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INSIGHT ON THE MOLECULAR MECHANISMS THAT CONTROL THE EXPRESSION OF PRRXL1 IN NOCICEPTIVE NEURONS

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Aos meus pais e à minha irmã

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Publication I. Regadas, I.; Matos, M.R.; Monteiro, F.A.; Gómez-Skarmeta, J.L.; Lima, D.; Bessa, J.; Casares, F.; Reguenga, C. Several cis-regulatory elements control mRNA stability, translation efficiency, and expression pattern of Prrxl1 (paired related homeobox protein-like 1). Journal of Biological Chemistry. 2013 December 20; 288(51):36285-301. doi: 10.1074/jbc.M113.491993. Epub 2013 November 8.

Publication II. Regadas, I.; Soares-dos-Reis, R.; Falcão, M.; Matos, M.R.; Monteiro, F.A.; Lima, D.; Reguenga, C. Dual role of Tlx3 as modulator of Prrxl1 transcription and phosphorylation. Biochimica et Biophysica Acta. 2014 November; 1839(11):1121-31. doi: 10.1016/j.bbagrm.2014.08.007. Epub 2014 August 17.

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ABBREVIATIONS

bHLH	basic Helix-Loop-Helix
CBP	CREB-binding protein
CGRP	Calcitonin gene-related peptide
CIAP	Calf intestinal alkaline phosphatase
CKO	Conditional knock-out
DRG	Dorsal root ganglia
dSC	Dorsal spinal cord
GABA	Gamma-amino butyric acid
GAD	Glutamate decarboxylase
GTO	Golgi tendon organs
HD	Homeodomain
IB4	Isolectin B4
MO	Morpholino oligonucleotide
Mrgpr	Mas-related G-protein coupled receptor
Ngn	Neurogenin
RRA	Regulatory region A
RRB	Regulatory region B
SP	Substance P
TALE	Three amino acid loop extension
TBP	TATA-binding protein
TG	Trigeminal ganglion
Trk	Tyrosine-kinase receptor
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPV	Transient receptor potential vanilloid
TSS	Transcription start site
UTR	Untranslated Region
VGLUT	Vesicular glutamate transporter
Viaat	Vesicular inhibitory amino acid transporter

ABSTRACT

The correct perception of nociceptive inputs relies on the proper neuronal connection between specialized receptors in the dorsal root ganglia (DRG) and neurons in the superficial laminas of the dorsal spinal cord. One gene intimately related with the establishment of this circuit is *Prrxl1*, which emerges as one of the few whose presence is observed in both tissues, as well as in functional equivalent supraspinal areas. The importance of this transcription factor is well demonstrated by the phenotype of *Prrxl1* null mouse embryos – spatiotemporal defects in the penetration of afferent sensory fibers in the spinal gray matter together with anomalies in the morphology of dorsal horn of the spinal cord are observed. All these abnormalities are correlated with a significant reduction in sensitivity to noxious stimuli. Despite Prrxl1 relevance for the development of the nociceptive system, little was known about the genetic cascade and molecular mechanisms that govern its gene expression and activity in these tissues. Evidence from previous studies pointed to the existence of tissue-specific mechanisms of *Prrxl1* transcriptional regulation – for instance, Tlx3 controls the expression of *Prrxl1* only in the dorsal spinal cord but not in the DRG and Islet1 induces the transcription of *Prrxl1* at early stages of DRG development but later on, *Prrxl1* expression is not affected in *Islet1* null mice. In order to further explore such issues, this thesis mainly focused on investigating the function of evolutionary conserved cis-regulatory elements present in the *Prrxl1* gene and trans-acting factors that may modulate those sequences. It was first shown here (Publication I) that *Prrxl1* transcription is regulated by an alternative promoter usage mechanism – three alternative promoters (named P1, P2 and P3) give rise to three distinct *Prrxl1* 5'-UTR variants, named 5'-UTR-A, 5'-UTR-B and 5'-UTR-C. These 5'-UTR sequences confer distinct rates of mRNA decay and translation efficiency to the *Prrxl1* transcript. The neuronal-specific promoter P3 is well conserved along the phylogenetic tree and contains a TATA-box, displaying *in vivo* enhancer activity in a pattern that overlaps with the zebrafish *Prrxl1* homologue, *drgx*. Regulatory elements present in this region were identified and are binding sites for Phox2b and Tlx3 transcription factors. It was demonstrated that zebrafish Phox2b is required for the expression of

drgx in the facial, glossopharyngeal, and vagal cranial ganglia. In Publication II it was depicted that Tlx3 induces the transcriptional activity of promoter P3 by directly binding to a bipartite DNA motif and synergistically interacts with Prrxl1 in order to indirectly act on the TATA-less promoters P1/P2, via the action of Brn3a. In addition to its action on *Prrxl1* alternative promoters, Tlx3 proved to have the ability to induce Prrxl1 phosphorylation. Altogether, these results demonstrated that Tlx3 uses distinct mechanisms to tightly modulate Prrxl1 activity, either by controlling its transcriptional levels or by increasing Prrxl1 phosphorylation status.

The outcome of this work adds new information to the literature about the transcriptional cascades involved in the neuronal specification and differentiation in primary sensory ganglia and respective relay centers in the central nervous system.

RESUMO

A perceção correta de estímulos nociceptivos depende do estabelecimento de conexões neuronais entre recetores especializados que formam os gânglios das raízes dorsais (DRG) e neurónios presentes nas lâminas superficiais da medula espinal dorsal. Um dos genes relacionado com o estabelecimento deste circuito é o *Prrxl1*, que surge como um dos poucos cuja presença é observada nestes dois tecidos, assim como em áreas supra-espinais funcionalmente equivalentes. A importância deste fator de transcrição é demonstrada pelo fenótipo de ratinhos mutantes para o *Prrxl1* – são observados defeitos espaço-temporais na penetração das fibras aferentes sensitivas na matéria cinzenta espinal, assim como anomalias na morfologia do corno dorsal da medula espinal. Todas estes defeitos estão relacionadas com uma redução significativa na sensibilidade a estímulos nóxicos. Apesar da relevância do Prrxl1 no desenvolvimento do sistema nociceptivo, pouca informação é conhecida sobre a cascata genética e os mecanismos moleculares que governam a sua expressão e atividade nestes tecidos. Informação recolhida em estudos anteriores aponta para a existência de diferentes mecanismos de regulação da transcrição do *Prrxl1* – por exemplo, o Tlx3 controla a expressão do *Prrxl1* apenas na medula espinal dorsal mas não nos DRG e o Islet1 induz a transcrição do *Prrxl1* em estados iniciais do desenvolvimento dos DRG, mas em estados embrionários posteriores, a expressão do *Prrxl1* não é afetada em ratinhos mutantes que não expressam o *Islet1*. De forma a explorar mais profundamente estas questões, esta tese focou-se principalmente na investigação da função de elementos *cis*-reguladores conservados evolutivamente e nos fatores de transcrição que modulam essas sequências. Foi primeiro demonstrado (*Publication I*) que a transcrição do *Prrxl1* é regulada por um mecanismo de promotores alternativos – três promotores (chamados P1, P2 e P3) originam três variantes *Prrxl1* 5'-UTR distintas, chamadas 5'-UTR-A, 5'-UTR-B e 5'-UTR-C. Estas sequências 5'-UTR conferem diferentes ritmos de degradação de mRNA e eficiência de tradução proteica ao *Prrxl1*. O promotor P3, com atividade específica de células neuronais, é bastante conservado ao longo da árvore filogenética e contém uma TATA-box, apresentando atividade *enhancer in vivo*, num padrão que se sobrepõe com o do

homólogo do *Prrxl1* no peixe-zebra, o *drgx*. Elementos reguladores presentes nessa região foram identificados e são locais de ligação para os fatores de transcrição Phox2b e Tlx3. Foi demonstrado que no peixe-zebra, o Phox2b é necessário para a expressão do *drgx* nos gânglios cranianos facial, glossofaríngeo e vago. Na *Publication II* foi demonstrado que o Tlx3 induz a atividade de transcrição do promotor P3 através da ligação direta a um motivo de DNA bipartido, e interage de forma sinérgica com o Prrxl1 para atuar indiretamente nos promotores P1/P2, através da ação do Brn3a. Para além da sua ação sobre os promotores alternativos do *Prrxl1*, o Tlx3 também tem capacidade para induzir a fosforilação do Prrxl1. Estes resultados demonstram que o Tlx3 recorre a mecanismos distintos para modular a atividade do Prrxl1, quer através do controlo dos seus níveis de transcrição, quer através do aumento do estado de fosforilação do Prrxl1.

O resultado deste trabalho traz nova informação à que é conhecida sobre cascatas de transcrição envolvidas na especificação e diferenciação de neurónios nos gânglios sensitivos primários e nos seus respetivos centros de transmissão de informação, no sistema nervoso central.

INTRODUCTION

1. INTRODUCTION

1.1. THE SOMATOSENSORY SYSTEM

According to the International Association for the Study of Pain, pain is defined as "a sensory and emotional experience associated with real or potential injuries, or described in terms of such injuries. Pain has an individual connotation and suffers the influence of previous experiences" (Merskey and Bogduk, 1994). Intense thermal or mechanical stimuli, and environmental or endogenous chemical irritation, capable of provoking real or potential injury, can be detected by specialized receptors (nociceptors), interpreted by the nervous system as a noxious input and generate acute pain (Basbaum et al., 2009). This is advantageous for the organism, as the acute manifestations of pain alert for the damage (impending or real) and proper mechanisms of defense and repair can be initiated. However, in certain circumstances pain symptoms persist over an extended period of time and become associated with chronic pathological processes, causing suffering in multiple systems and resulting in a chronic pain condition. All the findings that guide scientists to a better understanding of the development and function of the nociceptive system and associated molecular mechanisms, may be useful to improve the knowledge about novel therapies to ultimately relieve severe pain conditions.

A sensory stimulus can be defined as a physiologic "event or object that is received by the senses and elicits a response from a person. The stimulus can come in many forms such as light, heat, sound, touch, as well as from internal factors" and it is primary registered by the somatosensory system (Lestrude, 2013). The proper functioning of the somatosensory system depends on the accurate contact established between discrete populations of primary afferent neuronal fibers, whose cell bodies are located in the cranial trigeminal ganglion (TG) and trunk dorsal root ganglia (DRG), and second order neurons located in brainstem and spinal cord, respectively. These neurons will then relay the information to major integrative somatosensory centers in the thalamus and midbrain (McGlone and Reilly, 2010; Braz et al., 2014). TG and DRG are essentially clusters of afferent nerve cell bodies and supporting glial cells (satellite

and Schwann cells that myelinate the peripheral nerve fibers). The morphology of primary afferent neurons is characterized by the lack of dendrites and the presence of a single axon that bifurcates soon after leaving the ganglion (therefore named "pseudo-unipolar"). One branch of the axon projects peripherally and terminates in specialized sensory receptors that innervate skin, muscles, bones or viscera, whereas the other one centrally connects with secondary neurons and interneurons in spinal cord or brainstem (Kandel, 2012). There, neuronal populations are organized in discrete layers (laminas) that consist of a unique combination of cells distinguished by features such as their morphology, projections and gene expression (Rexed, 1952; Jessell, 2000; Caspary and Anderson, 2003). The layers of neuronal subpopulations located in the dorsal half of the spinal cord and hindbrain are responsible for the integration of the sensory inputs, whereas layers of neurons in the ventral half regulate the motor output. The existence of a wide range of sensory modalities urged the neurons in the DRG to evolve into a diversity of classes with receptors dedicated to the correct perception and transduction of electric impulses in response to different inputs. As a consequence, distinct modalities of somatic sensation can be classically defined, comprising tactile, thermal, pain and itch sensing, and proprioception (McGlone and Reilly, 2010; Le Pichon and Chesler, 2014). The diversity of neuronal phenotypes is mainly determined by the specific combination of early extracellular signaling and precise expression of diverse transcription factors during embryonic development (Wolpert, 2007). The unique transcriptional code of each neuronal population confers distinct molecular and functional features, resulting in cells possessing different morphologies and axonal projections, as well as specialized terminal receptors. Albeit considerable data is available nowadays, in regard to the network of transcription factors required for the determination and differentiation of each neuronal class, many issues still remain to be explored. One of them is the elucidation of the genetic cascade and molecular relationships that surround the expression of a set of transcription factors involved in the development of certain neuronal populations in the DRG and spinal cord, with the ultimate goal of understanding their contribution to the establishment of the nerve circuit responsible for the perception and integration of the pain (nociceptive) sensation. This was the focus of this thesis.

1.2. NEURONAL POPULATIONS AND NOCICEPTION IN THE ADULT DRG

Primary somatosensory neurons were classically organized according to defined morphological and functional features that include the size of cell body, degree of fiber myelination, laminar location of afferent projections to the spinal cord, and the morphology and specialization of the nerve endings (Kandel, 2012). The anatomical diversity is the consequence of differences in the genetic background of each class of neuronal fibers and correlates well with specific properties, such as the sensitivity to distinct types of stimuli, transduction of electrical impulses and neurotransmitter release. Additionally, the presence of specific ion receptors and sensory channels defines subsets of the main classes of sensory neurons. Adult somatosensory neurons can be categorized into four general major classes: **i.** the proprioceptors (or Aα), that sense body position; **ii.** the receptors that deal with cutaneous sensitivity and include the low threshold mechanoreceptors Aβ that respond to innocuous touch, pressure and vibration; **iii.** the Aδ and **iv.** C fibers that conduct innocuous cold or warm signals, as well as noxious and itch-inducing stimuli (Figure 1) (McGlone and Reilly, 2010; Le Pichon and Chesler, 2014).

Proprioceptors and Aβ fibers have the largest cell bodies (>50μm) and are heavily myelinated, a property that confers them the fastest conduction velocities (Le Pichon and Chesler, 2014). Proprioception deals with information related to body position, limb and trunk movements, sense of effort and heaviness. Mechanically sensitive proprioceptors are activated through the detection of muscle tension and contraction of muscle spindles and Golgi tendon organs at periphery (Proske and Gandevia, 2012). These nerve fibers centrally project to deep laminas in the motor-stretch reflex circuit in the ventral spinal cord. The Aβ are low-threshold mechanoreceptors and have different subclasses that are specialized in the transduction of discrete mechanical stimuli, such as touch, vibration and hair deflection, a competence conferred by nerve endings morphologically distinct. The Aβ fibers synapse predominantly with neurons in the internal dorsal spinal cord laminas (III-V), where the sense of touch is mediated (Le Pichon and Chesler, 2014).

Fibers dedicated to the perception of other senses besides innocuous touch, such as pain, itch and temperature comprise the Aδ and C categories. The lightly

myelinated Aδ have medium diameter and are slower conducting than the aforementioned nerves. Specific subpopulations of these fibers are thermosensory and respond to innocuous warmth or cold stimuli, whereas others are uniquely activated by noxious low and high temperatures (<20 °C and >45 °C). They project their axons centrally to the superficial layers of the spinal cord (laminas I and II) but also target deeper layers (lamina V), where they initiate nocifensive reflexes, such as the innate withdrawal provoked by a noxious stimuli (Basbaum et al., 2009; Gascon and Moqrich, 2010).

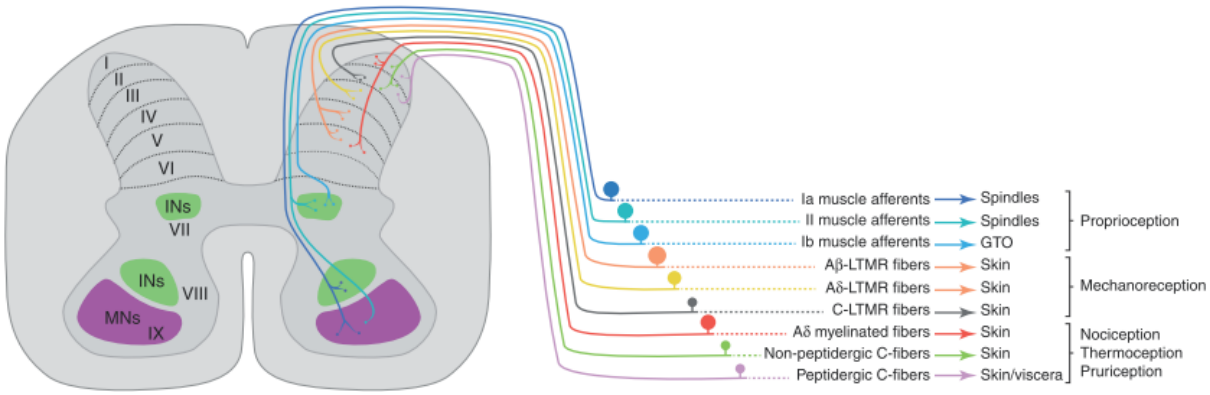

Figure 1: Schematic representation of the different subclasses of DRG afferent fibers centrally projecting to distinct laminas of the spinal cord. Ia, II and Ib comprise the proprioceptive fibers innervating muscle spindles and Golgi tendon organs (GTO) that project to motor neurons in the ventral spinal cord. The mechanoreceptors innervate the skin and establish synapses mostly with neurons in laminas III-V. Neuronal fibers that sense noxious, thermo and itchy (pruriception) stimuli comprise the Aδ and C (peptidergic and non-peptidergic) categories. Aδ centrally projects to laminas I, II and V, whereas C fibers mainly project to laminas I and II. Image withdrawn from (Lallemend and Ernfors, 2012).

C fibers are unmyelinated, slowly conducting and the most abundant type in the DRG. They have small cell bodies and terminate as free nerve endings in the skin, organs and bone. These are the primary afferent nerves mainly responsible for the detection of harmful stimuli such as tissue damage, chemical irritants and different combination of temperatures. Whereas some of these are discrete receptors that have been reported to respond to a unique type of stimuli, others are considered to be polymodal nociceptores, as they are activated by a combination of signals, including intense mechanical stimulation, heat and noxious chemicals. Within the class of C fibers, two major subclasses can be defined considering whether these neurons

express or not neuropeptides – the ones that express substance P and the calcitonin gene related-peptide (CGRP) are peptidergic neurons while the ones bound by the isolectin B4 (IB4) are the non-peptidergic class of C fibers. The mechanism of recognition and encoding the noxious stimuli into an electrical signal to be processed as pain sensation afterwards, is termed nociception (Basbaum et al., 2009; Gascon and Moqrich, 2010). Distinct noxious stimuli activate specific members of membrane receptors present in nociceptores, such as the acid-sensing ion channels (ASIC) and sodium channels (including Nav1.8 and Nav1.9) that generate the perception of sting and pain when activated by protons, or the capsaicin receptor subtype-1 of the family of thermo Transient Receptor Potentials (TRPV1), one of the most studied cation channels expressed in cutaneous sensory fibers and associated with pungent irritants and heat pain (Gascon and Moqrich, 2010). Similarly to Aδ, C fibers also target superficial layers of the spinal cord (laminae I-II), a region known to be critical for the first stage processing of noxious and thermal stimuli. A given stimulus sensed by a DRG neuron passes directly from the distal part of the axon to the proximal fiber, to enter the dorsal half of the spinal cord. There, pain and thermal Aδ fibers synapse ipsilaterally with second order neurons – forming the Lissauer's tract – that decussate at the entry level of primary afferents and relay the information to the thalamus trough the spinothalamic tract. Exceptionally, C fibers enter the lamina II and synapse on interneurons (which do not project outside the spinal cord) that relay the signal to secondary afferents in marginal nucleus (lamina I) and *nucleus proprius* in lamina III. The spinothalamic tract ascends the entire length of the spinal cord and brainstem to enter the thalamus, together with the medial lemniscus. The thalamus plays a crucial integrative role in the brain, relaying both sensory and affective pain information to primary sensory cortices (Basbaum et al., 2009).

Most important is the existence of a set of molecular traits that also define the classes of developing neurons in the DRG. Those are specific transmembrane receptors expressed at early embryonic stages that transduce diffusible signals from distinct neurotrophic factors, essential for cell survival and differentiation, and are tightly correlated with the diversity of primary sensory neuronal populations and their central projection pattern (Snider, 1994; Bibel and Barde, 2000). Hence, proprioceptors are well defined by the expression of the Tyrosine-kinase receptor C (TrkC; receptor for the

neurotrophin 3 or NT-3), whereas Aβ mechanoreceptors are marked by the expression of TrkB (the receptor for the brain-derived growth factor or BDNF) or Ret (the receptor for the glial-derived growth factor or GDNF family of neurotrophin factors). The small-diameter nociceptores, thermoceptors, pruriceptors and C-fiber low threshold mechanoreceptors are generally defined by the presence of TrkA (receptor for the nerve growth factor, NGF) (Barbacid, 1994). Furthermore, near postnatal stages, the class of TrkA-positive neurons segregates in two subpopulations, since some of the neurons downregulate TrkA and begin to express Ret. This recently expressing class of Ret-positive neurons comprises the non-peptidergic fibers that mainly innervate the skin, whereas the ones that maintain TrkA are the peptidergic and innervate most tissues.

1.3. EARLY NEURAL TUBE DEVELOPMENT

The birth and development of neuronal populations that will give rise to both DRG and spinal cord structures begin at an early developmental stage. The process starts during gastrulation, when the ectoderm lying along the dorsal midline and above the notochord of the vertebrate embryo becomes specified as neuroectoderm and is modified to a thickened plate of tissue, constituting the open and flat neural plate (Figure 2). Between 7.7 and 8.5 mouse embryonic days (E) the anterior part of the embryo grows and the neural plate invaginates and begins to close, creating a groove with the neural folds at the edges. As the neural folds fuse along the rostrocaudal axis of the embryo, a tube of epithelium is formed (the neural tube), in a process called neurulation, which initially leaves the anterior and posterior ends open. The midline of the dorsal neural tube becomes the roof-plate, a signaling structure that commands the induction of different dorsal cell types. In the region of the prospective head, the neural tube expands to form the brain, whereas the more caudal regions originate the spinal cord. Following neurulation, the neural tube detaches from the adjacent ectoderm, which becomes the epidermis. Another group of cells is induced at the folds of the dorsal neural tube to give rise to neural crest cells. They undergo an epithelial-to-mesenchymal transition, delaminate and migrate away from the neural tube

throughout the entire body to give rise to a wide range of descendants, including neurons and glia of the peripheral nervous system (somatic and autonomic), melanocytes, neuroendocrine cells and bone and cartilage from the facial skeleton. The neural crest cells that arise at trunk levels of the neural tube are the ones contributing to the formation of neurons in the DRG, whereas the cranial sensory ganglia originate partially from cranial neural crest cells (Butler and Bronner, 2015).

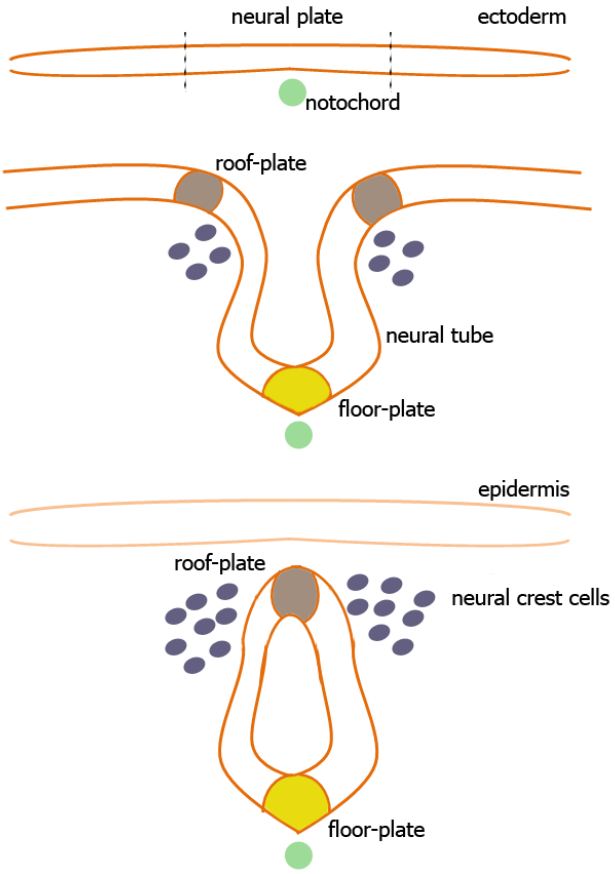

Figure 2: Scheme representing the formation of the neural tube. The ectoderm tissue that becomes specified as the open and flat neuroectoderm constitutes the neural plate. The invagination and closure of the neural plate creates the neural tube with neural folds at the edges. The most caudal regions of the neural tube originate the spinal cord. Signals from either the roof-plate or the floor-plate will orchestrate the formation of neuronal populations in the spinal cord. Neural crest cell delaminate from the neural folds and migrate away from the neural tube throughout the whole body. These cells will give rise to neurons and glia of the peripheral nervous system (among other tissues).

1.4. NEUROGENESIS AND PRIMARY SENSORY SPECIFICATION

The continuous progress of developmental biology studies associated with the emergence and priming of molecular biology experimentation revealed that the

creation of neuronal diversity is an intricate process that starts early in development to extend into postnatal life. The characterization of the genetic transcriptional cascades that regulate the specification and differentiation of peripheral somatosensory neurons from progenitor cells has been crucial to understand their diversification into functional subclasses. Sensory neurogenesis in the trunk mainly occurs in two successive waves and it is initiated in the transient neural crest population of cells. The progenitor neural crest cells are induced by a set of signalling molecules (BMP, Wnt or FGF) that are diffused from either the ventral ectoderm or the paraxial mesoderm, and initiate the transcription program that determines the fate of these cells (Mayanil, 2013). Early expression of a set of transcription factors that includes *Pax3*, *Pax7*, *Msx1*, *Zic1* and *AP-2* prevents premature cell differentiation and induces the neural crest cells by switching on the expression of another set of genes that establish and specify the prospective neural crest (Sato et al., 2005; Mayanil, 2013). Subsequently, the activation of *Snail1*, *Snail2*, *FoxD3* and members of the SoxE family prompts the cells to undergo an epithelial-to-mesenchymal transition and become highly motile due to the rearrangement in cytoskeletal organization (Cheung et al., 2005). This transition leads to alterations in cell shape and upregulation of cell surface low adhesion molecules (type II cadherins and the regulators RhoB) and signaling receptors, which confer cells the ability to detach and migrate away from the neural tube (Cheung et al., 2005; Lim and Thiery, 2012). Twist is required for cells to identify their correct new position, as they will invade different sites according to the distinct membrane receptors they express, before they differentiate into the various cell types they give rise to (O'Rourke and Tam, 2002). Eventually, trunk sensory neuronal precursors become fate restricted and migrate away from the neural folds in chain-like structures that then coalesce into ganglia at regular intervals in the anterior half of each somite, adjacent to the developing neural tube. Sox10 precedes the expression of two proneural genes encoding the basic-Helix-Loop-Helix (bHLH) transcription factors Neurogenin1 (Ngn1) and Neurogenin2 (Ngn2) (Carney et al., 2006), that instruct the progression from neurogenesis to the segregation into major classes of sensory populations (proprioceptors/mechanoreceptors and thermoceptors/nociceptors/pruriceptors) (Fode et al., 1998; Ma et al., 1999). It has been demonstrated that the expression of these factors is the imperative event in the decision of progenitor cells to become

sensory neurons. Cells that express *Mash1* at this point, instead of *Ngn1* or *Ngn2*, differentiate towards the autonomic lineage instead of to the somatosensory one (Johnson et al., 1990; Lo et al., 1998).

1.4.1. FIRST AND SECOND WAVES OF DRG NEUROGENESIS

The first wave of neurogenesis is marked by the presence of Ngn2, which is initially detected around E9 in a set of migratory neural crest cells near the edges of the neural tube (Figure 3). The expression is transient and maintained until early stages of DRG condensation, being extinguished around E10.5 (Sommer et al., 1996). This group of cells have acquired a sensory neuronal fate and exclusively gives rise to the large-diameter myelinated DRG neurons (the TrkB/TrkC-positive Aβ mechanoreceptors and proprioceptors). Due to limited cell division of the early migrating neural crest cells, the first wave of neurogenesis only gives rise to about 4% of the adult neurons in the DRG (Marmigere and Ernfors, 2007). The second wave of neurogenesis is controlled by Ngn1 and occurs at E10.5 in mouse, lasting until E13.5 (Lawson and Biscoe, 1979). *Ngn1* is expressed in the progenitor cells lying in the coalescing DRG that did not have undergone neurogenesis or express little or none *Ngn2* (Figure 3). These cells mainly generate the small-diameter lightly- or non-myelinated neurons that are mostly TrkA-positive at early embryonic stages (nociceptores/pruriceptors/ thermoceptors), although a residual birth of additional large proprioceptive/ mechanoreceptors is also observed (Ma et al., 1999; Zirlinger et al., 2002). This wave accounts for the largest bulk of adult DRG neurons – about 91% (Marmigere and Ernfors, 2007). Redundancy is observed during the development of sensory ganglia, as the absence of Ngn2 is later compensated by the onset of *Ngn1* expression, resulting in the formation of large-diameter neurons regardless the deficiency of Ngn2. Nevertheless, a similar mechanism is not observed the other way around, as mutant mice for *Ngn1* develop DRG with a diminished size, due to the loss of about 70% of neurons that populate this tissue. Mutants for both *Ngn1* and *Ngn2* fail to develop sensory ganglia at all, indicating that these transcription factors act together to promote the DRG formation (Ma et al., 1999). Indeed, Ngn1 is fundamental for the precursor cells in the DRG to adopt a neuronal fate, as the absence of it thrust these cells to become glia and not neurons (McGraw et al., 2008). In all of the fate-

determined sensory neurons, the expression of *Ngn1* and *Ngn2* is followed by the activation of the bHLH factor Neurod, which is dependent on the Neurogenins (Ma et al., 1999).

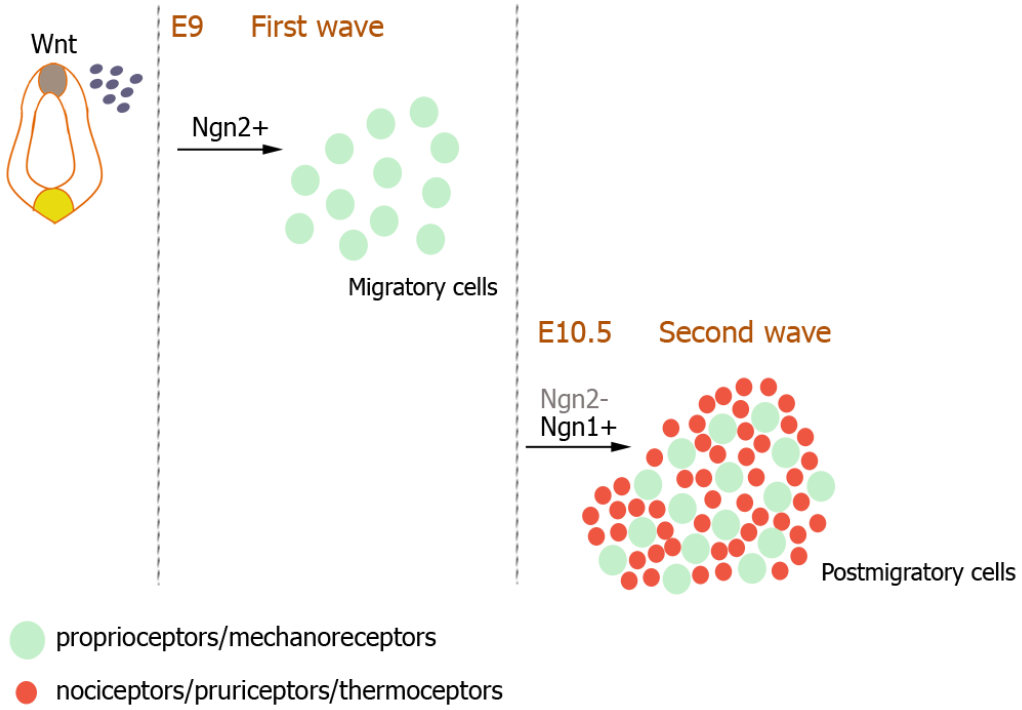

Figure 3: Schematic representation of the first and second waves of neurogenesis. The onset of Ngn2 by E9, in a set of migratory neural crest cells, marks the first wave of neurogenesis. Cells that contain Ngn2 will exclusively give rise to mechanoreceptors and proprioceptors expressing TrkB and TrkC. By E10.5, *Ngn2* is downregulated and *Ngn1* expression was initiated in cells that have already migrated to the place where prospective DRG cells will coalesce. Besides the large-diameter mechanoreceptors and proprioceptors, this second wave of neurogenesis originates the small-diameter nociceptors, pruriceptors and thermoceptors that are TrkA-positive. This wave accounts for 91% of neurons in the DRG.

1.4.2. TERMINAL DIFFERENTIATION OF SENSORY NEURONS

Following the early neurogenic phase, the differentiation programs that control the sensory neuronal fate determination are switched off and by E12.5 the specification in different classes of DRG neurons is initiated. This transition requires the combined expression of Homeobox genes (that encode Homeodomain transcription factors) and neurotrophic growth factors that regulate the activation of downstream key genes, crucial to ascertain the diverse subclasses of sensory neurons by ascribing the distinct physiologic functions to each population (Anderson, 1999). Two pan-neuronal transcription factors – Islet1 and Brn3a, members from the LIM and

a POU subfamilies of Homeodomain (HD) proteins, respectively – stand out as major players in this process (Sun et al., 2008; Lanier et al., 2009; Dykes et al., 2011). Genetic studies using mutant mice for these genes revealed that, as they influence the cell cycle exit, they hold a role in suppressing the expression of proneural genes (including *Ngn1*, *Neurod1*, *Neurod4* and *Neurod6*), as well as molecules specifically associated with dorsal spinal cord development (such as *Lhx1*, *Lhx2*, *Olig1* and *Olig2*) (Eng et al., 2004; Lanier et al., 2007; Sun et al., 2008; Lanier et al., 2009; Dykes et al., 2011). In contrast, they are intimately related with the activation of a set of genes that regulate the mechanisms that eventually result in the generation of neuronal diversity in sensory ganglia, through the activation of neural transmission mediators, such as ion channels, neuropeptides and membrane receptors that participate in signal transduction, or are involved in axon guidance and synapse establishment formation. Among these are the sodium channel Nav1.8, the capsaicin receptor TRPV1 and the neuropeptide galanin (Sun et al., 2008; Dykes et al., 2011). In *Islet1* conditional knock-out (CKO) embryos, the expression of *TrkA* in the DRG is reduced by E12.5, while the onset of *TrkC* is delayed by two days (Sun et al., 2008). As expected in systems lacking neurotrophic receptors, defect in neuronal survival is observed, resulting in mutant mice with sensory ganglia presenting smaller size than their control littermates. Although not observed in the DRG, the decrease of *TrkA* and *TrkB* expression is detected in the trigeminal ganglia of E13.5 *Brn3a* null mice, whereas the expression of *TrkC* is not initiated at all (Huang et al., 1999). This effect is enhanced when the expression of *Klf7*, a zinc finger transcription factor, is ablated. The activity of Klf7 is required for the correct expression of *TrkA* and consequent development of nociceptive sensory neurons. Loss of *Klf7* expression results in the exclusive apoptosis of nociceptive neurons, whereas other somatosensory populations develop normally (Lei et al., 2005). In *Brn3a/Klf7* double mutants, a severe reduction of TrkA-positive neurons is observed at E12.5, indicating that Klf7 acts together with Brn3a to promote the expression of *TrkA* in trigeminal sensory neurons (Lei et al., 2006). Moreover, at trunk level, both Brn3a and Islet1 have been shown to be required for the correct formation of sensory afferent axon projections to periphery and spinal cord (Dykes et al., 2011). *Brn3a* mutants exhibit defective axon trajectories – the TrkA-positive afferents are not able to enter the dorsal root entry zone, remaining outside of spinal

cord; the TrkC-positive fibers also fail to accurately project into the ventral horn of spinal cord (Zou et al., 2012). In *Islet1* CKO mice, loss of cutaneous innervation subserving pain, touch and temperature is apparent – probably due to excessive apoptosis – which is in accordance with a reduced response to mild noxious stimuli applied to the skin of newborn animals. Concomitantly, the inactivation of *Islet1* specifically affects the central projections of TrkA-positive fibers, which are markedly reduced in spinal cord by E14.5 (Sun et al., 2008). In zebrafish, Islet1 is also necessary to promote the axon growth of Rohon-Beard primary sensory neurons to spinal cord (Tanaka et al., 2011).

