Runx1t1 - Exploring its role as a transcriptional regulator in the dorsal root ganglion neuron specification

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Introduction

One of the most complex issues surrounding developmental neurobiology is the creation of diversity in the nervous system. During the process of development, time of generation of neurons and the different environments in the body give rise to subtypes of neurons which are functionally different. The peripheral nervous system is involved in the innervations of the skin, muscles and other peripheral organs and connects them with the central nervous system by conveying sensations of pain, touch, temperature, limb movements etc., through the somatic sensory neurons. Developmentally, these neurons derive from the neural crest cells (NCCs) that coalesce into dorsal root ganglia (DRG) adjacent to the neural tube.

**Neural crest cell migration**

The neural crest that forms at the border between neural and non-neural ectoderm during the neurulation process is under a tight control by complex molecular signals [including Wnt, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs)] derived from the neural tube, the non-neural ectoderm and the paraxial mesoderm. Later, changes in environmental signals and intrinsic properties induce an epithelia-mesenchymal transition that individualizes NCCs which now begin to migrate in a ventral to dorsal manner and form the dorsal root ganglia (DRGs)\(^1\). The migration starts at around E8.5 in the mouse\(^2\) and around stage 11 in the chicken\(^3-4\).

Fig1. a) Delamination and migration of neural crest cells to form the DRG. b) The three principle subtypes of sensory neurons arising within the DRG. Figure adopted from Frédéric Marmigère and Patrik Ernfors\(^5\) (2007).
**Sensory neuron types**

There are three main types of sensory neurons arising from the DRG namely the large diameter mechanoreceptive and proprioceptive neurons and the small diameter nociceptive neurons. The mechanoreceptive neurons are responsible for sensing mechanical sensations like touch, while the proprioceptive neurons sense limb movements and position. The nociceptive neurons are those which respond to stimuli like pain, cold, heat etc. Each of these neurons terminates in a specific laminar region in the spinal cord that is typical of each type and the function it serves. Specific to each neuron type are a class of high affinity tyrosine kinase receptors called Trks (tropomyosin-receptor kinase) as well as the receptor Ret, which are receptors for neurotrophins and glial-derived neurotrophic factor (GDNF) family ligands (GFLs) respectively. They are the earliest markers for neuron type specification and are involved in cell survival, diversification and target innervations.

<table>
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<tr>
<th>Sensory types</th>
<th>Receptors</th>
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<tr>
<td>Nociceptive</td>
<td>TrkA⁺</td>
<td>Nerve Growth Factor (NGF)</td>
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<td>Mechanoreceptive</td>
<td>TrkB⁺, Ret⁺, Ret⁺/TrkB⁺, Ret/TrkC⁺</td>
<td>Brain derived neurotrophic factor (BDNF) &amp; Neurotrophin 4 (NT4)</td>
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<tr>
<td>Proprioceptive</td>
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<tr>
<td>Nociceptive</td>
<td>Ret⁺</td>
<td>Glial derived neurotrophic factor (GDNF)</td>
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Table 1: Ligands that specify different types of sensory neurons. Nociceptive neurons are unmyelinated, small size neurons that express TrkA⁺ and or Ret⁺, mechanoreceptive and proprioceptive neurons are large-size myelinated neurons that are TrkB⁺, Ret⁺, Ret⁺/TrkB⁺ or Ret/TrkC⁺ and only TrkC⁺, respectively.

**Neurogenesis**

Sensory neurogenesis occurs as the NCCs migrate ventrally and coalesce by the neural tube to form the DRG. The neurogenesis has been observed to happen in three distinct waves of which the first two have been reported using retroviral tracing of single NCCs in the chicken³ and mouse⁶. The third wave was discovered more recently using genetic tracing methods⁷ in which the cells came from the boundary cap, the region bordering the central and peripheral nervous systems⁸.

During the first wave of neurogenesis, around one-third of the migrating NCCs, showing limited cell division, produce approximately 3.1 neurons each which are large in diameter and are either mechanoreceptive or proprioceptive⁹ neurons. During the second wave, the remaining NCCs give rise to an average of 35.9 neurons each, with the neurogenesis being longer³, and it is during this wave that many nociceptive neurons arise. The rate of proliferation is kept high due to the expression of the transcription factor SOX10 (Sex determining region Y box 10)¹⁰,¹¹.
The third and final wave arising from the boundary cap cells contributes to about 5% of the small TrkA nociceptive neurons. During all the waves of neurogenesis, peripheral glial cells are produced.

