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Prrxl1 and *Casz1* transcriptional regulation in the development of the DRG-spinal nociceptive circuitry

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Aos meus orientadores

A todos os meus colegas do Departamento de Biologia Experimental

Aos meus pais

Ao Romero e à Joana

Preface

March the thirty first, just a date, yet, the year is 1596. However, “just a date” becomes relevant when someone in an already civilized world is born, especially when that someone is Renè Descartes, one of the fathers of modern science thought. Even though his ideas shaped the way science is done nowadays, this was not a miracle, nor even a sole event, this was merely one of the major threads that descended from the most ancient of minds throughout our history, such as Aristotle, Archimedes or Plato.

But does this diminish Descartes’s birth or does it make any less relevant by any means? Of course not. In fact, if it hadn't been Renè Descartes, it would have been a Renè Artois for all it would matter, yet, the legacy would still spawn out of someone's brain, and therefore, the thread would still be there. This thread of extreme importance is one of the most divergent threads of all our history due to the fact that it installed doubt and method into the scientific thinking. Four centuries passed, thinkers thought, conclusions were made, leading to new paradigms. From these, new theories took the place to, afterwards, be broken and corrected once again, so all threads could flourish.

July the second and the year is 2010, again maybe just a date to mankind itself, but for me it has a very particular significance. In an exponentially yet not so predictably evolved world from what it once was four centuries ago, this was the day when one of those threads crossed my path, as I started working in the Department of Experimental Biology at the Faculty of Medicine of the University of Porto. A very important part of my career was beginning where I could just finally apply what I had grown to learn until that very day.

I started to work with Professor Filipe Monteiro for my Master Thesis. Through this phase, I gathered the support of my colleagues to go further on the work I presented back then. With further support of the one that’s now my current supervisor, Professor Deolinda Lima, I was thankfully awarded with a FCT scholarship that allowed me to pursue my scientific career. Notwithstanding, I have to thank Professor Deolinda and Professor Filipe for their guidance. Without them, I wouldn't have been able to either complete my degree with efficiency nor grown myself into the person I am today.

I would also like to thank Professor Sandra Rebelo and Professor Carlos Reguenga for all scientific brainstormings we shared. I would also have to thank to all my colleagues that always cheered me up in the bad times. A special thanks to Anabela and Ilda for never denying their technical support, always with a smiles upon their faces.

I would also like to thank all the scientists/thinkers that always inspired me, namely Philo of Alexandria, René Descartes, Charles Darwin, Albert Einstein, Marie Curie, Richard

Feynman, Richard Dawkins, Sam Harris, Daniel Dennet, Christopher Hitchens, Robert Wright, Yuval Noah Harari and António Damásio, even though some of them are not among us anymore but whose legacy still lives today.

Last but not the least, to my family, friends and lover for their unconditional support through these years.

«Je réputais presque pour faux tout ce qui n'était que vraisemblable»

René Descartes

Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados e redigi as publicações que fazem parte integrante desta dissertação.

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Prrxl1 and *Casz1* transcriptional regulation in the development of the DRG-spinal nociceptive circuitry

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Pain is a distressing feeling often caused by intense or damaging stimuli (noxious stimuli). Due to its complexity and subjectivity, defining pain has been a challenge. According to the International Association for the Study of Pain (IASP), "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Clinically, pain is normally taken as a symptom and is one of the most common reasons for physician consultation in developed countries. Chronic pain, however, for its persistence and abnormal characteristics is considered as a disease in itself.

Noxious stimuli are transduced by specific peripheral nociceptive neurons (nociceptors), with cell bodies located in the dorsal root ganglia (DRG) and central processes synapsing with dorsal spinal cord transmission and locally processing neurons. Unraveling the molecular mechanisms underlying the proliferation, differentiation and development of these neurons is essential to understand the functioning of the nociceptive circuitry.

The transcription factor Prrxl1 was found to play an important role in the formation of the DRG-spinal circuitry, dedicated to the transmission of peripheral nociceptive input to the central nervous system. *Prrxl1*-null mice exhibit spatiotemporal abnormalities in sensory afferent fibers ingrowth into the developing spinal cord dorsal horn, altered dorsal horn morphogenesis, increased apoptosis of superficial dorsal horn glutamatergic neurons and nociceptors apoptosis shortly after birth. Together, these findings were taken as suggestive of a role for Prrxl1 in the differentiation of an important fraction of excitatory superficial dorsal horn neurons on one hand, and in the guidance of their primary afferents to the establishment of central synapses on the other.

Despite of its pivot role in the development of the DRG-spinal excitatory nociceptive circuit, very little is known about the transcriptional network downstream of Prrxl1. In order to unravel Prrxl1 transcription network, our lab group previously combined genome-wide location analysis of Prrxl1 binding sites (ChIP-seq) with global gene expression profiling of *Prrxl1*-null mice (microarray analysis) in both DRG and dorsal spinal cord embryonic tissues.

By overlapping the data sets of Prrxl1-bound genes and deregulated genes, we identified a comprehensive list of genes likely to be Prrxl1 direct targets. This thesis deals with the transcriptional regulation and expression pattern of selected Prrxl1 target genes (i.e. *Prrxl1* itself and *Casz1*).

In the first study, we demonstrated that Prrxl1 binds both its own proximal promoter and 4th intron, displaying a differential occupancy in DRG and dorsal spinal cord. Prrxl1 was shown to control its own expression by a transcriptional autorepression mechanism. This work was published in FEBS Letters: *"Paired related homeobox protein-like 1 (Prrxl1) controls its own expression by a transcriptional autorepression mechanism"*.

In the second study, we addressed Casz1 expression regulation by Prrxl1 as well as its expression pattern throughout DRG and dorsal spinal cord mouse development. Prrxl1 was shown to positively control Casz1 expression in the spinal cord but not in the DRG. In the DRG, we found Casz1 expression in virtually all neurons. In the dorsal spinal cord, Casz1 is expressed in two waves: in between embryonic days 10.5 and 11.5, in the dorsal most portion of the neural tube, and from E13.5 onwards, in excitatory glutamatergic neurons of the superficial laminae of the spinal dorsal horn. These results were published in the European Journal of Neuroscience: *"Zinc finger transcription factor Casz1 expression is regulated by homeodomain transcription factor Prrxl1 in embryonic spinal dorsal horn late born excitatory interneurons"*.

The results presented in this thesis contribute to enrich our knowledge about the molecular mechanisms behind the nociceptive system development, in particular regarding the Prrxl1 transcriptional program. Nonetheless, the functional role of Casz1 and other Prrxl1 target genes remains unexplored. Future investigation on other Prrxl1 target genes will be important to fully understand the molecular basis underlying the formation of the nociceptive circuit.

Regulação Transcripcional do *Prrxl1* e *Casz1* durante o desenvolvimento do circuito nociceptivo DRG-medula espinhal

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A dor é uma sensação angustiante frequentemente causada por estímulos intensos ou lesões (estímulo nóxico). Devido à sua complexidade e subjectividade, definir tem sido um desafio. De acordo com a Associação Internacional para o Estudo da Dor (IASP), "A dor é uma experiência sensorial e emocional desagradável associada a uma lesão tecidual real ou potencial ou descrita em termos de tal lesão". Clinicamente, a dor é normalmente considerada como um sintoma, sendo uma das razões mais comuns das consultas médicas nos países desenvolvidos. A dor crónica, no entanto, pela sua persistência e características anormais é considerada, em si, uma doença.

Estímulos nóxicos, são transduzidos por neurónio nociceptivos periféricos específicos (nociceptores), cujos corpos celulares se encontram nos gânglios dorsais raquidianos (DRG) e, respetivas projeções centrais estabelecem sinapses com neurónios de projeção e interneurónios localizados no corno dorsal da medula espinhal. Desvendar os mecanismos moleculares subjacentes à proliferação, diferenciação e desenvolvimento destes neurónios é essencial para compreender o funcionamento do circuito nociceptivo.

O factor de transcrição Prrxl1 desempenha um papel importante na formação do circuito DRG-medula espinhal, dedicados à transmissão de *inputs* nociceptivos periféricos para o sistema nervoso central. Murganhos Prrxl1-nulos apresentam alterações espaço-temporais na orientação e crescimentos das fibras aferentes primárias para o corno dorsal da medula espinhal, morfogénese anormal do corno dorsal, aumento da apoptose em neurónios glutamatérgicos do corno dorsal da medula espinhal em desenvolvimento, bem como em nociceptores, logo após o nascimento. Juntos, estes resultados sugerem que o Prrxl1 tem um papel importante na diferenciação de uma fracção significativa de neurónios excitatórios do corno dorsal superficial, na orientação das aferências primárias e no estabelecimento de sinapses centrais.

Apesar do seu papel crucial no desenvolvimento do circuito nociceptivo DRG-medula espinhal, muito pouco se sabe sobre a rede de transcripcional dirigida pelo Prrxl1. De modo a

desvendá-la, o nosso grupo de investigação combinou uma análise *genome-wide* dos locais de ligação do Prrxl1 (*Chip-seq*) com o perfil global de expressão génica em murganhos Prrxl1-nulos (análise de *microarray*), tanto nos DRG como na medula espinhal dorsal embrionários. Sobrepondo o conjunto de genes *Prrxl1-bound* e genes desregulados, identificamos uma ampla lista de genes que poderão ser alvos diretos do Prrxl1. Esta tese analisa o padrão de expressão e a regulação da transcrição de uma seleção de genes alvo do Prrxl1 (o próprio Prrxl1 e o Casz1).

No primeiro estudo, foi demonstrado que o Prrxl1liga-se tanto seu próprio promotor proximal como ao seu quarto intrão, exibindo uma ocupação diferencial nos DRG medula espinal dorsal. Aqui, mostramos que o Prrxl1 controla a sua própria expressão por um mecanismo de autorepressão transcripcional. Este trabalho foi publicado na revista FEBS Letters: *"Paired related homeobox protein-like 1 (Prrxl1) controls its own expression by a transcriptional autorepression mechanism"*.

No segundo estudo, abordamos a regulação da expressão do Casz1 pelo Prrxl1, bem como o seu padrão de expressão em durante o desenvolvimento dos DRG e medula espinhal dorsal de murganhos. Além disso, revelámos que o Prrxl1 regula positivamente a expressão do Casz1 na medula espinhal, mas não nos DRG. Nos DRG, encontramos a expressão do Casz1 em praticamente todos os neurónios. Na medula espinal dorsal, o Casz1 apresenta duas vagas de expressão: uma entre dias embrionários (E) 10.5 e 11.5, na porção mais dorsal do tubo neural, e outra a partir de E13.5 em diante, em neurónios glutamatérgicos excitatórios localizados nas lâminas superficiais do corno dorsal da medula espinhal. Estes resultados foram publicados no European Journal of Neuroscience: *"Zinc finger transcription factor Casz1 expression is regulated by homeodomain transcription factor Prrxl1 in embryonic spinal dorsal horn late born excitatory interneurons"*.

Os resultados apresentados nesta tese contribuíram para enriquecer o nosso conhecimento sobre os mecanismos moleculares envolvidos no desenvolvimento do sistema nociceptivo, nomeadamente no que concerne ao programa transcripcional do Prrxl1. No entanto, o papel funcional da Casz1 e outros genes alvo do Prrxl1 permanece por explorar. Futuras investigações sobre outros genes alvo do Prxxl1 serão importantes para compreender a base molecular subjacente à formação do circuito nociceptivo.

1. Introduction

A common feature among all life forms is their capacity to sense variations in the external environment and to respond adaptively. In unicellular organisms such as *Paramecium*, these tasks are accomplished at the level of a single cell. When multicellular organisms emerged 600 million years ago, organisms progressively acquired more and more specialized cell types with dedicated functions (Mikhailov et al. 2009).

Neurons are highly differentiated and specialized cells, characterized by their high excitability and capacity to rapidly conduct electrical currents. Cnidarians (sea anemones, corals, jellyfish and hydroids) are the most primitive group of organisms to differentiate neurons. These neurons form a rudimentary and diffused network capable of transducing mechanical, chemical and, in some species, even light stimuli. However, none of the known species possess specialized sense organs or cephalized structures like the brain in vertebrates (Hartenstein and Stollewerk 2015; Mikhailov et al. 2009).

Unlike in primitive animals, vertebrate nervous system can accurately perceive, convey and discriminate different types of sensory stimuli. Processing such a wide variety of sensory information requires a well-organized synaptic connectivity between peripheral sensory neurons and their specific synaptic targets in the central nervous system. These systems can be subdivided in two sub-categories: the special sensory systems, which comprises vision, audition, taste and olfaction, and the somatic sensory system, which has two major subsystems: one for the detection of mechanical stimuli (light touch vibration pressure, and both cutaneous and muscular tension), and another for the detection of painful stimuli, itch and temperature. Together, these neurons give the organisms the ability to identify shapes and textures, monitor the internal and external forces acting on the body at any given moment, and detect potentially harmful circumstances (Purves et al., 2008).

1.1 The somatosensory system

In mammals, somatosensory neurons are located in dorsal root ganglia (DRG) and in trigeminal ganglia (TG). At the spinal level, these neurons are pseudo-unipolar, sending one of their axonal branches to the periphery and the other to specific laminae in the dorsal horn. TG neurons project either to the principal or spinal trigeminal nuclei in the brainstem.

Each dorsal root ganglion comprises a heterogeneous population of sensory neurons capable of transducing diverse sensory modalities, including pain (nociception), itch (pruriception), temperature (thermoception), touch (mechanoreception) and positional information (proprioception) (Lallemend & Ernfors, 2012). Most of these neurons project to the dorsal horn of the spinal cord or to cranial sensory nuclei. Thermo- and pruriceptive information is conveyed to lamina I-II neurons, nociceptive information to laminae I-II and V, mechanical information to lamina III-IV, and proprioceptive information to lamina V and ventral spinal cord (Graham *et al.*, 2007; Lallemend & Ernfors, 2012). In the spinal cord, sensory input is processed by local circuitries and descending pathways that can either facilitate or inhibit the transmission of sensory information to higher centers in the brain (Millan, 2002; Todd, 2010).

1.1.1 Mechanoreceptors

Mechanoreceptors are characterized by their capacity to generate action potentials upon weak mechanical (Low-threshold) stimulation such as touch pressure, vibration and cutaneous tension (Lewin & Moshourab, 2004). They form large myelinated Aβ-fibers and mostly innervate the dermis and epidermis, forming four major types of encapsulated nerve endings: Meissner's Corpuscles, Pacinian Corpuscles, Merkel's disks, and Ruffini's Corpuscles (Lewin & Moshourab, 2004; Lallemend & Ernfors, 2012; Abraira & Ginty, 2013). A small subset of low threshold mechanoreceptors, expressing the glutamate vesicular transporter 3 (Vglut3), identifies a poorly known population of unmyelinated C-fibers responsible for transducing light/pleasant touch stimuli (Bjornsdotter *et al.*, 2010; Abraira & Ginty, 2013). Peripherally, these neurons form a longitudinally arranged ring of nerve endings around hair follicles, called lanceolate endings (Abraira & Ginty, 2013).

1.1.2 Proprioceptors

Proprioceptors are a specific type of mechanoreceptors that transduce mechanical forces arising from the musculoskeletal system (Hasan & Stuart, 1988). Peripherally, they specific such as muscle spindles and Golgi tendon organs (Hasan & Stuart, 1988). The main biological function of the proprioceptive system is to give detailed and continuous

information to the brain about the position of the different body parts, which ultimately allow the organism to execute complex movements.

1.1.3 Nociceptors

Nociceptors comprise a class of sensory neurons that respond with a sole discharge to high threshold stimulation (Julius & Basbaum, 2001; Lewin & Moshourab, 2004; Basbaum *et al.*, 2009). They are commonly divided in two classes: Aδ-fiber and C-fiber nociceptors. Aδ-nociceptors have lightly myelinated axons and medium diameter cell bodies and transduce noxious mechanical and thermal stimuli (Julius & Basbaum, 2001). C-fiber nociceptors have unmyelinated axons and small diameter cell bodies (Julius & Basbaum, 2001). They mediate burning quality pain and are activated by mechanical, thermal, and chemical stimuli, therefore being commonly known as polymodal (McCarthy and Lawson 1990; Lawson et al., 1996). Nociceptors can also be divided into two major classes according to their neurotransmitter content: peptidergic neurons, which contain substance P (SP) or calcitonin gene-related peptide (CGRP), and non-peptidergic neurons, which are identified by their capacity to bind the lectin *Griffonia simplicifolia* (IB4) (Averill *et al.*, 1995; Michael *et al.*, 1997). Peripherally, peptidergic nociceptors mostly innervate the dermis and viscera, while the epidermis is innervated by both peptidergic and non-peptidergic nociceptors. Centrally, peptidergic neurons mostly project to lamina I and outer lamina II, while non-peptidergic neurons project to inner lamina II (Yoshikawa *et al.*, 2007).

1.1.4 Sensory processing in dorsal spinal cord

Conveying sensory information from the dorsal spinal cord to the brain depends on the coordinated action between local excitatory and inhibitory interneurons, and descending supraspinal afferents (Millan, 2002; D'Mello & Dickenson, 2008). Local excitatory neurons use glutamate as neurotransmitter, whereas inhibitory neurons use γ-aminobutyric acid (GABA). Some of these inhibitory neurons also use glycine, which acts as a cotransmitter (Takazawa & MacDermott, 2010b; Takazawa & MacDermott, 2010a).

Supraspinal descending fibers mainly originated in the brainstem can either inhibit or facilitate sensory transmission to higher centers in the brain (Millan, 2002; Todd, 2010). The output from local circuits and descending fibers activity is further conveyed by

supraspinally projection neurons, which form defined axonal bundles (also known as spinal tracts) within spinal cord white matter towards the brain (Watson & Harrison, 2012).

1.2 Development of the somatosensory system

The nervous system is originated from an ectodermal domain called neural ectoderm (also called neuroepithelium or neural plate), that extends almost the entire cranio-caudal axis of the vertebrate embryo (Serbedzija *et al.*, 1990; Lee & Jessell, 1999). During a process called neurulation, the left and right halves elevate and fuse to form the neural tube. Once closed, two gradients of morphogens are generated and will be responsible for the dorsal-ventral patterning of the central nervous system.

Dorsally-derived signals are produced by roof plate cells and overlaying ectodermal cells, and comprise members of the bone morphogenetic protein (BMPs) and wingless-related mouse mammary tumor virus integration site (Wnt) families, which are responsible for establishing dorsal neuronal fates (Lee & Jessell, 1999). Ventrally, floor plate and notochord cells secrete sonic hedgehog (Shh) that plays a key role in establishing ventral neuronal fates. In the posterior portion of the neural tube, these gradients are responsible for inducing six dorsal domains (dP1-dP6) and five ventral domains (p3, pMN, p2, p1 and p0) of progenitor cells, which will give rise to spinal cord neurons (Hollyday *et al.*, 1995; Liem *et al.*, 1995; Liem *et al.*, 1997; Megason & McMahon, 2002). Each one of these domains is characterized by the combinatorial expression of different sets of basic Helix-loop-Helix (bHLH) and homeodomain (HD) classes of transcription factors, which are required for cell fate determination (Briscoe *et al.*, 2000; Caspary & Anderson, 2003; Helms & Johnson, 2003b).

The peripheral nervous system is originated from a subpopulation of roof plate cells, located at the junction with the surface ectoderm, that undergoes an epithelial-to-mesenchymal transition and become Neural crest cells (NCCs) (Serbedzija *et al.*, 1990; Failli *et al.*, 2002; Chizhikov & Millen, 2004; Montelius *et al.*, 2007). Once they become delaminated from the roof plate, NCCs migrate to several regions of the embryo, contributing to the formation of several cell types, such as melanocytes and cardiomyocytes (Bhatt *et al.*, 2013). NCCs migrating ventrally alongside the neural tube will give rise to sensory and autonomic neurons (Anderson, 1999; Montelius *et al.*, 2007; Bhatt *et al.*, 2013).

1.2.1 Role of extrinsic and intrinsic factors in somatosensory system development.

The formation of the somatosensory system requires a spatio-temporal coordinated action of extrinsic (secreted morphogens) and intrinsic factors (transcription factors). Morphogens are essential do define neural tube dorso-ventral axis, and do so by triggering changes in the expression of transcription factors that will further contribute to the specification and proliferation of several classes of dorsal interneurons (Liu & Niswander, 2005; Zechner *et al.*, 2007). Transcription factors can positively or negatively regulate gene transcription by recognizing and binding to specific DNA motifs found in cis-regulatory sequences such as promoters and enhancers. In general, transcription factors have one or more DNA-binding domains and a regulatory domain that mediates interactions with transcriptional co-regulators. Especially during embryonic development, the orchestrated action of several transcription factors is required to control the generation of different cell types and normal organ morphogenesis (Castro *et al., 2006*; Kim *et al.*, 1998; Ma, 2006).