1.4.3. SEGREGATION OF TRKA-DERIVED CELLS INTO PEPTIDERGIC AND NON-PEPTIDERGIC NEURONS

The cooperative action of Brn3a and Islet1 also profoundly affects the transcription of *Runx1* and *Runx3*, members of the Runt domain-containing factors family that function as key regulators of lineage-specific genes (Dykes et al., 2011). Globally, the expression of *Runx1* is mainly associated with definitive hematopoiesis mechanisms and with certain occurrences of human leukemia (Ichikawa et al., 2013). Runx3 is a tumor suppressor and apoptosis inducer in many types of human cancer (Chuang et al., 2013; Yang et al., 2014). During peripheral nervous system development these factors act complementarily in the diversification of somatic sensory neurons, through the recruitment of known transcriptional regulators (Kramer et al., 2006). *Runx1* is selectively expressed in small-diameter TrkA-positive neurons and considered to be a master regulator of the terminal specification of nociceptive sensory neurons in DRG. Runx3 is found in large TrkC-positive neurons and its expression is fundamental for the correct consolidation of the proprioceptive phenotype (Inoue et al., 2002; Levanon et al., 2002). The postmigratory large-diameter precursors of proprioceptors and mechanoreceptors that arise from the first wave of neurogenesis, initiate the expression of *Runx3* and *TrkC* at early developmental stages (by E10.5). At later stages, cells that maintain *Runx3* expression remain TrkC-positive and become proprioceptors. Neurons that lose or strongly decrease *Runx3* expression become either TrkB/TrkC-positive, only TrkB-positive, only Ret-positive or TrkB/Ret-positive and acquire the mechanoreceptive trait (Chen et al., 2006a; Kramer et al., 2006). Further gain-of-function studies have shown that Runx3 acts to specify the

proprioceptive class by suppressing the *TrkB* expression while maintaining *TrkC* (Kramer et al., 2006). In addition, at postnatal stages, *Runx3* mutant mice exhibit altered axonal trajectories of proprioceptive fibers to spinal cord, which fail to project to the ventral and intermediate laminas, whereas the peripheral branch of the axons project to skin but not to muscle (Inoue et al., 2002; Nakamura et al., 2008).

In parallel with Runx3, Runx1 is involved in the diversification of nociceptive neurons arising from neural crest cells. Cells that are born during the second wave of neurogenesis are characterized by the presence of Ngn1, Brn3a and Foxs1 (Montelius et al., 2007) and at early embryonic stages, start to express either *Runx1* or *Runx3*. This moment is the defining event in the determination of the nociceptive or proprioceptive specification, respectively. The potential nociceptors initiate the expression of *TrkA* between E10.5-E11.5 and soon after that, 88% of them begin to express *Runx1* (Chen et al., 2006b). During embryonic development and until postnatal stages, *Runx1* expression is restricted to TrkA-positive neurons but afterwards Runx1 acts to segregate the Runx1/TrkA-positive population in two main subpopulations of nociceptors (Figure 4):

i) the class of **peptidergic** neurons, that extinguishes the expression of *Runx1*, maintains *TrkA* and upregulates *Met* receptor (Gascon et al., 2010). This is followed by the induction of the neuropeptides CGRP and substance P. The downregulation of *Runx1* is essential for the maintenance of TrkA, the expression of CGRP and thus, for the establishment of the nociceptive peptidergic phenotype (Chen et al., 2006b; Kramer et al., 2006; Yoshikawa et al., 2007).

ii) the class of **non-peptidergic** neurons that preserves the expression of *Runx1*, which in turn induces *Ret* and represses *TrkA*. The emergence of Ret in Ngn1-dependent population occurs at E17 (Molliver et al., 1997), resulting in the formation of a subpopulation of TrkA/Runx1/Ret-positive neurons. At perinatal stages, these neurons acquire the ability to bind the IB4. A few weeks after birth, Runx1 have progressively downregulated *TrkA* and suppressed the expression of *Met* and CGRP (Chen et al., 2006b; Kramer et al., 2006).

In the absence of Runx1, prospective non-peptidergic neurons acquire peptidergic identity because CGRP expression is upregulated (Chen et al., 2006b). Peptidergic neurons extend their central branches to lamina I and outer lamina II,

regions associated with the mediation of inflammatory pain. The non-peptidergic Runx1/Ret-positive afferent fibers centrally project to the inner lamina II, an area associated with the modulation and integration of neuropathic pain (Figure 4) (Snider and McMahon, 1998). Runx1 is also essential to coordinate these processes, as in its absence, projections of nociceptive afferent nerves from the periphery to spinal cord are impaired. In *Runx1* CKO, both CGRP/SP-positive and IB4-positive fibers reach the dorsal horn of the spinal cord. However, there is a dorsal shift in the projections of the Runx1-expressing non-peptidergic afferents, which no longer extend their processes to the inner lamina II but instead send projections to outer lamina II (Chen et al., 2006b).

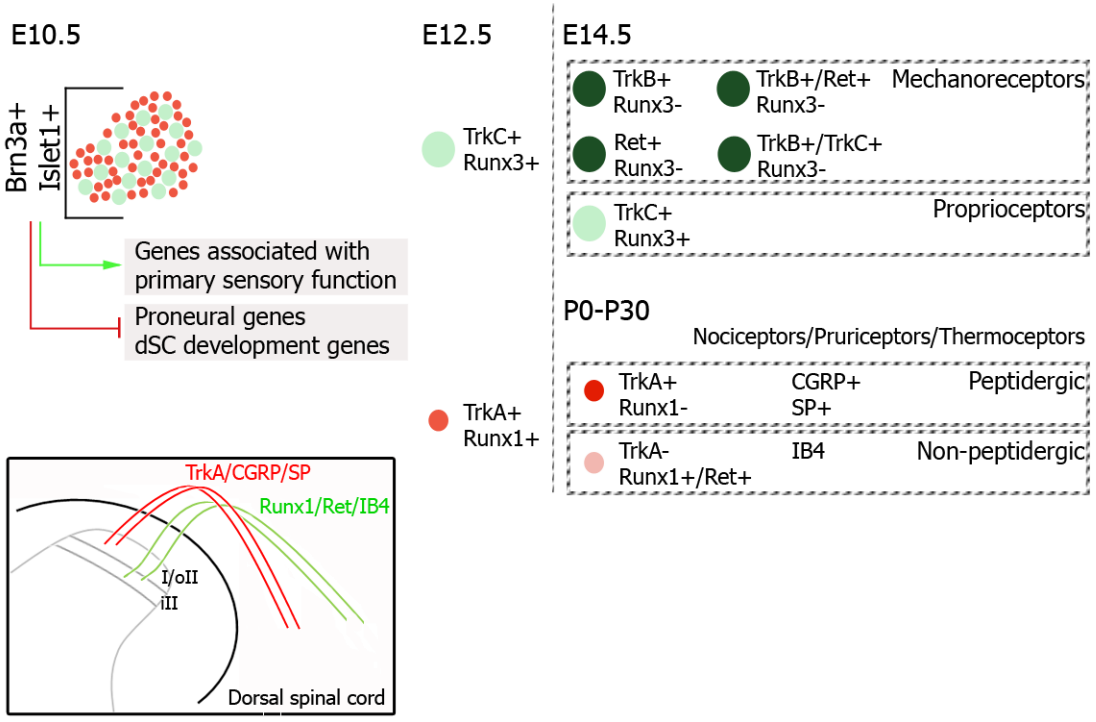

Figure 4: By E10.5, all the neurons in the DRG are Brn3a- and Islet1-positive. Brn3a and Islet1 donwregulate the expression of genes associated with primary neurogenesis and dorsal spinal cord (dSC) development and increase the expression of genes associated with primary sensory perception and transmission. Later on, by E12.5, the population of large-diameter neurons have upregulated *TrkC* and *Runx3*, whereas the small-diameter neurons have upregulated *TrkA* and *Runx1*. At E14.5, the initial population of TrkC-/Runx3-positive cells segregates into different neuronal classes: mechanoreceptors shut down *Runx3* expression, increase TrkB or Ret and in a few cases maintain TrkC; proprioceptors maintain the levels of TrkC and Runx3. At perinatal/postnatal ages, the TrkA-/Runx1-positive small-diameter neurons segregate into neurons that maintain the levels of TrkA, stop expressing *Runx1* and upregulate CGRP and SP (peptidergic neurons) or neurons that downregulate *TrkA*, maintain Runx1 levels, increase Ret and are bound by IB4 (Non-peptidergic neurons). Peptidergic neurons extend their central branches (in red) to dSC lamina I and outer lamina II (I/oII), regions associated with the mediation of inflammatory pain. The non-peptidergic Runx1/Ret-positive afferent fibers (in green) centrally project to the inner lamina II (iII), an area associated with the modulation and integration of neuropathic pain.

Highly consistent with the exclusion of *Runx1* expression from most neurons that innervate deep tissues in adult (mostly peptidergic), it was recently demonstrated that Runx1 suppresses the molecular program associated with these neurons (Yang et al., 2013). Hence, Runx1 is a seminal factor in the selective determination of the nociceptive cutaneous identity as it tightly activates the expression of many high threshold ion channels and thermal, pain and itch receptors in the Ret-positive DRG neurons – among the ones controlled by Runx1 are the cold receptors TRPM8 and TRPA1; capsaicin/heat receptors TRPV1, TRPV2 and TRPC3; the Mas-related G-protein coupled receptor members Mrgprd, Mrgprb4 and Mrgprb5 that sense mechanical pain and innervate exclusively cutaneous/epidermal tissues; the purinergic receptor P2X3 channel and sodium channel Nav1.9 (Chen et al., 2006b). In fact, Runx1-dependent genes are mainly expressed in Mrgprd-positive polymodal nociceptors. In contrast, no specific markers associated with deep tissue TrkA lineage neurons have been identified up to this date, as they express genes that are expressed as well in cutaneous sensory neurons (Yang et al., 2013).

Chen and colleagues (Chen et al., 2006b) also demonstrated that the induction of some of the pain-related receptors and ion channels activated by Runx1 does not require the cooperation of Ret or TrkA, suggesting that it was likely that Runx1 would work in cooperation with other unknowing factors to do so. This remained unclear until recently, when Lopes *et al.* showed that Runx1 is dependent on the Tlx3 presence to establish the Runx1-dependent nociceptive lineage in the DRG (Lopes et al., 2012). The pivotal role of Tlx3 in the development and fate specification of sensory relay neurons of the dorsal spinal cord and hindbrain has been widely reported (Shirasawa et al., 2000; Qian et al., 2002; Cheng et al., 2004). In contrast, and despite *Tlx3* is broadly expressed in the DRG neurons at embryonic stages, little relevance for the development of primary sensory neurons had been credited to this transcription factor up to this date (Qian et al., 2002). At postnatal stages, Tlx3 is selectively required to increase Ret levels and to suppress TrkA, as well as to contribute to the Runx1-dependent molecular identity in non-peptidergic neurons, as the ion channels and sensory receptors upregulated by Runx1 (such as the TRP, Mrgprd/b, P2X3 and Nav1.9), are decreased in the absence of Tlx3 (Lopes et al., 2012). The genetic

regulation of *Runx1* and *Tlx3* are independent of each other, suggesting that these two factors act in combination and not in a cascade fashion.

1.5. DEVELOPMENT OF SPINAL CORD NOCICEPTIVE PROCESSING NEURONS

The vertebrate dorsal spinal cord is considered the organizing and relay centre of the sensory inputs that arrive from the periphery via DRG primary afferent nerves. The proper assembly and transmission of the somatosensory information and the production of the consequent adequate response, depend on the existence of functionally distinct types of spinal neurons. The adult spinal cord is structured in ten discrete laminas, organized in a dorsoventral patterning (Rexed, 1952; Altman and Bayer, 1984). Each lamina contains a unique combination of cells with dedicated functions in the integration and transmission of sensory information to the appropriate centers in the brain (Figure 1). Neurons in the most superficial laminas (I and II) establish synapses with small-diameter DRG afferent fibers that sense nociceptive, itch and thermal stimuli, whereas other neurons located deeper in the dorsal spinal cord (laminas III, IV and V) integrate the mechanical inputs arriving from the periphery. Proprioceptive information can travel straightforward across the dorsal half and interact directly with motor neurons located in the *nucleus dorsalis* in the ventral spinal cord, to produce spinal reflexes without ascending to higher brain centers. The ventral half of the spinal cord is mainly associated with the response of the motor output that comes from the brain, and neurons laying there connect back to the muscle via efferent fibers to control movement (Bonanomi and Pfaff, 2010). The neuronal populations that define the laminas in the dorsal spinal cord are classified according to the well known expression of molecular markers, the synapses established with different classes of DRG projecting neurons, type of neurotransmitters, firing properties and physiological functions (Butler and Bronner, 2015). Nevertheless, the genetic cascade of events and the detailed molecular mechanisms that drive the specification of progenitor cells into distinct subpopulations of postmitotic neurons remain to be fully unveiled.

The laminar organization of the spinal cord is specified early in the development of the neural tube. At the trunk level of the organism, diffusible signals emitted from a dorsal and ventral midline cluster of neuroepithelial cells (the roof and the floor plate, respectively; see Figure 2) control the dorsoventral patterning of the spinal cord through the activation or repression of transcriptional regulators (Ulloa and Briscoe, 2007; Wolpert, 2007; Le Dreau and Marti, 2012). Rapidly dividing cells in the inner ventricular zone of the early neural tube are the mitotic progenitors for all the neurons and glia that will constitute the spinal cord. It is the combined action of extracellular signals secreted from either the roof-plate (BMP and Wnt) or floor-plate and the notochord (Shh) and downstream transcription factors that orchestrate the patterning and the identity of cells during spinal neurogenesis (Wolpert, 2007). Signaling in a dosage-dependent manner induces the expression of proneural genes, which encode bHLH transcription factors and are required between E9.5 and E12.5 to impel the differentiation of the mitotic progenitors in the ventricular zone (Bertrand et al., 2002). At E9.5, neural progenitors arise in 11 discrete population domains and migrate laterally towards the mantle layer of the developing spinal cord, soon after they exit the cell cycle and initiate differentiation. These progenitor populations are defined by the unique combinatorial code of bHLH and HD transcription factors they express, which coordinate their migration and posterior rearrangement of cellular patterns. At this stage, the dorsal part of the neural tube is subdivided in six domains of cell progenitors (dP1-dP6, dorsal to ventral), whereas the remaining five progenitor domains form the ventral half (p3, pMN, p2-p0, ventral to dorsal) (Caspary and Anderson, 2003; Helms and Johnson, 2003; Dessaud et al., 2008; Matise, 2013). In the dorsal half of the developing embryo, the combination of BMP and Wnt signaling with HD factors such as Pax3 and Pax7, induces the expression of the different proneural genes (*Math1*, *Ngn1*, *Mash1*) that define the six progenitor domains. *Math1* is expressed in the cells most adjacent to the roof plate (dP1), *Ngn1* expression defines the domain below (dP2) and *Mash1* is expressed in the dP3-dP5 progenitors. Ngn1 is also present in the dP6 progenitors, but does not restrict its expression to this population, expanding it to domains in the ventral part of the spinal cord (Ma et al., 1997; Gowan et al., 2001; Helms et al., 2005). Later on (at E10), the prospective neurons in the mantle layer that evolve from each progenitor population domain are

distinguished according to the Homeobox genes they begin to express and six populations of dorsal interneurons are characterized (dI1 to dI6). The dP1-dP3 progenitors give rise to dI1-dI3 neurons that will settle in the deep layers of the adult dorsal spinal cord (Caspary and Anderson, 2003; Helms and Johnson, 2003).

1.5.1. GENERATION OF CLASS A AND CLASS B EARLY-BORN NEURONS

Experiments consisting in the ablation of the roof plate resulted in the exclusive loss of the three topmost dorsal progenitor populations (dP1-dP3), pointing to the dependence of these populations on the signaling diffused through this tissue (Lee et al., 2000). These progenitors comprise the prospective Class A neurons (dI1-dI3), the ones that are born in the dorsal alar plate and require BMP and Wnt signaling in order to be generated. Early studies performed using explants cultures demonstrated that high and low concentration of BMP signaling, respectively determined the fate of dI1 and dI3 neurons (Liem et al., 1997) and that although levels of BMP family members are similar along the whole dorsoventral axis shortly before the closure of the neural tube, afterwards they become restricted to the region of the presumptive dP1-dP3 (Tozer et al., 2013). Ablation of BMP type 1 receptors, *Bmpr1a* and *Bmpr1b*, in mouse neural tube, results in the complete loss of the dP1 population as demonstrated by the absence of Math1. As a consequence, the population of dI1 interneurons is not formed, whereas the dI2 domain is strongly reduced, highlighting the influence of BMPs in the differentiation of these progenitors (Wine-Lee et al., 2004). In contrast, the dP4-dP6 domains are expanded, confirming that BMP signaling does not affect the generation of this group of neurons – hence, neurons that differentiate from these populations of progenitors are classified as Class B neurons. Moreover, BMP acts as an instructor to restrict the ventral spinal fate in the spinal cord since it functions as an activator of the expression of *Pax7* (Mansouri and Gruss, 1998). Analysis of mouse *Pax3/Pax7* double mutant demonstrated that these factors act together to impel the correct development of the neural crest lineage and of spinal cord progenitor cells that give rise to a subset of commissural interneurons, as well as to proper restrict the dorsal boundary of ventral cell types (Mansouri and Gruss, 1998).

Concurrently with the major role of Wnt signaling in the proliferation of dorsal progenitors, the canonical Wnt/β-catenin pathway is also involved in the patterning of

dorsal spinal neuronal populations. Members of the Wnt family, *Wnt1* and *Wnt3a*, start to be expressed in the roof plate soon after neural tube closure. Disruption of these genes results in mouse embryos with an altered number of dI1-dI3 interneurons at E10.5. A reduction in the number of dI1 to dI3 neurons is confirmed by a decrease in the expression of *Lhx2* and *Islet1*, which serve as molecular markers for two of these subpopulations. On the other hand, the dorsal expansion of *Pax2*, a transcription factor typically expressed in the dI4 interneurons, revealed the dorsal spreading of this subpopulation, at the expense of dI1-dI3 neurons (Muroyama et al., 2002). Moreover, the Wnt/β-catenin signaling is intimately related with the activation of *Olig3* (Figure 5) (Zechner et al., 2007), encoding a bHLH transcription factor common to the specification of all dP1-dP3 progenitors. The expression of *Olig3* is first detected in mouse spinal cord by E9 in a continuous broad stripe of cells that encompasses these progenitor domains, and later in a small set of postmitotic neurons in the ventral spinal cord (Takebayashi et al., 2002). Müller and colleagues (Muller et al., 2005) ascribed a crucial role for Olig3 in the generation of the dI1-dI3 neurons. They demonstrated that null mutant mice for the gene encoding this bHLH protein present a shortage in the number of dI1 neurons and that the reduced number of *Math1* expressing cells in *Olig3* mutants may contribute to this phenotype. More drastically, the dI2 and dI3 neurons failed to be generated at all, since the markers for these populations (FoxD3 and Tlx3/Islet1, respectively) are absent in these embryos. The fact that Olig3 is required to maintain the levels of Ngn1 and Ngn2 is the most likely reason for the failure in the formation of dI2 neurons in these mutants. Instead, these neurons appear to be misspecified and express molecular markers characteristic of dI4 interneurons, such as Lbx1, Pax2 and Lhx1/5 transcription factors. In addition, cell lineage analyses showed that neurons where *Olig3* expression was abolished went to occupy a more lateral position in the dorsal spinal cord, which is a place typically occupied by Class B neurons (Muller et al., 2005). All the data suggests that in the absence of Olig3, Class B neurons are formed at the expense of Class A.

The specification of the neurons that evolve from dP4-dP6 progenitors is regulated by a different genetic network, given the fact that these cells develop independently of the presence or signaling from the roof plate (Lee et al., 2000). This process was first unveiled by Müller and colleagues, who thoroughly characterized the

presence of the HD protein Lbx1 in the mouse embryonic spinal cord (Muller et al., 2002). They found out that Lbx1 appears at E10 in a group of postmitotic neurons that are outside the ventricular zone and no longer express *Pax7*, which marks proliferating neuronal progenitors. These correspond exclusively to the dI4-dI6 classes of interneurons, as demonstrated by co-localization of Lbx1 with other proteins – dI4 and dI6 are both defined by *Lbx1* and *Lhx1/5* expression and are intercalated by the dI5 population, which is defined by the presence of Lbx1, Lmx1b, Brn3a, Tlx3 and Prrxl1 (Figure 5). The mechanisms that regulate the differentiation of these neurons are intricate. Albeit dI4 and dI6 interneurons are defined by a similar code of HD transcription factors, they emerge at distinct positions in the spinal cord and evolve from progenitors whose fate determination is differentially regulated. Conversely, dI4 and dI5 *Lbx1*-expressing neurons emerge from the same Mash1- and Pax7^{high}-positive domain, despite the fact they later diverge in their molecular identities (Lbx1/Lhx1/5-positive vs. Lbx1/Lmx1b/Brn3a-positive neurons, respectively). Lbx1 emergence is favored in the presence of high doses of Pax7, since lower concentrations of Pax7 and Mash1 stimulate the specification of the Lbx1-negative dI3 interneurons. In the absence of Lbx1, Class B neurons assume the molecular characteristics of Class A neurons. Conversely, its ectopic expression in the most dorsal part of spinal cord forces prospective Class A neurons to differentiate towards the Class B phenotype, strongly resembling the *Olig3* null mutant mice. Therefore, the specification of the early-born neuronal populations in two main classes during dorsal spinal cord development, relies on the antagonistic role exerted by Olig3 and Lbx1.

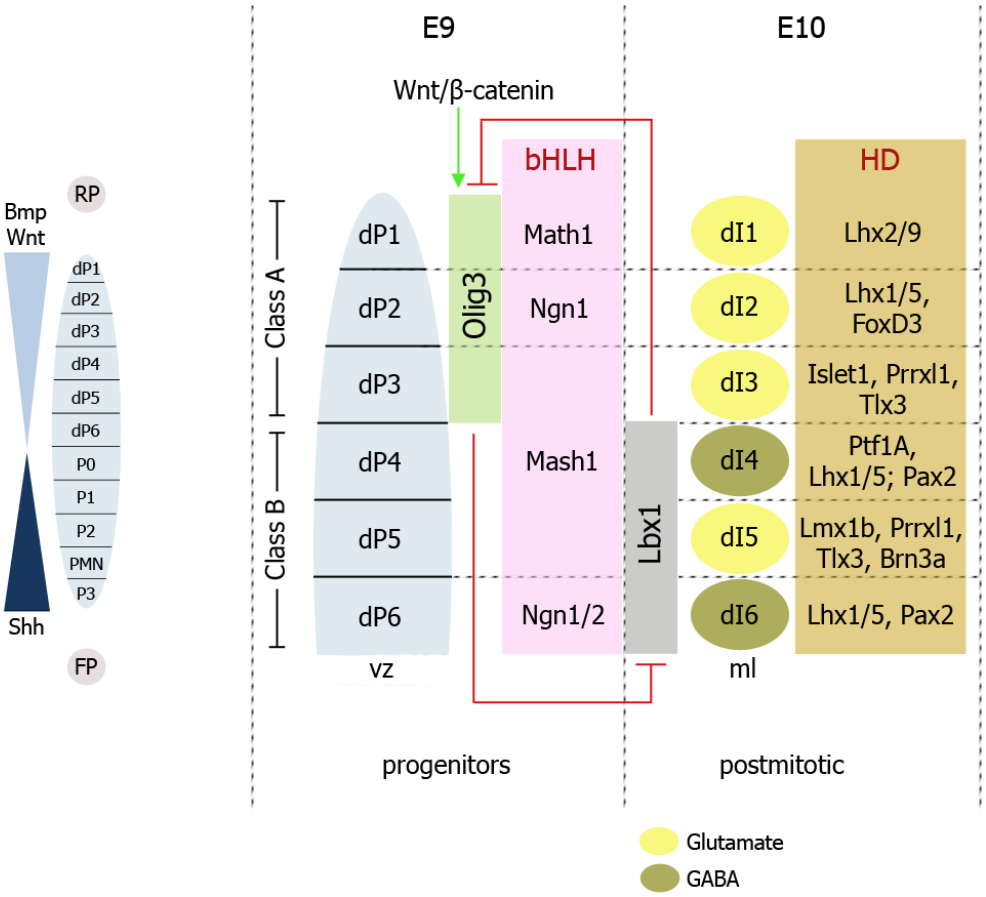

Figure 5: Schematic representation of the inductive signals and transcription factors acting during the early neurogenesis in the dorsal spinal cord. BMP and Wnt signals released from the roof-plate (RP) and Shh released from the floor-plate (FP) and the notochord orchestrate the initial patterning of the eleven progenitor populations (dP1-P3) in the developing spinal cord, in a dosage-dependent manner. In the dorsal half of the spinal cord, only the dorsal progenitors dP1-dP3 respond to the inductive signals from the roof-plate (Class A), whereas Class B progenitors (dP4-dP6) generate independently from those signals. The Wnt/β-catenin pathway is required to activate *Olig3*, which encodes a bHLH factor essential for the generation of Class A neurons. Complementarily, Lbx1 is required for the development of Class B neurons. Olig3 and Lbx1 are antagonist factors. By E9, the distinct populations of dorsal progenitors in the ventricular zone (vz) can be distinguished according to the bHLH transcription factors present there: dP1 – Math1; dP2 – Ngn1; dP3-dP5 – Mash1; dP6 – Ngn1/2. At E10, the dividing progenitor cells have exited the cell cycle (postmitotic neurons), migrated laterally to the mantle layer (ml) and have switched on the expression of several HD transcription factors that define the six populations of dorsal interneurons (dI1-dI6). dI1, dI2, dI3 and dI5 will originate glutamatergic excitatory neurons and dI4 and dI6, GABAergic inhibitory neurons.

1.5.2. GENERATION OF LATE-BORN NEURONS

Approximately two days later (by E12.5), the second wave of spinal neurogenesis is initiated. Two newly mutually exclusive populations of neurons emerge from the dI3-dI5 Lbx1/Mash1-positive domain of dividing progenitors and express the same molecular markers as dI4 and dI5 neurons (Gross et al., 2002; Muller et al., 2002). These late-born populations are named dIL^{A} and dIL^{B} and their primary

difference accounts for the distinct combinatory code of HD transcription factors: dIL^{A} neurons are defined by the presence of Lbx1, Lhx1/2 and Pax2, whilst dIL^{B} are Lbx1/Lmx1b/Tlx3 and Prrxl1-positive. Unlike their early-born counterparts, which are also Lbx1-positive, late-born neurons will migrate dorsally and populate the most superficial layers (laminas I-III) of the spinal cord dorsal horn in an intermingled pattern. There, dIL^{A} and dIL^{B} arise as association interneurons that express, respectively, gamma-amino butyric acid (GABA) and glutamate as predominant neurotransmitters in inhibitory and excitatory neurons, and are responsible for the integration of sensory information arriving from the periphery (Figure 6). In order to do it properly, the correct balance of these excitatory and inhibitory neurons is mandatory. Functional studies that consisted on the disruption of genes involved in the specification and differentiation of the late-born populations, resulted in animals with impaired nociceptive function (Chen et al., 2001; Gross et al., 2002; Muller et al., 2002; Xu et al., 2013). In *Lbx1* null mice, both the dI4/dIL^{A} (Lhx1/5-positive) and dI5/dIL^{B} (Lmx1b/Brn3a-positive) assume the molecular characteristics of the Class A dI2 (Lhx1/5/Brn3a-positive) and dI3 (Islet1/Brn3a-positive), respectively. This indicates that Lbx1 acts by repressing conditioners of Class A subtypes, such as *Islet1/2*. Unlike Class B neurons, these cells are faded to establish as commissural interneurons that relay the somatosensory information in the deep dorsal spinal cord. Therefore, a shortage of dorsally migrating cells to the surface is observed and, in consequence, these embryos develop a defective dorsal horn with impaired nociceptive integrative centers (Muller et al., 2002). This is reflected in the incomplete penetrance of peripherin- and TrkA-positive projecting afferent fibers to the spinal gray matter and in the reduced production of excitatory and inhibitory neurotransmitters (Muller et al., 2002).

Following early neuronal determination (E10 – E12.5) and further in dorsal spinal cord development, it is of great importance to activate transcription programs involved in terminal differentiation processes. These include genes that deal with maintenance of cell survival, axon guidance and synapse establishment, as well as with the activation of genes that control the expression of molecules that confer the proper identity and specify different neuron types (neurotransmitters synthesizing enzymes and transporters, ion channels, sensory receptors and signaling factors) (Hobert,

2011). Most of these specifiers are HD transcription factors. Although late-born neurons develop from a common Mash1/Lbx1-positive domain of progenitors, they soon divide into neuronal populations with opposing functions. By E13.5, the presence of the HD factors Pax2 and Tlx3 represent the two intermingled developing late-born populations in the dorsal horn of the spinal cord, in a way complementary to the mutually exclusive development of GABAergic and glutamatergic neurons (Cheng et al., 2004). *Tlx3* is solely expressed in VGLUT2-positive cells (a vesicular glutamate transporter) and almost never co-localizes with Pax2, which in its turn, highly co-localizes with two enzymes involved in GABA synthesis, the glutamic acid decarboxylases GAD67 and GAD65, as well as the inhibitory amino acid transporter that packages GABA or glycine into synaptic vesicles (encoded by the gene *Viaat*) (Cheng et al., 2004). By suppressing the activity of Lbx1 in dIL^{B} neurons, Tlx3 and its paralogue gene *Tlx1* are essential for the development of the glutamatergic neurotransmitter phenotype in the superficial laminas of the dorsal horn (Cheng et al., 2005). This regulation is specific of the late-born population dIL^{B}, as the disruption of both genes (*Tlx3* and *Tlx1*) does not affect the development of glutamatergic neurons that derive from the dP1 and dP2 progenitors and settle in the profound laminas of the dorsal spinal cord, which is somehow consistent with the lack of *Tlx1* and *Tlx3* expression in the early-born dI1 and dI2 populations (Caspary and Anderson, 2003; Helms and Johnson, 2003; Cheng et al., 2004). Opposed to the development of glutamatergic neurons, Ptf1a appears as a master regulator of the fate of GABAergic populations. This HD factor starts to be expressed at E10.5 in postmitotic cells in the Mash1-derived dI4 domain, but by E11.5 it has expanded its expression both dorsally and ventrally (dI3 to dI5). At E12.5, Ptf1a marks the Pax2/Lhx1/5-positive dILA population but not the dILB, which is Tlx3/Lmx1b/Prrxl1-positive. Indeed, Ptf1a is crucial for the development of the dI4 and dILA populations of interneurons, as seen by the loss of Pax2/Lhx1/5-positive cells in *Ptf1a* null mutants. Moreover, Ptf1a contributes to inhibit the generation of dILB neurons, as the number of cells that express *Tlx3* and *Lmx1b* are increased in *Ptf1a* null mice. In fact, a cell fate switch occurs in the absence of Ptf1a, as demonstrated by fate mapping analysis. In *Ptf1a* mutants, the presence of Tlx3 and Lmx1b is detected in cells where the expression of *Ptf1a* was ablated, contrasting with the non-overlapping expression of *Ptf1a* and

Tlx3/*Lmx1b* in mouse embryos heterozygous for *Ptf1a* (Glasgow et al., 2005). In accordance with this, dIL^{A} cells fail to become GABAergic, whereas the number of Tlx3/VGLUT2-positive neurons increase. The Class A dI2 and dI3 interneurons are not affected by the disruption of this gene (Glasgow et al., 2005). Thus, Ptf1a and Lbx1 are upstream targets of *Pax2*, and all act together to select and determine the GABAergic inhibitory fate of neurons that emerge from the Mash1-positive domain of the dorsal spinal cord progenitors (Figure 6).

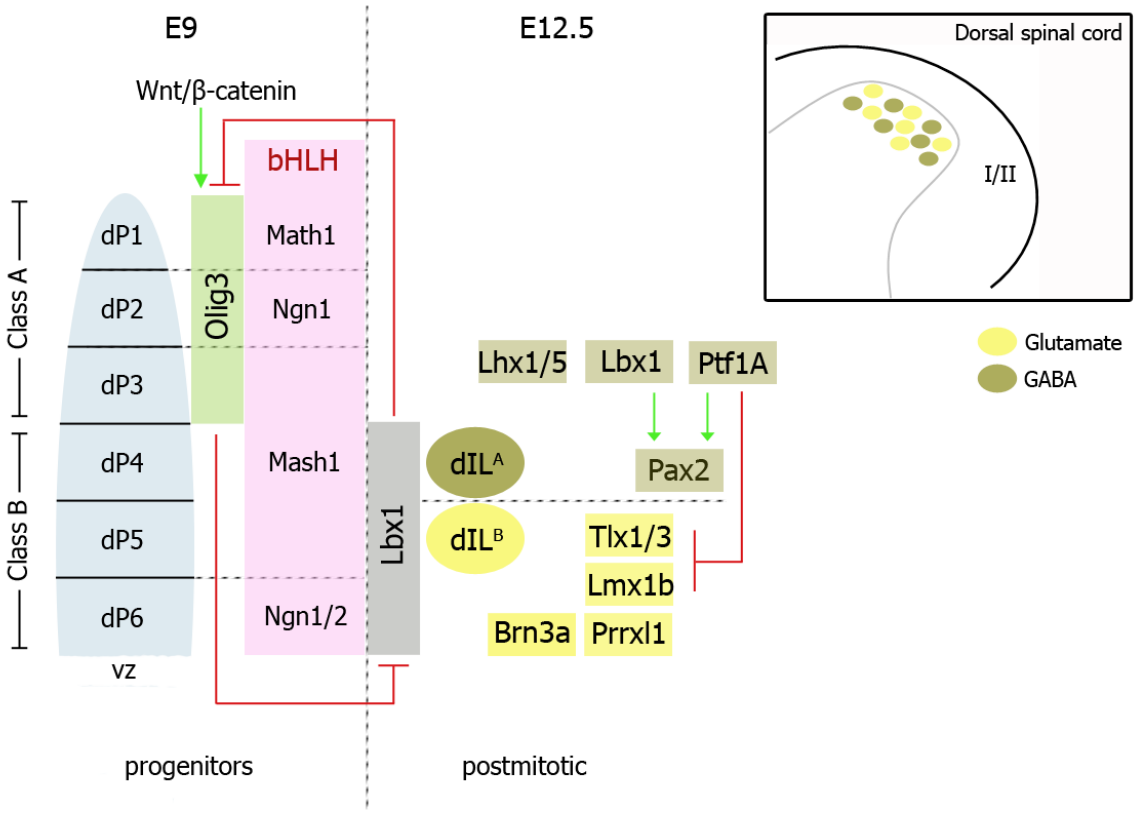

Figure 6: Schematic representation of transcription factors acting during the generation of late-born neurons in the dorsal spinal cord. At E12.5, two mutually exclusive populations of neurons emerge from the dI3-dI5 Lbx1/Mash1-positive domain of dividing progenitors and express the same molecular markers as dI4 and dI5 neurons (respectively, dIL^{A} expresses *Lhx1/5/Lbx1/Ptf1A/Pax2* and dIL^{B} expresses *Tlx1/3/Lmx1b/Brn3a/Prrxl1*). dIL^{A} neurons will express GABA and dIL^{B} will express glutamate as main neurotransmitters. Both populations migrate in a dorsolateral fashion and establish in the superficial laminas of the spinal cord dorsal horn, in an intermingled pattern of GABAergic inhibitory and glutamatergic excitatory neurons.

