ROLE OF TRANSCRIPTION FACTORS IN SENSORY NEURON SPECIFICATION

Andreas Montelius
ROLE OF TRANSCRIPTION FACTORS IN SENSORY NEURON SPECIFICATION

Andreas Montelius

Stockholm 2007
Till Anna, Edit, Lisen och Harry
ABSTRACT

The interface between our mind and the internal and external environments is the peripheral nervous system. A range of different sensory neuron subtypes detects cold, heat, pain, touch, and other stimuli. Specialized receptor molecules defining the sensory modality of the cell, specific target innervation, and central connectivity characterize the neuronal subtypes. Neural crest cells (NCCs) are a transient population of pluripotent cells in the embryo that arise between the neural ectoderm and the ectoderm, go through epithelial to mesenchymal transition, migrate through the embryo and give rise to a wide range of tissues and cell types. Among NCC derivatives are the neurons and glia of the peripheral nervous system, with sensory neurons populating the dorsal root ganglia (DRG) and sympathetic neurons populating the sympathetic chain; other cell types are melanocytes, and jawbone tissue. During migration, an individual NCC gradually goes through a series of fate restrictions, driven by extrinsic cues and intrinsic properties. How and when is the diversity of neural crest derived cells and more specifically sensory neuronal subtypes created?

This thesis explores the role of transcription factors in sensory neuron specification. We describe the transcription factor Foxs1 as an early sensory neuronal marker and use it to describe molecularly distinct waves of neurogenesis in the DRG.

Our work shows that Runx1 directs the establishment of the TrkA+ nociceptive subclass of neurons. We describe context-dependent downstream effects of Runx1 overexpression, as well as loss of function effects. These effects include direct transactivation of TrkA, incompatibility with multipotency marker Sox10, increased axonal growth, and branching, and effects on survival prior to the period of programmed cell death. We report the results of an unbiased screen for downstream targets of Runx transcriptional activities in sensory neuron development and apoptosis.

In conclusion, this thesis describes crucial aspects of sensory neuron development and subtype specification, focusing on the role of transcription factors.
LIST OF PUBLICATIONS


III. Andreas Montelius, Xin Li, Francois Lallemend, Patrik Ernfors. (2007) Downstream targets of Runx transcriptional activities in sensory neuron development and apoptosis. Manuscript

*) Equal contribution
CONTENTS

1 Research question ........................................................................................................ 1
How and when does a migrating neural crest cell turn into a sensory neuron?. 1
   Aims...................................................................................................................... 1
Reading instruction .......................................................................................... 1
   Box 1 What is differentiation?........................................................................ 2

2 Introduction .................................................................................................................. 3
2.1 The neural crest is a model system for the study of development .... 3
   The neural crest is a well-studied model system. ................................... 3
   Neural crest induction is driven by signalling from adjacent tissues 3
   Neural crest cells migrate along distinct routes. ................................... 3
   Is lineage choice driven by intrinsic or extrinsic factors? ................. 4
   Cellular markers reveal the state of an individual neural crest cell. . 5
   Molecular biology brings new power to a classical model system... 5
   Specification of the sensory neuron and the nociceptor. ................. 5
2.2 How is diversity generated in the sensory nervous system?............. 5
2.3 Robustness in Neural Crest differentiation............................................ 6
   Development of Neural Crest derived tissues is robust. ................. 6
   There are several paths leading to the sensory neuron fate............ 6
   Is there a cost associated with multiple paths in development?...... 7
   Box 2 Life – a stable property of the planet earth......................... 8
2.4 The function of transcription factors......................................................... 8
   How many transcription factors are needed and why? ............... 8
   A transcription factor is just a switch in the regulatory machinery .. 9
   Box 3 What is a transcription factor?............................................................ 10
2.5 Runx1 – a runt domain transcription factor ......................................... 10
   Runx transcription factors share a runt DNA-binding domain...... 10
   Runx transcription factor structure and isoforms.......................... 11
   Runx is implicated in developmental processes ......................... 12
   Runx acts together with coactivators or corepressors............... 12
   Runx coactivators ..................................................................................... 12
   Runx corepressors.................................................................................. 13
   Posttranslational regulation of Runx activities......................... 13
   Runx 1 and Runx3 are expressed in the developing DRG .......... 13
   Upstream regulation of Runx activity................................................. 14
2.6 What makes a functional subtype selector?............................................. 14
   What is the pattern of subtype segregation in the sensory lineage? 14
   Minimal requirements of a sensory neuron subtype selector....... 15
   Does Runx meet the criteria for a trk-receptor subtype selector?... 15
2.7 Foxs1 - A Forkhead transcription factor............................................. 15
   The forkhead family of transcription factors ................................. 15
3 Methods .................................................................................................................... 17
   3.1 Genetically altering neural crest cells by in ovo electroporation.... 17
   3.2 Imaging, 3D rendering and image analysis ........................................ 17
4 Results and discussion ............................................................................................ 19
   4.1 Paper I......................................................................................................... 19
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>CBF</td>
<td>Core binding factor</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglion</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic Day</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>Fkh</td>
<td>Forkhead</td>
</tr>
<tr>
<td>GGF</td>
<td>Glial Growth Factor</td>
</tr>
<tr>
<td>HH</td>
<td>Hamburger Hamilton developmental stage</td>
</tr>
<tr>
<td>HMG</td>
<td>High Motility Group</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>MAOB</td>
<td>Monoamine oxidase B</td>
</tr>
<tr>
<td>MyNF1</td>
<td>Myeloid nuclear factor 1</td>
</tr>
<tr>
<td>NCC</td>
<td>Neural Crest Cell</td>
</tr>
<tr>
<td>NF-deltaE3A</td>
<td>Nuclear factor delta E3A</td>
</tr>
<tr>
<td>Ngn</td>
<td>Neurogenin</td>
</tr>
<tr>
<td>NMP2</td>
<td>Nuclear matrix protein 2</td>
</tr>
<tr>
<td>OBSC</td>
<td>Osteoblast-specific complex</td>
</tr>
<tr>
<td>OSF2</td>
<td>Osteoblast-specific factor 2</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PEA2</td>
<td>Polyoma enhancer A-binding factor 2</td>
</tr>
<tr>
<td>PEBP2</td>
<td>Polyomavirus enhancer binding protein 2</td>
</tr>
<tr>
<td>POU</td>
<td>Bi-partite DNA-binding domain (Pit Oct Unc)</td>
</tr>
<tr>
<td>run</td>
<td>Runt domain-encoding gene</td>
</tr>
<tr>
<td>Runx</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>S/A-CBF</td>
<td>SL3-3 and AKV core-binding factor</td>
</tr>
<tr>
<td>SEF1</td>
<td>SL3-3 enhancer factor 1</td>
</tr>
<tr>
<td>SMAD</td>
<td>Combination of D. melanogaster MAD and C. elegans SMA</td>
</tr>
<tr>
<td>SN</td>
<td>Sensory Neuron</td>
</tr>
<tr>
<td>SOX</td>
<td>Sry Box HMG domain</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TGF beta</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>til-1</td>
<td>T-cell tumor integration locus 1 protein</td>
</tr>
<tr>
<td>TLE</td>
<td>Transducin-Like Enhancer of split</td>
</tr>
<tr>
<td>Trk</td>
<td>Tyrosine receptor kinase</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient Receptor Potential</td>
</tr>
<tr>
<td>Wnt</td>
<td>Combination of Wingless and Int</td>
</tr>
</tbody>
</table>
1 RESEARCH QUESTION

*How and when does a migrating neural crest cell turn into a sensory neuron?*

The thesis presents a number of answers, questions, and investigation strategies in relation to this research question. Developmental biology asks how a fertilized egg cell develops into a full grown individual. Our research question is deals with a special case of the egg to adult question. We are interested in sensory neurons because the neural crest, from which sensory neurons develop, is a well-studied model system for development and differentiation, but also because of the clinical implications concerning pain and regeneration.

*Aims*

Our goal has been to study the role of transcription factors in sensory neuron development and subtype specification. In papers I, and III we describe the functional role of the transcription factor Runx1 in nociceptor development. In paper II we describe the emergence of the sensory nervous system using the transcription factor Foxs1 as a marker.

*Reading instruction*

The important part of the text is the papers, accompanied by an introduction, and the intended readers are the members of the committee and the faculty opponent. Sections Box 1-3 and the Swedish summary are for the enthusiastic non-biologist.
What is differentiation?

Developmental biology asks how a fertilized egg cell can give rise to a mature adult individual. Bacteria multiply to generate identical progeny. Your egg cell on the other hand must divide to produce new similar egg cells plus a 70 kg protective cover made of extremely diverse and highly organized cells.

Differentiation is the process in which a stem cell goes through sequential changes to become a specialized cell.

What mechanisms generate diversity among differentiated cells?

A number of mechanisms that produce diversity in daughter cells or groups of cells have been described in the literature.

