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Vsx1 and Chx10 paralogs sequentially secure V2 interneuron identity during spinal cord development

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Summary

Locomotion is a complex behavior regulated by circuits, named central pattern generators, located in the ventral part of the spinal cord. These circuits are composed of motor neurons (MNs) and of different populations of interneurons: dorsal dI6 and ventral V0 to V3 cardinal populations. During development, they are produced from specific progenitor domains distributed along the dorso-ventral axis of the spinal cord. The p2 progenitor domain generates V2 interneurons that diversify at least into five populations: V2a, V2b, V2c, V2d and Pax6-V2 interneurons. This differentiation process involves fine genetic regulations and cross-repressive mechanisms that consolidate cell fate. Indeed, progenitor domains of V2 interneurons and of MNs are closely adjacent during development and share some molecular determinants. Cross and mutual repressions between *Ceh-10 homeobox* (*Chx10)* gene, expressed in differentiating V2a interneurons, and *Homeobox 9* (*Hb9*), expressed in early MNs, participate in the consolidation of V2 interneuron versus MN identity by preventing the activation of inappropriate differentiation program. However, *Chx10* starts to be expressed in differentiating V2a interneurons. Therefore, we could address the following question: which factor secures the identity of V2 interneurons at early stages of development. Studies demonstrate that the unique paralog gene of *Chx10*, the *Visual System homeoboX 1* (*Vsx1*), is also present in V2 interneuron compartment in a more medial part of the spinal cord. The aim of my thesis project is to characterize the expression profile of *Vsx1* in the spinal and determine whether it may, in collaboration with its paralog *Chx10*, contribute to the securisation of V2 interneuron identity.

In this work, we show that Vsx1 transiently labels an intermediate V2 precursor compartment. We provide evidence that this transcription factor is not necessary for V2 interneuron production but contributes to their development. We show that

the unique paralog factor of Chx10 identically prevents motor neuron differentiation in early V2 precursors. Furthermore, combined inactivation unveiled cooperativity between Vsx1 and Chx10 although they are not produced in the same cells.

Abbreviation list

Ascl1	Achaete-Scute Complex-Like 1
BC	Bipolar Cell
Bcl2	B cell lymphoma 2
BHLH	Basic-Helix-Loop-Helix
BMP	Bone Morphogenic protein
Cabp5	Calcium binding protein 5
CBP	histone acetyl transferase CREB binding protein
ChAT	Cholin acetyl transferase
Chx10	Ceh-10 homeobox
ChIP	Chromatin Immunoprecipitation
ChIPseq	Chromatin immunoprecipitation sequencing
CPG	Central Pattern Generator
CR1	Conserved Region 1
Dbx1/2	Developing brain homeobox 1/2
dI	dorsal interneuron
DMRT3	Doublesex and Mab-3 Related Transcription factors
Dll4	Delta-like ligand 4
dpf	Days post fertilization
E10.5	Embryonic day 10.5
En1	Engrailed 1
ERG	Electroretinogram
Evx1	Even-skipped homeobox 1
FACS	Fluorescent Activated Cell Sorting
FGF	Fibroblast Growth Factor
Foxn4	Forkhead box gene N4
FoxP1	Forkhead box protein P1
GABA	γ-aminobutyric acid
Gata3	GATA binding protein 3
GC	Ganglion Cell
Gdf11	Growth differentiation factor 11
Hb9	Homeobox 9
HCN4	Hyperpolarization activated cyclic nucleotide gated potassium chanel 4

HD	Homeodomain
HEK293	Human embryonic kidney 293
Hes1	Hes Family BHLH Transcription Factor 1
HMC	Hypaxial Motor Column
Hnf6	Hepatocyte Nuclear Factor-6
Hox	Homeobox gene family
HxRE	Hexam Response Element
IN	Interneuron
Insm1	Insulinoma associated protein 1
Irx3	Iroquois homeobox gene 3
Isl1	Islet 1
Lbx1	Ladybird homeobox 1
LC	Loosely Coupled
Lhx3	LIM homeobox gene 3
LMC	Lateral Motor Column
LMCl	lateral LMC
LMCm	medial LMC
LMO1	LIM-Only 1
LMO4	LIM-Only 4
MafA	MAF BZIP transcription factor A
Maml1	Mastermind-like protein 1
miR	microRNA
MLR	Mesensephalic locomotor region
MMC	Median Motor Column
MN	Motor neuron
N-CAM	Neural Cell Adhesion Molecule
NCOR	Nuclear receptor corepressor
NES	Nuclear Export Signal
Neto1	Neuropilin and tolloid like 1
Neurog	Neurogenin
NICD	Notch intracellular domain
NK3R	Neuromedin-K 3 Receptor
Nkx	NK homeobox
NLI	Nuclear LIM Interactor
NLS	Nuclear Localization Signal

OC	One Cut factor
Olig2	Oligodendrocyte transcription factor 2
Olig3	Oligodendrocyte transcription factor 3
Otp	Orthopedia Homeobox
Otx	Orthodenticle
$p27^{Kip1}$	Cyclin dependent kinase inhibitor protein
Pax6	Paired box 6
Pitx2	Pituitary homeobox 2
PGC	Preganglionic Column
pMN	Progenitor domain of motoneuron
POU3F1	POU domain class 3 transcription Factor 1
PPD	Posterior polymorphous dystrophy
Prd-L	Paired-like
Prdm8	PR/SET domain 8
Prox1	Prospero homeobox protein 1
Ptc1	Patched1
qPCR	Quantitative PCR
RA	Retinoic Acid
Raldh2	Retinaldehyde Dehydrogenase 2
Rbpj	recombination signal binding protein for immunoglobulin kappa J region complex
RbS	Rubrospinal
RC	Renshall Cell
RINX	Retinal Inner Nuclear homeoboX
RNAseq	RNA sequencing
RPC	Retinal Progenitor Cell
RtS	reticulospinal
Scl	Stem cell leukemia
Sey	Small eyes
Shh	Sonic Hedg Hog
Shox2	Short stature HomeoboX 2
Sim1	Single minded homolog 1
Sip1	Smad Interacting Protein 1
Sp1	Specificity protein 1
Slit3	Slit Guidance Ligand 3

Smo	Smoothened
Sox1	Sex determining Regions-Y Box 1
Sox14	Sex determining Regions-Y Box 14
Syt2	Synaptotagmin 2
TC	Tightly Coupled
TeRE	Tetramer Response Element
TGFβ	Transforming Growth Factor β
VGluT2	Vesicular Glutamate Transporter 2
VS	Vestibulospinal
Vsx1	Visual System Homeobox gene 1
Vsx2	Visual System Homeobox gene 2
Wnt	Wingless integration site
WT1	Wilms Tumor 1

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Foreword

Robustness is the ability of a biological system to maintain its functions despite genetic and environmental variations (Diss et al., 2014). It implicates functional changes in cellular network architecture due to two general properties: distributed robustness and redundancy. We speak about distributed robustness when a system uses different ways to achieve the same function in the cell. Whereas redundancy refers to the ability of several genes to perform common, overlapping functions. This property usually results from gene duplications during evolution leading to the birth of paralog genes. An obvious illustration of this redundancy is the absence of phenotype in more than 50 percents of gene knock-out murine lines in which the absence of a gene is compensated by paralog(s)(Diss et al., 2014; White et al., 2013). Even if paralog genes usually present fully overlapping functions directly after duplication, mutations or landing in a different genomic region can lead to novel or modified functions: loss of function (non-functionalization), acquisition of novel functions (neo-functionalization), retention of varying degrees of overlapping function (sub-functionalization) or a combination of these last two. In this work, we illustrate an original model in vertebrates wherein two paralog factors retained an identical function that they exert at successive steps of cell differentiation in a single lineage. We named this concept the “labor division” between two paralog genes.

During spinal cord development, different progenitor domains give rise to multiple neuronal subtypes. This process is controlled by fine genetic regulations. In particular, adjacent progenitor domains of V2 interneurons and of MNs share identical developmental determinants and therefore require additional control mechanisms to consolidate respective fate. *Vsx1* and its paralog factor *Vsx2*, or *Chx10*, are expressed in V2 population at different steps of differentiation. Vsx1 is

found in an early intermediate V2 compartment that gives rise to all V2 interneurons while *Chx10* is expressed in differentiating V2a subset. Our work shows that the two paralog genes inhibit MN production at two successive steps of differentiation and cooperate to consolidate V2 interneuron identity.

1. General introduction

1.1. Spinal locomotor system

Locomotion is a highly complex behavior characterized by different parameters: a controlled speed, a certain rhythm, alternation of left and right limbs and alternation of the contraction of flexor and extensor muscles. These parameters need to be finely regulated and coordinated by a robust neuronal system. The locomotor system structure is composed of a cerebral command system, local spinal networks called Central Pattern Generators (CPGs), and a sensory feedback system. First described in invertebrates (Goulding, 2009; Wilson and Wyman, 1965), CPGs, qualified as local control and command centers in the spinal cord, are capable of generating stereotyped locomotion independently of sensory inputs. Located in the ventral part of the spinal cord, they are composed of MNs that directly innervate muscles and different populations of interneurons that control the activity of MNs and modulate locomotion pattern. Even if CPGs generate an organized pattern of locomotion independently of sensory afferences, initiation, selection and shaping of their outputs, necessary for correct motor tasks, are controlled by descending sensory afferences (Eccles, 1968; Goulding, 2009; Jankowska, 2001; Lundberg, 1979).

The motor cortex initiates and refines motor tasks via the cortico-spinal pathway. Feedback from ongoing movements is integrated in the cerebellum and allows to refine the motor actions through descending afferences from the brainstem: the reticulospinal, the rubrospinal and the vestibulospinal pathways. In addition to its modulation function, the reticulospinal pathway is the primary activator of motor activity and receives inputs from mesencephalic locomotor

region, itself controlled by the thalamus and basal ganglia (Figure 1) (Acevedo and Diaz-Rios, 2013; Goulding, 2009). This work focuses on the developmental mechanisms that control the differentiation and the consolidation of ventral populations of the spinal cord.

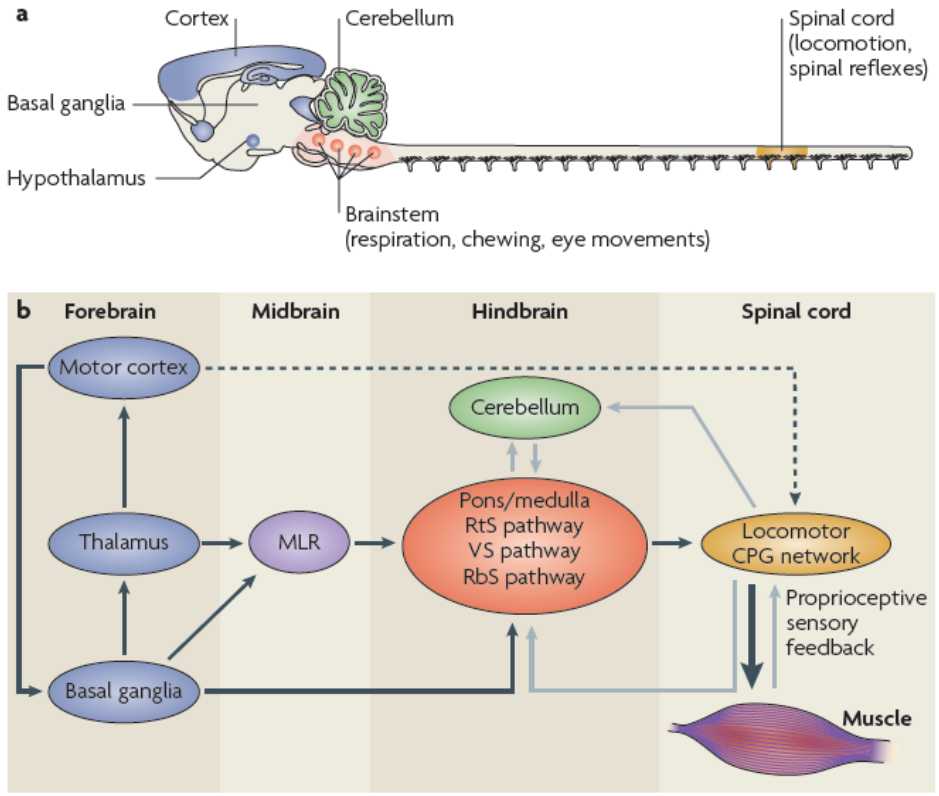


FIGURE 1 : Vertebrate locomotor system.
(A) Neuronal structures implicated in motion control. **(B)** Motor behavior, initiated in the motor cortex, is processed by local CPG networks in the spinal cord. CPGs activity is modulated by reticulospinal (RtS), vestibulospinal (VS) and Rubrospinal (RbS) pathways from the hindbrain. Moreover, the reticulospinal pathway can be activated by mesensephalic locomotor region (MLR) that receive inputs from the thalamus and the basal ganglia. Finally, the sensory afferences are modulated by the cerebellum that coordinates proprioceptive feedback information (Goulding, 2009).

1.2. Early spinal cord development

The spinal cord development is finely controlled in space and time and well conserved between vertebrates. Spinal cord development can be described according to three morphological axes: the rostro-caudal, the dorso-ventral and the medio-lateral axes (Dasen, 2009; Goulding, 2009; Grillner and Jessell, 2009; Jessell, 2000; Lee and Pfaff, 2001).

Specification along the rostro-caudal axis

In mammals, developmental processes are initiated at the beginning of gastrulation by the induction of the anterior neural plate leading to the formation of forebrain, midbrain, hindbrain and anterior spinal cord (Figure 2A). This process, called activation, is followed by a mesenchymo-epithelial transformation process along the rostro-caudal axis leading to the formation of posterior spinal cord. Recent data suggest that the neural plate is not the only source of cells for the central nervous system. Neuromesodermal progenitors, located in the node peak border, also participate to the formation of the posterior neural tube (Figure 2B)(Henrique et al., 2015; Wilson et al., 2009).

Caudal extension of the spinal cord is controlled by the balance between Fibroblast Growth Factors (FGFs) and Retinoic Acid (RA) that are mutually inhibitory. *FGFs*, in particular *FGF8*, are expressed by pre-somitic mesoderm, in the Hensen's node and the primitive streak. They maintain cells in proliferation state by maintaining *Cyclin D2* expression or by repressing the expression of several differentiation factors such as *Paired Box 6* (*Pax6)*, *Iroquois homeobox gene 3* (*Irx3*) or *Developing brain homeobox 1/2* (*Dbx1/2*)(Lobjois et al., 2004). On the contrary, RA, produced by the Retinaldehyde Dehydrogenase 2 (Raldh2) enzyme in somites,

promotes proneural genes expression and neuronal differentiation (Figure 2C) (Diez del Corral et al., 2003; Liu et al., 2001; Ulloa and Briscoe, 2007).

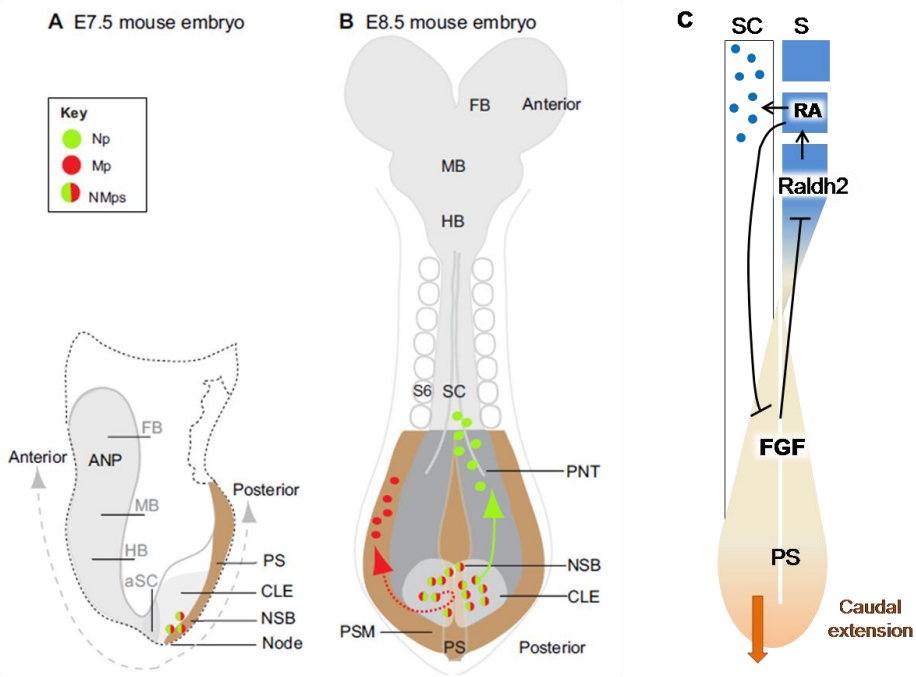


FIGURE 2 *:* Rostro-caudal extension of the central nervous system.
(A) During gastrulation, differentiation of the anterior neural plate leads to the formation of the brain and the anterior part of the spinal cord. **(B)** Through a mesenchymo-epithelial transformation process, neuromesodermal cells located in the primitive streak colonize the neural tube to allow the caudal extension of the spinal cord. **(C)** Opposite gradients of FGFs and RA, mutually inhibitory, allows the differentiation and the rostro-caudal extension of the spinal cord. ANP=anterior neural plate; FB=forebrain; MB=midbrain; HB=hindbrain; aSC=anterior spinal cord; PS=primitive streak; CLE=caudal lateral epiblast; NSB=node streak border; SC=spinal cord; S=somite; PNT=preneural tube; PSM=presomatic mesoderm (Diez del Corral et al., 2003; Henrique et al., 2015).

As mentioned above, the spinal cord is composed of different neuronal populations. Although interneuron subtypes are mostly produced along the entire rostro-caudal axis (Francius et al., 2013), recent data suggest that a segmental

specification occurs for some populations such as V1 and V2a interneurons (Azim et al., 2014b; Hayashi et al., 2018; Sweeney et al., 2018). Moreover, classes and subclasses of MNs are differentially produced along the antero-posterior axis, as represented by different motor columns and motor pools (Dasen and Jessell, 2009; Fetcho, 1987; Gutman et al., 1993; Prasad and Hollyday, 1991). This specification is controlled by a combination of FGFs, retinoid signaling and Growth differentiation factor 11 (Gdf11) that activates the expression of *Homeobox* gene family (*Hox*) along the rostro-caudal axis of the embryo (Bel-Vialar et al., 2002; Dasen and Jessell, 2009; Diez del Corral and Storey, 2004; Liu, 2006; Liu et al., 2001; Nordstrom et al., 2006). This developmental process is described in more details in the section "1.5.2. Motor neuron diversification".

Dorsa-ventral patterning of the spinal cord

In addition to rostro-caudal specification, the neural tube is divided along the dorso-ventral axis into progenitor domains that give rise to different neuronal populations (Figure 3). Neural tube is arbitrary divided into a dorsal and a ventral part. Eight dorsal progenitor domains, pd1 to pd6 and late-born progenitor domains pdILA and pdILB, produce dorsal populations of interneurons, dI1 to dI6 and dILA and B. While the ventral part of the spinal cord is divided into four progenitor domains of ventral interneurons, p0 to p3, and into a progenitor domain of MNs (pMN)(Alaynick et al., 2011). The dorsal part of the spinal cord mainly relays sensory information, while dorsal dI6 population, V0 to V3 ventral interneuron populations and MNs form complex networks, the CPGs, that control locomotor behaviors (Goulding, 2009; Grillner and Jessell, 2009; Grillner and Zangger, 1979)

The dorso-ventral patterning of the spinal cord is mediated by two opposite gradients of secreted signaling molecules called morphogens. Dorsalizing Wingless Integration site (Wnt) factors and Bone Morphogenic proteins (BMPs), members of

Transforming Growth factor β (TGFβ), are first released by the ectoderm and then by the roof plate. They activate the expression of *basic Helix-Loop-Helix* (*bHLH*) factors in a gradient dependent manner to regionalize most dorsal populations of interneurons (Hernandez-Miranda et al., 2017; Ulloa and Briscoe, 2007). On the other hand, the notochord and then the floor plate produce Sonic hedgehog (Shh), a ventralizing morphogen that activates the expression of different homeodomain (HD) patterning factors (Figure 3) (Ericson et al., 1996; Lee et al., 1998; Liem et al., 1995; Megason and McMahon, 2002; Muroyama et al., 2002; Roelink et al., 1994; Timmer et al., 2002).

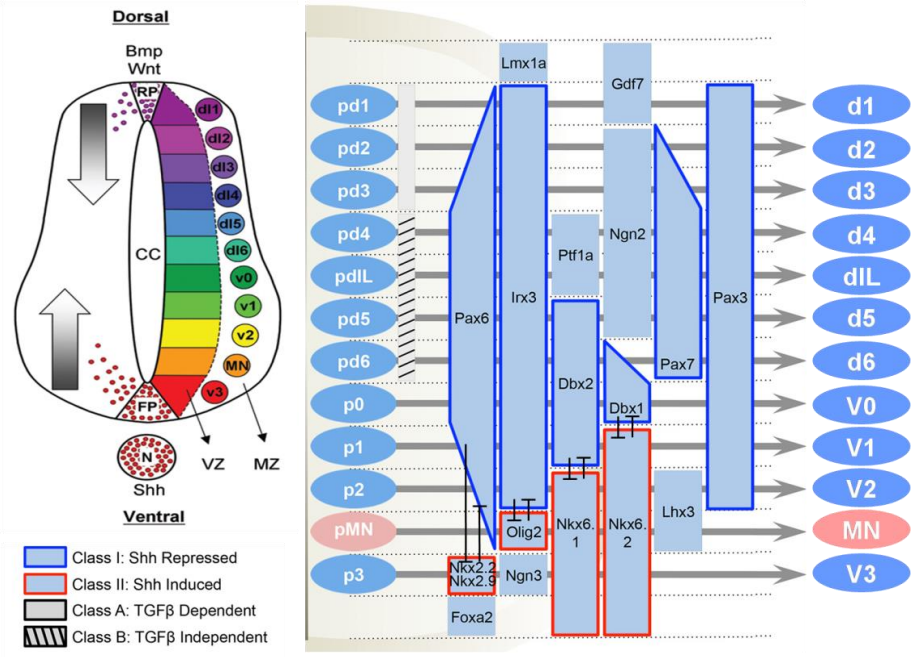


FIGURE 3 : Dorso-ventral patterning of the spinal cord.
During development, two opposite gradients of morphogens lead to the subdivision of the neural tube into 13 progenitor domains. Wnt and BMP signaling are released by ectoderm and then the roof plate to regionalize most dorsal progenitor populations. In the ventral spinal cord, Shh, released by the notochord and the floorplate, represses class I and activate class II patterning genes to ensure the subdivision of the ventral part of the neural tube. Cross-repressions between patterning genes precisely define progenitor domain boundaries (Alaynick et al., 2011).

Shh-induced ventral patterning

Identity of ventral progenitor domains mainly depends on the Shh gradient. First, Shh released by the notochord induces the floor plate to produce Shh in turn. This morphogen activates, in a concentration-dependent manner, expression of class II patterning genes, like *NK2 homeobox 2* (*Nkx2.2*), *Nkx6.1* or *Oligodendrocyte transcription factor 2* (*Olig2*), and inhibits the expression of class I patterning genes, as *Dbx1*, *Pax3* or *Pax6* (Figure 3 and Figure 4) (Briscoe et al., 2000). In addition to morphogen concentration, duration of exposure also influences the response of patterning factors to Shh. For example, *Nkx2.2*, that requires a high Shh concentration, is expressed in the most ventral p3 progenitor domain. However, it necessitates a longer Shh exposure and is expressed after *Olig2* that labels pMN progenitor domain (Danesin and Soula, 2017).

Activation of the Shh signaling pathway is well described. Shh binds a Patched1 (Ptc1) receptor present on receiving cells that release a Smoothened intracellular signal (Bijlsma et al., 2006; Ingham and McMahon, 2001; Passmore et al., 2012; Taipale et al., 2002; Ulloa and Briscoe, 2007). Smo regulates the expression of *Gli* family of zinc-finger-containing transcription factors that, in turn, ventrally activate or dorsally repress the expression of ventral patterning genes (Figure 4) (Huangfu and Anderson, 2006; Ulloa et al., 2007). Three Gli proteins are found in the developing spinal cord. Gli1 is a transcriptional activator while Gli2 and 3 are either activator or repressor depending respectively on the presence or the absence of Shh. While Shh activates the expression of *Gli1* and *Gli2*, Gli3 repressor activity is clearly repressed by the morphogen and the protein is found only in the most dorsal part of the ventral spinal cord (Jacob and Briscoe, 2003; Ulloa and Marti, 2010). It has also been shown that Wnt1/3a regulates Gli3 repressor activity that reciprocally inhibits Wnt/β-catenin pathway (Ulloa and Marti, 2010). In addition to its patterning function, Shh plays a primary role in proliferation and survival of

progenitor cells by regulating *Cyclin D1*, *N-myc* and *B cell lymphoma 2 (Bcl2)* expression (Bigelow et al., 2004; Kenney and Rowitch, 2000; Oliver et al., 2003; Ulloa and Briscoe, 2007). Ventral specification of progenitor domains also depends on opposite FGFs and RA gradients. They regulate *Pax6* and *Irx3* expression and specify, at least, progenitor domains of V1 and V2 interneurons and progenitor domain of MNs (Diez del Corral et al., 2003). In summary, Shh gradient differentially regulates the expression of patterning genes in ventral part of the spinal cord. Rough expression profile is then refined through cross-repression mechanisms between patterning factors leading to the establishment of precise boundaries between progenitor domains (Figure 4).

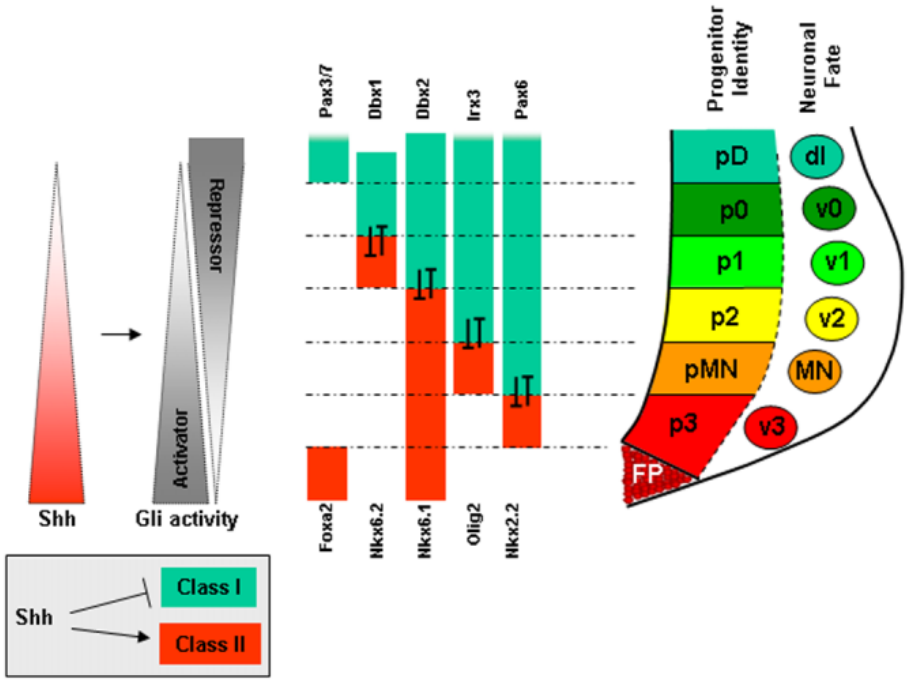


FIGURE 4 : Ventral spinal cord subdivision by Shh signaling.
Shh gradient regulates the activation of Gli factors that either ventrally activate class II or dorsally repress Class I patterning genes. Then cross-repression between patterning factors mechanisms sharp boundaries of progenitor domains and reinforces identity of progenitor cells (Ulloa and Marti, 2010).

Finally, a medio-lateral axis of specification is observed. Progenitor domains, located close to the lumen, differentiate into neuronal populations that migrate laterally. This developmental process requires fine genetic regulations that will be addressed in the following chapters.

1.3. Ventral interneuron populations

Each embryonic interneuron subtype can be distinguished by a specific molecular identity, by its connectivity and the kind of activity it displays. Spinal interneurons are either excitatory or inhibitory if they respectively use glutamate or acetyl choline as neurotransmitter to stimulate target activation, or glycine or γ-aminobutyric acid to inhibit target activity (Svensson et al., 2018; Tritsch et al., 2016). Finally, interneurons are classified as commissural or non-commissural if they respectively project axons to the contra-lateral side of the spinal or if they ipsilaterally project on neurons located on the same side of their cell body (Gosgnach et al., 2017). Ventral interneurons characteristics are summarized in Figure 5.

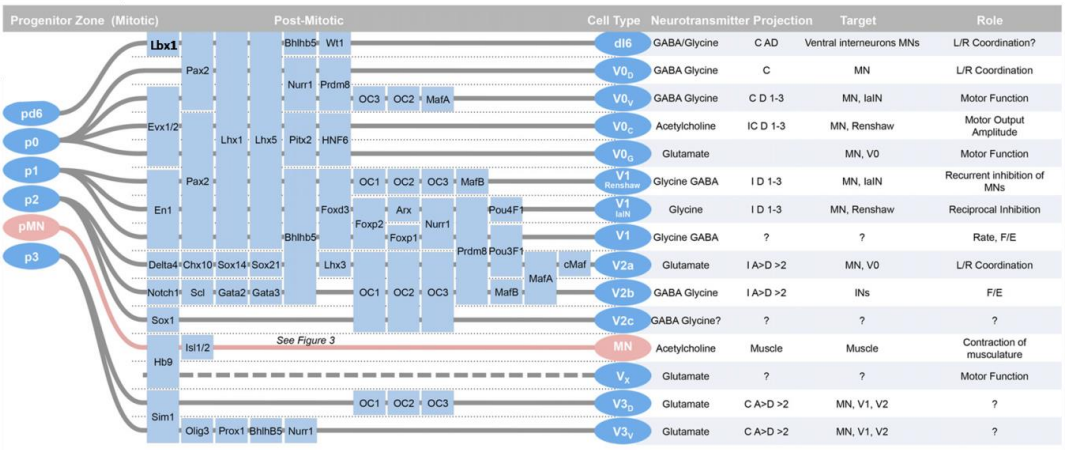


FIGURE 5 : Summary of ventral spinal population characteristics that compose CPGs. I=Ipsilateral; C=Contraleral; A=Ascending; D=Descending; L/R=Left/Right; F/E=Flexor/Extensor (Lu et al., 2015).

DI6 interneurons

As described above, mammalian CPGs are composed of MNs, dI6 interneurons and V0 to V3 interneuron populations. Even if dI6 interneurons are arbitrarily classified as a dorsal population, cells migrate ventrally in the CPGs and participate to the control of locomotion (Dyck et al., 2012). During development, dI6 interneurons, transiently labeled by Ladybird homeobox 1 (Lbx1), derive from a progenitor domain expressing *Pax7* and *Dbx1* located dorsal to the p0 progenitor domain (Figure 3). This population presents commissural inhibitory mono- or disynaptic projections on MNs. The major part of the population is labeled by Double sex and Mab-3 Related Transcription factors (DMRT3) and/or Wilms Tumor 1 (WT1) (Andersson et al., 2012; Griener et al., 2017). dI6 interneurons can be classified into two subsets depending on electrophysiological properties: Loosely Coupled (LC) and Tightly Coupled (TC) (Dyck et al., 2012). LC dI6 interneurons are

rhythmically active during fictive locomotion and are probably responsible of rhythm generation. In the contrary, TC dI6 subset receives rhythmic inputs from CPGs and probably coordinates MN activation. The involvement of dI6 interneurons in the coordination of MNs can be illustrated by the altered gait locomotion in some breeds of domesticated horses when *DMRT3* is mutated (Andersson et al., 2012).

V0 interneurons

Ventral to the pdI6, the p0 progenitor domain, characterized by the expression of *Pax6*, *Dbx1/2* and *Irx3,* produces four V0 populations. Its ventral boundary is delineated by cross repressive action of Dbx1 with Nkx6.2, present in p1 progenitor domain (Alaynick et al., 2011). While ventral $V0_V$ interneurons are glutamatergic excitatory and specifically express *Even-skipped homeobox 1* (*Evx1*), dorsal $V0_D$ interneurons are Glycinergic/GABAergic inhibitory and are not labeled by this factor. Inactivation of Dbx1 leads to a loss of the two V0 subsets and display defects in left/right limb alternation during locomotion. As $V0_D$ interneurons are active during fictive locomotion and clearly project on MNs, they are probably the main coordinators of left/right alternation (Griener et al., 2015). Two smaller ipsilateral subtypes, cholinergic $V0_C$ and gutamatergic $V0_G$, representing 5% of p0-derived cells, are specifically labeled by Pituitary homeobox 2 (Pitx2). Their role in locomotion is still unknown but given their transient expression of *Evx1*, they probably derive from $V0_V$ interneurons (Zagoraiou et al., 2009).

V1 interneurons

Ventral to the p0 progenitor domain, Pax6, Dbx2, Nkx6.2 and Irx3 define the p1 progenitor domain, ventrally delineated by cross-repression between Dbx2 and Nkx6.1. The p1 progenitor domain produces at least two inhibitory populations

labeled by Engrailed 1 (En1): Ia interneurons and Renshaw cells (RCs). However, lineage tracing analyses revealed that 75% of V1 interneurons are not characterized yet but present a high transcriptional diversity (Alvarez et al., 2005; Bikoff et al., 2016). Ia interneurons receive inputs from Ia sensory muscle afferences, or primary afferent fibers, that monitor stretch changes in muscle. They project on MNs and RCs. Recent data showed that Ia interneurons are necessary for limb extension and inhibit reciprocal motor pools that control flexor muscles (Britz et al., 2015). RCs receive input from intraspinal MN collaterals, they modulate proprioceptive sensory inputs and inhibit homonymous and synergistic MNs. They also project on Ia interneurons. Recent data demonstrated that RCs are already rhythmically active at early stages of development (Boeri et al., 2018). Pax6 is required for RC differentiation but not for Ia interneurons (Alvarez et al., 2013; Sapir et al., 2004; Wang et al., 2008).

V2 interneurons

During development the p2 progenitor domain, defined by the expression of *Pax6*, *Nkx6.1* and *Irx3* patterning genes, gives rise to 5 described V2 populations. V2a and V2b, respectively labeled by Chx10 and GATA binding protein 3 (Gata3), are firstly produced and represent around 60% of the total amount of V2 interneurons (Li et al., 2010). Other smaller populations are later produced: V2c, V2d and V2-Pax6 interneurons (Dougherty et al., 2013; Panayi et al., 2010; Panayiotou et al., 2013). This thesis project studies the mechanisms implicated in V2 interneuron consolidation. V2 interneuron differentiation process is described in detail in the part *"1.4.* V2 interneuron differentiation".

