Universidade de Aveiro Departamento de Biologia
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CÉSAR BRUNO DA CUNHA MONTEIRO

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CONTRIBUTOS PARA O ESTUDO DA BASE MOLECULAR DA FUNÇÃO DO PRRXL1

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Professor Doutor Filipe Almeida Monteiro, Professor Auxiliar convidado da Faculdade de Medicina da Universidade do Porto, e orientação da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

À memória do Professor Doutor Edgar Cruz e Silva.

o júri

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agradecimentos

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palavras-chave

Desenvolvimento do Sistema nociceptivo, Prrxl1, factor de transcrição homeodominio, programa transcriptional, gânglios raquidanos, medula espinhal, Yeast-Two-Hybrid.

resumo

A correcta percepção dos estímulos sensitivos pelo cérebro envolve tipos diferentes de neurónios sensitivos e requer circuitos funcionais específicos. Como se diferenciam os neurónios sensitivos nos vários subtipos e de que forma estabelecem sinapses funcionais são questões centrais que permanecem, em grande parte, sem resposta. Durante o desenvolvimento do sistema nervoso, os fatores de transcrição "*Basic helix-loop-helix*" (bHLH) e homeodomínio (HD) desempenham um papel essencial no controlo da proliferação, especificação, diferenciação e estabelecimento de circuitos neuroniais. Entre os fatores de transcrição envolvidos na diferenciação de neurónios sensitivos, o Prrxl1 (Paired related homeobox protein-like 1) tem merecido um interesse especial do nosso laboratório, uma vez que o Prrxl1 está especificamente envolvido na diferenciação de neurónios nociceptivos dos gânglios raquidianos (GRs) e da medula espinhal (ME). O Prrxl1 é um dos poucos factores de transcrição que se expressam especificamente em neurónios sensitivos periféricos e nos neurónios da medula espinhal e tronco cerebral com que aqueles se articulam. Este tipo de expressão foi entendido como sugestivo de que o Prrxl1 desempenhe papel especial no estabelecimento de conexões entre o primeiro e o segundo neurónio da via sensitiva. De facto, a análise de murganhos transgénicos, *knockout* para o gene Prrxl1, revelou que este factor de transcrição pode estar envolvido em vários aspectos da diferenciação neuronal, incluindo migração, desenvolvimento da projecção axonal, bem como no comportamento nociceptivo normal no animal adulto. As várias funções em que o Prrxl1 parece estar envolvido apontam para a activação de um programa genético complexo.

Resultados recentes do nosso laboratório mostram que o gene Prrxl1 origina dois transcritos, o Prrxl1a e o Prrxl1b, através do mecanismo de *splicing* alternativo. Além disto, a Prrxl1a apresenta vários estados de fosforilação cujo padrão varia ao longo do desenvolvimento do GR e da ME do murganho. Assim, a fosforilação parece ter um papel na regulação da actividade da Prrxl1a, no entanto, as cinases e fosfatases envolvidas neste processo, assim como outras proteínas que interajam com o Prrxl1a/b não são conhecidas. Um dos objectivos do presente estudo foi o de caracterizar o interatoma do Prrxl1 recorrendo ao sistema Yeast-Two-Hybrid. No entanto, tal não foi possível porque tanto a Prrxl1a como a sequência C-terminal específica da Prrxl1b usadas como iscos tinham por si só a capacidade de transactivar o gene repórter, sem o uso de presas em fusão com o domínio de activação. Deste modo, a estratégia do Yeast-Two-Hybrid utilizado não teve sucesso.

Por outro lado, tendo por objectivo a caracterização do programa genético do Prrxl1 na ME em desenvolvimento, foi realizada no nosso laboratório uma experiência de Imunoprecipitação da Cromatina seguida de uma sequenciação massiva em paralelo (ChIP-Seq) usando um anticorpo específico para a Prrxl1 e cromatina da região dorsal da espinal medula de embriões com 14,5 dias de desenvolvimento embrionário (E14,5). Esta abordagem permitiu a identificação de 939 genes candidatos, associados com os locais de ligação da Prrxl1 obtidos por ChIP-seq. No entanto, a interação entre uma proteína e o DNA não significa que exista regulação da transcrição. Assim, outro objectivo deste trabalho foi o de seleccionar um grupo de genes candidatos a partir da lista de ChIP-seq e de analisar a sua expressão em GRs e ME de murganhos "wild type" e *"knockout"* para o gene Prrxl1, usando como metodologia experimental a hibridação *in situ* e o PCR em tempo real. Neste trabalho, mostramos que os genes *Dscaml1* e

Sema3F são genes alvo diretos do Prrxl1 nos GRs e ME, e os genes *Cdh4* e *Efna5* são apenas nos GRs. Estes resultados sugerem que o Prrxl1 regula a expressão de genes envolvidos na orientação das projeções axonais e conetividade neuronial, estando em linha com o fenótipo observado nos murganhos Prrxl1-/-.

keywords

Nociceptive system development, Prrxl1, homeodomain transcription factors, transcriptional programme, dorsal root ganglia, spinal cord, Yeast-Two-Hybrid.

abstract

Correct perception of sensory stimuli by the brain involves different types of sensory neurons which require specific and functional networks. How do they differentiate into their specific subtypes, and how do they establish functional synapses, are questions that remain largely unsolved. Throughout the nervous system development, homeodomain transcription factors play a pivotal role in the control of proliferation, specificity, differentiation and establishing connectivity between neurons. Among the transcriptions factors involved in the differentiation of sensory neurons, Prrxl1 (paired related homeobox protein-like 1) had received a special interest in our laboratory since it is involved specifically in the differentiation of nociceptive neurons within dorsal root ganglia (DRG) and their central targets within dorsal spinal cord. Prrxl1 is one of the few transcriptions factors that exhibit an expression in the primary afferent neurons and their central targets. This expression pattern suggests that Prrxl1 have an important role establishing connectivity between first order neurons and second order neurons. Indeed, analysis of Prrxl1$^{-/-}$ mice phenotype revealed that Prrxl1 is involved in several aspects of neuronal differentiation such as migration, axon guidance and the proper nociceptive behavior in adult individuals. Hence, Prrxl1 function might be based in the activation of a complex genetic program.

Our recent findings had shown that Prrxl1 gene origin two transcripts, Prrxl1a and Prrxl1b, through the alternative splicing mechanism. Furthermore, Prrxl1a present several phosphorylation states which pattern throughout the development is variable. Hence, phosphorylation appear to have a regulatory role in Prrxl1 activity, however, kinases and phosphatases involved in this process, as well as other proteins that might interact with Prrxl1a/b remain unknown. The first objective of the present study was to characterize Prrxl1 interactome by the Yeast-Two-Hybrid system. However, it was not possible to achieve it due Prrxl1a and Prrxl1 specific C-terminal sequence, used as baits, to activate reporter genes transcription in the absence of any prey fused to an activation domain. Thus, the Yeast-Two-Hybrid approach used was not successful.

On the other hand, aiming to characterize Prrxl1 genetic program in the developing spinal cord, was performed Chromatin Immunopercipitation followed by massive parallel sequencing (ChIP-Seq) using a Prrxl1 specific antibody and chromatin from the murine E14.5 dorsal half of the spinal cord. This approach allowed the identification of 939 candidate genes associated with the Prrxl1 binding sites obtained by ChIP-Seq. However, DNA-protein interaction did not mean that there is a transcriptional regulation. Hence, the second objective of the present study was to select a set of candidate genes from ChIP-Seq list, and to analyze their expression pattern in spinal cord and DRG, in wild type and Prrxl1$^{-/-}$ mice by *in situ* hybridization and Real Time PCR. Our study revealed that *Dscaml1* and *Sema3F* are Prrxl1 direct targets in spinal cord and DRG, and *Cdh4* and *Efna5* are Prrxl1 direct targets only in DRG. These results suggest that Prrxl1 is involved in axon guidance and neuronal connectivity and, therefore, are in agreement with the Prrxl1$^{-/-}$ mice phenotype.

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Abbreviations

3-AT	3-aminotriazole
AD	Activating domain
APS	Ammonium persulfate
BD	Binding domain
BMP	Bone morphogenetic protein
Cart	Cartilage paired-class homeoprotein
Cdh4	Cadherin 4
ChIP	Chromatin Immunoprecipitation
ChIP-Seq	ChIP followed by parallel massive Sequencing
CK	Casein kinase
Cut	cut-like homeobox 1
CGRP	Calcitonin gene-related peptide
Dcc	Deleted in Colorectal Carcinomas
dI	Dorsal interneurons
dIL	Late dorsal interneurons
DMSO	Dimethylsulfoxide
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglia
DNA	Deoxyribonucleic acid
Dscaml1	Down Syndrome Cell adhesion molecule-like 1
E	Embryonic day
Efna5	Ephrin A5
Eph	Ephrin receptor
FRAP	Fluoride-resistant acid phosphatase
GPI	Glycosylphosphatidylinositol
HD	Homeodomain
His	Histidine
HRP	Horseradish peroxidase
Kcnma1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1
Islet	ISL LIM homeobox 1
LB	Loading Buffer
Lbx1	Ladybird homeobox homolog 1
LGB	Lower gel buffer

Lhx	LIM homeobox protein
Lmx1b	LIM homeobox transcription factor 1 β
LiAc	Lithium acetate
Mash1	Mammalian achaete-scute homolog 1
NCCs	Neural crest cells
NF200	Neurofilament 200
NGF	Nerve growth factor
NGN 1 and 2	Neurogenin 1 and 2
NS	Nociceptive specific cells
OAR	otp, aristaless, and rax
OB	Olfactory bulb
OD	Optical density
OSN	Olfactory sensory neurons
P	Post-natal day
PAG	Periaqueductal grey
PBA	Parabrachial area
PEG	Polyethylene glycol
PKC	Protein kinase C
PMSF	Phenylmethyl-sulfonyl fluoride
Prx	Paired related homeobox
Prrxl1	Paired related homeobox protein-like
YPD	Yeats extract, peptone and dextrose
YTH	Yeast Two Hybrid
RNA	Ribonucleic acid
RD	Repressor domain
RGMb	Repulsive guidance molecule b
Robo	Roundbout (Slit receptor)
Rora	RAR-related orphan receptor
RVM	Rostral ventromedial medulla
Runx	runt related transcription factor
SDS	Sodium dodecyl sulfate
Sema	Semaphorin
SP	Substance P
Shh	Sonic hedgehog
TBS-T	Tris-Buffered Saline and Tween 20

TE	Tris/EDTA solution
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Transcription factor
Tlx3	T-cell leukemia homeobox 3
TMP	Thiamine monophophatase
TrkA, B and C	Tyrosine receptor kinase A, B and C
Trp	Tryptophan
UAS	Upstream activating sequence
Unc	Uncoordinated
UGB	Upper gel buffer
Vz	Ventricular zone

Part I- GENERAL INTRODUCTION

1. THE SOMATOSENSORY SYSTEM

All living organisms are characterized by their capability to respond to external stimuli. However, such response requires a system specialized in the perception of the external environment. Metazoans have a highly specialized system capable of receive and process external stimuli, and then, develop a suitable motor response. Such system is known as the nervous system.

Spinal cord dorsal horn neurons convey sensory information from primary afferent neurons, which innervate the skin and deep tissues of the body. Primary afferents receive and transmit specific types of noxious and non-noxious stimuli to their spinal cord dorsal horn target neurons. Afferent central projections have a distribution pattern that is determined by their sensory modality and the region of the body that they innervate (Fig.1). Primary afferent neurons can be classified by their peripheral targets (i.e. cutaneous, articular or visceral afferents), conduction velocity (which is related to the diameter of the axons and degree of myelination), response properties (including sensory modalities and the intensity of stimulus necessary to activate them) and neurochemical phenotype (i.e. neuropeptide expression and presence of certain receptors) (Todd, 2010). These features are interrelated, as most large myelinated cutaneous afferents, known as Aβ fibers, are low-threshold mechanoreceptors, responding to touch or hair movement, whereas the majority of fine myelinated afferents, known as Aδ fibers, and unmyelinated afferents, known as C fibers, are nociceptors or thermoreceptors, i.e, conveying noxius and thermal stimuli, respectively (Todd, 2010).

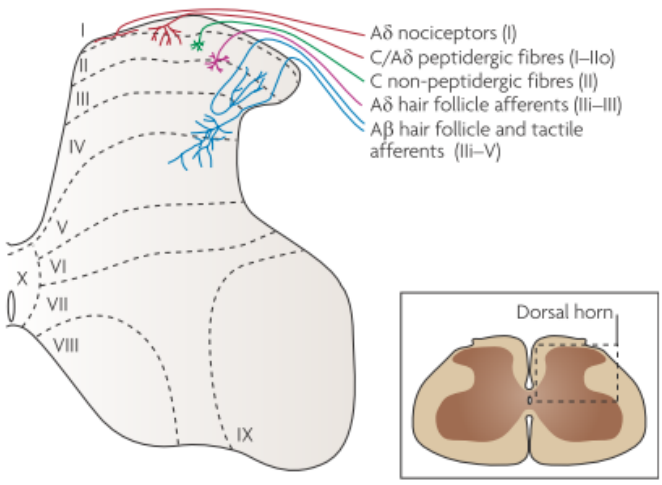


Figure 1: *Illustration of different types of primary afferent sensory fibers projections into their respective spinal cord dorsal horn projecting areas.* ***Adapted from Tood, 2010****.*

The balance between excitation and inhibition inputs from afferent fibers, spinal cord interneurons and supraspinal circuits is crucial for maintaining normal sensory function. For example, blocking inhibitory transmission at spinal levels, can lead to allodynia, which is pain following a normally non-painful tactile or thermal stimulus (Yaksh, 1989, Sivilotti and Woolf, 1994). Indeed, changes in the function of these circuits have been implicated in the development and maintenance of inflammatory and neuropathic chronic pain states. Together, these sensory modalities give animals the ability to identify shapes and textures of objects, to monitor the internal and external forces acting on the body at any moment, and to detect potentially harmful circumstances.

2. THE NOCICEPTIVE SYSTEM

The perception of pain, called nociception, plays a pivotal role alerting the brain for potentially threatening circumstances. Nociceptors, like other somatic sensory receptors, are unipolar neurons with cell bodies located in dorsal root ganglia (DRG) or trigeminal ganglion which sends one axonal process to periphery and the other into the dorsal spinal cord or brainstem, respectively. Nociceptors fall into either Aδ group of thin myelinated axons or C fiber group of unmyelinated axons (Julius and Basbaum, 2001). In general, Aδ nociceptors respond to dangerously intense mechanical stimuli or mechanothermal stimuli (Julius and Basbaum, 2001). The C nociceptors tend to respond to thermal, mechanical, and chemical stimuli, and are, therefore, known as polymodal (Julius and Basbaum, 2001, Purves et al., 2003). However, unlike normal thermal, mechanical, and chemical receptors, the nociceptive axons depolarize only when the strength of the stimulus reach high levels.

Nociceptive and thermoreceptive Aδ and C afferents innervate lamina I and outer lamina II (Liu et al., 2007, Seal et al., 2009). In addition, nociceptive information that is conveyed to the ventral horn contributes to spinally-mediated nocifensive reflexes (Sivilotti and Woolf, 1994). Nociceptive C-fibres can be divided into two major neurochemical groups: the peptidergic fibers that express neuropeptides such as substance P (Lawson et al., 1997), and non-peptidergic that do not express neuropeptides (Snider and McMahon, 1998). Non-peptidergic C-fibers are mainly associated with the skin, where they innervate the epidermis (Taylor et al., 2009), whereas peptidergic fibers innervate several other tissues as well as deeper regions of the skin (Plenderleith and Snow, 1993, Bennett et al., 1996, Perry and Lawson, 1998).

