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Specification of V2b and KA neurons in the zebrafish spinal cord.

Liwia Anna Andrzejczuk
Syracuse University

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Abstract

Spinal cord contains different types of neurons. A vast majority of these neurons are interneurons. Therefore, a better understanding of how interneurons in the spinal cord develop is particularly important, as it will hopefully enable researchers to establish better treatments for spinal cord injury patients. In this thesis, I used zebrafish to study development of two classes of spinal cord neurons – KA cells (which correspond to cerebrospinal fluid-contacting neurons, CSF-cNs) and V2b cells. Both KA and V2b neurons are functionally important in vertebrate locomotor circuitry.

Development of functional neurons involves progression of genetic cascades that lead to correct cell specification. Some of the most important genes expressed during cell development encode for transcription factors, which are regulatory proteins that can either activate or repress expression of downstream genes. Both V2b and KA neurons in zebrafish express a common set of transcription factors – Tal1, Gata2a, and Gata3. These proteins are not present in any other spinal cord cell type. Previous work used a knockdown approach (morpholinos) to show that gata2a and gata3 have different functions in specification of KA cell types – KA” cells require gata2a and KA’ cells require gata3 to develop correctly. In this thesis, I test whether the same phenotypes occur in null mutants. Also, I investigate the role of tal1 in specification of KA cells, and the role of all three (gata2a, gata3, tal1) genes in V2b specification. To do this, I used tal1, gata2a and gata3 zebrafish mutants.
KA and V2b neurons also share another characteristic – all these cells are GABAergic. Therefore, I investigate whether tal1, gata2a and gata3 genes are required for correct specification of V2b and/or KA global cell fate and/or GABAergic phenotypes of these cells. In addition, I identify a subset of genes expressed by either KA", KA' and/or V2b cells and analyze the expression of some of these in these mutants.

My results show that tal1, gata2a, and gata3 have distinct functions in each neuron type. In KA" cells, gata2a is required for correct expression of the majority of KA" markers and the GABAergic phenotype of these cells. Interestingly, both tal1 and gata3 are not required for correct specification of KA" cells. In KA’ cells, the situation seems to be reversed – tal1 and gata3 are required for correct expression of all KA’ markers, but gata2a is required for only some aspects of the KA’ cell fate in a subset of KA’ cells. In V2b cells, the phenotypic effects of these mutations are more complicated – neither tal1, gata2a nor gata3 are required for correct expression of all V2b genes. However, tal1 is required for expression of a subset of these genes, and for the GABAergic phenotype of V2b cells.

This thesis contributes to better understanding of KA", KA’ and V2b neuron specification. Also, the presented results have broader implications, as they underlie the importance of cell type specificity of genetic cascades that lead to correct development of spinal cord neurons.
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Specification of V2b and KA neurons in the zebrafish spinal cord

by

Liwia Anna Andrzejeczuk

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1. Introduction

The spinal cord consists of nerve cells (neurons) and glia, and together with the brain constitutes the central nervous system (CNS). Nerve cells in the CNS consist mainly of motoneurons (MNs) and interneurons (INs), while most sensory neurons belong to the peripheral nervous system (PNS). Together, these cell types form functional neuronal circuitry (see Fig. 1). Sensory neurons have cell bodies outside of the spinal cord, but their axons terminate in the spinal cord and they primarily function to convey sensory information from the body towards the spinal cord and brain. Motoneurons have their cell bodies in the spinal cord, but their axons project away from the spinal cord to connect to the muscles of the body. They play a primary role in conveying locomotory information to muscles and are the most broadly studied group of spinal cord cells, as they have been easiest to identify so far (Lewis and Eisen, 2003). However, most spinal cord neurons are interneurons – cells that have their cell bodies and axons within the CNS, and are crucial for processing and conveying information between the brain and rest of the body, as well as within the spinal cord as part of oscillatory networks known as central pattern generators (CPGs) (as reviewed in: Frigon, 2002). CPGs are neuronal networks that create timing and patterns of rhythmic muscle movements, without a need for sensory input (for reviews see: Marder and Bucher, 2001; Grillner et al., 2007). Interneurons in the dorsal part of the spinal cord are important for receiving and processing sensory information, while cells in the ventral part of spinal cord regulate locomotion (for review see: Goulding, 2009). In the case of some ventral cell populations, we already know their specific functions in locomotor circuits. For example, V0 cells regulate right-left alternation in mouse (Lanuza et
al., 2004; Griener et al., 2015). Also, V1 cells are important for fast movements (Gosgnach et al., 2006), and together with V2b cells regulate flexor/extensor muscle output in mouse (Zhang et al., 2014; Britz et al., 2015).

The physiological functions of many spinal interneuron populations are already known, but we still don’t understand the processes by which these cells form. This knowledge could help develop therapeutic applications, particularly for spinal cord injuries. For example, understanding the genetic networks that lead to distinct, fully functional neurons should help researchers develop stem-cell based therapies to replace damaged neurons and/or circuits. In one case, expression of developmentally-characterized morphogens at specific concentrations in cell culture has already led to the generation of electrophysiologically functional V2a cells (Brown et al., 2014). Another recent study demonstrated that expression of specific genes in stem cells led to development of neurons from glia that once grafted, improved the locomotion of paraplegic rats (Hong et al., 2014).

Some of the most important molecules for understanding development of particular cell types are transcription factors. Transcription factors are proteins that bind to DNA and regulate expression of other genes. Due to their regulatory role, they are crucially important in developmental processes and often act as ‘master regulators’, sitting at the top of genetic networks that lead to the development of functional cell types. Understanding the roles that transcription factors play in spinal cord development, is key to understanding how mature, fully functional neurons arise. The hypothesis that underlies my research is that the combinatorial code of transcription factors and the temporal
sequence in which they are expressed, is what specifies the development of particular neuronal subpopulations (Lewis, 2006). My study concentrates on V2b and KA (Kolmer-Agduhr) neurons that are located in the ventral part of the spinal cord. V2b and KA neurons share a GABAergic neurotransmitter phenotype, and several transcription factor genes are co-expressed by both of these cell types: *gata3, gata2a* and *tal1* are expressed by both KA and V2b cells, while *tal2* is expressed by KA cells and a subset of V2b cells (Batista *et al.*, 2008; Yang *et al.*, 2010). In this thesis, I will describe the expression patterns of additional transcription factor genes in these cells and I will also investigate the functions of *gata3, gata2a* and *tal1* in development of V2b and KA cells. Finally, I will also briefly examine the expression of additional genes that are good candidates for being expressed by either V2b and/or KA cells. As my work is informed by the findings from other model organisms, I will use the nomenclature that is specified in section 2.10 of the methods chapter.

### 1.1 Zebrafish as a model organism for understanding spinal cord development

Zebrafish (*Danio rerio*) is an excellent organism to study spinal cord development. The animals develop fast, gaining a heart, brain and spinal cord by 24 hours post-fertilization (24hpf). Importantly, external fertilization allows for easy observation of the embryos without the need to sacrifice adults. Also, compared to most other animals that are opaque, the optical transparency of zebrafish embryos makes it especially easy to visualize tissues deep inside the animal, such as the spinal cord. In addition, one pair of fish
can potentially lay several hundred embryos at one time. This facilitates working with double mutant animals and genetic analyses.

The translucent nature of zebrafish has enabled very detailed descriptions of individual cells in the spinal cord (e.g. Bernhardt et al., 1990) and as of now, all of the zebrafish spinal cord neuron types have been described based on their morphology (size and shape of the cell body and axon projections) and dorsoventral position in spinal cord (Fig. 2, adapted from: Lewis and Eisen, 2003). This is in contrast with the amniote spinal cord, which is more complicated and where individual cell types are more difficult to identify on the basis of morphology. Despite the zebrafish spinal cord being much smaller than that of mammals or birds, the distinct subtypes of interneurons found in amniotes seem to have corresponding cells in zebrafish: at least most of the currently known genes expressed by amniote spinal cord are also expressed in zebrafish spinal cord, and the dorsoventral position of those cells corresponds to that of amniote cell populations (as reviewed in: Lewis, 2006 and in Goulding, 2009). Therefore, it seems likely that the mechanism of spinal cord development is highly conserved between zebrafish, birds, and mammals.

The main differences between gene expression in amniote and zebrafish spinal cord result from a particular evolutionary event - in the course of evolution, zebrafish (together with all other teleosts) underwent an additional whole genome duplication (Postlethwait et al., 1998). As a consequence, some genes have duplicate copies in zebrafish, as compared to most other vertebrates. Most often, after genome duplication one copy of the gene would become nonfunctional (as reviewed in: Glasauer and Neuhauss, 2014). However,
sometimes both copies are retained either because one of the copies acquires a novel function (neofunctionalization), or the functions of the gene become split between both copies (subfunctionalization) (as reviewed in: Glasauer and Neuhauss, 2014). Nevertheless, the processes of spinal cord development in zebrafish and other animals are highly homologous (Goulding et al., 2009, Lewis, 2006), so the results of my research are likely to be highly relevant to other vertebrates, including mammals.

1.2 Overview of spinal cord development

The spinal cord of most vertebrate animals can be divided into two regions medial-laterally: ventricular zone/proliferating zone (medially) and post-mitotic domain (laterally), with floor plate and roof plate delineating the most distant boundaries of the spinal cord along the dorsoventral axis (Fig. 1). The most ventral part of the spinal cord, the floor plate, secretes sonic hedgehog (Shh), while the dorsally located roof plate and neural tube secrete bone morphogenetic protein (BMP) and Wnt signaling molecules (as described in: Lewis, 2006). In addition, retinoic acid plays an important role in both anterior-posterior and dorso-ventral neural tube patterning (Lupo et al., 2006; Maden, 2002). Combined gradients of these morphogens across the spinal cord lead to the existence of molecularly distinct cell domains (progenitor domains) within the ventricular zone of the spinal cord (Briscoe et al., 2000; Lewis, 2006).

In mouse, at least 5 types of ventral progenitor domains (p3, pMN, p2, p1, p0) and 6 types of dorsal progenitor domains (dP6, dP5, dP4, dP3, dP2, dP1) have been identified based on their dorsoventral position and the specific set of genes that they express (see Fig. 1B; also for review, see: Jessell, 2000 or Goulding and Pfaff, 2005). Progenitor cells in the
ventricular zone have potential to undergo mitosis (divide) and give rise to differentiated post-mitotic cells. When cells become post-mitotic, they exit the cell cycle, move laterally and begin to express a different combination of transcription factors that will lead to the formation of fully differentiated, functional neurons (as described in: Lewis, 2006). Specific progenitor domains have potential to give rise to at least one (usually more) post-mitotic cell types (as described in: Lewis, 2006; see Fig. 1B). In each case, these related populations of cells share at least a subset of transcription factors, and often share one or more functional characteristics (as described in: Lewis, 2006 and in: Goulding, 2009).

The post-mitotic interneuron subpopulations on the dorsal side are designated d11-d-6, while on the ventral side they include V0, V1, V2 and V3 cells. In addition, ventral spinal cord contains motoneurons that are located between the V3 and V2 subpopulations (as described in: Lewis, 2006; see Fig. 1). So far, homologous cells for all of the ventral post-mitotic cells and most of the dorsal ones have been described in zebrafish (as reviewed in: Lewis, 2006 and Goulding, 2009, unpublished data Lewis Lab). In addition, as mentioned above the zebrafish spinal cord contains KA cells, which form in the most ventral part of the spinal cord and contact the central canal (Bernhardt et al., 1992). While similar cerebrospinal-fluid contacting neurons (CSF-cNs) have been described in mouse and other species (as described in: Djenoune et al., 2014), it is not clear where they form in these animals. I will discuss both V2 cells and KA cells in more detail below.
Figure 1. Schematic representation of cross-section through the spinal cord. Dorsal up, ventral down. (A) shows relative position of neuron types in the cross-section through the spinal cord. Blue neurons are sensory neurons (dorsal) and motoneurons (ventral). Green neurons represent interneurons, which do not leave the central nervous system. (B) shows position of individual neuron subpopulations in vertebrate spinal cord. The positions of V2 (V2a and V2b) cells and KA (KA" and KA') cells in ventral spinal cord are indicated in green. (B) is adapted from: Lewis et al., 2006.
1.3 Specification of functional characteristics of post-mitotic spinal cord interneurons

Spinal cord cells can exhibit a variety of functional characteristics, including a specific axon trajectory, neurotransmitter chemical messenger, and specific electrophysiological properties like firing intensity and duration (as reviewed in: Goulding, 2009). Each of those characteristics is important for neurons to function correctly in circuitry, and therefore crucial for a correctly functioning neuronal network. In my research, I am interested in determining whether the transcription factors I examine are required for specification of global cell fate, or whether they just specify a subset of the functional characteristics of these cells. In terms of specific aspects of cell fate, I am particularly interested in identifying transcription factors that are required for the neurotransmitter phenotype of KA and V2b cells. However, I am also interested in determining whether the transcription factors I examine are required for specification of global cell fate, or whether they just specify a subset of the functional characteristics of these cells.

Neurotransmitters are small molecules that are synthesized and released by neurons into the synaptic cleft to elicit a response in the postsynaptic neuron or another cell. Neurotransmitters can be either inhibitory or excitatory in nature, depending on the receptors that they bind. An inhibitory effect decreases the likelihood of the postsynaptic cell firing an action potential, and an excitatory effect increases the chances of firing in postsynaptic cells. Out of over 20 types of neurotransmitters currently known to be synthesized by cells, the most abundant ones in the spinal cord are GABA.
(gamma-Aminobutyric acid), glycine and glutamate (reviewed in: Goulding, 2009). GABA and glycine are usually inhibitory, while glutamate is excitatory. Enzymes that limit the synthesis of any given neurotransmitter are usually characteristic of cells with that fate/phenotype and can be used to identify cells, e.g. GABA is formed by decarboxylation of glutamate by the enzyme glutamate decarboxylase (GAD), and GAD is therefore expressed in all GABAergic neurons (as reviewed in: Erlander and Tobin, 1991). Markers of GABAergic cells used in this thesis include gad1b (previously known as gad65; described in methods section 2.5), and gad2 (previously known as gad67; described in the methods section 2.5), and encode for GAD enzyme in zebrafish (Higashijima et al., 2004a, b).

In addition, it is important to remember that, even though usually mature cells use predominantly one neurotransmitter as their primary signaling molecule, a cell can use more than one neurotransmitter - for example, GABA and glycine are sometimes co-expressed by the same neurons in the spinal cord (Jonas et al., 1998; Batista and Lewis, 2008). The inhibitory action of GABA is due to hyperpolarization of the neuron membrane mediated by reduction of calcium levels in the postsynaptic neuron, which depends on the influx of chloride into the cell (as reviewed in: Li and Xu, 2008). However, GABA can also be excitatory very early in development, when the concentration of chloride ions inside the cells is higher than outside, and GABA-mediated opening of the channels causes a chloride efflux, which leads to the elevation of calcium concentration and depolarization of postsynaptic neuron membranes (as reviewed in: Ben-Ari, 2002).
Figure 2. Cell trajectory and neurotransmitter phenotype of neurons found in zebrafish spinal cord. Schematic showing morphologies and positions of cell somas and axon trajectories of interneurons in zebrafish spinal cord (lateral view). Full lines indicate axons that are ipsilateral, and dashed lines show axons that cross the midline of spinal cord, becoming contralateral. Neurons that synthesize inhibitory neurotransmitters (GABA, glycine) are shown in orange and red respectively, while excitatory (glutamatergic) neurons are blue. Note that some morphological classes of neurons contain cells that utilize different neurotransmitters. KA and V2b (VeLD) cells both use GABA. Figure adapted from: Lewis and Eisen, 2003.
Both cell populations that I am interested in, KA cells (called Cerebral Spinal Fluid Contacting Neurons, or CSF-cNs in most other species) and V2b cells, are GABAergic in mouse (Orts-Del’Immagine et al., 2014) and in zebrafish (Bernhardt et al., 1992). These are, however, only two of several populations of spinal cord GABAergic neurons. In amniotes, other GABAergic subpopulations include dI4 and dI6 and late-born dILA neurons in the dorsal spinal cord, and V1 neurons in the ventral spinal cord (Hori and Hoshino, 2012). In mouse, all of these dorsal populations express Pax2, Pax5 and Pax8, but Pax2 is required for the inhibitory phenotype of only the most dorsal spinal cord cells (Pillai et al., 2007).

Similarly, in zebrafish pax2a and pax2b (orthologs of mouse Pax2), and pax8, are expressed in most inhibitory cells within the V1 and dorsal spinal cord region (Batista and Lewis, 2008), and act redundantly in those inhibitory cells to specify the inhibitory phenotype (Batista and Lewis, 2008). At the same time, in zebrafish pax5 is not expressed in the spinal cord (Batista and Lewis, 2008). However, inhibitory KA and V2b cells in zebrafish do not express the pax2a, pax2b or pax8, which suggests that a different mechanism must specify the inhibitory phenotype of those cells. In this thesis, I will investigate whether any of three genes expressed by V2b and KA cells, tal1, gata2a and gata3, are required for specification of the neurotransmitter fates of these cells.

Another functional property of a neuron is its axon trajectory. Neurons can send their axons either rostrally, which is also called an ‘ascending’ phenotype, or caudally, which is also called a ‘descending’ phenotype (see Fig. 2, adapted from: Lewis and Eisen, 2003). Also, the axon can cross the midline of spinal cord (contralateral), or can stay on the same side of the spinal cord, (ipsilateral). Axon trajectory is especially tractable in optically clear zebrafish. Since the cell populations that I’m investigating have different axonal
trajectories (KA axons are ipsilateral ascending, and V2b axons are ventral lateral descending), it is highly unlikely that the shared transcription factors expressed by those cells are responsible for specifying particular axon trajectories of either V2b or KA cells.

Other functional properties, such as electrophysiological properties of the cell, or correct synapse-formation require more specialized assays and are not properties that I’m going to investigate. Nevertheless, they are important for a neuron to become a part of a fully functional neuronal circuit.

1.4 V2 cells

V2 cells are born from p2 progenitor domain cells. When a p2 cell divides for the last time, it gives rise to two immature V2 cells, which then usually develop into one V2a and one V2b cell (Kimura et al., 2008). In amniotes, immature V2 cells are characterized by the expression of transcription factor genes \( gata2a \) and \( lhx3 \), which begin to be expressed by late progenitor p2 cells and persist into early post-mitotic V2 cells (Zhou et al., 2000; Al-Mosawie et al., 2007). However, after the V2 cells begin to diversify, the binary choice between becoming either a V2a or V2b cell is regulated by Delta-Notch signaling in both zebrafish (Kimura et al., 2008; Batista et al., 2008) and in amniotes (Peng et al., 2007; Del Barrio et al., 2007; Joshi et al., 2009). In amniotes, the mechanism that leads to asymmetric activation of Delta-Notch signaling is mediated via direct binding of FOXN4 and ASCL1 to the enhancer of \( Delta-like 4 \) (\( Dll4 \)) receptor (Fig. 3, Misra et al., 2014). In amniotes, high levels of Delta expression (and low levels of Notch) in V2a cells leads to downregulation of \( Gata2 \) while high levels of \( Lhx3 \), and high levels of Notch in V2b cells results in upregulation of \( Gata2 \), followed by expression of \( Tal1 \) and concurrent suppression of \( Lhx3 \) (Del Barrio et al., 2007).
al., 2007; Peng et al., 2007). This leads to specification of V2a versus V2b cell fates, and a similar mechanism is likely to exist in zebrafish as in both amniote and zebrafish spinal cord attenuation of Delta-Notch signaling leads to overproduction of V2a cells at the expense of V2b cells (Kimura et al., 2008; Batista et al., 2008a; Joshi et al., 2009).