V3 interneurons

The most ventral progenitor domain is labeled by Nkx2.2, Nkx2.9 and Singleminded homolog 1 (Sim1). The p3 progenitor domain is dorsally delimitated by cross-repression between Nkx2.2 and Pax6. Nkx2.2 and Nkx2.9 are necessary for V3 interneurons production (Holz et al., 2010) while Sim1 probably plays a role in migration and axonal projections (Blacklaws et al., 2015). The p3 progenitor domain gives rise to two V3 subtypes identified by their body position in the spinal cord: the dorsal $V3_D$, labeled by One Cut factors (OC) and the ventral $V3_V$ interneurons, labeled by Olig3 and Bhlhb5 (Goulding, 2009). Both are glutamatergic excitatory and have a majority of commissural projections on MNs, V1 and V2a interneurons. V3 interneurons are active during locomotion. $V3_D$ cells produce rapid tonic firing spikes meanwhile $V3_V$ produce slow average spikes. However, their specific function remains to be identified (Borowska et al., 2013).

Hb9-expressing interneurons

Finally, a group of glutamatergic excitatory interneurons labeled by Hb9 is associated with locomotor rhythm. It generates membrane potentials in phase with rhythmic motor outputs (Hinckley et al., 2005). Moreover, the output silencing of this population leads to a reduction in locomotor frequency (Caldeira et al., 2017). Unfortunately, the origin of Hb9 expressing interneurons remains to be identified.

1.4. V2 interneuron differentiation

The aim of this work is to study the mechanisms implicated in the consolidation of V2 interneuron identity. As described above, these cells are produced from a p2 progenitor domain characterized by the expression of *Pax6, Nkx6.1, Irx3 and Bhlhb5*. The p2 domain boundaries are dorsally consolidated by cross-repressive

actions of Dbx2 and Nkx6.1 and ventrally delimitated by cross-repression of Irx3 and Bhlhb5 with Olig2, marker of the adjacent pMN (Novitch et al., 2001; Skaggs et al., 2011). The p2 progenitors also express *Forkhead box gene N4 (Foxn4), LIM homeobox gene 3 (Lhx3), Gata2* and proneural genes such as *Achaete-Scute Complex-Like 1 (Ascl1), neurogenin-1 and neurogenin-2 (neurog1 and 2) (Peng et al., 2007).* The p2 progenitor domain gives rise to at least five populations of V2 interneurons: V2a, V2b, V2c, V2d and V2-Pax6 interneurons. However, Foxn4-lineage tracing analyses suggest the existence of other p2-derived cells that remains to be identified (Li et al., 2005).

V2a and V2b interneurons specification

V2a and V2b interneurons are the earliest V2 populations to be produced. A single mitotic division of p2 progenitor leads to the production of two precursor daughter cells that give rise to either a V2a or a V2b interneuron (Del Barrio et al., 2007; Kimura et al., 2008; Li et al., 2005). In V2a interneuron, the expression of *Gata2* is down-regulated while Lhx3 is maintained and stimulates *Chx10* expression (Lee et al., 2008). On the contrary, the expression of *Lhx3* is down-regulated in V2b cells that maintain *Gata2* expression and starts to express *Stem cell leukemia* (Scl*)* and *Gata3* (Karunaratne et al.).

The specification in V2a or V2b interneuron depends on the asymmetric activation of the Dll/Notch signaling pathway. In p2 progenitor domain, a Delta-like ligand 4 (Dll4) binds a Notch1 receptor present at the surface of neighboring cells (Benedito and Duarte, 2005). The activation of Notch pathway in these cells leads to the expression of *Scl* and *Gata2* and promotes V2b interneuron fate while the Dll4-positive cells become V2a interneurons. Different factors regulate the activation of Dll/Notch signaling cascade. In addition to its role in the establishment and the maintenance of progenitor domain borders, Bhlhb5, stimulate *Dll4*

expression and promote V2a differentiation (Skaggs et al., 2011). Foxn4 is also a key regulator of V2a and V2b specification. On the one hand, it directly activates *Dll4* expression by binding to its Conserved Region (CR1) enhancer and, on the other hand, it stimulates the expression of *Ascl1*, also responsible of direct activation of *Dll4* expression (Li et al., 2005; Misra et al., 2014). *Neurog* proneural genes can also bind the CR1 enhancer of *Dll4* but repress its activity.

The Dll/Notch cascade is well described. Transmembrane Dll4 ligand binds to the extracellular domain of the Notch1 receptor present at the surface of neighboring cells. Dll4/Notch1 binding leads to the cleavage and the release of Notch intracellular domain (NICD) in the cytoplasm. NICD traverses to the nucleus where it forms a complex with a DNA-binding protein, the Recombination signal binding protein for immunoglobulin kappa J region complex (Rbpj). Binding of NICD to Rbpj complex releases the Nuclear receptor corepressor complex (NCOR) and leads to the recruitment of co-activators, as Mastermind-like protein1 (Maml1) and histone acetyltransferase p300, to enhance the expression of bHLH genes, *Hes Family BHLH Transcription Factor 1* and *5* (*Hes1* and *Hes5)*. Then, Hes1 and Hes5 repress the expression of proneural genes as *Ascl1* and *Neurog2* to maintain cells in an undifferentiated state (Figure 6). Thereby, Notch cascade, activated by proneural factors as Ascl1, stimulates the expression of *Hes1* and *Hes5* that, in turn, inhibit proneural gene expression leading to a oscillating activity of Notch signaling (Kageyama et al., 2009; Zhang et al., 2018).

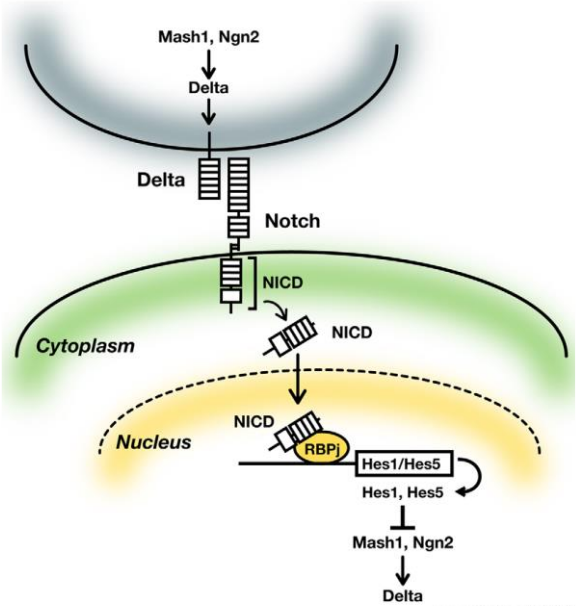


FIGURE 6 : Dll/Notch pathway.

In progenitors, proneural factors regulate the expression of Dll ligand that binds Notch receptor present at the surface of neighboring cell. Dll binding leads to the cleavage of NICD that translocates to the nucleus. There, NICD forms a complex with Rbpj and recruits co-activators to stimulate the expression of bHLH *Hes1* and *Hes5*. Then, Hes1 and Hes5 repress the expression of *Ascl1* (*Mash1*) and *Neurog2* (*Ngn2*) and maintain cells in proliferation (Kageyama et al., 2008).

In summary, V2a or V2b interneuron fate is determined by the mosaic expression of *Ascl1* and *Neurog* genes. *Foxn4*- and *Ascl1*-expressing p2 precursors strongly express *Dll4*, activate Notch cascade in neighboring cells and become V2a interneurons. While cells that weakly express these factors, or that express *Neurog* factors, poorly or do not activate *Dll4* expression and become V2b interneurons (Figure 7)(Misra et al., 2014).

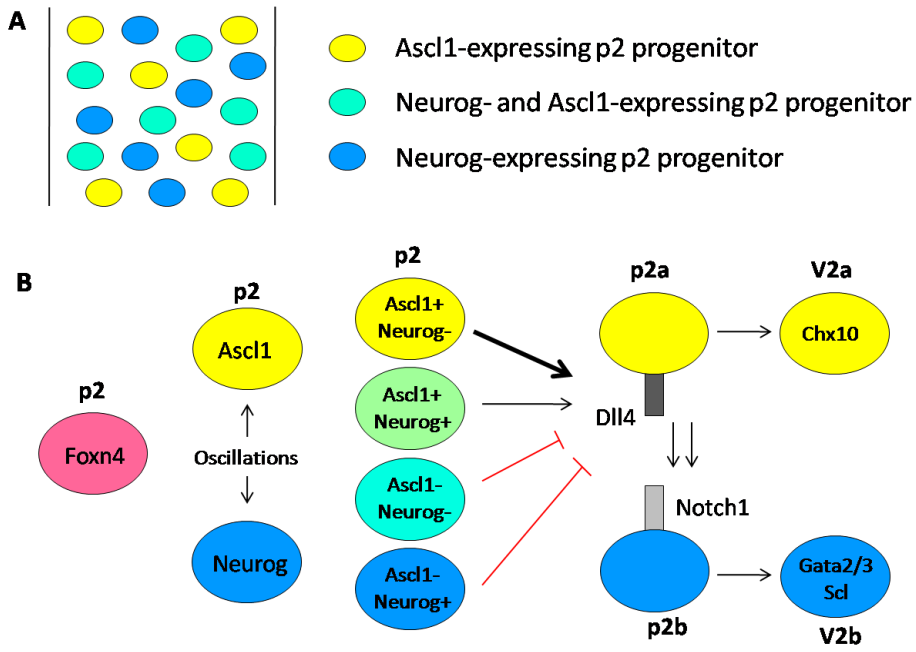


FIGURE 7 : V2a and V2b specification by asymmetric activation of Dll4/Notch1 signaling. **(A)** the p2 progenitor cells present a mosaic expression of *Ascl1* and *Neurog* proneural genes. (**B)** Composition in proneural factors leads to the asymmetric activation of Notch signaling and the segregation of V2a vs V2b interneurons (Misra et al., 2014).

In V2a precursors, maintained expression of *Lhx3* allows the formation of a complex composed of two Lhx3 molecules and a dimer of Nuclear LIM Interactor (NLI, also called Ldb1 or Clim2). The 2Lhx3:2NLI V2-tetramer complex binds a specific Tetramer Response Element (TeRE) to promote V2a gene expression. Indeed, it has been shown that V2-tetramer complex was able to directly activate *Chx10* expression by binding to a TeRE-Chx10 enhancer (Figure 8) (Lee et al., 2008) and promote the expression of V2a markers as *Sox14* and *Shox2*, also expressed in V2d interneurons (Al-Mosawie et al.; Clovis et al.; Dougherty et al., 2013; Ericson et al.; Hargrave et al.; Karunaratne et al., 2002). Different subsets of V2a interneurons are defined through the specific expression of *OCs*, *BhlhB5*, *POU domain class 3*

transcription factor 1 (*Pou3F1*), *PR/SET domain 8* (*Prdm8*), *MAF BZIP transcription factor A* (*MafA*) and *cMaf* (Francius et al., 2013).

During development, Chx10 plays a dual role in V2a identity consolidation. On the one hand, Chx10 prevents MN differentiation by repressing the expression of MN genes as *Hb9*, *LIM-Only 1* (*LMO1)* and *miR218-2* (Clovis et al., 2016). On the other hand, it directly represses the expression of non-V2a genes such as *Dbx1*, *Orthopedia Homeobox* (*Otp)* or *Olig3* and stimulates *Sox14*, *Sox21* or *Vesicular Glutamate Transporter 2* (*VGluT2)* expression (Clovis et al., 2016). The absence of Chx10 leads to a V2a interneuron differentiation defect, characterized by a decrease in the expression of other V2a markers. However, its inactivation is not sufficient to allow a complete activation of the MN differentiation program in presumptive V2a interneurons (Clovis et al., 2016; Lee et al., 2008).

Even if Gata2 is necessary for both V2a and V2b interneuron production when it is expressed in progenitors (Zhou et al., 2000), in V2b precursors, it also forms a complex with Scl and LMO4 to stimulate and maintain its own expression and to directly activate *Gata3* expression (Figure 8)(Joshi et al., 2009; Karunaratne et al., 2002; Muroyama et al., 2002). LMO4 also binds NLI and thereby competes with Lhx3 to trigger the V2-tetramer complex formation and the V2a specification (Joshi et al., 2009; Lee et al., 2008). Gata3 inhibits *Chx10* expression and thereby also secures the V2a/V2b segregation (Karunaratne et al., 2002).

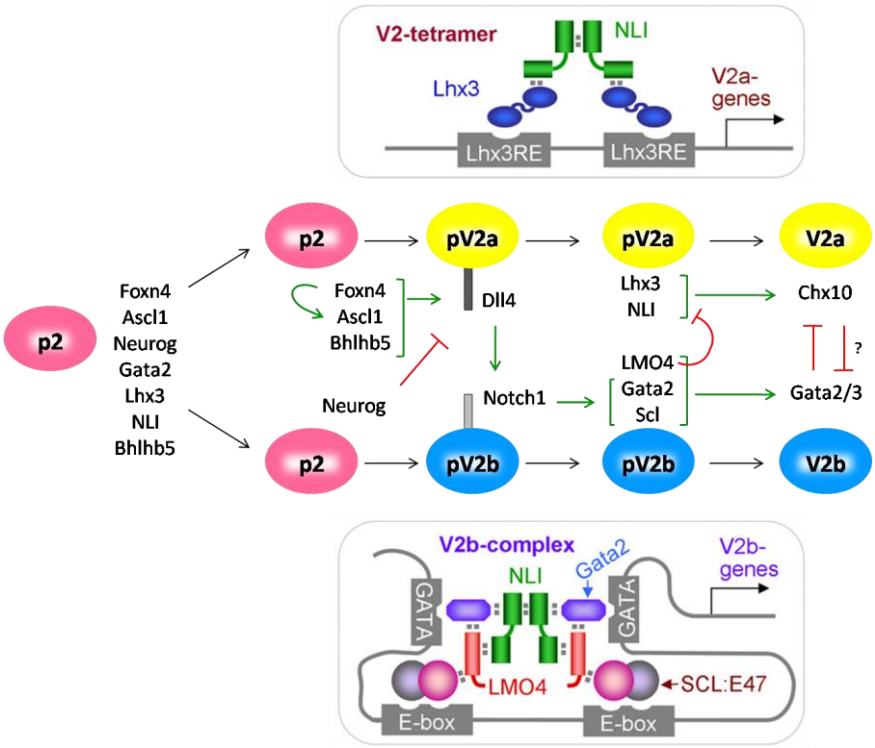


FIGURE 8 *:* V2a and V2 specification.
Developmental mechanisms of V2a and V2b interneurons specification implying tetramer and V2b complexes formation following asymmetric activation of Notch signaling (adapted from (Joshi et al., 2009; Lee et al., 2008).

Differentiation of other V2 interneurons

While V2a and V2b interneuron specification is well described, the differentiation of other V2 interneuron subsets is poorly studied. V2c interneurons are labeled by Sox1, which is also expressed in most neural progenitors of the spinal cord. Lineage tracing revealed that V2c interneurons transiently express *Gata3*. Moreover, Sox1 inactivation leads to the conversion of V2c in V2b cells and a decrease in V2c interneurons is observed in the absence of Foxn4, as for V2b interneurons (Panayi et al., 2010).

Shox2 defines different subsets of neurons in the ventral spinal cord. Two more dorsal populations derive from *Lbx1* or *Islet 1* (*Isl1*)-expressing cells while more ventral subsets correspond to a large part of V2a interneurons (~75%) and to V2d interneuron population, respectively. V2d interneurons probably derive from V2a cells but represent a distinct population with a specific function during locomotion (see section "1.4.3. V2 populations in locomotor system")(Dougherty et al., 2013).

Finally, Foxn4 lineage tracing analyses revealed that other V2 cells, produced later during development, were labeled by Pax6. Some of these *Pax6*-expressing cells express *Gata3* before migrating out of the ventricular zone and represent thereby a subset of V2b population. Nevertheless, Pax6 also labels V2 cells that do express neither *Chx10* nor *Gata3* and are named Pax6-V2 interneurons (Panayiotou et al., 2013).

In summary, p2 progenitor domain produces at least 5 populations of V2 interneurons characterized by the expression of specific markers and a specific function in locomotion that will be described in the following chapter. Moreover, lineage tracing analyses show that one or more additional V2 populations still need to be characterized (Li et al., 2005).

V2 populations in locomotor system

Each V2 population is integrated into a complex neuronal network to perform a specific role in locomotion or in movement control. V2 interneurons present ipsilateral projections (Crone et al., 2008; Lundfald et al., 2007). The large majority of V2a interneurons is constituted of glutamatergic excitatory interneurons. Consistently, it has been shown that all the *Shox2*-expressing cells, including V2d interneurons, are glutamatergic neurons (Dougherty et al., 2013). In contrast, V2b interneurons, and probably V2c interneurons, are GABAergic or glycinergic inhibitory neurons (Crone et al., 2008; Lundfald et al., 2007; Panayi et al., 2010).

V2a interneurons

The function of V2a interneurons in locomotor circuits has been already well studied. Even if the V2a interneurons are found along the entire spinal cord, they control the forelimbs and the hindlimbs in a different way. Recently, two main types of V2a interneurons, type I and type II, have been identified during development and at least eleven V2a-subtypes can be distinguished by their molecular composition at birth (Figure 9) (Hayashi et al., 2018). Type I V2a interneurons, that predominate at the lumbar level, maintain a high level of *Chx10* expression while type II interneurons, more numerous at the cervical level, downregulate it starting embryonic day 12. At each level of the spinal cord, V2a interneurons project on other V2a cells, on other ventral interneurons including V0 cells, and on MNs. However, synaptic connections with MNs, in the ipsilateral side or to the contralateral side through V0 interneurons, are numerously more robust for type I V2a interneurons at lumbar level. In contrast, type II V2a interneurons in the cervical region have ascending outputs to the cerebellum via the lateral reticular nucleus, a pre-cerebral relay that transmits the motor commands and enables a fine adjustment of the motor control (Azim et al., 2014b; Hayashi et al., 2018).

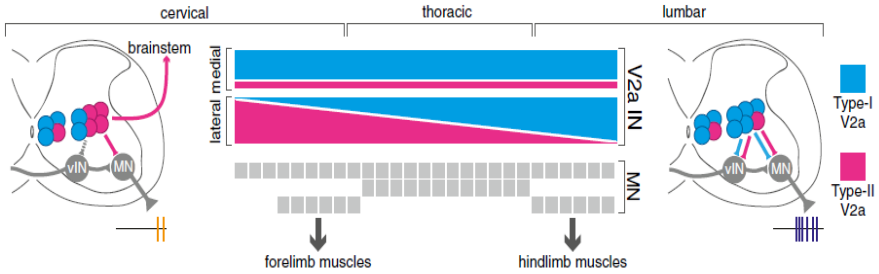


FIGURE 9 : Type I and Type II subsets of V2a interneurons.
Type I and Type II V2a subsets are differentially distributed along the rostrocaudal axis of the spinal cord. Type I V2a interneurons dominate at lumbar level and mainly stimulate ventral interneurons (vIN) and MNs. While Type II are more numerous at cervical level where they establish connections to the brainstem. Motor columns and motor pools are shown as grey boxes (Hayashi et al., 2018).

Transgenic lines in which V2a cells are ablated helped to identify their function in the spinal cord. First of all, locomotor behavioral tests and drug-induced locomotor-like activity assays realized at lumbar level showed that V2a interneurons are needed for a correct left/right limb alternation in a speed-dependent manner (Crone et al., 2008; Crone et al., 2012; Crone et al., 2009). Indeed, V2a-deficient mice progressively replace their left/right limb alternation by a synchronous gallop when the speed increases. As already mentioned, V2a interneurons project on MNs (Al-Mosawie et al., 2007) and on different subsets of inhibitory commissural interneurons, including V0 interneurons (Grillner et al., 1981; Jordan et al., 2008; Rossignol and Dubuc, 1994). The role of V0 interneurons in left-right alternation has been clearly determined. Talpalar *et al.* demonstrated that inhibitory $V0_D$ and excitatory $V0_V$ interneurons are required for left and right limb alternation at low and high speed of locomotion, respectively (Lanuza et al., 2004; Talpalar et al., 2013). Therefore, V2a interneurons are probably necessary to activate $V0_V$ interneurons and secure limb alternation at high locomotor frequency. As described above, Shox2 labels a part of V2a population (Dougherty et al., 2013). Deficiency in *Shox2*-expressing cells does not result in a a defect in left/right limbs alternation suggesting that this mechanism is controlled by the part of V2a interneurons that does not express *Shox2* (Dougherty et al., 2013).

Secondly, V2a population stabilizes the CPG activity as shown by the irregularities of burst frequency and amplitude in V2a-deficient mice although these cells are not directly implicated in rhythm regulation (Crone et al., 2008).

Thirdly, V2a interneurons participate in the induction of locomotor activity. In Chx10-defient spinal cord, sensory afferent stimulations fail to initiate motor-like activity. Indeed, V2a interneurons receive inputs from brainstem via descending

reticulospinal pathway necessary for locomotor induction. (Crone et al., 2008; Crone et al., 2009).

Finally, cervical V2a interneurons are implicated in the control of skilled forelimbs movement. Cervical V2a ablation leads to the inability of mice in reaching behavior. This mechanism is finely regulated by a feedback system composed of superior pathway and local spinal regulation. Caudal projection of V2a interneurons on MNs and rostral projection on lateral reticular nucleus, a pre-cerebral relay, can elicit a fine feedback loop regulation allowing skilled movements of the forelimbs (Azim et al., 2014a).

V2b interneurons

Although the left/right limb alternation defect is clear in V2a-deficient mice, the alternation of flexor and extensor muscle contractions do not seem to be affected (Crone et al., 2008; Crone et al., 2009). This mechanism is regulated by two other neuronal populations, the V1 and the V2b interneurons (Zhang et al., 2014). Both inhibitory populations ipsilaterally project on MNs. V1 interneurons, probably Ia cells (Myers et al., 2005), are necessary for the extension of limb and inhibit the flexor motor pools. In the contrary, V2b interneurons connect to the extensor motor pool and favor the flexor movement (Britz et al., 2015). They also probably project on excitatory interneurons to allow a positive feedback activation of flexor vs extensor MNs.

Other V2 interneuron populations

The role of the other V2 interneuron subsets is poorly studied. The function of V2c and Pax6-V2 interneuron remains to be identified.

Even if V2d interneurons share similarities with V2a interneurons, they have a distinct function during locomotion. V2d interneurons project on V2a interneurons,

on ipsi- and contralaterally projecting interneurons and on MNs that innervate flexor muscles. Moreover, recent data demonstrated that *Shox2*-expressing cells are interconnected (Ha and Dougherty, 2018). A depletion in *Shox2*-expressing cells leads to a clear rhythm defect while this aspect of locomotion is not affected in V2a-deficient mice. Thus, V2d interneurons significantly participate, probably with other not yet identified populations, to rhythm generation (Dougherty et al., 2013).

1.5. Motor neurons

In addition to interneurons, spinal neuronal networks are composed of cholinergic MNs that innervate muscles. Vertebrates possess hundreds of distinct muscle groups innervated by genetically distinct motor neurons well organized in spinal clusters. MNs are classified into clusters characterized by their specific position in the spinal cord, their expression profile, their axon trajectory and their target. During development, different levels of organization are observed. The pMN, specifically labeled by Olig2, initially produces generic MNs that early express *Hb9*, *Isl1*, *Isl2*, *Lhx3* and *Hepatocyte Nuclear Factor-6* (*Hnf6* also named *OC1*). They rapidly diversify in MN subtypes distributed into different motor columns, differentially organized along the rostro-caudal axis of the spinal cord. MNs that compose these motor columns innervate different parts of the body musculature. In a second level of organization, motor columns are subdivided into distinct divisions and finally into motor pools that each innervate one specific muscle (Dasen and Jessell, 2009). This complex organization is finely regulated by a specific genetic program that is either maintained or later activated in some motor groups.

Early MN differentiation

During development of the spinal cord, Shh signaling defines ventral progenitor domains including a pMN characterized by the expression of *Nkx6.1*, *Nkx6.2, Pax6* and the bHLH factor *Olig2*. Boundary between pMN and p2 progenitor domain is sharped by cross-repressive regulations of Olig2 with Irx3 and bHLHb5. While the limit with p3 progenitor domain is delineated through cross-repressive regulations of Olig2 with Nkx2.2 and Nkx2.9 (Figure 3 and 4). Olig2 is mandatory for the production of pMN progenitors. In the absence of Olig2, MNs are missing and replaced by ventral interneurons (Rousso et al., 2008; Zhou and Anderson, 2002). Olig2 stimulates the expression of another bHLH factor, *Neurog2* (Lee et al., 2005). With RA, Neurog2 recruit the histone acetyl transferase CREB binding protein (CBP) and p300 that stimulate the expression of neuronal genes and play a primary role in the MN differentiation (Lee et al., 2009). The balance between Olig2 and pro-neural factor Neurog2 defines the neuronal progression between progenitor cells and differentiating MNs (Lee et al., 2005). A marker of early MN differentiation is Hb9. Initially isolated in human (Deguchi et al., 1991), *Hb9* is found in different vertebrates and is represented in avian model by two paralog factors: *Hb9* and *MNR2* (Tanabe et al., 1998). Hb9 is able to regulate its own expression and is sufficient for MN production (Tanabe et al., 1998). It activates the expression of *Isl1, Isl2* and *Lhx3* LIM factors, essential for early MN differentiation, and stimulates the expression of *Cholin acetyl transferase* (*ChAT*) (Thaler et al., 1999b), necessary for cholinergic transmission (Tanabe et al., 1998). While its inactivation does not lead to a complete loss of MNs, it is accompanied by the inappropriate expression of V2a interneuron markers, demonstrating a role of Hb9 in the consolidation of MN versus V2 interneuron identity (Arber et al., 1999; Clovis et al., 2016; Lee et al.,

2008; Stifani, 2014; Thaler et al., 1999a; Francius and Clotman, 2014; Lee et al., 2004).

Hb9 expression is initiated in the last mitotic division of progenitor cells and is maintained at high level in a part of MNs (see section 1.5.2) (Francius and Clotman, 2014). *Hb9* promoter presents different enhancer regions stimulated by general activators or neuron-specific complexes. Indeed, Specificity protein 1 (Sp1) or E2F, general transcription factors, bind and activate a proximal region of the *Hb9* promoter while proneural factors such as Neurog2, NeuroM or Neuronal differentiation (NeuroD) bind an enhancer located in a more distal part of *Hb9* promoter. General activation of Hb9 expression is restricted in progenitor and in non-MNs by repressors such as Sox1, Nkx2.2, Irx3, Nkx6.1 and Pax6 (Figure 10) (Lee et al., 2004).

As mentioned above, Hb9 stimulates the expression of *Isl1* and *Lhx3* in postmitotic neurons. While Lhx3 and NLI form a V2-tetrameric complex, the presence of Isl1 leads to the formation of a MN-hexameric complex. This complex is composed of 2 molecules of Isl1, 2 molecules of Lhx3 and a NLI dimer and binds a distal enhancer of the *Hb9* promoter called Hexamer Response Element (HxRE) and located in the M250 distal region. Therefore, MN-Hexamer complex stimulates *Hb9* expression through a feedforward regulation mechanism (Figure 10) (Lee et al., 2008).

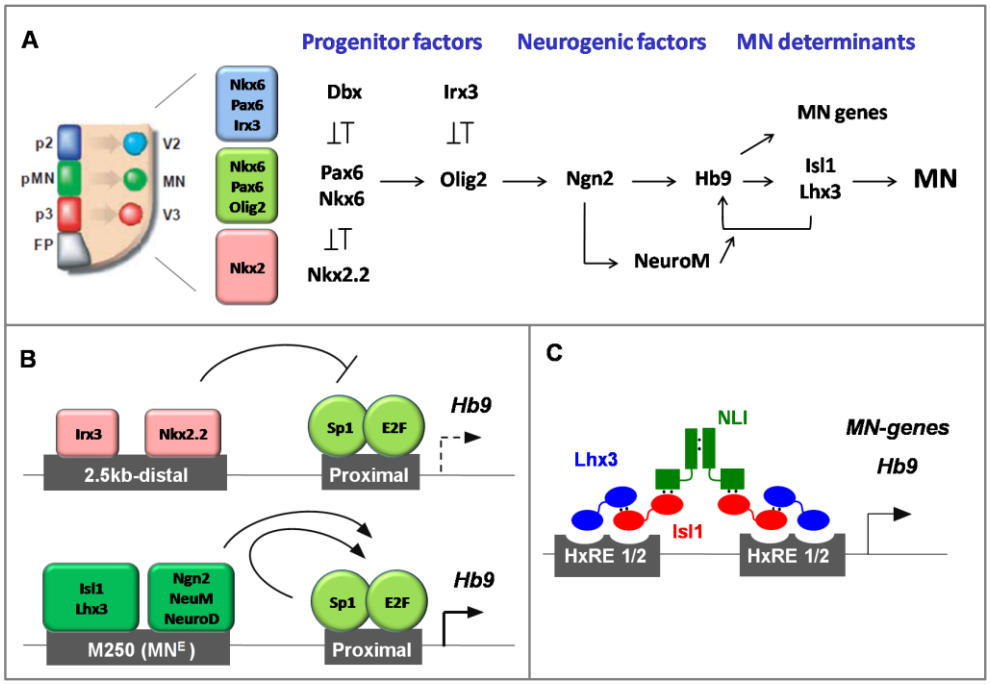


FIGURE 10: Early MN differentiation mechanism.
(A) General and neuronal factors stimulate the expression of *Hb9* to promote MN differentiation. Feedforward regulation by Isl1:NLI:Lhx3 complexes sustains this activation process. (**B)** Sp1 and E2F general activators stimulate *Hb9* expression by binding to a proximal promoter region. *Hb9* activation is inhibited by Irx3 and Nkx2.2 that bind a distal region of its promoter. Isl1-Lhx3 complexes, supported by Ngn2, NeuroM and NeuroD, stimulate *Hb9* expression by binding a more distal enhancer. (**C)** The Isl1:NLI:Lhx3 hexameric complex recognizes the distal HxRE located in the M250 region to stimulate *Hb9* expression (Lee et al., 2008; Lee et al., 2004).

Hexamer-mediated stimulation of *Hb9* expression is enhanced by different regulators. NeuroM forms a complex with E47 dimer and synergizes with Isl1 and Lhx3 to stimulate *Hb9* transcription (Lee and Pfaff, 2003). Similarly, Stat3 is recruited by STAT-binding motif and is associated with the Hexamer-complex (Lee et al., 2013). MN-Hexamer complex plays a critical role in the terminal differentiation of MNs by controlling MN gene expression, axonogenesis, axon guidance and synaptic function. Indeed, the absence of Isl1 leads to an important

cell death in the ventral spinal cord accompanied by the absence of ChAT and MN markers such as Hb9 and Lhx3. Moreover, a defect in the expression of the neural cell adhesion molecule allowing the synaptogenesis at muscular junction is observed (Pfaff et al., 1996).

While they are necessary for early differentiation of MN, Hb9, as well as Isl1 or Lhx3, are maintained at later stages of development in a part of MN subpopulations that start to diversify quickly after their production. The ability of Lhx3 to form 2 different complexes that stimulate either V2 interneuron or MN differentiation requires a strong consolidation system. In particular, activation of the V2 differentiation program in early motor neurons must be repressed, as Lhx3:NLI V2 tetramers can also form in these cells (Lee et al., 2004). The absence of Hb9 leads to the aberrant expression of V2a markers in MN cells but other factors are implicated in the consolidation of MN versus V2 interneuron identity. More details are given in the section “1.6. V2 interneuron versus MN consolidation”.

Motor neuron diversification

The early produced MNs rapidly diversify into subtypes presenting specific soma positions, axon trajectories and target innervations. A first subdivision is made between somatic and visceral MNs that innervate skeletal or smooth muscles of visceral organs, respectively. Visceral MNs are exclusively found at thoracic level and compose the Preganglionic Column (PGC). They establish connections with the noradrenergic neurons from the sympathetic ventral chain before innervating cardiac or smooth muscles of visceral organs. PGC is labeled by different markers as Isl1, Forkhead box protein P1 (FoxP1) or Smad Interacting Protein 1 (Sip1). Somatic MNs are subdivided into 3 motor columns, the Median Motor Column (MMC), the Hypaxial Motor Column (HMC) and the Lateral Motor Columns (LMC), differentially distributed along the rostro-caudal axis. MMC, present in the entire

spinal cord, is composed of somatic MNs that innervate the dorsal axial musculature. This column is labeled by Isl1/2, Lhx3 and Hb9. At thoracic level mainly, MNs that compose the HMC innervate intercostal and abdominal wall musculature. These cells also expressed *Isl1/2* and *Hb9* but are not labeled by Lhx3. Finally, at brachial and lumbar levels, MNs from the LMC project axons on limb musculature. Another level of organization takes place with the subdivision of LMC into lateral (LMCl) and medial (LMCm) divisions that target dorsal and ventral muscles of the limbs, respectively. Both motor columns are labelled by Hb9 and Foxp1 and can be distinguished by Isl1 and Lhx1 markers, respectively found in LMCm and LMCl (Figure 11) (Francius and Clotman, 2014). Diversification increases with the subdivision of LMC divisions into motor pools that each innervate a dedicated target muscle. Motor pools differ along the antero-posterior axis, more rostral pools mainly target rostral and proximal muscles while caudal pools innervate more caudal and distal targets (Dasen and Jessell, 2009; Hollyday and Jacobson, 1990; Landmesser, 1978). Identity of each motor column and their axonal connection is closely related to their position in the spinal cord and the signaling cues they receive.

MN diversification is finely controlled by combinations of transcription factors. *Hox* genes play a crucial role in MN regionalization along rostro caudal axis. They are organized in 13 conserved paralog clusters distributed in four complexes, A to D, on different chromosomes (Lappin et al., 2006). FGF activates their expression in a graded manner. Thereby, 3' *Hox* clusters are expressed in the anterior spinal cord where FGF concentration is low while higher caudal concentration of FGF activates 5' *Hox* clusters expression. Their expression profile is refined by RA signaling and Gdf11 regulation and through cross-repression between Hox factors themselves. Hence, Hox6, Hox9 and Hox10 respectively regulate the regionalization of brachial,

thoracic and lumbar MNs as observed by unappropriated column acquisition in Hox-deficient mice (Figure 11A)(Dasen et al., 2003).

Hox factors regulate motor columns identity by controlling the expression of molecular markers implicated in terminal differentiation and axon connectivity. FoxP1 for example, which serves as a Hox cofactor, is stimulated in LMC by Hox8 and Hox10 paralogs at brachial and lumbar, respectively, level while Hox9 paralogs activate its expression in PGC at thoracic level. Specific molecular determinants of each motor columns are summarized in Figure 11B (Dasen et al., 2003; Francius and Clotman, 2014).

