THE ROLE OF THE TRANSCRIPTION FACTOR ZIC2 IN NEURAL CREST DEVELOPMENT

Doctoral Thesis presented by

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Sant Joan d'Alacant, January 2020

To whom it may concern,

The doctoral thesis entitled "The role of transcription factor Zic2 in neural crest development" has been developed by myself, Gerald MUÇA. This thesis is presented in a conventional format. It is based on experimental studies undertaken at the Neuroscience Institute of Alicante during the PhD program in neuroscience of the Miguel Hernández University.

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The doctoral thesis entitled "The role of transcription factor Zic2 in neural crest development." has been developed by myself, Gerald MUÇA. This thesis includes the following publication, of which I am fifth author. I declare that the publication has not been used and will not be used in any other thesis in agreement with my thesis director Eloísa Herrera González de Molina:

Article: Uncoupling of EphA/ephrinA Signaling and Spontaneous Activity in Neural Circuit Wiring.

Authors: Isabel Benjumeda, Augusto Escalante, Chris Law, Daniel Morales, Geraud Chauvin, Gerald Muça, Yaiza Coca, Joaquín Márquez, Guillermina López-Bendito, Artur Kania, Luis Martínez, Eloísa Herrera.

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D. Eloísa Herrera González de Molina, Investigadora Científica del CSIC

Autoriza la presentación de la Tesis Doctoral titulada "The role of the transcription factor Zic2 in neural crest development" y realizada por D Gerald Muça bajo mi inmediata dirección y supervisión como director de su Tesis Doctoral 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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CERTIFICO:

Que la Tesis Doctoral titulada "The role of the transcription factor Zic2 in neural crest development" has sido realizada por D. Gerald Muça, bajo la dirección de D./Dª.Eloisa Herrera como director/directora, y doy mi conformidad para que sea presentada a la Comisión de Doctorado de la Universidad Miguel Hernández.

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To my brother Ardit!...

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INDEX

INDEX

LIST OF TABLES ... 21

LIST OF FIGURES ... 22

ABREVIATIONS ... 11

ABSTRACT ... 15

RESUMEN ... 17

INTRODUCTION ... 21

1. Neurulation and dorsoventral patterning of the neural tube. ... 23

2. The Neural Crest. ... 27

2.1. Neural crest induction. ... 28

2.2. Signalling pathways involved in neural crest induction. ... 30

2.3. Transcription factors involved in neural crest induction. ... 35

2.4. Transcription factors involved in the specification of NCCs. ... 36

2.5 The Epithelial-Mesenchymal Transition (EMT). ... 38

2.6. Regionalization of the neural crest along the anteroposterior axis. ... 41

2.7. Neural crest migration. ... 43

2. 8. The role of membrane proteins, extracellular matrix proteins and guidance cues in neural crest migration. ... 45

2. 9. NCCs specification. ... 49

3. The Zic family of transcription factors. ... 53

3. 2. Zic2 in early nervous system development ... 55

3. 3. Zic2 in late stages of development ... 57

OBJECTIVES ... 63

MATERIAL AND METHODS ... 67

1. Mice. ... 67

2. Embryo harvesting, dissection and fixation. ... 68

3. *In ovo* electroporation. ... 68

4. ShRNA sequences. ... 71

5. Immunohistochemistry in sections. ... 72

6. Whole mount Immunohistochemistry and clearing. ... 74

7. Whole mount In Situ Hybridization in chicken. ... 76

8. Whole mount In Situ Hybridization in mouse. ... 76

9. Microarrays analyses. ... 77

10. Quantification of migrating NCCs. ... 78

11. Statistical Analysis. ... 79

UNIVERSITAS Miguel Hernández

RESULTS 83
1. **Zic2 is expressed in premigratory NCCs and downregulated in migrating NCCs.** 83
2. **Zic2 is necessary for NCCs emigration from the neural tube.** 85
3. **Zic2 is sufficient to induce NCCs exit and alter their migration.** 89
4. **Zic2 does not determine melanocytic or sensory NCC fate.** 92
5. **Zic2 target genes in neural crest formation.** 94
6. **Zic2 regulates the expression of the secretable molecule Draxin/Neucrin.** 96

DISCUSSION 103
1. **Zic2 expression in pre-migratory neural crest during development.** 104
2. **The role of Zic2 in premigratory neural crest cells** 105
3. **Zic2 target genes during NCC formation.** 108

CONCLUSIONS 115
CONCLUSIONES 117
BIBLIOGRAPHY 121
ANNEX (ARTICLE) 149

LIST OF TABLES

TABLE	PAGE
Table 1. Hamburger and Hamilton stages.	43
Table 2. DNA constructs electroporated in this study.	70
Table 3. Oligonucleotides used in this study.	72
Table 4. Primary antibodies used in this study.	73
Table 5. Secondary antibodies used in this study.	74
Table 6 Probes used for ISH in chicken and mouse.	77

LIST OF FIGURES

FIGURE	PAGE
Figure 1. Gastrulation.	21
Figure 2. Neurulation.	25
Figure 3. Neural crest induction and migration.	30
Figure 4. WNT signaling pathways.	34
Figure 5. Gene regulatory network controlling neural crest formation.	38
Figure 6. The EMT process.	39
Figure 7. Cadherin structure and interconnecting elements.	40
Figure 8. Regionalization of the chick neural crest.	42
Figure 9. Trunk neural crest migration pathways.	45
Figure 10. Guidance cues involved in trunk neural crest migration.	49
Figure 11. Neural crest differentiation.	52
Figure 12. The role of Zic2 as a determinant of axon ipsilaterality.	59
Figure 13. Homemade Time-lapse plate.	75
Figure 14. Scheme of different ROIs used to quantify NCCs migration.	79
Figure 15. Immunostaining against Zic2 in transverse neural tube sections from E8-E10 mouse embryos.	83
Figure 16. Characterization of Zic2 positive cells during the formation of the neural tube.	85
Figure 17. NCC accumulate into the dorsal neural tube in *Zic2* mutant mice.	86
Figure 18. Transverse sections from E8.5 Tg(eGFP)::*Zic2*$^{kd/kd}$ and control littermate embryo.	86
Figure 19. The schema illustrates electroporation of plasmids and cell migration analysis at different time points.	87
Figure 20. Downregulation of Zic2 in the chick neural tube leads to a reduced number of migrating NCCs.	88
Figure 21. Zic2 downregulation does not induce cell death.	89
Figure 22. Overexpression of Zic2 in the chick neural tube leads to an accumulation of NCC in the mesenchyme.	90
Figure 23. Ectopic expression of Zic2 leads to accumulation of NCC in the dorsal mesenchyme.	91
Figure 24. PH3 in Zic2 GOF.	92
Figure 25. Zic2 does not induce melanocytic fate.	93
Figure 26. Zic2 does not induce sensory fate.	94
Figure 27. EphB1 does not reproduce Zic2 overexpression phenotype.	95
Figure 28. EphA4 reproduce partially Zic2 overexpression phenotype.	96
Figure 29. A. Hit map generated from the DNA microarray data from embryos electroporated with Zic2ireseGFP encoding plasmids or eGFP alone.	96
Figure 30. Draxin expression after manipulation of Zic2.	97
Figure 31. Transverse sections of E3 chick embryos electroporated at HH12/14 with control shRNA or Draxin shRNAs plus eGFP encoding plasmids.	98
Figure 32. Scheme representing Zic2 expression during neural crest development in mouse.	105
Figure 33. Scheme representing Zic2 phenotype.	108

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ABREVIATIONS

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Miguel
Hernández

ABREVIATIONS

APC: Adenomatous polyposis coli

BMP: Bone morphogenetic proteins

CNS: central neural system

DL: dorsolateral

Dm: dermomyotome

DM: dorsomedial

Dp: dorsal progenitors

DRG: dorsal root ganglia

Dvl: Dishevelled

EDNRB: Endothelin receptor B

EMT: Epithelial Mesenchymal Transition

FGF: Fibroblast Growth Factors

Fp: floor plate

Fz: Frizzled

GSK3β: glycogen synthase kinase 3β

HH: Hamburger and Hamilton

HPE: holoprosencephaly

LRP: low-density lipoprotein receptor related proteins

HMG: high mobility group

MTF: microphthalmia-associated transcription factor

NC: neural crest

NCCs: neura crest cells nc: notochord

NPB: neural plate border

NTD: neural tube defects

nt: neural tube

pMN: progenitor motor interneurons

PNA: Peanut agglutinin

PNS: peripheral neural system

Rf: roof plate

Scl: sclerotome

SG: sympathetic ganglia

SHH: Sonic hedgehog

S: somite

TRP1,2: Tyrosine related protein1 and 2

Vp: ventral progenitors

WNT: Wingless proteins

Zic2: Zinc Finger of the Cerebellum protein 2

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ABSTRACT

ABSTRACT

Neural Crest cells (NCCs) are pluripotent cells that originate in the dorsal neuroephithelium of the dorsal neural tube and travel all over the embryo to contribute to the formation of many organs. The zinc finger transcription factor Zic2 (Zinc Finger of the Cerebellum protein 2) is known to participate in several steps of neural development including neurulation and neural crest formation. However, its precise role in these processes has not been yet clearly stated. Here, we show that Zic2 is expressed in premigratory but not in migrating NCCs. Then, functional experiments *in vivo* demonstrate that Zic2 expression is crucial for the formation of the NNCs but does not regulate cell death or cell fate. In an unbiased gene-wide screen performed in the chick neural tube, we identified the secreted protein Draxin/Neucrin as a possible Zic2 effector. We then confirm that spatiotemporal expression of Zic2 and Draxin in the dorsal tube are very similar and, functional experiments in chick demonstrate that Zic2 regulates Draxin/Neucrin expression during NCC formation. Finally, loss of function and gain of function studies confirm that Draxin/Neucrin partially recapitulates Zic2 functions during NCC migration. All these experiments together demonstrate that Zic2 plays a critical role during the progression of NCCs to get out of the neural tube and reveal that Zic2 induces Draxin/Neucrin expression during this process.

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RESUMEN

Las células de la cresta neural (CCN) son células pluripotentes que se originan en el neuroepitelio dorsal del tubo neural y viajan por todo el embrión para contribuir a la formación de muchos órganos. Se sabe que el factor de transcripción Zic2 (la proteína del dedo zinc 2 del cerebelo) participa en varios pasos del desarrollo neural, incluido la neurulación y la formación de la cresta neural. Sin embargo, su papel preciso en estos procesos aún no se ha establecido claramente. En este trabajo, mostramos que Zic2 se expresa en las CCN premigratorias pero no en celulas migratorias. Luego, los experimentos funcionales *in vivo* demuestran que la expresión de Zic2 es crucial para la formación de las CCN pero no regula la muerte celular o la diferenciacion celular. En un cibrado amplio y equitativo de la genoma realizada en el tubo neural del pollitos, identificamos la proteína secretable Draxin / Neucrin como un posible efector de Zic2. Luego confirmamos que la expresión espacio-temporal de Zic2 y Draxin/Neucrin en el tubo dorsal es muy similar y, los experimentos funcionales en pollitos demuestran que Zic2 regula la expresión de Draxin / Neucrin durante la formación de las CCN. Finalmente, los estudios de pérdida de función y ganancia de función confirman que Draxin / Neucrin recapitula parcialmente las funciones de Zic2 durante la migración de CCN. Todos estos experimentos juntos demuestran que Zic2 juega un papel crítico durante la progresión de las CCN para salir del tubo neural y revelan que Zic2 induce la expresión de Draxin / Neucrin durante este proceso.

INTRODUCTION

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INTRODUCTION

The formation of the nervous system is one of the earliest events in development and the last to be completed after birth. The development of the nervous system generates the most complex structure within the embryo, the brain, and in utero insult during pregnancy may have consequences in adult brain function.

Once the zygote forms it begins to divide exponentially forming the morula and blastula, structures that are composed of pluripotent cells. In blastula, cells start to stratify in three germinal layers, forming the gastrula. These three laminas are the ectoderm (the upper layer), the mesoderm (the middle one) and the endoderm (the bottom one). Gastrulation starts with the formation of the primitive streak. Cells invaginate from the epiblast to form the endoderm and the mesoderm. The remaining cells from epiblast will form the ectoderm, (Figure 1). The ectoderm will give rise to the central and peripheral nervous system (CNS, PNS), epidermis and placodes, while mesodermal cells will give rise to the notochord (Acloque et al., 2009; Gilbert, 2013).

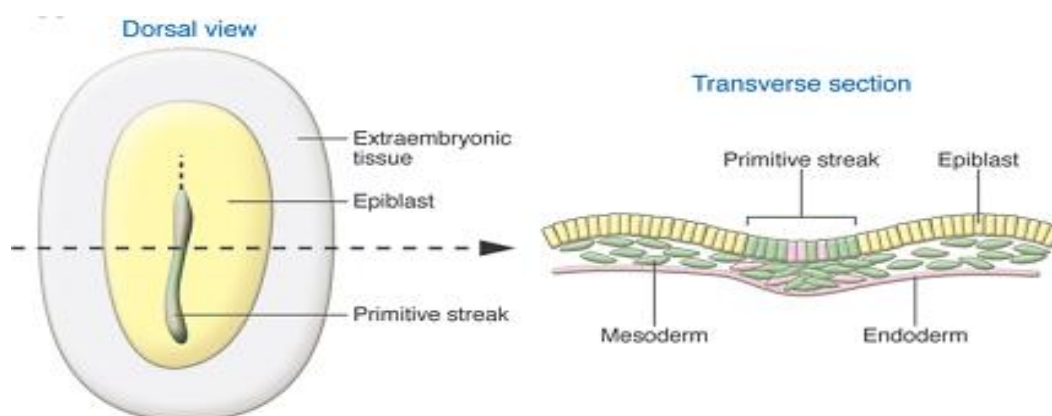

Figure 1: **Gastrulation.** After primitive streak the three germ layers (ectoderm, endoderm and mesoderm) are formed (Acloque et al., 2009).

The notochord is positioned in the same direction as the primitive streak, above the ectoderm. The central region of the ectoderm, under the influence of inductive signals secreted by the notochord, will develop into the neural ectoderm (neural plate), which is the structure that will give rise to the future CNS and PNS. These notochord inductive signals push the neural plate to fold and form the neural tube. The neural tube will subsequently differentiate into the CNS, the brain and the spinal cord. The lateral parts of the ectoderm, far from the notochord signalling, will transform in nonneural ectoderm that later leads to the epidermis (Gilbert, 2013).

The development of the nervous system could be divided into three main phases:

a) Cell division and morphogenesis. It starts with the higher proliferation of neural progenitors inside the neural plate. Then, under the influence of morphogen factors, the neural tube starts to regionalize forming the different parts of the future brain (prosencephalon, mesencephalon, rhombencephalon) and spinal cord.

b) Cell differentiation and migration. In this phase, progenitor cells differentiate into different types of neural and glial cells.

c) Axon guidance, cell connectivity and refinement. In this, phase cells extend their axons to finally reach their partners and establish definitive connections.

During the early phases of neural development, cell diversification is extraordinary. Neurodevelopment may be comparable to a growing tree. One cell type (the root and the trunk) will separate in several cell types (the branches). A given ectodermal cell will become a neural progenitor, a glial cell, a NCC or an epidermal cell. Then an NCCs will differentiate in melanocyte, sensory neuron or glial cell. In the end, each cell will be located in a specific location and will establish exclusive connections with

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particular cells. Complex signalling networks direct these processes (Gilbert, 2013, Purves, 2008).

The transcription factor Zic2 is known to play important functions in different stages of brain development ranging from gastrulation to axon guidance. In this thesis, we aimed to investigate the particular contribution of Zic2 and some of its target molecules to the early steps of nervous system development. In particular, to the formation of the neural crest.

1. Neurulation and dorsoventral patterning of the neural tube.

Neurulation is a key event during embryonic development. It includes the formation of the neural tube, a rudiment of all future structures of the CNS. Neurulation starts with the formation of the neural plate followed by its invagination. Then, the neural plate bends, forming the neural tube folds. Finally, the fusion of the neural folds takes place and the neural tube closes (Figure 2). This process completes by different bending and closure points at ventrodorsal and anteroposterior directions. The neural plate begins to bend ventrally at the midline and later bending process is spread upper to each neural fold at the dorsolateral direction. At the end the neural folds start to fusion at three different points in the anteroposterior direction that spread until neural tube closure is completed. Defects during neural tube closure provoke congenital malformations called neural tube defects (NTDs). They are named based on the anteroposterior axial localisation: Anencephaly, when the cephalic part fails to close; holoprosencephaly (HPE), when midline structures of the forebrain fail to form; craniorachischisis, when most of the brain and spinal cord fail to close and spina bifida, when the failing in neural tube closure is at the lumbosacral level (Brown et al., 2001; Copp et al., 2003; Ybot-Gonzalez et al., 2007).

Neurulation takes place in two steps: Primary neurulation: Is the folding of the neural plate from the cephalic region to the tail bud. In mouse, the primary neurulation is extended from E8.5 to E10. Secondary neurulation: It includes the formation of the remaining part of the neural tube in the tail bud region. During this process, mesenchymal cells of a rudimentary part of the primitive streak transform into epithelial cells to complete the closure of the ending part of the neural tube (Copp et al., 2003; Ybot-Gonzalez et al., 2002).

Concomitant with the closure of the neural tube, cells inside the tube differentiate in several neural progenitors and glial cells. Progenitors start to layer following a dorsoventral pattern that depends on the action of different signalling molecules. These molecules form a coordinated net along the anteroposterior and the dorsoventral axis (Lee and Jessell, 1999; Lumsden and Krumlauf, 1996). At the beginning of neurulation, cells within the neural tube are in continuous proliferation and while the neural tube is closing, cells differentiate in different types of progenitors. Cells in the dorsal part of the neural tube differentiate into six types of interneurons (dl1-dl6) that will receive sensory information. Cell progenitors in the ventral region of the tube will produce both interneurons (p0-p3) and motor neurons (pMN). From the most apical and ventral part of the tube cells will differentiate into the roof and floor plate respectively, (Figure 2) (Caspary and Anderson, 2003; Jessell, 2000; Lee and Jessell, 1999; Lupo et al., 2006).

Different families of diffusible proteins, as well as factors from the ectoderm, roof plate, notochord and floor plate, participate in neurulation and dorsoventral patterning. These families of diffusible proteins include Bone morphogenetic proteins (BMPs), Fibroblast growth factors (FGFs), Retinoic acid, Sonic hedgehog (SHH) and Wingless proteins (WNTs) (Wilson and Maden, 2005).

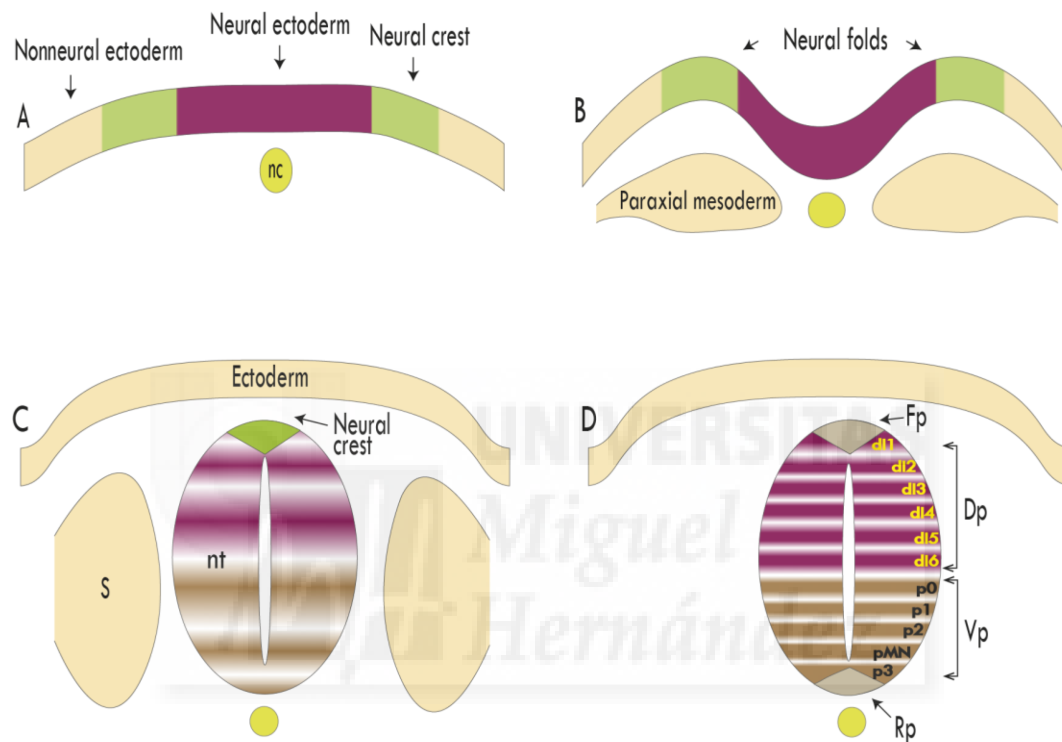

Figure 2: Neurulation. A, Neural plate and neural crest induction. **B**, Neural plate starts folding. **C**, Neural tube starts dorsoventral patterning after neural crest migration. **D**, Dorsal and ventral progenitors already regionalized. Dp-dorsal progenitors, Vp-ventral progenitors, nc-notochord, nt-neural tube, Rf- roof plate, Fp-floor plate, s-somite.

BMP signalling plays a complex role in neural tube development. It participates first in neural induction and neural crest formation but it is also fundamental in neural tube patterning. The early source of BMPs is the non-neural ectoderm and later, the roof plate. Cells from the dorsal neural tube respond to BMPs to differentiate into NCCs and later in distinct types of interneurons. BMP signalling is a key factor for cell differentiation and patterning in the dorsal neural tube but is also important for neural plate bending

(Lee and Jessell, 1999; Liu and Niswander, 2005). BMPs act in conjunction with other factors such as WNTs and SHH (Liu and Niswander, 2005).

SHH signalling is a morphogen produced by the notochord and later by the floor plate (Echelard et al., 1993). This protein is also responsible for cell differentiation and for the patterning of the ventral neural tube (Briscoe et al., 1999; Pierani et al., 1999). SHH has been described as necessary for medial neural plate bending but not for neural tube closure. *Shh* mutant mice close their neural tube properly (Ybot-Gonzalez et al., 2002) and it appears that in humans holoprosencephaly associated to mutations in *SHH* (Pierani et al., 1999) is related to cell patterning rather than to neural tube closure defects (Chiang et al., 1996).

FGFs are released from the caudal part of the mesoderm and have an inhibitory effect in cell differentiation. The inhibition of FGF signalling by Retinoic acid is necessary to initiate cell differentiation (Del Corral et al., 2003; Wilson and Maden, 2005).

WNTs are present in the dorsal part of the neural tube and are involved in the initiation of neurulation, neural tube closure and cell differentiation rather than in patterning. In *Xenopus* ectopic expression of the Dishevelled (Dvl), a component of the WNT pathway, disrupts neural tube closure (Copp et al., 2003; Jessen et al., 2002; Wallingford and Harland, 2002), and a similar phenotype is observed after mutation of mouse *Dvl*. The *Dvl1/2* double mutant mice exhibit defects in the neural tube closure and also show craniorachischisis (Copp et al., 2003; Hamblet et al., 2002).

Finally, teratogenic factors such as Retinoic acid or ethanol are also important for neurulation. Disturbance on their balance is accompanied by NTDs, although the mechanisms by which these factors participate in neurulation remain unclear. Essential for proper neurulation is a good balance between cell proliferation and cell death; both

of them have a key role during neural tube fusion (Copp et al., 2003; Pennimpede et al., 2010; Smith and Schoenwolf, 1997).

2. The Neural Crest.

The neural crest is the transitory structure located at the dorsal edge of the neural tube formed by pluripotent cells that will delaminate to spread and later differentiate in a huge variety of cell types (Le Douarin and Kalcheim, 1999). The neural crest was first observed in the chick embryo by Wilhelm His in 1868, who named it "Zwischenstrang", that means "the cord in between" (Hall, 2008). Wilhelm described this tissue as layers of cells between the presumptive epidermis and the neural tube. Arthur Marshall introduced the term “Neural Crest” for the first time in an article in 1879. He used this name for NCCs that give rise to chicken cranial and spinal ganglia. Before this publication, Marshall was using the term “neural ridge”, to the NCCs existing before neural tube closure. Later he adopted the term “neural crest” to describe all NCCs, those before and after neural tube closure (Hall, 2008). Among others, the neural crest has the following properties:

1. It is present in all vertebrates. Recent data show that its evolution was not a one-step process but rather a systematic process. The Urochordata is the nearest invertebrate relative to vertebrates having a "neural crest like" structure with some features similar to the vertebrate neural crest (Hall, 2008; Hall and Gillis, 2013). According to Gans and Northcutt the most important element that makes vertebrates different from their nearest relatives is the possession of the neural crest (Gans and Northcutt, 1983). Thanks to the acquisition of the neural crest, vertebrates developed a head with complex structures and sensory organs. Neural crest contributed to the formation of jaw and skull, paired sensory ganglia and bars of pharyngeal cartilaginous. This "new head” had a critical

importance in the adaptation of vertebrates to predatory life compared to passive feeding life characteristics of their nearest relatives (Gans and Northcutt, 1983).

2. NCCs are pluripotent. The neural crest gives rise to a huge variety of cell types. NCCs from the head region give rise to connective tissues: chondroblasts and osteoblasts, while NCCs cells from the rest of the body may differentiate into sensory ganglia, sympathetic ganglia and glial cells of the peripheral neural system, melanocyte and endocrine cells of adrenal and thyroid gland (Dupin et al., 2007; Ezin et al., 2009).

3. NCCs delaminate from the neural tube and migrate to their different target tissues. NCCs undergo an epithelial to mesenchymal transition (EMT) that allows them to detach from the neural tube and migrate to their destination (Acloque et al., 2009).

A significant number of congenital diseases called "neurocristopathies" are the consequence of defective neural crest formation. These pathologies, such as DiGeorge, Charge syndrome or Hirschsprung's disease, lead to craniofacial malformations, cleft palate, heart malformation, and problems with PNS (Simões-Costa and Bronner, 2013; Zhang et al., 2014). Research on how the neural crest develops and on the mechanisms of action of molecules involved in this process could lead to a better understanding of the pathogenesis of these diseases.