The two pro-neural transcription factors neurogenin 2 (NGN2) and NGN1 are required for the sequential generation of the first and the second waves of neurogenesis, respectively. The expression of Neurod1 and Neurod4 are dependent on the neurogenins. These proteins are responsible for the process of neurogenesis. Downstream to these are other Homeodomain transcription factors, Brn3a and Islet1 which act epistatically to repress NGNs and Neurod transcription factors. This act of co-repression is to initiate the process of neuronal differentiation into specific subtypes.
**RUNX transcription factors**

Runt related transcription factors (RUNX) include a small family of three proteins namely RUNX1, RUNX2 and RUNX3. All the three proteins have a homologous domain (RUNT) characterized by a conserved 128a.a motif and it is this domain that binds to DNA sequences. RUNX transcription factors play a crucial role in the embryonic development of several metazoans including hematopoiesis, osteogenesis, sensory neuron subtype specification etc. On the other hand, they are also involved in several forms of cancer. The α-subunit of the RUNX transcription factor has DNA binding capability and is termed polymovirus enhancer-binding protein 2 (PEBP2). The β-subunit called PEBP2β or core binding factor β (CBFβ) binds the RUNT domain and enhances the affinity of the α-subunit for DNA and protects it from degradation by proteosomes.

**RUNX3: Expression and role in proprioception**

Analysis of gene sequences in humans and mouse revealed that out of the three genes, Runx3 is the smallest and most probably the earliest to appear in the evolutionary ladder in this family. Its expression is first detected at E10.5 in the mouse and homozygous Runx3 knockout mice display severe limb ataxia. The analysis of these mice showed that proprioceptive neurons and therefore the monosynaptic connection between the Ia afferents and motor neurons were lost.

When the Runx3 expression begins during embryonic development, it is seen that around 85% of TrkC+ neurons co-express Runx3 and less than 5% of Runx3+ neurons co-expressed TrkB or Ret. TrkC+/TrkB+ hybrid neurons showed a low Runx3 expression level indicating that only TrkC+ neurons have a high Runx3 expression level during the early stages of development. The initial expression pattern of Runx3 does not depend on the expression of NGN1, TrkC & TrkA. The homozygous Runx3 mutants show a 2.1 fold increase in TrkB+ neurons while the TrkC+ neurons dwindled in numbers and their expression was also lowered. The remaining TrkC+ neurons (~95%) co-expressed TrkB. All these results further strengthen the fact that Runx3 transcription factor mainly controls the specification of proprioceptive neurons (TrkC+) from a population of TrkC+/TrkB+ hybrids. Runx3 is also responsible for controlling the axonal projections of proprioceptive neurons (NT-3 dependent) to central and peripheral targets.

**Runx1: Expression and role in nociception**

During embryogenesis, TrkA expression in the second wave of neurogenesis starts at E11.5 and becomes strong by E12 and following this, at E12.5, begins the expression of Runx1. The expression of Runx1 is restricted to TrkA+ neurons during embryogenesis with ~88% of TrkA neurons showing varying levels (high or low) of Runx1. During late embryogenesis in mouse, a population of TrkA neurons start expressing Ret and postnatally, repress TrkA, some of them maintaining Runx1. Neurons that maintain TrkA and do not express Ret lose Runx1 perinatally. In the Runx1 conditional knockout mice, the TrkA+ population at P60 increased from 28% to 69% while the Ret+ population decreased from 69% to 30%.

Also, most of TrkA+ neurons are peptidergic i.e. they express genes coding for two proteins called substance P (SP) and calcitonin gene related peptide (CGRP).
always linked with TrkA expression\textsuperscript{23} during embryogenesis, but \texttilde{}90\% of the DRG Runx1\textsuperscript{+} neurons do not co-express CGRP at E17.5. By using a Runx1 over-expression mouse model, it was seen that Runx1 plays a major role in the suppression of CGRP expression at the DRG level\textsuperscript{34}. Together with its function in regulating the expression of several ion channels and receptors that characterize the function of unmyelinated neurons\textsuperscript{38}, these results indicate the crucial role of the repressive and activator activity of Runx1 in driving correct differentiation of unmyelinated sensory neurons in the DRG.

The role of Runx1 in central afferent targeting is also significant and it is seen that in the wild type mice, IB4\textsuperscript{+} afferents (most of them being Runx1+) target the inner lamina II (IIi) of the spinal cord while TrkA\textsuperscript{+} afferents target the outer layer II (IIo) and lamina I\textsuperscript{37}. In the mutant mice, all the afferents reach the dorsal horn, but a closer look by double labelling revealed that the IB4\textsuperscript{+} afferents had projected into a position more in dorsal lamina of the dorsal horn than their usual targets while the TrkA\textsuperscript{+} afferents remained unaffected. The co-staining of a subset of IB4\textsuperscript{+} afferents with CGRP in the dorsal horn shows the impact of loss of Runx1 on the derepression of CGRP. But some of the afferents in the mutant mice in the superficial dorsal lamina express only CGRP or SP. These results indicate that the unmyelinated TrkA\textsuperscript{+} only afferents are unaffected in the Runx1 mutant mice.

![Diagram](image1)

Fig3. a| Afferents in wild type mice: CGRP+/SP+ afferents project to lamina I and IIo while IB4+ afferents project to IIi. b| Abnormal projection of IB4+ afferents to the most superficial dorsal lamina (I) in the Runx1 mutant mice. Figure adopted from Chen et. al., (2006).

