions (Figure 16). Given that at P7 most spinocerebellar axons have already reached the cerebellum (Nunes and Sotelo, 1985; Grishkat and Eisenman, 1995), the reduction in the number of axons that we observed in supraspinal regions suggest that most of the neurons targeted by electroporation at E12 are propriospinal neurons that project to different segments, forming an ipsilateral fast communication system between distant regions of the spinal cord (Petkó, 2012). The few axons found beyond the spinal cord were located in two areas: i) entering the inferior cerebellar peduncle and ii) projecting to the dorsal column nuclei (Figure 16).

These observations indicate that late born dorsal interneurons comprise propriospinal neurons, postsynaptic dorsal column cells and spinocerebellar neurons, all of them forming ipsilateral pathways running through the DF and DLF. Together with the experiments in Zic2^{EGFP} reporter mice, these results point to dIL$_B$ neurons being ipsilateral long-range projecting cells that include both propriospinal and supraspinal targets.

4.4 Zic2 is necessary to prevent midline crossing in the dorsal spinal cord

The dorsal spinal cord of Zic2 mutant mice is highly distorted due to the failure of neural tube closure, which results in holoprosencephaly, anencephaly and *spina bifida* (Brown, 1998; Nagai, 2000; Ybot-Gonzalez, 2007). This early and strong phenotype precludes the assessment of Zic2 function in the cord at later developmental stages. Therefore, to investigate the role of Zic2 in late-born spinal neurons we used and alternative approach. We designed shRNAs against Zic2 (Zic2RNAi; see Materials and Methods) and were electroporated unilaterally into the spinal cord of E12 mouse embryos, when most Zic2 postmitotic neurons are being generated (Figure 17. A). Four days later (E16) the axonal phenotype of targeted neurons was analyzed (Figure 17. A). We confirmed the downregulation of Zic2 expression after Zic2RNAi electroporation by quantifying Zic2 immunoreactivity in spinal cord sections. Zic2 protein expression was reduced 10% in the targeted side compared to the endogenous expression in the non-electroporated side (Non electroporated side: 100±2.71%, electroporated side: 90±2.91%, n=9 embryos) (Figure 17. B).

We hypothesized that if Zic2 was necessary for the determination of ipsilaterality in dorsal horn neurons, its downregulation should cause an increase in the number of axons crossing the midline to the other side, generating aberrant contralateral projections, a phenotype never observed in wild type dorsal horn neurons after electroporation of EGFP. As predicted, Zic2 downregulation lead to a significant increase in the number of targeted axons that aberrantly crossed the midline compared to the controls (Figure 17. C-E).

We also noticed that a number of Zic2RNAi/EGFP targeted neurons were mislocated in more superficial layers than control neurons targeted with EGFP alone (data not shown) suggesting that Zic2 keeps these neurons close to the midline and its downregulation may be necessary to reach the appropriate dorsal horn layer. We quantified the number of Zic2RNAi targeted cell bodies mislocated in the non-electroporated side and found that the number of cell bodies that crossed the midline was

more than twice compared to the controls (Figure 17. C-E).

These results indicate that Zic2 is critical to avoid midline crossing in dorsal horn neurons that normally project ipsilaterally through the DLF and the DF, and that Zic2 is also important to keep the cell bodies on the correct side.

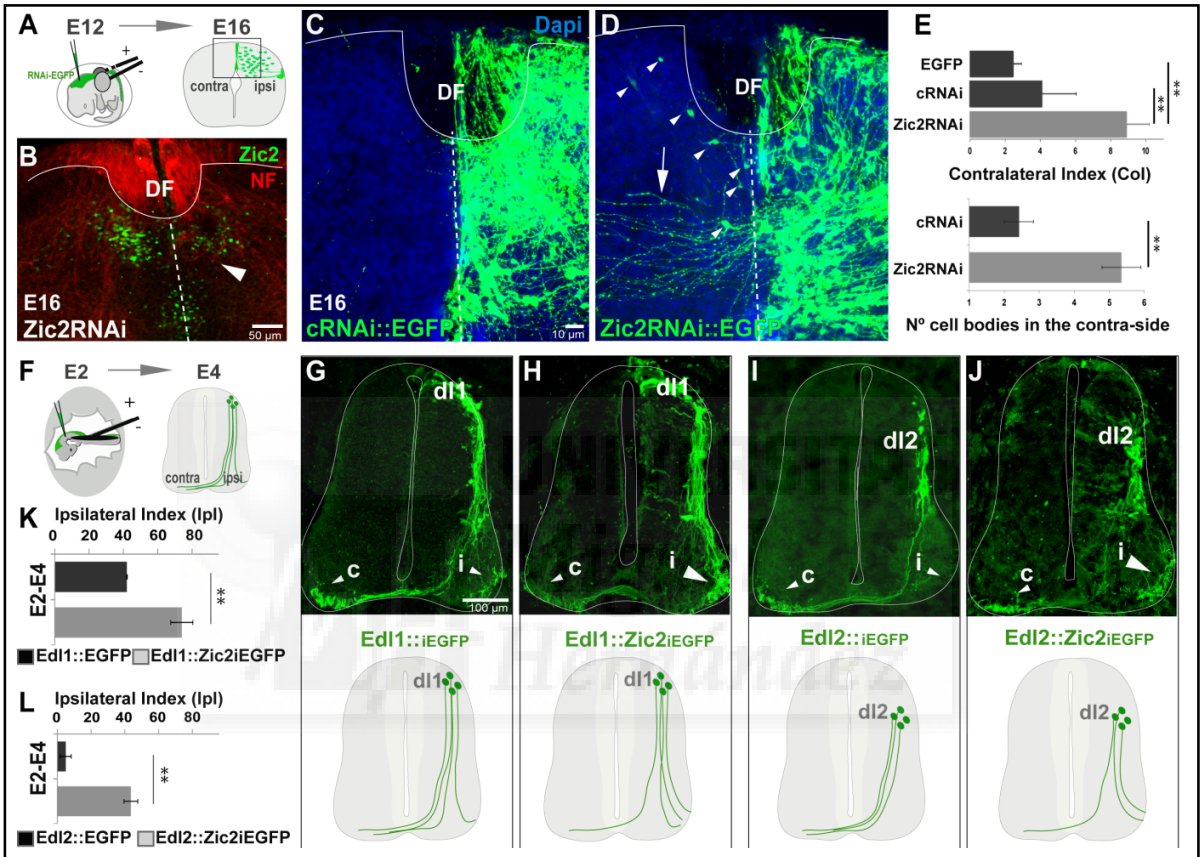

Figure 17. Zic2 is necessary and sufficient to determine axonal ipsilaterality in the spinal cord.

(A) Schematic representation of the experimental procedures. E12 embryos were electroporated with Zic2RNAi and harvested at E16 for analysis of the contralateral index (CoI). The square area marks the analyzed region. **(B)** A transverse spinal cord section of an E16 mouse embryo electroporated unilaterally with Zic2RNAi shows the effective downregulation of Zic2 protein (green) in the electroporated side (white arrow). Dashed line marks the midline. Neurofilament (NF) staining is shown in red. **(C, D)** Transverse spinal cord sections from E16 mouse embryos electroporated at E12 with plasmids encoding control RNAi and EGFP (cRNAi::EGFP) or Zic2RNAi and EGFP (Zic2RNAi::EGFP) show that the downregulation of Zic2 causes massive aberrant crossing (white arrow) at the dorsal midline (dashed white line). The arrowheads point to the cell bodies of mislocated neurons on the side contralateral to the electroporation. **(E)** Top, quantification of the contralateral index (CoI) in embryos electroporated with control plasmids (CAG-EGFP, control RNAi) or plasmids encoding Zic2RNAi. A significant increase of axons aberrantly crossing the dorsal midline after downregulation of Zic2 was observed. Bottom graph shows the quantification of the number of cell bodies found on the side contralateral to electroporation. Error bars indicate ±SEM (**p<0.01, Student's unpaired t-test). **(F)** Schematic representation of the experimental procedures. E2 embryos were electroporated and harvested at E4 for analysis and quantification of the ipsilateral index. **(G-H, I-J)** Transverse spinal cord sections from E4 chick embryos electroporated at E2 with plasmids driving EGFP expression to the dI1 domain (EdI1) or to the dI2 domain (EdI2) show an increase in ipsilateral axons after Zic2 induction compared to the controls. Schemes below summarize the results in each case. C, contralateral side; I, ipsilateral side. **(K, L)** Quantification of the ipsilateral index (Ipl) in E4 chick embryos electroporated at E2 with EdI1:EGFP or EdI2:EGFP plasmids revealed an increase in the number of axons projecting ipsilaterally after the induction of Zic2. Error bars indicate ±SEM (**p<0.01, Student's unpaired t-test).

To investigate whether Zic2 is not only necessary to avoid midline crossing, but also sufficient to switch the laterality of spinal cord axons, we performed *in vivo* gain-of-function experiments. The aim was to ectopically express Zic2 in spinal cells that under physiological circumstances project axons contralaterally across the midline. Considering that our electroporations in E12 mouse embryos rarely targeted contralateral interneurons, we turned to the chick spinal cord because electroporation *in ovo* would give us access to earlier developmental stages, when most of the generating cells are contralaterally-projecting neurons (Wentworth, 1984; Nandi, 1991; Altman and Bayer, 2001). First we confirmed that Zic2 electroporation in the chick spinal cord did not have any effect in cell differentiation or subtype specification. Expression vectors containing EGFP alone (pCAG-IRES-EGFP) or Zic2 plus EGFP (pCAG-Zic2-IRES-EGFP) coding sequences were unilaterally introduced into the spinal cord of chick embryos at HH14-16 and embryos were analyzed two days later by immunostaining with markers for the different dorsal interneuron types (dI1-6) generated at these stages (Lhx2/9, Lhx1/5, Isl1, Brn3a, Tlx3, Lbx1, Lmx1b and Pax2). Electroporated embryos sacrificed at E4 revealed that ectopic expression of Zic2 does not affect cell differentiation or fate (Figure 18) allowing thereby the examination of the axonal phenotype of neurons that ectopically express Zic2. To visualize the axons of a specific cell population better than labeling all interneurons unspecifically, we took advantage of a specific promoter previously used to drive gene expression to interneurons of the dI1 domain (EdI1) (Avraham, 2009). As expected from previous reports, electroporation of EdI1::IRES-EGFP plasmids at HH14-16 mainly labeled commissural axons at E4 with only a small population of ipsilaterally-projecting dI1 neurons. However, after electroporation of EdI1::Zic2-IRES-EGFP plasmid, approximately 50% of targeted commissural axons switched to project ipsilaterally (Figure 17. G, H,

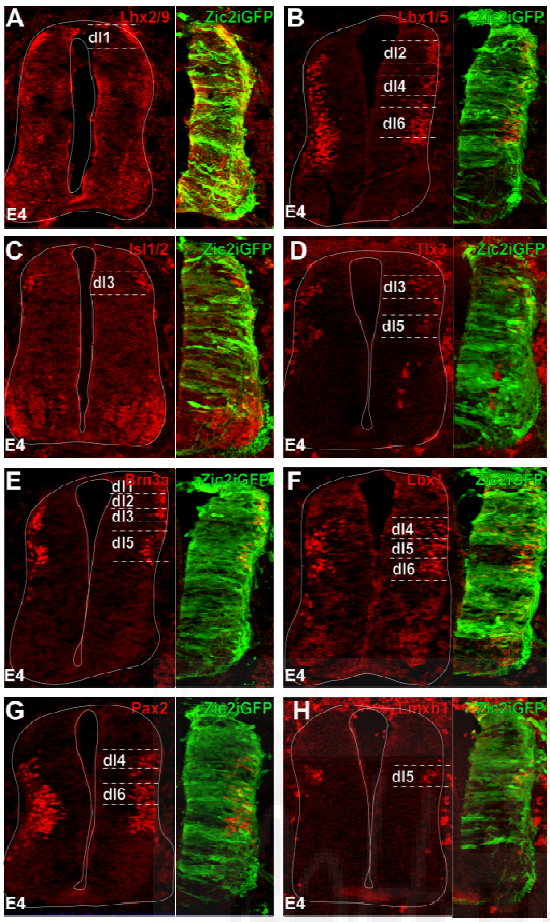

Figure 18. Alterations in the expression of Zic2 do not affect cell fate.

(A-H) 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 show no alteration in cell fate in the electroporated compared to the non-electroporated side. Electroporated sides are shown at the right of the corresponding images.

K). To address whether Zic2 is also sufficient to switch axonal laterality in a population of interneurons that does not contain ipsilaterally-projecting neurons endogenously, we used an enhancer that drive gene expression to interneurons from the dI2 domain (EdI2) (Avraham, 2009). As previously reported, HH14-16 chick embryos electroporated with EdI2::IRES-EGFP plasmid showed mostly crossed axons (Avraham, 2009). In contrast, after electroporation of EdI2::Zic2-IRES-EGFP plasmid, embryos exhibited a significant increase (GFP Ipl:4.99±3.23%, Zic2 Ipl: 43.56±4.07%) in the number of ipsilateral axons compared to the controls (Figure 17. I, J, L). Since dI1 and dI2 enhancers drive gene expression to postmitotic commissural neurons, these results demonstrate that postmitotic expression of Zic2 is sufficient to switch axonal laterality of commissural spinal neurons.

4.6 Zic2 is not expressed in ipsilateral neurons whose axons do not approach the midline

As shown above, some early born neurons contain ipsilaterally-projecting neurons. In particular, some neurons from the dI1 domain (dI1i) (Bermingham, 2001; Wilson, 2008; Ding, 2012) and dI3 neurons

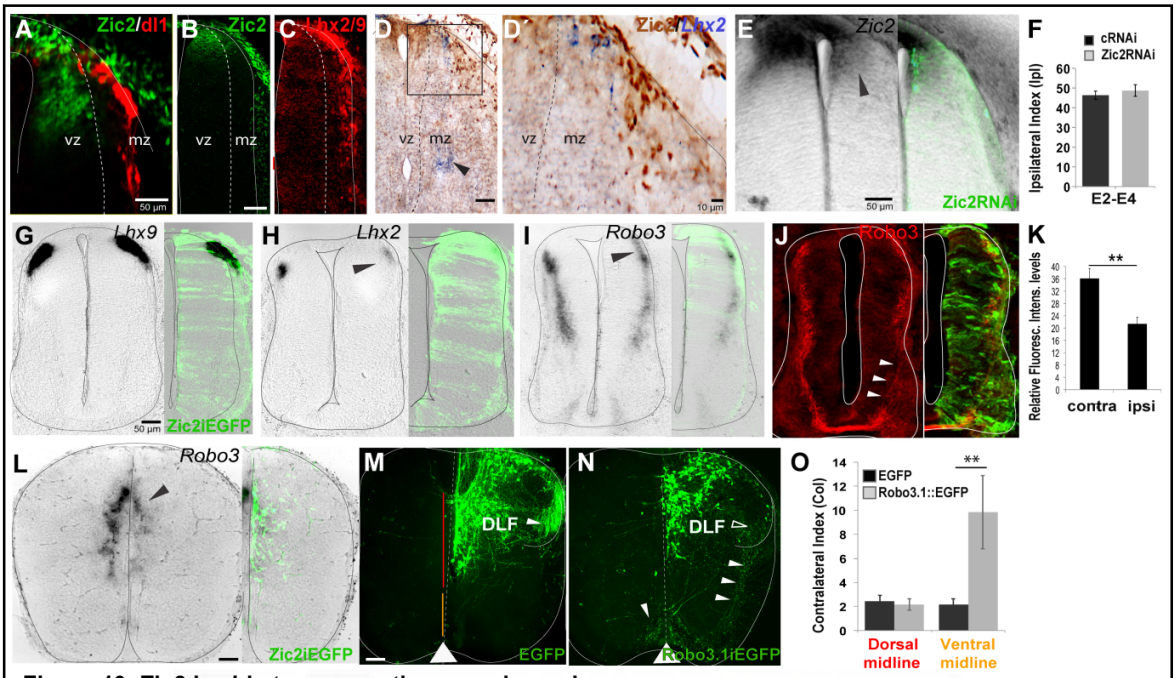

Figure 19. Zic2 is able to repress the commissural program.

(A) Dorsal quadrant of a transverse spinal section of a E4 chick embryo electroporated with EdI1::EGFP plasmids (red) and immunostained with Zic2 antibodies (green) shows Zic2 expression in progenitors but not in dI1 interneurons. **(B, C)** Consecutive transverse spinal cord sections from an E11 wildtype mouse embryo immunostained with Zic2 (green) and Lhx2/9 (red) antibodies show that these transcription factors do not localize at this stage. **(D)** Immunostaining against Zic2 (brown) combined with *in situ* hybridization for *Lhx2 mRNA* (blue) in spinal cord transverse sections of E12 wildtype mouse embryos. At E12 most Lhx2 neurons occupy medial positions in the deep horn while some others are still dorsally located. None of the Lhx2 populations colocalize with Zic2, which shows a more lateral location at this stage. **(d)** High magnification of the dorsal spinal cord section in (D) shows that Zic2 and Lhx2 do not colocalize in dorsal areas. **(E)** *In situ* hybridization against chick Zic2 in transverse spinal cord sections of E4 chick embryos electroporated with Zic2RNAi at E2 leads to effective Zic2 downregulation (black arrow) in the chick dorsal cord. **(F)** Quantification of axons projecting ipsilaterally versus total targeted axons in E4 chick embryos electroporated at E2 with controlRNAi (cRNAi) or Zic2RNAi. The graph shows that at this stage downregulation of Zic2 does not affect axonal laterality. **(G-I)** *In situ* hybridization against Lhx9, Lhx2 and Robo3 in transverse spinal sections from E4 chick embryos electroporated unilaterally with CAG-Zic2-IRES-EGFP (Zic2iEGFP) plasmid shows no alteration of Lhx9 mRNA levels while Lhx2 and Robo3 mRNA levels decreased (black arrows) after Zic2 induction. The electroporated side is shown at the right of each section. **(J)** Immunohistochemistry against Robo3 (red) in transverse spinal sections from E4 chick embryos electroporated unilaterally with Zic2iEGFP plasmid show decreased levels of Robo3 in the commissural axons of the electroporated side (white arrows). **(K)** Quantification (measured by fluorescence intensity levels) of Robo3 protein levels in the side electroporated with Zic2iEGFP plasmid compared to the non-electroporated side. Error bars indicate ±SEM (**p<0.01, student´s unpaired t-test). **(L)** *In situ* hybridization against Robo3 in transverse spinal sections from an E14 mouse embryo unilaterally electroporated with CAG-Zic2 and CAG-GFP shows a decrease of *Robo3mRNA* levels in the electroporated side (black arrow). **(M, N)** Transverse spinal sections of E16 mouse embryos electroporated at E12 with CAG-Robo3.1 and CAG-GFP plasmids show that most axons ectopically expressing Robo3.1 are not directed to the DLF but grow instead ventrally to cross the ventral midline (while arrows). **(O, P)** Quantification of the number of axons that cross the dorsal and the ventral midline in E16 mouse embryos electroporated at E12 with Robo3.1. Vz, ventricular zone; mz, mantle zone; DLF, dorsolateral fascicle.