2.1. Neural crest induction.

Neural crest induction is a multistep process that starts at gastrulation and continues until the neural tube is closed. Future NCCs lay in the border of the neural plate, limited laterally by the non-neural ectoderm and below by the paraxial mesoderm, (Figure 3). Both ectoderm and paraxial mesoderm have been proposed to participate in neural crest formation (Rogers et al., 2012). The interaction of non-neural ectoderm and neural

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ectoderm (neural plate) is crucial in neural crest formation. The juxtaposition between both parts of ectoderm induces the formation of the neural crest (Selleck and Bronner-Fraser, 1995). A number of neural fold cells will give rise to neurons and epidermal cells while some others will become premigratory NCCs (Collazo et al., 1993; Knecht and Bronner-Fraser, 2002; Selleck and Bronner-Fraser, 1995).

When NCCs are still inside the neural tube they are morphologically indistinguishable from other neuroepithelial cells located at the top of the neural tube. In fact, NCCs do not acquire a well-defined fate until they start their migration but at this time, they already start to express transcription factors to gain specific properties. At the time of delamination, these cells are morphologically and molecularly different from the rest of the neural tube cells (Barrallo-Gimeno and Nieto, 2006; Duband, 2006; Gammill and Bronner-Fraser, 2003). Neural crest induction requires the combination of multiple signalling pathways that include: BMPs, FGFs, WNTs, Notch/Delta and the Retinoic acid (Rogers et al., 2012).

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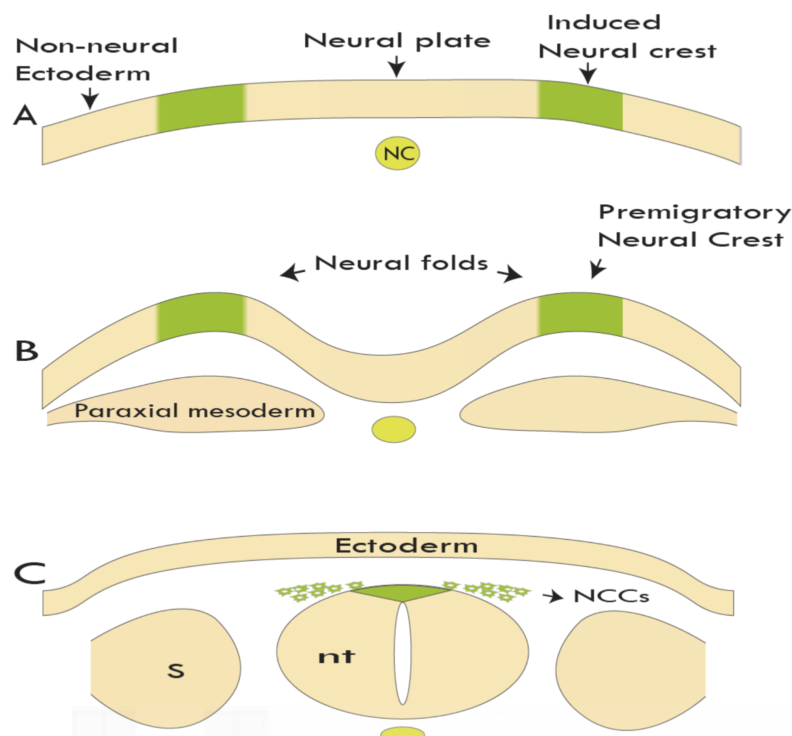

Figure 3: **Neural crest induction and migration.** **A**, Neural crest is induced between two parts of ectoderm, **B**, Neural tube folding and premigratory neural crest, **C**, NCCs delaminate from neural tube. nt-neural tube, nc-notochord, s-somite.

2.2. Signalling pathways involved in neural crest induction.

2.2.1. BMP proteins.

BMPs are secreted proteins, members of the Transforming Growth Factor-β (TGF-β) superfamily. BMPs are produced by the paraxial mesoderm and the non-neural part of the ectoderm (Rogers et al., 2012). These proteins bind two types of receptors (R type1 and type2). The interaction protein-receptor leads to SMAD1/5/8 phosphorylation which forms dimers with SMAD4 and translocate to the nucleus to induce or repress gene expression (Barrallo-Gimeno and Nieto, 2006; Kishigami and Mishina, 2005; Massagué, 2008; Stuhlmiller and Garcia-Castro, 2012).

High levels of BMPs induce ectodermal cells to the epidermis. In contrast, low levels of BMPs induce ectodermal cells to adopt a neural fate. Chordin, Noggin, and Follistatin are inhibitory molecules of the BMP pathway. They are secreted by the paraxial mesoderm and modulate the activity of BMPs (Knecht and Bronner-Fraser, 2002; Wilson and Hemmati Brivanlou, 1997). The combination of these proteins has an important influence in the differentiation of the dorsal part of the neural tube into NCCs and dorsal neural progenitors. The gradient model proposed the necessity of intermediate BMPs levels for neural crest induction (Betancur et al., 2010a; Knecht and Bronner-Fraser, 2002; Nguyen et al., 1998), but experiments in chicken demonstrated that in addition to the levels of BMPs, the timing of BMP exposure is also important to induce neural crest and neural differentiation. Neural crest explants initially treated with BMP4, differentiate into epidermis but if they are treated with BMP4 10 hours later they differentiate into NCCs (Patthey et al., 2009; Stuhlmiller and Garcia-Castro, 2012). In mice, the scenario is more complex and the results are difficult to interpret. In the beginning, it was thought that BMPs are important for neural crest migration rather than for neural crest induction because *Bmp5*/*Bmp7* double mutants showed a reduction of the trunk neural crest formation (Basch et al., 2004; Solloway and Robertson, 1999). It was later suggested that BMPs could be rather involved in NCCs migration because *Bmp2* null mutants exhibited defects in the cephalic part of neural crest but their early neural crest markers were not affected (Correia et al., 2007; Stuhlmiller and García-Castro, 2012). The analysis of BMP Receptor1A mutants have more recently supported the idea that BMP signalling is important for neural crest induction because the neural crest does not develop in these mice (Rogers et al., 2012; Stottmann and Klingensmith, 2011). It has also been demonstrated that intermediate levels of BMPs are necessary but not sufficient to induce

neural crest differentiation in any vertebrate model (Betancur et al., 2010a; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Wilson and Hemmati Brivanlou, 1997). Taking all those results into account the most recent model proposes that BMP-dependent neural crest induction takes place in two phases. During the first phase (gastrulation), partial inhibition of BMP would allow the expression of other signals like WNTs and FGFs to specify neural crest. Then, in a second phase, BMP signalling would complete the differentiation of the neural plate border to the neural crest cells (Ragland and Raible, 2004; Steventon et al., 2009; Stuhlmiller and García-Castro, 2012).

2.2.2. The WNT signalling pathway.

The WNT signalling pathway is an evolutionarily conserved pathway that regulates many different functions during development in vertebrates and invertebrates (Clevers and Nusse, 2012; Mayor and Theveneau, 2014). WNTs are secreted glycoproteins and a wide number of different experiments have demonstrated that WNT dependent pathways are essential for neural crest induction in vertebrates (Betancur et al., 2010b; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Lewis et al., 2004). In chick, WNTs are necessary and sufficient to induce neural crest. Wnt6 is secreted by ectodermal cells during neural crest induction and is able to induce neural crest differentiation when opposed to the neural plate (Betancur et al., 2010b; Garcia-Castro et al., 2002; Schubert et al., 2002). *Wnt1/Wnt3* double mutant mice show defects in several neural crest derivatives (Betancur et al., 2010b; Ikeya et al., 1997) and because they are expressed just before the initiation of neural crest migration, it was thought that they participate in migration rather than in neural crest induction (Ikeya et al., 1997; Stuhlmiller and García-Castro, 2012). However more recent evidence demonstrates that at least Wnt1, also

UNIVERSITAS Miguel Hernández

participates in neural crest specification (Brault et al., 2001; Stuhlmiller and Garc??a-Castro, 2012).

WNTs activity depend on the activation of downstream effectors. This activity is separated in two principal pathways: the canonical that relays on the activation of β-catenin-dependent transcription and the non-canonical that does not involve β-catenin-dependent transcription. The non-canonical pathway is further divided into two branches: The WNT/planar cell polarity (PCP) and the WNT/Ca2+ pathway (Komiya and Habas, 2008).

The activation of the canonical pathway starts with the interaction of WNTs with the transmembrane receptors Frizzled (Fz) and the low-density lipoprotein receptor-related proteins (LRP5 or LRP6). This event leads to the inhibition of the "destruction complex" which is integrated among others by GSK3β (glycogen synthase kinase 3β), Axin, the Adenomatous polyposis coli (APC) protein, and the E3-ubiquitin ligase β-TrCP. In the absence of WNTs, the "destruction complex" is in an active phase and consequently, the level of β-catenin inside the cell is kept low because the "destruction complex" phosphorylates and targets β-catenin for proteasome degradation, (Figure 4) (Bryan T. MacDonald; Keiko Tamai and Xi He, 2010; Gómez-Orte et al., 2013; Gordon and Nusse, 2006; Niehrs, 2012). In the presence of WNTs the "destruction complex" is inactive, leading to accumulation of β-catenin inside cell cytoplasm. The Accumulated β-catenin is then translocated to the nucleus where it regulates gene transcription by interacting with TCF/LEF DNA binding proteins (Bryan T. MacDonald; Keiko Tamai and Xi He, 2010; Gómez-Orte et al., 2013). This pathway appears to be active in proliferating cells and it has been shown that it must be turned off before NCCs delaminate from the dorsal neural tube (Rabadán et al., 2016). The non-canonical pathways are not as well characterized but it

has been mainly associated with cytoskeleton reorganization (Mayor and Theveneau, 2014).

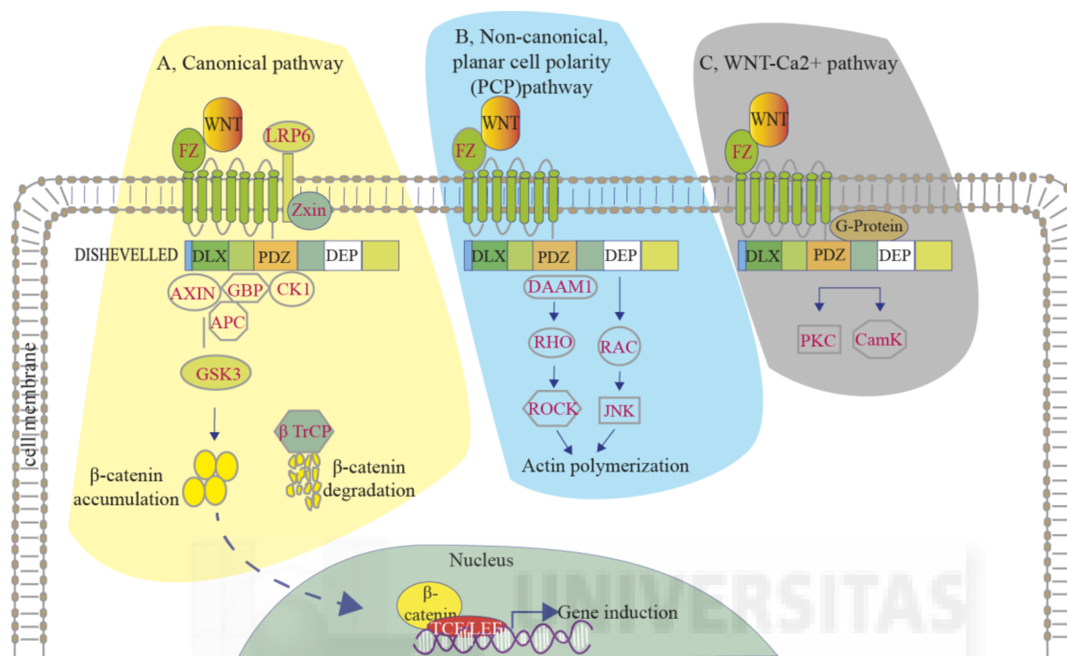

Figure 4: WNT signalling pathways. A, Canonical pathway B, Non-canonical PCP pathway and C, Non-canonical Ca^{2+} pathway.Adapted from (Wallingford and Habas, 2005).

There are 19 types of WNTs and 15 different types of WNT receptors and it is not possible to make a strict correlation of specific receptors with one or another pathway. Despite this complexity, it is widely accepted that Wnt1, Wnt3a, and Wnt8 activate the canonical pathway while Wnt5a and Wnt11 activate the non-canonical pathways (Kikuchi et al., 2011; Niehrs, 2012).

2.2.3. The FGF pathway.

The FGF pathway also participates in neural crest induction. During chick gastrulation, inhibition of FGF signalling leads to a reduction in the expression of the transcription factors Pax7 and Snail2 which result in the inhibition of neural crest induction (Stuhlmiller

and García-Castro, 2012). In mouse, members of the FGF family are active during early stages of development and a specific role during neural crest induction has not been clearly stated because mutant mice for the FGF signalling pathway die before neural crest formation (Itoh, 2007; Stuhlmiller and García-Castro, 2012).

In summary, it seems that during the first stages of neural crest differentiation WNTs and FGFs induce neural crest differentiation with a combination of low levels of BMPs, while in a second phase, BMPs levels increase and in combination with WNTs maintain neural crest differentiation (Betancur et al., 2010b; Stuhlmiller and García-Castro, 2012).

2.3. Transcription factors involved in neural crest induction.

UNIVERSITAS Miguel Hernández

BMPs, FGFs, WNTs and other secreted proteins induce the expression of a set of transcription factors called neural plate border specifiers or neural crest inducers. These specifiers induce the differentiation of neural crest progenitors located inside the neural tube. The best characterised neural plate border specifiers are: *Zic1, Msx1/2, Dlx3/5* and *Pax3/7* genes, (Figure 5) (Sauka-Spengler and Bronner-Fraser, 2008). All of them are expressed early during neural induction.

In general, due to rapid neural border specification and difficult accessibility the gene interaction, hierarchy organisation and relationships between neural crest inductors and neural border specifying factors, are not fully understood, although it is known that expression of each of these neural border specifiers need particular levels of BMPs, FGFs and WNTs at a particular moment of development (Betancur et al., 2010b). The activation of Zic1 and Pax3 for instance, depends on simultaneous BMP, WNT and FGF signalling activity (Betancur et al., 2010b; Sato et al., 2005).

2.4. Transcription factors involved in the specification of NCCs.

Neural plate border genes in cooperation with previous “neural crest inductors” upregulate the expression of other transcription factors called "neural crest specifiers" marking premigratory NCCs inside the neural tube. Neural crest specifiers are important to instruct cells for the EMT process, delamination and differentiation to the final fate. In this group of genes are: *Snail, Fox, Sox, Pax, Zic, Twist, c-Myc* and *Id* family members, (Figure 5) (Betancur et al., 2010b).

FoxD3 is a member of the Fox (fork head box) transcription factors family, which is associated with cell differentiation. FoxD3 is a pan-neural crest marker in all vertebrates, expressed in both neural and glial precursor cells (Cheung et al., 2005; Cuesta et al., 2007; Nelms and Labosky, 2010; Teng et al., 2008; Thomas and Erickson, 2009). Overexpression of FoxD3 or its inhibition lead to expansion or suppression of the neural crest (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001). FoxD3 expression is downregulated once NCCs differentiate to specific fates. FoxD3 promotes NCCs survival and multipotency, maintains NCCs in a progenitor stage, reduces apoptosis and promotes Slug/Snail activity (Cheung et al., 2005).

The family of zinc-finger transcription factors Snail1 (Snail) and Snail2 (Slug) also play an important role in neural crest development (Villarejo et al., 2014). Slug and Snail induce the EMT process by repressing the expression of E-cadherin (Barrallo-Gimeno, 2005; Bolós et al., 2003; Gammill and Bronner-Fraser, 2003). Their expression depends on the species. In fish and mice, Snail is expressed in premigratory and migratory NCCs, whereas Slug only in migratory NCCs. In contrast, chick Slug is expressed in premigratory and migratory NCCs while Snail is only expressed in migratory NCCs (Locascio et al., 2002).

Therefore, Slug plays a fundamental role in the EMT of premigratory neural crest in chick while in mouse, this function is played by Snail (del Barrio and Nieto, 2002; Knecht and Bronner-Fraser, 2002). Ectopic expression of both, Snail and Slug promote neural crest formation in chick (del Barrio and Nieto, 2002). The expression of Slug is controlled by BMP and WNT signalling (Betancur et al., 2010b; Sakai et al., 2005).

The SoxE (*Sox8, Sox9* and *Sox10*) family of genes are part of a larger family of a high mobility group (HMG) of transcription factors. They, as other neural crest specifier genes, are activated by neural crest induction signals and are expressed in a specific time-window during neural crest development (Hong and Saint-Jeannet, 2005). Their overexpression induces neural crest migration in chick (McKeown et al., 2005). Sox10 is member of this family and plays an essential role in neural crest development acting at multiple steps of the process. Sox10 interacts with other transcription factors controlling NCCs fate acquisition (Nelms and Labosky, 2010) and also promotes cell survival. *Sox10* mutation in zebrafish and mouse develop a normal neural crest but later, cells undergo apoptosis (Dutton et al., 2001; Gammill and Bronner-Fraser, 2003; Mollaaghababa and Pavan, 2003). Sox10 maintains cells undifferentiated and delay their differentiation at the beginning of neural crest migration (Kim et al., 2003). In addition, this transcription factor is essential for peripheral glia development and melanocyte lineage differentiation (Britsch et al., 2001; Hou et al., 2006; Mollaaghababa and Pavan, 2003).

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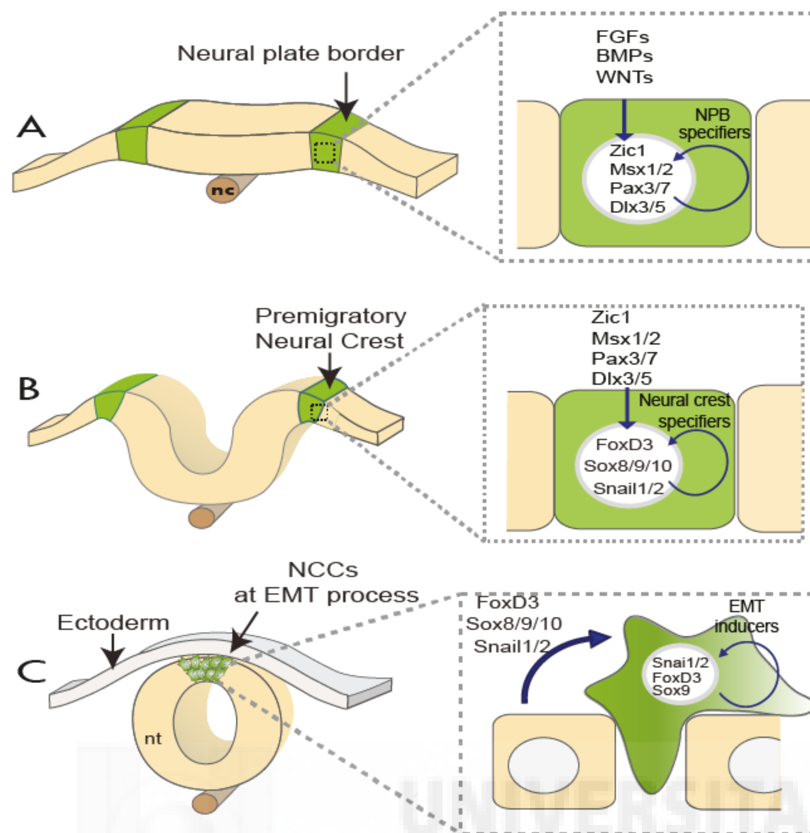

Figure 5: Gene regulatory network controlling neural crest formation. A, FGFs,WNTs, and BMPs induce the expression of neural plate border specifiers. B, Neural crest border specifiers induce the expression of the neural crest specifier genes. C, Neural crest specifiers induce the expression of EMT inducers. NPB-neural plate border, nc-notochord, nt-neural tube. Adapted from (Simões-Costa and Bronner, 2015).

2.5 The Epithelial-Mesenchymal Transition (EMT).

The EMT includes all the molecular and cytoskeletal rearrangements happening when a cell passes from epithelial to mesenchymal fate. EMT is a key process during neural crest formation but is also very important for the initiation of metastasis during tumorigenesis (Thiery et al., 2009). Epithelial and mesenchymal cells differ morphologically and functionally. Epithelial cells have close contacts with their neighbours through intercellular connections as adherent junctions, desmosomes and tight junctions. They have an apicobasal polarity, form laminar structures and attach to the basement membrane through integrins. Adherent junctions and other epithelial intracellular

connections contain Catenin, Cadherin and Actin components. In contrast, mesenchymal cells are loosely organised cells in a three-dimensional extracellular matrix, without apicobasal polarity and intracellular junctions. They are highly motile with invasive and migratory properties (Thiery et al., 2009), (Figure 6).

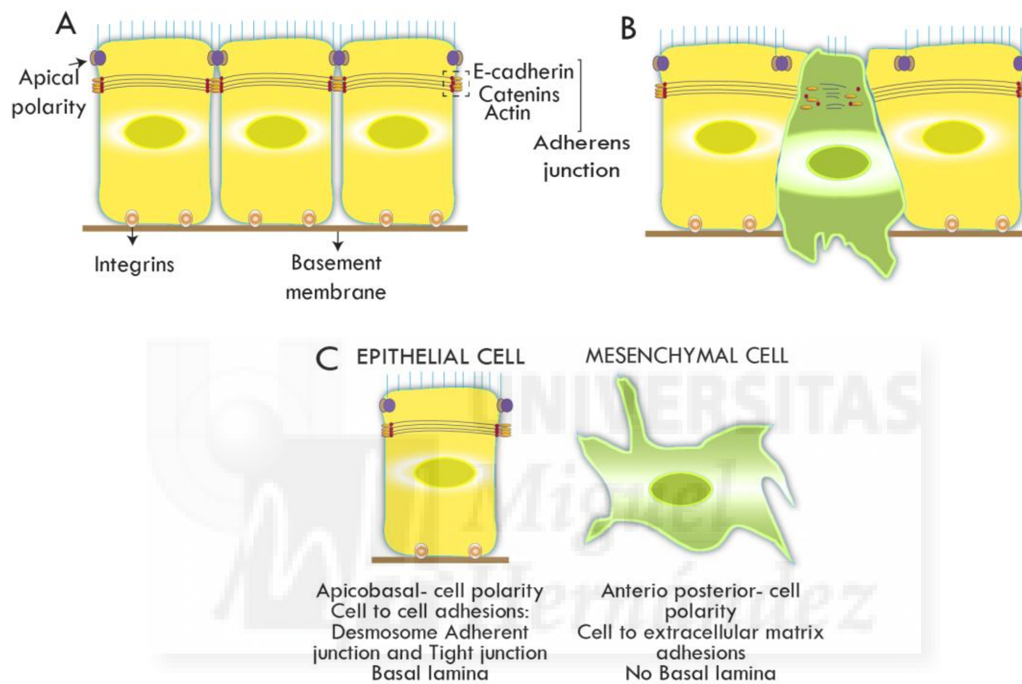

Figure 6: The EMT process. A, Epithelial cell B, Start of EMT process in a epithelial cells C, Differences between epithelial and mesenchymal cells. Adapted from (Acloque et al., 2009).

In the premigratory phase, NCCs have to undergo an EMT process to delaminate from the neural tube. A network of factors that promote neural crest delamination controls this process. The cascade of factors is triggered by the BMP, WNT and FGF signalling pathways. The BMP and WNT signalling pathways participate in the EMT process through activation of Snail, Rho proteins and other neural crest specifiers (Gilbert, 2013; LaBonne and Bronner-Fraser, 1998; Nieto et al., 1994; Thiery et al., 2009). The non-canonical WNT pathway, particularly WNT11 activates RhoB, which then changes

cytoskeletal architecture promoting actin polymerization (De Calisto et al., 2005; Gilbert, 2013).

Most of the neural crest specifiers are directly related to EMT induction. The main activity of these factors is spinning around Cadherin downregulation and other cytoskeletal rearrangements that allow NCCs to acquire a mesenchymal fate and migrate away from the neural tube (Cano et al., 2000; Cheung et al., 2005; Taneyhill et al., 2007; Villarejo et al., 2014). The Cadherins are membrane proteins that belong to a large family of calcium-dependent cell adhesion molecules. They attach inside the cells with other proteins, such as the Catenins. Cadherin-Catenin complex forms adherent junction that holds epithelial cells together. The Cadherin-Catenin complex also connects with the actin microfilaments and participates in cytoskeleton organisation (Gilbert, 2013; Hatta et al., 1987; Suzuki and Takeichi, 2008), (Figure 7).

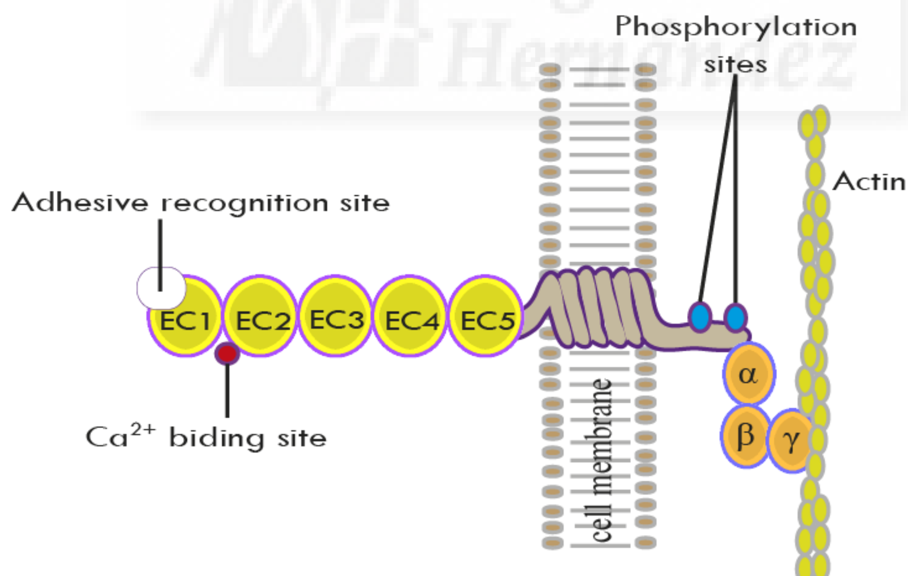

Figure 7: Cadherin structure and interconnecting elements. Adapted from (Gilbert, 2013).