- Early patterning resulting in 10 different cell types along some axis is often created by a gradient of a secreted, diffusing factor. Cells detect the gradient and generate segments with sharp boundaries containing cells with a certain fate. Examples of diversity generated by this process are insect body segments or motor neuron subclasses in mammalian neural tube.

- Some cells retain stem cell properties throughout the life of an individual. They reside in a so-called niche, a restricted location in the body, and divide asymmetrically. Time after time they divide to generate one copy of themselves (self-renewal) and one daughter cell that has lost stem cell properties and will turn into a specialized cell. An example being the stem cells creating the high turn over intestinal villi.

- In the nervous system, neurons and glia are generated from the same progenitor by a process called lateral inhibition. Identical neighboring cells signal to inhibit each other’s ability to become neuron. Stochastic events allow one cell to signal more strongly, forcing the neighbors to become glia.

This thesis explores two differentiation decision points of the sensory neuron precursor:

- A cell becomes a sensory neuron, the kind of cell we use to collect information about our internal and external environment.

- A sensory neuronal precursor becomes a nociceptor, the kind of cell that registers noxious stimuli, translated to pain in the central nervous system.
2 INTRODUCTION

2.1 THE NEURAL CREST IS A MODEL SYSTEM FOR THE STUDY OF DEVELOPMENT

The neural crest is a well-studied model system.

This thesis explores the differentiation of neural crest cells (NCC) to sensory neurons. The NC is a very attractive model system in developmental biology, allowing the study of lineage choice, migration, and presenting differentiation from multipotent cells to a diverse cell types and tissues, such as neural, mesenchymal, endocrine, pigment and bone (Douarin and Kalcheim, 1999). NCCs are a transient population of cells in the vertebrate embryo, situated between the dorsal ectoderm and the neural tube. It was first described in the chick embryo as the “Zwischenstrang” (His, 1868). Early work on migration of NCCs was performed through nuclear labeling by tritiated thymidine (Chibon, 1967). Limitations of this technology due to dilution of radioactive isotope through cell division was overcome by the introduction of the quail-chick marker system by LeDouarin (Le Douarin, 1973; Le Douarin and Barq, 1969). This technique is based on the fact that NCC from quail can be transplanted into the chicken embryo, fully integrating into the host, but retaining nuclear morphology differences readily detectable by electron microscopy or regular microscopy following various staining techniques. Using cell staining methods and the quail-chick model a lot of data was collected about potential and migration timing and pathways of NCCs. There is a generous literature covering extrinsic and intrinsic factors affecting NCC induction, fate, and migration. The combination of a vast body of knowledge generated with old methods and the new molecular biology tools that allow researchers to control activity of selected genes makes the neural crest a wonderful model system.

Neural crest induction is driven by signalling from adjacent tissues

The NC is induced from epithelial cells at the border between the neural plate and the nonneural ectoderm in response to BMP, Wnt and FGF signaling from the underlying mesoderm and adjacent nonneural ectoderm (Bonstein et al., 1998; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Liem et al., 1995; Marchant et al., 1998; Monsoro-Burq et al., 2003). Some of the NCC undergo epithelial to mesenchymal transition (EMT). Changes include a switch cell adhesion molecules expressed (Bronner-Fraser et al., 1992; Nakagawa and Takeichi, 1998; Newgreen and Gooday, 1985), and secretion of proteases (Valinsky and Le Douarin, 1985) that allow NCC to delaminate and start migrating through the embryo.

Neural crest cells migrate along distinct routes.

Trunk NCCs migrate through the embryo along distinct routes at defined time points (Fig.1A). Migration starts at E8.5 in the mouse (Serbedzija et al., 1990) and at stage 13 in the chicken (Serbedzija et al., 1989). The process of migration starts rostrally in the embryo followed by migration in more caudal parts. This means that collecting an E9.5 mouse allows the analysis of several stages of trunk NCC migration (eg. Paper II). NCCs migrate along distinct pathways. NCC following a ventromedial route through
the somatic mesoderm will become neurons and glia of the sympathetic chain. The migration is restricted to the rostral halves of the somites by caused by expression repulsive F-spondin (Debby-Brafman et al., 1999) and Sema3F (Gammill et al., 2006) in the caudal halves of the somites. The structures to be populated along the ventromedial pathway are completed in a ventral to dorsal order, such that completion of the sympathetic chain is followed by waves of migration to the DRG and finally the boundary cap. The boundary cap is a transient structure that contributes to satellite cells, schwann cells and sensory neurons (Hjerling-Leffler et al., 2005; Maro et al., 2004; Topilko et al., 1994). A dorsolateral migration pathway between the dermamyotome and the epidermis. These cells can be observed throughout the period of migration, they don’t migrate in a rostro-caudally segmented way and give rise to melanocytes.

Is lineage choice driven by intrinsic or extrinsic factors?

At some time point during migration an individual NCC will become, in some order of events, sensory or sympathetic, neuron or glia. To find out time points and mechanisms for these choices, the potential of NCC have been studied by means of staining NCC, grafting, clonal cultures and NCC cultures subjected to various diffusing factors. A question that has been addressed is whether fate decision occurs early and directs migration or if migration pathway choice is stochastic and fate is imposed by extrinsic factors a cell is subjected to in a certain pathway, such as diffusing factors, extracellular matrix or cell-cell interactions. There is data supporting the role of diffusing factors in lineage choice. Isolating migrating NCCs from the neural tube by implanting a silastic membrane cases selective death of NCC that would have formed the DRG without preventing formation of sympathetic ganglia (Kalcheim and Le Douarin, 1986). BMP and WNT signaling that are important for NC induction, are later important in lineage choice between sensory and sympathetic fate. High levels of BMP are expressed in the dorsal aorta, inducing a sympathetic fate (Reissmann et al., 1996; Schneider et al., 1999). Wnt1 in combination with lower BMP levels, as expressed in the dorsal neural tube (Liem et al., 1995; Shackleford and Varmus, 1987; Wilkinson et al., 1987) has been shown to instruct sensory neuronal fate. It is also possible that NCC are biased or restricted to a fate before delaminating and would be driven into the correct migration path by a genetic program. Data supporting this hypothesis include lineage tracing experiments demonstrating that some NCC are biased to a certain fate before migration (Lo et al., 2005). A large body of research supports the idea that a subset of the early NCC population is multipotent and that some early NCCs are more or less fate restricted. There are quail-chick transplantation experiments developed by Le Douarin (Le Douarin, 1973; Le Douarin and Barq, 1969), clonal culture analysis (Baroffio et al., 1988; Sieber-Blum and Cohen, 1980), regrafting clonal cultures (Bronner-Fraser et al., 1980) or infecting NCC with recombinant retrovirus (Frank and Sanes, 1991). Both grafting experiments and knockout studies show a remarkable robustness in the system. Regrafting late migrating NCCs in embryos with ablated NC yields in some cases early NCC derived tissues (Baker et al., 1997; Raible and Eisen, 1996). This implies the
existence of a mechanism that allows migrating NCC to adapt fate choice to presence of other NCC derived cells in the tissue. An observation that could offer a mechanism is that NCCs keep physical contact during migration (Kasemeier-Kulesa et al., 2005). Studies showing that expression in subsets of NCC confers lineage restriction or bias include Gdf7 (Lo et al., 2005) and Ngn3 (Perez et al., 1999; Zirlinger et al., 2002). Taken together there is evidence for heterogeneity of early NCCs, some being multipotent, and some being more restricted. It is notable that data showing that some marker biases an early NCC population to a certain fate never has been shown to be the sole source of that certain final fate. A striking property of the system is the robustness in generating the final set of cell types.

Cellular markers reveal the state of an individual neural crest cell.

In our studies we use a number of cellular markers to define the identity of NCCs. Some of the markers detectable by immunohistochemistry are shown in Fig.1B. Migrating multipotent NCC express Sox10 throughout the ventromedial pathway at E9 through E10.5 in the mouse. At later stages Sox10 is expressed in glia in the DRG and along nerves, probably no longer indicating multipotency. Sympathetic neurons are detected by expression of tyrosine hydroxylase in our studies. Boundary cap cells are detected by means of their MAOB expression. Neuronal precursors express panneuronal marker Beta-III-tubulin (Membrege and Hall, 1995). Sensory lineage neuronal precursors express Brn3a and Is11 that are also expressed in the neural tube and Foxs1, a marker unique to sensory neuronal precursors.

Molecular biology brings new power to a classical model system.

A solid framework of knowledge about the neural crest collected in classical experiments like grafting and vital dye injection (Fraser and Bronner-Fraser, 1991) can now be analyzed on molecular level and lineage tracing or gene targeting. By infecting cells with expression vector for fluorescent protein NCC migration can now be studied live on cellular level (Kasemeier-Kulesa et al., 2005).

Specification of the sensory neuron and the nociceptor.