Mature V2a cells are vsx1+/vsx2+/lhx3+ excitatory interneurons (Karunaratne et al., 2002; Kimura et al., 2006). Their sister cells, V2bs are gata2a+/gata3+/tal1+ inhibitory interneurons (Batista et al., 2008; Kimura et al., 2008; Joshi et al., 2009). While differing in their neurotransmitter phenotype, post-mitotic mature V2a and V2b cells share a similar ipsilateral descending axon trajectory, at least in zebrafish, in which V2a cells develop into excitatory Circumferential Descending (CiD) interneurons (Kimura et al., 2006b), while V2b cells develop into Ventral Lateral Descending (VeLD) interneurons (Batista et al., 2008). In their mature form, fully developed V2a and V2b cells are intermingled in a ‘salt and pepper’ manner and located adjacent to the p2 progenitor domain along the dorso-ventral axis in both amniotes (Li et al., 2005) and zebrafish (Batista et al., 2008).
Figure 3. Schematic representation of potential genetic hierarchy in amniote V2b cells. Schematic was constructed based on interpretation of results published so far in mouse and chicken. Arrows indicate genes that appear to be downstream of each other. Black color represents loss-of-function experiments, and orange arrows represent gain-of-function experiments. Pointed arrow indicates the activation of downstream genes, and line with a circle indicates downstream gene repression. Numbers indicate studies that are referenced by first author on the left.
Interestingly, experiments using a *Tg(vsx2:Kaede)* transgenic line that labels V2a cells show that in zebrafish these cells migrate dorsally after they form (Kimura *et al.*, 2006). Kaede is a protein that can be converted from green to red by a particular wavelength of light. This can be performed at a specific time point, so cells born before the conversion become red, while cells born after conversion time remain green (Kimura *et al.*, 2006). Conversion of the Kaede chromophore at 32hpf shows that cells labeled with *Tg(vsx2:Kaede)* that are green are located more ventrally, while red cells are more dorsal (Kimura *et al.*, 2006). Therefore, older V2a cells are positioned more dorsally than younger ones (Kimura *et al.*, 2006). So far, it is not known whether V2b cells might also migrate along the dorso-ventral axis once specified.

In addition, in some vertebrates additional V2 subpopulations have been described. V2c cells in mouse are born from the p2 progenitor domain, as are V2a and V2b cells, but they do not express *Vsx2* (V2a marker) or *Gata3* (V2b marker); instead, they express *Sox1* (Panayi *et al.*, 2010). V2c cells are likely derived from V2b cells, as *Sox1* is upregulated while *Gata3* is downregulated in a subset of V2b cells (Panayi *et al.*, 2010). In the absence of *Sox1* the number of *Gata3*+ V2b cells increases, while the number of V2a cells remains unchanged, further suggesting that V2c cells are specified from post-mitotic V2b cells and that *Sox1* is required for their specification (Panayi *et al.*, 2010). However, the precise mechanism by which V2c cells are specified remains to be elucidated. Also, whether V2c cells exist in zebrafish is currently unknown. To address this, I will investigate the expression patterns of *sox1a* and *sox1b*, the orthologs of *Sox1* in zebrafish, in this thesis.
In addition, at least in mouse, yet another V2 population exists. V2d cells are also born from the p2 domain, express Shox2 and contribute to central pattern generator (CPG) activity (Dougherty et al., 2013). V2d neurons are similar to V2a neurons in that they are also excitatory and project axons ipsilaterally (Dougherty et al., 2013). Even though some Shox2-expressing cells express V2a marker Vsx2, about a quarter of Shox2-positive cells do not express Vsx2 (Dougherty et al., 2013). So far, there is no evidence for presence of V2d interneurons in other species.

1.5 KA cells

A special class of spinal cord cells that contact cerebrospinal fluid was first described by W. Kolmer and E. Agduhr in the early 1920s and 1930s and shown to exist in over 200 vertebrate species (as described in (Djenoune et al., 2014). The cell bodies of KA neurons are contained within the spinal cord but they project their sensory cell tuft (dendrites) to directly contact the cerebrospinal fluid in the central canal (Bernhardt et al., 1990). In zebrafish, as well as in frog (Dale et al., 1987), these cerebrospinal-fluid contacting cells are named Kolmer-Agduhr (KA) cells, after the researchers that initially described them. In those species at least, KA cells are ciliated GABAergic cells that and have ventrally projecting ipsilateral ascending axons (Dale et al., 1987; Bernhardt et al., 1992). KA cells are important in locomotor behaviors. In zebrafish larva they can contribute to central pattern generator (CPG) activity and swimming behavior, as optical stimulation of those cells is sufficient to induce spontaneous swim-like behavior (Wyart et al., 2009).

KAs are derived from two distinct progenitor domains (p3 and pMN) and consequently occupy distinct dorsoventral positions (Park et al., 2004). Based on those
differences, they have been subdivided into KA" and KA’ subpopulations (Park et al., 2004). Cell lineage tracing experiments show that more dorsally located KA’ cells originate from the *olig2*-expressing pMN domain, while KA” originate ventral to this domain (Park et al., 2004). Notch signaling is important for specification of KA’ cells – in absence of Notch signaling primary MNs form at the expense of KA’ cells, and when there is an excess of Notch signaling, KA’s form at expense of primary MNs in zebrafish (Shin et al., 2007). KA” cells differentiate in the lateral floorplate (LFP) region (Schafer et al., 2007) and are intermingled with V3 (and potentially other) interneurons. KA” differentiation is also dependent on Notch signaling, as KA” cells are reduced in *mindbomb(mib)* mutants that are deficient in Notch signaling (Kang et al., 2013), and also after early (7hpf) heatshock-mediated Notch signaling ablation (Yeo and Chitnis, 2007). However, heatshock-mediated Notch signaling ablation at later stages (10-14hpf), as well as morpholino-induced knockdown of Notch receptor *jagged2*, both result in an increase of KA” and other cells including secondary MNs (Yeo and Chitnis, 2007). Interestingly, heatshock-mediated Notch signaling ablation at 17hpf has no effect on number of KA” cells (Yeo and Chitnis, 2007). This suggests that the timing of Notch signaling plays a pivotal role in specifying KA” cells from p3 cells (Yeo and Chitnis, 2007).

Even though KA” and KA’ cells differ in their dorsoventral position, many of their properties (morphology, electrophysiological properties and molecular markers) are similar (Yang et al., 2010; Djenoune et al., 2014). In addition, most genes that are expressed by these cell types are expressed by both KA and V2b cells.
1.6 Expression profiles of known markers of V2b and/or KA cells

Both KA and V2b cells express gata2a, gata3 and tal1 transcription factor genes in zebrafish (Batista et al., 2008). As I analyze mutations in all three of these genes in this thesis, here I will describe what is known about these genes in zebrafish and other vertebrate species. Since my project investigates role of those genes in the spinal cord development, in this introduction I will focus primarily on studies of the spinal cord and brain. When relevant, I will also describe briefly evidence from other tissues. In addition, I will describe tal2 as this is a homologue of tal1 (also called tal1) that is also expressed by both V2b and KA cells. Also, Table 1 outlines known genes expressed by V2b and/or KA cells, as well as transgenic lines used in my experiments to visualize V2b/KA cells as well as V2a cells.
**Table 1. Genes expressed by V2b and/or KA cells.** Summary of the reference genes and transgenic lines used in this thesis to visualize V2 and KA cells. All of these genes are expressed by the known cell populations, as described in the published literature. Note that *Tg(gata1:GFP)* might not label all of the V2b cells at 24hpf, a stage at which I performed my experiments. Also, I do not see clear labeling by the transgenic lines that label V2a cells (*Tg(vsx2:GFP), Tg(vsx2:RFP), Tg(vsx2:Kaede)*) before 27hpf.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Cells in which expressed</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gata3</em></td>
<td>KA and V2b cells</td>
<td>Batista <em>et al.</em>, 2008</td>
<td></td>
</tr>
<tr>
<td><em>gata2a</em></td>
<td>KA and V2b cells</td>
<td>Batista <em>et al.</em>, 2008</td>
<td></td>
</tr>
<tr>
<td><em>tal1</em></td>
<td>KA and V2b cells</td>
<td>Batista <em>et al.</em>, 2008</td>
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<tr>
<td><em>tal2</em></td>
<td>KA cells</td>
<td>Pinheiro <em>et al.</em>, 2004,</td>
<td>Not in all V2b cells at 24hpf</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Schafer <em>et al.</em>, 2007</td>
<td></td>
</tr>
<tr>
<td><em>gads</em></td>
<td>KA and V2b cells</td>
<td>Higahsijma <em>et al.</em>, 2004a, 2004b</td>
<td>In a few additional cells at 24hpf</td>
</tr>
<tr>
<td><em>Tg(gata1:GFP)</em></td>
<td>KA and V2b cells</td>
<td>Batista <em>et al.</em>, 2008</td>
<td>Not in all V2b cells at 24hpf</td>
</tr>
<tr>
<td><em>Tg(vsx2:GFP)</em></td>
<td>V2a cells</td>
<td>Kimura <em>et al.</em>, 2006</td>
<td>I don’t see cells labeled before 27hpf</td>
</tr>
<tr>
<td><em>Tg(vsx2:RFP)</em></td>
<td>V2a cells</td>
<td>Kimura <em>et al.</em>, 2006</td>
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<tr>
<td><em>Tg(vsx2:Kaede)</em></td>
<td>V2a cells</td>
<td>Kimura <em>et al.</em>, 2006</td>
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</table>
1.6.1 Gata2a

Gata2a (GATA-binding protein 2) is a C4 finger transcription factor from the GATA family of proteins which normally bind to a WGATAR (W = A or T and R = A or G) consensus sequences (Merika and Orkin, 1993). It was originally found as a regulatory sequence important in erythroid development (Yamamoto et al., 1990). It is expressed in a variety of cell lines and tissues, including pluripotent mouse progenitor cells, mast and megakaryocytic mouse cell lineages, endothelial cells, chicken early hematopoietic cells, and many other animal and human cell types and tissues (as reviewed in: Orkin, 1992 and in Burch, 2005). Gata2 has been especially widely studied in hematopoiesis, where it is important not only in proliferation of blood-forming cells, but also in specification of blood lineages as shown by cell culture experiments (Leonard et al., 1993; Briegel et al., 1993). It is also important for mouse urogenital development (Zhou et al., 1998) and for specifying mouse ventral pituitary cell types (Dasen et al., 1999). In neuroepithelial mouse cell culture, Gata2 acts in suppressing cell proliferation and promoting exit of the cell cycle (El Wakil et al., 1996). In mouse nervous system, Gata2 is expressed in both developing brain and spinal cord (Zhou et al., 1998). At first, it is expressed in rhombomeres 2 and 4 of the hindbrain, where it can be detected at day 9, and the expression in the remaining brain parts and in the spinal cord follows soon after that (Zhou et al., 1998).

In mouse neural tube, Gata2 expression begins between 10 and 12 dpc in both brain and spinal cord (Zhou et al., 1998). In the spinal cord, the gata2 expressing cells are located near the ventricular zone, but the labeling with BrdU shows that very few cells that express Gata2 are still dividing: the majority of Gata2-expressing cells are located more
laterally than the BrdU-positive cells of the ventricular zone (Zhou et al., 2000). At the early stage at which it was investigated (E10.5), Gata2 expression does not overlap with Nkx2.2, but it does overlap with dorsal Lim3- positive cells (expressed dorsally by V2 and more ventrally by MNs), and with Vsx2-expressing cells (Zhou et al., 2000), which were the only known V2 markers at the time.

In chick spinal cord, GATA2 is present in cells located within the V2 domain, with some of the GATA2-positive cells co-labeled with GATA3, but not by VSX2 (also known as CHX10) (Karunaratne et al., 2002). Interestingly, the GATA2-positive cells that are not labeled with GATA3 are localized to the more medial part of the chicken spinal cord, especially at earlier stages, suggesting that GATA2 could potentially be expressed before GATA3 (Karunaratne et al., 2002). A similar expression pattern is found in mouse, where Gata2 is expressed closer to ventricular zone than Gata3 is (Nardelli et al., 1999).

In zebrafish, the teleost genome duplication led to the existence of two Gata2 paralog genes, gata2a and gata2b (Gillis et al., 2009). gata2a used to be previously known as gata2a, and gata2b had the name zgc:91840 (ZFIN.org). These genes share only 57% identity and 67% similarity, and they play different functions in tissues in which they’re expressed (Butko et al., 2015). Transcription of gata2b is first detected in zebrafish at 16hpf, where it is expressed first in the posterior plate mesoderm (Butko et al., 2015). Later gata2b expression can be detected in dorsal aorta and branchimotor neurons (at 20hpf) and in hematopoietic cells (at 50hpf and 72hpf), but at any of the investigated stages it does not appear to be expressed in spinal cord cells (Butko et al., 2015; Lewis lab observations). This is in contrast to gata2a, which is transcribed earlier – it is already
expressed at 75% epiboly in ventral ectoderm (Detrich et al., 1995), and in presumptive hematopoietic progenitors by around 3-somite stage (approx. 10.3hpf) (Detrich et al., 1995; Li et al., 2009). \textit{gata2a} is detected in posterior plate mesoderm earlier than \textit{gata2b}, at 8 somite stage (13dpf) (Li et al., 2009). Later, \textit{gata2a} is also expressed in hematopoietic and endothelial stem cells, as well as in brain and the spinal cord (Yang et al., 2007; Batista et al., 2008; Kobayashi et al., 2010). Expression patterns of \textit{gata2a} and \textit{gata2b} are therefore different – for example, \textit{gata2a} is expressed throughout dorsal aorta, and \textit{gata2b} expression is limited to only the hematopoietic part of the dorsal aorta, and \textit{gata2b} is not present in the spinal cord (Butko et al., 2015). Functions of \textit{gata2a} and \textit{gata2b} are also different in the circulatory system – loss of \textit{gata2a} results in lack of trunk circulation and pooling of red blood cells in the trunk (Zhu et al., 2011; Butko et al., 2015), while \textit{gata2b} morphants show normal expression of several blood cell markers, including \textit{tal1} (which is also expressed in spinal cord cells) and \textit{gata2a} (Butko et al., 2015). \textit{gata2a} and \textit{gata2b} may represent a subfunctionalization of mammalian \textit{Gata2} gene functions, with \textit{gata2a} being required for vascular development, and \textit{gata2b} required for correct development of hematopoietic stem cells (HSCs) (Butko et al., 2015). Given the different expression patterns and functions of \textit{gata2a} and \textit{gata2b} in zebrafish, and no apparent expression of \textit{gata2b} in zebrafish spinal cord, in this thesis I will only look at expression of \textit{gata2a} and its role in the spinal cord.

**1.6.2 Gata3**

\textit{GATA3} (GATA-binding protein 3) also belongs to the GATA family of transcription factors that bind \textit{WGATAR} (\textit{W} = \textit{A} or \textit{T} and \textit{R} = \textit{A} or \textit{G}) consensus sequences (Merika and
GATA3 was first identified as a protein that binds to an enhancer of Delta gene of human T-cell receptor (Ho et al., 1991; Ko et al., 1991; Joulin et al., 1991). Since then, it was described to play multiple roles in development, proliferation and maintenance of T-cells, as well as innate lymphoid cells, with its important role in innate and adaptive immunity highly dependent on the dose, developmental stage, and cell lineage (reviewed in: Heicklen-Klein et al., 2005, in Wan et al., 2014, and in Tindemans et al., 2014).

In mouse spinal cord, Gata3 is expressed dorsally to motoneurons and ventrally to V1 cells, by the same cells as Lhx3 and Gata2 which are markers of V2 cells (Ericson 1997, Zhou et al., 2000). Later experiments in chicken show that GATA3 labels GATA2-positive cells that are intermingled with cells that express VSX2, and VSX2 and GATA3 are never co-expressed in the same cells of spinal cord (Karunaratne et al., 2002). In mouse spinal cord, GATA3 expression partially overlaps with TAL1 expression, with cells that express both markers located more medially than GATA3+/TAL1- cells (Smith et al., 2002). Similar to what was found in chicken, in mouse GATA3 and VSX2 expression are mutually exclusive but cells that express those two markers are intermingled with each other (Smith et al., 2002). Also, at least some gata3-expressing cells co-express gata2a and tal1 in zebrafish, and these cells are intermingled with vsx2-positive V2a cells (Batista et al., 2008). In conclusion, Gata3 is a well-established marker of inhibitory V2b cells in chicken, mouse and zebrafish (Karunaratne et al., 2002, Smith et al., 2002, Kimura et al., 2008; Batista et al., 2008).

In zebrafish gata3 is first expressed at 4hpf (Neave et al., 1995). During epiboly, gata3 expression is restricted mainly to the yolk syncytial layer, deep cell blastomeres, and
later to part of the blastula in a pattern resembling baseball stitching (Neave et al., 1995). Later, the expression is also detected in the intermediate mesoderm that will form pronephros, and in the pronephral ducts (Neave et al., 1995). In zebrafish central nervous system (CNS), *gata3* expression can be detected in brain and spinal cord. By 15hpf *gata3* is present in ventral spinal cord (Neave et al., 1995). At 16-somite stage *gata3* is already present in KA domains (together with *tal1* but before *gata2a*), begins only between 18hpf-24hpf (after *tal1* and *gata2a*; Batista et al, 2008). Expression in spinal cord persists until later stages but starts to diminish by 36hpf (Neave et al., 1995). *gata3* is also expressed in zebrafish brain, where it can be detected in ventral midbrain and diencephalon at 20hpf, as well as in optic tectum by 48hpf (Neave et al., 1995). *gata3* is also expressed in both newly formed neurons and glia of the telencephalon after injury, where it is required for both migration of newly born neurons and cell proliferation (Kizil et al., 2012).

### 1.6.3 Tal1

Tal1, (stem cell leukemia; also known as Scl or Tcl5), is a basic helix-loop-helix (bHLH) protein that was first discovered through its important role in T-cell acute lymphoblastic leukemia (Begley et al., 1989; Finger et al., 1989; Chen et al., 1990). Even though the protein contains a DNA binding domain, TAL1 has been shown in zebrafish, in embryonic stem cells and in human hematopoietic cell culture to be able to exert transcriptional activity without necessarily binding DNA (Porcher et al., 1999; Ravet et al., 2004). Also, in mouse TAL1 DNA-binding activity is dispensable for specification of hematopoietic cells, but is necessary for red cell maturation, suggesting that the mechanisms that TAL1 uses to mediate regulation of gene expression are dependent on cell
type and developmental process (Kassouf et al., 2008). Mechanisms that might contribute to a DNA-binding independent action of TAL1 involve protein-protein interactions and/or sequestration of other regulators that would otherwise inhibit developmental processes (Kassouf et al., 2008). It is possible that TAL1 is recruited to the enhancers of the target genes as part of a larger complex, and that the complex can not bind to DNA in absence of TAL1. Such a mechanism is in place in erythroid cell formation, where the TAL1-FOG-GATA1 complex enables expression of downstream genes (Wadman et al., 1997). Also, another possibility is that the proteins that would normally inhibit expression of downstream genes bind to TAL1 and are thus prevented from inhibiting gene expression.

Tal1 is essential for correct specification of all haematopoietic lineages and for differentiation of megakaryocytes from mesoderm (Porcher et al., 1996; Gering et al., 1998). Tal1 is also expressed in the developing nervous system, including midbrain, hindbrain, and spinal cord (Smith et al., 2002). In mouse spinal cord, tal1 is expressed in very late p2/early V2 cells, with the expression beginning at 10.5dpc in mouse, and fading by 14dpc (Smith et al., 2002; Muroyama et al., 2005).

In zebrafish, tal1 is expressed in the same cells as gata2a and gata3, which includes V2 cells but also the KA cells that are located more ventrally (Batista et al., 2008). tal1 mRNA is already present in zebrafish spinal cord at 16-somites in KA cells, but expression doesn't begin in the V2 domain until 18-somites (Batista et al., 2008).