The segregation of NCCs from roof plate cells is a good example of how the coordinated action of external and intrinsic signals play essential and complementary roles during development. Nowadays, a wide supported hypothesis (for review see Chizhikov and Millen, 2004) postulates that BMP and Wnt signals induce a common progenitor pool for neural crest and roof plate cells. These cells express the LIM-HD transcription factor Lmx1a, known to be a marker of roof plate cells, and markers of NCCs such as the snail family zinc-finger 2 (Slug) and the forkhead-box D3 (Foxd3) (Bronner-Fraser & Fraser, 1988; Selleck & Bronner-Fraser, 1995; Failli *et al.*, 2002; Chizhikov & Millen, 2004). The subpopulation that delaminates from the roof plate to form NCCs loses Lmx1a expression and starts to express the SRY-box10 transcription factor (Sox10) (Montelius *et al.*, 2007; Bhatt *et al.*, 2013). Last, cells that remain in the neural tube lose expression of NCCs markers and maintain Lmx1a expression, which promotes the expression of BMPs and Wnt families of morphogens, thus contributing to the induction of dorsal progenitors (Timmer *et al.*, 2002; Zechner *et al.*, 2007).

1.2.2 DRG development

Sensory neurons arise from a subpopulation of NCCs committed to a neuronal phenotype, which, together with glial cells, coalesce to form a pair of DRGs at each segmental level (Montelius *et al.*, 2007). NCCs express Sox10, which is required to maintain their multipotency but is rapidly downregulated upon neurogenesis (Kim *et al.*, 2003). In mouse, bHLH transcription factors Neurogenin1 (Ngn1) and Neurogenin2 (Ngn2) bias NCCs to the sensory lineage and trigger two wave of sensory neurogenesis between E9.5 and E13.5 (Ma *et al.*, 1999). The first wave is driven by Ngn2 and gives rise mostly to precursors of mechanoreceptive and proprioceptive neurons. The second wave of neurogenesis, driven by Ngn1, essentially gives rise to unmyelinated and thinly-myelinated nociceptive neurons. A subset of migrating boundary cap cells, expressing the zinc-finger transcription factor Krox20, is responsible for a third wave of neurogenesis, giving rise to thinly-myelinated nociceptors, contributing only to 5% of DRG neurons (Maro *et al.*, 2004; Hjerling-Leffler *et al.*, 2005). To date, the genetic cascade responsible for triggering the third wave of neurogenesis is still unknown.

As sensory progenitors exit cell-cycle, they lose Sox10 and Ngn1/2 expression and start to express the POU-domain class 4 transcription factor 1 (Pou4f1), also known as Brn3a, and the LIM-HD transcription factor Islet1 (Isl1) (Ninkina *et al.*, 1993; Montelius *et al.*, 2007; Sun *et al.*, 2008). These transcription factors act together to regulate the transition from neurogenesis to terminal differentiation and contribute to sensory subtype specification (Montelius *et al.*, 2007; Sun *et al.*, 2008; Lanier *et al.*, 2009; Dykes *et al.*, 2011). Following the birth of sensory neurons, immature neurons progress through a series of additional specification steps (see Figure 1) controlled by dedicated gene programs that, together with environmental cues, orchestrate axonal guidance and the segregation of neurons involved in specific sensory modalities (Chilton, 2006; Masuda *et al.*, 2007; Schmidt & Rathjen, 2010; Lallemend & Ernfors, 2012). By E13.5, at least five different types of neurons can be identified in mouse DRG according to the expression pattern of the tyrosine-kinase receptors TrkA, TrkB, TrkC, Met and c-Ret, which serve as receptors for the neurotrophins such as, respectively, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), hepatocyte growth factor (HGF) and glial-derived neurotrophic factor (GDNF) (Chen *et al.*, 2006b; Marmigere *et al.*, 2006;

Bourane *et al.*, 2009; Luo *et al.*, 2009; Gascon *et al.*, 2010; Samad *et al.*, 2010; Lopes *et al.*, 2012; Abraira & Ginty, 2013). These receptors are crucial for correct peripheral innervation and neuronal survival (Michael *et al.*, 1997; Eggert *et al.*, 2000; Schramm *et al.*, 2005; Bourane *et al.*, 2009; Schmidt & Rathjen, 2010). Nevertheless, several lines of evidence demonstrate that lineage-specific transcription factors regulate the expression of these receptors (for review see (Lallemend & Ernfors, 2012)).

1.2.2.1 Development of TrkB and TrkC lineage of sensory neurons

Most mechanoreceptors and proprioceptors are originated from the $TrkB^+$/$TrkC^+$- and c-Ret^+-cells (commonly known as early c-Ret cells), generated during the first wave of neurogenesis (Ma *et al.*, 1999; Bourane *et al.*, 2009) (Figure 1). By E13.5 in the mouse, TrkB and TrkC expression becomes restricted to different and non-overlapping sets of neurons (Inoue *et al.*, 2002; Kramer *et al.*, 2006; Marmigere *et al.*, 2006; Sun *et al.*, 2008; Abdo *et al.*, 2011; Wende *et al.*, 2012b). Neurons expressing TrkB will generate different types of mechanoreceptors (Abdo *et al.*, 2011), whereas TrkC-expressing cells will differentiate into proprioceptive neurons (Inoue *et al.*, 2002). The maintenance of TrkB and TrkC expression requires the activity of the transcription factors short stature homeobox 2 (Shox2) and runt related transcription factor 3 (Runx3), respectively. Shox2 was shown to promote TrkB expression and to negatively regulate Runx3 expression (Scott *et al.*, 2011). Conversely, Runx3 is sufficient to activate TrkC and repress TrkB and Shox2 expression (Inoue *et al.*, 2002; Levanon *et al.*, 2002; Kramer *et al.*, 2006). Recently a novel Ngn2-dependent population of Ret-expressing neurons was also characterized, which differentiates into mechanoreceptors forming hair follicle lanceolate endings and mechanoreceptors innervating Meissner and Pacinian corpuscles (Bourane *et al.*, 2009; Hu *et al.*, 2012; Wende *et al.*, 2012a; Wende *et al.*, 2012b). These early Ret^+-neurons selectively express the basic leucine-zipper transcription factors c-Maf and MafA, which are required for proper differentiation of Pacinian and Meissner corpuscles (Hu *et al.*, 2012; Wende *et al.*, 2012b).

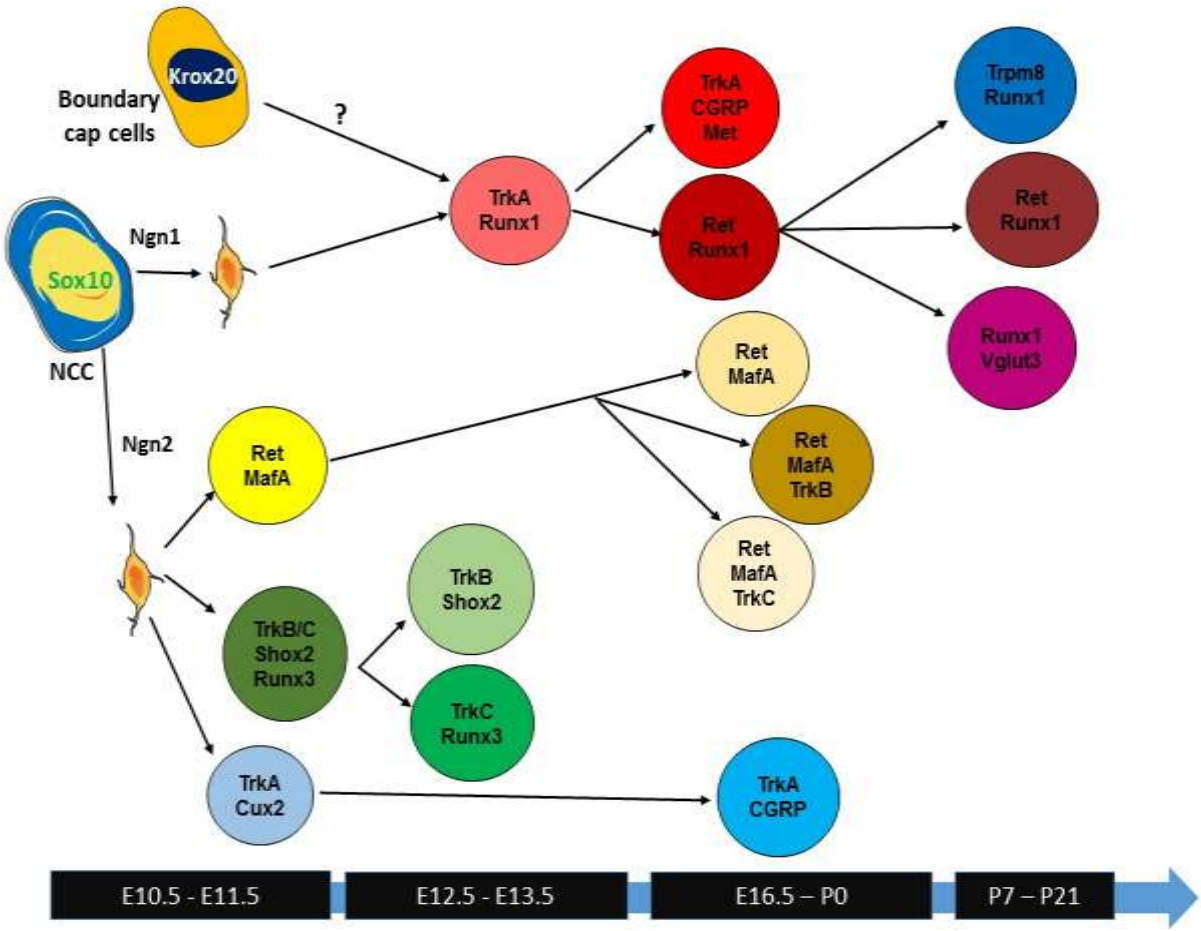

Figure 1: Schematic representation of DRG neurons diversification during development. NCCs expressing Sox10 are biased towards a sensory fate. The first wave of sensory neurogenesis is driven by Ngn2 and generates mostly large mechanreoceptive and proprioceptive neurons. By E13.5, these neurons can be subdivided in four populations: $Ret^+/MafA^+$, $TrkB^+/Shox2^+$, $TrkC^+/Runx3^+$ and $TrkA^+/Cux2^+$. $Ret^+/MafA^+$-population will further segregate in three different populations: $Ret^+/MafA^+$, $Ret^+/MafA^+/TrkB^+$, and $Ret^+/MafA^+/TrkC^+$. A small subset of post-mitotic neurons expressing TrkA and Cux2 gives rise to thinly myelinated peptidergic neurons. Most of TrkA neurons are generated during the second and third wave of neurogenesis, coexpressing Runx1 instead of Cux2. Peri- and postnataly, Runx1 is actively involved in suppressing TrkA and activate c-Ret expression, giving rise to the peptidergic and non-peptidergic nociceptors, respectively. Runx1 is also involved in the differentiation of cold-sensing neurons expressing Trpm8 and low-threshold mechanoreceptors.

1.2.2.2 Development of TrkA lineage of sensory neurons

$TrkA^+$-neurons comprise a small population of Ngn2-dependent neurons, also known as early $TrkA^+$-neurons, and a large population of neurons from the second (Ngn1-dependent) and third wave of neurogenesis (Ma *et al.*, 1999; Maro *et al.*, 2004; Hjerling-Leffler *et al.*, 2005; Bachy *et al.*, 2011). The early $TrkA^+$-population expresses the Cut-like

homeobox 2 transcription factor (Cux2) and differentiates into lightly myelinated peptidergic neurons (Bachy *et al.*, 2011). $TrkA^{+}$-neurons formed in the second and third waves of neurogenesis are segregated into two major subpopulations of sensory neurons, peptidergic and non-peptidergic. During perinatal and postnatal stages, a subset of this population switch off TrkA expression and starts to express c-Ret, giving rise to non-peptidergic nociceptors, whereas peptidergic neurons derive from a subpopulation that maintain TrkA expression and co-express the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP). The Runt-related transcription factor 1 (Runx1) was shown to play a key role in segregating these two populations (Chen & Rajewsky, 2007; Gascon *et al.*, 2010; Samad *et al.*, 2010). Runx1 suppresses TrkA and activates c-Ret expression, as well as most of nociceptive ion-channels and receptors such as Trpm8, Trpv1 and P2X3 (Ugarte *et al.*, 2012; Ugarte *et al.*, 2013). Non-peptidergic afferents of $Runx1^{-/-}$ mice abnormally project to lamina I and outer lamina II (like peptidergic neurons) instead of inner lamina II (Chen *et al.*, 2006a; Yoshikawa *et al.*, 2007). Runx1 is also required for the formation of $Vglut3^{+}$-mechanoreceptors, responsible for transducing light/pleasant touch stimuli, and to establish mechanosensitivity by controlling the expression of the mechanically gated ion channel Piezo2 (Lou *et al.*, 2013). Similarly, the T-cell leukemia homeobox protein 3 (Tlx3) was shown to be partially required to control most Runx1-dependent sensory phenotypes, including the segregation of TrkA- versus c-Ret-expressing neurons and, the expression of sensory channels and receptors implicated in nociception, itch and temperature such as Trpv1, Trpm8, TrpA1 and P2X3 (Lopes *et al.*, 2012).

1.2.3 Development of dorsal spinal cord

Dorsal spinal cord neurons are generated in two successive waves of neurogenesis from progenitor cell domains (dP) located in the ventricular zone of the dorsal neural tube (see figure 2). These cells are identified by the combinatorial expression of different bHLH and HD transcription factors such as Atoh1/Math1, Ngn1, Mash1 and Dbx2, which are specifically expressed in dP1, dP2, dP3-5 and dP6 cells, respectively (Müller *et al.*, 2002; Qian *et al.*, 2002; Zhou & Anderson, 2002; Caspary & Anderson, 2003; Cheng *et al.*, 2004; Ding *et al.*, 2004; Cheng *et al.*, 2005; (Helms et al. 2005)). In the mouse, the first wave of neurogenesis occurs between E10.5 and E11.5 and generates six different populations of

dorsal interneurons, which migrate ventrally to occupy the deep dorsal horn (dI1-dI6) (Lee & Jessell, 1999; Gowan *et al.*, 2001; Gross *et al.*, 2002; Müller *et al.*, 2002; Helms & Johnson, 2003a; Liu & Niswander, 2005). The second wave of neurogenesis occurs between E11 and E13 from cells expressing Mash1/Ascl1, also called late born domain (pdIL), and generate two populations of late-born dorsal interneurons, called dILA and dILB, which migrate dorsally to occupy the superficial dorsal horn (Gross *et al.*, 2002; Müller *et al.*, 2002).

Dorsal horn neurons can be further classified as class A or B, according to its dependency on roof plate signals and expression of the LIM-homeobox transcription factor Lbx1. Class A neurons (dI1-dI3) specification is dependent on roof plate signals. Gain- and Loss-of-function studies in chick embryos neural tube showed Bmp and Wnt to trigger β-catenin to induce the expression of transcriptions factors involved in Class A neurons specification such as Olig3, Atoh1 and Ngn1/2 (Zechner et al. 2007). Conversely, studies on Olig3-null mice embryos demonstrated that Olig3 represses the emergence of Class B neurons (Müller et al. 2005; Ding et al. 2005; Zechner et al. 2007). When Class A neurons become post-mitotic, they migrate to deep layers of the dorsal horn where they are thought to participate in the processing of proprioceptive information (Helms & Johnson, 1998; Bermingham *et al.*, 2001; Gowan *et al.*, 2001; Gross *et al.*, 2002; Bui *et al.*, 2013). Class B neurons (dI4-dI6, dILA and dILB) are derived from $Gsx1^{+}$-progenitors, do not require roof plate signals for their specification and, unlike Class A neurons, express Lbx1 when they exit cell-cycle (Müller *et al.*, 2002). Class B neurons can further be subdivided into inhibitory/GABAergic/glycinergic neurons (dI4, dI6 and dILA) and excitatory/glutamatergic neurons (dI5 and dILB). Lbx1 specifies the neuronal inhibitory (GABAergic) fate in dI4, dI6 and dILA neurons by enabling the expression of genes involved in GABAergic differentiation such as Pax2 and Lhx1/5 (Gross *et al.*, 2002; Glasgow *et al.*, 2005; Hoshino *et al.*, 2005; Huang *et al.*, 2008; Hanotel *et al.*, 2014). Specifically, in dI4 and dILA neurons, the bHLH Ptf1a was also shown to directly and positively regulate Pax2 and Lhx5/1, as well as components of GABA biosynthesis and transport (Mizuguchi et al., 2006; Huang et al., 2008; Borromeo et al., 2014). By contrast, the HD transcription factor Tlx3 specifies excitatory (glutamatergic) fate in dI5 and dILB neurons by antagonizing Lbx1 action (Cheng *et al.*, 2005). These neurons also co-express

the HD transcription factors Prrxl1 and Lmx1b. Analysis of Tlx3, Lmx1b and Prrxl1 mutants indicates that these HD are required for proper assembly of nociceptive dorsal horn circuits, required for processing of noxious signals (Qian *et al.*, 2001; Cheng *et al.*, 2004; Ding *et al.*, 2004; Cheng *et al.*, 2005; Mizuguchi *et al.*, 2006; Holstege *et al.*, 2008; Rebelo *et al.*, 2010; Xu *et al.*, 2013; Szabo *et al.*, 2015).

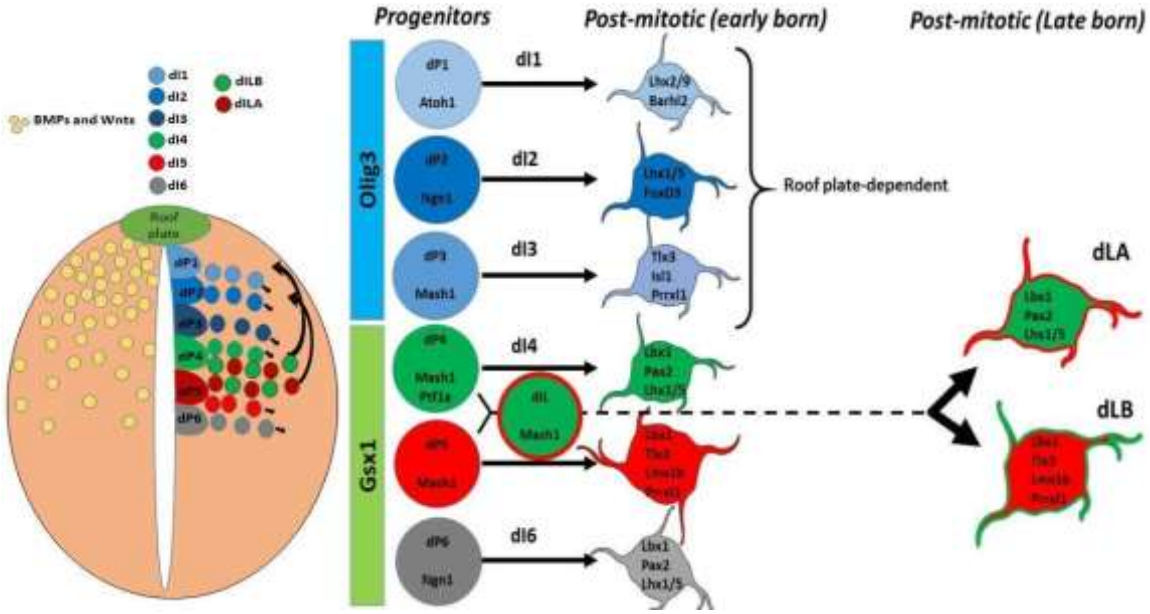

Figure 2: Schematic representation of dorsal spinal cord development. BMPs and Wnts morphogens define neural tube dorsal patterning and induce six dorsal progenitor domains (dP1-6) by triggering changes in the expression of bHLH and HD classes of transcription factors. In dP1-dP3, roof plate signals are required for proper specification of dI1-dI3 neurons. During the first wave of neurogenesis, each progenitor domain gives rise one populations of dorsal spinal neurons (dI1-6) that migrate ventrally to occupy spinal cord deep dorsal horn. Here, dI1-dI5 cells differentiate into glutamatergic neurons, while dI4 and dI6 cells differentiate into GABAergic neurons. In the second wave of neurogenesis, a pool of $Gsh1^{+}$/$Mash1^{+}$-progenitors (pdIL) generates two late born populations called dLA and dLB, which will further specifies into inhibitory and excitatory neurons, respectively. Newborn dorsal interneurons also express a unique set of transcription factors that required for their differentiation.

1.2.4 Importance of cis-regulatory sequences in regulating gene transcription

The precise control of gene transcription is crucial for all organisms' development. The genome contains the necessary information to ensure that genes are expressed in the right place, at the right time and at the proper rate. The presence of abnormal mRNA levels

during development can modify the adult phenotype and have detrimental fitness effects (Chen & Rajewsky, 2007; Borok et al., 2010; Rubinstein & de Souza, 2013). Genes implicated in developmental processes often possess long stretches of DNA flanking their consign sequence and/or large introns, which contain elements influencing their expression (Frankel, 2012). Most of these regulatory elements are relatively small and can be studied in isolation using *in vitro* reporter gene assays. Up to date, four types of elements have been described: transcriptional enhancers, transcriptional silencers, enhancer blocking insulators and promoter tethering elements (Banerji et al., 1981; Udvardy et al., 1985; Laimins et al., 1986; Calhoun et al., 2002).