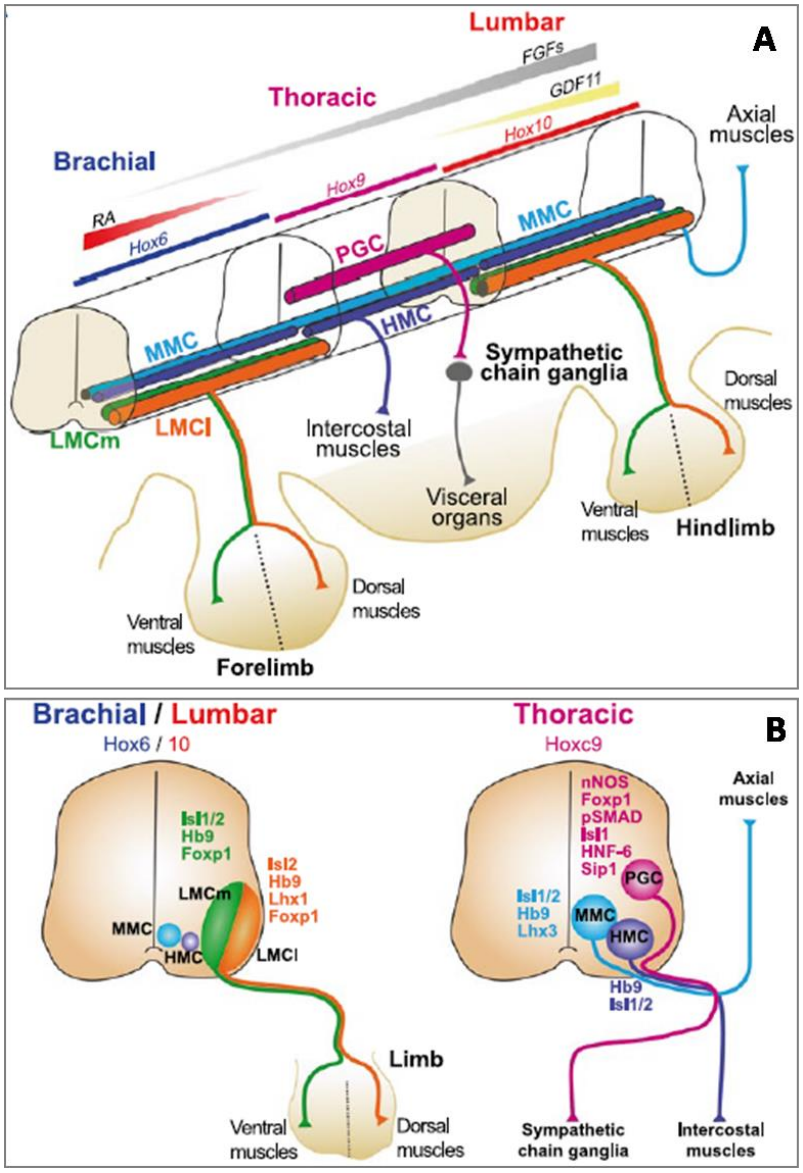


FIGURE 11 : Motor column organization along the rostro-caudal axis of the spinal cord
(A) Three-dimension scheme of motor column distribution and projections along the rostro caudal axis following Hox activation by FGF, RA signaling and Gdf11. (**B)** Localization and molecular composition of motor columns in schematic spinal transverse section (Francius and Clotman, 2014).

1.6. V2 interneuron versus MN consolidation

The p2 and the pMN progenitor domains are adjacent during development and share identical developmental determinants. *Lhx3* is early expressed in precursors of both populations and forms, either a Lhx3:NLI V2-tetramer or a Lhx3:NLI:Isl1 MN-hexamer complex that promotes V2a or MN identity, respectively (Figure 12) (Lee et al., 2008). As described earlier, different aspects of their differentiation process require consolidating regulations. On the one hand, V2-tetramer can be formed in both V2 and MN compartments. On the other hand, unlike the V2-tetramer, the MN-hexameric complex can activate either the TeRE and the HxRE although with different affinities (Lee et al., 2008). Therefore, mechanisms are necessary to consolidate V2 and MN identities and prevent the activation of inappropriate differentiation program.

A first level of consolidation is observed with the cross-repressive action of Irx3 and Olig2, leading to the segmentation of progenitor domains. The absence of Olig2 results in the substitution of MNs by V2a interneurons (Rousso et al., 2008). Then, *bHLHb5*, initially expressed in progenitors, is maintained in some differentiated populations such as V2a interneurons. It also inhibits MN formation through cross-repressive actions with Olig2 (Skaggs et al., 2011).

As described in the section 1.4.1, LMO4 consolidates the identity of V2b versus V2a interneurons by competing with Lhx3 for NLI binding. *LMO4* is highly expressed in MN progenitor and is mainly maintained in Lhx3 containing MMC where it participates to MN consolidation by preventing V2-complex formation (Lee et al., 2008; Lee et al., 2005). In fact, the absence of this LIM factor leads to an increase in the number of *Chx10*-expressing cells, and an increase in the number of hybrid cells expressing *Chx10* and *Isl1*. This increase in V2a markers expression is amplified in *LMO4* and *Hb9* double knock-out embryos (Lee et al., 2008). However, LMO4 competes for NLI binding, that also occurs in MN-hexameric complex. Analyses of

Isl1 hypomorphic mutant provided evidence that LMO4 is not able to displace Isl1 from NLI but that a low expression of *Isl1* leads to the inhibition of MN production that is not observed when LMO4 is inactivated (Song et al., 2009).

Hb9 expression is stimulated in MN compartment by the Isl1:NLI:Lhx3 complex that binds and activates an HxRE in its distal promoter region (Lee et al., 2008). The inactivation of Hb9, as already described, leads to the aberrant expression of V2a interneuron markers in prospective MN population. Indeed, a co-expression between V2a interneuron markers, like *Chx10* or *Sox14*, and *Isl1* is observed (Arber et al., 1999; Clovis et al., 2016; Lee et al., 2008; Thaler et al., 1999a). *In vivo* and *in vitro* analyzes revealed that Hb9 can bind the TeRE with a higher affinity than the V2-hexamer and inhibit its activation in the MN compartment (Figure 12) (Clovis et al., 2016; Lee et al., 2008).

In the same way, *in vitro* and *in ovo* analyzes showed that Chx10 binds and inhibits the activation of the HxRE in the V2 compartment (Figure 12) (Clovis et al., 2016). However, the absence of Chx10 leads to a decrease in V2a interneurons which is not balanced by an increase in MN markers. The double inactivation of Chx10 and Hb9 restores the V2a interneuron phenotype observed in the single Chx10 mutant but a decrease in MN markers, observed in *Hb9*$^{-}$ mutant, is still observed (Clovis et al., 2016). *Chx10* is expressed as soon as V2a and V2b start to segregate while *Lhx3* is expressed earlier, in the p2 progenitors. Therefore, it is speculated that another factor could play the role of Chx10 to secure V2 identity at earlier stages of development. As a possible candidate, we hypothesized that Vsx1, the single paralog of Chx10, could contribute to this process. In zebrafish, Vsx1 is found in p2 precursor cells and is transiently maintained in V2a interneurons (Batista et al., 2008). In mouse, *Vsx1* was initially described to be expressed in V2a interneurons deep analysis of its expression profile has never been performed. Vsx1 and Chx10 paralog factors recognize similar binding sites and both display repressor

activity (Dorval et al., 2005). Therefore, Vsx1 could contribute to consolidate the identity of the V2 interneurons during spinal cord development.

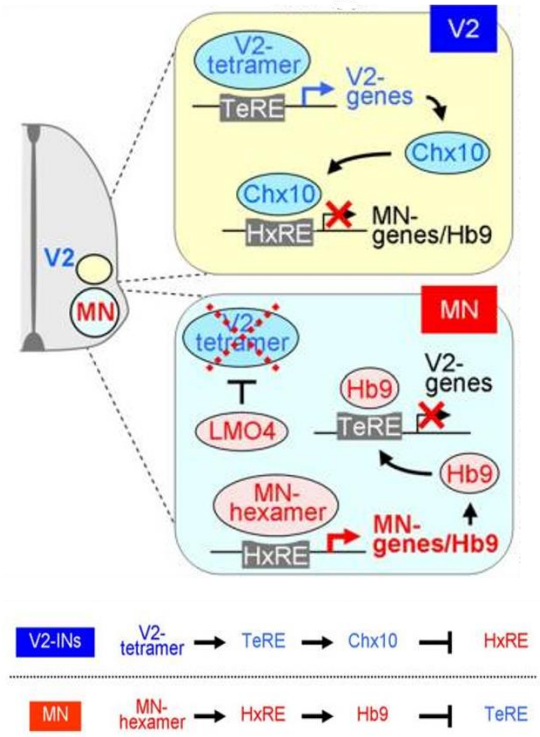


FIGURE 12 : V2 interneuron versus MN consolidation
In the V2 compartment, V2-tetramer complexes stimulate the expression of *Chx10* by binding to a TeRE while in MNs, the MN-hexamer complex activates the expression of Hb9. Both enhancer regions are kept silent by respective binding of Chx10 and Hb9 to HxRE and TeRE (Lee et al., 2008).

1.7. Vsx1

Vsx1 structure

Vsx1, firstly discovered in the goldfish retina (Levine et al., 1994), belongs to the *paired-like:CVC (Prd-L:CVC) homeobox* gene family, also composed of its unique paralog *Chx10* (Liu et al., 1994). It contains 5 exons and maps to chromosome 2 in mouse (Ohtoshi et al., 2001) and chromosome 20 in human (Hayashi et al., 2000). Homeobox genes, known to be crucial for many developmental events (Manak and Scott, 1994), encode transcription factors containing a 60-amino acid DNA binding

domain called homeodomain (Rohde et al., 2017). In comparison with conserved *paired* gene from *Drosophila*, it can be classified as *paired, paired-like* or *Orthodenticle (Otx)-like. Paired HD* encodes a 128-amino acid conserved *paired*-box while *paired*-like *HD* is more similar to *aristaless* or Drosophila retinal homeobox genes. Finally, *Otx-like HD* is similar to that of the Orthodenticle protein family (Passini et al., 1998). The Vsx1 HD belongs to the *paired*-like subset because of its similarity with *aristaless* gene that lacks the serine residue at position 50 (Gehring et al., 1994). Directly adjacent to the C-terminal part of the HD, Vsx1 displays an additional motif conserved between *Chx10* in mouse (Liu et al., 1994), *Vsx1/Vsx2* in goldfish and *Ceh-10* in nematodes (Svendsen and McGhee, 1995) and therefore named CVC domain (Figure 13). Regarding its proximity with the HD, it is suggested that CVC domain could influence transcriptional regulation. Dorval *et al.* demonstrated that this domain was necessary for the repressor activity of Vsx1 (Dorval et al., 2005).

Prd-L:CVC homeobox genes are classified into two families containing orthologs from several species: the Vsx1 group is composed of *Chx10-1* from chicken (Chen and Cepko, 2000), *Vsx1* from goldfish, zebrafish and mouse (Chow et al., 2001; Levine et al., 1997; Passini et al., 1997) and *Retinal Inner Nuclear homeoboX* gene (*RINX)* from human and bovine (Hayashi et al., 2000). It presents a proline-rich region and an acidic domain, described in other factors to have activation properties (Mermod et al., 1989; Williams and Tjian, 1991). It also contains a highly conserved domain between RINX and Vsx1 named RV domain (Hayashi et al., 2000). This last domain is not encoded by the Vsx2 group, composed of *Chx10* from chicken, human, mouse and zebrafish (Chen and Cepko, 2000; Hayashi et al., 2000; Liu et al., 1994), *Ceh10* from nematodes (Svendsen and McGhee, 1995) and *Vsx2* from goldfish (Levine et al., 1997). In place of the RV domain, the Vsx2 group

presents an OAR region conserved in Otp, Aristaless and Rax proteins (Furukawa et al., 1997).

Both groups contain a Nuclear Localization Signal (NLS) and a leucine-rich Nuclear Export Signal (NES), previously described as the octapeptide, which contribute to their cellular localization and their optimal and flexible transcription activities. (Hayashi et al., 2000; Knauer et al., 2005)

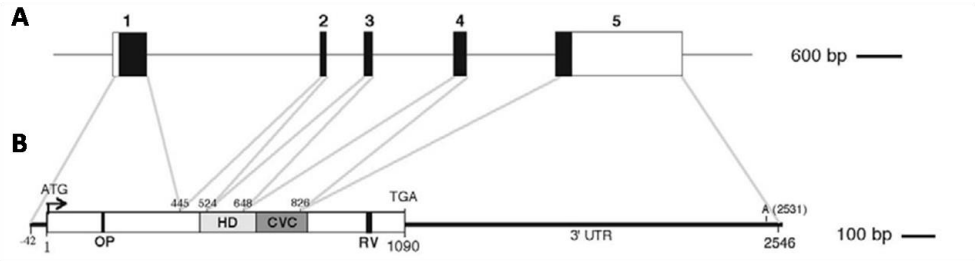


FIGURE 13: Vsx1 structure.
A) Schematic representation of mouse Vsx1 gene composed of 5 exons with untranslated regions (UTR) in white and the open reading frame as black boxes. **B)** Schematic representation mouse Vsx1 messenger RNA. OP=octapeptide (nuclear export signal); HD=Homeodomain (Chow et al., 2001).

Unlike its paralog factor Chx10, Vsx1 orthologs do not present a high conservation level. Mouse and human Vsx1, for example, only share 74% overall amino acids identity (Ohtoshi et al., 2001), which contrasts with the 96% conservation between mouse and human Chx10 sequences (Chow et al., 2001). Vsx1 HD and CVC domain also present a certain divergence, with conservation between 78 and 96% depending on the species (Table 1). This suggests that Vsx1 is evolving rapidly (Ohtoshi et al., 2001).

	N-Variable	HD	CVC Domain	C-Variable	Total
HsVsx1	69%	96%	90%	57%	74%
BtRINX	67%	96%	86%	53%	72%
GgChx10-1	51%	96%	80%	26%	56%
DrVsx1	37%	95%	78%	22%	52%
CaVsx1	38%	95%	78%	22%	51%
MmChx10	19%	91%	78%	12%	42%

TABLE 1 : Comparison of mouse Vsx1 peptidic sequence with other Prd-L:CVC containing-protein.
Hs=Homo sapiens; Bt=Bos Taurus; Gg=Gallus gallus; Dr=Danio rerio; Ca=Carassius auratus; Mm=Mus musculus. Adapted from (Ohtoshi et al., 2001).

Vsx1 expression profile and function

Vsx1 sequence comprises a prolin-rich region and an acidic domain described to have activation properties for other transcription factors (Hayashi et al., 2000). Nevertheless, *in vitro* experiments revealed that Vsx1 presents a repressor activity. As Chx10, Vsx1 is able to bind a P3 consensus motif composed of TAAT sites via its HD and to repress the activity of downstream genes. It has also been shown that, mutation in its CVC domain affect its repressor activity (Dorval et al., 2005).

Vsx1 messenger is firstly detected at very early stage of embryogenesis corresponding to gastrulation. Nevertheless, its role at this stage of development is not identified. Later, Vsx1 is expressed in the hindbrain and the spinal cord while its expression in the developing retina is initiated at developing postnatal stage. Its function in the eye has been deeply analyzed, in contrast with its role in the developing central nervous system (Ohtoshi et al., 2001).

In developing retina

Cell type composition and gross morphology of retina is well conserved (Avanesov and Malicki, 2004). Retina is divided into different layers mainly

composed of six cell types: photoreceptors, horizontal, amacrine, Müller glial, ganglion and bipolar cells (BCs). Photoreceptors and ganglion cell signals, mediated by bipolar interneurons, are fundamental for visual perception. In mouse, eleven types of BCs are described. Based on their synaptic connection to cone or rod photoreceptors, which respectively mediate photopic or scotopic vision, they are classified as cone or rod bipolar cells. Cone bipolar cells are classified as either OFF (type 1-4) or ON (type 5-9) depending on their response to glutamate (Ghosh et al., 2004).

Differentiation of photoreceptor, horizontal, amacrine and ganglion cells occurs mainly during embryogenesis, while BCs and Müller glial cells differentiate after birth. In comparison with mammalian eye development, retina development in teleosts occurs more rapidly, a visual function being observed from 3 days post-fertilization (3 dpf). Unlike its paralog factor *Chx10*, expressed in Retinal Progenitor Cells (RPCs) during embryogenesis and maintained in main BCs, *Vsx1* is present in differentiating and mature BCs from the inner nuclear layer (Chow et al., 2001; Hayashi et al., 2000; Passini et al., 1998). Its retinal expression profile is well conserved between species except for *Ch10-1* in chicken and *Vsx1* in xenopus that are present earlier, in the RPCs (Chen and Cepko, 2000; D'Autilia et al., 2006).

In mouse, Vsx1 is starts to be expressed after birth in type 1, 2, 3a OFF BCs and type 7 ON BCs but is absent from rod bipolar interneurons. Nevertheless, its expression in type 3a BCs is transient and not maintained at adult stage (Chow et al., 2001; Chow et al., 2004; Hayashi et al., 2000; Ohtoshi et al., 2004; Passini et al., 1998) (Chow et al., 2004; Shi et al., 2012; Shi et al., 2011). Chx10 and Vsx1 expression tend to be mutually exclusive in bipolar interneurons. A co-expression between both *Prd-L:CVC* factors can be observed but cells expressing *Chx10* poorly express *Vsx1* and vice versa (Clark et al., 2008). Chx10 inactivation leads to an

increase in *Vsx1* expression in the retina. Indeed, it has been shown that Chx10 is able to directly inhibit Vsx1 expression (Clark et al., 2008; Clovis et al.).

Vsx1 deficient mouse helped to identify its function in retina development. Gross morphology and global number of BCs stay unchanged in *Vsx1* mutants. Nevertheless, depending on the cellular context, Vsx1 either activates or inhibits the expression of specific genes. In OFF BCs, the absence of Vsx1 results in a down-regulation of specific markers such as *recoverin*, *Neuropilin and tolloid like 1 (Neto1)*, *Neuromedin-K 3 Receptor* (*NK3R)*, *Hyperpolarization activated cyclic nucleotide gated potassium chanel 4* (*HCN4)*, *Synaptotagmin 2* (*Syt2)* or *Calcium binding protein 5* (*Cabp5)*. In contrast, the deletion of Vsx1 induces an increased expression of *Cabp5* and *Chx10* in type 7 ON BCs. Thereby, even if Vsx1 is necessary for specific marker expression in OFF BCs, it uses different mechanisms to inhibit expression of the same genes in other cell types (figure 14) (Chow et al., 2004; Shi et al., 2012; Shi et al., 2011).

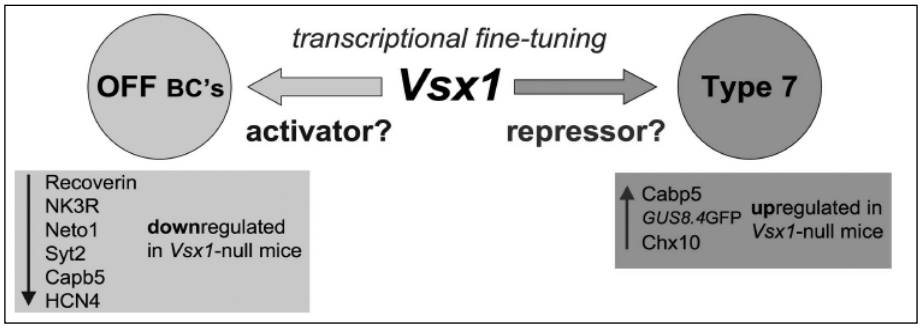


FIGURE 14 : Roles of Vsx1 in retinal gene expression
Vsx1 plays a dual role in regulation of BC markers. It activates the expression of specific markers of OFF bipolar interneurons but represses the expression of other markers in type 7 BCs (Chow et al., 2004).

Molecular abnormalities observed in absence of Vsx1 result in visual defects as observed by electroretinogram (ERG) analyses. A reduction in ERG b-wave is

observed with photopic stimuli, reflecting a defect in OFF and ON pathways (Chow et al., 2004; Ohtoshi et al., 2004). A defect in OFF BC synaptic arborization is also observed leading to defects in ganglion cells (GCs) activity (Chow et al., 2004). Moreover, the directionally selective GCs that receive input from type 7 BCs present a sustained response to stimuli (Shi et al., 2011). Finally, double mutant for *Vsx1* and *Irx5* allowed to unveil the role of Vsx1 in the contrast adaptation. Indeed, the double mutant mice present a reduced ability of GCs to adapt to temporal contrast (Kerschensteiner et al., 2008).

In human, although it is not found in the cornea, different *Vsx1* mutations are responsible of inherited corneal dystrophies, the posterior polymorphous dystrophy (PPD) and the keratoconus disease (Heon et al., 2002). In addition, additional ERG b-wave defects confirm what was observed in mouse *Vsx1* mutant lines (Heon et al., 2002).

In central nervous system

While the expression profile and the role of Vsx1 in the retina is well described, its role in the brain and the spinal cord is poorly studied. In zebrafish, Vsx1 is found in the hindbrain and the spinal cord at early stages of development. Its expression is observed in putative V2 precursors that give rise, after a single division, to V2a and V2b interneurons. Few *Vsx1*-expressing cells directly differentiate in V2a interneurons without cell division; a majority of them is glutamatergic and expresses *Lhx3* (Batista et al., 2008). In mouse, *Vsx1* was supposed to be expressed in V2a interneurons. Recent paper from Clovis et al. suggested that Vsx1 presents the same expression profile than Chx10 but is downregulated in the more lateral part of the spinal cord (Clovis et al., 2016).

1.8. Chx10 and Vsx1 paralog factors

Chx10 and *Vsx1* are the unique members of the *Prd-L*:*CVC* homeobox family in the mammalian genome. Paralog factors usually have redundant function implying an overlapping expression profile and structure. In this chapter, we compare the structure and the expression profile and function of these two paralog factors.

Total peptidic Vsx1 and Chx10 sequences present a low homology, 42% in mouse for example. Nevertheless, their CVC and HD are quite well conserved with a respective homology of 91 and 78% (Chow et al., 2004). Their structures differ by a RV and an OAR domain respectively found in Vsx1 and Chx10 only (Furukawa et al., 1997; Hayashi et al., 2000). Their HD is primordial for DNA binding and transcriptional activity. Chx10 and Vsx1 HD recognize same consensus sequences characterized by TAAT motifs and both paralogs present a repressor activity (Dorval et al., 2005).

Vsx1 and Chx10 are most of the time mutually exclusive. In zebrafish spinal cord, if a co-expression is observed in V2a interneurons, the expression level of one is complementary to the other. In mouse, *Vsx1* expression seems to be restricted to the medial part of the spinal cord while Chx10 appears in differentiated lateral V2a interneurons. In the same way, in the retina, Vsx1 is found in bipolar interneurons containing a minimal amount of Chx10. The *Vsx1* repression by Chx10 has been already addressed above. *In vitro* and *in ovo* analyses showed that Chx10 directly inhibits *Vsx1* expression by binding to a P3 motif located at position -567 in the *Vsx1* promoter (Clark et al., 2008; Clovis et al., 2016). In the retina, an upregulation of *Vsx1* is observed in *Chx10*$^{or J}$ deficient line. Nevertheless, this overexpression does not compensate the absence of Chx10 in RPC, leading to a high decrease in RPC proliferation and a loss of BCs (Burmeister et al., 1996b; Clark et al., 2008). Moreover, the absence of Vsx1 in retina leads to an overexpression of *Chx10* in type 7 BCs, leaving open the possibility that Vsx1 also represses *Chx10* expression (Chow

et al., 2004). Regarding their mutually exclusive expression profile, the probability that Chx10 and Vsx1 have a redundant function in the retina is low.

As already described, Chx10 consolidate V2a interneuron fate through the repression of the HxRE-mediated *Hb9* expression (Clovis et al., 2016; Lee et al., 2004). However, Lhx3, that composes V2-tetramer and MN-hexamer, is produced before *Chx10* expression, in V2 precursor cells. As for *Chx10*, misexpression of *Vsx1* in chicken spinal cord leads to a down-regulation of *Hb9* (Clovis et al., 2016). Therefore, we assess whether the unique paralog factor of Chx10, presenting the same kind of repressor activity, could play a similar role at earlier stage of development.

2. Objectives and strategies

During spinal cord development, Chx10 and Hb9 contribute to the consolidation of V2 interneuron versus MN differentiation by respectively competing with hexamer- and tetramer-complexes, for binding to their cognate sequence. Nevertheless, *Chx10* is expressed in differentiated V2a interneurons while *Lhx3*, that contributes to both complexes, is expressed in p2 and pMN (Peng et al., 2007). This strongly suggests that another factor anticipates and/or cooperates with Chx10, before the onset of V2a and V2b interneuron differentiation, to consolidate V2 interneuron identity. Paralog genes often present overlapping function. *Vsx1*, the unique paralog factor of *Chx10*, is also described as expressed in the V2 compartment, in zebrafish and in mouse, and could therefore constitute a good candidate to participate to this consolidation process. However, even if its distribution is quite well described in the zebrafish spinal cord (Batista et al., 2008), it is poorly studied in mammal models.

In this context, the first objective of this work was to characterize in details the distribution of Vsx1 in the murine spinal cord and to characterize the *Vsx1*-expressing cells. The second objective was to identify the roles of this transcription factor during V2 interneuron development and finally evaluate its contribution to the consolidation mechanism of V2 interneuron versus MN identity.

To do this, we firstly characterized the distribution of Vsx1 in murine spinal cord at different stages of development in combination with specific markers of V2 interneurons and neighbouring populations. Molecular composition of *Vsx1*-expressing cells was deeply assessed by characterization of the expression of specific V2 subset markers and of by characterization of proliferation and differentiation marker expression.

Secondly, to identify potential regulators of *Vsx1* expression or of newly-born V2 interneuron development, the number of *Vsx1*-expressing cells in the spinal cord was compared between control and different mutant lines: *Ascl1*$^{-/-}$, *PS1*$^{-/-}$, *Small eye* mutant (*Pax6*$^{Sey/Sey}$) and *HNF6/OC2*$^{-/-}$. Ascl1 and Dll/Notch signalling, affected in *PS1*-deficient line, are essential for the balanced segregation of V2a and V2b interneurons (Peng et al., 2007). In the same way, deficiency in *Pax6* expression leads to a decrease in V2a interneurons (Ericson et al., 1997; Gosgnach et al., 2006). Finally, *OC* factors are expressed in the spinal cord where they regulate different aspects of development (Audouard et al., 2012; Francius and Clotman, 2010; Roy et al., 2012), and our analyses uncover that Hnf6 is present in *Vsx1*-expressing cells and could therefore be implicated in its regulation.

Thirdly, to identify the function of Vsx1 in V2 interneuron differentiation, I produced two different transgenic lines: a *Vsx1*-deficient line and a conditional *Rosa26R::Vsx1-IRES-EGFP* line. The consequences of Vsx1 inactivation were evaluated by comparison of the number of p2 progenitor cells and the size of each V2 subset in control and in mutant spinal cords. We also analysed the expression of key regulators of V2 interneuron differentiation. In the same way, the conditional *Rosa26R::Vsx1-IRES-EGFP* line allowed us to analyzed the phenotype of V2 interneurons after ectopic production or overexpression of *Vsx1* in the MNs or in the V2 compartment, respectively.

Fourth, to evaluate the potential implication of Vsx1 in the consolidation of V2 interneuron versus MN identity, *in vitro*, *in ovo* and *in vivo* analyses were performed. We firstly assessed the ability of Vsx1 to bind the HxRE by chromatin immunoprecipitation assay. Then, we analyzed the capacity of Vsx1 to repress activation of the HxRE by luciferase assay performed on Human embryonic kidney 293 (HEK293) cells transfected with *Vsx1*-expressing constructs and HxRE::luciferase reporter plasmid. These *in vitro* observations were confirmed in

whole embryos using *in ovo* electroporation of chicken spinal cord with *Vsx1*-expression constructs and a HxRE::GFP reporter plasmid. Moreover, we assessed the effects of *Vsx1* misexpression on *Hb9* expression and MN production. Finally, the conditional *Rosa26R::Vsx1-IRES-EGFP* mouse line was used to drive the ectopic expression of *Vsx1* in MN or to increase and prolong *Vsx1* expression in V2 interneurons. These lines allow to analyze the consequences of Vsx1 gain-of-function on MN or V2 interneuron differentiation.

In the last part of this project, to evaluate the contribution of Vsx1 and Chx10 to the consolidation of V2 interneuron fate, we analysed *Chx10^{orJ}* and *Vsx1^{-}* mutant lines. We assessed the consequences of the inactivation of both paralog factors on V2 interneuron and MN differentiation. It was demonstrated that the inactivation of Chx10 is not sufficient to restore the MN decrease observed in *Hb9^{-}* mutant line. In this work, we assessed the contribution of Vsx1 in this consolidation mechanism by the analysis of the double mutant for *Hb9* and *Vsx1*.

3. Results

3.1. Vsx1 Transiently Defines an Early Intermediate V2 Interneuron Precursor Compartment in the Mouse Developing Spinal Cord

Foreword

Locomotion is a stereotyped behaviour mainly controlled by MNs and different populations of interneurons, dI6 and V0 to V3 interneurons, located in the ventral spinal cord. During development, these different populations arise from specific progenitor domains, pMN, pdI6 and p0 to p3 progenitor domains, following a well-regulated differentiation process. In particular, p2 progenitor domain gives rise at least to five populations of V2 interneurons: V2a, V2b, V2c, V2d and pax6-V2 interneurons.

In mouse spinal cord, it was supposed that *Vsx1* was expressed in V2a interneurons meanwhile no systematic analysis of its expression profile was performed. In this study, we demonstrate that Vsx1 transcription factor transiently labels early intermediate V2 precursors. We provide evidence that these cells derive from p2 progenitors that underwent their last mitotic division but did not start to initiate their differentiation process. On the one hand, we demonstrate that generation of V2 intermediate precursors and Vsx1 production does not require Dll/Notch signalling pathway activation although it is necessary for V2a and V2b segregation. On the other hand, we provide evidence that *Pax6*, expressed in V2 intermediate cells, is necessary for *Vsx1* expression. Finally, we show that the V2 interneuron production is not affected by the absence of Vsx1 which is probably compensated by other factors.

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Vsx1 Transiently Defines an Early Intermediate V2 Interneuron Precursor Compartment in the Mouse Developing Spinal Cord

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Abstract

Spinal ventral interneurons regulate the activity of motor neurons, thereby controlling motor activities. Interneurons arise during embryonic development from distinct progenitor domains distributed orderly along the dorso-ventral axis of the neural tube. A single ventral progenitor population named p2 generates at least five V2 interneuron subsets. Whether the diversification of V2 precursors into multiple subsets occurs within the p2 progenitor domain or involves a later compartment of early-born V2 interneurons remains unsolved. Here, we provide evidence that the p2 domain produces an intermediate V2 precursor compartment characterized by the transient expression of the transcriptional repressor *Vsx1*. These cells display an original repertoire of cellular markers distinct from that of any V2 interneuron population. They have exited the cell cycle but have not initiated neuronal differentiation. They co express *Vsx1* and *Foxn4*, suggesting that they can generate the known V2 interneuron populations as well as possible additional V2 subsets. Unlike V2 interneurons, the generation of Vsx1-positive precursors does not depend on the Notch signaling pathway but expression of *Vsx1* in these cells requires Pax6. Hence, the p2 progenitor domain generates an intermediate V2 precursor compartment, characterized by the presence of the transcriptional repressor Vsx1, that contributes to V2 interneuron development.

Introduction

During CNS development, the generation of numerous neuronal populations distinct in their functional properties, location and connectivity is a prerequisite for building circuitries that regulate complex behaviors like locomotion. In vertebrates, locomotor activity is controlled by multiple regions of the CNS but is eventually triggered by local neuronal circuits that are established in the ventral spinal cord. These circuits are composed of motor neurons and of several types of interneurons that regulate motor neuron activity (Garcia-Campmany et al., 2010; Goulding, 2009; Grillner and Jessell, 2009; Grossmann et al., 2010; Lu et al., 2015). Although cardinal populations of ventral interneurons have been thoroughly described, the molecular mechanisms that regulate the generation and the diversification of these cells remain only partly elucidated. Upon activation of unique combinations of transcription factors in response to morphogen gradients, distinct ventral interneuron progenitor domains called p0 to p3 are defined in the developing spinal cord. These progenitor domains generate four cardinal classes of ventral interneurons termed V0 toV3 (Garcia-Campmany et al., 2010; Grillner and Jessell, 2009; Lu et al., 2015). Interestingly, these cardinal populations further diversify in to numerous subsets characterized by distinct combinations of molecular markers, use of different neurotransmitters, specific projection patterns and differential contributions to the locomotor circuits (Al-Mosawie et al., 2007; Azim et al., 2014b; Britz et al., 2015; Crone et al., 2008; Dougherty et al., 2013; Francius et al., 2013; Karunaratne et al., 2002; Moran-Rivard et al., 2001; Panayi et al., 2010; Panayiotou et al., 2013; Pierani et al., 2001; Sapir et al., 2004; Zagoraiou et al., 2009; Zhang et al., 2014; Zhang et al., 2015). For example, V2 interneurons eventually subdivide into at least five different subtypes including V2a, V2b, V2c, V2d and Pax6+ V2 interneurons (Al-Mosawie et al., 2007; Dougherty et al., 2013; Karunaratne et al.,

2002; Panayiotou et al., 2013; Zhou et al., 2000). This diversification process enables ventral interneuron subpopulations to ensure different aspects of the stereotypic pattern of locomotor activity, including left-right alternation (V0 + V2a), flexor-extensor alternation (V1 + V2b), speed (V1) and robustness and rhythmicity (V2d + V3) (Azim et al., 2014b; Britz et al., 2015; Crone et al., 2008; Dougherty et al., 2013; Gosgnach et al., 2006; Lanuza et al.; Talpalar et al., 2013; Zhang et al., 2014; Zhang et al., 2008). Within the p2 domain, segregation of the V2a vs. V2b lineages depends on differential activation of the Dll4/Notch pathway (Del Barrio et al., 2007; Joshi et al., 2009; Peng et al., 2007; Skaggs et al., 2011), which relies on the mosaic expression of several transcriptional regulators including Foxn4 and Ascl1 (Del Barrio et al., 2007; Li et al., 2005; Misra et al., 2014). However, it remains unclear whether activation of the Notch pathway, V2a/V2b segregation and generation of the other V2 interneuron subsets occur within the p2 progenitor domain or in a later cell compartment of early-born V2 interneurons. In the present study, we identify an early intermediate V2 precursor compartment characterized by the transient expression of the transcription factor *Vsx1*. Vsx1 is a transcriptional repressor of the Paired-like CVC (Prd-L:CVC) homeobox gene family (Chow et al., 2001; Ohtoshi et al., 2001). In the mouse, it is expressed in gastrula stage embryos (Ohtoshi et al., 2001) and in several bipolar cone interneurons of the retina where it regulates different aspects of their terminal differentiation (Chow et al., 2004; Kerschensteiner et al., 2008; Ohtoshi et al., 2004; Shi et al., 2012). Here we show that, in the mouse developing spinal cord, Vsx1-positive cells are transiently detected from e9.5 onward, are molecularly distinct from any V2 interneuron populations and generate all the V2 populations and possibly additional V2 subsets that remain to be characterized. Loss-of-function analyses indicate that Pax6 is required for the expression of *Vsx1* in this cell compartment.

