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In embryo and *in silico* mapping of transcription factors expression during early sensory differentiation of the dorsal root ganglion

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Orientadora: Profª. Drª. Chao Yun Irene Yan

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In embryo **and** ***in silico*** **mapping of transcription factors expression during early sensory differentiation of the dorsal root ganglion**

Thesis presented to the Master's Program in System Biology of the Institute of Biomedical Sciences at the University of São Paulo, to obtain the Title of Master of Science.

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RESUMO

BOTEZELLI, V.S. Mapeamento da expressão de fatores de transcrição do gânglio da raiz dorsal *in embryo* e *in silico* durante a diferenciação sensorial inicial. 2021. (102). Dissertação de Mestrado em Biologia de Sistemas – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2021.

O gânglio da raiz dorsal (GRD) é um dos componentes do sistema nervoso periférico que contém o corpo celular dos neurônios sensoriais – sendo eles: mecanoceptivos, proprioceptivos ou nociceptivos. A diferenciação dessas células depende da expressão sequencial de genes específicos, desde o comprometimento sensorial até a diferenciação inicial e tardia das linhagens. O objetivo deste estudo foi definir a identidade de células positivas para SCRT2 e sua posição na cascata gênica de diferenciação do GRD. Primeiramente analisamos o transcriptoma de células positivas para SCRT2 com os dados de scRNA-seq de GRD de camundongo nos estágios E11.5 e E12.5 disponíveis publicamente (Sharma et al., 2020). Observamos que o SCRT2 não está relacionado especificamente a uma população de células em ambos os estágios analisados. Além disso, em estágios avançados, o número de células positivas para SCRT2 e o nível médio de expressão diminuem, o mesmo acontece com POU4F1. Além disso, as células que expressam SCRT2 não expressam marcadores de comprometimento sensorial, como NEUROG2. Juntos, esses dados sugerem que SCRT2 é um componente do módulo gênico de diferenciação inicial. Para confirmar *in embryo* os resultados acima, analisamos o padrão de expressão espacial dos fatores de transcrição SCRT2, ISLET-1, POU4F1, NEUROG2 e PAX3 em GRDs embrionários de galinha. Nossos dados de imunofluorescência e hibridização *in situ* mostram que em estágios anteriores, HH20 e HH25, SCRT2 é de fato co-expresso com ISLET-1 e POU4F1. Além disso, em HH25, o padrão de expressão de SCRT2 é complementar ao de NEUROG2. Em HH30, NEUROG2 não é mais expresso no GRD e SCRT2 e POU4F1 tornam-se restritos à região apical do GRD, enquanto ISLET-1 expande sua área de expressão por quase todo o GRD. Especificamente, já foi mostrado que NEUROG2, ISLET-1 e POU4F1 participam de uma rede gênica regulatória (GRN) neuronal, com diversas conexões de feedback. Assim, nosso próximo passo foi verificar se SCRT2 também faz parte dessa rede e se este fator de transcrição poderia regular a expressão de NEUROG2, ISLET-1 e POU4F1. Utilizamos dados de *Cut and Run* de tubo neural para identificar sítios alvo

de SCRT2 no genoma de galinha. Identificamos sítios alvo de SCRT2 na região genômica dos três genes citados anteriormente. Essas regiões possuíam sítios de ligação de outros fatores de transcrição neurais, sugerindo que essas regiões são potencialmente regiões regulatórias. SCRT2 pode se ligar a esses sítios de ligação de fatores de transcrição e regular a atividade de outros genes presentes nesta GRN. Para confirmar se SCRT2 regula a atividade de ISLET-1, superexpressamos SCRT2 em embriões de galinha e contamos o número de células positivas para ISLET-1 no GRD. O SCRT2 exógeno aumentou o número de células positivas para ISLET-1, indicando que o SCRT2 de fato regula a expressão de ISLET-1. Assim, concluímos que o SCRT2 não está relacionado a uma linhagem sensorial específica no GRD, além disso, o SCRT2 está posicionado na fase inicial de diferenciação na cascata gênica do GRD, regulando a expressão de ISLET-1.

Palavras chave: Gânglio da raiz dorsal. Embrião de galinha. Fator de transcrição neural. scRNA-seq. Rede gênica.

ABSTRACT

BOTEZELLI, V.S. *In embryo* and *in silico* mapping of transcription factors expression during early sensory differentiation of the dorsal root ganglion. 2021. (102). Masters thesis in Systems Biology – Institute of Biomedical Sciences, University of São Paulo, São Paulo, 2021.

The dorsal root ganglion (DRG) is a component of the peripheral nervous system that contains the cell body of sensory neurons- which are divided mainly into: mechanoceptive, proprioceptive and nociceptive. The development of these cells depends on the sequential expression of specific genes from commitment to early and late sensory lineage differentiation. The aim of this study was to define the identity of SCRT2-positive cells, its position in the DRG gene cascade. For the first aim, we analyzed the transcriptome of SCRT2 positive cells with the publicly available E11.5 and E12.5 mouse DRG scRNA-seq data (Sharma et al., 2020). We observed that SCRT2 is not specifically related with a cell population in both stages analyzed. Also, the evolution of the temporal expression pattern of SCRT2 and the early commitment gene POU4F1 suggest a positive covariance. For instance, in advanced stages, the number of SCRT2 positive cells and average single-cell expression level decrease. Further, cells that express SCRT2 do not express commitment markers, as NEUROG2. Together these data suggest that SCRT2 is a component of the early differentiation gene module. To confirm *in embryo* the above results, we analyzed the spatial expression pattern of the transcription factors SCRT2, ISLET-1, POU4F1, NEUROG2 and PAX3 in chick embryonic DRGs. Our immunofluorescence and *in situ* hybridization data show that in earlier stages, HH20 and HH25, SCRT2 is indeed co-expressed with ISLET-1 and POU4F1. Also, at HH25, SCRT2 expression pattern is complementary to that of NEUROG2. At HH30, NEUROG2 is no longer expressed in the DRG and SCRT2 and POU4F1 became restricted to the apical region of the DRG, while ISLET-1 expand its expression area through almost the entire DRG. Specifically, NEUROG2, ISLET-1 and POU4F1 have been shown by others to participate in a neuronal genetic regulatory network (GRN), with diverse feedback connections. Thus, our next step was to verify if SCRT2 is also part of this network and could regulate the expression of NEUROG2, ISLET-1 and POU4F1. We used neural tube *Cut and Run* data to identify SCRT2 target sites in the chick genome. We identified SCRT2-target sites in the genomic region of all three genes. These sites were all located in