In the present thesis we studied molecular characteristics of NCCs at the sensory lineage restriction point using a mouse knock-in model (paper II). We also studied a later time point in NCC development, the nociceptor subtype specification. Here we used in ovo electroporation to genetically alter NCC (papers I and III). As I will briefly present in section 5, we aim to perfect our methods by creating fluorescent reporter constructs. This would enable in vivo visualization of marker expression onset, and isolation of specific sensory neuronal subtypes for analysis of their active transcriptome.

2.2 HOW IS DIVERSITY GENERATED IN THE SENSORY NERVOUS SYSTEM?

In this thesis, we present evidence that transcription factors Foxs1 and Runx1 mark sensory neuronal progenitors with certain fates. We also show downstream effects of Runx expression relevant to specification: incompatibility with multipotency marked by Sox10 expression and subtype specific functionality marked by TrkA receptor
expression. Those two observations are relevant for the function of Runx in lineage choice given that expression of a Trk receptor can control fate (Moqrich et al., 2004), and that Sox10 is essential for the glial fate where Runx is not expressed (Aquino et al., 2006; Britsch et al., 2001; Stolt et al., 2002). When approaching the mechanism generating diversity in progeny it is important to keep in mind the basic properties of the diversification event. How many different cell types are generated? At what time and during how long time? In a sequential or hierarchical way? Are the final cell types organized in groups like along a gradient, or intermixed like as a result of lateral inhibition. In some cases very large number of cell types must be generated in one tissue, such as olfactory neurons, where stochastic mechanisms are a likely candidate. In the case of sensory neuronal subtype segregation, time of migration is an attractive generator of diversity. The underlying mechanism could be intrinsic or extrinsic and most probably a combination (Edlund and Jessell, 1999). An intrinsic mechanism could be an internal clock in the genomic machinery making later emigrating cells expressing Ngn1 instead of Ngn2 or if the decision point occurs later, Runx1 instead of Runx3. It is imaginable that early migrating NCCs pass through a different environment of diffusing factors that later emigrating, constituting an extrinsic mechanism for lineage segregation. Very importantly cells keep in touch during the process of migration, in the sympathetic chain moving along the rostrocaudal axis after reaching correct dorsoventral position (Kasemeier-Kulesa et al., 2005). Sensory neurons are generated in excess and are pruned during the time of programmed cell death to make up the correct number in relation to target tissue. There is data showing that even if the number of cells created initially is the double of the number needed after programmed cell death, the percentage of each subtype is roughly correct before pruning (T. Jessel, Wenner-Gren Conference 2005).

### 2.3 ROBUSTNESS IN NEURAL CREST DIFFERENTIATION

*Development of Neural Crest derived tissues is robust.*

In grafting experiments where part of the early migrating neural crest is removed, and late neural crest cells are regrafted, early-derived structures can form (Baker et al., 1997; Raible and Eisen, 1996). Ngn expression define two waves of neurogenesis (Ma et al., 1999). In the Ngn2 knockout, sensory neurons are born in correct numbers but delayed, suggesting that the later Ngn1 wave compensates. Only in the Ngn1, 2 double knockout the DRGs are absent. These data show redundancy in the formation of neural crest derives tissues.

*There are several paths leading to the sensory neuron fate.*

There are studies claiming that some early marker, already expressed in premigratory NCC, bias cells to a certain fate, like Gdf7 biasing cells to sensory lineage (Lo et al., 2005). Interestingly, only a minor proportion of sensory neurons is derived from Gdf7-expressing NCCs. Boundary cap cells, the last wave of NCC migration is reported to contribute a part of the final population of sensory neurons, mainly Swann cells, satellite cells and nociceptors (Maro et al., 2004). These data suggest a possibility that subgroup restrictions early in differentiation, eventually partially merge into the same final fate. If that were true, there would be several roads for a NCC to become a
sensory neuron. How should an experiment be designed to test the hypothesis that there are several roads to the sensory neuron subtype? One strategy would be to map “cell pedigree” in one individual from stem cell to final adult functional subtype. This should be repeated in several animals to measure the degree of overlap between pedigree and functional subtype (personal communication, Sten Linnarsson). A good start that could be would be to map in great detail the intermediate cell types. Present at different time points, defined by gene expression profile. Measuring expression levels of 200 well selected genes at cellular level would be appropriate.

Is there a cost associated with multiple paths in development?

Let’s imagine that there are several differentiation routes to one fate. How could this be understood in a functional way? A common way of addressing this kind of issue is taking the engineer point of view: To think about every decision point in the process as one more step towards a final optimal product, in this case being the functional adult. With this view, robustness seems like the obvious benefit of redundant paths. To me it seems difficult to understand how multiple redundant pathways could give a higher fitness to an individual. I would like to propose a different way to think about differentiation.

Imagine that there are two routes from NCC to nociceptor in a mouse, and three routes in a second mouse. Each differentiation road is controlled by a set of interacting genes. I hypothesize that the difference in energy cost in Joule to maintain two or three alternative routes equals zero, given that the cells are confined in a controlled temperature environment producing the same total amount of protein. I would further hypothesize that the benefit of having more than one back-up route probably is close to zero. Even if there is no energetic cost to multiple differentiation routes, there must be a selection pressure protecting the involved genes and regulatory sequences from the erosion of random mutation. I propose that these sequences are preserved because of the heavy re-usage of regulatory components that is so apparent throughout development and evolution. A third wave of neurogenesis in the DRG may be preserved in evolution because the involved genes and regulatory sequences are crucial at some other time point of development.
Box 2  *Life – a stable property of the planet earth.*

Cellular life may have existed on earth more than 3 billion years. During this period of time the physical properties of the planet have gone through major changes: The composition of the atmosphere, the temperature, the magnetic field. Continents have surfaced, split and chased around the globe. It is remarkable that individual living beings are so fragile, yet the biosphere as a whole seems extremely robust.

A key physical property of cellular life is that it has an inside separated from the outside by a membrane. Inside is a machinery that can process and react to internal and external information. What is this machinery and how does it work?

You can think about life as an unbroken process of cell division beginning 3.7 billion years ago. You would study evolution and focus on long-term changes in the genome in response to environmental changes. The present study is within the field of developmental biology, looking at how individual cells change in concert with neighboring cells during the embryonic development of one individual. We focus on how the state of the genome-machine changes, manifested as changes in gene expression and cellular morphology.

2.4  **THE FUNCTION OF TRANSCRIPTION FACTORS**

*How many transcription factors are needed and why?*

Roughly 5%, that is 1500, of the genes in the mouse genome are transcription factors. 223 out of 1444 transcription factors analyzed in the mouse by *in situ* hybridization were found to be expressed in developing sensory ganglia (Gray et al., 2004). That’s the tissue where we have studied the temporal, spatial and in some cases functional connections between a number of transcription factors and a few downstream effectors during the period when NCCs are specified into rather few subtypes. The total number of genes we address in some detail is not more than a handful (Foxs1, Sox10, Isl1, Brn3a, Ngn1,2, Runx1, TrkA,B,C, NeuN). With our precision, the number of specified cell types is approximately the same as the number of genes detected: A few distinct migrating subgroups defined by Ngn expression, stages of neuronal precursors defined by Brn3a, Foxs1 and proliferation rate. At the time point our study ends we define three sensory neuronal (SN) subtypes, based on Trk receptor expression. Why are there 223 transcription factors expressed in this tissue? There are likely answers that could be tested:

A) A lot of transcription factors are redundant. B) We underestimate the number of distinct cell types that exists in the studied tissue. C) The way transcription factors works to regulate gene activity requires a large number of similar but not identical transcription factors to be present.

I would propose that the first experimental question to ask in order to address the issue is: What is the variation in gene expression on cellular level. The first parameter to list would be how many cell types we can define in the developing DRG at a given time point.
A transcription factor is just a switch in the regulatory machinery

A striking observation in our and others studies of transcription factors is the lack of predictable meaning a transcription factor has by itself. Engrailed may be a transcriptional repressor and VP16 an activator. But the genes we present here change function according to the presence of cofactors. Runx can drive TrkA expression in one context (paper I) fail to do it in a different (paper I) and act to suppress TrkA in a third (Chen et al., 2006). We see examples of one transcription factor Sox10, at early time points keeping cells multipotent (Kim et al., 2003), and then later define a certain fate (Aquino et al., 2006; Britsch et al., 2001; Stolt et al., 2002). But we also see how a Foxs1 is expressed in cells of different origin (placodal and neural crest) that share the same functional fate, sensory neuronal (paper I). Combinatorial codes of transcription factors define borders and regions in CNS and other tissues. Here also, the same combinations can drive similar downstream genetic programs in otherwise different cells (Ma, 2006). Major classes of transcription factors are listed in table 1, somewhat adapted from (Gray et al., 2004).