(Avraham, 2010) project ipsilaterally. The LIM homeodomain transcription factors Lhx2 and Lhx9 are expressed in spinal interneurons from the dI1 domain and distinguish commissural (dI1c) and

ipsilateral (dI1i) pools. At early stages, when postmitotic neurons migrate out of the ventricular zone, Lhx2 and Lhx9 are strongly expressed by all dI1 neurons. Later, Lhx2 but not Lhx9, disappears from the dI1i. Since dI1i neurons project ipsilaterally and we observed that some Zic2 neurons are born at E11 (Figure 11), we considered the possibility that postmitotic expression of Zic2 was also responsible for the axonal laterality of early-born dI1i interneurons. To address this question we performed Zic2 immunostaining in chick embryos electroporated with the EdI1::EGFP plasmid to label neurons from the dI1 domain and found that dI1 targeted neurons never express Zic2 (Figure 19. A). Immunostaining with Lhx2/9 and Zic2 antibodies at different stages showed that when Lhx2/9 neurons are already differentiated, Zic2 is expressed only in the ventricular layer (Figure 19. B, C) and later, when the first postmitotic Zic2 neurons leave the cell cycle, most Lhx2/9 neurons are already located in the medial deep dorsal horn (Figure 19. D). A low number of Lhx2 cells were still located in dorsal areas at E12 but Zic2 and Lhx2 never colocalized in this region (Figure 19. d). Moreover, downregulation of Zic2 by electroporation of cZic2RNAi at early stages of chick spinal cord development yield similar ratio of ipsilaterally-projecting axons than control RNAis (Figure 19. E, F), supporting the idea that Zic2 does not control the axonal ipsilaterality of dI1i interneurons.

These results demonstrate that Zic2 is not required to determine the ipsilateral trajectory of dI1i neurons and suggest that Zic2-dependent mechanisms are used exclusively by those ipsilaterally projecting neurons that must actively avoid midline crossing.

4.7 Zic2 represses the expression of Lhx2 and Robo3

To understand the mechanisms underlying the ability of Zic2 to yield an ipsilateral phenotype we analyzed its relationship with the *Lhx* genes that are thought to promote dI1 axon crossing in the spinal cord. Zic2 was ectopically induced in chick spinal cord and the expression pattern of Lhx genes examined (Wilson, 2008; Ding, 2012). Ectopic induction of Zic2 into the spinal cord of chick embryos

did not affect *Lhx9 mRNA* expression but produced a strong downregulation in the expression of *Lhx2 mRNA* (Figure 19. G, H). According to previous reports, Lhx2 controls the expression of Robo3/Rig1 (Robo3 from now on) in dI1c neurons (Ding, 2012). Robo3 is a guidance receptor essential to cross the midline (Sabatier, 2004). We reasoned that downregulation of Lhx2 expression should cause a loss of Robo3 expression that would be responsible for the ipsilateral phenotype induced by Zic2. Both in situ hybridization and immunostaining showed a clear decrease in mRNA and protein levels of Robo3 after ectopic expression of Zic2 (Figure 19. H-K). These results show that Zic2 is able to efficiently repress the commissural program mediated by Lhx2.

At later stages, we noticed a previously undescribed expression of Robo3 in a population of dorsal neurons (Figure 19. L). This late expression of Robo3 at E14 was also reduced after ectopic induction of Zic2 in E12 mouse embryos (Figure 19. L). These results lead us to postulate that deactivation of Robo3 is a prerequisite in dorsal horn neurons to avoid midline crossing. To test this possibility we ectopically expressed Robo3.1, the Robo3 isoform that promotes commissural axon crossing (Chen, 2008), in dorsal cord cells by *in utero* electroporation. Strikingly, after ectopic expression of Robo3.1, axons from dorsal horn neurons aberrantly grew towards ventral cord regions instead of following their normal path to the ipsilateral DLF. A significant number of these Robo3-misrouted axons crossed the ventral midline through the floor plate compared to the controls (Figure 19. M-O) as commissural axons normally do. Together these results suggest that downregulation of Robo3 is necessary to prevent axon extension into ventral cord regions and across the ventral midline.

4.8 Zic2 regulates the expression of EphA4 to prevent dorsal midline crossing

Our results suggest that downregulation of Robo3 is essential to avoid dorsal axons from growing into the ventral spinal cord. However, Robo3 overexpression did not cause axon crossing at the dorsal midline as Zic2-downregulation did, suggesting that downregulation of Robo3 is not sufficient to induce

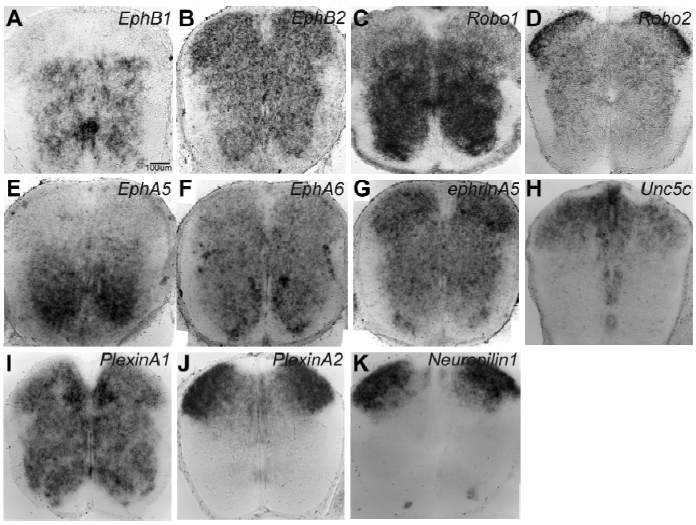

Figure 20. Expression pattern of repulsive guidance receptors in the developing spinal cord.

(A-H) *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.

midline avoidance. Therefore, to avoid dorsal midline crossing, Zic2 must be regulating the expression of other effector molecules. Although, the tyrosine kinase receptor EphB1 has been described as the main effector of Zic2 in ipsilateral retinal fibers (Garcia-Frigola, 2008), we found that EphB1 is not expressed in the dorsal cord at the peak of Zic2 expression (Figure 20). We turned then to analyze the expression of other guidance receptors known to mediate axon repulsion in different contexts such as EphB2, EphA4, EphA5, EphA6, ephrinA5, Robo1, Robo2, PlexinA1, PlexinA2, Neuropilin1 and Unc5c. None of these molecules matched Zic2 expression in the developing dorsal horn (Figure 20) except EphA4 that showed a similar pattern to Zic2 at E14 and E16 (Figure 21. A).

EphA4 is an atypical Eph receptor since it is the only member of the EphA family able to interact with both ephrinA and ephrinB ligands (Kullander and Klein, 2002; Pasquale, 2004). EphA4 has been previously implicated in determining the ipsilaterality of excitatory spinal neurons by ephrinB3-mediated repulsion to the midline (Kullander, 2003). Consequently, we analyzed the expression patterns of ephrinB ligands in the spinal cord. *In situ* hybridization for ephrinB1, ephrinB2 and ephrinB3 mRNAs showed that these genes are highly expressed in the spinal dorsal midline (Figure 21. B, D), moreover ephrinB2 and ephrinB3 were also expressed in the superficial laminae of dorsal horns showing a complementary pattern to EphA4. Therefore, EphA4/ephrinBs signaling

seemed a good candidate to mediate midline avoidance controlled by Zic2 in dIL_B spinal neurons. To evaluate this possibility we analyzed EphA4 mRNA levels after alteration of Zic2 expression in the dorsal cord and found that EphA4 mRNA levels significantly increased on the side electroporated after ectopic introduction of Zic2 (Figure 21. E-F) while it was significantly downregulated after electroporation of Zic2RNAi (Figure 21. G).

These results together indicate that Zic2 regulates EphA4 and suggest that EphA4/ephrinB signaling may mediate midline avoidance at the dorsal spinal cord.

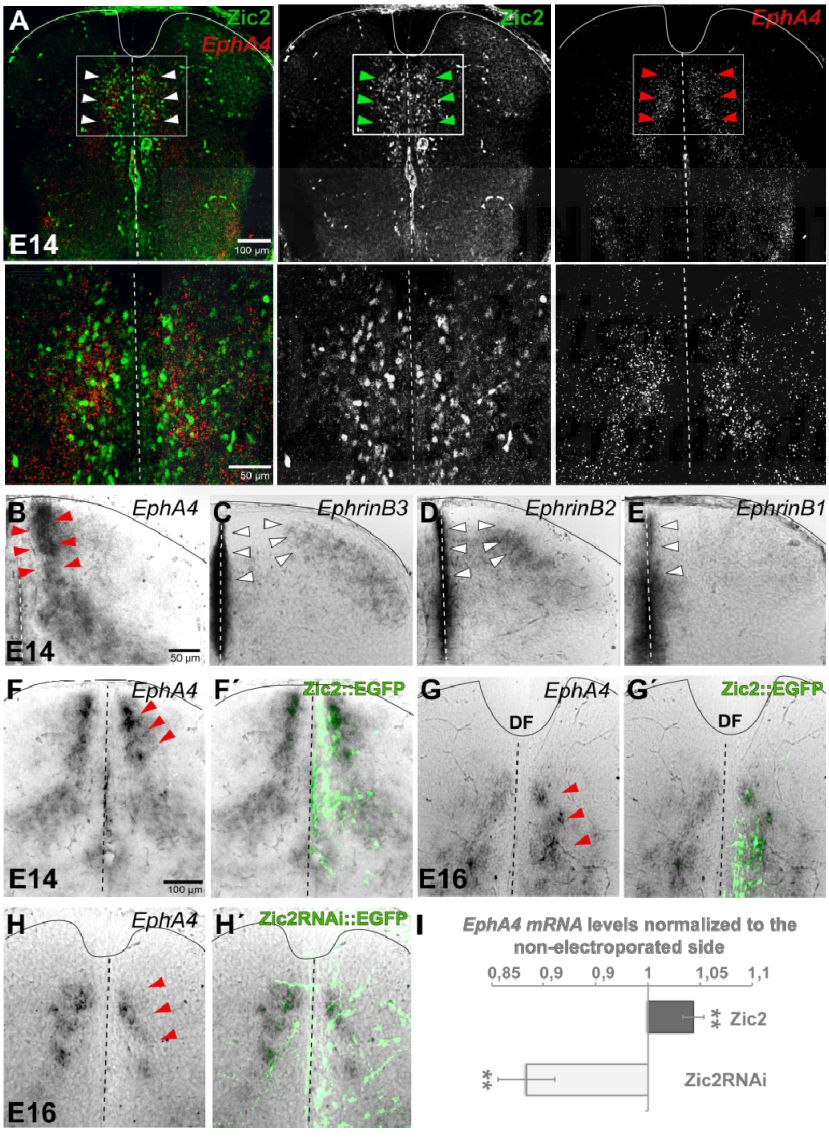

Figure 21. Zic2 induces EphA4 in the dorsal spinal cord

(A) Immunostaining of Zic2 (green arrows) in transverse spinal cord sections from E16 wild-type embryos combined with *in situ* hybridization against EphA4 (red). **(B-E)** *In situ* hybridization for EphA4 (red arrowheads), ephrinB3, ephrinB2 and ephrinB1 mRNAs (white arrowheads) in transverse spinal cord sections from E14 embryos. All three ephrinBs were expressed at the dorsal midline. In addition, ephrinB3 and ephrinB2 were expressed in the prospective dorsal horns (white arrowheads). **(F-G)** *In situ* hybridization against EphA4 in transverse spinal cord sections of E14 and E16 embryos electroporated at E12 with CAG-Zic2 plasmids show increased EphA4 mRNA on the electroporated side (red arrows) compared to the non-electroporated side. **(F´, G)** Same sections depicting the electroporated side (green). **(G)** *In situ* hybridization against EphA4 in a representative transverse spinal cord section of an E16 embryo electroporated at E12 with Zic2RNAi showed …

… decreased levels of EphA4 mRNA in the electroporated side (red arrows) compared to the non-electroporated side. **(G´)** Same section depicting the electroporated side (green). **(H)** Quantification of EphA4 mRNA levels in a representative transverse spinal cord section from an E16 embryo electroporated at E12. EphA4 mRNA levels increased after Zic2 ectopic expression (dark grey column) and decreased after Zic2RNAi electroporation (light grey column). EphA4 mRNA levels in the electroporated side were normalized to the levels of the respective non-electroporated side. Error bars indicate ±SEM (**p<0.01, Student's unpaired t-test).

Chapter V

Discussion

Our findings shed some light onto the problem of how axons connect brain hemispheres in bilaterally organized organisms. The choice of crossing or not the midline that many developing axons confront during the formation of the nervous system has served as an useful model to understand the mechanisms of axon guidance over the last decades. Cumulative efforts have revealed a complex landscape of different guidance cues and receptor families implicated in midline crossing. However, information regarding the control of the equally important ipsilateral pathway is sparse, with few reports restricted to the mammalian visual system. We show here that the transcription factor Zic2, identified in the visual system as a determinant of the ipsilateral pathway, also specifies axon midline avoidance in dorsal horn spinal neurons. However, Zic2 expression is not required in ipsilateral neurons that extend axons from a lateral position and never confront midline cues such as dI1i interneurons. These results demonstrate that Zic2 plays a general and highly conserved role in the establishment of bilateral circuits in the CNS by preventing the crossing of those ipsilateral neurons whose axons approach to the midline at some point of their trajectory.

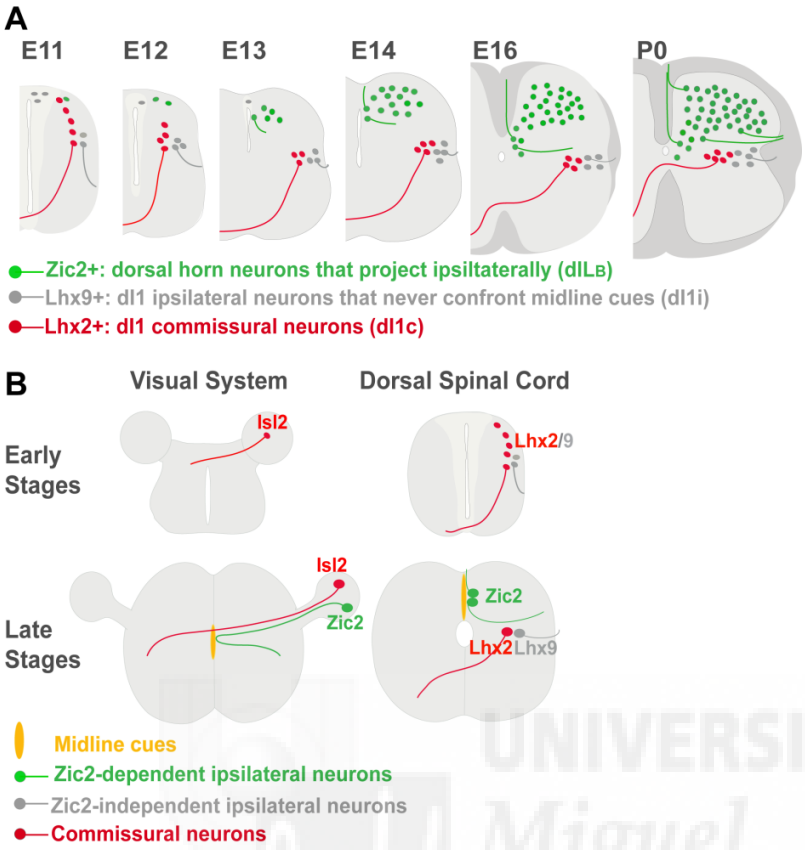

Figure 22. Summary of Zic2 expression in the developing spinal cord and the visual system

(A) dI1 interneurons become postmitotic as early as E10 and migrate and extend axons ventrally, in the case of Lhx2 neurons (red), or laterally, in the case of Lhx9 neurons (grey). The first postmitotic Zic2 neurons (green) appear around E11, at the time that dI1 neurons are still being generated, but they populate the dorsal horns instead of migrating ventrally. From E13 to E16, a high number of Zic2 neurons extend their axons to the DLF and to the DF. By P0, most dorsal horn neurons are already in their final location. We propose that the dorsal horn neurons that differentiate and start to extend their axons close to the midline need to develop mechanisms to actively avoid crossing. In contrast, axons from other ipsi-laterally projecting neurons that never approach the midline, such as the dI1i neurons, do not express Zic2 because they do not need to avoid midline crossing.
(B) In the visual system, Isl2 is expressed in contralateral retinal ganglion cells (RGCs) and Zic2 is postmitotically upregulated only in the RGCs that will be repelled to project ipsilaterally. In the early spinal cord, Lhx2/9 are both expressed in dI1 interneurons. dI1 interneurons migrate ventrally, and their axons navigate laterally and never confront midline cues. In contrast, later-born interneurons (dIL_B) express Zic2, which triggers the expression of a set of molecules that are essential for responding to dorsal midline cues.

5.1 Zic2 impedes midline crossing of dorsal spinal horn neurons

As soon as gastrulation is finished, Zic transcription factors are expressed at the neural plate borders and they seem to be essential to induce neurulation (Aruga, 2004). After neural tube closure, Zic2 continues to be expressed in dorsal neural progenitors although its role at this stage is unclear. At later stages, when neurogenesis is mostly finished in the cord, Zic2 is transiently expressed near the midline in a population of postmitotic dorsal neurons.

Previous work has shown that downregulation of Zic2 at early stages of development produces severe defects in neurulation (Aruga, 2006; Merzdorf, 2007). These defects in the neural tube closure of Zic2 mutant mice complicated the investigation of a possible role for Zic2 in postmitotic neurons. Here, our *in utero* electroporation approach combined with efficient delivery of Zic2RNAi at late developmental stages, when Zic2 is expressed postmititocally, demonstrates that expression of Zic2 in differentiated neurons is essential to avoid axon midline crossing in the dorsal cord. In addition, ectopic expression of Zic2 in postmitotic dI1 and dI2 neurons indicates that postmitotic expression of Zic2 is also sufficient to switch axonal laterality at the midline. Together all these results prove that the main function of this transcription factor in postmitotic dorsal cord neurons consist on impeding midline crossing.