Transcriptional enhancers are, by far, the most studied genetic elements (Levine, 2010). When inserted upstream of a minimal promoter and a reporter gene, enhancers often recapitulate the native expression pattern of a given gene. Enhancer activity is generated by transcription factors binding to short DNA motifs and interactions with enhancers and their target core promoter through the formation of chromatin loops (Chen & Rajewsky, 2007; Frankel, 2012). Silencers repress gene transcription through interactions with the core promoter and/or enhancer. Insulators isolate enhancers from nearby genes, restricting their influence to the target core promoter. Promoter tethering elements facilitate the interaction between enhancers and target core promoters.

Nowadays, it is clear that reversible covalent modifications of DNA and DNA-binding proteins, known as epigenome, are also important for gene transcription regulation. Epigenetic processes influence chromatin structure of regulatory elements and their flanking regions by: 1) methylation of 5' position of cytosine in 5'-Cytosine-Guanine-3' dinucleotides (known as CpGs islands) (Nakao, 2001); and 2) posttranslational modifications of nucleossomal proteins such as histone methylation and histone acetylation (Murr, 2010; Aday et al., 2011; Mazzio & Soliman, 2012). CpGs islands reinforce transcriptional silencing either by inhibition of transcriptional initiation complexes or recruitment of corepressor complexes that bind through C2H2 Zinc-Finger transcription factors (Murr, 2010; Sasai et al., 2010). Histone modifications (commonly known as the histone code) are recognized by diverse nucleosomal proteins that perpetuate either nucleosomal constriction or relaxation, controlling transcription factor accessibility to cis-regulatory regions (Frankel, 2012; Mazzio & Soliman, 2012). For instance, the mono- and

trimethylation of the histone H3 lysine 4 (H3K4me1 and H3K4me3, respectively) are usually found in active promoter regions in close vicinity to open chromatin, whereas trimethylation of H3 at lysine 9 or 27 (H3K9me3 or H3K27me3) is associated with constricted chromatin (Heintzman et al., 2009; Aday et al., 2011; Whyte et al., 2012).

1.3 Prrxl1: A HD transcription factor involved in nociceptive system development

1.3.1. Prrxl1 gene and protein functional domains

Prrxl1 is a 47kb gene in the mouse chromosome 14, formerly known as Drg11, which encodes a paired-like homeobox transcription factor specifically expressed in nociceptors located in DRG and TG, and their putative central targets in the dorsal spinal cord and in the trigeminal complex subnuclues *Caudalis* (Saito *et al.*, 1995; Rebelo et al. 2007). Prrxl1 transcription is controlled by three alternative promoters called P1, P2 and P3), that are responsible for the transcription of three *Prrxl1* 5'-UTR variants (5'-UTR-A, 5'-UTR-B and 5'-UTR-C) (Regadas et al. 2013) . These variants confer different mRNA stability and translation efficiency, being the 5'-UTR-A variant the most stable and with the highest translation rate.

By alternative splicing on the 3' end, *Prrxl1* pre-mRNA transcript originates two mRNAs, *Prrxl1a* and *Prrxl1b* (Rebelo *et al.*, 2009). *Prrxl1a* has seven exons and encodes a protein with 263 residues, whereas in *Prrxl1b*, the 7th exon is substituted by two alternative exons, and encodes a 220 residues protein. Both share the initial 175 residues in the N-terminus, which contains a HD of 60 amino acids with a helix-loop-helix conformation, required for DNA binding. In addition, Prrxl1a has an OAR motif in the C-terminus, which is missings in Prrxl1b (Rebelo *et al.*, 2009). The OAR domain in other HD transcription factors, such as Cart1 and Prx1, function as an intra-molecular switch that attenuates its own transcriptional activity (Norris & Kern, 2001a; b; Brouwer *et al.*, 2003). Due to the lack of an isoform-specific antibody, Prrxl1b protein presence *in vivo* remains to be confirmed.

1.3.2 Prrxl1 expression pattern along development

Prrxl1 expression is first detected at E10.5 in DRG and dorsal spinal cord in post-mitotic neurons (Rebelo *et al.*, 2007). During neurogenesis the number of Prrxl1-expressing neurons increases and then persists until perinatal ages with no apparent changes. In the DRG, Prrxl1 expression is restricted to $TrkA^+$-, $IB4^+$- and c-Ret^+-sensory neurons which correspond to the nociceptive population. In the spinal cord, Prrxl1 expression is firstly detected in early-born dI3 and dI5 neurons (E10.5-E11.5) and in a large subset of late-born excitatory neurons (dILB) (after E12.5) located in the dorsal spinal cord (Rebelo *et al.*, 2010). At E18.5, when spinal cord lamination is distinguishable, Prrxl1 expression extends from lamina I to III and is maintained at initial postnatal life, although decreasing along time. Prrxl1 expression is also detectable in the TG, spinal Trigeminal complex (subnuclei *caudalis* and *oralis*) Principal trigeminal nucleus *Principalis*, nucleus of the solitary tract, and *nucleus prepositus* from E12.5 to Postnatal day (P) 14 (Rebelo et al., 2006). In summary, Prrxl1 expression is restricted to the nervous system, specifically in nociceptors and in spinal and brainstem second order areas involved in the processing of nociceptive information.

1.3.3 Prrxl1 function in developing nociceptive system

Prrxl1 is simultaneously expressed in nociceptors and in their putative central targets neurons in spinal laminae I-III (Rebelo *et al.*, 2007). Such coordinated expression pattern suggests that Prrxl1 could be involved in establishing the connectivity between first and second order nociceptive neurons. *Prrxl1*$^{-/-}$ mice display temporal and spatial abnormalities in the initial penetration of sensory afferent fibers into the dorsal spinal cord and abnormal migration of superficial dorsal horn neurons. Afterwards, approximately 70% of Lamina I-III neurons, corresponding to the 53% of glutamatergic neurons that normally express Prrxl1, are lost by apoptosis around E17.5, while the GABAergic population is spared (Chen *et al.*, 2001; Rebelo *et al.*, 2010). In addition, about 30% of peptidergic and non-peptidergic nociceptors are lost in *Prrxl1*$^{-/-}$ mice by P7, which causes significant reduction in peripheral innervation of several tissues such as skin, bladder and deep tissues (Rebelo *et al.*, 2006), Accordingly, *Prrxl1*$^{-/-}$ mice present abnormally increased latency responses upon noxious stimuli (Chen *et al.*, 2001). Such developmental and nociceptive abnormalities imply that

Prrxl1 plays an important role in the establishment of the nociceptive circuit during embryogenesis, putatively controlling several developmental processes, namely neuronal differentiation, migration and axon guidance.

Thus, to understand how Prrxl1 exerts its function at the molecular level, it is of utmost importance to identify its target genes. Despite the diversity of its putative functions, very little is known regarding the Prrxl1-downstream transcriptional network. The only Prrxl1-target gene identified so far is *Rgmb*, which encodes a membrane-associated glycosyl-phosphatidylinositol-anchored protein involved in axon guidance (Samad *et al.*, 2004).

1.3.4 Preliminary data on Prrxl1 transcriptional program

Next-generation sequencing has greatly increased the scope and the resolution of transcriptional regulation studies. Chromatin Immunoprecipitation followed by massive parallel sequencing (ChIP-Seq) generates comprehensive data that allow whole-genome localization of DNA binding sites of various transcription factors, histone modifications, and other proteins, *in vivo* (Valouev *et al.*, 2008; Mundade *et al.*, 2014).

In order to unveil Prrxl1 transcriptional program, our group undertook an experimental strategy that combined ChIP-Seq with microarray expression analysis, profiling global gene expression in DRG and dorsal spinal cord of *Prrxl1*$^{-/-}$ and control mouse embryos (at E14.5). Through ChIP-Seq, it was found that most Prrxl1-binding events occur in genomic regions enriched in the TAAT or ATTA consensus motif, as expected for a HD transcription factor (Wilson *et al.*, 1993). Prrxl1 binding sites were annotated to the nearest transcription start site, generating a comprehensive list of putative target genes. Microarray expression analyses showed that a large subset of Prrxl1-bound genes was deregulated in *Prrxl1*$^{-/-}$ embryos, suggesting that they are direct Prrxl1-transcriptional targets. Gene ontology analysis of this gene list revealed that the most enriched functional categories are associated with late aspects of neural development, such as neuronal differentiation, migration and axon guidance, which are in line with the developmental abnormalities observed in *Prrxl1*$^{-/-}$ mice.

Objectives

The main goal of the present thesis is to give insight into the transcriptional regulation and expression pattern of selected Prrxl1 target genes. One of the targets analyzed in this thesis was *Prrxl1* itself. ChIP-on-chip data showed that Prrxl1 binds its own promoter and 4th intron, raising the question "Does Prrxl1 regulate its own transcription?". This issue was addressed in Publication I, where we demonstrate that Prrxl1 regulates its own transcription through a repressive autoregulatory loop.

Another target analyzed in this thesis was *Casz1*. The criteria for selecting *Casz1* among Prrxl1 putative target genes was based on: 1) Prrxl1 robust binding (ChIP-Seq data); 2) marked expression deregulation in dorsal spinal cord of *Prrxl1*-null mice (microarrays data); 3) co-expression with Prrxl1; and 4) unknown role in the DRG-spinal nociceptive development. In Publication II, we provide a detailed characterization of Casz1 spatio-temporal expression pattern and molecular identity of Casz1-expressing cells at various developmental time points in the DRG and dorsal spinal cord, and its transcriptional dependency on Prrxl1 in dILB neurons.

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Paired related homeobox protein-like 1 (Prrxl1) controls its own expression by a transcriptional autorepression mechanism

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ABSTRACT

The homeodomain factor paired related homeobox protein-like 1 (Prrxl1) is crucial for proper assembly of dorsal root ganglia (DRG)–dorsal spinal cord (SC) pain-sensing circuit. By performing chromatin immunoprecipitation with either embryonic DRG or dorsal SC, we identified two evolutionarily conserved regions (i.e. proximal promoter and intron 4) of *Prrxl1* locus that show tissue-specific binding of Prrxl1. Transcriptional assays confirm the identified regions can mediate repression by Prrxl1, while gain-of-function studies in Prrxl1 expressing ND7/23 cells indicate Prrxl1 can down-regulate its own expression. Altogether, our results suggest that *Prrxl1* uses distinct regulatory regions to repress its own expression in DRG and dorsal SC.

1. Introduction

Nociceptive information is transmitted from the periphery to the spinal cord (SC) dorsal horn by small-diameter neurons of dorsal root ganglia (DRG), called nociceptors [2]. DRG and SC sensory neurons are specified early in development, long before establishing synapses with their peripheral and central targets [12]. During development, distinct sets of transcription factors determine a variety of neuronal phenotypes in the DRG and SC dorsal horn [3,15,17]. One of such factors is paired related homeobox protein-like 1 (Prrxl1), previously known as DRG11.

Analyses of *Prrxl1*$^{-/-}$ embryos have shown spatio-temporal abnormalities in embryonic development. Small-diameter afferent fibers terminating in the superficial dorsal horn develop normally up to birth, although with retarded penetration in the spinal dorsal horn, and a significant fraction of which undergoes apoptosis at early post natal life [5,20]; at the spinal level, abnormal migration and differentiation followed by marked death of superficial dorsal horn neurons takes place during embryonic development [5,9,22]. In line with these nociceptive circuitry developmental defects, adult *Prrxl1*$^{-/-}$ mice displayed a substantial reduction in sensitivity to a broad range of noxious stimuli [5]. These tissue specific cellular defects suggest a differential role played by Prrxl1 in DRG and dorsal SC that is yet unexplored.

Prrxl1 is expressed in both peripheral nociceptive neurons and their putative central synaptic targets [25]. Genetic studies in mice have shown that Prrxl1 expression in developing sensory neurons depends on the presence of several transcription factors such as Isl1 [11,30] and Pou4f1 (Brn3a) [11] in DRG, and Tlx1/3 [19] and Lmx1b [9] in dorsal SC. In addition, in cranial ganglia, *Phox2b* down-regulates *Prrxl1*, as revealed by increased *Prrxl1* expression in *Phox2b*$^{-/-}$ mice [6]. However, no evidence on direct transcriptional regulation has been demonstrated, except for Phox2b, which binds to a regulatory element near *Prrxl1* TATA box promoter [24]. Transcription of *Prrxl1* is controlled by three alternative promoters giving rise to distinct *Prrxl1* 5′UTR variants that impact on mRNA stability and translation efficiency but not on translation initiation [24]. On the other hand, alternative splicing of *Prrxl1* transcript at

Abbreviations: ChIP, chromatin immunoprecipitation; DRG, dorsal root ganglia; HD, homeodomain; NLS, nuclear localization signal; OAR, Otp-Aristaless-Rax domain; Prrxl1, paired related homeobox protein-like 1; SC, spinal cord

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the 3′ end gives rise to two Prrxl1 homeoproteins (i.e. Prrxl1 and Prrxl1b). Both variants retain the N-terminal homeodomain (HD), whereas Prrxl1b lacks the C-terminal Otp-Aristaless-Rax domain (OAR) [21]. Although the regulatory elements controlling *Prrxl1* transcription are starting to be identified, the mechanisms underlying spatio-temporal control of *Prrxl1* expression are still scarce.

A detailed search for DNA-binding consensus sequences revealed several evolutionarily conserved TAAT-containing sites on *Prrxl1* proximal promoter, including two HD motifs in close vicinity of *Prrxl1* TATA box promoter [24]. This observation led us to question whether an auto-regulatory mechanism is involved in *Prrxl1* transcription. Here we describe an autorepression mechanism controlling *Prrxl1* expression, based on the tissue-specific recruitment of this transcription factor to two regulatory regions within its own locus.

2. Materials and methods

2.1. Mice

NMRI mouse strain was bred and housed at the IBMC 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. Pregnant females were subjected to isoflurane anesthesia, euthanized by cervical dislocation and E14.5 embryos were collected. Afterwards, DRG were collected and dorsal SC tissues were dissected in ice-cold PBS. Experiments were carried out in agreement with European Community Council Directive (86/609/EEC) and the animal ethics guidelines of the IBMC, and approved by the Portuguese Veterinary Ethics Committee.

2.2. Plasmid construction

Prrxl1 genomic sequences retrieval and analysis of conservation across vertebrates species were performed using the UCSC Genome Browser (http://genome-euro.ucsc.edu/) [31]. For expression plasmids, the sequences corresponding to *Prrxl1* (NCBI acc. n. EU670677) and *Prrxl1b* (EU670678) open reading frames were amplified by PCR from mouse embryonic SC cDNA, subcloned in the pCR2.1 TOPO (Invitrogen) and then transferred to pcDNA3 (Invitrogen) using EcoRI/XbaI and EcoRI restriction sites for Prrxl1 and Prrxl1b, respectively. Prrxl1-ΔHD sequence was amplified by PCR using a forward primer containing Kozak and nuclear localization signal (NLS) sequences and a reverse primer containing a HA tag sequence, and cloned into pcDNA3.3-TOPO (Invitrogen). For luciferase reporter plasmids, *Prrxl1* proximal region [−1777/−672] and intron 4 region [+5267/+6969] were amplified by PCR using genomic DNA extracted from mouse tail. They were subcloned in pCR2.1 TOPO (Invitrogen) and then cloned into p-βglob-Luc using NheI and XhoI restriction sites. Constructs for *Prrxl1* proximal region [−604/−50] and minimal promoter region [−772/−584] were previously reported [24]. Plasmid construction primers are listed in Table 1. Site-directed mutagenesis of either P3 HD or hs HD binding sites or both on *Prrxl1* minimal promoter region construct were performed using the KAPA HiFi HotStart PCR kit (Kapa Biosystems). Double HD motif mutant was produced using the construct containing mutated P3 HD binding site as DNA template. Mutagenesis primers are listed in Table 1. All constructs were validated by sequencing.

2.3. Cell culture, transfection and reporter assays

ND7/23 (mouse neuroblastoma × rat neurone hybrid) and HEK (Human Embryonic Kidney) 293 cell lines were from the European Collection of Cell Cultures. ND7/23 and HEK-293 cells were propagated in 25 cm^2 flasks in monolayer and maintained at 37 °C in a 95% humidified atmosphere and 5% CO_2. Cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamax (Gibco) and without antibiotics (complete media). For luciferase reporter assays, ND7/23 and HEK-293 cell transient transfections were performed in triplicate in 96 well plates using Lipofectamine 2000 (Invitrogen). Cells were seeded one day earlier and co-transfected at 80% confluence with 100 ng of appropriate expression plasmid, 50 ng of luciferase reporter plasmid, and 50 ng of CMV-β-gal plasmid as internal control to normalize the transfection efficiencies. Before adding the transfection mixture, fresh complete culture media was added. After 24 h, cells were lysed in 50 μL of lysis buffer (0.1 M Phosphate buffer, 1 mM EDTA, 1% Triton X-100, 10% glycerol and 2 mM DTT), cleared by centrifugation and extracts were assayed for luciferase and β-galactosidase activities using a plate reader (Tecan). The substrates used were luciferin (Promega) and ONPG (Sigma). Data are represented as means of triplicates, and experiments were repeated at least three times. Student's *t*-test analysis was used to determine statistical significance upon Prrxl1 overexpression between cells transfected with wild type *Prrxl1* minimal region [−772/−584] construct and cells transfected with the same construct but containing mutated versions of either P3 HD or hs HD binding sites or both sites. For mRNA expression assays, transfections of ND7/23 cells were performed essentially as above with minor modifications: transfections were performed in duplicate using 6 well plates and in each condition 2 μg of expression plasmid was used.

2.4. DNA-affinity pull down assays (DAPA)

Biotinylated DNA fragments were generated by PCR and agarose gel purified. Primers used to amplify *Prrxl1* TATA box promoter region (Prom) and *Prrxl1* open reading frame (ORF) of exon 6–7, as a negative control region, are listed in Table 2. Prrxl1 or mock-transfected ND7/23 cells were lysed in DAPA buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 10% glicerol, 1%

Table 1
Sequences of primers used for plasmid construction and mutagenesis.

Gene symbol	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Prrxl1	CCGGAATT*CGCCACCATG*TTTTATTTCCACTGTCCGCCAC	GCTCTAGATCATACACTCTTCTCTCCC
Prrxl1b	CCGGAATT*CGCCACCATG*TTTTATTTCCACTGTCCGCCAC	CGCTCTAGATCATCCTTGTGTCATATCTAC
Prrxl1 proximal region [−1777/−672]	AGGCCTTTTGTTTCCAAAGC	CTCACGCATAATTGGCCTTA
Prrxl1 intron 4 region [+5268/+6969]	CCTCACAGAAGCCAGAGTGC	CTTTGAGGCTGAGCCCTCTG
Prrxl1-ΔHD	*CGCCACCATG*CCAAAAAAGAAGAGAAAGGTA GCAGAGGTGACACCACCG	TCAAGCGTAATCTGGAACATCGTATGGGTATACACTCTTCTCTCCCTCGC
P3 HD mut.	TTACTCGCTGTAGACAGCGCTGGAAAT***CC***TCAGA***GG***AA GGCCAATTATGCGTG	CACGCATAATTGGCCTT***CC***TCTGA***GG***ATTTCCAGCGCTGTCT ACAGCGAGTAA
hs HD mut.	GGAAATAATCAGATTAAGGCCAA***CC***ATGCGTGAGATTATA	TATAATCTCACGCAT***GG***TTGGCCTTAATCTGATTATTTCC
P3 & hs HD mut.	CCTCAGAGGAAGGCCAA***GG***ATGCGTGAGATTATAAAGG	CCTTTATAATCTCACGCAT***CC***TTGGCCTTCCTCTGAGG

Kozak sequence is italic, NLS and HA sequences are underlined, and mutated bases are highlighted in bold and italic.

Table 2
Sequences of primers used for DAPA.

Gene symbol	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Prom	TACACCACGTGGGCCATTTGTAA	CAGGACCAGAGAAGTGACTG
ORF	CCCATGTGGCATCTCTGAAAG	TCATACACTCTTCTCTCCCTCGC

TritonX-100, 0.5 mM DTT and Protease Inhibitor cocktail (Sigma–Aldrich)) and cleared by centrifugation. Equal amounts of protein extracts (100 μg) were incubated with 150 ng of biotinylated DNA fragment at room temperature for 2 h in 100 μl of DAPA buffer supplemented with 1 μg of poli-dIdC (Sigma–Aldrich). The DNA–protein complexes were recovered with 20 μL of equilibrated streptavidin-agarose resin (Sigma–Aldrich) after incubation for 2 h at room temperature with agitation and washed 5 times with DAPA buffer. The proteins bound to the DNA fragments were eluted in SDS–PAGE sample buffer and analysed by Western-blotting using rabbit polyclonal anti-Prrxl1 antibody [23].

2.5. Chromatin immunoprecipitation assays

Prrxl1 chromatin immunoprecipitation (ChIP) assays were performed essentially as previously described [24], with the following modifications: (i) chromatin samples were extracted from about 16 dissected E14.5 mouse dorsal SC; (ii) antibody used was a home-made rabbit polyclonal anti-Prrxl1 antibody [23]; (iii) salt concentration of IP buffer was 500 mM; (iv) immunoprecipitates were washed with wash buffer II containing 500 mM NaCl. For quantitative PCR (qPCR), sets of primers were used for assessing ChIP enrichment and designed using primer3 software (http://biotools.umassmed.edu). The primer sequences are listed in Table 3. Results are shown as the mean of triplicates ± S.D. of at least two independent experiments.