<table>
<thead>
<tr>
<th>Transcription factor class</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>bHLH</td>
<td>116</td>
</tr>
<tr>
<td>bzip</td>
<td>57</td>
</tr>
<tr>
<td>ets</td>
<td>28</td>
</tr>
<tr>
<td>forkhead</td>
<td>40</td>
</tr>
<tr>
<td>HMG</td>
<td>58</td>
</tr>
<tr>
<td>homeobox</td>
<td>227</td>
</tr>
<tr>
<td>jmjc</td>
<td>15</td>
</tr>
<tr>
<td>nucrec</td>
<td>50</td>
</tr>
<tr>
<td>POU</td>
<td>15</td>
</tr>
<tr>
<td>Tbox</td>
<td>17</td>
</tr>
<tr>
<td>ZN Tfs</td>
<td>678</td>
</tr>
<tr>
<td>others</td>
<td>144</td>
</tr>
<tr>
<td><strong>sum</strong></td>
<td><strong>1445</strong></td>
</tr>
</tbody>
</table>
Box 3  What is a transcription factor?

Mammalian cells hold a genome of roughly 30,000 genes. 5% of these are transcription factors. They encode proteins that control the activity of genes. Transcription factors often regulate the activity of other transcription factors in hierarchical patterns. Groups of transcription factors serve to integrate signals such as gradients of diffusing molecules in the embryo. Through mutually repressive loops they create and sharpen borders between groups of cells. These cells gain group-specific properties, imposed by the same set of transcription factors.

In the present thesis we describe the transcription factor Foxs1 as a unique marker for cells that will become sensory neurons. We show how the transcription factor Runx1 regulates development of a sensory neuron subtype, the nociceptor.

2.5 RUNX1 – A RUNT DOMAIN TRANSCRIPTION FACTOR

The transcription factor Runx1b and the truncated splice form Runx1d are studied in papers I and III. The reason why Runx1 caught our interest was the reported expression in a subset of sensory neuronal precursors (Levanon et al., 2001a) and an implication of Runx proteins in developmental processes. This section gives a background to Runx1 and the Runx family of transcription factors, their structure and function. The multiple names that are used in the literature for Runx genes can be confusing. The nomenclature has been standardized (van Wijnen et al., 2004). Table 2 and 3 list Runx1 homologues in different species. Tables 4 lists alternative names in the mouse with corresponding names specific to human and chicken. Table 5 lists names of runt related proteins in different species.

Runx transcription factors share a runt DNA-binding domain

Runx1 belongs to the group of three mammalian runx transcription factors Runx1, 2 and 3. Runx proteins share 128 aa runt DNA-binding domain named from the highly homologous domain in the Drosophila pair-rule gene runt (Daga et al., 1992; Ogawa et al., 1993b; Wang et al., 1993). The first purification of Runx was as CBF alpha in a heterodimer called Core binding factor (CBF) together with CBFbeta (Wang and Speck, 1992). CBF subunits were subsequently cloned and characterized (Wang et al., 1993) and CBF alpha was defined as homolog to human AML1 (Bae et al., 1994; Bae et al., 1993). The CBF heterodimer binds a consensus sequence (R/TACCRCRA) (Kamachi et al., 1990; Meyers et al., 1993) in the polyomavirus enhancer or Moloney leukemia virus enhancer (Ogawa et al., 1993a; Wang and Speck, 1992). Drosophila runt encodes a protein important for segmentation that regulates hairy, evenskipped and fushi-tarazu (Gergen and Butler, 1988; Kania et al., 1990).
### Table 2 Alignment scores for Runx1 genes in different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene Symbol</th>
<th>Protein Identity (%)</th>
<th>DNA Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. sapiens</td>
<td>RUNX1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vs. P. troglodytes</td>
<td>LOC473981</td>
<td>100.0</td>
<td>99.9</td>
</tr>
<tr>
<td>vs. C. familiaris</td>
<td>LOC487746</td>
<td>98.0</td>
<td>93.4</td>
</tr>
<tr>
<td>vs. R. norvegicus</td>
<td>Runx1</td>
<td>96.0</td>
<td>90.5</td>
</tr>
<tr>
<td>vs. D. melanogaster</td>
<td>run</td>
<td>43.0</td>
<td>53.1</td>
</tr>
<tr>
<td>vs. A. gambiae</td>
<td>ENSANGG00000011627</td>
<td>71.7</td>
<td>66.4</td>
</tr>
<tr>
<td>G. gallus</td>
<td>LOC396152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vs. M. musculus</td>
<td>Runx1</td>
<td>89.0</td>
<td>79.5</td>
</tr>
</tbody>
</table>

### Table 3 Runx1 homologues in different species, with highlighted runt domain

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene Symbol</th>
<th>Protein Identity (%)</th>
<th>DNA Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. sapiens</td>
<td>RUNX1</td>
<td>NP_001745.2</td>
<td>480 aa</td>
</tr>
<tr>
<td></td>
<td>runt-related</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>factor 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(acute myeloid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>leukemia 1; aml1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>oncogene)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. troglodytes</td>
<td>LOC473981</td>
<td>XP_001168297.1</td>
<td>480 aa</td>
</tr>
<tr>
<td></td>
<td>similar to PEBP2aB2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. familiaris</td>
<td>LOC487746</td>
<td>XP_544871.2</td>
<td>485 aa</td>
</tr>
<tr>
<td></td>
<td>similar to runt-related transcription factor 1 isoform a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>Runx1</td>
<td>NP_059021.1</td>
<td>450 aa</td>
</tr>
<tr>
<td></td>
<td>runt related</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>factor 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>run</td>
<td>NP_523424.2</td>
<td>510 aa</td>
</tr>
<tr>
<td></td>
<td>runt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. gambiae</td>
<td>ENSANGG00000011627</td>
<td>XP_318339.2</td>
<td>134 aa</td>
</tr>
<tr>
<td></td>
<td>ENSANGP00000014116</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. musculus</td>
<td>Runx1</td>
<td>NP_033951.1</td>
<td>387 aa</td>
</tr>
<tr>
<td></td>
<td>runt related</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>factor 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. gallus</td>
<td>LOC396152</td>
<td>NP_990558.1</td>
<td>408 aa</td>
</tr>
<tr>
<td></td>
<td>ch-runtB2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Runx transcription factor structure and isoforms**

The Runx family of transcription factors in mammals consist of Runx1, 2 and 3 (Coffman, 2003; Levanon et al., 2001b; Levanon and Groner, 2004). In addition there is alternative splicing of Runx transcripts (Levanon et al., 1996; Miyoshi et al., 1995; Tanaka et al., 1995). A splice isoform that lacks the transactivation domain has been shown to bind stronger and can act as dominant negative to runx signalling (Aziz-
Aloya et al., 1998; Bae et al., 1994; Miyoshi et al., 1991; Tanaka et al., 1998). We use Runx1d, that is truncated c-terminal to the runt domain, to suppress Runx signalling in papers I and II. Alternative promoter regions are part of the Runx protein diversity (Ghozi et al., 1996). Functional domains of Runx proteins include the runt DNA binding domain, a nuclear localization domain and a number of motifs that confers binding to specific protein partners are important for transactivation and repression (Ito, 1999; Kurokawa, 2006) (Fig. 2). Runx1 is commonly disrupted between exons five and six in leukaemia (Nisson et al., 1992), or fused with a different gene, hence the name Acute Myeloid Leukaemia (AML) (Miyoshi et al., 1991).

**Runx is implicated in developmental processes**

The transcription factors of the Runx family have been established as essential in developmental processes. The runx1 knockout lacked fetal liver hematopoiesis and died E12.5 (Ogawa et al., 1993b; Okuda et al., 1996; Wang et al., 1996a). Runx2 is essential for skeletal development (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). Context dependence is key to Runx genes and they have been described as lineage specific oncogenes and tumor suppressors (Cameron and Neil, 2004). Runx proteins regulate lineage-specific gene expression in developmental pathways (Cameron and Neil, 2004; Levanon and Groner, 2004).

**Runx acts together with coactivators or corepressors**

One of the striking conclusions of our work in paper I is how context dependent the Runx activities are. Overexpression of Runx1b in the chicken neural tube leads to ectopic TrkA expression at stage E3. No such effect on TrkA expression was seen at later stages, such as E4 or E5. Thus at stages when Runx1 is endogenously expressed in the neural tube, Runx1 has lost it’s ability to drive TrkA expression in this tissue. A very probable explanation for this context dependence is that Runx activities depend on the presence of coactivators and corepressors. Below, a number of reported Runx and Runx1 modulators will be listed.