evolutionarily conserved non-coding regions. Finally, these regions were enriched for binding sites of other transcription factors, suggesting that these are potential regulatory regions. Thus, with this analysis, we conclude that SCRT2 can bind to those transcription factors binding sites and regulate the activity of other genes present in this GRN. To confirm if SCRT2 regulates the activity of ISLET-1 we overexpressed SCRT2 in chick embryos and counted the number of ISLET-1 positive cells in the DRG. Exogenous SCRT2 increased the number of ISLET-1 positive cells, indicating that SCRT2 indeed regulates the expression of ISLET-1. Thus, we conclude that SCRT2 is not related to a specific sensory lineage in the DRG, besides that, SCRT2 is positioned in the early differentiation phase in DRG gene cascade, regulating the expression of ISLET-1.

Keywords: Dorsal root ganglion. Chick embryo. Neural transcription factor. scRNA-seq. Gene regulatory network.

1. INTRODUCTION

1.1 Mouse and chicken embryos as experimental models for DRG research

The majority of research on dorsal root ganglion (DRG) development are in mouse or chicken embryos. Here, we will explore conclusions derived from both animal models. To facilitate understanding and comparison of DRG development and embryonic stages of those two organisms, we created a timeline that correlates developmental phases of DRG development with developmental stages of mouse and chicken (Figure 1). Mouse embryonic stages are defined by the letter E (e.g. E11.5) and chicken embryonic stages by HH (e.g. HH25).

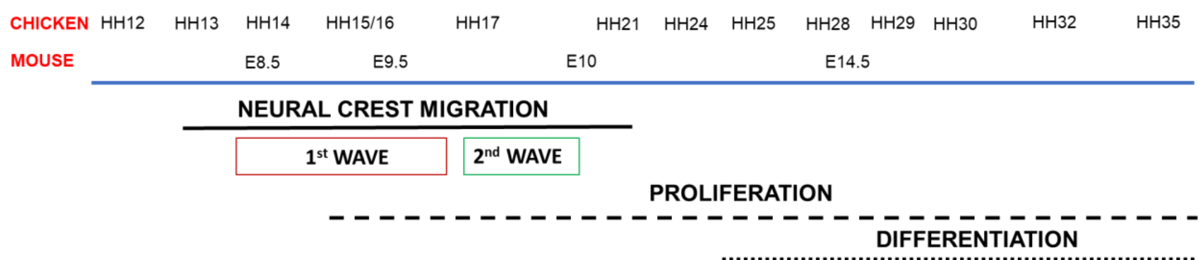


Figure 1 – Timeline in DRG neurogenesis of chicken and mouse embryo. Neural crest cells migration occurs mainly in two waves, at the same time that proliferation in the DRG begins. Sensory neurons differentiation follows onset of proliferation and remains concomitant with mitotic activity in the stages represented here.

1.2 Neural properties of the Dorsal Root Ganglion (DRG)

The somatosensorial system mediates sensations perceived by receptors located in the skin and muscles that are retransmitted to specific targets in the central nervous system (CNS). This system presents three major modalities: proprioception (sense of self-movement and body position), exteroception (sense of the direct interaction existent between the external world and the body) and enteroception (sense of the internal state of the body).

The somatic sensations from the somatosensorial system are mediated by a class of sensory neurons, in which the cell bodies are grouped in the dorsal root ganglia (DRG). Anatomically, the DRG can be identified as a bilateral cell aggregate next to the spinal cord, between the dorsal (composed by sensory fibers) and ventral (composed by motor fibers) root junction (Figure 2). These neurons respond to specific sensory stimuli and present a diversity of morphological and molecular specializations

in their peripheral endings. Mainly, they have two principal functions, transduction of the stimuli received into electrical signals and posteriorly, its transmission to CNS.

The axon of the DRG neurons is bipolar, i.e. present two branches, one projecting to the peripheral tissues and the other projecting to the CNS, the peripheral terminals of these neurons innervate muscles, skin and viscera, and have specific receptors for each type of stimuli. Differently, the central ramifications innervate spinal cord interneurons or the brainstem. In this way, the axon present in each cell serves as a unique line of transmission. Transmission is unidirectional, relaying the signal from the terminal receptor to the CNS. Therefore, the axons are named primary afferent fibers. Different modalities of somatic sensations are mediated by fibers that can differ in diameter and velocity of neurotransmission (KANDEL et al., 2013).

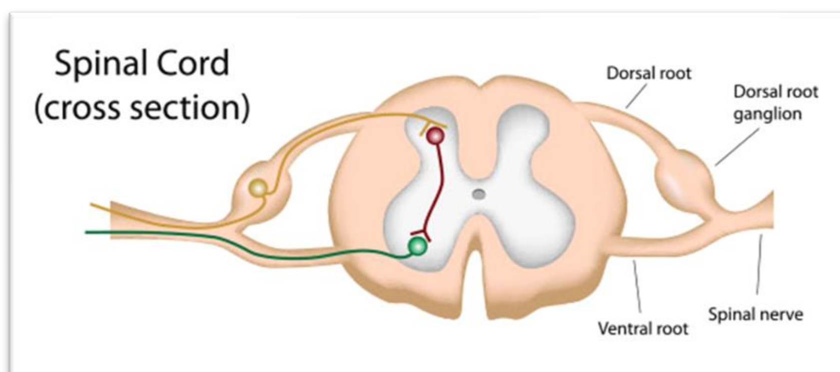


Figure 2 – Illustration of anatomical position of the DRG and a simple representation of the connection between sensory neurons (brown), interneurons (red) and motor neurons (green). Centrally is located the spinal cord, bilaterally, the dorsal and ventral roots, and between them, the DRG. (Dreamstime)

Neurotransduction of the different sensory modalities are carried out by different subtypes of DRG neurons. Accordingly, the sensory neurons subtypes present different characteristics. Small diameter neurons, express the membrane receptor TrkA (TrkA+), and emit thin myelinated or unmyelinated axons. These cells respond to nociceptive sensations of pain and temperature (MARMIGÈRE; ERNFORS, 2007b). The neurons of large diameter, express either TrkB (TrkB+) or TrkC (TrkC+) receptors, and mediate the transmission of mechanoceptive information, related to mechanical shocks and proprioceptive, related to postural state, respectively (RIFKIN et al., 2000).