At a physiological level, the loss of several nociceptive ion channels and certainly of correct central targeting in the Runx1 mutant mice make them less responsive to thermal and neuropathic pain but they respond normally to mechanical pain. Peripheral nerve injury induces increased pain sensitivity where harmless stimuli can cause pain or pain like withdrawal response\textsuperscript{39}. By using the spared nerve injury model\textsuperscript{40} (SNI), sensitivity to
neuropathic pain was assessed in the Runx1 mutant mice. It was seen that the paw withdrawal threshold level was not lowered in the mutant mice compared to the control mice which became very sensitive. Together the Runx transcription factors are involved in controlling three important factors of DRG neuron phenotype- sensitivity to neurotrophin levels, axonal targeting and neurotransmitter profile.

**Runx1t1**

In humans, acute myeloid leukemia is characterised by the chromosomal translocation (t8;21). As a result of the translocation, the two genes AML1 (Runx1) and ETO (Eight Twenty One) fuse and express the protein AML1-ETO. The fusion protein acts as a constitutive transcriptional repressor and represses genes that AML1 mainly activates and as a consequence the hematopoietic precursor cells fail to differentiate.

ETO is also known as MTG8 or Runx1t1 (HGNC: 1535). Its role in cancer has been well studied but its natural role in development is still unclear. A homolog of the human Runx1t1 (ETO) protein named XETOR was identified in Xenopus laevis and it is seen that it plays a role in controlling the size of the proneural domain. The truncated form of protein was used to test if one of the forms performs antimorphically to the wild type XETOR but surprisingly all the truncated forms showed repression making it clear that XETOR was strong transcriptional repressor. Functional knockouts of XETOR resulted in the increase in the size of the proneural domain.

Lateral inhibition in Drosophila is defined as the specific cell-cell signalling where one cell attaining a neuronal fate inhibits its neighbouring cells to remain non-neural. Two proteins, Delta and Notch, are involved in this cell-cell signalling. It has been suggested that both XETOR and lateral inhibition must work together to define the proneural domain size and the number of primary neurons. The role of Runx1t1 in other organisms like chicken and mouse is yet to be explored but it is speculated that it might have similar functions as in Xenopus laevis.

**Plan of Action**

To understand the role of Runx1t1 in the DRG neuron differentiation by

- Subcloning Runx1t1 insert into new plasmid for generating riboprobes.
- Expression pattern studies of Runx1t1 by synthesis of riboprobes for in-situ hybridization analysis.
- Immunostaining.
- Functional analyses of Runx1t1 in DRG neuron differentiation: over-expression studies by in-ovo electroporation.
MATERIALS AND METHODS

Plasmids

Plasmids for *Gallus gallus* (pCAGGS & pCAeGFP) & *Mus musculus* (pBSK) were gifts from Yoram Groner and Ditsa Levanon. The Runx1t1 construct was obtained from Epoch Life Science. Plasmid pCDNA3.1 (gift from Yoram Groner and Ditsa Levanon) was obtained to sub-clone the Runx1t1 insert from pCAGGS to enable riboprobe synthesis for in-situ hybridization experiments in *Gallus gallus*.

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![Fig4. Plasmid pCAGGS with the Runx1t1 insert (marked here as GST1063-7).](image-url)
Fig 5. Plasmid pBSK for Runx1t1 expression in *Mus musculus*.

Fig 6. Plasmid pCDNA3.1
Subcloning

Plasmids pCAGGS and pCDNA3.1 were subjected to restriction enzyme digestion with the same enzymes *HindIII* & *XhoI* (20,000 U/mL, NEB) in two separate reaction tubes overnight at 37°C. The open vector was dephosphorylated with *Antarctic phosphatase* (5000 U/mL, NEB) in Antarctic buffer (10x, NEB) for 20 mins at 37°C and then ligated with the Runx1t1 insert using ligase enzyme (*T4 DNA ligase* from NEB) in quick ligase buffer (2x, NEB) for 5 mins at 16°C. Restriction digested and ligated samples were checked on 0.8% agarose (High melting, Dutscher Scientific) gel to confirm if the reaction was proceeding as expected. The ligated plasmid was then sent for DNA sequencing to check if the sequence and orientation of the insert DNA was correct.

Fig7. Process of subcloning the Runx1t1 insert into pCDNA3.1
**Transformation and Maxi prep**

Plasmids were thawed at room temperature and the One Shot® TOP10 competent cells (Invitrogen) were thawed on ice before use. Approximately 2-5 µL of DNA was added to one vial of TOP ten cells. The cells were gently mixed by tapping with a finger and incubated on ice for 30 mins. The cells were then subjected to a heat shock (42°C for 30 seconds) and immediately placed on ice. S.O.C medium (500 µL, Invitrogen) was added to the cells and incubated at 37°C for 1 hour on a shaking incubator.

Nutrient agar plates with the appropriate antibiotic were prepared and kept at room temperature. The cells were serially diluted up to 100 times and then plated. Plates were incubated at 37°C overnight.

An isolated colony of bacteria was picked up using a sterile tip and dropped it into the mini prep tube containing 2.5 mL LB medium and 50 µg/µL appropriate antibiotics. Tubes were incubated at 37°C for 8-9 hours at 220 rpm.