Regarding the neuronal populations in the dorsal horn of the spinal cord, the combinatorial code that leads to the generation and maintenance of excitatory and inhibitory neurons, is relatively well-known. The entire pool of GABAergic neurons

derives from Lbx1-positive postmitotic cells and requires the presence of Ptf1a and Pax2 to maintain the inhibitory features, as these factors regulate the transcription of genes characteristic of the GABAergic phenotype. The coordinated action of these genes promote the expression of *GABA*, *glycine* and a set of peptides essential for the inhibitory phenotype, such as *neuropeptide Y* (NPY), *dynorphin* (DYN), *galanin* (GAL), *somatostatin* (Sst) and *enkephalin* (ENK) (Figure 7) (Glasgow et al., 2005; Huang et al., 2008). The genetic mechanisms involved in the differentiation and specification of glutamatergic excitatory neurons are mainly regulated by the differential action of Tlx1 and Tlx3, Lmx1b, Brn3a and Prrxl1. From those genes, Tlx3 emerges as the master regulator in the determination of the glutamatergic excitatory phenotype in the dIL^{B} neuronal population. Indeed, in *Tlx3* null mutant mice, typical markers of GABAergic inhibitory neurons are increased, including *Pax2*, *Gad1* and *Viaat* (Cheng et al., 2004). A set of neuropeptides responsible for the modulation of nociceptive sensory information are expressed in Tlx3-positive neurons or their derivatives (Xu et al., 2008). Among them, are included the *CCK* (which originates the anti-opioid peptide cholecystokinin) and *Tac1* (encoding the precursor for SP and Neurokinin A), whose expression is abolished in double *Tlx3/Tlx1* null mutants, from E13.5 to E18.75, in intermediate and deep laminas, respectively. Remarkably, though the expression of *Tlx3* is transient in most of these neurons, a strong requirement of Tlx3 (and the redundant role of Tlx1 in the rostral spinal cord) to establish the peptidergic transmitter phenotype is acknowledged. However, Tlx3 needs to act in combination with other factors in order to accomplish such functions. Specifically, it has been reported that Tlx3 first activates the transcription of *Brn3a* (as verified by the partial loss of *Brn3a* expression in *Tlx3/Tlx1* double mutants), which then acts to positively control the *Tac1* expression in *Tlx3*-expressing neurons. The expression of other peptides controlled by Tlx3 appears to be more intricately regulated. For instance, Tlx3 exerts both a positive and a negative regulation in the transcription of the *Sst* encoding gene. In the double *Tlx3*/*Tlx1* null mice, the number of *Sst*-expressing neurons is prominently decreased in the superficial dorsal spinal cord by E18.75, whereas its expression in deeper laminas is not affected. Nevertheless, only the late wave of *Sst*-positive neurons is affected in these mutants. At earlier stages, the number of *Sst*-

expressing neurons increases by five-fold in *Tlx3/Tlx1* mutant embryos and are confined to the intermediate and deep dorsal laminas (Xu et al., 2008).

Once the glutamatergic cell fate is determined in the dorsal horn of the spinal cord, other molecular players are required to maintain neurons in a functional excitatory condition. Nonetheless, the nature of the genetic relationships of the transcription factors involved in the proper integration and transmission of noxious stimuli remains elusive. Albeit not involved in the initial specification of the glutamatergic fate, Lmx1b stands out as an essential regulator of the transcriptional program that coordinates the assembly of the nociceptive circuitry. In the developing spinal cord, the expression of this gene begins at E10.5 in the dI5 and dIL^{B} and is mediated by Lbx1 (Ding et al., 2004). Subsequently, it is required for the spinal cord specifier *Prrxl1* (Chen et al., 2001) to initiate its expression, as this is completely absent in *Lmx1b* null mutants, from its time of onset until E15.5; it is not essential for the onset but for the maintenance of *Tlx3* and *Ebf3* expression, which are drastically downregulated or absent by E15.5 in the developing dorsal horn of *Lmx1b* mutants; it controls the expression of several molecular cues important for axon guidance and neuronal migration, such as receptors for the chemorepellent *Sema3a*, *Neuropilin1* and *PlexinA2*, *Slit1*, *Robo2* and *Netrin1* (Ding et al., 2004), as well as the extracellular matrix glycoprotein *Reelin* in laminas I and II (Szabo et al., 2015). Altogether, *Lmx1b* disruption conducts to severe anatomical deficiencies in the superficial spinal cord due to the incorrect differentiation and patterning of *Lmx1b*-expressing neurons (lamina I and II become indistinguishable in *Lmx1b* mutants) and absent projections of TrkA-positive sensory afferent fibers to the prospective laminas I and II (Ding et al., 2004). Moreover, Lmx1b is required for the correct development and survival of dorsal horn excitatory neurons without affecting the number of inhibitory neurons (Szabo et al., 2015), contrasting to observations in the trigeminal nucleus principalis (PrV) where Lmxb1 facilitates the glutamatergic phenotype and suppresses the GABAergic one by reducing the expression of *Pax2* at late embryonic stages (after E14.5) (Xiang et al., 2012). The absence of Lmx1b in pain-processing spinal cord neurons affects mechanical (possibly related to the loss of *Sst* expression) and thermal nociception (Szabo et al., 2015).

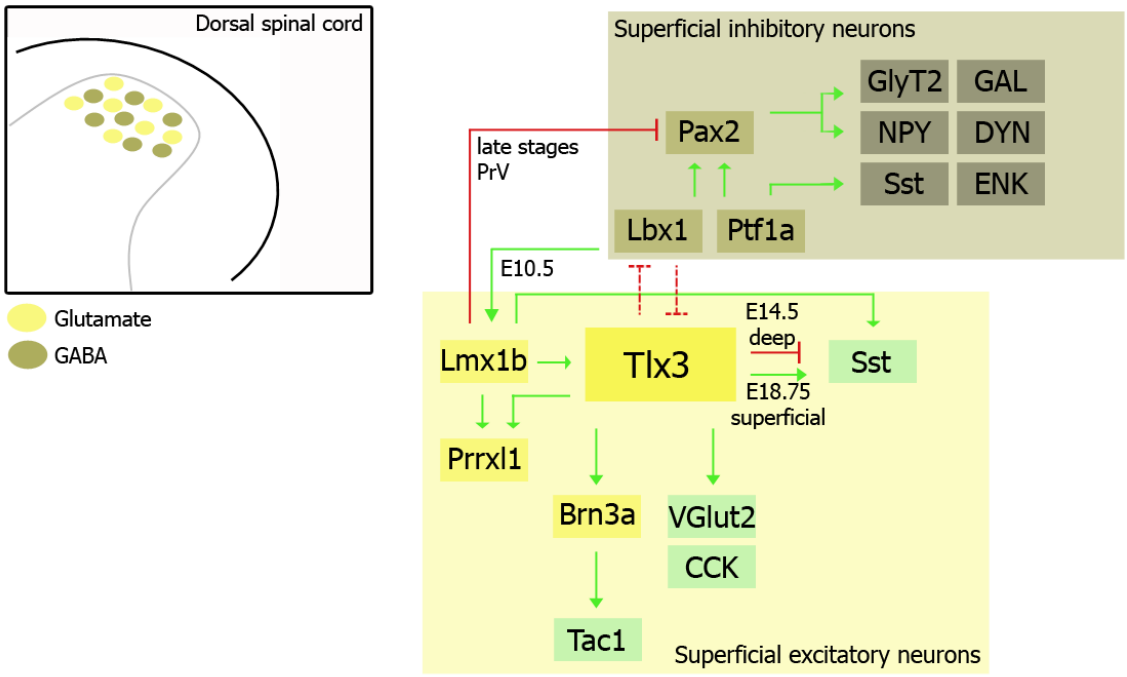

Figure 7: Schematic representation of the genetic relationships established between transcription factors acting on the terminal differentiation processes of glutamatergic (yellow) and GABAergic (green) neurons in the dorsal spinal cord. *Lbx1*, *Ptf1A* and *Pax2* are essential genes in the generation and maintenance of inhibitory features in GABAergic neurons, as they promote the expression of genes characteristic of GABAergic phenotype - *neuropeptide Y* (NPY), *dynorphin* (DYN), *galanin* (GAL), *somatostatin* (Sst) and *enkephalin* (ENK), and the glycine transporter GlyT2. Tlx3 emerges as the master regulator of the glutamatergic fate, inducing or maintaining the expression of *Lmx1b*, *Prrxl1* and *Brn3a*, as well as genes essential for the establishment of the excitatory and peptidergic phenotype, such as *VGlut2*, *CCK*, *Sst* (only at E18.75 but not at E14.5) and *Tac1*, via activation of *Brn3a*. Lbx1 activates the transcription of *Lmx1b* at E10.5, which is in its turn, is required for the onset of *Prrxl1*. At late stages of the trigeminal nucleus principalis (PrV) development, Lmx1b facilitates the glutamatergic phenotype and suppresses the GABAergic one, by antagonizing the expression of *Pax2*.

All the information gathered in the past years points towards the major role of Tlx3 coordinated with other HD transcription factors, such as Lmx1b and Prrxl1, in the control of differentiation, migration and generation of distinct subpopulations of nociceptive glutamatergic excitatory lamina I and II neurons, as well as the in establishment of synapses with the incoming primary afferent neurons. Nevertheless, whether and when these factors act together or in transcriptional cascades, and what are the precise molecular mechanisms that regulate their relationship and define their tissue-specific function are questions that still remain to be unraveled. In order to pursue this issue, this thesis focused on the study of the molecular mechanisms that regulate *Prrxl1* expression, since it is, among the many genes playing important roles in

the development and establishment of the pain circuit, one of the few exclusively present in nociceptive sensory neurons.

1.6. THE HOMEODOMAIN TRANSCRIPTION FACTOR PRRXL1

During the assembly of the peripheral-to-central pain circuit, Prrxl1 stands out as an important regulator of the nociceptive neuronal identity both in DRG and spinal cord. This is remarkable, as many critical nociceptive determinants are present exclusively in DRG (such as Runx1) or dorsal spinal cord (such as Lbx1 and Lmx1b). Prrxl1 (formerly known as Drg11) is a HD transcription factor expressed from E10.5 in mouse peripheral sensory ganglia (DRG and TG), as well as in neurons that will populate the dorsal horn of the spinal cord and trigeminal nucleus, responsible for the modulation of the nociceptive sensory information. There, its expression persists at significant levels until P14-P21 (Saito et al., 1995; Rebelo et al., 2007). In the DRG, *Prrxl1* initiates its expression in TrkA-positive small-diameter neurons and later on it accompanies the segregation of C fibers into peptidergic and non-peptidergic, maintaining its expression in both subpopulations (Rebelo et al., 2006). Although the onset of *Prrxl1* is observed quite early during the development of primary afferent sensory neurons, this factor is not apparently involved in the initial differentiation or specification of these fibers. Instead, Prrxl1 is required at postnatal stages for the survival of peptidergic and non-peptidergic small primary neurons in the DRG, as verified by the decrease of both CGRP- and IB4-positive cells in *Prrxl1* null mice by P7 (Rebelo et al., 2006). Concomitantly with the equally distributed levels of Prrxl1 in these fibers, the innervation of cutaneous, visceral and deep tissues is affected (Rebelo et al., 2006).

Similar to the functionally related proteins Tlx3 and Lmx1b, Prrxl1 is detected in the developing spinal cord at E10.5 in postmitotic dI3 (akin to Tlx3) and dI5 (akin to Tlx3 and Lmx1b) interneurons (Rebelo et al., 2007). By E12.5, *Prrxl1* expression is observed in a subset of late-born Tlx3-positive neurons (dIL^{B}) that will migrate dorsolaterally to establish in the superficial laminas of the dorsal horn. Indeed, Prrxl1 acts alongside Tlx3 and Lmx1b to define various subpopulations of both glutamatergic

early- and late-born neurons, in accordance with their differential expression (Rebelo et al., 2010). *Prrxl1* expression appears to be particularly required in lamina I and II neurons. *Prrxl1* null mouse embryos display defects in the spatio-temporal projection patterning of small-diameter sensory afferent fibers to the dorsal horn. Cutaneous afferent projections fail to properly enter the spinal gray matter, as determined by the delay in the penetrance of calbindin-positive fibers in the dorsal horn of E13.5 *Prrxl1* mutants, a stage when no apparent morphological defects are observed in the developing spinal cord. In contrast, in wild-type mice, afferent fibers expressing *calbindin* already have penetrated the gray matter by this age. Although the ingrowth of afferent fibers occurs at last (by E16.5), these enter the gray matter in a disorganized manner, towards the spinal midline instead of to the lateral-most portion of the dorsal horn, as observed in wild-type embryos. A similar derangement is detected when the expression of *TrkA* is analysed (Chen et al., 2001). The impaired connection established between primary and second order neurons may be the cause of posterior cell death, evident in the dorsal horn of mutant mice, beginning at E17.5. Cell death persists by postnatal stages, as a reduction of 60-70% of mostly lateral dorsal horn neurons is observed in adult mice. Moreover, the absence of PKCγ-positive cells in *Prrxl1* mutants is indicative of the failure in the correct formation of second order neurons involved in the processing of the nociceptive information. Altogether, these anatomical deficiencies lead to alterations in pain sensitivity in adult mutants. In comparison to wild-types, these animals display a significant delay in the perception of thermal stimuli (as assessed in the hot plate, tail-flick and paw withdrawal tests), as well as a reduced sensitivity to chemical noxious inputs (as demonstrated by the use of formalin or capsaicin) (Chen et al., 2001). These abnormalities are strikingly similar to the *Tlx3* CKO mice, whose *Tlx3* expression was specifically removed from the dI5 and dIL^{B} lineage (Xu et al., 2013) – the sensorimotor and proprioceptive function are intact but normal sensitivity to noxious thermal, chemical and mechanical is compromised.

As Prrxl1 is essential for the survival of TrkA-derived neurons and establishment of nociceptive circuit, one would wonder that its transcription in primary sensory neurons could be controlled at least by one of the main effectors of the nociceptive phenotype described above – Runx1 or Tlx3. However, genetic studies performed in mice with disturbed expression of *Tlx3* (Qian et al., 2002) or *Runx1* (Yang et al., 2013)

in the DRG, did not demonstrate any evidence pointing to the regulation of *Prrxl1* gene expression by these transcription factors. In contrast, Islet1 and Brn3a do have an important role in its transcriptional activation, as double mutant mice for these genes display a strong decrease in the expression of *Prrxl1* (Dykes et al., 2011). Specifically, Islet1 control of *Prrxl1* transcription in the DRG is observed at E12.5, but not at later stages, since by E14.5 *Prrxl1* expression is no longer decreased in *Islet1* mutants (Sun et al., 2008). In the cranial visceral sensory neurons, it was demonstrated that Phox2b has the potential to shut down *Prrxl1*, after an early wave of expression, in a process mediated by the direct repression of *Brn3a* (D'Autreaux et al., 2011). Phox2b is a strong regulator of the visceral sensory phenotype in cranial ganglia, and in its absence these neurons switch to a somatic fate. Hence, Phox2b has an important role in the repression of genes characteristic of the somatic phenotype, such as *Brn3a*, *Runx1* and *Prrxl1* (D'Autreaux et al., 2011). Nonetheless, *Prrxl1* expression in the nucleus of the solitary tract (nTS) of the hindbrain is not affected by *Phox2b* inactivation, suggesting that the repression of *Prrxl1* by Phox2b only occurs at a specific time frame in the visceral sensory ganglia. In spinal cord, *Prrxl1* requires Lmx1b to trigger its transcription, as *Prrxl1* expression is not initiated at all in *Lmx1b* mutant mice (Ding et al., 2004). In contrast to what is observed in the DRG, *Tlx3* inactivation strongly affects *Prrxl1* expression in spinal cord, resulting in the downregulation of *Prrxl1* (Qian et al., 2002). Altogether, this data indicates that *Prrxl1* expression is regulated by distinct tissue-specific genetic programs.

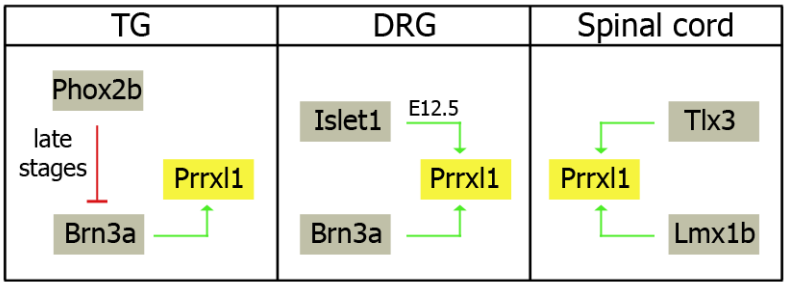

Figure 8: Available data regarding transcriptional pathways involved in the regulation of *Prrxl1* transcription. In the trigeminal ganglia (TG), *Prrxl1* expression is inhibited by Phox2b, possibly via direct repression of Brn3a. In the dorsal root ganglia (DRG), it is known that *Prrxl1* transcription is induced both by Islet1 and Brn3a. In the spinal cord, *Prrxl1* expression is activated by Lmx1b and requires Tlx3 in order to be maintained.

2. OBJECTIVES

Despite the findings regarding the importance of Prrxl1 for the proper differentiation and assembly of the nociceptive circuit, little is known about the way Prrxl1 controls such processes. In order to address these questions, a better understanding of the genetic transcriptional programs and detailed mechanisms that control the expression of *Prrxl1* would be required, to put Prrxl1 in context with other players, whose functions are better known. To add some information on this subject, different approaches were used in order to dissect the molecular machinery that controls the spatiotemporal expression of *Prrxl1* during the development of nociceptive neurons. Therefore, in this study we mainly sought:

I. To identify and characterize conserved tissue-specific cis-regulatory elements that control *Prrxl1* transcription during the development of nociceptive neurons;

II. To identify and characterize trans-acting factors that bind the elements identified in I. and to ascribe a functional significance to such binding.

Most of these analyses were performed *in vitro* using an immortalized cell line (ND7/23) that displays nociceptive properties characteristic of sensory ganglia and recapitulates some molecular mechanisms occurring in DRG or spinal cord nociceptive neurons. In addition, considering that *Prrxl1* expression had been described in the zebrafish and displays a pattern similar to what is observed in mouse (McCormick et al., 2007), we concluded this would be a suitable model to evaluate the functional relevance of the conserved regions identified in the first tasks of this study.

The data collected during this study was published in two original papers and is presented in this thesis in the form of Publication I and Publication II. In the first publication (Publication I), the identification of several cis-regulatory elements that control *Prrxl1* transcription and translation is described. Among them, there are three alternative promoter regions and a binding motif for Phox2b, which is required for *Prrxl1* expression in zebrafish visceral sensory neurons. In this publication, binding sites

recognized by the HD transcription factors Tlx3 and Brn3a were also identified. The relationship between Prrxl1, Tlx3 and Brn3a was further explored in Publication II. There, we reported that Tlx3 controls the expression and activity of Prrxl1 by two different mechanisms, either by controlling its transcription or by inducing alterations in its phosphorylation state.

PUBLICATIONS

3.1. PUBLICATION I

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Several Cis-regulatory Elements Control mRNA Stability, Translation Efficiency, and Expression Pattern of Prrxl1 (Paired Related Homeobox Protein-like 1)*[S]

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Background: The mechanisms that control the *Prrxl1* expression are poorly understood.
Results: Several regulatory elements present in *Prrxl1* alternative promoters are functionally characterized, including a binding motif for Phox2b required for *Prrxl1* expression in visceral sensory neurons.
Conclusion: We define diverse regulatory modules, which control the spatiotemporal expression of *Prrxl1* in nociceptive neurons.
Significance: A new mechanism involved in the ganglion specific action of *Prrxl1* is described.

The homeodomain transcription factor Prrxl1/DRG11 has emerged as a crucial molecule in the establishment of the pain circuitry, in particular spinal cord targeting of dorsal root ganglia (DRG) axons and differentiation of nociceptive glutamatergic spinal cord neurons. Despite Prrxl1 importance in the establishment of the DRG-spinal nociceptive circuit, the molecular mechanisms that regulate its expression along development remain largely unknown. Here, we show that *Prrxl1* transcription is regulated by three alternative promoters (named P1, P2, and P3), which control the expression of three distinct *Prrxl1* 5′-UTR variants, named 5′-UTR-A, 5′-UTR-B, and 5′-UTR-C. These 5′-UTR sequences confer distinct mRNA stability and translation efficiency to the *Prrxl1* transcript. The most conserved promoter (P3) contains a TATA-box and displays *in vivo* enhancer activity in a pattern that overlaps with the zebrafish Prrxl1 homologue, *drgx*. Regulatory modules present in this sequence were identified and characterized, including a binding site for Phox2b. Concomitantly, we demonstrate that zebrafish Phox2b is required for the expression of *drgx* in the facial, glossopharyngeal, and vagal cranial ganglia.

Sensory perception of peripheral stimuli is primarily mediated by different types of afferent neurons, which are located in the trunk dorsal root ganglia (DRG)[2] and send processes to the periphery and the spinal cord. In the spinal cord, specialized neurons integrate and relay the information to somatosensory centers in the brain where appropriate responses are generated (1, 2). Spinal sensory neurons differentiate from several classes of proliferating progenitor cells, whose establishment requires the expression of proneural genes encoding for basic helix-loop-helix (bHLH) transcription factors (3–5). The various cell lineages are specified according to the combined expression of a set of homedomain transcription factors that confer neural identity to each class (4, 5). One of these factors is Prrxl1 (also known as DRG11). Prrxl1 has emerged as a crucial molecule in the development of the pain-perception circuitry, especially in the establishment of the nociceptive DRG-spinal pathway. Prrxl1 is also expressed in sensory cranial ganglia and their target relay neurons in the hindbrain (6, 7). Nevertheless, the role of Prrxl1 in these tissues is poorly studied.

Prrxl1 null mutant mice present a distorted spinal dorsal horn with scarce superficial nociceptive-responsive neurons (8–10), reduced DRG neuronal population (10), and a marked decrease in nociceptive response capacity in various pain tests (8). Interestingly, although involved in the embryonic differentiation of various subpopulations of superficial dorsal horn excitatory neurons (10), Prrxl1 appears not to be required for the normal development of DRG neurons before birth but rather to be essential for their survival in early postnatal life (9). Although Prrxl1 expression in various cell lineages in DRG and spinal cord is well known, the mechanisms of transcriptional control exerted by different bHLH and homeodomain proteins that modulate *Prrxl1* transcription are still poorly understood.

Recently, a *Prrxl1* alternative spliced variant was identified, and multiple variants of exon 1 in both *Prrxl1* mRNA isoform sequences were discovered, suggesting the existence of various 5′-untranslated regions (5′-UTRs) controlled by distinct promoters (11). Modulation of gene expression through alternative promoter usage is now widely accepted following evidence gathered in the past years (12, 13). According to Baek *et al.* (14),

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[S] This article contains supplemental Table S1.
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[2] The abbreviations used are: DRG, dorsal root ganglia; bHLH, helix-loop-helix; TSS, transcription start site; HD, homeodomain; TBP, TATA-binding protein; MO, morpholino oligonucleotide; RRA, regulatory region A; RRB, regulatory region B; eGFP, enhanced-GFP; hpf, hours post fertilization; Ngn1, neurogenin1.

about 40–50% of human and mouse genes contain alternative promoters, a condition that seems to be required to initiate transcription in a tissue-specific manner (15–17). The use of multiple promoters, each one controlling at least one transcription start site (TSS), usually originates different 5′-UTRs that might have a role in the control of mRNA stability or translation efficiency (18, 19).

Here, we characterize three *Prrxl1* 5′-UTRs variants and the corresponding promoter regions, which may explain the differential involvement of Prrxl1 in the DRG and spinal cord development. We also present *in vitro* and *in vivo* evidence that the most evolutionarily conserved *Prrxl1* promoter region is sufficient to drive expression to neuronal cells and is regulated by Phox2b specifically in primary afferent neurons.

EXPERIMENTAL PROCEDURES

Animal Care—NMRI mice were bred and housed at the Instituto de Biologia Molecular e Celular, Porto, animal facility under temperature- and light-controlled conditions. The embryonic day 0.5 (E0.5) was considered to be the midday of the vaginal plug. The animals were euthanized (isoflurane anesthesia followed by cervical dislocation), and tissues were collected. Experiments were carried out in compliance with the animal ethics guidelines at Instituto de Biologia Molecular e Celular and approved by the Portuguese Veterinary Ethics Committee.

Wild-type AB/Tuebingen (AB/TU) zebrafish strain were maintained in the breeding colony in CABD, Seville, according to standard procedures. Fertilized eggs were kept at 28 °C in E3 medium with 0.003% 1-phenyl-2-thiourea to prevent pigmentation and were staged according to Kimmel *et al.* (20).

Reverse Transcriptase-PCR—The different 5′-UTR-*Prrxl1* molecules were amplified by reverse transcriptase-PCR (see supplemental Table S1 for primers) from spinal cord total RNA, extracted from mice at different developmental stages (E11.0, E12.5, E14.5, and E16.5) using the Micro-to-midi total RNA purification System (Invitrogen) following the manufacturer's instructions. The first-strand cDNA synthesis was prepared at 42 °C during 1 h from 1 μg of total RNA using 200 units of transcriptase enzyme (Bioline) and 500 ng of oligo(dT)$_{12-18}$ (Bioline). To assess for potential contaminants, a control containing all reagents except the reverse transcriptase enzyme was included for each sample. Normalization was performed by amplification of mouse β-actin using the primers pair listed in supplemental Table S1. The PCR conditions were the following: denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 45 s. Thirty-two cycles were performed for the amplification of *Prrxl1* 5′-UTR-B and 5′-UTR-C, 29 for 5′-UTR-A and ORF, and 20 cycles for β-actin. The amplification for each gene was in the linear curve (data not shown). Equal amounts of the PCR products were subjected to a 1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light source. The signals were acquired by a Kodak digital camera DC290, and the densitometric analyses were conducted using the computational program Kodak 1D Image Analysis Software.

In Vitro Transcription-Translation Assay—The different full-length 5′-UTR-*Prrxl1* cDNA sequences (see supplemental Table S1 for primers) were PCR-amplified from mouse E14.5 spinal cord cDNA and cloned by TA overhangs in the pCR2.1 (Invitrogen). The resulting vectors were selected for orientation and used to perform the coupled transcription-translation assay in rabbit reticulocyte lysates using the PROTEINscript® II T7 kit (Ambion). A sequence corresponding to nucleotides −1 to −50 shared by all isoforms was also cloned into the pCR2.1 plasmid and used as a control. The Prrxl1 expression was measured by Western blotting using our homemade rabbit anti-Prrxl1 antibody as described previously (6) and normalized with a mouse anti-tubulin antibody (Sigma).

Expression Vectors—Plasmids used in this work were pRSK-Brn3a (a gift from Dr. Mengqing Xiang), pcDNA3.3-Tlx3, pCAGGS-mPhox2b (a gift from Dr. Christo Goridis), pcDNA3-Islet1 (a gift from Dr. Chunyan Zhou), pcDNA3.1-His-Ngn1 (a gift from Dr. Soyeon Kim) pcDNA3.3-Lmx1b, and pCAGGS-FLAG-Mash1 (a gift from Dr. Diogo S. Castro). The sequences corresponding to the Tlx3 and Lmx1b open reading frame were amplified from mouse E14.5 spinal cord cDNA (for primers see supplemental Table S1) and cloned in the pCDNA3.3-TOPO TA cloning vector (Invitrogen). Protein expression in transfected cells was assessed by Western blotting using the antibodies mouse anti-Brn3a (Santa Cruz Biotechnology), rabbit anti-Tlx3 (Santa Cruz Biotechnology), rabbit anti-Phox2b (a gift from Dr. Qiufu Ma), mouse anti-Islet-1 (40.2D6, Developmental Studies Hybridoma Bank), mouse anti-polyhistidines (Sigma), rabbit anti-Lmx1b (a gift from Dr. Thomas Müller), and mouse anti-FLAG (Sigma).

For the mRNA stability assays, the different 5′-UTR-specific sequences were amplified from E14.5 mouse spinal cord cDNA and cloned by TA overhangs in the pCR2.1 plasmid (Invitrogen). These sequences were then subcloned in the HindIII site of the pGL3-Control vector (Promega) between the SV40 promoter and the luciferase coding region.

To determine the *Prrxl1* alternative promoters, the entire and overlapping fragments of the −1401/−50-bp region upstream of the start codon were PCR-amplified (for primers see supplemental Table S1) from mouse genomic DNA and cloned in the pBlue-TOPO vector (Invitrogen). The sequences were then subcloned in the HindIII site of the promoter-less vector, pGL3-Basic (Promega).

Site-directed mutagenesis of TATA box and HD element were performed from pGL3-REG1 using the QuikChange Site-directed Mutagenesis kit from Stratagene and following the manufacturer's instructions. Primers used are depicted in supplemental Table S1.

Cell Culture—ND7/23, HeLa, HEK293, and PC12 cell lines were maintained and grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 50 units/ml penicillin and streptomycin (Invitrogen). All cells were kept at 37 °C and 5% CO_2 gas phase. Transfection was performed using Lipofectamine2000 agent (Invitrogen), and 24 h later cells were harvested for posterior analysis. Overexpression in differentiated PC12 cells was performed by transfecting PC12 cells, and 6 h later differentiation was induced with 100 ng/ml NGF (Sigma). Luciferase reporter assays were performed 2 days after transfection. To infer the mRNA stability, 24 h after transfection, 100 μg/ml actinomycin D (A9415, Sigma) was added to DMEM

(Invitrogen), and luciferase activity was measured 0, 3, and 6 h later.

DRG Primary Culture and Cell Electroporation—DRG were extracted from newly born mice and, after a 2-h treatment with 10% collagenase (Sigma), were electroporated using the Neon™ Transfection System (Invitrogen) according to the manufacturer's instructions. Afterward, cells were cultured in polyornithine-coated wells in DMEM-F-12 (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 2 mM glutamine (Invitrogen), 4% Ultroser G (Pall), 1× B27 (Sigma), and 10 ng/ml NGF (Sigma). Twenty-four hours later the cells were harvested and processed for luciferase reporter assays.

Luciferase Reporter Assays—Transfected cells from a 96-well plate format were resuspended in 50 μl of lysis buffer (Promega), and the protein extract was cleared by centrifugation. 5 μl of the extract were mixed with the luciferase reagent (Promega), and the signals were measured using a luminometer reader (Tecan). Transfection efficiency was normalized by assessing the β-galactosidase activity using 2-nitrophenyl β-D-galactopyranoside (Sigma) as substrate.

Electrophoretic Mobility Shift Assay—Recombinant TATA-binding protein (sc-4000, Santa Cruz Biotechnology, Inc.) was incubated with 50 fmol of the double-stranded oligonucleotide TATA containing the TATAbox promoter sequence (5′-TTATGCGTGAGAT**TATA**AAGGCGAGTGCTGAGCGGCGGCGCGCGCTG-3′) and end-labeled with a dyomic dye DY682 (Thermo Scientific) in a buffer containing 12 mM Tris-HCl, pH 8.0, 0.15 mM EDTA, 6 mM $MgCl_2$, 90 mM KCl, 1 mM DTT, 10% glycerol, 0.5 mg/ml BSA, and 0.4 μg/μl poly(dI/dC) (adapted from Riquet *et al.* 21). Competition experiments were performed using a non-labeled oligonucleotide with the same sequence. For supershift experiments, 200 ng of the anti-TBP antibody (sc-273, Santa Cruz Biotechnology, Inc.) were used. All samples were run in a 5% PAGE with 10 mM $MgCl_2$.

Nuclear proteins were extracted from ND7/23 cells previously transfected with pcDNA3.3, pcDNA3.3-Tlx3, pCAGGS-mPhox2b, or pRSK-Brn3a using first a low salt lysis buffer (30 mM Tris-HCl, pH 7.8, 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 0,1% Triton X-100, and proteases and phosphatases inhibitors cocktails). After nuclear fractionation, proteins were resuspended in 10 mM Tris-HCl, pH 7.5, 60 mM KCl, 200 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and 0.1% Triton X-100, cleared by centrifugation, and incubated with 50 fmol of the double-stranded oligonucleotide HD (5′-CTGGAAA**TAAT**CAG**ATTA**AGGC-3′) end-labeled with dyomic dye DY682. The samples were run in a 5% polyacrylamide electrophoresis get. The fluorescent signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Chromatin Immunoprecipitation Assays—For TATA-binding protein (TBP) chromatin immunoprecipitation (ChIP) assays, dorsal spinal cords from E14.5 mouse embryos were dissected and fixed with 2 mM di(*N*-succinimidyl) glutarate (Sigma) in phosphate buffer saline (PBS) for 45 min followed by 1% formaldehyde in PBS for 10 min and lysed in 50 mM Tris-HCl, pH 8.0, 1% SDS, and 10 mM EDTA. Chromatin shearing was performed using Bioruptor (Diagenode) at high power settings for 60 cycles (30 s on/30 s off). ChIP assays with or without (mock control) mouse monoclonal anti-TBP antibody (Mab-002–100, Diagenode) were performed using 80 μg of chromatin/assay in ChIP buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 5 mg/ml BSA), and protease inhibitor mixture (Roche Applied Science). Immunoprecipitates were retrieved with 50 μl of Protein G Dynabeads (Invitrogen) per assay and washed once with wash buffer I (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with wash buffer II (20 mM Tris, pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), twice with wash buffer III (10 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate), and once with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and eluted with lysis buffer at 65 °C for 10 min. Eluted and input chromatin were subjected to proteinase K (Roche Applied Science) treatment for 2 h at 42 °C and reverse-cross-linked at 65 °C overnight. Immunoprecipitated and input DNA samples were purified by phenol-chloroform extractions followed by isopropyl alcohol precipitation. DNA sequences were quantified by real-time PCR (primers are listed in supplemental Table S1) using a StepOnePlus Real Time PCR system (Applied Biosystems) and a SYBR Green chemistry for quantitative PCR (Maxima master mix, Fermentas). Quantities of immunoprecipitated DNA were calculated by comparison with a standard curve generated by serial dilutions of input DNA. Data were plotted as the means of at least two independent ChIP assays and three independent amplifications; *error bars* represent S.E.