The diversity of sensory modalities emerges during early embryonic development as subtypes of sensory are generated from a common progenitor population, the neural crest cells (FAURE et al., 2020). A lot of analysis has identified more than twelve functionally distinct subtypes of DRG somatosensory neurons that

collectively enable the detection of a broad range of salient features of the internal and external world (ABRAIRA et al., 2014). Whit the analysis of single cell RNA sequencing (scRNA-seq) data, it is possible to group diverse subtype of cell populations and characterize them by their differentially expressed genes. In our case, we were capable of identify mainly the three sensory subpopulations, nociceptors, mechanoceptors and proprioceptors and some glia populations.

1.3 Development of sensory populations in the DRG

The first neurons of the DRG derive from neural crest cells that delaminate laterally to the neural tube (GEORGE et al., 2010). The neural crest cells undergo epithelial-mesenchymal transition and delaminate from the neural tube (BRONNER, 2012). Next, these cells migrate dorsolaterally to the ectoderm and continues until the ventral median line of abdomen or ventromedially, between the dermomyotome and the neural tube to generate the DRG (LORING; ERICKSON, 1987). Posteriorly, the expression of NEUROG2+ and NEUROG1- contributes to the segregation of differentiated neurons in the center of the DRG, and non-differentiated progenitors in DRG periphery (GEORGE et al., 2010). In summary, the early DRG is organized with proliferating cells in the periphery and differentiated cells in central region.

During DRG development, differentiation events of neuronal subtypes are segregated temporal and spatially. Sensory neurogenesis in GRD occurs mainly in three temporally distinct waves. In a first moment, the neural crest cells that in future will differentiate in large diameter neurons, mechanoceptive and proprioceptive, migrate between the stages HH14-16 in chick embryos (RIFKIN et al., 2000). This neuronal subpopulation is positive for the neurotrophic receptor TrkC and/or TrkB, respectively. The second migration wave occurs between stage HH16-17, and the cells that will form the nociceptive neurons, which are more abundant in the mature DRG. The nociceptive neurons express the TrkA receptor (GEORGE et al., 2007; MA et al., 1999). Finally, the third wave contributes to the multipotent stem cells of the outer limiting membrane of the DRG, located at the dorso-apical region of the DRG (GEORGE et al., 2010).

Proliferation and differentiation of the cells that are already in the DRG occurs concomitantly with the addition of new cells through neural crest migration. The stage

HH24 in chick embryo DRG is organized in concentric pattern that reflects the chronological order of those neurogenesis waves: the precursors generated later are located more peripherally and the differentiated cells more centrally (GEORGE et al., 2010). As development progresses, the organization of the DRG evolves to compartmentalize different sensory modalities in different locations: TrkA neurons became confined to the dorsomedial region and the TrkB and TrkC to the ventrolateral (HAMBURGER; LEVI-MONTALCINI, 1949). These events can be presented as space-temporal line that shows the overlap of migration, proliferation and differentiation together with the expression onset of different molecular markers both n mice and chicken (Figure 3).

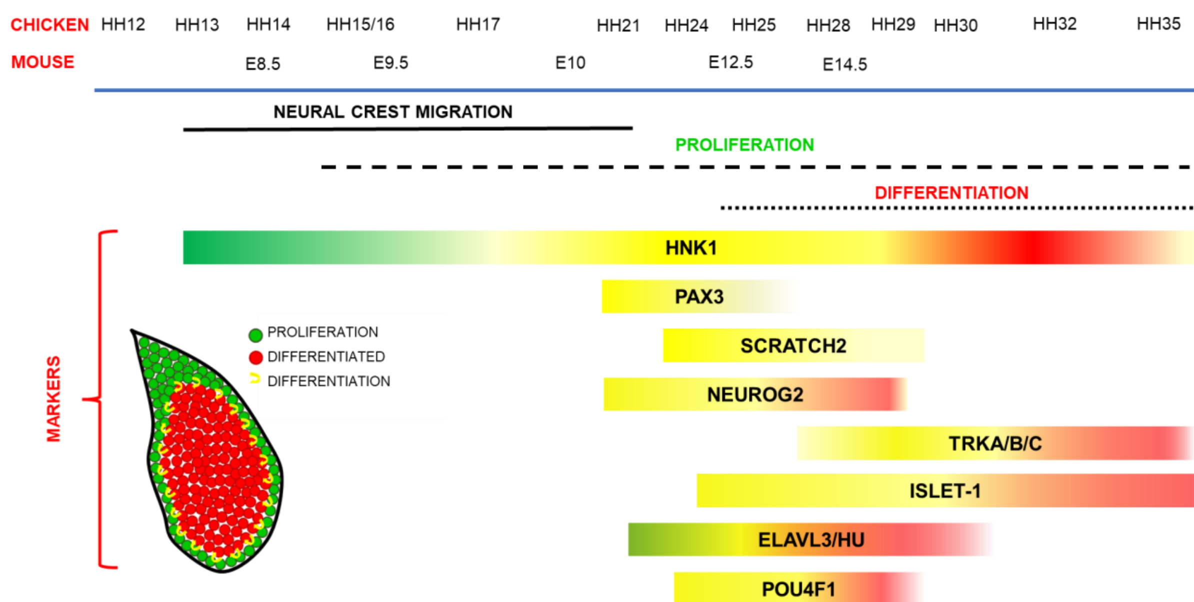


Figure 3 – Timeline of transcription factor expression during DRG development in mouse and chicken. PAX3 and NEUROG2 are expressed right after neural crest migration. SCRT2, ISLET-1, POU4F1 and ELAVL3/HU starts to be expressed almost with the start of differentiation of sensory neurons. The tyrosine kinase receptors TRKA/B/C are expressed only in differentiated cells. In this scheme of HH30 chick DRG, green represents the proliferating cells, yellow the cells in differentiation and red the differentiated cells.