**Runx coactivators**

The non-DNA-binding subunit of CBF, CBFβ, associates with Runx through the N-terminal runt homology domain and stabilizes binding of the complex to DNA by 5- to 10-fold (Wang et al., 1996b) (Ogawa et al., 1993; Wang et al., 1993). CBFβ enhances the effect of Runx in both repressing and activating complexes. Yes-associated protein (YAP) is a coactivator of Runx proteins. It contains a module named the WW domain that binds to a PY motif, the oligopeptide PPxY, present in Runx (Yagi et al., 1999). ALY is a ubiquitously expressed, nuclear protein that associates with the activation domains of Runx1. ALY can increase the affinity Runx1 DNA complex. Overexpression of ALY stimulates the activity of the TCR alpha enhancer complex (Bruhn et al., 1997). The c-terminal region of Runx interacts with p300. Overexpression of p300 stimulates Runx1-dependent transcription (Kitabayashi et al., 1998). Myb binds Runx and is necessary for transcriptional activation from the transcriptional enhancer for the human t-cell receptor (Hernandez-Munain and Krangel, 1994). Ets binds Runx 1 and acts to activate transcription (Petrovick et al., 1998). SMADs are activated by phosphorylation upon transforming growth factor-beta ligand...
stimulation. SMADSs bind Runx and acts as coactivator of transcription (Hanai et al., 1999).

Runx corepressors

Runx interacts through the C-terminal WWRPY motif with the SP domain of TLE1, a mammalian homologue of Drosophila Groucho (Stifani et al., 1992). Through the WWRPY interaction, TLE1 inhibits Runx1-induced transactivation (Imai et al., 1998) (Levanon et al., 1998). In Drosophila, Groucho interacts with basic Helix-Loop-Helix (bHLH) proteins through a WRPW domain (Paroush et al., 1994). Sin3A is another stabilizer of the Runx1-DNA complex repressor of Runx1 (Lutterbach et al., 2000).

Posttranslational regulation of Runx activities

Posttranslational regulation has been reported to regulate Runx activities (Bae and Lee, 2006). A few will be mentioned briefly. PIM-I kinase enhances the activity of Runx proteins by phosphorylation (Aho et al., 2006). EGF and IL-3 stimulate Runx1 transcriptional activity through ERK-mediated phosphorylation (Tanaka et al., 1996; Zhang et al., 2004). Dimerization to with CBFb is crucial to protect Runx1 from ubiquitin mediated degradation (Huang et al., 2001). Runx1 is acetylated by p300 (Kitabayashi et al., 1998; Yamaguchi et al., 2004).

Runx1 and Runx3 are expressed in the developing DRG

Runx1 is expressed in the TrkA subpopulation of DRG neuronal precursors shown by others (Levanon et al., 2001a) and by us in paper I. Runx3 is expressed in the TrkC population of sensory neuronal precursors. Knockout of Runx3 leads to decreased proprioceptor marker expression, axonal deficits and death of proprioceptors (Inoue et al., 2002; Levanon et al., 2002).

<table>
<thead>
<tr>
<th>Homo sapiens</th>
<th>Mus musculus</th>
<th>Gallus gallus</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML1</td>
<td>Runx1</td>
<td>PEBP2αB1</td>
</tr>
<tr>
<td>AML3</td>
<td>Runx2</td>
<td>PEBP2αA</td>
</tr>
<tr>
<td>AML2</td>
<td>Runx3</td>
<td>PEBP2αC</td>
</tr>
</tbody>
</table>
Table 5. Names for Runt-related genes in different species, adapted from (van Wijnen et al., 2004)

<table>
<thead>
<tr>
<th>Species</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>AML</td>
</tr>
<tr>
<td></td>
<td>NF-deltaE3A</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Runt, Lozenge</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>PEBP2αB1</td>
</tr>
<tr>
<td></td>
<td>CBFalpha</td>
</tr>
<tr>
<td></td>
<td>SEF1</td>
</tr>
<tr>
<td></td>
<td>S/A-CBF</td>
</tr>
<tr>
<td></td>
<td>MyNF1</td>
</tr>
<tr>
<td></td>
<td>OSF2</td>
</tr>
<tr>
<td></td>
<td>til-1</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>run</td>
</tr>
<tr>
<td>Gallus gallus</td>
<td>RuntB</td>
</tr>
<tr>
<td>Rattus</td>
<td>NMP2</td>
</tr>
<tr>
<td></td>
<td>OBSC</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>run</td>
</tr>
</tbody>
</table>

Upstream regulation of Runx activity

How are Runx proteins regulated and how is the diversity of sensory neuronal precursors as defined by Runx isoform expression regulated. Looking at the segregation of TrkA positive neuronal precursor in the E6 chicken DRG (paper I) or TG (Fig supplementary fred). It is hardly a lateral inhibition, if not cell migrate after fate segregation, although notch has been shown to act upstream of runx in hematopoietic stem cell specification (Burns et al., 2005).

2.6 WHAT MAKES A FUNCTIONAL SUBTYPE SELECTOR?

Within a given lineage, there are a certain number of cellular subtypes that have to be made in the correct proportions. We expect to find a selector - a gene or a small number of interacting genes - that governs the selection of sensory neuronal subtypes. What are the main characteristics of the sensory lineage subtype selection pathway that can be drawn from the discussion in the preceding sections? Can we list the minimal requirements of a subtype selector that we expect to find in the sensory lineage?

What is the pattern of subtype segregation in the sensory lineage?

The starting point in our thinking is the multipotent neural crest cell. According to the literature discussed above these cells differentiate in a stepwise hierarchical manner, from multipotent to increasingly restricted fates, eventually making up more than 20 sensory neuronal subtypes and some glial cell types with no particular spatial organisation in the mature DRG. To repeat a few key steps, Ngn:s biases fate at an early time point to sensory as supposed to sympathetic lineage, but does not completely restrict cells to neuronal subtype or even neuronal fate, since these cells also become glia. Segregation between subtypes defined by expression of a certain Trk receptor happens shortly after Ngn expression, but long before selection of TRP-receptor
identity. Throughout the time line there is data showing a redundancy or flexibility, such that Ngn1 positive cells that normally would bias to nociceptor fate, can, in an Ngn2 knockout, fully compensate the proprioceptors usually formed from the Ngn2 population. One could think of the process as a decision tree with some branches possibly merging at certain points.

**Minimal requirements of a sensory neuron subtype selector**

1. The selector genes must be present in active form in the right tissue at the right time point for subtype specification
2. The selector must directly or indirectly detect subtype bias imposed by upstream factors like Ngn expression, early or late migration.
3. The selector must have a mechanism that suppresses alternative fates.
4. The selector must drive a subtype specific genetic program.

**Does Runx meet the criteria for a trk-receptor subtype selector?**

From the work of others and us it is clear that Runx1 and Runx3 drive subtype specific genetic programmes. Interestingly Runx1 seems to control subtype selection and downstream effectors at several different time points, such as promoting TrkA in chicken E5 (paper I) and downregulating TrkA a at postnatal day 60 (Chen et al., 2006). We have shown that Runx1 suppresses Sox10 (paper I); which may be part of a segregation function, since those cells are restricted from a glial fate. It is tempting to hypothesise that Runx proteins should repress each other’s expression, but this may not be so (Chen et al., 2006). In that case, a different protein must drive the segregation mechanism, either downstream or parallel to Runx. Interestingly Notch signalling has been reported to be able to switch neurogenesis to gliogenesis in neural crest stem cells (Morrison et al., 2000), and there seems to be a possible link between Runx1 and Notch signalling in hematopoiesis (Nakagawa et al., 2006). Less is known about the connection between Runx activity and upstream events. One interesting piece of data is the dependence of Runx activity on BMP and TGFbeta signalling via SMAD (Hanai et al., 1999).

**2.7 FOXS1 - A FORKHEAD TRANSCRIPTION FACTOR**

The transcription factor Foxs1 in the development of the sensory nervous system is the topic of paper II. In this section a short background to Foxs1 and the family of forkhead transcription factors is presented.

**The forkhead family of transcription factors**

The forkhead family of transcription factors are named after the Drosophila mutant fork head (Weigel and Jackle, 1990). They share a 110 bp conserved DNA-binding domain but are otherwise quite divergent (Carlsson and Mahlapuu, 2002; Kaufmann and Knochel, 1996). Among Fox transcription factors there are both transcription activators and repressors. The nomenclature is organized (Kaestner et al., 2000) and a total of 100 forkhead proteins from different species are listed on the nomenclature website (http://www.biology.pomona.edu/fox.html). The mouse genome has some 30 forkhead genes, 10 of which are expressed in sensory ganglia during embryogenesis (Gray et al.,
2004). A different name sometimes used for forkhead transcription factors is “winged helix”, a term coined by researchers who used X-ray to map FoxA3 structure (Clark et al., 1993). Other than Forkhead transcription factors, linker histone H5 found in chicken erythrocytes, and the ubiquitous H1 linker histone both have winged-helix structures, although lacking one of the wings (Cerf et al., 1994; Ramakrishnan et al., 1993). Fox genes was shown to be organized in genomic clusters, similar to HOX genes (Wotton and Shimeld, 2006). Previously FoxsI was reported to be related to human FKHL18 (Cederberg et al., 1997) and mouse Fkh3 (Kaestner et al., 1993). Recently Foxs1 orthologues have been defined in chimp, cow, dog and chicken (Wotton and Shimeld, 2006).
3 METHODS