Phox2b ChIP assays were performed essentially as described above, with the following modifications: (i) chromatin samples were extracted from dorsal medulla oblongata of E14.5 mouse embryos; (ii) mouse monoclonal anti-Phox2b antibody (sc-376997, Santa Cruz Biotechnology) was used; (iii) NaCl concentration of ChIP buffer was reduced to 20 mM; (iv) immunoprecipitates were washed 6 times with wash buffer III containing only 0.7% sodium deoxycholate.

Production of Transgenic Zebrafish—A sequence (−751/−584 bp) that includes the P3 core promoter was PCR amplified from mouse genomic DNA, cloned in the pCR/GW/TOPO vector (Invitrogen), and then recombined by the Gateway *in vitro* recombination technology using the Gateway LR Clonase II Enzyme mix (Invitrogen) into a Tol2 vector (22) containing an *iroquois* enhancer with midbrain activity (Z48; Refs. 23 and 24) and the enhanced GFP reporter gene. This vector was assembled by cloning a SalI/NotI fragment of pCS2eGFP (25) containing the CMV promoter, the enhanced GFP reporter gene, and the poly(A) of SV40 into SalI/NotI restriction sites of a modified pminiTol2/MCS vector (26) that has a fragment of the pUC19 polylinker that goes from EcoRI to HindIII. Z48 *iroquois* enhancer was isolated from the Z48 TOPO vector (23) by cutting with EcoRI and cloned into NotI restriction site after blunting with Klenow. Finally, a gateway cassette (Invitrogen) was cloned in blunt between the SalI/BamHI restriction sites, replacing the CMV promoter. This vector lacks a promoter and does not drive GFP reporter expression. Therefore, it is useful to test for promoter activity of selected DNA sequences. The following primers were used for amplification: 5′-TAAGGCCCAATAGACCTATC-3′ and 5′-CAGGACCAGAGAAGTGACTG-3′). About 5 nl of the reaction mix containing 50 ng/μl transposase mRNA, 50 ng/μl phenol/chloroform purified vec-

tor, and 0.05% phenol red were injected in the cell of one-stage zebrafish embryos. The GFP expression was then documented from the next 24 to 72 h.

The fragment containing the Ebox and the HD element was PCR-amplified from human genomic DNA, cloned in the pCR/GW/TOPO plasmid (Invitrogen), and then recombined to the ZED vector (23) by the Gateway *in vitro* recombination technology described above. The following primers were used for amplification: 5′-GTGGTGGTTGTATCGTTCTC-3′ and 5′-GCATAATTGGCCTTAATCTG-3′. The injections were performed as described above. Positive transgenic embryos strongly expressing red fluorescent protein were selected 72 h later. Those F0 embryos were raised, and the F1 generation of embryos expressing GFP was analyzed.

RNA Probe Synthesis and Whole-mount in Situ Hybridization—The *drgx* and *tlx3b* sequences were amplified from zebrafish cDNA using the following primers: 5′-ATGTTTTACTTTCACTGTCCTCCA-3′ and 5′-CATTTCTTATCCGGACCCTC-3′ for *drgx* and 5′-TTCGGGTGGTGAGGATGGAC-3′ and 5′-GATTTTGGGATGCAACAGCA-3′ for *tlx3b*. PCR products were cloned in the pGEM-T Easy vector (Promega), and a phenol-chloroform purification was performed after linearization with NsiI (for drgx) or NcoI (for tlx3b). Each vector was used as a template for the *in vitro* synthesis of a DIG-labeled RNA probe for zebrafish *drgx* and *tlx3b*.

Wild-type embryos at 48 and 72 h post fertilization were fixed overnight with 4% paraformaldehyde at 4 °C. After brief washes with PBS, 0.1% Tween 20 (PBST), they were treated with 10 μg/ml proteinase K and fixed for 20 min with 4% paraformaldehyde at room temperature. After a 20-min wash with PBST, embryos were incubated in hybridization buffer for 1 h at 70 °C. The respective RNA probe was then added to the hybridization buffer (50% formamide, 2× SSC, and 0.1% Tween 20) to a final concentration of 1 ng/μl, and embryos were incubated overnight at 70 °C. The next day embryos were sequentially washed at 70 °C in solutions containing different concentrations (75, 50, 25, and 0%) of hybridization buffer diluted in 2× SSC (75 mM NaCl and 7.5 mM sodium citrate, pH 7.0). After a further wash with 0.05× SSC for at least 1 h at 70 °C, embryos were finally washed with PBST for 10 min at room temperature. Embryos were blocked in 2% normal goat serum/PBST for at least 1 h at room temperature. Anti-digoxigenin coupled with alkaline phosphatase was added in fresh 2% normal goat serum, PBST (1:5000) for 2 h and then allowed to wash overnight in PBST. The next day embryos were washed with AP reaction buffer without $MgCl_2$ (100 mM Tris, pH 9.5, 100 mM NaCl, and 0.1% Tween 20). Detection was performed with 3.5 μl of nitro blue tetrazolium (50 mg/ml) and 1 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml) per 1 ml of complete AP-reaction buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, and 0.1% Tween 20). The signal was allowed to develop for 3–5 h.

Antisense Morpholino Oligonucleotide (MO) Analysis—Antisense MO targeted to the translation initiation site of Phox2b (CATTGAAAAGGCTCAGTGGAGAAGG) was obtained from Gene Tools, LLC, diluted to a working concentration in Milli-Q water (0.4 ng/nl) with 0.05% phenol red, and about 5 nl were injected into 1- to 2-cell-stage embryos.

Statistical Analysis—In the present study, all the data presented (except for ChIP experiments; see above) were derived from at least three independent experiments with three replicates. When necessary, a two-tailed *t* test was performed. Both mean and S.D. values were calculated and included in the figures.

RESULTS

Prrxl1 5′-UTR Variants Present Distinct mRNA Stability and Translation Efficiency—Prrxl1 is a transcription factor first identified by Saito (27) in a subtractive hybridization screening with rat DRG. The *Prrxl1* mRNA sequence described by these authors contained the start codon in exon 2, whereas exon 1 corresponded to a 5′-UTR. More recently, by the use of 5′-rapid amplification of cDNA ends assays with mice spinal cord RNA extracts, we described two novel variants of *Prrxl1* containing alternative exon 1 that gives rise to distinct 5′-UTRs (11). BLAST searches of GenBank™ database led us to identify sequences that correspond to all the three 5′-UTRs that contained both the first and the last coding exons of the annotated *Prrxl1*. We named these *Prrxl1* 5′-UTR variants 5′-UTR-A, 5′-UTR-B, and 5′-UTR-C, which encompass, respectively, nucleotides −622 to −485, −484 to −298, and −148 to −85 relative to the start codon (Fig. 1*A*).

To assess for the presence of the different *Prrxl1* 5′-UTR variants, we performed reverse transcriptase-PCR experiments using a common reverse primer mapping within exon 7 (containing the stop codon), shared by all isoforms, and isoform-specific forward primers mapping within alternative exon 1 (*arrows* in Fig. 1*A*). With this primer design, we wanted to make sure that the amplicons contained the entire coding region and to exclude from our analysis other putative non-annotated *Prrxl1* splicing variants. The three transcripts are detected in the spinal cord at developmental stages where Prrxl1 expression has been previously reported (6). The levels of amplification are suggestive that 5′-UTR-A variant is the most abundant transcript, being detectable three PCR cycles earlier than the other two 5′-UTR transcripts (Fig. 1*B*). The semiquantitative reverse transcriptase-PCR analysis with spinal cord extracts from E11.0 to E16.5, a period that includes the early-born (E10.5-E12.0) and late-born (E12.5-E14.5) neurogenesis waves, showed that 5′-UTR-A reaches the maximum level at E14.5, a profile similar to the ORF (Fig. 1*B*). On the other hand, the highest expression levels of 5′-UTR-B and 5′-UTR-C are reached at E12.5 (Fig. 1*B*), which is suggestive of a more preponderant role of these two isoforms during early neurogenesis.

Prrxl1 5′-UTR variants result from alternative processing of non-coding exon 1 and, therefore, have no consequences in the *Prrxl1* coding region. Because mRNA-untranslated regions have been associated to post-transcriptional regulation mechanisms (18, 19), we wondered if 5′-UTR sequences confer distinct mRNA stability or translation efficiency to *Prrxl1* transcripts. To address this question, we performed coupled transcription-translation *in vitro* assays using different vectors containing the *Prrxl1* ORF associated to each 5′-UTR under the control of the bacterial T7 RNA polymerase. The amount of Prrxl1 translated was determined by Western blotting (Fig. 1*C*). *Prrxl1* transcript containing 5′-UTR-A was about three times

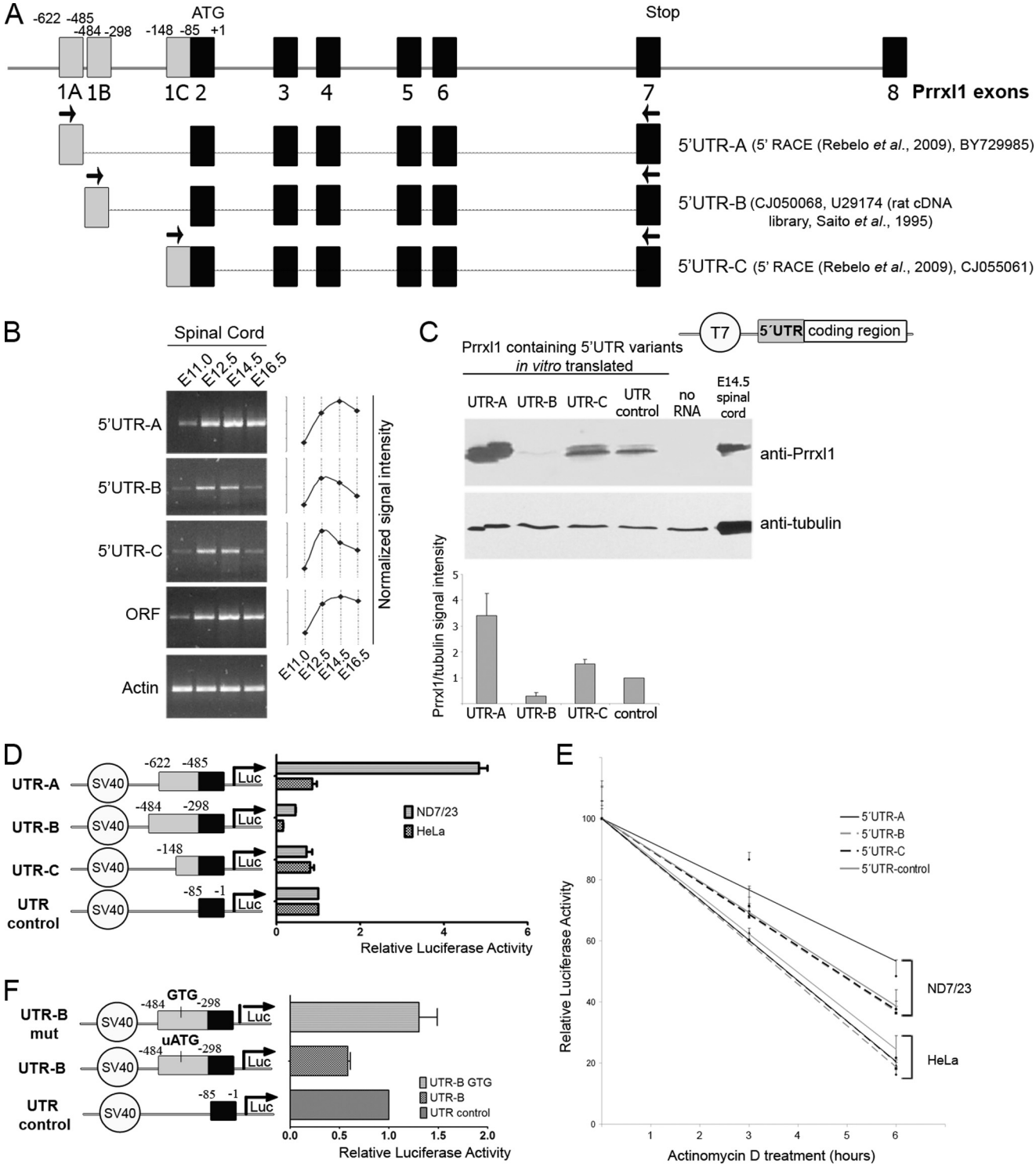

FIGURE 1. **Differential expression and stability of *Prrxl1* 5′-UTR variants.** *A*, scheme of the *Prrxl1* gene. The gene gives rise to three alternative transcripts that are composed by eight exons and differ on their untranslated first exon (*1A*, *1B*, or *1C*). Those untranslated regions were named 5′-UTR-A, 5′-UTR-B, and 5′-UTR-C. *B*, expression studies of *Prrxl1* transcripts containing different 5′-UTRs (5′-UTR-A, 5′-UTR-B, and 5′-UTR-C) and *Prrxl1* ORF by reverse transcriptase-PCR and gel electrophoresis analysis using mouse spinal cord at different developmental ages (E11 to E16.5). The graph illustrates a typical expression profile, normalized with β-actin signal intensity, from three independent experiments. *C*, Western blot analysis of Prrxl1 *in vitro* translated from mRNA containing distinct 5′-UTR and transcribed by T7 RNA Polymerase. The graph represents the mean of signal intensity (normalized with β-tubulin) from three independent experiments. *D*, luciferase reporter assays in ND7/23 and HeLa cells transfected with vectors containing the different 5′-UTR fused to the luciferase encoding gene. *E*, analysis of the stability of the luciferase mRNA molecule conferred by the distinct 5′-UTR. The constructs used in *D* were used to transfect the ND7/23 and HeLa cell lines. Transcription was halted using actinomycin D and luciferase activity measured at different time points (0, 3, and 6 h of treatment). *F*, comparison of luciferase expression regulated by different versions of 5′-UTR-B. 5′-UTR-B contains an upstream ATG (*uATG*), which was replaced by a GTG sequence (*5′-UTR-B mut*). In *C–E*, 5′-UTR control represents a fragment encompassing nucleotides −1 to −85 and shared by all isoforms.

more efficiently translated than the UTR control (a fragment immediately upstream of the start codon encompassing nucleotides −1 to −85 and shared by all isoforms). On the contrary, 5′-UTR-B led to a loss of translational rate, whereas 5′-UTR-C was as expressed as the 5′-UTR control. Similar results were obtained in luciferase reporter assays using the ND7/23 cell line transfected with each 5′-UTR cloned between the SV40 promoter and the firefly luciferase coding region (Fig. 1*D*). The ND7/23 cells are an appropriate *in vitro* model for the study of Prrxl1-associated mechanisms as they endogenously express this transcription factor and display a phenotype characteristic of nociceptive neurons (28). Interestingly, the increased luciferase activity of the 5′-UTR-A construct observed in ND7/23 cells was not observed in the non-neuronal HeLa cell line. To test if the neuron-specific activity induced by 5′-UTR-A could be due to an increase in the transcript stability, mRNA decay associated to each 5′-UTR variant was inferred by measuring the luciferase activity at different time points upon treatment with actinomycin D in both cell lines (Fig. 1*E*). All the different 5′-UTRs promoted a similar mRNA decreasing rate in HeLa cells. In ND7/23 cells, 5′-UTR-B- and 5′-UTR-C-containing transcripts displayed decay similar to the control, whereas the 5′-UTR-A was more stable. This result suggested that the 5′-UTR-A sole effect, both in the mRNA translation rate and stability, is conferred by a neuronal context likely mediated by specific RNA-binding proteins.

On the contrary, the 5′-UTR-B variant reduced the *in vitro* translation efficiency (Fig. 1*C*) and protein expression both in ND7/23 and in HeLa cells (Fig. 1*D*) without interfering with mRNA stability (Fig. 1*E*). A careful analysis of the 5′-UTR-B sequence led us to the identification of an ATG located upstream (*uATG*) of the *Prrxl1* main ATG that could be mistaken as an alternative start codon, modifying the reading frame and thereby explaining the feature of this variant (Fig. 1*F*). Upstream ORFs are widely recognized as cis-regulatory elements that can affect mRNA translation and thus are the molecular base of severe disorders (29, 30). By changing this upstream ATG to GTG, the luciferase activity of the mutated 5′-UTR-B increased to values similar to the control sequence (Fig. 1*F*), suggesting that 5′-UTR-B sequence works as a negative modulator of Prrxl1 expression levels. 5′-UTR-C did not confer any particular trait to the mRNA molecule.

Identification of Prrxl1 Alternative Promoter Regions and Evolutionarily Conserved Regulatory Elements—The existence of TSSs specific to each *Prrxl* 5′-UTR suggested that *Prrxl1* expression is controlled by a mechanism of alternative promoter usage. To identify these promoter regions and further dissect the mechanisms of *Prrxl1* regulation, we selected a region of 1351 bp (−1401/−50) upstream of the *Prrxl1* translation initiation site (+1) based on the high degree of conservation observed in a genomic alignment of homologous region in various species (human, chick, *Xenopus tropicalis*, and zebrafish) (Fig. 2). This sequence (named REG-1) was amplified and cloned into the promoter-less pGL3-basic vector. Upon transient transfection into mouse DRG primary cell culture and neuronal derived ND7/23 cells, the luciferase reporter gene expression was activated indicating the presence of promoter activity in the cloned region (Fig. 2). We then evaluated the luciferase activity of shorter overlapping sequences (named REG-2 to REG-16) in ND7/23 cells. When the entire fragment was divided in two (REG-2 and REG-11), we verified that both sequences were able to drive the transcription of the luciferase gene. Because we used a promoter-less vector, this result indicated that each fragment harbored at least one promoter. The reduction of the most distal fragment (REG-2) from its 5′ end (REG-3 to REG-7) led to the identification of a minimum sequence displaying transcriptional activity (REG-7, −772/−584). This sequence is adjacent to 5′-UTR-A TSS and, therefore, was considered to be a promoter region. The same analysis was performed for the fragment most proximal to the Prrxl1 start codon (REG-11), and two minimal fragments eliciting luciferase activity (REG-13, −622/-481 and REG-14, −157/−50), located in the vicinity of the TSS of 5′-UTR-B and 5′-UTR-C, were considered as promoter regions. By this analysis, we identified three alternative promoters, named P1 (−85/−157), P2 (−485/−604), and P3 (−622/−772) that likely control, respectively, the transcription of Prrxl1 5′-UTR-C, 5′-UTR-B, and 5′-UTR-A variants.

Some fragments, even though containing promoter regions, did not display any transcriptional activity (REG-6 and REG-9) (Fig. 2). When these fragments were shortened (REG-7 and REG-10), the luciferase activity increased to values closer to fragments of similar size, which is suggestive of the existence, in the region encompassing nucleotides −811/−772, of a regulatory element (termed regulatory region A (RRA)) with the capacity to strongly suppress the transcription of the three alternative promoters. Interestingly, by the 5′ expansion (REG-4 and REG-8) of the fragments containing the RRA, the luciferase activity increased, again revealing the presence of a new regulatory element in the region −891/−922 (termed Regulatory Region B, RRB), with the potential to inhibit the action of the repressive motif RRA and consequently to activate *Prrxl1* transcription.

It is also important to note the presence of transcriptional regulatory elements located in the sequence between −1401 and −958 bp. The abrogation of this region, which originates REG-8 construct (−958/−50), resulted in a strong decrease (about 75%) in the luciferase activity when compared with the full sequence (REG-1). Given that the deletion of the region between −1401 and −958 bp did not significantly alter the reporter activity of fragments containing P3 as sole promoter (compare the activity of REG-2 with REG-3 and REG-4 in Fig. 2), we concluded that these elements are required for transcription driven by promoters P1 and P2 rather than for the P3 promoter activity.

Because Prrxl1 expression has only been detected in neuronal tissues, namely all sensory ganglia and second order relay sensory neurons (6), we found it pertinent to investigate whether the promoter regions here identified could also drive transcription in non-neuronal cell models. Thus, we performed similar luciferase reporter assays using HeLa and HEK293 cells and compared the results with those obtained for the ND7/23 cells (Fig. 3*A*). The longer sequence in analysis (REG-1) exhibited the capacity to drive the luciferase transcription 10 (HeLa) to 15 (HEK293) times lower than when transcription was promoted in the neuronal-derived cells ND7/23 (Fig. 3*A*). An

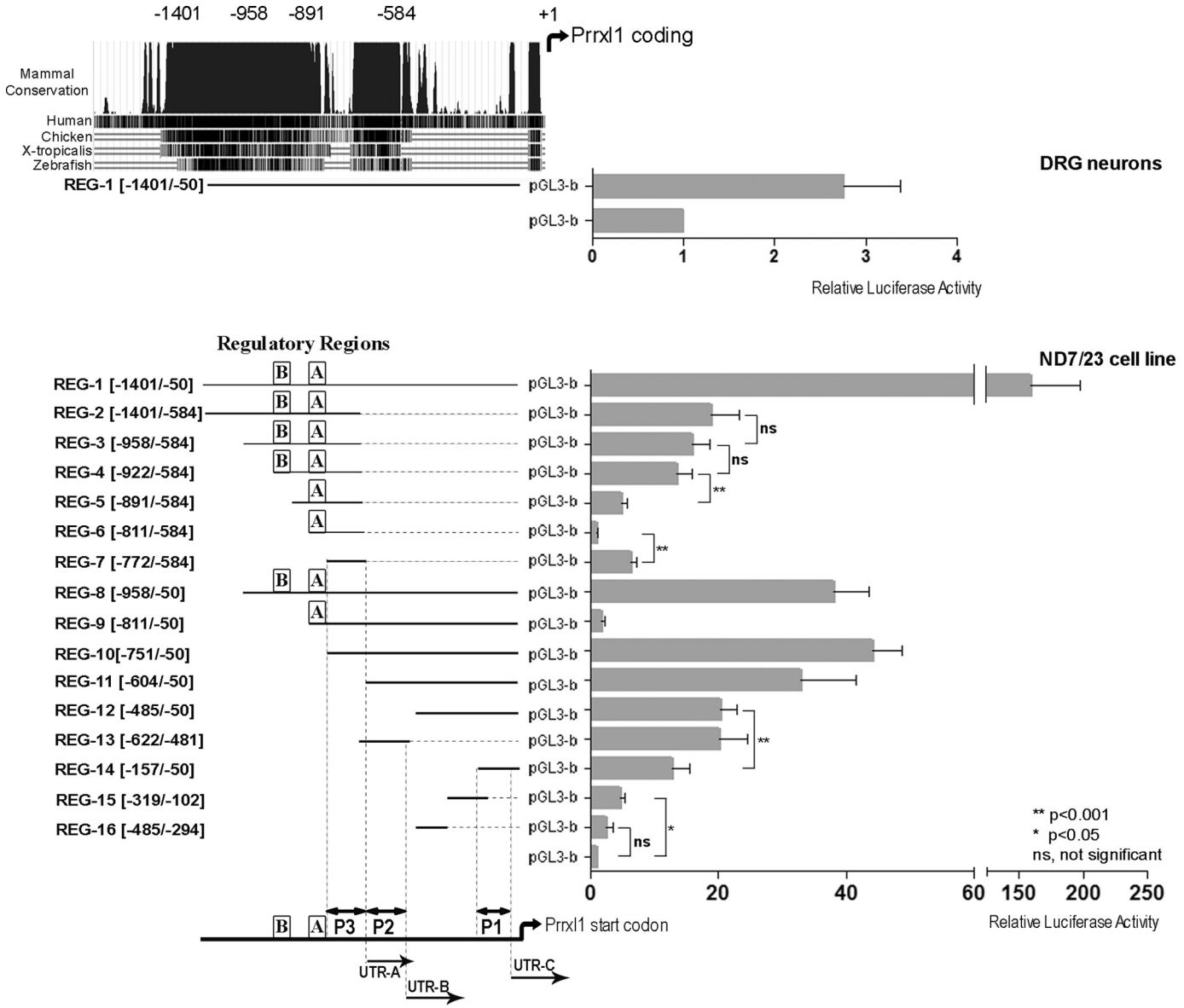

FIGURE 2. **Identification of regulatory cis-elements involved in the modulation of *Prrxl1* transcription.** Genomic alignment using the UCSC Genome Browser of the 1401-bp sequence upstream of Prrxl1 coding region from evolutionarily distant species (human, chick, *X. tropicalis* and zebrafish) is shown. *Black peaks* correspond to mammal conservation. Luciferase reporter assays using the −1401/−50-bp region were performed in mouse E15.5 DRG primary cultures and ND7/23 cells to test its transcriptional capacity. By successive deletion analysis from the longer region, three regions displaying promoter activity (termed P1, P2, and P3) were identified as well as two regulatory regions (*A* and *B*). Each promoter is located in the vicinity of the transcription start site of Prrxl1 5′-UTR.

individual analysis of each promoter and its correspondent luciferase activity demonstrated a neuron-specific activity for promoters P1 (REG-14) and P3 (REG-7), as no relevant activity in the HeLa and HEK293 cells was detected. On the other hand, promoter P2 still led to some expression of the reporter enzyme in non neuronal cells. We assume that the potential displayed by promoter P2 to drive transcription in these cells is probably responsible for the transcriptional activity exhibited by the whole fragment (REG-1) due to the presence of constitutive regulatory elements (see below).

Additionally, both regulatory regions RRA and RRB appeared to act exclusively in the neuronal model, as little differences in the transcriptional activity of the fragments REG-8 (−958/−50) and REG-9 (−811/−50) in comparison to REG-10 (−751/−50) were observed in HeLa and HEK293 cells (Fig. 3*A*).

Interestingly, a careful search for chromatin modifications in the human REG-1 sequence (chr10:50,603,551–50,604,877; GRCh37/hg19 assembly) using the UCSC Genome Browser and ENCODE annotations (31) of ChIP-seq assays revealed trimethylation in the lysine 4 of the histone H3 (H3K4me3) in all the three promoter regions (Fig. 3*B*). This is a chromatin signature of promoters actively transcribing protein-coding genes (32, 33) and was detected in human embryonic stem cells (H7/H1-hESC) and in different types of neuronal cells, such as a neuroblastoma cell line (SK-N-SH) and neurons derived from embryonic stem cells (H1-neurons) but not in HeLa cells (Fig. 3*B*). Moreover, binding events for RNA Polymerase II, TBP, and the enhancer-associated protein P300 (34, 35) are also detected in the *Prrxl1* promoter regions in H1-neurons, H1-hESC, and SK-N-SH cells but not in HeLa cells. Note that

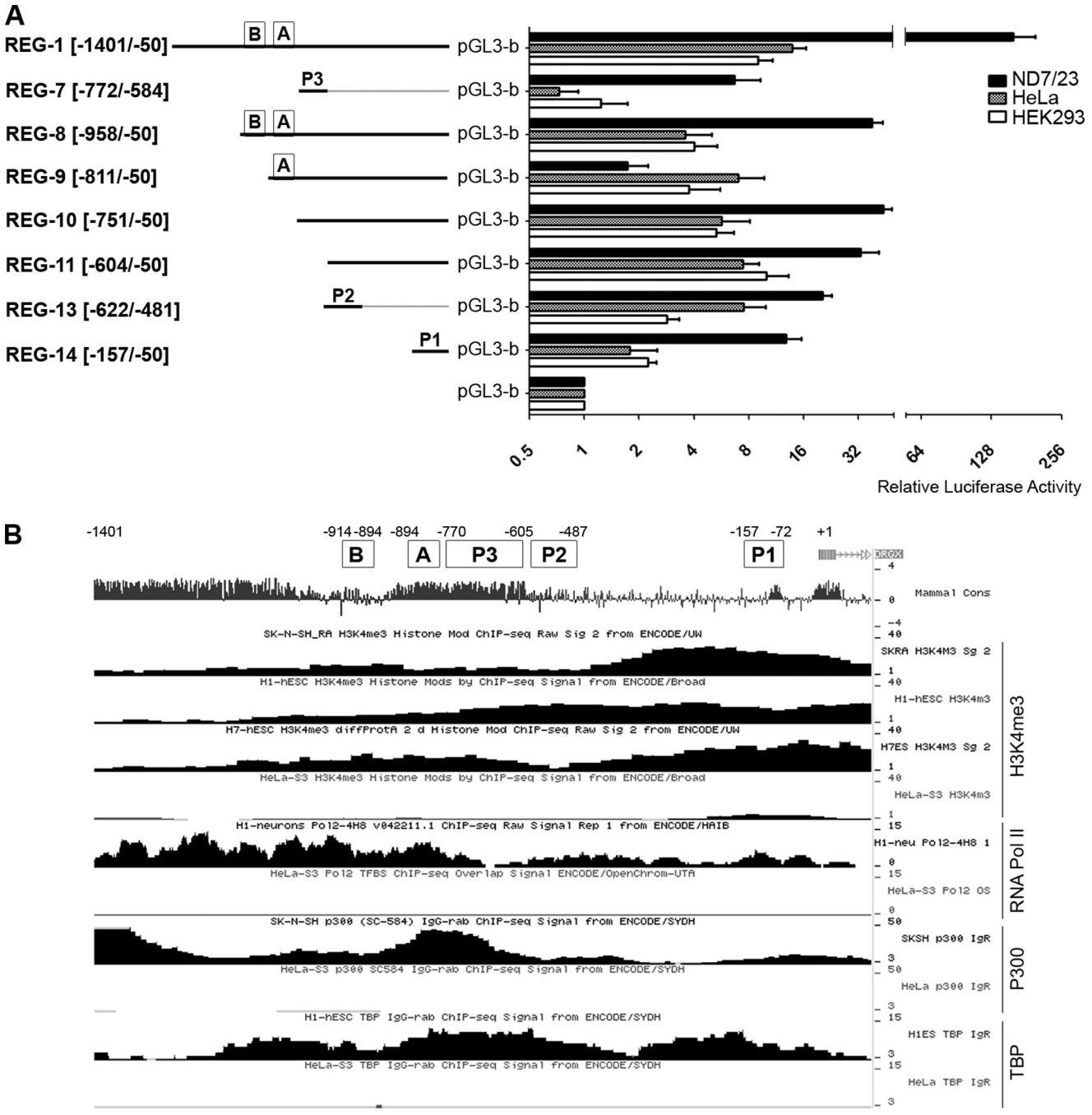

FIGURE 3. **Dependence on neuronal context for the transcriptional activity of *Prrxl1* alternative promoters.** *A*, the luciferase activity induced by some selected fragments was compared in ND7/23 and the non-neuronal HeLa and HEK293 cells. P1 and P3 only promote significant transcription in neuronal-derived cells, whereas P2 exhibits activity in the three cell lines. The repressive or activator transcriptional effect induced by the regulatory elements A and B in ND7/23 cells was no longer observed in HeLa and HEK293 cells. *B*, the presence of binding peaks of H3K4me3, RNA Polymerase II, P300, and TBP on the *Prrxl1* promoters was specifically detected in neuronal cells, using the UCSC genome Browser and ENCODE annotations of ChIP-seq assays.

the three-peak pattern observed with RNA Polymerase II and P300 overlaps with the three alternative promoter regions (Fig. 3*B*). These data reinforce our previous observation that *Prrxl1* promoters displayed neuron-specific activity.

The Promoter P3 Displays Neuron-specific Activity—Nucleotide alignment of *Prrxl1* alternative promoter sequences from different species revealed that the region comprising promoter P3 presents a high degree of conservation, from zebrafish to human, whereas promoters P1 and P2 appeared to be specific to mammals (Fig. 4). We screened these sequences to identify conserved DNA binding elements for transcription factors using the bioinformatics prediction tool MatInspector from Genomatix. Some putative motifs known to be important for transcriptional regulation during embryonic development were identified.

Promoter P1 has no evident conserved motifs, whereas promoter P2 contains a GC box element (Fig. 4), known to be a binding site for Sp1, Sp3, and Sp4 transcription factors (36). CpG islands are elements often associated with the transcription of genes whose expression is ubiquitous and feature >50%

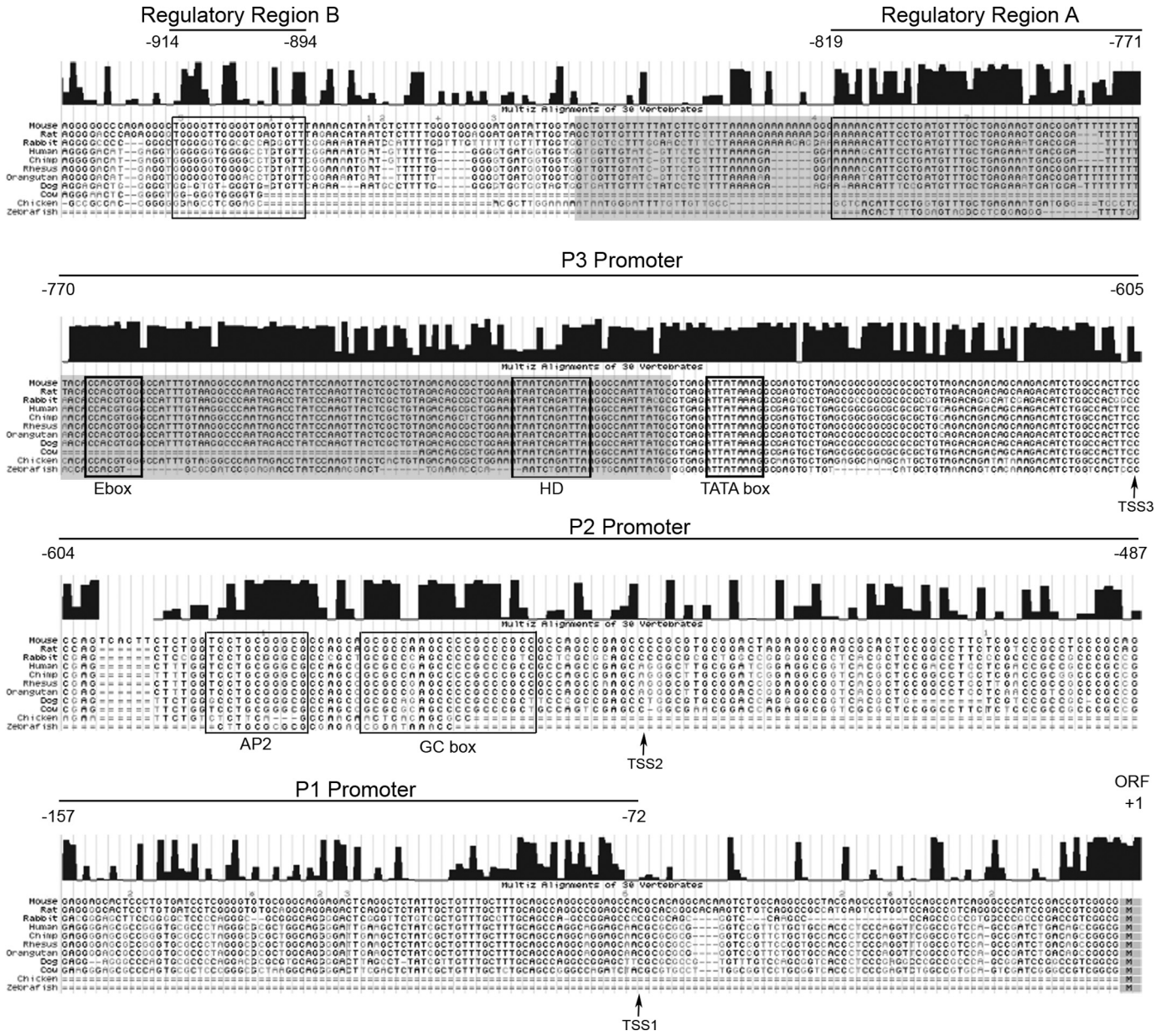

FIGURE 4. **Nucleotide alignment of the regulatory regions A and B and the *Prrxl1* alternative promoters.** The nucleotide alignment was performed using the UCSC Genome Browser and sequences from several mammals, chick, and zebrafish. The *boxes* that delimit the regulatory regions A and B are depicted. P3 is the most conserved promoter and contains binding sites for the TFIID complex (*TATA box*), bHLH (*Ebox*), and homeodomain transcription factors (*HD*). P2 and P1 are only present in mammals. P2 contains putative motifs for the binding of SP1-like family transcription factors (*GC box*) and for the activation protein 2 (*AP2*). The TSSs of each 5′-UTR are also represented. TSS1 corresponds to 5′-UTR-C, TSS2 to 5′-UTR-B, and TSS3 to 5′-UTR-A. Binding sites were predicted by the bioinformatics tool MatInspector (Genomatix). *Black peaks* represent mammal conservation. The *gray box* highlights the human sequence used to produce the zebrafish transgenic line (see Fig. 6). Not to scale.

of vertebrate regulatory sequences (37). The presence of this GC-box may explain the activity displayed by the promoter P2 in HeLa cells (Fig. 3*A*). Moreover, a conserved binding site for the activator protein 2 family was also predicted on the P2 promoter (Fig. 4). Activator protein 2 transcription factors are general regulators of vertebrate development, controlling the balance between proliferation and differentiation during embryogenesis (38, 39).