There are different Cadherins involved in EMT: E-cadherin, N-cadherin, P-cadherin, Cadherin6B and R-cadherin. They are expressed before EMT induction and when EMT

starts they downregulate and rearrange to allow cell delamination (Chu et al., 2006; Hatta et al., 1987; Nakagawa and Takeichi, 1995; Strobl-Mazzulla and Bronner, 2012).

2.6. Regionalization of the neural crest along the anteroposterior axis.

From the anterior to the posterior axis, NCCs differentiate into cranial, cardiac, truncal and vagal regions. Cells coming from these regions exhibit different migration pathways and cell types. Some NCCs such as those giving rise to neurons of sensory and sympathetic ganglia or melanocytes cells have common characteristics along the different regions while some others NCCs have special features depending on the region (Basch et al., 2004; Rogers et al., 2012).

Cranial (cephalic) neural crest migrates on the anterior part of the neural tube. These cells limit caudally with the cardiac neural crest at the optic vesicle. Cells migrating from the cephalic neural crest will form the craniofacial mesenchyme and connective tissue of the face. These cells differentiate in cartilage, bone, glia, neuronal cells of cranial ganglia and pigmented cells. They form craniofacial structures such as the jaw, bones of the middle ear and odontoblast of the tooth (Gilbert, 2013; Le Douarin and Kalcheim, 1999).

The cardiac neural crest extends from the optic vesicle to the third somite (Kirby, 1988; Kirby and Waldo, 1990). These NCCs differentiate into neurons, connective tissue (pharyngeal arches), cartilage and melanocyte. This region gives rise to some components of the cardiovascular system. It produces large arteries of "outflow tract" and the septum between the pulmonary vein and aorta (Gilbert, 2013; Le Douarin and Kalcheim, 1999; Le Lièvre and Le Douarin, 1975).

The trunk neural crest extends between somites 8-28. Most of these cells will differentiate into sensory neurons of dorsal root ganglia (DRG), sympathetic ganglia, Schwann cells, neural plexus of the aorta and melanocyte cells (Gilbert, 2013; Kuriyama and Mayor, 2008; Le Douarin and Teillet, 1974). A small group of cells will differentiate in secretory cells of adrenal and thyroid glands, such as chromaffin and parafollicular cells (Bronner and Simões-Costa, 2016). The vagal and sacral neural crest generates the sympathetic ganglia of the gastrointestinal tract. The vagal region extends between somites 2-7 while sacral cells extend posteriorly from somite 28 to the ending part (Le Douarin and Teillet, 1973; Pomeranz et al., 1991), (Figure 8).

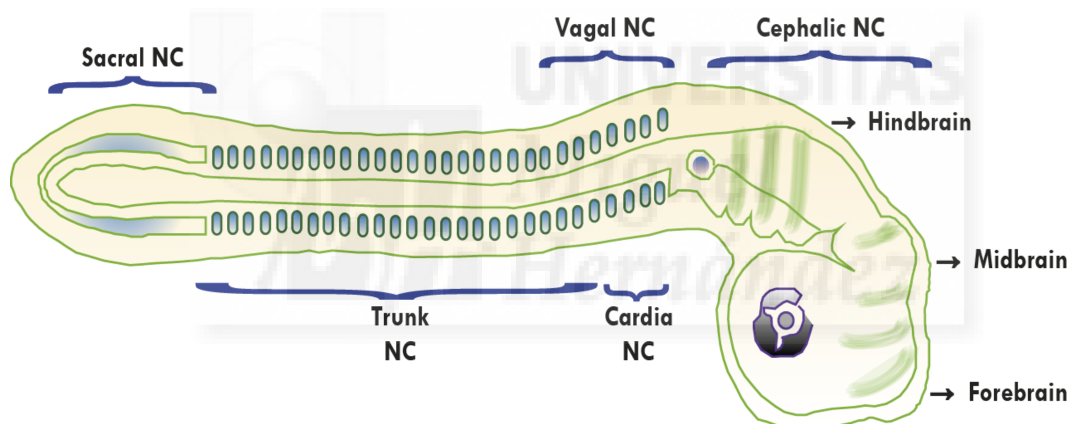

Figure 8: **Regionalisation of the chick neural crest.** It can be divided into five main regions depending on cell derivatives and functions. Adapted from (Gilbert, 2010). NC-neural crest.

The development of chick embryos has a duration of 21 days. It has a fast development with more than one stage within a day. For this reason, Hamburger and Hamilton established a series of embryonic stages starting from laying of the egg to hatched chick. These stages are named with HH symbols from HH1 to HH46, (table 1) (Hamburger and Hamilton, 1992).

Table 1. Hamburger and Hamilton stages

Hamburger Hamilton Stages	Age	Hamburger Hamilton Stages	Age	Hamburger Hamilton Stages	Age
HH1	0 hours	HH17	66 hours	HH33	7-8 days
HH2	6 hours	HH18	68 hours	HH34	8 days
HH3	12 hours	HH19	3 days	HH35	8-9 days
HH4	16 hours	HH20	3+ days	HH36	10 days
HH5	19 hours	HH21	3.5 days	HH37	11 days
HH6	23 hours	HH22	4- days	HH38	12 days
HH7	24 hours	HH23	4 days	HH39	13 days
HH8	26 hours	HH24	4 days	HH40	14 days
HH9	29 hours	HH25	4+ days	HH41	15 days
HH10	33 hours	HH26	4.5 days	HH42	16 days
HH11	42 hours	HH27	5 days	HH43	17 days
HH12	47 hours	HH28	5.5 days	HH44	18 days
HH13	50 hours	HH29	6 days	HH45	19-20 days
HH14	52 hours	HH30	6.5 days	HH46	21 days
HH15	56 hours	HH31	7 days		
HH16	62 hours	HH32	7.5 days		

UNIVERSITAS Miguel Hernández

2.7. Neural crest migration.

The factors involved in neural tube patterning along the rostro caudal axis also participate in regionalizing NCCs along the same axis. Once NCCs leave the roof plate they migrate in a stereotyped manner forming specific migratory pathways (Gilbert, 2013; Le Douarin et al., 1992).

NCCs migration first starts in the cephalic region and goes progressively caudal to the other regions. From the cephalic, region cells have a collective migration, forming chains and sheets of cells (McLennan et al., 2012; Theveneau and Mayor, 2012a). In this region, all NCCs migrate around the same time. Quickly after leaving the neural tube, NCCs

split in different migratory pathways. They migrate through hindbrain rhombomeres to the adjacent branchial arches. The pattern of migration in this region shows little differences between species (Kulesa and Fraser, 2000; Theveneau and Mayor, 2012b).

However, in the trunk region, NCCs migrate as individual cells where they contact with neighbour cells through filopodial prolongations. Depending on attractive-repulsive forces cells form small groups of 2-4 cells (Krull et al., 1995; Li et al., 2019). There are differences in the timing of migration between mice and chicken at the trunk level. In mice, cell migration starts before neural tube closure, having a peak of migration around E8.5-E9.5 and both dorsolateral and dorsomedial pathways start around the same time (Serbedzija et al., 1990; Trainor, 2005). In chicken, cell migration starts right after neural tube closure, having a peak of migration around HH15-HH19 (Giovannone et al., 2015), whereas dorsolateral pathway starts 24 hours after cells in the dorsomedial pathway initiate migration (Erickson and Goins, 1995; Theveneau and Mayor, 2012c). NCCs migration extends progressively in time in this region. Cells migrating in the dorsomedial pathway travel through the anterior part of somite to mainly form the sympathetic ganglia and the DRG and glial cells. Cells in the dorsolateral pathway move between dermomyotome and the overlying ectoderm and mainly differentiate into skin melanocytes (Kasemeier-Kulesa et al., 2005, 2006; Krull, 2001; Serbedzija et al., 1990), (Figure 9) .

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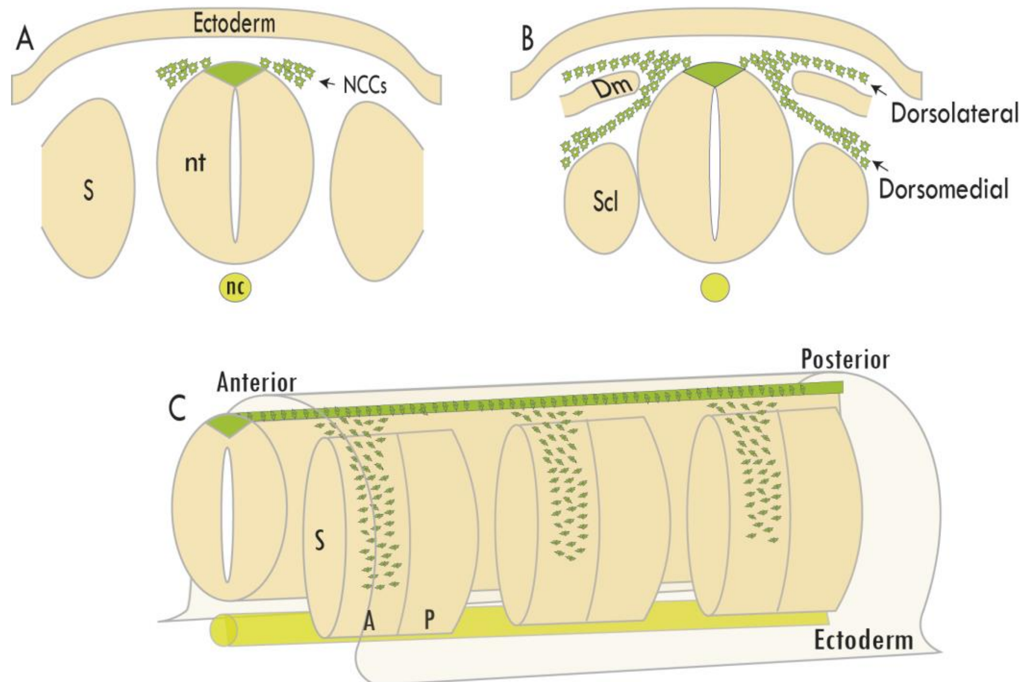

Figure 9: Trunk neural crest migration pathways. A, Neural crest delamination B, Neural crest dorsoventral and dorsolateral migratory pathway. C, Neural crest migration thought anterior somite. nc-notochord, nt-neural tube, A-anterior, P-posterior, S-somite, Scl-sclerotome.

2. 8. The role of membrane proteins, extracellular matrix proteins and guidance cues in neural crest migration.

2. 8. 1. Cadherins. As mentioned before, the downregulation of E-Cadherin and other types of Cadherins is essential for neural crest cells to delaminate from the neural tube. Cadherins are also involved in NCCs migration. Each Cadherin changes during NCCs migration in a different way, that depends on the axial level and animal species (Strobl-Mazzulla and Bronner, 2012; Taneyhill et al., 2007). After NCCs delaminate from the neural tube, as a condition to form clusters during migration or once they reach destination, they upregulate the expression of other Cadherins such as Cadherin7 and Cadherin11 (Acloque et al., 2009; Dottori et al., 2001; Suzuki and Takeichi, 2008; Vallin et al., 1998). The newly discovered Procadherins, specifically Procadherin1 was shown to control NCCs localization during the formation of DRG in chicken (Bononi et al., 2008),

whereas N-cadherin was demonstrated to control NCCs segregation during the formation of sympathetic ganglia (Mckinney et al., 2011).

2. 8. 2. Extracellular matrix (ECM) molecules.

A group of molecules faced by NCCs during migration are the extracellular matrix molecules. The principal members of this group are laminin, fibronectin, collagen and proteoglycan. Some of them do not just build the substrate for NCC migration but also participate actively during migration (Gilbert, 2013; Testaz and Duband, 2001). The NCCs which express α4integrin are favourable to migrate in a substrate that contains α4integrin bound like elements: fibronectin and thrombospondin-1 (Kil et al., 1998; Kuo and Erickson, 2010). F-spondin situated in the posterior somite, peri-notochord area and dermomyotome, has an inhibitory effect on NCCs migrating in these areas (Debby-Brafman et al., 1999; Kuo and Erickson, 2010). Other extracellular matrix molecules are Peanut agglutinin (PNA) binding molecules and Chondroitin-6-sulfate Proteoglycan which inhibit early migrating NCCs to enter in the dorsolateral pathway (Kerr and Newgreen, 1997; Kuo and Erickson, 2010; Oakley et al., 1994).

2. 8. 3. Guidance cues.

They include a huge group of interacting proteins which work as a receptor-ligand combination. Depending on the circumstances, the interaction receptor ligand could be attractive or repulsive. Guidance cues are involved in several processes like: cell migration, growth cone formation and axon guidance, some of them such as Ephrin/Eph, Slit/Robo and Semaphorin/Neuropilin, also participate in neural crest migration, guiding NCCs to a specific pathway as follows:

2.8.3.1. Semaphorin/Neuropilin. The Semaphorins (Semas) are transmembrane and secreted molecules that bind to neuropilin (Nrp) receptors. A well-known member of this family involved in the guidance of NCCs are Sema3F, Sema3A and their receptors Nrp1, Nrp2. They coordinate NCCs migration through the dorsomedial pathway and participate in the sorting of NCCs migration to their final fate localization (DRG and sympathetic ganglia) (Lumb et al., 2014; Schwarz et al., 2009). In the trunk region NCCs expressing Nrp1 differentiate in DRG and sympathetic ganglia, while those expressing Nrp2 exclusively differentiate to DRG cells (Lumb et al., 2014). The interaction Sema3A/Nrp1 is important for NCCs to switch migration from the inter-somitic space to the anterior somite and restrict their migration to the dorsomedial pathway. Cells expressing Nrp1 migrate through the anterior part of the somite because Sema3A is expressed in the posterior region. Sema3A is also expressed by dermomyotome where restricts later wave of migratory NCCs to migrate to the dorsolateral pathway (Schwarz et al., 2009). NCCs expressing Nrp2 enter the anterior somite because they are repelled by Sema3F expressed in the posterior part (Gammill et al., 2006), (Figure 10).

2.8.3.2. Slit/Robo. Slits are secreted proteins that bind to Robo receptors to mediate repulsion (Dickson and Gilestro, 2006; Hammond et al., 2005). They prevent Robo1/2 NCCs from migrating into the dorsolateral pathway, particularly in the trunk region, because NCCs expressing Robo1/2 receptors are repelled from the dorsolateral pathway because dermomyotome cells express Slits proteins (Jia et al., 2005; Kuriyama and Mayor, 2008), (Figure 10).

2.8.3.3. Ephrin/Eph. The ephrin ligands (ephrins) and ephrin receptors (Ephs) are cell surface bidirectional signalling proteins. Eph/ephrin signalling cascades can be activated by Eph recpetors (forward pathway) or by ephrin ligand acting as a receptor (reverse pathway) (Kania and Klein, 2016). With few exceptions, Eph/ephrin signalling generally mediates repulsion. Eph/ephrin signalling participates in NCCs migration through the dorsomedial and dorsolateral pathways and they are involved in migration in the anterior somite (Kuriyama and Mayor, 2008; Santiago and Erickson, 2002; Theveneau and Mayor, 2012b).

NCCs expressing EphB3/B2 are repelled by ephrinB1 expressed by the caudal somite. This repulsive interaction restricts NCCs to migrate through the anterior somites (Kasemeier-Kulesa et al., 2006; Krull et al., 1997). EphrinB1 ligand has a dual role in NCCs migration. It restricts cells to migrate into the anterior somite but it is also expressed in the dermomyotome and repulses early migrating NCCs to travel through the dorsolateral pathway to later promote dorsolateral migration (Santiago and Erickson, 2002). In addition, ephrinB2 also seems to play a key role in NCCs migration restricting NCCs to the anterior somite in the cranial part. EphA4, a receptor for ephrinB2 and for ephrinA2/A5, is expressed in the anterior part of somite and in the ventral dermomyotome (Baker and Antin, 2003; Mellott and Burke, 2008; Nakajima et al., 2006; Santiago and Erickson, 2002; Smith et al., 1997), (Figure 10). It has been proposed that EphA4 promotes neural crest migration through the rostral somites but the interactions of EphA4 with its different ligands is not clear (Davy and Soriano, 2007; McLennan and Krull, 2002). In the trunk, EphA4 has a weak expression at early stages and later, it is strongly detected around the dorsomedial migratory pathway and the dermomyotome area (McLennan and Krull, 2002; Santiago and Erickson, 2002). EphB1 is expressed by migrating NCCs of the dorsomedial

and dorsolateral pathway (Santiago and Erickson, 2002). As in the case of EphA4, EphB1 interaction is better characterized in the anterior part at the brachial level where it has been reported that interacts with ephrinB2 (Smith et al., 1997). There is no evidence for EphB1 function during trunk neural crest formation or migration.

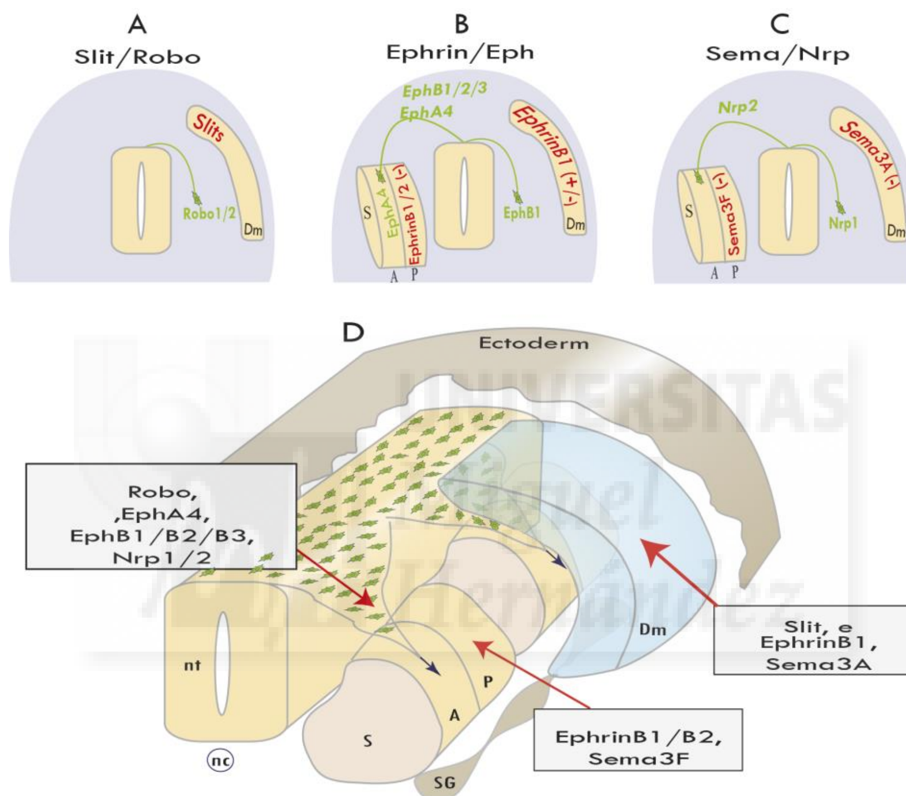

Figure 10: **Guidance cues involved in trunk neural crest migration. A**, Slit/Robo signalling. **B**, Ephrin/Eph signalling. **C**, Sema/Nrp signalling. **D**, General summary. A-anterior, nt-neural tube, P-posterior, S-somite, SG-sympathetic ganglia.

2. 9. NCCs specification.

The question of whether NCCs are specified before or after migration has been the focus of investigators for long time and it is now accepted that NCCs fate is decided before and during cell migration. This process is governed by both intrinsic factors and extrinsic elements related with the surrounding tissues that cells face during migration. Before

migration the NCCs are in different stages of differentiation (Le Douarin and Kalcheim, 1999). Each pathway is associated to a specific cell fate segregation and behind this process there is a set of transcription factors that will specify every cell type (Gilbert, 2013; Le Douarin and Kalcheim, 1999). Most migrating cells, exit from the dorsal midline of neural tube but their position inside the neural tube is not related to their fate except for the cells that will form the sympathetic ganglia that are positioned at the top and are the first that migrate (McKinney et al., 2013).

2. 9. 1. Differentiation of NCCs in the dorsomedial pathway.

The Sensory Nervous System (SNS) and the Autonomic Nervous System (ANS) build PNS. The SNS is integrated by glial cells and different sensory neurons that convey information related to pain, touch, temperature and body position from the periphery to the spinal cord and brain. In the trunk region, the SNS is composed by a metameric series of ganglia, called dorsal root ganglia (DRG). The ANS is built by motor neurons that also form ganglia carrying information from CNS to various organs in the body, providing involuntary control for the visceral organs. The ANS has three components: the sympathetic and parasympathetic ganglia that work in coordination to control body homeostasis, and the enteric ganglia that controls gut motility. Trunk region of the neural crest will give rise mainly to sympathetic ganglia (Kalcheim, 2018; Le Douarin and Kalcheim, 1999). The first wave of migration travels through the intersomitic space and stops above the dorsal aorta to form the sympathetic ganglia (localized under DRG level). The same wave of cell will form adrenal chromatofin cells and Schwann cells (Kasemeier-Kulesa et al., 2005; Lallier and Bronner-Fraser, 1988; Le Douarin and Kalcheim, 1999). The other migratory cells of this pathway go through the anterior part of the somite, stop at dorsal positions where

and will become sensory neurons forming the DRG and Schwann cells of the peripheral nerves (Kasemeier-Kulesa et al., 2005; Le Douarin and Kalcheim, 1999) (Figure 11).

Gene regulatory networks participating in cell differentiation of sensory progenitors is triggered by the WNT signalling pathway (Lee and Hari, 2012; Thomas and Erickson, 2008). The first NCCs that differentiate in sensory progenitors upregulate Neurogenin 1/2 (Pavan and Raible, 2012; Perez et al., 1999). NCCs that in addition to Neurogenin2 express Mash1 will not become DRG cells but will differentiate in sympathetic ganglia cells (Parras et al., 2002; Pavan and Raible, 2012). Once inside the DRG area, sensory progenitors differentiate in postmitotic neurons and start to express other transcription factors like Brn3A and Isl1 (Fedtsova and Turner, 1995; Nitzan et al., 2013; Pavan and Raible, 2012).

Schwann cells precursors first express Neurogenin1, Sox10 and FoxD3. Then, those cells expressing Oct6, Krox20 and Egr2 will differentiate in myelinating Schwann cells while those expressing Pax3 will become non-myelinating Schwann cells (Kipanyula et al., 2014).

Regarding ANS, it has been reported that Bmp2 and Bmp4 induce sympathetic neuronal cell differentiation (Shah et al., 1996) and the expression of a combination of different transcription factors, Phox2a/2b, Hand2, Gata3 and Mash1 lead to sympathetic cell fate (Bhatt et al., 2013).

2. 9. 2. Differentiation of NCC in the dorsolateral pathway.

NCCs that migrate through the dorsolateral pathway colonize the ectoderm to finally differentiate in skin melanocyte cells. This migration involves a set of transcription factors that induce NCCs to acquire melanocytic identity (Le Douarin and Kalcheim, 1999).

The upregulation of transcription factors that are essential for melanocyte fate is also initiated by the WNT and BMP signalling pathways (Sommer, 2011; Thomas and Erickson, 2008). NCCs differentiate first to melanocyte precursor (melanoblast) which requires upregulation of Sox10 (Hou et al., 2006) and downregulation of FoxD3, Sox9 and Snail (Krispin et al., 2010; Pavan and Raible, 2012). This combination induces the expression of the microphthalmia-associated transcription factor (MITF) to specify melanocytic fate (Hornyak et al., 2001; Pavan and Raible, 2012; Thomas and Erickson, 2008). At the same time, once NCCs are differentiated in melanoblasts, they start to express melanocyte specific receptors such as the Tyrosine related protein1 and 2 (TRP1, TRP2) (Cichorek et al., 2013), Endothelin receptor B (EDNRB), or the Tyrosine kinase receptor KIT (Hou et al., 2000; Le Douarin and Kalcheim, 1999; Lee et al., 2003; Pavan and Raible, 2012; Wilson et al., 2004).

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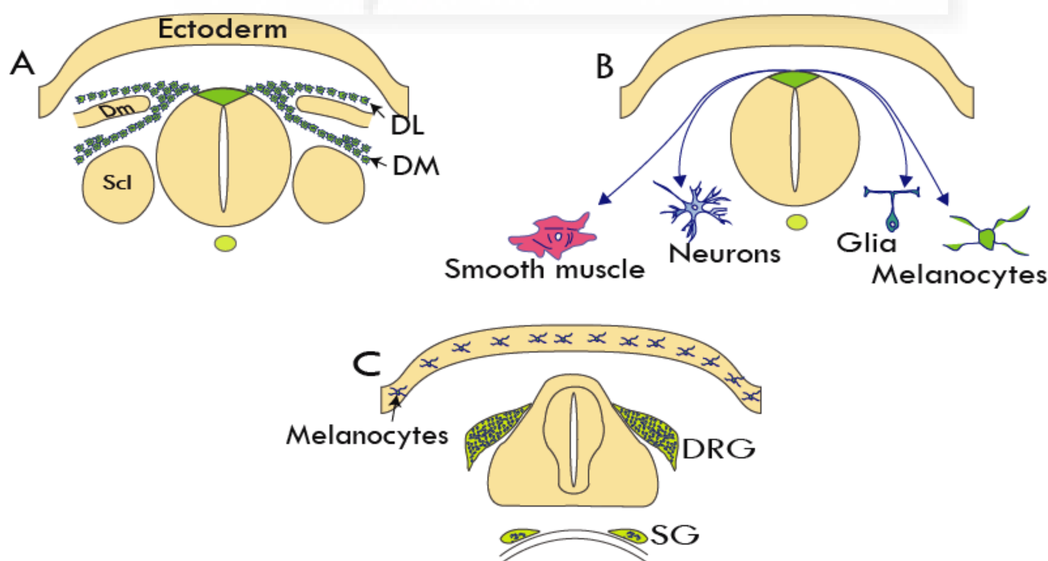

Figure 11: **Neural crest differentiation.** **A**, Dorsomedial and dorsolateral migratory pathway. **B**, Different cell types originated form NCCs. **C**, NCCs localization after final differentiation. Dm-dermomyotome, DRG-dorsal root ganglia, Scl-sclerotome, SG-sympathetic ganglia.