The mini-prep DNA was extracted using mini-prep kits (QIAprep® Spin miniprep kit, Qiagen) and the amount of plasmid quantified. Based on the best concentration, the mini-prep tube was chosen for maxi-prep. One litre flasks with 250 mL LB medium, 500 µL mini-prep culture, 1% glucose and 125 µL appropriate antibiotics (50 µg/µL) were prepared and incubated at 37°C for 18 hours at 220 rpm. The culture was spun at 25364 xg and the pellet was used for maxi-prep DNA extraction (EndoFree® Plasmid maxi kit, Qiagen). A small sample of the maxi-prep culture was saved for glycerol stock preparation.

**Expression Analysis**

**Riboprobe synthesis**

Ribonucleotide probes (riboprobes) are RNAs which are usually synthesized by in-vitro transcription and used for in-situ hybridization studies. Both the sense and anti-sense probes are generated from the same insert cloned in a plasmid downstream of a promoter, usually a viral promoter.

Plasmids pBSK and pCDNA3.1 sub-clone containing the Runx1t1 insert were digested with restriction enzymes overnight at 37°C. The restriction enzymes were chosen based on the availability of restriction enzyme sites before and after the insert sequence. The reaction products were checked on a 1% agarose gel to confirm the presence of linearized plasmids. The linearized plasmids were then purified from the reaction mixture using spin columns (Qiagen) or by using phenol/chloroform extraction (see Appendix). The amount of plasmid was quantified in a spectrophotometer (NANODROP 2000, Thermo Scientific) and around 1 µg of DNA was taken for the in-vitro transcription. For a reaction mixture of volume 20 µL, 4 µL of 5x transcription buffer (Promega), 2 µL of DTT (100 mM, Promega) and 2 µL DIG lab mix (10x, Roche) were added. Based on whether the reaction generated the sense or anti-sense strand the enzyme was added (T3, T7 or SP6, 2.4µL, Promega) and the reaction mix was made up to 20 µL using RNase free water. The reaction mix was incubated at 20°C overnight and the transcripts were precipitated at -20°C using LiCl (4 M), absolute ethanol and Tris-EDTA buffer (pH 7.9-8.1). After 2 hours or later, the precipitates were spun at 25364 xg for 30 mins. The pellet was washed once with 70% ethanol and air-dried. The pellet
was then reconstituted in T.E buffer or dH₂O. A sample of the probes were denatured at 95°C for one min and cooled on ice before being checked on a 1% agarose gel.

**In-situ hybridization**

In-situ hybridization is a technique widely used to analyse the mRNA expression pattern (localization) within a given tissue sample. Ribonucleotide probes were prepared as mentioned earlier. The tissue sections were defrosted at room temperature for 1 hour before use. For strong probes (in terms of expression), 10-12 µL of probes were diluted in 1 mL of hybridization buffer while 25-30 µL of weaker probes/mL of hybridization buffer were used. Probes were denatured at 95°C for 1 min and placed on ice immediately. Hybridization buffers are usually very viscous. Therefore, they were kept at 65°C for 5 mins before use. The denatured probes were mixed with appropriate amounts of hybridization buffer and about 180 µL of this solution applied per slide. The slides were covered carefully with glass cover slips (24x50 mm, Menzel Gläser) and placed in a box with the lower chamber humidified with washing buffer. The box was incubated at 70°C overnight. The following day, the slides were washed with washing buffer (4x30 mins) and with MABT 1x (3x20 mins). They were then blocked with a blocking buffer for 1 hour at room temperature. Alkaline phosphatase-conjugated anti-DIG antibodies (Roche) were prepared in blocking buffer and added to the slides and incubated overnight at 4°C. The slides were then washed with MABT 1x (6x30 mins) and then with buffer B3 (3x10 mins). The bottom of the slides was dried and using an immunopen, a hydrophobic line was drawn on the slide such that it bordered the tissue sections. Sections were stained using nitro-blue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3'-indolylphosphate (BCIP, Roche). Once the sections showed sufficient staining, the reaction was halted by washing several times in 1x PBS.

**Immunostaining**

Immunostaining refers to the staining of samples, usually tissues, with specific antibodies directed against particular proteins. The cryosections were defrosted for at least 30 mins at room temperature before use and using an immunopen, a hydrophobic line was drawn bordering the tissue sections. The slides were then covered with PBS buffer containing triton serum (refer Appendix) for at least 5 minutes before the primary antibodies were added.