The incoming information is processed by complex spinal cord circuits involving excitatory and inhibitory interneurons, and then eventually transmitted to the thalamus and to the parabrachial area (PBA) (Fig.2) (D'Mello and Dickenson, 2008, Schmidtko et al., 2009). From the thalamus, cortical regions forming the 'pain matrix' (somatosensory cortex, insula, anterior cingulate and prefrontal cortices) are activated, which detect the sensory discriminatory aspects of nociception (D'Mello and Dickenson, 2008, Schmidtko et al., 2009). The processing of pain stimulus also initiates the affective reactions mediated by complex circuits, which include the limbic system, hypothalamus, periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM) (D'Mello and Dickenson, 2008, Schmidtko et al., 2009). Thus, unpleasant feelings and negative emotions are generated. In addition, these affective reactions activate descending pathways that control the spinal nociceptive processing. Therefore, the dorsal horn of the spinal cord integrates peripheral, spinal and supraspinal components of pain processing.

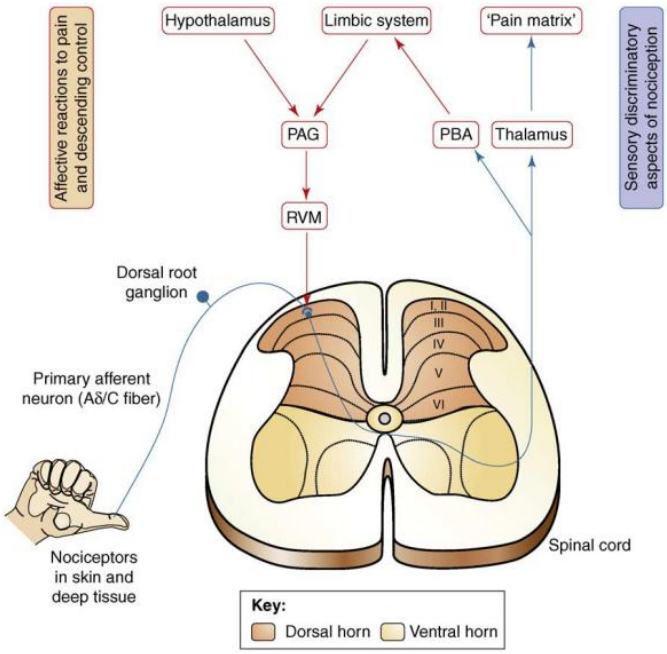


Figure 2: *Illustration of important pain processing circuits. Stimulation of Aδ and C nociceptors relays noxious sensory information to their terminals in the superficial dorsal horn of the spinal cord. Projection neurons of the spinal cord forward the excitation to supraspinal areas where is detected sensory discriminatory aspects of nociception, unpleasant feelings and activation descending pathways that control the spinal processing. Abbreviations: I-VI, laminae of the dorsal horn; PBA, parabrachial area; PAG, periaqueductal grey; RVM, rostral ventromedial medulla.* ***Adapted from Schmidtko et al, 2009****.*

3. NEURAL TUBE AND NEURAL CREST CELLS FORMATION

The nervous system development starts with the neural tube formation (Gilbert, 2000). In mammals, shortly after the neural plate has been formed, its edges thicken and move upward to form the neural folds, while a U-shaped neural groove appears in the center of the plate, dividing the future right and left sides of the embryo (Fig.3). The neural folds migrate toward the midline of the embryo, eventually fusing to form the neural tube beneath the overlying ectoderm. The cells at the dorsal most portion of the neural tube become the neural crest cells (NCCs) (Gilbert, 2000).

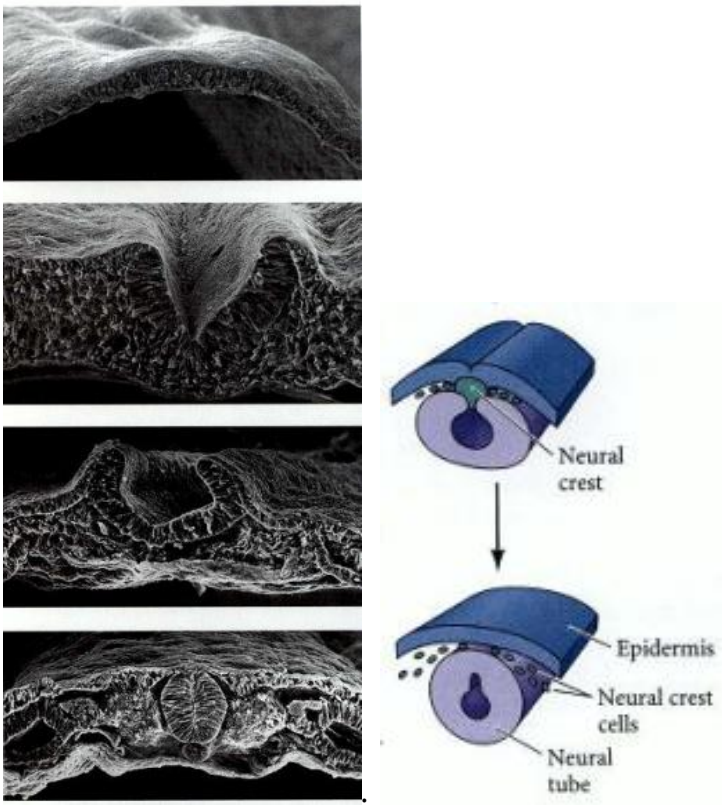


Figure 3: *Neural tube formation (Left) and the migration of the neural crest cells (right). The neural plate starts to form a groove which will close in order to form the neural tube. After that, one of the lineages of neural crest cells starts to migrate ventrally in order to generate the dorsal root ganglia.* ***Adapted from Gilbert, 2000****.*

Once closed, the neural tube is polarized along its dorsal-ventral axis. The polarity is induced by signals coming from its surrounding environment. The dorsal pattern is imposed by BMPs (a member of the TGF-β morfogene family) secreted by ectoderm and roof plate cells (Liem et al., 1995), while the ventral pattern is induced by Shh morphogene secreted by the notochord and floor plate cells (Yamada et al., 1993). These factors generate an extracelular gradient which is

responsible for the formation of sensory and motor neurons at dorsal and ventral spinal cord, respectively, and delamination of the spinal cord in different populations which will be responsible for the generation of different types of differentiated neurons (Helms and Johnson, 2003, Liu and Niswander, 2005).

3.1. NEUROGENESIS OF DORSAL ROOT GANLGIA (DRG) NEURONS

In mice, DRGs neurons are generated from E9.5 to E13.5 from NCCs in the dorsal region of the spinal cord (Serbedzija et al., 1990, Kasemeier-Kulesa et al., 2005). During NCC migration, sensory neurogenesis occurs in three successive waves which are differentially exposed to signals from the adjacent somites and spinal cord, driving the generation of multiple types of sensory neuron in the DRG (Ma et al., 1999, Maro et al., 2004).

Neurogenesis and specification to the sensory lineage are linked and involves setting into place the transcriptional programs necessary for a specific path of differentiation. Furthermore, there is also a correlation between proneural transcription factors (TFs) driving neurogenesis in the sequential waves (Fig.4) and the appearance of specific sensory subtypes (Marmigere and Ernfors, 2007). In the mouse, NCCs begin to leave the dorsal most portion of the neural tube, between E8.5-10, to generate DRGs (Serbedzija et al., 1990, Kasemeier-Kulesa et al., 2005). The basic helix-loop-helix (bHLH) TFs neurogenin 1 (Ngn1) and neurogenin 2 (Ngn2) are required for neurogenesis and the specification of afferent sensory neurons (Anderson, 1999, Ma et al., 1999, Perez et al., 1999, Bertrand et al., 2002, Lo et al., 2002). Ngn2 initiates a first wave of neurogenesis (E 9.5) which contribute approximately for 4% of the DRG neuronal population which encompass $TrkB^+$ (mechanoreceptive) and $TrkC^+$ (proprioceptive) neurons (Ma et al., 1999). Ngn1 initiates a second wave of neurogenesis (at E10) within the DRG, which contributes for approximately 91% of the DRG neuronal population that encompass mostly small size $TrkA^+$ neurons (nociceptive) (Ma et al., 1999). In the third wave of neurogenesis are generated solely nociceptors expressing $TrkA^+$. This third wave contributes for approximately 5% of DRG neuronal population and, unlike the previous waves, came from the neural crest-derived multipotent boundary cap cells at E11 (for review: Marmigére and Ernfors, 2007).

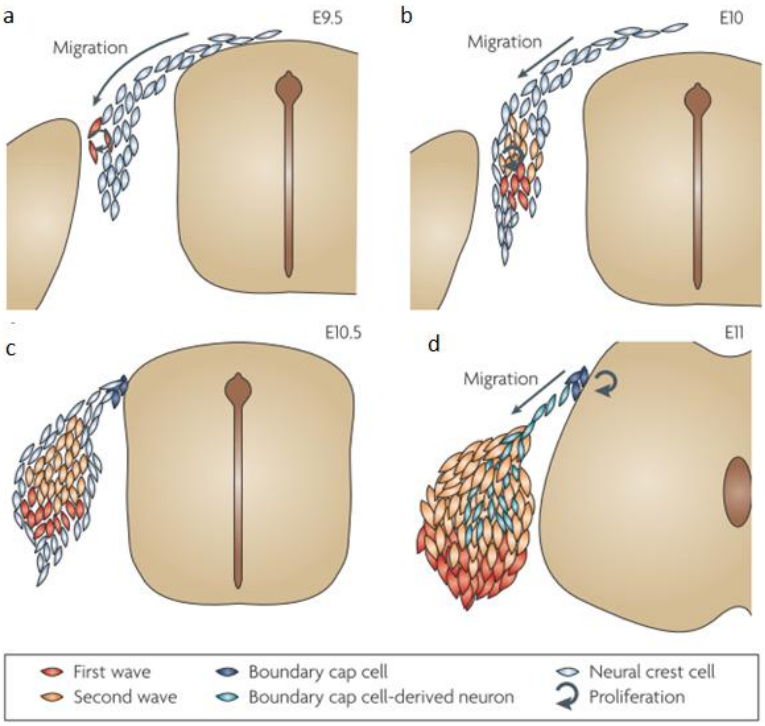


Figure 4:.*Three waves of neurogenesis in the sensory lineage. a) The first wave of neurogenesis takes place during migration of the NCCs at the level of the rostral part of the dorsal somatic lip. These cells will generate mechanoreceptive and proprioceptive neurons in DRG (~4% of DRG neurons). b) The NCCs involved on the second wave of neurogenesis are postmigratory cells on the DRG which contribute largely to the generation of TrkA$^+$ neurons, i.e, nociceptors. c) The neural crest-derived multipotent boundary cap cells can be identified at E10.5 in the mouse at the dorsal root entry zone of the spinal cord. d) Boundary cap cells proliferate and their progenies migrate into the DRG and assume a nociceptive neuron fate (mainly TrkA$^+$ neurons).* ***Adapted from Marmigére and Ernfors, 2007***.

3.2. NEUROGENESIS IN THE DORSAL SPINAL CORD

As to the embryonic development of the spinal dorsal horn, different populations of progenitors and differentiating neurons were identified based on the combinatorial expression of various classes of TFs, mainly bHLH and Homeodomain (HD) (Caspary and Anderson, 2003). In mouse, during the first wave of neurogenesis within dorsal spinal cord, progenitors cells located at ventricular zone generate six different populations of post-mitotic neurons called dI1-dI6 (dI- dorsal interneuron) (Helms and Johnson, 2003, Liu and Niswander, 2005) that express different combinations of TFs (Fig.5)(Caspary and Anderson, 2003, Helms and Johnson, 2003). Progenitors cells that express Math1 and Ngn1 generate dI1 and dI2 neurons, respectively. dI3-dI5 are generated from progenitors cells expressing Mash1, while dI6 neurons are generated from Pax7 and Dbx2 (Helms and Johnson, 2003, Liu and Niswander, 2005). Lbx1 TF is

also involved on the specification of dorsal horn neurons. This TF is initially expressed on dI4-6 but since E11.5 it is also expressed in dI3 neurons (Gross et al., 2002, Müller et al., 2002). Lbx1 determinates a basal neuronal inhibitory GABAergic phenotype (Gross et al., 2002). dI3 and dI5 neurons express Islet1 and Lmx1b, respectively, but both of them express Tlx3 (Qian et al., 2001). Tlx3 determine the neuronal excitatory glutamatergic phenotype (Cheng et al., 2004). The dI4 and dI6 domains are defined by the expression of Pax2 that is related to a GABAergic phenotype. From the second wave of neurogenesis, cells expressing Mash1 generate two more populations called dILA and dILB (dorsal late interneuron). dILA neurons express Pax2, Lhx1/5, while dILB neurons express Tlx3 and Lmx1b generating GABAergic and glutamatergic neurons, respectively (Müller et al., 2002).

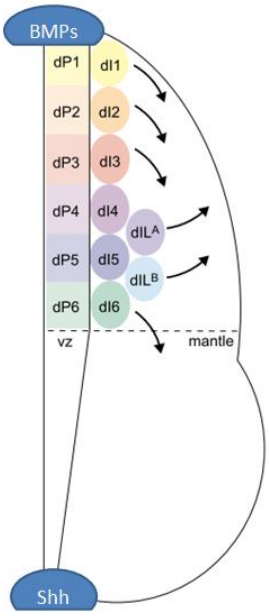


Figure 5: *Diagram of a neural tube showing the organization of the dorsal progenitors domains (dP1-dP6) in the ventricular zone (vz). At E10 starts the first wave of neurogenesis when are generated six types of post-mitotic dorsal neurons: dI1-dI6. dI1-dI3 neurons migrate ventrally , while dI4 neurons and the a subset of the dI5 population migrate laterally in order to generate the deeper region of the dorsal horn (lamina IV-V). The other subset of the dI5 and the dI6 population migrate ventrally towards ventral horn. At E12, are generated two more neuronal populations, the dILA and dILB, which generate GABAergic and glutamatergic neurons, respectively. Both populations will occupy superficial laminae. Class A and Class B neurons are distinguished by their dependence and independence, respectively, on roof plate signals secreting BMPs.* ***Adapted from (2005; Helms and Johnson, 2003).***

3.3. DEVELOPMENT OF PHERIPHERAL AND CENTRAL PROJECTIONS

As NCCs undergo neurogenesis and diversify into principal sensory subtypes, DRG axons grow rapidly to the dorsolateral margin of the spinal cord and pheripheral areas such as the limbs (Ozaki and Snider, 1997, Marmigere and Ernfors, 2007). Murine primary afferent axons reach the thoracic spinal cord at E10.5 and grow rostro-caudally for at least 48 hours, called the "waiting period", prior to extending collateral branches into the spinal gray matter (Fig.6)(Ozaki and Snider, 1997). The "waiting period" reflects the repulsive effects of Semaphorin3A (Sema3A) and netrin-1 secreted by dorsal spinal cord at these stages (Fu et al., 2000, Watanabe et al., 2006). Between E12.5 and E13.5, Sema3A and netrin-1 secretion decreases and the morphology of the sensory axons changes dramatically (Ozaki and Snider, 1997, Fu et al., 2000, Watanabe et al., 2006). In mice, pioneer cutaneous nociceptive fibres begin to enter into the spinal gray matter around E12.5. From the outset, each DRG neuron innervates characteristic skin dermatomes and projects in a precise somatotopic pattern in the dorsal horn (Ozaki and Snider, 1997). Most nociceptors, including Aδ and C axons, project to lamina I and II, although a few reach deeper laminae (Light et al., 1979, Schmidt and Rathjen, 2010). Such topography is accomplished by the differential expression of cell-cell adhesion molecules and responses to chemotropic cues (Gaillard et al., 2005, Inatani, 2005).