3. The Zic family of transcription factors.

The zinc finger transcription factors (Zic1, Zic2, Zic3, Zic4 and Zic5) are orthologues of the *Drosophila melanogaster* odd-paired (*opa*) gene and have been proposed to play an important role in neurulation, neural crest differentiation and migration (Aruga, 2004; Grinberg and Millen, 2005; Houtmeyers et al., 2013). In vertebrates, ZIC proteins were described for the first time after a cDNA screening of murine cerebellum (Aruga et al., 1994; Houtmeyers et al., 2013). Zic transcription factors are defined by the presence of a zinc finger domain that consists of five Cys2His2 zinc fingers. This domain is highly conserved except in the case of Zic4 and Zic5 that show some divergences. The Zinc finger domain is able to bind to DNA and to interact with other proteins. The five proteins also contain a short highly conserved domain (14–21 amino acids), immediately upstream of the zinc fingers. Three of the proteins (ZIC1, ZIC2, and ZIC3) share another small domain (9–10 amino acid) of homology. Zic2 is the only member of the family with an expansion of alanine repeats located at the C-terminal and mutations in this region is associated with human pathologies such as holoprosencephaly, exencephaly and spina bifida (Brown et al., 2005; Houtmeyers et al., 2013; Lim et al., 2010; Mizugishi et al., 2001; Pourebrahim et al., 2011; Salero et al., 2001).

Zic2 is located on chromosome 14 in mouse (Grinberg and Millen, 2005), on chromosome 13 in human (Brown et al., 1998) and on chromosome 4 in chick (McMahon and Merzdorf, 2010). In mice and humans, Zic5 is located in a paired but inverted configuration in the same locus than Zic2 (Aruga et al., 1996; Inoue et al., 2004).

3. 1. Zic family in neurulation and neural crest development.

ZIC proteins have similar expression patterns and biological functions in many tissues, although their expression differs in particular regions (Aruga, 2004). They are expressed during neurulation, neural crest formation and somite development (Aruga et al., 1994, 1996; Merzdorf, 2007). It has been shown that inhibition of BMP signalling creates permissive conditions to induce the expression of *Zic* genes while Msx1 and Dlx1 maintain *Zic* genes expression regionalized to the dorsal region of neural plate. It has been proposed that once the neural tube closes, a dorsal BMP signal stops Zic activation. On the contrary, ventral SHH signalling appears to have an inhibitory effect on *Zic* genes limiting their expression to dorsal neural tube areas (Aruga, 2004; Merzdorf, 2007). WNT signalling pathway is also related to the function of ZIC proteins although the nature of their interaction is still unclear. In zebrafish, it has been suggested that Zic2a and Zic5b act downstream of the Wnt pathway (Merzdorf, 2007; Nyholm et al., 2007). However, in *Xenopus,* it has been recently reported that *Zic2* and *Zic3* genes inhibit the canonical WNT signalling pathway (Fujimi et al., 2012; Houtmeyers et al., 2013; Pourebrahim et al., 2011).

It has been proposed that overexpression of ZIC proteins promotes cell proliferation, inhibit neural differentiation and induce neural crest expansion (Elms et al., 2003; Inoue et al., 2007a; Mizuseki et al., 1998; Nakata et al., 1998, 2000; TeSlaa et al., 2013). However, it was also postulated that Zic1 and Zic3 overexpression promotes neural crest progenitors differentiation and pigment cells formation (Nakata et al., 1998). *Zic3* mutant mice exhibit defects in neural tube closure, organ formation and left-right symmetry (Purandare et al., 2002) and, in collaboration with Zic2, Zic3 appears to be important for neurulation and mesoderm formation (Inoue et al., 2007b; Nagai et al., 2000). As *Zic2* mutants, mutant mice for *Zic5* exhibit defects in the closure of the anterior

region of the neural tube and they also show reduction in the production of cephalic NCCs as well as defects in neural crest derivates as the jaw (Inoue et al., 2004). In zebrafish and Xenopus respectively, injection of Zic5 morpholinos lead to a reduction of cell proliferation, while ectopic expression Zic5 seems to induce neural crest differentiation (Nakata et al., 2000; Nyholm et al., 2007). Therefore, although it is clear that ZIC proteins play important functions in neurulation and neural crest formation, their precise function remains unclear and controversial.

3. 2. Zic2 in early nervous system development

In the developing chick *Zic2 mRNA is* expressed at low levels along the anterior-posterior axis of the neural plate at stage HH7. Around the stage HH9, Zic2 expression is downregulated at the forebrain and midbrain regions. At the stage HH11-12, *Zic2 mRNA* is expressed along the dorsal neural tube and starts to be expressed also in the anterior part of the somite. At the stage of HH14-15 Zic2 is expressed at the dorsal neural tube, dermomyotome, sclerotome and the anterior part of somite. Later, at stage HH18-19, Zic2 expresses in the limb buds, without changing the expression level in other regions (McMahon and Merzdorf, 2010).

In mouse, *Zic2 mRNA* is expressed before gastrulation at blastula stage around E4 on the blastocyst (Brown and Brown, 2009). By E7 is expressed in the primitive streak, neural plate and mesoderm (Elms et al., 2004). As development proceeds, from E7 to E9, *Zic2 mRNA* expression becomes stronger on the head region. Later, *Zic2 mRNA* expression increases in the dorsal neural tube and somites (Inoue et al., 2007b). *Zic2 mRNA* is not expressed in the DRG but it was detected in the surrounding sclerotome. Also, Zic2 transcripts are weakly expressed in the dermomyotome (Nagai et al., 1997).

This wide expression of Zic2 during early development suggests that it is involved in different developmental steps. The first cue about its function in early development came from humans. Point mutations of *ZIC2* in humans produce forebrain malformation and holoprosencephaly (HPE) (Brown et al., 1998; Cheng et al., 2006). In mouse, the range of neural tube defects provoked by Zic2 mimic human phenotypes, hypomorphic *Zic2* mice show HPE, exencephaly as well as neural tube defects in the caudal part (spina bifida) followed by other skeletal malformations (Nagai et al., 2000). Similar but more severe phenotypes were also observed in another mouse mutant line (Kumba mutant), (Elms et al., 2003).

The mechanisms used by Zic2 in provoking neural tube defects have been proposed to relay on organizers centers, dorsoventral cell pattering and cell differentiation during the neurulation process. The HPE phenotype was initially proposed to occur because Zic2 was interfering with SHH signaling (Brown et al., 2003). However, it was later discovered that Zic2 does not directly interact with SHH signaling during this process and HPE seems to be provoked by a malformation originating from the organizer region. In mice lacking Zic2, are observed defects in prechordal plate, which is an important organizing center for forebrain formation (Warr et al., 2008). Similarly, Zic2 has been described as downstream of NODAL signaling that could be interacting with SMAD2 and SMAD3 proteins (Houtmeyers et al., 2016) which are important for EMT.

As for other ZIC genes, the results about a role for Zic2 in proliferation during the neurulation process are ambiguous. In *Xenopus*, it was first proposed that ectopic expression of Zic2 induces the expansion of progenitors in the neural plate, particularly in the anterior part of the embryo and an expansion of neural crest concomitant with an increase of melanocytes production (Nakata et al., 1998). However, in the same year, it

was also suggested that Zic2 inhibits neural differentiation (Brewster et al., 1998). In Zebrafish, ectopic expression of both Zic2a and Zic2b seems to induce neural crest production and conversely, Zic2a and Zic2b morpholinos injection lead to a reduction in the number of migrating neural crest cells (TeSlaa et al., 2013). In mice, downregulation of Zic2 produces a delay in the neurulation process, lack of Wnt3a in roof plate cells but not changes in cell proliferation or cell death (Nagai et al., 2000). Also, the expression of Foxd3, a premigratory neural crest marker, and Sox10 a general marker for migrating NCCs were both reduced but these defects were not followed by alterations in proliferation or cell death (Elms et al., 2003). The dorsoventral patterning of cells inside the neural tube does not change at the trunk region after Zic2 downregulation, which has been demonstrated using Pax3 and Pax6 as progenitor markers (Nagai et al., 2000). In addition, *Zic2* mutant mice exhibit white spots in their belly which may be a reflect of defects on NCCs giving rise to melanocytes (Elms et al., 2003).

Therefore, although it is clear that Zic2 plays an important role in neurulation and neural crest generation, the precise expression of Zic2 during neural crest migration, its relation with premigratory/migratory NCCs or with specific NCCs path is not well defined. At the same time the role of Zic2 in neural crest and/or other neurulation processes, is still not completely elucidated and in some studies still appears incongruent.

3. 3. Zic2 in late stages of development

Zic2 has also been implicated in later developmental processes. In the last few years, our laboratory has described a new role for Zic2 in cell migration. Zic2 is required for proper migration of at least three different populations of forebrain migrating cells: 1) The Cajal Retzius cells (CRCs) that are early born neurons coming from different sources to colonise

and disperse across the entire cortical surface. 2) Dorsal pallium cells that migrate to the lateral olfactory tract nucleus. 3) Zic2 migratory diencephalic interneurons that migrate from the prethalamus to the ventral lateral geniculate nucleus (Figure 12), (Murillo et al., 2015).

During the last decade, our group has also demonstrated that Zic2 plays an important role in axon guidance (Figure 12), (Herrera et al., 2003). In mice, most retinal ganglion cell (RGC) axons project across the midline to innervate targets on the opposite side of the brain (contralateral RGCs) and only a small subset of RGCs project to the same side (ipsilateral RGCs). Zic2 is specifically expressed in ipsilaterally-projecting RGCs and determines axon midline repulsion at the optic chiasm by inducing the expression of the Eph tyrosine kinase receptor EphB1 (García-Frigola et al., 2008). The binding of EphB1, expressed in ipsilateral but not in contralateral RGC axons, to its ligand, ephrinB2, expressed by midline cells mediates a repulsive response that forces visual axons to turn at the midline and project ipsilaterally (Williams et al., 2003).

More recently it has also been demonstrated that Zic2 determines axonal laterality in other CNS circuits (Escalante et al., 2013). Zic2 is expressed at least in one population of late-born dorsal horn interneurons that ascend ipsilaterally to the brain (Escalante et al., 2013; Paixão et al., 2013). In these neurons Zic2 has a similar function than in the visual system: it determines ipsilateral axon projection. Zic2 upregulates EphA4 and they are repelled by ephrinBs that are expressed at the midline (Figure 12). Chromatin immunoprecipitation experiments demonstrated that Zic2 directly binds to the EphA4 promoter in spinal cord extracts (Escalante et al., 2013)., an observation that has been later confirmed (Luo et al., 2015). *EphA4* mutant mice have a hopping gate movement caused basically by midline ectopic crossing from interneurons that form part of central

pattern generator (CPG), which are neurons distributed throughout the lower thoracic and lumbar regions of the spinal cord (Paixão et al., 2013; Serradj et al., 2014).

Therefore, Zic2 and its target molecules are essential in different stages of neural circuits development.

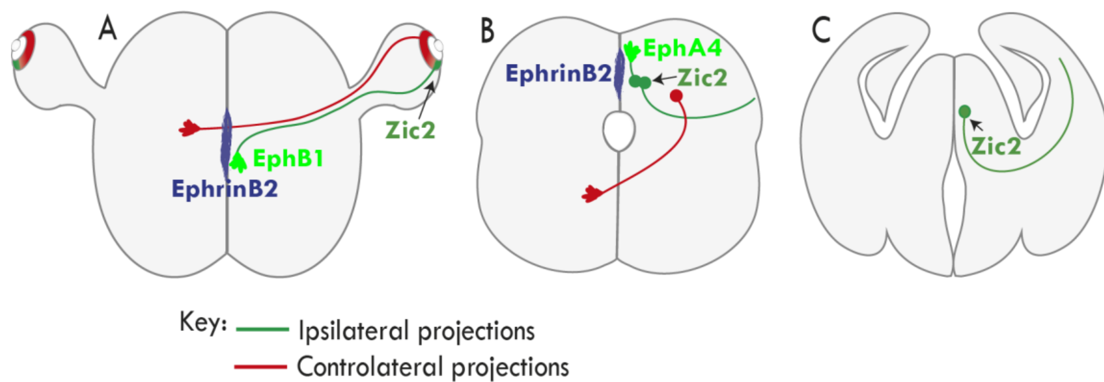

Figure 12: The role of Zic2 as a determinant of axon ipsilaterality. A, visual system B, spinal cord and C, thalamocortical projections. Zic2 positive ipsilateral axons are in green. Adapted from (Escalante et al., 2013; Herrera et al., 2003; Murillo et al., 2015).

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OBJECTIVES

OBJECTIVES

Many studies have demonstrated the necessity of Zic2 during neural crest formation, but the specific involvement of Zic2 during this process is still not clear. The two main objectives of this work were:

1. To define the role of Zic2 in neural crest development and,
2. To identify Zic2 target genes during this process

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MATERIAL AND METHODS

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MATERIAL AND METHODS

1. Mice.

The experiments of this study were performed in tow mouse strains. Mutant mice were maintained in an inbred strain, C57BL/6 and wild type mice in outbred strain, ICR, at the animal facilities of the Servicio de Experimentacion Animal (RGM-SEA) in the Instituto de Neurociencias de Alicante, a mixed centre of Miguel Hernandez University and Spanish National Research Council (INA-UMH-CSIC).

The care and handling of animals prior to or during the experimental procedures were done following European Union directive (2010/63/UE) and current Spanish Legislation (Real Decreto 53/2013). The Animal Care and Use Committee of Miguel Hernandez University (IACUC) approved them. Animals were kept on 12 hours light/dark cycle with constant ambient temperature and humidity. Food and water were available *ad libitum*.

Zic2 $^{kd/kd}$ mouse line was generated by gene targeting event designed to replace the first exon containing the start codon and encoding region for three of the five zinc fingers. However, unexpected recombination resulted in an allele with reduced expression. The allele retained exon 1 with the neomycin-resistance marker in the first intron. This resulted in a reduction (knockdown, kd) of Zic2 expression. The mice line (Zic2tm1Jaru) was obtained from the RIKEN repository, are the same mice as described in (Nagai et al., 2000).

Transgenic-Tg(Zic2^{eGFP}) mouse lines was generated by genotype modification to contain multiple copies of a modified BAC bacterial artificial chromosome (BAC) in which Enhanced green fluorescent protein (EGFP) reporter gene is inserted immediately upstream of the coding sequence of the targeted (Zic2) gene. The line

(identification number: RP23-158G6) was generated by GENSAT (Gonget al., 2003) and obtained from the Mutant Mouse Regional Resource Centre (http:// www.mmrrc.org/strains/17260/017260.html). The Tg mice (Zic2^{eGFP}) were crossed with the heterozygous mice for Zic2 (Zic2$^{kd/+}$) and the resulting F1 progeny was crossed [(Zic2$^{+/-}$; Tg(Zic2^{eGFP}) x (Zic2$^{+/-}$; Tg(Zic2^{eGFP})] to generate [Zic2+/+; Tg(Zic2^{eGFP}] and [*Zic2*$^{kd/kd}$; Tg(Zic2^{eGFP}) embryos into the same litter. We characterized them and we found that, GFP reproduce Zic2 expression in premigratory and not in migratory neural crest cells. This allowed us to visualize, by eGFP fluorescence, both Zic2 cells in the wild-type and cells with very low levels of Zic2 in the *Zic2*$^{kd/kd}$ embryos.

2. Embryo harvesting, dissection and fixation.

For staging of embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). Timed-pregnant females were killed by cervical dislocation and the embryos were extracted by Caesarean section. The uterine horns were cut and placed on cold Phosphate-buffered saline (PBS) in a Petri dish. The embryos were extracted from the uterus and removing by using two stainless steel forceps (13820078 Fisher Scientific) No5 under stereo microscope (S6E Leica) and microscope light source (KL 1500 Leica). After dissection the embryos were fixed in 4% paraformaldehyde (PFA) at 4°C in overnight (ON) agitation (ON).

3. *In ovo* electroporation.

Fertile White Leghorn chick eggs were obtained from the Santa Isabel chicken farm (Cordoba-Spain). Immediately after arrived, eggshell was wiped with ethanol 70% to avoid contamination and then stored to 16 °C. To avoid losing their viability, eggs were

not stored more than one week. The incubation was done by putting eggs in a vertical position inside a 70% humidified incubator chamber, settled to 38.5°C (101.3°F). Eggs were left incubating until desired HH stage, according to ((Hamburger and Hamilton, 1992). The eggs, in general, were electroporated 48 hours after incubation started, when the embryos were around stage HH12. Depending on which season was, the incubation time was extended or reduced with two hours regarding established time. Two hours more in winter and two hours less in summer.

Some hours before electroporation, eggs were wiped again with 70% ethanol after that the eggs were sealed with scotch tape. A small hole was made with blunt forceps on the narrow end of the egg through which 4-5 ml of watery albumen were removed. This was done by inserting a 5ml syringe fitted with a 20-G needle through the shell at the blunt end of the egg. The hole that was done, latter was used as a starting point for eggshell windowing. The windowing was done through the longitudinal axis by carefully breaking away pieces of the eggshell with curved scissors, holding them in a horizontal position to avoid yolk perforation. After that the egg was sealed using scotch tape following the same longitudinal axis. In the end, and the eggs were left in a horizontal position inside the incubator, switching of shaking mode, so the embryo could be properly positioned for electroporation.

The electroporations were done using electroporator (TSS20 IntraCell), to visualise better embryonic neural tube, was used a stereomicroscope (MZ6 Leica) and a manipulator to hold electrodes. Electrodes were placed around trunk region of the neural tube, they were placed in parallel on each side of the neural tube and in a horizontal position, with a distance of 3mm between + and – electrode. Plasmidic DNA solution was then injected through the base of the tail in the lumen of the neural tube.

Every plasmidic DNA solution was prepared at 15µl of final volume. The solution was composed by plasmidic DNA constructs, each one in a specific concentration, (Table 2), adding 0.02% fast green (Sigma) and at the end was added double-distilled water (ddH_2O) until final volume was reached. At early stages, around HH10-12, a solution of 10% India ink (A08029423- Pelikan ink 523) diluted in PBS solution was injected below the blastodisc to visualise the embryos and facilitate electroporation.

Table 2. DNA constructs electroporated in this study

Name	Constructs	Specie	Source	Vector	Electropo. concentration
GFP	pCAG-EGFP	Medusa	Cristina Garcia	pCAGGS	0.5 µg/ul
ZIC2	pCAGGS/SE -Zic2T	Mammalian expression vector	Cristina Garcia	pCAGGSSE	1 µg/ul
DRAXIN	pCAGSE-cDraxin-IRES2EGFP	Chicken	Augusto Escalante	pCAGSE	1 µg/ul
RNA_I CONTR.	pRFPRNAiC	---	Augusto Escalante	pRFPRNAiC	3 µg/ul
ZIC2 RNA_I	pRFPRNAiC-cZic2RNAi7	Chicken Zic2 Rna_i	This study	pRFPRNAiC	3 µg/ul
DRAXIN RNA_I	pRFPRNAiC-cDraxinRNAi	Chicken Draxin Rna_i	This study	pCAGGS	3 µg/ul
EPHA4	pCAGES EphA4	Chicken	Angela Nieto	pCAGES	1 µg/ul
EPHB1	pCAG-SE-EphB1	Chicken	Augusto Escalante		1 µg/ul

The electroporation mix was injected using standard glass capillaries (1B120F-4 World Precision Instruments), electroporation parameters were settled; 25 volts, 5 widths, 5 space and distance 500 m/s. After the electroporation procedure was finished 3-5 drops of PBS were thrown on top of the embryo using a 5ml plastic transfer pipettes (137010, Globe Scientific) then the egg was sealed again with scotch

tape and putted again at the incubator chamber until was reached the desired HH stage.

Eggs dissection procedure was done at specific HH stage required for each experimental group. The egg was taken out from incubator chamber and put in homemade wood holder with blunted end at horizontal position. Stainless steel scissors with sharp tip (08940 Fisher Scientific) were used to cut the scotch tape around egg window. Then using stereo microscope, microscope light source and the same scissors the embryonic disc was cut around and the embryo was removed from the egg and put in a Petri dish filled with PBS inside an ice box. After that using two stainless steel forceps No5 the embryos were carefully dissected. It was removed the head, tail and formed organs from abdominal and thoracic cavity, leaving just the trunk part of the spinal cord and limb bubs. At the end the embryos were fixed in 4% PFA.

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4. ShRNA sequences.

Short hairpin shRNA used in this study were transferred into a clone which contains U6 promoter from chicken chromosome 28. Chick RNA interference experiments were performed using the pRFPRNAiC plasmid as it is described at (Das et al., 2006). The target sequences (table 3) were designed using the GenScript siRNA Target Finder tool located at https:// www.genscript.com/ssl-bin/app/rnai and was cloned into the pSilencer2.1 plasmid using the pSilencer Kit (Life Technologies) in accordance with the manufacturer’s recommendation.

Table 3. Oligonucleotides used in this study

Name	Sequence (5` to 3`)
Draxin shRNA Fw	GAGAGGTGCTGCTGAGCGCAGCAGAACCGAGAGCAGCGATAGTGAAGCCACAGATGTA
Draxin shRNA Rv	ATTCACCACCACTAGGCAAAGCAGAACCGAGAGCAGCGATACATCTGTGGCTTCACT
Zic2 shRNA Fw	GAGAGGTGCTGCTGAGCGGTCAAGGCCAAGTACAAACTGTAGTGAAGCCACAGATGTA
Zic2 shRNA Rv	ATTCACCACCACTAGGCATTCAAGGCCAAGTACAAACTGTACATCTGTGGCTTCACT

5. Immunohistochemistry in sections.

Mouse and chick embryos were fixed ON at 4ºC in 4% Paraformaldehyde (PFA)/PBS solution. Then were embedded in 30% agarose (8010.01 Conda) and sectioned at 75 um sections with a vibratome (VT1000S Leica). Samples were blocked in 0.02% pig gelatine (G6144-500G Sigma)/PBT (0.5% Triton100x (1001211492 Sigma)/PBS) solution. Then slices were incubated with primary antibodies, (Table 4) at corresponding concentration, using the same solution that was used for blocking. Posteriorly slices were washed 3 times with PBS and incubated with appropriate secondary antibodies, (Table 5) at 0.5% Triton100x/PBS. At the same time with the secondary antibody were added 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI). After staining, sections were mounted, analyzed and photographed using a Leica SP5 Confocal.

For immunohistochemistry on slices, embryos were fixed at 4ºC in 4% PFA/PBS solution, 2 hours for E9 embryos, 3 hours for E10 and E11 embryos. For vibratome sections, embryos were embedded in 30% agarose. For cryostat, sectioning embryos

were embedded in 7% pig gelatine and 10% sucrose (4072-JT Baker) in PBS solution. Vibratome slices were cut at 75µm and while cryostat slices were cut at 30um, using (Slee MNT) cryostat. The 30 µm sections were blocked with 5%BSA (bovine serum albumin)/PBT (0.25%Triton100x + PBS). The incubation with primary and secondary antibody (Table 4 and Table 5) for cryostat slices was done in 3%BSA/PBT (0.25% Triton100X + PBS) solution. For vibratome sections was followed the same procedure, using 0,02% pig gelatine/PBT (0.50% Tritonx100 + PBS).

Table 4. Primary antibodies used in this study

Antigen	Provided by	Catalogue No.	Description	Concentration
HNK1	DSHB	3H5	mouse monoclonal	1/300
SOX10	Santa Cruz	SC-17342	goat polyclonal	1/250
FOXD3	R&D System	AF-5090	goat polyclonal	1/1000
ZIC2	Home made		rabbit monoclonal	1/700
MITF	Abcam	AB-12039	mouse monoclonal	1/200
GFP	Aves Lab	GFP-1020	chicken polyclonal	1/2000
BRN3A	Millipore	MAB-1585	mouse monoclonal	1/300
PH-H3	Cell Signaling Technology	9706	mouse monoclonal	1/800
CASPASE 3	Cell Signaling Technology	9661	rabbit polyclonal	1/500

Table 5. Secondary antibodies used in this study

Trget Species	Target Isotype	Host Specie	Concentration	Company
647 Alexa Fluor, Anti-mouse	IgG	Donkey	1:1000	Invitrogen, A31571
546 Alexa Fluor, Anti-mouse	IgG	Donkey	1:1000	Invitrogen, A10036
488 Alexa Fluor, Anti-chicken	IgY	Donkey	1:1000	Invitrogen, A21206

6. Whole mount Immunohistochemistry and clearing.

Whole-mount immunohistochemistry was performed based on a protocol from Prof. Carol Meson group who kindly send as, except small modifications. Embryos were previously fixed ON at 4ºC in 4% PFA solution. Then embryos were dehydrated with different methanol concentrations, starting from 25%methanol/PBS, 50%methanol/ddH2O2, 80%methanol/ddH2O2 and 100%ethanol. After that, embryos were bleached with 3%H2O2/ethanol solution. Again, embryos were rehydrated starting at 80%methanol/PBS, 50%methanol/PBS, 25%methanol/PBS. Then embryos were blocked in 5%BSA (bovine serum albumin) (10735078001 Roche) and 3%Tween/PBS solution. The incubation with primary antibody was done in 1%BSA/PBT (3%Tween +PBS) solution, for 3 days at 4ºC. After embryos were washed in 3%Tween/PBS solution. Then embryos were incubated with secondary antibody for 2 days in 1%BSA/PBT (3%Tween +PBS) solutions and at the end washed again in PBT (3%Tween +PBS) and save for clearing process.

The tissue clearing in chicken was performed as previously described (Kuwajima et al., 2013), modifying jus incubation time. Embryos were put in 25% and 50% Formamide/40% and 20% Polyethylene Glycol (PEG) 800MW solutions. The time was reduced from one hour to thirty minutes for each step. When the clarification was sufficient, embryos were almost transparent, were analysed and photographed using stereomicroscope (MZ16 F Leica).

For whole mount, immunohistochemistry embryos were processed in the same way as for IH in slices. For clearing was used an i-Disco protocol, performed as previously described (Renier et al., 2014).

Embryos were putted in homemade plate (Figure 13), that was built with glass bottom and wholes at different dimensions surrounded by plastic resistant of c photographed using Olympus FV1000 Confocal Microscope. For analysing was used IMARIS 8.1 Bitplane software. orrosive liquid used during i-Disco protocol. After embryos were clarified they were

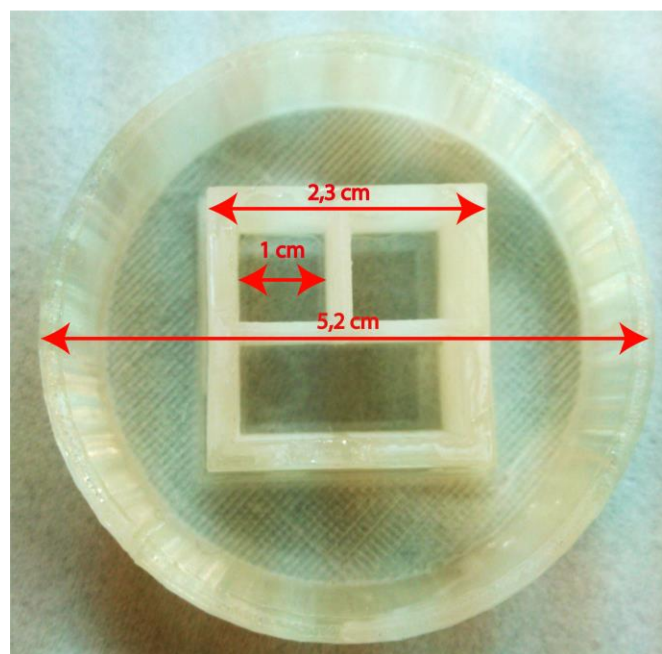

Figure 13: Homemade Time-lapse plate.