2.6. Array design and hybridization

Agilent custom microarrays tiling the desired regions were designed using Earray software (Agilent), with a probe spacing of approx. 200 bp. ChIP-on-chip array hybridization, data extraction and peak calling were essentially performed as previously described [4].

2.7. RNA extraction and real-time quantitative PCR

Total RNA from transfected ND7/23 cells was isolated using Genelute mammalian total RNA miniprep kit (Sigma), quantified in Nanodrop (Thermo Scientific) and verified for integrity by agarose gel electrophoresis. cDNA was prepared using oligo dT primers (Bioline) and Tetro reverse transcriptase (Bioline) according to the manufacturer's instructions. A control containing all reagents except the reverse transcriptase enzyme was included (minus RT control) to assess potential residual genomic DNA in the RNA samples. Real-time qPCR analysis was performed using the Maxima SYBR Green/ROX master mix (Thermo Scientific) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Sets of primers were designed in different exons for assessing gene expression levels using primer3 software. The primer sequences are listed in Table 4. Expression of endogenous *Prrxl1* and *Prrxl1b* transcripts was normalized to the endogenous control gene *Hprt*. Results are shown as the mean of triplicates ± S.D. of at least two independent experiments. Student's *t*-test statistical analysis was used to determine statistical significance between cells transfected with empty vector (vector) and cells transfected with either Prrxl1 or Prrxl1b.

Table 4
Sequences of primers used for RT-qPCR.

Gene symbol	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Prrxl1	TCCTGAACACAGCCACGTAT	TTGTGGTTCAGAGGATGGCA
Prrxl1b	CCCATGTGGCATCTCTGAAAG	TCATCCTTGTGTCATATCTACTGC
Hprt	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA

3. Results

3.1. Prrxl1 binds to its own genomic locus

Sequence alignment of *Prrxl1* promoter region across vertebrate species showed multiple HD DNA binding motifs (data not show), two of which located immediately upstream of the TATA box promoter [24]. The presence of these multiple sites prompted us to investigate whether an auto-regulatory feedback loop contributes to Prrxl1 gene regulation. To determine if Prrxl1 homeoprotein is able to interact with its own minimal promoter, we performed DNA affinity pull-down assays (DAPA) in the ND7/23 cell line. ND7/23 cells are derived from neonatal DRG, display nociceptive-like properties [33] and express both Prrxl1 isoforms (Fig. S1, Supplemental Data), making them a good model system to study *Prrxl1* function. By performing DAPA with Prrxl1-transfected ND7/23 cell extracts, we found that Prrxl1 binds to a DNA fragment of 186 bp flanking the TATA box element corresponding to the *Prrxl1* minimal promoter region (Prom). This interaction is specific, as Prrxl1 did not bind to a DNA fragment of 287 bp spanning exons 6 and 7, corresponding to the *Prrxl1* coding region (ORF) (Fig. 1A). These results demonstrate that Prrxl1 interacts with a region containing HD motifs, located in the vicinity of the TATA box promoter of *Prrxl1* gene.

Table 3
Sequences of primers used for ChIP-qPCR.

Gene symbol	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
RGMb	GATAGTTGGAGGGGGTTTGC	GAGAACGGGAGTCAGGGATT
Calb1	TAAACAGCCACGTGATGGTC	CAGATGGAAGGGAAAGCTGA
Prrxl1 ORF	TTGGTTCCAGAACCGAAGAG	CAGGGCCTCCTTCTTGCT
RGMb ORF	TGCCAACAGCCTACTCAATG	GTGGAAGATGTGGGTCCATC
Prrxl1 (−3568)	TTTAATGTCTCCCGCAGCTT	CACTTGATGTTCACCAGACTCA
Prrxl1 (−1297)	TTATGCGCCATTAGACTTGC	CTCTCTGCCTGGGTGAAAAT
Prrxl1 (−737)	TGCTGAGAAGTGACGGATTT	TCACGCATAATTGGCCTTAAT
Prrxl1 (+3247)	GAAGTCGGCAGGGTTTTCTA	AGCCTTTCACACCTCTTTCC
Prrxl1 (+5601)	ATCCATTTCCTTGCTTTGGA	ATCGCGGTTCTCTGCAAT
Prrxl1 (+6054)	TTTTATGTAGGAGCTTGGGGTTA	TCCATTATCTCTCTTCAGCCATT
Prrxl1 (+6869)	CTGCATTTTGCTTTGCATTT	TGCCATCCCACCTTATCTCT
Prrxl1 (+9787)	ACTCCAGCACATTTTTGCAC	TGTGGACCCTTCTACCCTTC

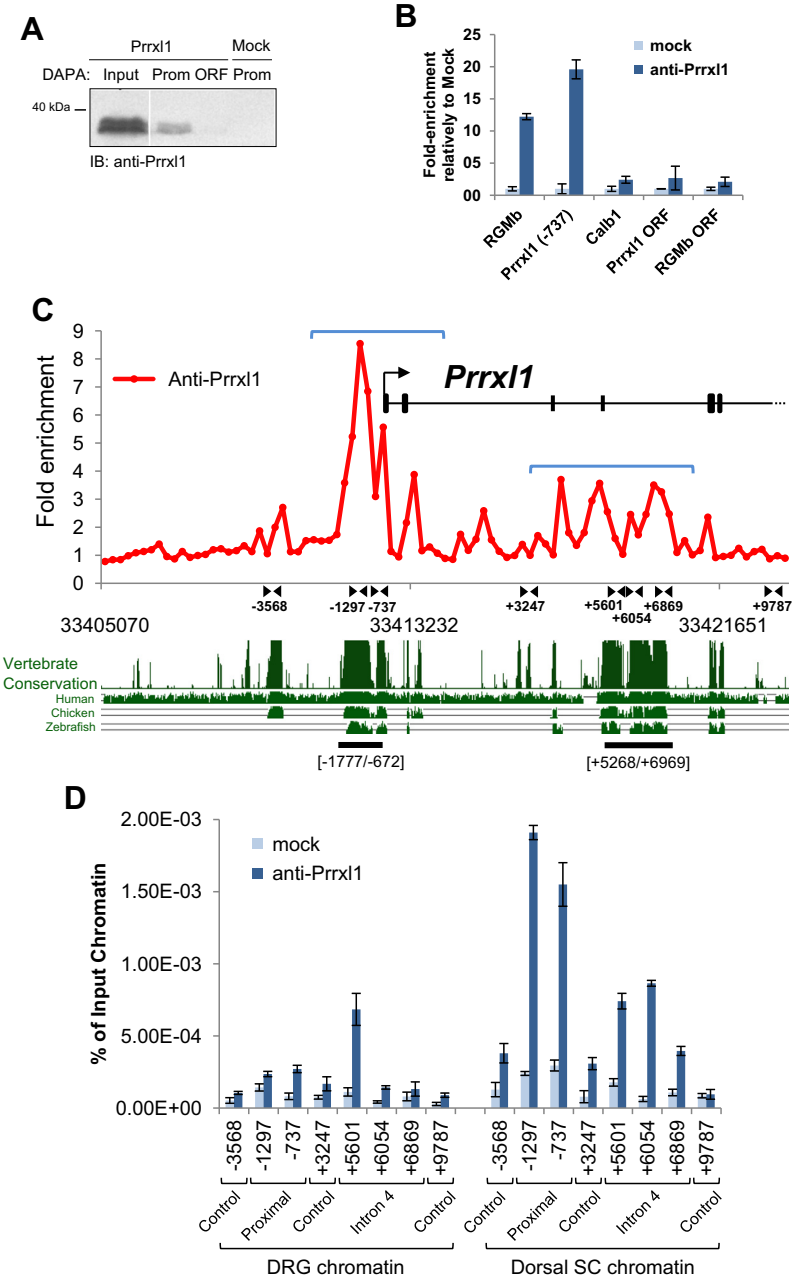

Fig. 1. Prrxl1 interacts with its own locus in developing DRG and dorsal SC. (A) DNA pull-down assays revealed binding of Prrxl1 within its minimal TATA box promoter region. Prrxl1 and mock-transfected ND7/23 cells protein extracts were incubated with 5′ biotinylated double-stranded DNA probes from *Prrxl1* promoter (Prom) or *Prrxl1* exon 6–7 (ORF). The complex was pulled down with streptavidin-agarose and the bound fraction analysed by Western blotting and probed with anti-Prrxl1 antibody. (B) Prrxl1 binds to *Prrxl1* minimal TATA box promoter region in embryonic dorsal SC. ChIP-qPCR analysis of Prrxl1 binding to *Prrxl1*, *RGMb* and *Calb1* promoter regions in chromatin prepared from E14.5 dorsal SC. *RGMb* promoter region was used as positive control and *Prrxl1* and *RGMb* exonic regions (ORF) were used as negative controls. Data are presented as fold-enrichment relatively to ChIP control without antibody (mock), which was set as 1, and as the mean ± S.D. of triplicate quantifications. (C) Prrxl1 binds to two segments on *Prrxl1* locus in embryonic dorsal SC. ChIP-on-chip analysis of Prrxl1 binding to *Prrxl1* mouse promoter in chromatin prepared from E14.5 dorsal SC. The plots display ChIP enrichment ratios for Prrxl1 (red) relatively to control samples. Blue brackets represent bound regions identified by peak calling algorithm. *Prrxl1* gene is shown to scale above the plot (exons represented as boxes and introns represented as lines) and genomic regions (NCBI build 37 of the mouse genome) below the plot. Black arrow indicates the transcription start site and direction of transcription. Arrowheads indicate the position of primers, relatively to the translation initiation site, used for ChIP-qPCR validation. Multispecies vertebrate conservation plots (in green; human, chick and zebrafish are displayed) were obtained from UCSC genome browser. Highly conserved non-coding genomic sequences of the *Prrxl1* proximal promoter [−1777/−672] and intron 4 [+5268/+6969] cloned in luciferase-reporter constructs are displayed at the bottom (black lines). (D) Prrxl1 occupancy of *Prrxl1* locus is tissue-dependent. ChIP-qPCR analysis of Prrxl1 binding at proximal promoter (−1297 and −737), intron 4 (+5601, +6054 and +6869) and control regions (−3568, +3247 and +9787) in chromatin prepared from the DRG and dorsal SC of E14.5 wild-type embryos. Data are presented as mean ± S.D. of triplicate quantifications.

To assess binding of Prrxl1 to its own promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays on mouse dorsal SC tissue at embryonic stage E14.5 of development. As positive control for ChIP-qPCR, we used primers that amplify a promoter region of *RGMb* (also known as *Dragon*) that has been previously shown to contain a Prrxl1 response element [26]. Both the *Prrxl1* and *RGMb* promoter regions were enriched in the material immunoprecipitated with anti-Prrxl1 antibody (but not in the absence of antibody) as compared to negative control regions, located within the coding regions (ORF) (Fig. 1B). In contrast, binding was not detected in a region spanning a HD motif located proximal to *Calb1* promoter, a candidate Prrxl1 target gene [5].

Thus, ChIP results confirmed that Prrxl1 binds to its own proximal promoter in embryonic dorsal SC.

To expand our analysis of the autoregulation of the *Prrxl1* gene, we performed ChIP on chromatin extracted from E14.5 mouse dorsal SC, followed by hybridization to custom-made genomic microarrays (ChIP-on-chip) tiling the *Prrxl1* locus. In addition to previously identified binding site at the proximal promoter of Prrxl1 gene, peak calling identified a second bound region spanning exon 2, exon 3 and intron 4 (Fig. 1C, blue brackets). As binding to exonic sequences is unlikely to have a regulatory role, we decided to focus on intron 4. Proximal promoter and intron 4 regions are very well conserved across vertebrate species (i.e. human, mouse, chicken and zebrafish), which may be indicative of a regulatory function. Prrxl1 occupancy at the proximal promoter region was more robust, reaching 8.5-fold enrichment, which compares to a maximal peak of 3.5-fold enrichment at the intron 4 region (Fig. 1C). Overall, results demonstrate that Prrxl1 interacts with its own genomic locus at an extended proximal promoter region containing the TATA box and to a second region at intron 4, suggesting that *Prrxl1* transcription may be subject to autoregulation.

3.2. Prrxl1 recruitment to its own genomic locus is tissue-dependent

To analyse whether binding of Prrxl1 to its own genomic locus occurs in a tissue-dependent manner, we performed ChIP-qPCR using chromatin extracted from either DRG or dorsal SC tissues of E14.5 mouse embryos, using primers targeting both evolutionarily conserved Prrxl1-bound regions identified by ChIP-on-chip (Fig. 1C, arrowheads). In the DRG, binding of Prrxl1 was stronger at intron 4 (in particular at position +5601) as compared to the proximal promoter region (positions −1297 and −737). In the dorsal SC, a strong Prrxl1 enrichment is obtained for both the proximal promoter (positions −1297 and −737) and intron 4 regions (positions +5601, +6054 and +6869), though more pronounced at the proximal promoter (Fig. 1D). Thus, our results indicate that Prrxl1 is differentially recruited to the proximal promoter and intron 4 regions in developing DRG and dorsal SC.

3.3. Prrxl1 acts as a repressor of its own transcription

To assess the regulatory potential of Prrxl1-bound regions, we performed transcriptional assays in ND7/23 cells transfected with promoter-reporter constructs bearing the β-globulin minimal promoter and the firefly-luciferase gene under the regulation of either the *Prrxl1* proximal promoter [−1777/−672] or the *Prrxl1* intron 4 [+5268/+6969] regions. When designing these constructs, we took into consideration the evolutionary conservation of putative regulatory regions across vertebrate species (Fig. 1C, bottom black lines and green plots). Transcriptional assays were performed expressing either Prrxl1 isoforms (i.e. Prrxl1 and Prrxl1b), both of which are recognized by the anti-Prrxl1 antibody used in ChIP (Figs. 2A and S1). Co-expression of either Prrxl1 or Prrxl1b repressed the activity of both *Prrxl1* proximal promoter [−1777/−672] and intron 4 [+5268/+6969] regulatory regions (Fig. 2B), with Prrxl1 displaying the strongest effect. Accordingly, recent data showed that Prrxl1 has a repressor domain spanning amino acids 227–263 of Prrxl1, which is not present in Prrxl1b [28]. No repression was detected when using a region spanning *Prrxl1* alternative promoters downstream of the TATA box [−604/−50]. In addition, Prrxl1 and Prrxl1b repression activity on these regulatory regions was not observed in HEK-293 cells, which do not express *Prrxl1* endogenously (data not shown), indicating that their activity is dependent on cellular context (Fig. 2B).

To identify Prrxl1 binding sites mediating its autorepression, we narrowed down our analysis to the *Prrxl1* minimal promoter region [−772/−584] bound by Prrxl1 (Fig. 1A–C), which contains two putative HD motifs (Fig. 2D). Co-expression of either Prrxl1 or Prrxl1b repressed the transcriptional activity of this regulatory region, whereas a mutant form of Prrxl1 protein with the HD replaced by a NLS sequence (Prrxl1-ΔHD) had no effect, suggesting that Prrxl1 DNA binding domain is necessary for this activity (Fig. 1C). Next we performed site-directed mutagenesis of one HD binding bipartite site (P3 HD) and one HD binding half-site (hs HD) (Fig. 2D). We verified that Prrxl1 mediated autorepression was significantly attenuated to similar levels, when either or both HD binding sites were mutated. Moreover, no repression was observed in *Prrxl1* promoter control region [−604/−50] (Fig. 2E). These data indicate that both HD binding sites are required for Prrxl1 mediated autorepression. However, since repression was not completely abolished, additional binding sites cannot be excluded.

3.4. Prrxl1 isoforms repress the expression of their isoform counterpart

To determine whether Prrxl1 and Prrxl1b can modulate endogenous expression of their isoform counterparts, we overexpressed either Prrxl1 or Prrxl1b in ND7/23 cells and assessed endogenous expression of, respectively, Prrxl1b or Prrxl1 through RT-qPCR. Overexpression of Prrxl1 resulted in decreased expression of Prrxl1b transcript by 7.1-fold (Fig. 3A), whereas overexpression of Prrxl1b decreased expression of Prrxl1 transcript by 2.4-fold (Fig. 3B), when compared to mock-transfected ND7/23 cells. Noteworthy, the repressive effect was stronger when overexpressing Prrxl1, in line with data from the transcriptional assays. Overall, our results strongly suggest autorepression as a control mechanism of *Prrxl1* transcription.

4. Discussion

This study addresses the transcriptional control mechanisms of *Prrxl1*, a homeobox gene critical for proper assembly of DRG-dorsal SC pain circuitry [5,20,22]. ChIP performed with chromatin derived from embryonic DRG and dorsal SC identified two regions (in the proximal promoter and intron 4 of *Prrxl1* locus) that show tissue specific recruitment of Prrxl1. Transcriptional assays and gene expression studies in ND27/23 cells suggest these regions control *Prrxl1* expression by a negative auto-regulatory mechanism.

A striking finding of the present work was that occupancy by Prrxl1 on its locus occurs in a tissue-dependent manner. In the DRG, Prrxl1 was preferentially recruited to intron 4, while in the dorsal SC Prrxl1 was strongly recruited to the proximal promoter and moderately to intron 4. Differential recruitment to regulatory regions may be attributed to different chromatin accessibility and/or interaction with tissue-specific transcription factors. ChIP-based studies using histone mark antibodies would answer whether chromatin structure differs in these two regions. The other possibility is also plausible, as differential expression of transcription factors exist between tissues. Another example of cell context dependency was obtained with the transcriptional assay based on transfection of plasmid DNA in HEK-293 cells, where Prrxl1 did not show any transcriptional activity. This result is more likely due to the absence of appropriate factors that are not expressed in this cellular context. Such factors may be required for appropriate binding of Prrxl1 to its sites or, alternatively, they may be part of the repressor complex recruited by Prrxl1. As any Prrxl1 co-regulators or co-factors are presently unknown, we cannot distinguish between these two possibilities. We have previously shown that Prrxl1 is the most expressed isoform in developing DRG and dorsal SC [21]. The expression ratio between Prrxl1 and Prrxl1b is kept constant in the DRG, but varies in the

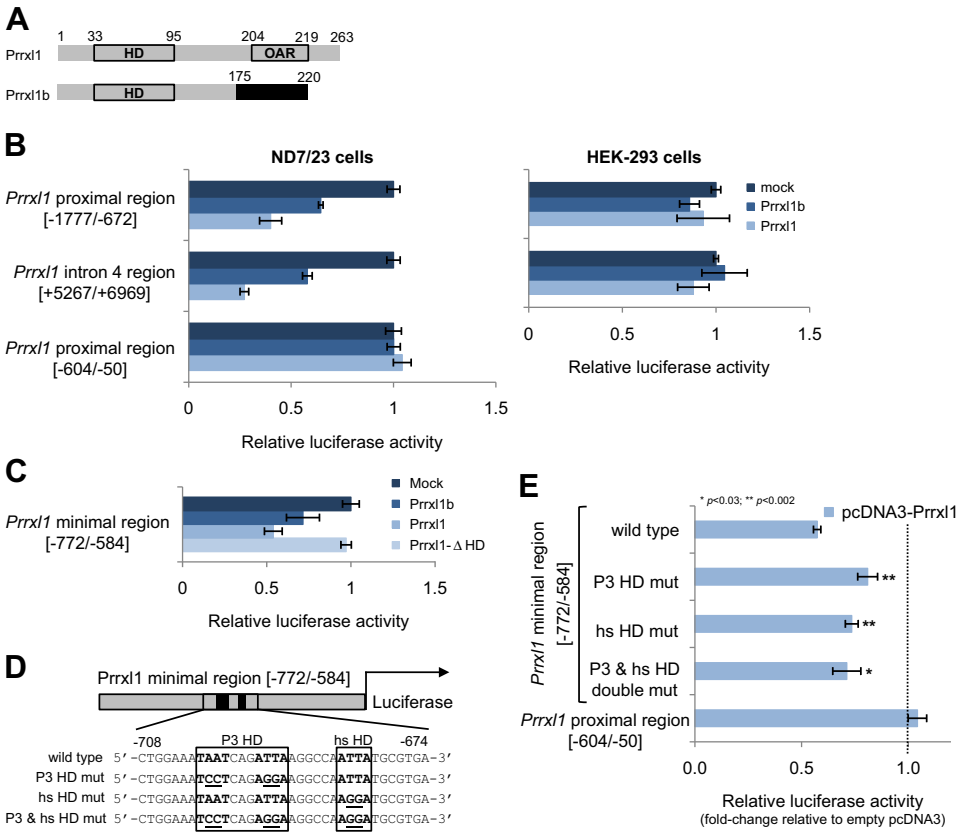

Fig. 2. Autorepression of Prrxl1-bound regions is cell-context dependent. (A) Schematic representation of Prrxl1 and Prrxl1b. The numbers indicate positions of the amino acid residues. The HD and OAR domains (in boxes) and Prrxl1b-specific C-terminal peptide sequence (in black) are shown. (B) Transcription assays in ND7/23 and HEK-293 cells co-transfected with luciferase reporter constructs containing *Prrxl1* proximal promoter [−1777/−672], [−604/−50] or intron 4 [+5267/+6969] regions and either an expression construct empty (mock), Prrxl1b (Prrxl1b) or Prrxl1 (Prrxl1). (C) Transcription assays in ND7/23 cells co-transfected with luciferase reporter construct containing *Prrxl1* minimal region [−772/−584] and either an expression construct empty (Mock), Prrxl1b (Prrxl1b), Prrxl1 (Prrxl1) or Prrxl1 lacking the HD (Prrxl1-ΔHD). (D) Schematic representation of *Prrxl1* minimal region [−772/−584] in luciferase reporter construct. DNA sequences displayed correspond to the wild type sequence spanning the two putative HD binding motifs and the respective mutant versions of the bipartite site (P3 HD mut) and half-site (hs HD mut) as well as both sites (P3 & hs HD mut) used in the luciferase assays. (E) Transcription assays in ND7/23 cells co-transfected with a Prrxl1 expression plasmid and luciferase reporter constructs containing *Prrxl1* minimal region [−772/−584] or the same construct with mutated HD binding sites displayed in Fig. 2D. Data are presented as fold-change of each reporter construct relative to empty pcDNA3, which was set as 1, and as the mean ± S.D. of triplicate assays.