Peripheral neuropathies caused by developmental errors, are characterized by loss of temperature and pain perception, in combination with other sensory and autonomic abnormalities. Some of those disorders results from abnormal sensory neurons development. Many cases of congenital insensitivity to pain (CIP) are caused by mutations of components of the nociceptive neurons signaling pathway. Recently, it was suggested that regulation of early gene expression can cause CIP as well. PRDM12 is an evolutionarily conserved gene expressed in sensory neurons that has

been found mutated in CIP patients (DESIDERIO et al., 2019). PRDM12 is required for the initiation and maintenance of TrkA expression (DESIDERIO et al., 2019). It was suggested that PRDM12 has an epigenetic role in modifying histones and thereby, regulating the expression of genes during neurogenesis (NAHORSKI et al., 2015). Pathologic PRDM12 mutations reported in pain-less patients significantly impair protein function by causing PRDM12 aggregation, disrupting its interaction with binding partners. Due to the PRDM12 impairment, the downstream nuclear signaling controlled by PRDM12 is abolished, and consequently, sensory neurons are not properly formed (MATSUKAWA et al., 2015). This study underscores the importance of identifying components and regulatory elements of the gene cascade relevant for sensory lineage differentiation

1.4 Gene regulatory networks (GRN) in development

Diversity in cell fate depends on a complex combinatorial system centered on the partnership between different transcription factors. In this system, the differentiation of a neural subtype is restricted and refined by the combined availability of factors and co-transcription factors. Therefore, the panel of transcription factors concomitantly expressed in a certain moment determines the spectrum of available differentiation pathways. The concept of GRN, which is growing in the functional genomic field, maps the regulatory interactions between transcription factors. Specifically, in embryonic development, typically the transcription factors target downstream genes and the retro-regulation relation are studied. The GRN are built on in vivo experimental data about transcription factors and theirs binding sites (TFBS) in target genes (LONGABAUGH;DAVIDSON; BOLOURI, 2005).

1.4.1 Genomic Regulatory Elements

Genomic regulatory elements mediate a variety of regulatory processes at the transcriptional (DOANE, ELEMENTO, 2017). A variety of DNA regulatory elements are involved in the regulation of gene expression and rely on the biochemical interactions involving the DNA and nuclear proteins. Among the regulatory elements, promoters and enhancers are the primary genomic regulatory components of gene expression. Promoters are DNA regions located next to the gene transcription start site, that contain short regulatory elements (motifs) necessary to assemble RNA polymerase

machinery. However, transcription do not occur without the contribution of DNA regulatory elements located more distant to the gene transcription start site. These regulatory elements are called enhancers, and they interact with site-specific transcription factors to establish cell identity and regulate gene expression (SHLYUEVA, STAMPFEL, STARK, 2014).

In evolution, the conservation of those non-coding regions suggests an important role in gene expression. As those regions will not be translated into proteins, the conservation of those regions is related with the establishment of gene expression (BERTHELOT et al., 2018). Cellular development, morphology and function are governed by specific patterns of gene expression. These events are established by coordinate action of genomic regulatory elements. In this way, the different action of regulatory elements in different tissue will lead to cellular specific differentiation (SHLYUEVA, STAMPFEL, STARK, 2014).

Genes are regulated by interactions with multiple transcription factors (DOANE, ELEMENTO, 2017). Transcription factors bind to promoters and enhancers and stimulate or inhibits gene transcription through interaction with the DNA and the nuclear transcriptional machinery. Transcription factors form regulatory networks that interact genetically to modulate the cellular transcriptome profile (PAPAVASSILIOU, 1995). The accessibility of a binding site for a transcription factor depends on chromatin availability (HOBERT, 2008). Transcription factors can be classified by their mechanism of action, regulatory function, or sequence homology of their binding domains (LATCHMAN, 1999). The ability of transcription factors to activate transcription has been shown to be dependent of specific regions of the protein, which are distinct from the region mediating DNA binding (PTASHNE, 1988). Several types of activation domains have been described and it is likely that the different activation domains act by interacting with other protein factors to facilitate transcription. Although most transcription factors so far identified act as activators, some of them inhibit transcription (LATCHMAN, 1997).

1.4.2 Types of Transcription Factors

Multiple types of transcription factors already were described as important for different developmental events. Amongst these, here we will focus on zinc-finger (Zn-

finger or ZF), basic helix-loop-helix (bHLH) and POU domain proteins. bHLH proteins have been implicated in the specification of neuronal subtype identities in different model systems (GUILLEMOT, 2007). The bHLH family members NEUROG1 and NEUROG2 were described as required for development of DRG (MA et al., 1999). The members of bHLH family of transcription factors present two highly conserved and functionally distinct domains, one of them, the basic domain, binds the transcription factor to the DNA (Figure 4A). Different members of bHLH family recognize different target sequences, determining their ability to control diverse developmental functions through transcriptional regulation (JONES, 2004).