7. Whole mount In Situ Hybridization in chicken.

Whole-mount in situ hybridization of chick embryos was performed as previously described (Nieto et al., 1996), with small modifications. The RNA probes used for whole-mount ISH during this thesis are noted in (Table 6). For the probe synthesis, was digested 5µg of, DNA with 1 µm of appropriate restriction enzyme + 4 µl restriction buffer + 30 µl RNase-free water and was left ON at 37ºC at Bain-Marie. The Next day plasmid linearization was checked running in 1% agarose gel, 1 µl digestion and 1 µl of the original probe, in parallel. After digestion was checked, it was precipitated, adding 1/10th NaOAc and 3x 100% Ethanol of the digestion volume. The Digestion was left to precipitate ON ta -20^{0}C. Then the next day digestion was washed with 70% Ethanol at maximum speed in 4^{0}C centrifuge. After the digestion was suspended with 30 µl RNase-free water. The DNA concentration was checked running 1µl on Spectrophotometer (ND-1000 NadoDrop). The Rna labelled probe synthesis was the same as is described (Nieto et al., 1996). The synthesis efficacy was checked running, in 1% agarose gel, 1µl of the probe. Then the probe was precipitated, adding 80µl TE buffer pH=8 + 1µl glycogen (10901393001 Roche) + 5µl LiCl 8M + 300µl Ethanol 100% and was left ON at 20^{0}C. The Next day the probe was washed, at the same way was done with digestion. At the end, the probe was precipitated with 50µl Deionized Formamide (AM9344 Ambion) and 50µl RNase free water.

8. Whole mount In Situ Hybridization in mouse.

For whole mount ISH in mouse, embryos were left with 10 mg/ml proteinaseK/PBT (0.50% Tritonx100 + PBS), 15 min for mouse E9, E10 and 20 min for embryos E11. Other steps were the same used for whole mount ISH in chicken.

The chicken embryos were dissected and fixed ON at 4ºC in 4% PFA solution. Embryos were dehydrated with different Methanol/PBT solutions (25, 50, 75 and 100% ethanol/PBT (0.2%Triton100x + PBS). Every step was 10 min long for chicken around E3 and 12 min long for chicken around E5. After dehydration, embryos were stored at -20ºC. At the experimental day after rehydration embryos were left 20 min at 1% H2O2/PBT. Immediately after embryos were washed at least 5 times in PBT solution. Then were incubated with protein kinaseK 10 mg/ml, 7 min for embryos E3 and 20 min for embryos E5. The other steps were the same as previously described (Nieto et al., 1996). At the end, embryos were post-fixed with 4% PFA for 20 min. They were analysed and photographed using stereomicroscope (MZ16F Leica). After post-fixation embryos were embed in agarose 30% and sectioned at 75 µm. ISH expression in slices was analysed and photographed using a microscope (DM 2500 Leica). When embryos were electroporated after sectioning was continued with corresponding IH. After staining, sections were mounted, analysed and photographed using a Confocal (SP5 Leica).

Table 6. Probes used during ISH in chicken and mouse.

Name	Description	Gene ID	Source	Digestion enzyme	Retro. trascrip enzyme
DRAXIN	Chicken Draxin	419492	Hideaki Tanaka	EcoRI	T7
DRAXIN	Mouse Sox10	70433	Hideaki Tanaka	SpeI	T7

9. Microarrays analyses.

Fifteen embryo neural tubes were electroporated with EGFP or Zic2EGFP for each of the three replicates. Isolated neural tubes were trypsinized and100 000 GFP-positive cells were FACS purified for each replicate. Candidate genes are found by correlating

differences in the expression level of transcripts, represented on the microarray data analysis that were performed using the GENESPRING software 10.0 (Silicon Genetics, Redwood City, CA, USA). The statistical significance of the differences was evaluated by using unpaired t-test. Differentially expressed genes were identified when the absolute FC. 1.5 and p, 0.05.

10. Quantification of migrating NCCs.

For quantification of functional experiments (ectopic expression of Zic2 or downregulation of Zic2 [Zic2 Rna_i]) in chicks, ImageJ software was used to select three mirror regions of interest (ROIs). The electroporated side of neural tube [ROI^1], cells migrating through dorsolateral pathway [ROI^2] and cells migrating through ventromedial pathway ROI^3) per image, cells were labelled with GFP and DsRed antibodies. The migration Index was obtained as a measure of EGFP or DsRed levels in the ROI^1s, ROI^2s and ROI^3s, expressed as a function of ($RO^{2,3}$/iRO^1+ ROI^2 + ROI^3) X 100 (Figure 14). For the quantification of cell proliferation and cell death, were selected two mirror regions of interest (ROIs) with the same dimension. The fluorescence intensity of labelled cells with PH3 and Caspase-3 antibodies of the electroporated side of neural tube [ROI^1] and fluorescence intensity of labelled cells with PH3 and Caspase3 [ROI3] of the non-electroporated side of neural tube. The fluorescence Index was obtained as a measure of PH3 and Caspase3 levels in the ROI^1s and ROI^3s, expressed as a function of ($RO^{1,2}$/RO^1+ ROI2 + ROI^2) X 100 (Figure 14). Fluorescence intensities were measured, compared and represented in different graphs.

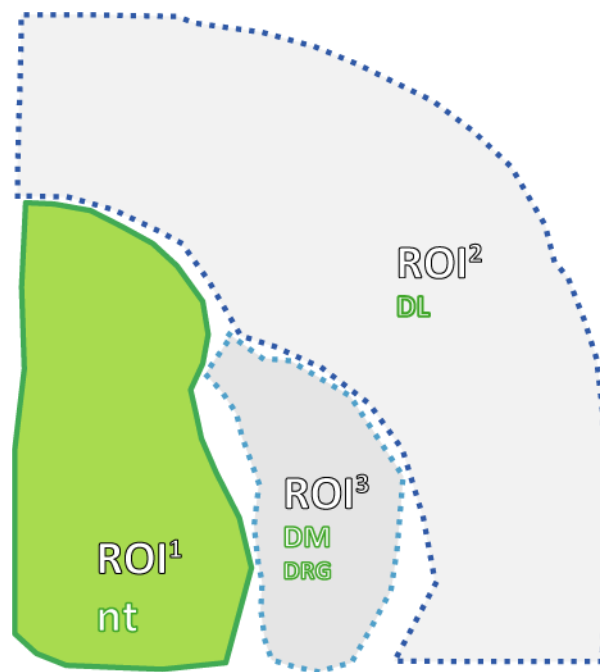

Figure 14: Scheme of different ROIs used to quantify NCCs migration

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11. Statistical Analysis.

Student's *t*-test or one-way ANOVA followed by appropriate Fisher's post hoc analysis. Quantitative data were expressed as mean ± s.e.m. significant differeneural crestes among groups were tested by Student´s t-test for independent and equal varianeural creste samples. Quantitative data are presented as the standard error of the mean (s.e.m.) (*P<0.05, **P<0.01 and ***P<0.001).

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RESULTS

RESULTS

1. Zic2 is expressed in premigratory NCCs and downregulated in migrating NCCs.

Previous studies have shown that Zic transcripts are expressed in the dorsal area of the developing neural tube in several vertebrates (Escalante et al., 2013; Li et al., 2014; McMahon and Merzdorf, 2010; Nagai et al., 1997) and expression of ZIC proteins using a pan-Zic antibody has been reported in the dorsal neural tube of mice embryos (Inoue et al., 2007b). However, specific expression of Zic2 during NC development has not been yet reported. To determine the spatiotemporal expression pattern of Zic2 during neurulation and neural crest cell formation stages, we incubated transverse sections from mouse embryos with antibodies specific for Zic2 (Herrera et al., 2003). At E8.0, prior to neural tube closure, we found some Zic2 positive cells surrounding the neural groove but cells in the neural folds were not positive for Zic2 (Figure 15). Once the neural tube closes (E8.5), Zic2 is highly express in a population of dorsal cells and continues being expressed in cells outside the tube (Figure 15). Between E9.5 and 10.0, Zic2 is mostly expressed outside the tube although some cells at the dorsal neural tube still express residual levels of this protein. Therefore, Zic2 expression is quite dynamic at the developing dorsal neural tube.

Figure 15. Zic2 expression pattern. Immunostaining against Zic2 in transverse neural tube sections from E8-E10 mouse embryos.

In order to characterize Zic2 positive cells at these stages, we used a reporter mouse line that expresses eGFP under the regulatory sequences of Zic2 [Tg(Zic2^{eGFP})]. In this mice line, eGFP expression is detected longer than Zic2 expression (Escalante et al., 2013; Marcucci et al., 2016; Murillo et al., 2015) allowing the tracking of the trajectories followed by Zic2 positive cells once that Zic2 expression has been turned off or in other words, allowing fate-mapping of Zic2 positive cells. At E.8.5, total overlap between Zic2 and eGFP was confirmed in cells inside the tube. However, those Zic2 cells located outside the neural tube, were not positive for eGFP (Figure 16). Immunostaining in transverse sections from these reporter mouse embryos, demonstrated that Zic2 cells inside the neural tube are premigratory NCCs because they express the marker FoxD3 (Figure 16). At E9.0, immunostaining for Sox10, a marker for migratory NCCs, showed that eGFP cells outside the tube are positive for

Sox10 (Figure 16). We also observed that eGFP cells outside the tube travel through both the dorsolateral (DL) and the ventromedial (VM) pathway (Figure 16).

These results showed that: 1/ Zic2 is transiently expressed in premigratory NCCs but they turn off Zic2 as they leave the tube. 2/ Zic2 is also expressed in dermomyotome cells. 3/ Migratory NCCs derived from Zic2 cells travel through the DL as well as the VM pathways.

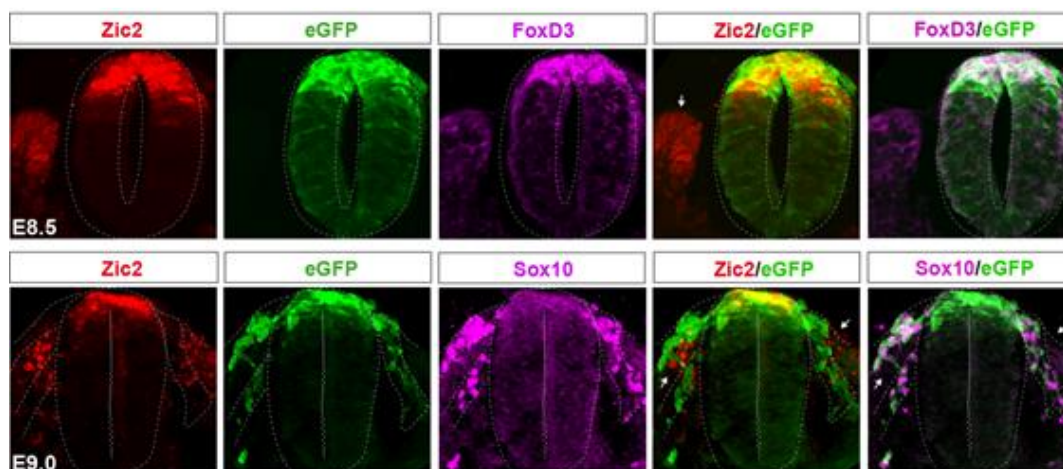

Figure 16. Characterization of Zic2 positive cells during the formation of the neural tube. Upper panels: Immunostaining against Zic2, eGFP and FoxD3 in transverse sections through the neural tube of E8.5 E9.0 Tg(Zic2) mouse embryos. Note that cells positive for Zic2 outside the neural tube are not eGFP positive (white arrow). In contrast, inside the tube Zic2 cells are eGFP and also positive for FoxD3. Bottom panels: Double immunostaining for Zic2 and Sox10 in transverse sections of E9.0 Tg(Zic2) embryos. Cells positive for Zic2 outside the neural tube are not eGFP positive or Sox10 positive (white arrows). In contrast, eGFP cells outside the neural tube are positive for Sox10 (pink arrows).

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2. Zic2 is necessary for NCCs emigration from the neural tube.

Then, to define the role of Zic2 in these cells we performed loss of function experiments by analyzing *Zic2* mutant embryos crossed with the (Tg(Zic2eGFP) line as in (Escalante et al., 2013). Analysis of E8.5/9.0 clarified embryos in 3D revealed that while eGFP cells migrate in a segmented manner in control embryos, in *Zic2* mutant embryos *Zic2*$^{kd/kd}$::Tg(Zic2eGFP) eGFP cells do not show such stereotyped migration pattern (Figure 17A, B). In fact, an aberrant accumulation of eGFP cells that were not positive for Sox10 was observed in the most dorsal aspect of the neural tube in *Zic2* mutants (Figure 17C, D), suggesting that in these embryos NCCs do not exit the tube.

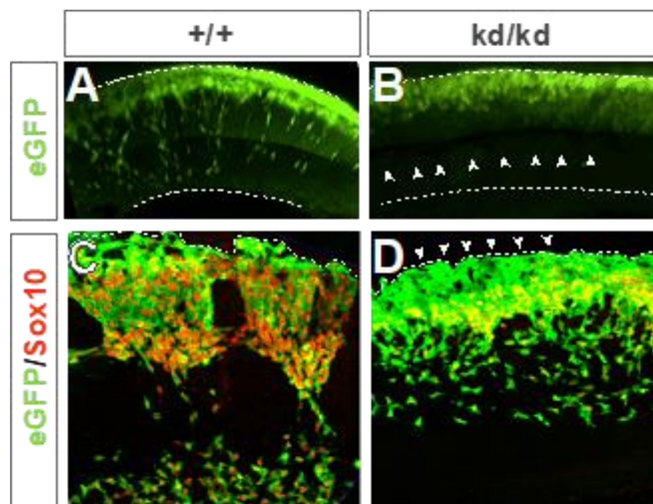

Figure 17. **NCC accumulate into the dorsal neural tube in *Zic2* mutant mice. A, B**. Wholemount preparations of E9.0 embryos reveal fewer GFP cells migrating in Tg(eGFP)::*Zic2*$^{kd/kd}$ embryos contrasting to the controls in which eGFP cells are visualized migrating in a segmented manner. Arrowheads point to the place where eGFP should be migrating as in controls embryos. **C, D**. Immunostaining against Sox10 in a wholemount E10.5 Tg(eGFP)::*Zic2*$^{kd/kd}$ embryo and a control littermate. Arrowheads point to eGFP cells that do not express Sox10 in the dorsal part of the tube in Zic2 mutant embryos.

In sections, we confirmed an accumulation of eGFP cells in the dorsal tube of ZIC2 mutants compared to the control embryos and fewer eGFP migrating cells in *Zic2*$^{kd/kd}$:(Tg(Zic2eGFP) embryos with almost no cells migrating superficially (Figure 18).

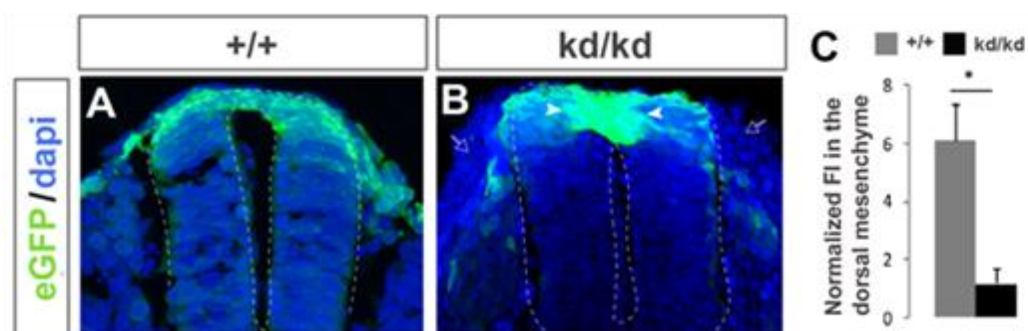

Figure 18. Transverse sections from E8.5 Tg(eGFP)::*Zic2*$^{kd/kd}$ and control littermate embryos. White arrowheads point to accumulation of eGFP cells inside the dorsal neural tube in Zic2 mutants. Notice the strong reduction in the number of eGFP migrating cells in the Zic2 mutants compared to the controls (empty arrows). Graph quantifies fluorescence intensity in the dorsal mesenchyme, outside the neural tube.

During gastrulation stages (E6-E7.5) *Zic2* mRNA is present in epiblast stem cells (Elms et al., 2004). Therefore, the defects on neural crest migration found in *Zic2*$^{kd/kd}$ embryos could be the consequence of Zic2 downregulation at these early stages. To test this possibility and precisely define the role of Zic2 in NCCs, we performed loss of function experiments by electroporating chick embryos that allows temporal manipulation of Zic2 expression at the time that NCCs are going to leave the tube and initiate migration (Figure 19).

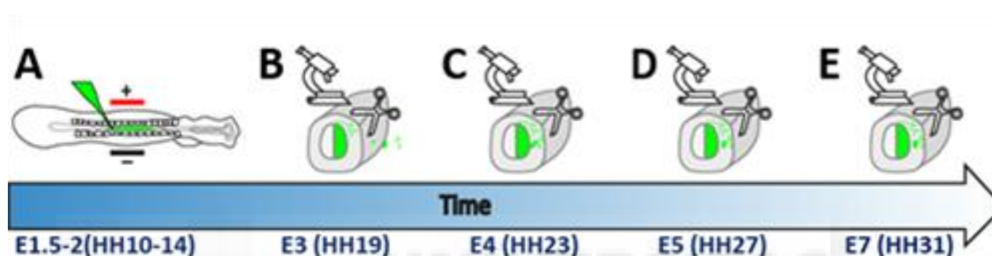

Figure 19. **The schema illustrates electroporation of plasmids and cell migration analysis at different time points.** A, Injection of chicken embryos in the neural tube and electroporation at E1.5 or E2 (HH10-14). B-E, Embryos dissection and analysis of cell migration at E3 (HH19), E4 (HH23), E5 (HH27) and/or E7 (HH31).

Plasmids bearing Zic2 short hairpin mRNA (Zic2 shRNAs) or control shRNAs were electroporated in HH12/14 chick embryos and analyzed at E3 and E4 in sections. Downregulation of Zic2 lead to a significant decreased in the number of NCCs at the dorsal mesenchyme compared to the controls (Figure 20). At E3 both, the DL and the VM pathways were dramatically affected and at E4 the number of eGFP in the dorsal root ganglia (DRG) was also reduced (Figure 20).

These results, together with the observations obtained in *Zic2*$^{kd/kd}$ mice demonstrate that Zic2 is an essential factor for neural crest formation in both mice and chicken.

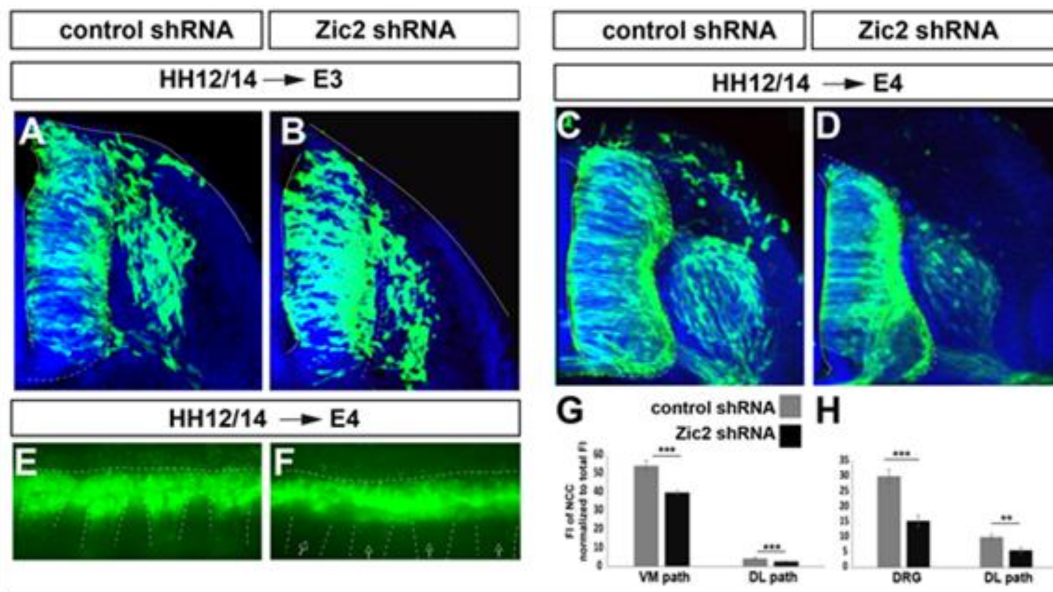

Figure 20. **Downregulation of Zic2 in the chick neural tube leads to a reduced number of migrating NCCs. A-D**. Transverse sections of E3 and E4 chick embryos electroporated at HH12/14 with control shRNA or Zic2shRNAs plus eGFP encoding plasmids. At both stages the number of migrating NCC is reduced in embryos with downregulated expression of Zic2. **E-F**. Dorsal view of wholemount E4 chick embryos electroporated at HH12/14 with control shRNA or Zic2shRNAs plus eGFP encoding plasmids. Empty arrows point to the segments where eGFP should be migrating as in control embryos. **G-H**. Graphs represent fluorescence intensity across the dorsolateral (DL) path or in the dorsal root ganglia (DRG) region normalized to total fluorescence in transverse sections of E3 (**G**) and E4 (**H**) chick embryos electroporated at HH12/14 with control shRNA or Zic2shRNAs plus eGFP encoding plasmids.

To investigate the possibility that the lower number of NCCs observed after downregulation of Zic2 manipulations is due to cell death, we analyzed the expression Caspase3 in chick embryos after electroporation of plasmids encoding Zic2 shRNA. E5 embryos electroporated with Zic2 shRNA at HH12/14 did not show any difference in the number of cells positive for Caspase3 compared to the control embryos electroporated with control shRNA or eGFP encoding plasmids (Figure 21). These results indicate that the absence of Zic2 does not induce cell death, confirming previous data obtained in other contexts or animal models (Elms et al., 2003; Murillo et al., 2015; Nagai et al., 2000).

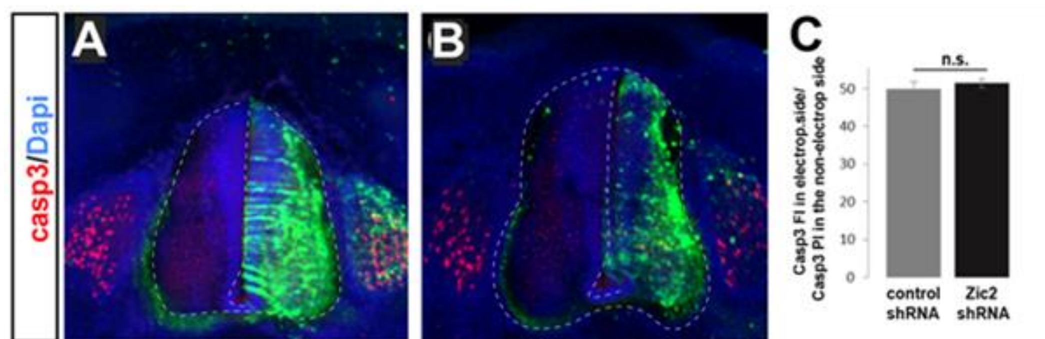

Figure 21. **Zic2 downregulation does not induce cell death. A-B**, Transverse sections of neural tube stained for Caspase3, DsRed and Dapi in E5 (HH27) embryos, electroporated at E2 (HH12-14) with control shRNA (A) and Zic2shRNA (B). Zic2 downregulation does not induce more Caspase3 expression. **C**, Quantification of Caspase3 expression in E5 embryos. No significant difference in Caspase3 fluorescence intensity is observed between control shRNA and Zic2 shRNA embryos.

3. Zic2 is sufficient to induce NCCs exit and alter their migration.

It has been previously described that overexpression of Zic2 in *Xenopus* induces neural crest expansion (Brewster et al., 1998; Nakata et al., 1998). To test whether this is also the case in chick, vectors driving the expression of eGFP alone (pCAG-eGFP) or Zic2eGFP (pCAG-eGFP + pCAGGS/SE-Zic2T) were unilaterally electroporated into the trunk neural tube at E1.5-2 (HH10-14) embryonic stages. Then, embryos were sacrificed and analyzed at different time points (Figure 19). 36 hours after Zic2 overexpression, a higher number of migrating cells in the DL path was observed compared to the controls together with a strong reduction in the number of cells in the DRGs path (Figure 22).