Similarly, dorsal horn projection neurons (called commissural neurons) extend their axons to several brain areas. The pathway taken by commissural axons in the developing spinal cord starts when they initiate the differentiation process (Fig.6) (Schmidt and Rathjen, 2010). Firstly, commissures are attracted to the floor plate by netrin-1, which is secreted by the floor-plate cells and binds to receptor DCC (deleted in colorectal carcinomas) in the axonal membrane (Stein and Tessier-Lavigne, 2001, Alberts et al., 2008). As axons cross the floor plate, the growth cones upregulate the expression of Roundabout (Robo), which is the receptor for the repellent proteins, Slits. These proteins are also secreted by the floor plate cells, and their interaction with Robo not only acts as a repellent to keep the cells from re-entering the floor plate, but also blocks the attractive responsiveness to netrin-1 (Stein and Tessier-Lavigne, 2001). Simultaneously, the growth cones switch on expression of receptors for other repellent proteins, semaphorins, that are secreted by the cells in the side walls of the neural tube (Fu et al., 2000). Trapped between two repellent territories, the growth cones, having crossed the midline, travel in a tight fascicle up toward the brain.

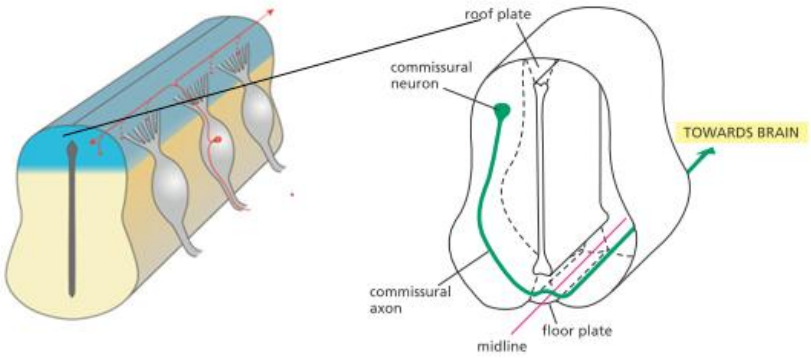


Figure 6: *The guidance of primary afferent fibers (left) and commissural axons (right). Afferent sensory axons bifurcate at the dorsal entry zone of the spinal cord. The two resulting daughter axons extend longitudinally in opposite directions at the dorsolateral margin of the cord. After waiting period collaterals grow from the stem axons and terminate in their respective laminae. Commissural axons are attracted by netrin-1 that is secreted in the floor plate cells. As they cross the floor plate, they start to respond to the repellents Semaphorin3A and Slits in order to cross the midline. Then, commissural axons travel toward the brain.* ***Adapted from Alberts et al., 2008; Schmidt and Rathjen, 2010.***

4. PRRXL1 GENE IN THE DEVELOPMENT OF THE NOCICEPTIVE SYSTEM

4.1. PRRXL1: GENE AND PROTEIN CHARACTERIZATION

Prrxl1 is 47kb gene in the mouse chromosome 14 (Rebelo et al., 2007) that encodes a paired-like Homeodomain transcription factor. By alternative splicing, its pre-mRNA transcript originates two mRNAs (Fig.7). One of them contain seven exons and encodes Prrxl1a and in the other the 7th exon is substituted by new different exons encoding Prrxl1b (Rebelo et al., 2009). Both encode proteins that have in common the initial 175 aminoacid residues in the N-terminal.

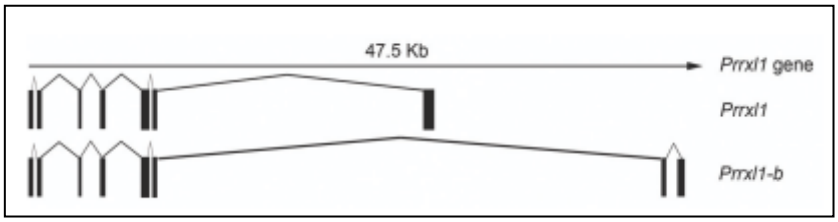


Figure 7: *Genomic organization of the murine Prrxl1 gene, and of the Prrxl1-b isoform. Black boxes indicate the exons.* ***Adapted from Rebelo et al., 2009****.*

As many other paired-like HD TFs, Prrxl1a contains a HD of 60 aminoacids with a helix-loop-helix conformation which mediates DNA binding (Rebelo et al., 2007) (Fig.8). In addition, Prrxl1a possesses a 14 aminoacids conserved motif on the C-terminal, called OAR, previously described in Otp (Semina et al., 1996) and Aristaless homeobox TFs (Gage and Camper, 1997). This domain was associated with brain vertebral malformations in transgenic mice lacking this domain on Cart1 homeoprotein (Brouwer et al., 2003). OAR domain in Cart1 as well as in other HD TF, Prx1, function as an intra-molecular switch that attenuates the activity of the transcription factor. Indeed, OAR deletion increases DNA binding (Norris and Kern, 2001a, b, Brouwer et al., 2003).

Prrxl1a, contains 263 residues while the recently found Prrxl1b contains 220 residues. Prrxl1b just differ from Prrxl1a in the C-terminal, where it lacks the OAR motif (Rebelo et al., 2009). To date, only one anti-Prrxl1 antibody was reported and recognizes Prrxl1 isoforms (Rebelo et al., 2007). However, an anti-Prrxl1b antibody is still lacking.

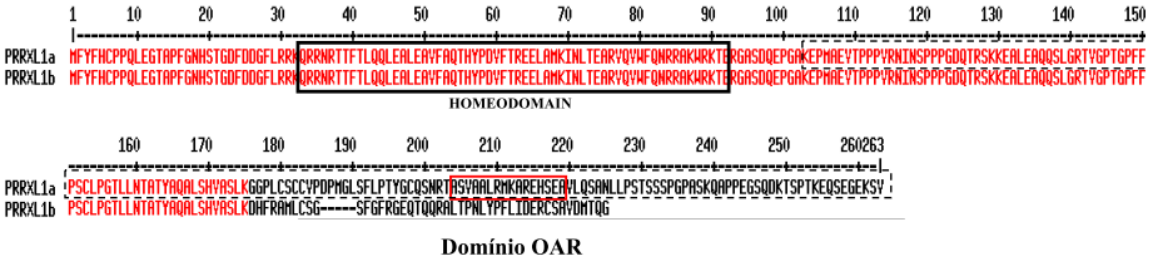


Figure 8: *Prrxl1a and Prrxl1b protein sequences alignment. Both isoforms contain the HD domain, whereas only Prrxl1a contain the OAR motif. The red-colored residues represent the common aminoacids of both isoforms while the black-colored residues represent the region that is different between the isoforms. The region spanning aminoacids 103–263 of Prrxl1a was used for antibody generation (indicated by the broken lined box).* ***Adapted from Rebelo et al., 2007****.*

4.2. PRRXL1 EXPRESSION PATTERN THROGHOUT THE DEVELOPMENT

The expression of Prrxl1 throughout the development had been described by Rebelo et al. (2007) through immunohistochemistry (Fig.9A-I) and by Chen et al. (2001) through *in situ* hybridization (ISH) (Fig.9J-L). Prrxl1 expression is first detected at E10.5 in DRG and dorsal spinal cord (Fig.9A). In DRG, Prrxl1-expressing neurons are generated between E10.5 and E11.5 (Fig. 9A and B) and the expression persists with no apparent changes until perinatal age (Fig.9C-G). In the spinal cord, Prrxl1 is

expressed in dI3 and dI5 neurons early-born neurons, which correspond to the first wave of neurogenesis (Fig.9A). At E11.5 Prrxl1 is detected in early born neurons that are migrating (Fig.9B). Between E12.5 and E13.5, the number of Prrxl1-expressing cells increases when the second wave of neurogenesis is occurring(Fig.9C and D) and neurons migrate from dorsal periventricular area to the dorsolateral spinal cord region (Fig. 9C-E).

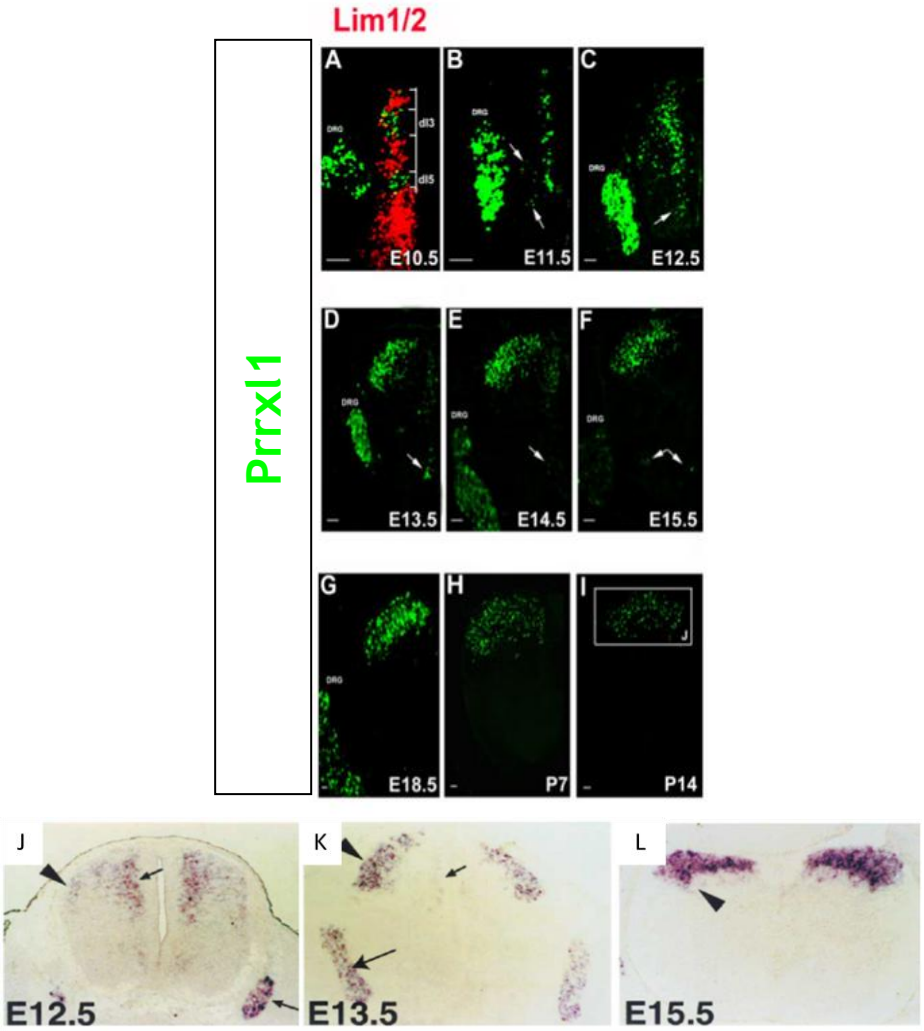


Figure 9: *Spatio-temporal analysis of Prrxl1 expression on developing neural tube and dorsal root ganglia (DRG) by immunohistochemistry (A-I) and in situ Hybridization (J-L). Prrxl1 is initially expressed at E10.5(A) in dI3 and dI5 interneurons and DRGs (green labeling). Lim1/2 labels dI2, dI4 and dI6 (red labeling). Prrxl1 is detected in migrating post-mitotic neurons located in the periventricular zone at E11.5 and E12.5 (arrows in B,C and J) and also in DRG. Prrxl1^{+} expression starts to converge into laminae I-III (D-G, K and L). Postnatally, Prrxl1 expression decreases in superficial dorsal horn (H and I). Scale bars=50 μm.* ***Adapted from Rebelo et al., 2007 (A-I) and Chen et al., 2001 (J-L).***

From E15.5 onwards, Prrxl1^{+} neurons occupy the most superficial region of the spinal cord (Fig.9F-L). At E18.5, when spinal cord lamination is distinguishable, Prrxl1 expression extended from lamina I to III and it is maintained postnatally, although decreasing along time (Fig.9 F-I). In some brainstem areas, Prrxl1 expression is also detectable from E12.5 to P14. Such areas are the Principal trigeminal nucleus, Spinal trigeminal nucleus, nucleus of the solitary tract, and *nucleus prepositus*. In summary, Prrxl1 expression is restricted to the nervous system and occurs in peripheral ganglia as well as in spinal cord and brainstem areas related to the processing of somatosensory and viscerosensory information.

4.3. ROLE OF PRRXL1 HOMEODOMAIN TRANSCRIPTION FACTOR IN THE NOCICEPTIVE SYSTEM DEVELOPMENT

Prrxl1 is simultaneously expressed in peripheral nociceptors and in their putative central targets neurons (Rebelo et al., 2006). Such coordinated expression pattern suggested that Prrxl1 could be involved in establishing connectivity between first and second order nociceptive neurons. Prrxl1 null mice exhibit temporal and spatial abnormalities in the initial penetration of afferent fibers into the dorsal spinal cord, and, afterwards an increased cell death on Lamina I-III of the spinal cord exists (Chen et al., 2001, Rebelo et al., 2006). In fact, about 85% of glutamatergic populations that normally express Prrxl1 are lost (Rebelo et al., 2010). Glutamatergic subpopulation that does not express Prrxl1 as well as the GABAergic population are spared. Thus, such data suggests that Prrxl1-dependent neurons in the superficial dorsal horn die instead of following another differentiation pathway. Furthermore, in DRG occur a 30% reduction of peptidergic and non-peptidergic neurons around P7 by apoptosis that might be due the absence of their neuronal targets in the superficial dorsal horn (Rebelo et al., 2006). Accordingly, afferent innervation of several tissues such as skin and bladder are significantly reduced (Rebelo et al., 2006). This phenotype suggests that Prrxl1 is involved on the development of the nociceptive system. Indeed, several behavioral tests have shown reduced noxious responses on Prrxl1 null mice. Such developmental and nociceptive imply that defects Prrxl1 TF is crucial for the establishment of the nociceptive system.

To understand how Prrxl1 exerts its function, it is of utmost importance to identify its target genes. The only Prrxl1 target gene identified so far is DRAGON

which is implicated in axon guidance (Monnier et al., 2002) and coexpressed with Prrxl1 in developing DRG and spinal cord (Samad et al., 2004). DRAGON expression is decreased in Prrxl1 null mice, however, its expression starts before the onset of Prrxl1 expression suggesting that Prrxl1 is not the only TF to be involved on the regulation of *DRAGON* transcription. Nevertheless, little is known about the Prrxl1 transcriptional program.

4.4. YEAST-TWO-HYBRID: A TECHNOLOGY TO IDENTIFY PROTEIN BINDING PARTNERS

The function and regulatory mechanisms of several proteins are often addressed via Yeast Two Hybrid (YTH) approach. The YTH system allows the characterization a protein interactome, i.e, the set of proteins that interact with a protein under study. Based in the identification of the binding partners, it is possible to infer the putative functions of the prey proteins. This technology is based on the fact that most eukaryotic transcription activators have two functionally independent domains, the N-terminal containing a DNA-binding domain (BD) that recognizes a specific DNA sequence in the promoters of different genes, and a C-terminal DNA-activation domain (AD) that brings the transcriptional machinery to the promoter closeness.