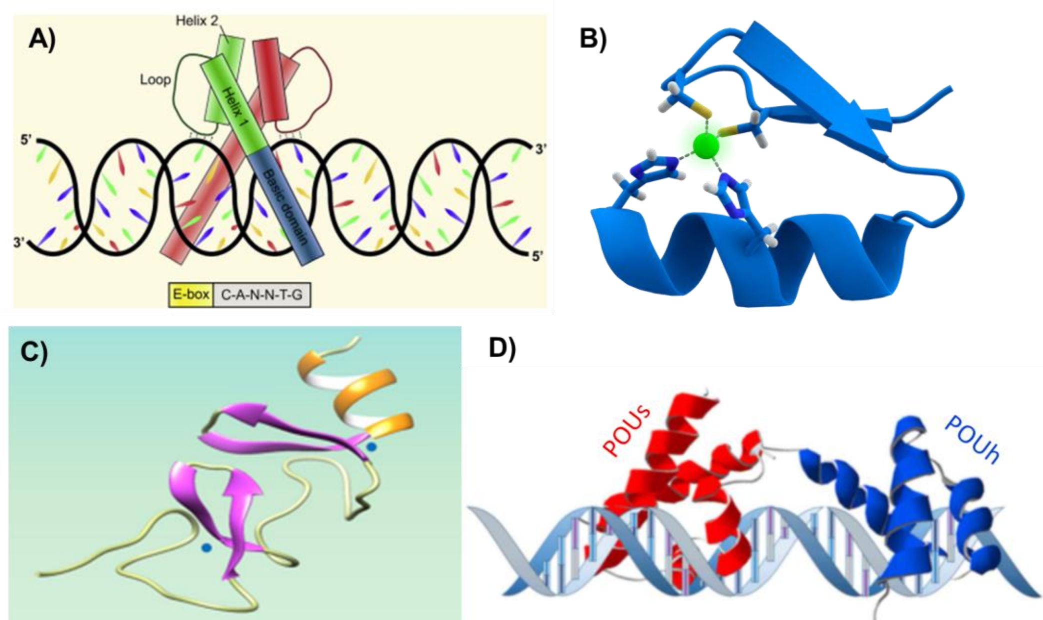


Figure 4 - Types of transcription factors. (A) Structure of bHLH transcription factors is comprised of two alpha helices, which mediate dimerization, and a basic domain, that binds to the E-Box sequences in the DNA; (B) The Zn-finger motif consists of a short antiparallel sheet formed by two strands and a hairpin turn, followed by an alpha helix. The alpha helices bind to the E-Box sequences in the DNA; (C) LIM domains are composed of two Zn-fingers, separated by a two amino acid residue linker. LIM is a protein interaction domain that is involved in binding to may structurally and functionally diverse partners; (D) The POU domain is a bipartite domain composed of two subunits separated by a non-conserved region. The N-terminal subunit is known as POU-specific (POUs) domain, while the C-terminal subunit is a homeobox (POUh) domain. Both subdomains contain the helix-turn-helix motif. (Adapted from DENNIS et al., 2019; GANSS; JHEON, 2004; IQBAL et al., 2020; WU et al., 2021).

Zinc-finger proteins are one of the most abundant groups of proteins and have a wide range of molecular functions (Figure 4B). They can interact with DNA, RNA and proteins. They were described as having a key role in development and differentiation of several tissues. Many ZFs contain multiple and different types of zinc-finger

domains, for example, domains of LIM type (CASSANDRI et al., 2017). ISLET-1 is a transcription factor of the LIM-homeodomain protein family. LIM proteins contain protein-interacting domains, a DNA-binding homeodomain, and a C-terminal region (Figure 4C). SCRT2 is another example of transcription factor of ZF type. SCRT2 belongs to the Snail superfamily of transcription factors, that can be divided into two groups, the Snail and the Scratch families. All the members of Snail superfamily of TFs recognize sites that contains six bases (E-box) the binding site of bHLH transcription factors (NIETO, 2002).

Finally, the members of POU domain family have been shown to be important for cell type specification and for regulating sensory neuron development (HUANG et al., 1999). The POU domain constitutes of a DNA binding domain of the protein and a POU-specific domain which is unique to this class of transcription factors, and a POU homeodomain (Figure 4D). Analysis of members of POU family if transcription factors revealed that those TFs play a critical role in regulating gene expression, particularly in cells of the nervous system (LATCHMAN, 1999).

1.4.3 Interactions between transcription factors

The neuronal subtypes are defined by the expression of a unique combination of transcription factors. The alteration in the expression of key transcription factors can change the identity of a specific neuronal subtype (HOBERT, 2008). The sensory neuronal diversity is large, but the transcription factors involved in the differentiation of those neurons is not. In this way, the conversion of sensory progenitors in specialized subtypes necessarily involves the expression of subtype-specific transcription factors (SHARMA et al., 2020). Besides that, the cooperation between transcription factors in modulating target genes transcription adds complexity in the resulting transcriptome. In other words, the synergism between different transcription factors is essential to the occurrence of cell differentiation and consequently the development.

When two or more transcription factors are expressed in a cell population at the same time and occupy the same hierarchical level in a gene regulatory network, exists the possibility of some types of interactions (Figure 5). First, in the biochemical interaction, one transcription factor forms a complex with another transcription factor that is already in a protein-DNA interaction (Figure 5A) (MARTINEZ; RAO, 2012). In

the joint modulation interaction, one transcription factor binds to a target site and another transcription factor bind to other target site and together, both regulate transcription (Figure 5B) (MORGUNOVA; TAIPALE, 2017). Finally, in the cross-regulation interaction, one transcription factor binds to a target regulatory site near another transcription factor and modulates its transcription and vice-versa (Figure 5C).