Spinal cord sections from $Zic2^{EGFP}$ embryos show that by E13 Zic2–expressing neurons are already extending their axons to the ipsilateral DF and DLF. Mature dorsal horn neurons do not express Zic2. However, because many dorsal horn neurons express EGFP in the $Zic2^{EGFP}$ mice we assume that dorsal horns neurons expressing EGFP arise from the postmitotic Zic2 population. These observations suggest a rapid downregulation of Zic2 once axons are expelled from the midline and before EGFP-expressing neurons reach their final location in the dorsal horns.

Zic2 neurons belong to the B-subtype of late born interneurons as demonstrated by immunohistochemical analyses of $Zic2^{EGFP}$ reporter mice and fate-switch experiments. dIL neurons populate the dorsal horns and are subdivided in excitatory (dIL_B) and inhibitory (dIL_A) subtypes. Together with investigations on the organization of the dorsal horn connections (Petkó, 2012), our data suggest an important role for Zic2 in determining the laterality of excitatory dorsal horn neurons.

Our findings on the projection patterns of Zic2-spinal interneurons, together with previous results (Giesler, 1978; Willis, 1978), suggest that Zic2-dorsal horn cells are ipsilateral neurons composed by propriospinal and supraspinal pathways. Further efforts using specific reporters for

mature dIL neurons will reveal whether Zic2 dL_B neurons are divided in different subpulations regarding their targeting projection pattern.

Molecular programs controlling axon guidance have been shown to also play important functions in the migration of the cell body (Marillat, 2004; DiMeglio, 2008). Here, we observed that downregulation of Zic2 in the spinal cord caused an increase in the number of neurons that cross the dorsal midline. This result, together with the loss of axon repulsion at the midline, points to Zic2 as a mechanism that could be controlling the mediolateral positioning of the cell bodies. It is possible that lack of Zic2 in spinal interneurons primarily causes movement of somas and that axon crossing is an indirect consequence of cell reorganization. However, since ephrinB3 is a cell-membrane protein and does not diffuse in a gradient, this is an unlikely scenario, being more probable that axons, that generally explore the surrounding territory, touch the ephrinB3 positive midline cells to be repelled. Supporting this idea, many observations suggest that cell bodies of migrating interneurons follow their axon and not the other way around (Marin, 2010; O'Leary and McDermott, 2011). Therefore, we believe that the loss of Zic2 causes interneurons to aberrantly project their axons across the midline pulling their cell bodies to then follow the same abnormal path.

5.2 Zic2 is able to repress the contralateral program

The dI1 population of interneurons has been extensively used to analyze the molecular programs that control the mechanisms of midline crossing (Wilson, 2008; Kawauchi, 2010; Ding, 2012). Although Zic2 is not implicated in determining axonal laterality in dI1 neurons, our experiments help to understand the molecular control of axon guidance in this neural type. The dI1 domain comprises two subtypes of cells regarding their axonal projection and cell body position in the dorsal horn: Axons from dI1c neurons cross the floor plate midline and their somas settle medially at the base of the dorsal horns. In contrast, the cell bodies of dI1i neurons are laterally positioned and their axons project ipsilaterally to the lateral

fasciculus. These differences have been attributed to the differential expression of the LIM-HD transcription factors Lhx2 and Lhx9. However, the precise role of these proteins in mediating axonal laterality is not clear because although dI1c axons are not able to cross the midline in double Lhx2/9 mutant mice, single KO mice for each of these transcription factors do not exhibit any phenotype regarding dI1 neurons (Wilson, 2008). We show that ectopic expression of Zic2 downregulates Lhx2 and Robo3 to impede axon crossing, but do not affect Lhx9 reinforcing recent data suggesting that Lhx2 but not Lhx9 is implicated in midline crossing (Ding, 2012).

Downregulation of Robo3 after loss of Lhx2/9 (Wilson 2008) together with the ectopic expression of Robo3 found after loss of Barhl2 (Ding 2012) demonstrate the importance of this atypical robo receptor (Camurri, 2005) in the guidance of commissural neurons (Chedotal, 2011). Robo3 is expressed through two splice variants with different functions. While Robo3.2 promotes axonal repulsion to Slits and expels axons away from the midline, Robo3.1 silences the repulsive response triggered by Robo1/2 to floor plate derived Slits, allowing axon growth towards the midline (Sabatier 2004; Chen 2008). It is then reasonable to expect that any mechanism controlling the guidance of ipsilateral axons would block, directly or indirectly the expression of Robo3.1, as it seems to be an important part of the contralateral program. Accordingly we observed that Zic2-neurons do not express Robo3.1 and that ectopic induction of Zic2 into contralateral neurons represses Robo3.1 expression. However, ectopic expression of Robo3.1 in dorsal horn neurons does not cause axons to ectopically cross through the dorsal midline (as Zic2 downregulation does). Instead, ectopic expression of Robo3.1 promotes axons to grow towards the ventral midline and cross at the floor plate. It has been proposed that instead of, or together with, functioning as a repulsion silencer, Robo3.1 would act as an attractant to the floor plate (Sabatier, 2004; Chen, 2008; Jaworski, 2010). Our results on the ectopic expression of Robo3.1 favor this hypothesis and suggest that deactivation of Robo3 is required to establish a proper ipsilateral projection. This idea is also supported by Robo3 loss of functions

experiments through downregulation by RNAi injections (Philipp, 2012 and E. Stoeckli personal communication) in which aberrant ipsilateral axons turn laterally to project near the DLF.

5.3 Transcriptional control of guidance receptors in different systems

In the visual system, Zic2 acts through EphB1 to determine the ipsilateral pathway (Garcia-Frigola, 2008). Based on this observation, we checked EphB1 expression pattern in the spinal cord but in contrast to the expected, EphB1 was not detected in the dorsal horns. However, we found that EphA4, another member of the EphA family, shows an expression pattern compatible with a possible regulation by Zic2. Although we cannot discard that receptors such as EphB2, Robo1/2 and PlexinA1, play a role in the prevention of midline crossing, it is unlikely that they depend on Zic2 because they are ubiquitously expressed in the developing cord (Figure 20). By functional *in vivo* experiments we demonstrate that Zic2 is necessary and sufficient to modulate EphA4 expression in the dorsal cord. EphrinB1, B2 and B3, three main ligands for EphA4, are highly expressed in the dorsal midline in a complementary pattern to EphA4, suggesting that EphA4/ephrinB repulsive signaling prevents axons from crossing the dorsal midline. Because the only EphA receptor that binds to ephrinBs ligands is EphA4 (Kullander and Klein, 2002; Murai and Pasquale, 2003), it is not surprising that instead of EphB1 (the main Zic2 effector in the visual system) the Eph receptor regulated by Zic2 in the dorsal cord is EphA4. EphA4 and EphB1 bind to ephrinB2, and it is likely that, in both cases, regulatory sequences that respond to Zic2 activity have been evolutionarily selected.

EphrinB2/B3 are also expressed in the dorsal horns and they also may prevent axons from invading the dorsal horns. In agreement with this hypothesis, downregulation of Zic2 leads to premature cell body and axon invasion into the dorsal horns. Indeed, not only ephrinBs but also ephrinA5 is expressed in the dorsal horns possibly establishing a repulsive territory for dorsal horn

axons. According to this, a role for Eph/ephrin signaling has been previously proposed for the positioning of axonal tracts in the white matter (Imondi and Kaprielian, 2001).

5.4 Zic2 regulates midline avoidance but not ipsilaterality per se

During development, different distances separate early-born (dI1-6) and late-born (dIL) neurons from the midline. By the time that dI1-6 interneurons start axonogenesis they are located laterally in the mantle and separated from the midline by the ventricular zone. At the early stages, the dorsal ventricular zone occupies more than half of the thickness of the neural tube wall. Early-born neurons extend their axons circumferentially along the basal membrane to finally settle in the deep dorsal horn, never encountering short-range midline cues along their trajectory (Avraham, 2010; Bermingham, 2001; Helms and Johnson, 1998; Wilson, 2008). Under these circumstances, establishing an ipsilateral projection may rely on fasciculation with the contralateral axons coming from the other side, a process that could depend on Lhx9 function. In contrast, during the differentiation of dL_B neurons, the progenitor layer is dramatically reduced in size and, as a consequence, neurons are born in close proximity to the midline. We propose that dIL_B neurons, express Zic2 to actively avoid the midline due to its proximal midline location at the time of axon extension.

A similar explanation may apply to the ventral spinal cord (V0-V3) and to the thalamic neurons. A number of ventral interneurons project ipsilaterally (reviewed in Goulding, 2009) but Zic2 is not expressed in ventral spinal cells. During the generation of ventral spinal neurons (between E10 and E12), progenitors occupy medial neural tube regions and the ventricle physically separates the two sides of the tube. At this time, pioneering axons extend laterally to contact with motor neurons or project into the ventro-lateral bundles never approaching the midline. In ventral regions a second round of neural differentiation proximal to the midline never takes place and therefore there would be no need

for Zic2 expression.

In the thalamus, early-born neurons would never have the opportunity of crossing the midline due to the existence of third ventricle and progenitors located in medial thalamic regions. At later stages, the ventricle shrinks, progenitors become postmitotic neurons and the two thalamic leaves fuse in a caudo-rostral manner. At this time the late-born population of TC cells (rmTC neurons) would need Zic2 to avoid crossing because they are located close to the midline. In contrast caudolateral TC neurons in the thalamus extend their axons distally from the midline and therefore their laterality would be determined by a different mechanism.

Further investigations comparing gene profiles of postmitotic Zic2 cells from retina and dorsal spinal cord will clarify the similarities and differences between the mechanisms governing Zic2-dependent axon guidance laterality in different areas of the CNS. Nevertheless our results show that through regulation of a variety of guidance receptors, Zic2 determines midline avoidance in at least two different systems, setting this transcription factor in a unique position in the control and formation of bilateral circuits.

Chapter VI

Conclusions

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1. Zic2 is expressed in late-born excitatory interneurons (dIL_B) of the dorsal horns in the spinal cord.
2. Axons from dIL_B-Zic2 neurons run ipsilaterally into the dorsal and dorsolateral funiculi.
3. Zic2 is necessary in dorsal spinal cord neurons to avoid midline crossing.
4. Zic2 is sufficient to switch axonal laterality in commissural spinal cord interneurons.
5. Zic2 is able to repress genes that control the commissural trajectory such as Lhx2 and Robo3.
6. Robo3.1 is sufficient to induce commissural misprojection of dorsal spinal neurons.
7. Zic2 does not determine ipsilaterality *per se* but rather regulates axon midline avoidance.
8. EphrinBs are expressed at the dorsal midline.
9. Zic2 controls the expression of the tyrosine kinase receptor EphA4 in dorsal horn neurons, which suggests that EphA4/ephrinB binding mediate the repulsive axonal response that impedes axon crossing at the dorsal midline.

Chapter VII

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Addendum. Full page resolution figures.

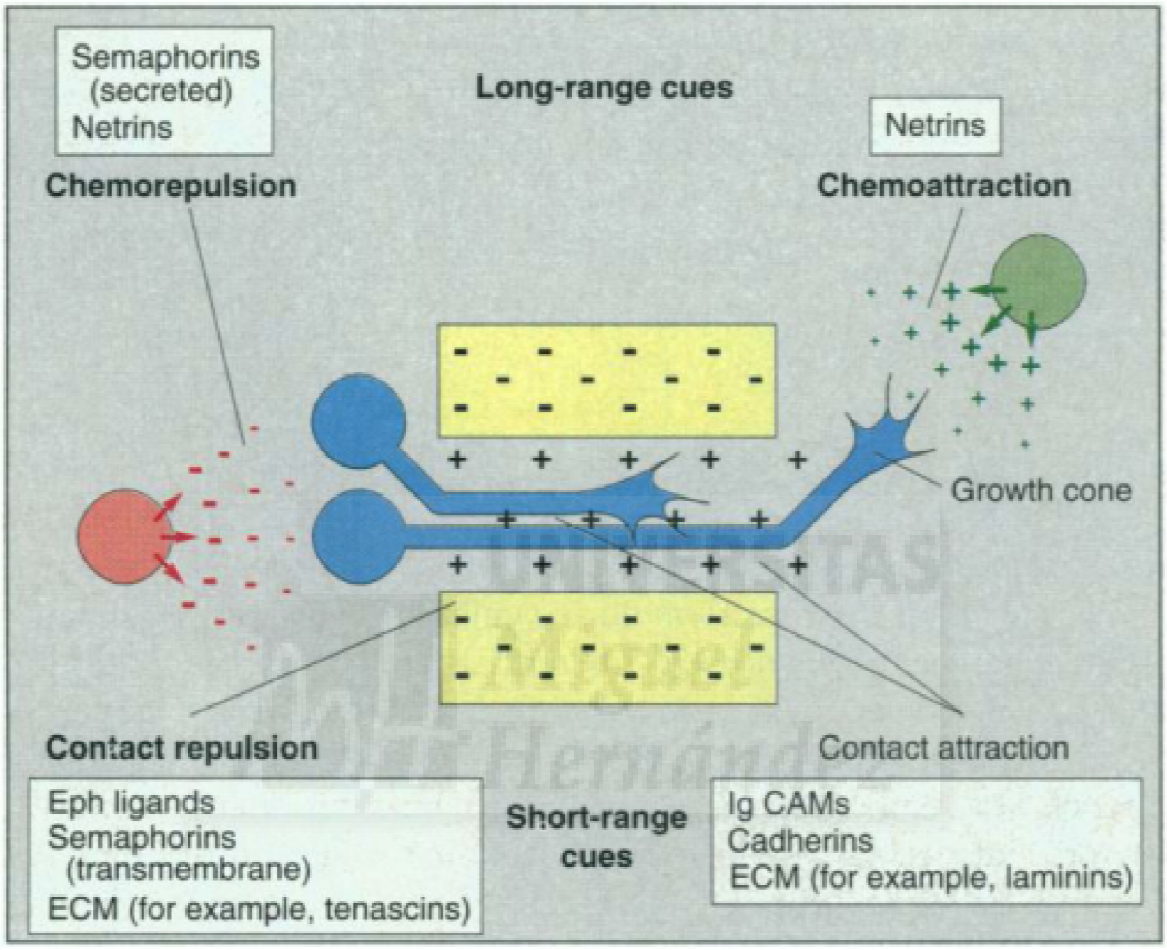

Figure 1. Guidance forces.

Four types of mechanisms contribute to guiding growth cones: contact attraction, chemoattraction, contact repulsion and chemorepulsion. Attraction refers here to a range of permissive and attractive effects while repulsion refers to inhibitory repulsive effects. Growth cones might be "pushed" from behind by a chemorepellent (red), "pulled" from a distance by a chemoattractant (green) and confined by attractive and repulsive local cues (yellow).

From Tessier-Lavigne and Goodman, 1996.

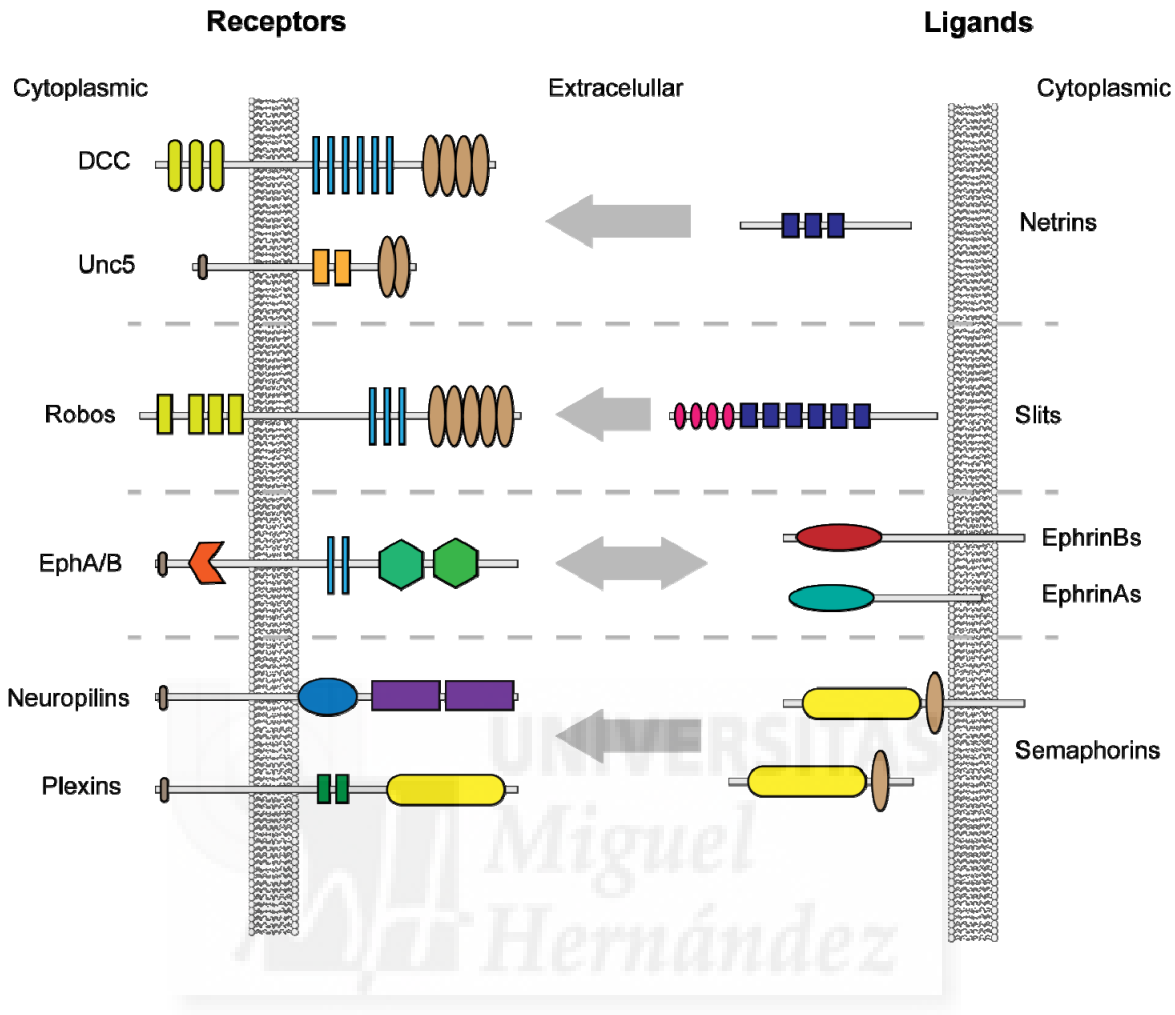

Figure 2. Major families of receptor/ligand pairs in axon guidance.

Representative members of the main families of axon guidance molecules and their receptors. Flow of the signaling cascade is indicated by a thick light grey arrow.