**1.6.4 Tal2**

TAL2, like TAL1 (TAL1), is a bHLH transcription factor and it is also implicated in T cell acute lymphoblastic leukemia (T-ALL) (Baer, 1993). It was first identified as another
protein that is activated by the common chromosomal translocation implicated in human T-ALL, and it is highly homologous to TAL1 (Xia et al., 1991). In mouse, Tal2 is expressed in testes (Xia et al., 1991), as well as in developing brain tissue including midbrain, diencephalon and anterior pons (Mori et al., 1999). Even though TAL2 mutations are associated with T-cell tumors, Tal2 is not normally expressed in blood and mouse TAL2 mutants do not display any obvious changes in blood formation (Bucher et al., 2000). However, mutants display widespread malformations in CNS development including midbrain malformations, and do not survive longer than 32 days after birth (Bucher et al., 2000).

Brain expression of Tal2 in mouse partly overlaps with expression of Gata2, Gata3 and Tal1/Tal1 (Achim et al., 2013). Interestingly, all those genes are expressed by GABAergic neurons of the brain, but the overlap in their expression patterns differs between specific areas of the brain. In the midbrain area, Tal2 is expressed more broadly than Tal1 (Achim et al., 2013). The opposite is true for the neighboring rhombomere 1 region, where Tal1 is expressed more broadly than Tal2 (Achim et al., 2013). Also, in the midbrain almost all Tal2-expressing cells are labeled by GATA2 antibody, but only very few Tal2-expressing cells coincide with GATA3-positive cells (Achim et al., 2013). As will be described later, those differences result in different functions of those transcription factor genes in GABAergic neurogenesis of mouse brain (Achim et al., 2013).

In zebrafish, tal2 is also expressed in brain, but it is also present in developing spinal cord (Pinheiro et al., 2004). So far spinal cord expression has not been reported in any other organism. It is first detected in a few cells of head and spinal cord at 13 somite
(15.5hpf) stage (Pinheiro et al., 2004). Expression in head proceeds from the developing midbrain (at 22hpf), to diencephalon of the forebrain, tectum of the midbrain and hindbrain structures (at 47hpf) (Pinheiro et al., 2004). The diencephalon and tectum expression resemble that of tal1 (tal1) (Pinheiro et al., 2004, Sinclair et al., 1999), and are conserved between mouse and zebrafish (Pinheiro et al., 2004; Bucher et al., 2000). Tal2 is visible throughout the entire length of the spinal cord at 22hpf, in lateral floor plate cells, and in more dorsal sonic hedgehog-dependent cells that are located approximately in the V2/V1 domain (Pinheiro et al., 2004). By 44hpf, tal2 expression is already significantly reduced in the spinal cord (Schafer et al., 2007), and no expression of tal2 in spinal cord was detected at 47hpf (Pinheiro et al., 2004). tal2-expressing cells at 18-20 hpf do not divide, as detected by M-phase marker PhH3, suggesting that those cells may already be post-mitotic at this stage (Schafer et al., 2007). In addition, at 24hpf, tal2 at least partly co-localizes with the neuronal marker Elavl3 (previously known as HuC), indicating that some of the tal2-expressing cells are already post-mitotic neurons (Schafer et al., 2007). At 24hpf, two-thirds of the cells that express tal2 also express nkx2.9 (Yang et al., 2010). Some of the tal2-positive cells express p3 marker nkx2.2b, which suggests that tal2 is expressed by some p3 progenitor cells (Schafer et al., 2007). However, other researchers also show that at 24hpf all of the tal2-expressing cells are already GABAergic (Yang et al., 2010), which would argue against the tal2-expressing cells being progenitor cells. In addition, at least a subset of the tal2-expressing cells develop later into V3 cells (Schafer et al., 2007). In fact, at 44hpf a subset of tal2-expressing cells co-expresses sim1a (previously known as sim1). sim1a is an ortholog of mouse Sim1 gene, which is a marker of V3 cells (Borowska et
al., 2013) required for correct V3 specification (Blacklaws et al., 2015). Also, earlier at 36hpf about 25% of the tal2-positive cells also express sim1a (Yang et al., 2010).

However, the majority of cells that express tal2 in the floor plate region seem to correspond to KA" cells, and more dorsally located cells correspond to KA’ cells (Yang et al., 2010) and possibly other unidentified cell types. As mentioned before, all of the tal2-expressing cells are GABAergic at 24hpf (Yang et al., 2010). Also, tal2 expression co-localizes with gata2a, with the majority of cells in the floor plate region expressing both markers, but only a fraction of more dorsally-located gata2a+ cells expressing tal2 (Yang et al., 2010). Similarly, tal2 is expressed in a number of gata3-expressing cells (Yang et al., 2010), but it is not clear whether it’s present in all or only a subset of them. Overall, these results suggest that tal2 is expressed in at least a subset of p3 cells, KA" and KA’ cells, possibly other GABAergic cells, and later in V3 cells in the floor plate region.

1.7.1 Interactions between TAL1, GATA2, GATA3, and TAL2 in the CNS

The role of TAL1 in amniotic spinal cord was extensively tested in mouse and chick, but has not been investigated in zebrafish spinal cord prior to this thesis research. In amniotes, TAL1 is expressed at the same time as GATA3 in a subset of mouse spinal cord cells (Smith et al., 2002), which raises possibility that those two proteins might interact in those cells. At 11.5dpc, TAL1+/GATA3+ cells are located more medially, while TAL1-/GATA3+ cells are restricted to the more lateral edges of the spinal cord (Smith et al., 2002), which suggests that Tal1 may stop being expressed by V2b cells earlier than Gata3 and may be upstream of Gata3. Since TAL1 null mutant mice lack blood cells and die early at E8.5 (Porcher et al., 1996), a special nerve-tissue specific knockout line was constructed
to test the function of TAL1 in mouse spinal cord (Muroyama et al., 2005). The resulting experiments show that nerve tissue-specific ablation of Tal1 results in loss of Gata3 expression in V2 cells (which was not associated with cell death), and significant attenuation of Gata2 expression (Muroyama et al., 2005). It’s important to note that gata2a attenuation in mouse could result from the mosaic nature of tissue-specific tal1 ablation, and gata2a might be completely lost if non-mosaic V2 cell Tal1 deletion was possible. In addition, the same study revealed that Tal1 ablation results in dorsal expansion of Olig2-expressing pMN cells, and Olig2 mutation results in expansion of Tal1, Gata2 and Gata3, suggesting that Tal1 and Olig2 cross-repress each other (Muroyama et al., 2005).

Also, in chicken spinal cord overexpression of full length mouse TAL1 results in suppression of endogenous VSX2-expression by V2 cells, and ectopic expression of GATA3 (Fig. 3; Muroyama et al., 2005). This suggests that TAL1 may be sufficient to potentially change V2a cells to V2b cells in chicken embryos (Muroyama et al., 2005). Taken together, these results suggest that with respect to V2b cells in amniote spinal cord, Tal1 is both sufficient and required for Gata3+ V2b development, and is required for maintenance of normal levels of Gata2 expression (Muroyama et al., 2005).

Mutant Gata2 mice investigated at the early (E10.5) stage show a reduction in both Vsx2 and more dorsal Lim3, suggesting that Gata2 is required for formation of V2 cells (Fig. 3; Zhou et al., 2000). Also, in mouse Gata3 is expressed outside of the ventricular zone (as opposed to Gata2 which is present closer to ventricular zone), and its expression might depend on presence of Gata2, as in Gata2 mutants Gata3 cannot be detected, at least in whole mount embryos that were analyzed (Fig. 3; Nardelli et al., 1999). In addition, a very
recent study performed in mouse (Fig. 3; Francius et al., 2014), confirms that Gata2 is required for formation of normal numbers of both V2a (Vsx2-positive) and V2b (Gata3-positive) cells in mouse spinal cord, as the expression of each of these markers is reduced by about 60% in Gata2 mutant embryos. In addition, use of conditional Cre-induced knockdown in a Gata2loxp/loxp mouse, which removes the start codon of Gata2, shows that Gata2 is crucial for consolidation of the V2a/V2b fate, as many cells express both Vsx2 and Gata3 simultaneously in the spinal cord of E12.5 mouse when Gata2 is knocked down at E9.5 (Francius et al., 2014). However, it seems that Gata3-expressing neurons still form in large numbers in Gata2 conditional knockout mice, which is a different result to the Gata2 complete knockout mouse reported earlier (Zhu et al., 2000), and the above study (Francius et al., 2014). Unfortunately, Gata3-expressing cells in the conditional knockout were not counted, and it is not clear how many cells still express this marker (Francius et al., 2014). Overall, this suggests however that Gata2 is required for correct development of both V2a cells and V2b cells in mouse, and that Gata2 is upstream of Gata3 in mouse spinal cord. The function of Gata3 in the mouse mutant spinal cord has not been investigated so far.

Overexpression experiments in chicken also show that ectopic GATA2 expression is sufficient to induce GATA3 expression while reducing the expression of VSX2 by V2 cells, as well as reducing the number of ISLET1-expressing MNs in the chicken spinal cord (Fig. 3, Karunaratne et al., 2002). In addition, the same study shows that overexpression of GATA3 can result in ectopic expression of GATA2, this effect is, however, less prominent. The expression patterns of GATA2 and GATA3, together with these overexpression experiments, suggest that in chicken GATA2 is upstream of GATA3 in the V2 cell domain and that once
expressed, \textit{GATA3} can either induce or maintain the expression of \textit{GATA2 in vivo} (Karunaratne \textit{et al.}, 2002). Interestingly, both \textit{Gata2} and \textit{Gata3} have 5’ sequences that would potentially be able to bind proteins of the GATA family, supporting the idea of a possible ‘feedback loop’ (Karunaratne \textit{et al.}, 2002).

During brain development in mouse, \textit{Gata2} is required for specification of GABAergic neurons in the midbrain (Kala \textit{et al.}, 2009), and both required and sufficient for specification of serotonergic neurons in rhombomere 1 of the hindbrain (Craven \textit{et al.}, 2004). It is also required for normal development and correct migration of optic tectum cells in rat brain (Willett and Greene, 2011). In chick midbrain and hindbrain, \textit{Gata2} expression precedes that of \textit{Tal1} and \textit{Gata3}, suggesting that \textit{Gata2} might be upstream of the other two genes in chick brain (Herberth \textit{et al.}, 2005). This suggests that the hierarchies of those transcription factors differ between the amniote spinal cord and brain. In fact, recent research shows that interactions between GATA-family transcription factors and TAL-transcription factors within mouse brain may be tissue-dependent. Formation of midbrain GABAergic neurons seems to be differently regulated between specific subpopulations of cells (Achim \textit{et al.}, 2013).

In midbrain, \textit{Tal2} expression seems to be \textit{Gata2a}-independent, while \textit{Tal1}-expression is affected by loss of \textit{Gata2} in midbrain (and not affected in the rhombomere 1), as shown in \textit{gata2}^{floX/floX} conditional knockout (Achim \textit{et al.}, 2013). In \textit{Tal2} knockout mouse midbrains, \textit{Gata3} and \textit{Gad1} are still present but downregulated, whereas \textit{Gata2} expression persists fully (Achim \textit{et al.}, 2013). This expression of \textit{Gata2} remains unaffected even in \textit{Tal2}/\textit{Tal1} double knockout embryos, but in those embryos expression of \textit{Gad1} and \textit{Gata3} is
completely gone (Achim et al., 2013). In addition, expression of glutamatergic markers PAX6 and Slc17a6 are upregulated in Tal2 single knockout, and Tal2/Tal1 double knockout embryos, suggesting that TAL-factors, especially TAL2, are crucial for the correct specification of GABAergic cells in mouse midbrain (Achim et al., 2013). Overall, in the mouse midbrain, where Gata2 seems to be required for formation of all GABAergic neurons in that region, requirement for Tal2 in specification of GABAergic cells seem to be varied between different midbrain sub-regions, and it could be potentially explained by partial redundancy with Tal1 (Achim et al., 2013). Since the expression patterns vary between different regions of the brain, it remains to be elucidated whether Tal2 is required for formation of other GABAergic cells in the brain, and in the spinal cord.

In zebrafish, roles of gata2a, gata3 and tal1 in V2b cells of spinal cord remain unclear. Preliminary data in zebrafish gathered by a previous student in the lab, Jeffrey Jacobstein, suggests that tal1 might be upstream of gata3, but not of gata2a, in zebrafish spinal cord (Jeffrey Jacobstein, MSc dissertation, 2008). He observed that in tal1 mutant fish, gata3 is significantly reduced, while gata2a expression remains unaffected. However, when I started my research, this result still needed to be confirmed, and it was not known whether loss of tal1 affects gata3 in all of the cells that express it, or just a subset of them. In my research, I repeat these experiments, and test the functions of tal1 in V2b and KA cells using tal1 mutant zebrafish.

In addition, I also test the functions of gata2a and gata3 in specifying KA and V2b cells. Functional analysis of those transcription factor genes was previously investigated by a different lab using morpholino antisense knockdown experiments, as discussed below.
However, since the results were surprising and using morpholinos can produce non-specific effects, I decided to further test the functions of these genes in mutant embryos. The function of tal2 was also tested by this other group using a knockdown morpholino approach (as discussed below), but currently there is no zebrafish mutant available to confirm these results.

1.7.2 Functional analyses of gata2a, gata3 and tal2

Surprisingly, evidence from another study performed in zebrafish suggests that gata2a and gata3 act differently in KA" cells versus KA' cells (Yang et al., 2010).

Knockdown of gata2a using morpholinos resulted in a loss of KA" cells and what appeared to be normal numbers of KA' cells, while the opposite was true for gata3 morphants, in which KA' cells did not form and KA" cells formed in what appeared to be normal numbers (as detected with tal2 and gad67 at 24hpf in both cases; cells were not counted in either case) (Yang et al., 2010). In contrast, knockdown of tal2 had no effect on expression of either gata2a or gata3 in KA cells (Yang et al., 2010).

Interestingly though, tal2 knockdown via morpholino injections resulted in a loss of the GABAergic phenotype in KA" cells, with the KA' and V2b GABAergic phenotype remaining unaffected (Yang et al., 2010). Given that, as mentioned above, injections with this morpholino do not affect gata2a (and gata3) expression in any of the cells (Yang et al., 2010), but injections with gata2a abolish tal2 expression in KA" cells, these results suggest that tal2 is downstream of gata2a or acts in an independent pathway to specify the GABAergic phenotype of the cells.
Since morpholinos are known to sometimes have non-specific binding off-target effects, or induce apoptosis (Eisen and Smith, 2008), I decided to re-confirm these results using mutants. In addition, I examined the roles of gata2a and gata3 in V2 cells as this was not addressed by this previous study. Finally, I also used many more markers of KA and V2b cells than this previous study, which used only two molecular markers to look at KA cells, tal2 and gad. These additional markers enable me to assess whether KA cells require either gata2a or gata3 for either their global fate specification or for their GABAergic phenotype.

### 1.7.3 Mutant alleles

The tal1 mutant used in this study, tal1\(^{t21384}\) (Bussmann et al., 2007; kindly provided by Dr. Varga at University College of London, UK) carries a nonsense mutation at amino acid 183, leading to deletion of the C-terminus of the protein, including the entire bHLH domain (also see Fig. 4). This suggests that it might be a null mutant. The mutation results in abnormal vessel formation between somites, loss of tal1 expression in erythroid cells and reduction in tal1 spinal cord expression; all abnormal phenotypes can be rescued by injecting wild-type tal1 mRNA (Bussmann et al., 2007).

The gata2\(^{um27}\) mutant used in this study was kindly provided by Dr. Lawson at University of Massachusetts Medical School, MA, US. It was created with zinc-finger nuclease and results in a 10bp deletion that leads to a protein truncation upstream of both zinc finger domains (Zhu et al., 2011; also see Fig. 4). The mutant phenotype includes defects in morphogenesis of dorsal aorta, and loss of trunk blood circulation (Zhu et al., 2011).
The \textit{gata3aa0234} mutant used in this study was generated using zinc-finger-nucleases, and kindly provided by Dr. Steven Harvey at Wellcome Trust Sanger Institute, UK. The mutation causes deletion followed by an insertion at position 264aa, which leads to addition of 13 extra amino acids before reaching the stop codon (personal communication and my sequencing results). This causes only 8 amino acids of the first zinc finger domain to remain intact, and completely removes the second zinc finger from the truncated protein.
Figure 4.
Figure 4. Schematics showing location of *gata2a*<sup>um27</sup>, *tal1*<sup>t21384</sup> and *gata3*<sup>sa0234</sup> mutations. Numbers indicate amino acid positions of particular protein domains. DNA-binding domains are indicated in red (zinc finger domains) or yellow (bHLH domain). (A) *gata2a*<sup>um27</sup> mutation is a 10bp deletion that leads to a premature stop codon. If a protein is still made it will not contain either of the two zinc finger domains normally present in Gata2a (Zhu et al., 2011). (B) *tal1*<sup>t21384</sup> mutation is an A→T change that leads to formation of premature stop codon. If the protein is still made, it will not contain the bHLH DNA-binding domain (Bussmann et al., 2007). (C) *gata3*<sup>sa0234</sup> is a small deletion and insertion that leads to formation of a premature stop codon before the two zinc finger domains (unpublished, courtesy of Dr. Harvey and Dr. Stemple at Wellcome Trust Sanger Institute, UK).
1.8 Discovering novel candidates that may be expressed by V2 and/or KA cells

In addition to analyzing the functions of \textit{tal1, gata2a} and \textit{gata3} in V2b and KA cells, I was also interested in finding new potential markers of these cells. I was particularly interested in finding genes that are expressed solely by either V2b or KA cells, because at least when I started my project there were only a few transcription factor genes known to be expressed by these cells, and all of them were shared between both populations. Literature in other organisms suggested that \textit{foxn4} and \textit{sox1a/sox1b} are expressed by V2 cells, but it was not known whether KA cells and V2b cells in zebrafish also express these genes. In the following sections, I will outline the current knowledge about expression patterns of these genes and about their role in the spinal cord development.

1.8.1 \textit{foxn4}

\textit{Foxn4} (Forkead box N4) is a forkhead helix-loop-helix transcription factor that is expressed in mouse eye and spinal cord during embryonic development (Gouge \textit{et al.}, 2001). It is required for correct specification of particular cell types in the eye (Li \textit{et al.}, 2004). In mouse spinal cord, \textit{Foxn4} is expressed in a subset of p2 progenitor cells, and a small subset of \textit{Foxn4} cells also co-expresses \textit{Gata2} and \textit{Tal1} (Li \textit{et al.}, 2005; delBarrio \textit{et al.}, 2007). Later, it was also shown that \textit{Foxn4}-expressing cells give rise to all V2 cells in mouse spinal cord (Li \textit{et al.}, 2010; Misra \textit{et al.}, 2014; Panayi \textit{et al.}, 2010). In mouse spinal cord, \textit{Foxn4} acts upstream of \textit{Tal1}, as \textit{Foxn4} null mutants show loss of \textit{Tal1} expression, while \textit{Tal1} knockout mice have normal \textit{Foxn4} expression (Li \textit{et al.}, 2005; Del Barrio \textit{et al.}, 2007).
Also, early studies in chicken indicated that foxn4 on its own may not be sufficient to specify V2b cells (Li et al., 2005), but later experiments show that overexpression of Foxn4 is sufficient to induce Gata3, Gata2 and Tal1 in chicken embryos, while suppressing Vsx2 expression, suggesting that Foxn4 is sufficient for V2b formation, at the expense of V2a cells (Del Barrio et al., 2007). This study suggests that Foxn4 is both sufficient and required for V2b formation in amniotes (Li et al., 2005; Del Barrio et al., 2007).