Analysis of the P3 sequence highlighted a putative TATA motif, a conserved element of some basal promoters (Fig. 4). To test if this sequence could bind a TBP, the binding of a recombinant TBP to an oligonucleotide spanning the P3 TATA motif present in the promoter P3 was tested by EMSA (Fig. 5*A*). A gel shift, which was impaired by the use of competitor oligonucleotides, was observed, demonstrating the specificity of the binding to this region (Fig. 5*A*). The incubation with an antibody directed to TBP resulted in a supershift (*arrow* in Fig. 5*A*), strongly indicating that P3 is a TATA box-containing promoter. The *in vivo* association of the TBP to the TATA motif of the P3 sequence was tested in mouse embryonic spinal cord by performing ChIP assays combined with real-time PCR (Fig. 5*B*). In these experiments a clear enrichment in the binding of the TBP was observed in the P3 region comprising the TATA motif compared with the

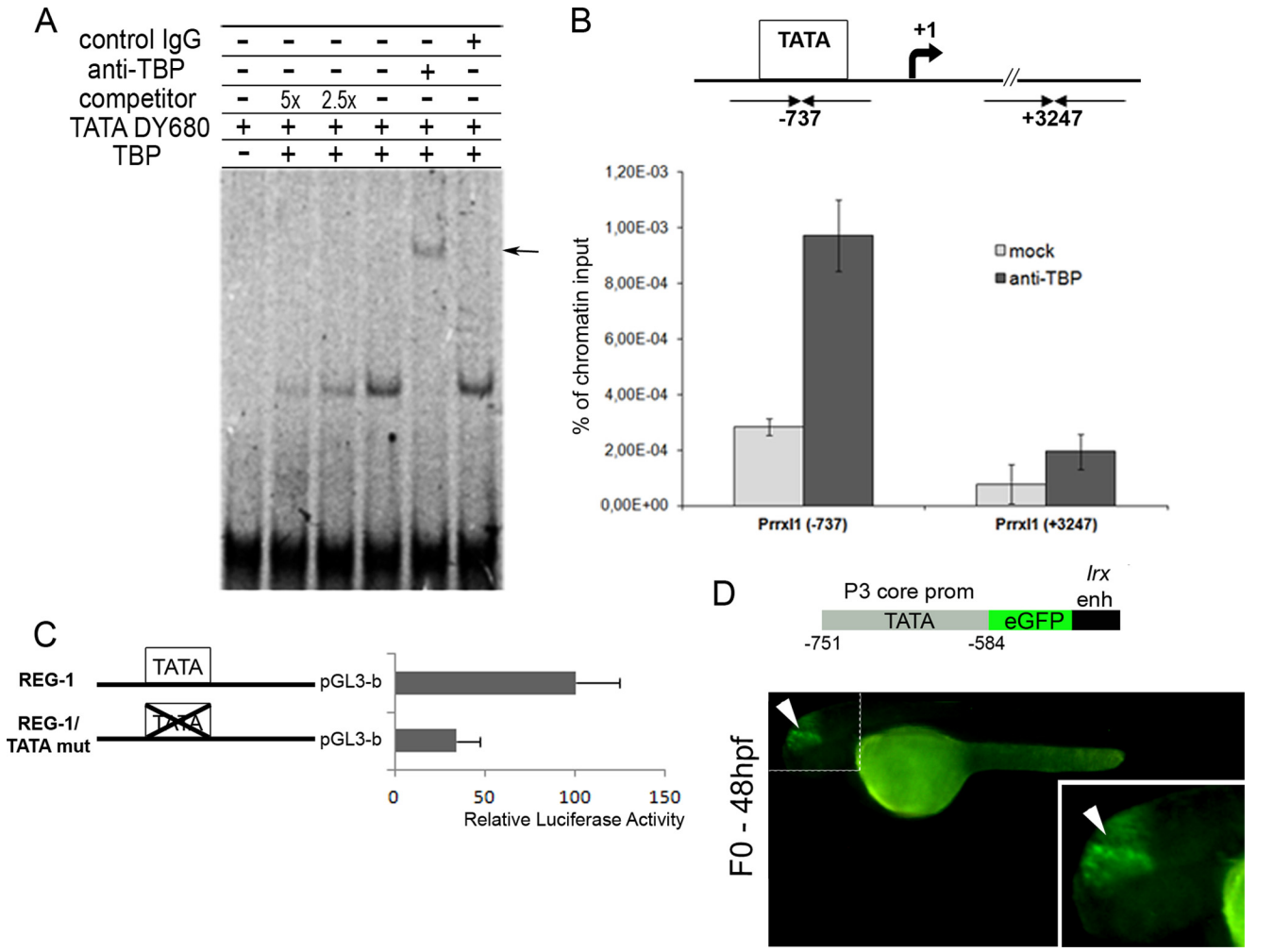

FIGURE 5. **Validation of P3 as a TATA-containing promoter.** *A*, electrophoretic mobility shift assay using recombinant TBP and a fluorescent probe (*TATA DY680*) corresponding to a region of P3 with the TATA box motif. A shift, corresponding to a complex formed between TBP and the probe, was detected, and decreases of intensity when different amounts (2.5× and 5×) of non-labeled competitor were mixed. The supershift (*arrow*) represents the binding of anti-TBP to the protein-DNA complex. *B*, chromatin immunoprecipitation-quantitative PCR assays with (*anti-TBP*) or without (*mock*) an anti-TBP antibody were performed using dorsal spinal cord chromatins from E14.5 mouse embryos followed by quantitative PCR using primers targeting P3 region (Prrxl1 −737) and a downstream region (Prrxl1 + 3247). Enrichment is observed in the region where the TATA box is present (−737). A region corresponding to *Prrxl1* intron was used as control (+3247). *Mock* represents the condition without antibody. *C*, the evaluation of the functional importance of the TATA box for the transcriptional potential of the fragment REG-1 was assessed by luciferase reporter assays in ND7/23 cells, comparing the REG-1 fragment with a sequence containing a mutated TATA motif (REG-1/TATA mut). *D*, to evaluate the ability of the P3 core promoter to activate transcription in zebrafish, a region encompassing nucleotides −584/−751 bp was cloned upstream to an *Irx* (*Iroquois*) enhancer, which drives expression to the zebrafish midbrain (*white arrowheads*). The vector was injected in zebrafish embryos at one-cell stage, and 48 hpf GFP signal was recorded.

downstream site (Fig. 5*B*, compare position −737 to + 3247) and the control ChIP (*mock*).

Moreover, to evaluate the importance of the TATA box in the transcriptional activity displayed by the fragment REG-1 in the ND7/23 cells, we compared the luciferase activity of this fragment with a fragment containing a mutated TATA motif (Fig. 5*C*). This mutation resulted in a 3-fold decrease of luciferase expression, indicating that this element is required for correct transcription. The remaining luciferase expression observed for the fragment REG-1/TATAmut is probably due to the activity of the alternative promoters P1 and P2, which was unaltered.

Taking into account that the P3 sequence is well conserved among species, we used the zebrafish model to test the potential of this TATA promoter to drive transcription *in vivo*. One- to two-cell- stage embryos were injected with a vector containing the enhanced-GFP (eGFP) reporter gene under the control of the P3 minimum region combined with a zebrafish *iroquois* enhancer that could direct the expression of eGFP to the midbrain (24). Twenty-four hours post fertilization (hpf), eGFP was already observed in the midbrain of the embryos (Fig. 5*D*), an expression that lasted at least until 72 hpf. This result indicated that the P3 region exhibits promoter activity *in vivo* in this transgenic zebrafish assay.

Adjacent to the TATA motif, the P3 region contains evolutionarily conserved motifs potentially bound by bHLH (Ebox motif) and homeodomain (HD motif) transcription factors (Fig. 4). Transcription factors belonging to these families play important roles in the determination of neuronal fates from undifferentiated progenitor cells (for review, see Refs. 5 and 40). To evaluate their functional relevance, a region of 172 bp containing these sites but excluding the TATA motif was cloned in a Tol2 vector, specifically designed to analyze cis-regulatory elements in zebrafish (23) and carrying the eGFP reporter gene

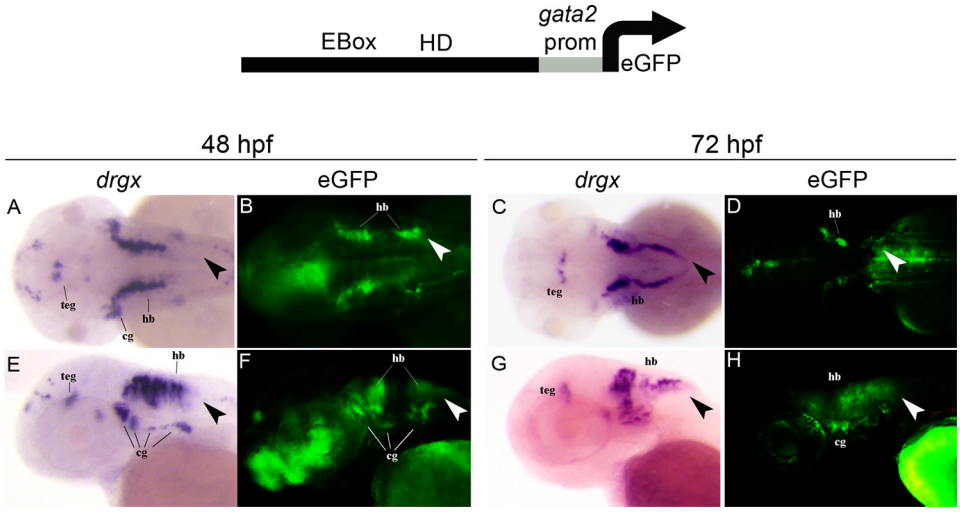

FIGURE 6. **Expression patterns of endogenous *drgx* and eGFP in the head region of a P3 promoter-eGFP zebrafish stable line.** *A*, *C*, *E*, and *G*, analysis of endogenous *drgx* expression by *in situ* hybridization. *drgx* expression is detected in embryos at 48 hpf in the anterior region of the hindbrain (*hb*), in the tegmentum (*teg*) (see dorsal view (*A*) and lateral view (*E*)), and in cranial ganglia (*cg*) (see *E*). At 72 hpf, *drgx* expression is more widespread in the hindbrain, and it still maintains its expression in the cranial ganglions and in the tegmentum (see dorsal view (*C*) and lateral view (G)). *B*, *D*, *F*, and *H*, analysis of eGFP expression in transgenic zebrafish embryos. eGFP expression is regulated by a module that encloses the *gata2* minimal promoter and a region of the *Prrxl1* P3 promoter that contains the Ebox and HD elements, excluding the TATA motif (see the *scheme*). In 48 hpf embryos, eGFP expression is distinguished in the hindbrain and in cranial ganglia (see dorsal view (*B*), lateral view (*F*)). The *arrowhead* indicates a region where eGFP is apparently activated prematurely in the transgenic embryos at 48 hpf (*B* and *F*) but whose *drgx* expression is only detected at 72 hpf (*C* and *G*). Although eGFP expression is reduced at 72 hpf, it is still detected in a small region of the anterior hindbrain and in the cranial ganglia (see dorsal view (*D*) and lateral view (*H*)).

under the control of the *gata2a* minimal promoter (Fig. 6). This vector was used to generate a zebrafish stable transgenic line. eGFP signal was recorded in F1 embryos at different developmental ages and compared with endogenous expression of *drgx*, the *Prrxl1* zebrafish homologue. The expression of *drgx* was previously reported (41) as restricted to dorsal spinal cord, DRG, and, in the developing brain, to sensory neuron populations of the midbrain and hindbrain, cranial sensory ganglia, and the habenula. From 48 hpf, a strong eGFP signal was detected in the developing hindbrain and cranial ganglia (Fig. 6, *B* and *F*) in a pattern that only partially overlaps the expression of endogenous *drgx*, as revealed by *in situ* hybridization (Fig. 6, *A* and *E*). Indeed, eGFP expression was prematurely observed in a posterior region of the hindbrain (marked by an *arrowhead* in Fig. 6, *B* and *F*) that only expresses *drgx* later at 72 hpf (compare the *arrowheads* in Fig. 6, *C* and *G*, with the *arrowheads* in Fig. 6, *B* and *F*). Due to the very small length of this fragment (172 bp), it is conceivable that repressive elements may be missing. Expression of eGFP in cranial ganglia displayed a spatiotemporal pattern that perfectly matched with *drgx* staining (figure 6*E*-H). At 72hpf, a decrease in the eGFP staining was observed (compare Fig. 6, *D* and *H*, with Fig. 6, *panels C* and *G*). Albeit the expression of *drgx* in DRG and spinal cord is well reported in zebrafish (41), the 172-bp sequence does not drive eGFP transcription to these tissues (data not shown). Together, these experiments pointed out that the region of 172 bp containing the conserved Ebox and HD elements was sufficient to drive expression of the reporter gene in *drgx*-expressing neurons.

Phox2b Controls Prrxl1 Expression by Binding the HD Motif in P3 Promoter—To unravel the trans-acting factors that may be responsible for the modulation of *Prrxl1* transcription via Ebox or HD element, we tested the overexpression effect of a set of transcription factors previously implicated in the control of Prrxl1 expression, as described in diverse epistatic studies employing mutant mice. In *Tlx3* and *Lmx1b* null mutant mice, Prrxl1 expression is affected in the spinal cord but not in the DRG (42, 43), whereas studies performed with DRG of *islet1* inducible conditional knock-out mice suggested that Prrxl1 expression is regulated by islet1 only at an early stage of neurogenesis (44). Moreover, Prrxl1 is activated by Brn3a in the DRG and trigeminal ganglion (45) and repressed by Phox2b in the facial, glossopharyngeal, and vagal cranial ganglia (7). Mash1 and Neurogenin1 (Ngn1) are proneuronal genes implicated in the specification of progenitors cells from which Prrxl1-expressing neurons arise either in the DRG or the neural tube (3, 46–48). Ngn1 and Mash1 belong to the bHLH transcription factor family and bind the Ebox consensus motif CANNTG (49), whereas the remaining proteins belong to homeodomain family and recognize the HD bipartite element TAATNNNATTA (50, 51). Thus, we assessed the expression of luciferase under the control of the REG-1 fragment in ND7/23 cells overexpressing Brn3a, Tlx3, Phox2b, Islet1, Ngn1, Lmx1b, and Mash1 (Fig. 7*A*). Three transcription factors strongly induced the transcriptional activity of the fragment REG-1. Those were Brn3a, Tlx3, and Phox2b. As homeodomain proteins, they are candidates to bind the HD element present in the P3 promoter. Therefore, we evaluated by luciferase reporter assays the effect of the overexpression of these transcription factors on the REG-1 construct containing the mutated HD motif compared with wild-type REG-1 (Fig. 7*B*). Tlx3 and Brn3a overexpression displayed the same luciferase activity in both constructs, whereas the induction of Phox2b decreased about 50% when the HD motif is mutated. To ascertain if Phox2b binds directly to the HD element present in *Prrxl1* P3 promoter, EMSA and ChIP-PCR were performed (Fig. 7, *C* and *D*). By EMSA, a DNA-protein shift was detected after the incubation of an oligonucleotide comprising the HD motif sequence and nuclear protein extracts from ND7/23 cells overexpressing Phox2b. On the

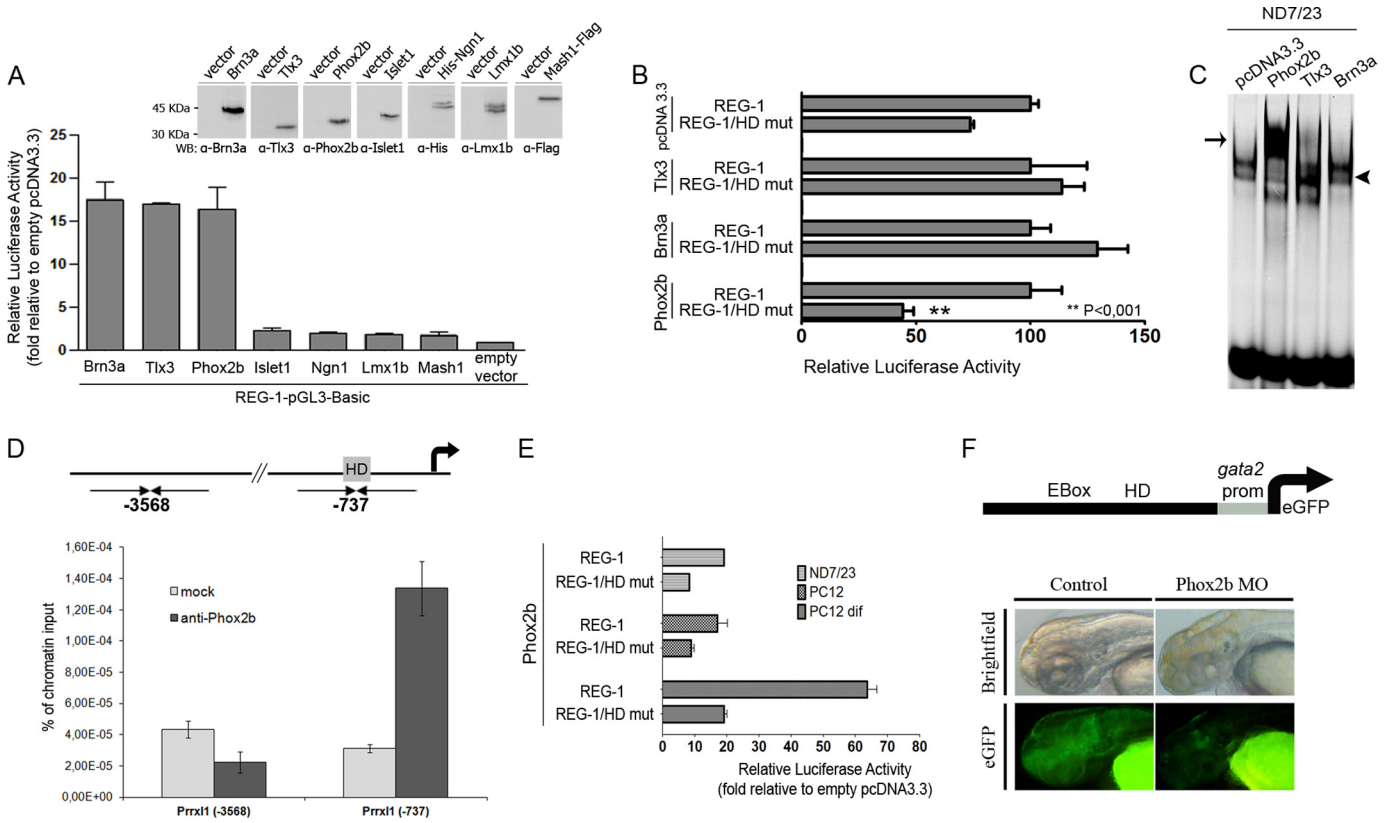

FIGURE 7. **Phox2b modulates Prrxl1 transcription by binding to the HD element in the P3 promoter.** *A*, the effect on REG-1 transcriptional activity by overexpressing the bHLH transcription factors Mash1 and Ngn1 and the homeodomain proteins Lmx1b, Islet1, Phox2b, Tlx3, and Brn3a in ND7/23 cells was assessed by luciferase reporter assays. The immunoblot confirms the protein expression and corresponds to one representative experiment. *B*, luciferase activity induced by REG-1 and REG-1/HD mut was measured in ND7/23 cells overexpressing Tlx3, Brn3a, and Phox2b or transfected with an empty pcDNA3.3. *C*, electrophoretic mobility shift assay using nuclear extracts from ND7/23 cells overexpressing Brn3a, Tlx3, or Phox2b or transfected with an empty pcDNA3.3 and a fluorescent probe (HD DY680) that comprises the HD motif present in the P3 promoter. The shift (*arrow*) is only observed in ND7/23 cells that overexpressed Phox2b. The *arrowhead* indicates a basal shift detected in all samples. *D*, chromatin immunoprecipitation assays with (*anti-Phox2b*) or without (*mock*) an anti-Phox2b antibody were performed using dorsal medulla oblongata chromatins from E14.5 mouse embryos followed by quantitative PCR using primers targeting regions, which comprises the HD motif (*Prrxl1 −737*) and an upstream control sequence (*Prrxl1 −3568*). An enrichment was only observed in the region where the HD element was present (*−737*). *E*, luciferase activity induced by REG-1 and REG-1/HD mut was measured in ND7/23, PC12, and differentiated PC12 cells overexpressing Phox2b. *F*, analysis of eGFP expression in transgenic zebrafish embryos at 72 hpf, containing the module that comprises the Phox2b-binding site (HD motif) present in the P3 promoter. Expression of eGFP was reduced in Phox2b MO-injected embryos when compared with controls. The fluorescence acquisition settings were exactly the same in both images.

contrary, overexpression of Tlx3 or Brn3a only led to a weak basal shift in the gel, which is also observed in extracts containing Phox2b overexpressed (marked by an *arrowhead* in Fig. 7*C*). We assume that this interaction represents a binding with another homeodomain protein that remains to be identified. The binding of Phox2b on *Prrxl1* promoter was further validated by ChIP-PCR assays (Fig. 7*D*). A chromatin enrichment (relative to the no antibody control, *mock*) was only observed with primers that amplified the region comprising the HD element (Fig. 7*D*, position −737).

Co-expression of Phox2b and Prrxl1 or its zebrafish orthologue, *drgx*, was only reported in some sensory ganglia, namely in the facial, glossopharyngeal, and vagal cranial ganglia, and their target relay neurons in the hindbrain (6, 7). As Phox2b is not detected in the ND7/23 cell line, we performed the same analysis using non-differentiated and differentiated PC12 cells, which endogenously expressed Phox2b and Prrxl1 (52). Again, overexpression of Phox2b increased the luciferase activity of REG-1 fragment, which is impaired by mutating the HD motif (Fig. 7*E*).

To better investigate the regulation of Prrxl1 expression by Phox2b *in vivo*, we used a validated specific antisense MO to interfere with the translation of zebrafish Phox2b protein. According to what was previously reported by Elworthy *et al.* (53), our zebrafish Phox2b morphants also presented scarce Hu-positive cells in the hindgut at 5 dpf (53%, 8 in 15 embryos). We injected Phox2b MO in the transgenic zebrafish line described above (Fig. 6), which contains eGFP under the control of the 172-bp sequence comprising the Phox2b binding element (HD motif). A decrease in the eGFP expression was observed (Fig. 7*F*), supporting our hypothesis that Phox2b binds to the HD element present in the P3 promoter region of *Prrxl1* and is required for this promoter activity.

Phox2b Is Required for drgx Expression in the Glossopharyngeal, Vagal, and Facial Ganglia—To further address the role of Phox2b in the control of *drgx* expression, we injected Phox2b MO in wild-type zebrafish embryos at one- to two-cell stage. Expression analysis of *drgx* by *in situ* hybridization showed that in 48 hpf morphant animals, *drgx* is still maintained in the hindbrain, in the tegmentum, and in the trigeminal ganglia (Fig. 8,

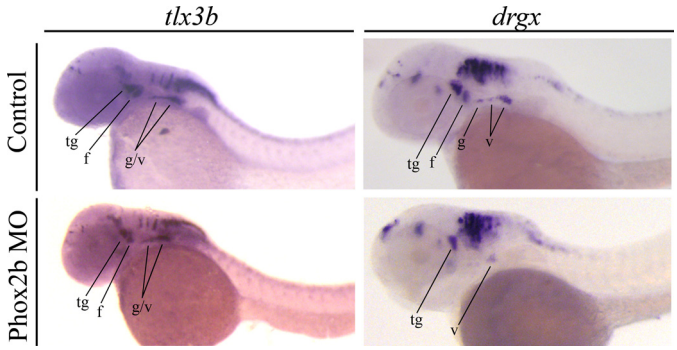

FIGURE 8. **Phox2b is required for the expression of *drgx* in the glossopharyngeal, vagal, and facial ganglia of zebrafish embryos.** Analysis of the *tlx3b* and *drgx* expression by *in situ* hybridization in control or Phox2b MO-injected embryos is shown. The *upper panels* show the control embryos, and the *lower panels* show representative images of morphant embryos. *tlx3b* and *drgx* expression are maintained in the hindbrain and trigeminal ganglion (*tg*) of both control and Phox2b MO-injected animals. Expression of *drgx* in the facial (*f*), glossopharyngeal (*g*), and vagal (*v*) ganglia is greatly reduced in Phox2b MO-injected animals, whereas *tlx3b* expression was still observed in all these ganglia.

compare *drgx* expression in control with Phox2b MO). Nevertheless, in 44% of embryos, *drgx* expression is much weaker or absent in the glossopharyngeal, vagal, and facial ganglia (Fig. 8), suggesting that, as predicted by *in vitro* assays, Phox2b is required for the transcriptional control of *drgx*.

To discard the hypothesis that the absence of *drgx* expression was due to a failure in the development of these ganglia induced by Phox2b knockdown, *in situ* hybridizations were performed using a *tlx3b* probe. Tlx3 expression has been shown to extensively overlap with Prrxl1 (10, 42) and thus was used here as a reliable marker of neuronal differentiation. As expected, *tlx3b* expression is maintained in Phox2b morphants (Fig. 8, *Phox2b MO tlx3b*).

DISCUSSION

Prrxl1 is a transcription factor with an important role in the establishment and maintenance of the nociceptive DRG-spinal cord neuronal circuit. The function of Prrxl1 in this process has been characterized in detail, but the molecular determinants causing its activation or repression are not yet understood. To shed some light on the molecular mechanisms regulating *Prrxl1* gene expression, we isolated and characterized the alternative promoters that control the expression of three distinct *Prrxl1* 5′-UTR variants, named 5′-UTR-A, 5′-UTR-B, and 5′-UTR-C. These variants are originated from different exon 1 during *Prrxl1* splicing and do not have any consequence in the protein reading frame as the AUG start codon is present in exon 2.

The alternative use of different exon 1 has been recognized as another mechanism of control of gene expression. Within the human and mouse genome, >3000 genes with multiple first exons have been identified (54). 5′-UTR-mediated regulation were shown to modulate gene expression through mechanisms that influence post transcriptional modification of RNA (secondary structure and mRNA stability) and translational efficiency (18). This proved here to be also the case for *Prrxl1* 5′-UTR variants. The 5′-UTR-A displayed a neuron-specific effect, increasing both the rate of Prrxl1 protein translation and the stability of the mRNA molecule. This observation implies a contribution of neuronal specific RNA-binding proteins. Taking into account that Prrxl1 has an important role in the development of the neuronal circuit connecting the DRG to the spinal dorsal horn (8, 9), possible candidates are Hu RNA-binding proteins (human homologues of *Drosophila* ELAV), namely the neuronal specific HuC and HuD isoforms, which are long used as markers of neuronal differentiation. Overexpression of HuC or HuD in PC12 cells increased the rate of neuronal differentiation, whereas down-regulation resulted in an impairment of neurite growth (55, 56). Likewise, a decrease in neuronal differentiation was observed in HuD null mutant mice (57). Although HuC/D proteins are strong candidates to modulate the neuron-specific activity of *Prrxl1* 5′-UTR-A, their involvement remains to be demonstrated.

On the contrary, the 5′-UTR-B reduced mRNA half-life and suppressed mRNA translation, resulting in a decrease in the luciferase expression both in the neuronal-derived ND7/23 and HeLa cells. This was due to the presence of an AUG in the 5′-UTR-B, as shown by luciferase reporter gene assays and site-directed mutagenesis. This AUG is used as an alternative start codon, modifying, therefore, the protein reading frame. AUG codons upstream of the main open reading frame are present in ~10% of all mRNAs (58). Although the functional impact of this mechanism has not been investigated in detail, a recent study on *male-specific lethal-2* mRNA suggested an important role in negative translational control by increasing initiation of scanning ribosomes at the upstream open reading frame and blocking downstream translation (58). The cis-regulatory upstream ORF present in the *Prrxl1* 5′ UTR-B exerts a negative influence on translational efficacy, as seen in Fig. 1*C*, and is probably responsible for controlling the amount of Prrxl1 protein during early neurogenesis, the stage where 5′-UTR-B is most expressed.

By studying deletion derivatives of the *Prrxl1* 5′-flanking region, we demonstrated that transcription of each *Prrxl1* 5′-UTR variants is controlled by specific promoters. The here-named promoter P1, P2, and P3 controlled, respectively, the expression of 5′-UTR-C, 5′-UTR-B, and 5′-UTR-A. Recent genome wide analyses indicated that alternative promoter usage is a common event that occurs at least with the same order of frequency as alternative splicing, affecting about 52% of human genes (37). On average, there are 3.1 alternative promoters per gene, with the composition of one CpG-island-containing promoter per 2.6 CpG-less promoters. Moreover, it was demonstrated that genes that undergo complex transcriptional regulation often include at least one CpG island-containing promoter, expressed ubiquitously, and are accompanied by other promoters used for tissue-specific or signal-dependent expression (37). Our results on the *Prrxl1* promoters are in accordance with these observations. Indeed, a GC-rich region is contained within promoter P2, whereas P3 is a TATA promoter. P2 displayed activity in all cell lines tested, whereas P3 and P1 were regulated depending on the cellular context being only active in neurons (for a summary of these findings see the model in Fig. 9).

Among the three *Prrxl1* alternative promoters, P3 is the most conserved and displays *in vivo* activity in the zebrafish. Reverse

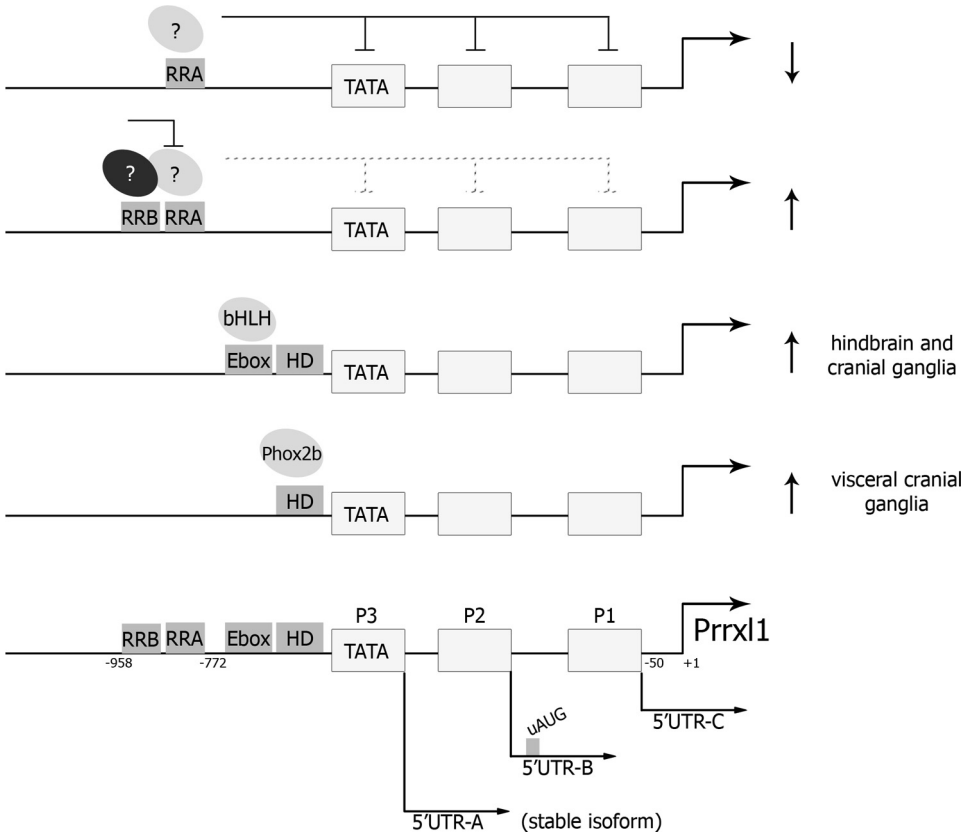

FIGURE 9. **Schematic representation of the regulatory elements that control the expression of *Prrxl1* 5′-UTR variants.** Promoter regions and other regulatory elements were identified upstream of *Prrxl1* translation start site (+1). Transcription of the three *Prrxl1* mRNA variants 5′-UTR-C, 5′-UTR-B, and 5′-UTR-A is controlled by distinct promoters, termed *P1*, *P2*, and *P3*, respectively. 5′-UTR-A is the most stable *Prrxl1* transcript and is enriched at E14.5 spinal cord, a developmental age associated with late neurogenesis. Located between −958 and −772 bp, two preponderant elements were identified: RRA is able to totally suppress the transcriptional activity regulated by the three promoters, and RRB inhibits the repressive effect of RRA resulting in an increase of transcription. In addition, the Ebox and HD motifs located upstream of the TATA box drive the transcription of *Prrxl1* to hindbrain and cranial ganglia. Although the bHLH transcription factor that binds the Ebox motif remains to be identified, the homeodomain protein Phox2b binds the HD element and is required for Prrxl1 expression in visceral cranial ganglia. *uAUG*, AUG codons upstream.

transcriptase-PCR experiments also showed that the P3-derived transcript, *Prrxl1* 5′-UTR-A, is more abundant than the other two variants. These two observations led us to conclude that promoter P3 may play a more prominent role in *Prrxl1* transcriptional regulation and prompted us to focus our study on transcriptional mechanisms modulating P3 activity. Core promoters were shown to comprise DNA sequence motifs, such as the TATA box, the Initiator (Inr), and the downstream promoter element, located within −30 to +30 nucleotides relative to the TSS and to mediate the recognition and recruitment of the RNA polymerase II to the transcriptional apparatus (59, 60). *Prrxl1* P3 core promoter contained a TATA motif located at 35 bp upstream of 5′-UTR-A TSS. The functional validation of this motif was attained by *in vitro* and *in vivo* evidence, such as (i) TBP interacted with the TATA motif as detected by EMSA and ChIP using mouse spinal cord samples, (ii) mutation of the TATA motif greatly reduced promoter activity, and (iii) P3 core promoter, which contains the TATA element, is sufficient to drive the reporter gene expression in the zebrafish.