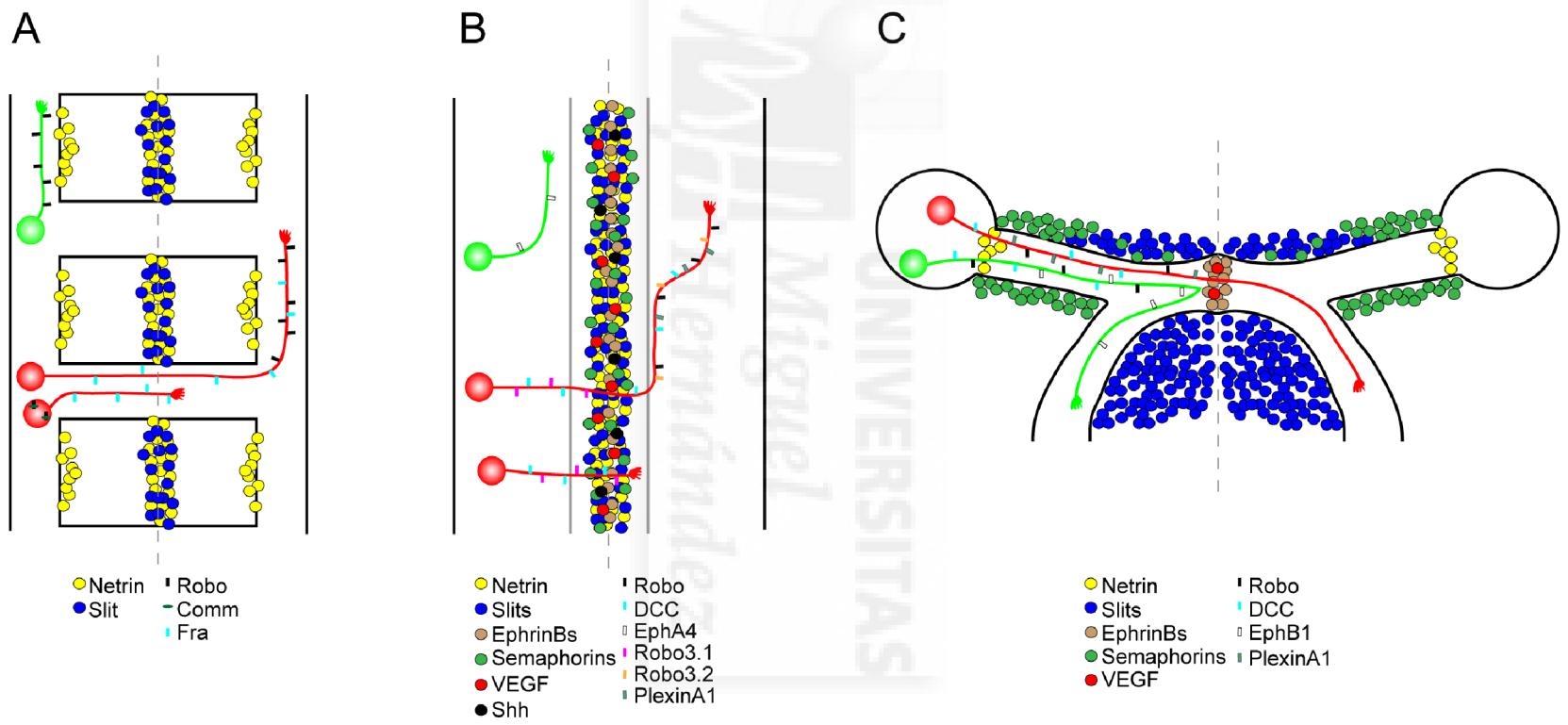
A
Netrin
Slit
Robo
Comm
Fra
B
Netrin
Slits
EphrinBs
Semaphorins
VEGF
Shh
Robo
DCC
EphA4
Robo3.1
Robo3.2
PlexinA1
C
Netrin
Slits
EphrinBs
Semaphorins
VEGF
Robo
DCC
EphB1
PlexinA1

Figure 3. Three models frequently used to study axon guidance at the midline.

(A) Several segments of the ventral nerve cord of *Drosophila* are represented in a ventral view. Ipsilateral (green) neurons express Robo receptors, but no Comm, causing a repulsive response to the slits expressed at the midline. Commissural (red) neurons are first attracted to the midline by Netrin-Frazzled (the *Drosophila* ortholog of the vertebrate DCC) and allowed to overcome the repulsive Slit territory due to the lack of Robo expression in the growth cone which is kept in endosomal compartments by Comm. Following exit of the floor plate on the contralateral side Comm expression is downregulated and thus Robo expression upregulated so that axons can no longer cross.

(B) The vertebrate spinal cord is depicted in an open-book configuration. Similar to the ventral nerve cord of insects, Netrin-DCC and Slit-Robo determine the attraction and later repulsion of commissural axons. Here, another member of the Robo family, Robo3, is responsible of keeping commissural axons from becoming prematurely responsive to midline repellents. However, several other families of guidance molecules have been shown to play a role in the guidance of commissural axons (see text). The knowledge about the guidance of vertebrate spinal ipsilateral axons on the other hand is sparse. Till today, only the expression of EphA4 has been shown to be necessary for avoiding ventral midline crossing.

(C) The mouse optic chiasm is shown in a horizontal section. A different arrangement of the same guidance molecules control the guidance of the RGCs. While in this model Netrin-DCC is responsible for the exit of axons from the retina and Slit-Robo for keeping the axons within the limits of the tract, it is the repulsive signaling mediated by ephrinB2-EphB1 binding the determinant of midline avoidance.

Dotted lines indicate the midline.

Adapted from Williams, 2004.

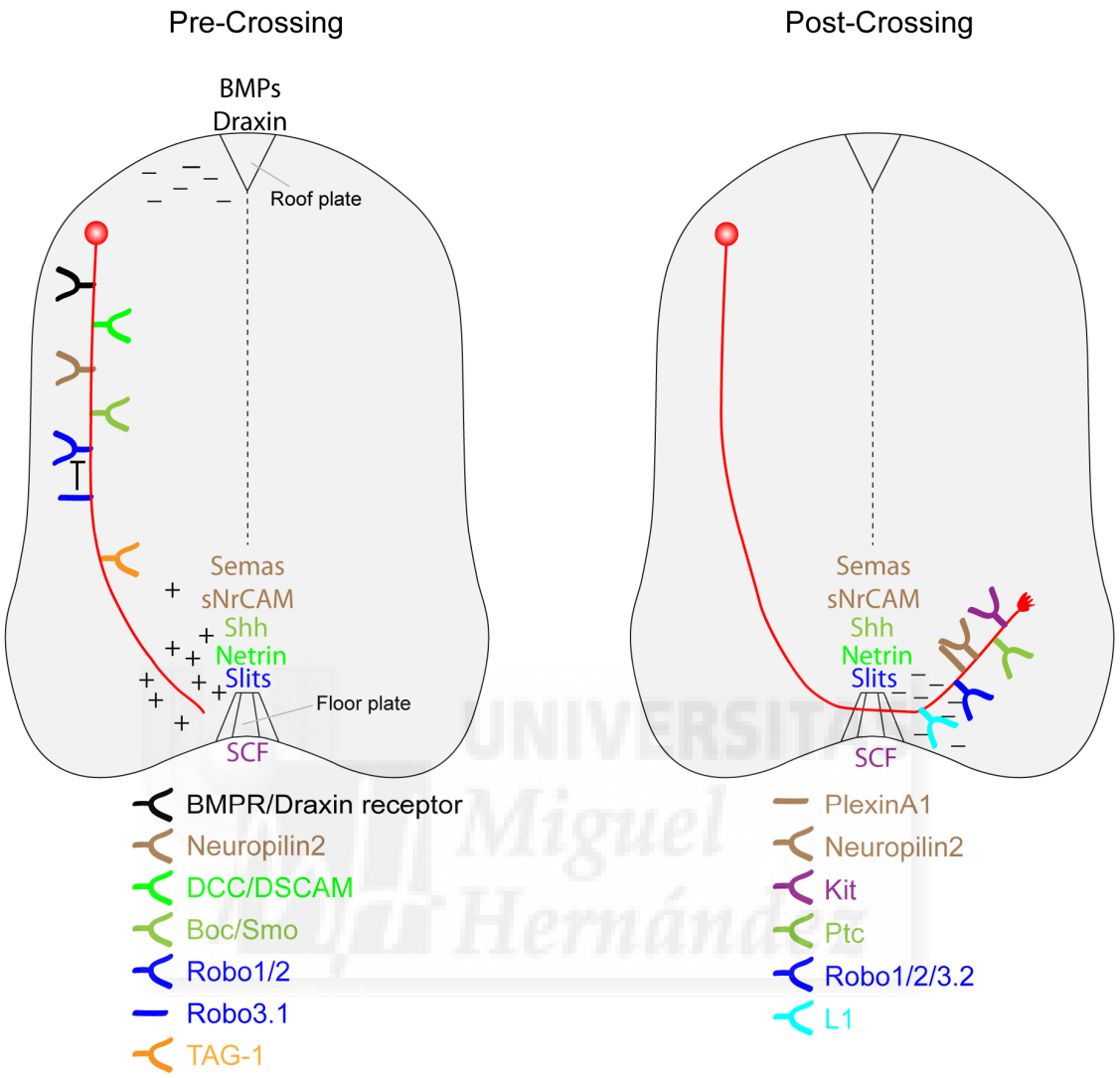

Figure 4. Molecular guidance of spinal commissural axons.

Pre-crossing axons are repelled from the dorsal part of the spinal cord by Draxin and BMPs. They are attracted to the floor plate by Netrin1 and Shh. In pre-crossing axons, Slit/Robo repulsion is inactivated by Robo3.1. At the floor plate, SCF promotes midline exit and DCC/Netrin1 attraction is silenced by Robo1 in a Slit dependent manner. The expression of the other Robo receptors is upregulated and Slit repulsion is activated. In parallel, the inhibition of Calpain1 induced by soluble NrCAM leads to an upregulation of PlexinA1 expression on post-crossing commissural axons allowing it to form a complex with Neuropilin2 that mediates Sema3B repulsion. Shh is also involved in switching Sema3B repulsion and, in chick, may also directly repel post-crossing axons.

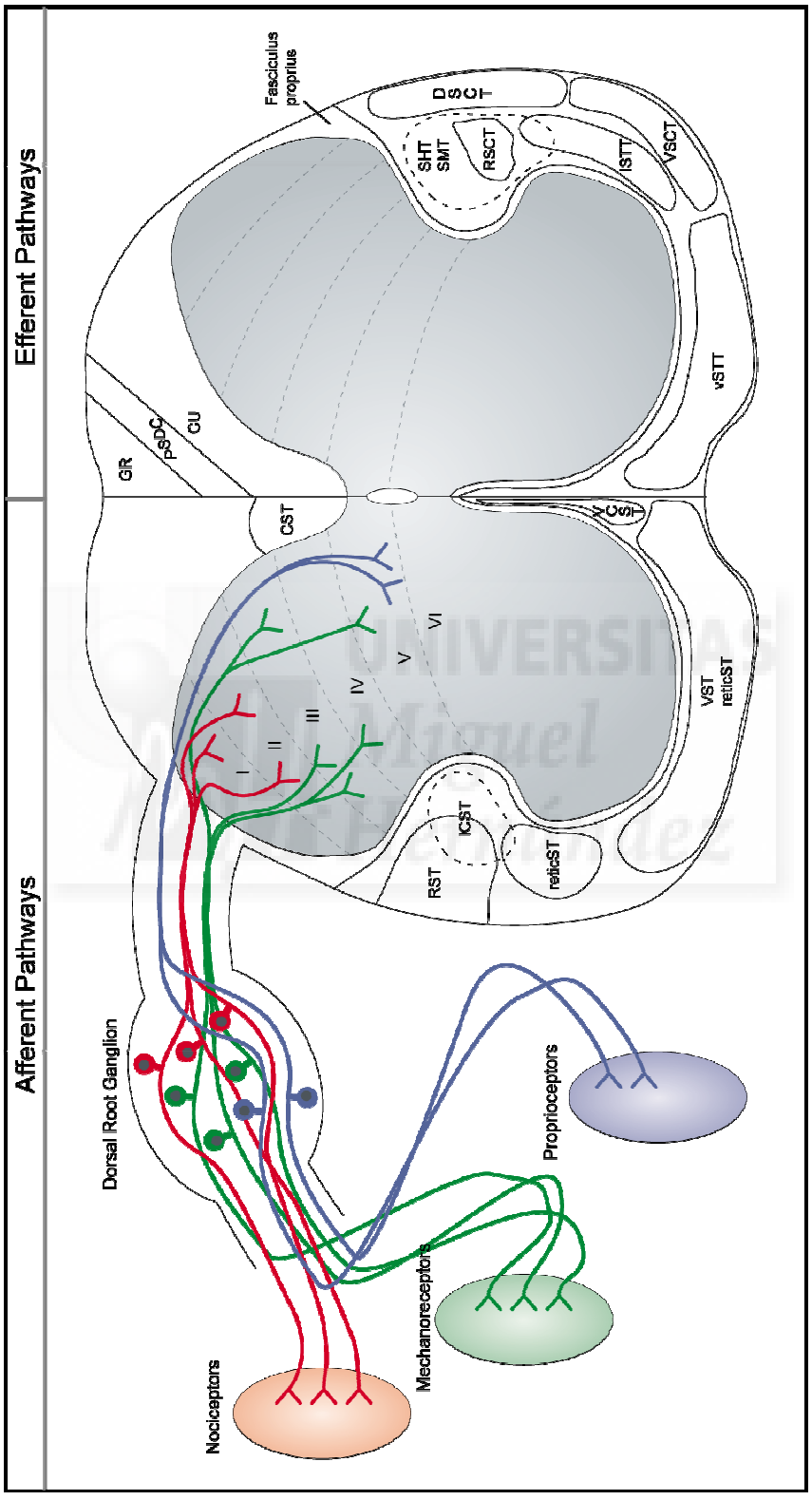
Efferent Pathways
Afferent Pathways
Fasciculus proprius
DSCT
SHT
SMT
RSCT
ISTT
VSCT
vSTT
GR
psDC
CU
CST
vCST
I
II
III
IV
V
VI
VST
reticST
ICST
RST
reticST
Dorsal Root Ganglion
Proprioceptors
Mechanoreceptors
Nociceptors

Figure 5. Spinal cord structure, afferent and efferent pathways.

Schematic representation of the neuronal circuits in the mouse spinal cord. Sensory information from dorsal root ganglion cells is delivered to specific laminae in the dorsal horns. Supraspinal centers also provide information to the spinal cord through several pathways (left side): corticospinal tract (CST), lateral corticospinal tract (lCST), ventral corticospinal tract (VSCT), rubrospinal tract (RST), reticulospinal tract (reticST) and vestibulospinal tract (VST). Spinal neurons then drive the information to supraspinal targets through different pathways (right side): gracile tract (GR), cuneate tract (CU), postsynaptic dorsal column tract (PSDC), dorsal spinocerebellar tract (DSCT), ventral spinocerebellar tract (VSCT), rostral spinocerebellar tract (RSCT), spinohypothalamic tract (SHT), spinomesencephalic tract (SMT), lateral spinothalamic tract (lSTT), ventral spinothalamic tract (vSTT).

Compiled from Caspary and Anderson, 2003; Webb and Muir, 2004; Watson and Harrison, 2012.

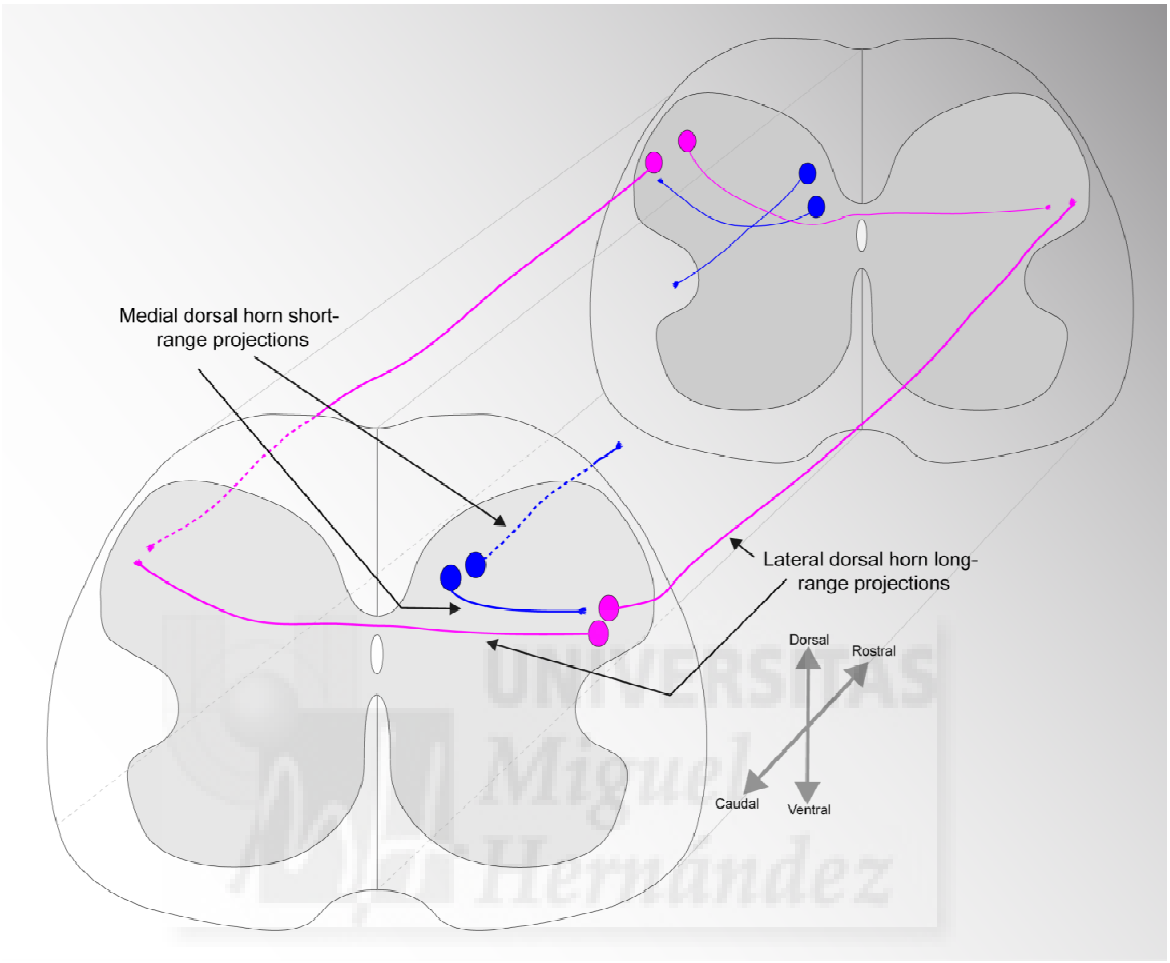

Figure 6. Propriospinal pathways in the dorsal horns

The medial and lateral aspects of the dorsal horns show differential patterns of projections. Propriospinal neurons whose somas are located in the medial dorsal horn (blue) posses short projections to the lateral aspects and to close rostrocaudal levels on the same side of the horn. However, propriospinal neurons on lateral positions of the dorsal horns (pink) show long range projections both to the same side of the spinal cord and to the contralateral dorsal horn.