Results

The p2 Progenitor Domain Generates an Early V2 Population

In mouse embryonic spinal cord, neurogenesis starts around e9.5. The earliest differentiating neurons are motor neurons, which originate from the pMN progenitor domain. We recently described that the production of V1 interneurons is also initiated at e9.5 (Stam et al., 2012). At this early stage, the presence of the OC transcription factors, namely OC-1 (or HNF-6), OC-2 and OC-3, was restricted to differentiating neurons and was observed in motor neurons and V1 interneurons (Francius and Clotman, 2010; Francius et al., 2013; Roy et al., 2012; Stam et al., 2012). Surprisingly, OC-1 was additionally detected in cells located between motor neurons and prospective Renshaw cells (Figures 15A–A’’’; Stam et al., 2012). As the p2 progenitors are located between the pMN and the p1 domains we postulated that these unidentified cells were most likely early-born V2 cells containing OC factors. To test this hypothesis, we assessed the distribution of Ascl1, which is specifically detected in the p2 domain and early V2 interneurons (Gong et al., 2003; Parras et al., 2007; Wildner et al., 2006). Co-detection of OC-1 and Ascl1 confirmed that early-born OC-1+ cells derived from the p2 domain (Figures15B–B’’’). Given that OC factors are detected only in post-mitotic neurons (Francius and Clotman, 2010), we concluded that these cells were early V2 interneurons. However, V2 interneurons are reported to be generated from e10.5 onward (Li et al., 2005; Peng et al., 2007; Zhou et al., 2000). Accordingly, the number of V2a or V2b identified by the presence of Chx10 or Gata3, respectively, was restricted at e9.5 (1–5 cells per hemisection; Figures 15C–C’’, F–F’’, 16C) but significantly increased from e10.5 on (Figures 16A–C) (Nardelli et al., 1999; Smith et al., 2002; Zhou et al., 2000). At e9.5, OC-1 was present in V2a and V2b interneurons, as well as in other cells located lateral to the p2 progenitor domain (Figures 15C–C’’). Taken together, these

observations suggested either that OC-1 was detected in early V2a/V2b interneurons before the onset of *Chx10*/*Gata3* expression or that OC-1 was present in an additional unknown V2 subset. The latter hypothesis was consistent with results from a genetic lineage-tracing of Foxn4+ progenitors, which indicated that the p2 domain produces additional unidentified V2 interneurons (Li et al., 2010).

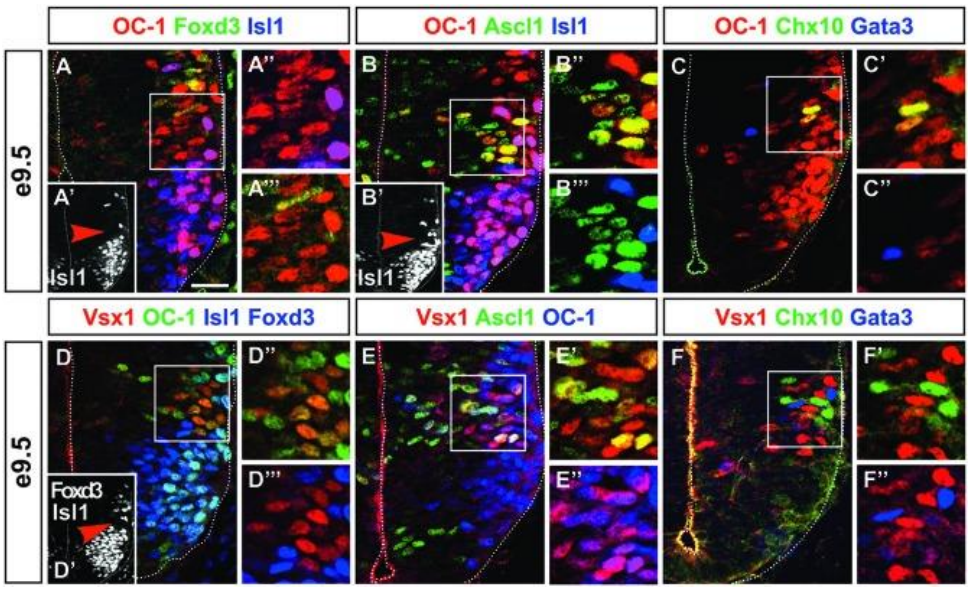


FIGURE 15 : Onecut (OC)-1 and Vsx1 are present in an early V2 interneuron population. **(A–A''')** At e9.5, OC-1 is detected in cells located between motor neurons (Isl1+, inset A'; arrowhead indicates the location of A'' and A''' insets) and V1 interneurons (Foxd3+). **(B–B''')** These cells derive from the p2 progenitor domain (Ascl1+) and are not motor neurons (Isl1+, inset B'; arrowhead indicates the location of B'' and B''' insets). **(C–C'')** OC-1 is detected in the V2 domain in cells that are neither V2a (Chx10+) nor V2b (Gata3+) interneurons. **(D–D''')** Vsx1 is detected in cells containing OC-1 located between motor neurons (Isl1+, inset D'; arrowhead indicates the location of D'' and D''' insets) and V1 interneurons (Foxd3+). **(E–E'')** Vsx1 is present in cells that derive from the p2 domain (Ascl1+) and contain OC-1. **(F–F'')** Vsx1+ cells are distinct from early V2a (Chx10+) and V2b (Gata3+) interneurons. Scale bar = 50 µm.

To distinguish between these possibilities, we searched for specific markers of these cells. Several transcription factors present in V2 interneurons are also

detected in differentiating neurons of the retina (Glaser et al., 1992; Gouge et al., 2001; Liu et al., 1994; Tomita et al., 1996). Thus, specific markers of this V2 subset may be found among retinal proteins that are not yet associated with a specific spinal population. Vsx1 is the single paralog of the V2a marker Chx10 in the mouse genome and is expressed as Chx10 in the developing retina (Chow et al., 2001; Ohtoshi et al., 2001). Interestingly, *Vsx1* expression in retinal cells is inhibited by Chx10 (Clark et al., 2008) and *Chx10* expression is repressed by Vsx1 in Type7 cone bipolar cells (Shi et al., 2011). In the zebrafish embryonic spinal cord, the Vsx1 ortholog is detected in common V2a/V2b progenitors and is transiently maintained in the V2a lineage (Batista et al., 2008; Kimura et al., 2008). In Xenopus and chick, *Vsx1* expression was attributed to V2a interneurons based on its distribution similar to Chx10 (Chen and Cepko, 2000; D'Autilia et al., 2006). However, co-labeling of Vsx1 and other V2 markers in the mouse embryonic spinal cord was not reported yet.

Therefore, we characterized the distribution of Vsx1 in the mouse developing spinal cord. First, we assessed whether Vsx1 is present in the early OC-1+ V2 cells identified at e9.5. At this stage, Vsx1 was detected in cells containing OC-1 that were neither motor neurons nor early V1 interneurons (Figures 15D–D’’’). As observed for OC-1 (Figures 15B–B’’’), these cells contained Ascl1, confirming that they derived from the p2 domain (Figures 15E–E’’). To determine whether these may correspond to V2a or V2b interneurons, the distribution of Vsx1 was compared to that of Chx10 and Gata3. However, Vsx1 was detected in cells lacking Chx10 or Gata3 (Figures 15F–F’’), leaving open the question whether they corresponded to early V2 precursors or to a distinct population of V2 interneurons.

Vsx1 Defines a Specific V2 Subset

To further investigate this question, we compared the distribution of Vsx1 and V2 markers at later developmental stages (Figures 16A–E). We first noticed that the number of Vsx1+ cells increased from e9.5 to e10.5, then decreased to e12.5 and became undetectable at later stages (Figures 16A–C and data not shown), indicating a transient expression of *Vsx1* in the mouse spinal cord as observed in zebrafish embryos (Passini et al., 1998). At all developmental stages, Vsx1 was found in cells located in close vicinity to V2a and V2b interneurons. However, co-detection of Vsx1 with Chx10 or Gata3 was never observed (Figures 16A–B''). Vsx1 was also present at e11.5 in cells located nearby V2d interneurons, characterized by the presence of Shox2 in the absence of Chx10 (Dougherty et al., 2013), but Shox2 was not detected in Vsx1+ cells (Figures 16D–D''). Finally, Vsx1 distribution was compared to that of Sox1, which labels V2c interneurons in addition to spinal progenitors (Panayi et al., 2010). However, V2c are located more ventrally and were distinct from cells containing Vsx1 (Figure 16E, arrow). Hence, Vsx1 is a specific, albeit transient, marker of a novel cell subset distinct from described V2 populations.

Spinal neurons that share lineage relationship or developmental origin of often exhibit common markers. To assess whether Vsx1+ cells might relate to any V2 population, we established a repertoire of markers detected in these cells. As OC-1 was detected in Vsx1+ cells at early developmental stages (Figures 15D–D''), we assessed whether OC factors were co-detected with Vsx1 in more differentiated cells. At e10.5, OC-1 levels decreased sharply but the protein was still detected in 66% of the Vsx1+ cells (Figures 16F–F''), while OC-2 (Figures 16F–F'') and OC-3 (data not shown) were absent. As observed at e9.5, Ascl1 was present at e10.5 in the Vsx1+ cells, confirming their p2 origin (Figures 16G–G'''). Lhx3 is present in pMN and in p2 progenitors and is maintained in the medial motor column and in V2a

interneurons but not in other V2 subsets. Lhx3 was detected in 95% of the Vsx1+ cells (Figures 16H–H’’’), suggesting that they might relate to V2a interneurons. Accordingly, Prdm8 and Bhlhb5, which are present in V2a interneurons (Francius et al., 2013; Skaggs et al., 2011), were detected in 97% and 87% of the Vsx1+ cells, respectively (Figures 16G–H’’’). In contrast Lhx1/5, which is present in V2b but is excluded from V2a interneurons, was barely detectable in 7% of the Vsx1+ complement (Figure 16I, arrowheads). Thus, as previously reported in other species (Batista et al., 2008; Chen and Cepko, 2000; D'Autilia et al., 2006; Kimura et al., 2008), Vsx1+ cells share some characteristics with V2a interneurons but Vsx1 is not detected in known V2 populations.

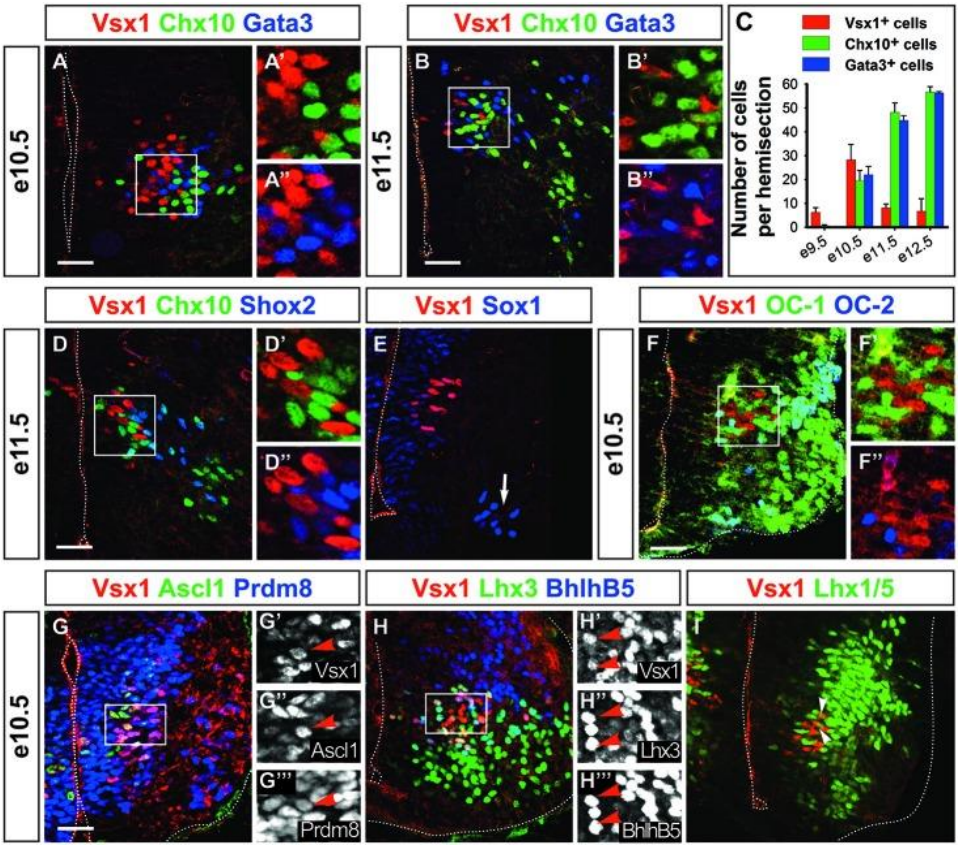


FIGURE 16: Vsx1 identifies a population distinct from V2 interneurons.
At e10.5 **(A–A'')** and e11.5 **(B–B'')**, Vsx1 is present in cells that intermingle with, but are distinct from V2a (Chx10+) and V2b (Gata3+) interneurons. **(C)** Quantification of V2a, V2b and Vsx1+ cells at different stages (mean values ± SEM, n = 3). **(D–D'')** Vsx1 is not detected in V2d interneurons (Shox2 + Chx10−). **(E)** Vsx1+ cells are also distinct from V2c cells (Sox1+, arrow). **(F–F'')** At e10.5, OC-1 is present at low levels in Vsx1+ cells, while OC-2 is undetectable. **(G–G''')** Ascl1 is present in Vsx1+ cells, confirming their p2 origin. Proteins present in V2a, including Prdm8 **(G–G''')**, Lhx3 and Bhlhb5 **(H–H''')**, are also detected. **(I)** In contrast, Lhx1/5, which is found in V2b, is barely detectable (arrowheads). Scale bars = 50 µm.

Vsx1 Is Restricted to an Early V2 Compartment Prior to Neuronal Differentiation

The lack of differentiated V2 interneuron markers in Vsx1+ cells and their medial location in the intermediate zone (Figures 15, 16) prompted us to assess whether they were differentiating neurons. Co-labeling experiments from e9.5 to e11.5 showed that only a minority of the Vsx1+ cells, decreasing from 14% to 0%, respectively, contained the neuronal differentiation marker b-III-tubulin (Figures 17A–D). This suggested that Vsx1 was present before the onset of neuronal differentiation in p2-derived cells. Accordingly, Sox1, which labels the spinal progenitors in the ventricular zone, was detected in medial Vsx1+ cells located within the subventricular zone (Figures 16E, 17C–C''). These observations supported the hypothesis that *Vsx1* is expressed in early V2 precursors before the onset of expression of their population-specific differentiation markers. Consistent with this idea, b-III-tubulin was detected in all the V2a and V2b cells at e10.5 (Figures 17E–E'') and the cyclin-dependent kinase inhibitor protein p27 Kip1 was present in V2a and V2b interneurons but not in Vsx1+ cells (Figures 17F–F'', Chx10 and Gata3 both in blue). This suggested that *Vsx1* expression occurs earlier than that of *Chx10* and *Gata3* in V2 interneurons. Hence, Vsx1 may be restricted to an early V2 compartment prior to V2 interneuron diversification.

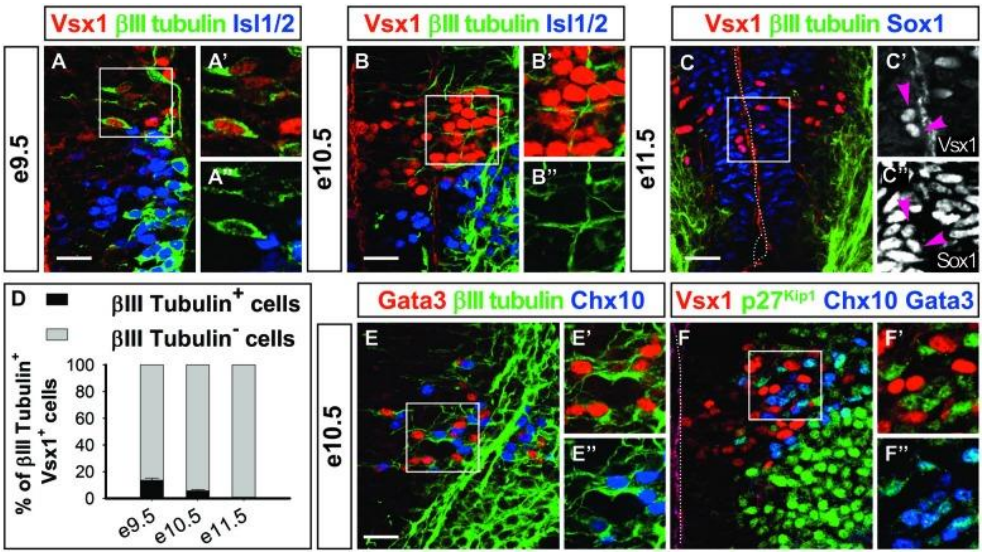


FIGURE 17 : Vsx1 is present before the onset of neuronal differentiation.
(A–B'') At e9.5 **(A–A'')** and e10.5 **(B–B'')**, the majority of the Vsx1+ cells are devoid of β-III-tubulin. In contrast, most of the motor neurons (Isl1/2+) contain β-III-tubulin at this stage. **(C–C'')** Co-labeling of β-III-tubulin and Vsx1 is never observed at e11.5, and the Vsx1+ cells are located between the neural progenitors (Sox1+) and the differentiating neurons (β-III-tubulin+). Vsx1 is detected in some Sox1+ progenitors (arrowheads). **(D)** Quantifications (mean percentage ± SD, n = 3) shows that only a minority of Vsx1+ cells contain β-III-tubulin at e9.5 and e10.5 and none at e11.5. **(E–E'')** In contrast, at e10.5, β-III-tubulin is readily detected in differentiating V2a (Chx10+) or V2b (Gata3+) interneurons. **(F–F'')** Accordingly, p27Kip is present in V2a and V2b interneurons (Chx10+ and Gata3+, both in blue) but not in Vsx1+ cells. Scale bars = 50 µm.

To test this idea, we compared the distribution of Vsx1 with that of Prospero homeobox protein 1 (Prox1) and Insulinoma associated protein 1 (Insm1). These two factors define an early intermediate compartment containing late progenitors (i.e., progenitors in their last division) and newborn neurons (Duggan et al., 2008; Misra et al., 2008). Indeed, we found that Vsx1 was present in cells containing both Prox1 and Insm1 (Figures 18A–A''), demonstrating that Vsx1 is restricted to an early intermediate V2 subset. The presence of Ascl1 in this subset but not in differentiating V2a or V2b interneurons (Figures 15B–B'', 16G–G'', 18B–B'', Chx10

and Gata3 both in blue) suggested that the Vsx1+ cells may correspond to precursors of the described V2 populations. We thus investigated whether Vsx1+ cells contained other determinants of V2 development. The segregation of V2a and V2b interneurons depends on a regulatory network involving Ascl1 and Foxn4 that eventually controls production of the Notch ligand Dll4 and thereby differentially modulates activation of Notch signaling in V2 precursors (Del Barrio et al., 2007; Li et al., 2005; Misra et al., 2014; Peng et al., 2007; Zou et al., 2015). Dll4 was present in Vsx1+ cells at the time of V2a/V2b production (Figures 18C–C'', arrowheads), although it was not exclusive to the Vsx1+ population. Similarly, *Foxn4* expression was detected by double ISH in the cells expressing *Vsx1* (Figures 18D–F'). As V2 precursors expressing *Foxn4* have been shown to generate all the described V2 interneuron populations as well as additional V2 subsets (Li et al., 2010), our data demonstrate that Vsx1+ cells are precursors of V2a/V2b interneurons and of other V2 subsets that also derive from Foxn4- and Dll4-containing progenitors (Panayi et al., 2010; Panayiotou et al., 2013; Zou et al., 2015).

We then assessed whether Vsx1 is detected in dividing cells of the ventricular zone or in intermediate precursor cells. Pax6 and Nkx6.1, which are present in the p2 progenitors, were detected in the Vsx1+ cells. However, these cells were clustered nearby the lateral border of the ventricular zone rather than the lumen (Figures 18G–G'''). Consistently, although Sox1 was present at low levels (Figures (Figures 16E, 18H–H'''), the proliferation marker Ki-67 was barely detectable in Vsx1+ cells (Figures 18H–H''') and only remaining traces of phosphorylated-Histone H3, specific to the metaphase of mitotic division, were found (Figures 18I,I', arrowhead). Taken together, these observations suggest that Vsx1 is present in cells that just completed their last mitotic division but did not yet initiate neuronal differentiation, defining an early intermediate V2 interneuron compartment. As

observed respectively in motor neurons and in V1 interneurons, Nkx6.1 and Pax6 are maintained in these cells even though they are no longer dividing.

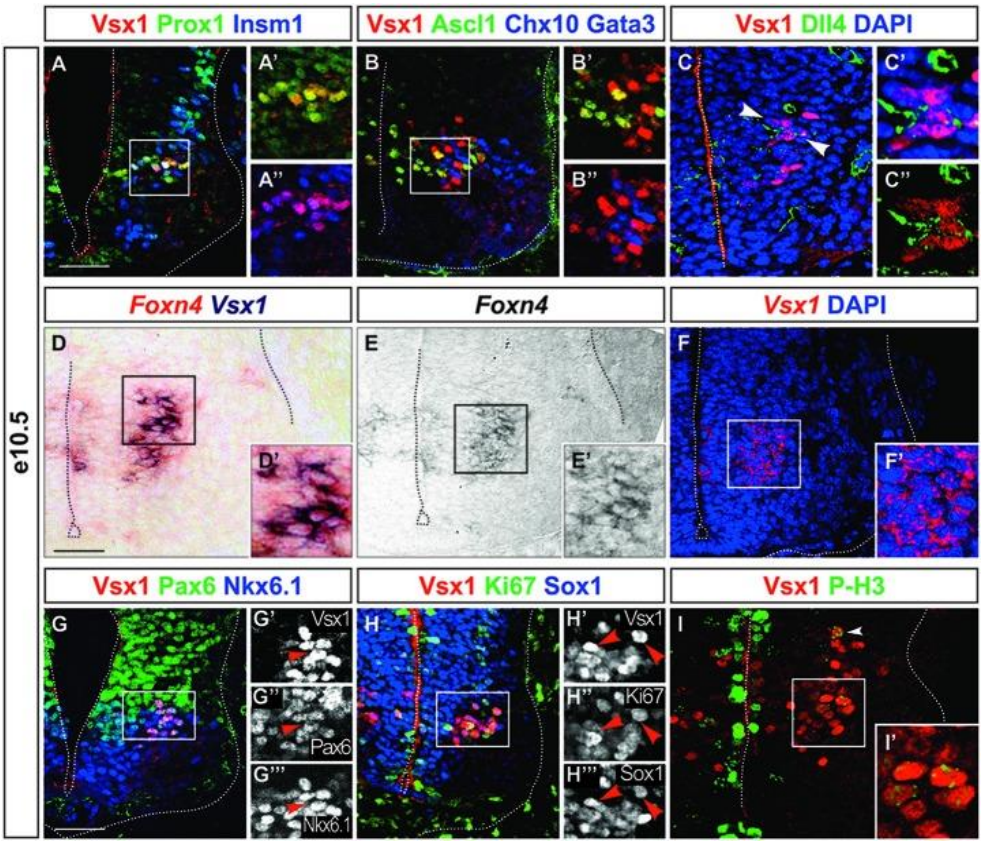


FIGURE 18 : Vsx1 is present in an early intermediate compartment during V2 interneuron differentiation.

(A–A'') Vsx1 is detected in the intermediate compartment characterized by the presence of Prox1 and Insm1. **(B–B'')** Ascl1 is co-detected with Vsx1, which is not the case for Chx10 and Gata3 (both in blue). **(C–C'')** Dll4 is detected in Vsx1+ cells (arrowheads). **(D–F')** Vsx1 mRNA is detected by double in situ hybridization (ISH) in cells expressing Foxn4. **(G–G''')** Pax6 and Nkx6.1 are maintained in cells containing Vsx1. **(H–H''')** Although Sox1 is present at low levels in some Vsx1+ cells, Ki-67 is barely detectable. **(I, I')** Only remaining traces (arrowhead) of phospho-HistoneH3 is detected in cells containing Vsx1, as compared to the strong labelling of the dividing progenitors in the ventricular zone. Scale bars = 50 µm.

The Developmental Determinants of V2 Interneurons Are not Required for the Production of Vsx1^{+} Cells

Given the possibility that Vsx1+ cells may constitute precursors of V2 populations, we assessed whether their production depends on factors necessary for proper V2 interneuron development. The segregation of the V2a and V2b lineages relies on the asymmetrical activation of the Notch pathway by Dll4, which stimulates Notch signaling in surrounding cells and thereby promotes V2b differentiation, restricting V2a differentiation to cells with lower Notch activity (Del Barrio et al., 2007; Li et al., 2005; Misra et al., 2014; Peng et al., 2007; Zou et al., 2015). Proper activation of this pathway requires mosaic expression of several transcriptional regulators including Foxn4 and Ascl1 in the p2 domain (Del Barrio et al., 2007; Li et al., 2005; Misra et al., 2014; Xiang and Li, 2013). To assess whether the production of the Vsx1+ cells also depends on this network, we evaluated the phenotype of these populations in embryos mutant for *Foxn4*, *Ascl1* or *PS1*. Lack of *Foxn4* resulted as expected in a strong imbalance in the generation of V2 interneuron subsets, with V2a being produced in excess at the expense of V2b cells (Figures 19A–B″). However, the generation of Vsx1+ cells was not affected (Figures 19A–C). Similarly, inactivation of *Ascl1* resulted in excessive production of V2a interneurons at the expense of V2b, but the number of Vsx1+ cells was not altered (Figures 19D–F). Consistently, loss of PS1, which results in severe diminution of Notch activation (Struhl and Greenwald, 2001), did not alter the specification of Vsx1+ cells (Figures 19G–I). These observations indicate that the generation of Vsx1+ cells does not depend on Notch signaling but remain consistent with the hypothesis that these cells may constitute the precursor population wherein differential activation of the Notch pathway determines further V2 interneuron diversification.

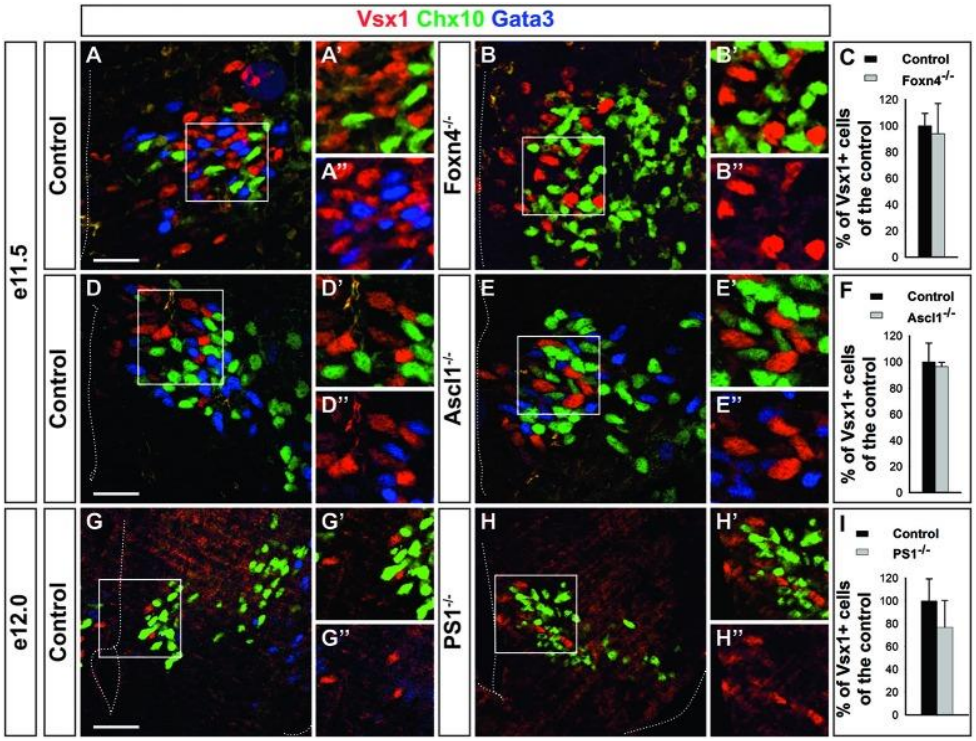


FIGURE 19 : The generation of Vsx1+ cells does not require Notch signaling.
(A–C) The generation of Vsx1+ cells is not altered in Foxn4 mutant embryos, although production of V2b interneurons (Gata3+) is abolished in favor of V2a interneurons (Chx10+). **(D–F)** Vsx1+ cell production is not altered in Ascl1 mutant embryos whereas supernumerary V2a interneurons are generated at the expense of V2b cells. **(G–I)** In Presenilin-1 (PS1) mutant embryos, the number of Vsx1+ cells was similar to that of control embryos whereas V2b interneurons were replaced by V2a interneurons. Scale bars = 50 µm. Quantifications are expressed as mean percentage ± SD, n = 4.

Pax6 Is Required for Proper Expression of *Vsx1* in V2 Precursors

We then turned our analyses to the other transcription factors expressed during Vsx1+ cell development. OC factors are present in the early Vsx1+ cells (Figures 15, 16). The role of OC factors has been evaluated in *Oc1/Oc2−/−* double-mutant embryos, which also lack Oc3 in the CNS (Roy et al., 2012). However, the number

of Vsx1+ cells was not changed in the spinal cord of these mutants, suggesting that OC proteins are not necessary for their production (Figures 20A–C).

Next, we investigated the role of Pax6 in Vsx1+ cell generation. Pax6 is required for patterning the ventral spinal cord during neurogenesis (Ericson et al., 1997) and for proper generation of V1 interneurons (Burrill et al., 1997; Gosgnach et al., 2006; Sapir et al., 2004). In Small eye (Sey/Sey) mutant embryos, which lack Pax6 (Glaser et al., 1992), the production of V0 and V2b populations is not affected (Burrill et al., 1997; Gosgnach et al., 2006; Sapir et al., 2004; Zhang et al., 2014) while the number of V2a interneurons is only slightly decreased (Ericson et al., 1997; Gosgnach et al., 2006). The persistence of Pax6 in the early Vsx1+ cells suggested that this factor may contribute to their development. Accordingly, Vsx1 was barely detectable in Sey/Sey embryos at e11.5 (Figures 20D–F) and e12.5 (data not shown), although the production of V2a and V2b interneurons was conserved. The presence of V2a and V2b interneurons indicated that the p2 progenitor domain was not re-specified and that the V2a/V2b precursors were preserved. In contrast, the loss of Vsx1 unveiled a selective requirement for Pax6 in the expression of Vsx1 in this early intermediate V2 compartment. Furthermore, these observations suggest that Vsx1 may be dispensable for proper generation of V2 interneuron subsets.

To test this hypothesis, we assessed the role of Vsx1 in V2 interneuron development. In the absence of any other specific marker of the Vsx1+ cells, this compartment could not be analyzed in the Vsx1 mutant embryos. In contrast, as Vsx1-containing precursors generate all the V2 subsets, we addressed a possible role for Vsx1 in V2 interneuron development. An inverse relationship between the levels of Chx10 and Vsx1 was previously reported in bipolar cells of the retina (Clark et al., 2008). To investigate whether a similar relationship also exists in spinal cord, the distribution of Chx10 was first assessed in Vsx1 mutant embryos.

However, the number of Chx10-positive cells was similar at e12.5 in control and in mutant embryos, indicating that the absence of Vsx1 does neither impact on V2a interneuron production nor on Chx10 expression. Accordingly, the number of V2b interneurons was not affected and the V2a/V2b ratio was preserved (Figures 20G–I). Taken together, these observations confirm that Vsx1 is not required for the production of described V2 interneuron populations, suggesting that the integrity of the early V2 precursor compartment is also preserved in Vsx1 mutant embryos. Consistently, Vsx1 mutant mice did not display any alteration in their motor behavior (data not shown).

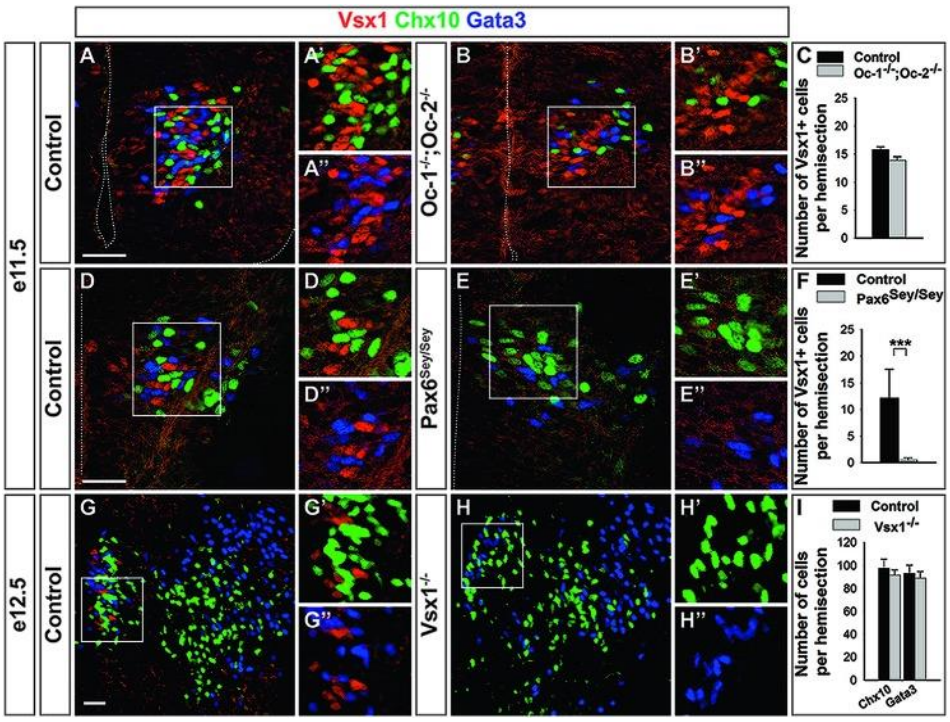


FIGURE 20 : Pax6 is necessary for Vsx1 expression.
(A–C) The generation of Vsx1+, Chx10+ and Gata3+ cells, is not affected in $Oc1^{-/-}Oc2^{-/-}$ embryos. **(D–F)** In $Pax6^{Sey/Sey}$ embryos, the number of Vsx1+ cells is dramatically reduced (***$p < 0.001$) without similar alteration of V2a or V2b production. **(G–I)** In embryos lacking Vsx1, the production of V2a and of V2b interneurons is unaffected. Scale bars = 50 µm. Quantifications are expressed as mean values ± SEM, n = 3.