Located upstream of the P3 promoter, a highly conserved region (−1401/−958) was identified as an important modulator of *Prrxl1* transcription. This region contains binding sites for actively transcribing protein such as RNA Polymerase II, TBP, and P300. By sequence deletion experiments, the presence of still uncharacterized regulatory modules was predicted. These elements appeared to be mainly associated with P1 and P2 promoters and thereby to the transcription of 5′-UTR-B and 5′-UTR-C. Because these Prrxl1 mRNA variants are enriched in early-born neurons of the developing spinal cord, we hypothesize that the modules included in the −1401/−958 bp region may be responsible for enhancing temporal-specific transcription controlled by promoters P1 and/or P2 promoters.

We also defined two neuron-specific elements, RRA (−811/−772) and RRB (−891/−922), which exhibit the opposite effect on *Prrxl1* transcription. RRA has the potential to strongly suppress the transcription of the three alternative promoters, whereas RRB counteracts the action of the RRA repressive motif and consequently induces *Prrxl1* transcription (see the model in Fig. 9). Such tight regulation could be understood as a way to modulate Prrxl1 expression in different neuronal types, namely in glutamatergic (Prrxl1-positive) over GABAergic (Prrxl1-negative) neurons during the developing spinal cord. Because these two neuronal populations derived from the same progenitor domain and are mutually exclusive, molecular interrepression between inhibitory and excitatory interneurons has been suggested. For instance, Tlx3 acts as an inhibitor of Lbx1 expression inducing the glutamatergic transmitter phenotype (61), whereas Ptf1a represses Tlx3 specifying a GABAergic cell

fate (62). A similar mechanism could be envisaged for Prrxl1. In GABAergic neurons, Prrxl1 expression could be prevented by a specific transcription factor acting on the RRA element. This repressive effect could be suppressed in glutamatergic neurons through the RRB element. It would be interesting to assess if one possible RRB binding candidate could be Tlx3 as this transcription factor induces *Prrxl1* promoter activity (Fig. 7*A*) and highly co-localizes with Prrxl1 (10).

Furthermore, the 172-bp 5′ region adjacent to the P3 core promoter was sufficient to drive specific neuronal activity in zebrafish. GFP expression under the control of putative regulatory elements present in this 172-bp region was only observed in the area in which the transcript of the zebrafish *Prrxl1* ortologue, *drgx*, is detected. Such specificity suggested that important cis-regulatory modules are present in this region, and further analysis of this sequence revealed the presence of a highly conserved putative binding site for homeodomain transcription factors, proteins that are expected to control the expression of Prrxl1 as a function of developmental age and neuronal context. Among the tested candidates, Tlx3, Brn3a, and Phox2b induced *Prrxl1* promoter activity, but only Phox2b was able to bind to this HD motif. Co-expression of Phox2b and Prrxl1 or its zebrafish ortologue, *drgx,* was only detected in the visceral sensory pathway, namely in the facial, glossopharyngeal, and vagal cranial ganglia and their target relay neurons in the hindbrain (6, 7, 63).We, therefore, presume that the P3 activity observed in the transgenic line (Fig. 6) may be controlled, at least to some extent, by Phox2b. This positive regulation is further supported by silencing experiments with Phox2b morphants (Fig. 7*F*).

Nonetheless, in the mouse, visceral sensory neurons switch to a somatic fate in the absence of Phox2b acquiring a molecular profile similar to that of somatic sensory neurons at later developmental stages, with higher expression of Prrxl1 and Brn3a (7). This increase in Prrxl1 expression was suggested to be mediated by the increase of Brn3a, which, in turn, is directly repressed by Phox2b (7). On the contrary, Prrxl1 expression in the nuclear TS progenitor domains of the hindbrain is not affected by Phox2b inactivation, suggesting that the repression of Prrxl1 by Phox2b only occurs in the visceral sensory ganglia (7). In accordance, Phox2b is not required for *drgx* expression in the zebrafish hindbrain. However, contrary to the mouse, Phox2b down-regulation induced a loss of *drgx* expression in the facial, glossopharyngeal, and vagal cranial ganglia. Binding of Phox2b on the HD motif increased *Prrxl1* promoter activity in ND7/23 and PC12 cells. Our data strongly suggest that Phox2b has the potential to work as a direct Prrxl1 activator (see model in Fig. 9).

Although Phox2b is a determinant of visceral fate, its mode of action varies with neuronal types (64, 65) likely due to cell type-specific combinations of transcription factor complexes. On the other hand, Prrxl1 is transiently expressed at early stages of the development of the facial-glossopharyngeal ganglion and the distal part of the vagal ganglion, as observed in mice (7) and zebrafish (41). During this particular developmental window, co-expression of Prrxl1, Phox2b, Tlx3, and Islet1 is observed (7). Recently, studies performed in Islet1 inducible conditional knock-out mice (44) suggested that Prrxl1 expression in the DRG is regulated by Islet1 only at an early stage of neurogenesis. Thus, it is conceivable that Phox2b, in combination with Islet1, binds to the HD motif on the P3 *Prrxl1* alternative promoter to activate *Prrxl1* expression at early stages of sensory ganglia development, whereas later (from E13.5 on), as part of a distinct transcriptional machinery, Phox2b shuts down Prrxl1 expression likely through the repression of Brn3a. Understanding how Brn3a acts on *Prrxl1* promoters will add new insight on this mechanism. The present data thus support that the P3 alternative promoter is involved in the ganglion specific action of Prrxl1 (see the model in Fig. 9), which appear to be controlled by Phox2b in the case of visceral sensory neurons.

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3.2. PUBLICATION II

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Dual role of Tlx3 as modulator of Prrxl1 transcription and phosphorylation

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ABSTRACT

The proper establishment of the dorsal root ganglion/spinal cord nociceptive circuitry depends on a group of homeodomain transcription factors that includes Prrxl1, Brn3a and Tlx3. By the use of epistatic analysis, it was suggested that Tlx3 and Brn3a, which highly co-localize with Prrxl1 in these tissues, are required to maintain Prrxl1 expression. Here, we report two Tlx3-dependent transcriptional mechanisms acting on *Prrxl1* alternative promoters, referred to as P3 and P1/P2 promoters. We demonstrate that (i) Tlx3 induces the transcriptional activity of the TATA-containing promoter P3 by directly binding to a bipartite DNA motif and (ii) it synergistically interacts with Prrxl1 by indirectly activating the *Prrxl1* TATA-less promoters P1/P2 via the action of Brn3a. The Tlx3 N-terminal domain 1–38 was shown to have a major role on the overall Tlx3 transcriptional activity and the C-terminus domain (amino acids 256–291) to mediate the Tlx3 effect on promoters P1/P2. On the other hand, the 76–111 domain was shown to decrease Tlx3 activity on the TATA-promoter P3. In addition to its action on *Prrxl1* alternative promoters, Tlx3 proved to have the ability to induce Prrxl1 phosphorylation. The Tlx3 domain responsible for Prrxl1 hyperphosphorylation was mapped and encompasses amino acid residues 76 to 111. Altogether, our results suggest that Tlx3 uses distinct mechanisms to tightly modulate Prrxl1 activity, either by controlling its transcriptional levels or by increasing Prrxl1 phosphorylation state.

1. Introduction

The establishment of the neuronal circuit connecting the dorsal root ganglion (DRG) and the spinal dorsal horn constitutes the basis for the transmission of sensory input from the periphery to the central nervous system, to be ultimately processed at specified centers in the brain [1]. Various classes of sensory afferent fibers dwell in the DRG and synapse with second-order neurons and interneurons in the dorsal spinal cord [2–4]. The distinctive phenotypes of the various classes of DRG and spinal sensory neurons are ascribed to the combinatorial expression of a set of transcription factors [3,5,6]. One of these is the paired-like homeodomain (HD) transcription factor Prrxl1 (mouse orthologue of human DRGX). *Prrxl1* null embryos display spatiotemporal defects in the spinal gray matter penetration of afferent sensory fibers together with defects in spinal dorsal horn morphogenesis [7,8]. Such early developmental abnormalities are correlated with a significant reduction in sensitivity to noxious stimuli [7]. Despite the recognized importance of Prrxl1 in the establishment of the nociceptive circuitry, the molecular mechanisms that specifically control Prrxl1 expression and function along embryonic development remain largely unknown.

Recently, we defined multiple *cis*-regulatory modules that control the spatiotemporal expression of *Prrxl1* during the development of nociceptive neurons. These include three alternative promoters (referred to as P1, P2 and P3 promoters) implicated in the transcription of distinct *Prrxl1* 5′-UTR variants, which confer distinct mRNA stability and translation efficiency to the *Prrxl1* transcript. In addition, we showed that *Prrxl1* promoter activity is induced by the HD proteins Tlx3 and Brn3a [9]. Both Tlx3 and Brn3a co-express extensively with Prrxl1 [10–14]. Ablation of *Tlx3* gene affects *Prrxl1* expression only at late stages of spinal cord development (from E13.5 onwards) but not at the DRG [13]. When *Tlx3* ablation is restricted to a subset of glutamatergic neurons that evolve from Lbx1-positive cells, mice develop a phenotype that strongly resembles that of *Prrxl1* null mutants, with spinal cord abnormalities and weak responses to pain-related stimuli [15]. The disruption of the *Brn3a* gene results in impaired projection of DRG and trigeminal ganglion axons to their central target neurons and subsequent apoptosis [16,17], abnormalities that are also observed in *Prrxl1* null mutant

Abbreviations: ChIP, chromatin immunoprecipitation; CIAP, calf intestinal alkaline phosphatase; DRG, dorsal root ganglion; HD, homeodomain; pI, isoelectric point; TALE, three amino acid loop extension

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mice [7,8]. Microarray analysis demonstrated that *Brn3a* null mice have a decreased expression of *Prrxl1* in primary afferent neurons [18], strongly suggesting that Brn3a holds a role in the control of *Prrxl1* transcription. Altogether, these observations not only imply that Tlx3 and Brn3a control *Prrxl1* expression, but also point to the use of distinct tissue-specific genetic programs by each one.

In the present study, we shed some light on the mechanisms underlying Brn3a and Tlx3 activity on Prrxl1 functioning. We provide evidence that Tlx3 positively regulates *Prrxl1* expression by modulating *Prrxl1* promoter activity and also induces Prrxl1 hyperphosphorylation. At the transcription level, Tlx3 binds directly to *Prrxl1* promoter P3 and acts indirectly on promoters P1/P2 by forming a transcriptional complex with Prrxl1, which activates Brn3a. Tlx3 functional domains involved either in the dual transcriptional control of *Prrxl1* or in the induction of Prrxl1 phosphorylation were identified. Altogether, our data demonstrate that Prrxl1 and Tlx3 are intimately related and share a genetic pathway, which may be essential for the regulation of downstream effectors responsible for the establishment of the nociceptive phenotype in the spinal cord.

2. Materials and methods

2.1. Animal care

NMRI mice were bred and housed at IBMC, Porto, animal facility under temperature- and light-controlled conditions. The embryonic day 0.5 (E0.5) was considered to be the midday of the vaginal plug. The animals were euthanized (isoflurane anaesthesia followed by cervical dislocation) and tissues were collected. Experiments were carried out in compliance with the animal ethics guidelines at IBMC and approved by the Portuguese Veterinary Ethics Committee.

2.2. Cell culture, plasmid transfection and luciferase reporter assays

ND7/23 and HeLa cells were maintained and grown in DMEM with high glucose, supplemented with GlutaMAX™ (Gibco), 10% fetal bovine serum (Gibco) and 50 U/mL penicillin and streptomycin (Gibco). All cells were kept at 37 °C and 5% CO_2 gas phase. Transfection was performed using Lipofectamine®2000 (Invitrogen), and 24 h later, cells were harvested for posterior luciferase reporter assays. Transfected cells from a 96-well plate format were resuspended in 50 μl of lysis buffer (Promega) and the protein extract cleared by centrifugation. Five microliters of the extract were mixed with the luciferase reagent (Promega) and the signal measured using a luminometer reader (Tecan). Transfection efficiency was normalized by assessing β-galactosidase activity using ONPG (Sigma-Aldrich) as substrate.

2.3. Plasmids

Expression vectors previously constructed were pRSK-Brn3a (a gift from Dr. Mengqing Xiang), pcDNA3.3-Tlx3 [9], pcDNA3.3-Prrxl1 [19], pcDNA3.3-HA-Prrxl1 [19] and pCMVβ (Clontech). The sequences used to clone Tlx3-FLAG or the truncated variants ($Tlx3_{38–291}$-FLAG, $Tlx3_{76–291}$-FLAG, $Tlx3_{111–291}$-FLAG, $Tlx3_{156–291}$-FLAG, $Tlx3_{1–256}$-FLAG and $Tlx3_{1–224}$-FLAG) into pCDNA3.3 were PCR amplified from pcDNA3.3-Tlx3 with primers depicted in Table 1.

The following luciferase reporter constructs were previously used in [9]: pGL3-REG-1, pGL3-P3-prom (former pGL3-REG-2), pGL3-P2/P1-prom (former pGL3-REG-11), pGL3-P3-A (former pGL3-REG-4), pGL3-P3-B (former pGL3-REG-5) and pGL3-P3-C (former pGL3-REG-6).

Site-directed mutagenesis of HD3 element was performed from pGL3-P3-prom using the NZY Mutagenesis Kit (NZYTech). The primers used were the following: 5′-GGAAATAATCAGATTAAGGCCAACCATGCG TGAGATTATA-3′ and 5′-TATAATCTCACGCATGG TTGGCCTTAATCTGAT TATTTCC-3′.

2.4. Small interfering RNA transfection

The appropriate siRNA sequence for *Prrxl1* was chosen from a set of three RNA oligos (Invitrogen). A pre-designed siRNA oligo for *Brn3a* was purchased from Ambion. ND7/23 cells were transfected with the RNA oligos using the Lipofectamine®RNAiMAX (Invitrogen) and 48 h later were co-transfected with the appropriate expression and reporter vectors using Lipofectamine®2000 (Invitrogen). Cells were harvested for luciferase assays on the next day. Scrambled sequences were used as control siRNA.

2.5. Quantitative PCR

Following transfection of ND7/23 cells with the siRNA for *Prrxl1* or *Brn3a*, RNA was extracted using the GenElute™ mRNA Miniprep Kit (Sigma-Aldrich). The first-strand cDNA synthesis was prepared at

Table 1
List of primers.

	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Mapping of functional Tlx3 domains		
Tlx3-FLAG	GCCACCATGGAGGCGCCCGCCAGC	TCATTTATCGTCATCGTCTTTGTAGTCTGCGGCCTGTCACACCAGAGAGGTGACAGCGG
$Tlx3_{38–291}$-FLAG	GCCACCATGCCCGACGGCGCCAGCTAC	*Idem*
$Tlx3_{76–291}$-FLAG	GCCACCATGAGTGTCAACCTAAGCTTGGCCC	*Idem*
$Tlx3_{111–291}$-FLAG	GCCACCATGCCCTCGGTGCCCACGGTCTCC	*Idem*
$Tlx3_{156–291}$-FLAG	GCCACCATGCACCCCTACCAGAACCGGACGCC	*Idem*
$Tlx3_{1–256}$-FLAG	GCCACCATGGAGGCGCCCGCCAGC	TCATTTATCGTCATCGTCTTTGTAGTCTGCGGCCTGGATGGAATCGTTGAGGC
$Tlx3_{1–224}$-FLAG	GCCACCATGGAGGCGCCCGCCAGC	TCATTTATCGTCATCGTCTTTGTAGTCTGCGGCCTGCCGCCGCCACTTGGTC
Chromatin immunoprecipitation		
RGMb ORF	TGCCAACAGCCTACTCAATG	GTGGAAGATGTGGGTCCATC
Prrxl1 (−3568)	TTTAATGTCTCCCGCAGCTT	CACTTGATGTTCACCAGACTCA
Prrxl1 (−737)	TGCTGAGAAGTGACGGATTT	TCACGCATAATTGGCCTTAAT
Prrxl1 (+3247)	GAAGTCGGCAGGGTTTTCTA	AGCCTTTCACACCTCTTTCC
Prrxl1 (+9787)	ACTCCAGCACATTTTTGCAC	TGTGGACCCTTCTACCCTTC
DNA pull-down assay		
P3-prom/P3-promHD3mut	CCGTAACGGGCTATTATCCTGG	Biotin-CAGGACCAGAGAAGTGACTG
P3-A	GAGGGCTGGGGTTGGGGTG	*Idem*
P3-B	ATAATCTCTTTTGGGTGGGG	*Idem*
P3-C	CCTGATGTTTGCTGAGAAGTG	*Idem*
P3B-short	ATAATCTCTTTTGGGTGGGG	Biotin-GAATGTTTTTCCCTTTTTTT
P2/P1-prom	CAGTCACTTCTCTGGTCCTG	Biotin-CTGGCAGACTTGTGCCTGTGC
Control	ATGTTTTATTTCCACTGTCC	Biotin-CTCTCTCTGTCTTCCTCCAT

42 °C during 1 h, from 1 μg of total RNA using 200 U of reverse transcriptase enzyme (Bioline) and 500 ng of oligo(dT)12–18 (Bioline). To assess for potential contaminants, a control containing all reagents except the reverse transcriptase enzyme was included for each sample. The levels of mRNA expression were then quantified by PCR using a StepOnePlus Real-Time PCR system (Applied Biosystems). For *Prrxl1*, a SYBR Green chemistry for quantitative PCR (Maxima Master Mix, Fermentas) and the following primers 5′-CCCATGTGGCATCTCTGAAAG-3′ and 5′-TCATACACTCTTCTCTCCCTCGC-3′ were used. Normalization was performed by amplification of mouse β-actin using the primers 5′-TCATGAAGTGTGACGTTGACATCC-3′ and 5′-GTAAAACGCAGCTCAGTAACAGTC-3′. For *Brn3a*, the quantitative PCR was performed using a Taqman® Gene Expression Assay (Mm02343791_m1, Life Technologies) with a Taqman® Fast Advanced Master Mix (Life Technologies). Normalization was performed using a Taqman® Gene Expression Assay for *Hprt* (Mm00446968_m1).

2.6. DNA affinity pull-down assay

Nuclear proteins were extracted from ND7/23 cells transfected with pCDNA3.3-Tlx3 using first a low salt lysis buffer (30 mM Tris-HCl (pH 7.8), 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and protease/phosphatase inhibitors cocktail (Sigma-Aldrich)). After nuclear fractionation at 1000 × *g* for 10 min at 4 °C, proteins were resuspended in 10 mM Tris-HCl (pH 7.5), 60 mM KCl, 200 mM NaCl, 10% glycerol, 5 mM MgCl2 and 0.1% Triton X-100, cleared by centrifugation and incubated with biotinylated probes for one hour at room temperature. Afterwards, the complexes were incubated with NanoLinK™ Streptavidin Magnetic Beads (Solulink) for two hours at room temperature. After washing and elution, samples were analysed by Western blotting using rabbit anti-Tlx3 (sc-30185, Santa Cruz Biotechnology). The DNA fragments used as probes were PCR-amplified from pGL3-REG-1, pGL3-P3-prom/HD3mut and pCDNA3.3-Prrxl1 (for control probe, which corresponds to a fragment amplified from the *Prrxl1* coding sequence) using biotinylated reverse primers (see Table 1).

2.7. Chromatin immunoprecipitation assays

Tlx3 chromatin immunoprecipitation (ChIP) assays using E14.5 mouse dorsal spinal cord tissue were performed essentially as previously described [9], with the following modifications: (i) a mixture of 0.5 μg of each anti-Tlx3 antibodies (sc-23397 and sc-30185, Santa Cruz Biotechnology) was used; (ii) immunoprecipitates were washed twice with wash buffer I followed by two washes with wash buffer III. For quantitative PCR, sets of primers were used for assessing ChIP enrichment and designed using Primer 3 software (http://biotools.umassmed.edu). The primer sequences are listed in Table 1. Results are shown as the mean of triplicates ± SD of at least two independent experiments.

2.8. Co-immunoprecipitation assays

ND7/23 cells transfected with pcDNA3.3-Tlx3-FLAG and pcDNA3.3-HA-Prrxl1 or an empty pcDNA3.3 were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl and 0.1% Triton X-100 with protease/phosphatase inhibitor cocktails (Sigma-Aldrich). One hundred micrograms of cell lysate were immunoprecipitated using ANTI-FLAG® M2 magnetic beads (Sigma-Aldrich) for two hours. The beads were washed and the proteins were then eluted in 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM DTT and 0.1% bromophenol blue and resolved by SDS-PAGE. Primary antibodies used in the Western blotting were mouse anti-FLAG M2 (Sigma-Aldrich) and rabbit anti-HA (Invitrogen).

2.9. Dephosphorylation assays

Extracts from ND7/23 cells transfected with pcDNA3.3-Prrxl1 and pcDNA3.3-Tlx3 or an empty pcDNA3.3 were homogenized in dephosphorylation buffer [20] and incubated with calf intestinal alkaline phosphatase (CIAP; Invitrogen) for 2 h at 37 °C. An inhibitor (20 mM Na_2HPO_4) was added to a replicate reaction mix as control.

2.10. 2D-PAGE

ND7/23 cells transfected with pcDNA3.3-Tlx3 and pcDNA3.3-Prrxl1 were homogenized in 20 mM phosphate buffer (pH 8.0), 0.1% Triton X-100, supplemented with phosphatase and protease inhibitors and Benzonase nuclease (Novagen), sonicated and cleared by centrifugation. Isoelectric focusing was performed on a Protean i12 Cell (Bio-Rad) using 11 cm immobilized pH gradient strips pH 3–10 non-linear (Bio-Rad) for 38,000 Vh and held at 750 V as previously described [19]. Afterwards, SDS–polyacrylamide gels (12%) were run, and Western blotting was performed with home-made rabbit anti-Prrxl1 antibody [10]. Blots were directly imaged using Chemidoc MP (Bio-Rad).

2.11. Statistical analysis

In the present study, all the data presented (except for ChIP experiments; see above) were derived from at least three independent experiments with three replicates. When necessary, a two-tailed unpaired *t*-test was performed.

3. Results

3.1. Tlx3 and Prrxl1 synergistically act to activate Prrxl1 promoter activity

Recently, we studied the DNA region, referred to as REG-1 (Fig. 1A), located immediately upstream of the Prrxl1 start codon (−1401/−50), which encloses three alternative promoters (named P1, P2 and P3). Promoter P3 gives rise to 5′UTR-A mRNA variant, which displays distinct stability and translation efficiency compared to the 5′UTR-C and -B, transcripts that originated from the activity of promoters P1 and P2, respectively. Moreover, 5′UTR-A mRNA is expressed at late stages of spinal cord neurogenesis, while 5′UTR-B and -C are mainly present at early stages [9]. The HD proteins Tlx3 and Brn3a were shown in that study to enhance the activity of *Prrxl1* alternative promoters [9].

To better understand the regulatory mechanisms implicated in Tlx3 and Brn3a control of *Prrxl1* expression, we performed gain- and loss-of-function experiments in ND7/23 cells, a DRG-derived cell line previously shown to be a suitable model for studying Prrxl1 [9,19]. Given that promoters P1 and P2 share many features, we decided to analyse these DNA regions as a single module. Tlx3 overexpression increased the transcriptional capacity of *Prrxl1* promoters to more than 20-fold when compared to mock conditions (control/Tlx3, $p < 0.01$; Fig. 1B). This effect was enhanced 2 times (42-fold increase in the luciferase activity compared to control values) by the overexpression of Prrxl1 itself. It is likely dependent on other neuronal factors since no significant induction was observed in HeLa cells (control/Tlx3, ns; Fig. 1B). Silencing endogenous *Prrxl1* expression reduced Tlx3 induction of luciferase activity by 3 times (siPrrxl1/Tlx3; Fig. 1C) in comparison with the control condition (siControl/Tlx3; Fig. 1C). This result suggests that the Tlx3-inductive effect on *Prrxl1* promoters is dependent on the synergistic action of endogenous Prrxl1. Thus, it is likely that the induction observed for Prrxl1/Tlx3 co-transfection, when compared to Tlx3 alone, would be superior, as the latter is potentiated by endogenous Prrxl1.

This is quite surprising as the overexpression of Prrxl1 alone results in the repression of the reporter enzyme (0.58-fold decrease, $p < 0.01$), and implies that the increase in the activity of Tlx3 by Prrxl1 is due to the combination of both transcription factors, rather than the sum of their independent actions.

To dissect how Tlx3 alone or in combination with Prrxl1 acts upon the *Prrxl1* regulatory regions, we divided the entire region analysed (REG-1) into two smaller fragments containing promoter P3 (P3-prom) or promoters P1 and P2 (P1/P2-prom). We then analysed the

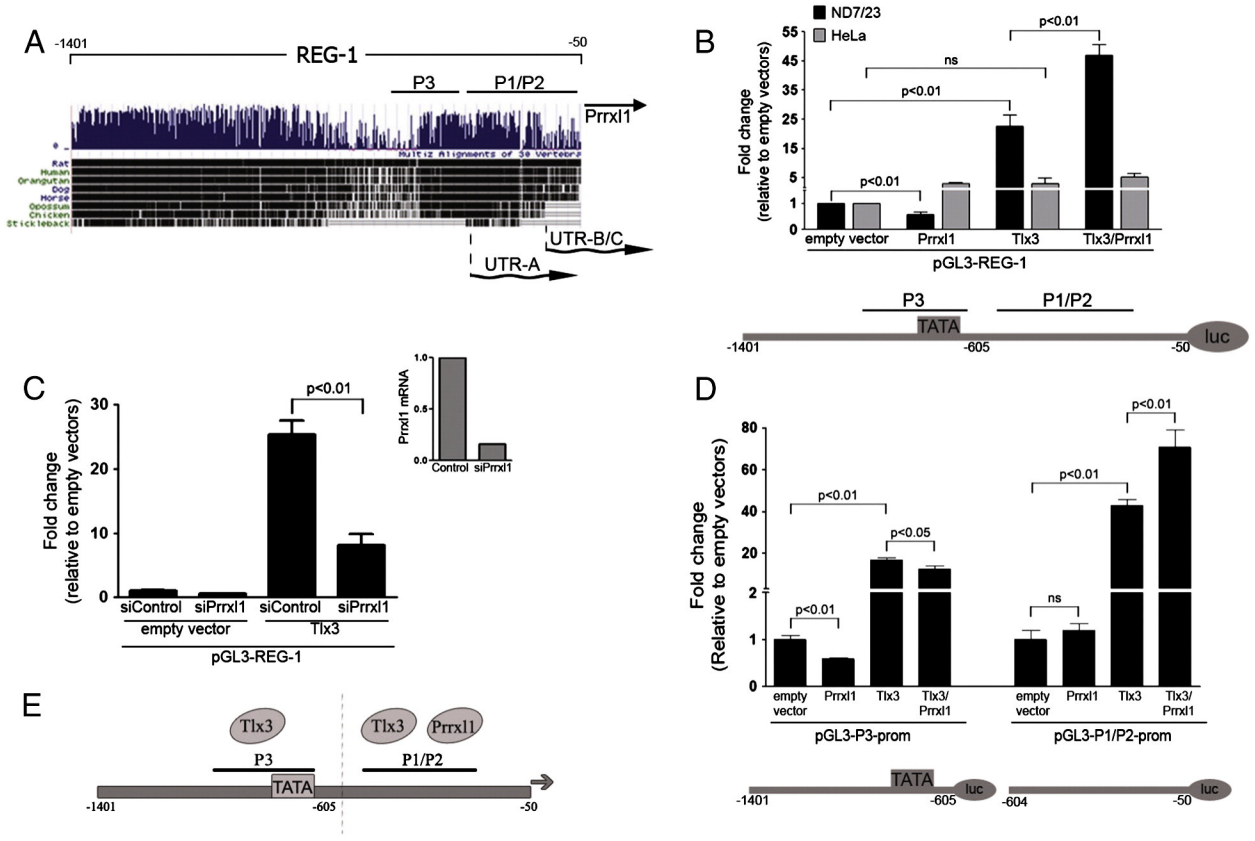

Fig. 1. Tlx3 uses multiple mechanisms to modulate Prrxl1 transcription. A. UCSC genomic alignment of evolutionarily distant species of a previously characterized sequence (referred to as REG-1; [9]) positioned upstream of the Prrxl1 translation start site. Blue peaks correspond to conservation in mammals. Bottom panel displays conservation across vertebrate species. This sequence has been shown to comprise three promoters (P1, P2 and P3), which control the transcription of the mRNA variants 5′UTR-C, 5′UTR-B and 5′UTR-A, respectively [9]. B. Luciferase reporter assays were performed in ND7/23 and HeLa cells using the pGL3 luciferase vector carrying the REG-1 sequence. The transcriptional activity of this sequence was assessed after overexpression of Prrxl1 and Tlx3, alone or in combination, in both cell lines (ns, not significant). C. In similar reporter assays, the effect of Tlx3 overexpression upon the transcriptional activity of the REG-1 sequence was tested after transfection with siRNA oligos for Prrxl1 (siPrrxl1), and compared to control conditions (Control; scrambled oligo). The Prrxl1 silencing efficiency was assessed by quantitative PCR and is shown in the graph with gray bars. D. Luciferase reporter assays were performed using two different fragments originating from the partition of REG-1 sequence. One of the fragments contains the promoter P3 (pGL3-P3-prom, −1401/−605) and the other contains the promoters P1/P2 (pGL3-P1/P2-prom, −604/−50). The effect of Tlx3 and/or Prrxl1 overexpression upon the transcriptional activity of these fragments was assessed (ns, not significant). E. Schematic representation of the distinct transcriptional mechanisms that control Prrxl1 gene expression. Working hypotheses are that Tlx3 acts in combination with Prrxl1 upon the promoters P1/P2 region and independently of Prrxl1 upon promoter P3 region.

effect of Prrxl1 and Tlx3 overexpression upon these two distinct regulatory regions in ND7/23 cells (Fig. 1D). When overexpressed alone, Prrxl1 exerted its repressive action only on the DNA fragment that contained the promoter P3 ($p < 0.01$, pGL3-P3-prom; Fig. 1D). No effect was observed in the P1/P2-prom fragment (ns; Fig. 1D). Tlx3 overexpression caused an increase in the transcriptional activity of both fragments, although with higher impact on P1/P2 (pGL3-P1/P2-prom; 42-fold increase, $p < 0.01$) than on P3 (P3-prom; 17-fold increase, $p < 0.01$). Combined overexpression of Tlx3 and Prrxl1 resulted in a 70-fold increase in the activity of P1/P2 (pGL3-P1/P2-prom), confirming the synergistic effect of both transcription factors at these promoters. Similar to the P3, Tlx3/Prrxl1 overexpression led to a 13-fold increase in transcriptional activity, which is the result of the isolated effects provoked by Prrxl1 (0.6-fold) and Tlx3 (18-fold) overexpression (Tlx3/Prrxl1/pGL3-P3-prom; Fig. 1D).

Up to this point, our results strongly indicated that Tlx3 induces transcription from *Prrxl1* promoters by two distinct mechanisms (see model in Fig. 1E). Tlx3 (i) activates the TATA-containing promoter P3, independently of Prrxl1 and (ii) works in combination with Prrxl1 to enhance the activity of the promoters P1/P2.

3.2. Tlx3 action on Prrxl1 promoter P3 requires a bipartite regulatory module

To better understand how Tlx3 specifically acts on the *Prrxl1* promoter P3, we focused our study on the identification of *cis*-regulatory elements important for this modulation. We performed successive sequence deletions of the P3-prom fragment (Fig. 2A) and measured the capability of Tlx3 to enhance luciferase expression in each construct. The transcriptional activity of P3 progressively increased with the successive trimming of P3-prom fragment, reaching maximum activity with P3-B (2.8-fold increase in comparison with P3-prom fragment, $p < 0.01$). The data suggest the presence of important repressor motifs located upstream of position −889 bp. The deletion of the region encompassing nucleotides −889 to −809, which gave rise to P3-C fragment, induced a strong decrease in activity (0.5-fold decrease in comparison with P3-prom fragment, $p < 0.01$; Fig. 2B). We conclude that this region contains important elements mainly responsible for the modulation of Tlx3-induced promoter activity. The fact that the P3-C fragment (−809 to −605) still responded to Tlx3 induction points to the presence of additional enhancing motifs in this region.