Based on Petkó, 2012.

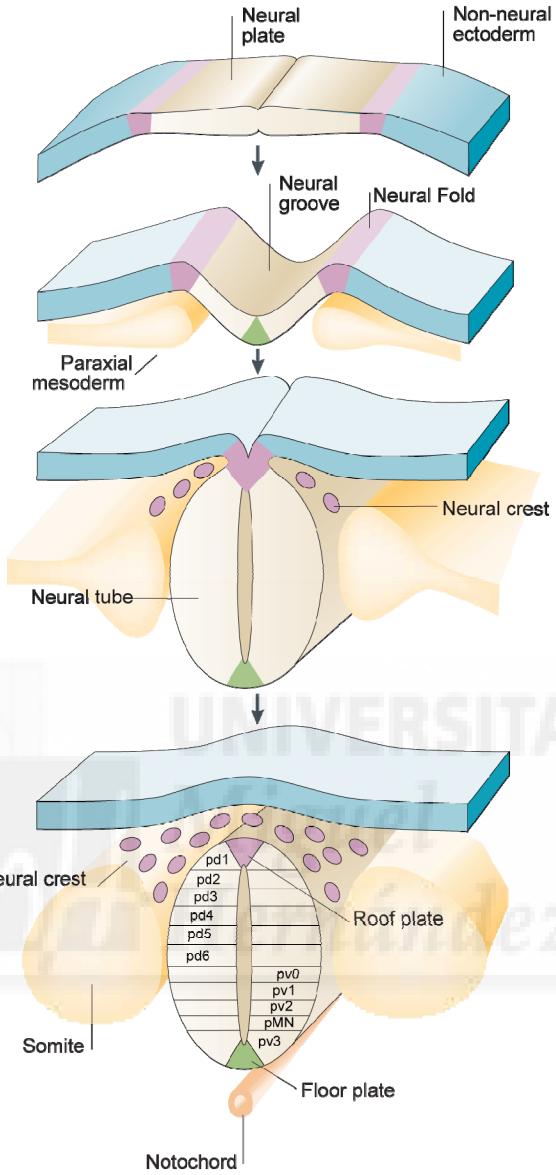

Figure 7. The formation of the neural tube.

The neural tube arises from invagination of the neuroectoderm induced by signals coming from the non-neural ectoderm (blue) and the underlying paraxial mesoderm (yellow). The neural plate borders fold and elevate causing the neural plate to roll into a neural tube. Neural crest cells delaminate from the dorsal neural tube and the remaining cells become the roof plate which constitutes a new signalling center.

Adapted from Gammill and Bronner-Fraser, 2003 and Liu and Niswander, 2005.

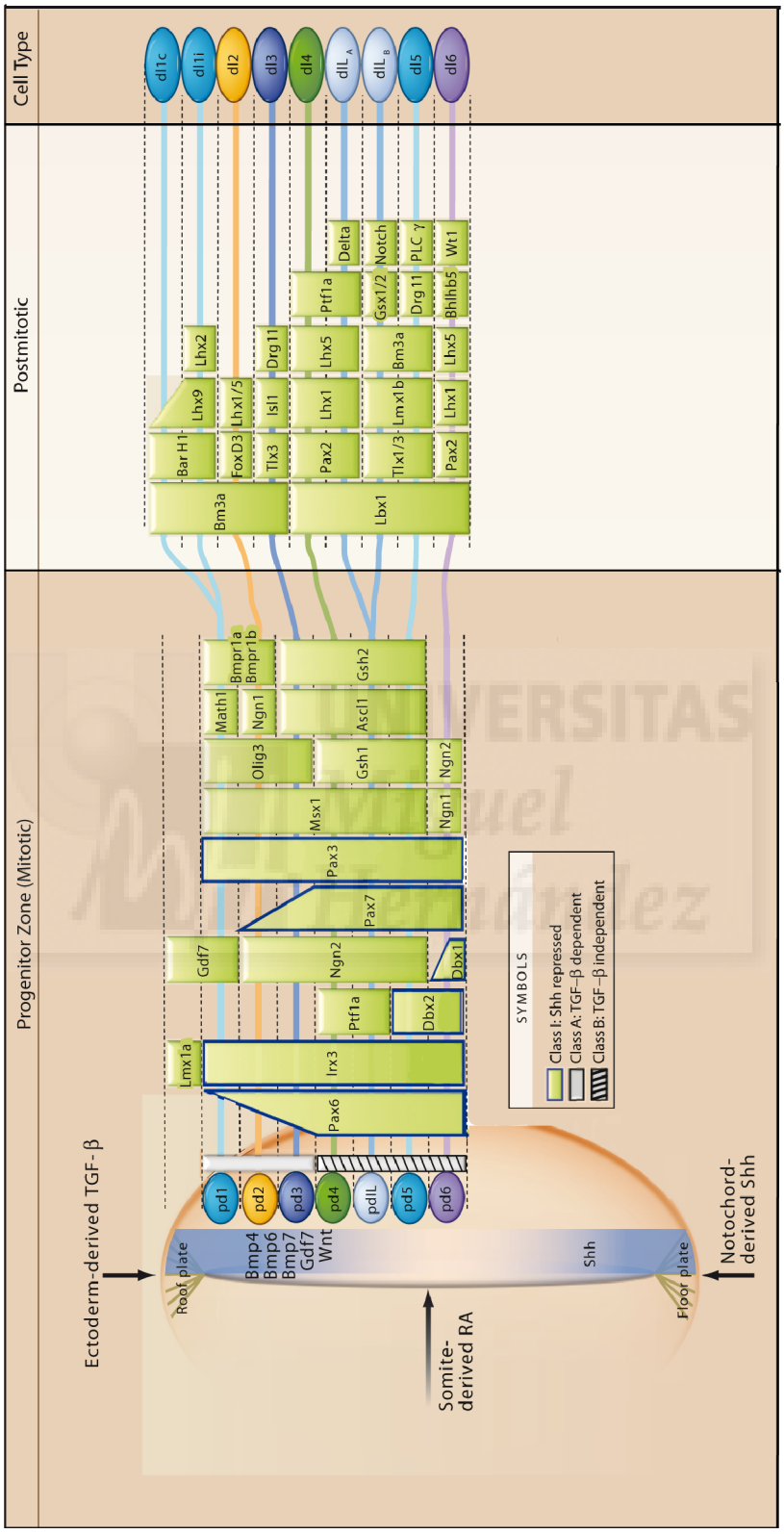
Cell Type
Postmitotic
Progenitor Zone (Mitotic)
dI1c
dI1i
dI2
dI3
dI4
dIL A
dIL B
dI5
dI6
Bar H1
Lhx9
Lhx2
FoxD3
Lhx1/5
Tlx3
Isl1
Drg11
Bm3a
Pax2
Lhx1
Lhx5
Tlx1/3
Lmx1b
Bm3a
Lbx1
Ptf1a
Gsx1/2
Drg11
Bhlhb5
Delta
Notch
PLC γ
Wt1
Bmpr1a
Bmpr1b
Gsh2
Math1
Ngn1
Ascl1
Olig3
Gsh1
Ngn2
Msx1
Pax3
Pax7
Gdf7
Dbx1
Dbx2
Lmx1a
Irx3
Pax6
pd1
pd2
pd3
pd4
pdIL
pd5
pd6
Roof plate
Floor plate
Bmp4
Bmp6
Bmp7
Gdf7
Wnt
Shh
Ectoderm-derived TGF-β
Somite-derived RA
Notochord-derived Shh
SYMBOLS
Class I: Shh repressed
Class A: TGF-β dependent
Class B: TGF-β independent

Figure 8. Genetic specification of dorsal interneuron populations.

Cellular identities are defined by the influences of a two-dimensional system of morphogen gradients acting on the neuroepithelia of the ventricular zone. Floor plate and notochord derived Shh repress the expression of dorsal identities while ectoderm and roof plate derived BMPs and Wnts induce their expression. Crossinhibitory mechanisms between transcription factors of neighboring progenitor domains prevents cells from developing hybrid identities.

Adapted from Alaynick, 2011.

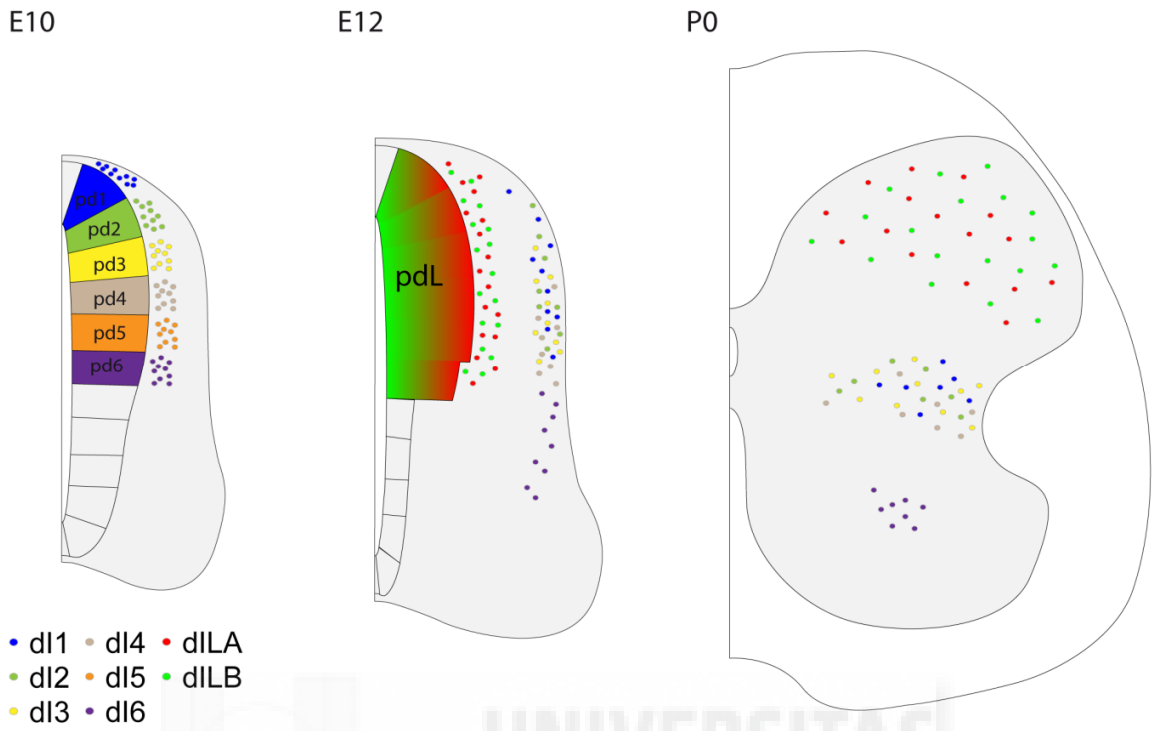

Figure 9. Migratory paths and final location of dorsal spinal interneurons.

Early born (E10-E12) neurons in the dorsal neural tube (dI1-6) differentiate laterally from the ventricular zone into the marginal zone and follow a ventral migration to their finallocation. Meanwhile, late born (E12-E14) neurons (dIL) give rise to the dorsal horns.

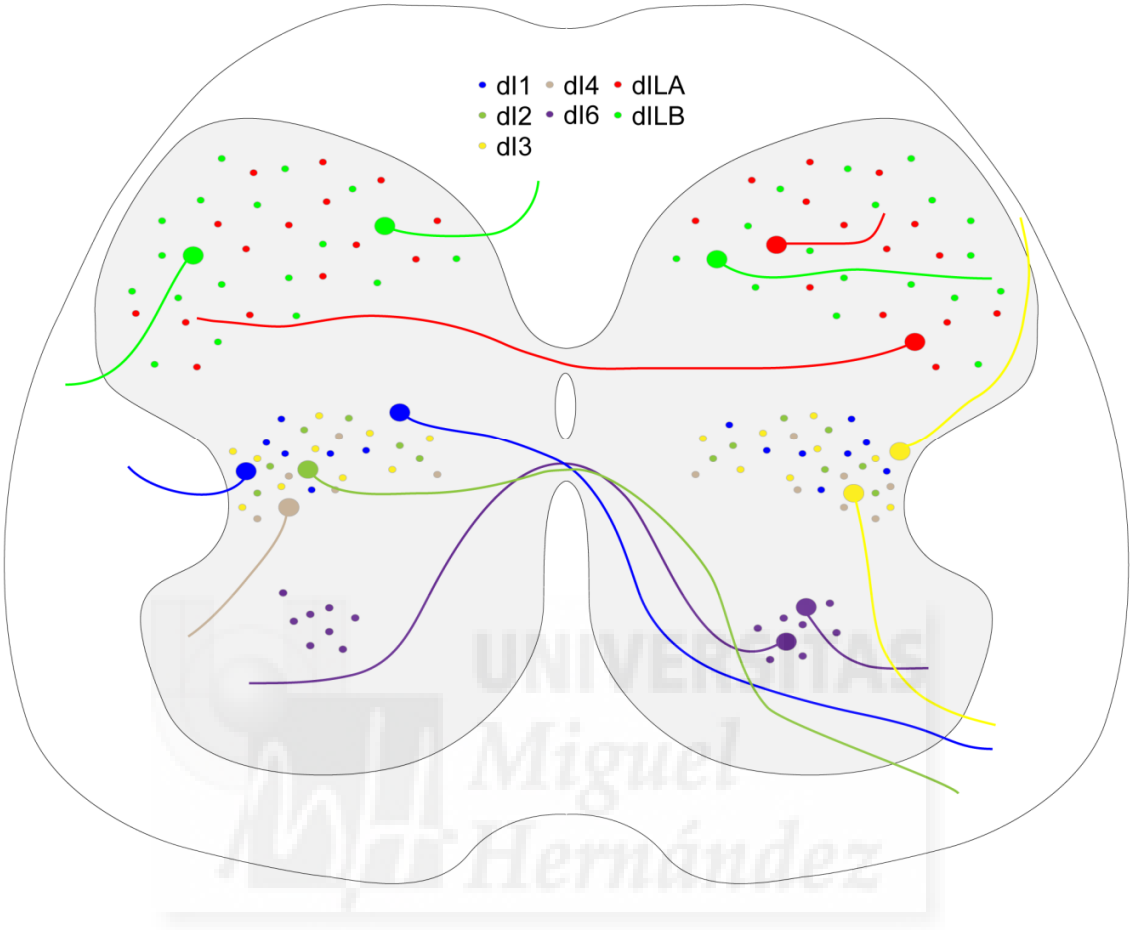

Figure 10. Projection patterns of dorsal spinal interneurons.

Representation of the known axonal trajectories with respect to the midline of dorsal interneurons populations. dI1 and dI6 form both ipsi- and contralateral projections, dI2 is considered to be almost exclusively contralateral while dI3 and dI4 have been shown to project only ipsilaterally. dILA neurons are local and contralateral neurons. dILB are ipsilateral neurons which project to different fascicles into the white matter.

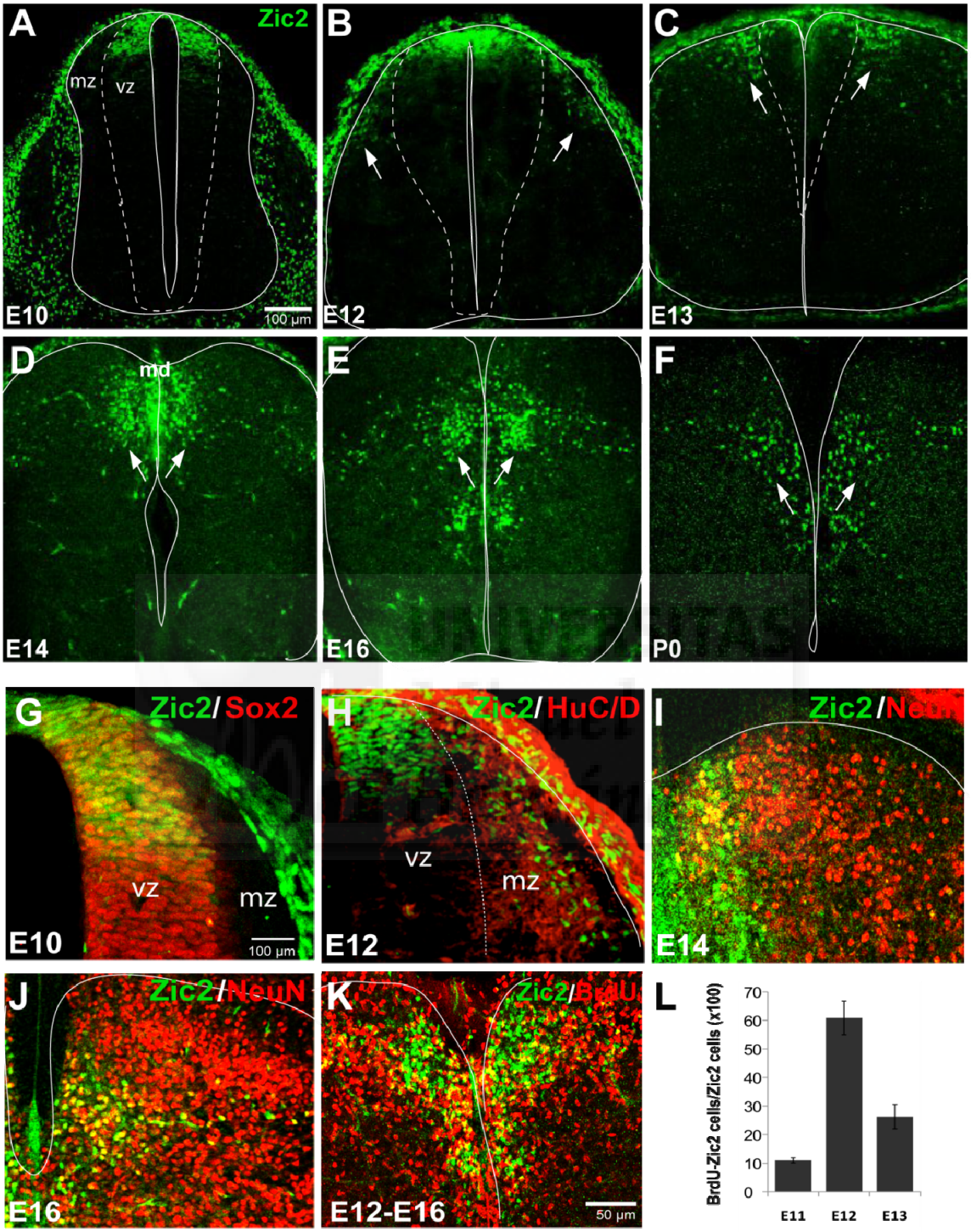

Figure 11. Spatiotemporal expression of Zic2 in the developing spinal cord.