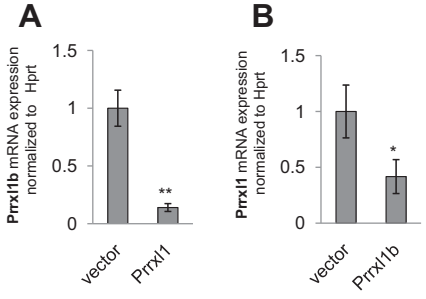

Fig. 3. Overexpression of Prrxl1 isoforms induces repression of the endogenous expression of their isoform counterpart in ND7/23 cells. ND7/23 cells were transfected with an expression construct for Prrxl1 (Prrxl1), Prrxl1b (Prrxl1b) and empty vector (vector) and after 24 h the endogenous expression of Prrxl1b (A) and Prrxl1 (B) mRNA was assessed by RT-qPCR. mRNA level intensities were normalized to *Hprt* housekeeping gene. Mean ± S.D.; (*) $P < 0.02$ and (**) $P < 0.003$ with Student's *t*-test; $n = 3$.

dorsal SC during later embryonic and postnatal development [21]. Transcriptional autorepression may thus control the expression ratio of Prrxl1 isoforms, being Prrxl1b more effectively repressed than Prrxl1.

Positive regulators of *Prrxl1* transcription have been described in the DRG and the dorsal SC. In the DRG, the pan-sensory HD transcription factors Pou4f1 and Isl1 are both required for *Prrxl1* expression [11]. On the other hand, in developing dorsal SC, Tlx1/3 and Lmx1b are extensively co-expressed with Prrxl1 [22,23]. Prrxl1 appears to depend more on Lmx1b than on Tlx1/3, as in *Lmx1b* null mice *Prrxl1* expression was completely abolished [9], whereas in *Tlx1/3* null mutant mice *Prrxl1* expression was normally initiated but completely lost by E14.5 [19]. Our data, showing Prrxl1 represses its own expression, point to a role for this transcription factor in fine-tuning positive transcriptional activity of aforementioned transcription factors in developing DRG and dorsal SC.

To date, Phox2b HD transcription factor, which is transiently co-expressed with Prrxl1 at early development (from E10.5 to 13.5) of facial-glossopharyngeal and vagal ganglia [6,23], is the only known direct regulator of *Prrxl1* transcription. Both Prrxl1 and Phox2 bind to the minimal TATA box promoter region of *Prrxl1*, but have opposing transcriptional activities [24] and (Fig. 2B) possibly by recruiting distinct cofactor complexes. It was previously suggested that Phox2b may be directly responsible for the initiation of *Prrxl1* expression, possibly in cooperation with Isl1 [24], while the mechanism leading to *Prrxl1* extinction at E13.5 is still elusive. It is possible that *Prrxl1* transcriptional autorepression plays a role in this silencing process.

Homeoproteins of the paired class bind DNA most efficiently as homo or heterodimers to a palindromic TAAT-$(N)_{2-3}$ATTA motif, and the spacing between the half-sites is predicted by the nature of the residue at position 50 of the HD [32]. In the HD of Prrxl1, this residue is a glutamine (Gln^{50}), suggesting that Prrxl1 binds to

bipartite sequence TAAT-$(N)_3$ATTA, as described for paired-like homeoprotein Chx10 [10]. Consistent with this, two of such bipartite sites are found in a Prrxl1 response element within a 363 bp fragment of the *RGMb/Dragon* proximal promoter [26]. In fact, *RGMb* expression was shown to be deregulated in the DRG and dorsal SC of *Prrxl1* null mouse embryos [26]. Here, we confirm by chromatin immunoprecipitation that *RGMb* is a direct target of Prrxl1 in developing dorsal SC. RGMb is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that was implicated in axon guidance [18], corroborating Prrxl1 putative function in the guidance of small-diameter primary afferent neurons.

In the present study, we addressed the functional relevance of two putative HD binding motifs (i.e. a bipartite and a half-site HD binding motifs) located in the vicinity of *Prrxl1* TATA box promoter and showed that these motifs mediate Prrxl1 repression activity. The fact that repression was not completely abolished by mutating these sites suggests that other Prrxl1 binding site/s differing from the TAAT consensus may also intervene. In fact, binding of HD proteins to variations of their consensus sequences is often observed, as exemplified by binding of Six3 with similar affinities to TAATTGTC and TGATAC sequences [29].

Other homeobox genes were shown to be also transcriptionally regulated by autorepression, such as *Drosophila Engrailed* [14], *Ultrabithorax* [16], *Suppressor of Hairless* [1], *Xenopus Goosecoid* [7], mouse *Six3* [34], *Msx1* [27] and *Msx2* [8]. This supports the idea that many genes need to maintain stable levels of expression in development, as a way to guarantee the correct expression of target genes sensible to HD factor's concentration, through transcriptional autorepression mechanisms.

The transcriptional control mechanisms involved in *Prrxl1* spatio-temporal expression are not fully understood. In this work, we gained new insight into these transcriptional mechanisms by demonstrating that Prrxl1 binds to its locus and negatively regulates its own transcription. It is likely that such negative feedback is used to suppress fluctuations of gene expression, as it has been shown to be the case with other transcriptional regulators that are also subject to autorepression [13], ensuring in this way that *Prrxl1* is maintained at the appropriate level during development of the nociceptive circuitry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.08.006.

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PUBLICATION II

Zinc finger transcription factor Casz1 expression is regulated by homeodomain transcription factor Prrxl1 in embryonic spinal dorsal horn late born excitatory interneurons

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Zinc finger transcription factor Casz1 expression is regulated by homeodomain transcription factor Prrxl1 in embryonic spinal dorsal horn late-born excitatory interneurons

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Abstract

The transcription factor Casz1 is required for proper assembly of vertebrate vasculature and heart morphogenesis as well as for temporal control of *Drosophila* neuroblasts and mouse retina progenitors in the generation of different cell types. Although Casz1 function in the mammalian nervous system remains largely unexplored, Casz1 is expressed in several regions of this system. Here we provide a detailed spatiotemporal characterization of Casz1 expression along mouse dorsal root ganglion (DRG) and dorsal spinal cord development by immunochemistry. In the DRG, Casz1 is broadly expressed in sensory neurons since they are born until perinatal age. In the dorsal spinal cord, Casz1 displays a more dynamic pattern being first expressed in dorsal interneuron 1 (dI1) progenitors and their derived neurons and then in a large subset of embryonic dorsal late-born excitatory (dILB) neurons that narrows gradually to become restricted perinatally to the inner portion. Strikingly, expression analyses using *Prrxl1*-knockout mice revealed that Prrxl1, a key transcription factor in the differentiation of dILB neurons, is a positive regulator of Casz1 expression in the embryonic dorsal spinal cord but not in the DRG. By performing chromatin immunoprecipitation in the dorsal spinal cord, we identified two Prrxl1-bound regions within *Casz1* introns, suggesting that Prrxl1 directly regulates *Casz1* transcription. Our work reveals that *Casz1* lies downstream of Prrxl1 in the differentiation pathway of a large subset of dILB neurons and provides a framework for further studies of Casz1 in assembly of the DRG–spinal circuit.

Introduction

Proper perception of internal and external stimuli requires appropriate connections between peripheral afferents and their target neurons in the dorsal spinal cord. The correct wiring of such circuitries relies on the interplay between environmental cues and cell-intrinsic information along development. Cell-intrinsic genetic programmes are controlled by the highly orchestrated action of transcription factors, which contribute to the generation of the neuronal diversity and connectivity required to receive, modulate and convey multiple types of sensory information (Caspary & Anderson, 2003; Helms & Johnson, 2003; Marmigere & Ernfors, 2007). The expression of basic helix–loop–helix (bHLH) and homeodomain family transcription factors segregates the dorsal root ganglion (DRG) neurons into their specific sensory modalities and shapes the dorsal spinal cord into its laminated cellular pattern (Caspary & Anderson, 2003; Liu & Ma, 2011). Understanding the mechanisms behind neuronal diversity generation and the establishment of proper synaptic connections is a current challenge, in particular the molecular mechanisms underlying the specification processes.

In mouse, sensory neurons arise from a subpopulation of neural crest cells, expressing Sox10, in three successive waves of neurogenesis (Marmigere & Ernfors, 2007; Liu & Ma, 2011). As proliferating precursors become post-mitotic, they start to express Islet1 (Isl1) and Brn3a, which are required for sensory subtype specification (Sun *et al.*, 2008; Lanier *et al.*, 2009; Dykes *et al.*, 2011). The first wave of neurogenesis occurs between embryonic day (E) 9.5 and E11.5, generates mainly Aβ/Aδ mechanoreceptive and Aβ proprioceptive populations and is driven by the bHLH neurogenin 2 (Ngn2) (Ma *et al.*, 1999). These are defined by the expression of tyrosine kinase receptor B (TrkB) and TrkC, respectively, while the tyrosine kinase receptor c-Ret is expressed in subsets of both populations (Ma *et al.*, 1999; Marmigere & Ernfors, 2007; Lallemend & Ernfors, 2012). About 5% of the neurons generated in the first wave

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of neurogenesis express TrkA, and give rise to lightly myelinated Aδ sensory neurons (Bachy *et al.*, 2011). Around E10, Ngn1 drives the second wave of neurogenesis, generating mostly small-diameter TrkA-expressing neurons (Ma *et al.*, 1999). The third wave of neurogenesis starts at E11.5 from migrating and differentiating Krox20$^+$ boundary cap cells that differentiate into TrkA$^+$ nociceptive neurons (Maro *et al.*, 2004; Hjerling-Leffler *et al.*, 2005).

Dorsal spinal cord neurons are generated from progenitor domains, located in the ventricular zone of the neural tube, in two neurogenic waves between E10 and E14 (Gross *et al.*, 2002; Müller *et al.*, 2002; Helms & Johnson, 2003). These progenitor domains are identified by the expression of different sets of transcription factors, mainly from bHLH and homeodomain classes (Müller *et al.*, 2002; Caspary & Anderson, 2003; Helms & Johnson, 2003). The first wave of neurogenesis takes place at E10–11.5 and generates six subpopulations of early−born dorsal interneurons (dI1–6) that will populate the deep dorsal horn. In the second wave (E12–14.5), two late−born neuron populations (dILA and dILB) arise, both expressing Lbx1, which will migrate towards the superficial laminae of the dorsal horn (Gross *et al.*, 2002; Müller *et al.*, 2002). dILA neurons express Lhx1/5, Pax2 and Ptf1a, which are necessary for the differentiation of GABAergic and glycinergic inhibitory neurons (Glasgow *et al.*, 2005; Huang *et al.*, 2008; Chang *et al.*, 2013; Hanotel *et al.*, 2014). The dILB population is characterized by the expression of Prrxl1, Tlx3 and Lmx1b, which are required for differentiation of glutamatergic excitatory neurons (Qian *et al.*, 2002; Ding *et al.*, 2004; Rebelo *et al.*, 2010).

Prrxl1 encodes a paired-like homeodomain transcription factor specifically expressed in developing nociceptors and their putative synaptic targets in the dorsal spinal cord (Chen *et al.*, 2001; Rebelo *et al.*, 2007). Analysis of *Prrxl1*-knockout mouse embryos revealed several developmental abnormalities in the nociceptive DRG–dorsal spinal cord circuitry, namely misguided primary afferent ingrowth into dorsal spinal cord grey matter, abnormal migration of newborn superficial dorsal horn neurons followed, at E17.5, by loss of ~70% of the glutamatergic population and postnatal loss of ~30% of DRG nociceptors (Chen *et al.*, 2001; Rebelo *et al.*, 2006, 2010). These defects ultimately lead to a diminished response to noxious stimuli with sensorimotor function unaltered (Chen *et al.*, 2001). Given the multiple abnormalities affecting, in particular, nociceptive differentiation, Prrxl1 is thought to be implicated in the orchestration of a complex, still poorly understood genetic programme of the DRG and dorsal spinal cord nociceptive circuit.

Casz1 is an evolutionarily conserved C2H2 zinc-finger transcription factor required for heart morphogenesis and blood vessel assembly in vertebrates (Christinel & Conlon, 2008; Charpentier *et al.*, 2013; Amin *et al.*, 2014; Liu *et al.*, 2014). Global *Casz1*-knockout mice show abnormal heart development and mimic features of the human 1p36 deletion syndrome, including non-compaction cardiomyopathy and a ventricular septal defect (Liu *et al.*, 2014; Dorr *et al.*, 2015). Human mutations in the *Casz1* locus are significantly associated with hypertension (Kato *et al.*, 2011; Simino *et al.*, 2014; Lu *et al.*, 2015). Moreover, loss of Casz1 is associated with neuroblastoma poor prognosis, whereas restoration of Casz1 function suppresses tumorigenicity by triggering cell cycle exit and promoting some aspects of the differentiation process (Liu *et al.*, 2011b, 2013; Virden *et al.*, 2012). *Drosophila castor*, the fly orthologue of vertebrate *Casz1*, regulates temporal cell fate acquisition within neuroblast cell lineages (Mellerick *et al.*, 1992; Kambadur *et al.*, 1998; Isshiki *et al.*, 2001). In mouse retinal progenitors, Casz1 is also implicated in the regulation of temporal progression through a conserved transcriptional cascade as in fly neuroblasts (Mattar *et al.*, 2015). It was also shown that Casz1 is expressed in mouse DRG and spinal cord (Amin *et al.*, 2014; Liu *et al.*, 2014; Mattar *et al.*, 2015), although neither its spatiotemporal expression pattern nor its function has been addressed. In the present study, we characterize neuronal populations expressing Casz1 along mouse DRG and dorsal spinal cord development. In addition, we reveal that Casz1 expression is regulated by Prrxl1 in the embryonic dorsal spinal cord, but not in the DRG.

Materials and methods

Mouse strains and tissue preparation

CD1 *Prrxl1*-knockout mice (Chen *et al.*, 2001) and the NMRI mouse strain were bred and housed at the I3S animal facility, under temperature- and light-controlled conditions. E0.5 was considered to be the midday of the vaginal plug. Pregnant females were subjected to isoflurane anaesthesia, killed by cervical dislocation and embryos were collected at different developmental stages. For postnatal stages (i.e. P7 and P14), animals were anaesthetized with 50 mg/kg of pentobarbital sodium (Eutasil) and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). When necessary, DRG and dorsal spinal cord were dissected for downstream analysis. For immunofluorescence, previously fixed embryos, postnatal DRGs and spinal cords were incubated overnight in 30% sucrose in PBS and embedded in OCT (Surgipath). All tissues were sectioned at 12 μm on a cryostat (Leica) and collected on Superfrost Plus microscope slides (Menzel-Glaser). Experiments were carried out in agreement with European Community Council Directive (2010/63/EU) and the animal ethics guidelines of the I3S, and were approved by the Portuguese Veterinary Ethics Committee.

Histological analysis

Frozen tissue sections were air-dried, rinsed in 0.1 M PBS and, when necessary, antigens were retrieved by microwaving in 10 mM citrate buffer (pH 3.0 or 6.0) for 15 min, permeabilized in 0.1 M PBS with 0.1% Triton X-100 (T) and incubated in blocking buffer [4% fetal bovine serum (FBS) and 1% bovine serum albumin (BSA) in 0.1 M PBS-T] for 1 h at 37 °C in a moist chamber. Incubation with primary antibody at the appropriate dilution in blocking buffer was performed overnight at 4 °C in a humidified chamber. Primary antibodies were rabbit anti-Prrxl1 (1 : 1000 dilution) generated by us (Rebelo *et al.*, 2007), rabbit and guinea pig anti-Casz1 (Atlas antibodies and a gift from Johan Erickson from Karolinska Institute, respectively; 1 : 200), rabbit anti-TrkA (Millipore, 1 : 1000), goat anti-TrkB (AF1494, 1 : 500), TrkC (AF1404, 1 : 500) and c-Ret (AF482, 1 : 500) from R&D Biosystems, mouse anti-Lhx1/5 (4F2, 1 : 100) and anti-Islet1 (40.2D6, 1 : 100) from Developmental Studies Hybridoma Bank, goat anti-Ngn1 from Santa Cruz (A-20, 1 : 50), rabbit anti-Atoh1 (1 : 200) (gift from Jane Johnson, University of Texas, USA), guinea-pig anti-Lbx1, Tlx3 and Lmx1b (1 : 1000) (gifts from Thomas Müller, Max-Delbrück Molecular Medicine Center, Germany), and guinea-pig anti-Sox10 (1 : 500) (gift from Christian Schmitt, Erlangen-Nurnberg University, Germany). On parallel control tissue sections, primary antibody was omitted and resulted in the complete absence of staining. The antigen signal was detected by Alexa-conjugated secondary antibodies (Invitrogen).

For double *in situ* hybridization/immunofluorescence, cryosections were air-dried, washed in PBS–0.1% diethylpyrocarbonate, post-fixed in 4% paraformaldehyde in PBS-diethylpyrocarbonate for 15 min, treated with 100 mM acetylated triethanolamine pH 8.0 for

15 min and pre-hybridized with hybridization buffer [50% formamide, 1 × Denhart's solution (Sigma), 500 μg/mL salmon sperm DNA (Sigma), 250 μg/mL yeast RNA (Sigma), 750 mM NaCl and 75 mM sodium acetate] for 1 h at 60 °C. Hybridizations were performed with a 1 μg/mL digoxigenin (Roche)-labelled *Barhl2* probe (gift from Jane Johnson, University of Texas, USA) in hybridization buffer and incubated overnight at 60 °C. Non-specific hybridization of riboprobe was removed by two successive washes in washing buffer (50% formamide, 300 mM NaCl, 30 mM sodium acetate and 0.1% Tween-20) for 1 h at 60 °C. For immunodetection of hybridized riboprobes, sections were incubated with a goat anti-digoxigenin antibody coupled to alkaline phosphatase (Roche; 1 : 2000) overnight at 4 °C. Signal revelation was performed using nitro blue tetrazolium and chloride-5-bromo-4-chloro-3-indoly phosphate (NBT-BCIP) substrates (Sigma) diluted in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20) until the appearance of the desired signal. Afterwards, sections were immunostained with guinea pig anti-Casz1 antibody using the procedure described above. Fluorescent samples were captured with a Zeiss Z1 Apotome microscope. The bright-field images of *in situ* hybridization signals were converted into pseudo-green fluorescent colour and then merged with the fluorescent images.

To quantify the co-localization between Casz1 and different molecular markers, we analysed three to six DRG and alternate dorsal spinal cord lumbar transverse sections (L2–L6) from two to four mice. Casz1 expression displayed either a full or punctiform nuclear staining pattern. When punctiform, we considered a cell immunoreactive for Casz1 if at least two dots were present. For each section analysed, we manually counted immunoreactive cells in the green and red channels, and double-stained cells in merged channels, using IMAGEJ open source software (http://imagej.nih.gov/ij/). Results are shown as mean percentage (%) ± standard deviations (SD).

Chromatin immunoprecipitation

For Prrxl1, chromatin immunoprecipitation (ChIP) assays were performed as previously described (Monteiro *et al.*, 2014). Briefly, chromatin samples were extracted from about 12 dissected E14.5 mouse dorsal spinal cords and fixed with 2 mM di(*N*-succinimidyl) glutarate (Sigma) in 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 rabbit polyclonal anti-Prrxl1 antibody (Rebelo *et al.*, 2007) were performed using 80 μg of chromatin per assay in ChIP buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 5 mg/mL BSA) and protease inhibitor cocktail (Roche). Immunoprecipitates were retrieved with 50 μL 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, 500 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% NP-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) treatment for 2 h at 42 °C, and reverse cross-linked overnight at 65 °C. Immunoprecipitated and input DNA samples were purified by phenol-chloroform extractions followed by isopropanol precipitation. For quantitative PCR (qPCR), sets of primers, designed using PRIMER3 software (http://biotools.umassmed.edu), were used for assessing ChIP enrichment. The primer sequences are listed in Table 1. *Dscaml1* (ORF) and *Casz1* (+ 134 655) exonic regions were used as negative controls. ChIP-qPCR results are shown as the mean of triplicates ± SD of three independent experiments.