In 1989, Fields and Song created two fusion proteins, one containing the AD of yeast GAL4 (a yeast transcription factor involved in galactose metabolism) fused with a protein X (prey), and another containing the GAL4-BD, which recognizes the UASG sequence, fused with a protein Y (bait) (Fields and Song, 1989). These two elements were cointroduced into yeast with one or more reporter genes, with independent promoters, that were made to be transcriptionally dependent on activation through a binding site recognized by the BD (for review see Bruckner et al., 2009). The binding of fusion X with fusion Y leads to transcriptional activation of the reporter genes (Fig. 10). Nowadays, are widely used reporter genes such as MEL1 gene (encoding α-galactosidase that is secreted into the culture medium), LacZ gene (encoding β-galatosidase) and auxotrophic genes such as HIS3 and ADE2 that allow the yeast to grow in medium lacking histidine (his) or adenine (ade), respectively. Currently, in this method a yeast strain expressing the bait protein is mated with another yeast strain of opposite mating type pretransformed with the cDNA library. Interaction between two proteins can then be determined by the activation of one or more reporter genes in the diploid strain. The advantages of this approach are the possibility of

using frozen aliquots of pretransformed yeast cells saving time and resources, and that diploid cells are more tolerant to expression of toxic proteins and less false positives will appear since the diploids have reporter genes less sensitive to transcription (Kolonin and Finley, 1998).

Before proceeding to the mating, it must be ensured that protein fusions are expressed from the plasmids at appropriate levels (high enough to generate an interaction signal but not so high to cause toxicity). Also, it must be checked whether the bait itself has the capacity of autoactivation of the reporter gene. Thus, if the protein is properly expressed and no autoactivation or toxicity is observed, mating can be performed.

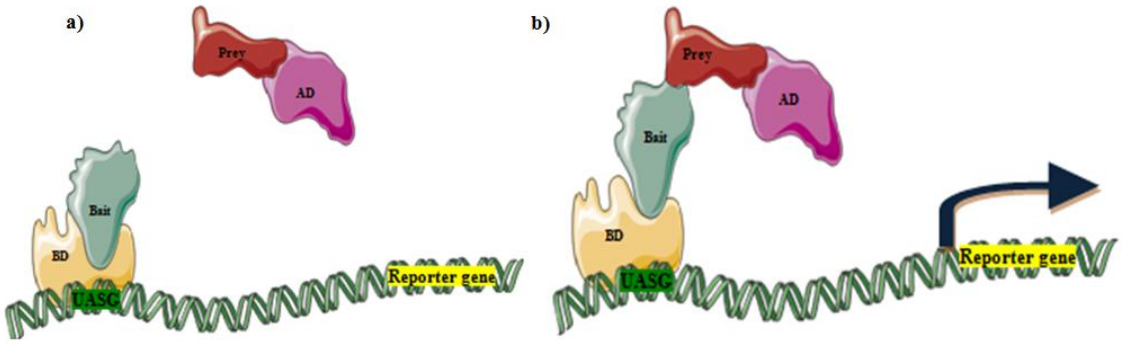


Figure 10: *The yeast two-hybrid system. Two fusion proteins are expressed in yeast: a) GAL4 DNA-binding domain (BD) fused to a bait protein and an activation domain (AD) (for example: the GAL4 AD) fused to a prey protein. The BD-bait hybrid protein can bind to upstream activation sites (UASG) but cannot activate transcription. The AD-prey protein cannot recognize the UASG, thus, alone is not capable of initiating transcription. b) When the bait and the prey interact, the BD and AD are brought together and can activate reporter gene transcription.*

5. OBJECTIVES

The objective of the present study was to unveil some aspects of the Prrxl1 regulation and function. Thus, we aimed to identify Prrxl1 interacting partners through Yeast-Two-Hybrid approach (Part II, chapter I) and validate a group of Prrxl1 putative target genes previously identified by chromatin immunopercipitation followed by massive parallel sequencing (ChIP-Seq) (Part II, Chapter II).

Part II – RESEARCH PROJECT

Chapter I- SEARCH FOR PRRXL1A FULL LENGHT AND PRRXL1B C-terminal INTERACTING-PROTEINS BY THE YEAST-TWO-HYBRID SYSTEM

1. INTRODUCTION

Analysis of mouse embryonic spinal cord and DRG extracts shows a pattern of four bands for Prrxl1a differing approximately 1kDa from each other, which are differentially expressed across development, suggesting that post-translational modifications may have a functional role in modulating Prrxl1 function at different stages of development (Rebelo et al., 2007). Recently, we have shown that this Prrxl1a bands pattern is a result of several sates of phosphorylation using dephosphorylation assays (Soares dos Reis et al., 2010). In addition, cell culture dephosphorylation assays have shown that both Prrxl1a and Prrxl1b are phosphorylated (Soares dos Reis et al., 2010).

Phosphorylation in HD TFs has been described to affect protein-protein interaction, DNA binding and nuclear translocation (Kapiloff et al., 1991, Segil et al., 1991, Bourbon et al., 1995, Coqueret et al., 1996, Coqueret et al., 1998b, Kasahara and Izumo, 1999, Ploski et al., 2004). For example, the human Cut is phosphorylated by protein kinase C (PKC) and by casein kinase II (CKII) (Coqueret et al., 1996, Coqueret et al., 1998b). It was also reported HD-interacting kinases that phosphorylates homeoproteins (Kim et al., 1998). In addition, phosphorylation of homeoproteins may be regulated by cell cycle (Coqueret et al., 1998a) or temporally and spatially during development (Gavis and Hogness, 1991, Lopez and Hogness, 1991). Thus, Prrxl1 phosphorylation might regulate its capacity to bind DNA, interact with others TFs and co-factors or even nuclear carriers. In fact, *In silico* analysis of Prrxl1 protein sequence (Soares dos Reis et al., 2010) had identified several evolutionary conserved putative phosphorylation and kinase sites. These sites are located within the HD (DNA-binding domain), OAR domain (likely regulator of transcriptional activity) and other portions of the protein.

Beyond interactions with protein kinases (Kim et al., 1998) and phosphatases (Alcalay et al., 2007), homeoproteins can also interact with other TFs forming homo- or heterodimers (Wilson et al., 1993). The cooperative dimerization of HDs increases the

binding specificity to palindromic sites and thus occurs more rarely in the genome (Wilson et al., 1993). It was also described an HD-specify importin, called Karyopherin 13, that address Pax6 TF into nucleus via a nuclear localization sequence (NLS), which is located within a segment of 80 amino acid residues that includes the HD (Ploski et al., 2004). Therefore, Prrxl1 interaction with kinases, phosphatases and importins are likely to occur. In addition, it seems likely that Prrxl1 homo- or heterodymerize with other TFs. Accordingly, in order to understand the molecular mechanisms that control Prrx1 activity, it is crucial to identify its interacting proteins.

In the present study, we aimed at cloning mouse Prrxl1a full length (1-263) and Prrxl1b-specific C-terminal sequences (175-222) in pAS2.1 plasmid in frame with the GAL4 DNA-BD in order to perform an YTH, using a commercial library from E17 mouse embryos. With this approach, we expect to characterize the interactome of both Prrxl1 isoforms and give insights into their regulatory mechanisms.

2. MATERIALS AND METHODS

Molecular Cloning

Prrxl1a full lenght and Prrxl1b C-terminal (175-220) ORFs (open reading frames) were amplified by PCR (see primers in Table 1) and each product was ligated in an opened pCR2.1-TA cloning plasmid (Invitrogen) following the manufacturer's instructions. Subsequently, the constructs were sequenced and used to transform chemically competent *E.coli*. After cloning, the constructs were purified from the transformed cells. The pCR2.1 constructs and final pAS2.1 plasmid were sequentially digested with SalI and EcoRI restriction enzymes. Finally, Prrxl1a (1-263) and Prrxl1b-C-terminal (175-220) were ligated to pAS2.1 vector (Fig.1). After sequencing the constructs, we verified that GAL4 DNA BD was in frame with the inserts without mutations.

Table 1. Primers used for amplification of Prrxl1a full length and Prrxl1b C-terminal

ORF	Forward Primer	Reverse primer
Prrxl1a Full Length	3'GAATTCTTTTATTTCCACTGTCCG 5'	3'GTCGACTCATACACTCTTCTCTCC 5'
Prrxl1b C-Terminal	3'GAATTCCTGAAAGATCATTTCCAG 3'	3' GTCGACTCATCCTTGTGTCATACC 5'

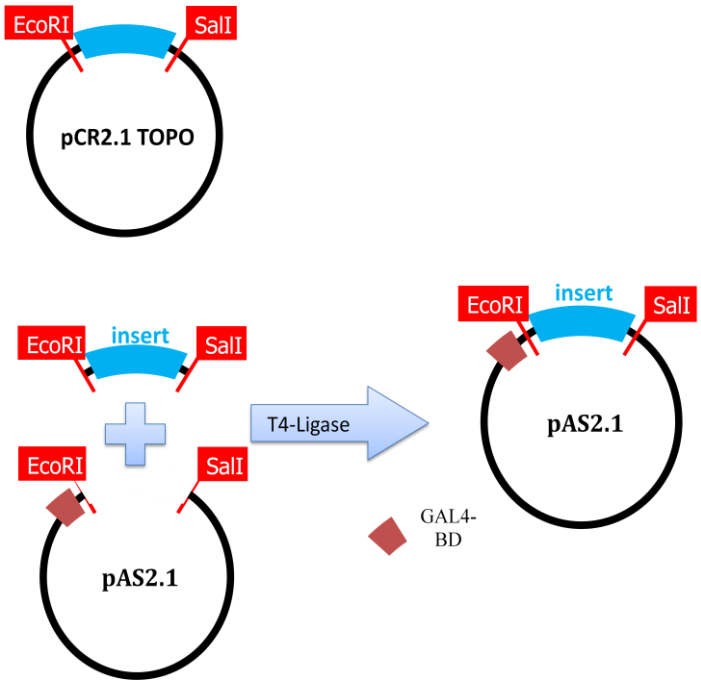


Figure 1: *Diagram showing the molecular clonning strategy used to construct pAS2.1-Prrxl1a and pAS2.1-Prrxl1b-C-terminal plasmids.*

Yeast transformation with plasmid DNA

Preparation of competent yeast cells

Yeasts strain AH109 colonies, previously cultured in agar plates with the appropriate selection media, were inoculated into a 1ml of YPD (Yeast extract, Peptone and Dextrose medium for *S. cerevisiae*) in a 1.5ml microtube and vortexed vigorously to disperse cell clumps. The culture was transferred into a 250ml flask containing 50ml of YPD and incubated at 30ºC with shaking at 230rpm overnight until it reached an OD_{600} higher than 1 unit (corresponding to the stationary phase). An amount (30ml) of this culture was transferred into 300ml YPD, in a 1L flask, to an OD_{600} ranging 0.2-0.3 units. The culture was incubated 3h at 30ºC with shaking at 230rpm. This culture was centrifuged at 2,200rpm for 5min at room temperature and the supernatant was discarded and the cells resuspended in 25ml H_2O. Cells were washed, recentrifuged and the pellet was resuspended in 1.5ml of freshly prepared, sterile Tris-EDTA/Lithium-Acetate (TE/LiAc).

Yeast transformation by the Lithium-Acetate method

In a microtube, 0.1µg of pAS2.1 plasmid was added to 100µg of herring testes carrier DNA. Then, 100µl of freshly prepared competent cells were added to the

microtube, followed by 600µl of sterile PEG/LiAc (40% PEG 4000/TE/LiAc). The solution was incubated at 30ºC for 30min with shaking (200rpm). After adding 70µl of DMSO, the solution was mixed gently and then heat shocked for 15min in a 42ºC water bath. The cells were pelleted, after being chilled on ice, by centrifugation for 5sec at 14,000rpm and resuspended in 0.5ml of 1xTE buffer. Then, 100µl of these cells were plated in SD(Minimal and synthetically medium) -trp (lacking tryptophan) medium (where only the yeasts transformed with pAS2.1 plasmid can grow), and incubated at 30ºC for 2 days (this procedure was done for two transformations of pAS2.1 containing either Prrxl1a (1-263) or Prrxl1b-C-terminal (175-222)).

Assessement of baits toxicity and autoactivation

For each pretransformed cultures, subcultures were made in four different media: -trp; + X-α-Gal and - trp; + X-α-Gal - trp - his; - trp and -his. The cultures were incubated for 3 days at 30ºC. After this procedure, new subcultures were performed from the initial culture, containing 3-aminotriazole (3AT) at different concentrations- 10, 20, 30, 40, 50 and 60mM. 3AT inhibits the activity of the HIS3 reporter product (i.e. imidazole glycerol phosphatase dehydrogenase, allowing only the growth of the culture that express higher levels of this gene required for the synthesis of his).

Bait expression

Preparation of yeasts for protein extraction

For each transformed yeast to be assayed by immunoblotting, 5ml overnight cultures in an appropriate medium (-trp) were prepared by inoculating a colony of the previously transformed yeast (see section 2.2.2). As a negative control an untransformed yeast colony was inoculated in YPD. The overnight cultures were vortexed and added to 50ml of YPD. These cultures were incubated at 30ºC with shaking (220rpm) until OD_{600} reached 0.4-0.6 units (3-6h). The culture was quickly chilled by pouring it into a prechilled 50ml centrifuge tube halfway filled with ice. The tube was immediately centrifuged at 1,000g for 5min at 4ºC. The supernatant was discarded and the pellet washed in 50ml of ice-cold water. The pellet was recovered by centrifugation at 1,000g for 5min at 4ºC and immediately frozen by storing the microtube at –80ºC.

Preparation of protein extracts

Complete cracking buffer was prepared (100µl of cracking buffer were used per 7.5 OD_{600} units of cells) and pre-warmed to 60ºC. The cell pellets were quickly thawed by resuspending in the pre-warmed cracking buffer. The samples were briefly placed at 60ºC to hasten melting. An additional aliquot of the PMSF(0.1M) stock solution was added to the samples every 7min during the procedure (1µl of PMSF 0.1M per 100µl of cracking buffer). Each cell suspension was transferred into a 1.5ml microtube containing 80µl of glass beads per 7.5 OD_{600} units of cells. The samples were heated at 70ºC for 10min to release the membrane-associated proteins. Then, the microtubes were vortexed vigorously for 1 minute and the debris pelleted at 14,000rpm for 5min at 4ºC. The supernatants were transferred to fresh microtubes and placed on ice. The supernatants were stored at –70ºC (or prepared for SDS-PAGE analysis).

Western-blot

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations were carried out using well established methods (Laemmli, 1970). The percentage of acrylamide was 15% in order to resolve GAL4-BD-Prrxl1a(1-263) and GAL4-BD-Prrxl1b-C-terminal(175-220) fusion proteins, which have a estimated molecular weight of 44 and 21 kDa, respectively. After electrophoresis, proteins were transferred into a nitrocellulose membrane (Whatman). The membrane was blocked in 5% of nonfat milk in TBS-T (saline Tris buffer with 0.1% Tween-20) during 1h at room temperature with shaking. After blocking, the membranes were washed with TBS-T and incubated 4h with the respective primary antibodies diluted in 3% of nonfat milk on TBS-T (mouse anti-GAL4-BD 1:150 (CLONETECH); rabbit anti-Prrxl1a (Rebelo et al., 2007) 1:500). Then, the membranes were washed and incubated with the necessary secondary antibodies: anti-mouse IgGs anti-rabbit IgGs conjugated with HRP (horseradish peroxidase; Sigma) diluted 1:1000 in 3% of nonfat milk on TBS-T for 1 hour at room temperature. Blots were revealed exposing an autoradiography film after incubating the membrane with a chemiluminescence substrate (ECL Plus Western Blotting Detection Reagents- GE Healthcare).