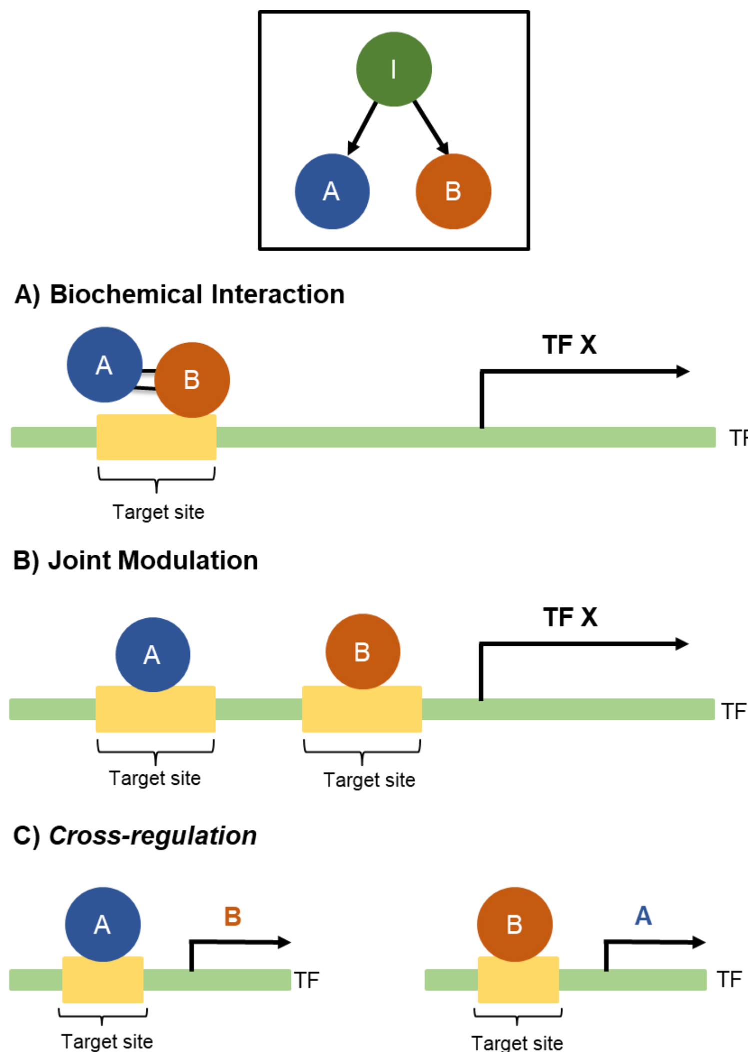


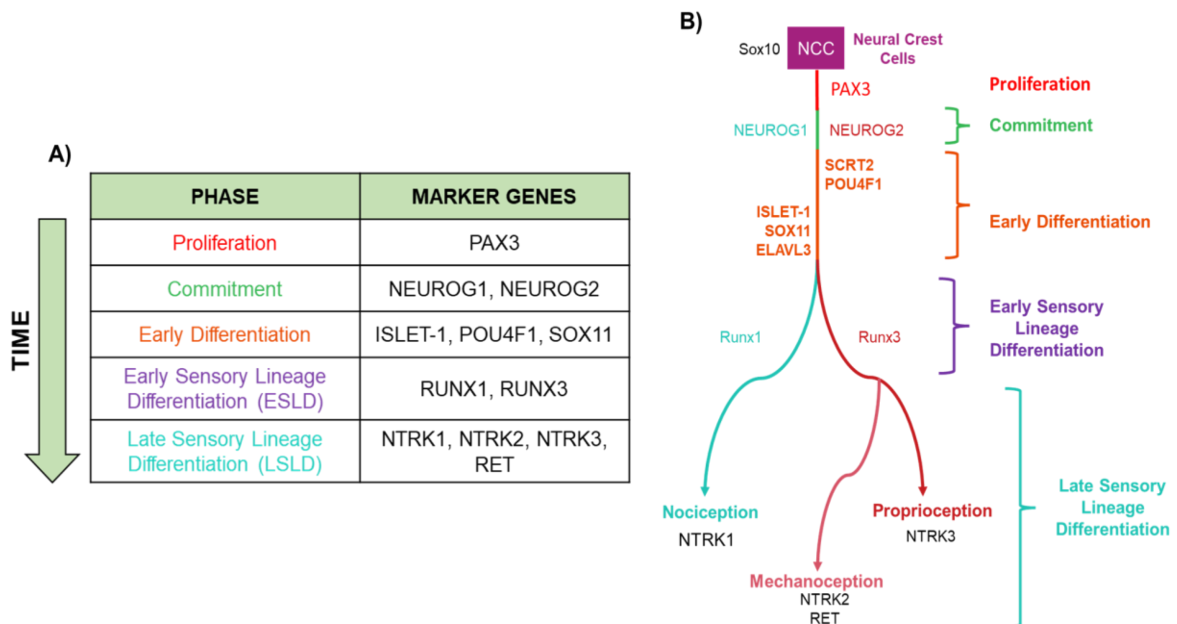
Figure 5 – Representation of three types of possible interactions between transcription factors. Considering two TFs located in the same hierarchical position (first scheme), it is possible to occur three types of interactions. A) Biochemical interaction between two TFs that occupy the same hierarchical level where only one of the TFs binds to the DNA B) Interaction of the type joint modulation between two TFs where both bind to the DNA C) Interaction of *Cross-regulation* between two TFs. Where one TF regulates the expression of the other.

For example, LIM proteins represent one of the biggest classes of transcriptional activators. They cooperate with other activators during cell differentiation, and interact biochemically with bHLH domains of other transcription factors (JOHNSON et al., 1997). Besides that, the combinatorial expression of LIM proteins, as ISLET-1, ISLET-2, LHX1 and LHX3 in motor neurons is correlates with the future innervation targets of these cells (JURATA; PFAFF; GILL, 1998). Another type of cooperation during embryo development occurs between transcription factors of types bHLH and Zn-finger (ACAR et al., 2006). To better understand the complexity and map the underlying GRN of the different differentiation paths to

generate the sensorial cell subtypes, it is necessary to evaluate the possible interactions between transcription factors in embryo.

1.5 Gene cascade in the DRG.

As mentioned above, the differentiation of sensory neurons in the DRG relies on fine temporal control of gene expression for each step (Figure 6). After migration of neural crest cells, the cells proliferate and express markers as PAX3. After mitotic arrest, the transcription factors NEUROG1 and NEUROG2 are expressed. Both specify sensory lineage. Here we will refer to this phase as sensory commitment. After commitment, the DRG cells will express markers for early differentiation, as ISLET-1, POU4F1 and SOX11. During this phase, the cells will start the process of differentiation in neurons but will not define their specific sensory subtype yet. We refer to this moment as early sensory differentiation. Neurons from all the sensory modalities must undergo early sensory differentiation. In other words, all sensory neurons – independent of sensorial modality- evolve from proliferation to early sensory differentiation similarly.



A)

PHASE	MARKER GENES
Proliferation	PAX3
Commitment	NEUROG1, NEUROG2
Early Differentiation	ISLET-1, POU4F1, SOX11
Early Sensory Lineage Differentiation (ESLD)	RUNX1, RUNX3
Late Sensory Lineage Differentiation (LSLD)	NTRK1, NTRK2, NTRK3, RET

Figure 6 – Phases of DRG development. (A) Table containing the phases of DRG development in the first column and the marker genes of each phase in the second column. (B) Gene cascade co-relating the evolution of the phases of DRG development and their markers.