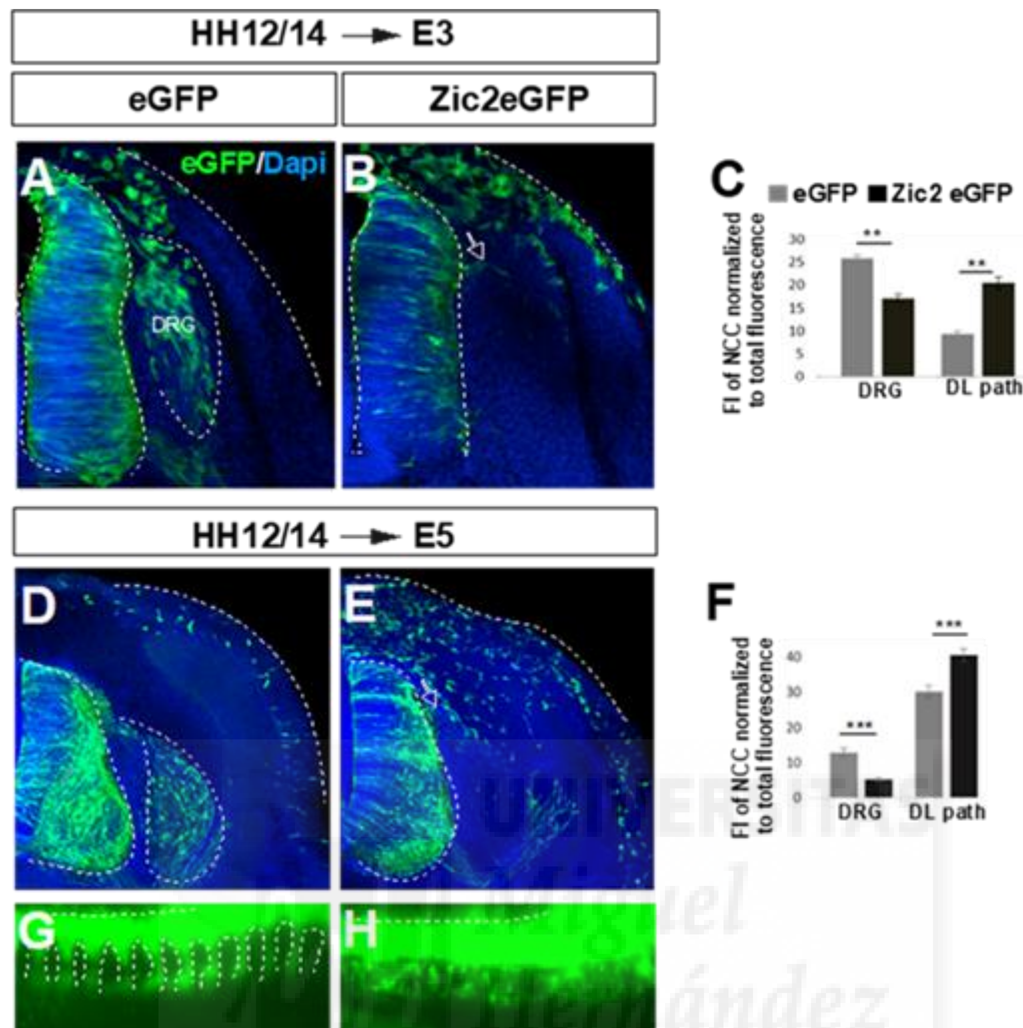

Figure22. Overxpression of Zic2 in the chick neural tube leads to an accumulation of NCC in the mesenchyme. A-B, D-E. Transverse sections of E3 and E5 chick embryos electroporated at HH12/14 with plasmids encoding Zic2eGFP or CAG (empty) eGFP plasmids. At both stages an accumulation of eGFP cells is observed after electroporation of Zic2 plasmids. Empty arrows point to the initial point of the dorsal root ganglia **E-F**. Dorsal view of wholemount E4 chick embryos electroporated at HH12/14 with plasmids encoding Zic2ireseGFP or eGFP plasmids alone. Note that electroporated NCC follow a segmented stereotyped path in the controls they migrate in a desorganised manner after electroporation of Zic2 plasmids. **G-H**. Graphs represent fluorescence intensity across the dorsolateral (DL) path or in the dorsal root ganglia (DRG) region normalized to total fluorescence in transverse sections of E3 (**G**) and E5 (**H**) chick embryos electroporated at HH12/14 with plasmids encoding Zic2ireseGFP or eGFP plasmids alone.

To better visualize the effect of Zic2 in the entire neural tube, we analyzed wholemount preparations from the same experimental group. Zic2eGFP electroporated embryos showed disruption of segmental cell migration. Cells did not migrate through the stereotyped segmented paths as they do in control embryos.

Instead, they homogenously spreader around the dorsal part of the neural tube and covered shorter distances compared to the controls. A similar but even more evident result was observed when embryos were analyzed three days after electroporation (E5; HH27). Many Zic2 electroporated cells remained at the mesodermal area around the dorsal part of the neural tube and there were almost not electroporated cells located in the DRGs, contrasting to the eGFP controls in which few electroporated cells were present in dorsal mesoderm and a lot of them populated the DRGs (Figure 22).

Analysis of E7 (HH31) embryos also electroporated at HH12/14 with Zic2 plasmids showed that NNCs ectopically expressing Zic2 stay in the dorsal mesenchymal area suggesting a defect in their migration (Figure 23).

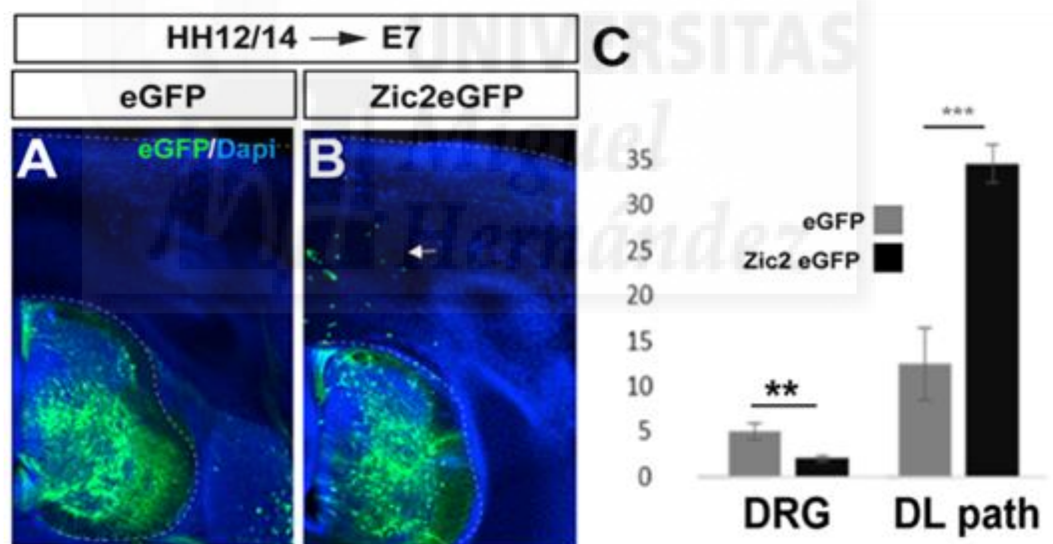

Figure 23. Ectopic expression of Zic2 leads to accumulation of NCC in the dorsal mesenchyme.

Gain of function experiments suggested that Zic2 may control NCCs exit from the neural tube. However, it is also possible that the accumulation of NNCs observed after Zic2 ectopic induction results from an overproduction of NNCs. To address this possibility, we analyzed the number of proliferating cells by counting the number of PH3 positive cells in embryos electroporated with Zic2-encoding plasmids and observed no differences with controls embryos (Figure 24).

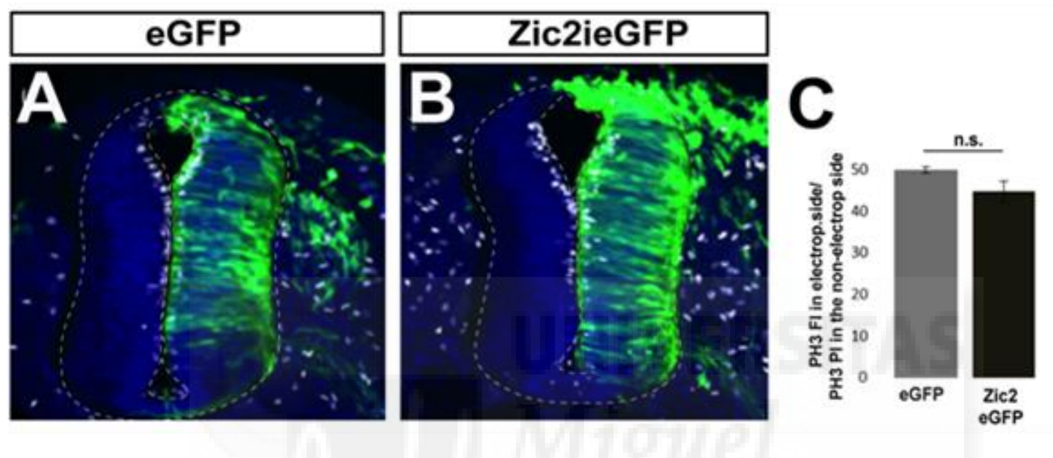

Figure 24. PH3 in Zic2 GOF. A, B. Phosphohistone (PH3) immunostaining (white) in transverse sections of E3 chick embryos electroporated at HH12/14 with plasmids encoding Zic2ireseGFP or eGFP alone. **C**. Graph represents fluorescence intensity of PH3 positive cells in the electroporated side normalized to the non-electroporated side in transverse sections of E3 chick embryos electroporated at HH12/14.

These results indicate that Zic2 does induce an increase in the number of proliferating cells and suggest that the increased number of cells observed in the dorsal mesenchyme after Zic2 ectopic expression likely is the consequence of premature exit of NCCs from the tube combined with misguidance problems.

4. Zic2 does not determine melanocytic or sensory NCC fate.

Similar to our Zic2 gain of function results in chick, previous experiments in *Xenopus* described an accumulation of NCC in the dorsal mesenchyme after ectopic expression of Zic2 (Brewster et al., 1998; Nakata et al., 1998). This, together with the observation

that *Zic2* mutant mice exhibit a reduction in the number of cells in the DRGs (Nagai et al., 2000) previously led to the idea that Zic2 is a determinant of melanocytic fate. To test this hypothesis, we analyzed the expression of the transcription factor Mitf, a marker for melanocytic progenitors (Hornyak et al., 2001; McGill et al., 2002), as well as the expression of Brn3a, a marker for sensory neurons (Fedtsova and Turner, 1995; Nitzan et al., 2013) after manipulation of Zic2 levels. As expected, in control embryos, many eGFP cells migrating dorsally expressed Mitf and eGFP cells located in the DRG expressed Brn3a (Figure 25, 26). After ectopic electroporation of Zic2, some eGFP cells expressed Mitf but most of them were not positive for this transcription factor (Figure 25). In addition, none of the eGFP cells expressed Brn3a in Zic2 electroporated embryos (Figure 26).

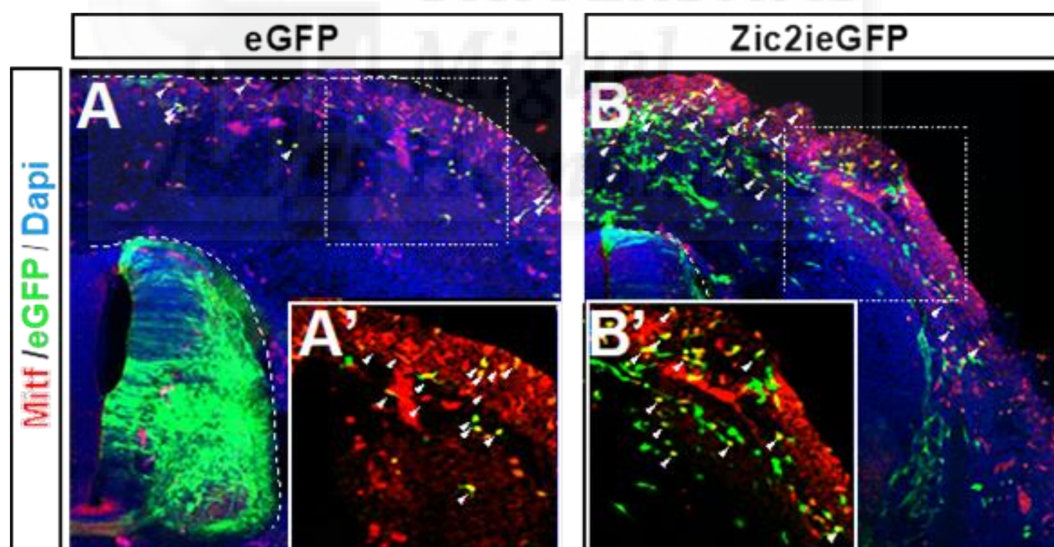

Figure 25. **Zic2 does not induce melanocytic fate. A, B**. Mitf immunostaining in transverse sections of E5 chicken embryos electroporated at HH12/14 with plasmids encoding for Zic2ireseGFP or eGFP alone. Note that in both Zic2 electroporated embryos and controls some eGFP cells are positive for Mitf (arrows) but the mayority of eGFP cells did not express Mitf after electroporation of Zic2. **A' B'**. Inset of the boxed area without Dapi to better appreciate that many eGFP cells are negative for Mitf.

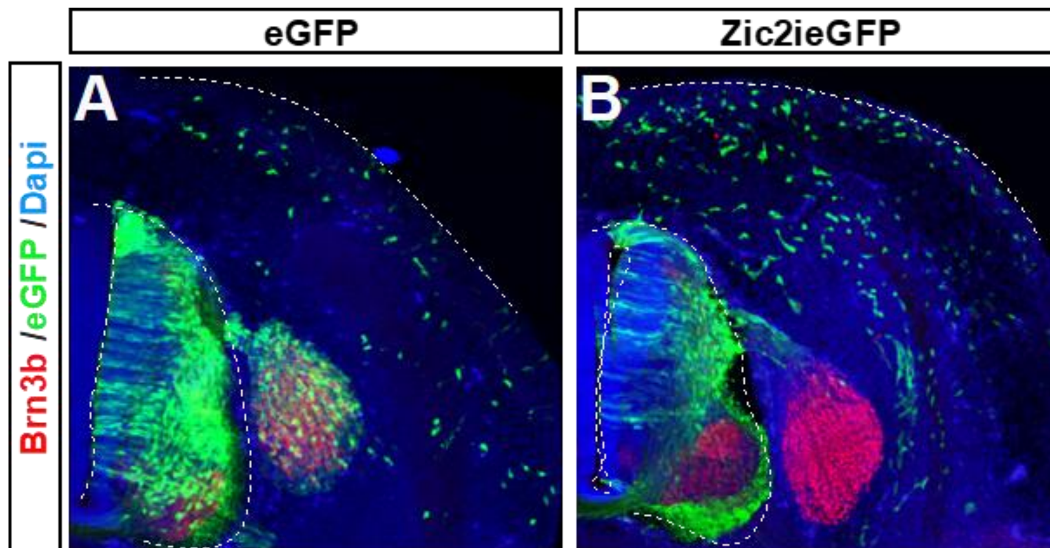

Figure 26. Zic2 does not induce sensory fate. Brna3a immunostaining in transverse sections of E5 chicken embryos electroporated at HH12/14 with plasmids encoding for Zic2ireseGFP or eGFP alone. eGFP cells in the dorsal root ganglia of control embryos are positive for Brn3a. In embryos electroporated with Zic2ireseGFP, eGFP cells do not express Brn3a.

Therefore, in contrast to previous reports performed in *Xenopus* embryos suggesting that Zic2 induces melanocytic fate, our results rather support the hypothesis that Zic2 is not an inducer of any particular NCC fate and that accumulation of NCCs in the dorsal mesenchyme is due to a premature NCCs exit.

5. Zic2 target genes in neural crest formation.

The tyrosine kinase receptor EphB1 has been described as a target of Zic2 during the formation of the visual circuit (García-Frigola et al., 2008) and in cell migration during forebrain formation (Murillo et al., 2015). In the spinal cord it has been shown that Zic2 controls the expression of another receptor from the same tyrosine kinase family, EphA4 (Escalante et al., 2013).

Based on these previous works and on the fact that Eph receptors have been involved in neural crest formation (McLennan and Krull, 2002; Santiago and Erickson,

2002; Smith et al., 1997), we wondered whether Zic2 regulates the expression of these receptors also during neural crest formation. To address this hypothesis, we analyzed the phenotypes of embryos electroporated with plasmids encoding EphB1 or EphA4 and compared them with embryos electroporated with Zic2 plasmids. Vectors driving the expression of eGFP alone (pCAG-EGFP) as a control group and EphB1 (pCAG-SE-EphB1) or EphA4 (pCAG-SE EphA4) plus eGFP were electroporated at E2 (HH12-14) and analyzed at E5 (HH27). Overexpression of EphB1 did not produce any differences compared to the controls (Figure 27).

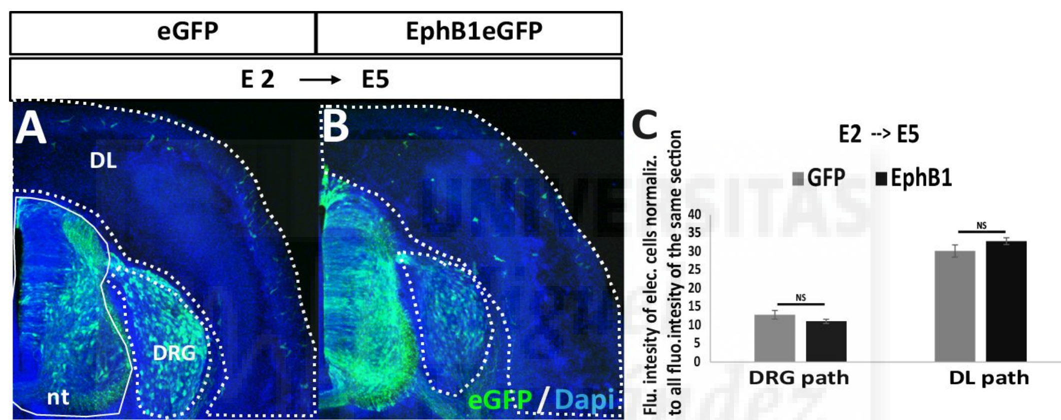

Figure 27. **EphB1 does not reproduce Zic2 overexpression phenotype. A-B**, Transverse sections of neural tube from E5 chicken embryos electroporated at E2 with eGFP or EphB1eGFP. The EphB1 overexpression does not lead to more migration in DRG and DL path compare to control GFP group. **C**, Graph summarizing migrating NNCs in electroporated embryos. No significant difference in migrating cells is observed in DRG and DL path after EphB1 ectopic expression compares to GFP control group. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t-test), nt (neural tube).

Embryos electroporated with EphA4 exhibited a higher number of neural crest cells in the DL path although not significant differences in the DRG path (Figure 28).

These results show that ectopic electroporation of EphB1 do not affect NCCs exit or initial NCCs migration. However, ectopic introduction of EphA4 in NCCs alters the behavior of NCCs and partially recapitulates the phenotype observed after Zic2

ectopic induction, suggesting that EphA4 may be a target Zic2 molecule. Further experiments are needed to address whether this is indeed the case.

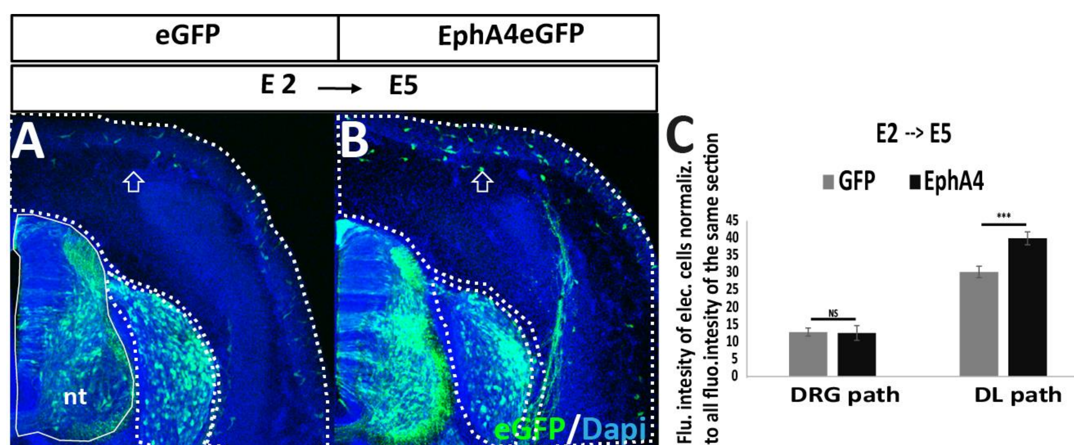

Figure 28. **EphA4 reproduce partially Zic2 overexpression phenotype**. **A-B**, Transverse sections of neural tube from E5 chicken embryos electroporated at E2 with eGFP or EphA4eGFP. EphA4 overexpression leads to more cells migrating through the DL path (arrows). In the DRG path cells migrate more or less at the same level. **C**, Graph summarizing migrating NNCs in electroporated embryos. A significant more migrating cell in DL path is observed after EphA4 ectopic expression compares to GFP control group. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t-test), nt (neural tube).

6. Zic2 regulates the expression of the secretable molecule Draxin/Neucrin.

To get further insight into the molecular mechanisms by which Zic2 allows NCC migration, we searched for Zic2 target molecules in an unbiased manner by comparing

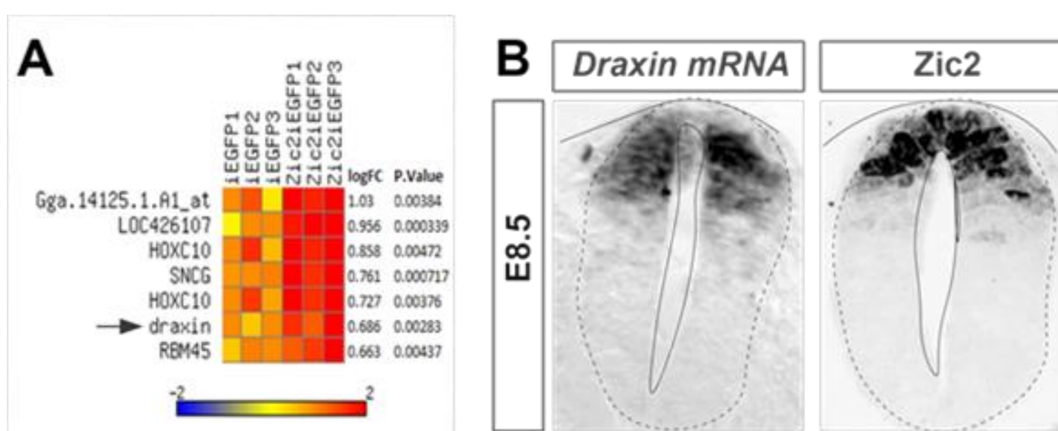

Figure 29. A. Hit map generated from the DNA microarray data from embryos electroporated with Zic2ireseGFP encoding plasmids or eGFP alone. Seven probe-sets/transcripts were significantly upregulated on samples overexpressing Zic2 with a fold-change larger than 0.5 (p<0.05). The probe-set ID, gene symbol, fold change and P values for each transcript are indicated. The colour scale bar indicates up-regulation in red. **B**. Draxin/Neucrin mRNA detected by in situ hybridization in sections from E8.5 mouse embryos (left) shows a similar pattern to Zic2 expression at the same stage (right).

the transcriptome profile of E4 chick embryos electroporated at HH12-14 with eGFP alone versus embryos electroporated with Zic2 encoding plamids. Applying a fold-change of >0.5 and a pvalue <0.005, we found only 7 genes differentially overexpressed in the Zic2 electroporated samples (Figure 29A).

One of these genes was *Draxin/Neucrin*, which encodes for a soluble protein previously reported to be expressed in the developing dorsal neural tube (Miyake et al., 2012; Su et al., 2009). In situ hybridization for Draxin/Neucrin compared with Zic2 immunostaining confirmed that they are expressed in a similar pattern in the dorsal neural tube of E8.5 embryos (Figure 29, B). To confirm that Zic2 regulates Draxin/Neucrin, we analyzed the expression of *Draxin/Neucrin* mRNA in *Zic2*$^{kd/kd}$ embryos and found a strong downregulation compared to wildtype littermates (Figure 30, A).

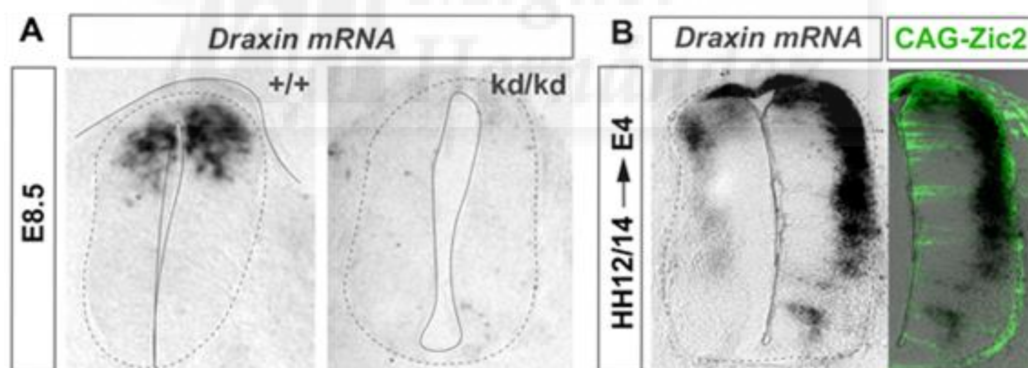

Figure 30. **Draxin expression after manipulation of Zic2. A**. In situ hybridization for Draxin mRNA in sections from E8.5 *Zic2* mutant mice and control littermates reveals a significant reduction in Draxin expression compared to control embryos. **B**. In situ hybridization for Draxin mRNA in sections from E4 chick embryos electroporated with Zic2ireseGFP plasmids at E2 shows an strong upregulation of Draxin mRNA levels in the electroporated side.

Conversely, E4 chick embryos electroporated at HH12-14 with Zic2 encoding plasmids revealed a strong induction of *Draxin/Neucrin* mRNA expression in the electroporated side compared to the non-electroporated half of the embryo (Figure

30B) or, to embryos electroporated only with eGFP encoding plasmids (data not shown). These functional experiments demonstrate that Zic2 is necessary and sufficient to regulate Draxin/Neucrin expression in the dorsal neural tube at the time that NCC are leaving the neural tube.

Previous reports *in vitro* and *in vivo* have implicated Draxin/Neucrin in neural crest migration (Hutchins and Bronner, 2018; Lu et al., 2017; Su et al., 2009). To confirm these results and compare them with Zic2 functional experiments we electroporated Draxin/Neucrin shRNAs (pRFPRNAiC-cDraxinRNAi/pCAG-EGFP) or control shRNA plus EGFP (pRFPRNAiC/pCAG-EGFP) in HH12-14 chick embryos and processed the embryos two days later (E3). As expected from previous analysis (Hutchins and Bronner, 2018), we observed a lower number of NCC in the mesenchyme compared to the controls.

This decrease was also similar to the reduction in the number of NCCs that we observed after downregulation of Zic2 in the same conditions (Figure 31).