3. RESULTS

3.1. Expression of the bait proteins

In order to verify the ability of pAS2.1 recombinant constructs to express Prrxl1a (1-263) and Prrxl1b-C-terminal (175-220) fusion proteins we transformed them into yeast strain AH109 and performed an immunobloting analysis. One band of the expected molecular mass was detect for each fusion proteins using protein extracts from yeast cells containing the pAS2.1-Prrxl1a, pAS2.1-Prrxl1b-C-terminal (175-220) and pAS2.1-PP1α, which was used as a positive control for the anti-GAL4-BD (Fig.2A). In addition, we could verify that GAL4-BD-Prrxl1a fusion protein is expressed using an anti-Prrxl1a antibody (Fig.2B). Therefore, expression of fusion proteins was confirmed.

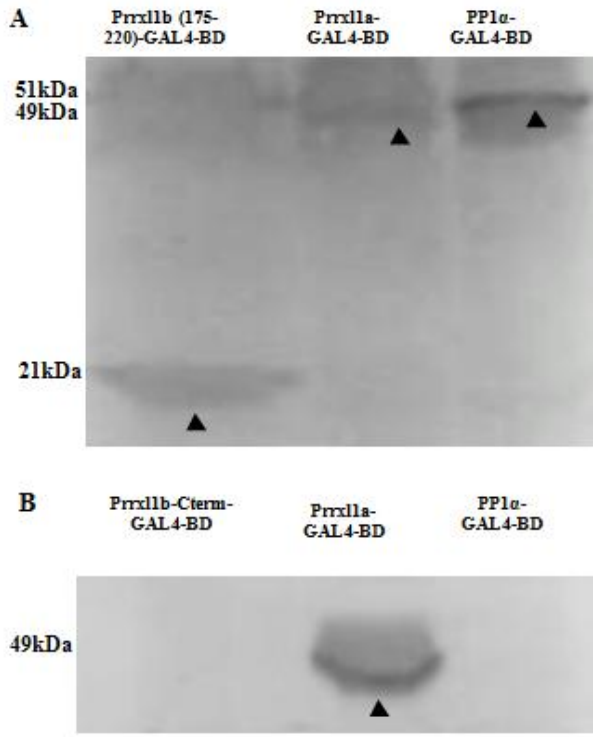


Figure 2: *Confirmation of the GAL4-BD-Prrxl1a and GAL4-BD-Prrxl1b (175-220) fusion proteins expression by Immunoblot analysis. A), Immunoblot using an anti-GAL4BD. B) Immunoblot using an anti-Prrxl1a. Each lane represents the insert product of each strain from where the protein extract was performed. The calculated molecular masses (in kDa) of the PP1α-GAL4-BD, Prrxl1a-GAL4-BD and Prrxl1b-Cterm-GAL4-BD fusion proteins are 51kDa, 49kDa and 21kDa, respectivly .*

3.2. Bait toxicity and autoactivation tests

Before proceeding to mating with a strain containing prey plasmids was performed a toxicity test in order to guarantee that the insert products are not toxic to

the yeasts cells. Thus, the strains containing the pAS-Prrxl1a and pAS-Prrxl1b-C-term plasmids were cultured in an appropriate medium, -trp, which is selective for pAS2.1 plasmid containing strains. This test showed that both inserts did not induce toxicity for the yeasts cells allowing the culture growth (Fig. 3).

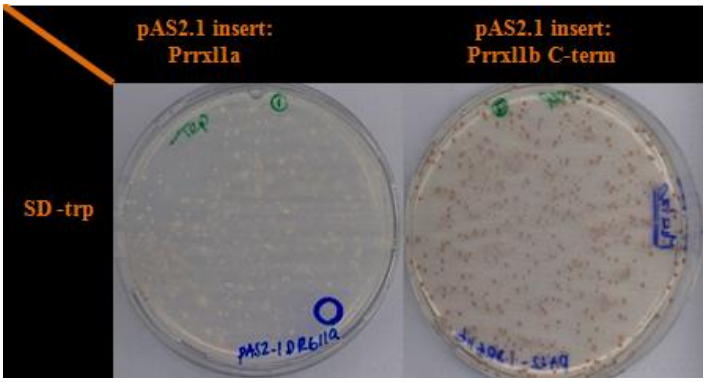
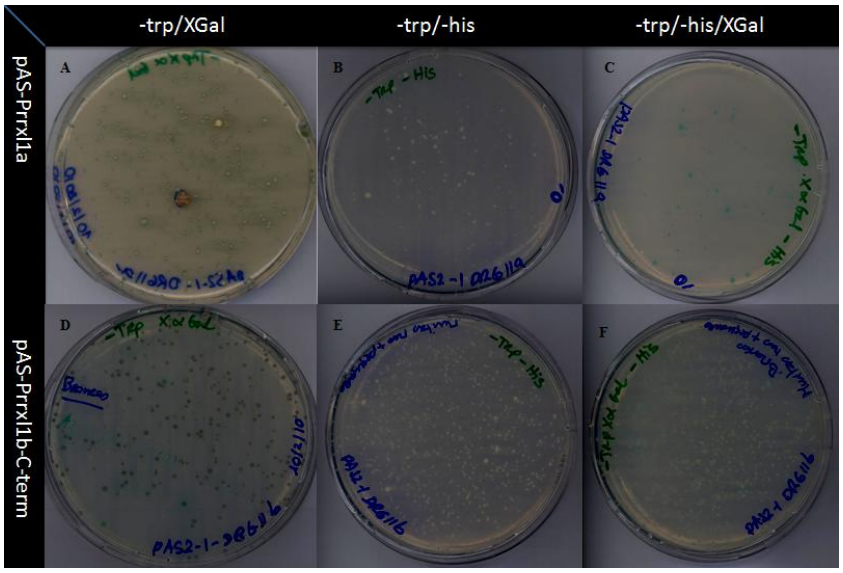


Figure 3: *GAL4-BD-Prrxl1a and GAL4-BD-Prrxl1b-(175-220) fusion proteins are not toxic to yeast. Toxicity test was performed by allowing the pAS2.1-Prrxl1a (left) and pAS2.1-Prrxl1b-C-term (right) transformed yeasts grow in a medium lacking trp which is selective for pAS2.1-transformed strains. Both strains had grown in this medium.*

After the toxicity test, an autoactivation test was performed do determine whether the bait could activate the transcription of the MEL1 and HIS3 reporter genes. Thereby, the transformed strains were cultured in selective media, 1: -trp and –his; 2: + X-α-Gal and – his; 3: + X-α-Gal , -trp and - his. As observed in Fig. 4, yeasts were able to grown in all culture media tested, therefore, the HIS3 reporter gene is transcriptionally activated. Furthermore, in both cultured strains, we observed the formation of blue colonies (Fig.4A, C, D and F) in the media containing X-α-Gal suggesting that the MEL1 reporter gene is also activated.

Due to the activation of the reporter genes by both fusion proteins, cultures were made, using media containing 3AT (which reduces the production of his) at different concentrations ranging 10-60mM and lacking trp and his, to cover the possibility of a residual expression of the HIS3 reporter gene. However, even adding 3AT to the culture media, there was growing of both yeasts strains (Fig.5).



	-trp/XGal	-trp/-his	-trp/-his/XGal
pAS-Prrxl1a	+	-	+
pAS-Prrxl1 C-Term	++	-	+

Figure 4: *GAL4-BD-Prrxl1a and GAL4-BD-Prrxl1b-C-term fusion proteins activate the expression of the reporter genes. Upper panel***:** *The strains transformed with pAS-Prrxl1a (A-C) grown in a medium lacking trp and his (B and C) and form blue colonies in the presence of X-Gal (A and C) which mean that this bait autoactivates the transcription of the HIS3 and Mel1 reporter genes. In the strains transformed with pAS-Prrxl1b-C-term (D-F) there was also an activation of the HIS3 and Mel1 reporter genes. Lower panel***:** *The table below the picture summarizes the relative frequency of blue colonies in each condition ([+], low number of blue colonies; [++], high number of blue colonies; [-], without blue colonies)*

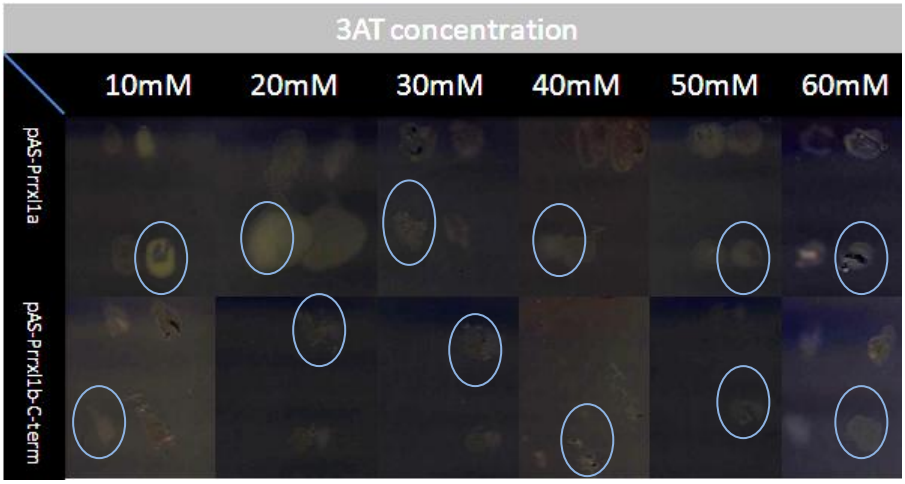


Figure 5: *Both pAS2.1-GAL4-BD-Prrxl1a and pAS2.1-GAL4-BD-Prrxl1b-C-term transformed strains grow in medium containing 3AT. The strains transformed with pAS-Prrxl1a (upper) grown in a medium containing 3AT at different concentrations and lacking trp and his. Thus, this means that the HIS3 was expressed at rates higher enough to resist against 3AT. In the strains transformed with pAS-Prrxl1b-C-term (below) there was also an activation of the HIS3 at rates which overcomes the inhibitory effect of 3AT.*

4. DISCUSSION

The YTH approach allows the characterization of proteins interactomes. Even knowing that some false positives can occur, this method has been greatly improved allowing to overcome such limitation (Bruckner et al., 2009). In this study, our results show another limitation of this technique. Once the YTH system is based on reconstitution of a transcription factor, it is not possible to proceed to an YTH screen if the bait itself activates transcription (although this can be overcome, see below). This was the case in the present study where Prrxl1a and Prrxl1b (175-220) overexpressed in AH109 activated transcription of the reporter genes. Therefore, the YTH screen could not be performed.

Prrxl1 is a member of the large family of HD proteins (Saito et al., 1995) which are characterized by a conserved 60 aminoacid DNA-binding structure, known as the HD that forms three alfa-helices (Gehring et al., 1994, Dragan et al., 2006). This structure binds cooperatively as homo- or heterodimers to palindromic DNA sequences (Wilson et al., 1993). More recently, as it was referred in General Introduction, it was described a new splice variant of this transcription factor (Rebelo et al., 2009). Both Prrxl1a and Prrxl1b contain a HD whereas only Prrxl1a contains a conserved 14 aminoacids motif known as OAR located in the C-terminal (Rebelo et al., 2009). Here, we showed that the cloned baits are transcriptionally active through the transcription of the reporter genes in *Sacharomyces cerevisae*, even the Prrxl1b specific C-terminal sequence that lacks the OAR motif. However, transcriptionaly active domains of both isoforms remain to be determinated.

In another paired-like homeobox gene-Prx1, it was described two isoforms with opposing functions in the regulation of chondrogenesis (Peterson et al., 2005). The Prx1a contains the C-terminal OAR domain and the Prx1b lacks this domain(Norris and Kern, 2001b). The function of the OAR domain is poorly understood, but a deletion of this domain in the Prx1a, as well as in another homeobox transcription factors such as Cart1 and Prx2, leads to an increase in DNA binding and transactivation potential (Norris and Kern, 2001a, b, Brouwer et al., 2003). Such analysis was not yet been performed for Prrxl1a and Prrxl1b. Nevertheless, this result might be due to different transcriptional machinery in the yeasts that besides interacting with Prrxl1 also induce the transcription of the reporter genes

Chapter II- EXPRESSION ANALYSIS OF PRRXL1-PUTATIVE TARGET GENES BY RT-PCR AND TISSUE IN SITU *HYBRIDIZATION*

1. INTRODUCTION

Prrxl1 is specifically expressed in nociceptive neurons and is one of a relatively small number of TFs that are expressed in both peripheral sensory neurons and their putative central targets in the spinal cord. Such coordinated expression suggested a functional role for Prrxl1 in establishing connectivity between first order and second order nociceptive neurons. Indeed, analyses of *Prrxl1*$^{-/-}$ embryos have shown this TF may be involved in migration (Ding et al., 2004), differentiation, axon guidance (Chen et al., 2001) and survival (Rebelo et al., 2006, Rebelo et al., 2010) of nociceptive neurons, as well as for proper adult nociceptive behaviour (Chen et al., 2001). These multiple functions must be based on the Prrxl1-dependent activation of a complex genetic program. However, very little is known about the transcriptional network downstream of this factor. Aiming to give insights into this issue, our group recently performed chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) using a home-made anti-Prrxl1 antibody. ChIP-Seq has been shown to enable genome-wide mapping of protein binding (Valouev et al., 2008). This strategy has identified 2595 *in vivo* binding sites of Prrxl1 homeoprotein in E14.5 mouse dorsal spinal cord using a false discovery rate threshold of <0,05, provided a long list of 939 putative Prrxl1 target genes (Monteiro et al., 2011). Moreover, an *in silico* search for enriched DNA motifs in Prrxl1 bound regions found the TAAT(N3)ATTA consensus sequence, which is associated with binding of HD proteins (Wilson et al., 1993). Gene ontology analysis, of identified Prrxl1 target genes, suggests that Prrxl1 is involved in biological processes related to later aspects of neuronal differentiation, such as neuronal migration, differentiation, axon guidance, and synaptic transmission of dorsal spinal neurons. This preliminary finding is consistent with previous analyses of *Prrxl1*$^{-/-}$ mice and show the potential of this experimental approach in gathering important insights into the biological function of Prrxl1.

As protein-DNA binding alone is not indicative of a regulatory event, expression data from *Prrxl1* mutant mice is needed. On the present study, we selected a small set of

Prrxl1-putative target genes and assessed whether they are under the regulation of Prrxl1. For that, we assessed target genes expression in *Prrx1*$^{-/-}$ and wild type E14.5 mouse embryos by real-time RT-PCR and *in situ* hybridization.

2. MATERIALS AND METHODS

Animals and dissection

Animals used in this study were bred and maintained at the IBMC animal facility. The day when the vaginal plug was formed was considered to be the E0.5. The ethical guidelines for investigation of experimental pain in animals (Zimmermann, 1983) and the European Community Council Directive of 24 November 1986 (86/609/EEC) were followed. Embryos were removed by cesarian surgery of pregnant females.

E14.5 mouse embryonic DRG and dorsal spinal cord tissues were dissected in ice-cold PBS, ressuspended in Trizol (Invitrogen), frozen in liquid nitrogen and stored at -80ºC until completion of genotyping. Prior to total RNA extraction, three samples of individual embryos were pooled according to the genotype (wild type or *Prrxl1*$^{-/-}$ mice).