(A-F) Transverse spinal cord sections from wildtype mouse embryos at the indicated stages stained with antiZic2 antibodies show the spatiotemporal exprssion pattern of Zic2 throughout spinal cord development.

(G-J) Transverse spinal cord sections double-stained with Sox2, HuC/D, NeuN and Zic2 show that Zic2 is expressed in progenitors at early stages and in postmitotic neurons at later stages. Arrows points to the region of double-labeled cells

(K) Transverse spinal cord sections from E16 embryos injected with BrdU at E11, E12 and E13 and double stained with anti-BrdU and anti-Zic2 antibodies.

(L) Quantification of the number of double-labelled $Zic2^+/BrdU^+$ cells in E16 embryos injected at E11, E12 and E13 indicates that most of the $Zic2^+$ cells are born at E12. At least five sections/embryo and four embryos/stage were used to quantify. Scale bar: mm. Error bars indicate ±SEM (**p<0.01, Student´s unpaired t-test).

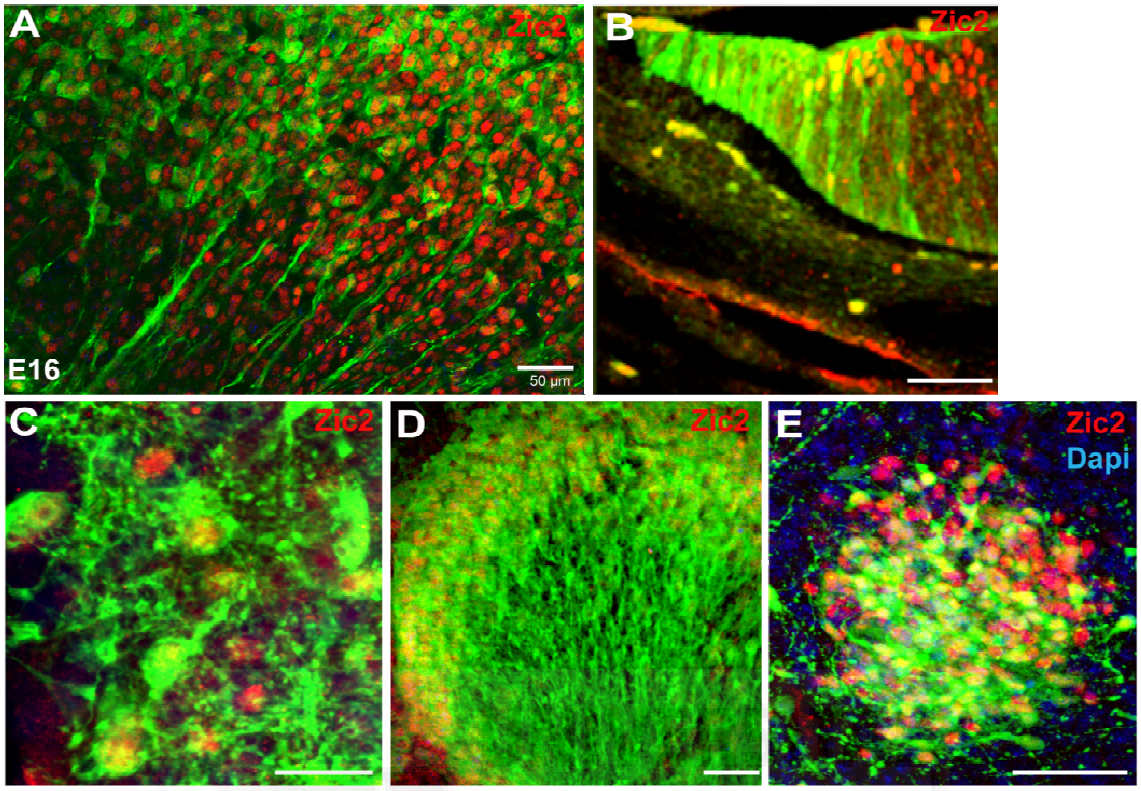

Figure 12. Zic2 reporter mice: EGFP expression recapitulates the endogenous expression of Zic2.

Zic2 and EGFP co-localize in different brain areas such the thalamus **(A),** ciliary margin and ipsilateral ganglion cells in the retina **(B),** Cajal-Reltzius cells **(C),** hippocampus **(D)** or amygdala **(E)**.

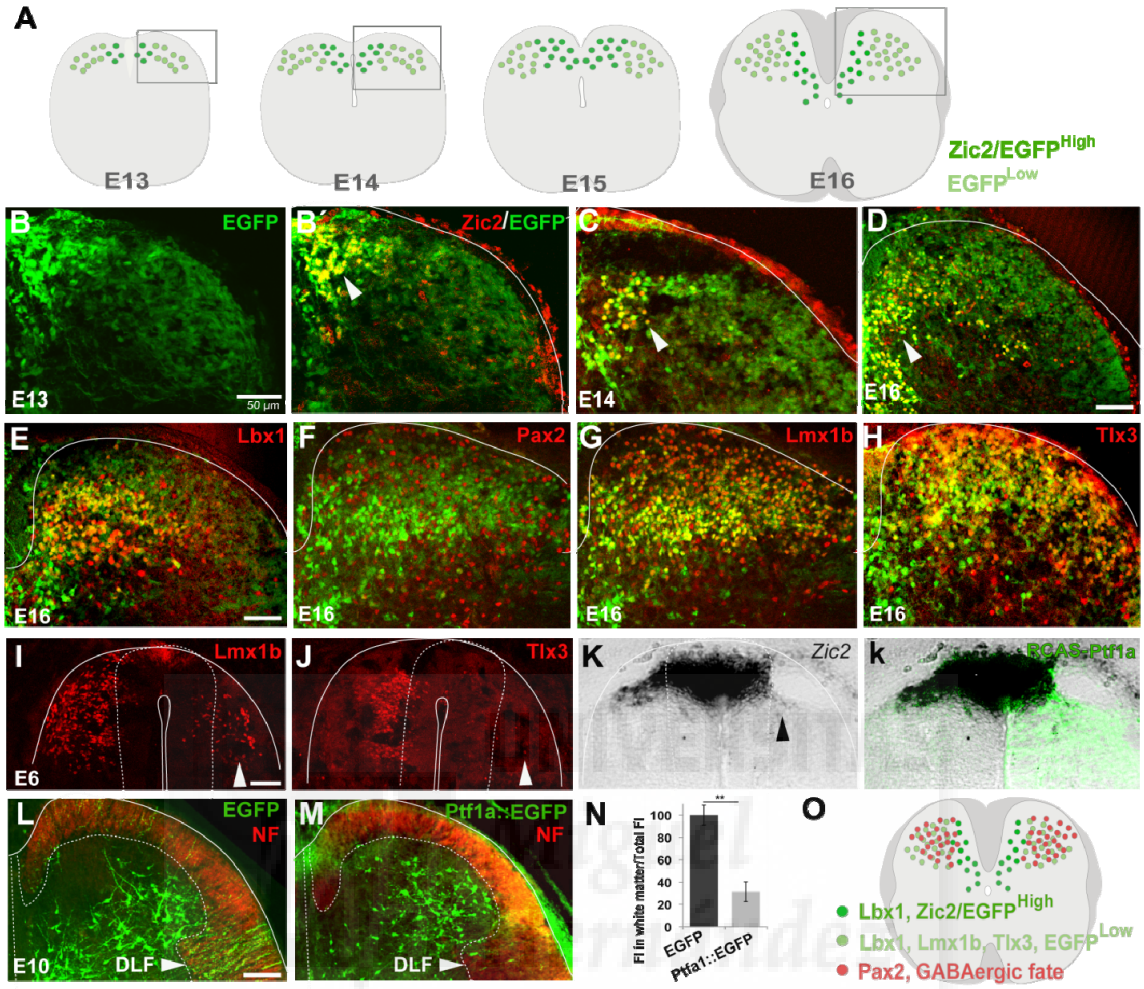

Figure 13. Spinal neurons that transiently express Zic2 are dIL$_B$ projection neurons.

(A-C) Dorsal quadrant of transverse spinal cord sections of Zic2^{EGFP} embryos stained with Zic2 antibodies shows that Zic2 cells are also positive for EGFP. Arrows point to the region with a larger concentration of double-labeled Zic2/EGFP cells.

(D) Scheme summarizing the spatiotemporal expression of Zic2 and EGFP in Zic2^{EGFP} embryos. At early stages Zic2^{+}/EGFP^{+} cells (yellow) are located in the dorsocentral region. Through development, cells expressing high levels of EGFP (dark green) separate from the midline and go to more dorsal and lateral locations. Cells expressing low levels of EGFP (light green) are in the superficial dorsal horn layers.

(E-H) Dorsal quadrant of transverse spinal cord sections from Zic2^{EGFP} E16 embryos stained with the indicated markers. Bar scale: 100 µm.

(I-K) Transverse spinal sections of E6 chick embryos that were electroporated *in ovo* at E4 with plasmids driving the coding sequence of Ptf1a (RCAS-Ptf1a) reduces the expression of glutamatergic markers such as Lmx1b and Tlx3 (red, white arrows) as well as postmitotic expression of *Zic2* mRNA in the dorsal cord (black arrow). Bar scale: 100 µm.

(L-M) Dorsal quadrant of transversal spinal cord sections from E10 chick embryos electroporated *in ovo* at E6 with plasmids driving EGFP (CAG-EGFP) alone or together with Ptf1a expressing plasmids (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) but rather project locally. Bar scale: 100 µm.

(N) Quantification of the amount of axons into the white matter after electroporation of RCAS-Ptf1a/CAG-EGFP or CAG-EGFP plasmids.

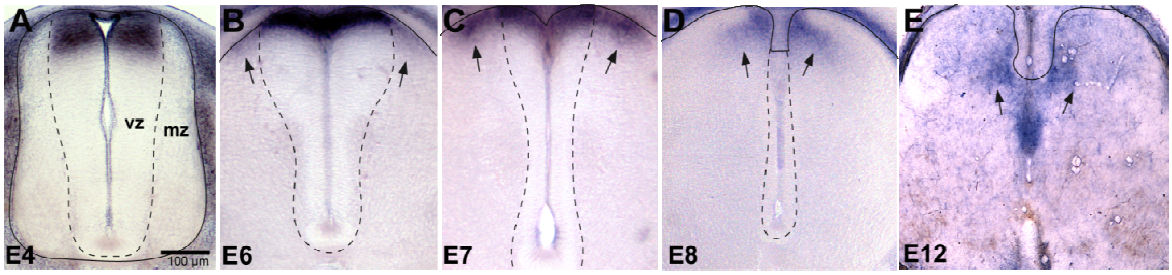

Figure 14. Spatiotemporal expression pattern of Zic2 in the chick spinal cord.

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

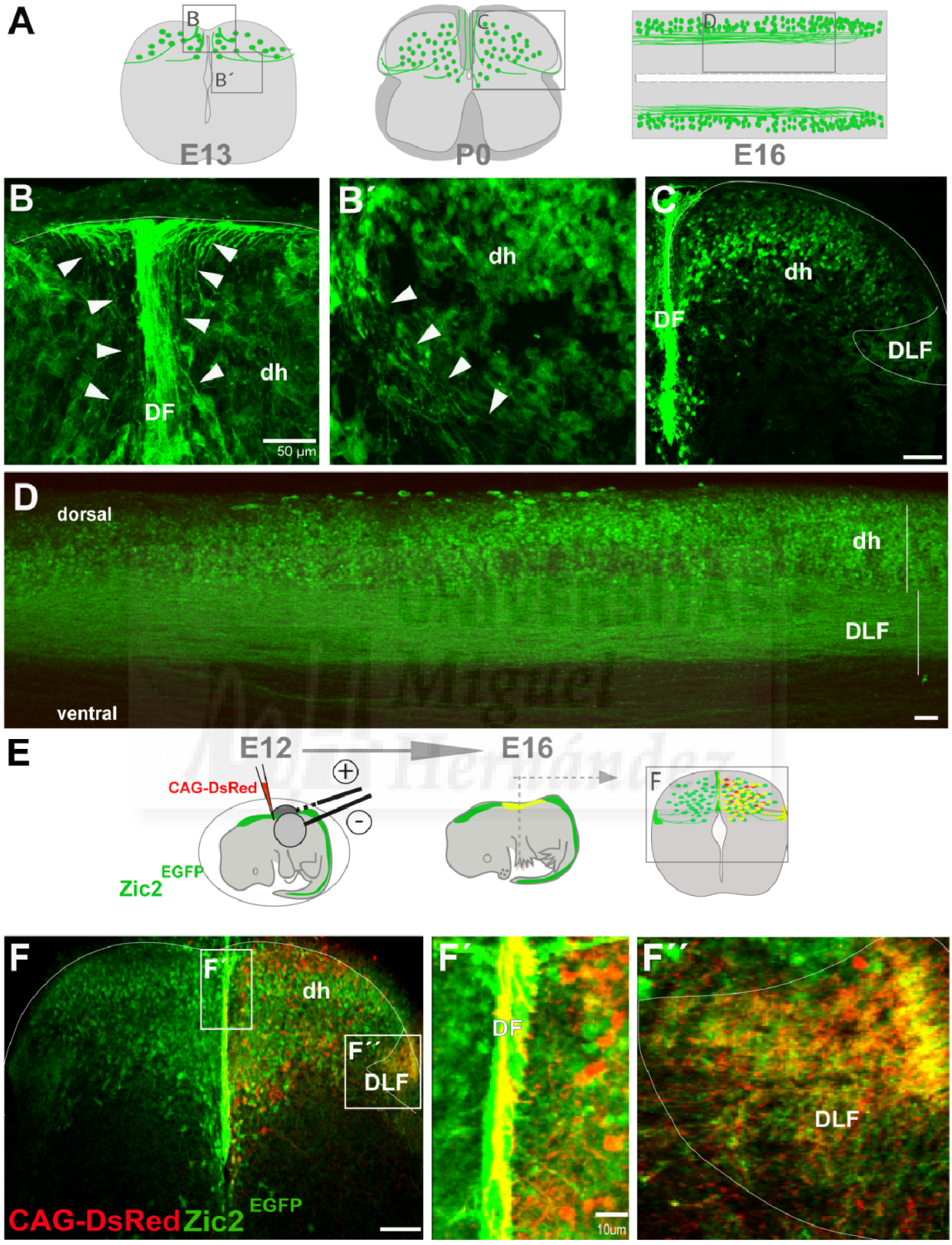
A
B
B´
E13
C
P0
D
E16
B
B´
C
dh
DF
50 µm
dh
DF
DLF
D
dorsal
ventral
dh
DLF
E
E12
E16
CAG-DsRed
EGFP
Zic2
F
F
F´
dh
F´´
DLF
CAG-DsRed
Zic2
EGFP
F´
DF
10um
F´´
DLF

Figure 15. Zic2 spinal neurons project ipsilaterally.

(A-C) Schematic representation that illustrates the expression of Zic2 (green) at E14, P0 in transversal sections and at E15 in open-book preparations. **(a)** Dorsal quadrant of a transverse section from an E14 Zic2^{EGFP} embryo shows that axons from Zic2 neurons are located outside the spinal cord (white arrows) in regions that correspond to the future DF. **(a´)** In the same transverse section, another population of EGFP axons grows to the DLF. **(b)** Dorsal quadrant of a transverse spinal cord section from a Zic2^{EGFP} newborn mouse shows that many Zic2/EGFP^{+} axons are in the DLF while some others are in the DF. **(c)** Open-book preparation from an E15 Zic2^{EGFP} embryo shows that Zic2/EGFP axons run ipsilaterally into the DLF.

(D) Schematic representation of the experimental procedures. CAG-DsRed plasmids were unilaterally injected in the spinal cord of E12 Zic2^{EGFP} embryos by *in utero* electroporation and transverse spinal cord sections from electroporated embryos were analyzed four days later. The trajectory of axons double labelled with DsRed and EGFP (yellow) was analyzed.

(E) Transverse spinal cord section of an E16 Zic2^{EGFP} embryo unilaterally electroporated with CAG-DsRed plasmids shows that double-labeled DsRed/Zic2^{EGFP} axons project ipsilaterally into the DF as well as into the DLF. **(e, e´)** Higher magnifications of squared areas in E.

(F) An open-book preparation of an E15 Zic2^{EGFP} embryo electroporated with CAG-DsRed plasmid show that DsRed/EGFP axons run ipsilaterally along the DLF.

(G) A transverse spinal cord section from a P0 mouse stained with Zic2 (green) and Parvalbumin (red) antibodies show that the arrival of PV afferents coincide with the location of Zic2 neurons in the dorsal cord. DLF, dorsolateral fascicle; DF, dorsal fascicle; dh, dorsal horns.