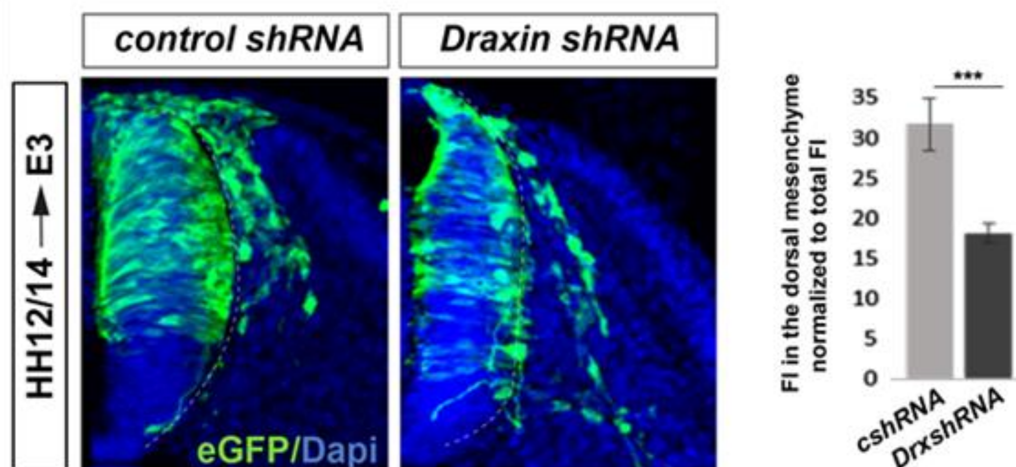

Figure 31. Transverse sections of E3 chick embryos electroporated at HH12/14 with control shRNA or Draxin shRNAs plus eGFP encoding plasmids. At this stage the number of migrating NCC is reduced in embryos with downregulated expression Draxin. Graph represents fluorescence intensity of eGFP cells in the dorsal mesenchyme of E3 chick embryos after manipulation of Draxin levels at HH12/14.

These results demonstrate that although Zic2 likely regulates the expression of many different factors to promote neural crest cell delamination, Draxin/Neucrin is a Zic2 target molecule during this process.

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DISCUSSION

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DISCUSSION

The involvement of Zic2 during neural crest development is described in different animal models. However, many aspects about molecular basis and specific mechanisms controlled by this transcription factor during the development of this structure were not well understood. This study provides evidences regarding the specific contribution of Zic2 during neural crest development. We define for the first time the expression pattern of ZIC2 protein from the beginning to the end of neural crest development. Our functional experiments demonstrate that downregulation of Zic2 reduces cell migration and disrupts normal cell migratory pattern, demonstrating that Zic2 is necessary for neural crest formation. The overexpression of this transcription factor strongly suggests that it is sufficient to induce NCC delamination. Our results also support the idea that rather than determining any particular neural crest cell fate Zic2 appears to be important for general specification of NCCs and subsequent delamination.

Our data also suggest that during neural crest development Zic2 does not appear to regulate EphB1 tyrosine kinase receptors as in the visual system. However, it could be regulating EphA4, as occurs during later stages of spinal cord development. Further, searching for novel Zic2 target genes during NC development, we identified Draxin/Neucrin and demonstrated that this secretable protein, proposed to be an antagonist of the Wnt canonical pathway, is indeed regulated by Zic2.

1. Zic2 expression in pre-migratory neural crest during development.

The expression of Zic2 transcripts during development starts prior to gastrulation until the early phase of organogenesis. This description included the neural crest of several vertebrate species (Elms et al., 2004; McMahon and Merzdorf, 2010; Merzdorf, 2007; Nakata et al., 1998; Warr et al., 2008). Although these studies provided a general view of Zic2 expression at mRNA levels, there were no details about the expression of the protein during specific phases of neural crest development. Furthermore, there was not a comparison between Zic2 and any neural crest marker.

Our immunostaining experiments with antibodies specific for Zic2 combined with the analysis of the Zic2 reporter mice confirm previous studies (Elms et al., 2004; Inoue et al., 2007b; Nakata et al., 1998) but they also provide a detailed expression pattern of ZIC2 protein through specific phases during neural crest development. Our results reveal for the first time that migrating NCCs populate both the dorsal and the ventral paths and although they are not positive for Zic2 while migrating, NCCs express Zic2 transitorily just before leaving the neural tube. Once NCCs go out of the neural tube and express Sox10 they turn off Zic2 expression. In the meantime, Zic2 expression continues in the surrounding sclerotome, in a complementarity pattern to Sox10 (Figure 32). These findings already lead as to the idea that Zic2 could be necessary for the generation and delamination of neural crest but stops being necessary for NCCs once they delaminate from the neural tube.

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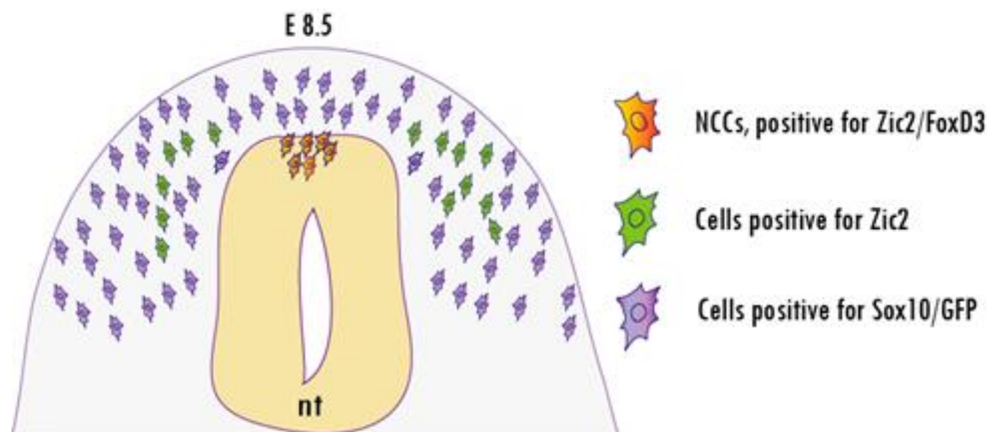

Figure 32. Scheme representing Zic2 expression during neural crest development in mouse. A- E8.5, B- E10.5.

2. The role of Zic2 in premigratory neural crest cells

The analysis of whole mount preparations and transverse sections through *Zic2*$^{kd/kd}$ mouse embryos both demonstrated an aberrant accumulation of NCCs in the dorsal part of the neural tube and suggested that NCCs are not able to leave the neural tube. NCCs accumulate in the dorsal part of the neural tube and consequently the number of migrating NCCs was reduced. Our functional experiments in chicken where also in harmony with these results in mouse because downregulation of Zic2 showed a reduction in the number of migrating NCCs in both migratory pathways [DL and DM (DRG)] at different stages. These results were also in coherence with previous results (Nagai et al., 2000) and our own observations showing a reduction in the size of DRGs in hypomorphic *Zic2* mutant mice. A reduction of migrating NCCs was also described in a different line of *Zic2* mutants, the “Kumba mutants” (Elms et al., 2003). Also consistent with previous results, we did not find any increase in the number of dying cells in *Zic2* mutant mice. In previous analysis performed in *Zic2* mutant mice NCCs were not labeled and therefore the accumulation of NCCs in the dorsal tube was not

detected. The crossing of *Zic2* mutant mice with the reporter Zic2 mice allowed us to visualize the accumulation of NCCs in the dorsal tube revealing this effect as the most likely cause of a reduction in the number of migrating NCCs.

Our Zic2 overexpression experiments in chick showed a higher number of migrating NCCs in the DL path and almost no cells in the DM (DRG) pathway (Figure 33). This phenotype was also persistent at latter ages. Previous experiments in *Xenopus*, suggested that Zic2 induces melanocytic fate because they observed an increase in the number of NCCs in the dorsal pathway (Brewster et al., 1998; Nakata et al., 1998). However, these studies only showed a global view from the top of the embryos. They did not analyze transversal sections or performed quantifications of any specific migratory pathway. Our results showing that NCCs ectopically expressing Zic2 do not express melanocytic markers suggest that Zic2 does not induce any particular fate but rather induces the delamination of NCCs.

Our results also explain previous observations showing a reduction in the number of cells in the DRGs in *Zic2* mutants. DRG cells do no express Zic2 but they are populated by NCC that expressed Zic2 when they still were inside the neural tube (McMahon and Merzdorf, 2010; Nagai et al., 1997). In the absence of Zic2, NCCs are not specified and they do not delaminate properly and cannot migrate to their final destinations leading to a reduced number of cells in the DRGs. We also observe a reduction in the number of cells populating the DRGs after overexpression of Zic2. This phenotype likely is produced because maintained expression of Zic2 in NCCs once they leave the tube interferes with their migration and leads to their accumulation in the dorsal mesenchyme. This accumulation of cells in the DL pathway after Zic2

overexpression could be also due to an overproduction of cells. However, our results for phospho histone H3 did not show differences compared to the control group, indicating that the accumulation of NCC in the dorsal mesenchyme is likely the result of a precocious delamination of the NCCs from the neural tube.

Notably, it has been shown that the temporary deactivation of the canonical-Wnt pathway is essential for NCCs delamination from the neural tube (Rabadán et al., 2016). On the other hand, although it was initially proposed by experiments in zebrafish that Zic2a is a downstream effector of the canonical Wnt pathway (Nyholm et al., 2007), it has been later demonstrated that Zic2 inhibits the Wnt/β-Catenin signaling (Pourebrahim et al., 2011). Therefore, it seems likely that Zic2 is transitorily expressed in the dorsal neural tube to switch off the canonical pathway and stop proliferation.

In *Xenopus*, the results related to the role of Zic2 in neural crest were contradictory. Results from Nakata and colleagues (Nakata et al., 1998) claimed that Zic2 overexpression induces neurogenesis whereas the study of (Brewster et al., 1998) claimed that Zic2 overexpression reduces neural tissue and maintains cells in an undifferentiated stage. Together with previous observations indicating that Zic2 blocks the canonical Wnt signaling the results presented here strongly suggest that the actual role of Zic2 in NCCs is stopping their proliferation and inducing their delamination. If Zic2 is not expressed, NCCs continue proliferating and cannot leave the tube leading to an accumulation of cells inside the dorsal tube, as in fact we observe in the Zic2 mutant embryos. Conversely, in gain of function experiments, cells in the dorsal tube precociously leave the tube and produce an increased number of

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cells outside. These Zic2-expressing cells accumulated in the dorsal mesenchyme likely are a mix of cells that were not meant to be NCCs but delaminated because ectopic expression of Zic2 and cells that because they continue expressing Zic2 once they are out of the tube cannot migrate properly.

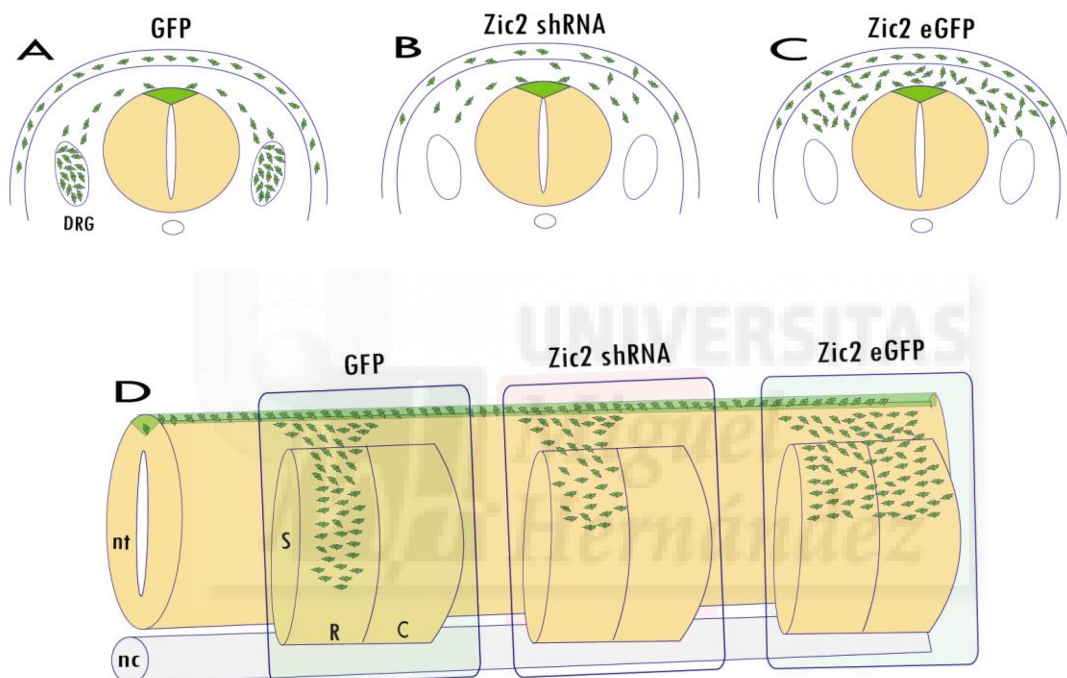

Figure 33. Scheme representing Zic2 phenotype after down regulation and expression. nc-notochord, nt- neural tube, S-somite, R-rostral, C-caudal.

3. Zic2 target genes during NCC formation.

Very few Zic2 target effectors have been described to date. EphB1 was identified as a Zic2 target in differentiated retinal ganglion cells (García-Frigola et al., 2008) and EphA4, also a member of the Eph family that binds to the same ligand (ephrinB2), is regulated by Zic2 in dorsal spinal cord neurons (Escalante et al., 2013). Previous studies have shown that NCCs express EphB1 during early stages of their migration

preventing them to go onto the dorsolateral path (Santiago and Erickson, 2002) and avoiding areas where ephrinB1 is expressed (Mellott and Burke, 2008; Smith et al., 1997). These observations lead us to the idea that Zic2 could be regulating NCC migration through the regulation of this guidance receptor. However, the fact that EphB1 does not show a similar expression pattern to Zic2 in premigratory NCCs, together with our results showing that EphB1 overexpression do not have a significant effect in cell migration on any of the analyzed paths, suggested that Zic2 does not regulate the expression of EphB1 in this context.

EphA4 is expressed very weakly at the beginning of neural crest development with a peak around HH18-21 (McLennan and Krull, 2002; Santiago and Erickson, 2002). EphA4 has been proposed to promote neural crest migration (McLennan and Krull, 2002). In agreement with this study we also observe a higher migration of NCCss on DL pathway after EphA4 gain of function, similar, although more modest, to the phenotype observed after Zic2 ectopic expression. Therefore, it is possible that Zic2 regulates the expression of EphA4 during this process. Arguing against this, we did not find upregulation of EphA4 in the microarrays gain of function analysis. Therefore, further experiments are needed to test whether Zic2 regulates the expression of EphA4 in NCCs.

Nevertheless, our gain of function screen revealed a strong upregulation of the secreted protein Draxin also known as Neucrin (neural tissue specific cysteine-rich protein) after Zic2 ectopic expression. Draxin is a soluble protein that was initially identified as a repulsive molecule for commissural axons in the spinal cord and cortical forebrain commissures (Islam et al., 2008). Later, the same year, it was described as an antagonist of the canonical/WNT signaling pathway (Miyake et al., 2009).

This protein is expressed early during embryonic development, starting around gastrulation and the early stages of neural crest development. In chick it is expressed in the dorsal part of the spinal cord and future brain areas, concentrated in the central midline of the roof plate, it is also expressed in the dorsal lip of dermomyotome. In mouse Draxin is expressed in the DRG and there is no expression in the dermomyotome region and it shows an anteroposterior gradient, being weaker in the caudal part of the neural tube (Su et al., 2009; Zhang et al., 2016).

It has been proposed that in chick, Draxin reduces NCCs polarity and migratory velocity and it is able to change the trajectory of some early migrating NCCs from the dorsomedial to dorsolateral pathway (Su et al., 2009). In mouse, Draxin mutants showed a reduced cell polarity although their cells did not show a significant difference in NCCs migration (Zhang et al., 2016).

Zic2 and Draxin have a similar expression pattern in E8.5 mouse embryos, being both molecules expressed at the dorsal part of the tube. In *Zic2* $^{kd/kd}$ was seen a notable reduction in Draxin mRNA expression compared to wild type. The results obtained by DNA microarray gain of function experiments were also confirmed by in situ hybridization showing an evident upregulation of Draxin mRNA levels after Zic2 electroporation. The results presented here describe for the first time Draxin as Zic2 target molecule. There are no previous data either about the relation of Draxin with other Zic family members.

Overexpression of Draxin at E4 resulted in a reduction of migrating NCCs, on line with a previous study showing similar results (Su et al., 2009). At a first sight, this observation appears to contradict the fact that Zic2 induces neural crest cell

delamination at the time that induces Draxin. However, it is likely that Zic2 induces Draxin to block the canonical Wnt signaling but Draxin does not have an additional function in delamination, although other Zic2 targets do. This would explain that Draxin expression at early stages, turns off the canonical Wnt pathway stopping proliferation and producing a reduced number of NCCs.

Despite these interesting results, further experiments are needed to unveil the relationship between Zic2 and Draxin during NCCs. It will be also interesting to investigate whether Zic2 regulates Draxin in other contexts where Zic2 is expressed such as the retinal ganglion cells, spinal cord neurons or cerebellar cells.

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CONCLUSIONS

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CONCLUSIONS

1. Zic2 is expressed in premigratory neural crest cells and downregulated when they are already migrating.

2. Zic2 is necessary for neural crest cells delamination from the neural tube

3. Zic2 is sufficient to induce neural crest cells exit and is able to alter their migration pathways

4. Zic2 does not induce neural crest cells proliferation or determines melanocytic or sensory neural crest cells fate

5. EphA4 may be a Zic2 target molecule during the formation of the neural crest

6. Draxin is a novel Zic2 effector during neurulation stages.

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CONCLUSIONES

1. Zic2 se expresa en células premigratorias de la cresta neural y pierde la expresion cuando las celulas ya están migrando.

2. Zic2 es necesario para la delaminación de las células de la cresta neural.

3. Zic2 es suficiente para inducir la salida de las células de la cresta neural y puede alterar sus rutas de migración.

4. Zic2 no induce la proliferación de las células de la cresta neural, ni determina su diferenciacion en melanocítos o neurones sensoriales.

5. EphA4 puede ser una molécula diana del Zic2 durante la formación de la cresta neural.

6. Draxina es un nuevo efector de Zic2 durante la formacion de la cresta neural.

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ANNEX

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ANNEX (ARTICLE)

Uncoupling of EphA/ephrinA Signaling and Spontaneous Activity in Neural Circuit Wiring

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Classic studies have proposed that genetically encoded programs and spontaneous activity play complementary but independent roles in the development of neural circuits. Recent evidence, however, suggests that these two mechanisms could interact extensively, with spontaneous activity affecting the expression and function of guidance molecules at early developmental stages. Here, using the developing chick spinal cord and the mouse visual system to ectopically express the inwardly rectifying potassium channel Kir2.1 in individual embryonic neurons, we demonstrate that cell-intrinsic blockade of spontaneous activity *in vivo* does not affect neuronal identity specification, axon pathfinding, or EphA/ephrinA signaling during the development of topographic maps. However, intrinsic spontaneous activity is critical for axon branching and pruning once axonal growth cones reach their correct topographic position in the target tissues. Our experiments argue for the dissociation of spontaneous activity from hard-wired

developmental programs in early phases of neural circuit formation.

Introduction

The formation and refinement of vertebrate neural circuits involve neural identity specification, axon targeting, and synaptogenesis, processes that are primarily controlled by hard-wired developmental programs. However, developing neurons exhibit spontaneous electrical activity (Spitzer, 2006), and the extent of its influence over genetically encoded developmental mechanisms is currently under debate.

The classical view posits that activity-independent and activity-dependent programs sequentially regulate different aspects of neural development (Katz and Shatz, 1996; Er-zurumlu and Kind, 2001). However, recent evidence suggests that spontaneous activity may be more of a critical player at earlier developmental stages than previously thought, influencing the expression and function of transcription factors and axon guidance molecules as well as affecting the concentration of axon guidance receptor secondary messengers such as calcium or cAMP (Nishiyama et al., 2003; Hanson and Landmesser, 2004; Nicol et al., 2007). The representative experiments that raised this idea examined the impact of spontaneous activity on the repulsive signaling mediated by the tyrosine kinase receptor EphA and its ephrinA ligand in both the developing chick spinal motor neurons and the mammalian visual system. Limb-innervating motor neurons of the spinal lateral motor column (LMC) are segregated into dorsal limb muscle-innervating lateral LMC motor neurons and ventral limb muscle-innervating medial LMC motor neurons. The binary decision of motor axon projection to the ventral or to the dorsal limb mesenchyme is mediated, at least in part, by the repulsive signaling from ephrins expressed in the limb to axonally expressed EphA4 receptors (Kao et al., 2012). These motor neurons are electrically active even before they form synapses with their target muscles (O'Donovan and Landmesser, 1987), and it has been proposed that this spontaneous activity may influence their guidance by modulating the expression and/or function of axon guidance receptors such as EphA4 (Hanson and Landmesser, 2004). In the visual system, the classical view proposes that EphA/ephrinA signaling and spontaneous activ-

ity in the form of retinal waves act independently and sequentially to form the retinotopic map in the visual targets (McLaughlin et al., 2003; Pfeiffenberger et al., 2006; Cang et al., 2008). However, a set of in vitro experiments challenges this view and suggests that early spontaneous activ-ity might be essential for the action of EphA/ephrinA signaling (Nicol et al., 2007).

In the last few years, these and other results have stirred the debate on the influence of spontaneous activity in early stages of development, particularly its effects on EphA/ephrinA-mediated signaling. Here, we analyze in vivo the contribution of spontaneous activity in early (neural specification and pathfinding) and late (topography and axonal refinement) phases of development in two topographically organized systems, the chick spinal motor neurons and the mouse visual system. Our results show that ec-topic expression of EphA is sufficient to change axon trajectories and mapping, even in the absence of neural activity, and that activity is, in turn, needed for local axon branching and pruning. These results demonstrate that, in the developing motor and vi-sual systems, EphA/ephrinA signaling and neural activity interact in a very limited manner.

Materials and Methods

Animals. ICR female mice obtained from a timed pregnancy-breeding colony at the Instituto de Neurociencias (IN) were used in all the visual system experiments. E0 was defined as midnight before a plug was found. Animal protocols were approved by the IN Animal Care and Use Committee and National and European laws. Fertilized chick eggs (Couvoir Simetin) were stored for a maximum of 1 week at 18°C, then incubated at 38°C and staged according to standard protocols (Hamburger and Ham-ilton, 1992).

In ovo electroporation. Chick spinal cord electroporation of expression plasmids was performed at Hamburger and Hamilton stage (HH st.) 18/19, generally as described previously (Kao et al., 2012). In brief, a 5 mg/ml solution of plasmid DNA in distilled water was injected into the lumbar neural tube through a small eggshell window under a Discovery V12 stereomicroscope (Zeiss). Lower bodies of chick embryos were then electroporated with platinum/iridium electrodes (FHC) and the TSS20 Ovodyne electroporator (Intracel; settings: 30 V, 5 pulses 50 ms wide in a 1 s interval). Shell windows were sealed

with Parafilm (Pechiney Plastic Packaging Company) and incubated at 37°C until harvesting at HH st. 29/30. The efficiency of electroporation varied between 5 and 30% of total LMC neurons electroporated, depending on the construct and DNA concentration used.

HRP retrograde labeling of motor neurons. Retrograde labeling of chick motor neurons (MN) was performed as described previously (Luria et al., 2008). In brief, chick HH st. 29/30 embryos were harvested and incubated in Tyrode's solution (140 mM NaCl, 3 mM KCl, 17 mM NaHCO3, 12 mM glucose, 2 mM CaCl2, 1 mM MgCl2; Fisher Scientific) at room temperature, and were aerated with 95% oxygen and 5% CO2. The retrograde tracer used was a 20% solution of HRP (Roche) made by dissolving 100 mg of HRP in 450 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4) with 50 ml of 10% lysophosphatidylcholine (Fluka) in PBS. The HRP solution was injected into either dorsal or ventral hindlimb shank musculature of chick embryos; embryos were incubated in oxygenated Tyrode's solution at 30°C for 5 h before fixation.

Immunostaining. Sectioned tissue was first washed in PBS, incubated in blocking solution (1% heat-inactivated horse serum in 0.1% Triton-X/PBS; Sigma) for 5 min, followed by incubation overnight at 4°C in selected primary antibodies diluted in blocking solution. The following primary antibodies were used: mouse anti-Isl1 (1:100; Tsuchida et al., 1994); mouse anti-Lim1/2 (1:100; Tsuchida et al., 1994); goat anti-HRP (1:2000; Jackson ImmunoResearch Laboratory); rabbit anti-EphA4 (1: 500; Santa Cruz Biotechnology); guinea-pig Foxp1 (1:1000; gift from Dr. Bennett Novitch, UCLA, Los Angeles, CA); and mouse anti-neurofilament (1:100; DSHB). After incubation in primary antibodies, samples were washed with PBS and incubated with appropriate secondary antibodies for 1 h at room temperature. Secondary antibodies manufactured by either Jackson ImmunoResearch (CY5) or Invitrogen (Alexa Fluor 488 and Alexa Fluor 568) against appropriate species were used at 1:500. The protocol for tissue clearing described in Kuwajima et al. (2013) was followed to acquire images from wholemount limbs of chick embryos containing EGFP or Kir2.1/EGFP-expressing axons.

Image acquisition, quantification, and statistical analysis of motor neuron data. Images were acquired using either a Zeiss LSM 700 or LSM 710 confocal microscope with Zen imaging software (Zeiss). For analysis of EphA4 expression levels, a

region of interest that covered Lim1, Foxp1, and Kir2.1/EGFP neurons was drawn. This drawn region was fit into the Lim1, Foxp1 region of the unelectroporated side. The mean pixel intensity of the electroporated and unelectroporated sides of the spinal cord was then calculated using ImageJ (NIH Image), and a ratio of these two values was quantified per section. Similar methods were used to quantify EphA4 levels in axons, but the region of interest was defined by neurofilament staining of the spinal ventral root. To assay misprojections, motor neurons that were GFP, HRP, and either Lim1 or Isl1 were quantified by combining cell counts of a series of spinal cord section images (5–30 12 mm sections from each embryo) using Photoshop (Adobe). Data from the experimental replicate sets were evaluated using Prism. Means of the combined proportions or cell numbers were compared with two-tailed, unpaired Student’s t tests with the threshold for statistical significance set at 0.05.