Afterwards, we performed binding assays to investigate whether Tlx3 could directly bind these promoter regions. DNA pull-down experiments were done using biotinylated DNA fragments similar to the ones previously examined (scheme in Fig. 2A). Equal amounts of each fragment were incubated with protein extracts from ND7/23 cells overexpressing Tlx3 and then submitted to a streptavidin-agarose pull-down procedure. Following Western blotting analysis of the precipitates, we observed that Tlx3, as expected, did not bind the control region (a portion of the *Prrxl1* coding sequence), nor the DNA fragment containing the P1/P2 promoters (Fig. 2B), suggesting that Tlx3 induction on the P1/P2 alternative

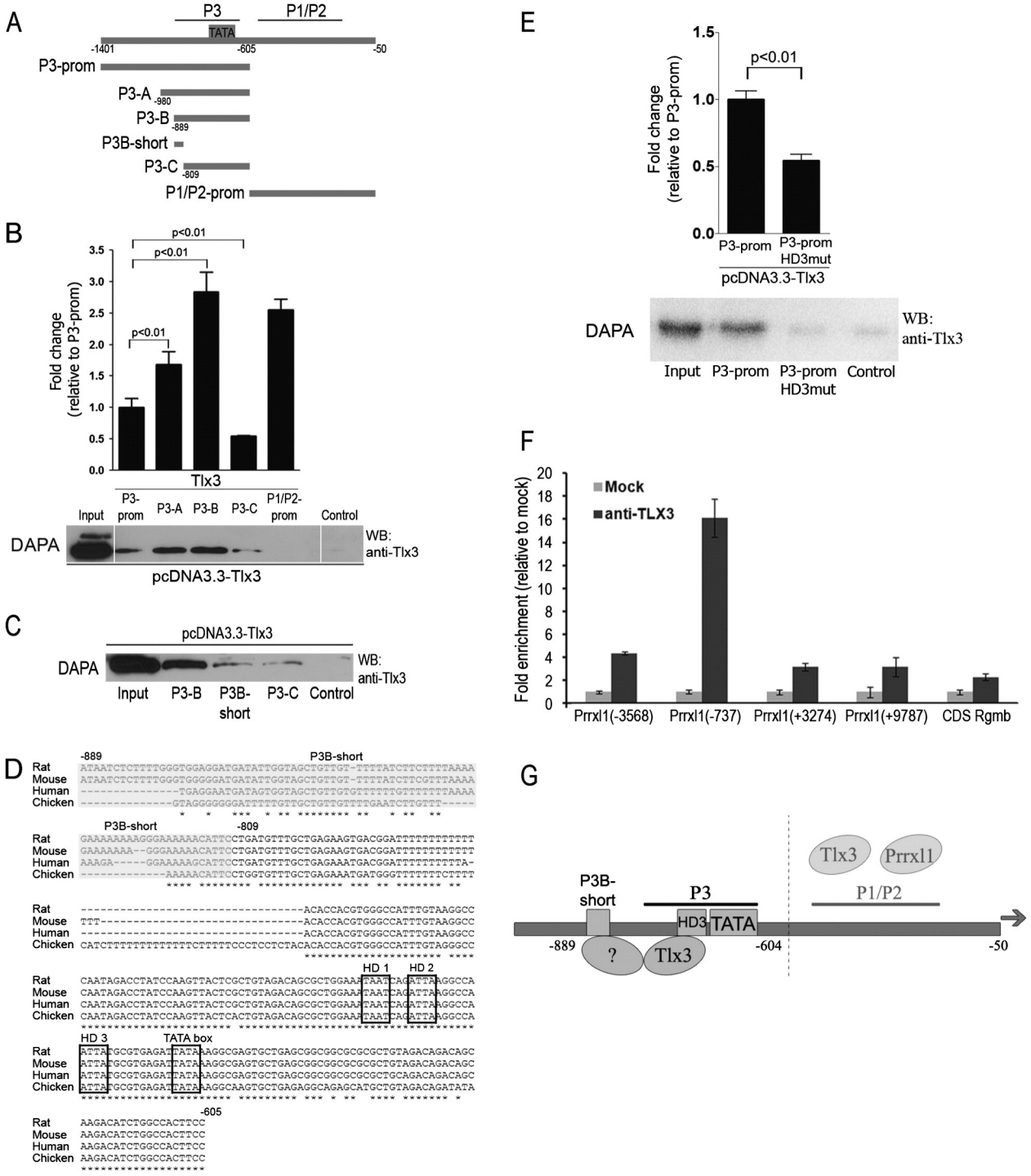

Fig. 2. Tlx3 activates *Prrxl1* P3 promoter by acting on a bipartite DNA element. A. Schematic representation of different fragments originating from successive deletions of the fragment REG-1. B. DNA fragments derived from REG-1 sequence, depicted in A, were cloned into the pGL3 plasmid. These constructs were used to transfect ND7/23 cells and luciferase reporter assays were performed with the cell lysates to evaluate the impact of Tlx3 on the transcriptional activity of each DNA fragment. To evaluate the Tlx3 binding capacity to these regions, ND7/23 cells overexpressing Tlx3 were incubated with biotinylated DNA probes corresponding to fragments P3-prom, P3-A, P3-B, P3-C, and P1/P2-prom. As a control, a region corresponding to part of the Prrxl1 coding sequence was used. The samples were submitted to affinity pull-down assays and the protein eluates analysed by Western blotting with an anti-Tlx3 antibody. A region between −889/−809 bp is required for Tlx3 activity on P3 promoter. C. DNA pull-down assays were performed as described in B with biotinylated DNA probes corresponding to fragments P3-B, P3-C and P3B-short. The Tlx3 action depends on a bipartite module. D. CLUSTALW nucleotide alignment of P3-B sequences from rat, mouse, human and chicken. The stars represent conserved nucleotides. The boxes delimit the TATA-box and three putative binding sites for homeodomain transcription factors (HD1, HD2 and HD3). The gray box highlights the "P3B-short" motif (−889/−809) used in DNA pull-down assays in C. E. Luciferase activity induced by the sequence P3-prom containing a mutated HD3 motif (P3-prom/HD3-mut) relative to P3-prom was measured in ND7/23 cells overexpressing Tlx3. The same DNA fragments were biotinylated and used in DNA pull-down assays using ND7/23 cells overexpressing Tlx3. The eluates were analysed by Western blotting using an anti-Tlx3 antibody. The Tlx3 binding affinity to the P3-prom region decreases when the HD3 motif is mutated. F. Chromatin immunoprecipitation assays with (anti-TLX3) or without (Mock) an anti-Tlx3 antibody were performed using dorsal spinal cord chromatins from E14.5 mouse embryos, followed by quantitative PCR using primers targeting the P3 region (Prrxl1 −737), an upstream region (Prrxl1 −3568), two downstream regions (Prrxl1 +3274 and Prrxl1 +9787) and a portion of the coding sequence of a non-related gene (CDS *Rgmb*). Strong enrichment of Tlx3 binding is observed in the region where the HD3 is present (−737). G. Schematic representation of the distinct transcriptional mechanisms that control the Prrxl1 gene expression. Tlx3 positive effect on *Prrxl1* promoter P3 depends on two distinct modules: the HD3 motif and an element within the P3B-short region, which is the binding site of a still unknown transcription factor. The mechanisms that regulate the transcription induced by Prrxl1/Tlx3 on promoters P1/P2 will be studied afterwards.

promoters is indirect. On the other hand, Tlx3 bound all the fragments that encompassed the promoter P3 (P3-prom, P3-A, P3-B and P3-C; Fig. 2B) with a progressive increase in the binding affinity that correlates to what was observed with transcriptional activity (Fig. 2B, graph). Likewise, although the band intensity decreased significantly when fragment P3-B was trimmed to originate fragment P3-C, some degree of binding was still observed. This raises the hypothesis that Tlx3-dependent induction requires two separate elements, one located in region −889/−809 and the other within region −809/−605, which is supported by the fact that Tlx3 is also not able to strongly bind the DNA fragment −889/−809 (P3B-short motif; Fig. 2C). In fact, when comparing the Tlx3 binding affinity of the fragments P3-C and P3B-short with the entire sequence P3-B, we observed that the band intensity for fragments P3B-short and P3-C were similar, but significantly weaker than the band intensity detected for P3-B (Fig. 2C).

By looking at the nucleotide sequence alignment of fragment P3-B (−889 to −605), we only identified three conserved motifs that could be binding sites for HD transcription factors (Fig. 2D; HD1, HD2 and HD3 boxes), including Tlx3. Recently, we showed that mutations on the HD1 and HD2 motifs did not compromise Tlx3-induction on *Prrxl1* promoters [9], pointing to the remaining HD motif (HD3) as a good candidate for Tlx3 binding. To test this hypothesis, we compared the transcriptional activity displayed by the fragment P3-B in ND7/23 cells overexpressing Tlx3 with that of a fragment containing a mutated HD3 motif (A**TT**A for A**CC**A; Fig. 2E). This mutation resulted in a two-fold decrease of luciferase activity ($p < 0.01$), indicating that this element is required for Tlx3-dependent induction. Likewise, a significant decrease in the Tlx3 binding affinity was observed by DNA pull-down assays using a P3-B biotinylated fragment harboring a mutation on HD3 motif (Fig. 2E).

To further prove that Tlx3 is able to bind the DNA region containing the HD3 motif *in vivo*, ChIP with anti-Tlx3 antibody was performed using dorsal spinal cord extracts from E14.5 mouse embryos. Immunoprecipitated genomic DNA was analysed by quantitative PCR using primers amplifying the vicinity of P3 promoter, as well as control primers for upstream and downstream of *Prrxl1* promoter regions and a non-related (*Rgmb*) coding region (Fig. 2F). Binding of Tlx3 was much stronger at the P3 promoter region (position −737), compared to control regions. Thus, our results indicate that Tlx3 is recruited to the DNA region comprising the promoter P3 in the developing spinal cord, most likely through interaction with the HD3 element.

It is important to mention that no conserved putative HD motif was detected inside the sequence encompassing nucleotides −889 to −809 (P3B-short), a region previously shown to be required for Tlx3 binding. Taking into account this observation and the fact that Tlx3 induction of *Prrxl1* promoter activity is dependent on the neuronal cell context (see Fig. 1B), we draw the hypothesis that Tlx3 binding on this region is indirect and requires the presence of another transcription factor (see model, Fig. 2G).

Altogether, our results indicate that a bipartite regulatory module is required for the positive action of Tlx3 on the *Prrxl1* promoter P3. Tlx3 binds directly to the HD3 motif (position −683), localized in the vicinity of the TATA box, and in combination with a putative neuronal specific transcription factor, which recognizes an element present within region −889/−809 (see model in Fig. 2G).

3.3. Brn3a is required for the transcriptional activation of the Prrxl1 promoters P1 and P2

The cooperative effect of Prrxl1 and Tlx3 on the activity of promoters P1/P2 raised the question of whether these two proteins could be part of the same transcriptional machinery. To unveil the existence of a physical interaction between Prrxl1 and Tlx3, co-immunoprecipitation assays were performed using protein extracts from ND7/23 cells transfected with pcDNA3.3-Tlx3-FLAG and pcDNA3.3-HA-Prrxl1 or an empty pcDNA3.3 (Fig. 3A). The immunoprecipitation of FLAG-Tlx3 with an anti-FLAG affinity matrix followed by Western blotting demonstrated that these two transcription factors are part of the same interacting complex (Fig. 3A). It remains to be investigated whether the complex contains other proteins.

After having established the Prrxl1/Tlx3 interaction, we focused our attention on understanding the specific mechanisms that modulate the regulation promoted by this complex. As shown by DNA pull-down assays, Tlx3 is not able to bind the DNA fragment containing P1/P2 promoters (P1/P2-prom; Fig. 2B). This result suggests that Tlx3-dependent induction on these promoters is indirect and thereby requires other molecular players. We have shown elsewhere that Brn3a induces an increase in the luciferase gene transcription regulated by the sequence REG-1 [9]. This protein co-expresses with Prrxl1 in primary sensory neurons and in spinal glutamatergic neurons of early developmental stages. Prrxl1 expression is decreased in *Brn3a* null mice [18], indicating that Brn3a has a role in the control of *Prrxl1* transcription. Considering these data, we tested the effect of Brn3a overexpression in the transcriptional activity of promoter P3 or promoters P1/P2 (Fig. 3B). We observed a prominent activation in luciferase activity for the region that comprised promoters P1/P2 (30-fold induction, $p < 0.01$; pGL3-P1/P2-prom in Fig. 3B), at values that resembled the activation induced by Tlx3 and Prrxl1 for this region (Fig. 1D). An effect was also displayed on promoter P3, although less pronounced (10-fold, $p < 0.01$; pGL3-P3-prom in Fig. 3B). To investigate whether Brn3a induction on P1/P2 could be related to transcriptional activation promoted by Tlx3 and Prrxl1, we silenced endogenous Brn3a expression and assessed the transcriptional activity of P3-prom and P1/P2-prom induced either by Tlx3 or Tlx3/Prrxl1 (Fig. 3C). Transcription induced by combined overexpression of Tlx3 and Prrxl1 was affected by Brn3a silencing mainly at promoters P1/P2 (compare the decrease in luciferase activity from siControl to siBrn3a in Tlx3/Prrxl1/pGL3-P1/P2-prom, $p < 0.01$; Fig. 3C). However, downregulation of Brn3a did not affect Tlx3 activation per se of any *Prrxl1* alternative promoters (see Tlx3/pGL3-P3-prom and Tlx3/pGL3-P1/P2-prom, ns), implying that Tlx3 also acts on P1/P2 promoters through a Brn3a-independent mechanism.

A model for the dual action of Tlx3 on *Prrxl1* alternative promoters is proposed (see Fig. 3D). Tlx3 exerts its positive effect by directly binding to promoter P3 (see Fig. 2) or by acting in combination with Prrxl1 to modulate the transcriptional activity of promoters P1/P2 via Brn3a. Alternative mechanisms for regulation of *Prrxl1* promoters by Tlx3 and Brn3a need to be investigated in the future.

3.4. Tlx3 induces Prrxl1 hyperphosphorylation

Recently, we have shown that Prrxl1 is differentially phosphorylated in the dorsal spinal cord and DRG along development [19]. The increase in phosphorylation state is accompanied by conformational changes resulting in a multiple band pattern on electrophoretic analysis [19]. Western blotting analysis of Prrxl1 in ND7/23 cells showed a clear duplet: the lower band accounted for hypophosphorylated Prrxl1 while the upper band corresponded to hyperphosphorylated Prrxl1 (Fig. 4A; [19]). Surprisingly, overexpression of Tlx3 altered the phosphorylation state of Prrxl1. In Western blotting experiments, co-expression of Prrxl1 with increasing amounts of Tlx3 resulted in a progressive disappearance of Prrxl1's lower band while the upper band was maintained (Fig. 4A). This Prrxl1 upper band corresponded to higher phosphorylated states as demonstrated by CIAP treatment of protein extracts of ND7/23 cells transfected with Prrxl1 with or without Tlx3 (Fig. 4B). CIAP dephosphorylation promoted the conversion of the Prrxl1 uppermost band into the lower band while the addition of an inhibitor abolished this effect. These data point to the upper migrating band being the result of phosphorylation. Likewise, taking advantage of the isoelectric focusing technique to detect different levels of phosphorylation, we analysed protein extracts from ND7/23 cells overexpressing Prrxl1 or Prrxl1 together with Tlx3. 2D electrophoresis resolved Prrxl1 in multiple spots, corresponding to different levels of

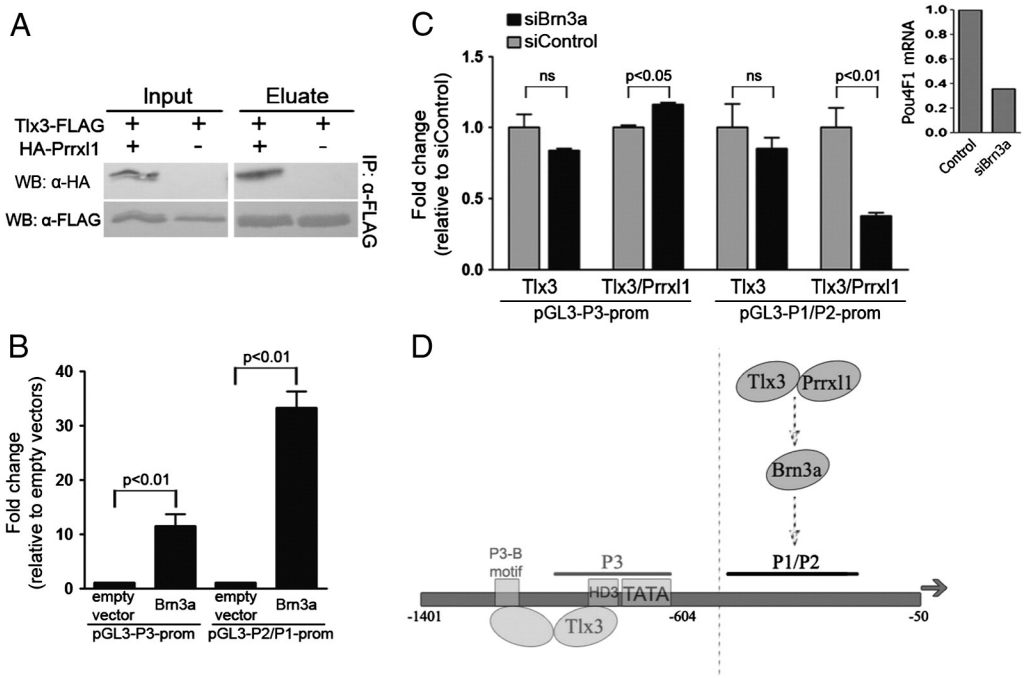

Fig. 3. The Tlx3/Prrxl1 protein complex activates *Prrxl1* P1/P2 promoters via the action of Brn3a. A. Co-immunoprecipitation experiment to test the interaction between Prrxl1 and Tlx3. ND7/23 cells were transfected with Tlx3-FLAG together or in the absence of HA-Prrxl1. Immunoprecipitation was performed against the FLAG tag and the presence of Prrxl1 in the eluate was confirmed by Western blotting using an anti-HA antibody. B. Luciferase activity induced by the sequence P3-prom or P1/P2-prom was measured in ND7/23 cells overexpressing Brn3a or not (empty vector). Brn3a overexpression displays a stronger effect on promoters P1/P2. C. Luciferase reporter assays were performed in ND7/23 cells previously transfected with either siRNA oligos for *Brn3a* (siBrn3a) or with a scrambled sequence (siControl). The relative luciferase activity induced by Tlx3 or Tlx3/Prrxl1 overexpression on P3-prom or P1/P2-prom DNA sequences was assessed. The *Pou4F* silencing efficiency was determined by quantitative PCR and is shown in the upper right graph. The induction by Tlx3/Prrxl1 complex depends on the presence of Brn3a specifically on promoters P1/P2 region (ns, not significant). D. Schematic representation of the distinct transcriptional mechanisms that control the Prrxl1 gene expression. Besides the Tlx3 action on promoter P3 described in Fig. 2, we now add the inductive effect of Tlx3 on promoters P1/P2 activity, which requires the synergistic interaction between Tlx3 and Prrxl1 and the presence of Brn3a.

phosphorylation (Fig. 4C). Prrxl1 has a theoretical isolelectric point (pI) of 8.74 and each phosphate adds a negative charge to the protein modifying the pI and thereby shifting the protein migration toward the anode. In the presence of Tlx3, Prrxl1 displayed more intense spots in the acidic region of the strip, likely due to the presence of more phosphorylated residues (Fig. 4C). Altogether, our results strongly suggest that Tlx3 promotes Prrxl1 hyperphosphorylation.

3.5. Mapping the functional Tlx3 domains related to Prrxl1 activity

To gain insight into the Tlx3 domains implicated in the control of Prrxl1 transcription and phosphorylation, we generated truncated versions of Tlx3 based on the degree of homology of Tlx3 primary sequence from different species (Fig. 5A). We then tested these constructs by luciferase reporter assays, in which the reporter gene was controlled by *Prrxl1* alternative promoters P3 or P1/P2. Deletion of the C-terminal domain (amino acid residues 256−291) reduced Tlx3-induced activity in the promoters P1/P2 (black bars; Fig. 5B), while no impact was observed on the *Prrxl1* promoter P3 (white bars; Fig. 5B). Deletion of the N-terminal (amino acid residues 1−38) greatly interfered with the overall Tlx3 transcriptional activity (strong decrease of luciferase expression on all the promoter regions under analysis; Fig. 5B), which is indicative of the presence of an important activator domain for Tlx3 action. Interestingly, removal of the region encompassing amino acids 76−111 restored the Tlx3 transcriptional activity on P3 promoter (Fig. 5B), pointing to the presence of a repressor domain in this region. This regulatory domain was not implicated in the transcriptional control of *Prrxl1* promoter P1/P2, since all the Tlx3 versions with N-terminal deletions displayed low values of luciferase activity on P1/P2 *Prrxl1* promoters (Fig. 5B).

In order to define the Tlx3 domain involved in the induction of Prrxl1 hyperphosphorylation, co-expression of Prrxl1 with Tlx3 truncated versions was carried out in the ND7/23 cell line. The Prrxl1 band profile was then assessed by Western blotting (Fig. 5C). Quantification of the upper (hyperphosphorylated Prrxl1) and lower (hypophosphorylated Prrxl1) band intensity relatively to the total Prrxl1 signal was depicted in a graph (Fig. 5C). As previously observed in Fig. 4A, the presence of the wild-type Tlx3 induced the hyperphosphorylation of Prrxl1, converting Prrxl1 from a double band pattern to a sole upper band (black bar in the graph; Fig. 5B). While the deletion of Tlx3 C- or N-extremity did not significantly interfere with the Prrxl1 phosphorylation state (compare bars for $Tlx3_{(1-224)}$, $Tlx3_{(1-256)}$, $Tlx3_{(38-291)}$, $Tlx3_{(76-291)}$ with Tlx3 in Fig. 5C), removal of the region encompassing amino acids 76 to 111 resulted in a Prrxl1 band pattern similar to what was observed in the absence of Tlx3 (compare $Tlx3_{(111-291)}$ with pcDNA3.3 in Fig. 5C). This result maps the Tlx3 domain responsible for Prrxl1 hyperphosphorylation between amino acids 76 and 111.

Altogether, we defined multiple Tlx3 domains controlling different aspects of Prrxl1 functioning. These domains are highly conserved in the two other members of the Tlx family (Tx1 and Tlx2), as shown in the amino acid sequence alignment in Fig. 5D. A summary of the activity of each domain is depicted in Fig. 5E: Domain I (1–38)—N-terminal activator domain implicated in the overall Tlx3 transcriptional activity, which contains a highly conserved Eh1 motif; Domain II (76–111)—includes a conserved Pro-rich region, induces Prrxl1 hyperphosphorylation and reduces Tlx3 induction of *Prrxl1* promoter P3; Domain III (256–291)—C-terminal activator domain that modulates *Prrxl1* promoter P1/P2 activity. While Domains I and III were already reported to have a transcriptional action on Tlx1-target genes [21,22], the relevance of Domain II for the function of Tlx genes was defined here for the first time.

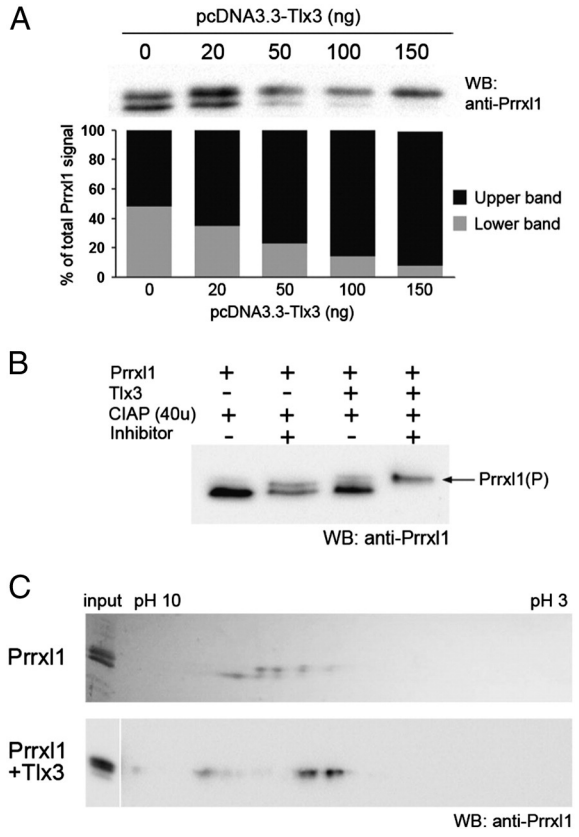

Fig. 4. Tlx3 induces Prrxl1 hyperphosphorylation. A. Western blotting analysis of the Prrxl1 band pattern when co-expressed with increasing amounts of Tlx3 (none to 150 ng of plasmid) in ND7/23 cells. The Prrxl1 band intensity was quantified and the values are graphically presented. Note the progressive disappearance of the lower band (hypophosphorylated Prrxl1) as the concentration of Tlx3 plasmid increases. The upper band, which corresponds to a higher phosphorylated state [19] is enriched with the increase of Tlx3 levels. B. ND7/23 cells transfected with Prrxl1 and Tlx3 (+) or an empty vector (−) were treated with 40 U of CIAP in the presence (+) or absence (−) of a competitive inhibitor (Na_2HPO_4). The CIAP activity converts the Prrxl1 upper band into the lower band, suggesting that the Tlx3-induced upper band is the result of phosphorylation. Prrxl1(P) represents the hyperphosphorylated form of the protein. C. 2D electrophoresis analysis of Prrxl1 from ND7/23 cells overexpressing Prrxl1 alone (Prrxl1) or together with Tlx3 (Prrxl1 + Tlx3). Prrxl1 spots correspond to different numbers of phosphorylated residues. By comparing Prrxl1 with Prrxl1 + Tlx3 samples, we observe that in the presence of Tlx3, Prrxl1 spots are dislocated towards a lower pH indicating a higher degree of phosphorylation. Five percent of the total extract was used as the input sample.

4. Discussion

The present study addresses the molecular mechanisms that control the expression of Prrxl1, an HD transcription factor that plays a crucial role in the establishment of the DRG/dorsal spinal cord nociceptive circuitry. It departs from previous data showing that Prrxl1 expression depends on Tlx3 at late stages of spinal cord development [13], and investigates how Tlx3 acts on the recently identified *Prrxl1* promoters P1, P2 and P3 [9]. Tlx3 was here proved to control *Prrxl1* transcription at P1/P2 and at P3 in two different ways: indirectly, at P1/P2 promoter, as a Tlx3/Prrxl1 complex via the action of Brn3a; and directly, by binding to an HD motif at P3 promoter. Tlx3 was also shown to induce Prrxl1 post-translational modifications, namely, phosphorylation (see proposed model; Fig. 6).

4.1. Tlx3 uses transcriptional mechanisms to control Prrxl1 expression

At the *Prrxl1* TATA-containing promoter P3, Tlx3 enhances *Prrxl1* transcription through a bipartite regulatory module. One element corresponds to a newly identified HD motif (position −683) recognized directly by Tlx3, and the other one is located within the DNA region −889/−809 bp. This region should be the binding site of a still unknown transcription factor. Putative candidates may be among the TALE (three amino acid loop extension) protein family, a class of transcription factors associated with developmental gene regulation that often interact with HOX factors, such as Tlx3 [23,24]. Tlx3 forms a DNA-binding complex with the TALE protein Pbx3 to enhance the transcription of Tlx3-responsive elements *in vitro* [25]. Tlx3/Pbx3 interaction is also likely to occur *in vivo*, as *Pbx3* null mice have a respiratory phenotype similar to that occurring in *Tlx3* null mice [26]. The overlapping expression of Tlx3, Pbx3 and Prrxl1 in second-order sensory neurons additionally suggests that Tlx3 may complex with Pbx3 to regulate *Prrxl1* transcription [14,27].

On *Prrxl1* promoters P1/P2, Tlx3 was shown to act in combination with Prrxl1. However, Tlx3/Prrxl1 modulation was not performed through direct DNA binding but rather required the presence of Brn3a. Brn3a silencing in ND7/23 cells impaired *Prrxl1* expression enhancement through Tlx3/Prrxl1 overexpression. Interestingly, Prrxl1 alone exerts no effect on promoters P1/P2 and acts mainly as an autorepressor on P3. This observation has relevant implications and will be addressed elsewhere (Monteiro F.A., unpublished data).

Although studies approaching Brn3a's implication on the control of Prrxl1 spinal cord levels are missing, *Brn3a* null mice present a marked decrease in *Prrxl1* expression in primary afferent neurons [18], pointing to the important role of Brn3a in the positive modulation of *Prrxl1* transcription *in vivo*. Promoters P1 and P2 control the transcription of two distinct *Prrxl1* 5′UTR variants (5′UTR-B and 5′UTR-C, respectively), which are predominantly expressed at early stages of spinal cord neurogenesis (maximum expression peak at E12.5; [9]). On the other hand, Prrxl1 expression is maintained in the developing spinal cord up to E14.5 in *Tlx3* null mice, and only later on does it start to vanish. This implies that at early spinal cord developmental stages, expression of *Prrxl1* may be triggered by DNA elements that do not respond directly to Tlx3. The possibility that Prrxl1 early stage transcription does not require the presence of Tlx3, but instead relies on Brn3a, should not be ruled out. Indeed, in the developing spinal cord, co-localization between Brn3a, Prrxl1 and Tlx3 is only observed in glutamatergic neurons at early stages [13,14,28,29]. Brn3a would then be primarily required to initiate the transcription of promoters P1/P2 and the Tlx3/Prrxl1 complex would, through a positive feedback mechanism, later enhance Brn3a activity (see model; Fig. 6). This hypothesis is further supported by genome-wide ChIP assays with anti-Tlx3 and anti-Prrxl1 antibodies, which resulted in the identification of shared binding events for Tlx3 and Prrxl1 transcription factors on the *Brn3a* gene (Monteiro F.A., unpublished data).

4.2. Distinct Tlx3 protein domains are required to control Prrxl1 transcription

Using protein sequence alignment and Tlx1 biochemical data already reported by others [22], we were able to define three Tlx3 functional domains (Fig. 5D) besides the already known Homeodomain (amino acids 167–224) and PIM motif (amino acids 125–129). These are the NH_2-terminus Domain I, which contains an Engrailed homology 1 motif (Eh1; amino acids 17–24), the COOH-terminal Domain II (amino acids 256–291) and a newly identified proline-rich domain III (PRD; amino acids 76–111). We then investigated whether the various Tlx3 protein domains are differentially implicated in the multi-component transcriptional activity of the *Prrxl1* alternative promoters. The N-terminal domain was shown to be responsible for the overall transcriptional activity of the Tlx3 protein, inducing promoters P3 and P1/P2. This domain includes an Eh1 motif, a short peptide sequence that is totally conserved in the other two members of the Tlx family (Tlx1 and Tlx2). In Tlx1 protein, this Eh1 motif is required for Tlx1-mediated target gene activation through interaction with the co-repressor Groucho/transducin-like enhancer of split protein (Gro/TLE), a co-regulator for many developmental transcription factors [21,22]. It is therefore reasonable to postulate that the Tlx3 N-terminal domain

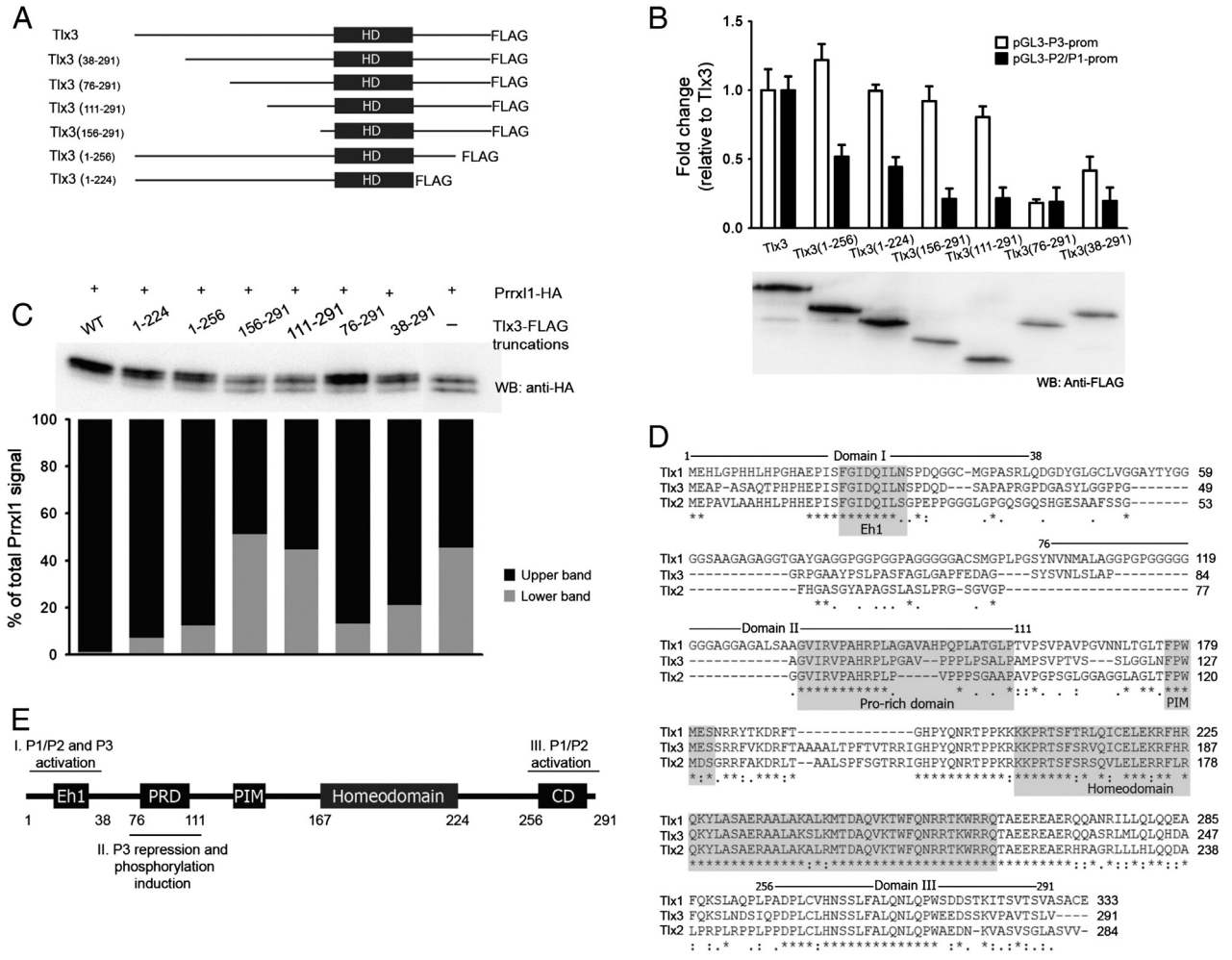

Fig. 5. Mapping of functional Tlx3 domains relevant for Prrxl1 transcription and phosphorylation. A. Schematic representation of Tlx3-FLAG truncated versions (HD, homeodomain). B. Relative luciferase activity from ND7/23 cells transfected with pGL3-P3-prom or pGL3-P2/P1-prom as luciferase reporter constructs, together with wild-type or truncated versions of Tlx3. Multiple Tlx3 functional domains are revealed. The representative immunoblot confirms the correct expression of all Tlx3 constructs. C. Western blotting analysis of the Prrxl1 band pattern when co-expressed with different Tlx3 truncated forms in ND7/23 cells. The graph depicts the amount of hyperphosphorylated (black bar, upper band) and hypophosphorylated Prrxl1 (gray bar, lower band), normalized for the total Prrxl1 signal in each lane. Tlx3-induced hyperphosphorylation is not observed with $Tlx3_{(111–291)}$ and $Tlx3_{(156–291)}$. D. Alignment of mouse Tlx1, Tlx2 and Tlx3 primary sequences. The alignment was performed using the CLUSTALW algorithm and the stars represent conserved amino acids. Highlighted in gray are the homeodomain, the Eh1 (engrailed homology 1 motif) and PIM (Pbx-interaction motif) domains, previously characterized in Tlx1 protein, and the newly characterized proline-rich domain. Tlx3 functional domains (Domains I, II and III) with impact on *Prrxl1* alternative promoters are indicated. E. Schematic representation of conserved functional domains identified in the Tlx3 primary sequence. The N-terminal domain (amino acids 1–38), which contains the Eh1 motif, is required for the overall transcriptional induction of *Prrxl1* promoters, whereas C-terminal domain (CD; amino acids 256–291) is exclusively required for P1/P2 activity. A proline-rich domain (PRD; amino acids 76–111) is essential for transcriptional repression of *Prrxl1* promoter P3 and also for the induction of Prrxl1 hyperphosphorylation.

may activate *Prrxl1* alternative promoter transcription in a similar way, by recruiting Gro/TLE protein via the Eh1 motif.

A C-terminal domain identical to that here identified is also present on Tlx1 protein, where it is required for Tlx1 efficient transcriptional transactivation [22]. Likewise, the analogous Tlx3 domain (amino acids 256–291) was shown here to be responsible for *Prrxl1* transcription only at promoters P1/P2, when transcription is accomplished by Tlx3/Prrxl1 protein complex. The fact that this C-terminal domain does not have any influence on the modulation of promoter P3 reinforces the occurrence of distinct transcriptional mechanisms at each promoter region.