The following primary antibodies were used for immunostaining of various mouse and chicken tissue sections: mouse anti-Islet1 (1/200, DSHB), rabbit anti-Runx1 (1/500), rabbit anti-Runx3 (1/500), guinea pig anti-Runx3 (1/500) [Runx1 and Runx3 antibodies were gifts from Thomas M. Jessel, Columbia University Medical Center], goat anti-TrkA (1/250, R&D systems), goat anti-TrkC (1/500, R&D systems) and mouse anti-NF-200 (1/400, R&D systems). The different antibodies for a particular slide were diluted in the PBS-triton buffer (refer Appendix) as mentioned (total volume 500 µL). Different combinations of primary antibodies were made for different tissue samples. Once the antibodies were added, the slides were stored at +4°C for at least one overnight. It was then washed twice in 1x PBS and the last wash was in PBS containing 1% donkey serum. The serum was added to act as a surfactant and ease the spread of the secondary antibodies. The secondary antibodies that were used are as follows: Alexa Fluor® donkey anti-mouse-555 (1/500, Invitrogen), Alexa Fluor® donkey anti-rabbit-555 (1/500, Invitrogen), Alexa Fluor® goat anti-guinea pig- 488 (1/500, Invitrogen) and Alexa Fluor® donkey anti- mouse-647 (1/500, Invitrogen). The secondary antibodies were also diluted in PBS-triton buffer and 500 µL applied per slide and stored at +4°C overnight or incubated at room temperature for 2 hours (for short
experiments). The combinations of secondary antibodies were chosen based on the where the primary antibodies were raised. For example, if the primary antibody was raised in mouse, then using an anti-mouse secondary antibody would be optimal. The secondary antibodies were then washed thrice using 1x PBS and then with water. Finally, they were air dried and mounted with glass cover slips (24x50 mm, Menzel Gläser) using Dako fluorescence mounting medium (Dako). Slides were stored at +4°C until further use for confocal imaging.

**Confocal analysis**

Confocal microscopy is a sophisticated imaging technique giving high optical resolution and contrast. It eliminates unwanted light (usually the one not in focus) by using pin holes through which the specimen is illuminated by a laser beam. The confocal imaging of the immunostained specimens was carried out using Zeiss LSM5 exciter. LSM stands for laser scanning microscope and is widely used for obtaining images by activated fluorescence and also creates 3-D image stacks (Z-stack). All the images obtained with the LSM5 confocal microscope had the same configuration (10x objective, multi track- GFP [green], Cy3 [red], Cy5 [blue] & DIC [differential interference contrast] with no bleed through) and the images were analyzed using LSM image browser (Carl Zeiss).

**Functional Analysis**

**In-ovo Electroporation & cryostat tissue sectioning**

Chicken eggs were incubated at 37°C with relative humidity at 55% in an incubator. The day they were placed into the incubator was considered as the starting point. The process of embryonic development happening during the period of incubation has extensively studied. The staging of the chicken embryos follows the procedure as established by Hamburger and Hamilton.

Plasmids for electroporation were prepared as mentioned in Appendix. When the eggs reached embryonic day 2 (E2), they were removed from incubation and sterilized with a spray of alcohol to prevent any post-electroporation infections that may develop in the incubator. Carefully, a lateral hole was punched into the egg with the help of forceps (World Precision Instruments) and around 3-5 mL of the albumen aspirated with a syringe. A small portion of the shell overlying the embryo was then cut out with clean scissors. The embryo was checked under the microscope to confirm if it was at the right stage and then about 1µL of the plasmid (pCAGGS) was injected into the neural tube of the embryo using a glass capillary. The injected plasmid was electroporated using an electroporator (BTX® Electro Square Porator™ ECM 830) and gold coated electrodes (BTX genetrodes, 5 mm length, 3-4.5 mm distance between the electrodes) placed parallel to the embryo at 25 V in 5 pulses (each 50 msec with 1 sec interval). To ease the process of electroporation and to keep the embryo free from infections a few drops of PBS with Penicillin-Streptomycin were added before placing and while removing the electrodes. During electroporation, the plasmid DNA migrates towards the anode and hence driven into cells positioned near the positive electrode, including neural crest cells (which later migrate down to form the DRG). The eggs were now sealed with a special tape and placed back in the incubator. Plasmid pCAeGFP was used as the control in this experiment.
When the eggs reached stage E5 or E6, they were taken out and checked under the microscope to confirm their stage of development. The embryos were separated from the eggs using fine forceps and placed in 1x PBS. The intensity of the green fluorescence under a fluorescence microscope of each embryo was checked to see if the electroporation was successful. Following this, the good embryos were placed in 4% para-formaldehyde (PFA) for 2 hours for fixation. Once fixed, they were washed twice with 1x PBS to remove the PFA and then stored overnight in 20% sucrose-PBS. Following the overnight incubation, the embryos were transferred to another tube with 30% sucrose-PBS. They were then mounted in O.C.T™ medium [Sakura Tissue-Tek] and subjected to cryostat tissue sectioning (14 µm thick sections, Microm HM 560).

* - It must be kept in mind that the neural crest cells begin to migrate only after HH13 for the next 36 hours and form the early DRGs. The embryos were analysed when they reached stages E5 and E6. The images were adopted and modified from Hamburger and Hamilton (1992).
RESULTS AND DISCUSSION

Generation of constructs for in-situ hybridization

The Runx1t1 riboprobes for in-situ hybridization studies in Gallus gallus could not be synthesized from the plasmid pCAGGS. Hence I had to subclone the Runx1t1 insert into another plasmid, pCDNA3.1, suitable for riboprobe synthesis (Fig.7).