Discussion

During spinal cord development, ventral progenitor domains produce multiple neuronal populations that variously contribute to the activity of motor circuits. Here, we provide evidence that the p2 progenitor domain generates an early intermediate compartment characterized by the presence of Vsx1 prior to neuronal differentiation. This early compartment very likely produces the collection of known V2 interneuron populations, and may also generate additional V2 subsets that remain to be identified (Figure 21).

In the zebrafish embryonic spinal cord, Vsx1 is detected in common V2a/V2b progenitors and its expression is transiently maintained in the V2a lineage (Batista et al., 2008; Kimura et al., 2008). In Xenopus and chick, the expression of *Vsx1* was attributed to V2a interneurons based on its distribution similar to that of Chx10 (Chen and Cepko, 2000; D'Autilia et al., 2006). Here, we demonstrate that, in contrast to other species, murine Vsx1 is restricted to an early intermediate compartment that likely comprises precursors of the known V2 subsets. Although they contain factors known as progenitor markers, including Sox1, Nkx6.1 and Pax6, $Vsx1^+$ cells are not proliferating as evidenced by the absence of proliferation or mitosis markers. Conversely, $Vsx1^+$ cells are not differentiating neurons, as they neither display cyclin-dependent kinase inhibitor protein of V2 interneurons (Gui et al., 2007) and very few of them produce β-$_{III}$-tubulin. Therefore, they constitute an early intermediate compartment of V2 postmitotic precursors (Figure 21).

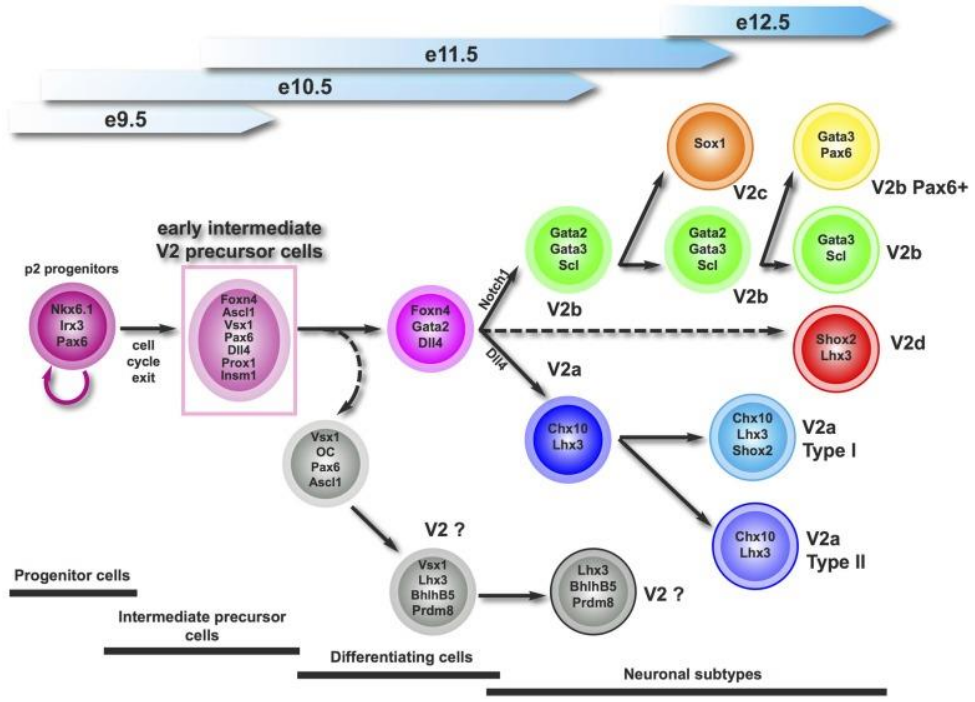


FIGURE 21 : Proposed working model for the generation of V2 interneuron subtypes.
Vsx1 defines an early intermediate precursor compartment that generates all the described V2 interneuron subsets and may produce additional V2 subpopulation(s) that remain(s) to be characterized.

Consistent with this idea, Vsx1^{+} cells contain Prox1 and Insm1, which specifically label compartments comprising late progenitors and newborn neurons and are transiently expressed before the onset of neuronal differentiation (Duggan et al., 2008; Misra et al., 2008). Prox1 acts downstream of the pro-neural proteins Ascl1 and Neurog-2 to implement neurogenesis in the spinal interneurons and may antagonize the anti-neurogenic activity of Sox proteins (Misra et al., 2008) including Sox1, which is present in spinal progenitors. Insm1 is thought to play a role in the termination of progenitor proliferation throughout the nervous system (Duggan et al., 2008). Prox1 and Insm1 may affect the onset and extent of terminal and penultimate neurogenic divisions, and thus the total number of neurons produced,

in any area and stage of neurogenesis. In the spinal intermediate V2 compartment defined by the presence of Vsx1, one may additionally propose that Prox1 and Insm1 could delay the onset of neuronal differentiation and thereby open a time-window necessary for the activation of signaling pathways, including Notch, that will determine the diversification of V2 precursors in multiple V2 populations.

Our data suggest that $Vsx1^+$ cells are the precursors of most of the V2 interneurons (Figure 21) including V2a and V2b subsets (Li et al., 2005; Del Barrio et al., 2007; Peng et al., 2007; Misra et al., 2014; Zou et al., 2015). Vsx1 is never co-detected with the V2a or V2b specific markers Chx10 or Gata3 although some $Vsx1^+$ cells very likely differentiate into V2a interneurons as they contain Dll4 (Zou et al., 2015). This indicates a sharp downregulation of *Vsx1* expression at the onset of V2 diversification. Interestingly, *Chx10* expression is repressed by Vsx1 in Type 7 cone bipolar cells (Shi et al., 2011) and *Vsx1* expression in retinal cells is inhibited by Chx10 (Clark et al., 2008). Such a cross-repression loop may also exist between these two Prd-L:CVC factors during V2a differentiation and may contribute to rapidly inhibiting *Vsx1* expression in differentiating V2a cells. However, Chx10 distribution was not expanded in the absence of Vsx1, indicating that the latter is not required to restrict *Chx10* expression to the differentiating V2a interneurons. In contrast, lack of co-detection of Vsx1 and Gata3 suggests either that other mechanisms downregulate *Vsx1* expression at the onset of V2b differentiation or that V2b interneurons do not derive from $Vsx1^+$ cells. The latter possibility is unlikely since we detected *Vsx1* in the cells expressing *Foxn4* and V2b interneurons derive from *Foxn4*-positive precursors (Del Barrio et al., 2007; Li et al., 2010). Hence, the mechanisms that sharply downregulate *Vsx1* expression at the onset of V2 diversification and the necessity of this tight regulation require further investigations.

Our observations strongly suggest that Vsx1 defines an early intermediate V2 compartment that generates multiple V2 subsets. Whether Vsx1$^+$ cells constitute only precursors of the known V2 populations or additionally produce other V2 subsets (Figure 21) remains an open question. The existence of an additional V2 population that derives from *Foxn4*-expressing cells has been demonstrated (Li et al., 2010), and *Vsx1* and *Foxn4* are extensively coexpressed in the early V2 precursors. Therefore, spinal Vsx1$^+$ cells very likely produce yet unknown V2 subsets.

Our study of the developmental determinants of the Vsx1$^+$ cells identified a novel role for Pax6 in the developing spinal cord. Pax6 is initially present in all progenitor domains of the neural tube and, following neural patterning, is excluded from the p3 domain (Ericson et al., 1997). It is also transiently maintained in V1 (Stam et al., 2012) and in Vsx1$^+$ cells (this study). It is necessary for the production of V1 interneurons and slightly impacts on the generation of V2a cells but not of the adjacent V0 or V2b populations (Burrill et al., 1997; Ericson et al., 1997; Sapir et al., 2004; Gosgnach et al., 2006; Zhang et al., 2014). Here we provide evidence that Pax6 is additionally necessary for the expression of *Vsx1* in an early intermediate V2 compartment. The loss of *Vsx1* expression in the spinal cord of *Sey* mutants is unlikely to result from defective dorsoventral patterning or altered neurogenesis in the p2 domain since V2b cells are generated properly and V2a interneurons are only slightly decreased (Burrill et al., 1997; Ericson et al., 1997; Sapir et al., 2004; Gosgnach et al., 2006; Zhang et al., 2014). It more likely manifests a requirement of Pax6 for the expression of *Vsx1* in this early V2 compartment. Identification of other specific markers for these cells will be required to test this hypothesis. In the retina, Pax6 and Vsx1 are present in different cellular compartments (de Melo et al., 2003b), suggesting that Vsx1 in the spinal cord may have been recruited into genetic networks different from those that operate in the eye. Consistently, *Vsx1*

expression is reactivated after spinal cord injury in the adult zebrafish and a large majority of the Vsx1^{+} cells observed after lesion also contains Pax6 (Kuscha et al., 2012).

In the zebrafish embryonic spinal cord, Vsx1 is detected in common V2a/V2b progenitors and is transiently maintained in part of the V2a lineage. The onset of *Vsx1* expression seems to occur in committed intermediate progenitors, i.e., cells that will undergo a last division to produce pairs of neurons between which Notch signaling will be activated to segregate V2a and V2b interneurons. However, these studies did not exclude the existence of an additional V2 population wherein Vsx1 would be individually present (Batista et al., 2008; Kimura et al., 2008). Compared to our data, these observations suggest that *Vsx1* is initially expressed in a pattern reminiscent of its paralog Chx10, although with an earlier onset, and has been recruited during vertebrate evolution to earlier multipotent neuronal precursors able to generate a large array of V2 subsets. Increased neuronal production and diversification in this early intermediate precursor compartment may have provided motor circuits with specific properties necessary for terrestrial tetrapod locomotion. The detection of murine Vsx1 in this early compartment and the presence in these cells of several markers also found in V2a interneurons are consistent with this late evolution. Functional studies will enable to determine the roles of their derivative populations in the spinal motor circuits and to assess whether they contribute to the execution of original motor capacities present in higher vertebrates.

Materials and methods

Mouse Strains

All experiments were performed in accordance with the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC). Mice were treated according to the principles of laboratory animal care, and experiments and mouse housing were approved by the Animal Welfare Committee of the Université catholique de Louvain (Permit Number: 2013/UCL/MD/11). The day of vaginal plug was considered as embryonic day (e)0.5. Minimum numbers of three embryos of the same genotype were analyzed in each experiment. The *Ascl1*$^{+/-}$, *Hnf6*$^{+/-}$; onecut 2(*Oc2*)$^{+/-}$, Presenilin-1 (*PS1*)$^{+/-}$, *Pax6*$^{+/Sey}$ and *Vsx1*$^{+/\tau LacZ}$ mutant mice were previously described (Hill et al., 1992; Guillemot et al., 1993; Wong et al., 1997; Jacquemin et al., 2000; Gong et al., 2003; Chow et al., 2004; Li et al., 2004; Clotman et al., 2005). Although β-galactosidase production was evident in a ventral population in *Vsx1*$^{\tau LacZ/\tau LacZ}$ spinal cords, it was barely detectable in *Vsx1*$^{+/\tau LacZ}$ heterozygous embryos, probably due to the negative auto-regulatory loop reported to control *Vsx1* expression levels in the retina (Chow et al., 2004). Furthermore, β-galactosidase distribution was diffuse and punctuated, hindering the identification of the cells wherein it was present (data not shown). Therefore, a novel *Vsx1*$^{+/nlsLacZ}$ line was generated using the PG00233_Z_5_A10 allele developed by the Knock-Out Mouse Project (KOMP). *Vsx1* inactivation was confirmed by genotyping PCR and by complete loss of the Vsx1 protein. However, β-galactosidase was never detected in this line (data not shown). Nevertheless, *Vsx1*$^{nlsLacZ/nlsLacZ}$ embryos were analyzed for the development of V2 interneuron populations.

Immunofluorescence Labelings

Mouse embryos were fixed in PBS/4% PFA at 4°C for 15–30 min according to the developmental stage. Fixed mouse embryos were washed in PBS before incubation in PBS/30% sucrose overnight at 4°C. They were embedded in PBS/7.5% gelatin/15% sucrose and frozen at −55°C. Embryos were cut at 14 µm in a Leica CM3050 cryostat.

Cryosections were saturated with PBS/0.1% Triton/10% horse serum for 30 min and incubated with the primary antibodies diluted in the same solution at 4°C overnight. For Vsx1 labeling, cryosections were permeabilized with PBS/1% Triton for 30 min at room temperature and saturated for 30 min with PBS/0.1% Triton/1% horse serum. Anti-Vsx1 antibody diluted in the same solution was incubated for 2 h at room temperature. After three washes in PBS/0.1% Triton, the secondary antibodies, diluted in PBS/0.1% Triton/10% horse serum, were added for 30 min at room temperature. Slides were washed three times in PBS/0.1% Triton before a final wash in PBS/DAPI and were mounted with Fluorescent mounting medium (DAKO).

The following primary antibodies and dilution were used: mouse anti-Ascl1 at 1:200 (BD #556604), guinea-pig anti-Ascl1 at 1:10,000 (Kim et al., 2008), mouse anti-beta III tubulin at 1:5000 (Chemicon #MAB1637), goat anti-BhlhB5 at 1:1000 (Santa Cruz #sc-6045), rabbit or rat anti-BhlhB5 at 1:2000 (Ross et al., 2012), sheep anti-Chx10 (Vsx2) at 1:500 (Exalpha Biologicals #X1179P), goat anti Dll4 at 1:200 (R&D System #AF1389), rabbit or guinea pig anti-Foxd3 at 1:5000 (Müller et al., 2005), rat anti-Gata3 at 1: 20 (Panayi et al., 2010), guinea-pig anti-Insm1 at 1:10,000 (Welcker et al., 2013), mouse anti-Islet 1/2 at 1:6000 (DSHB #39.4D5), mouse anti-Lhx3 at 1:1000 (DSHB #67.4E12), mouse anti-Lhx1/5 at 1:2000 (DSHB # 4F2), rat anti-Nkx6.1 at 1:2 (Ono et al., 2007), guinea pig anti-OC-1 at 1:6000 (Espana and Clotman, 2012), sheep anti-OC-1 at 1:250 (R&D System #AF6277), rat anti-OC-2 at

1:400 (Clotman et al., 2005), mouse anti-$p27^{kip1}$ at 1:2000 (Velasco et al., 2016), mouse anti-Pax6 at 1:1000 (DSHB #PAX6), mouse anti-phospho-Histone H3 at 1:1000 (Abcam ab14955), rabbit or guinea pig anti-Prdm8 at 1:1000 (Ross et al., 2012), goat anti-Prox1 at 1:100 (R&D Systems # AF2727), mouse anti-Shox2 at 1:500 (Abcam #ab55740), goat anti-Sox 1 at 1:500 (Santa Cruz #sc-17318), rabbit anti-Vsx1 at 1:500 (Clark et al., 2008).

Secondary antibodies from Life Science were used at 1:2000 and were donkey anti-mouse/AlexaFluor 594 or 488 or 647, anti-rabbit/AlexaFluor 647, anti-rat/AlexaFluor 594, anti-goat/AlexaFluor 488, anti-sheep/AlexaFluor 594, anti-mouse IgG_1/AlexaFluor 594 or anti-mouse IgG_{2a}/AlexaFluor 488. Secondary antibodies from Jackson ImmunoResearch were used at 1:1000 and were donkey-anti chicken/Dylight 488 or 594, anti-mouse/AlexaFluor 647, anti-rat/AlexaFluor 647, anti-sheep/AlexaFluor 594 or 647, anti-rabbit/AlexaFluor 488 or 594, anti-guinea pig/AlexaFluor 488 or 647. All secondary antibodies gave signals in the spinal cord only in the presence of corresponding primary antibodies.

Double *in situ* Hybridization (ISH)

Mouse embryos were fixed overnight in PBS/4% PFA at 4°C and processed as for immunolabeling experiments. Fourteen micrometer cryostat sections were cut and double *in situ* hybridization (ISH) protocol was performed essentially as previously described with slight modifications (Beguin et al., 2013; Pelosi et al., 2014). Sections were simultaneously hybridized overnight at 65°C with a DIG-conjugated *Foxn4* (NM_148935.2, nucleotides 78–1643 (Francius et al., 2015)) and a fluorescein-labeled *Vsx1* (NM_054068.2, nucleotides 121–2728, provided by C. Cepko) riboprobe. After hybridization, sections were washed four times in 50% Formamide, 1× SSC, 0.1% Tween-20 for 1 h at 65°C, twice in MABT buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween20, pH 7.5) for 30 min before blocking in

blocking buffer (MABT, 2% blocking reagent from Roche, 20% inactivated horse serum) for 2 h at room temperature. Sections were then incubated overnight with anti-DIG-alkaline phosphatase (AP)-conjugate antibody (Roche) at 4°C. After washing for 30 min in MABT, *Foxn4* probe was visualized by AP-catalyzed chromogenic reaction using NBT-BCIP substrates (Roche) according to manufacturer's instructions. The color reaction was stopped in 1× PBS and AP was inactivated by incubating the slides for 30 min with 0.1 M glycine/HCl pH 2.2. The sections were then washed twice in MABT buffer for 30 min, blocked again in blocking buffer for 2 h, and incubated to a 1:2000 dilution of anti-Fluo-AP-conjugate antibody (Roche) in blocking buffer overnight at 4°C. Slides were washed and incubated with HNPP/Fast Red kit (Roche) according to the manufacturer's instructions to visualize *Vsx1* expression. The reaction was stopped by washing in 1× PBS. Sections were then counterstained with DAPI and slides were mounted with Fluorescent mounting medium (DAKO).

Imaging and Quantitative Analyses

Immunofluorescence and double ISH images of cryosections at thoracic level of the developing spinal cord were acquired on a Zeiss Axio Cell Observer Z1 confocal microscope with the Zeiss AxioVision Rel. 4.8 software and processed with Adobe Photoshop CS5 software. Double ISH images were also acquired using an EVOS® FL Auto Imaging System (ThermoFisher Scientific) and related software. Quantifications were performed on red or green or blue layer of acquired confocal images and double or triple labeling cells were processed by subtractive method (Francius and Clotman, 2010). For each embryo ($n \geq 3$), both sides of three sections at thoracic level were quantified using the count analysis tool of Adobe Photoshop CS5 software.

Raw data were exported from Adobe Photoshop CS5 software to SigmaPlot v12.3 software and processed in order to generate histogram figures. All data were analyzed and histograms were made with SigmaPlot software. Adequate statistical tests were applied based on the number of comparisons and on the variance in each group. For analysis of cell quantification based on comparison of two groups (control or mutant), standard Student's *t*-tests were performed. Quantitative analyses were considered significant at $p < 0.05$. Three asterisks (***) indicate $p < 0.001$.

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3.2. Vsx1 and Chx10 paralogs sequentially secure V2 interneuron identity during spinal cord development

Foreword

Spinal cord development is a highly complex process that requires strong consolidation mechanisms to insure robustness and reproducibility. Robustness relies on the concept of compensation by genes that present overlapping functions. Redundancy is often ensured by paralog factors, originated from gene duplication during evolution. In this work, we propose a novel concept, namely labor division, wherein two paralog factors perform the same function in a single lineage but at different steps of development. In the ventral part of the developing spinal cord, V2 interneurons and MNs differentiate from contiguous progenitor domains that share molecular determinants including Lhx3 and NLI. *Chx10* and *Hb9*, respectively expressed in V2a interneurons and MNs, are mutually cross-repressed and therefore consolidate their respective population identity. Nevertheless, *Chx10* is expressed in a subset of differentiated V2 interneurons (V2a) but not in the early precursors that generate all the V2 interneuron subsets. Vsx1, the unique paralog factor of Chx10, specifically labels these early precursors but is not necessary for V2 interneuron production (see “paper 1”). Therefore, we wonder if this factor could secure the identity of all V2 interneurons and prevent the activation of MN program at early stage of development.

In this work, we demonstrate that Vsx1, as its paralog factor Chx10, binds the HxRE and prevent its activation. We show that Vsx1 inhibits *Hb9* expression and prevent the activation of MN program in early V2 compartment. In addition, we provide evidence that Vsx1 stimulate the differentiation of all V2 interneurons. Our analyses demonstrate that Vsx1 and Chx10 successively secure the identity of V2 and V2a interneurons versus MNs. These two genes retained redundant

functionality although they are expressed at different steps of development, illustrating an original model of “labor division” between two paralog factors.

This work is submitted in eLife. I carried out almost all the experiments shown in this paper, including the production of two new transgenic lines: the *Rosa26R::Vsx1-IRES-EGFP* murine line and the *Vsx1*$^{-/-}$ deficient mice. Charlotte Baudouin, that I supervised during her master thesis, realized most of the analyses of the *Nestin::Cre* x *Rosa26R::Vsx1-IRES-EGFP* line.

Vsx1 and Chx10 paralogs sequentially secure V2 interneuron identity during spinal cord development

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Abstract

Paralog factors are usually described as consolidating biological systems by displaying redundant functionality in the same cells. Here, we report that paralogs can also cooperate in distinct cell populations at successive stages of differentiation. In mouse embryonic spinal cord, motor neurons and V2 interneurons differentiate from adjacent progenitor domains that share identical developmental determinants. Therefore, additional strategies secure respective cell fate. In particular, Hb9 promotes motor neuron identity while inhibiting V2 differentiation, whereas Chx10 stimulates V2a differentiation while repressing motor neuron fate. However, Chx10 is not present at the onset of V2 differentiation. We show that Vsx1, the single paralog of Chx10, identically prevents motor neuron differentiation in early V2 precursors. Furthermore, combined inactivation unveiled cooperativity between Vsx1 and Chx10 although they are not produced in the same cells. Hence, this study uncovers a novel paradigm, namely labor division, exerted by paralog genes at successive steps of neuronal development.

Introduction

Robustness of a biological system is defined as the ability to maintain its functions despite perturbations. The mechanistic bases of robustness are not fully understood, but seem to notably rely on the fact that several genes or groups of genes have partially overlapping functions, ensuring compensation when perturbations occur (Diss et al., 2014). A major source of compensation is gene duplication, which results in the birth of paralog genes, and logically supposes at least partial overlapping expression of the two paralogs in the same cells. However, newly-generated paralogs can evolve towards loss of function (non-functionalization), acquisition of novel functions (neo-functionalization) or retention of varying degrees of overlapping function (sub-functionalization) (Diss et al., 2014; Ewen-Campen et al., 2017). Here, we uncover an original situation of labor division wherein two paralogs exert seemingly identical functions in a single cell lineage at successive steps of differentiation.

In the developing spinal cord, different neuronal populations are generated from distinct progenitor domains orderly distributed along the dorso-ventral axis of the ventricular zone (Lai et al., 2016; Lu et al., 2015). Adjacent progenitor domains often share identical developmental determinants. Therefore, additional strategies have been developed to segregate and consolidate respective cell fate in neighboring populations, as observed for motor neurons (MNs) and V2 interneurons (INs) (Figure 22A). In the adjacent progenitor domains of MNs (pMN) and of V2 INs (p2), the *LIM-homeodomain transcription factor Lhx3* is upregulated shortly before the onset of neuronal differentiation. In differentiating MNs, Lhx3 associates with its LIM-homeodomain partner Isl1 and the nuclear LIM interactor (NLI, also called LDB-1 or CLIM2) to form a MN-hexameric complex. This complex binds to hexamer-response elements (HxREs), stimulates expression of a large array

of MN genes including *Hb9* and promotes MN differentiation (Lee et al., 2012; Lee et al., 2008; Lee et al., 2013; Lee et al., 2004; Lee and Pfaff, 2003; Mazzoni et al., 2011; Thaler et al., 2002). Additionally, it inhibits multiple IN determinants (Lee et al., 2012). In contrast, in differentiating V2 INs, Lhx3 associates only with NLI to form a V2-tetrameric complex that binds tetramer-response elements (TeREs) (Lee et al., 2008; Thaler et al., 2002). This complex stimulates V2 genes including *Chx10*, which promotes the differentiation of V2a INs, one of the 2 main V2 populations (Clovis et al., 2016; Lee et al., 2008; Thaler et al., 2002). However, additional mechanisms are necessary to consolidate MN and V2 fates. In particular, MNs must be protected against aberrant activation of the V2 differentiation program by the Lhx3-NLI tetramer complex, which can also form in MNs. Hb9, which is specifically produced in MNs upon stimulation by the MN-hexameric complex (Lee et al., 2004; Lee and Pfaff, 2003), silences TeREs by replacing the V2-tetrameric complex and actively suppresses its activation, thereby preventing aberrant activation of the V2 program(Lee et al., 2008). Consistently, absence of Hb9 results in ectopic activation of Chx10 in early MNs and production of a hybrid lineage coexpressing MN and V2a markers (Arber et al., 1999; Clovis et al., 2016; Thaler et al., 1999a). Symmetrically, Chx10 secures cell fate in V2a INs by binding to and preventing the activation of HxREs, thereby inhibiting ectopic activation of the MN differentiation program (Lee et al., 2008) and enabling the expression of IN determinants (Lee et al., 2012). Accordingly, absence of Chx10 in the *Chx10*$^{orJ/orJ}$ mutant results in a reduction in the V2a IN population (Clovis et al., 2016).

However, *Chx10* expression is activated exclusively in the V2a population after segregation of the V2a and V2b lineages (Karunaratne et al., 2002) (Figure 22A). This raises the question of the consolidation of V2 identity before *Chx10* activation and in the other V2 populations. Recently, we showed that *Vsx1*, the single paralog of Chx10 in the mammalian genomes, is expressed in V2 precursors before the

segregation of the V2a/V2b lineages and the onset of *Chx10* expression (Figure 22A)(Francius et al., 2016). Vsx1 is a transcriptional repressor of the Paired-like CVC (Prd-L:CVC) homeobox gene family (Chow et al., 2001; Ohtoshi et al., 2001). In the mouse, it is expressed in gastrula stage embryos (Ohtoshi et al., 2001) and in several types of bipolar cone INs of the retina wherein it regulates different aspects of terminal differentiation (Chow et al., 2004; Kerschensteiner et al., 2008; Ohtoshi et al., 2004; Shi et al., 2012). In the spinal cord, it is transiently detected after cell cycle exit of p2 progenitors but before the onset of neuronal differentiation. The role of Vsx1 in the developing spinal cord remains elusive but this factor is not required for V2 IN production or for the segregation of the V2a and V2b lineages (Francius et al., 2016).

As *Vsx1* is paralog to *Chx10* and is expressed prior to *Chx10* in the V2 lineage, we hypothesized that it may anticipate Chx10 action in V2 INs to secure V2 identity. Here, we demonstrate that Vsx1 can bind HxREs and inhibit their activation by the MN Isl1-NLI-Lhx3 hexamer. Consistently, Vsx1 is sufficient to inhibit MN differentiation and to promote V2 IN production. However, the absence of Vsx1 does not impact on V2 fate consolidation, suggesting that lack of Vsx1 may be compensated by other factors. Nevertheless, combined inactivation of *Vsx1* and *Chx10* induces MN/V2 differentiation imbalance that was not observed in single mutants, confirming that Vsx1 and Chx10 paralogs sequentially secure V2 IN identity during spinal cord development. Hence, this study uncovers a novel paradigm, namely labor division, exerted by paralog genes at successive steps of neuronal differentiation.

Results

Vsx1 inhibits HxRE activation and stimulation of MN differentiation by the Isl1-NLI-Lhx3 complex

In early-born MNs, the Isl1-NLI-Lhx3 hexameric complex binds HxREs, stimulates Hb9 production and promotes MN differentiation. In V2a INs, Chx10 secures V2a identity by preventing activation of HxREs and the MN differentiation program (Lee et al., 2008) and by stimulating V2a gene expression (Figure 22A)(Clovis et al., 2016). However, early V2 precursors lack the expression of *Chx10*. Recently, we showed that *Vsx1*, the single paralog of *Chx10* in the mammalian genomes, is transiently expressed in V2 precursors during spinal cord development (Figure 22B) (Francius et al., 2016). Therefore, we reasoned that Vsx1 may anticipate V2a-restricted Chx10 action and consolidate V2 fate in V2 precursors. To test this hypothesis, we first investigated whether Vsx1 can bind the *Hb9* HxRE and prevent its activation. Chromatin immunoprecipitation assays in HEK293 cells transfected with a pEF::Vsx1-HSV expression vector showed that Vsx1 is able to bind the HxRE (Figure 22C). In MNs, the *Hb9* HxRE is activated by the MN-specific hexamer complex Isl1-NLI-Lhx3 (Figure 22A), which promotes *Hb9* expression and MN differentiation (Lee et al., 2008). Using a HxRE::LUC reporter in HEK293 cells, we showed that Vsx1 is able to inhibit the activation of the HxRE by the Isl1-NLI-Lhx3 complex (Figure 22D). This inhibition required binding of Vsx1 to DNA as a binding-defective version of this protein, Vsx1R166W (Dorval et al., 2005), failed to downregulate HxRE activity (Figure 22D). Thus, Vsx1 can bind the *Hb9* HxRE and prevent its activation by the MN Isl1-NLI-Lhx3 complex.

To assess whether a similar regulation can take place in spinal neuronal populations, we studied activation of an HxRE::GFP reporter construct after chicken embryonic spinal cord electroporation. Consistently, Vsx1 was able to inhibit

ectopic HxRE activation by the Isl1-NLI-Lhx3 complex and endogenous HxRE activity in MNs (Figure 22E). In contrast, the presence of the mutated Vsx1R166W did not affect HxRE activity (brackets and arrows in Figure 22E). To evaluate the impact of HxRE regulation on MN production, we studied the distribution of MNR2, an early marker of chicken MNs, in similar experimental conditions. Wild type Vsx1, but not the Vsx1R166W mutant, inhibited the ectopic production of MNR2-positive MNs induced by the Isl1-Lhx3 fusion protein (brackets in Figure 22F-G). Taken together, these results demonstrate that Vsx1 is able as its paralog Chx10 to bind the *Hb9* HxRE, to inhibit its activation and to prevent the stimulation of MNR2 production and of MN differentiation by Isl1-Lhx3-containing complexes.

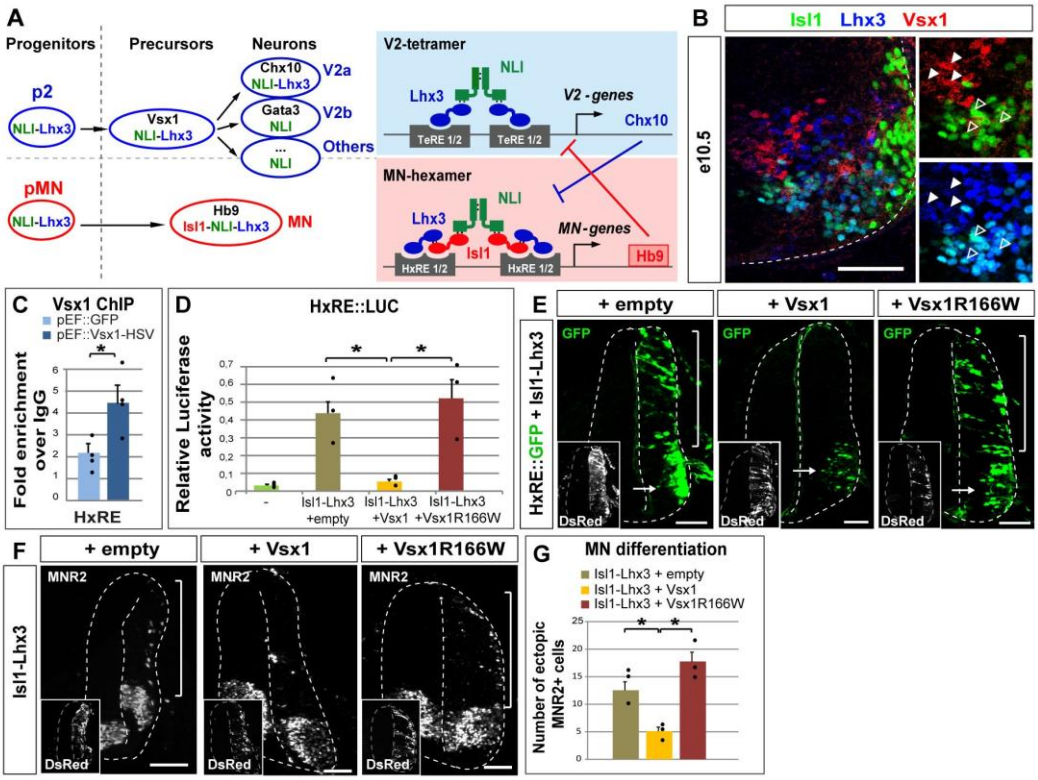


FIGURE 22 : Vsx1 binds the Hb9 HxRE and prevents its activation and stimulation of MN differentiation by Isl1-Lhx3 containing complexes.

(A) Schematic representation of V2 IN and MN specification during spinal cord development. **(B)** By immunofluorescence on transverse sections of wild type spinal cord, Vsx1 is co-detected with Lhx3 in V2 precursors (plain arrowheads) but is not present in Isl1+ MNs (open arrowheads). **(C)** Chromatin immunoprecipitation (ChIP) experiment demonstrates that Vsx1 can bind the Hb9 HxRE (n=3). **(D)** In HEK293 cells, activation of the Hb9 HxRE by the Isl1-Lhx3 fusion protein is suppressed by wildtype Vsx1 but not by the DNA binding-deficient Vsx1R166W mutant (n=3). **(E)** Following chicken embryonic spinal cord electroporation, activation of the HxRE by the Isl1-Lhx3 fusion protein (brackets) as well as endogenous activation in MNs (Miller et al., 2000) is inhibited by wildtype Vsx1 but not by the Vsx1R166W mutant (co-electroporated DsRed is shown as an electroporation control). **(F-G)** In electroporated chicken embryonic spinal cord, ectopic motor neuron differentiation induced by the Isl1-Lhx3 fusion protein (brackets) is reduced by Vsx1 but not by its Vsx1R166W variant (n=3). Mean values ± SEM; * p≤ 0.05. Scale bars = 50 µm.