RNA extraction and real-time qPCR

Total RNA from E14.5 DRGs and dorsal spinal cords was isolated using GenElute Mammalian Total RNA Miniprep kit (Sigma), quantified in Nanodrop (Thermo Scientific) and verified for integrity using an Agilent 2100 Bioanalyzer. cDNA was prepared using oligo-dT primers (NZYTech, Lisbon, Portugal) and Tetro reverse transcriptase (Bioline) according to the manufacturer's instructions. A control containing all reagents except the reverse transcriptase enzyme was included (minus RT control) to assess potential residual genomic DNA in the RNA samples. Real-time qPCR analysis was performed using the Maxima SYBR Green/ROX master mix (Thermo Scientific) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Each primer pair was separated by at least one intron and designed, using PRIMER3 software, to amplify *Casz1*, *Casz1a*, *Casz1b*, *Npy1r* and *Grp* transcripts (see primers in Table 1). Expression of *Casz1* transcript variants was normalized to the endogenous control gene encoding hypoxanthine guanine phosphoribosyl transferase (*Hprt*), while *Npy1R* and *Grp* transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Molecular sizes of each PCR amplicon were confirmed by agarose gel electrophoresis. Results are shown as the mean of triplicates ± SD of three independent experiments.

TABLE 1. Primers used to perform ChIP–qPCR and reverse transcriptase qPCR

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
Casz1 + 55 905	ATTACCGCCGTCTTTTCATC	GACGCAAGGGAGCTTCTATC
Casz1 + 117 505	CCGGAGTGGGGAGTTTTAT	TGATTTGGGAGCATTTCATC
Casz1 + 134 655	AGCCTCCCAATAGCAAGATG	GAGGGAAATAGGGTGTGGTG
ORF	GTGTCCCCAGACAAAGGAAG	AGGAGCTGTCCGAGATGAAG
Casz1	GGATTGCCCACAGATAAACC	TCCAGGCAGTGATAGTGCTC
Casz1a	GTGCGAGACCAGTTTGCTTA	TGGAAGTTTGGTGGTGATGT
Casz1b	GCGCATTTCCATTGTGTAGT	GACCCCCATACCTAGCTCAC
Npy1r	CGGCGTTCAAGGACAAGTAT	TGATTCGCTTGGTCTCACTG
Grp	CACGGTCCTGGCTAAGCT	ATCCCTTGCAGCTTCTTCC
Hprt	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA
Gapdh	GCATGGACTGTGGTCCTCAG	CCATCACCATCTTCCAGGAG

Statistical analysis

Group values, expressed as the mean ± SD, were compared by Student's *t*-test, and *P*-values of less than 0.05 were considered significant.

Results

Casz1 is expressed throughout mouse DRG and dorsal spinal cord development

Casz1 spatiotemporal expression pattern was characterized by immunofluorescence using antibodies that recognize both Casz1a/b isoforms. In the DRG, Casz1 expression was first detected at E10.5. At E13.5, when sensory neurogenesis is finishing, Casz1 was broadly expressed, a pattern that was maintained throughout DRG embryonic development and, at P14, Casz1 expression was nearly absent (Fig. 1). In the spinal cord, Casz1 expression onset occurred dorsally at E10.5 and was maintained until E11.5. At E13.5, Casz1 was expressed again in a small subset of migrating cells, which expanded to a larger subset of neurons occupying the superficial laminae by E14.5–15.5. From E15.5 until P7, Casz1 expression gradually became restricted to a narrow layer in its deeper portion (Fig. 1). At P14, Casz1 expression ceased in the dorsal horn (Fig. 1). Casz1 protein was also detected in a small group of cells located ventrally at E10.5, which expression was maintained until P7 (Fig. 1 and data not shown). Notably, co-staining with DAPI in both DRG and spinal cord neurons showed that Casz1 expression is located in specific domains within the nucleus (Fig. 1), which is in accordance with previous observations (Dorr *et al.*, 2015; Mattar *et al.*, 2015). Taken together, the data show that Casz1 is broadly expressed in the DRG as soon as sensory neurons are generated up to P14, while in the dorsal spinal cord it displays two non-overlapping phases of transient and restricted expression.

Characterization of Casz1-expressing neurons in developing DRG

As Casz1 expression was detected during early DRG neurogenesis, we questioned whether Casz1 is expressed in DRG progenitors and/or in post-mitotic neurons. Casz1 immunofluorescence analysis at E10.5, 11.5 and 12.5 showed a mutually exclusive expression pattern with progenitor marker Sox10 and a high level of co-localization with early pan-sensory marker Isl1 [82% (± 10.5), 92% (± 4.1) and 94% (± 1.8) at E10.5, E11.5 and E12.5, respectively)] (Fig. 2A and C). At E14.5, the expression of tyrosine kinase receptors is restricted to specific neuron lineages conveying different types of sensory information (Lallemend & Ernfors, 2012). To define the identity of Casz1^{+} neurons in developing DRG, we stained DRG tissues with

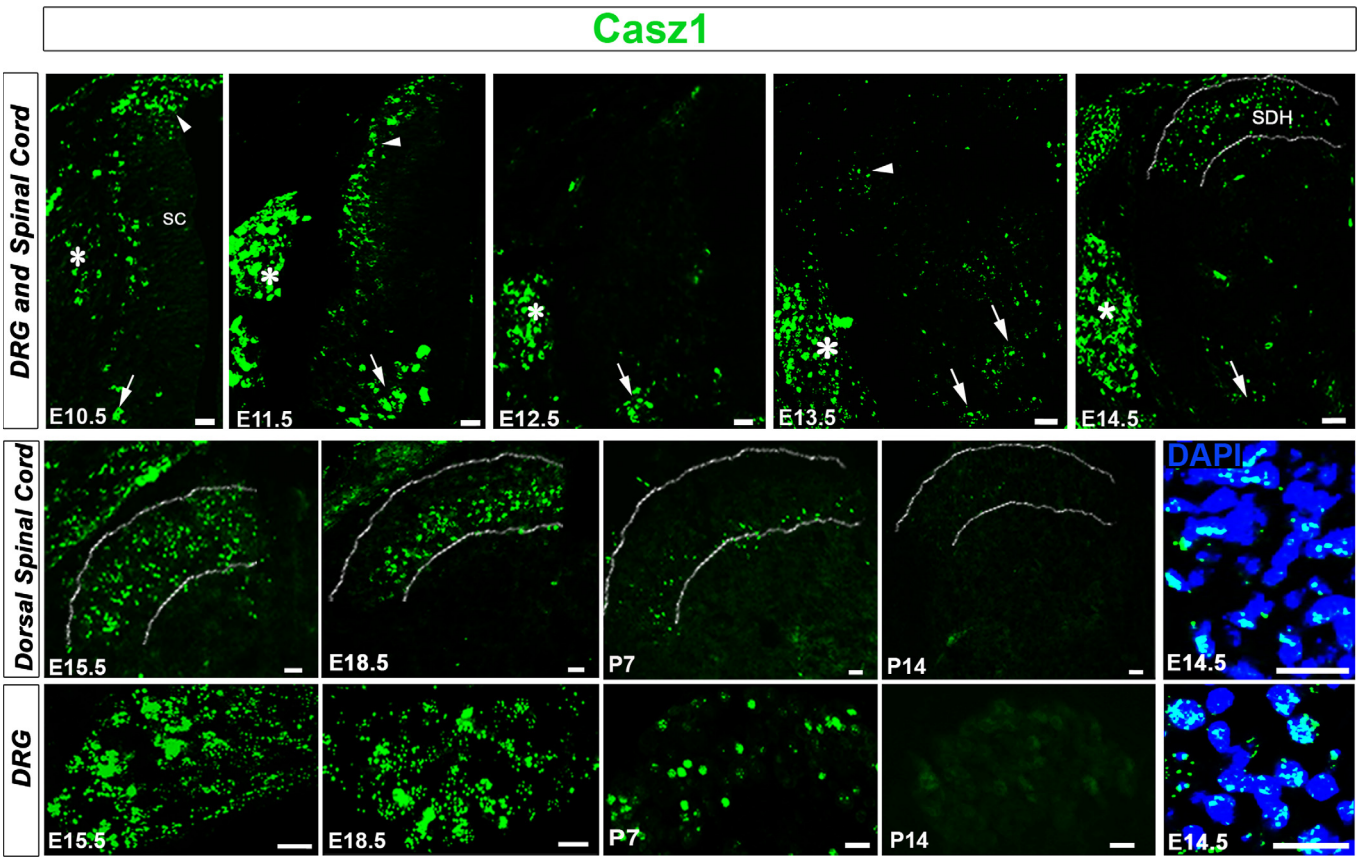

FIG. 1. Time course of expression of Casz1 in mouse developing DRG and dorsal spinal cord. In the mouse DRG (*), Casz1 protein expression onset occurs at E10.5 and peaks around E13.5 after the neurogenic waves. Afterwards, Casz1 expression level is sustained and diminishing after birth, being extinguished by P14. In the ventral spinal cord (arrows), Casz1 expression is detected. In the dorsal spinal cord (arrowheads), a first phase of Casz1 expression is observed from E10.5 to E11.5. In a second phase, Casz1 expression initiates at E13.5 and peaks at E14.5–15.5 in the superficial laminae. From E18.5 to P7, Casz1 expression starts to gradually silence from outer to inner layers of the superficial dorsal horn (SDH), until expression is extinguished at P14. Casz1 protein is located in defined regions within the nucleus, as shown by the co-localization with DAPI, presenting a punctate staining pattern in both DRG and dorsal spinal cord neurons. SDH (from E14.5 to P14) is delimited with a continuous line. SC, spinal cord. Scale bar = 25 μm.

antibodies against Casz1 and the sensory markers TrkA, TrkB, TrkC and c-Ret at embryonic (E14.5) and early postnatal (P7) stages. Casz1 was expressed in all sensory populations at E14.5 and P7. At E14.5, Casz1 was detected in 92% (± 3.0), 100%, 87% (± 7.8) and 100% of the TrkA$^+$-, TrkB$^+$-, TrkC$^+$- and c-Ret$^+$-neurons, respectively. Similarly, at P7, Casz1 was detected in 95% (± 5.6), 90% (± 10.1), 81% (±7.1) and 93% (± 9.3) of the TrkA$^+$-, TrkB$^+$-, TrkC$^+$- and c-Ret$^+$-neurons, respectively (Fig. 2B and D). As in early DRG neurogenic stages (E10.5–12.5), Casz1 was expressed in the majority of Isl1$^+$ cells at E14.5 [88% (± 7.6)] and P7 [89% (± 4.0)] (Fig. 2B and D). These results indicate that Casz1 presents a pan-sensory expression pattern in early sensory neurons and is kept in the majority of DRG neurons after neurogenesis, a pattern similar to that of the sensory marker Isl1 (Sun *et al.*, 2008).

Characterization of Casz1-expressing neurons in developing dorsal spinal cord

To define Casz1$^+$ cells in dorsal spinal cord throughout development, we performed Casz1 co-localizations with dorsal spinal cord population markers specifically expressed during and after neurogenic waves. At E10.5–11.5, Casz1 co-localized with Atoh1 but not with Ngn1 (Fig. 3A–F), which suggests Casz1 is expressed in dI1 progenitors (dP1). By E11.5, Casz1 is expressed in ventrally migrating *Barhl2*$^+$ cells, which labels post-mitotic neurons from the dP1. As Casz1 expression from E13.5 onwards was observed in the superficial dorsal horn, we reasoned these Casz1$^+$ neurons were born during the second wave of neurogenesis. We therefore analysed at E14.5 the expression of Lbx1, a marker of late-born dorsal interneurons (dIL), in Casz1-expressing neurons and observed 46% (± 8.2) of co-localization (Fig. 3G and Q). We next determined whether Casz1 is expressed in dILA and/or dILB neurons. Casz1 virtually did not co-express with Lhx1/5, a dILA marker (Fig. 3H and R), but extensively co-expressed with Lmx1b, Tlx3 and Prrxl1 [93% (± 4.0), 69% (± 5.9) and 82 (± 4.6), respectively], which label dILB neurons (Fig. 3I–K and S–U), suggesting that Casz1 is exclusively expressed in excitatory dorsal horn neurons. Early postnatally (P7), Casz1 expression was restricted to a narrow, more ventral layer within the dorsal horn. As at this developmental stage dorsal spinal cord laminae are well defined, we sought to identify the laminar localization of Casz1-expressing neurons. We found that the majority of Casz1$^+$ neurons co-expressed Lbx1 (expressed in lamina III), Lmx1b and Prrxl1 [both expressed in laminae I–III (Del Barrio *et al.*, 2013)] [94% (± 2.9), 98% (± 3.9) and 78% (± 8.0), respectively) (Fig. 3L,N,P,Q,S and U), but only a very small subset of 6% (± 3.0) expressed Tlx3 [expressed in laminae I–II (Del Barrio *et al.*, 2013)] (Fig. 3O and T). In addition, virtually none of the Casz1$^+$ neurons expressed Lhx1/5 [1% (± 0.7)] (Fig. 3M and R). Thus, at P7, Casz1 is expressed in glutamatergic neurons mostly located within lamina III. In summary, Casz1 expression in dorsal spinal cord is transient and restricted to dI1 progenitors and derived neurons during the first wave of neurogenesis, and to a subpopulation of differentiating dILB neurons, which gradually loses Casz1 expression dorso-ventrally so that, at P7, they are mostly restricted to lamina III.

Casz1 expression is regulated by Prrxl1 in embryonic dorsal spinal cord

Given that Prrxl1 expression in dILB neurons precedes Casz1 and both factors extensively co-express at E14.5, we investigated whether Casz1 expression could be under regulation of Prrxl1. We used a global expression profiling data set that we had previously generated from wild-type and *Prrxl1*-knockout mice embryonic DRG and dorsal spinal cord (F. A. Monteiro, unpubl. data). Both *Casz1a* [fold-change (FC) = 1.20, P = 1.30E-02] and *Casz1b* (FC = 1.50, P = 1.14E-06) transcripts were downregulated in E14.5 dorsal spinal cord of *Prrxl1*-knockout as compared to wild-type mice, while in the DRG no differences were observed in both transcripts (*Casz1a*, FC = 1.01 and P = 8.91E-01; *Casz1b*, FC = 1.03 and P = 7.57E-01), albeit also there Prrxl1 extensively co-expresses with Casz1 (Fig. 2B and D). Quantitative real-time PCR experiments confirmed downregulation of *Casz1* transcripts only in embryonic dorsal spinal cords of *Prrxl1* knockouts (Fig. 4A). These results were validated by immunostaining using antibodies against Casz1 (Fig. 4B). Note that the reduction in Casz1 expression cannot be attributed to abnormal cell death in the dorsal spinal cord of *Prrxl1* knockouts, as increased apoptosis was shown only to occur at E17.5 (Chen *et al.*, 2001; Rebelo *et al.*, 2010). These results thus demonstrate that Prrxl1 is a positive regulator of *Casz1* expression in the embryonic dorsal spinal cord but not in the DRG.

As at P7, Prrxl1 and Casz1 expression domain overlaps in lamina III, we investigated whether Casz1 would still be under the control of Prrxl1. No differences were observed in either expression levels or number of cells expressing Casz1 between *Prrxl1*-knockout and wild-type mice dorsal spinal cord (Fig. 4C and D) and DRG tissues (data not shown). This result indicates that Prrxl1 does not regulate Casz1 in this subset of lamina III neurons that maintain Casz1 expression until early postnatal life.

To assess whether Casz1 may be directly regulated by Prrxl1, we searched for Prrxl1 binding sites in the genomic locus of *Casz1* using a previously generated Prrxl1 ChIP-seq data set from E14.5 dorsal spinal cord (F. A. Monteiro, unpubl. data). Peak calling identified two Prrxl1-bound regions located at evolutionary conserved intronic sequences (Fig. 4E, asterisks). Enrichment of *Casz1* intronic regions (positions + 55 905 and + 117 505) was confirmed in Prrxl1 ChIP-qPCR assays. These data suggest that Prrxl1 directly controls *Casz1* expression in a large subset of dLB neurons during dorsal spinal cord development.

To gain new insight into the molecular control of dorsal horn neuron differentiation, we searched in our comprehensive list of Prrxl1 target genes identified in dorsal spinal cord for genes that are controlled by Casz1 in other systems, namely genes identified in Casz1-inducible neuroblastoma cell lines (Liu *et al.*, 2011b) and *Casz1*$^{-/-}$ embryonic mouse hearts (Liu *et al.*, 2014) through microarray expression profiling. From the intersection of the data sets (using FC > 1.3 and P < 5.0E-02 as cut-offs), we found solely the target gene neuropeptide Y receptor Y1 (*Npy1r*), which was shown previously to be mainly expressed in dILB neurons (Guo *et al.*, 2012). We found that *Npy1r* was downregulated (FC = −1.63, P = 1.65E-06) in the dorsal spinal cord of *Prrxl1* knockouts. By reverse transcriptase qPCR experiments, we confirmed decreased expression of *Npy1r* transcript in embryonic dorsal spinal cords of *Prrxl1* knockouts (Fig. 5). The neuropeptide *Grp* was used as a positive control, as *Grp* expression was previously shown to be markedly decreased in *Prrxl1* knockouts (Li *et al.*, 2006). Thus, using microarray analysis and real-time qPCR, we identified the *Npy1r* gene, which is enriched in dILB neurons, to be a target of Prrxl1, a regulatory pathway possibly mediated by Casz1.

Discussion

In the present study, we characterized the expression pattern of Casz1 in mouse developing DRG and dorsal spinal cord. The data point to an important role of Casz1 in the morphogenesis of both

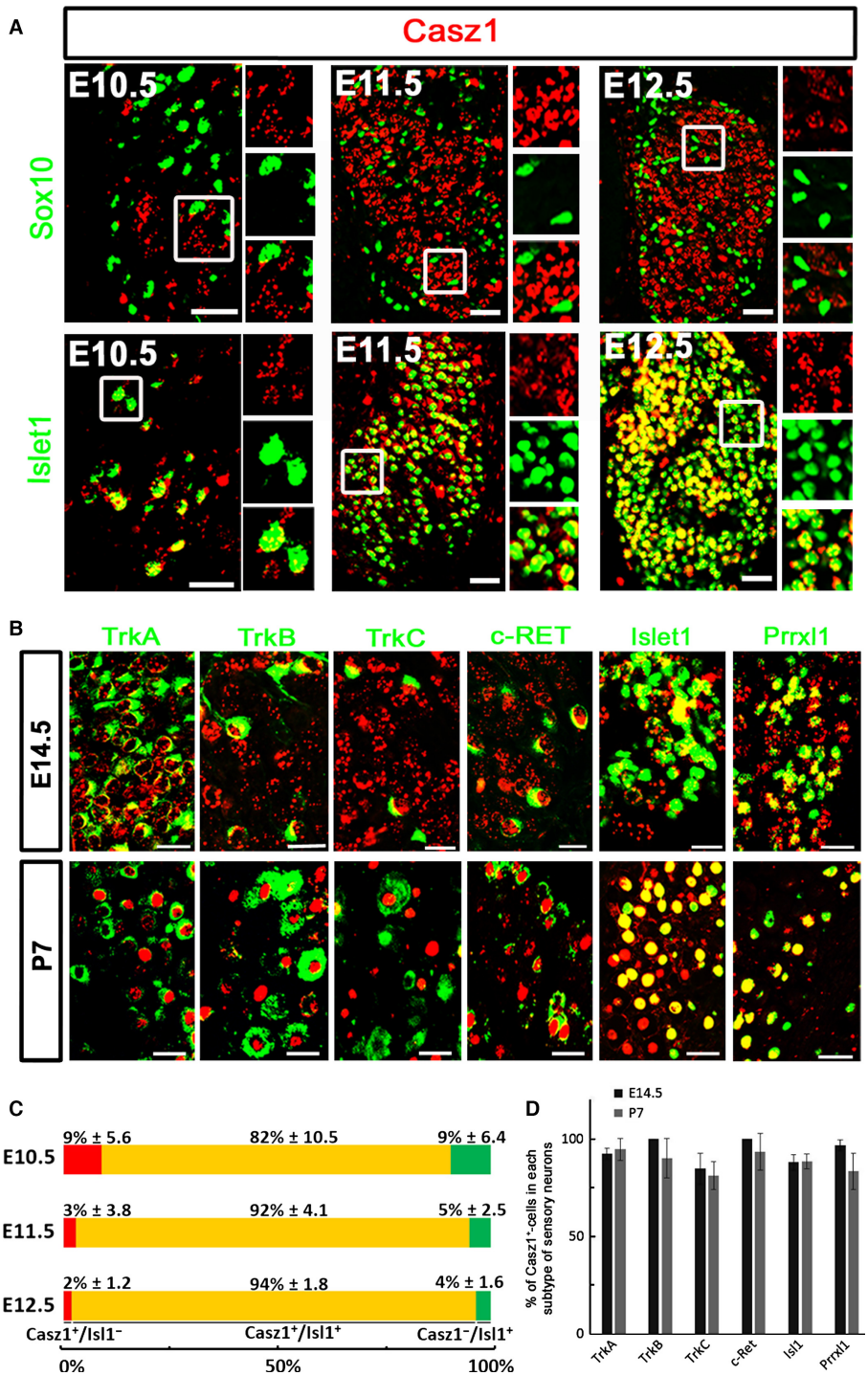

FIG. 2. Casz1 is broadly expressed in DRG sensory neurons. Immunohistochemical analysis of E10.5, E11.5, E12.5 (A), E14.5 and P7 (B) lumbar DRG. (A) Casz1 co-expresses with the pan-sensory marker Isl1 but never with the DRG progenitor cell marker Sox10. Insets: a magnification of the white-boxed areas, where individual and merged fluorescence images are shown to better observe Casz1 punctate staining. (B) Casz1 broadly co-expresses with TrkA, TrkB, TrkC, c-Ret, Isl1 and Prrxl1. (C) Quantitative analysis (related to A) showing that the majority of Casz1$^+$ cells co-express Isl1. This quantification was performed using six tissue sections (n = 6) from two embryos at each developmental stage. The immunoreactive cell counts were as follows: at E10.5, 32 ± 10; at E11.5, 98 ± 35; and at E12.5, 134 ± 33. Data are represented as percentage ± SD of the cell populations Casz1$^+$/Isl1$^-$, Casz1$^+$/Isl1$^+$ and Casz1$^-$/Isl1$^+$ in the DRG at E10.5, E11.5 and E12.5. (D) Quantitative analysis (related to B) showing co-expression of Casz1 with different neurotrophic factor receptor types: TrkA (at E14.5, n = 6, two embryos; at P7, n = 30, four animals), TrkB (at E14.5, n = 11, three embryos; at P7, n = 13, three animals), TrkC (at E14.5, n = 12, four embryos; at P7, n = 3, two animals) and c-Ret (at E14.5, n = 7, three embryos; at P7, n = 12, three animals), and the transcription factors Isl1 (at E14.5, n = 11, three embryos; at P7, n = 8, two animals) and Prrxl1 (at E14.5, n = 10, three embryos; at P7, n = 6, two animals). The immunoreactive cell counts were as follows: at E14.5, TrkA$^+$ (85 ± 58), TrkB$^+$ (6 ± 3), TrkC$^+$ (9 ± 7), c-Ret$^+$ (10 ± 4), Isl1$^+$ (80 ± 25) and Prrxl1$^+$ (72 ± 35); at P7, TrkA$^+$ (39 ± 19), TrkB$^+$ (6 ± 2), TrkC$^+$ (10 ± 6), c-Ret$^+$ (24 ± 14), Isl1$^+$ (68 ± 41) and Prrxl1$^+$ (72 ± 35). Data are represented as percentage ± SD of cells that express Casz1 in each sensory subtype population analysed in the DRG at E14.5 and P7. Scale bar = 25 μm.