Subcloning

Plasmids pCDNA3.1 and pCAGGS were digested overnight with enzymes XhoI and HindIII. The digested plasmids were checked on a 0.8% agarose gel to confirm the result (Fig 9). The gel was imaged using BioRad Molecular Imager® and analysed using ChemiDoc™ XRS+ Imaging System.

![Gel image of restriction digested plasmids pCDNA3.1 and pCAGGS. The open vector and the insert were gel extracted and used for subcloning.](image)

From the gel image, it was clear that the digestion was not complete i.e. not all the plasmids were digested uniformly. So the bands corresponding to the correct molecular weights of the expected digested fragments were gel extracted using GeneJET™ Gel extraction kit (Fermentas Life Sciences). The open vector was dephosphorylated to make sure then open ends do not self-ligate before the Runx1t1 construct was inserted. The open dephosphorylated
vectors were then checked on gel to confirm complete dephosphorylation to ensure there were no closed vectors (data not shown). Upon confirmation, the insert sequence was ligated using \textit{T4 DNA ligase} (NEB). The ligated plasmid was then double digested with two sets of different enzymes. One set consisted of \textit{XhoI} and \textit{HindIII} which yielded two products; the open vector and the insert sequence. The other set consisted of \textit{XbaI} and \textit{SmaI} which yielded a large fragment of size approximately 6.2 Kb and a smaller fragment of about 1 Kb.

![Gel Image](image)

**Fig10.** Gel image confirming successful ligation of the Runx1t1 insert in to pCDNA3.1

From figure 10, it was confirmed that the plasmid was intact with the Runx1t1 insert. But to confirm that the orientation of the insert was proper, the subcloned plasmid was sent for DNA sequencing. The results (data not shown) confirmed that the insert was indeed in the right orientation.

**Transformation and Maxi Prep**

Maxi prep was performed to increase the quantity of plasmid DNA. In separate vials, the different plasmids (pCDNA3.1 subclone and pCAGGS as well as pBSK and pCAeGFP) were transformed into competent bacterial cells (One Shot\textsuperscript{®} TOP10 competent cells). The miniprep culture was then used for the maxi prep culture from which large quantities of plasmid DNA were extracted.
Fig11. Gel image showing digested maxi-prep DNA of pCDNA3.1 subclone.

Fig12. Gel image showing digested maxi-prep DNA of pBSK.
**Riboprobe synthesis**

Usually, in-vitro transcription experiments are carried out at 37°C which is the most optimum temperature for enzymes involved in this reaction. But due to problems with synthesis at this temperature, I had to lower it to 20°C to check if synthesis was possible and it worked. The probes were synthesized successfully at this lower temperature and its specificity confirmed by an in-situ hybridization experiment.

![Gel images showing the synthesized sense and anti-sense probes of pCDNA3.1 subclone and pBSK.](image)

**In-situ hybridization & Immunostaining**

In-situ hybridization was performed on several tissue sections of mouse and chicken from different stages to understand the expression pattern of Runx1t1 and its influence on the other genes in the RUNX and Trk families.

E10.5 was chosen as the earliest stage for mouse tissue sections and the latest being P0, while E5 was chosen as the earliest stage for chicken and latest being E6. Probes for mouse were used in the concentration of 2.4 µL per 100 µL of hybridization buffer (2x). Probes for
chicken were used in 2 concentrations; 2.4 µL and 6 µL per 100 µL of hybridization buffer (2x & 5x).

Immunostaining was done on top of the in-situ staining to analyze the distribution of different proteins in the tissue sections. For this purpose, the anti-sense slides were used for the immunostaining.

Fig14. Cross section of E5 chicken embryos at the brachial level showing the neural tube and the DRGs. Top panel is the in-situ hybridization for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for Islet1 (in green, marking post-mitotic neurons) including a merged image. Dashed line marks the DRG.
Fig 15. Cross section of E6 chicken embryos at the brachial level. Top panel is the in-situ hybridization (2x) for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for Islet1 (in blue, marking post-mitotic neurons) including a merged image.

Embryonic stages E5 in chicken and E10.5 in the mouse mark the beginning of expression of Runx3 and the stabilized expression of the Trk receptors. Hence these stages were chosen as the lower embryonic stage limit for the experiment. From the in-situ hybridization images it is evident that the expression of Runx1t1 just begins around E5 for chicken and E10.5 for the mouse. From the merged in-situ and immunostaining image, it is possible to say that the expression of Runx1t1 seems to be localized to Islet1⁺ neurons in the DRG. The expression from other types of neurons can be witnessed in the spinal cord. Only a few sections at stage E5 showed faint positive staining for Runx1t1 in the in-situ hybridization experiments.
Fig 16. Cross section of E6 chicken embryos at the brachial level. Top panel is the in-situ hybridization (5x) for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for Islet1 (in blue, marking post-mitotic neurons) including a merged image.