In addition, Foxn4 is expressed in mitotically active cells that express Notch ligand delta-like 4 (Dll4) in mouse, and is often found in pairs of cells that presumably have recently undergone division (Del Barrio et al., 2007). A Foxn4 null mutation in mouse causes loss of Dll4 expression, and studies in chicken show that electroporation of Foxn4 causes ectopic expression of Dll4, suggesting that Foxn4 is both required and sufficient for Dll4 expression (Del Barrio et al., 2007). It is possible that the Foxn4 and Delta-Notch signaling participate in a regulatory loop that specifies V2b cells, as electroporation of Dll4 in chicken embryos causes an increase in Foxn4 and Gata2 expression, while attenuating the number of Vsx2-positive cells (Misra et al., 2014). Also, Notch signaling plays a crucial role in V2a/V2b division in both mouse and zebrafish, with Notch signaling being required for correct V2b specification (Del Barrio et al., 2007; Peng et al., 2007; Batista et al., 2008). In absence of Foxn4, more V2a cells form and V2b cells are reduced in number (Del Barrio et al., 2007; Peng et al., 2007; Misra et al., 2014). Taken together, these results suggest that FOXN4 may be a ‘master regulator’ of V2b versus V2a cell fate in p2 progenitor cells and that it may exert its influence through Dll4 and Notch/Delta signaling.
In zebrafish, *foxn4* is already expressed at the one cell stage, with early expression being presumably maternally derived (Danilova et al., 2004). Expression of Foxn4 continues but gradually decreases during early development (Danilova et al., 2004). Later, at 10hpf *foxn4* is expressed in the forebrain, and by 19hpf midbrain structures also express *foxn4* (Danilova et al., 2004). At 22hpf *foxn4* continues to be expressed in the retina, olfactory placode, and various parts of the brain including the area where cranial motoneurons form (Danilova et al., 2004), but from looking at photographed embryos in this study I think it might also be in anterior ventral spinal cord neurons at this stage. Expression in the head continues until at least 7dpf (Danilova et al., 2004). *foxn4* is also expressed in the atrioventricular canal of heart between 24hpf-72hpf, where is required for correct formation of this structure, as shown by morpholino-induced knockdown experiments (Chi et al., 2008). In zebrafish spinal cord, expression of *foxn4* has only been described by the one study which indicates that at 18hpf, *foxn4* is expressed in spinal cord cells that express vsx1 corresponding to very late p2 or very early V2a/V2b cells (Kimura et al., 2008; Supplementary Material). *foxn4* is expressed often in neighboring pairs of cells, and sometimes remains in only one of the cells out of the pair, which is suggested to become in future a V2a cell (Kimura et al., 2008). However, some of the Tg(Vsx1:GFP) pairs of cells express vsx1 but do not express *foxn4*, suggesting that *foxn4* expression might be transient and perhaps it could be downregulated before vsx1 (Kimura et al., 2008).

In this thesis, I will examine the expression of *foxn4* in zebrafish spinal cord over time, and try to identify cells that express this gene. I will also test whether any of the *tal1*, *gata2a* or *gata3* mutants affect the expression of *foxn4* in zebrafish spinal cord.
### 1.8.2 sox1a and sox1b

Zebrafish sox1a and sox1b are orthologs of mouse Sox1, and belong to the SOX family of transcription factors. Sox genes were first described in the mammalian genome and are divided into distinct groups based on their sequence similarity (Schepers et al., 2002). SoxB members share 85% sequence similarity within their DNA-binding domains, and the evolutionarily conserved SoxB1 group consists of Sox1, Sox2 and Sox3 (Harley et al., 1994; Bowles et al., 2000). In Drosophila, chicken and Xenopus, orthologs of these genes are expressed in neural primordium cells and are thought to play important role in specification and determination of neural cells (as described in Kan et al., 2004). In chicken, members of the SoxB1 family play an important role in interpretation of the morphogen signal in both the limb bud and neural tube (Oosterveen et al., 2013). For example, one of SoxB1 family members (Sox3) appears to be sufficient to give mesodermal cells the potential to respond to morphogens and later express transcription factor genes that form neurons (Oosterveen et al., 2013).

In mouse, Sox2 and Sox3 are expressed very early in development and are thought to contribute to maintenance of neural progenitor cells while inhibiting neurogenesis (Bylund et al., 2003; Graham et al., 2003). Sox1 is expressed later than other SoxB1 members, at the beginning of the formation of neural plate, and is required for formation of GABAergic cells in several mouse brain structures (Malas et al., 2003), as well as being sufficient for neuronal differentiation of cells from the P19 cell line (Pevny et al., 1998). Also, SOX1 is the only SoxB1 transcription factor that promotes neurogenesis via several different mechanisms, including binding inhibition of Notch signaling (binding to Hes1), suppressing
beta-catenin mediated signaling, promoting pro-neural *neurogenin1* expression and promoting exit from the cell cycle in cell culture (Kan *et al.*, 2004). However, although *Sox1* seems to be downregulated in majority of post-mitotic cells, it is still expressed in scattered cells in several adult brain structures (Malas *et al.*, 2003; Kan *et al.*, 2004).

In mouse spinal cord during early developmental stages (i.e. E9.5), *Sox1* is expressed in the ventricular zone (VZ), and not in post-mitotic differentiated cells (Pevny *et al.*, 1998; Genethliou *et al.*, 2009). However, at later stages starting from E10.5 and peaking at E12.5, *Sox1*-expressing cells are present in the differentiated cell domain (Panayi *et al.*, 2010). The origin of these cells can be traced to the progenitor p2 domain, as some of the *Sox1*-expressing cells overlap with *Foxn4-iCre_Rosa26stopYFP* –expressing cells (Panayi *et al.*, 2010). Furthermore, *Sox1*-expressing cells seem to have once expressed *Gata3* (as demonstrated by using GATA3-<sup>eGFP</sup> transgenic mouse) (Panayi *et al.*, 2010). Overall, this suggests, that in mouse an additional group of V2 cells (V2c cells) might exist, which derives from V2b cells (Panayi *et al.*, 2010). This remains to be confirmed in other organisms.

In this thesis, I have investigated expression patterns of *Sox1* orthologs, *sox1a* and *sox1b* in zebrafish, and examined whether their expression depends on presence of *gata2a*, *gata3* and/or *tal1*.

### 1.8.3 Other markers (found from microarray & literature)

In this thesis, I will also briefly describe the expression pattern of several transcription factors that have been identified by other lab members as being potential candidate markers of either V2b and/or KA cells. Those markers have been found either
from literature sources or via microarray analyses performed by the lab, which profiled the expression pattern of V2b/KA cells versus other cells in the spinal cord, using microarrays that contained at least most transcription factors in the zebrafish genome. Some of these (sox1a/sox1b/foxn4) are also included as KA and V2b markers in my mutant studies.
2. Methods

2.1 Fish husbandry

Adult zebrafish (*Danio rerio*) were maintained on a 14 hours light/10 hours dark cycle. Embryos were collected and maintained in Embryo Medium (EM: 5mM NaCl, 0.17mM KCl, 0.33mM CaCl$_2$·2H$_2$O, 0.33mM MgSO$_4$·7H$_2$O + 10g HEPES/liter). For all experiments, embryos were staged by hours post-fertilization at 28.5°C) or position of lateral line primordium over the somites (e.g. 24hpf=prim5 (Kimmel *et al.*, 1995)). Occasionally, embryos were grown at 25°C or 32°C to achieve desired stages at particular times. In these cases developmental stages were calculated and confirmed with morphological criteria as described in Kimmel *et al.*, 2005.

2.2 Fish lines

Wild-type embryos were obtained by mating wild type adults (AB, TL, or AB/TL hybrids). Transgenic embryos were obtained by mating heterozygous carriers of *Tg(8.1kGata1:eGFP)* (Kobayashi *et al.*, 2001) and homozygous/heterozygous *Tg(vsx2:GFP)*, *Tg(vsx2:Kaede)*, *Tg(vsx2:RFP)* (Kimura *et al.*, 2006) fish. Mutant embryos were obtained from matings of heterozygous *gata2a*um27 (Zhu *et al.*, 2011), *gata3*sa0234 (unpublished fish line kindly provided by Dr. Steven Harvey at Sanger Institute, Cambridge, UK) or *tal1*t21384 (Bussmann *et al.*, 2007) mutants. Adult *gata2a* and *gata3* mutant carriers (heterozygous fish) were identified by fin-clipping and PCR, followed by a restriction enzyme digest and/or sequencing whenever appropriate. Adult *tal1* mutation carriers were identified by
the above method or by the observation of the morphological phenotype in approximately 25% embryos (assumed to be homozygous mutants). The mutant morphology includes curved tails, smaller eyes, heart edema and no blood circulation in the trunk of embryos at around 48hpf (Bussmann et al., 2007).

2.3 Embryo fixation

All embryos were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich cat. # P6148) overnight at 4°C or for 4 hours at room temperature with shaking. Embryos that were to be used for antibody staining were washed out of 4% PFA with PBST (PBS (PBS, Sigma-Aldrich, cat. # P4417) + 0.1% Tween 20 (Sigma-Aldrich, cat. # P1379)) 2 x 5 minutes and 2 x 10 minutes and stored at 4°C in PBST. Embryos for in situ hybridization (ISH) were washed out of 4% PFA with PBST 2 x 5 minutes and 2 x 10 minutes, followed by dehydrating in 100% methanol (Fisher Scientific, cat. # BP1105) for 2 x 5 minutes and 2 x 10 minutes, and stored at -20°C in 100% methanol. Embryos that were used for in situ hybridization followed by immunohistochemistry (ISH+IHC) were washed 2 x 5 minutes and 2 x 10 minutes with PBST. Then these embryos were permeabilized with proteinase K 10μg/ml (Roche, cat. # 03115879001) diluted in distilled water for 24 minutes. The embryos were then fixed in 4% PFA for 20 minutes to inactivate the proteinase K, and 4% PFA was washed off with PBST for 2 x 5 minutes, 2 x 10 minutes. Finally the embryos were equilibrated in 50% cheap hybridization buffer/50% PBST. Cheap hybridization buffer is 50% formamide (Sigma-Aldrich, cat. # F9037) plus 50% 5x SSC (from a 20x SSC stock which is 3M sodium chloride (Sigma-Aldrich, cat. #S5886) + 0.3M tri-sodium citrate (Sigma-Aldrich, cat. # W302600) and 0.1% Tween 20. The embryos were then equilibrated
in 100% cheap hybridization buffer and stored -20°C for less than a week in hybridization buffer (cheap hybridization buffer + 500 μg/ml yeast RNA (Roche, cat. # 10109223001) + 50 μg/ml heparin (Sigma-Aldrich, cat. # H3393), final pH 6.0).

2.4 Plasmid preparation

All plasmids were purified using Qiagen’s QIAfilter Plasmid Midi Kit, (cat. #12243) according to the manufacturer’s instructions. Cells from a 150 ml (high copy plasmid) or 250 ml (low copy plasmid) culture of an *E. coli* transformant were lysed by alkaline lysis. The protein precipitate was separated using the filter provided in the kit. The cleared filtered solution containing the DNA was then applied to an anion exchange column. After several wash steps the plasmid DNA was eluted from the column and precipitated using isopropanol. The pellet, obtained by high speed centrifugation, was washed with 70% ethanol, air dried, resuspended in dH₂O and stored at -20°C.

2.5 Probe preparation

All probes were prepared to recognize genes described in the zebrafish community database (zfin.org). References to the gene ZFIN identifier number, and to the reference for each probe can be found in Table 2.
Table 2. Gene names and ZFIN identifiers. ZFIN identifiers (middle column) are provided for each of the genes used in this thesis along with common previous names. The column on the right provides the references for the RNA probes used for in situ hybridization experiments.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>ZFIN ID</th>
<th>References for probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gata2a</em> (previously called <em>gata2</em>)</td>
<td>ZDB-GENE-980526-260</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>gata3</em></td>
<td>ZDB-GENE-990415-82</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>tal1</em> (previously called <em>scl</em>)</td>
<td>ZDB-GENE-980526-501</td>
<td>(Peng <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td><em>tal2</em></td>
<td>ZDB-GENE-040115-1</td>
<td>(Pinheiro <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>foxn4</em></td>
<td>ZDB-GENE-990415-277</td>
<td>(Danilova <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>sox1a</em></td>
<td>ZDB-GENE-040718-186</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>sox1b</em></td>
<td>ZDB-GENE-060322-5</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>gad2</em> (previously called <em>gad65</em>)</td>
<td>ZDB-GENE-030909-9</td>
<td>(Higashijima <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>gad1b</em> (previously called <em>gad67</em>)</td>
<td>ZDB-GENE-030909-3</td>
<td>(Higashijima <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>slc32a1</em> (previously called <em>viaat</em>)</td>
<td>ZDB-GENE-061201-1</td>
<td>(Kimura <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td><em>nkh6.1</em></td>
<td>ZDB-GENE-040718-178</td>
<td>(Cheesman <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>nkh6.2</em></td>
<td>ZDB-GENE-070626-1</td>
<td>(Hutchinson <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td><em>dbx1b</em></td>
<td>ZDB-GENE-000128-11</td>
<td>(Seo <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td><em>dbx2</em></td>
<td>ZDB-GENE-000128-13</td>
<td>(Seo <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td><em>insm1a</em></td>
<td>ZDB-GENE-040426-1810</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>crb1</em></td>
<td>ZDB-GENE-050208-382</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>her6</em></td>
<td>ZDB-GENE-980526-144</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>mnx1</em></td>
<td>ZDB-GENE-040409-1</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td><em>sp8a</em></td>
<td>ZDB-GENE-030131-9849</td>
<td>(England <em>et al.</em>, 2014)</td>
</tr>
</tbody>
</table>
Probes were synthesized using template from either a Polymerase Chain Reaction (PCR) product or linearized plasmid DNA (Table 2, Table 3), followed by a probe reaction. For PCR, primers were designed by myself or other members of the Lewis lab and obtained from Integrated DNA Technologies, and the following reaction was carried out to obtain the template:

**PCR mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>27.25μL</td>
</tr>
<tr>
<td>5x Phusion HF Buffer</td>
<td>10μL</td>
</tr>
<tr>
<td>cDNA</td>
<td>5μL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1μL</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>1.25μL</td>
</tr>
<tr>
<td>Forward primer (10mM)</td>
<td>2.5μL</td>
</tr>
<tr>
<td>Reverse primer (10mM)</td>
<td>2.5μL</td>
</tr>
<tr>
<td>Phusion DNA polymerase</td>
<td>0.5μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50μL</td>
</tr>
</tbody>
</table>

**PCR conditions:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>94°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>56.5°C</td>
<td>30 seconds x35</td>
</tr>
<tr>
<td>72°C</td>
<td>90 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

2.5μL of the PCR reaction was run on a 1% TAE agarose gel at 125 mV for 45 minutes.

If the PCR resulted in a product was of the correct size, the remaining PCR mix volume was increased to 200μL with dH₂O. DNA was extracted using equal volume of
Phenol:Chloroform:Isoamyl Alcohol followed by equal volume of Chloroform:Isoamyl Alcohol. After each step, aqueous layer was removed and transferred to fresh tubes, vortexed for at least 20 seconds, and centrifuged for 5 minutes at 13500rpm. Finally, the DNA was precipitated by adding 1/10 volume of 4M sodium chloride and 2 x volume of ethanol and placing the reaction at -20°C overnight or longer. After spinning the reaction at 13,500 rpm for 30 minutes, the supernatant was removed and the pellet was washed with 70% ice cold ethanol. Finally, I resuspended the DNA in 20μL of dH2O.

When using plasmid DNA to prepare probe template, plasmid DNA in excess of 1μg of insert DNA was linearized with the appropriate restriction enzyme (Table 3). All restriction endonucleases were obtained from New England Biolabs or Roche Diagnostics Ltd. A 20μl reaction included 2μl buffer, DNA to give required concentration, dH2O to make up the volume and 0.5 μl enzyme (or 1 unit/μg DNA). The reaction mix was put at 37°C for 2 hours then treated with proteinase K (0.05μg/μl) for 30 minutes at 37°C. DNA was extracted with phenol chloroform followed by chloroform and cut DNA was precipitated with ethanol and salts (1/10 volume of 4M NaCl + 2 volumes of ethanol) by placing the solution at ~20°C overnight or longer, then centrifuged at maximum speed for 30 minutes and resuspended in dH2O.

Probe reactions were conducted in 20μl total volume containing linearized DNA equivalent to 1μg insert DNA, 2μl of 10X digoxigenin-UTP from DIG RNA Labeling Mix (Roche cat. # 11277073910) or 2μl of 10X fluorescein-UTP from Fluorescein RNA Labeling mix (Roche cat. # 11685619910) nucleotide mix, 2μl transcription buffer (40 units of the appropriate RNA polymerase (see Table 3), T7 RNA Polymerase (New England Biolabs, cat. # M0251S), T3 RNA Polymerase (Roche cat. # 11031171001), SP6 RNA
Polymerase (Roche cat. # 10810274001) and RNAs free dH2O. The reaction mixtures were put at 37°C for 2 hours, then 40 units DNase I (Roche, cat. # 04716728001) were added and incubated for 15 minutes. This reaction was stopped with 2μl of 200 mM EDTA pH 8.0 (Sigma-Aldrich, cat # E5134). RNA was precipitated with 2.5μl 4M LiCl (Sigma-Aldrich, L9650) and 75μl pre-chilled ethanol at −20°C overnight. The solution was then centrifuged at maximum speed for 30 minutes at 4°C and re-suspended in 100μl RNAs free water containing 40 units RNase inhibitor. 2.5 μl of this was electrophoresed in 1x TAE buffer at 180 mV for 7 minutes to check that a reasonable quantity of RNA had been synthesized and had not degraded. Then 400μl of hybridization buffer (50% formamide; 50% 5x SSC; 0.1% Tween 20; 500 μg/ml yeast tRNA + 50 μg/ml heparin final pH 6.0) was added to the rest of the RNA solution (probe) before storing at −20°C.
Table 3. Restriction enzymes, primer sequences and RNA polymerases used to prepare in situ hybridization probes used in this thesis. Reagents that were used for making each of the in situ hybridization probes used in this thesis are listed. RNA polymerases are listed in each case. When PCR primers were used for making in situ hybridization probes, each forward and reverse primers are shown. Whenever plasmid constructs were used, restriction enzymes used to linearize the plasmid are provided.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Polymerase</th>
<th>PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>gata2a</td>
<td>T3</td>
<td>GTGAGGGTTTCGAGGAGCTC AATTAACCCTCACTAAAGGGAAGCGAACCACATCGCCCTTTGCTAG</td>
</tr>
<tr>
<td>gata3</td>
<td>T3</td>
<td>CTGCTACCTCACAATCTCCAC AATTAACCCTCACTAAAGGGAACCCATTCGATCTGCAATTACATAAG</td>
</tr>
<tr>
<td>insm1a</td>
<td>T3</td>
<td>GCGAAATAAGAAAAGCGGACACCTG AATTAACCCTCACTAAAGGGAATGCTCCGCGGAGCTATAAAC</td>
</tr>
<tr>
<td>crb1</td>
<td>T3</td>
<td>TACTCAAGACCTCAACACTCTGC AATTAACCCTCACTAAAGGGAACCTCAGATCTGCTACCTCAG</td>
</tr>
<tr>
<td>her6</td>
<td>T3</td>
<td>ACCAGTGGAACTCGGGACAC AATTAACCCTCACTAAAGGGAATCATAAAAGGCGAAGT</td>
</tr>
<tr>
<td>mnx1</td>
<td>T3</td>
<td>TCCATATCCTCTCTCTCCCGACAC AATTAACCCTCACTAAAGGGAATGCTATGCTTCTGACCTTCAG</td>
</tr>
<tr>
<td>sp8a</td>
<td>T3</td>
<td>ACACAGAACCAGTCCAGAAC AATTAACCCTCACTAAAGGGAAGCGGTTCCTTTAACCTCAGATG</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Polymerase</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>tal1</td>
<td>T7</td>
<td>SalI</td>
</tr>
<tr>
<td>tal2</td>
<td>T3</td>
<td>EcoRI</td>
</tr>
<tr>
<td>foxn4</td>
<td>T3</td>
<td>XhoI</td>
</tr>
<tr>
<td>sox1a</td>
<td>T3</td>
<td>XhoI</td>
</tr>
<tr>
<td>sox1b</td>
<td>T3</td>
<td>HindIII</td>
</tr>
<tr>
<td>gad2</td>
<td>T3</td>
<td>EcoRI</td>
</tr>
<tr>
<td>gad1b</td>
<td>T3</td>
<td>EcoRI</td>
</tr>
<tr>
<td>slc32a1</td>
<td>T3</td>
<td>XhoI</td>
</tr>
<tr>
<td>nkx6.1</td>
<td>T7</td>
<td>XbaI</td>
</tr>
<tr>
<td>nkx6.2</td>
<td>T7</td>
<td>NotI</td>
</tr>
<tr>
<td>dbx1b</td>
<td>T7</td>
<td>BamHI</td>
</tr>
<tr>
<td>dbx2</td>
<td>T7</td>
<td>BamHI</td>
</tr>
</tbody>
</table>
2.6 *in situ* hybridization and immunohistochemistry