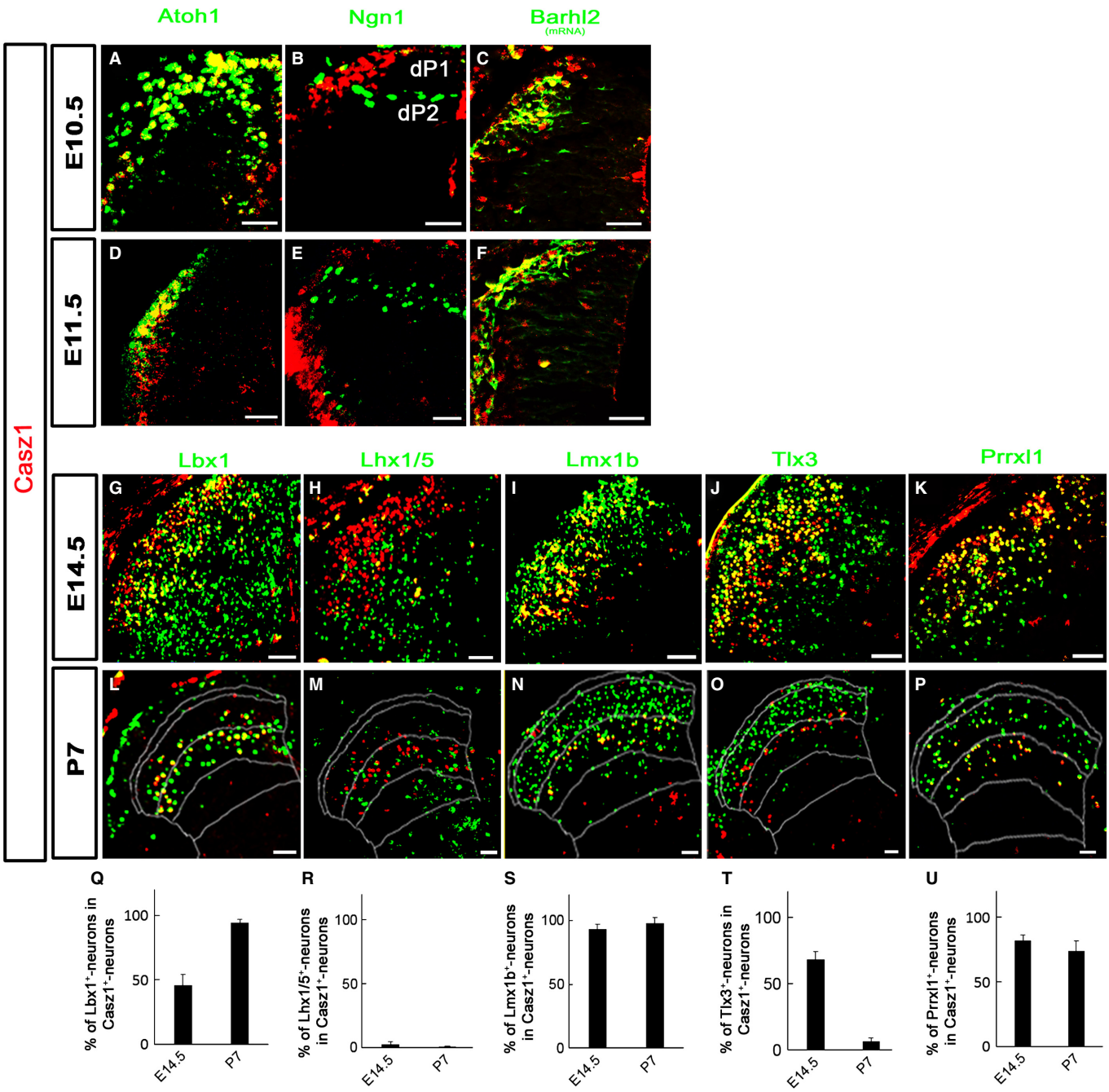

FIG. 3. Casz1 is expressed in early- and late-born subpopulations of dorsal spinal interneurons. Immunohistochemical analysis of E10.5 (A–C), E11.5 (D–F), E14.5 (G–K) and P7 (L–P) lumbar dorsal spinal cord. (A–F) Casz1 co-expresses with Atoh1 [marker of dI1 progenitors (dP1)] and *Barhl2* (marker of dI1 neurons) but not with Ngn1 [marker of dI2 progenitors (dP2)]. (G–K) Casz1 partially co-expresses with Lbx1 (marker of dIL neurons) but not with Lhx1/5 (marker of GABAergic neurons), and co-expresses with Lmx1b, Tlx3 and Prrxl1 (markers of glutamatergic neurons). (L–P) At P7, Casz1 co-expresses with Lbx1, Lmx1b and Prrxl1, but not with Lhx1/5 and little with Tlx3. (Q–U) Quantitative analysis showing co-expression of transcription factors Lbx1 [at E14.5, using nine tissue sections (n = 9) from three embryos; at P7, n = 18, four animals], Lhx1/5 (at E14.5, n = 12, three embryos; at P7, n = 9, three animals), Lmx1b (at E14.5, n = 8, two embryos; at P7, n = 17, four animals), Tlx3 (at E14.5, n = 13, three embryos; at P7, n = 12, three animals) and Prrxl1 (at E14.5, n = 10, three embryos; at P7, n = 12, three animals) with Casz1. The Casz1-immunoreactive cell counts were as follows: (Q) at E14.5 (131 ± 20) and at P7 (47 ± 12); (R) at E14.5 (82 ± 16) and at P7 (50 ± 16); (S) at E14.5 (94 ± 35) and at P7 (29 ± 9); (T) at E14.5 (111 ± 23) and at P7 (38 ± 10); and (U) at E14.5 (95 ± 27) and at P7 (39 ± 9). Data are represented as percentage ± SD of cells that express each transcription factor analysed in the Casz1$^+$ cell population in the dorsal horn at E14.5 and P7. Scale bar = 50 μm.

tissues. In the DRG, Casz1 is broadly expressed from early embryonic development to the perinatal stage, while in the dorsal spinal cord it is first expressed in dI1 progenitors and their derived neurons and then in a large subset of dILB neurons. In addition, we found that Prrxl1 positively regulates *Casz1* expression in a large subset of dILB spinal cord neurons. However, perinatally, Casz1 expression is only maintained in a narrow layer of cells mostly located within lamina III of the dorsal horn and is Prrxl1-independent.

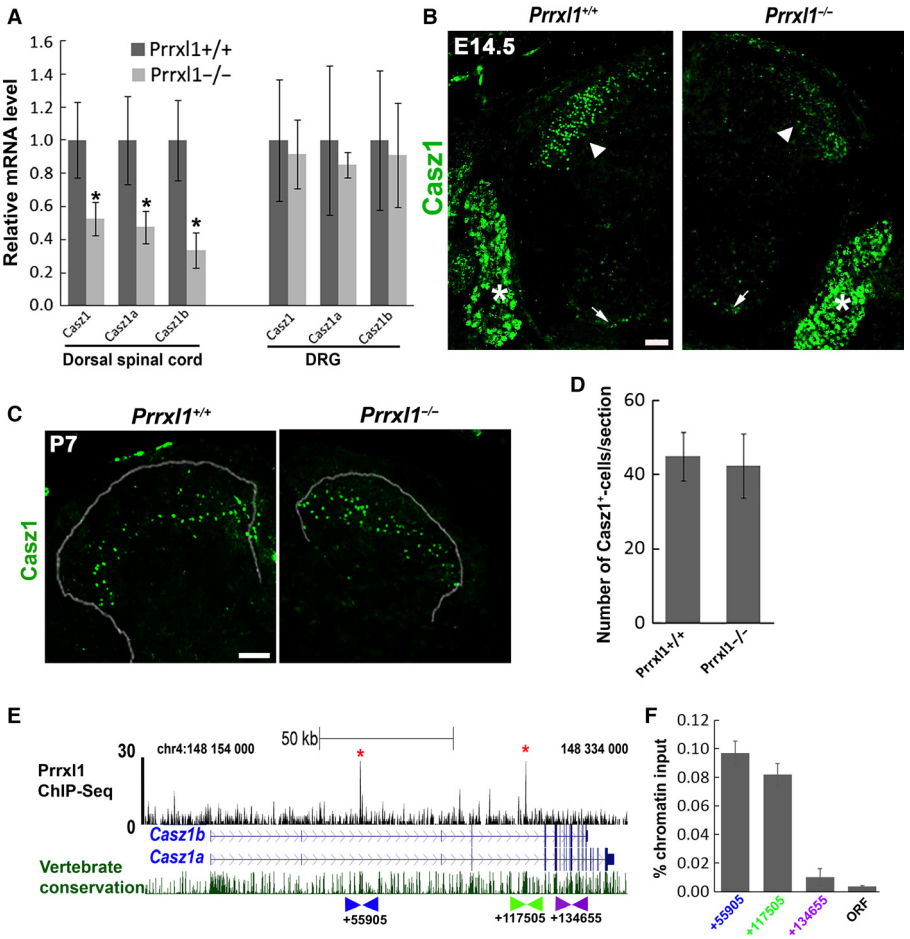

FIG. 4. Prrxl1 promotes Casz1 expression in embryonic dLB neurons. (A) *Casz1* transcripts are downregulated in dorsal spinal cord of E14.5 *Prrxl1*$^{-/-}$ embryos, but not in the DRG. Expression levels of both *Casz1* transcripts (Casz1) and either *Casz1a* or *Casz1b* were assessed by real time qPCR using E14.5 dorsal spinal cord and DRG tissues from wild-type (n = 3) and *Prrxl1* $^{-/-}$ (n = 3), being each n a pool of three embryos. *P < 0.0001, compared with control group. (B and C) Immunohistochemical analysis of E14.5 (B) and P7 (C) lumbar spinal cord. At E14.5, Casz1 protein expression levels are remarkably decreased in the dorsal spinal cord of *Prrxl1*$^{-/-}$ mouse but not in DRG or ventral spinal cord. At P7, Casz1 expression levels are similar in the dorsal spinal cord of *Prrxl1*$^{-/-}$ as compared with wild-type controls. (D) Graph showing similar numbers of Casz1-expressing neurons in the dorsal spinal cords of *Prrxl1*$^{-/-}$ (n = 3 animals) and wild-type controls (n = 3) at P7. (E) Prrxl1 binds to conserved intronic regions of *Casz1* in the dorsal spinal cord of E14.5 embryos. Dorsal spinal cord chromatins were previously immunoprecipitated with an anti-Prrxl1 antibody and subjected to next-generation sequencing (ChIP-seq). Prrxl1 binding profile (in black) within *Casz1* locus is shown. Casz1 gene structure and direction of transcription (in blue) and multispecies vertebrate conservation plot (in green) were obtained from the UCSC genome browser. Binding sites validated by ChIP-qPCR are labelled by red asterisks. These two binding sites fall into highly conserved regions within *Casz1* 2nd and 4th introns. Arrowheads represent the position of primers (+ 55 905, + 117 505 and + 134 655), relative to the transcription start site, used for ChIP-qPCR analysis. (F) Prrxl1 binding to *Casz1* intronic regions (+ 55 905 and + 117 505) is enriched. As negative controls, primers for *Casz1* (+ 134 655) and *Dscaml1* (ORF) exonic regions were used. Scale bar = 50 μm.

All newly born DRG neurons express the Brn3a and Isl1 homeodomain factors, which are necessary to suppress the expression of Ngn1/2 and to promote the expression of genes related to early sensory subtype specification (Sun *et al.*, 2008; Lanier *et al.*, 2009; Dykes *et al.*, 2011). We show that Casz1 also presents a pan-sensory expression pattern throughout DRG embryonic and perinatal development, as Casz1 extensively co-expresses with Isl1 and is maintained in the large majority of neurons of sensory lineages analysed (i.e. TrkA, TrkB, TrkC and c-Ret). This spatiotemporal window of expression suggests that Casz1 may control functions associated with both early and late aspects of differentiation of DRG neurons subserving nociception, mechanoreception and proprioception. Casz1 is not regulated by Prrxl1 in the DRG, as we show here, nor regulated by Pou4f1 or Isl1, as Casz1 deregulation was not observed in expression profiling studies using knockout DRGs for each or both homeobox genes (Dykes *et al.*, 2011). Thus, upstream regulators of *Casz1* in DRG sensory neurons still remain to be identified.

In the dorsal spinal cord, we show that Casz1 displays a more dynamic expression pattern. During the first wave of neurogenesis, Casz1 is expressed in Atoh1^{+} cells. Atoh1 is a bHLH transcription factor required for the specification and migration of proprioceptive commissural neurons forming the spinocerebellar tract (Helms & Johnson, 1998; Bermingham *et al.*, 2001; Gowan *et al.*, 2001). Atoh1 is expressed in both dI1 progenitors and their derivatives, although, similar to Casz1, its expression ceases by E12.5 (Helms & Johnson, 1998). Previously, it was proposed that Casz1 expression leads to the lengthening of cell cycle progression in neuroblastoma cells and cardiomyocytes and a subsequent decrease in cell proliferation (Liu *et al.*, 2013; Dorr *et al.*, 2015). It would be of interest to analyse whether Casz1 also controls proliferation of dI1 progenitors.

Immediately after the second wave of neurogenesis in the dorsal spinal cord, Casz1 expression is found in a large subset of dILB neurons occupying the entire superficial dorsal horn (presumptive laminae I–III), and then is gradually extinguished, from outer to

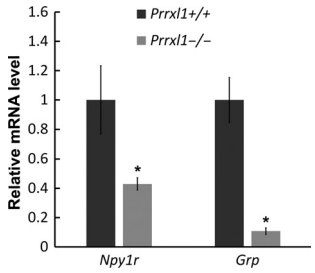

FIG. 5. Expression of *Npy1r* is decreased in embryonic dorsal horn of *Prrxl1*-knockout mice. Reverse transcriptase followed by qPCR was performed using RNA extracted from dorsal spinal cord tissue of wild-type ($n = 3$) and *Prrxl* $^{-/-}$ ($n = 3$) E14.5 embryos, being each n a pool of three embryos. $*P < 0.0002$, compared to wild-type group. *Grp* expression is known to be markedly decreased in dorsal spinal cord of *Prrxl1*-knockout embryos (Li *et al.*, 2006) and therefore was used as a positive control.

inner laminae, until it narrows down to a layer of neurons mostly within lamina III. This dynamic expression of Casz1 protein correlates well with the levels of *Casz1a* transcript along spinal cord development as previously assessed by real-time qPCR (Liu *et al.*, 2011a). Lamination of dorsal spinal cord neurons becomes apparent from E15.5 onwards (Ding *et al.*, 2004) and each lamina is responsible for receiving different types of sensory information. Lamina I and II neurons receive pruri-, thermo- and nociceptive inputs while lamina III neurons receive mostly mechanosensory information (Gillespie & Walker, 2001; Hunt & Mantyh, 2001; Julius & Basbaum, 2001). It is tempting to hypothesize that Casz1 spatiotemporal graded expression may be related to the somatosensory phenotype acquisition of the neurons composing laminae I–III. Despite the marked reduction in Casz1 expression levels in the embryonic dorsal spinal cord of *Prrxl1* knockouts, a small subset of cells maintains Casz1 expression unaltered. We provide two possible explanations: (i) a small subset of Casz1^{+} cells does not co-express Prrxl1 (Fig. 3K and U), and (ii) although both factors are coexpressed, Casz1 does not depend on Prrxl1. Our hypothesis is that on those cases other transcription factors control Casz1 expression. These factors may be Tlx3 and/or Lmx1b, which together with Prrxl1 define four subpopulations of superficial dorsal horn glutamatergic neurons (Rebelo *et al.*, 2010). In addition, the study of the respective knockout mice showed that these factors are functionally related regulating common target genes, such as neuropeptide Grp (Ding *et al.*, 2004; Li *et al.*, 2006). So far, the zinc finger transcription factors Ikzf1 is the only well-established regulator of *Casz1* transcription, acting as a repressor during mouse retinogenesis (Mattar *et al.*, 2015), an epistatic cascade similar to that of *hunchback* and *castor* in fly neuroblasts (Cleary & Doe, 2006). In non-neuronal tissues, upstream regulators of *Casz1* have not yet been reported. We found that Prrxl1 acts as a positive regulator of *Casz1* in embryonic dorsal spinal cord, an action most likely mediated by direct interaction with evolutionary conserved elements within *Casz1* introns 2 and 4. As Casz1 is downstream of Prrxl1 in a large subset of dILB neurons, it is likely that Casz1 function in dorsal spinal cord is related to Prrxl1. Analysis of the *Prrxl1*-knockout mice revealed that this factor is involved in some aspects of neuronal differentiation, such as migration and axon guidance (Chen *et al.*, 2001; Ding *et al.*, 2004). Regarding Casz1, previous studies using global gene expression analysis showed that Casz1 controls genes with roles in cell–cell adhesion and cell differentiation (Liu *et al.*, 2011b, 2014). In this regard, we show that *Npy1r*, a downstream gene of Casz1 in developing heart (Liu *et al.*, 2014), is regulated by Prrxl1 in the spinal cord dorsal horn. Thus, gene expression profiling studies in *Casz1*-knockout dILB neurons are needed to determine the overlap between the programmes of gene expression downstream of these factors.

Although Casz1 has been shown to have vital functions in heart and vasculature development (Charpentier *et al.*, 2013; Liu *et al.*, 2014), its function in the mammalian nervous system was recently addressed for the first time in retinogenesis (Mattar *et al.*, 2015). This work provides a detailed characterization of Casz1 expression in developing mouse DRG and dorsal spinal cord and demonstrates that Casz1 is transcriptionally regulated by Prrxl1, through direct binding, in embryonic dorsal spinal cord. Additional studies in *Casz1*-knockout mice are essential to unveil Casz1 function in both DRG and dorsal spinal cord neurons. However, recent studies reported that *Casz1*-knockout mice die during embryogenesis due to cardiovascular abnormalities (Liu *et al.*, 2014; Dorr *et al.*, 2015), implying the need to generate conditional knockout mice, specifically ablating Casz1 expression in either the DRG or the dorsal spinal cord.

Conflict of interest

The authors have no conflicts of interest to declare.