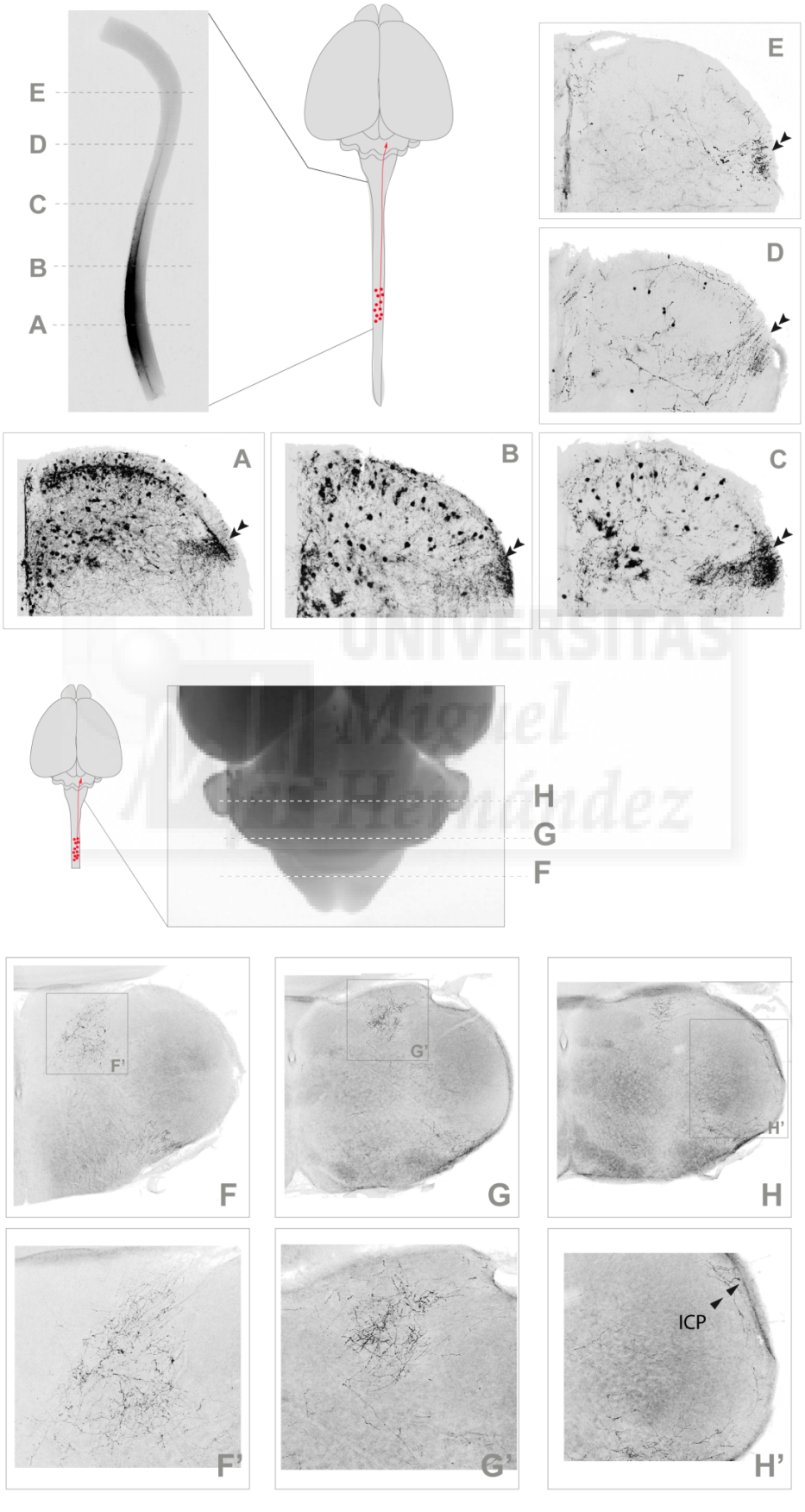
E
D
C
B
A
E
D
A
B
C
H
G
F
F'
G'
H'
F
G
H
F'
G'
ICP
H'

Figure 16. Late-born dorsal spinal neurons project propriospinally and supraspinally.

(A-E) E12 embryos were focally electroporated with DsRed2 encoding plasmids in the lumbar region. Coronal sections at the indicated levels from P7 electroporated spinal cords were analyzed.

(A-C) Transverse sections of the same spinal cord at different rostrocaudal levels in the electroporated region where targeted cell bodies reside. DLF is intensively labeled (arrowheads).

(D-E) Transverse sections rostral to the targeted area show a reduction in the number of axons projecting in the DLF (arrowheads).

(F-H) A P7 mouse brain electroporated locally at E12 in the spinal thoracic region. Coronal sections taken at the indicated levels.

(F-G) Tranverse sections show axons projecting to the cuneate nucleus.

(H) Axons could be followed to the inferior cerebellar peduncle (ICP).

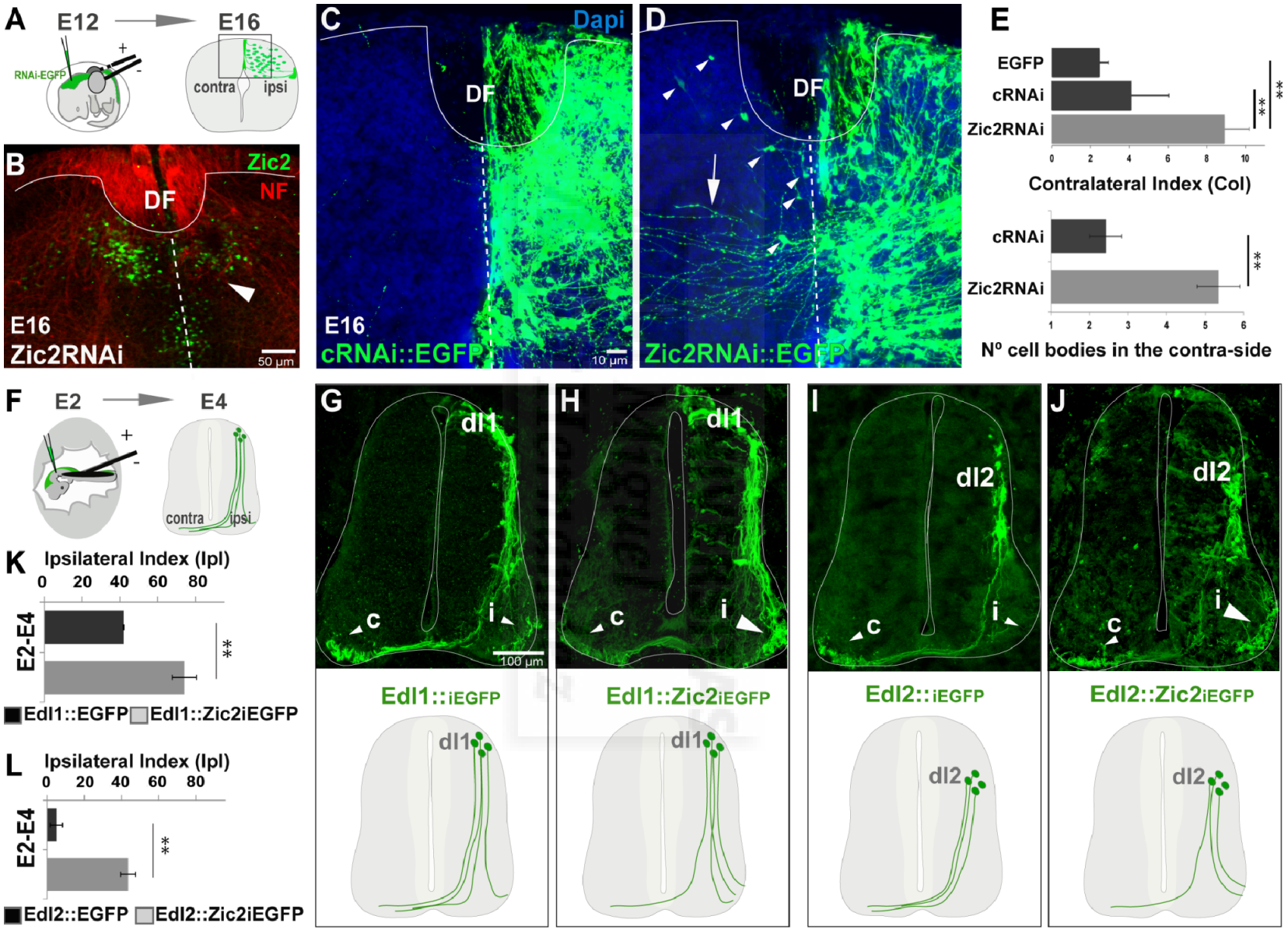
A
E12
E16
RNAi-EGFP
+
-
contra
ipsi
B
Zic2
NF
DF
E16
Zic2RNAi
50 µm
C
Dapi
DF
E16
cRNAi::EGFP
10 µm
D
DF
Zic2RNAi::EGFP
E
EGFP
cRNAi
Zic2RNAi
0 2 4 6 8 10
**
**
Contralateral Index (CoI)
cRNAi
Zic2RNAi
1 2 3 4 5 6
**
Nº cell bodies in the contra-side
F
E2
E4
+
-
contra
ipsi
G
dl1
c
i
100 µm
Edl1::iEGFP
dl1
H
dl1
c
i
Edl1::Zic2iEGFP
dl1
I
dl2
c
i
Edl2::iEGFP
dl2
J
dl2
c
i
Edl2::Zic2iEGFP
dl2
K
Ipsilateral Index (Ipl)
0 20 40 60 80
E2-E4
**
Edl1::EGFP
Edl1::Zic2iEGFP
L
Ipsilateral Index (Ipl)
0 20 40 60 80
E2-E4
**
Edl2::EGFP
Edl2::Zic2iEGFP

Figure 17. Zic2 is necessary and sufficient to determine axonal ipsilaterality in the spinal cord.

(A) Schematic representation of the experimental procedures. E12 embryos were electroporated with Zic2RNAi and harvested at E16 for analysis of the contralateral index (CoI). The square area marks the analyzed region.

(B) A transverse spinal cord section of an E16 mouse embryo electroporated unilaterally with Zic2RNAi shows the effective downregulation of Zic2 protein (green) in the electroporated side (white arrow). Dashed line marks the midline. Neurofilament (NF) staining is shown in red.

(C, D) Transverse spinal cord sections from E16 mouse embryos electroporated at E12 with plasmids encoding control RNAi and EGFP (cRNAi::EGFP) or Zic2RNAi and EGFP (Zic2RNAi::EGFP) show that the downregulation of Zic2 causes massive aberrant crossing (white arrow) at the dorsal midline (dashed white line). The arrowheads point to the cell bodies of mislocated neurons on the side contralateral to the electroporation.

(E) Top, quantification of the contralateral index (CoI) in embryos electroporated with control plasmids (CAG-EGFP, control RNAi) or plasmids encoding Zic2RNAi. A significant increase of axons aberrantly crossing the dorsal midline after downregulation of Zic2 was observed. Bottom graph shows the quantification of the number of cell bodies found on the side contralateral to electroporation. Error bars indicate ±SEM (**p<0.01, Student's unpaired t-test).

(F) Schematic representation of the experimental procedures. E2 embryos were electroporated and harvested at E4 for analysis and quantification of the ipsilateral index.

(G-H, I-J) Transverse spinal cord sections from E4 chick embryos electroporated at E2 with plasmids driving EGFP expression to the dI1 domain (EdI1) or to the dI2 domain (EdI2) show an increase in ipsilateral axons after Zic2 induction compared to the controls. Schemes below summarize the results in each case. C, contralateral side; I, ipsilateral side.

(K, L) Quantification of the ipsilateral index (IpI) in E4 chick embryos electroporated at E2 with EdI1:EGFP or EdI2:EGFP plasmids revealed an increase in the number of axons projecting ipsilaterally after the induction of Zic2. Error bars indicate ±SEM (**p<0.01, Student's unpaired t-test).

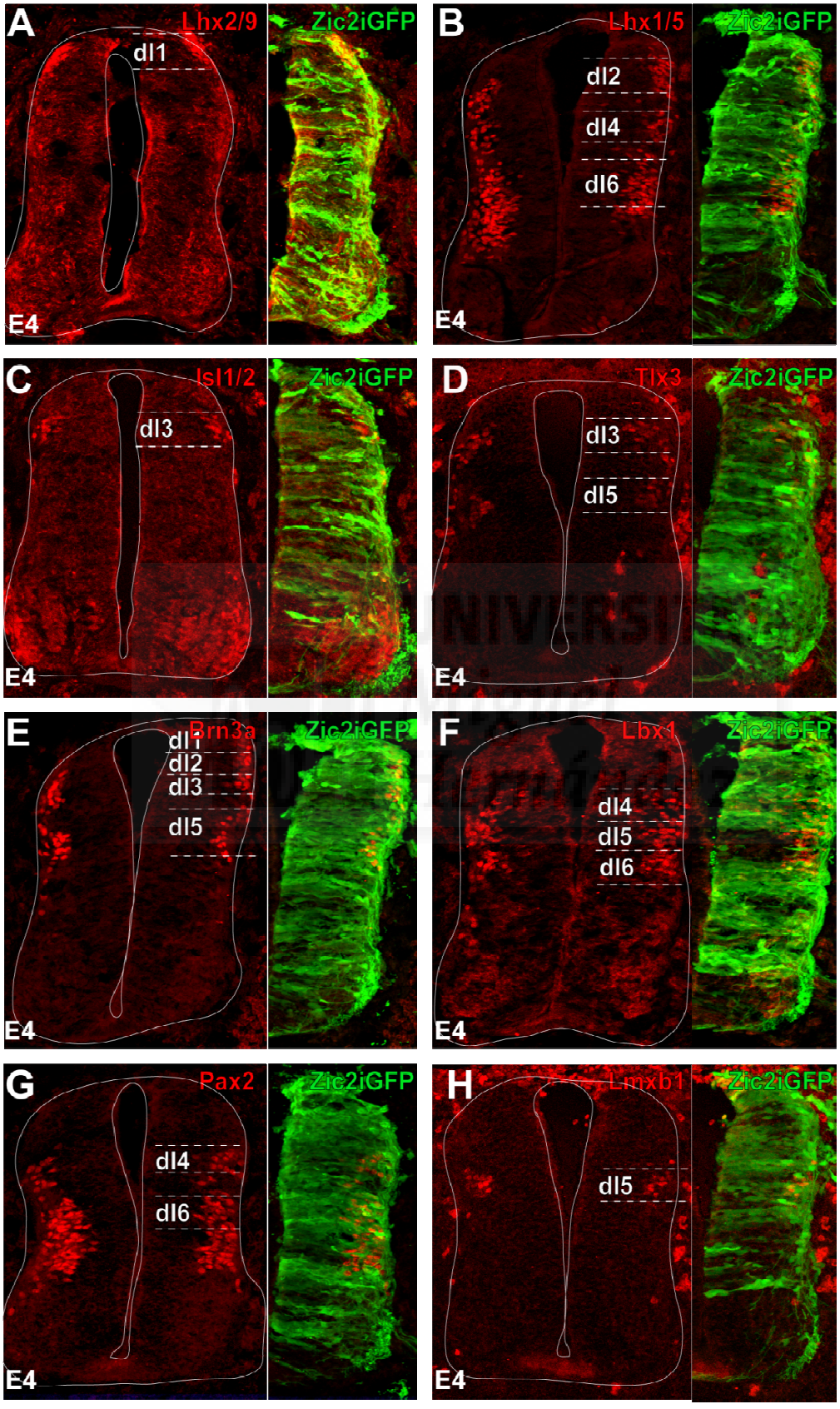
A
Lhx2/9
Zic2iGFP
dI1
E4
B
Lhx1/5
Zic2iGFP
dI2
dI4
dI6
E4
C
Isl1/2
Zic2iGFP
dI3
E4
D
Tlx3
Zic2iGFP
dI3
dI5
E4
E
Brn3a
Zic2iGFP
dI1
dI2
dI3
dI5
E4
F
Lbx1
Zic2iGFP
dI4
dI5
dI6
E4
G
Pax2
Zic2iGFP
dI4
dI6
E4
H
Lmxb1
Zic2iGFP
dI5
E4

Figure 18. Alterations in the expression of Zic2 do not affect cell fate.

(A-H) 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 show no alteration in cell fate in the electroporated compared to the non-electroporated side. Electroporated sides are shown at the right of the corresponding images.

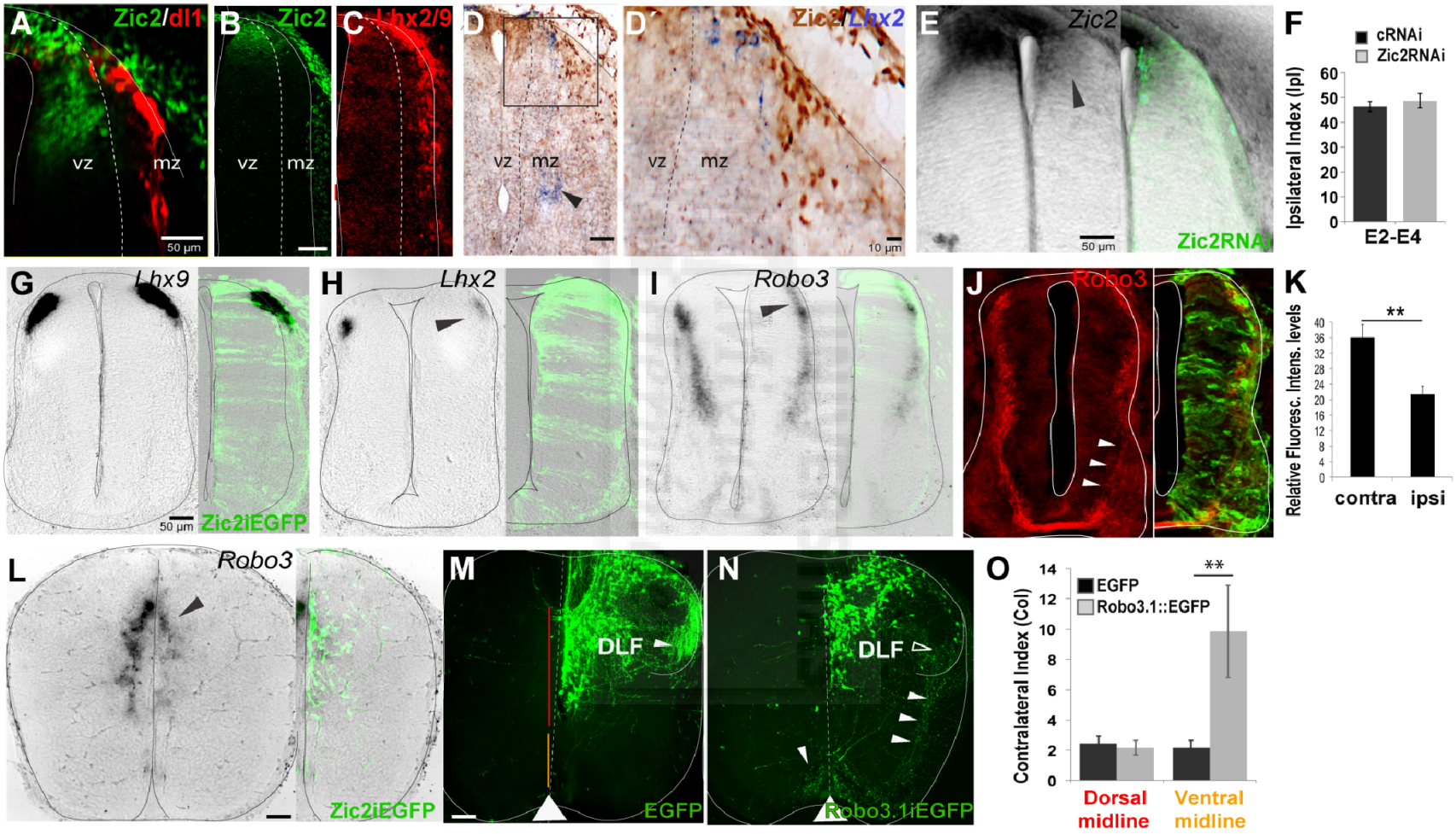
A
Zic2/dl1
vz
mz
50 µm
B
Zic2
vz
mz
C
Lhx2/9
D
vz
mz
D´
Zic2/Lhx2
vz
mz
10 µm
E
Zic2
50 µm
Zic2RNAi
F
cRNAi
Zic2RNAi
Ipsilateral Index (Ipl)
60
50
40
30
20
10
0
E2-E4
G
Lhx9
50 µm
Zic2iEGFP
H
Lhx2
I
Robo3
J
Robo3
K
Relative Fluoresc. Intens. levels
**
40
36
32
28
24
20
16
12
8
4
0
contra
ipsi
L
Robo3
Zic2iEGFP
M
DLF
EGFP
N
DLF
Robo3.1iEGFP
O
EGFP
Robo3.1::EGFP
**
Contralateral Index (CoI)
14
12
10
8
6
4
2
0
Dorsal midline
Ventral midline

Figure 19. Zic2 is able to repress the commissural program.