RNA extraction

Total RNA samples were extracted following RNeasy Mini Kit (Qiagen) protocol. RNA was quantified using Nanodrop (Thermo Scientific) and analyzed on agarose gel to check RNA integrity.

cDNA synthesis

cDNA synthesis was performed according to Biolone manufacturerer's instructions. Briefly, 2µg of dorsal spinal cord or 1 µg of DRG total RNA were warmed 3 minutes at 70ºC to solubilize and then subjected to reverse transcription. cDNAs were used for RT-PCR reactions and synthesis of RNA probes for tissue *in situ* hybridization.

Real-Time RT PCR

Real-time reverse transcriptase (RT) PCR was performed in order to quantify the expression of Prrxl1 candidate target genes relatively to the housekeeping gene *β-actin* in *Prrxl1*$^{-/-}$ and wild type mice. Each 16 µL reaction contained 8.0µL of IQ SYBR

Green Supermix (BioRad), primer mix (designed using Primer3 Plus software) at proper concentration (see table 2) and 1 µL of cDNA template previously diluted in water 1:30 (for dorsal spinal cord samples) or 1:10 (for DRG samples). For all genes, each condition analyzed was made in triplicates. The reactions were run in a Real-Time PCR device (BioRad) with the following programme: one cycle of 5 minutes at 95ºC; 35 cycles of 30 seconds at 95ºC, 45 seconds at 60ºC and 45 seconds at 72ºC. The specificity of the reaction was analyzed by the achievement of an unique melting peak and verifying amplicon molecular weight in agarose electrophoresis. The primers used, as well as their concentration, are summarized on table 2. Relative fold mRNA expression and standard deviation values of Prrxl1 null and Wild type embryos were calculated as Livak and Schmittgen (2001) by normalizing with housekeeping *β-actin* mRNA levels. The results are shown as the mean ± SE of three replicates from one representative experiment. Real-time PCRs were repeated at least three times. As negative control we used a RNA sample subjected to cDNA synthesis without the Reverse transcriptase to exclude possible genomic contamination.

Table 2. Exon spanning primers used for Real-time RT PCR expression analysis

Gene	Forward Primer	Reverse primer	Final Concentration (µM)
β-actin	5' TCATGAAGTGTGACGTTGACATCC 3'	5' GTAAAACGCAGCTCAGTAACAGTC 3'	0.312
DsCAMl1	5' ATGCAGAATGTCACCACCAC 3'	5' TGATGAAGGGACAGCTTTGA 3'	0.156
Cdh4	5' TCGTCAGCACACTCTGAACA 3'	5' GTCACGCTTCTGTCTCCTCA 3'	0.312
Kcnm1a	5' ATTCCTCCCACTCCTCACAG 3'	5' ATCCGGCTCATCTGTAAACC 3'	0.312
Rora	5' AAGAACCACCGAGAAGATGG 3'	5' AATGGAGGAAAATGGAGTCG 3'	0.312
Sema3F	5' GATGCCCTTCTCAGGAAAGA 3'	5' GGTACATGAGCGGATGAGTG 3'	0.312
Dcc	5' CATAAGCCGGATGAAGGACT 3'	5' GTCTGGGAAAGGAACCTCAA 3'	0.312
Efna5	5' ACGTCCAAAGGGTTCAAGAG 3'	5' TTCTTCCGTTGTCTGGGATT 3'	0.312

Statistical analysis of the RT-PCR results

Statistically significant differences on the expression at analyzed genes between *Prrxl1*$^{+/+}$ and *Prrxl1*$^{-/-}$ conditions were averiguated using Student's *t* test. Significant difference were assumed for $P<0.05$.

Riboprobes synthesis for ISH

Riboprobes synthesis by PCR Method

Riboprobes sequences were obtained, as well the primers suitable for their amplification, from previously published articles and databases (GenePaint and MSI). In addition, some probes were designed using Primer3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). In both cases, reverse primers had an extra sequence necessary for *In vitro* transcription which were different for different RNA polymerases (see table 3). Riboprobes sequences were amplified by PCR using cDNA as template and 0.5μL of Proofreading Taq DNA Polymerase (Roche). On this PCR, were also added, 0.5 μL of DMSO, 4 μL of 0.5μM primer mix, 1 μL of dNTPs and of pure water up to 50μL. PCR products were runned in an agaroses gel and purified using DNA gel extraction kit (Easy spin) . Using these purified products as template, we made another PCR under the same conditions of the first PCR. *In vitro* transcription reaction (containing 1 μL of the suitable RNA polymerase -according with the promoter sequence on the reverse primer summarized in the table 3-, 4μL of the proper buffer, 2μL of the 100mM DTT, 2μL of the Dioxygenenin RNA labeling mix (Roche), 0.5μL of the rRNAsin (Promega) and 10.5 μL of the purified PCR product) took 2h at 37ºC. Afterwards, template DNA incubating the reaction 20 minutes at 37 ºC with 1.5μL of DNAse (Promega). Riboprobes were then purified Ilustra Microspinn G-50 columns (GE Healthcare) following the manufacturer's instructions.

Riboprobes were quantified on a *NanoDrop* ND- 1000 UV-*Vis* spectrophotometer (Thermo Scientific) and then diluted in 1ml of hybridization buffer (50% Formamide, saline-sodium citrate (SSC) solution, Denharts solution, 500μg/mL salmon testis DNA and 250μg/mL yeast RNA).

Table 3. Primers for amplification of antisense riboprobes and their adaptor sequence

Gene	Forward Primer	Reverse primer	Adaptor sequence
DsCAMl1	5' GTACTCCTTCAACAAGATCGGG 3'	5'GGTAATACGACTCACTATAGGGTAGCTGATGAAGGGACAGC 3'	T7
Cdh4	5' TACTCCAAACTGTCTGATCCC 3'	5' GGTAATACGACTCACTATAGGGAGTAGAACCGCTGCCTTCATA 3'	T7
Kcnm1a	5' ATGACCCCAAGCTGGACA 3'	5' GGTAATACGACTCACTATAGGGTCACCTCCGCTCTTTTGACT 3'	T7
Rora	5'ACCAGTCGGGATTGGACA 3'	5' GCGATTTAGGTGACACTATAGGCGCGACATTTACCCATC 3'	SP6
Sema3F	5' ATGACCCCAAGCTGGACA 3'	5' GCGATTTAGGTGACACTATAGTCTGTGCCCAGGAAAAGC 3'	SP6
Dcc	5' ATGGTGACCAAGAACAGAAGGT 3'	5' GCGATTTAGGTGACACTATAGCCAACACAGTGAGAACACCAAC 3'	T7
Efna5	5' GACTCCTCTCATCCCCTTCC 3'	5' GGTAATACGACTCACTATAGGGACTGCCCGGTCCTAAAAAGT 3'	T7

Embryo preparation and sectioning

Prrxl1 mutant and wild type E14.5 embryos were obtained by breeding heterozygous mice. The limb buds were excised and sent to IBMC genotyping facility to determine each embryo genotype. Each embryo was fixated overnight with 4% PFA in PBS and then incubated 1 day in 15% sucrose at 4ºC. Subsequently, embryos were embed in O.C.T and frozen, by gradually immersing them in isophentan (-70ºC) and then stored at -80ºC. O.C.T. blocks containing the embryos were criosectioned with a 12µm thickness and collected on Superfrost/Plus laminas (ThermoScientific) and strored at -80 ºC. All solutions used on this procedure were RNAse-free. Embryo sections from *Prrxl1*$^{-/-}$ and wild type mice representing the same body structures were collected in parallel lines on the same lamina.

In situ hybridization (ISH)

Frozen sections were air-dried and the sequentially washed 5 minutes in 1xPBS, post-fixed 10 minutes in 4% PFA/PBS and washed again 5 minutes with PBS for 3 times. Then, sections were treated with 100mM triethanolamine acetylated pH8 by adding drop wise 0.25% acetic anhydride for 15 minutes at room temperature. Finally, sections were pre-hybridized with pre-warmed hybridization buffer at least 60 minutes at 70 ºC.

All pre-hybridized sections were incubated overnight with 200µL of RNA probe (600-1000ng/mL of RNA probe, diluted in Hybridization buffer and pre-warmed at 70ºC) in a wet chamber. Afterwards, sections were washed 60 minutes with pre-warmed washing buffer (50% formamide, 2xSSC buffer, 0,1%Tween20) twice. Then, sections were washed for 15 minutes with buffer 1 (100mM Tris-HCl pH7.5, 150mM NaCl and 0.1%Tween20) twice. Sections were blocked in buffer 2 (10% goat serum diluted in buffer1) at least 30 minutes at room temperature. After that, sections were incubated overnight with an anti-DIG antibody coupled to alkaline phosphatase (Roche) diluted 1:2000 in buffer 2 at 4ºC. After incubation with anti-DIG, sections were washed for 5 minutes with buffer 1 and incubated 30 minutes with buffer 3 (100mM Tris-HCl pH7.5, 100mM NaCl, 50mM $MgCl_2$, 0.1%Tween20) twice. Then, sections were overlaid with 200µL of filtered NBT/BCIP-0.1%Tween solution (Sigma) and covered with coverlslips and incubated at 4ºC in dark until the desired signal was achieved. To stop the developing reaction, sections were washed 5 minutes with PBS 3 times, post fixed

in 4% PFA/PBS, washed with distilled water, air-dried and finaly, mounted in Glycergel mounting medium (DAKO labometer). Photographs were taken using Zeiss Axioskope 40 photomicroscope at 50x magnification.

3. RESULTS

3.1. Expression pattern of selected *Prrxl1*-putative target genes

We have started by selecting a restricted group of seven Prrxl1 candidate target genes based on the following criteria: high enrichment in dorsal spinal cord Prrxl1 ChIP-Seq data set, co-expression with Prrxl1 and putative biological function, especially axon guidance molecules. Gene expression was first analyzed by consulting sagittal sections of E14.5 mouse embryos from the public *In situ* hybridization (ISH) database GenePaint (http://www.genepaint.org). In addition, we performed ISH with some genes in embryo transverse sections. The genes analyzed in the present study were: *Dscaml1*, *Cdh4*, *Sema3F*, *Dcc* and *Efna5*, which are involved in neuronal projection morphogenesis and axon guidance (Ranscht, 2000, Barlow et al., 2002, Dickson, 2002, Gaillard et al., 2005, Chilton, 2006, Fuerst et al., 2009, McIntyre et al., 2010), being *Cdh4* and *Dscaml1* also involved in homophilic cell adhesion (Ranscht, 2000, Fuerst et al., 2009), and *Rora* and *Kcnma1* that are involved in neuron differentiation processes (Nakagawa et al., 1996, Engel et al., 2006).

In transverse and sagittal sections, *Rora* mRNA expression is apparently absent from DRG and spinal cord (Fig 1). However, *Rora* expression was previously reported in lamina II in dorsal spinal cord (Li et al., 2006). Therefore, ISH in transverse sections have de advantage to reveal lamina-specific gene expression in spinal cord. To date, no *Kcnma1* expression is present in public ISH GenePaint database. Hence, we performed ISH and found *Kcnma1* expression sections in whole spinal cord and DRG (Fig.2). *Dcc* mRNA expression was detected in sagittal sections near the midline in dorsal spinal cord. Transverse sections showed that *Dcc* expression is restricted to the periventricular zone (Fig.1 and 2). In DRG, no *Dcc* expression was observed in both sagittal and transverse sections. *Efna5* and *Sema3F* have a similar expression pattern, being cell restricted in the DRG and enriched in the dorsal spinal cord (Fig. 1, 2). *Dscaml1* expression is detected in DRG and superficial dorsal horn of the spinal cord in both sagittal and transverse sections (Fig.1 and 3A). *Cdh4* expression is present in DRG and diffusely present in whole spinal cord (Fig.1). Such expression is in agreement with the labeling observed in transverse sections (Figure 3C).

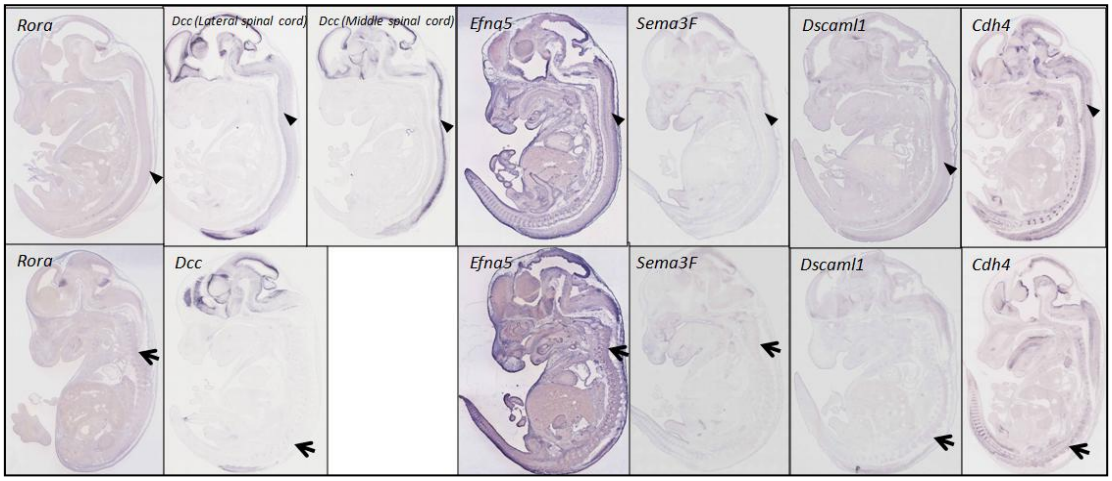


Figure 1: In situ *hybridization showing mRNA expression in the spinal cord (arrowheads in the upper panel) and DRG (arrows in lower panel) of the Cdh4, Dscaml1, Efna5, Sema3F and Dcc in E14.5 wild type mouse embryos. The expression patterns on sagittal sections of an E14.5 mouse embryo are from the public in situ hybridization database. GenePaint (http://www.genepaint.org).*

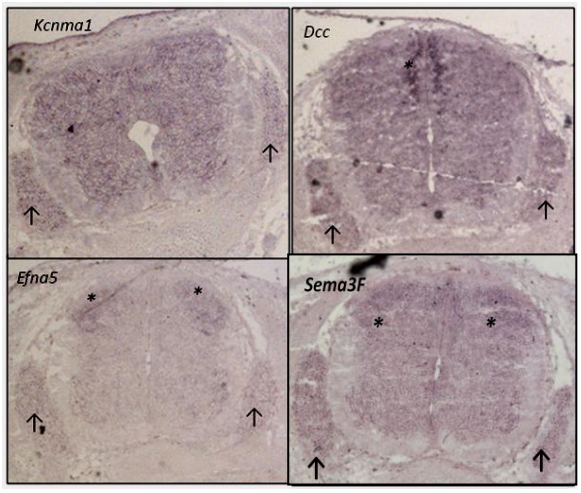


Figure 2: In situ *hybridization showing a mRNA expression in the DRG (arrow) and the spinal cord (asterisks) of Efna5, Sema3F, Dcc and Kcnma1 genes in Prrxl1^{+}/$^{+}$ mice at E14.5.*

3.2. *Dscaml1* and *Cdh4* expression analysis by ISH in E14.5 *Prrxl1*$^{-/-}$ and *Prrxl1*$^{+/+}$ mice

In order to check whether the expression of *Dscaml1* and *Cdh4* are altered in *Prrxl1*$^{-/-}$ mice were made ISH in transverse sections of E14.5 *Prrxl1*$^{-/-}$ and wild type mice. *Cdh4* expression in spinal cord and DRG was similar in *Prrxl1*$^{-/-}$ and wild type embryos (Fig.3C and D). In other hand, *Dscaml1* mRNA expression was apparently downregulated in DRG and dorsal spinal cord of E14.5 *Prrxl1*$^{-/-}$ mice (Fig.3A and B). These results suggest that *Dscaml1* is a Prrxl1 direct target gene in DRG and dorsal

spinal cord, whereas *Cdh4* is not. In addition, Prrxl1 seems to act as transcriptional activator of *Dscaml1* expression.