procedures

2.6.1 Single *in situ* hybridization

Embryos (including some for antibody pre-absorption) were rehydrated through a MeOH/PBS series: 5-10 minutes each in 75:25 50:50 25:75 and 100% PBST. Embryos fixed at 24 h or older stages were treated with proteinase K at 10μg/ml for the length of time that was pre-determined as most suitable by other lab members (our Proteinase K stock solutions are calibrated each time a new stock is made), or for 1-2 minutes longer in cases where staining had been weak in previous experiments and I wanted to check if more permeabilization would improve probe penetrance. The following times were normally used: 22hpf embryos 12 minutes, 24hpf embryos 24 minutes, 27hpf embryos 27 minutes, 72hpf embryos (for preabsorption of antibody) 1 hour (also see Table 4). Then all embryos were re-fixed in 4% PFA for 20 minutes and washed 2 times for 5 minutes and 1 time for 10 minutes in PBST. All embryos were equilibrated in 50% cheap hybridization buffer (50% formamide; 5x SSC; 0.1% Tween 20): 50% PBST. They were then equilibrated in 100% cheap hybridization buffer. This was replaced with fish hybridization buffer (cheap hybridization buffer + 500 μg/ml yeast tRNA + 50 μg/ml heparin, final pH 6.0) in which embryos were pre-hybridized at 70°C for at least 4 hours. Embryos were hybridized with RNA probes (1:20000 dilution of the synthesized probe) in hybridization buffer overnight. Probes in hybridization buffer solution were placed at 70°C for at least one hour before use. Washes were also prepared and placed at 70°C overnight to equilibrate to temperature (70°C). On the next day the embryos were washed at 70°C with 1 ml of the following
solutions: 2 times with cheap hybridization buffer for 5 minutes each; 1 time with 50% cheap hybridization buffer: 50% 2x SSC for 5 minutes; 3 times with 2x SSC for 20 minutes each; 2 times with 0.2x SSC for 20 minutes each; 1 time with 0.1x SSC for 20 minutes; 3 times with PBST 5 minutes each. The embryos were then washed with 1 ml of PBST at room temperature and incubated with sheep *in situ* block (PBST + 2 mg/ml BSA (Sigma-Aldrich, cat. # A7906) + 5 % sheep serum (Sigma-Aldrich, cat. # S2263) + 1% DMSO (Sigma-Aldrich, Cat. # D5879)) for 1 hour at room temperature. Sheep anti-Dig AP conjugated antibody (Roche, cat # 11093274910), which had previously been added to permeabilized embryos and preabsorbed over night at 4°C in sheep *in situ* block, was then added to the experimental embryos at a concentration of 1/2000 and incubated for two hours at room temperature. The embryos were then washed 8 x 15 minutes with PBST and left washing over night at 4°C in PBST. Next morning the embryos were washed 3 times in NTMT (0.1 M NaCl + 0.05M MgCl + 0.1M Tris pH 9.5 + 0.1% Tween 20, all diluted in dH2O) buffer and stained with 20 μl of NBT/BCIP stock solution (Roche, cat. # 11681451001) per ml of NTMT. To stop the staining reaction, embryos were washed twice for 5 minutes and then twice for a minimum of 10 minutes in NTMT, followed by being washed twice for 5 minutes and then twice for a minimum of 10 minutes in PBST.

Embryos were then kept in PBST at 4°C for short-term storage and or in PBST+azide for long-term storage. For photography, embryos were passed through a glycerol series (30% glycerol in PBS; 50% glycerol in PBS; 70% glycerol in dH2O). However embryos that needed to be genotyped were kept in PBST and then put straight into 70% glycerol in dH2O after being genotyped, before counting cells and photographing.
Table 4. Permeabilization times used for *in situ* hybridization and immunohistochemistry experiments in this thesis. Permeabilization times at room temperature with proteinase K (10μg/ml) times are stated for *in situ* hybridization experiments. Permeabilization times at -20°C with acetone are stated for immunohistochemistry experiments. All stages are shown in hours post-fertilization (hpf), and all times are shown in minutes (mins).

<table>
<thead>
<tr>
<th>Stage of embryo (hpf)</th>
<th>Incubation with Proteinase K at 10μg/ml (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>less than 24hpf</td>
<td>10 mins</td>
</tr>
<tr>
<td>24 hpf</td>
<td>24 mins</td>
</tr>
<tr>
<td>27 hpf</td>
<td>27 mins</td>
</tr>
<tr>
<td>30 hpf</td>
<td>30 mins</td>
</tr>
<tr>
<td>36 hpf</td>
<td>36 mins</td>
</tr>
<tr>
<td>72hpf and older</td>
<td>60 mins</td>
</tr>
<tr>
<td>(for preabsorption)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage of embryo (hpf)</th>
<th>Acetone permeabilization (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hpf</td>
<td>12 mins</td>
</tr>
<tr>
<td>27 hpf</td>
<td>18 mins</td>
</tr>
<tr>
<td>30 hpf</td>
<td>21 mins</td>
</tr>
<tr>
<td>36 hpf</td>
<td>25 mins</td>
</tr>
<tr>
<td>48 hpf</td>
<td>30 mins</td>
</tr>
</tbody>
</table>
2.6.2 Double fluorescent in situ hybridization

For double fluorescent in situ hybridization, both RNA probes (one digoxigenin and the other fluorescein labeled) were hybridized simultaneously and the protocol was initially the same as for single in situ hybridization (see above). However before incubation with blocking solution, embryos were treated with Image-iT FX Signal Enhancer (Invitrogen, cat. # I36933), by removing all PBST from the embryos and adding 2 drops of Image-iT FX Signal Enhancer and incubating at room temperature for 30 minutes. The embryos were then washed with PBST twice for 5 minutes, and once for 10 minutes. Embryos were then incubated with goat in situ blocking solution (1x PBST + 2 mg/ml BSA + 5% goat serum (Sigma-Aldrich, cat. # G6767) + 1% DMSO diluted in PBST) for 1 hour at room temperature. The embryos were then incubated with the primary antibodies Mouse anti-Dig (1/5000, Jackson ImmunoResearch, West Grove PA, cat. # 200-002-156) and Rabbit anti-Flu (1/2500, Molecular Probes, cat # A889) simultaneously for 4 hours at room temperature. The embryos were washed in PBST 8 x 15 minute intervals and left washing over night at 4°C in PBST. One of the primary antibodies was detected with Anti-Mouse-HRP or Anti-Rabbit-HRP (both from Invitrogen TSA kit number 5 cat. # T20922 and kit number 12 cat. # T20915, respectively) at a concentration of 1/200 in blocking solution during a 5 hour incubation period at room temperature. The embryos were again washed in PBST 8 times for 15 minutes and left washing over night at 4°C in PBST.

The next morning embryos were then incubated 10 minutes with amplification buffer (Invitrogen TSA kits mentioned before) and then incubated for 52 minutes with Alexa Fluor 488 (Invitrogen TSA kit number 12 cat. # T20915) or Alexa Fluor 594 (Invitrogen TSA kit number 5 cat. # T20922) tyramide reagents at a concentration of 1/100
diluted in amplification buffer with 0.0015% hydrogen peroxide. The embryos were then washed with PBST 8 times for 15 minutes. The HRP antibody from the first antibody staining was then inactivated before starting the second antibody incubation, by treating the embryos for 30 minutes with 3% hydrogen peroxide in PBST. After the inactivation step the embryos were washed with PBST 8 times for 15 minutes and left over night at 4°C in PBST. On the following morning the embryos were incubated with the complementary antibody to the previous reaction, either Anti-Mouse-HRP or Anti-Rabbit-HRP (both from Invitrogen TSA kit number 5 cat. # T20922 and kit number 12 cat. # T20915, respectively) for 2 hours at room temperature. The embryos were washed 8 times for 15 minutes and then left washing overnight at 4°C in PBST. Finally, the next morning the staining procedure with the tyramide reagent was repeated as described above with the other Alexa reagent. The embryos were washed for 8 times for 15 minutes with PBST at room temperature and then all the PBST was removed and 2 drops of DABCO were added to protect the embryos’ fluorescence from quenching. Embryos were mounted in DABCO for photography and analysis on a compound microscope and/or confocal microscope. Yolks were always removed from embryos for further analysis and photography.
2.6.3 Immunohistochemistry

Embryos stored at 4°C in PBST (or PBST + sodium azide if stored for longer than 2 weeks) and were washed 3 times with PBST. The embryos were then incubated with distilled water for 5 minutes at room temperature, then incubated with acetone at -20°C for the time indicated in Table 4, and then incubated again with distilled water for 5 minutes at room temperature. The embryos were then washed in PBS for 5 minutes and treated with Image-iT FX Signal Enhancer (Invitrogen, I36933), by removing all PBS from the embryos and adding 2 drops of Image-iT FX Signal Enhancer to the embryos and incubating at room temperature for 30 minutes. The embryos were then washed with PBS twice for 5 minutes, and once for 10 minutes. The embryos were incubated with goat antibody blocking solution (2% goat serum, 1% BSA, 2% DMSO, 0.2% Triton-X in PBS) for 1 hour at room temperature. Then a primary antibody diluted in goat antibody block at the appropriate concentration was added to the embryos and incubated for 5 hours at room temperature. The embryos were then washed 8 times every 15-20 minutes with PDT (2% DMSO; 0.1% triton-X in PBS). The embryos were left washing over night at 4°C in PDT. The next morning, the secondary antibody diluted in goat antibody block at the appropriate concentration was added to the embryos and incubated for 5 hours at room temperature. The embryos were then once again washed 8 times every 20 minutes with PDT and finally all the PDT was removed from the embryos and they were stored in DABCO (Acros Organics, AC11247-1000).
2.6.4 Single fluorescent in situ hybridization followed by immunohistochemistry

This protocol is similar to the double fluorescent in situ hybridization protocol described above, however, before the first antibody incubation and staining was inactivated with 3% hydrogen peroxide in PBST, the immunohistochemistry procedure was started. A primary antibody was added to the embryos (usually chicken anti-GFP (Abcam, ab13970) diluted 1:1000) in goat antibody block solution (PBS + 2% DMSO + 1% BSA + 2% goat serum + 0.2% Triton-X (Sigma-Aldrich, cat. # X100)) and incubated for 5 hours at room temperature. The embryos were then washed at least 8 times for 15 minutes each with PDT (PBS + 2% DMSO + 0.1% Triton X) over a period of two hours. The embryos were left washing over night at 4°C in PDT. The next morning the secondary antibody was added to the embryos (usually a Goat Anti-Rabbit Alexa 488 diluted 1:1000 in goat antibody block solution) and incubated for 5 hours at room temperature. The embryos were then once again washed 8 times every 15 minutes with PDT and finally all the PDT was removed from the embryos and they were stored in 2 drops of Vectashield Mounting Medium (Vector laboratories H-1000).

2.6.5 Use of antibodies in immunohistochemistry

GFP was detected with Rabbit Anti-GFP (Molecular Probes, A-6465), or Chicken anti-GFP (Abcam, ab13970). Kaede was detected with Rabbit Anti-Kaede (MBL International Corporation (cat. # PM012), RFP was detected with Living Colors anti-mCherry antibody (Clontech, 632496). All of the above primary antibodies were used at a
concentration. The primary antibodies used in this work were revealed using complementary combinations of the following secondary antibodies:

- Alexa-Fluor Goat-Anti-Chicken 488 (1/1000) from Invitrogen Corp., (cat. # A11039);
- Alexa-Fluor Goat-Anti-Rabbit 488 (1/1000) from Molecular Probes (cat. # A11034);
- Alexa-Fluor Goat-Anti-Mouse 488 (1/1000) from Molecular Probes (cat. # A11029);
- Alexa-Fluor Goat-Anti-Rabbit 568 (1/1000) from Molecular Probes (cat. # A11036);
- Alexa-Fluor Goat-Anti-Mouse 568 (1/1000) from Molecular Probes (cat. # A11031).

### 2.7 Genotyping

For the first part of research leading to this thesis I used one DNA isolation method, and later I used the second method. Both methods are described below, and I have used the new method since 28th November 2014. I verified that the new method worked as well as the older method by testing the same samples side by side but since the newer method was much more reproducible (PCR worked with newer method much more frequently than with the older one), as well as being more time-efficient, easier and requiring fewer reagents, I then switched to always using it.

#### 2.7.1 Fin-clipping adult zebrafish

Adult fish were placed into tricaine solution and approximately one-third of the caudal fin was clipped off.

**Older method of DNA isolation**: Fins were placed in lysis buffer (10 mM Tris-HCl, pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, 200 µg/ml proteinase K in dH2O) + 100 µg/ml Proteinase K and incubated at 55°C for 2 hours. The Proteinase K was inactivated by
heating to 90°C for 10 minutes. The debris was centrifuged for 10 minutes at 13500rpm, and the supernatant transferred to a new tube. The DNA was precipitated by adding 200μL isopropanol at -20°C overnight. Next day, the solution was centrifuged at 4°C for 30 minutes. The pellet was washed with 70% ethanol and centrifuged for another 20 minutes at 4°C. The DNA was resuspended in 100μL dH2O and stored at -20°C. Supernatant was transferred to fresh tubes, and stored at 4°C (short-term) or at -20°C (long-term) and 3μL of this was used for a PCR reaction.

**New method of DNA isolation:** Fins were placed into 100μL 50 mM sodium hydroxide and incubated at 90°C for 20 minutes. Tubes were cooled by placing them on ice for 10 minutes, followed by adding 10 μL (1/10 volume) 1M Tris-HCl, pH 7.4 to neutralize the sodium hydroxide and centrifuged at 13500rpm for 10 minutes at room temperature. Supernatant was transferred to fresh tubes, and stored at 4°C (short-term) or at -20°C (long-term) and 0.5-1μL of supernatant was used for a PCR reaction.

**2.7.2 Tissue extraction from individual zebrafish embryos**

Embryos were placed in 70% glycerol on a glass coverslip and part of the tissue was carefully removed usually from head, but sometimes from tail region. Procedure to extract DNA from tissue was very similar to that described above for fin-clipping, with following exceptions: In the newer method, 20μL of 50 mM sodium hydroxide was used for incubation at 90°C, and 1M Tris-HCl was reduced to 2μL. As with fin-clips, 3μL of supernatant extracted with older method, and 0.5-1μL of supernatant from newer method was used for a PCR reaction.
2.7.3 PCR protocols

2.7.3.1 gata2a

Primers used:

Forward: 3254- gata2genoFOR: AATTCTGCACAGAGGGGCGTGAATGTGTG (Tm = 64.2°C)
Reverse: 3255- gata2genoREV: GTACAGGCCGCACGCTGTTGCAGA (Tm = 66.4°C)

PCR mix:

10xTaq buffer 2μL
50mM MgCl₂ 1μL (old protocol) or 0.6μL (new protocol)
10mM dNTPs 0.5μL
10μM Fwd primer 1μL
10μM Rev primer 1μL
H₂O to 20 μL
Taq Polymerase 0.1μL
DNA 0.5-3μL (usually 1μL with new protocol)

Total: 20μL

PCR program used:

98°C 1 minute
94°C 30 seconds
65°C 45 seconds
72°C 45 seconds
72°C 5 minutes

The resulting PCR product was run on a high resolution 2% SFR gel (Amresco, J234-100G) for at least 60 minutes at 60 mV, and the resulting bands were 108bp for wild-type, and 98 bp for mutant (Fig. 5; as described in: Zhu et al., 2011).
Figure 5.
Figure 5. Genotyping $gata^{2a_{um27}}$, $tal^{1t21384}$ and $gata^{3sa0234}$ mutants. (A) $gata^{2a_{um27}}$ mutation results in 10bp deletion, which produces a 98bp PCR band (mutant) in comparison to 108bp PCR band (wild-type). Bands are separated on a 2% SFR agarose gel. (B) $tal^{1t21384}$ mutation produces a $DdeI$ restriction site, which after PCR amplification and restriction enzyme digestion produces 180+20bp cut bands (mutant), in comparison to 180bp uncut band (wild-type). The 20bp fragment is too small to see on a 2.5% agarose gel. (C) $gata^{3sa0234}$ ‘method 1’ protocol inserts the $BserI$ restriction enzyme site into the wild-type sequence after a nested PCR. Digestion with $BserI$ enzyme produces 233+300bp bands (wild-type), as opposed to uncut 533 band (mutant) visible on 1% agarose gel. As shown in figure, some bands are ambiguous and in these cases PCR products were sequenced to confirm the genotype. (D) $gata^{3sa0234}$ ‘method 2’ protocol takes advantage of $HinfI$ restriction enzyme site present in the wild-type, but removed after mutation (which is a deletion followed by an insertion). Digestion results in 159bp+81bp bands (wild-type), as opposed to uncut 240bp band (mutant). All PCR bands are visible on a 2% SFR agarose gel. (E) Example of sequencing result after using $gata^{3sa0234}$ ‘method 1’ protocol. Upper panel shows the wild-type sequence, in which $BserI$ restriction enzyme cutting site is introduced by the first primer set of the protocol. Lower panel shows the mutant sequence, in which the $BserI$ restriction site is not introduced. Second PCR in this protocol adds M13(-21) primer site which enables efficient sequencing.
2.7.3.2 tal1

Primers used:

Forward: tttcatgcatatccaaa (Tm = 50.7°C)
Reverse: gaaaatcgtgcacaactt (Tm = 53.9°C)

PCR mix:

- 10×Taq buffer: 2 μL
- 50 mM MgCl₂: 1 μL
- 10 mM dNTPs: 0.6 μL
- 10 μM Fwd primer: 1 μL
- 10 μM Rev primer: 1 μL
- H₂O: to 20 μL
- Taq Polymerase: 0.1 μL
- DNA: 0.5 μL - 3 μL (usually 1 μL with new protocol)

Total: 20 μL

PCR program used:

94°C 3 minutes
72°C 5 minutes
94°C 30 seconds
54°C 45 seconds
72°C 30 seconds

Digest:

- 10×Cutsmart buffer: 2 μL
- H₂O: 13.6 μL
- DdeI enzyme (10 000 U/μL): 0.3 μL
- DNA (PCR product): 4 μL

Total: 20 μL
The resulting digested product was run on a 2.5% agarose gel for 60 minutes at 80mV, and the resulting bands were 180bp for wild-type, and 160bp+20bp for mutant (Fig. 5; as described in: Bussmann et al., 2007).