(A) Dorsal quadrant of a transverse spinal section of a E4 chick embryo electroporated with EdI1::EGFP plasmids (red) and immunostained with Zic2 antibodies (green) shows Zic2 expression in progenitors but not in dI1 interneurons.

(B, C) Consecutive transverse spinal cord sections from an E11 wildtype mouse embryo immunostained with Zic2 (green) and Lhx2/9 (red) antibodies show that these transcription factors do not localize at this stage.

(D) Immunostaining against Zic2 (brown) combined with *in situ* hybridization for *Lhx2 mRNA* (blue) in spinal cord transverse sections of E12 wildtype mouse embryos. At E12 most Lhx2 neurons occupy medial positions in the deep horn while some others are still dorsally located. None of the Lhx2 populations colocalize with Zic2, which shows a more lateral location at this stage. **(d)** High magnification of the dorsal spinal cord section in (D) shows that Zic2 and Lhx2 do not colocalize in dorsal areas.

(E) *In situ* hybridization against chick Zic2 in transverse spinal cord sections of E4 chick embryos electroporated with Zic2RNAi at E2 leads to effective Zic2 downregulation (black arrow) in the chick dorsal cord.

(F) Quantification of axons projecting ipsilaterally versus total targeted axons in E4 chick embryos electroporated at E2 with controlRNAi (cRNAi) or Zic2RNAi. The graph shows that at this stage downregulation of Zic2 does not affect axonal laterality.

(G-I) *In situ* hybridization against Lhx9, Lhx2 and Robo3 in transverse spinal sections from E4 chick embryos electroporated unilaterally with CAG-Zic2-IRES-EGFP (Zic2iEGFP) plasmid shows no alteration of Lhx9 mRNA levels while Lhx2 and Robo3 mRNA levels decreased (black arrows) after Zic2 induction. The electroporated side is shown at the right of each section.

(J) Immunohistochemistry against Robo3 (red) in transverse spinal sections from E4 chick embryos electroporated unilaterally with Zic2iEGFP plasmid show decreased levels of Robo3 in the commissural axons of the electroporated side (white arrows).

(K) Quantification (measured by fluorescence intensity levels) of Robo3 protein levels in the side electroporated with Zic2iEGFP plasmid compared to the non-electroporated side. Error bars indicate ±SEM (**p<0.01, student´s unpaired t-test).

(L) *In situ* hybridization against Robo3 in transverse spinal sections from an E14 mouse embryo unilaterally electroporated with CAG-Zic2 and CAG-GFP shows a decrease of *Robo3mRNA* levels in the electroporated side (black arrow).

(M, N) Transverse spinal sections of E16 mouse embryos electroporated at E12 with CAG-Robo3.1 and CAG-GFP plasmids show that most axons ectopically expressing Robo3.1 are not directed to the DLF but grow instead ventrally to cross the ventral midline (while arrows).

(O, P) Quantification of the number of axons that cross the dorsal and the ventral midline in E16 mouse embryos electroporated at E12 with Robo3.1.

Vz, ventricular zone; mz, mantle zone; DLF, dorsolateral fascicle.

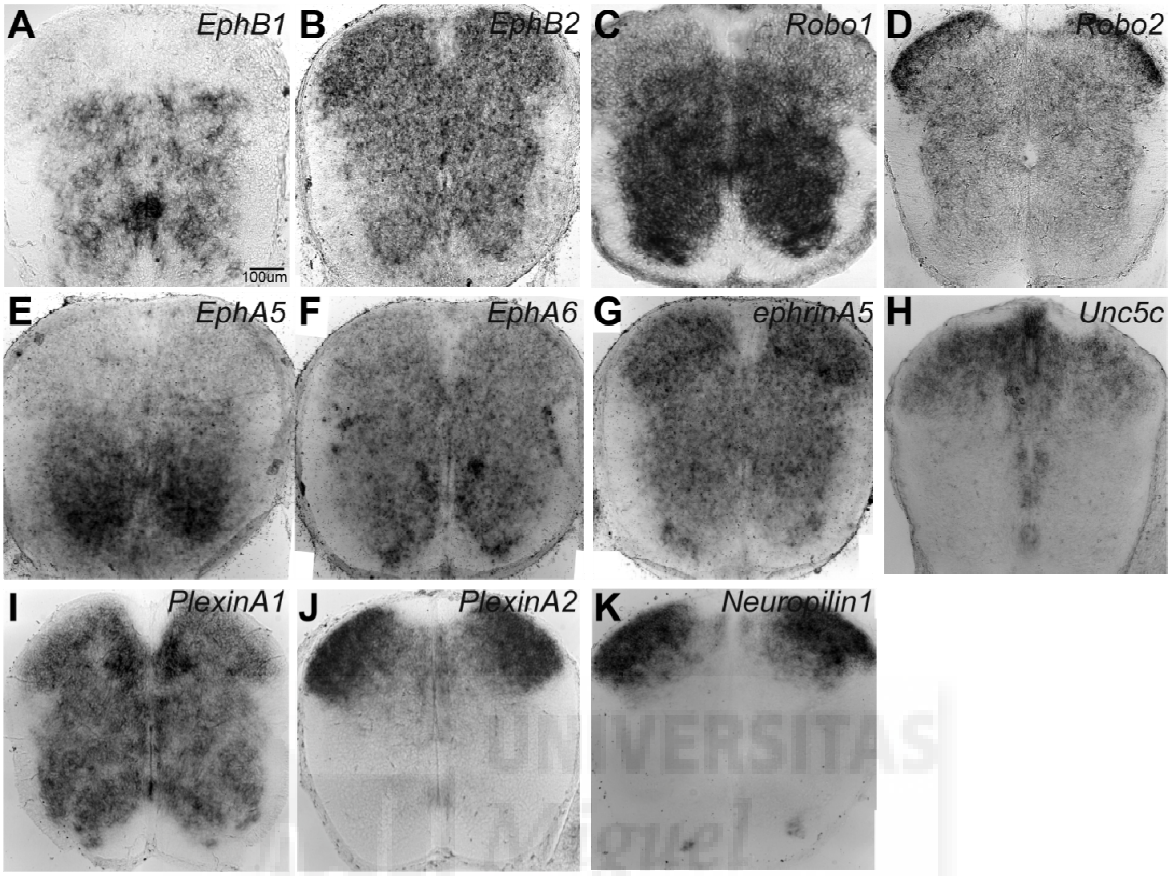

Figure 20. Expression pattern of repulsive guidance receptors in the developing spinal cord.

(A-H) *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.

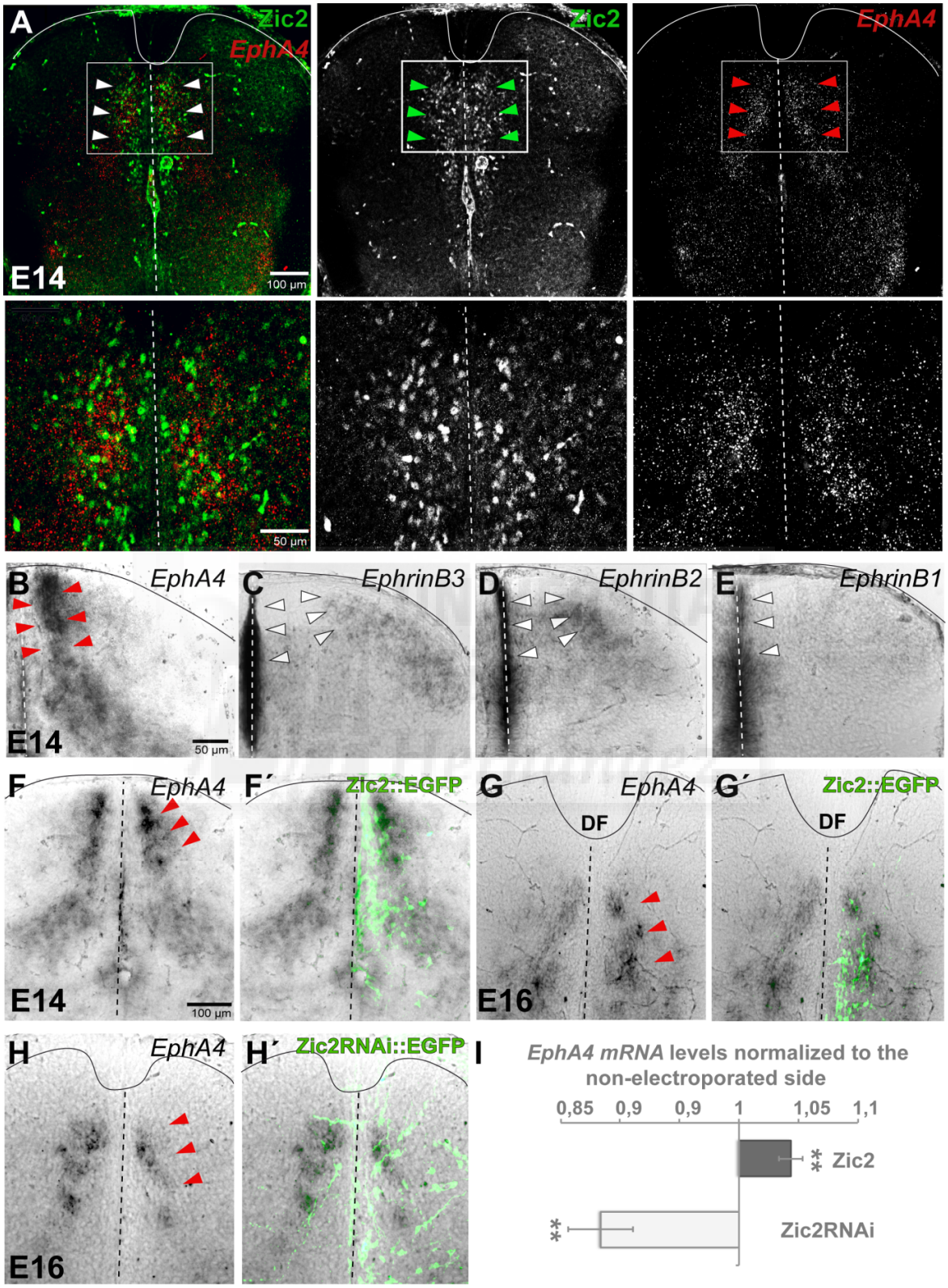
A
Zic2
EphA4
E14
100 µm
50 µm
Zic2
EphA4
B
EphA4
E14
50 µm
C
EphrinB3
D
EphrinB2
E
EphrinB1
F
EphA4
E14
100 µm
F´
Zic2::EGFP
G
EphA4
DF
E16
G´
Zic2::EGFP
DF
H
EphA4
E16
H´
Zic2RNAi::EGFP
I
EphA4 mRNA levels normalized to the non-electroporated side
0,85
0,9
0,9
1
1,05
1,1
Zic2
Zic2RNAi

Figure 21. Zic2 induces EphA4 in the dorsal spinal cord

(A) Immunostaining of Zic2 (green arrows) in transverse spinal cord sections from E16 wild-type embryos combined with *in situ* hybridization against EphA4 (red).

(B-E) *In situ* hybridization for EphA4 (red arrowheads), ephrinB3, ephrinB2 and ephrinB1 mRNAs (white arrowheads) in transverse spinal cord sections from E14 embryos. All three ephrinBs were expressed at the dorsal midline. In addition, ephrinB3 and ephrinB2 were expressed in the prospective dorsal horns (white arrowheads).

(F-G) *In situ* hybridization against EphA4 in transverse spinal cord sections of E14 and E16 embryos electroporated at E12 with CAG-Zic2 plasmids show increased EphA4 mRNA on the electroporated side (red arrows) compared to the non-electroporated side. **(F´, G)** Same sections depicting the electroporated side (green).

(G) *In situ* hybridization against EphA4 in a representative transverse spinal cord section of an E16 embryo electroporated at E12 with Zic2RNAi showed decreased levels of EphA4 mRNA in the electroporated side (red arrows) compared to the non-electroporated side. **(G´)** Same section depicting the electroporated side (green).
(H) Quantification of EphA4 mRNA levels in a representative transverse spinal cord section from an E16 embryo electroporated at E12. EphA4 mRNA levels increased after Zic2 ectopic expression (dark grey column) and decreased after Zic2RNAi electroporation (light grey column). EphA4 mRNA levels in the electroporated side were normalized to the levels of the respective non-electroporated side. Error bars indicate ±SEM (**p<0.01, Student's unpaired t-test).

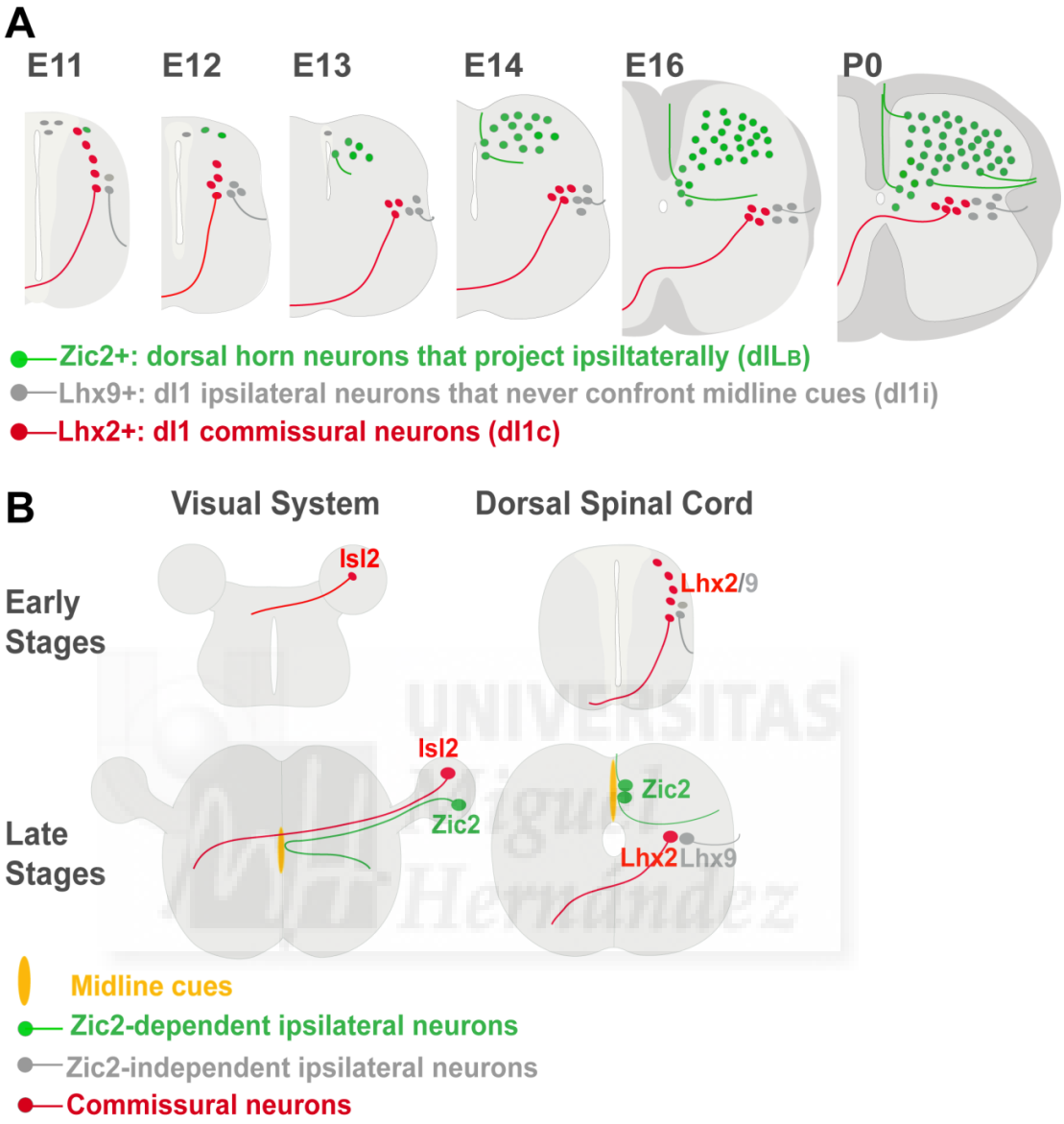

Figure 22. Summary of Zic2 expression in the developing spinal cord and the visual system

(A) dI1 interneurons become postmitotic as early as E10 and migrate and extend axons ventrally, in the case of Lhx2 neurons (red), or laterally, in the case of Lhx9 neurons (grey). The first postmitotic Zic2 neurons (green) appear around E11, at the time that dI1 neurons are still being generated, but they populate the dorsal horns instead of migrating ventrally. From E13 to E16, a high number of Zic2 neurons extend their axons to the DLF and to the DF. By P0, most dorsal horn neurons are already in their final location. We propose that the dorsal horn neurons that differentiate and start to extend their axons close to the midline need to develop mechanisms to actively avoid crossing. In contrast, axons from other ipsilaterally projecting neurons that never approach the midline, such as the dI1i neurons, do not express Zic2 because they do not need to avoid midline crossing.

(B) In the visual system, Isl2 is expressed in contralateral retinal ganglion cells (RGCs) and Zic2 is postmitotically upregulated only in the RGCs that will be repelled to project ipsilaterally. In the early spinal cord, Lhx2/9 are both expressed in dI1 interneurons. dI1 interneurons migrate ventrally, and their axons navigate laterally and never confront midline cues. In contrast, later-born interneurons (dIL_B) express Zic2, which triggers the expression of a set of molecules that are essential for responding to dorsal midline cues.