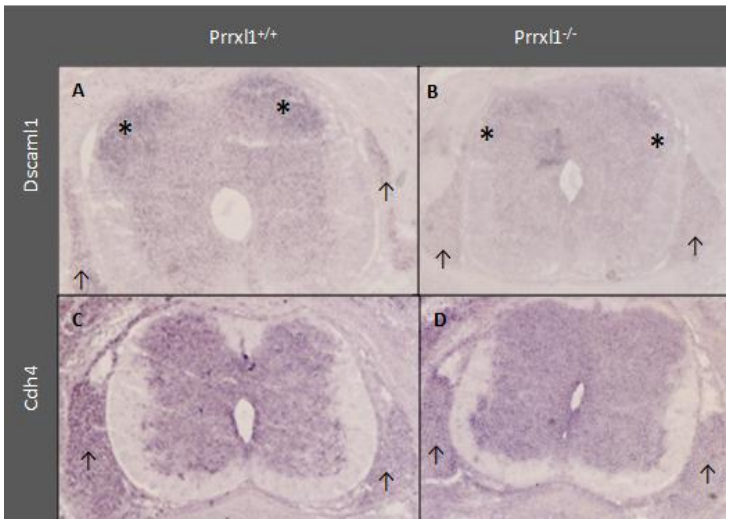


Figure 3: *In situ hybridization showing Cdh4 and Dscaml1 mRNA expression in the DRG (arrow) and the dorsal spinal cord (asterisk) in E14.5 Prrxl1*$^{+}$*/*$^{+}$ *and Prrxl1*$^{-}$*/*$^{-}$ *mice at. Dscaml1 mRNA expression in the DRG and dorsal spinal cord is decreased in Prrxl1 null as compared to wild type mice. In contrast, no significant difference in Cdh4 expression was observed comparing Prrxl1 null with wild type mice.*

3.3. Expression analysis of selected Prrxl1-putative targets by Real-time RT-PCR

In order to find genes directly regulated by Prrxl1, we also performed RT-PCR to assess whether the expression of such genes are altered in E14.5 *Prrxl1*$^{-/-}$ mouse embryos. We found that both *Dscaml1* and *Sema3F* mRNA expression is downregulated in *Prrxl1*$^{-/-}$ mice dorsal spinal cord and DRG (student's t test: $p<0.0001$ for *Dscaml1*; $p<0.01$ for *Sema3F*) at E14.5 (Fig.4). Moreover, *Cdh4* and *Efna5* mRNA expression is downregulated solely in DRG of *Prrxl1*$^{-/-}$ mice (student's t test: $p<0,01$). *Kcnma1*, *Rora* and *Dcc* mRNA expression on the dorsal spinal cord and DRG is not altered in *Prrxl1*$^{-/-}$ mouse embryos, at least in this stage of development. Together, *Dscaml1* and *Sema3F* appear to be Prrxl1 direct targets in DRG and spinal cord, and *Cdh4* and *Efna5* only in DRG. In addition, Prrxl1 seems to work as a transcriptional activator, at least for these genes.

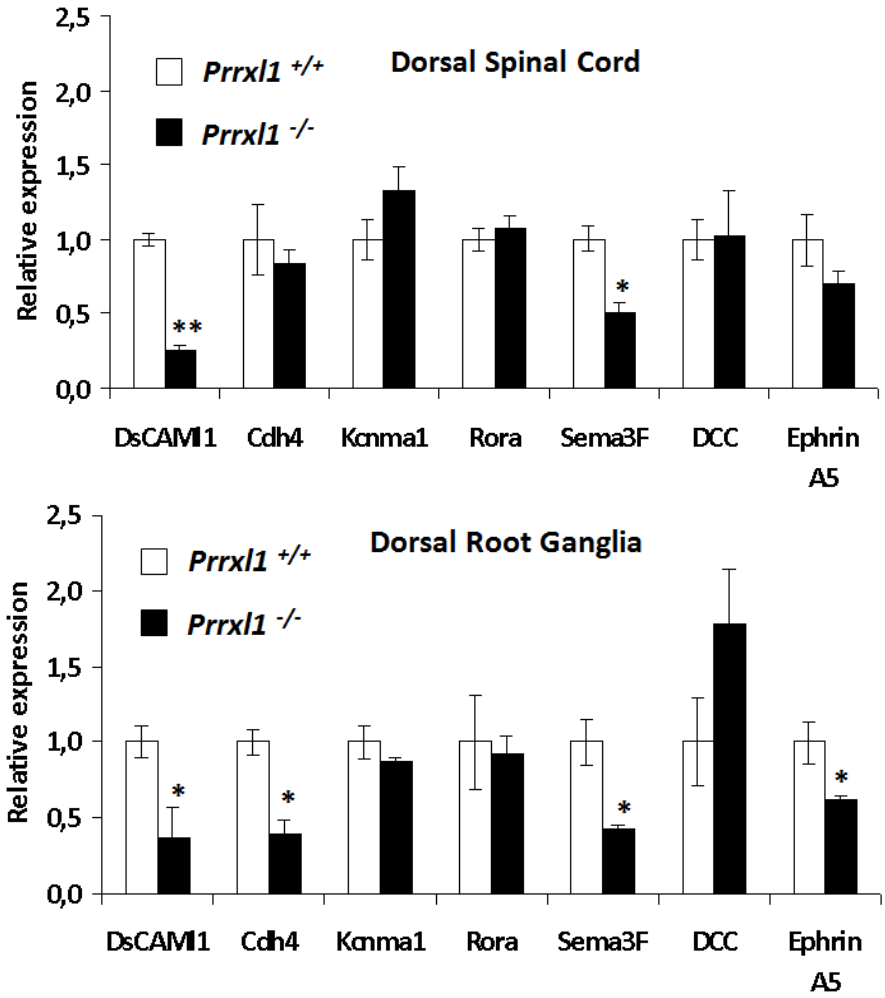


Figure 4: *Expression analysis of selected Prrxl1-putative target genes in Prrxl1$^{-/-}$ mice. RT-PCR was performed on total RNA from dorsal spinal cord (A) and DRG (B) of a pool of three E14.5 embryos either wild type (white bars) or knockout mice (black bars). Gene Expression was determined and the data is shown as relative fold expression of mRNA levels compared to the wild type control and normalized to β-actin expression level. Data represent mean ±SD of triplicate quantifications. Statistical analysis was performed using Student's t test (*, p<0.01; **, p<0.0001).*

4. DISCUSSION

Neuronal recognition involves the balance of attractive and repulsive cues as well as cell adhesion molecules interactions. Together, such cues play a pivotal role for many aspects of the neuronal circuit formation throughout the development. Numerous adhesion/axon guidance systems have been explored and a major consideration is the molecular diversity and binding specificity needed for a molecular code that determines the establishment of neural networks (Dickson, 2002, Chilton, 2006, Schmidt and Rathjen, 2010). Some of such molecules appear to be regulated by Prrxl1 in DRG and dorsal spinal cord (i.e. *Dscaml1* and *Sema3F*) or only in DRG (i.e. *Efna5* and *Cdh4*).

These results suggest that Prrxl1 transcriptional program in DRG and dorsal spinal cord might be in part different. However, we cannot exclude the possibility that *Cdh4* and *Efna5* genes could be regulated by Prrxl1 only in a small subset of Prrxl1-expressing spinal cord cells, where differences in their expression would be difficult to detect by real-time RT-PCR. Therefore, ISH analysis of these genes and the others that did not show differences is also needed.

Dscaml1 is an Ig super family neural cell-adhesion molecule involved in the self-avoidance of several cell types in the developing mouse retina (Fuerst et al., 2009). Its expression was also observed in others neural networks such as developing olfactory sensory neurons, cortex, cerebellum, DRG and dorsal spinal cord (Barlow et al., 2002, McIntyre et al., 2010). Here, we show that Dscaml1 expression is decreased in DRG and dorsal spinal cord of *Prrxl1*$^{-/-}$ mice at E14.5 by ISH and RT-PCR. Thus, Dscaml1 is a Prrxl1 direct target. Such result is in agreement with the morphological abnormalities observed in *Prrxl1*$^{-/-}$ mice, in particular the aberrant migration of the dorsal horn neurons (Ding et al., 2004) as Dscaml1 could be, like in developing retina, related with cell clumping and mosaic spacing between cells (Fuerst et al., 2009). However, such features were not yet investigated neither in spinal cord or DRG in *Dscaml1* mutant mice. Hence, further studies will be essential to clarify the role of Dscaml1 in the nociceptive system development.

Sema3F is a protein secreted into the extracellular space (Messersmith et al., 1995, Takeuchi et al., 2010, Torre et al., 2010) that binds to Neuropilin receptors 2 (Npn2) that affects axon and cell guidance in the developing nervous system (Messersmith et al., 1995, Takeuchi et al., 2010, Torre et al., 2010). Cells expressing Sema3F were found in developing olfactory system where it is secreted by early-arriving olfactory sensory neurons (OSN) axons deposited in the anterodorsal Olfactory bulb to serve as a guidance cue to repel late-arriving OSN axons that express Npn2 receptor (Takeuchi et al., 2010). Sequential arrival of projecting axons and complementary expression of Nrp2 and Sema3F by OSNs appear to contribute to dorsal-ventral patterning in the OB (olfactory bulb). A similar mechanism may occur in the guidance of DRG axons into the dorsal spinal cord, i.e, the first axons reaching the proper lamina in developing spinal cord could repel other late arriving axons from evading their projecting fields by the secretion of Sema3F. In fact, *Prrxl1* mutant embryos display a misguidance phenotype, with a lateral-to-medial shift of central primary afferent projections into the dorsal spinal cord, therefore it would be interesting

to investigate whether downregulation of *Sema3F* expression in both DRG and dorsal spinal cord could be the cause.

Cdh4, also known as R-cadherin, is a poorly studied transmembrane cell adhesion molecule with homophilic binding specificity (Ranscht, 2000, Shibuya et al., 2005). Our observations regarding Cdh4 mRNA expression by ISH in *Prrxl1*$^{-/-}$ mice did not show a significant downregulation in dorsal spinal cord. On the other hand, we were expecting decrease in *Cdh4* mRNA expression in DRG by ISH, as it was observed by RT-PCR. Such differences were not observed maybe due to the lack of ISH methodology sensibility to detect small changes of expression. Nevertheless, we observed that Cdh4 is, in fact, expressed in DRG but also in whole spinal cord at E14.5. This result could explain the lack of differences in the Cdh4 expression between dorsal spinal cord of *Prrxl1*$^{+}$/$^{+}$ and *Prrxl1*$^{-/-}$ mice obtained by RT-PCR.

Another molecule that is downregulated in *Prrxl1*$^{-/-}$ mice DRG is Efna5. Efna5 is a glycosylphosphatidylinositol (GPI)-bound ephrin which binds EphA receptors that are involved in contact-dependent bidirectional signaling between adjacent cells (Wilkinson, 2001). The EphA/ephrin-A class of proteins are expressed throughout many tissues in vertebrate embryos (Gale et al., 1996), and are implicated in several contexts during nervous system development as repellents that prevent cells or axons from entering inappropriate territory, thus stabilizing or establishing patterns of tissue organization (Wilkinson, 2001). Here, it was also shown that EfnA5 expression occurs in some DRG cells and in dorsal spinal cord. Therefore, deregulation of EfnA5 expression in *Prrxl1*$^{-/-}$ mice is a potential candidate to explain the abnormalities in the establishment of proper connectivity between DRG neurons and dorsal spinal cord neurons (Chen et al., 2001). Moreover, ChIP-Seq data have revealed that EfnA5 receptors, i.e, Ephs, such as EphA3 and EphA4 are Prrxl1 candidate targets.

Dcc is a netrin-1's receptor that is expressed in commissural neurons and it is involved in commissural axon guidance towards the spinal cord midline (Serafini et al., 1996, Fazeli et al., 1997). Here, we present its expression pattern in E14.5 mouse spinal cord transverse sections. *Dcc* is weakly expressed in all spinal cord but we detected a stronger expression in the dorsal part of the periventricular zone in transverse sections. Such expression is not related with Prrxl1 expression pattern. This result suggests two possibilities: 1) Prrxl1 could act as a transcriptional repressor of *Dcc*, and 2) Prrxl1 could be regulating Dcc expression at early stages of development.

In summary, we have started to validate some of the Prrxl1 candidate target genes by RT-PCR and ISH in *Prrxl1*$^{-/-}$ embryos and, therefore, these targets are putatively involved in nociceptive system formation. So far, the only Prrxl1 target identified was RGMb (Samad et al., 2004). Here, we confirmed *Dscaml1* and *Sema3F* as direct targets of the Prrxl1 at E14.5 in dorsal spinal cord and DRG. Moreover, *Cdh4* and *Efna5* genes appear to be regulated by Prrxl1 but only in the DRG, suggesting that different transcriptional programs are governed by Prrxl1 in peripheral nociceptors and central spinal neurons.

CONCLUDING REMARKS

Although YTH have been a successful approach to characterize the interactome of several proteins, its application to do such analysis for transcriptionaly active proteins, such as Prrxl1, appear to not be suitable. However, based in recent improvements of this technology (for review see Bruckner et al., 2009), it is possible to identify TFs-interacting proteins. This may be achieved using YTH based on the repression of transactivation preys fused with a repressor domain which interaction with the bait, unlike the canonical YTH, will lead to a lack of expression of the reporter genes. Other possibility, also based in YTH system, would be to use a prey in which its AD induces a RNA polymerase III-mediated transcription in opposition to the classic YTH that uses a RNA Polymerase II-mediated transcription of the reporter genes. Another different approach would be co-immunoprecipitation of Prrxl1 from embryonic tissue, followed by MALDI/MS analysis to identify Prrxl1 partners. In summary, it will be useful, in the future, betake such approaches to study Prrxl1a and Prrxl1b interactome.

On the other hand, our second goal was achieved once we have identified *Dscaml1*, *Cdh4*, *Efna5* and *Sema3F* as Prrxl1 direct targets. Moreover, our results suggest that Prrxl1 might be involved in different transcriptional programs within DRG and spinal cord. Moreover, these validated Prrxl1 targets will be used as positive controls to test RNA samples that will be used for gene expression microarrays analysis. This analysis will allow a global gene expression profiling of DRG and dorsal spinal cord from E14.5 *Prrxl1*$^{-/-}$ mouse. This is an important step in order to combine genome-wide binding (ChIP-Seq) and expression (gene microarrays) data to define Prrxl1 direct target genes and, therefore, reveal the molecular basis for Prrxl1 functions in the establishment of DRG-dorsal spinal cord nociceptive circuit. As future perspectives, we will study the functional role of Prrxl1 targets in nociceptive system development using chick embryo and/or knockout mouse as models organisms.

REFERENCES

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (eds.) (2008) Molecular Biology of the cell. New York: Garland Science.

Alcalay NI, Brantley JG, Sharma M, Gooch JL, Vanden Heuvel GB (Ectopic expression of the homeobox gene Cux-1 rescues calcineurin inhibition in mouse embryonic kidney cultures. Developmental Dynamics 236:184-191.2007).

Anderson DJ (Lineages and transcription factors in the specification of vertebrate primary sensory neurons. Current Opinion in Neurobiology 9:517-524.1999).