4.3. Tlx3 induces Prrxl1 hyperphosphorylation

A domain displaying a specific repressive trait of Tlx3 action upon promoter P3 was delimited (amino acids 76–111). This domain is rich in proline residues. Because the overall effect of Tlx3 on P3 promoter results in transcriptional activation, it is possible that this repressor domain is inhibited by another part of Tlx3 protein, such as the N-terminal Eh1 domain. Although being an intrinsic limitation of truncation approaches, we cannot exclude structural destabilization on Tlx3 by the removal of an important part of the protein. Thus, deletion of amino acids 1–38 may disturb the internal region 76–111, which may lead to Tlx3 unfolding and subsequent decrease in transcriptional activity. An alternative explanation is that the domain 76–111 reduces Tlx3 activation of the Prrxl1 promoter by binding to cofactors. This interaction could be modulated by the N-terminal Eh1 domain through intramolecular events. This protein complex, composed of Tlx3 and putative molecular partners, may act as transcriptional repressors on specific DNA target regions. This hypothesis would explain why domain 76–111 only negatively regulates Tlx3 action on the P3 promoter and has no effect on P1/P2 promoter. In fact, N-terminal deletions of Tlx1 have different effects when we consider different promoters. Tlx1 action on the Aldh1 and SV40 promoters requires a fully functional homeodomain. However, the same N-term deletion that eliminates Tlx1 induction of the Aldh1 promoter bears no effect on Tlx1-

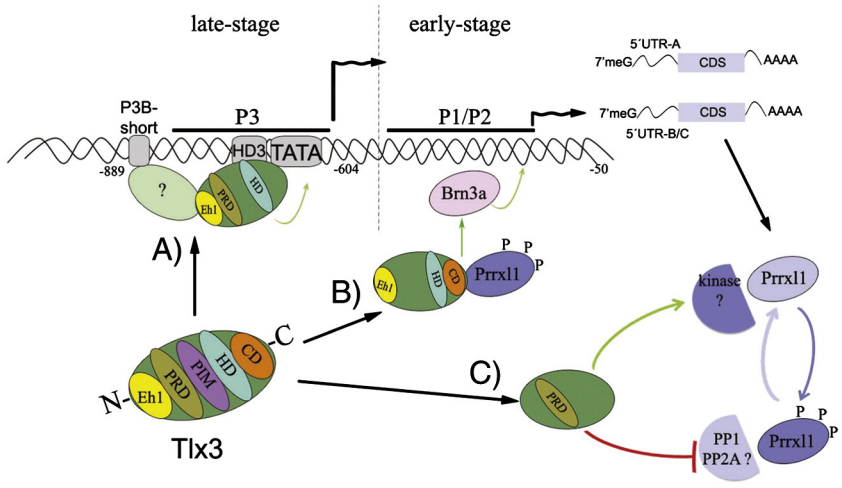

Fig. 6. Tlx3 displays a central role in the control of Prrxl1 expression and activity. A. Tlx3 positive effect on Prrxl1 promoter P3 requires a bipartite module composed by an HD3 motif (position −683/−679), which is the binding site of Tlx3, and an element within the P3B-short region (position −889/−809), which is recognized by a still unknown transcription factor. The N-terminal Eh1-containing domain activates the P3 promoter while the proline-rich domain (PRD) antagonizes this effect. P3 promoter was shown to transcribe the 5′UTR-A mRNA variant, which is enriched at late-stage neurogenesis [9]. B. The promoters P1/P2 activity is enhanced by a synergistic interaction between Tlx3 and Prrxl1 and requires the presence of Brn3a. Both Tlx3 N- and C-terminal domains (CD) are necessary for this regulatory mechanism. P1/P2 promoters were shown to transcribe the 5′UTR-B and -C mRNA variants, which are enriched at early stage neurogenesis [9]. C. The Tlx3 proline-rich domain (PRD) is required for the induction of Prrxl1 hyperphosphorylation through an unknown mechanism. Tlx3 modulation of kinase and/or phosphatase activity is hypothesized. Eh1, engrailed homology 1 motif; PRD, proline-rich domain; PIM, Pbx interaction motif; HD, homeodomain; CD, C-terminal domain.

mediated repression of the SV40 promoter. This shows that although the protein structure is preserved, as there is still repression and thus HD integrity, the Tlx1 N-terminus is capable of modulating Tlx1 activity [22].

In addition to its action in the repression of *Prrxl1* transcription, we present strong evidence that this Tlx3 Pro-rich domain is also responsible for increasing Prrxl1 phosphorylation state and thereby altering its conformation to a more exposed structure [19]. We have recently shown that this phospho-dependent conformational change has a significant impact on Prrxl1 transcriptional activity [19]. Prrxl1 is hyperphosphorylated in the dorsal spinal cord from E10.5 until E14.5 [19], a developmental window where spinal cord neurogenesis is occurring and extensive co-localization between Prrxl1 and Tlx3 is observed [14]. Thus, we suggest that until E14.5, the high Prrxl1 phosphorylated state is sustained by the presence of Tlx3. From E16.5 onwards, Prrxl1 is progressively dephosphorylated [19], which correlates well with the vanishing expression of Tlx3 in spinal neurons. Indeed, it was shown by us and others [13,14] that, at later stages of spinal cord development, part of the Tlx3/Prrxl1-positive neuronal population loses Tlx3 expression.

We here ascribe a new role for Tlx3 as a positive modulator of Prrxl1 phosphorylation. However, the mechanism by which Tlx3 induces Prrxl1 hyperphosphorylation remains elusive. How Tlx3 exerts this effect will be investigated in future studies. Nevertheless, it is well established that the balance between kinases and phosphatases activity acts as a molecular switch on protein function. The effect of Tlx1 in the activity of serine-threonine phosphatases, namely, PP1 and PP2A, was already reported by the use of enzymatic assays [30]. It is possible that the Pro-rich domain is implicated in the repression of PP1 and PP2A transcription and/or activity, and therefore in maintaining high levels of phosphorylated Prrxl1. Future studies should determine whether Prrxl1 is indeed a substrate for PP1 and PP2A activity. We also cannot exclude the possibility that Tlx3 promotes Prrxl1 phosphorylation via kinase recruitment. A recent work gave new insight on how the complex of TALE/Hox proteins function to regulate transcription [31]. The authors showed that following promoter occupancy, TALE proteins promote an active chromatin profile and recruit RNA polymerase II, which is maintained in a poised state until the Hoxb1b protein binds the target promoter to drive an efficient transcription. Hoxb1b triggers the recruitment of P-TEFb kinase, which in turn phosphorylates RNA polymerase II and other associated factors. This converts the RNA polymerase II poised state into an active elongating one. It is tempting to speculate that Tlx3, similarly to Hoxb1b, holds the capacity to recruit the P-TEFb or other kinase, which could promote the induction of Prrxl1 hyperphosphorylation, switching Prrxl1 to a more active transcriptional conformation.

Collectively, the present findings give important insights into the close relationship between Tlx3 and Prrxl1 transcription factors. They also point out that Prrxl1 depends on Tlx3 not only for the regulation of its transcription but also for the modulation of its phosphorylation levels (see model; Fig. 6). This novel finding has important implications in the molecular understanding of Tlx3 biology. The fact that Tlx3 has an impact on phosphorylation-dependent mechanisms can be helpful to explain the role of Tlx3 in other systems, such as in the establishment and maintenance of the T-cell acute lymphoblastic leukemia, a pathology derived from the aberrant expression of TLX genes.

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DISCUSSION

4. DISCUSSION

The general purpose of the investigation presented in this dissertation was to shed additional light on the molecular mechanisms and genetic relationships that govern the expression of *Prrxl1* during the development of pain-processing neurons. The study was motivated by the defective phenotype exhibited by *Prrxl1* null mutant embryos, a feature that encouraged our team to believe in the crucial requirement of this gene for the correct development and assembly of the pain circuit.

MOLECULAR MECHANISMS REGULATING *PRRXL1* TRANSCRIPTION AND POST-TRANSCRIPTION

Previously to the beginning of this study, three discrete *Prrxl1* 5'-UTRs were identified by RACE analysis (Rebelo et al., 2009). These variants would only differ at their 5' mRNA sequences positioned in exon 1, without interfering with the primary structure of the protein, since the Prrxl1 start codon is located in exon 2. This feature motivated the attempt to investigate their function and related post-transcriptional regulatory mechanisms that could be controlling *Prrxl1* expression. Indeed, in this study we successfully identified differences in the distinct 5'-UTRs concerning mRNA stability and translation efficiency. The control of mRNA rate of synthesis and decay is among the mechanisms by which a cell tightly defines the precise amount of mRNA, in a spatiotemporal way, conferring an additional step in the programmed fine-tune regulation of gene expression. A few mechanisms that may be involved in the regulation of mRNA stability include the mRNA binding by specific proteins (RNA-binding proteins), mRNA targeting by microRNAs (Filipowicz et al., 2008) and non-sense mRNA decay (Mendell et al., 2004). Although this is an issue that still remains to be explored in detail, we suggest in Publication I that RNA-binding proteins bind cis-regulatory elements present in the 5'-UTR region of the *Prrxl1* molecules and affect their stability in the developing neurons. This assumption is the most reasonable given the large number of RNA-binding proteins present in the eukaryote proteome (Lasko, 2000; Keene, 2001). Among them there is the family of the ELAV-like Hu RNA-binding proteins, which are involved in various aspects of mRNA regulation, from transport and stability to translation, being competent to control several features of neuronal

differentiation (Wakamatsu and Weston, 1997; Akamatsu et al., 1999). In fact, neuron-specific Hu proteins, such as HuC and HuD, are likely to bind the 5'-UTR-A and confer this variant the capacity to last longer than the other *Prrxl1* mRNA molecules and to have a higher translation rate (Perrone-Bizzozero and Bolognani, 2002). Post-transcriptional regulation is a common mechanism employed by the cell to control the amount and duration of transcript expression during nervous system development. Variation in the half-life of the different *Prrxl1* 5'-UTRs could be understood as a mechanism Prrxl1 use in situations where the precise level of this protein is determinant to accomplish distinct functions. Indeed, we previously observed that the levels of Prrxl1 change according to neuronal subtype and developmental age. We hypothesize that *Prrxl1* 5'-UTR-A mRNA gives rise to high amount of protein in neurons where its presence is needed to regulate genetic programs associated to processes that occur during an extended developmental period, such as axon guidance and cell adhesion. In opposition, Prrxl1-dependent transcriptional program active during a narrow developmental stage, such is the one that regulates the differentiation of spinal cord dI3 and dI5 early-born neurons (E10.5 - E11.5), requires a transient Prrxl1 presence, which may be accomplished by the use of the less stable 5'-UTR-B/-C mRNA variants. Further experiments consisting in the assessment of *Prrxl1* 5'-UTRs stability in cells lacking or with decreased expression of HuC and HuD would help to demonstrate their involvement in the regulation of *Prrxl1* mRNA decay.

The existence of 5'-UTR mRNA variants is usually indicative of a mechanism of alternative promoter usage. Indeed, in the selected region of the highly conserved 1351 bp upstream of Prrxl1 translation initiation site, we identified three promoter regions, each one adjacent to each of the 5'-UTR sequences previously characterized. These promoters were named P1, P2 and P3 and control, respectively, the transcription of *Prrxl1* 5'-UTR-C, 5'-UTR-B and 5'-UTR-A sequences. The transcriptional potential of the most conserved promoter (P3) was thoroughly analysed and evidence that it displays *in vivo* activity was presented. Further studies using transgenic zebrafish showed that the region adjacent to the TATA box, that comprises a highly conserved module of 172 bp containing the cis-regulatory HD and EBox motifs, is transcriptionally active in the developing hindbrain and cranial ganglia (Publication I, Figure 6), regions where the expression of zebrafish *drgx* had been previously

observed (McCormick et al., 2007). Additional analysis of the transcriptional activity of each promoter in distinct cell models revealed that only P1 and P3 display neuronal-specific properties, whereas the CpG island-containing promoter P2 is constitutive. Because the TATA-containing promoter P3 activates the transcription of the also neuronal-specific 5'-UTR-A, the existence of a promoter-mediated coordination mechanism of mRNA decay is suggested. In the past years, strong evidence of the intimate relationship between DNA promoters and transcription factors and the stability of the transcripts they give rise to, have been reported (Bregman et al., 2011; Trcek et al., 2011). These and other large-scale studies demonstrated that DNA regulatory elements present in yeast promoters are coordinated with mRNA degradation in the cytoplasm (Dori-Bachash et al., 2012), a graceful mechanism that appears to have evolved to become a fundamental aspect of gene regulation. When specific promoters elements (the upstream activating sequence) responsible for the transcription of a certain gene are replaced by the upstream activating sequence of another gene, the decay rate of the transcript is affected (Bregman et al., 2011; Trcek et al., 2011). Although the majority of these studies are performed in yeast, there is evidence that a similar process is applied to gene regulation in mammals (Enssle et al., 1993; Dori-Bachash et al., 2012). A strong evidence of the coordinated regulation of transcription and mRNA degradation is demonstrated in cases when an increase in mRNA levels occurs following the increase in the rate of mRNA degradation, rather than the expected decrease (Dori-Bachash et al., 2012). This positive correlation points to the existence of a cross-talk between the nuclear DNA promoter elements and the stability of cytoplasmic transcripts. Thus, variation in mRNA levels shapes the kinetics of gene transcription (Elkon et al., 2010), in a manner that guarantees the rapid response of promoters and continuous expression of transcripts when these are quickly degraded but their presence is still required. Unpublished results gathered during the research that conducted to this thesis showed that interference with transcription by Actinomycin D in primary cultures of mouse DRG, led to an increase in the levels of endogenous *Prrxl1* mRNA. This strongly suggests the existence of a direct response from *Prrxl1* promoters that leads to an increase in the transcriptional activity when a decay in the mRNA levels is sensed. This observation contrasts to what is observed in ND7/23 and HeLa cells (Publication I, Figure 1E) transfected with

constructs containing the different P*rrxl1* 5'-UTRs coupled to a SV40-containing promoter, where a decrease in the amount of mRNA was observed over time. Nevertheless, this is logically explained. As these constructs do not contain the *Prrxl1* 5'-UTRs linked to their wild-type promoters, the coordinated transcription/mRNA-decay regulation could not be observed.

REGULATORY ELEMENTS CONTROLLING *PRRXL1* TRANSCRIPTION

The search for alternative promoters in the conserved sequence of 1351 bp led to the finding that some DNA regions, even though containing core promoter elements, did not display any transcriptional activity. This is explained by the presence of an element (RRA) that completely abolishes the transcription of the three alternative promoters (Publication I, Figure 2). Another element (RRB) suppresses the negative action of RRA, resulting in the activation of *Prrxl1* transcription as the overall effect. Despite the fact that still no transcription factors that may act on these sequences have been identified, the existence of these elements with such antagonistic behavior suggests they may be relevant to the tight modulation of *Prrxl1* expression in distinct neuronal populations. This is the case observed during the development of the dorsal spinal cord, where the expression of *Prrxl1* is positively regulated in the glutamatergic neurons and suppressed in the population of GABAergic neurons. Although these two populations derive from the same progenitor domain, they are molecularly and functionally mutually exclusive. Thus, it is suggested that molecular players in either inhibitory or excitatory neurons, suppress each other expression. For instance, Tlx3 acts as an inhibitor of Lbx1 action inducing the glutamatergic transmitter phenotype (Cheng et al., 2005), whereas Ptf1a represses *Tlx3*, specifying a GABAergic cell fate (Glasgow et al., 2005). As already referred in Publication I, it is possible that in GABAergic neurons, *Prrxl1* expression could be prevented by a specific transcription factor acting on the RRA element. This repressive effect could be suppressed in glutamatergic neurons through the RRB element. In this light, it is reasonable that the transcriptional activity of the entire *Prrxl1* promoter region analysed was elevated (see Publication I, Figure 2), as the experiments were performed in the DRG-derived ND7/23 neurons, which are glutamatergic. This

supports our hypothesis that the inhibitory RRA element is repressed in this system. Among the candidates likely to bind the RRA element, are the transcription factors involved in the establishment of the GABAergic neuron cell fate in the dorsal spinal horn, such as Lbx1, Pax2 or Ptf1a. Lbx1 is present in both populations of late-born spinal cord neurons (Gross et al., 2002; Muller et al., 2002) but its activity is antagonized by Tlx3 in dIL^{B}, an action essential for the specification of glutamatergic fate (Cheng et al., 2005). In these neurons, the binding of Lbx1 to the RRA element would be prevented by the presence of Tlx3, bound to *Prrxl1* gene in a bipartite motif nearby (close to RRB), and *Prrxl1* transcription would normally occur. Concomitantly, in GABAergic neurons lacking Tlx3, Lbx1 would be able to bind the RRA element and *Prrxl1* transcription would be repressed. Alternatively, another protein present in the glutamatergic but not in the GABAergic neurons, could bind the DNA at the RRB motif. Similarly, Pax2 or Ptf1a could bind the RRA element and inhibit *Prrxl1* transcription in GABAergic neurons. Experiments consisting in the capturing and posterior identification of the transcription factors bound to RRA and RRB elements are required to understand the context of this transcriptional regulation.

TRANSCRIPTION FACTORS CONTROLLING *PRRXL1* TRANSCRIPTION

We demonstrated that Phox2b is required for *Prrxl1* transcription in the zebrafish visceral sensory neurons. Nonetheless, it has been described that mouse visceral sensory neurons where the expression of *Phox2b* was suppressed, acquire a molecular profile similar to that of somatic sensory neurons, with increased expression of *Brn3a* and *Prrxl1* (D'Autreaux et al., 2011). The increase in *Prrxl1* expression was suggested to be mediated by the increase of Brn3a levels, which in its turn, is directly repressed by Phox2b. This appears to conflict with results presented in Publication I, where *Prrxl1* expression in zebrafish glossopharyngeal, vagal and facial ganglia neurons is severely diminished when *Phox2b* expression is affected. However, it was demonstrated that Phox2b only functions as a *Prrxl1* repressor in the visceral sensory ganglia, since *Prrxl1* expression in the solitary nucleus is not affected by *Phox2b* inactivation (D'Autreaux et al., 2011). Hence, the hypothesis that in zebrafish and at that particular stage, Phox2b can work as a *Prrxl1* transcriptional activator instead of a repressor cannot be excluded. Indeed, Phox2b is mainly regarded a transcriptional

activator rather than a repressor. This is in accordance with the fact that this protein lacks the Eh1 domain responsible for conferring repressor activity to other HD transcription factors (Dubreuil et al., 2002). Moreover, Phox2b promotes general neuronal differentiation by increasing the levels of Ngn2 and Mash1 in cranial motor neurons (Dubreuil et al., 2002). It is then conceivable that when *Phox2* is suppressed, the downregulation of these or other bHLH transcription factors, could lead to a decrease in *Prrxl1* expression.

Subsequently, we dissected in more detail the molecular mechanisms by which Tlx3 and Brn3a regulate the expression of *Prrxl1*. Tlx3 induces *Prrxl1* transcription by two distinct mechanism – either by acting directly on the promoter P3 or indirectly on the region that comprises promoters P1/P2, via Brn3a action. It is shown here that at the *Prrxl1* TATA-containing promoter P3, Tlx3 acts upon a bipartite regulatory module, recognized directly by Tlx3 and by another transcription factor that remains to identify. One of the candidates considered in Publication II is a member belonging to the TALE (Three Amino acid Loop Extension) family of transcription co-factors. TALE factors are associated with developmental gene regulation and interact often with HOX genes (Moens and Selleri, 2006; Penkov et al., 2013). In particular, Tlx3 forms a transcriptional complex with the TALE protein Pbx3 to enhance the transcription of Tlx3-responsive elements *in vitro* (Rhee et al., 2004) and this complex is also likely to occur in respiratory neurons *in vivo* (Shirasawa et al., 2000). Expression of *Pbx3* is detected both in cranial ganglia and in DRG from E13.5 to E16.5 (Di Giacomo et al., 2006) and in several classes of spinal cord postmitotic neurons by E15 (Rottkamp et al., 2008). Given the high degree of overlapping expression of *Pbx3* and *Tlx3* and the phenotype of *Pbx3* null mutant mice in spinal cord, it has been hypothesized that Pbx3 cooperates with Tlx3 in the specification of glutamatergic fate in the superficial dorsal horn (Rottkamp et al., 2008). Because *Tlx3*, *Pbx3* and *Prrxl1* expression also overlap in certain populations of these neurons, we suggested that Pbx3 could be the factor interacting with Tlx3, to regulate *Prrxl1* transcription. In fact, similarly to *Prrxl1* (Chen et al., 2001) and *Tlx3* mutants (Xu et al., 2013), *Pbx3* mutant mice have reduced sensitivity to noxious and thermal stimuli (Rottkamp et al., 2008) as well as decreased number of cells expressing *calbindin*, *calretinin* and *PKC-γ*. Moreover, a recent work (Shimomura et al., 2015) reinforced the importance of the Tlx3/Pbx3 complex,

demonstrating that Tlx3 requires the presence of Pbx3 to enhance the interaction with the transcriptional co-activator CREB-binding protein (CBP) in order to promote the glutamatergic neuronal fate in embryonic stem cells. Binding events for the closely related protein p300 have been observed around the *Prrxl1* promoter P3 region in neurons, but not in HeLa cells (Publication I, Figure 3B). This supports the suggestion that Tlx3 binds the *Prrxl1* gene together with Pbx3, with the help of the chromatin modifier and transcriptional adaptor p300. In order to verify our hypothesis it would be interesting to determine whether *Prrxl1* expression is affected in *Pbx3* null mice.

The employment of a distinct regulatory mechanism was observed on the *Prrxl1* P1/P2 regulatory region. There, Tlx3 was shown to act in combination with Prrxl1 to enhance the transcriptional activity of these promoters. Nevertheless, the Tlx3/Prrxl1 protein complex does not activate *Prrxl1* promoters P1/P2 directly, but rather requires the presence of Brn3a in order to modulate their activity. The control of *Prrxl1* transcription by Brn3a has been observed before. Although studies approaching Brn3a's implication on the control of Prrxl1 spinal cord levels are missing, *Brn3a* null mice present a marked decrease in *Prrxl1* expression in primary afferent neurons (Eng et al., 2007). Moreover, it has been suggested that Brn3a controls the expression of *Prrxl1* in the somatic sensory cranial ganglia, since the increased expression of *Prrxl1* following *Phox2b* inactivation is likely to occur due to *Brn3a* de-repression (see Figure 8, Introduction) (D'Autreaux et al., 2011). In the developing spinal cord, co-localization between Brn3a, Prrxl1 and Tlx3 is only observed in glutamatergic neurons at early stages (Gross et al., 2002; Muller et al., 2002; Qian et al., 2002; Rebelo et al., 2010). As Tlx3 is required to maintain the expression of *Prrxl1* only from E14.5 in the developing spinal cord (Qian et al., 2002), it is hypothesized that Brn3a would be primarily required to initiate the transcription of promoters P1/P2 and the Tlx3/Prrxl1 complex would, through a positive feedback mechanism, later enhance Brn3a activity or transcription. Indeed, Tlx3 is able to activate the transcription of *Brn3a*, as demonstrated by the partial loss of *Brn3a* expression in *Tlx3/Tlx1* double mutants (Xu et al., 2008). It was also reported that the transcriptional induction of *Brn3a* by Tlx3 is a required condition for the expression of *Tac1* in Tlx3-containing neurons (Xu et al., 2008). Hence, it is reasonable to consider that a subsequent activation or maintenance of *Brn3a* expression by Tlx3 (together with Prrxl1) is a

condition for the Tlx3-dependent transcriptional activation of *Prrxl1* promoters P1/P2 at late stages of spinal cord neurogenesis. Corroborating this hypothesis, there is data from the group collected by genome-wide ChIP assays with anti-Tlx3 and anti-Prrxl1 antibodies pointing to the existence of shared binding events for Tlx3 and Prrxl1 transcription factors on the *Brn3a* gene (Monteiro, F.A. *et al.*, unpublished results).

POST-TRANSLATIONAL REGULATION OF PRRXL1 BY TLX3

One of the most important findings in this thesis unravels the dual role of Tlx3 as modulator of Prrxl1 transcription and phosphorylation. By reporting a novel role for Tlx3 as inducer of Prrxl1 hyperphosphorylation, rather than only enhancing its transcription, we add relevant information to the current state of the art. Ours is a sensible observation since Prrxl1 is hyperphosphorylated in the dorsal spinal cord from E10.5 until E14.5 (Soares-Dos-Reis et al., 2014), a time that encompasses the occurrence of spinal cord neurogenesis and extensive co-localization between Prrxl1 and Tlx3 is observed (Rebelo et al., 2010). Considering this, it is hypothesized in Publication II that the Prrxl1 high phosphorylated state is induced and maintained by the presence of Tlx3. From E16.5 onwards, Prrxl1 is progressively dephosphorylated, an observation that correlates well with the vanishing expression of *Tlx3* in dorsal spinal neurons. To clarify this issue, it would be valuable to assess Prrxl1 phosphorylation pattern in the dorsal spinal cord of *Tlx3* null mutant embryos.

Although the role of Tlx3 as modulator of protein phosphorylation has not been reported before, the effect of the functionally related Tlx homeobox gene Tlx1 in the activity of serine-threonine phosphatases PP1 and PP2A has already been described (Riz and Hawley, 2005). It is likely that similar Tlx3 protein domains are implicated in the repression of PP1 and PP2A transcription or activity, in order to maintain the high levels of phosphorylated Prrxl1. In addition, it cannot be excluded the recruitment of kinases by Tlx3 to promote Prrxl1 phosphorylation, which would be in accordance with what has been observed before for a complex of Hox/TALE proteins. In that case, TALE proteins promote an active chromatin profile and recruit RNA polymerase II, which is kept nearby in a poised state, until Hoxb1b triggers the recruitment of P-TEFb kinase, which in turn phosphorylates RNA polymerase II and other associated factors in order

to initiate active elongation (Choe et al., 2014). Genome-wide ChIP assays with anti-Tlx3 and anti-Prrxl1 antibodies showed great overlap in chromatin occupancy for these two transcription factors (Monteiro, F.A. *et al.*, unpublished results). Because it is known that alterations in Prrxl1 phosphorylated state significantly impact on Prrxl1 transcriptional activity (Soares-Dos-Reis et al., 2014), one may hypothesize that Tlx3 promotes the phosphorylation of Prrxl1 bound to the DNA in its vicinity, in order to endorse an active transcriptional state of Prrxl1. The observation that some kinases are able to directly bind DNA helps to support our hypothesis. For instance, Erk2 is recruited to certain DNA motifs to specifically phosphorylate poised RNA polymerase II on Polycomb Repressive Complex 2 (PRC2)-targeted developmental genes (Tee et al., 2014). Prrxl1 contains five phosphorylation sites predicted to be substrates for Erk kinases (Soares-dos-Reis *et al.*, unpublished data.). Thus, it is conceivable to hypothesize that Tlx3 recruits Erk2 (or other kinase) to occupy DNA motifs nearby, in order to phosphorylate Prrxl1 and thereby modulate its activity. ChIP-Seq experiments in mouse embryos with disrupted *Tlx3* expression would provide valuable information regarding the importance of Tlx3 on the chromatin deposition of Erk or other candidate kinases.

The fact that Tlx3 can modify the phosphorylation status of Prrxl1, raises the hypothesis that this could be a general feature of Tlx3 mechanism of action. Indeed, the potential role of Tlx3 as strong modulator of protein activity has been mentioned before. Tlx3 antagonizes Lbx1 in the dorsal spinal cord neurons, allowing them to differentiate towards a glutamatergic fate, and in the absence of Tlx3 those neurons become GABAergic (Cheng et al., 2005). Because Tlx3 does not repress the expression of *Lbx1*, it has been suggested that Tlx3 antagonizes Lbx1 protein function. However, it remained to further elucidate which mechanisms were responsible for this suppression. It would be interesting to disclose whether Lbx1 is phosphorylated and the contribution of Tlx3 on that feature, with additional studies evaluating the functional significance of such alterations on Lbx1 function.

In another situation, because Tlx3 was shown to act in cooperation with Runx1 to establish the Runx1-dependent nociceptive lineage in the DRG (Lopes et al., 2012), one may argue that Tlx3 also modifies the Runx1 phosphorylation pattern to promote this pathway. The effect of Runx1 phosphorylation on its transactivation potential has

been widely reported over the years in hematopoiesis and leukemia (Kurokawa et al., 1996; Tanaka et al., 1996; Yoshimi et al., 2012), but information regarding the specification of DRG neurons is still missing. In addition, ChIP-Seq analysis in E14.5 mouse DRG embryos demonstrated that in DNA regions occupied by Prrxl1 and Tlx3 there is an enrichment in a DNA-binding motif recognized by Runx1 (Monteiro, F. A. *et al.*, unpublished data). This data suggests that a transcriptional protein complex may be formed by these three factors, with Tlx3 being the molecule responsible for switching on the transcription of their common targets by activating Prrxl1 and Runx1 through the modulation of their phosphorylation state. This hypothesis should be further explored, primarily by assessing potential alterations in the phosphorylation pattern of Prrxl1 and Runx1 in the DRG of *Tlx3* mutant mice and then by identifying the intermediary players in this mechanism.

Collectively, the present findings bring important insight on the molecular mechanisms that control Prrxl1 at several levels and add interesting and novel information to the current state of the art dealing with the genetic and molecular network involved in the development of neurons in the somatosensory system.

CONCLUSIONS

5. CONCLUSIONS

The scientific results achieved during this work led to the following main conclusions (depicted in Figure 9):

1. *Prrxl1* transcription is controlled by a mechanism of alternative promoter usage. Three alternative promoters – named P1, P2 and P3 – are located immediately upstream of the Prrxl1 start codon, in the evolutionary conserved region of 1351 bp. P1 and P3 present promoter activity only in neuronal cells, whereas P2 is constitutive. Each promoter is responsible for the transcription of one of three *Prrxl1* 5'-UTR variants (5'-UTR-A, 5'-UTR-B and 5'-UTR-C). These 5'-UTR variants confer distinct mRNA stability and translation efficiency to the *Prrxl1* transcript. The 5'-UTR-A variant is the most stable in neuronal cells and presents the highest translation rate.

2. The most conserved promoter is P3 and it displays *in vivo* enhancer activity in zebrafish, in a pattern that overlaps with the endogenous *Prrxl1* homologue, *drgx*. Regulatory modules present in this sequence were identified and characterized. These include one motif that completely abolishes the transcriptional activity regulated by the three promoters (RRA), one that inhibits the repressive effect of RRA (RRB) and binding sites for Phox2b and Tlx3.

3. Phox2b is required in zebrafish for the expression of *drgx* in the facial, glossopharyngeal and vagal visceral cranial nerves.

4. Tlx3 induces the transcriptional activity of the *Prrxl1* promoters by two distinct mechanisms – It promotes the activity of P3 by directly binding to a bipartite DNA element and it synergistically interacts with Prrxl1 to indirectly activate the *Prrxl1* TATA-less promoters P1/P2 via the action of Brn3a.

5. A novel mechanism is described here, where it is shown that Tlx3 also modulates Prrxl1 activity by promoting its hyperphosphorylation. Altogether, these data demonstrate that Prrxl1 and Tlx3 are intimately related and share a genetic pathway, which may be essential for the regulation of downstream effectors responsible for the establishment of the nociceptive phenotype in the spinal cord.

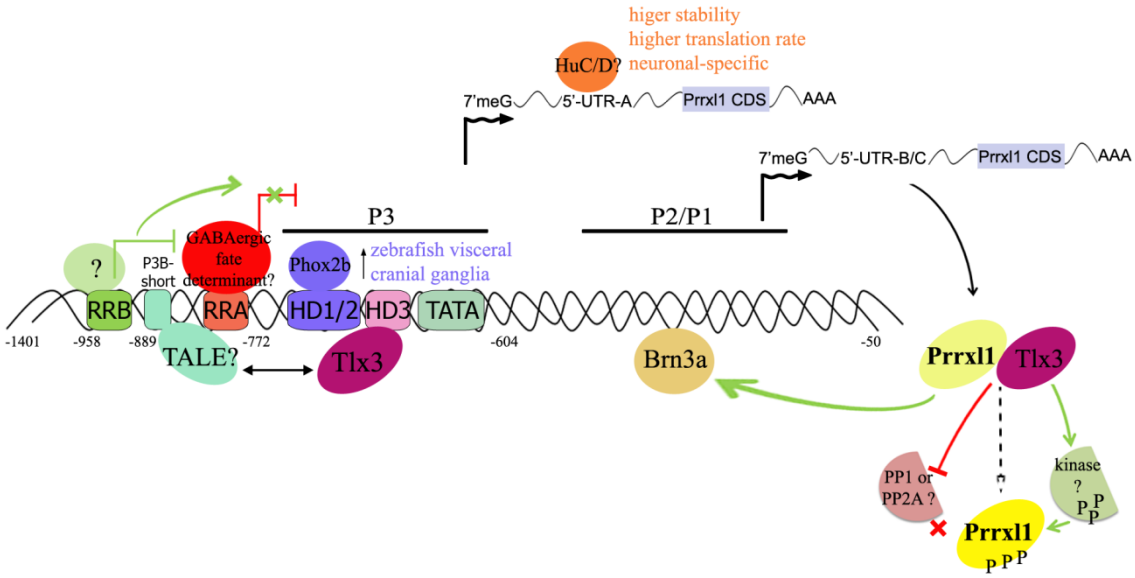

Figure 9: Distinct molecular mechanisms control the transcription and activity of Prrxl1. Three alternative promoters – P1, P2 and P3 – control the transcription of three *Prrxl1* 5'-UTR mRNA variants. P1/P2 regulates 5'-UTR-B and -C, whereas P3 regulates the transcription of 5'-UTR-A. The latter is the mRNA variant that displays the highest stability and translation rate in neuronal cells, probably due to the binding of RNA-binding proteins such as HuC or HuD. Two elements positioned between -958 and -772 exert a strong modulation upon *Prrxl1* transcription: RRA totally suppresses the transcriptional activity of the three promoters and RRB inhibits the negative effect of RRA, resulting in an overall increase of transcription. We suggest that RRA may be the binding site of transcription factors that promote the GABAergic neuronal fate, thus repressing the expression of *Prrxl1* in this population. The most conserved promoter is the TATA-containing P3 and includes binding sites for the HD transcription factors Phox2b (HD1/2) and Tlx3 (HD3). Phox2b is required in zebrafish for the expression of *drgx* in the facial, glossopharyngeal and vagal cranial ganglia. Tlx3 activates the transcriptional activity of promoter P3 by directly binding to a bipartite DNA element composed by the HD3 motif (the binding site of Tlx3) and an element within the P3B-short region. Pbx3 or other TALE factor are among the candidates that may bind the P3B-short motif. Tlx3 synergistically interacts with Prrxl1 to indirectly activate the *Prrxl1* TATA-less promoters P1/P2 via the action of Brn3a. In addition, Tlx3 modulates Prrxl1 activity by promoting its hyperphosphorylation. The mechanism remains to be characterized but Tlx3 modulation of kinases or phosphatases activity is suggested.

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