Ex vivo calcium imaging in chick spinal cord. Chick embryos were co-electroporated with either G-GECO and Kir2.1/mCherry under control of the CMV promoter or R-GECO, and either EGFP, under the control of the Hb9 promoter-enhancer, or Kir2.1/EGFP, under the control of the CAG promoter, at a ratio of 1:5, as described above, then incubated untilHH St. 29/30. Lumbar spinal cords were then dissected and placed ven-tral side down into an imaging chamber with circulating oxygenated Tyrode’s solution (described above) held at 30°C. Spinal cords were im-aged at 1 Hz for 10 min using a Zeiss inverted microscope with a Yok-ogawa spinning disk confocal system installed. Calcium activity was assessed using ImageJ (NIH) and a custom MATLAB (Mathworks) algorithm that looks for large changes in calcium indicator fluorescent inten-sity, defined here as “events.”

DNA plasmids, in utero electroporation, immunohistochemistry, and qRT-PCR in the retina. The coding sequences of human Kir2.1 and mouse EphA6 were cloned in a plasmid to drive their expression under the potent general CAG promoter. All the in-utero electroporation exper-iments were performed by electroporating CAG-EGFP and CAG-Kir2.1iresEGFP (Kir2.1/EGFP) as described by Garcia-Frigola et al. (2007), except those in Figure 5 in which the concentrations were 0.2 and 0.5 mg/ml, respectively, for CAG-EGFP and Kir2.1/EGFP. Mice were perfused with 4% paraformaldehyde in PBS. Cuts were made in the

retina to maintain orientation before wholemounting. Vibratome sections (80 mm) from the SC of electroporated mice were washed in PBS-0.1% Tri-ton X-100 (PBT) and incubated for 2 h at room temperature with 1% BSA-PBT and 10% horse serum. Sections were then inmunostained with rabbit anti-GFP (1:2000; Abcam) and mounted serially. Retinas and sections were photographed using a TCS SP2 AOBS Laser Scanning Confocal Microscope (Leica Microsystems). qRT-PCR for EphA6 and EphA5 was performed using protocol and primers as previously described (Carreres et al., 2011). To specifically detect the human Kir2.1 introduced by in utero electroporation, the following primers were used to perform qRT-PCR: forward, AAC-CAACCGCTACAGCATCGT; reverse, TTCT TCACAAAGCGGCTCCTG. Anti-Kir2.1 antiserum (Abcam; 1:800) was used to confirm protein expression.

Ex vivo calcium imaging in the retina. Retinas from embryos coelectroporated with CAG-DsRed and CAG-Kir2.1 plasmids were bulk loaded with the calcium indicator Fluo4 (Invitrogen) using a variant of the multicell bolus loading technique (Stosiek et al., 2003). Fluo4 solution was injected into the vitreous chamber with a micropipette in anesthetized mice. Up to four injections were applied to each animal. Mice were placed on a warm surface at 37°C for up to 3 h and anesthetized for decapitation and retina extraction. Retinas were placed in Ringer's solution containing 135 mM NaCl, 5 mM KCL, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, and then mounted with the RGC layer facing upward on a cellulose nitrate membrane while applying a vacuum to have direct access to the RGC layer. Epifluorescent calcium imaging was performed on a DM LFSA microscope (Spectra-Physics; KMC 100 immersion cooler, Neslab) using a 10 or 20 water-immersion objective with illumination provided by a Leica TCS resonant scanner. Calcium transients were identified in 3 min epochs, using a custom MATLAB (MathWorks) algorithm, which looks for large changes in calcium indicator fluorescent intensity, defined here as events. Analysis of targeted axons in the optic chiasm and in the SC.

To quantify the number of CAGEGFP- or CAG-Kir2.1/EGFP-expressing axons at the optic chiasm region, square bins were superimposed on the width of the labeled optic nerve proximal to the chiasm and on the contralateral optic tract. Fluorescence intensity within the bin covering the optic nerve [optic

nerve fluorescence intensity (ONFI)] and the optic tract contralateral to the electroporated side [optic tract fluorescence intensity (OTFI)] was measured using ImageJ software and standard methods described by Herrera et al. (2003). These two fluorescence measures were normalized to the fluorescence intensity in the corresponding electroporated retina [retinal fluorescence intensity (RFI)]: ONFI/RFI and OTFI/RFI. To quantify the number of CAG-EGFP- or CAG-Kir2.1/EGFP-expressing axons in the SC of postnatal day 0 (P0) mice, circular bins were superimposed over the entire SC area. Fluorescence intensity within each bin SC [SC superior colliculus fluorescence intensity (SCFI)] was measured using ImageJ software. Fluorescence measures were normalized to the fluorescence intensity in the corresponding electroporated retina (RFI): SCFI/RFI. To quantify electroporated retinal axons in the SC of P9 mice, three reconstructed serial sections from each animal were conformed to a single common template of the SC. The common template represents a sagittal slice cut through the center of the SC at a mediolateral coordinate where the dorsal layer is relatively homogeneous and flat. The common template was chosen to minimize the mean square distortion arising from piecewise-linear conformation of tissue sections from individual animals. Sections were linearly scaled to the mean thickness of the superficial layer of the SC, and fluorescence levels were measured along the rostrocaudal axis of the SC. Collicular sections were divided in 124 homogeneous bins to gauge the spatial distribution of the terminal arbors. We used a bin-by-bin one-way ANOVA to quantify the differences between the distributions of fluorescence intensity. Individual axons in electroporated P9 mice were reconstructed using an Imaris work station, and width, density, and distance to the SC surface were measured.

Results

Motor neuron specification and axon pathfinding are activity-independent processes

To determine whether intrinsic electrical activity is required for neural specification and axon navigation, we first focused on LMC neurons, which have been used to study the role of neural activity in early neuronal development and whose molecular identity is linked to myotopically organized axon pro-

jections (Tsuchida et al., 1994; Kania and Jessell, 2003; Hanson andLandmesser, 2004, 2006; Hanson et al., 2008; Plazas et al., 2013). We first examined how overexpression of the inwardly rectifying potassium channel Kir2.1, previously used in other systems (Burrone et al., 2002; Yamada et al., 2010), affected spontaneous activity in developing chick LMC neurons. Mixtures of plasmids encoding either the R-GECO or G-GECO calcium indicators (Zhao et al., 2011) alongside those encoding EGFP driven by the Hb9 promoter (Hb9-EGFP) or Kir2.1 driven by either the strong CAG promoter enhancer (Fig. 1A, Kir2.1/EGFP) or the weaker CMV promoter enhancer (Kir2.1/mCherry) were coelectroporated into the chick spinal cord at HH st. 18/19, and the neural activity of LMC neurons was assayed at HH st. 29 (Hamburger and Hamilton, 1992). Neurons expressing Hb9-EGFP were robustly active (1.6 events/min), but expression of the CAG-driven Kir2.1 (Kir2.1/EGFP) almost entirely blocked activity in chick neurons (0.05 events/min), although CMV-driven expression (Kir2.1/mCherry) was not sufficient to block activity (1.53 events/min; Fig. 1B, C). These data demonstrate that overexpression of Kir2.1/EGFP from the CAG promoter enhancer is sufficient to block spontaneous activity, allowing us to examine how this activity influences the development of LMC neurons in a cell-autonomous fashion.

The myotopically relationship between LMC neurons and their limb targets is revealed by the differential expression of LIM homeodomain transcription factors Lim1 and Isl1. Lateral LMC neurons express Lim1 and innervate dorsal limb muscles, while medial LMC neurons express Isl1 and innervate ventral limb muscles (Tsuchida et al., 1994). Because pharmacological manipulation of neuronal activity appears to affect the expression of Isl1 and Lim1 in motor neurons (Hanson and Landmesser, 2004, 2006), we reasoned that spontaneous activity may be important for the specification of LMC neuron molecular identity and therefore that blocking activity with Kir2.1/EGFP might alter the numbers of LMC neurons that express Isl1 or Lim1. To assess this, we electroporated chick embryos, as described above, and monitored the expression of the general LMC neuron marker Foxp1 (Dasen et al., 2008; Rousso et al., 2008), alongside Lim1 and Isl1 as markers of medial and lateral LMC neurons, respectively. The number of Foxp1 neurons did not differ significantly between embryos electroporated with EGFP and Kir2.1/EGFP (79 Foxp1 cells per LMC section in EGFP vs 70 in Kir2.1/EGFP-electroporated embryos; Fig. 1 D, E). In

EGFP-expressing spinal cords, 44% of Foxp1, EGFP LMC neurons expressed Lim1 (Fig. 1 D, E). Similarly, in Kir2.1/EGFP-expressing embryos, 48% of FoxP1, EGFP LMC neurons expressed Lim1 (Fig. 1 D, E). The proportion of Isl1-expressing LMC neurons was not different between the two groups either (52% in EGFP LMC neurons and 50% in Kir2.1/EGFP LMC neurons; Fig. 1E), demonstrating that LMC identity is not altered by spontaneous activity blockade.

Previous studies have demonstrated that a global, pharmacologically induced decrease in activity lowers the expression of Eph receptors in LMC neurons and leads to LMC axon guid-ance errors (Hanson and Landmesser, 2004; Kastanenka and Landmesser, 2010), raising the question of whether blocking neural activity in a cell-autonomous manner might have a similar effect. Chick embryos were electroporated as described above with either EGFP or Kir2.1/EGFP, and the expression of EphA4 was analyzed in EGFP, Lim1 LMC neurons at HH st. 29 (Fig. 1F). EphA4 expression levels, measured as fluorescence intensity in regions containing high numbers of EGFP, Lim1 neurons on the electroporated side, were compared with corresponding regions on the unelectroporated side. The ratio between EphA4 signal on the electroporated and unelectroporated sides in EGFP-expressing embryos was 1.002:1, which was not significantly different from the ratio in Kir2.1/EGFP-expressing embryos (1.07: 1), demonstrating that blockade of activity does not alter EphA4 expression in the LMC cell body. Axonal EphA4 expression was assessed in a similar fashion, in spinal ventral roots showing robust EGFP expression. The electroporated/unelectroporated ratio was not significantly different: 0.95:1 in EGFP-treated embryos, and 1.02:1 in Kir2.1/EGFP-treated embryos. Thus, cell-autonomous spontaneous activity blockade does not affect EphA4 protein expression on cell bodies or axons (Fig. 1 F, G).

We next asked whether, as previously suggested, spontaneous activity is important for the guidance of LMC axons, possibly by modulating EphA4 function (Hanson and Landmesser, 2004). Thus, we examined the limb trajectories of LMC axons expressing either EGFP or Kir2.1/EGFP in embryos electroporated with expression constructs, as described above. Termini of EGFP axons in EGFP and Kir2.1/EGFP were found at a similar distance from the spinal cord as those of neurofilament-expressing unelectroporated axons, and in untreated embryos (Fig. 2A; data

not shown), suggesting that spontaneous activity is not required for LMC axon outgrowth. Next, we examined whether lateral and medial LMC neurons select their appropriate limb nerve trajectory in the absence of spontaneous activity. To do this, we injected the retrograde tracer HRP into the dorsal or ventral limb of electroporated embryos, and determined the molecular identity of labeled LMC neurons. If spontaneous activity is important for limb nerve selection by LMC axons, then dorsal limb injections are expected to label a significant number of medial LMC neurons that normally innervate the ventral limb, and ventral limb injections are expected to label a significant number of lateral LMC neurons that normally innervate the dorsal limb. In embryos with a ventral limb HRP injection, the proportion of all EGFP, HRP LMC neurons that were also Lim1 was not significantly different between EGFP- and Kir2.1/EGFP-electroporated embryos (4% and 7%, respectively; p 0.05, Student's t test; Fig. 2B). Similarly, in embryos with a dorsal limb HRP injection, the proportion of all EGFP, HRP LMC neurons that were also Isl1 was not significantly different between EGFP- and Kir2.1/EGFP-electroporated embryos (6% for both; p 0.05, Student's t test; Fig. 2C). Together, these results argue strongly that the molecular specification of LMC neurons and the selection of axon trajectory in the limb occur independently of spontaneous activity.

Universitas Miguel Hernández

Spontaneous activity is dispensable for RGC axon pathfinding and SC targeting

We next probed, in vivo, the role of spontaneous activity in the development of graded topographic projections of RGC neurons, where it has been previously suggested to be important in an in vitro setting (Nicol et al., 2007). Because spontaneous activity has been reported in the embryonic retina (Galli and Maffei, 1988), we first analyzed whether activity plays a role in the processes that occur between embryogenesis and birth such as RGC axon guidance in the optic nerve and chiasm or targeting to the SC. Using in utero electroporation, Kir2.1/EGFP- or EGFP-encoding plasmids were ectopically expressed in mouse RGCs at embryonic day 13 (E13) and their axonal projections analyzed at two different stages: at E16, when most axons leave the optic chiasm; and at P0, upon axon arrival to the SC. Ectopic expression of Kir2.1 and functional blockade of spontaneous activity were confirmed in electroporated RGCs, and calcium imaging experiments demonstrated

that, as in the spinal cord, Kir2.1 expression blocks activity in retinal cells (0.7 events/min in control vs 0 events/min in Kir2.1/EGFP-expressing cells; p 0.001; Fig. 3A–C).

We then examined the behavior of RGC axons within the optic chiasm. Quantification of the number of axons expressing Kir2.1/EGFP visualized at the optic chiasm normalized to the number of electroporated RGC in the retina showed no significant differences in growth or projection patterns when co pared with control axons expressing EGFP (p 0.42 for measures at the optic nerve and p 0.27 for measures at the optic tract; Fig. 3D). Upon arrival at the SC, rather than directly targeting their correct topographical site, RGC axons initially overshoot their prospective termination zone (TZ) and extend caudally. Comparison of SCs from P0 mice electroporated with Kir2.1/EGFP to those electroporated with EGFP revealed no significant differences in timing or quantity of axons reaching the caudal SC of newborn mice (p 0.44; Fig. 3E). This demonstrates that the blockade of RGC activity at early stages of development does not affect the pathfinding of their axons, or their arrival at the optic chiasm or the SC; therefore, it is highly unlikely that the expression of, and the signaling mediated by, the guidance molecules controlling these processes require spontaneous activity.

EphA can redirect RGC axons in the absence of spontaneous activity

We next asked whether the expression and function of EphA/ephrinAs requires spontaneous activity. First, we tested whether the expression of EphA receptors is affected by activity blockade. In the mouse retina, the only members of the EphA family ex-pressed in a low-nasal to high-temporal gradient that control the establishment of topographic maps are EphA5 and EphA6 (Feld-heim et al., 2004; Reber et al., 2004; Carreres et al., 2011). It has been already shown that the expression of EphA5 does not change after postnatal injections of epibitadine into the eye or after treatment with tetrodotoxin (Pfeiffenberger et al., 2005; Nicol et al., 2007). However, the expression of neither EphA5 nor EphA6 receptors has been analyzed after activity blockade at early embryonic stages, when these molecules are already highly expressed in the temporal retina. To investigate whether activity may alter the expression of EphA5/A6 receptors at early stages, retinas

electroporated at E13 with Kir2.1/EGFP or EGFP expression plasmids were isolated to measure the levels of EphA6 and EphA5 mRNAs by qRT-PCR. DNA primers targeting Kir2.1 mRNA were used as controls to be sure that ectopic Kir2.1 was induced in the tested samples. While the levels of Kir2.1 mRNA increased 10% in retinas electroporated with Kir2.1/EGFP plasmids compared with those electroporated with EGFP, the levels of EphA5 and EphA6 mRNA were similar to those electroporated with EGFP plasmids, confirming that spontaneous activity is not required to induce EphA5 or EphA6 mRNA (Fig. 4F) and that the lack of activity does not affect endogenous retinal EphA transcription.

Given that axon guidance receptors relevant to RGC targeting were normally expressed in the absence of activity, we asked whether activity is required for RGC axon guidance, possibly at the level of EphA receptor signaling. After birth, following initial target overshoot, RGC axons in the caudal SC retract to their topographically accurate TZ and form interstitial branches rostral to it (Nakamura and O'Leary, 1989; Simon and O'Leary, 1992). Thus, during this initial phase, the precise TZ where individual axons will arborize in superficial layers is determined (Phase 1: retraction/topography phase). Then, complex arbors develop at the TZ, followed by the elimination of major segments of RGC axons that are distal to the TZ, together with branches and arbors that formed at topographically incorrect positions (Phase 2; remodeling phase; Nakamura and O'Leary, 1989). We reasoned that according to the classical model of activity-independent axon guidance, Kir2.1 expression should only affect the remodeling phase of RGC termination in the SC. In contrast, if activity were important for the retraction/topography phase, silencing RGC activity with Kir2.1 expression should result in a TZ position shift. To resolve between these alternatives, terminals of axons expressing Kir2.1/EGFP or EGFP were analyzed in the SC of P9 mice. Both EGFP- and Kir2.1/EGFP-expressing axons projected to equivalent central areas of the SC. The distance of the TZ (TZd; peak of fluorescence) to the rostral SC was 52% of the total SC length for EGFP-expressing axons and 54% for Kir2.1/EGFP-expressing axons (p 0.2), although the termini distribution at the TZ of Kir2.1/EGFP-expressing mice was wider than that of controls (half-width– half-height of the fluorescence peak; 16% vs 21% of total SC length, respectively; p 0.0004; Fig. 4A).

We then asked whether signaling downstream of EphA/ephrinAs is modulated by activity by testing whether a phenotype caused by overexpression of Eph receptors can be suppressed by activity blockade. Ectopic electroporation of EphA6 in embryonic central retina resulted in RGC axonal projection to rostral collicular areas (TZd, 5%; Fig. 4C; Carreres et al., 2011) after overshooting their normal TZ at birth (Fig. 4B). Axons expressing Kir2.1/EGFP and EphA6 also projected to the rostral colliculus (TZd, 4%), a behavior not significantly different (p 0.6) from that of axons expressing only EphA6/EGFP (Fig. 4C–E). Together, our findings demonstrate that RGC axon guidance and EphA/ephrinA signaling occur normally in the absence of spontaneous activity.

Spontaneous activity is essential for axon arborization and pruning at the RGC terminal zone

Activation of EphA/ephrinA signaling in axon retraction and establishment of the TZ do not depend on spontaneous activity. However, as in previous results reporting defects in collicular organization of RGC terminals in the absence of spontaneous activity (McLaughlin et al., 2003; Dhande et al., 2011), we found that in the SC of P9 mice, the termini of axons expressing Kir2.1/ EGFP were more dispersed and showed lower fluorescence intensity compared with the controls (Fig. 4A), suggesting a significant change in their morphology and indicating that spontaneous activity is important for RGC mapping. To define the precise function of spontaneous activity in this process, we examined the axonal arbor structure of individual RGCs in the SC (see Experimental procedures). Reconstruction analysis revealed that arbors of Kir2.1/EGFP-expressing axons occupy a wider TZ area (arbor width: 276 mm) that is deeper relative to the surface of the SC (length to the surface: 102 mm) than in controls (arbor width: 198 mm; length to the surface: 30 mm; p 0.007 and p 0.002, respectively; Fig. 5 A, B). In general, Kir2.1/EGFP-expressing arbors were less elaborate (arbor density: 19%) than those from cells expressing EGFP (arbor density: 56%; p 0.001); however, the point along the rostrocaudal axis of the SC at which axons establish their TZ was located at a similar distance from the rostral SC in both conditions (Figs. 4, 5 A, B). Collaterals outside the terminal zones were rarely found in Kir2.1/EGFP- or in EGFP-electroporated axons. These results clearly demonstrate a role of spontaneous activity in the formation of the visual

circuits at the level of local pruning and branching of RGC axons once they reach their TZ.

Discussion

For decades, experimental and computational studies have suggested that genetic factors and spontaneous activity play complementary but independent roles in the development of precise neural circuits and topographic maps. More recent experimental evidence suggests that these two mechanisms could interact extensively, with spontaneous activity regulating earlier events during the development of neural circuits. The in vivo results presented here in a setting that comprises all steps of neural development from early neural specification to late refinement processes, demonstrate that in birds and mammals, early and late phases of topo-graphic map development differ markedly in their requirement for spontaneous activity. Activity does not play a significant role in neural specification, axonal pathfinding decisions, or gross topography but is fundamental at a later stage to properly refine the axonal terminals once at the termination zone in the target tissues. Furthermore, our results in chick and mouse dis-engage the postulated coupling of EphA/ephrinA signaling to spontaneous activity in axon guidance and initial map formation.

Spontaneous activity in early stages of circuit formation

The improved techniques that detect modest changes in Ca 2 levels have been used as surrogate indicators of embryonic neural activity (Goodman and Spitzer, 1981; Maffei and Galli-Resta, 1990). The current robust evidence on the existence of early neural activity raised the possibility that different developmental processes such as cell specification, cell migration, axonal growth rate, axon pathfinding, and axon targeting could be also instructed by spontaneous activity (Spitzer, 2006). Thus, in recent years, spontaneous activity has been postulated as an important mechanism that may modulate early developmental stages in different brain systems and species. In contrast to this mounting evidence, we find that silencing neural activity in individual LMC neurons or RGCs does not alter the neuronal fate or axon guidance decisions of LMC

neurons, or affect the choices of visual axons in decision points such as the optic chiasm.

The discrepancy between our observations and the previous experiments in chick motor neurons could be attributed to the method used to block spontaneous activity; we studied the effect of the expression of Kir2.1 at the level of single cells, whereas Hanson and Landmesser (2004) used a pharmacological treatment using picrotoxin. Picrotoxin inhibits GABAA receptors, and it is known that GABA can act as an axon guidance cue (Ferguson and McFarlane, 2002). Therefore, it is possible that picrotoxin treatment influences axon guidance processes via GABA signaling rather than through activity inhibition. However, more recently, it has been shown that blocking activity in individual zebrafish motor neurons by ectopic expression of Kir2.1 causes pathfinding errors as well (Plazas et al., 2013). One critical difference between these results and ours is the influence of the species-specific embryonic environment. In our study, we examined mouse and chick embryos, which are cosseted in a very highly regulated environment to develop into terrestrial animals. In contrast, most of the experiments in which activity seems to influence early developmental events have been performed in organisms that develop and subsequently live in aquatic environments, which may be subject to changes in salt concentration (Nishiyama et al., 2003; Demarque and Spitzer, 2010; Plazas et al., 2013). By allowing the environment to influence the number of excitatory and inhibitory neurons in the nervous system as in the study of Borodinsky and Spitzer (2007), aquatic embryos may be able to adapt the wiring of their nervous system to their environment more readily than avian or mammalian embryos.

In mammals, the loss of neural activity has been reported to affect the expression of axon guidance molecules. In the embryonic thalamus, for example, ectopic expression of Kir2.1 increases the expression of Robo1 receptor (Mire et al., 2012). It is possible that the expression of some families of guidance molecules, such as Robo/Slits, is more sensitive to neural activity than others such as EphA/ephrinA. However, Robo receptors are essential for RGC axon guidance (Plump et al., 2002; Thompson et al., 2009), and since we do not find defects in RGC innervation of the SC in Kir2.1-expressing RGC axons until they navigate to the correct TZ, it is unlikely that this is the case. An alternative explanation for this apparent neural type-specific requirement of activity for guidance molecule

UNIVERSITAS Miguel Hernández

expression is that expression and/or function of some guidance molecules may rely on activity synchrony. In some developing neural circuits, spontaneous activity is initially nonsynchronous, meaning that neighboring neurons that eventually innervate adjacent targets do not exhibit simultaneous trains of activity. If activity patterns bear information instructive for axon guidance, neurons with asynchronous activity might misinterpret important guidance cues. Eventually, activity patterns become organized and synchronized, and in a system where this happens late in development such as the mouse visual system or the chick motor system, pathfinding could be uncoupled from electrical activity to prevent the stochastic nature of early asynchronous activity from causing a high level of heterogeneity of axon guidance fidelity. Thus, in systems with robust synchrony of activity at earlier stages, of which the thalamus may be an example, activity could play an essential role at earlier developmental stages.

Spontaneous activity in topography and map formation

In vitro studies suggested that during the establishment of retinocollicular topography spontaneous activity must be present for the correct functioning of EphA/ephrinA signaling (Nicol et al., 2007). However, blocking activity with tetrodotoxin (TTX) does not affect EphA transcription (Nicol et al., 2007), and we now show that activity is not required to modulate EphA/ephrinA signaling in vivo either. How can we reconcile the apparent discrepancy concerning the role of activity in the establishment of visual topography between past in vitro and our current in vivo results? First, there are significant differences between using TTX in culture and delivering Kir2.1 specifically in RGCs in vivo to block activity. TTX abolished spontaneous activity in the whole coculture, including the retinal explants and the SC slices. The use of Kir2.1, however, allows the blocking of spontaneous activity specifically in a restricted population of RGCs, leaving the rest of the retinal population and the SC unaffected. Second, the organotypic retinotectal cocultures used in the in vitro experiments corresponded to different retinal and collicular stages. E15 retinas were confronted to P6 SC slices, which makes it difficult to interpret the results since retinal axons at this early stage may not be ready to respond to signals from a more mature SC. Third, the ability to distinguish between the first (topography) and the second phase (remodeling) of the map maturation process using cocultures in vitro is very poor when

compared with our in vivo approach, with which we can easily visualize and distinguish the retraction of the principal axon from axonal pruning and arborization in the correct TZ. Therefore, our in vivo results using a much more reliable approach demonstrate that activity is only required at later phases of development once retinal axons start to establish connections with other neurons in the SC.

Recent reports have shown that in newborn mice, retinal waves originate preferentially in the binocular region, the ventral–temporal retina, and propagate following a biased direction toward the dorsal–nasal retina (Ackman et al., 2012). This finding, although compatible with a role for retinal waves in topography, suggests an important function for spontaneous activity in eye-specific refinement. b2KO mice exhibit dramatically enlarged axonal arbors that fail to refine in the SC but exhibit a nearly normal topography (Dhande et al., 2011), and genetically modified mice with altered patterns of retinal activity exhibit defects in eye-specific refinement but an approximately normal rostro-caudal topography (Xu et al., 2011). Although none of these studies directly test a possible relationship between the ex-pression of EphA/ephrinAs and spontaneous activity in the formation of topographic maps, they favor the idea of a prominent role for spontaneous activity in eye-specific segregation, which depends on fine-tuning of axonal terminals, rather than on topography.

In summary, our in vivo results clarify the long-standing debate over the precise roles of EphA/ephrinA signaling and spontaneous activity in neural circuit wiring, and finally confirm, at least in the motor and visual systems, the model initially pro-posed by Goodman and Shatz (1993) in which activity-independent mechanisms are sufficient to generate a coarse topographic map relying exclusively on molecular cues, whereas the fine-tuning of this map requires subsequent patterned activity.