2.7.3 gata3

gata3 mutants were genotyped using two separate protocols. The first protocol was developed by Henry Putz, and involved a nested PCR that produces a final product of 533bp, which can be cut into 2 fragments of 233bp and 300bp length (wild-type), or remain uncut (mutant). However, the BseRI enzyme used in this method is not always effective, so this method can produce ambiguous results (Fig. 5, details described above). In these cases, we re-confirmed the results by sequencing the PCR products. The sequencing method is reliable, but overall it makes the experiment time-consuming and expensive.

To avoid this issue, Dr. Santanu Banerjee developed a method that is faster and does not need sequencing. The final PCR product is 240bp in length, and it will be cut with Hinf1 enzyme into 159+81bp (wild-type), or will not be cut (mutant). After incubation for at least 6 hours at 37°C, the digest can be easily resolved on gel 2% SFR agarose (run for 60 minutes at 80 mV) in 0.5 x TBE (Fig. 5).

Primers used:

Method 1

10030 Forward 1:  tgtttagatccagcgcattg (Tm = 53.3°C)
10031 Reverse 1:  tgtccctgatgaatggcata (Tm = 53.6°C)
10026 Forward 2:  tgtgtaaaacgacggccagtagctgacacttctcagctgt (Tm = 67.7°C)
10027 Reverse 2:  tacaggaacagctagtgcagctgctgcctgctgg (Tm = 68.5°C)
Method 2

11341 Forward 1:  GGTTGTGTAGTTGTGCTTGC (Tm = 53.3°C)
11342 Reverse 1:  TTCTGTCCGTTCATTTGTG (Tm = 52.6°C)

PCR Mix:

Method 1

<table>
<thead>
<tr>
<th>PCR1:</th>
<th>PCR2:</th>
</tr>
</thead>
<tbody>
<tr>
<td>10xTaq buffer</td>
<td>10xTaq buffer</td>
</tr>
<tr>
<td>2µL</td>
<td>2µL</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>10mM dNTPs</td>
</tr>
<tr>
<td>1µL</td>
<td>0.6µL</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>10µM Fwd primer</td>
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<tr>
<td>0.6µL</td>
<td>1µL</td>
</tr>
<tr>
<td>10µM Fwd primer</td>
<td>10µM Rev primer</td>
</tr>
<tr>
<td>1µL</td>
<td>1µL</td>
</tr>
<tr>
<td>10µM Rev primer</td>
<td>H₂O</td>
</tr>
<tr>
<td>1µL</td>
<td>14.3µL</td>
</tr>
<tr>
<td>H₂O</td>
<td>Taq Polymerase</td>
</tr>
<tr>
<td>11.3µL (heads)</td>
<td>0.1µL</td>
</tr>
<tr>
<td>OR 14.3 µL (fins)</td>
<td>DNA (1:3 diluted PCR1)</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>1 µL</td>
</tr>
<tr>
<td>0.1µL</td>
<td>Total: 20µL</td>
</tr>
<tr>
<td>DNA</td>
<td>3µL (fins) OR 1µL (heads)</td>
</tr>
<tr>
<td>Total: 20µL</td>
<td></td>
</tr>
</tbody>
</table>
Method 2

PCR:
10xTaq buffer  2μL
50mM MgCl₂  1μL
10mM dNTPs  0.5μL
10μM Fwd primer  1μL
10μM Rev primer  1μL
H₂O  to 20μL
Taq Polymerase  0.1μL
DNA  3μL (fin) OR 1 μL (heads)

Total: 20μL

PCR programs used:

Method 1

PCR1:
94°C 3 minutes
94°C 30 seconds
52°C 30 seconds
72°C 75 seconds
72°C 5 minutes

PCR2:
98°C 1 minute
94°C 30 seconds
58°C 45 seconds
72°C 30 seconds
72°C 5 minutes
Method 2

98°C 1 minute

94°C 30 seconds
58°C 45 seconds
72°C 30 seconds
72°C 5 minutes

\[ \text{Method 2} \]

Digest:

Method 1

For Method 1, the amount of PCR product used in the digest was varied between experiments to maximize the efficiency of the enzyme. Concentration of DNA was estimated depending on the brightness of the band in a PCR gel and usually the amount was chosen to be around 200ng. Ambiguous results were always confirmed with genotyping (M13(-21) forward primer). Original recommendations from Henry Putz were to use 10 µl of PCR product in a 20µl reaction.

\[ \text{Method 2} \]

\textit{Hinf1} enzyme \quad 0.3 \mu l \\
10xCutsmart buffer \quad 3 \mu l \\
H_2O \quad 21.7 \mu l \\
DNA (PCR product) \quad 3\mu L \\
Total: 30\mu L
2.8 Imaging

Photographs were taken using a Zeiss Axio Imager M1 compound microscope or a Zeiss LSM 710 confocal microscope and processed using Adobe Photoshop and ImageJ software. Most images are projections of multiple focal planes, unless otherwise stated.

2.9 Cell counts and row numbers

In all cases, cell counts are for both sides of a 5-somite length of spinal cord adjacent to somites 6-10. Cell row numbers are assigned ventral to dorsal (e.g. cells directly above the notochord are in row 1). Error bars show standard error of the mean (SEM). Results were analyzed using the students’ T test. All statistical analyses was performed using Microsoft Excel. When appropriate, the following symbols were used to denote significance found level found by the Student’s t-test (Table 5).

Table 5. Symbols used to indicate different p-values. All p-values were obtained from the Student’s t-test. NS (not significant) test results are not denoted on graphs. All other values were represented by the stars as shown here.

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
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<tr>
<td>NS</td>
<td>p ≥ 0.05</td>
</tr>
<tr>
<td>*</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>**</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>***</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
2.10 Nomenclature for gene, mutant and protein names

The nomenclature for gene and protein names throughout this thesis is consistent with current guidelines for each organism. The basic nomenclature rules are outlined in Table 6.

**Table 6. Nomenclature guidelines.** Summary of nomenclature guidelines for gene and protein names for specific organisms. Rules are illustrated for a fictional gene/protein ABC. Gene symbols are always italicized, while protein symbols are not italicized. Rules about capital letters differ between organisms. When I refer to a protein or gene in more than one animal, I use just one of these conventions (usually the mouse or human). Current as of November 2015.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene symbols (always italicized)</th>
<th>Protein symbols (not italicized)</th>
<th>Reference</th>
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<td>Frog</td>
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<td>Only first letter uppercase (e.g. Abc)</td>
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</tr>
<tr>
<td>Mouse</td>
<td>Only first letter uppercase (e.g. Abc)</td>
<td>All letters uppercase (e.g. ABC)</td>
<td><a href="http://www.informatics.jax.org">www.informatics.jax.org</a></td>
</tr>
<tr>
<td>Rat</td>
<td>Only first letter uppercase (e.g. Abc)</td>
<td>All letters uppercase (e.g. ABC)</td>
<td>rgd.mcw.edu</td>
</tr>
<tr>
<td>Chicken</td>
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<td>All letters uppercase (e.g. ABC)</td>
<td>birdgenenames.org</td>
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<tr>
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<td>All letters uppercase (e.g. ABC)</td>
<td><a href="http://www.genenames.org">www.genenames.org</a></td>
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</tbody>
</table>
3. Results

3.1 Identifying and characterizing the expression of genes that label V2 and/or KA cells in zebrafish embryos

3.1.1 Investigating the relative numbers of KA’, KA’’ and V2b cells

3.1.1.1 Expression of gata2a, gata3 and tal1

As mentioned in the introduction, gata2a, gata3 and tal1 are expressed in KA and V2 cells in zebrafish spinal cord (Batista et al., 2008). To characterize in detail where these cell types are located dorso-ventrally in the spinal cord, I performed in situ hybridization with all three of these genes at 24hpf (Fig. 6A-C). I then counted how many cells were present in the region of the spinal cord adjacent to somites 6-10, on both sides of the spinal cord, in each dorsal-ventral row from the most ventral row just above the notochord (row 1) until there were no more labeled cells. As shown in Fig. 7, my cell counts show that at 24hpf, tal1, gata2a and gata3 are primarily expressed in rows 1-5, with very occasional cells found in row 6. This is consistent with these markers being expressed by both of the KA subpopulations (KA’ and KA’’; probably all cells in rows 1 and 2 and some cells in row 3; Batista et al., 2008), as well as V2b cells (probably some cells in row 3 and almost all cells more dorsal; Batista et al., 2008). This established an expression profile that I could then compare to, to establish whether other genes are also expressed in V2 cells and/or KA cells.
Figure 6. Genes expressed by V2b and/or KA cells in the wild-type embryos at 24hpf. Lateral views of zebrafish spinal cords at 24hpf. (A-C) show expression of established genes expressed by both V2b and KA cells, gata3, gata2a and tal1, respectively. (D) tal2 is expressed by KA cells and only some V2b cells. (E, F) sox1a and sox1b respectively are expressed in KA and V2b cell domains. (G) gads label GABAergic cells. All panels show the merged view of several planes from one side of the spinal cord from the compound microscope. Scale bar: 50μm
Figure 7. Number of cells that express established V2b and KA genes in wild-type embryos at 24hpf. Graphs shows average number of cells in each row labeled by in situ hybridization for different genes (represented by different colors). This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Lines above the bars indicate the expected positions of KA", KA’ and V2b cells in the zebrafish spinal cord based on current knowledge.
Table 7. Number of cells labeled by *in situ* hybridization experiments in wild-type embryos. Numbers represent average values of cells labeled by *in situ* hybridization experiments in genotyped wild-type embryos. This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Number of embryos counted is indicated in the last column. Numbers represent average ± SEM.

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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>TOTAL</th>
<th>4+ values</th>
<th>n</th>
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<td><em>gata3</em></td>
<td>20.75±0.45</td>
<td>8.75±0.59</td>
<td>15.38±0.42</td>
<td>11.38±0.73</td>
<td>6.13±0.44</td>
<td>1.75±0.31</td>
<td>0</td>
<td>0</td>
<td>64.13±0.67</td>
<td>19.25±0.53</td>
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<tr>
<td><em>gata2a</em></td>
<td>20.4±0.43</td>
<td>10.7±0.56</td>
<td>14.1±0.69</td>
<td>12.7±0.56</td>
<td>5.5±0.37</td>
<td>0.8±0.25</td>
<td>0</td>
<td>0</td>
<td>64.2±0.88</td>
<td>19±0.6</td>
<td>n=10</td>
</tr>
<tr>
<td><em>tal1</em></td>
<td>20.33±0.45</td>
<td>8.42±0.72</td>
<td>12.75±0.34</td>
<td>11.92±0.34</td>
<td>7±0.73</td>
<td>2.17±0.41</td>
<td>0.25±0.13</td>
<td>0</td>
<td>62.83±1.16</td>
<td>21.33±1.1</td>
<td>n=12</td>
</tr>
<tr>
<td><em>tal2</em></td>
<td>21.07±0.46</td>
<td>9.33±0.42</td>
<td>9.87±0.6</td>
<td>5.73±0.45</td>
<td>3.8±0.37</td>
<td>0.67±0.25</td>
<td>0</td>
<td>0</td>
<td>50.53±0.89</td>
<td>10.2±0.82</td>
<td>n=15</td>
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<tr>
<td><em>sox1a</em></td>
<td>20.93±0.35</td>
<td>8.57±0.48</td>
<td>12.43±0.5</td>
<td>10.93±0.45</td>
<td>8.07±0.58</td>
<td>3.57±0.44</td>
<td>0.5±0.14</td>
<td>0</td>
<td>65±1.23</td>
<td>23.21±0.91</td>
<td>n=14</td>
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<tr>
<td><em>sox1b</em></td>
<td>21.17±0.63</td>
<td>9±0.66</td>
<td>12.67±0.74</td>
<td>9.58±0.51</td>
<td>6.08±0.43</td>
<td>2.33±0.26</td>
<td>0.92±0.34</td>
<td>0</td>
<td>61.75±1.19</td>
<td>18.92±0.89</td>
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<td><em>foxn4</em></td>
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<td>0</td>
<td>3.33±0.47</td>
<td>10.33±0.39</td>
<td>1.93±0.21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15.6±0.25</td>
<td>12.27±0.44</td>
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<tr>
<td><em>gads</em></td>
<td>21.3±0.56</td>
<td>10.75±0.51</td>
<td>13.67±0.61</td>
<td>12.58±0.71</td>
<td>7.25±0.43</td>
<td>3.83±0.8</td>
<td>1.33±0.48</td>
<td>2.5±0.48</td>
<td>73.42±1.48</td>
<td>27.5±1.11</td>
<td>n=12</td>
</tr>
</tbody>
</table>
My cell counts in the region of the spinal cord adjacent to somites 6-10 in wild-type embryos suggest that at 24hpf the largest number of cells that express all three *gata3*, *gata2a* and *tal1* genes are located in row 1 (Fig. 7, Table 7, also see Fig 6A-C). *gata3* is expressed by an average of 20.75 cells, *gata2a* by an average of 20.4 cells, and *tal1* by an average of 20.3 cells (Fig. 7, Table 7). These results show that at 24hpf there are about 21 KA” cells in the region of spinal cord adjacent to somites 6-10. There are fewer labeled cells in row 2 where KA’ cells are located, with an average of 8.75 cells expressing *gata3*, 10.75 cells expressing *gata2a* and 8.4 cells expressing *tal1* (Fig. 7, Table 7). The slightly larger number of *gata2a*-expressing cells in this row compared to other markers suggests that *gata2a* might be expressed slightly earlier in KA’ cells than *tal1* and *gata3*. Row 3 probably corresponds to a mixed population of V2b and KA’ cells, as KA’ cells can also be found in row 3, although they are still located medially and are in contact with central canal (Dr. Claire Wyart, personal communication; Djenoune et al., 2014; my cell counts discussed later and shown in sections 3.2.1.2 and 3.2.1.3). In row 3, more cells express *gata3*, (15.3 cells), followed by *gata2a* (14.1 cells), and *tal1* (12.7 cells) (Fig. 7, Table 7; also Fig. 6 A-C; 13A-C, 15A-C; 19A-C). Expression of all of these genes is approximately equally distributed between medially located cells (KA’) and more laterally positioned cells (V2b), suggesting that KA’ and V2b cells form in similar numbers in this row (As discussed later in chapters 3.2.1.1 and 3.2.1.3). This suggests that the overall number of KA’ cells is slightly smaller than number of KA”s (approximately 18 versus 21). Alternatively, KA’ cells may be born later than KA” cells, in which case some KA’ cells may not yet express the investigated genes at 24hpf. Rows 4 and above contain no KA cells based on our current knowledge, and
all of the cells labeled by *tal1*, *gata2a* and *gata3* in these dorsoventral rows should be V2b cells (Batista *et al.*, 2008). Similar numbers of cells express all three markers in row 4 (Fig 7, Table 7). An average of 11.3 cells express *gata3*, 12.7 cells express *gata2a*, and 11.9 cells express *tal1* (Fig. 7, Table 7). Row 5 contains less V2b cells, with *gata3* being expressed by only around 6.1 cells, *gata2a* by 5.5 and *tal1* by 7.25 cells (Fig. 7, Table 7). Only occasional cells are labeled by these genes in row 6, with no more than 3 cells labeled by either of the genes (Fig. 7, Table 7). These results provide a reference point for the approximate numbers of KA and V2b cells present in this region of zebrafish spinal cord at 24hpf throughout the rest of this thesis.
3.1.1.2 Expression of tal2

As discussed in the introduction, tal2 is expressed by at least some KA cells and also by other cells, some of which are likely to be V2b cells, whereas others may be p3 or V3 cells in the lateral floor plate (Pinheiro et al., 2004; Schafer et al., 2007). This data does not clearly establish whether this gene is expressed by all KA and V2b cells. Therefore, I performed tal2 in situ hybridizations and compared the number of cells that express this gene in each dorsal-ventral row with the number of cells labeled with known KA/V2b markers. My cell counts suggest that at 24hpf tal2 is expressed by a similar number of cells to those expressing gata3/gata2a/tal1 in row 1 (Fig. 8, Table 7). At this developmental stage, V3 cells have probably not yet formed (Schafer at al., 2007), which could explain why there are not more cells that express tal2 in this row. Similarly, in row 2 an equivalent number of cells express tal2 as express other KA’ markers (Fig. 8, Table 7). However, in rows 3 and above, tal2 is expressed by far fewer cells than those expressing gata3, gata2a and tal1. In row 3 around 9.8 cells express tal2, compared to 12.7-15.4 cells labeled by tal1, gata2a and gata3. The difference is even more prominent dorsal to row 3, with tal2 only being expressed by around half of the cells that are labeled by other markers (Fig. 8, Table 7). This leads me to conclude that even though tal2 is expressed by both KA cell populations and probably by all KA cells, it is only expressed by a small subset of V2b cells.
Figure 8. Number of cells that express *tal2* in wild-type embryos at 24hpf. Graph shows average number of cells in each row labeled by *in situ* hybridization for different genes (represented by different colors). *tal2* is expressed by KA cells and some V2b cells (Pinheiro *et al*., 2004; Schafer *et al*., 2007). This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Lines above the bars indicate the expected positions of KA”, KA’ and V2b cells in the zebrafish spinal cord based on current knowledge.
3.1.1.3 Expression of sox1a and sox1b

As mentioned in the introduction, Sox1 is expressed in early spinal cord progenitor cells (Pevny et al., 1998), but also in post-mitotic spinal cells in mouse (V2c cells, Panayi et al., 2010). To investigate whether sox1a and sox1b, zebrafish orthologs of mouse Sox1, are expressed by V2 cells in zebrafish I performed in situ hybridization for each of these genes at 24h and compared the number and location of labeled cells to the expression patterns of tal1, gata2a and gata3. Interestingly, I found that sox1a and sox1b are expressed not only in the V2 domain but also in KA domains (Fig. 9, Table 7). The spinal cord expression patterns of sox1a and sox1b are very similar to each other, and to that of other KA and V2b markers (tal1, gata2a and gata3). In rows 1 and 2, similar numbers of cells express each of these sox1 genes, and similar numbers of cells are labeled by tal1, gata2a and gata3 (Fig. 9, Table 7). In row 3, the average number of cells that express sox1a (12.4), and sox1b (12.6), is closest to the number of tal1-expressing cells (12.75) than to that of other V2b/KA established markers which are expressed in more cells (Fig. 9, Table 7). This suggests that both tal1 and sox1 genes may be expressed later in row 3 cells, or not in all cells. In row 4, sox1a and sox1b are expressed by only approximately 1-2 cells less than other V2b markers. Interestingly, in rows 5 and 6, similar number of cells expresses sox1b to the number expressing tal1, gata2a and gata3, but sox1a is expressed by approximately 1-3 cells more (Fig. 9, Table 7). Occasional sox1a and sox1b-expressing cells can also be found in row 7, which is more dorsal than expression of the other markers.
Figure 9. Number of cells that express sox1a and sox1b in wild-type embryos at 24hpf. Graph shows average number of cells in each row labeled by in situ hybridization for different genes (represented by different colors). This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Lines above the bars indicate the expected positions of KA", KA' and V2b cells in the zebrafish spinal cord based on current knowledge. Both sox1a and sox1b appear to be expressed by a similar number of KA and V2b cells as the established KA and V2b genes, and potentially be a few more V2b cells in most dorsal rows.
To further confirm that *sox1a* and *sox1b* are expressed by KA and V2b cells I performed double labeling experiments with the *Tg(gata1:GFP)* line that labels V2bs and KAs in zebrafish spinal cord (Batista *et al.*, 2008). Even though *gata1* is not normally expressed in zebrafish spinal cord (Detrich *et al.*, 1995), the *Tg(gata1:GFP)* line used in this study labels *gata3*-expressing KA and V2b cells (Batista *et al.*, 2008). Most likely, *Tg(gata1:GFP)* is missing a repressor element that would normally prevent *gata1* from being expressed in a similar way to other spinal cord *gata* genes – *gata2a* and *gata3*, or it could act as an enhancer trap (Batista *et al.*, 2008). However, it seems that at 24hpf, the stage at which my experiments were performed, *Tg(gata1:GFP)* labels only a small subset of V2b cells. This might be due to delay in the labeling of the cells by GFP. In the region of the spinal cord that I usually analyze I could only identify about 5-10 GFP-expressing V2b cells on the basis of their dorsoventral position and characteristic axon trajectory. Therefore, we cannot exclude that additional cells that do not co-localize with *Tg(gata1:GFP)*-positive cells are V2b cells not labeled at this stage by this transgenic line. However, the results of my *in situ* hybridization followed by GFP immunohistochemistry staining experiment still help us to understand the identity of the *sox1a*- and *sox1b*-expressing cells. As shown in Fig. 10, both *sox1a*-expressing and *sox1b*-expressing cells co-localize with *Tg(gata1:GFP)* cells. In fact, when I analyzed three representative embryos for each experiment and counted the number of cells that co-localize, I discovered that all of the GFP-positive *Tg(gata1:GFP)* most ventral cells (corresponding to rows 1, 2, and to a lesser extent row 3) also express *sox1a* (Fig. 7A, Table 7), and *sox1b* (Fig. 7B, Table 7). This demonstrates that all KA cells express both *sox1a* and *sox1b* at 24hpf.
However, the identity of the more dorsal sox1-expressing cells is less clear. All of the more dorsal Tg(gata1:GFP) GFP-positive cells also express sox1a (Fig. 10A), and sox1b (Fig. 10B), showing that at least a subset of the dorsal sox1-expressing cells are V2bs. There are, however, many sox1a or sox1b-positive cells at a similar dorsoventral position that do not co-express Tg(gata1:GFP). My analyses show that the number of non GFP-positive cells that express sox1a is on average 16, while on average 10 cells express sox1b but not GFP (Table 8). The difference in these numbers probably reflects the fact that generally sox1a labels a few more cells than sox1b at this stage, as indicated by cell counts in single in situ hybridization experiments on wild-type embryos (Fig. 9; Table 7). These non-GFP cells could be additional V2b cells (that don’t yet express Tg(gata1:GFP), V2c cells if such cells exist in zebrafish spinal cord, another spinal cord cell type or a combination of these.