The expression of Runx1t1 at stage E6 (Fig. 15 & 16) is clear in the DRG compared to stage E5 (Fig. 14). The sense probe does not give any staining in both stages as expected. As the other antibodies (Runx3 and Runx1) failed to work in the immunostaining for the chicken embryonic sections, it is difficult to identify the expression pattern of Runx1t1 from the different types of sensory neurons. Also, as no results were obtained from the in-ovo electroporation experiments, it is not possible to comment on the functional aspects of Runx1t1 and its effect on Trks and RUNXs.
Fig 17. Cross section of E10.5 mouse embryos at the brachial level. Top panel is the in-situ hybridization for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for Islet1 (in green, marking post-mitotic neurons) including a merged image.

The increase in the number of Runx1t1 expressing Islet1<sup>+</sup> neurons can be clearly seen from figs. 17 & 18. The trend in expression follows a more dorso-medial path even though there are other parts of the DRG that show staining at the same stage. But since its expression is not so strong at stage E10.5 and also the fact that Runx1t1 is being expressed in the DRG makes it less possible for its involvement in the migration of the NCCs and neurogenesis but probably in specification of sensory neurons in the DRG.
Fig 18. Cross section of E11.5 mouse embryos at the brachial level. Top panel is the in-situ hybridization for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for Islet1 (in green, marking post-mitotic neurons) including a merged image.
Fig19. In Cross section of E12.5 mouse embryos at the brachial level. Top panel is the in-situ hybridization for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for Islet1 (in green, marking post-mitotic neurons) including a merged image.

At E12.5 the expression of Runx1t1 becomes strong in the DRG and in the spinal cord. But not all Islet1+ neurons express Runx1t1.
The other markers including Runx1, Runx3, TrkA and TrkC did not work and it is difficult to get more information from images. But faint NF200 staining at P0 (Fig.20) marks a few large diameter neurons (marked by arrows) that showed that the expression of Runx1t1 occurs in both the large and the small diameter neuron (marked by arrow heads) populations of the DRG. The sense probes did not give any staining at E10.5, E11.5, E12.5 and at P0 as expected.

Fig20. Cross section of P0 mouse embryos at the brachial level. Top panel is the in-situ hybridization for Runx1t1; staining showing the expression pattern & bottom panel shows immunostaining for NF200 (in green, marking large diameter myelinated neurons) including a merged image. Arrows indicate staining in large diameter neurons (NF200+) while arrow heads indicate staining in small diameter neurons.
**In-ovo electroporation**

Chick embryos were microinjected with the plasmids of interest and electroporated successfully. Upon reaching embryonic day E5 or E6, the surviving embryos in good condition were taken out of the incubator and fixed in 4% PFA and later mounted using OCT and sectioned in a cryostat machine. The idea behind this experiment was that, by over expressing Runx1t1 in the dorsal root ganglion, its effect on Runx1, Runx3, TrkA and TrkC could be studied in transfected cells. But unfortunately none of the survivors had strong reporter expression in the DRG cells and nothing could be concluded from this experiment.

**Conclusions**

Lack of sufficient results has made it difficult to conclude anything, but from the expression pattern of Runx1t1 in the mouse and chicken DRGs at these developmental stages, it is suggestive that it has a role in specification of sensory neurons. Runx1t1 is not expressed in the progenitor or glial cells, but only Islet1$^+$ neurons. Also, not all Islet1$^+$ neurons express Runx1t1. Due to its late expression during development (after E10.5 in mouse and E5 in chicken) it is less likely that it is involved in early neurogenesis and migration of NCCs. Very strong expression at E12 suggests it might have a role in specification of sensory neurons & both large and small diameter neurons express Runx1t1. One important aspect of this research is to develop good antibodies to specifically map the expression pattern of Runx1t1 in the DRG neurons. Since a lot of functional data is missing at this time point, it is crucial that functional aspects of Runx1t1 are studied by over-expression of this gene and its knockdown by using siRNA (in-ovo electroporation experiments). Also, conditional mutant mice have to be generated to better understand the physiological functions of Runx1t1. Use of sophisticated bioinformatics tools will help us understand and short list candidate molecules that interact and influence the expression and functions of Runx1t1. From the work of Ying Cao et. al., (2002), it is clear that Runx1t1 has a role as a transcriptional repressor in the nervous system very similar to how it functions in the hematopoietic system. By regulating other factors, such as the expression of Runx1 or Runx3 or any of the Trks receptors, Runx1t1 could possibly define the identity of a sensory neuron. Understanding these interactions will generate new knowledge about the various complex molecular networks that orchestrate neuronal identity during the process of embryonic development.
Acknowledgements

I would like to take this opportunity to sincerely thank all the people who, directly or indirectly, have supported me and helped me during my Masters programme. I would like to express my deepest appreciation to my supervisor Asst. Prof. Francois Lallemand, who continually and convincingly conveyed a spirit of adventure in regard to research. I also thank Post Doc. Saida Hadjab for giving me moral support; thoughtful criticism and encouragement that fuelled my thoughts and let me gain quality research experience. Without their guidance and persistent help this dissertation would not have been possible.