Barlow GM, Micales B, Chen XN, Lyons GE, Korenberg JR (Mammalian DSCAMs: roles in the development of the spinal cord, cortex, and cerebellum? Biochem Biophys Res Commun 293:881-891.2002).

Bennett DLH, Dmietrieva N, Priestley JV, Clary D, McMahon SB (trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. Neuroscience Letters 206:33-36.1996).

Bertrand N, Castro DS, Guillemot F (Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3:517-530.2002).

Bourbon HM, Martin-Blanco E, Rosen D, Kornberg TB (Phosphorylation of the Drosophila engrailed protein at a site outside its homeodomain enhances DNA binding. J Biol Chem 270:11130-11139.1995).

Brouwer A, ten Berge D, Wiegerinck R, Meijlink F (The OAR/aristaless domain of the homeodomain protein Cart1 has an attenuating role in vivo. Mech Dev 120:241-252.2003).

Bruckner A, Polge C, Lentze N, Auerbach D, Schlattner U (Yeast Two-Hybrid, a Powerful Tool for Systems Biology. International Journal of Molecular Sciences 10:2763-2788.2009).

Caspary T, Anderson KV (Patterning cell types in the dorsal spinal cord: what the mouse mutants say. Nat Rev Neurosci 4:289-297.2003).

Chen Z-F, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ (The Paired Homeodomain Protein DRG11 Is Required for the Projection of Cutaneous Sensory Afferent Fibers to the Dorsal Spinal Cord. Neuron 31:59-73.2001).

Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, Arata S, Shirasawa S, Bouchard M, Luo P, Chen C-L, Busslinger M, Goulding M, Onimaru H, Ma Q (Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci 7:510-517.2004).

Chilton JK (Molecular mechanisms of axon guidance. Dev Biol 292:13-24.2006).

Coqueret O, Berube G, Nepveu A (DNA binding by cut homeodomain proteins is down-modulated by protein kinase C. J Biol Chem 271:24862-24868.1996).

Coqueret O, Berube G, Nepveu A (The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates p21WAF1/CIP1/SDI1 in S phase. EMBO J 17:4680-4694.1998a).

Coqueret O, Martin N, Berube G, Rabbat M, Litchfield DW, Nepveu A (DNA binding by cut homeodomain proteins is down-modulated by casein kinase II. J Biol Chem 273:2561-2566.1998b).

D'Mello R, Dickenson AH (Spinal cord mechanisms of pain. British Journal of Anaesthesia 101:8-16.2008).

Dickson BJ (Molecular mechanisms of axon guidance. Science 298:1959-1964.2002).

Ding YQ, Yin J, Kania A, Zhao ZQ, Johnson RL, Chen ZF (Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. Development 131:3693-3703.2004).

Dragan AI, Li ZL, Makeyeva EN, Milgotina EI, Liu YY, Crane-Robinson C, Privalov PL (Forces driving the binding of homeodomains to DNA. Biochemistry 45:141-151.2006).

Engel J, Braig C, Ruttiger L, Kuhn S, Zimmermann U, Blin N, Sausbier M, Kalbacher H, Munkner S, Rohbock K, Ruth P, Winter H, Knipper M (Two classes of outer hair cells along the tonotopic axis of the cochlea. Neuroscience 143:837-849.2006).

Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H, Simons J, Bronson RT, Gordon JI, Tessier-Lavigne M, Weinberg RA (Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 386:796-804.1997).

Fields S, Song OK (A novel genetic system to detect protein protein interactions. Nature 340:245-246.1989).

Fu SY, Sharma K, Luo Y, Raper JA, Frank E (SEMA3A regulates developing sensory projections in the chicken spinal cord. J Neurobiol 45:227-236.2000).

Fuerst PG, Bruce F, Tian M, Wei W, Elstrott J, Feller MB, Erskine L, Singer JH, Burgess RW (DSCAM and DSCAML1 function in self-avoidance in multiple cell types in the developing mouse retina. Neuron 64:484-497.2009).

Gage PJ, Camper SA (Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. Hum Mol Genet 6:457-464.1997).

Gaillard S, Nasarre C, Gonthier B, Bagnard D (The cellular and molecular basis of axonal growth. Revue Neurologique 161:153-172.2005).

Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Strebhardt K, Hirai H, Wilkinson DG, Pawson T, Davis S, Yancopoulos GD (Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron 17:9-19.1996).

Gavis ER, Hogness DS (Phosphorylation, expression and function of the Ultrabithorax protein family in Drosophila melanogaster. Development 112:1077-1093.1991).

Gehring WJ, Affolter M, Burglin T (Homeodomain Proteins. Annual Review of Biochemistry 63:487-526.1994).

Gilbert SF (ed.) (2000) Developmental Biology. Sunderland: Sinauer Associates.

Gross MK, Dottori M, Goulding M (Lbx1 Specifies Somatosensory Association Interneurons in the Dorsal Spinal Cord. Neuron 34:535-549.2002).

Helms AW, Johnson JE (Specification of dorsal spinal cord interneurons. Curr Opin Neurobiol 13:42-49.2003).

Inatani M (Molecular mechanisms of optic axon guidance. Naturwissenschaften 92:549-561.2005).

Julius D, Basbaum AI (Molecular mechanisms of nociception. Nature 413:203-210.2001).

Kapiloff MS, Farkash Y, Wegner M, Rosenfeld MG (Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science 253:786-789.1991).

Kasahara H, Izumo S (Identification of the in vivo casein kinase II phosphorylation site within the homeodomain of the cardiac tissue-specifying homeobox gene product Csx/Nkx2.5. Mol Cell Biol 19:526-536.1999).

Kasemeier-Kulesa JC, Kulesa PM, Lefcort F (Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. Development 132:235-245.2005).

Kim YH, Choi CY, Lee SJ, Conti MA, Kim Y (Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. Journal of Biological Chemistry 273:25875-25879.1998).

Kolonin MG, Finley RL (Targeting cyclin-dependent kinases in Drosophila with peptide aptamers. Proc Natl Acad Sci U S A 95:14266-14271.1998).

Laemmli UK (Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227:680-&.1970).

Lawson SN, Crepps BA, Perl ER (Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. The Journal of Physiology 505:177-191.1997).

Li MZ, Wang JS, Jiang DJ, Xiang CX, Wang FY, Zhang KH, Williams PR, Chen ZF (Molecular mapping of developing dorsal horn-enriched genes by microarray and dorsal/ventral subtractive screening. Dev Biol 292:555-564.2006).

Liem KF, Tremml G, Roelink H, Jessell TM (Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 82:969-979.1995).

Light AR, Trevino DL, Perl ER (Morphological features of functionally defined neurons in the marginal zone and substantia gelatinosa of the spinal dorsal horn. J Comp Neurol 186:151-171.1979).

Liu A, Niswander LA (Bone morphogenetic protein signalling and vertebrate nervous system development. Nature Reviews Neuroscience 6:945-954.2005).

Liu Q, Vrontou S, Rice FL, Zylka MJ, Dong X, Anderson DJ (Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. Nat Neurosci 10:946-948.2007).

Lo L, Dormand E, Greenwood A, Anderson DJ (Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development 129:1553-1567.2002).

Lopez AJ, Hogness DS (Immunochemical dissection of the Ultrabithorax homeoprotein family in Drosophila melanogaster. Proc Natl Acad Sci U S A 88:9924-9928.1991).

Ma Q, Fode C, Guillemot F, Anderson DJ (NEUROGENIN1 and NEUROGENIN2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes & Development 13:1717-1728.1999).

Marmigere F, Ernfors P (Specification and connectivity of neuronal subtypes in the sensory lineage. Nat Rev Neurosci 8:114-127.2007).

Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P (Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7:930-938.2004).

McIntyre JC, Titlow WB, McClintock TS (Axon growth and guidance genes identify nascent, immature, and mature olfactory sensory neurons. J Neurosci Res 88:3243-3256.2010).

Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL (Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14:949-959.1995).

Monnier PP, Sierra A, Macchi P, Deitinghoff L, Andersen JS, Mann M, Flad M, Hornberger MR, Stahl B, Bonhoeffer F, Mueller BK (RGM is a repulsive guidance molecule for retinal axons. Nature 419:392-395.2002).

Monteiro FA, Monteiro CB, Rebelo S, Matos M, Reguenga C, Guillemot F, Castro DS, Lima D (2011) Characterization of Prrxl1 neuronal differentiation program in mouse embryonic dorsal spinal cord In: XII Meeting of the Portuguese Neuroscience Society Lisboa.

Müller T, Brohmann H, Pierani A, Heppenstall PA, Lewin GR, Jessell TM, Birchmeier C (The Homeodomain Factor Lbx1 Distinguishes Two Major Programs of Neuronal Differentiation in the Dorsal Spinal Cord. Neuron 34:551-562.2002).

Nakagawa S, Watanabe M, Inoue Y (Altered gene expression of the N-methyl-D-aspartate receptor channel subunits in Purkinje cells of the staggerer mutant mouse. Eur J Neurosci 8:2644-2651.1996).

Norris RA, Kern MJ (Identification of domains mediating transcription activation, repression, and inhibition in the paired-related homeobox protein, Prx2 (S8). DNA and Cell Biology 20:89-99.2001a).

Norris RA, Kern MJ (The identification of Prx1 transcription regulatory domains provides a mechanism for unequal compensation by the Prx1 and Prx2 loci. Journal of Biological Chemistry 276:26829-26837.2001b).

Ozaki S, Snider WD (Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J Comp Neurol 380:215-229.1997).

Perez SE, Rebelo S, Anderson DJ (Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. Development 126:1715-1728.1999).

Perry MJ, Lawson SN (Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. Neuroscience 85:293-310.1998).

Peterson RE, Hoffman S, Kern MJ (Opposing roles of two Isoforms of the Prx1 homeobox gene in chondrogenesis. Developmental Dynamics 233:811-821.2005).

Plenderleith MB, Snow PJ (The plant lectin Bandeiraea simplicifolia I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat. Neurosci Lett 159:17-20.1993).

Ploski JE, Shamsher MK, Radu A (Paired-Type Homeodomain Transcription Factors Are Imported into the Nucleus by Karyopherin 13. Mol Cell Biol 24:4824-4834.2004).

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, Lamantia A-S, James OM, Williams SM (eds.) (2003) Neuroscience. Sunderland, Massachusetts U.S.A.: Sinauer Associates, Inc.

Qian Y, Fritzsch B, Shirasawa S, Chen C-L, Choi Y, Ma Q (Formation of brainstem (nor)adrenergic centers and first-order relay visceral sensory neurons is dependent on homeodomain protein Rnx/Tlx3. Genes & Development 15:2533-2545.2001).

Ranscht B (Cadherins: molecular codes for axon guidance and synapse formation. Int J Dev Neurosci 18:643-651.2000).

Rebelo S, Chen ZF, Anderson DJ, Lima D (Involvement of DRG11 in the development of the primary afferent nociceptive system. Mol Cell Neurosci 33:236-246.2006).

Rebelo S, Lopes C, Lima D, Reguenga C (Expression of a Prrxl1 alternative splice variant during the development of the mouse nociceptive system. Int J Dev Biol 53:1089-1095.2009).

Rebelo S, Reguenga C, Lopes C, Lima D (Prrxl1 is required for the generation of a subset of nociceptive glutamatergic superficial spinal dorsal horn neurons. Developmental Dynamics 239:1684-1694.2010).

Rebelo S, Reguenga C, Osorio L, Pereira C, Lopes C, Lima D (DRG11 immunohistochemical expression during embryonic development in the mouse. Dev Dyn 236:2653-2660.2007).

Saito T, Greenwood A, Sun Q, Anderson DJ (Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol Cell Neurosci 6:280-292.1995).

Samad TA, Srinivasan A, Karchewski LA, Jeong SJ, Campagna JA, Ji RR, Fabrizio DA, Zhang Y, Lin HY, Bell E, Woolf CJ (DRAGON: A member of the repulsive guidance molecule-related family of neuronal- and muscle-expressed membrane proteins is regulated by DRG11 and has neuronal adhesive properties. J Neurosci 24:2027-2036.2004).

Schmidt H, Rathjen FG (Signalling mechanisms regulating axonal branching in vivo. Bioessays 32:977-985.2010).

Schmidtko A, Tegeder I, Geisslinger G (No NO, no pain? The role of nitric oxide and cGMP in spinal pain processing. Trends in Neurosciences 32:339-346.2009).

Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH (Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462:651-655.2009).

Segil N, Roberts SB, Heintz N (Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. Science 254:1814-1816.1991).

Semina EV, Reiter R, Leysens NJ, Alward WLM, Small KW, Datson NA, Siegel-Bartelt J, Bierke-Nelson D, Bitoun P, Zabel BU, Carey JC, Murray JC (Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. Nat Genet 14:392-399.1996).

Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M (Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87:1001-1014.1996).

Serbedzija GN, Fraser SE, Bronner-Fraser M (Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. Development 108:605-612.1990).

Shibuya Y, Takeuchi J, Kobayashi M, Murata M, Munemoto S, Yokoo S, Umeda M, Komori T (N-/R-cadherin expression in dorsal root ganglia and spinal cord. Kobe J Med Sci 51:35-40.2005).

Sivilotti L, Woolf CJ (The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. Journal of Neurophysiology 72:169-179.1994).

Snider WD, McMahon SB (Tackling Pain at the Source: New Ideas about Nociceptors. Neuron 20:629-632.1998).

Soares dos Reis R, Pessoa AS, Reguenga C, Monteiro FA, LIMA D (2010) Prrxl1, a homeodomain transcription factor, is phosphorylated at multiple sites. In: Society for Neuroscience 40th Annual Meeting San Diego.

Stein E, Tessier-Lavigne M (Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science 291:1928-1938.2001).

Takeuchi H, Inokuchi K, Aoki M, Suto F, Tsuboi A, Matsuda I, Suzuki M, Aiba A, Serizawa S, Yoshihara Y, Fujisawa H, Sakano H (Sequential arrival and graded secretion of Sema3F by olfactory neuron axons specify map topography at the bulb. Cell 141:1056-1067.2010).

Taylor AMW, Peleshok JC, Ribeiro-da-Silva A (Distribution of P2X3-immunoreactive fibers in hairy and glabrous skin of the rat. The Journal of Comparative Neurology 514:555-566.2009).

Todd AJ (Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11:823-836.2010).

Torre ER, Gutekunst CA, Gross RE (Expression by midbrain dopamine neurons of Sema3A and 3F receptors is associated with chemorepulsion in vitro but a mild in vivo phenotype. Mol Cell Neurosci 44:135-153.2010).

Valouev A, Johnson DS, Sundquist A, Medina C, Anton E, Batzoglou S, Myers RM, Sidow A (Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. Nat Meth 5:829-834.2008).

Watanabe K, Tamamaki N, Furuta T, Ackerman SL, Ikenaka K, Ono K (Dorsally derived netrin 1 provides an inhibitory cue and elaborates the 'waiting period' for primary sensory axons in the developing spinal cord. Development 133:1379-1387.2006).

Wilkinson DG (Multiple roles of eph receptors and ephrins in neural development. Nat Rev Neurosci 2:155-164.2001).

Wilson D, Sheng GJ, Lecuit T, Dostatni N, Desplan C (Cooperative dimerization of paired class homeodomains on DNA. Genes & Development 7:2120-2134.1993).

Yaksh TL (Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. Pain 37:111-123.1989).

Yamada T, Pfaff SL, Edlund T, Jessell TM (Control of cell pattern in the neural tube: Motor neuron induction by diffusible factors from notochord and floor plate. Cell 73:673-686.1993).