Table 8. Number of co-labeled cells in Tg(gata1:GFP) with sox1a and sox1b. Number of cells labeled within in a 5 somite length of spinal cord adjacent to somites 6-10. Green indicates number of Tg(gata1:GFP) cells, and red indicates sox1a or sox1b-labeled cells at 24hpf. Number of embryos counted is indicated at the end of each row. Numbers represent average ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>GREEN</th>
<th>GREEN ONLY</th>
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<tr>
<td>sox1a</td>
<td>43±1.15</td>
<td>0</td>
<td>59.33±0.33</td>
<td>16±1.15</td>
<td>n=3</td>
</tr>
<tr>
<td>sox1b</td>
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<td>0</td>
<td>51.67±1.45</td>
<td>10±0.58</td>
<td>n=3</td>
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Figure 10. Expression of sox1a and sox1b in Tg(gata1:GFP) embryos at 24hpf. sox1a and sox1b label KA cells, a subset of V2b cells and potentially an additional population of cells. Lateral views of spinal cords of Tg(gata1:GFP) embryos after immunohistochemistry followed by in situ hybridization labeling sox1a (A), or sox1b (B) mRNA. Anterior left, dorsal up. Panels in A and B show merged view of multiple confocal planes in half of the spinal cord. In each case, expression of sox1a or sox1b (red) and Tg(gata1:GFP) (green) are followed by the merged image of both red and green channels. Panels in A' and B' show single confocal plane of the area marked with white box in panels A and B, respectively. Stars (*) mark cells that are double labeled by both Tg(gata1:GFP) and sox1a or sox1b probes. Scale bar: 50μm
3.1.1.4 Expression of foxn4

As discussed in the introduction, foxn4 is expressed in the V2 domain of zebrafish spinal cord, possibly early in the differentiation of V2 cells. Co-expression of foxn4 and the V2a specific gene vsx1 was shown by Kimura et al., (2006). However, the V2b marker gata2a was also shown to be expressed by Tg(vsx1:GFP) cells (Kimura et al., 2006), so this transgenic line may label both V2a and V2b cells, making it unclear exactly which cells express foxn4. Whether foxn4 is co-expressed with any markers of V2b cells has not been investigated. To further confirm that foxn4 is expressed by V2 cells and to investigate whether foxn4 is expressed by V2b cells and/or KA cells, I performed in situ hybridization at 24hpf. My results and cell count data show that foxn4 is not expressed in rows 1 and 2 where KA cells form, but it is expressed in a subset of cells in the usual location of V2b cells (Fig. 11; Table 7; also photographs in Fig. 12C and in section 3.2). However, cell counts at 24hpf in wild-type embryos (Fig. 11; Table 7) show that foxn4 is expressed primarily in row 4, with only a few cells in rows 3 and 5. Only about 3 cells in row 3 express foxn4 at this stage, while an average of 1.7 cells in row 5 express foxn4. In row 4, where the majority of foxn4-expressing cells are found, the average number of cells (10.3 cells) is comparable with that of other V2b markers tal1, gata2a and gata3. Given that V2b cells are also found in other rows, and that foxn4 may also be expressed by V2a cells (Kimura et al., 2006), foxn4 appears to be expressed only in a small subset of V2 cells.
Figure 11. Number of cells that express foxn4 in wild-type embryos at 24hpf. Graph shows average number of cells in each row labeled by in situ hybridization for different genes (represented by different colors). This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Lines above the bars indicate the expected positions of KA", KA' and V2b cells in the zebrafish spinal cord based on current knowledge. Only a subset of cells in V2b domain express foxn4. Note that this panel may be compared to the cell counts in Fig. 12 (Expression of foxn4 in zebrafish wild-type embryos at different stages), but the numbers in this figure and in Fig. 12 come from separate experiments.
Figure 12.
Figure 12. Expression of foxn4 in zebrafish wild-type embryos at different stages. (A-E) show lateral views of spinal cords expressing foxn4 at 20hpf (A), 22hpf (B), 24hpf (C), 27hpf (D) and 30hpf (E). Anterior left, dorsal up. (F-H) show examples of cells labeled with foxn4 that appear to be in the process of division at 24hpf. (I) shows number of cells labeled with foxn4 in situ hybridization in wild-type embryos at indicated stages. Cell counts are an average of 4 embryos for each stage. (J) shows the total number of cells that express foxn4 at any given stage. The only statistically significant difference found by Student’s t-test is indicated by a bracket and star. Values in (I) and (J) come from the same cell count data. In both cases, error bars represent SEM. Scale bar: 50μm
To test whether foxn4 is expressed by V2b cells I performed double labels with 
Tg(gata1:GFP). To my surprise, Tg(gata1:GFP) and foxn4-expression did not overlap at 24h (Fig. 13). In addition, foxn4 was expressed more dorsally than Tg(gata1:GFP) cells, suggesting that either foxn4-expressing cells are more dorsal than V2b cells, or that the Tg(gata1:GFP) line labeled only KA cells and a small ventral subset of V2b cells in this experiment (which is possible – see also earlier discussion in 3.1.1.3 section describing sox1a and sox1b expression in this transgenic line). To further test whether foxn4 might be expressed by V2b cells I also performed double labels with slc32a1, which labels all inhibitory post-mitotic cells (as V2b cells are GABAergic). As shown in Fig. 14F, foxn4 is not co-expressed with slc32a1. Even though the cells that express slc32a1 are in the same dorsoventral position as the cells that express foxn4, none of the inhibitory cells co-express foxn4. Given the results of Kimura at al., 2006 that suggest that foxn4 is expressed by early forming V2 cells one possible explanation of my results would be that foxn4 is downregulated in older V2b cells. If this is the case, then zebrafish V2b cells appear be specified primarily in row 4 at 24hpf.
Figure 13. Expression of *foxn4* in *Tg(gata1:GFP)* at 24hpf. Lateral view of 
*Tg(gata1:GFP)* embryo after GFP immunohistochemistry and *in situ* hybridization for *foxn4* mRNA. Panels show a projection of multiple planes taken from half of the spinal cord. 
Anterior left, dorsal up. Expression of *foxn4* (red) and cells labeled by *Tg(gata1:GFP)* (green) are followed by a merged image of both channels. No cells were found to co-express *Tg(gata1:GFP)* and *foxn4*. Scale bar: 50μm
Figure 14. Expression of *foxn4* and other markers of the cell populations in the zebrafish spinal cord at 24hpf. Lateral views of spinal cords expressing *foxn4* in green and *dbx1b* (A), *dbx2* (B), *nkh6.1* (C), *nkh6.2* (D), *gata2* (E), and *slc32a1* (F) in red at 24hpf. Anterior left, dorsal up. (A-F) show a merge of multiple projections from one side of the spinal cord followed by a merged image of both green and red channels. White stars indicate cells that are double-labeled. (C'-E') show close-up single confocal planes of the area marked by the white dashed box in (C-E) respectively. Close-ups are shown only in cases where co-labeled cells were identified in single confocal planes. Scale bar: 50μm
Since foxn4 is expressed more dorsally than Tg(gata1:GFP) cells, it is also possible that the V2b cells form more dorsally over time or migrate ventrally once they have formed. To try and investigate this I examined foxn4 expression and counted the number of cells at 20hpf, 22hpf, 24hpf, 27hpf and 30hpf (Fig. 12). I predicted that if foxn4 is expressed only transiently by V2b cells, the total number of foxn4-expressing cells might not change much over time as “older” cells would be turning it off as “younger” cells are turning it on. This would be in contrast to other genes that are expressed for longer in these cells, where the number of cells expressing the gene increases over time (Batista et al., 2008). In addition, if the dorsal-ventral position at which V2b cells form changes over time, the location of foxn4-expressing cells should also change. In contrast, if “older” V2b cells migrate ventrally, the location of foxn4-expressing cells should remain the same at different stages.

As shown in Fig. 12A, foxn4 is expressed already at 20hpf in cells located mainly in row 3, with several cells expressed in row 4 (Fig. 12I). A similar pattern of expression is observed at 22hpf (Fig. 12B), however, more cells are labeled in row 4 at this stage (Fig. 12I). By 24hpf, foxn4 is expressed mainly in row 4, with some cells still in row 3, and some cells in row 5 starting to express foxn4 (Fig. 12C, I). Since both post-mitotic V2 cells and p2 progenitor domain cells are found in row 4 at 24 hpf (Batista et al., 2008; England et al., 2011), this would be consistent with foxn4 being expressed mainly by early V2 cells, or late p2 progenitor cells. Interestingly, by 27hpf similar numbers of cells in rows 4 and 5 express foxn4, with an occasional cell in row 6 expressing this marker (Fig. 12D, I). By 30hpf expression of foxn4 persists mainly in row 5, but there are also similar number of
cells labeled by foxn4 in rows 4 and row 6 (Fig. 12E, I). This shows that the dorsoventral position at which foxn4 is expressed does indeed move dorsal over time.

I also compared the total number of foxn4-expressing cells at different stages (Fig. 12J). Although there is a slight trend towards an increase in the total number of cells, the only statistically significant difference was between 22hpf and 30hpf (Appendix Table 2, bolded value). However, the differences between 20hpf and 30hpf, and between 22hpf and 27hpf are approaching the significance threshold of $p=0.05$ ($p=0.069$ for 20hpf vs 30hpf, and $p=0.063$ for 22hpf vs 27hpf, see Appendix Table 1). This suggests that the number of foxn4-expressing cells may increase slightly in zebrafish spinal cord over these time points, but it is a very subtle trend. Since the difference is so subtle and I only counted cells in 4 embryos at each stage, it is possible that examining a larger number of embryos would result in more of these differences becoming statistically significant.
Also, I observed that *foxn4* is often present in cells that are either very large, compared to their neighboring cells, or by pairs of neighboring cells, as shown in (Fig. 12F-H). This suggests that *foxn4* is expressed by cells that are undergoing division, or have just divided and both sister cells are still expressing *foxn4*. This is consistent with data from the previous zebrafish study (Kimura *et al.*, 2008 supplementary material), which observed *foxn4* in *Tg(vsx1:GFP)*-expressing cells, often in pairs of neighboring cells that both expressed *vsx1* at 18hpf.

Since *foxn4* is expressed soon after cell division, it is possible that it is already expressed by late stage progenitor cells. In order to investigate whether *foxn4* is expressed in the p2/V2 region, and also to determine whether it is expressed in progenitor cells, in post-mitotic cells, or in both populations, I performed double *in situ* hybridizations with molecular markers that mark different dorsal-ventral progenitor domains. First, I tested whether the dorso-ventral position of *foxn4* coincides with V0 progenitor markers, *dbx1b* and *dbx2*. While *dbx1a* is expressed solely by V0 progenitor cells, *dbx2* has a wider expression domain, which includes the p0 and p1 and part of the dp6 progenitor domains (see England *et al.*, 2011). As shown in Figs 14A and 14B, *foxn4* is expressed more ventrally than these markers.
Therefore, I used markers of p2 progenitor cells \textit{nkh6.1} and \textit{nkh6.2} to test whether \textit{foxn4} is expressed by V2 cells. \textit{nkh6.1} is expressed by p2 cells and also by more ventrally located pMN cells and p3 cells, while \textit{nkh6.2} is expressed by all those cells but also more dorsally located p1 cells (see England \textit{et al.}, 2011). In addition to being expressed in progenitor cells, at least \textit{nkh6.1} is also thought to persist in a subset of very early V2 interneurons (Cheesman \textit{et al.}, 2004; Kimura \textit{et al.}, 2006). As shown in Fig. 14C, many \textit{nkh6.1}-expressing cells do not express \textit{foxn4}. However, there is a slight overlap between the dorso-ventral position of \textit{nkh6.1} and \textit{foxn4}. This prompted me to examine single confocal microscope focal planes and investigate whether any of the \textit{foxn4}-expressing cells also express \textit{nkh6.1}. I found that several cells co-expressed both markers, an example of which is shown in panel 10C'. Similarly, \textit{foxn4}-expressing cells overlap with the dorso-ventral position of \textit{nkh6.2}, as shown in panel 10D. Even though many progenitor \textit{nkh6.2}-expressing cells do not express \textit{foxn4}, some \textit{foxn4}-cells express \textit{nkh6.2}, as shown in single confocal section panel 10D'. This suggests that \textit{foxn4} is expressed by very late p2 progenitor cells, and/or by very early post-mitotic V2 cells.

To further test whether \textit{foxn4} is expressed by early V2 cells, particularly early V2b cells I performed double \textit{in situ} hybridization experiments with \textit{gata2a}. \textit{gata2a} is expressed before \textit{gata3} in V2b cells in zebrafish (Batista \textit{et al.}, 2008), and it is also expressed early on by Tg\textit{(vsx1:GFP)} cells (Kimura \textit{et al.}, 2008), suggesting that it might be expressed initially by all early V2 cells, as it is in amniotes (Peng \textit{et al.}, 2007) before it resolves into just being expressed by V2b cells. I found that all of the \textit{foxn4}-expressing cells co-express \textit{gata2a}, although there are many more \textit{gata2a}-positive cells that do not express \textit{foxn4} (Fig. 14E).
This is consistent with *foxn4* being expressed only in more recently-born V2b cells and *gata2a* single-expressing cells down-regulating *foxn4*.

To summarize, my results show that *foxn4* is expressed in very early V2 cells that express *gata2a, nk6.1* and *nkx6.2*. It also can not be ruled out that *foxn4* may also be expressed by very late p2 cells, since *nk6.1* and *nkx6.2* are expressed by p2 progenitor cells. However, *foxn4* is not expressed by post-mitotic, *gata3*-expressing inhibitory V2b cells, or any other inhibitory cells in zebrafish spinal cord at 24hpf. Expression of *foxn4* is located more dorsally over time, suggesting that position at which V2 cells form changes over time. However, the gene appears to be expressed only transiently by maturing V2 cells.

### 3.1.1.5 GABAergic cells

Since V2b and KA cells are GABAergic, as discussed in the introduction, I decided to also use expression of *gad* genes (which are expressed by GABAergic cells; Higashijima *et al.*, 2004) as a method of examining both of these cell populations. However, there are also other GABAergic cells in zebrafish spinal cord, including V1 cells that are generally found slightly dorsal to V2 cells, but that probably also partly overlap with the V2 domain (Batista and Lewis, 2008; England *et al.*, 2011). According to my cell count data, at 24hpf *gads* are expressed by numbers of cells that are very comparable to the number of cells that express *gata2a/gata3/tal1* in rows 1-5, with the differences between the average number of labeled cells never being larger than 2 cells in each of those rows (Fig. 15; Table 7). Even in row 6, the number of GABAergic cells seems to be larger than that of V2b markers, but only slightly. As expected, there are additional sporadic GABAergic cells present in rows 7 and 8,
where V2b cells are not found. However, these are only few (average of 1.5 cells in row 7, and 3.5 cells in row 8), and likely reflect more dorsally located cells that are beginning to acquire a GABAergic phenotype (many of the dorsal inhibitory cells in zebrafish spinal cord are glycinegic (Higashijima et al., 2004a). Overall, I conclude that gads are a good marker for estimating the number of V2b and KA cells at 24hpf, and that V2b and KA cells can be easily distinguished at this stage from other GABAergic cell populations based on the dorsoventral location of the cells. More importantly though, use of this marker in experiments on mutant embryos discussed later will also help me to assess whether KA and V2b cells have lost their GABAergic phenotype, which is a key functional aspect of both of these cell types.
Figure 15. Number of GABAergic cells in wild-type embryos at 24hpf. Graph shows average number of cells in each row labeled by in situ hybridization for different genes, as indicated by colors. GABAergic cells are labeled by mixture of gads probes, and the numbers are shown in comparison to most established KA and V2b markers (gata3, gata2a, tal1). Only very few additional GABAergic cells are present at 24hpf in the zebrafish spinal cord. This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Lines above the bars indicate the expected positions of KA”, KA’ and V2b cells in the zebrafish spinal cord based on current knowledge.
3.1.2 Transgenic lines that could potentially label V2a cells.

V2b cells are formed by a division of one p2 cell into a V2a cell and a V2b cell (Batista et al., 2008; Kimura et al., 2008). Some genes are expressed by both V2 cell types early in differentiation (e.g. *lhx3, vsx1, gata2a*; Batista et al., 2008; Kimura et al., 2008). I was, therefore, interested in whether the new markers of V2b cells that I had identified (*sox1a, sox1b, foxn4*) are also expressed by V2a cells. I hoped to perform double labeling experiments with a transgenic line that labels V2a cells, *Tg(vsx1:GFP)* (Kimura et al., 2006) to test this. In addition, since V2a/V2b cells form from a single division, I was hoping to establish whether mutations in genes expressed in V2b cells (analyses discussed later in thesis) might result in V2a cells forming at the expense of V2b cells. For these purposes, I was hoping to use the *Tg(vsx1:GFP)* line (Kimura et al., 2008). To my surprise, my first experiments at 24hpf (when many V2a cells are already mature) did not reveal many cells labeled in the spinal cord by this transgenic line. To test whether this was the result of a delay in GFP expression I examined this line at 27hpf, 30hpf, 36hpf and 48hpf.