3.1 GENETICALLY ALTERING NEURAL CREST CELLS BY IN OVO ELECTROPORATION

We optimized a method to electroporate NCCs before delamination and migration. Eggs were incubated 52 to 54 hours at 38°C and higher than 60% humidity. By this time they reach stage HH13 (Hamburger and Hamilton, 1992), with around 22 somite pairs formed. An opening in the shell was cut with scissors to allow the injection of expression vector or siRNA into the neural tube. Plasmid DNA was prepared to concentrations ranging from 0.5 to 8 ug/ul in PBS with MgCl2 and for visibility, FAST green. After injection of DNA or RNA into the neural tube, electrodes were applied adjacent to the embryo at 4-5 mm distance. A square wave pulse generator (BTX) was used to electroporate at 40-75 V/cm, five pulses of 50 ms with 1 second intervals. Eggs were sealed with tape and incubated until embryonic day 3, 4, 5 or 6. Transversal cuts of an E6 chicken embryo, transfected with a chicken actin promoter driven EGFP expression vector shows the targeted cell types (see paper I, Supplementary Figure 3a). EGFP-expressing cells (green) include cells of the neural tube and NCC derivatives: Melanocytes, DRG neurons, Swann cells aligning the nerves. Embryos collected 24 hours after electroporation, at E3, transfected cells are in the neural tube and migrating through the rostral halves of the somites (Displayed in glow scale, red; Paper I, Supplementary Figure 3b-c, confocal microscopy scan of whole mount embryo in 3b; and in green in 3b projections of sets of transversal sections scanned by confocal scanner.)

3.2 IMAGING, 3D RENDERING AND IMAGE ANALYSIS

One striking aspect with the model systems used in the present thesis is that development of the DRGs occur in a rostral to caudal temporal order. This means that collecting a mouse E9 embryo or a chick E3 embryo for the analysis of trunk NCC migration and development yields information about several hours of development if the full potential of the material is used. At these stages, one somite pair is formed roughly every two hours. This can be achieved by whole mount staining as in paper II (paper II, Fig. 1, 2, 5) or by reconstructing 3D images from sectioned and processed tissue, like in paper I (paper I, Suppl. Fig 3c). Reconstructing 3D images from sectioned DRGs can be crucial for other reasons than capturing the rostro-caudal time dimension. Both in the mouse, shown by Brn3a and Foxs1 expression (paper II, Fig.7) and in the chicken, shown by Beta-II-tubulin, NeuN and Trk expression (paper I, Fig. 3), neuronal precursors are spatially organized, such that more mature cells are situated more ventrally in the forming DRG. Since the embryo is naturally curved, it is difficult to cut perfect transversal sections of more than a few pairs of DRGs. To get around difficulties and save time in the data collection, histological sections were routinely documented using a confocal scanner (TECAN) at 4 um resolution (Fig. 3). With very little hands on time, four regular microscopy glasses can be scanned in several channels within hours. The images were used to get a overview for planning high resolution imaging and for 3D stack projections reconstructing mm thick tissue sections. Both in paper I (tunel and activated casp-3) and for cell counting in culture (Ethidium bromide,
nuclear beta-galactosidase) we used 4 μm resolution imaging for cell counting, after validation by comparison with high-resolution images (0.5 μm). As it turns out, 4 μm resolution imaging can be efficient for counting 5 μm objects, like tunnel stained nuclei, provided the contrast is high and very few neighboring cells are stained. Weak leakage of signal into neighboring pixels due to broader diameter of the incoming laser beam than pixel step length, and the size of the object of study (Approximately 5 μm diameter nuclei) always yields the same signal signature: Strong central pixel with weak neighboring pixels. This signature allows efficient filtering of shotgun noise that from the detector. The imaging software ImageJ was used for automatic counting and 3D projections. A Visual Basic script by Arno Pihlak, TiffReader, was used and modified for various processing and filtering of images.
4 RESULTS AND DISCUSSION

4.1 PAPER I

Runx1 caught our interest because it is expressed in the right time and place to be relevant for sensory neuronal subtype specification. Runt domain transcription factors in other tissues and species play important roles in developmental pathways, controlling lineage-specific gene expression in proliferation and differentiation. We used bNCSC from mouse for in vitro experiments and in ovo electroporation of the neural tube, including NCCs, at HH13 as experimental models to analyze Runx, Runx1b and RuntB activities in the development of NCCs to TrkA positive nociceptors.

Developmental expression of Runx1 in mice and chick DRG

RuntB expression colocalizes with TrkA in neural crest-derived sensory neurons, including neurons in the dorsal root, trigeminal and superior/jugular ganglia. Runx1 expression was preceded by Ngn2 expression. No RuntB expression was detected in migrating NCC.

Runx1b promotes survival and axonal growth and branching

Runx1b overexpression in bNCSC was found to protect neuronal precursors from apoptosis and promote axonal growth and branching. These effects were dependent on Ngn2 overexpression. Runx1d, a truncated dominant negative splice form of Runx1, suppressed the effects in a dose dependent manner. This implies that transactivation or repression domains of Runx1 are crucial.

Blocking Runx activities leads to Trk downregulation and death

Runx activities were blocked by expression of Runx1d in NCCs in chicken embryo, leading to depletion of TrkA, downregulation of TrkC and complete death of transfected sensory neuronal precursors between E5 and E6. Selective targeting of RuntB by siRNA lead to loss of TrkA positive neuronal precursors.

Runx1b – incompatible with Sox10, sufficient for TrkA

Overexpression of Runx1 in chicken neural tube leads to ectopic and premature TrkA expression and suppression of Sox10, a transcription factor expressed in NCC cells that retain potency to become both neurons and glia (Kim et al., 2003). Interestingly there is no effect of Runx1 overexpression at later time points, when endogenous RuntB expression occurs in the neural tube. The switch in functionality is not surprising. The simplest explanation is that different comodulators are present at the different time points and tissues, a common feature of transcription factors described by example of Runx1 by other authors (Chen et al., 2006) and about ETS to mention another example (Hippenmeyer et al., 2005). The downregulation of Sox10 expression is very interesting, since it shows the ability of Runx1 not only to induce subtype specific effectors, like TrkA but also to restrict alternative fates through suppression of a multipotency marker. NCC that differentiate in to glia maintains Sox10 expression (Aquino et al., 2006; Britsch et al., 2001; Stolt et al., 2002). The current data does not reveal whether Runx1 affects Sox10 expression directly or via some intermediate.
4.2 PAPER II

We have analyzed the expression pattern of the forkhead family transcription factor Foxs1 in the developing mouse embryo and we report results from stages E8 through E12 in the paper. To map expression of Foxs1 we used a reporter gene knock in mouse where β-galactosidase was fused in frame with the Foxs1 coding sequence 16 amino acids downstream of the initiation codon (Heglind et al., 2005; Hjerling-Leffler et al., 2005). For the study presented in paper II, we used the heterozygote, where one Foxs1 allele is replaced by β-galactosidase. In the paper we use the expression of β-galactosidase, detected using its enzymatic activity or by immunohistochemistry, to describe the expression pattern of Foxs1. It has been shown by comparision with Foxs1 in situ hybridization that β-galactosidase faithfully reproduces Foxs1 distribution (Heglind et al., 2005). No overt phenotype was discovered at the studied time points. Mapping of Foxs1 expression in relation to known markers reveal molecularly distinct waves of neurogenesis in the DRG. We find that Foxs1 is expressed in all neuronal precursors of the emerging sensory nervous system, regardless of cellular origin. This is to say that neurons both of neural crest and placodal origin equally express Foxs1. Although no phenotype was detected, this fact implies that Foxs1 has importance for sensory neuronal identity, common to cells from different origin.

Foxs1 expression in the developing sensory nervous system

The first Foxs1 expression was detected by E9, at the 14 somite stage, where cranial ganglia start to form. Expression is then strengthened and appears in forming DRGs in a rostro-caudal direction chasing the somite formation. Cells with placodal origin such as neurons of the olfactory pit express Foxs1. Most Foxs1 positive cells appear in condensing or condensed ganglion structures in the embryo, but in the mesencephalon we discovered dispersed Foxs1 positive cells. These turned out to be Brn3a positive and thus constitute neurons of the mesencephalic trigeminal nuclei (mesV) (Hunter et al., 2001).