Vsx1 inhibits MN differentiation and promotes V2 IN identity

To assess the impact of Vsx1 on MN and V2 IN differentiation *in vivo*, we first crossed a conditional *Rosa26R::Vsx1-IRES-EGFP* line (Figure S1A) (Haenebalcke et al., 2013) with *Olig2::Cre* mice (Dessaud et al., 2007) to ectopically express *Vsx1* in the spinal MNs (Figure S1). Immunofluorescence for Vsx1 and for EGFP evidenced ectopic expression in MNs but also in more ventral populations of the spinal cord including V3 INs (Figure S1B-C"), likely owing to the transient expression of *Olig2* in the p3 progenitor domain (Chen et al., 2011). Combined immunofluorescence analyses at e12.5 for the MN markers Isl1, Lhx3, Foxp1 and Sip1 demonstrated that Vsx1 inhibits the differentiation of spinal MNs (Figure 23A-C; Figure S1). To confirm that this effect was specific to MNs and did not result from altered neurogenesis or general impairment of neuronal differentiation, we quantified the number of V3 INs also subjected to ectopic Vsx1 production (Figure S1B-C"). Nkx2.2 immunofluorescence labeling unveiled no change in V3 numbers between control and mutant embryos (Figure S1Q-S; n=3, p=0.26), supporting the interpretation that the reduction in MNs upon ectopic Vsx1 production resulted from specific alteration of MN differentiation. Altered MN differentiation in the presence of Vsx1 may be counterbalanced by increased differentiation of V2 INs or production of MN/V2 hybrid cells (Arber et al., 1999; Clovis et al., 2016; Lee et al., 2008; Thaler et al., 1999a). To assess this possibility and to evaluate whether Vsx1 is sufficient to stimulate V2 differentiation in MN precursors, we analyzed the distribution of Chx10 and Gata3, specific markers of V2a and V2b INs, respectively, in *Olig2::Cre* x *Rosa26R::Vsx1-IRES-EGFP* mutant embryos. However, no change was observed in the number of V2a or V2b INs (Figure 23D-F; n=3, p=0.65 or 0.69 for V2a or V2b INs, respectively). Moreover, hybrid cells containing MN and V2 IN markers were not detected (Figure S1H-I). These observations suggest that Vsx1 is not sufficient to stimulate V2 differentiation or the expression of V2 markers in a MN context.

In contrast, Vsx1 may promote V2 differentiation in its endogenous V2 context. To address this hypothesis, we increased Vsx1 production in all the spinal neurons by crossing the *Rosa26R::Vsx1-IRES-EGFP* line with a *Nestin::Cre* line (Figure S2) (Tronche et al., 1999). Consistent with our previous observations, we detected a decrease in the number of MNs at e12.5, although to a lesser extent than with the *Olig2-Cre* driver (Figure 23G-I). In contrast, quantifications of Chx10+ and Gata3+ cells unveiled a significant increase in V2a and V2b INs (Figure 23J-L), supporting the hypothesis that upregulated *Vsx1* expression in V2 precursors stimulates V2 differentiation. Taken together, these data demonstrate that Vsx1 is able to prevent MN differentiation and to promote V2 IN fate during spinal cord development.

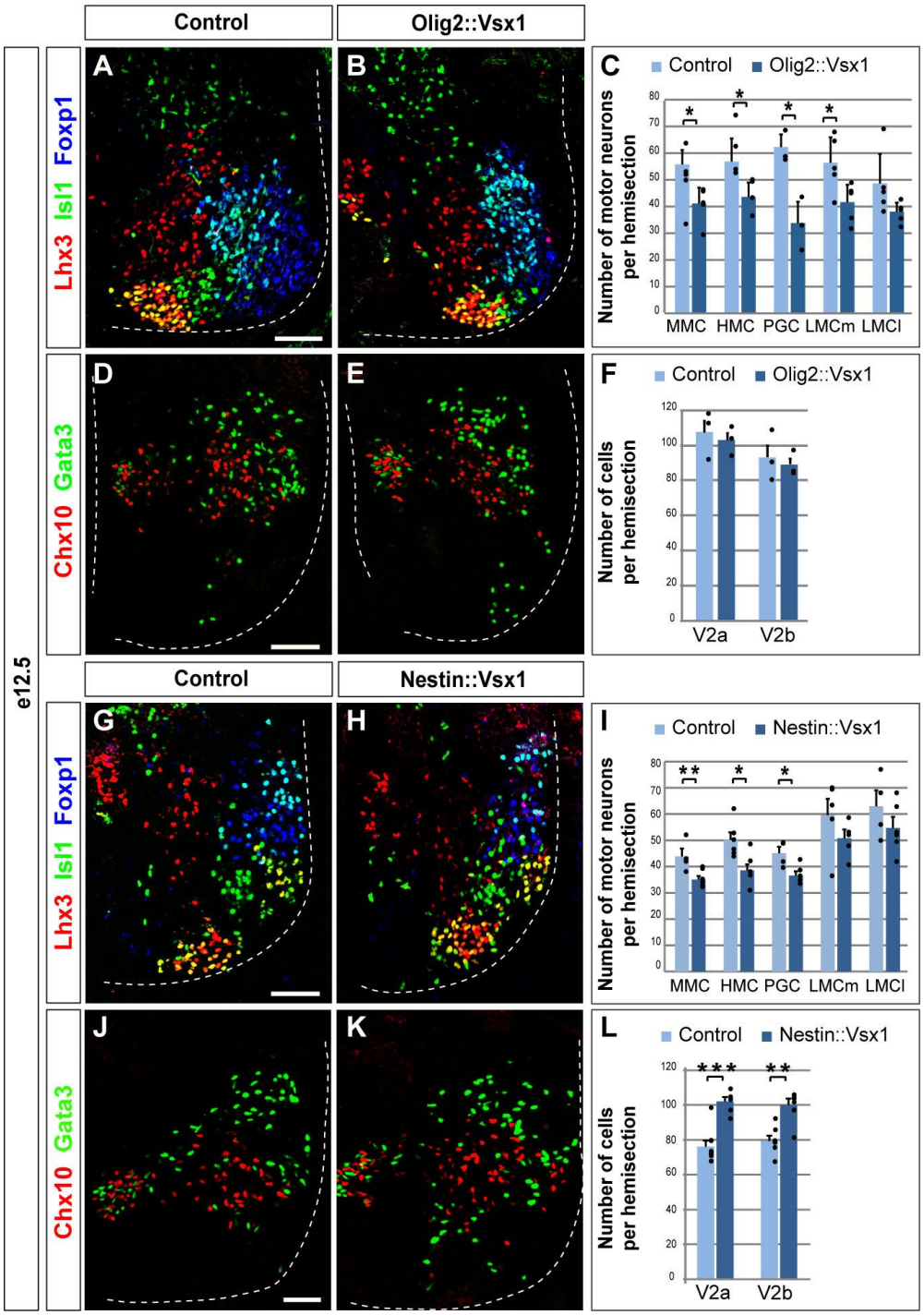

e12.5
Control
Olig2::Vsx1
Lhx3 Isl1 Foxp1
Chx10 Gata3
A
B
C
Control
Olig2::Vsx1
Number of motor neurons per hemisection
MMC HMC PGC LMCm LMCl
D
E
F
Number of cells per hemisection
V2a V2b
Control
Nestin::Vsx1
G
H
I
J
K
L

FIGURE 23. Vsx1 can inhibit MN differentiation and stimulate V2 IN production.
Immunofluorescence for MN (Isl1, Lhx3, Foxp1) or V2 IN (Chx10 for V2a and Gata3 for V2b) markers on transverse spinal cord sections of Vsx1 gain-of-function embryos. (**A-C**) In *Olig2::Cre* x *Rosa26R::Vsx1* embryos at e12.5, ectopic production of Vsx1 in MNs inhibits MN generation in each motor column (n=3). (**D-F**) Inhibition of MN production is not compensated by increased V2 IN generation (n=3). (**G-I**) In *Nestin::Cre* x *Rosa26R::Vsx1* embryos at e12.5, ectopic production of Vsx1 in MN also inhibits MN generation (n=4). (**J-L**) In the same embryos, increased expression of Vsx1 in V2 precursors promotes V2 IN generation (n=4). Mean values ± SEM; *** p≤0.001, ** p≤0.01 and * p≤0.05. Scale bars = 50 µm. MMC=medial motor column; HMC=hypaxial motor column; PGC=pre-ganglionic motor column; LMCm=medial portion of the lateral motor column; LMCl=lateral portion of the lateral motor column.

Vsx1 is not necessary for proper V2 IN differentiation

To assess whether Vsx1 is necessary for V2 fate consolidation, we studied in detail V2 production in the absence of Vsx1 (Figure 24A-B; Figure S3) at e10.5 and e12.5. In control embryos, Vsx1 is present in an intermediate V2 precursor compartment (Francius et al., 2016) wherein it partly overlaps with Sox14, which also labels V2a INs (Figure 24A)(Clovis et al., 2016). Therefore, we first studied Sox14 distribution in the absence of Vsx1. However, the number of Sox14+ cells was not significantly altered in mutant embryos (Figure 24A-B, E, K-L", O). Second, we evaluated whether the lack of Vsx1 impacts on the dual production of V2a and V2b INs from common precursors (Batista et al., 2008; Del Barrio et al., 2007). However, the number of V2a and V2b cells as well as the ratio between these two V2 subtypes were preserved (Figure 24C-E, I-J", M-O). Consistently, production of other V2 subsets including V2c and V2d was unaffected (Figure 24I-J", M-O). Third, to exclude any impact of Vsx1 on the p2 progenitor domain that could mask an influence on V2 differentiation, we labeled the p2 domain at e10.5 using a triple

immunolabeling of Nkx6.1, Sox1 and Olig2. However, the number of p2 progenitors (Sox1+ Nkx6.1+ Olig2- cells) was similar between control and mutant embryos (Figure S3; n=3, p=0.46). Moreover, no change was observed in the distribution of Ascl1, a key determinant of V2 differentiation (Del Barrio et al., 2007; Misra et al., 2014)(Figure S3; n=4, p=0.55). Thus, Vsx1 is not required for p2 domain integrity or for proper production of the multiple V2 IN subsets. Consistently, the absence of Vsx1 had no effect on the production of MNs (Figure 24F-H, P-R; n=3, p=0.93 or 0.77 for Isl1+ or Hb9+ cells, respectively). Taken together, these observations suggest that Vsx1 is not necessary for early differentiation of V2 INs or to prevent activation of the MN differentiation program in the V2 populations.

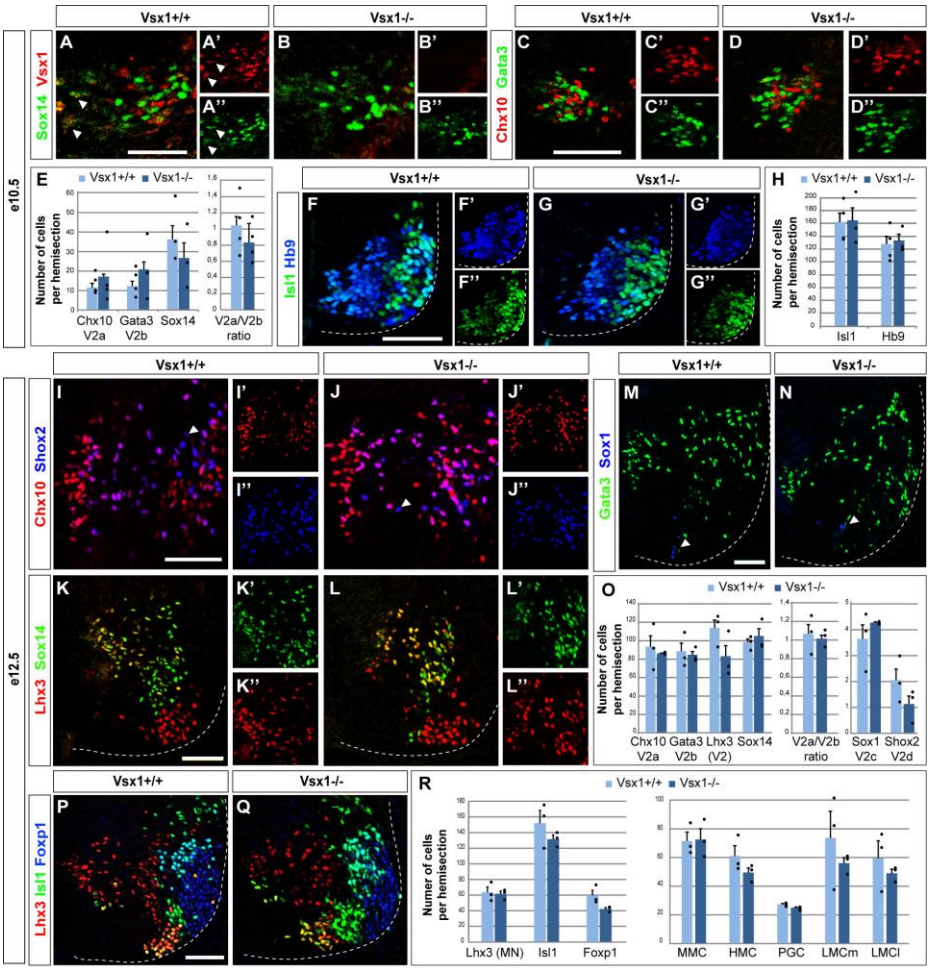


FIGURE 24: Vsx1 is not necessary for V2 IN production and diversification.
Immunofluorescence for MN (Hb9, Isl1, Lhx3, Foxp1) or V2 IN (Chx10, Sox14, Lhx3, Gata3, Sox1, Shox2) markers on transverse spinal cord sections of Vsx1 loss-of-function embryos. **(A)** At e10.5 in control embryos, Sox14 in detected in part of the V2 precursors (arrowheads), which contain Vsx1. (**B, E**) In *Vsx1-/-* mutant embryos, Vsx1 is lost whereas Sox14 distribution is not altered (n=3). (**C-E**) Similarly, the development of V2a and V2b INs is unaffected (n=4). (Attias et al., 2004) Absence of Vsx1 does not impact on MN production (n=4). (**I-O**) At e12.5, the lack of Vsx1 does not alter the production of V2 INs nor the diversification of V2 INs into V2a, V2b, V2c (arrowheads in **M-N**) and V2d (arrowheads in **I-J"**) subsets (n=3). (**P-R**) Consistently, absence of Vsx1 does not impact on MN differentiation (n=3). Mean values ± SEM; Scale bars = 50 µm. MMC=medial motor column; HMC=hypaxial motor column; PGC=pre-ganglionic motor column; LMCm=medial portion of the lateral motor column; LMCl=lateral portion of the lateral motor column.

However, the impact of Vsx1 on MN might appear in a context wherein MN differentiation is eroded. As an example, the *Vsx1* paralog *Chx10* is ectopically expressed in MNs upon inactivation of *Hb9* (Arber et al., 1999; Clovis et al., 2016; Thaler et al., 1999a). Interestingly, the number of Vsx1+ cells was also increased in the absence of Hb9 (Figure 25A-C). However, no Vsx1/Isl1 hybrid cells were observed in *Hb9* mutants (Figure S4A-B") and Vsx1 was not co-detected with Chx10 in MN/V2 hybrid cells (Figure S4C-D"), possibly owing to the repressive activity of Chx10 on *Vsx1* expression (Clark et al., 2008; Clovis et al., 2016; Shi et al., 2011) (and see below). Nevertheless, this suggested that absence of Hb9 or impairment of the MN differentiation program releases *Vsx1* repression in prospective MNs. Therefore, we assessed whether the absence of Vsx1 may rescue the MN differentiation defects observed in *Hb9* mutant embryos. However, reduction in the number of MNs, expansion of V2 populations and aberrant production of Isl1/Chx10 hybrid cells were similar in *Hb9*$^{-/-}$*Vsx1*$^{-/-}$double mutant embryos and in *Hb9*$^{-/-}$ littermates (Figure 25D-S), indicating that Vsx1 does not contribute to decrease MN production or to stimulate V2 differentiation in the absence of Hb9. Surprisingly, V2b INs were not impacted by the absence of Hb9 (Figure 25L-O), suggesting that the ectopic activation of Vsx1 and Chx10 biases excessive V2 differentiation towards the V2a lineage. Taken together, these data indicate that Vsx1 is not required for early consolidation of V2 identity, suggesting that the lack of Vsx1 may be compensated by other factors.

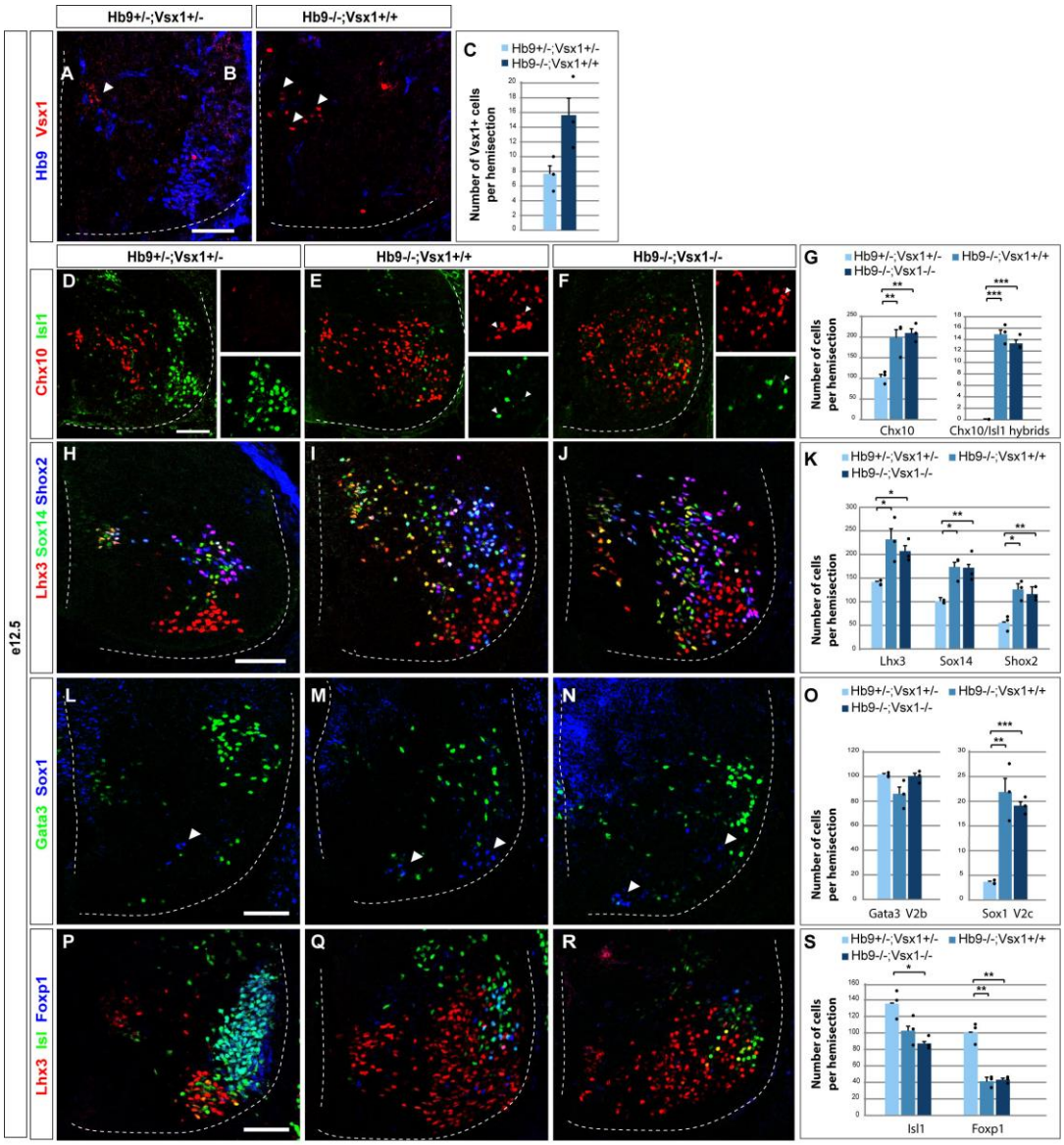

e12.5
Hb9+/-;Vsx1+/-
Hb9-/-;Vsx1+/+
Hb9-/-;Vsx1-/-
Hb9 Vsx1
Chx10 Isl1
Lhx3 Sox14 Shox2
Gata3 Sox1
Lhx3 Isl Foxp1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
Number of Vsx1+ cells per hemisection
Number of cells per hemisection
Chx10
Chx10/Isl1 hybrids
Lhx3
Sox14
Shox2
Gata3 V2b
Sox1 V2c
Isl1
Foxp1

FIGURE 25 Vsx1 does not contribute to decrease MN production or to stimulate V2 differentiation in the absence of Hb9.
Immunofluorescence for MN (Hb9, Isl1, Lhx3, Foxp1) or V2 IN (Chx10, Sox14, Lhx3, Gata3, Sox1, Shox2) markers on transverse spinal cord sections of *Hb9* single mutant or *Hb9/Vsx1* double mutant embryos. (**A-C**) In *Hb9-/-* single mutants at e12.5, the number of Vsx1+ cells is increased (arrowheads), indicating that Hb9 prevents Vsx1 production in MNs (n=3). (**D-E, G**) Absence of Hb9 results in increased production of cells containing Chx10 and in aberrant generation of hybrid MN/V2 cells (arrowheads) containing the MN marker Isl1 and the V2a marker Chx10 (n=3). (**F-G**) Chx10 expansion and hybrid cell production (arrowheads) are similar in double *Hb9/Vsx1* mutant embryos and in single *Hb9* mutants (n=3). (**H-K**) Accordingly, other markers of V2a INs are similarly expanded in *Hb9* single mutant and in *Hb9/Vsx1* double mutant embryos (n=3). (**L-O**) Surprisingly, the V2b marker Gata3 is not upregulated in the absence of Hb9 or in the combined absence of Hb9 and Vsx1 (n=3) whereas the V2c marker Sox1 (arrowheads) is similarly expanded in both mutants (n=3). (**P-S**) Consistently, the number of MNs is similarly decreased both in single and in double mutants (n=3; Isl1+ cells: p=0.08 for single mutants). Mean values ± SEM; *** p≤0.001, ** p≤0.01 and * p≤0.05. Scale bars = 50 µm.

Nkx6.1 may cooperate with Vsx1 to prevent activation of the MN program in V2 precursors

Nkx6.1 and Pax6 are present in defined neural progenitor domains during development and contribute to the dorso-ventral patterning of the neural tube (Burrill et al., 1997; Sander et al., 2000; Vallstedt et al., 2001). We previously showed that Nkx6.1 and Pax6 are maintained in the V2 precursors containing Vsx1 (Francius et al., 2016). Interestingly, Pax6 and Nkx6.1 are able to inhibit *Hb9* expression in transient transfection experiments (Lee et al., 2004). Furthermore, Nkx6.1 can inhibit MN differentiation and stimulate V2 differentiation (Francius et al., 2015). To assess whether Pax6 and/or Nkx6.1 could cooperate with Vsx1 to prevent *Hb9* expression and activation of the MN program, we used chicken embryonic spinal cord co-electroporation. First, we established experimental

conditions wherein overexpression of *Pax6* or *Nkx6.1* did not significantly (or marginally, see Nkx6.1+ Vsx1 in Figure 26B) alter dorso-ventral patterning by quantifying the number of Olig2+ MN progenitors (Figure 26A-B). Second, we used these conditions to determine if Pax6 or Nkx6.1, alone or in combination with Vsx1, could inhibit MN differentiation. Electroporation of high concentrations of *Pax6* or *Nkx6.1* expression vectors alone (0.5µg/µl) reduced the number of MNs (Figure 26C-F), consistent with the idea that both factors inhibit *Hb9* expression and MN differentiation (Lee et al., 2004). However, combining Vsx1 with such concentrations of Pax6 or Nkx6.1 led to severe patterning defects (not shown). Therefore, we used lower concentrations of each expression vector (0.25µg/µl) to investigate a potential synergic effect on MN differentiation without affecting patterning (Figure 26A-B). At low concentrations, none of these factors alone affected MN development. However, combination of Vsx1 and Nkx6.1, but not Pax6, did reduce MN production, and this inhibition trended to increase when Vsx1 and Nkx6.1 were combined with Pax6 (Figure 26C-F). Hence, these results support the hypothesis that Nkx6.1, and possibly Pax6, cooperate with Vsx1 in V2 precursors to prevent *Hb9* expression and activation of the MN program and that the absence of Vsx1 in *Vsx1*$^{-/-}$ mutants may be compensated by Nkx6.1 and Pax6.

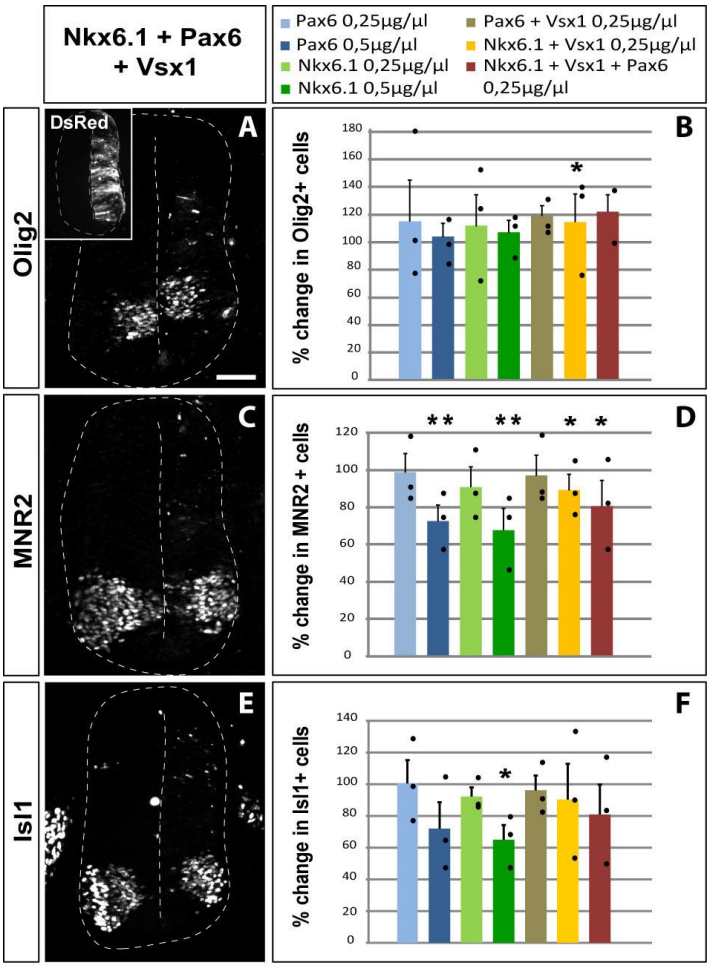


Figure 26: Nkx6.1 may cooperate with Vsx1 to prevent activation of the MN program in V2 precursors

Immunofluorescence for MN progenitor (Olig2) or MN (MNR2, Isl1) markers on transverse sections of chicken embryonic spinal cord electroporated with expression vectors for Nkx6.1, Pax6 or Vsx1, alone or in combination. (de Melo et al.) Assessment of electroporation conditions that do not affect dorso-ventral patterning of the spinal cord. (**C-F**) Nkx6.1 or Pax6 alone (0.5 µg/µl) or combinations of Nkx6.1+Vsx1 or Nkx6.1+Pax6+Vsx1 (0.25 µg/µl) reduce MN differentiation (n=3) (co-electroporated DsRed is shown as an electroporation control). Mean values ± SEM; ** p≤0.01 and * p≤0.05. Scale bar = 50 µm.

Vsx1 and Chx10 act successively to secure V2 IN identity

The absence of Vsx1 may also be compensated by its paralog Chx10. Our data demonstrate that Vsx1 and Chx10 use the same mechanism to prevent activation of the HxRE and of the MN differentiation program (Lee et al., 2008). In normal conditions, Vsx1 and Chx10 are not detected in the same cells (Francius et al., 2016). Therefore, they would exert this activity in distinct although lineage-related cell types, namely V2 precursors and V2a differentiating INs, respectively. However, Vsx1 represses *Chx10* expression in type-7 ON bipolar cells (Shi et al., 2011) and Chx10 represses *Vsx1* in retinal progenitor cells (Clark et al., 2008) and in ES cell-derived neuronal populations (Clovis et al., 2016). Therefore, we reasoned that each paralog may partly compensate for V2 differentiation defects caused by the lack of the other. The number of Chx10+ cells was not increased in the absence of Vsx1 (Figure 24), suggesting that Chx10 does not compensate for the absence of Vsx1. In contrast, the number of Vsx1+ cells was increased in *Chx10$^{orJ/orJ}$* single mutant spinal cord (Figure 27A-C). This indicates that, as previously reported in other cell types (Clark et al., 2008; Clovis et al., 2016), Chx10 prevents Vsx1 production in V2a IN and explains mutually exclusive expression of the 2 paralogs in wildtype (Francius et al., 2016) and in *Hb9* mutant spinal cord (Figure S4). This additionally suggests that prolonged *Vsx1* expression may prevent MN vs. V2 differentiation defects in the absence of Chx10.

To address this hypothesis, we studied V2 IN and MN production in *Vsx1/Chx10^{orJ}* double mutant embryos at e12.5. Using Sox14 as marker to label V2 precursors and V2a INs (Figure 27) (Clovis et al., 2016), we observed that combined absence of both Prd-L:CVC factors resulted in a reduction in the number of Sox14+ cells (Figure 27D-F, P) that was not observed in single mutants (Figure 24; Figure 27E, P). Consistently, the number of cells containing Lhx3 but not Hb9 or Isl1, corresponding to V2a, was similarly smaller (Figure 27J-P). Furthermore, cells

containing Shox2, which consists in a majority of V2a and in the V2d INs (Dougherty et al., 2013), were also significantly reduced (Figure 27D-F, P). This demonstrates that Vsx1 and Chx10 act successively to promote V2a IN differentiation. Surprisingly, although they derive from Vsx1-containing cells and although their number was increased upon *Vsx1* overexpression in V2 INs (Figure 23J-L), the number of V2b cells was not affected by the combined absence of Vsx1 and Chx10. Similarly, V2c INs, which derive from V2b (Panayi et al., 2010), were not impacted by the lack of Prd-L:CVC factors (Figure 27G-I, P). These observations confirm a bias in Vsx1 activity towards V2a fate (Figure 25D-G; L-O), as observed for Chx10 (Clovis et al., 2016). To assess whether this decrease in V2a INs was compensated by increased MN production, MN were quantified in *Vsx1/Chx10*^{orJ} double mutant embryos. A significant increase in the number of MNs (Figure 27J-O, Q) was observed, that is neither detected in the absence of Chx10 alone (Figure 28K,N,Q) (Clovis et al., 2016) nor in the absence of Vsx1 (Figure 24). Taken together, these observations indicate that Vsx1 and Chx10 cooperate during spinal cord development to prevent MN differentiation and to activate the V2a differentiation program in the V2 lineage.

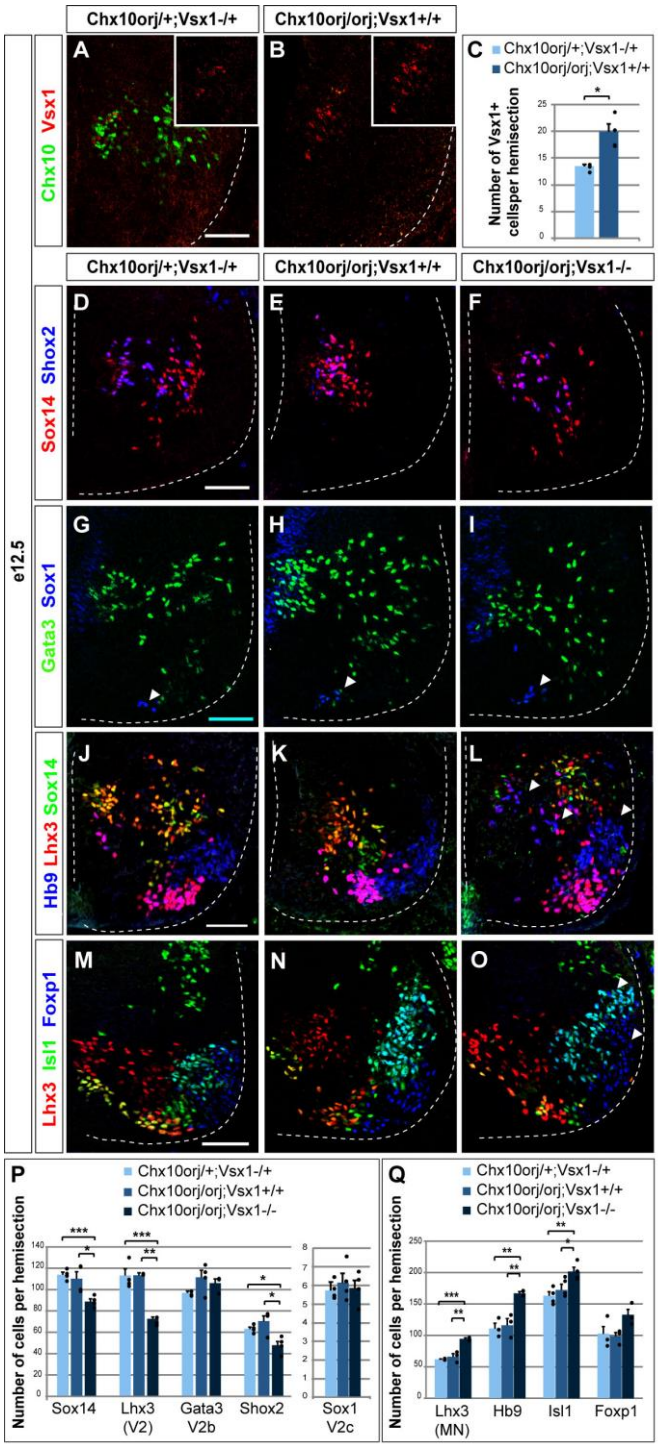

Chx10orj/+;Vsx1-/+
Chx10orj/orj;Vsx1+/+
Chx10orj/orj;Vsx1-/-
e12.5
Chx10 Vsx1
Sox14 Shox2
Gata3 Sox1
Hb9 Lhx3 Sox14
Lhx3 Isl1 Foxp1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Number of Vsx1+ cellsper hemisection
P
Q
Number of cells per hemisection
Sox14
Lhx3 (V2)
Gata3 V2b
Shox2
Sox1 V2c
Lhx3 (MN)
Hb9
Isl1
Foxp1

FIGURE 27 : Vsx1 and Chx10 cooperate to prevent MN differentiation and to activate the V2a differentiation program in the V2 lineage.
Immunofluorescence for MN (Hb9, Isl1, Lhx3, Foxp1) or V2 IN (Chx10, Sox14, Lhx3, Gata3, Sox1, Shox2) markers on transverse spinal cord sections of *Chx10^{orJ}* single mutant or *Chx10^{orJ}/Vsx1* double mutant embryos. (**A-C**) In *Chx10$^{orJ/orJ}$* single mutants at e12.5, the number of Vsx1+ cells is increased, indicating that Chx10 represses Vsx1 production in V2a INs (n=3). (**D-F, P**) The number of V2a INs labeled by Lhx3 (Lhx3+Isl1- cells), Sox14 or Shox2 is not changed in the *Chx10$^{orJ/orJ}$* single mutant but is significantly reduced in the combined absence of Vsx1 and Chx10 (n=3). (**G-I, P**) In contrast, the generation of V2b and of V2c (arrowheads) INs is unaffected (n=3). (**J-O, Q**) Consistently, the number of MNs is not modified in the *Chx10$^{orJ/orJ}$* single mutant but is significantly increased (arrowheads) in the combined absence of Vsx1 and Chx10 (n=3). Mean values ± SEM; *** p≤0.001, ** p≤0.01 and * p≤0.05. Scale bars = 50 µm

Discussion

Paralog genes are usually reported to evolve towards non-functionalization, neofunctionalization or sub-functionalization (Diss et al., 2014; Ewen-Campen et al., 2017). Here, we show that two paralogs of the Prd-L:CVC homeobox gene family use identical mechanisms to prevent activation of the MN differentiation program and secure V2 identity at successive stages of V2 IN differentiation (Figure 28). Hence, we uncover a novel paradigm of labor division wherein two paralogs exert seemingly identical functions in a single cell lineage at successive steps of development.