I would also like to thank all my friends at Karolinska Institute for making the lab an enjoyable place to come back to every day. I convey my heartfelt thanks to Dr. Lars Liljas and Dr. Staffan Svärd for their encouragement and support throughout the program.
Appendix

**Protocol for preparation of plasmids for electroporation**

For electroporation experiments, the plasmid with the reporter gene can have a concentration of 0.5 µg/µL while the plasmid of interest is usually higher (2 µg/µL). The final volume of the solution was kept as 10 µL and with the concentration of the plasmid known; the volume was calculated and added to two separate tubes, one containing only the control plasmid and the other with the control and plasmid of interest. Around 1/10th volume of 5 M NaCl was added followed by 2.5 volumes of absolute alcohol. This solution was kept at -20°C for 20 mins or longer and then spun at 25364 xg to precipitate the DNA. The pellet was washed once with 70% alcohol and then air dried. The pellet was resuspended in 10 µL of 1xPBS and 1 µL of dye added.

**Protocol for phenol-chloroform extraction of nucleic acids**

To 15 µL of DNA solution, 180 µL of dH₂O and 200 µL of phenol were added and then spun at 3340 xg for 5 minutes. The aqueous phase was extracted carefully and transferred to a fresh tube. To this 100 µL of chloroform and 100 µL of phenol were added and then spun at 3340 xg for 5 mins. The aqueous phase was again carefully aspirated and then transferred to a fresh tube. To this, 1/10th volume of 5 M NaCl and 2.5 volumes of absolute ethanol were added and left at -20°C for 30 minutes. It was then spun at 15682 xg for 30 mins and washed once with 70% ethanol. The pellet was then air dried and resuspended in 10 µL T.E buffer or dH₂O.

**Solutions and buffers**

*For use in in-situ hybridization*

*Salt: This must be autoclaved before use.*

<table>
<thead>
<tr>
<th>For 1 L</th>
<th>10X</th>
<th>1X conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>114 g</td>
<td>0.19 M</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>14.04 g</td>
<td>10 mM Tris pH 7.2</td>
</tr>
<tr>
<td>Tris Base</td>
<td>1.34 g</td>
<td></td>
</tr>
<tr>
<td>Na₂(H₂PO₄)₂.H₂O</td>
<td></td>
<td>5 mM pH 6.8</td>
</tr>
<tr>
<td>Na₂HPO₄.2H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>100 mL</td>
<td>50 mM</td>
</tr>
<tr>
<td>H₂O</td>
<td>qsp</td>
<td>qsp</td>
</tr>
</tbody>
</table>
Hybridization buffer:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration</th>
<th>1 mL</th>
<th>10 mL</th>
<th>5 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X salt (from above)</td>
<td>1X</td>
<td>100 µL</td>
<td>1 mL</td>
<td>500 µL</td>
</tr>
<tr>
<td>Formamide</td>
<td>50%</td>
<td>500 µL</td>
<td>5 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>50% Dextran Sulphate (in water)</td>
<td>10%</td>
<td>200 µL</td>
<td>2 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Yeast rRNA (10 mg/mL)</td>
<td>1 mg/mL</td>
<td>40 µL</td>
<td>4 mL</td>
<td>200 µL</td>
</tr>
<tr>
<td>50X Denhardt’s</td>
<td>1X</td>
<td>10 µL</td>
<td>100 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>H₂O RNase Free</td>
<td></td>
<td>150 µL</td>
<td>1.5 mL</td>
<td>0.75 mL</td>
</tr>
</tbody>
</table>

Washing buffer:
- Formamide (50% final) - 500 mL
- **SSC 20X pH 7.5 (2X final)** - 20 mL (Autoclaved)
- Tween 20 (0.1% final) - 1 mL

Adjust with autoclaved water up to 1 L

**MABT 5x (1 L):**
- Maleic Acid (116.07 g/mol) - 58 g
- NaCl - 44 g
- H₂O - up to 800 mL
- pH with NaOH pellets (approx 37-38 g) until pH 7.5
- H₂O - up to 1 L

**MABT 1X (1 L): Prepared on the day of use**
- MAB 5X - 200 mL
- H₂O - 800 mL
- Tween 20 (0.1%) - 1 mL

Blocking Solution:
- 2% Blocking agent : 0.6 g
- 20% Sheep Serum : 3 mL of Goat Serum (Jackson Research Immunolaboratories Inc.) and 3 mL of Donkey Serum (Jackson Research Immunolaboratories Inc.)
- MABT : 24 mL

**B3 (NTMT) 500 mL:**
- Tris HCl 1 M pH 9.5 (100 mM final) - 50 mL
- NaCl 5 M (100 mM final) - 10 mL
- MgCl₂ 1 M (50 mM final) - 25 mL
- Tween 20 (0.1% final) - 500 µL
- H₂O - 414.5 mL
**NBT-BCIP solution (10 mL): Prepared on the day of use**

- Tris HCl 1 M pH 9.5 - 1 mL
- NaCl 5 M - 0.2 mL
- Tween 20 - 0.01 mL
- dH$_2$O - 8.59 mL
- NBT-BCIP stock solution - 0.2 mL

*For use in immunostaining*

PBS-Triton Buffer: PBS + 0.2% Triton + 2% donkey serum (Jackson Research Immunolaboratories Inc.) + 0.025% NaAz
REFERENCES