Foxs1 colocalizes with neuronal markers, but not with Sox10

The main focus of paper II is neuronal precursors in developing DRGs at the fore limb level. The first Foxs1 expressing cells in the forelimb level forming DRG are at the dorsal somatic lip, here referred to as a pioneering neuronal precursor. All Foxs1 positive cells express β-III-tubulin and Brn3a. Since there are β-III-tubulin and Brn3a with no Foxs1 expression we conclude that Foxs1 appears just after β-III-tubulin and Brn3a. In contrast to these markers (Fanarraga et al., 1999; Fedtsova and Turner, 1995; Jiang and Oblinger, 1992), Foxs1 is exclusive to sensory neurons at this stage. Localization of Foxs1 positive cells and staining with markers for other NCC derivatives: tyrosine hydroxylase for sympathetic lineage (Ernsberger et al., 1995), Sox10, Sox2 for glial cells aligning the serve. The NCC derived boundary cap (Hjerling-Leffler et al., 2005; Maro et al., 2004) as defined by MAOB expression (Vitalis et al., 2003) was negative for Foxs1, probably reflecting that these cells were not yet specified to become neuronal. Another interesting observation was that Foxs1 never colocalized with multipotency marker Sox10 (Kim et al., 2003). This was also in contrast to Brn3a, which showed quite some overlap with Sox10 at this early stage.
A Foxs1 positive cluster of pioneering neuronal precursors

The pioneering Foxs1 positive neuronal precursors formed a dense cluster in the center of the DRG anlage, surrounded by migrating Sox10 positive cells and arriving neuronal precursors. The early arriving neuronal precursors show almost concomitant expression of Brn3a and Foxs1. At slightly later stages we observed that a core of Foxs1/Brn3a positive precursors become surrounded by a second wave of arriving neuronal precursors that express Brn3a and Isl1 for some time before expressing Foxs1. We set out to measure cell division by BrdU pulsing, and we conclude that Foxs1 cells divide very little, whereas the Brn3a positive second wave neuronal progenitors continue to divide. It has been shown that before that Brn3a is a postmitotic marker in the CNS but not in the PNS (Fedtsova and Turner, 1995).

At later stages Foxs1 is no longer exclusive to sensory neurons
At E11 and E12 Foxs1 is expressed in virtually all sensory neuronal precursors, as shown by a total overlap with β-III-tubulin expression. Soon after Foxs1 expression appears in other tissues, and at E18, Foxs1 expression still appears in many different sensory neuronal subtypes, but not in 100% of the cells in each class.

Foxs1 marks sensory and neuronally committed cells

An experiment concerning the properties of Foxs1 positive cells that is not reported in paper II tests the commitment of these cells to a neuronal fate. We set up primary cultures containing Foxs1 positive neuronal precursors by dissecting, dissociating and plating E9 trunk tissue and E11 dissected DRG from Foxs1 knock in heterozygotes. All Foxs1 positive cells in the cultures showed neuronal morphology and were β-III-tubulin positive, also when exposed to glial growth factor (GGF), a factor that has been shown to instruct glial fate (Shah et al., 1994).

Further studies

We have shown that Foxs1 is a very early marker for sensory and neuronally committed cells, and that Foxs1 can be used to distinguish two molecularly distinct waves of neurogenesis in the DRG. The exclusivity of Foxs1 expression would make it possible to specifically analyze sensory neuronal precursors. One way to take advantage of this is the strategy we used in our GGF exposure experiment. A minority of the dissected cells are relevant, but they can be analyzed easily among large amounts of irrelevant cells by automatic microscopy. Efforts have been made in our laboratory to make an identical knock-in mouse where EGFP is used as reporter construct. This could allow isolation of large amounts of early neuronal precursors by FACS for further analysis.

4.3 PAPER III

The aim of paper III is to answer some outstanding questions in paper I. What is the mechanism behind the survival effect of Runx1 in Ngn2 fated neural crest cells? What genes mediate the apoptosis that follows blocking of Runx signaling in the chicken? In paper I we were limited to analysis of downstream effects by immunohistochemistry.
and in situ hybridization. In paper II we set out to take measure downstream broader gene expression effects.

**Collecting tissue for gene expression analysis**

To allow measurement of a larger set of genes we set up an experiment to collect RNA from E3 spinal cord and E5 DRG in the chicken. Various expression vectors always in combination with a EGFP expression vector as control were injected in the HH13 chicken embryo neural tube. Tissue was electroporated tissue, marked by EGFP expression, was dissected under UV-light for visibility. The time points for collection were E3, spinal cord, and E5 DRGs. The E3 time point is when we see activation of TrkA by Runx1 overexpression (paper I). E5 DRG is just before the time point of apoptosis for cells transfected with Runx1d (paper I). Tissues were frozen on dry ice for RNA extraction. From a piece of highly fluorescent neural tube we would typically get 1-5 ug total RNA, and from 2-4 DRG the yield would be 300 – 900 ng.

**Candidate gene approach**

Initially we used Q-PCR to test the effect of Runx signaling on candidate genes related to apoptosis. The induction of TrkA by Runx1b was readily detectable and was used as a quality control step. None of the genes we tested in our search for a mechanistic connection between Runx signaling and apoptosis/survival turned out to be regulated by Runx. Those genes were BclxS/L, for which the knockout leads to massive sensory neuronal death before the time of programmed cell death (Motoyama et al., 1995) and p53 that regulates apoptosis in neuronal cells through Noxa and Bax expression (Yang et al., 2004). We also measured Brn3a because Brn3a is known to regulate Bclx (Budhram-Mahadeo et al., 1999; Smith et al., 1998; Sugars et al., 2001).

**Identification of Runx downstream targets**

Runx downstream targets were identified by measuring changes in global gene expression with Affymetrix gene chip. Interesting targets were analyzed by Q-PCR for validation. A validated gene relevant for apoptosis was ANXA1, chicken homologue to human Annexin A1, a proapoptotic gene (Hsiang et al., 2006; Perretti and Solito, 2004; Sakamoto et al., 1996). Interestingly ANXA1 was downregulated by Runx1 at E3 and upregulated by blocking Runx activities E5. This makes ANXA1 a very interesting gene for us. We look for the mechanism behind Runx1 survival effect in Ngn2 fated bNCSC and the mechanism that links blocking of Runx activities to apoptosis in E5.5 chicken DRG. Could it be that the same set of genes is active in both contexts and that ANXA1 is one of them? We discovered that Nkx2.8 is a target of Runx signaling. This is highly interesting, and before we know more about this connection I want to point out two aspects of Nkx and Runx signaling that may be of importance. Nkx genes are involved in governing cell fate by patterning, for instance in the spinal cord. Chicken Nkx2.8 is implicated in organ formation, in the heart (Brand et al., 1997; Reecy et al., 1997). Runx1 has been reported to regulate subtype specific effectors, like subtype specific receptors (paper I) (Chen et al., 2006). We discovered that Runx1 affects Sox10 expression, showing how Runx1 acts by regulating other transcription factors. It will be interesting to see if the effect of Runx1 on Nkx2.8 belongs to this category of Runx action. The second interesting aspect is that many transcription factors are known
to be expressed both in the spinal cord and in the DRG, often in subsets of cells (Dasen et al., 2003; Dasen et al., 2005; Gray et al., 2004). Among those are both Runx and Nkx genes. It is possible that the expression of the same set of transcription factors in the DRG and in the central innervation target tissue could play a role in connectivity. Is there a role for an interaction between Runx and Nkx in that context? The strongest regulated downstream target we found was a gene we call cyclin F similar (CyFsim) because of the sequence similarity to human cyclin F, a gene implicated in cell cycle control (Fung et al., 2002; Movsesyan et al., 1996; Tetzlaff et al., 2004). This is highly interesting given the connection between Runx and proliferation. There are also reports that Runx affects the timing of the cell cycle (Strom et al., 2000).

More confirmation to be done

There are more genes to analyse by Q-PCR in the large datasets from our gene chip experiments. Interesting genes will be confirmed by in situ and possibly by gain-of-function /loss-of-function experiments, that are readily done in the chicken system.
5 UPCOMING EXPERIMENTAL AND ANALYTICAL TOOLS

Our data from a systems biology viewpoint

In the following section I would like to describe some of our published and unpublished experiments in the light of a distant goal that may not be evident from the papers of the thesis. First I would like to rephrase the work:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Function</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxs1</td>
<td>One gene</td>
<td>Loss-of-function</td>
<td>No effect</td>
</tr>
<tr>
<td>Runx</td>
<td>Set of genes</td>
<td>Loss-of-function</td>
<td>Strong effect</td>
</tr>
<tr>
<td>Runx1b</td>
<td>One gene</td>
<td>Gain-of-function</td>
<td>Multiple effects</td>
</tr>
<tr>
<td>Runx1b</td>
<td>One gene</td>
<td>Loss-of-function</td>
<td>Weak effect</td>
</tr>
</tbody>
</table>

We have not yet been able to identify a functional phenotype for the Foxs1 knockout in the developing sensory nervous system, despite its striking expression pattern. What could be the reason for this? One possibility is redundancy. Ten out of thirty mouse Fox proteins are expressed in the embryonic DRG (Gray et al., 2004). These or other proteins could have redundant function with Foxs1. Runx1 on the other hand is a protein with detectable effects both in gain- and loss-of-function experiments. Our strongest effects were detected in the experiments where the activities of at least two Runx genes were blocked.