Prd-L:CVC paralogs sequentially secure V2 interneuron identity

Here, we demonstrate that Vsx1 and Chx10 are jointly required to prevent ectopic activation of the MN differentiation program in V2 cells and to secure V2 identity. Indeed, combined absence of these 2 Prd-L:CVC factors results in an increase in MN production that is not observed in corresponding single mutants. This suggests that, although the hexameric Isl1-NLI-Lhx3 complex cannot form in

V2 interneurons since Isl1 is not produced in these cells, Vsx1 and Chx10 are jointly necessary to prevent activation of HxREs and of the motor neuron differentiation program in the V2 lineage. Several observations support this hypothesis. First, Vsx1 and Chx10 are able to bind the *Hb9* HxRE and to prevent its activation by the MN-hexameric complex. Second, both factors suppress MN production induced by the hexameric complex. Third, ectopic production of Vsx1 in MN in 2 independent transgenic mouse lines results in a reduction in MN differentiation. Taken together, these observations suggest that Vsx1 and Chx10 actively suppress HxRE activation and MN differentiation in the V2 lineage. Since HxRE activation results in inhibition of the expression of multiple INs determinants (Lee et al., 2012), joined repression of HxREs is also likely required to enable IN differentiation. However, Chx10 is present in the V2a INs but is not produced in earlier V2 precursors cells (Figure 29)(Francius et al., 2016). Therefore, we hypothesized that Vsx1 could anticipate V2a-restricted Chx10 action and secure V2 fate in V2 precursors. In support of this possibility, increased *Vsx1* expression in V2 precursors resulted in increased production of V2 cells. However, loss-of-function experiments demonstrated that Vsx1 alone is not required for proper V2 IN generation. In contrast, combined inactivation of the 2 Prd-L:CVC genes resulted in V2 and in MN defects that were not observed in single mutants of the same mouse line, although the lack of Chx10 induced a mild reduction in V2 cells in a different genetic background (Clovis et al., 2016). This apparent cooperativity between these 2 factors is surprising since Vsx1 and Chx10 are not present in the same cell compartment. However, Chx10 represses *Vsx1* expression in differentiating V2a INs and loss of Chx10 results in prolonged production of Vsx1. Given their similar activity, persistence of Vsx1 in V2a likely compensate for the loss of Chx10 (Clovis et al., 2016). In contrast, *Chx10* expression is not anticipated in embryos lacking Vsx1 and can therefore not substitute for its absent paralog in V2 precursors. However, we provide evidence

that loss of Vsx1 can be compensated by the homeodomain-containing transcription factors Nkx6.1 and Pax6 (Francius et al., 2015) (see below). Hence, alterations of MN and of V2 IN development in *Vsx1/orJ* double mutants support the hypothesis that Vsx1 and Chx10 cooperatively contribute to inhibit MN differentiation and to promote V2 fate at successive stages of differentiation in the developing V2 INs (Figure 28).

Distribution and function of Prd-L:CVC paralogs during V2 differentiation

In the embryonic zebrafish spinal cord, Vsx1 is initially detected in V2 precursors before the V2a/V2b segregation and is retained in V2a but not in V2b interneurons (Batista et al., 2008; Kimura et al., 2008). In contrast, in the mouse, Vsx1 is exclusively detected in V2 precursors but not in V2a or V2b cells (Francius et al., 2016), suggesting that the functions of the two paralogs have progressively been segregated during evolution. This raises the question of how mutually exclusive production of Vsx1 and Chx10 during murine V2 differentiation is ensured. Intriguingly, in the retina, Prd-L:CVC factors show an opposite sequence of expression, as Chx10 is present in progenitors before the onset of Vsx1 production, which is restricted to differentiating cone bipolar cells (Chow et al., 2001). Inverse relationship between *Chx10* and *Vsx1* expression levels suggested that mutual repression could contribute to segregate production of the Prd-L:CVC paralogs. Accordingly, Vsx1 represses *Chx10* expression in type 7 ON bipolar cells {Shi, 2011 #2002} whereas Chx10 represses *Vsx1* in retinal progenitor cells {Clark, 2008 #2006}. Furthermore, *Vsx1* is also downregulated by Chx10 in ES cell-derived neuronal populations (Clovis et al., 2016). Taken together, these observations suggest that, in the developing spinal cord, Chx10 may restrict Vsx1 production to V2 precursors, i.e. before the segregation of V2a and V2b subsets. This is in agreement with our observation that the number of Vsx1-containing cells was

increased in *orJ* homozygous embryos. It also explains the absence of Vsx1/Isl1 hybrid cells in *Hb9* mutant embryos, as ectopic production of Chx10 in prospective MNs likely prevents *Vsx1* expression in these cells. This could account for the preferential production of supernumerary V2a interneurons instead of a combination of V2a and V2b cells in the absence of Hb9, as could be expected if Vsx1 would have been present in these cells. Hence, we propose a scenario wherein two paralog genes that were initially expressed in the same cells, i.e. V2 precursors and V2a differentiating interneurons as observed in zebrafish, have been progressively segregated into successive but distinct populations of the same lineage to exert seemingly identical function at different stages of development (Figure 28). Consistent with this model, phylogenetic analyses have suggested that Chx10 and Vsx1 did evolve rapidly (Chow et al., 2001). How Vsx1 expression is inhibited in the other V2 subsets, particularly in V2b interneurons, remains to be investigated.

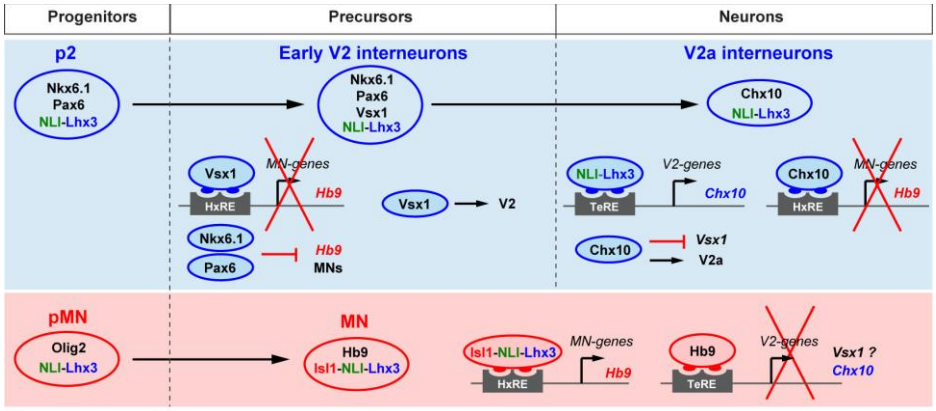


FIGURE 28 : Labor division between Vsx1 and Chx10 at successive stages of V2 IN differentiation.

Schematic representation of MN and V2 IN identity specification and consolidation. In V2 precursors (early V2 INs), Vsx1 binds HxREs and inhibits their activation and *Hb9* and MN gene expression, thereby securing V2 identity. Nkx6.1, and possibly Pax6, may cooperate with Vsx1 in this process. In addition, Vsx1 can stimulate V2 differentiation.

Evolution of paralog genes is supposed to result in non-functionalization, neo-functionalization or sub-functionalization (Diss et al., 2014; Ewen-Campen et al., 2017). Despite their rapid evolution (Chow et al., 2001), multiple pieces of evidence suggest that Vsx1 and Chx10 retained broadly overlapping functions in the mammalian spinal cord. Both factors can bind HxREs, prevent their activation and inhibit MN production (Clovis et al., 2016; Lee et al., 2008; Thaler et al., 2002) and this study). Furthermore, they are both able to promote V2 production ((Clovis et al., 2016) and this study). Surprisingly, absence of Vsx1 did not impact the number of V2b INs in any of the mouse lines we studied. Two hypotheses can support these observations. Firstly, Vsx1 may retain the capacity of Chx10 to specifically stimulate V2a IN fate (Clovis et al., 2016). However, increased Vsx1 production in the V2 lineage stimulated both V2a and V2b production, consistent with the idea that Vsx1 consolidate V2 identity before V2 diversification. Secondly, Chx10 may contribute to inhibit V2b differentiation, as observed after overexpression in the chicken embryonic spinal cord (data not shown). This could account for the lack of V2b perturbations in the single *Hb9* or compound *Vsx1/Hb9* mutants, wherein Chx10 is ectopically produced, and in the *Vsx1/orJ* compound mutant wherein the absence of Chx10 may release the inhibition on V2b IN production. Careful investigations of the respective roles of Vsx1 and Chx10 in V2 precursors and regarding V2b differentiation will be required to address these hypotheses.

Compensation of *Vsx1* inactivation by Nkx6.1 and Pax6

Although Vsx1 and Chx10 both contribute to secure V2 identity and to promote V2a differentiation, absence of Vsx1 alone did not impact on V2 production or diversification, suggesting that other factors present in V2 precursors may compensate for the loss of Vsx1. *Nkx6.1* and *Pax6* are patterning genes that

contribute to the dorso-ventral regionalization of the neural tube (Briscoe et al., 2000; Ericson et al., 1997; Sander et al., 2000; Vallstedt et al., 2001). They are also maintained or reexpressed in distinct neuronal subsets, namely V1 interneurons for Pax6 and specific MN pools for Nkx6.1, wherein they regulate neuronal specification and axon guidance, respectively (Burrill et al., 1997; De Marco Garcia and Jessell, 2008; Sapir et al., 2004). Additionally, they are both maintained in V2 precursors wherein they are co-detected with Vsx1 (Francius et al., 2016). Interestingly, Nkx6.1 and Pax6 are able to inhibit *Hb9* expression through a 2.5 kb distal region upstream of the *Hb9* minimal promoter (Lee et al., 2004). Although they are less active than exclusive progenitor factors including Nkx2.2 and Irx3, their presence in V2 precursors combined with that of Vsx1 may contribute to prevent activation of Hb9 and of the MN differentiation program until Chx10 takes over in differentiating V2a (Figure 28). Owing to the involvement of Nkx6.1 and Pax6 in dorso-ventral patterning, inactivating these factors was not an option to study their contribution to MN fate inhibition in V2 precursors. Therefore, we relied on overexpression in chicken embryonic spinal cord. In conditions wherein dorso-ventral patterning of the spinal cord was not perturbed, combined expression of *Vsx1* and *Nkx6.1* inhibited MN development, which was not observed with each factor separately and was increased when *Pax6* was added. This suggests that Nkx6.1, and possibly Pax6, may cooperate with Vsx1 in V2 precursors to prevent irrelevant activation of *Hb9*expression and of MN differentiation. Interestingly, Gata2 is downstream of Nkx6.1 in V2 interneurons and is also able to inhibit MN differentiation (Francius et al., 2015). Accordingly, these factors could compensate for the loss of Vsx1 regarding repression of the MN program in the V2 lineage.

Hb9 represses Prd-L:CVC gene expression to secure HxRE activation and MN differentiation

Hb9 is critical for proper differentiation of the spinal MN. Accordingly, recent studies indicate that the MN hexameric complex initially binds a specific set of enhancers, including regulatory *Hb9* sequences, at early stages of MN development (Rhee et al., 2016; Velasco et al., 2016), then is recruited by Ebf and Onecut transcription factors to another set of targets that promote terminal MN differentiation (Velasco et al., 2016). Hb9, which is an early target of the MN-hexameric complex (Rhee et al., 2016), secures MN differentiation using two complementary strategies. Firstly, it prevents activation of the TeRE by the Lhx3-NLI tetrameric complex, which can also form in MN, and by the MN Isl1-NLI-Lhx3 hexameric complex, which is also able to bind and activate the TeRE (Lee et al., 2008). It is assisted in this function by STAT factors, which enhance the transcriptional activity of the MN–hexamer in an upstream signal-dependent manner (Lee et al., 2013), and by LMO4, which blocks V2-tetramer assembly (Lee et al., 2008; Song et al., 2009). Secondly, doing so, it also prevents possible inhibition of the HxRE by the Prd-L:CVC paralogs. Indeed, absence of Hb9 results in ectopic activation of Vsx1 (this study) and of Chx10 (Arber et al., 1999; Clovis et al., 2016; Thaler et al., 1999a), indicating that Hb9 inhibits *Vsx1* and *Chx10* expression in early MNs. Repression of *Chx10* is direct, as Hb9 binds to the *Chx10*-TeRE and prevents its activation by the MN hexameric complex (Lee et al., 2008). Whether a similar mechanism accounts for *Vsx1* repression in MNs remains to be investigated. Hence, Hb9 secures MN fate by preventing Vsx1 and Chx10 activation (Figure 28).

Labor division between Prd-L:CVC paralog genes

Within multigenic families, pairs of paralogs including Emx, Otx, Dlx or Dvl family members have been repeatedly shown to act redundantly in the regulation of CNS

development. These pairs of genes are usually expressed in partly overlapping expression patterns, and often display redundant functionality in cells or tissues wherein they are coexpressed and divergent functionality in specific expression areas (Cecchi and Boncinelli, 2000; Cecchi et al., 2000; Gentzel and Schambony, 2017; Simeone et al., 2011; Solomon and Fritz, 2002). Here, we provide evidence that the murine Prd-L:CVC paralogs Vsx1 and Chx10 retained redundant functionality although their expression has been segregated at distinct stages of V2 interneuron differentiation. Active paralogous compensation by transcriptional reprogramming is at least one of the strategy used by the Prd-L:CVC factors to secure V2 identity. Direct or indirect repression of *Vsx1* expression by Chx10 enables compensation for a loss of Chx10 in V2a interneurons, reminiscent of similar compensation previously shown for *PDC1* and *5* or *NHP6A* and *B* in yeast or *RPL22* and *RPL22l1* in mouse (Diss et al., 2014). However, our data also suggest that the Prd-L:CVC paralogs additionally acquired divergent functionality, as Vsx1 can stimulate V2b differentiation whereas Chx10 may rather repress it. Although showing conserved primary sequences, functional promiscuity of these factors may rely on versatile conformational flexibility (Canzio et al., 2014) enabling variable interactions with cofactors or the transcriptional machinery. Hence, our observations emphasize the critical importance of paralog redundancy for the robustness of biological systems. They are also consistent with surveys demonstrating in *S. cervisiae* or in *C. elegans* that redundancy is often an evolutionary stable state (Vavouri et al., 2008) and showing in mouse or human that paralog genes are less likely to harbor mutations associated to lethality or diseases, respectively (White et al., 2013).

Materials and methods

Mouse strains

All experiments were performed in accordance with the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87- 848/EEC). Mice were treated according to the principles of laboratory animal care, and mouse housing and experiments were approved by the Animal Welfare Committee of the Université catholique de Louvain (Permit Number: 2013/UCL/MD/11 and 2017/UCL/MD/008). The day of the vaginal plug was considered to be embryonic day (e) 0.5. The embryos were collected at e10.5 and e12.5, a minimum of 3 embryos of each genotype were used in each experiment. *Olig2-Cre, Nestin-Cre*, *Vsx1*$^{-}$, *Chx10*^{orJ} and *Hb9*$^{-}$ mutant lines have been previously described (Burmeister et al., 1996b; Dessaud et al., 2007; Francius et al., 2016; Thaler et al., 1999a; Tronche et al., 1999). The *Rosa26-Vsx1* line was generated using a gateway- and a recombinase-mediated cassette exchange-based system targeting the G4 ROSALUC embryonic stem cells, as previously described (Figure S1A)(Haenebalcke et al., 2013). The *Vsx1* coding sequence, flanked by *Att* recombination sites, was amplified from embryonic cDNA at e10.5 by PCR with GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAACCATGACTGGACGGG ATGGGCTTTCG and GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATGAGGCTCCCAC CTGTGG primers (5'-3'). Primer sequences available on request.

Chromatin immunoprecipitation (ChIP) and quantitative (q)-PCR assays

ChIP experiments were performed using Human Embryonic Kidney 293 (HEK293) cells. Cells were cultured in DMEM high glucose media (Thermo Fisher Scientific #11965092) supplemented with fetal bovine serum 10% (Thermo Fisher Scientific #10500064) and Penicillin-Streptomycin (Thermo Fisher Scientific #15070063). For ChIP assays, cells were seeded in 6-wells plates and transfected

with pEF::Vsx1-HSV (625 ng/well) or pEF::GFP plasmid (625 ng/well), and pCAGGS::DsRed2 (625 ng/well) using Lipofectamine 2000 (Thermo Fisher Scientific #11668027). Cells were collected 16 to 24h after transfection and ChIP was performed using the EZ-ChIP™ kit (Millipore #17-371) according to manufacturer's instructions. Chromatin was fragmented to 200 to 600 bp by sonication (high power, 30 cycles of 30 seconds with 1 minute between pulses) and incubated with anti-HSV antibody (goat; 1:500; Novus Biolabs #NB600-513) or species-matched IgG overnight at 4°C. *HxRE* sequence enrichment was assessed by quantitative PCR assays with GCAACACTTCCAGGCTCAGCCAG and CTGTTCTTGCAGACTAGCAGG primers (5'-3'). Fold enrichment was calculated over IgG using $2^{(-\Delta\Delta CT)}$, where $\Delta\Delta CT=(Ct_{ip}–Ct_{Input})–(Ct_{IgG}–Ct_{Input})$.

Luciferase assays

Luciferase assays were performed using the Dual-luciferase Reporter Assay System (Promega #E1910) in HEK293 cells. Cells were seeded in 24-wells plates and transfected with HxRE::Luciferase (125ng/well) (Lee et al., 2008), renilla luciferase control vector (5 ng/well) used for reporter activity normalization, Isl1-Lhx3 (30 ng/well), and pCS2::Vsx1 or pCS2::Vsx1R166W (60ng/well) or an empty vector (60 to 220ng/well). After 24h of treatment, cells were collected and prepared according to manufacturer's instructions. Luciferase reporter activities were measured with a tube luminometer (Titertek Berthold).

***In ovo* electroporation**

In ovo electroporations were performed at stage HH12 and embryos were collected 48h after electroporation. HxRE::GFP (1µg/µl) (Lee et al., 2008), Isl1-Lhx3 (0,25 to 0,5 µg/µl) (Lee et al., 2008), pCS2::Vsx1 and pCS2::Vsx1R166W (1,5 µg/µl) (Dorval et al., 2005), pCAGGS::Nkx6.1 (0.25 to 0.5 µg/µl; kindly provided by J. Briscoe) (Briscoe et al., 2000), pMxig-Pax6 (0,25 to 0,5 µg/µl; kindly provided by M.

Gotz) (Ninkovic et al., 2010), pEF::Vsx1-HSV (0,25 µg/µl) and empty pCMV (0,25 to 1,5 µg/µl, kindly provided by C. Pierreux) were co-electroporated with the pCAGGS::DsRed2 plasmid (0,25 µg/µl; gift of Y. Takahashi) (Watanabe et al., 2007) to visualize electroporated cells. Collected embryos were fixed in ice-cold PBS/4% PFA for 25 min and processed as above. Labeled cells were counted on both sides of 5 to 10 sections per embryo. For *Vsx1*, *Nkx6.1* and *Pax6* overexpression experiments, the ratio between electroporated and non-electroporated sides of each embryo was calculated to normalize for developmental stage and experimental variations.

Immunofluorescence labelings

Collected embryos were fixed in ice-cold phosphate buffered saline (PBS)/4% paraformaldehyde (PFA) for 15 to 25 min, according to the developmental stage. After washes in PBS, fixed embryos were incubated in PBS/30% sucrose overnight at 4°C, embedded and frozen in PBS/7.5% gelatin/15% sucrose. Immunostainings were performed on 14µm serial sections as previously described (Francius et al., 2016).

Primary antibodies against the following proteins were used: activated Caspase-3 (rabbit; 1:100; Cell Signaling #ASP175), Ascl1 (mouse; 1:200; BD #556604), Chx10 (sheep; 1:500; Exalpha Biologicals #X1179P), Foxp1 (goat; 1:1000; R&D Systems #AF4534; or mouse; 1:250; Origene #UM800020), Gata3 (rat; 1:50 ; Absea Biotechnology #111214D02), GFP (chick; 1:1000; Aves Lab #GFP-1020), chicken Hb9 (rabbit; 1:1000; kindly provided by S. Morton), Isl1/2 (goat; 1:3000; Neuromics #GT15051; or mouse; 1:6000; DSHB #39.4D5), Lhx3 (rabbit; 1:2000; DSHB #G7.4E12), MNR2 (mouse; 1:2000; DSHB #81.5C10), Nkx2.2 (mouse; 1:1000; DSHB #74.5A5), Nkx6.1 (mouse; 1:2000; DSHB #F55A10), Olig2 (rabbit; 1:4000; Millipore #AB9610), Shox2 (mouse; 1:500 ; Abcam #ab55740), Sip1 (rabbit; 1:500) (Van de Putte et al., 2003), Sox1 (goat; 1:500; Santa Cruz #sc-17318), Sox14 (guinea-pig;

1:1000) (Clovis et al., 2016), Vsx1 (rabbit, 1:500; kindly provided by E. Levine) (Clark et al., 2008).

Following secondary antibodies were used: donkey anti-goat/alexaFluor 488, donkey anti-guinea-pig/AlexaFluor 594, 488 or 647, donkey anti-mouse/AlexaFluor 488, 594 or 647, goat anti-mouse, IgG1/ AlexaFluor 594, goat anti-mouse, IgG2a/AlexaFluor 488, goat anti-mouse IgG3/AlexaFluor 488, donkey anti-rabbit/AlexaFluor 488, 594 or 647, donkey anti-rat/AlexaFluor 647, donkey anti-sheep/alexaFluor 594 or 647 purchased from ThermoFisher Scientific or Jackson Laboratories and used at dilution 1:1000.

Imaging, quantitative and statistical analyses

Acquisition of immunofluorescence images were performed on Evos FL, Evos FL Auto cell imaging system or Olympus FluoView FV1000 confocal microscope. Adobe Photoshop CS3 program was used for cell quantifications and image modifications. Brightness and contrast were adjusted uniformly in all replicate panels within an experiment to match with observation. Labeled cells were counted on both sides of 3 to 5 sections at brachial or thoracic levels at e10.5 and at brachial, thoracic or lumbar levels at e12.5. Raw data were exported from Adobe Photoshop CS3 software to SigmaPlot v11.0 software and processed for statistical analyses. T-test or Wilcoxon-Mann-Whitney test were used for statistical comparison of mouse section quantifications, luciferase assay data and qPCR data. Paired-test or Wilcoxon-signed-rank-test was used for chicken section quantifications.

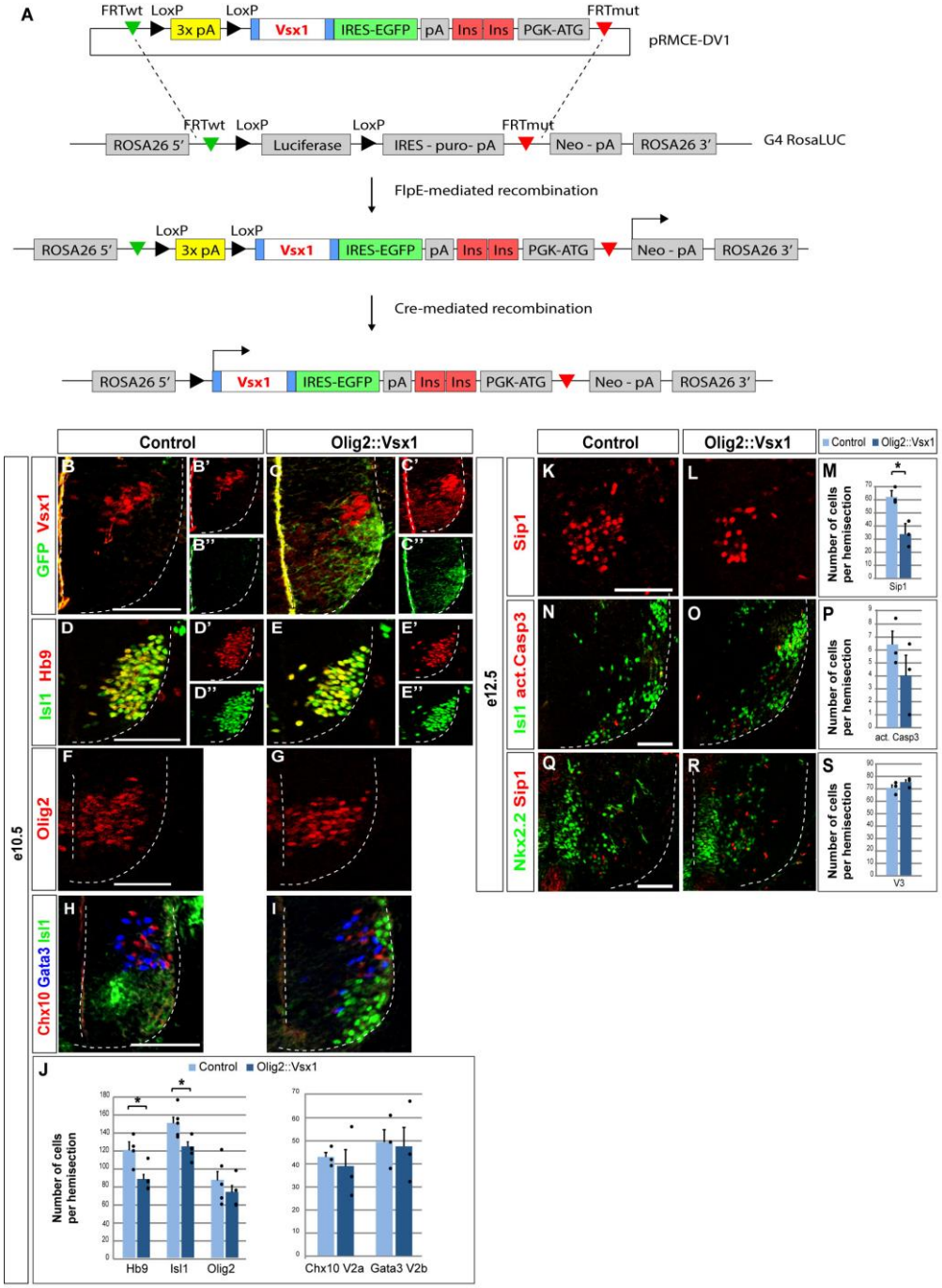
Acknowledgments

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for *Renilla* luciferase control and empty pCMV plasmids, to Y. Takahashi for the *pCAGGS::DsRed2* plasmid, to J. Briscoe for the *pCAGGS::Nkx6.1* plasmid, to M. Gotz for the*pMxig-Pax6* plasmid and to E. Hermans for luciferase assay kit. The antibodies anti Isl1/2 (.4D5), anti Lhx3 (#G7.4E12), anti MNR2 (.5C10), anti Nkx2.2 (.5A5) and anti Nkx6.1 (#F55A10) were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

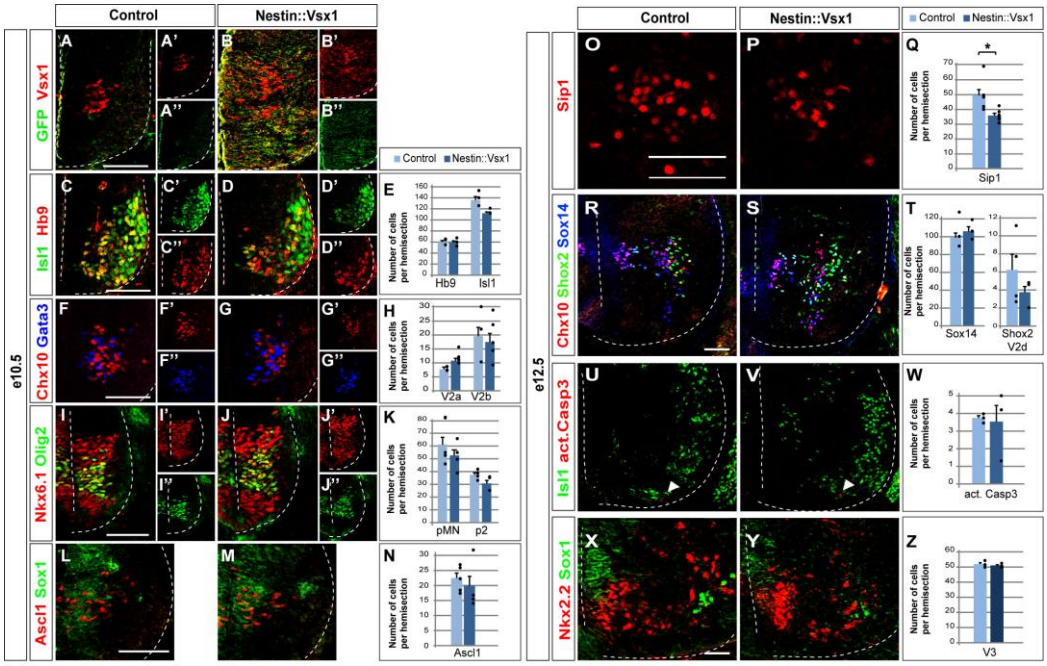
This work was supported by grants from the "Fonds spéciaux de recherche" (FSR) of the Université catholique de Louvain, by a "Projet de recherche (PDR)" #T.0117.13 and an "Equipement (EQP)" funding #U.N027.14 of the Fonds de la Recherche Scientifique (F.R.S.-FNRS), by the "Actions de Recherche Concertées (ARC)" #10/15-026 of the "Direction générale de l'Enseignement non obligatoire et de la Recherche scientifique – Direction de la Recherche scientifique – Communauté française de Belgique" and granted by the "Académie universitaire 'Louvain'" and by the Association Belge contre les Maladies neuro-Musculaires (ABMM, Belgium). S.D. and C.B. hold PhD grants from the FRIA (F.R.S.-FNRS, Belgium), M.H.-F. was a Postdoctoral Researcher of the F.R.S.-FNRS, F.C. and F.T. are Senior Research Associate and Research Director of the F.R.S.-FNRS, respectively.

Supplementary figures



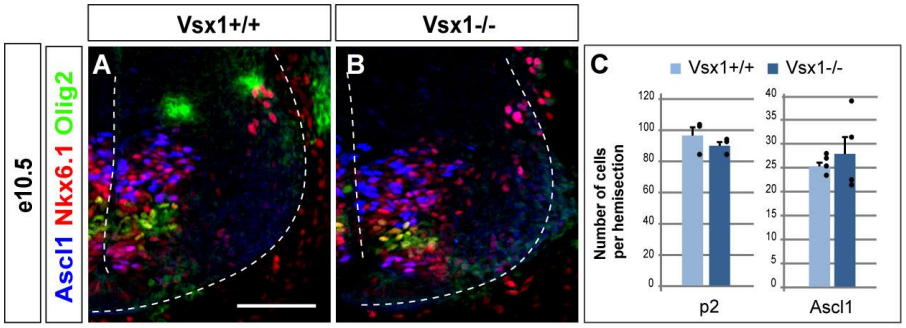
Supplementary Figure 1 : Related to Figure 22 and to the "Mouse strains" section of Materials and Methods. Vsx1 specifically inhibits MN differentiation without affecting the pMN progenitor domain.

(**A**) Schematic representation of the strategy to produce the *Rosa26R::Vsx1* allele. The mouse *Vsx1* coding sequence was inserted into the pRMCE-DV1 vector using the Gateway system (blue rectangles). The wild-type and mutated Frt site-flanked cassette was integrated at the modified Rosa26 locus in G4 RosaLUC ES cells by Recombination-Mediated Cassette Exchange (co-electroporation with a Flp-expressing vector). Recombination restores neomycin resistance for selection of recombinant ES cells. Insulator (Ins) prevents interference in *Vsx1* expression by the strong ubiquitous PGK promoter (PGK). See (Haenebalcke et al., 2013) for additional details. (**B-S**) Immunofluorescence for EGFP and Vsx1, and markers for progenitors and visceral MN (Sip1), MN progenitors (Olig2), MNs (Isl1, Hb9), V2 INs (Chx10 for V2a and Gata3 for V2b), V3 progenitors and INs (Nkx2.2) or apoptosis (activated Caspase-3) on transverse spinal cord sections of MN-specific Vsx1 gain-of-function embryos. (**B-C**) In *Olig2::Cre* x *Rosa26R::Vsx1* embryos at e10.5, Vsx1 and EGFP are ectopically produced in the ventral part of the spinal cord, including MN (compare with **D-E"**) and more ventral cells (V3 INs). (**D-E, J**) Ectopic production of Vsx1 in MN at e10.5 reduces the number of Isl1+ or Hb9+ MNs (n=3). (**F-G, J**) However, the number of Olig2+ MN progenitors and the integrity of the pMN domain are preserved (n=5; p=0.40). (**H-J**) The number of V2a and V2b INs is not changed, and no hybrid cell containing MN and V2 IN markers is observed (n=3; V2a: p=0.68; V2b: p=0.88). (Witczak-Malinowska et al., 2001) In *Olig2::Cre* x *Rosa26R::Vsx1* embryos at e12.5, ectopic production of Vsx1 reduces the number of Sip1-positive visceral MNs (n=3). (Eccles et al., 1968) The reduction in MNs observed after ectopic expression of *Vsx1* is not due to increased apoptosis as the number of cells containing activated Caspase-3 is not upregulated in the *Olig2::Cre* x *Rosa26::Vsx1* embryos (n=3; p=0.27). (**Q-S**) Ectopic production of Vsx1 in the most ventral part of the spinal cord does not affect Nkx2.2+Sip1- V3 IN differentiation (n=3; p=0.26). Mean values ± SEM; * p≤ 0.05. Scale bars = 50 µm.



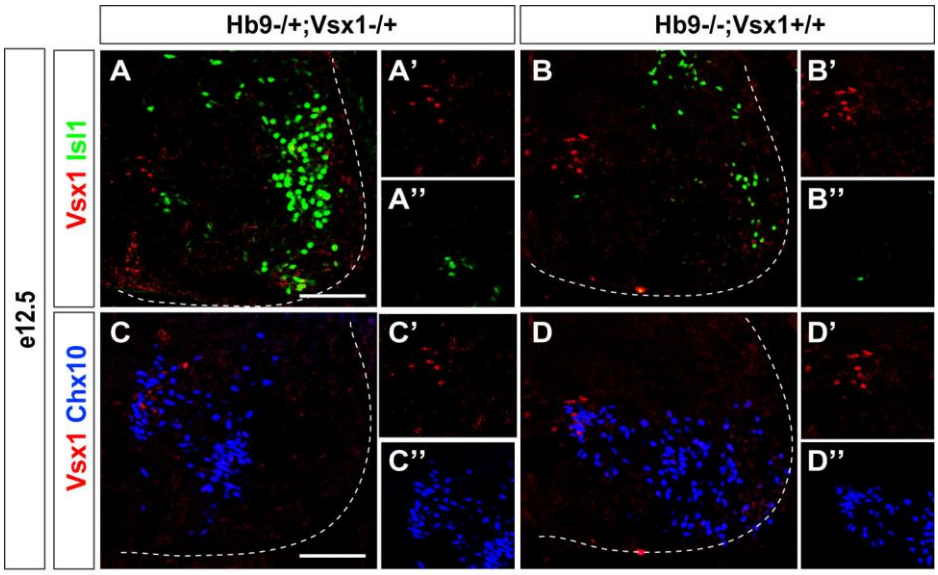
Supplementary Figure 2 : Related to Figure 23. Vsx1 inhibits MN differentiation and stimulates V2 IN differentiation without affecting dorso-ventral patterning.