The definition of neuronal subtype into the different sensory modalities depends on the transcription factors RUNX1 or RUNX3. Here, the lineages/modalities differ

significantly in their transcriptome. Cells that express RUNX1 will differentiate in nociceptive neurons, NTRK1/TrkA positive. Differently, the cells that express RUNX3 will differentiate in two sensory subtypes, mechanoceptive and proprioceptive neurons, NTRK2/TrkB and NTRK3/TrkC positive, respectively. Here, we will refer to this phase as late sensory differentiation phase.

1.5.1 Proliferation marker PAX3

PAX3 is specifically expressed in the DRG undergoing formation, but its expression is not maintained during sensory neurons differentiation (MONSORO-BURQ, 2015). The period when PAX3 is expressed (GOULDING et al., 1991), corresponds to the phase where occurs cellular proliferation in mice DRG (GOULDING et al., 1991; MARMIGÈRE; ERNFORS, 2007a). The expression levels increase between the first and second waves of neurogenesis and decrease when differentiation begins.

1.5.2 Commitment markers

NEUROG1 and NEUROG2 are both transcription factors of bHLH type. NEUROG2 starts the first neurogenesis wave and it is expressed in a subpopulation of migratory neural crest cells and continues to be expressed throughout the migration that populates the DRG (MA et al., 1999). NEUROG1 is expressed in a larger number of cells, including the cells that express low levels or do not express NEUROG2 at all. Those two commitment markers are essential for sensory commitment in the DRG. When NEUROG2 is removed, there is an increase in cell death at earlier stages, but the DRG development occurs normally, raising the hypothesis that in the absence of NEUROG2, the transcription factor NEUROG1 acts in a compensatory manner, resulting in the normal DRG formation (ZIRLINGER et al., 2002). Differently, the knockout of both, NEUROG1 and NEUROG2, results in the absence of DRG in embryos (MA et al., 1999).

However, NEUROG1/2 do not control the expression of neurotrophic receptors TrkA, TrkB and TrkC (ZIRLINGER et al., 2002). In conclusion, the transcription factors NEUROG1/2 control the formation of sensory neurons, but do not define the sensory lineage, indicating that definition of sensory modalities requires other transcription factors.

1.5.3 Early differentiation markers

1.5.3.1 POU4F1

POU4F1 is also known as Brn3a and is expressed in all sensory neurons (XIANG et al., 1995). The knockout of POU4F1 results in an increase in the number of nociceptive and mechanoceptive neurons and decrease in the number of proprioceptive neurons (ZOU et al., 2012). Besides that, the authors observed that occurs an increase in the levels of cleaved caspase-3 in DRG cells at E15.5 and a decrease in the expression levels of RUNX1 and RUNX3 (ZOU et al., 2012). It was already shown that POU4F1 directly represses the bHLH transcription factors NEUROD1 and NEUROD4, by analysis of locus-ChIP (LANIER et al., 2007). Also, POU4F1 is a negative modulator of its own expression, and this autoregulation is mediated by a direct interaction between POU4F1 and its recognition elements within the POU4F1 sensory enhancer region. Comparison of the POU4F1 loci reveals that this regulatory region is conserved between species (TRIEU et al., 2003). Besides that, POU4F1 activates TRKA expression directly by binding to two binding sites in the TRKA promoter (MA et al., 2003).

1.5.3.2 ISLET-1

The LIM transcription factor ISLET-1 is one of the first neuronal differentiation markers (AVIVI et al., 2002). Besides its expression in CNS, this transcription factor is also expressed in neural crest cells that will form the DRG and sympathetic ganglion (ERICSON et al., 1992). Experiments in chick embryo shown ISLET-1 expression is detected since DRG condensation and it is maintained until late sensory lineage differentiation (CUI; GOLDSTEIN, 2000). The knockout of ISLET-1results in underdeveloped DRG and decreases the expression of the RUNX1 but the expression levels of RUNX3 remains the same. Also, the KO of ISLET-1 is earlier stages, as E12.5, the number of nociceptive neurons decrease. ISLET-1 was shown as a repressor of the bHLH transcription factors NEUROD1, NEUROD4 and NEUROG2, by microarray analysis in E12.5 (SUN et al., 2008).

1.5.3.3 SOX11

The transcription factor SOX11 has an activator regulatory role during neuronal

maturation (BERGSLAND et al., 2006). This gene is expressed in both in peripheral and central nervous system, but presents high expression levels in the PNS (UWANOGHO et al., 1995). The misexpression of the transcription factor SOX11 in mice resulted in a decrease of sensory subtypes and axon emission at later stages of development and in absence of all the sensory subtypes in DRG one day after birth. But, at initial stages of development, the absence of SOX11 does not affect the relative distribution of sensory subtypes. In this way, SOX11 is important for neuronal maturation, but not for the determination of sensory lineages in the DRG (LIN et al., 2011). Overexpression of SOX11 in neural tube did not alters the expression of the bHLH proteins NEUROG2 or NEUROM, with those results, the authors suggest that SOX11 is expressed before the commitment marker NEUROG2 in sensory neurons (BERGSLAND et al., 2006). Also in developing spinal cord, SOX11 is required for expression of the pan-neuronal gene TUJ1(BERGSLAND et al., 2006).

1.5.3.4 SCRATCH2 (SCRT2)

SCRT2 is a member of SNAIL superfamily of transcription factors that is specifically expressed in embryonic neural tissue. In the chick, mouse and zebrafish embryos, SCRT2 is expressed in pos-mitotic progenitor cells of the neural tube (ELLIS, HORVITZ, 1991; ROARK et al., 1995; VIECELI et al., 2013). In the chick DRG, its expression was seen in only after the second wave of neural crest cells migration, but during differentiation stages presented an expression pattern enriched in the dorsomedial region. SCRT2 expression in the neural tube do not overlap with the expression of proliferation markers, but with the expression of differentiation markers, as ISLET-1, both in the neural tube and in the DRG. Overexpression of SCRT2 generates extra neurons in Drosophila embryos (ELLIS, HORVITZ, 1991) and inhibits neural cell death of C. elegans nematode (ROARK et al., 1995). Also, the overexpression of SCRT2 anticipate migration of neural precursors to external cortex layers, while the suppression of SCRT2 retain the precursors in ventricular layer (ITOH et al., 2013). SCRT2 also inhibits the migration of neural precursors from the subventricular to intermediate region (PAUL et al., 2014). In mouse, SCRT2 is represses E-cadherin transcription and contributes with postmitotic migration to the outer layers of the cortex (PAUL et al., 2014). In C. elegans, the SCRT2 orthologous gene, called CES-1, repressed programmed cell death during asymmetric division of neural progenitors (METZSTEIN; HORVITZ, 1999). A previous published work shown

that the sequence preferentially recognized by SCRT2 is a modified version of the E-Box (CANNTG), called CES-Box (CMACAGGTK) Where M can be an adenine or a cytosine and K a guanine or a thymine. (REECE-HOYES et al., 2009).