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Abbreviations

Atoh1, atonal homolog 1 (*Drosophila*); Barhl2, BarH-like 2 (*Drosophila*); bHLH, basic helix–loop–helix; Casz1, castor zinc finger 1; ChIP, chromatin immunoprecipitation; c-Ret, ret proto-oncogene; dI1, dorsal interneurons 1; dIL, dorsal late-born interneurons; DRG, dorsal root ganglion; E, embryonic day; Isl1, ISL1 transcription factor; Lmx1b, LIM homeobox transcription factor 1 beta; Ngn2, neurogenin 2; P, postnatal day; PBS, phosphate-buffered saline; Prrxl1, paired related homeobox protein-like 1; qPCR, quantitative PCR; Sox10, SRY (sex determining region Y)-box 10; Tlx3, T cell leukaemia, homeobox 3; Trk, tyrosine kinase receptor.

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Discussion

Prrxl1 is a paired-like HD transcription factor firstly recognized by (Saito et al. 1995) to be expressed in embryonic DRG and spinal cord dorsal horn. Further immunohistochemical studies showed that Prrxl1 expression in these tissues starts during early stages of development, at E10.5, and is maintained until shortly after birth (Rebelo et al. 2007; Rebelo et al. 2006; Chen et al. 2001a). Strikingly, Prrxl1 expression is claimed to be exclusive of differentiating nociceptive neurons, since its expression was never detected in other cell types, being limited to small size DRG and superficial dorsal horn laminae neurons and *Prrxl1* knockouts display marked nociceptive impairment (Chen et al., 2001; Rebelo et al., 2006; Rebelo et al. 2007). Research undertaken in our lab during the last decade clearly demonstrated that Prrxl1 is essential for proper assembly and maturation of the nociceptive system (Rebelo et al. 2010; Rebelo et al. 2006; Chen et al. 2001). Analysis of *Prrxl1*-null mice revealed spatial and temporally altered dorsal horn primary afferent innervation pattern (Chen et al., 2001), abnormal migration and programmed cell death of laminae I-III glutamatergic neurons (Chen et al., 2001; Rebelo et al., 2010), early postnatal death of about 1/3 small diameter primary afferents (Rebelo et al., 2006), and adult individuals exhibited decreased responsiveness to noxious stimuli. Proper development of spinal neurons implicated in pain and itch processing also requires other HD transcription factors, such as Lmx1b and Tlx3, which are co-expressed with Prrxl1 (Szabo et al. 2015; Ding et al. 2004; Xu et al. 2008; Rebelo et al. 2010; Chen et al. 2001)(Cheng *et al.*, 2004; Cheng *et al.*, 2005). Altered primary afferents spinal innervation pattern, similar to the one observed in *Prrxl1*$^{-/-}$ mice, is also observed in *Lmx1b* and *Tlx3* mutants, suggesting that both transcription factors are required to establish connections between peripheral and spinal nociceptive neurons (Qian *et al.*, 2002; Ding *et al.*, 2004; Xu *et al.*, 2008; Xu *et al.*, 2013; Szabo *et al.*, 2015). The combined action of Prrxl1, Lmx1b and Tlx3 is indeed necessary for the correct assembly of the nociceptive system in the superficial dorsal horn. Several mouse genetic studies suggest that Prrxl1, Lmx1b, and Tlx3 cooperate to drive migration, lamination, differentiation, axonal guidance, and specification of neurotransmitter phenotype in spinal dorsal horn neurons (Chen et al. 2001b; Rebelo et al. 2006; Rebelo et al. 2010; Szabo et al. 2015; Ding

et al. 2004; Xu et al. 2008; Cheng et al. 2004). Tlx3 also acts as a genetic switch that selects glutamatergic over a GABAergic neurotransmitter phenotype (Cheng et al. 2004; Cheng et al. 2005). Prrxl1 and Lmx1b appear to support Tlx3 in the regulation of neuropeptide expression in dorsal horn neurons and are required for the survival and phenotype maintenance of these neurons (Chen *et al.*, 2001; Ding *et al.*, 2004; Rebelo *et al.*, 2006; Rebelo *et al.*, 2010; Szabo *et al.*, 2015). Li and collaborators (2006) showed that Tlx3, Prrxl1 and Lmx1b are required for the expression of a structural gene (*Enc1*, neuropeptides (*Grp* and *Pcp4*) and a transient receptor potential cation channel (*Trpc3)*.

Although Prrxl1 functions may be inferred from studies in respective mutant mice, little was known about Prrxl1 downstream direct target genes in DRG and dorsal spinal cord. In the beginning of the present work, the only known Prrxl1 target gene was *Rgmb*, which encods an extracellular membrane-associated protein involved in axon guidance (Samad *et al.*, 2004). Using a ChIP-seq/expression microarray combined approach, a comprehensive list of putative Prrxl1-direct target genes was produced for both the dorsal spinal cord and the DRG (our own group unpublished results), which set up the basis for the work presented in this thesis.

Thus, the main goal of this thesis was therefore to deepen our knowledge on Prrxl1 transcriptional program in nociceptive neurons. In Publication I, we showed that Prrxl1 regulates its own expression through a repressive loop mechanism. In the Publication II, we demonstrated that Prrxl1 directly regulates Casz1 expression in dorsal spinal cord and characterized Casz1 spatio-temporal expression pattern in mouse developing DRG and spinal cord dorsal horn.

Mechanisms regulating Prrxl1 expression

During the course of the present work, studies from my colleagues provided important insight on the mechanisms that modulate *Prrxl1* transcription. By studying the region located 1400 bp upstream of *Prrxl1* coding region, it was shown that it is controlled by three alternative promoters called P1 [-72/-157], P2 [-487/604] and P3 [-605/-770], which are responsible for

the transcription of three *Prrxl1* 5'-UTR variants that impact on mRNA stability and translation efficiency, but not in translation initiation (Regadas et al. 2013). Promoters P1 and P2 are conserved only in mammals. P1 has a neuron-specific activity but has no evident conserved motifs, while P2 is constitutively active and contains a GC-box element. The most conserved element identified by Regadas studies is Promoter P3, which, like Promoter P1, has a neuron-specific activity. These regions contain evolutionarily conserved motifs that are thought to be bound by bHLH and HD transcription factors. In fact, genetic studies in mice showed that *Prrxl1* expression depends on the presence of Isl1 and Brn3a in DRG (Dykes *et al.*, 2011) and on Tlx1/3 and Lmx1b in dorsal spinal cord (Qian *et al.*, 2002; Ding *et al.*, 2004). However, data on the mechanisms by which they modulate Prrxl1 expression are still scarce. In Publication I, we found that Prrxl1 binds in the vicinity of the TATA-box (i.e. promoter P3) and on two novel evolutionarily conserved regions, one located about 1297 bp upstream of *Prrxl1* translation initiation site and another within *Prrxl1* 4th intron, 5601-6869 bp downstream. Like minimal promoter P3, these two novel regions contain several conserved HD bipartite binding motifs TAAT-N_3-ATTA, suggesting that Prrxl1 and, likely, other HD transcription factors modulate *Prrxl1* transcription. By the use of reporter gene assays and gene expression studies in ND7/23 cells, we here demonstrated that these regions mediate Prrxl1 transcriptional auto-repression. Regadas et al. (2015) revealed that Tlx3 also binds to *Prrxl1* proximal promoter using embryonic dorsal spinal cord chromatins. In addition, transcriptional assays in ND7/23 cells showed that Tlx3, as Brn3a, acts as a positive regulator of *Prrxl1* transcription (Regadas et al., 2004), which is in line with previous evidence, obtained from *Tlx3*- and *Brn3a*-mutant mice showing a strong downregulation of *Prrxl1* expression in dorsal spinal cord and DRG, respectively (Qian *et al.*, 2002; Dykes *et al.*, 2011). In addition to these mechanisms, our group recently revealed that Prrxl1 transcriptional activity is modulated by phosphorylation and Tlx3 increases Prrxl1 phosphorylation levels(Regadas *et al.*; Soares dos Reis *et al.*, 2010). Electrophoretic analysis of DRG and dorsal spinal cord protein extracts showed that Prrxl1 presents a multiple band pattern resulting from phosphorylation and conformational changes (Soares dos Reis *et al.*, 2010).

Noteworthy, Prrxl1 tends to exist in less phosphorylated states as development progresses. In dorsal spinal cord, Prrxl1 is mainly found in a hyperphosphorylated state until E14.5, but from E16.5 on Prrxl1 shifts to less phosphorylated states (Soares dos Reis *et al.*, 2010). This observation correlates well with the vanishing expression of Tlx3 in Prrxl1^{+}-neurons of lamina III during late embryonic developmental stages (Rebelo *et al.*, 2007; Rebelo *et al.*, 2010). In the DRG, Prrxl1 presents a hyperphosphorylated state until P0 but, from P7 onwards, Prrxl1 exists mostly in less phosphorylated states (Soares dos Reis *et al.*, 2010). This differential expression of Prrxl1 phospho-states observed along development in dorsal spinal cord and DRG may contribute to Prrxl1 differential role in these tissues. To date, only phosphorylation on serine 119 has been shown to influence Prrxl1 transcriptional activity on the *Rgmb* proximal promoter region by transcriptional assays (Soares dos Reis *et al.*, 2010). Future studies will be required to clarify the relationship between Prrxl1 phosphorylation states and its own transcriptional regulation. The multiplicity of the above mentioned mechanisms controlling *Prrxl1* tissue-specific and temporal expression, is quite expected for biologically important genes.

Analysis of *Prrxl1*-null mouse embryos suggests that Prrxl1 function in nociceptors differs from that on their central target neurons in the superficial spinal cord dorsal horn and in spinal trigeminal complex nuclei (Chen et al. 2001; Rebelo et al. 2010; Rebelo et al. 2006; Ding et al. 2003). While in DRG Prrxl1 appears not to influence nociceptors embryonic development, Prrxl1 deletion disrupts normal patterning and differentiation of glutamatergic neurons located in spinal cord superficial dorsal horn. Through ChIP-seq and microarray studies, our research team revealed a tissue-specific genetic program for Prrxl1, distinctive in the DRG and dorsal spinal cord (our own group unpublished results). Nonetheless, Prrxl1 is similarly necessary for DRG/Trigeminal Ganglia and dorsal spinal cord/trigeminal nucleus neurons survival at late developmental stages (Rebelo *et al.*, 2006; Rebelo *et al.*, 2010). Based on these results, Prrxl1 is thought to orchestrate different, but complementary, genetic programs in peripheral and central nociceptive neurons. The results presented in this thesis provided some insight into tissue-specific mechanisms controlling *Prrxl1* expression in DRG and

dorsal spinal cord. In Publication I, we show that Prrxl1 binding to its own promoter region and 4th intron displayed a different occupancy in DRG and dorsal spinal cord chromatins. In DRG, Prrxl1 preferentially binds to its 4th intron, whereas in dorsal spinal cord, Prrxl1 strongly interacts both with its own proximal promoter and 4th intron, though more pronouncedly at the proximal promoter. In publication II, we demonstrate that, although Casz1 is expressed in DRG and the spinal dorsal horn, its expression appears to be Prrxl1-dependent solely in the dorsal horn. Hence, we hypothesize that Prrxl1 differential role in these tissues is related with tissue-specific interactions with cofactors and/or co-regulators, and differential chromatin accessibility. In DRG, Prrxl1 expression depends on the presence of Brn3a and Isl1, although there is no evidence that they directly regulate *Prrxl1* (Dykes *et al.*, 2011). In dorsal spinal cord, Prrxl1 expression is dependent on the presence of the HD transcription factors Tlx3 and Lmx1b, as revealed by Prrxl1 strong downregulation in *Tlx3*- and *Lmx1b*-mutant mice (Qian *et al.*, 2002; Ding *et al.*, 2004). In cranial ganglia, Phox2b represses *Prrxl1* as revealed by increased Prrxl1 expression in *Phox2b*-mutants (D'Autreaux *et al.*, 2011). Moreover, Phox2b was shown to bind *Prrxl1* proximal promoter in dorsal *medulla oblongata* of mouse embryos (Regadas *et al.*, 2013). Considering the presence of several HD motifs in Prrxl1-binding sites, we cannot discard the hypothesis that the HDs Lmx1b and Isl1 also bind *cis*-regulatory elements controlling *Prrxl1* transcription in the dorsal spinal cord and DRG, respectively. In this context, the Prrxl1 autoregulatory loop mechanism described in Publication I may contribute to fine-tune *Prrxl1* expression levels in both the DRG and spinal components of the nociceptive circuitry along development.

Prrxl1 contributes to the differentiation of glutamatergic neurons in spinal cord dorsal horn

In the present thesis (Publication II), we show evidence that Prrxl1 directly regulates *Casz1*, a gene involved in cell differentiation, and is required for proper expression of genes involved in pain processing (*Npy1r*) and itch (*Grp*), which is in line with the predicted functions of Prrxl1 (Chen et al. 2001a; Rebelo et al. 2010; Rebelo et al. 2006). *Casz1* encodes a Zinc-finger transcription factor known for its role in neuronal differentiation of *Droshophila* neuroblasts and mouse retina (Mellerick *et al.*, 1992; Isshiki *et al.*, 2001; Mattar *et al.*, 2015). In E14.5 *Prrxl1*-mutant spinal cords, Casz1 expression is downregulated but not totally lost. At this stage, Casz1 expression is found in a large subset of dILB neurons in the superficial dorsal horn by E14 (presumptive laminae I–III), however, it is gradually extinguished, from outer to inner laminae, until it narrows down to a layer of neurons within lamina III at P7. However, Casz1 expression in these cells is independent of Prrxl1, suggesting that other factors are required, at least in this subpopulation of dILB neurons. Noteworthy, Mattar and collaborators (2015) recently revealed several regulatory modules rich in HD binding sites within *Casz1* introns 2 and 3. In addition, Prrxl1-binding sites within Casz1 2nd and 4th introns contain several HD binding motifs (data not published). These observations support the possibility that other HD transcription factors may intervene in the control of Casz1 expression. Lbx1 is a good candidate as its expression in dILB neurons accompanies that of Casz1 (Gross *et al.*, 2002), co-localizes with several dILB markers by E14.5 (Gross *et al.*, 2002; Müller *et al.*, 2002), only persists in a small subset of Lmx1b^{+}/Tlx3^{-}-neurons located in lamina III (Müller et al. 2002; Del Barrio et al. 2013) and almost all Casz1^{+}-neurons co-express Lbx1 and Lmx1b at P7. Another plausible regulator of *Casz1* transcription is Lmx1b, since virtually all Casz1^{+}-neurons in dorsal spinal cord are also Lmx1b^{+} both at E14.5 and P7. These possibilities, as the identification of other regulators of *Casz1* transcription in dorsal spinal cord needs, however, future investigation.

To determine if Prrxl1 and Casz1 shared commonly dependent genes, we intersect our previously generated list of Prrxl1 target genes in dorsal spinal cord, using as cut-offs a fold-change > 1.3 and *P*-value < 5.0E-02, with data sets of Casz1-dependent genes in another systems, namely human neuroblastoma cells and differentiating cardiomyocytes. Thus, we

identified one possible common gene encoding neuropeptide Y receptor Y1 (*Npy1r*), which was previously shown to be expressed in dILB neurons of spinal cord (Guo et al., 2012). *Npy1R* expression onset occurs before E14.5 (Xu et al., 2008; Liu et al., 2014), which corresponds to the stage when Casz1 reaches maximal expression levels. Through expression analysis, we showed that *Npy1r* is downregulated in dorsal spinal cord of *Prrxl1*-mutant embryos. Hence, it is conceivable that *Npy1r* expression is Casz1-dependent, as is the case in differentiating cardiomyocytes (Liu et al., 2015). Worth mentioning, Npy1r expression is also dependent on the presence of Tlx3 and Lbx1 in dorsal spinal cord (Guo et al., 2012), which raises the possibility that Casz1 may also act downstream of these transcription factors besides Prrxl1. Future investigations are necessary to understand the mechanisms involving Prrxl1 and Casz1 in the control of *Npy1r* expression.

Epigenetic processes influence chromatin structure of regulatory elements and their flanking regions through methylation of 5' position of cytosine in 5'-Cytosine-Guanine-3' dinucleotides (known as CpGs islands), as well as posttranslational modifications of nucleossomal proteins such as histone methylation and histone acetylation (Mazzio and Soliman 2012). Recently, Liu *et al.* (2015) demonstrated that Casz1 interacts with the nucleosome remodeling and histone deacetylase (NuRD) complex, histones and DNA repair proteins, suggesting that Casz1 may have an epigenetic role. In Publication II, we advance the hypothesis that Casz1 is involved in somatosensory phenotype acquisition of neurons in laminae I–III. It is also plausible that Casz1 acts downstream of Prrxl1 to induce chromatin changes necessary for proper differentiation of superficial dorsal horn glutamatergic neurons. Differential exposure to Casz1 action could thus contribute to the neurochemical, electrophysiological and morphological heterogeneity of Lamina I-III neurons. In order to unveil Casz1 role in dILB ontogeny, future studies should address Casz1 DNA binding landscape in dorsal horn neurons and identify its dependent genes. As we propose in Publication II, the *Casz1* conditional mutants in DRG and dorsal spinal cord will be an important tool to elucidate Casz1 role in somatosensory circuit assembly during embryogenesis.

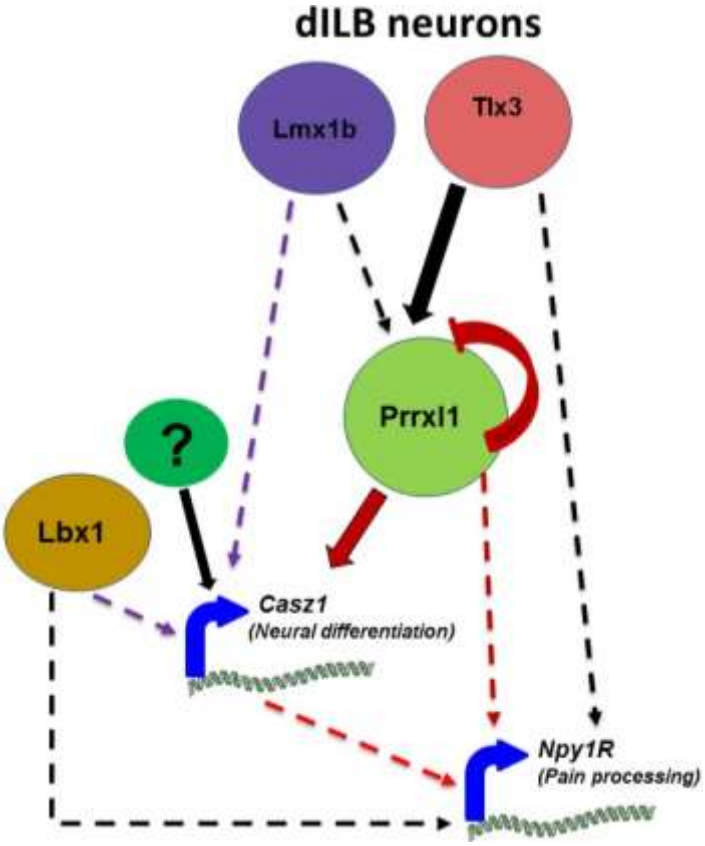

Figure 3: Genetic interactions coordinated by Prrxl1 in differentiating dILB neurons. In dILB neurons, Prrxl1 expression is dependent on Tlx3 and Lmx1b. Prrxl1 directly and positively regulates Casz1 expression in a subset of dILB neurons during embryonic stages. Casz1 role in dILB neurons in unknown, but it has an important role in neuronal differentiation of *Drosophila* neuroblasts and mouse retinal cells. *Npy1r* is a Prrxl1-dependent gene in dILB neurons and hypothetically, Casz1-dependent as well. **Arrows:** positive regulation; **Blunt arrow**: negative regulation; **Line:** direct regulation; **Dashed line:** direct or indirect regulation; **Black**: interactions based on previous evidence; **Red**: interactions revealed in this thesis; **Purple**: hypothetical interactions.

Conclusions

Results presented in this thesis allow drawing the following conclusions:

- Prrxl1 represses its own transcription through by direct binding to its promoter region and 4th intron. This negative feedback mechanism likely contributes to fine tune *Prrxl1* expression levels, counterbalancing the positive regulation action of Brn3a and Isl1 in DRG, and Tlx3 and Lmx1b in dorsal spinal cord.
- Casz1 displays two waves of expression in dorsal spinal cord. Firstly, Casz1 is present between E10.5 and E11.5, in dP1 cells and their derivatives (dI1 neurons), and, by E13.5, its expression restarts in neurons reaching the superficial dorsal horn. Until P7, Casz1 expression in these neurons is gradually extinguished, from outer to inner laminae, until it narrows down to a layer of neurons mostly located in lamina III.
- Casz1 expression starts around E10.5 and presents a pan-sensory expression pattern throughout DRG embryonic and perinatal development (until P7).
- Prrxl1 promotes *Casz1* transcription in differentiating dILB neurons by direct binding to DNA elements located at 2nd and 4th introns. Postnatally, Casz1 expression in lamina III neurons is not Prrxl1-dependent.
- Casz1 expression during DRG development is not Prrxl1-dependent.

Overall, the data presented in this thesis shed new light into the mechanisms involved in the modulation of *Prrxl1* transcription and the Prrxl1-downstream transcriptional program. We revealed that Prrxl1 binds its promoter region and 4th intron to repress its own transcription. Moreover, we identified the Zinc-finger transcription factor *Casz1* as a new Prrxl1-direct target gene in embryonic dorsal spinal cord.

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