Immunofluorescence for EGFP and Vsx1, and markers for progenitors and V2c INs (Sox1), MN progenitors (Olig2), V2 progenitors (Ascl1), p2-pMN-p3 progenitors (Nkx6.1), MNs (Isl1, Hb9), visceral MN (Sip1), V2 INs (Chx10, Sox14, Gata3, Shox2), V3 progenitors and INs (Nkx2.2) or apoptosis (activated Caspase-3) on transverse spinal cord sections of Vsx1 gain-of-function embryos. (de Melo et al.) In the *Nestin::Cre* x *Rosa26R::Vsx1* embryos at e10.5, Vsx1 and EGFP are produced in the entire spinal cord. (**C-E**) Ectopic production of Vsx1 in the entire spinal cord mildly inhibit MN differentiation (n=3; Hb9: p=0.82; Isl1: p=0.84). (Attias et al., 2004) Increased expression of *Vsx1* in V2 INs does not significantly change V2a or V2b production (n=4; V2a: p=0.053; V2b: p=0.75). (**I-N**) The dorso-ventral patterning is not affected by the ectopic expression of Vsx1 in the entire spinal cord, as the pMN and p2 progenitor domains are normal and Ascl1 production in p2 is not modified (n=4; p2: p=0.09; pMN: p=0.38; Ascl1: p=0.56). (**O-Q**) At e12.5, the production of visceral MNs labeled by Sip1 is reduced upon ectopic expression of Vsx1 in MNs (n=6). (**R-T**) In contrast, the number of Sox14+ and Shox2+ V2 INs is not changed (n=4; Sox14+ cells: p=0.26; Shox2+ cells: p=0.57). (**U-W**) The reduction in MNs observed after ectopic expression of *Vsx1* is not due to increased apoptosis as the number of cells containing activated Caspase-3 (arrowheads) is not upregulated in the *Nestin::Cre* x *Rosa26R::Vsx1* embryos (n=3; p=0.96). (**X-Z**) V3 IN differentiation is not affected by ectopic expression of Vsx1 (n=3; p=0.81). Mean values ± SEM; * p≤ 0.05. Scale bars = 50 µm.



Supplementary Figure 3 : Related to Figure 24. Absence of Vsx1 does not alter p2 progenitor domain or dorso-ventral patterning.

Immunofluorescence for markers of MN progenitors (Olig2), V2 progenitors (Ascl1) and p2-pMN-p3 progenitors (Nkx6.1) on transverse spinal cord sections of *Vsx1*$^{-/-}$ embryos. (A-C) The absence of Vsx1 does not modify the size or the relative location of the p2, pMN or p3 progenitor domains (n=3; p2 progenitors, i.e. Nkx6.1+ cells dorsal to Olig2 cells: p=0.40; Ascl1: p=0.55). Mean values ± SEM. Scale bar = 50 µm.



Supplementary Figure 4 : Related to Figure 25. Absence of Hb9 does not generate Vsx1/Isl1 or Vsx1/Chx10 hybrid cells.

Immunofluorescence for Vsx1 and markers of MN (Isl1) or V2a INs (Chx10) on transverse spinal cord sections of *Hb9*$^{-/-}$ embryos. (**A-D"**) No Vsx1/Isl1 or Vsx1/Chx10 hybrid cell is observed in the *Hb9* mutant, suggesting that Chx10 may repress *Vsx1* expression in MN/V2 hybrid cells. Mean values ± SEM. Scale bars = 50 µm.

4. General discussion

Locomotion is the ability of an organism to move in its environment. Vertebrates evolution was accompanied by a transition from water to land-based locomotion that resulted in a change in locomotion mode and an increase in neural diversity. In terrestrial vertebrates, CPGs located in the ventral spinal cord control the coordination of limb movements and posture characterized by a certain rhythm, a defined speed, left/right alternation and alternation of the contraction of antagonistic flexor and extensor muscles. Spinal CPGs generate a rhythmic and stereotyped locomotor pattern without descending and sensory afferences. However, a stimulation by neuromodulators is required to observe this activity. During development, MNs and interneuron populations that compose these networks arise from different progenitor domains orderly distributed along the dorso-ventral axis of the neural tube. They diversify and migrate inside the spinal cord to establish connections with neuronal targets and form complex networks. The network organization and function, illustrated by cell diversification, acquisition of molecular characteristics, cell body position, synapse establishment and selection of targets, are controlled by finely regulated genetic programs.

In this work, we show that Vsx1 is transiently produced by an early intermediate V2 population that probably give rise to all V2 interneuron subsets. We provide evidence that Vsx1 stimulates V2 interneuron differentiation although it is not necessary for their production. Moreover, we demonstrate that Vsx1 contributes, in collaboration with its unique paralog factor Chx10, to the consolidation of V2 interneuron versus MN fate. Cross- and mutual repressions between Hb9 and Vsx1 in V2 precursors or Chx10 in differentiating V2a interneurons, prevent the activation of inappropriate program of differentiation in each neuronal population.

Therefore, our observations suggest that the two Prd-L:CVC paralog factors retained redundant functionality but have been segregated at distinct steps of V2 interneuron differentiation..

4.1. Vsx1 transiently labels an early V2 intermediate population that give rise to all V2 subsets

Specification and differentiation processes, controlled by combination of morphogens, cell cycle regulators and transcriptional programs, imply the transition from proliferative to differentiating cells. These events are coordinated in time, but cell cycle arrest is neither necessary nor sufficient to induce differentiation. Lobjois *et al.* showed that overexpression of cyclin D in developing chicken spinal cord maintains progenitors in cell cycle but that, at later stages, cells migrate and extend axons while still being in proliferation (Lobjois et al., 2004). Conversely, the arrest of cell cycle is not sufficient to initiate differentiation. In developing spinal cord, proneuronal gene *Gata2* starts to be expressed in p2 progenitor domain where it promotes cell cycle exit. However, gain of function analyses revealed that even if Gata2 is sufficient to inhibit proliferation, it does not systematically promote differentiation (El Wakil et al., 2006). In this work, we provide evidence that the p2 progenitor domain first produces V2 intermediate precursors, labelled by Vsx1, that exited cell cycle but did not start to initiate their differentiation.

In zebrafish, *Vsx1* is expressed in V2 precursors and is maintained in V2a interneurons (Batista et al., 2008). Based on this observation, *Vsx1* was supposed to be expressed in V2a interneurons in mouse. However, our studies demonstrate that Vsx1 is never co-detected with Chx10 in the mouse spinal cord. We suggest that *Vsx1*-expressing precursors produce all described V2 subsets and probably give rise to additional V2 cells that remains to be characterized. This hypothesis was

based on the strong co-expression of *Vsx1* with *Foxn4*. Indeed, Li *et al.* demonstrated that *Foxn4*-expressing progenitors produce V2a, V2b and V2c interneurons but that these populations represent only two-thirds of cells deriving from the Foxn4-positive population, leaving open the possibility that other V2 subset(s) are produced from p2 progenitor domain (Li et al., 2010). Recently, a Tamoxifen-dependent Vsx1-CreER^{T2} transgenic line was produced in my thesis laboratory. First lineage tracing analyses, through crossing with a *Rosa26R::tandem dimer-Tomato* (*td-Tomato*) reporter line, revealed that Vsx1-expressing precursors produce all described V2 subsets and that around 30% of Vsx1-deriving cells, at e14.5, are neither V2a nor V2b interneurons (Baudouin C., Pelosi B. *et al.,* in progress). These preliminary data confirm that *Vsx1* is transiently expressed in early V2 intermediate cells that produce all V2 interneurons and that some Vsx1-deriving cells are not identified yet. Vsx1 reporter line could be used to identify new markers of V2 subsets, in particular those that remain to be characterized, and to study in more details the diversification of V2 interneurons. Isolation of Vsx1-deriving cells could be performed by Fluorescent Activated Cell Sorting (FACS) based on *td-tomato* expression, followed by the analysis of their transcriptome.

Vsx1 transiently labels V2 precursors. This raises the question of which factors control its sharp expression. We could speculate that some genes expressed in p2 progenitor domain stimulate its expression. In this work, we demonstrate that factors that control V2a and V2b segregation, as Foxn4, Ascl1 and the Dll/Notch pathway, do not regulate *Vsx1* expression. However, our data suggest that Pax6 promotes Vsx1 production as observed by the decrease in *Vsx1*-expressing cells in Pax6^{Sey} mutant line. *Nkx6.1*, another patterning gene expressed in p2 progenitor domain, is maintained in early V2 intermediate cells. Its inactivation leads to a reduction in V2 interneuron number (Sander et al., 2000). Therefore, Nkx6.1 could also be a good candidate for the activation of *Vsx1* expression. Gain-of-function

analyses show that *Lhx3*, expressed in p2 progenitor domain and maintained in V2a interneurons, promotes V2a interneuron markers expression (Clovis et al., 2016) (Lee et al.). It could be speculated that this LIM factor also activates the expression of *Vsx1*. Gain-of-function in chicken spinal cord and deeper analysis of mutant lines could allow to challenge these different hypotheses (Sharma et al., 1998).

The short time-window of *Vsx1* expression in V2 precursors implicates rapid transcriptional repression and/or degradation mechanisms. First, *Vsx1* transcription could be inhibited by repressor factors. The mutually exclusive expression of *Vsx1* and *Chx10* in the retina and the spinal cord suggests cross-repression mechanisms between these two paralog factors. *In vitro* and *in vivo* analyses revealed that Chx10 binds two consensus regions in the *Vsx1* regulating sequences and inhibit its activation (Clark et al.; Clovis et al.; Dorval et al.). *Vsx1* is overexpressed in Chx10^{orJ} mutant retina (Clark et al., 2008) and our data show that it is also the case in the Chx10^{orj} spinal cord. Conversely, in the developing retina, Vsx1 inhibits the expression of *Chx10* in Type 7 bipolar cells (Chow et al., 2004), demonstrating mutual repression in this cell type. However, our results showed that the inactivation of Vsx1 in the spinal cord does not lead to an increase in *Chx10* expression. Finally, *Vsx1*-expressing cells give rise to all V2 subsets, indicating that other factors may inhibit *Vsx1* expression in other V2 interneurons. Karunaratne et al. suggests that Gata3 could inhibit *Chx10* expression in V2b interneurons (Karunaratne et al., 2002). Whether this could be also the case for *Vsx1* remains to be investigated.

Second, post transcriptional regulation is often required to restrict gene expression to a specific developmental stage or to a cellular subset. MicroRNAs repress transcription through messenger RNA degradation or inactivation of translation. In goldfish and zebrafish, microRNA-20a (miR-20a) represses *Vsx1* expression by binding to its 3'-UTR region. Buffering this miR leads to an excessive

production of *Vsx1*-expressing neurons, while its overexpression inhibits *Vsx1* expression in the spinal cord (Sun et al., 2015). This data demonstrated that post-transcriptional regulation by miR-20a restricts *Vsx1* expression in teleost. Moreover, in Xenopus retina, *Vsx1* expression is repressed by miR-129, miR-155 and miR-222 that regulate bipolar cell proportion (Decembrini et al., 2009). Post-transcriptional regulation is also important during mouse spinal cord development. MiR-17-3p, for example, is required to repress *Olig2* expression in p2 progenitor domain and consolidates p2/pMN specification (Chen et al., 2011). In chicken spinal cord, miR-9 represses c*Oc1* expression and controls early- and late-born MN development of the LMC (Luxenhofer et al.; Radhakrishnan and Alwin Prem Anand, 2016). Therefore, miRs could be involved in the sharp repression of *Vsx1* in differentiating mouse V2 subsets or to the inhibition of Vsx1 production in other cell types.

Third, Kurtzman *et al*. demonstrated that Vsx1 is a substrate for the ubiquitin/proteasome pathway. Its C-terminal peptide sequence is ubiquitinated, leading to the degradation of the protein by the 26S proteasome (Kurtzman et al., 2000). This mechanism of degradation predominantly occurs in the cytoplasm. Thanks to a NLS and a NES located in its peptide sequence, Vsx1 can shuttle between the nucleus and the cytoplasm and therefore modulate its transcriptional activity or its degradation (Knauer et al., 2005). Our analyses of the *Nestin::Cre* x *Rosa26R::Vsx1-IRES-EGFP* line transgenic line suggests that the increase in Vsx1 in neuronal spinal cells is not maintained at later stages of development (data not shown) which suggest that Vsx1 degradation by the proteasome may significantly contribute to the regulation of Vsx1 distribution in the developing spinal cord. The sharp time-window of *Vsx1* expression suggests a fine regulation by transcriptional, post-transcriptional and degradation mechanisms.

In the present work, we identified a population of V2 intermediate cells that exited the cell cycle but did not start to initiate their differentiation process. This intermediate state between proliferative and differentiating cells is probably required for the consolidation of V2 interneuron identity and the correct establishment of differentiation mechanism. V2a and V2b interneurons segregation, for example, requires the asymmetric activation of the Notch signalling pathway. It has been demonstrated that Notch signalling activates the expression of *Hes1* in an oscillating manner through negative feedback regulation (Kageyama et al., 2008). A delay in the differentiation process of V2 interneurons could be required for the correct acquisition of V2 identity following mosaic expression of proneural genes and the oscillating Notch signal.

4.2. Vsx1 is not necessary for V2 interneuron differentiation

Even if the role of Vsx1 in the mouse retina is quite well described, very few was known about its function during spinal cord development. Its inactivation does not lead to a decrease in V2 interneurons or an expansion of MNs. This suggests that the absence of Vsx1 can be compensated by other factors, either expressed by the early V2 intermediate population or misexpressed when Vsx1 is inactivated. A co-expression of *Vsx1* with *bHLHb5* is observed in V2 precursors. This factor consolidates V2 interneuron fate through cross-repression with Olig2 (Skaggs et al., 2011). bHLHb5 stimulates Dll4 expression and promotes the differentiation of V2a interneurons where it is maintained (Skaggs et al., 2011). Interestingly, this factor is also co-expressed with *Vsx1* in the retina where it regulates Type 2 OFF bipolar cell differentiation (Feng et al., 2006). Therefore, bHLHb5 could potentially compensate the absence of Vsx1 for the inactivation of MN program by a mechanism that remains to be identified.

In this work, we show that Pax6 and Nkx6.1 are maintained in *Vsx1*-expressing cells although these are not proliferating progenitors and that the absence of Vsx1 does not affect their expression. Lee *et al.* suggested that these two factors are able to inhibit *Hb9* expression to maintain pMN progenitors in proliferation and thereby prevent MN differentiation. Our gain of function analyses, conducted in chicken embryonic spinal cord, confirmed the repression of *Hb9* by these factors and unveiled a possible cooperation of Vsx1 with Nkx6.1 and, less efficiently, with Pax6. Nkx6.1 mutant embryos display patterning defects including a ventral expansion of the p1 progenitor domain and a decrease in V2 interneurons and MNs (Sander et al., 2000). In contrast, a dorsal expansion of p3 progenitor domain is observed in Pax6 mutant individuals (Ericson et al., 1997). Therefore, the analysis of a triple constitutive mutant line for Nxk6.1, Pax6 and Vsx1 is not feasible. Compound conditional mutants, or specific repression of these three factors in the V2 compartment of chicken spinal cord, could help us to confirm a potential compensatory mechanism involving these factors in the inhibition of the MN program in V2 compartment.

4.3. Vsx1 and Chx10 sequentially consolidate V2 interneuron versus MN fate

In this work, we provide evidence that Vsx1 consolidate V2 interneuron fate by acting at 2 different levels. On the one hand, it stimulates V2 interneuron differentiation, as observed by our analyses of Vsx1 gain-of-function. On the other hand, it prevents the inappropriate activation of MN program by repressing the expression of *Hb9*. The role of Vsx1 is closely related to the function of its unique paralog factor Chx10, which also prevents MN differentiation but promotes the differentiation of the V2a interneuron subset. In this work we partially elucidated

the mechanism of action of Vsx1. However, downstream Vsx1 targets remain to be identified.

As its paralog factor Chx10, Vsx1 is described as a repressor of transcription. Chx10 drives V2a interneuron fate through the direct repression of non-V2a genes, such as *Dbx1*, *Olig3* or *Otp,* and indirectly stimulates *Sox14*, *Shox2* or *VGluT2* (Clovis et al., 2016). Vsx1 targets are not identified in the spinal cord but Dorval *et al*. demonstrated that the protein recognizes P3 consensus, as its paralog factor Chx10, and that the integrity of the CVC domain is necessary for its repression activity (Dorval et al., 2005). Moreover, Clovis *et al.* showed that overexpression of *Vsx1* in chicken spinal cord results in an increase in the number of *Sox14*-expressing cells, i.e. V2 precursors and V2a interneurons (Clovis et al., 2016). Therefore, this together with our data suggest that, as its paralog factor Chx10, Vsx1 stimulates V2 interneuron differentiation by supressing V2 repressors. To demonstrate the ability of Chx10 to repress MN program and to activate V2a interneuron differentiation, Clovis *et al* produced a model of Chx10-inducible embryonic stem cell line, directed to differentiate in MNs (Clovis et al., 2016). Chromatin immunoprecipitation sequencing (ChIPseq) and RNA sequencing (RNAseq) were performed after the activation of Chx10 and allowed to identify multiple genes downstream of this Pdr-L:CVC factor. This model could be transposed to our study to identify genes downstream of Vsx1 in spinal neurons. Moreover, the recently produced Vsx1-CreER^{T2} transgenic line could be used to isolate *Vsx1*-expressing cells by FACS and realize a transcriptome comparison of Vsx1 mutant vs control V2 precursors. These experiments could allow the identification of Vsx1 downstream targets and the identification of the Vsx1-dependant mechanisms that stimulate V2 interneuron development.

As described above, Vsx1 stimulates the differentiation of both V2a and V2b interneurons. In contrast, its paralog factor Chx10 could prevent V2b interneuron

fate by repressing Gata3 expression. Different observations support this hypothesis. First, we show in this work that a trend for an increase in *Gata3* expression is observed in Chx10^{orJ} mutant line. We performed deeper analyses of the *Gata3* expression profile at different levels of the spinal cord. A significant increase in the number of *Gata3*-expressing cells was observed at lumbar level (p=0.04, data not shown). Our data also demonstrate that *Vsx1* is overexpressed in the absence of Chx10. However, the increase in *Gata3* expression is not observed in the double mutant for Chx10 and Vsx1. Therefore, the increase in Gata3 could reflect different regulation mechanisms. The overexpression of *Vsx1* in Chx10^{orJ} mutant line could stimulate *Gata3* expression, in agreement with our results from Vsx1 gain-of-function analyses. Furthermore, *Gata3* overexpression could also be due to the inactivation of Chx10 that probably prevents its expression. Hence, the most likely hypothesis is that this phenotype is due to the combined action of Vsx1 and Chx10 that respectively activates and represses *Gata3* expression. Furthermore, *Gata3* repression by Chx10 is supported by data from the laboratory of Soo-Kyung Lee (Oregon Health and Science University). They showed that misexpression of Chx10 in the chicken spinal cord leads to a decrease in Gata3 expression (personal communication). Moreover, deeper analyses of the *Hb9*$^{-}$ mutant line revealed a significant decrease in *Gata3* expression at lumbar level of the spinal cord (p=0.02, data not shown), while only a trend was observed for the total number of V2b interneurons. In this Hb9-deficient line, *Chx10* is overexpressed and could therefore inhibit *Gata3* expression. Surprisingly, a restoration of Gata3 phenotype is observed in double mutant for Vsx1 and Hb9. Therefore, meanwhile Vsx1 stimulates Gata3 expression in V2 compartment, it does not display the same function in the MN context. Together, our different observations strongly suggest a downregulation of *Gata3* expression by Chx10 and

demonstrate that Vsx1 stimulates Gata3 expression in V2 interneuron compartment.

During spinal cord development, consolidation mechanisms are required to properly segregate V2 interneurons and MNs. The cross- and mutual repressions of *Chx10* and *Hb9* are well characterized (Clovis et al., 2016) (Lee et al.). The requirement of MN program inhibition by Chx10 suggests that factors, or complexes, are able to activate the HxRE in V2 compartment. Dessaud *et al*. showed that *Olig2* is early and transiently expressed by some cells belonging to p2 progenitor domain (Dessaud et al., 2010). Consequently, some activators of MN differentiation could be produced at early stage of development. However, *Chx10* is expressed in differentiating V2a interneurons. Therefore, the activation of the HxRE has to be kept silent by other factors at earlier stages of development. In this work we provide evidence that Vsx1 displays the same functionality than Chx10 to prevent HxRE activation and *Hb9* expression at earlier stages of V2 interneuron development. We used different techniques to address this hypothesis. We firstly demonstrated by *in vitro* experiments that Vsx1 binds and inhibits the activation of the HxRE. Then, chicken spinal cord electroporation experiments were used to show that Vsx1 prevents HxRE activation in vivo and inhibits *Hb9* expression. Finally, we demonstrated that misexpression of Vsx1 in the MN compartment in two independent murine transgenic line led to a global decrease in the number of MNs. These different data strongly support the repression of HxRE activation and *Hb9* expression by Vsx1. Clovis *et al.* identified more than 900 genomic regions that recruit both Chx10 and Isl1:Lhx3 complex. Among them, 12 corresponds to MN-genes and are respectively downregulated or upregulated by Chx10 or the MN hexameric complex (Clovis et al., 2016). As Vsx1 recognizes similar sequences than Chx10 (Dorval et al., 2005), it is also likely that it does not exclusively represses *Hb9* expression but also other MN-specific genes, as *LMO1* or *Slit Guidance Ligand 3*

(*Slit3*) for example. Here again, ChIPseq and transcriptome analyses would be very useful to identify Vsx1 targets.

Our results demonstrate that the two Prd-L:CVC paralog factors act successively to robustly secure V2 interneuron fate and prevent activation of the MN program. While a decrease in V2a markers is observed by Clovis *et al.* in Chx10^{orJ} deficient mice (Clovis et al.), it does not correlate with an expansion of MNs, suggesting that other factors could compensate its absence to consolidate V2a interneuron fate. Our analysis of the double mutant for Chx10 and Vsx1 demonstrates that the inactivation of both factors presents a stronger phenotype corresponding to a decrease in V2a interneurons and an expansion of MN markers that is not observed in *Vsx1* or *Chx10* single mutants. The inactivation of Chx10 is accompanied by an expansion of *Vsx1* expression. Therefore, Vsx1 could partly compensate the absence of its paralog factor and attenuate the reduction in V2a interneuron differentiation due to the lack of Chx10.

In the MN compartment, Hb9 binds and inhibits the activation of TeRE to prevent Chx10 expression (Lee et al., 2008). In *Hb9* mutant embryos, a decrease in MNs is accompanied by an expansion of V2a markers as Chx10, Shox2 and Sox14. Our data show that *Vsx1* expression is also expanded, suggesting that Hb9 represses this transcription factor. Hb9 gain-of-function analysis performed in chicken spinal cord could help us to confirm our hypothesis. Nevertheless, the increased expression of *Vsx1* seems to be restricted to the medial part of the spinal cord and no hybrid cell expressing Vsx1 and MN markers was detected. As described above, Chx10 directly represses *Vsx1* expression, suggesting that it could also prevent Vsx1 expansion in the MN compartment. Therefore, we could expect a higher increase in *Vsx1* expression in the double mutant for *Chx10* and *Hb9*.

The expansion of phenotype observed in the *Hb9* single mutant line is partially restored in *Chx10^{orJ}/Hb9^{-}* double mutant. The expanded expression of V2a

interneuron markers, as *Sox14* and *Lhx3*, observed in the absence of Hb9, returns to normal when Chx10 is inactivated, while a decrease in MNs is still observed (Clovis et al., 2016). As already proposed, we suggest that Vsx1 is aberrantly expressed in double mutant embryos for *Chx10* and *Hb9* and could therefore prevent activation of the MN program. This hypothesis could be confirmed by the analysis of *Vsx1* expression profile in *Chx10^{orJ}/Hb9^{-}* double mutant and the analysis of the phenotype of the triple mutant for *Chx10*, *Hb9* and *Vsx1*. After combined inactivation of Chx10, Vsx1 and Hb9, different scenarii could be foreseen. We can speculate that the abolishment of cross-repression between Hb9 and Chx10 or Vsx1 could lead to a complete normalization of V2 interneuron and MN phenotypes. However, Chx10, and probably Vsx1, prevent the expression of non V2-genes as *Dbx1* or *Olig3* (Clovis et al., 2016). Therefore, an expansion of more dorsal populations could also be observed in the presumptive V2 compartment. In addition, we can also elaborate on the development of the V2b interneurons in this triple mutant. The analysis of *Gata3* expression profile in Chx10 and Hb9 deficient mice, together with the analysis of the triple mutant for *Chx10*, *Vsx1* and *Hb9*, could further enlighten our understanding of Vsx1 function important for the development of all the V2 interneuron populations.

In summary, Vsx1 transiently labels a V2 intermediate population of immature cells that give rise to all V2 interneurons. In this compartment, Vsx1 prevent the activation of MN program and stimulates V2 interneurons differentiation. We show that Vsx1 presents the same functionality than its paralog factor Chx10, expressed in V2a interneurons, and allows on robust consolidation of V2 interneuron fate.

4.4. Labor division between Prd-L:CVC paralog genes

Developmental processes follow most of the time the same trajectories despite genetic or environmental perturbations. In general, robustness results from

functional compensation that often implies genetic redundancy. Genetic redundancy is defined as a situation wherein paralog factors, originating from gene duplication, or unrelated factors, display overlapping functions and compensate for the absence of the other. Gene duplication frequently occurred during evolution. However, most of duplicated genes became non-functional through loss-of-function mutations and were progressively lost with time. In contrast, paralog factors can acquire beneficial mutations that confer them novel functions (neo-functionalization) while others only retain partial or fully overlapping properties (sub-functionalization)(Diss et al., 2014). Compensation by paralog factors can be characterized as passive or active. A passive compensation is observed when the basal production and function of a protein is sufficient to compensate the absence of its related factor. While in active compensation, the loss-of-function of one paralog leads to the overexpression of the related gene, the delocalization of its protein or the rewiring of protein interactions. In this work, we demonstrate that the robust consolidation of V2 interneuron identity implies functional compensation by different factors.

We demonstrated that Prd-L:CVC paralog factors Chx10 and Vsx1 conserved partly redundant functionalities. Both present a repressor activity and recognize same nucleic region (Dorval et al., 2005). Dorval *et al* demonstrated that Chx10 and Vsx1 binding to DNA sequence, through their HD, require CVC domain integrity. Sequence conservation between these two paralog factors is not high, with only 42% of identity between Chx10 and Vsx1 peptidic sequences in mouse. However, their HD and Prd-L:CVC domains are more conserved, namely 91 and 78% respectively (Ohtoshi et al., 2001). We provided evidence that Vsx1, as its paralog factor Chx10, binds and inhibits the activation of the HxRE to prevent the expression of *Hb9* in early V2 precursors. Therefore, both factors could compensate for the absence of each other. We demonstrated that the inactivation of Chx10, in

Chx10^{orJ} mutant, was accompanied by an expansion of *Vsx1* expression. The transcriptional reprogramming of *Vsx1* partly compensates for the loss-of-function of its paralog factor. This is illustrated by the mild phenotype observed in *Chx10*^{orJ} mutant and the obvious defect in V2a and V2d interneuron markers in the double mutant for *Chx10* and *Vsx1*. Moreover, an expansion of MNs is observed when both paralog factors are inactivated. Therefore, a functional active compensation is ensured by Vsx1 in the absence of Chx10. Gene upregulation mechanisms in the context of phenotypic compensation are poorly studied. Change in the expression profile of a factor in the absence of its paralog implies that this factor is downregulated in normal condition, through feedback regulation by downstream product or direct repression by the paralog factor. It is demonstrated that Chx10 directly represses *Vsx1* expression (Clovis et al., 2016; Dorval et al., 2005), suggesting that its absence allows an expansion in *Vsx1* expression that partly compensates for the Chx10 loss.

After gene duplication, beneficial mutations can lead to the acquisition of novel functions (neo-functionalization) while functions of the ancestral gene can be partitioned between paralogs (sub-functionalization). In this work, we demonstrate that Vsx1 promotes the differentiation of all V2 interneurons, in contrast to Chx10 that only stimulates V2a interneuron fate and potentially represses V2b identity, likely illustrating a partial sub-functionalization between paralogs. Moreover, we provide evidence that both duplicated genes retained the ability to prevent MN program activation and that this function is exerted by these paralogs at two successive steps of neuron differentiation. To our best knowledge, it is the first example of such a paralog cooperation in vertebrate embryos. The temporal segregation in their distribution profile confers to Vsx1 and Chx10 the ability to strongly consolidate V2 interneuron fate. We named this original example of an

identical function performed by two paralog factors at successive steps of cell differentiation in a single lineage the “labor division”.

Functional compensation does not systematically involve paralog factors. The absence of phenotype in *Vsx1* mutant spinal cord suggests the existence of compensatory mechanisms. In contrast to the overexpression of *Vsx1* in the *Chx10*^{orJ} mutant, no change regarding *Chx10* expression was observed in the *Vsx1*$^{-}$ mutant. As both paralog factors are not expressed in the same cells, other factors probably compensate the absence of Vsx1. Our results obtained by gain-of-function analyses in chicken spinal cord support the hypothesis that Nkx6.1, and possibly Pax6, cooperate with Vsx1 to inhibit the expression of *Hb9* and prevent the activation of MN program in early V2 precursors. The absence of Vsx1 does not lead to an increase in Nkx6.1 or Pax6 in V2 compartment. Therefore, it is suggested that these patterning factors compensate, in a passive manner, for the absence of Vsx1 (Figure 29)(Diss et al., 2014).

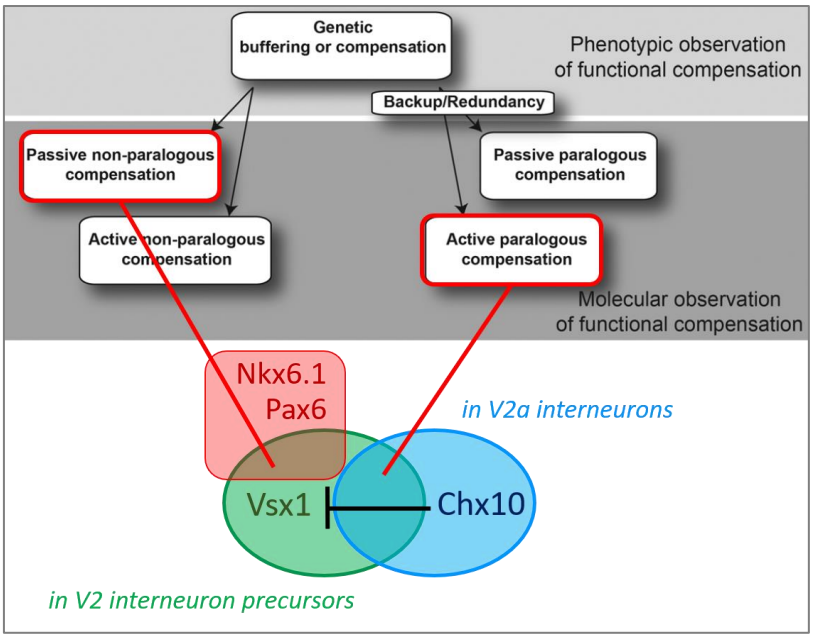


FIGURE 29 : Functional compensation model that consolidates V2 interneuron identity. Robustness in a biological system involves passive or active compensation performed by paralog or non-paralog factors. Objects in the bottom of the figure represent biological functions performed either by paralog genes (oval object) or non-paralog factors (rectangular object) (Diss et al., 2014).

4.5. Conclusion and afterword

During this thesis project, we highlighted the complexity of neuron differentiation process and the importance of genetic robustness. We provided further information about an important consolidation mechanism, based on cross-repressions between two paralog factors, Vsx1 and Chx10, and the early MN marker Hb9. This model shows that Vsx1 and Chx10 strongly consolidate V2 interneuron identity through MN fate inhibition at two successive steps of cell differentiation, a situation that we called "labor division". The V2a interneuron defect and the expansion of MNs, observed in the double mutant for *Vsx1* and

Chx10, illustrate the importance of compensation mechanisms that secure differentiation process.

While our observations are relevant to clarify V2 interneuron versus MN fate consolidation mechanisms, the project met some limits. The study of the double mutant for *Vsx1* and *Chx10* should be completed by a locomotor phenotype analysis, either by locomotor behavioural tests or by fictive locomotion experiments. In the same way, the production of a triple mutant for *Vsx1, Chx10* and *Hb9* is in progress in the host laboratory. The analysis of this mutant line is clearly missing to complete our understanding of the consolidation model that involves these three factors.

It is always interesting to make a connection between what we observed in studied animal model and human. Improve our knowledge about differentiation mechanisms activated during development could allow us to *in vitro* differentiate cells to perform cell therapy, either for spinal cord or for retina injuries. Very few is known about Vsx1, its distribution profile or its function in human locomotor control. Human and mouse Vsx1 peptide sequence present 96 and 90% of conservation for their HD and CVC domain, respectively (Ohtoshi et al., 2001). Human Vsx1 is found in embryonic craniofacial tissues and adult cornea. However, due to obvious ethical limits, a deeper analysis of its expression profile during embryogenesis is difficult.

In this work, we compare the distribution and the functions of two paralog factors in mouse model that diverged during evolution. It could be very interesting to study this divergence in other animal models and make a connection with the complexity of their locomotor behaviour. As already described, Vsx1 orthologs are found in animals presenting different locomotor behaviour. In teleost model, Vsx1 is present in p2 progenitor domain and is transiently maintained in V2a interneurons (Batista et al., 2008), in contrast with mouse Vsx1 that appears in

post-mitotic cells. The chicken ortholog, Chx10-1, is also found in the embryonic spinal cord but a deep analysis of its distribution is missing, such as for Xvsx1 in Xenopus (Chen and Cepko, 2000; D'Autilia et al., 2006). Identify Vsx1 distribution and functions in these different animal models could allow us to make a connection between the locomotor behaviour and the complexity of neuronal population acquired during evolution.

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