1.5.3.5 ELAVL3/4

The family of neural proteins Hu (ELAV) interact with RNAs and have an important role in development and maintenance of vertebrate neurons (MARUSICH et al., 1994). Neural Hu proteins (HuB/C/D) binds to introns of target pre-mRNAs in the brain to regulate alternative splicing and to 3'UTR sequences to regulate mRNA levels (INCE-DUNN et al., 2012). HuD is a key component in multiple regulatory processes, including pre-mRNA processing, mRNA stability and translation, governing the fate of a substantial number of neuronal mRNAs (BRONICKI; JASMIN, 2013). The over-expression of HuD, accelerate neurite outgrowth in rat cortical neurons (ANDERSON et al., 2000). In the chick embryos, HuC/D is expressed in differentiating neurons of the neural tube, DRG, enteric ganglion and sympathetic ganglion, both in nucleus and cytoplasm of these cells. In chick embryo its expression starts after the end of second wave of neural crest cells migration and remains in post-mitotic cells (MARUSICH; WESTON,1994). At earlier stages, HuD (ELAVL4) expression was observed in differentiating neurons located in neural tube periphery. Besides that, in the DRG, it was seen that its expression is restrict to the central region, where the differentiating cells are located (WAKAMATSU; WESTON, 1997).

1.5.4 Early and late sensory lineage differentiation markers

1.5.4.1 RUNX1/3

The runt-related (RUNX) genes are evolutionarily conserved developmental regulators, where they play diverse roles in different biological systems, including cell differentiation (INOUE et al., 2008). One of the Drosophila pair-rule genes, Runt, controls segmentation, sex-determination and neuronal development (DUFFY; GERGEN, 1994). RUNX1 is essential is synthesized in both central and peripheral nervous system, differently, RUNX3 is confined to the peripheral nervous system, specifically to the DRG and cranial ganglion (LEVANON et al., 2001). In the absence of RUNX3, the neurotrophin receptors (TrkA and TrkB) synthesis goes through changes (KRAMER et al., 2006). The mammalian transcription factors RUNX1 and

RUNX3 are expressed and essential for distinct cell subpopulations in the DRG (INOUE et al., 2008) The knockout of RUNX3, that is expressed in all proprioceptive neurons, results in cell death of all proprioceptive neurons in the DRG (LEVANON et al., 2001). During late embryonic and early post-natal periods, neurons expressing TrkA differentiate into two subpopulations of nociceptive neurons, TrkA-retaining peptidergic neurons, and non-peptidergic neurons that represses TrkA and instead, activate RET, a receptor for glial-derived neurotrophic factor (GNDF). The knockout of RUNX1 did not affect the total number of cells in the DRG, but decreased the number of RET-positive neurons (CHEN et al., 2006). Also, the knockout of RUNX1 decreases the total number of nociceptive neurons in the DRG and the number of ISLET-1 and Hu positive neurons (KOBAYASHI et al., 2012). Runx protein works both, as activator or as a repressor, depending on the molecular context (KUROKAWA, 2006). For example, RUNX3 represses TRKB in DRG gene regulatory network (DURST; HIEBERT, 2004).

1.5.4.2 TRKA/B/C

The expression of tyrosine kinase receptors (TRK) is necessary for sensory neurons responsiveness to neurotrophins (HUANG; REICHARDT, 2003). Right after the end of neural crest cells migration to form the DRG, TrkC is highly expressed by most of the cells located in DRG central region, and TrkB is expressed in an irregular manner. Between the stages of proliferation and differentiation, there is an increase in TrkA expression and a decrease of TrkC expression in the DRG (PHILLIPS; ARMANINI, 1996). Neurotrophins are a family of polypeptide growth factors that use specific receptor tyrosine kinases (the Trk family) to exert their diverse functions in the developing and mature nervous system. Specifically, the nerve growth factor (NGF) is the preferred ligand of TrkA; brain-derived neurotrophic factor (BNDF) and neurotrophin-4 (NT-4) are the preferred ligand for TrkB; and neurotrophin-3 (NT-3) shows high affinity for TrkC. The signaling by BNDF via the TrkB receptor, or by NT-3 through the TrkC receptor support distinct populations of sensory neurons (POSTIGO et al., 2002). Mice double mutant for TrkB and TrkC had a significantly shorter lifespan and display more sensory defects than their single mutants. The most dramatic sensory deficit observed in the double KO mice was the absence of vestibular and cochlear ganglion (SILOS-SANTIAGO et al., 1997). The results obtained by these researchers corroborate with the idea of sensory neurons distinct populations requiring

distinct neurotrophins to differentiate. Here, we will refer to TrkA+ neurons as nociceptive, TrkB+ as mechanoceptive and TrkC+ as proprioceptive. Also, in scRNA-seq, TrkA is equivalent to NTRK1, TrkB to NTRK2 and TrkC to NTRK3.

Development of the sensory nervous system requires a temporal and sequential activation of specific genes, that will lead to differentiation of a specific sensory lineage. Although some of the genes that act in the gene cascade of sensory neurons differentiation are already known, the complexity of the sensory system and the diversity of its components clearly indicate that many other genes have not yet been identified as acting in this gene cascade. Therefore, the work presented here searched for a possible role or participation of SCRT2 in the gene cascade of sensory neurons development.

2. CONCLUSION

With the present work we conclude:

1. SCRT2 is expressed during early differentiation phase of DRG development, is downstream of proliferation and commitment genes and upstream to early and late lineage differentiation phases.

2. SCRT2 regulates ISLET-1 expression in DRG, and possibly the expression of POU4F1 and NEUROG2 during development.

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