Scale-free networks and knockout mice with no phenotype

Foxs1 is not the first gene described that has as striking expression pattern but no overt phenotype. It is attempting to think that this is a phenomenon that reveals important properties of the genome and its regulatory machinery. Genetic and other naturally occurring networks have for a long time been modeled as random, implying that the number of nodes with k links would follow a Poisson distribution. All genes would have similar number of connections. Recent literature demonstrates that many real world networks can be described as scale-free. The number of nodes with k links then follows a power law distribution, $P(k) \sim k^{-\text{degree exponent}}$ (Barabasi and Albert, 1999). As a result of emerging high throughput experimental methods there are now studies describing scale-free biological networks (Barabasi and Oltvai, 2004; Jeong et al., 2000). One interesting property of scale-free networks is their inherent robustness and error-tolerance, similar to the behavior of the genome as a whole. Think about the robustness internet. The overall performance is extremely robust to random failure, but if key sites such as Yahoo, MSN and Google are affected, most users will notice. This may be similar to genetic networks.

Next step in the neural crest

The neural crest provides a great model system for studying differentiation and lineage segregation, because of the wide potential of the neural crest cells, the vast knowledge from decades of intense study and the potential of new methods. It will be very fruitful to bring systems biology thinking into the field of neural crest development. If genetic networks in development are best described as a scale free network, then the classical
approach would only detect the super nodes, key genes that control the activity of a large number of downstream effectors. A systems biology approach asks questions about multiple interactions in networks of genes. What are the experimental design factors that lead to a successful systems biology approach? Recent studies attempting to recreate and expand knowledge about signaling networks have shown the importance of interfering with the system and measuring a large number of cells (Sachs et al., 2005). During my thesis work I aimed towards meeting a number of criteria I believe are crucial to bring the neural crest study to the systems biology level.

1. Perturb more than one component at a time
2. Measure a large set of downstream components
3. Measure effects on single cell level to get high n and the possibility to exploit stochastic variation in screening for correlations between genes.

We have made systematic attempts too meet these criteria, although we have not reach the goal yet. I would like to briefly mention some of our attempts that may be successful in the future.

1. Blocking the activity of all runx proteins (paper I) was a successful strategy in reaching more than one component. A possible future experiment would be to study the effect of electroporating chicken neural tube with expression vector for Forkhead DNA-binding domain coupled to VP16 activation and engrailed repression domain respectively, to ask if there are redundant functions among Fox-genes expressed in the DRG.
2. We have moved from measuring a handful components in paper I to larger numbers in paper III. The best approach will be to measure a set of 100 to 300 genes with the accuracy of Q-PCR.
3. The main weakness of paper III in my mind is that we measure gene expression in a mixed population of cells. We are working on two projects to overcome this problem: A) FACS sorting. So far we got good quality RNA from a cleaner population of cells, but with too low cell numbers for measuring low copy number genes. We are currently working on a fluorescent reporter construct driven by a minimal TrkA promoter (Ma et al., 2000) that will allow us to FACS, or analyze by automated microscopy, the transfected TrkA population of neuronal precursors specifically.

Needless to say that all efforts to automate image analysis and speeding up data collection by replacing microscope with scanner ultimately aim at collecting more single cell date faster.
6 CONCLUSIONS

- Runx1 regulates development and survival of TrkA nociceptors

- Runx1 promotes survival and axonal growth and branching in Ngn2-fated bNCSCs.

- Runx1 is expressed in neural crest derived TrkA+ neuronal precursors.

- Runx1 overexpression transactivates TrkA in migratory NCC and is incompatible with multipotency defined by Sox10 expression.

- Blocking Runx activity in the chick depletes TrkA expression, downregulates TrkC expression and leads to neuronal death

- Specific targeting of RuntB activity downregualtes TrkA expression

- Foxs1 is an early marker for sensory neuronal precursors, both of neural crest and placodal origin.

- Foxs1 expression reveals molecularly distinct waves of neurogenesis in the DRG

- Runx effects on neuronal precursor survival are not mediated by p53 expression or Bclx expression or alternative splicing.

- Runx1 downregulates the pro-apoptotic gene ANXA1

- Blocking Runx activities leads to upregulation of ANXA1

- Transcription factor Nkx2.8 and possible cell cycle regulator CyFsim are targets of Runx signaling
7 ACKNOWLEDGEMENTS

Thank you all for making it possible! for making it a pleasure

Supervisor
Patrik. Thank you for excellent scientific supervision, friendly support and tremendous enthusiasm. It has been a privilege to be your student, and a lot of fun.

Coworker
Fred. Thank you for the fun time we spent together electroporating, sectioning, listening to music, collecting embryos late at night and then again in the morning. Thanks for doing science with me. Thank you also for sharing the hard times, with stress, winter and the dark side.

Project coworkers
Christel, Jorge, Francois, Li. Struggling together.

Lab
Past and present. I love the mix of constant scientific discussion and something else. I love you all in different ways and I’ve told you this. Special thanks to PhD student reception team for taking good care of me in the beginning: Karin, Anna, Jens. Special thanks to Anna for tears and laughers. Special thanks to Emma for good networking. Special thanks to Michael for bringing in the wild. Julianna, thanks for patience nightly work.

Support team
Sten, Ellef, Ats, Arno. For giving me asylum, unlimited access to instruments and resources, for discussion and perspective. Thanks Arno for baby-sitting the scanner and for company during crazy working hours.

Trappkollektivet
Hurra för alla gångna äventyr och de nya!

Folks
Allt stöd, referenshantering, bibliotekstjänster, barnpassning

Family ❤️❤️❤️
Anna, Edit, Lisen och Harry. Ni är min högsta prioritet och jag älskar er.

Motto
I want to work with you everyday,
towards one world
in peace and sustainable prosperity.
Driven by passion,
guided by the best available evidence.
8 APPENDIX: SAMMANFATTNING


Vi har studerat var, hur och när en känselnervcell eller en smärtkänselcell bildas. Vi har koncentrerat oss på effekten av transkriptionsfaktorer – en klass av gener som kontrollerar andra generations aktivitet.

Vårt arbete visar hur transkriptionsfaktorn Runx1 är viktig för bildandet av smärtkänselceller. Vi har beskrivit transkriptionsfaktorn Foxs1, och visat att den är en markör för celler som skall bli känselceller av alla olika sorter. Med hjälp av Foxs1 kan man visualisera framväxten av perifera nervsystemet.
9 FIGURE LEGENDS

Figure 1. Trunk neural crest migratory pathways and markers

(A) Green arrow shows the migration pathways of trunk neural crest cells. Cells delaminate from the neural tube and migrate along a ventromedial pathway through the rostral halves of somites. The first cells migrate through to the ventral side and become enteric nervous system, and sympathetic chain (1). Later arriving cells stop, aggregate and form the DRG (2). The last migrating cells will form the boundary cap (3). During the period of migration some cells follow a dorsolateral pathway (m) and give rise to melanocytes of the skin. Black and white rendering of confocal micrograph showing a transversal section of a E9 mouse embryo. Montage. Left and right sides are from different original images. (B) The same image in colour shows some of the markers studied in this thesis. Sox10 (blue, nuclear) present in all migrating NCC at this stage. Foxs1 (red, nuclear) in sensory neuronally committed cells. Beta-III-tubulin (green to the right) an early pan-neuronal marker. Brn3a (green to the left, nuclear) a marker for sensory neurons, but also present in the CNS.

Figure 2. Runx structure and functional domains

(A) 3D rendering of Runx1 runt domain in complex with CBFbeta and DNA. Projection was dome with CN3D (from NCBI) based on a 2.6Å structure (Bravo et al., 2001). CBFbeta does not contact DNA. (B) Runx1 runt domain and som additional aa. (C) Runx1 secondary structure. Runt domain in black. (NLS) Nuclear localisation signal. Interacting proteins, acetylation sites, phosphorylation sites.

Figure 3. The confocal scanner saves time

(A) Confocal scanner image at 4 um pixel resolution. Green channel image of microscope glass with transversal sections of three transfected chicken embryos collected at stage E4 and mounted in one block of OCT. The background is enhanced to show the structure of the tissue. Dark stain is EGFP expressed in transfected half of neural tube and in migrating NCCs. The image is used in low resolution print out for orientation and documentation. (B) Inset from (A) showing a more balanced contrast. EGFP positive cells are seen as black dots. Stained cells are one side of the neural tube, in the forming DRG, along the nerve and also migrating along dorsolateral pathway towards the skin. (C) An inset similar to (B) but rendered in gloscale, different colours representing different intensities. This can be a way of illustrating both the tissue structure and the signal from the rare EGFP positive cells. The 3D rendering in Supplementary Figure 3C in paper I is a stack projection of 84 consecutive images like the inset in (B).
REFERENCES


Runx1 aa 1-183 out of 453 with aa 1-135 of CBFbeta and DNA PEBP2 site

Runx1 secondary structure, functional domains and interactions

Figure 2