As shown in Fig. 16A, at 27hpf I could see only a background-like haze and only 1-2 cells in the anterior of the embryo on the compound microscope. At 30hpf I could see many more cells (see also Kimura et al., 2006). However, the background haziness did not allow me to clearly see cells and their axons on the compound microscope (Fig. 16B), and a similar effect was observed at 36hpf on the compound microscope (Fig. 16C). The experiment on embryos at 48hpf did not work, most likely because it is harder to permeabilize the embryos at this stage, and I was not able to see the GFP in the spinal cord (Fig. 16D). Therefore, I examined the embryos where the results appeared most promising
(30hpf and 36hpf) on the confocal microscope to see whether I could see cells with better resolution. As shown in Fig. 16E, at 30hpf even the confocal microscope did not let me see cell axons very clearly (likely due to the surrounding haze), but I was able to better see cell somas. At 36hpf the cell soma was completely visible (Fig. 16F), and the largely reduced haze at that stage enabled me to examine the axons of Tg(vsx2:GFP) cells. However, the number of cells that are labeled at that stage made it very difficult to distinguish between individual cells and/or follow individual axons. These limitations would make it very difficult to perform double labels, count cells or examine axon phenotypes in mutant embryos.
Figure 16. Assessment of Tg(vsx2:GFP) zebrafish line. Lateral views of spinal cord of transgenic Tg(vsx2:GFP) embryos following GFP immunohistochemistry at 27hpf (A), 30hpf (B, E), 36hpf (C, F) and 48hpf (D). Anterior left, dorsal up. (A-D) were taken using a compound microscope. (E and F) show pictures of embryos from the same experiment taken using a confocal microscope. Scale bar: 50μm
Other alternatives for labeling V2a cells are two other transgenic lines, $Tg(vsx2:RFP)$, and $Tg(vsx2:Kaede)$ (Kimura et al., 2006). These lines were made in a similar way to $Tg(vsx2:GFP)$, but as they are the result of separate BAC insertions, they might have slightly different expression patterns. I was hoping that at the earlier stages (27hpf, 30hpf, 36hpf), the haziness seen in $Tg(vsx2:GFP)$ would be reduced and I would be able to see cells more clearly, in numbers that would allow for easier counts and/or analyzing axons. Unfortunately, this is not the case (Fig. 17), although these embryos were analyzed only on the compound microscope. At 27hpf $Tg(vsx2:Kaede)$-labeled cells are slightly more visible (Fig. 17A, compare to Fig. 16A) but they still have a ‘halo’ around their cell bodies, and the cell axons are not clearly visible. $Tg(vsx2:RFP)$ cells look similar but are even dimmer at 27hpf, as shown in Fig. 17D. The haze is still present at 30hpf in $Tg(vsx2:Kaede)$. Even though more cells seem to be labeled at this stage (Fig. 17B), I still could not see cell axons in those embryos. In $Tg(vsx2:RFP)$ at 30hpf, the cells begin to be brighter and more cells are labeled, but the haze makes it possible to see only a few clear cell body shapes and none of the axons (Fig. 17E). At 36hpf the $Tg(vsx2:Kaede)$-labeled cells have largely reduced background (Fig. 17C), and many more cells are visible. This looks more suitable for experiments, as compared to $Tg(vsx2:GFP)$ pictures from the compound microscope (Fig. 16C). The $Tg(vsx2:RFP)$ line shows many cells at 36hpf (Fig. 17F), but since the cells are much dimmer and more difficult to see, even at this stage this line seems the least suitable for experiments.
Figure 17. Assessment of Tg(vsx2:Kaede) and Tg(vsx2:RFP) zebrafish lines. Lateral views of spinal cords of transgenic Tg(vsx2:Kaede) and Tg(vsx2:RFP) embryos following RFP or Kaede immunohistochemistry. Anterior left, dorsal up. (A-C) show Tg(vsx2:Kaede) embryos at 27hpf (A), 30hpf (B), 36hpf (C). (D-F) show Tg(vsx2:RFP) embryos at 27hpf (D), 30hpf (E) and 36hpf (F). All photographs were taken using a compound microscope. Scale bar: 50μm.
Overall, I conclude that the \( Tg(vsx2:Kaede) \) line might be the best out of the three lines for examining V2a cells, and the likely best stages to investigate would be between 30hpf and 36hpf. However, at those stages cells might be difficult to count, and it is still not clear whether all of the V2a cells are labeled. The \( Tg(vsx2:GFP) \) line could potentially be used between 30hpf and 36hpf depending on nature of experiments, but it is probably less clear than the \( Tg(vsx2:Kaede) \) line. The \( Tg(vsx2:RFP) \) line seems least likely to clearly show V2a cells due to both the background and how dim the cells are, making it even more difficult to distinguish cells from the surrounding background. However, at 24hpf those lines do not label sufficient number of V2a cells to perform double-staining, or to assess the phenotype caused by the mutation in the gene. None of the lines seemed sufficiently suitable for my experiments and I did not pursue using these lines any further.

### 3.2 Roles of \( gata2a \), \( tal1 \) and \( gata3 \) transcription factor genes in development of zebrafish spinal cord

#### 3.2.1 Roles of \( gata2a \), \( tal1 \) and \( gata3 \) in V2b and KA cell specification

As mentioned several times previously, \( gata2 \), \( tal1 \) and \( gata3 \) are all expressed in post-mitotic V2b and KA cells in zebrafish spinal cord at 24hpf (Batista et al., 2008; this thesis). To test whether any of these transcription factor genes are required for correct specification of these cells, I examined the expression of different V2b and/or KA marker genes in mutants for each of these genes. In each case, I performed \textit{in situ} hybridization in embryos from an incross of two heterozygous parents and then determined the number and location of labeled cells in the region of the spinal cord adjacent to somites 6-10.
3.2.1.1 gata2a

As discussed in the introduction, the gata2a mutant used in this study should result in a truncated protein that lacks both of the functional zinc finger domains (Fig. 4A; Zhu et al., 2011). Therefore, it is highly likely that gata2um27 is a null mutant allele. Based on results from a previous study in zebrafish using morpholinos designed to knock down gata2a (Yang et al., 2010), we would expect KA” cells not to form in gata2a mutants, and KA' cells to form normally. The effects of loss of Gata2a function on zebrafish V2b cells, however, were unknown before my study.

In gata2a mutants, the number of cells expressing gata3, tal1, sox1a, and gads seems to be severely reduced in the row directly above the notochord where KA” cells are located (Figs 18 and 19) but there is no statistically significant change in the number of cells expressing most of these markers in more dorsal rows. More specifically, gata3 and tal1 expression is only altered in row 1, where on average only about 1 cell remains in the mutants (Figs 18A, 18I, 18C, 18K, 19A, and 19E). In addition, sox1a and gad expressions are lost in row 1 (about 1 sox1a-expressing cell remains and about 3 gad-expressing cells), and also significantly decreased in row 2 (Figs 18D, 18L, 18G, 18O, 19C and 19D). These results are consistent with the prediction that gata2a is required for KA” cells to form properly and they also suggest that gata2a may be required for the correct expression of some genes in KA' cells.
Figure 18.
Figure 18. Expression of V2b and/or KA markers in \textit{gata2a}^{um27} mutant and sibling embryos at 24hpf. Lateral views of zebrafish spinal cords at 24hpf. \textbf{(A-H)} show expression of indicated genes in wild-type sibling embryos, and \textbf{(I-P)} show expression in mutant embryos. In most cases (\textit{tal1}, \textit{sox1a}, \textit{sox1b}, \textit{tal2}, \textit{gads}, \textit{foxn4}) embryos shown here were genotyped as wild-type or mutant. Occasional photographs (\textit{gata3}, \textit{gata2a}) are of embryos that were not genotyped. In these cases, pictures are representative of the phenotype that was observed in genotyped wild-type and mutant embryos and these phenotypes were observed in appropriate Mendelian ratios in the photographed experiment. Embryos were always genotyped if there was no obvious phenotype. All panels show the merged view of several planes from one side of the spinal cord from the compound microscope. Scale bar: 50\textmu m.
Figure 19.
Figure 19. Number of cells labeled in the \textit{gata2a}^{um27} mutant and sibling embryos at 24hpf. Graphs show average number of cells expressing indicated gene in the spinal cord region adjacent to somites 6-10. Cell counts were performed on genotyped mutant and wild-type embryos, apart from one case (\textit{gata2a}) where the cell count of mutant embryos is compared to a mixture of heterozygous mutant and wild-type embryos at 24hpf. At least four embryos were counted in each case. Cell counts from rows 4 and above are shown as ‘4+’. Statistically significant (\(p<0.05\)) differences as assessed by Student’s t-test are indicated by brackets and stars (\(* p<0.05, ** p<0.01, *** p<0.001\)). Error bars indicate SEM.
To my surprise however, *tal2* expression in the KA” domain is unchanged in mutant embryos (Figs 18F, 18N and 19F). My cell counts show that there is only a small, not statistically significant decrease in the number of *tal2*-expressing cells in row 1 where KA” cells are found (Fig. 19F). This very small difference is in sharp contrast to the loss of other markers discussed above. These results also contrast with findings from *gata2a* morpholino knockdown studies that showed loss of *tal2* expression in row 1 (Yang et al., 2010).

Similarly, the number of cells expressing *sox1b* in this domain remains unchanged (Figs 18E, 18M and 19G) and, there is no obvious *sox1b* expression phenotype in *gata2a* mutant embryos (Fig. 19G). My cell counts show that the numbers of cells labeled with *sox1b* do not differ significantly in any row. Given that *sox1b* is expressed by KA” cells, as I previously demonstrated with the *Tg(gata1:GFP)* co-expression experiments in Fig. 10B, this result is also surprising. It shows that Gata2a function is dispensable for expression of *sox1b* in either KA or V2b cells. It also suggests that *sox1a* and *sox1b* are regulated differently in KA” cells.

In addition, I found no change in the number of *foxn4*-expressing cells in mutant and wild-type embryos (Figs 18H, P and 19H), despite the fact that *gata2a* and *foxn4* are co-expressed in the spinal cord (Fig. 14). This suggests that Foxn4 might be upstream of Gata2a in those cells – or that these two transcription factors act independently of each other.

Taken together, my data suggest that either KA” cells still form in the absence of *gata2a*, but the cells lose expression of some of the markers normally expressed by those
cells (i.e. *gata3*, *tal1* and *sox1a*), as well as their GABAergic phenotype, or that KA” cells become a different type of cell that does not express *gata3*, *tal1* or *sox1a* but still expresses *tal2* and *sox1b*.

I also examined at the expression of *gata2a* in *gata2a* mutants (Figs 18B, 18J and 19B). Because of time constraints, in this experiment I did not genotype sibling embryos but just identified them by their phenotype (this is the only marker that I did this for with this mutant). Therefore, while the mutant cell counts from this experiment are genotyped mutant embryos, the ‘sibling’ cell counts are a mixture of wild-type and heterozygous mutant embryos. As shown in Fig. 19B, I saw a decrease in number of *gata2a*-expressing cells in all rows, compared with sibling embryos. Also, the expression is much weaker in all remaining cells (Figs 18B and 18J). This could be due to nonsense-mediated decay of the RNA, where the RNA molecule within the cell is detected as erroneous and targeted for degradation. However, if this is the case, it is surprising that while expression of *gata2a* is almost completely lost in KA” and KA’ cells, expression in V2b cells is diminished but persists in many cells. All of the above cell counts in *gata2a* wild-type and mutant embryos are summarized in Table 9.
Table 9. Summary of the phenotype observed in *gata2*um27 mutant embryos and their siblings. Values represent average numbers of cells expressed in each row of the spinal cord for any given marker. First value corresponds to genotyped mutant embryos, and second value represents number of cells in genotyped wild-type embryos, apart from *gata2* expressing cells, where values are an average from heterozygote and wild-type embryos. Values of rows 1-8 show cell numbers in individual spinal cord rows. Row ‘4+’ represents values from rows 4-8 grouped together, and ‘2+’ represents values from rows 2-8 grouped together. Total number of cells from rows 1-8 is shown in the last row, but no colors were used in this case. In other cases, colors indicate whether the differences are statistically significant. Red and grey colors show no statistically significant difference between wild-type and mutant cell counts using Student’s t-test. Also, grey color indicates values where very few cells were present in either wild-type or mutant embryos (less than 2 cells). Other colors denote a statistically significant difference ($p \leq 0.05$) between the numbers of cells in wild-type and mutant embryos. Dark green = cells almost completely lost in mutants (less than 15% cells left in mutants), light green = cells significantly reduced (less than 33% cells left in mutants), blue = cells reduced (more than 33% cells left but statistically significant difference between mutant and wild-type). $p$-values that approach 0.05 significance cut-off are indicated in brackets.
Table 9.

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4+ TOGETHER | R     | R      | ✔    | ✔     | ✔     | ✔    | ✔ |
|            | 15.6/19.5 | 15.75/23.8 | 24/24.75 | 28.4/23.8 | 20.25/21 | 9.2/8.2 | 17.75/25.25 |

2+ TOGETHER | R     | R      | ✔    | ✔     | ✔     | ✔    | ✔ |
|            | 38.8/44.25 | 19.5/41.8 | 38.25/43.75 | 46.6/47.4 | 44.75/42.25 | 27.8/26.6 | 38/50 |

TOTAL | R     | R      | R    | ✔     | ✔     | ✔    | R |
|      | 40/65 | 24.25/61.8 | 38.75/65 | 47.8/69.2 | 65.75/64.75 | 46.8/48.2 | 41.25/71.75 |
In addition, I examined whether the \textit{gata2a} mutation causes a phenotype in heterozygous embryos. In order to test this, I examined expression of \textit{gads} in genotyped heterozygous embryos and compared them to genotyped wild-types. I chose to examine expression of \textit{gads} because they label the largest number of cells, including all KA and V2b cells, and these genes have a pronounced and obvious expression phenotype in homozygous mutants. No statistically significant difference was found between heterozygous \textit{gata2um27} carriers and wild-type embryos (Fig. 20D), suggesting that there is no heterozygous phenotype caused by this mutation in spinal cord.
Figure 20. Heterozygous mutants have no phenotype. No heterozygous mutant phenotype was found in tal1t21384, gata2aum27 or gata3sa0234 mutants. Graphs show number of cells that express the gene indicated, as assessed by in situ hybridization. Embryos were identified as heterozygous carriers of mutations in tal1t21384 (A-C), gata2aum27 (D), or gata3sa0234 (E) and compared with genotyped wild-type siblings. Values show average of cell counts from at least 4 embryos in each case. Error bars represent SEM. No significant differences were found with Student’s t-test (p>0.05 in all cases).
3.2.1.2 tal1

As described in the introduction, the tal1 mutant used in this study results in a protein truncation, which removes the entire helix-loop-helix DNA binding domain (Fig. 4B; Bussman et al 2007). Homozygous mutants also lack blood circulation, and have heart edema at 48hpf (Bussmann et al., 2007). Given the loss of functional domains and the severe phenotype in homozygous mutant embryos, it is likely that the tal1 mutant used in this study is a null mutant. I used the blood phenotype to identify mutant embryos in in situ hybridizations with genes expressed in blood where the blood phenotype is obvious (tal1), but also genotyped those embryos afterwards for cell counting. The heart edema phenotype was visible too late for my analysis, so I genotyped embryos expressing genes not expressed in blood, to identify mutants. Before this work, the role of tal1 in zebrafish spinal cord development had not been investigated, even though we know from previously published studies that tal1 is expressed in V2b and KA cells at 24hpf in zebrafish (Batista et al., 2008).

My studies show that expression of gata3, gata2a, sox1a, sox1b, tal2 and gads in tal1 mutants is unchanged in row 1 where KA” cells are located, but is severely reduced in rows 2 and 3, where KA’ cells are found (Figs 21A-B, 21D-I, 21K-O and 22A-D, 22F, 22G). However, the degree to which expression in those rows is reduced varies between different markers. To resolve whether remaining cells are medial and belong to the KA’ population, or lateral and are more likely to be V2b cells, I recounted a subset of genotyped embryos looking at the medial/lateral position of cells. The cells that remain in rows 2 and 3 in mutant embryos are located laterally, suggesting that they belong to V2b population, and
are not KA’ cells (Fig. 23, Table 10). Therefore, expression of all of these genes is lost in KA’ cells in tal1 mutants. This data is also summarized in Table 11, in which a lack of medially located cells in mutant embryos is indicated by ‘(0m)’. In rows 4 and above, no significant difference was found in the number of cells expressing gata3 or gata2a (Fig. 22A and 22B, respectively). In contrast, expression of sox1a was slightly reduced, and the number of GABAergic cells was reduced to an even larger extent (Fig. 22C and 22D, respectively). Interestingly, expression of sox1b and tal2 was almost completely abolished in rows 4 and above in tal1 mutants (Fig. 22F and 22G, respectively).
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<td>foxn4</td>
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Figure 21. Expression of V2b and/or KA markers in the tal1<sup>121384</sup> mutant and sibling embryos at 24hpf. Lateral views of zebrafish spinal cords at 24hpf. (A-H) show expression of indicated genes in wild-type sibling embryos, and (I-P) show expression in mutant embryos. In some cases (gata2a, tal1, foxn4) embryos shown here were genotyped as wild-type or mutant. Other photographs (gata3, sox1a, sox1b, tal2, gads) are of embryos that were not genotyped. In these cases, pictures are representative of the phenotype that was observed in genotyped wild-type and mutant embryos and these phenotypes were observed in appropriate Mendelian ratios in the photographed experiment. Embryos were always genotyped if there was no obvious phenotype. All panels show the merged view of several planes from one side of the spinal cord from the compound microscope. Scale bar: 50μm
Figure 22.

Key:  
- wild-types
- mutants
Figure 22. Number of cells labeled in the *tal1*t21384 mutant and sibling embryos at 24hpf. Graphs show average number of cells expressing indicated gene in the spinal cord region adjacent to somites 6-10. Cell counts were performed on genotyped mutant and wild-type embryos at 24hpf, and at least four embryos were counted in each case. Cell counts from rows 4 are shown as ‘4+’. Statistically significant (* p<0.05) differences as assessed by Student’s t-test are indicated by brackets and stars (** p<0.01, *** p<0.001). Error bars indicate SEM.
Figure 23.
Figure 23. Number of cells labeled in the \textit{tal1}$^{t21384}$ mutant and sibling embryos at 24hpf with distinction between lateral and medial position of the cells. Graphs show the average number of cells expressing indicated genes in the spinal cord region adjacent to somites 6-10. Cell counts were performed noting the medial/lateral position of labeled cells. These counts are independent of the previous cell counts that did not address medial/lateral position of the cells. Whenever possible, embryos from the previous cell count were included. If this was not possible, additional embryos were genotyped. For \textit{scl} expression, only sibling (mixture of genotyped heterozygous mutant and wild-type) embryos were available for cell counts, as indicated in the graph. Cell counts from rows 4 and above were gathered together and are shown as ‘4+’. At least four embryos were counted in each case.
Table 10. Lateral/medial cells counts in *tal1*<sup>121384</sup> mutant embryos and their siblings. Values represent average numbers of cells expressed in each row of the spinal cord for any given marker. Columns in yellow color show mutant values, and no color shows wild-type (or sibling, in a case of *tal1* marker) values. First value in each cell represents number of medial cells, and second value represents total number of cells in a specific row. Number of embryos counted is indicated at the bottom of each column.

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Table 11. Summary of the phenotype observed in *tal1*21384 mutant embryos and their siblings. Values represent average numbers of cells expressed in each row of the spinal cord for any given marker. First value corresponds to genotyped mutant embryos, and second value represents number of cells in genotyped wild-type embryos. Values of rows 1-8 show cell numbers in individual spinal cord rows. Row ‘4+’ represents values from rows 4-8 grouped together. Total number of cells from rows 1-8 is shown in the last row, but no colors were used in this case. In other cases, colors indicate whether the differences are statistically significant. Red and grey colors show no statistically significant difference between wild-type and mutant cell counts using Student’s t-test. Also, grey color indicates values where very few cells were present in either wild-type or mutant embryos (less than 2 cells). Other colors denote a statistically significant difference ($p \leq 0.05$) between the numbers of cells in wild-type and mutant embryos. Dark green = cells almost completely lost in mutants (less than 15% cells left in mutants), light green = cells significantly reduced (less than 33% cells left in mutants), blue = cells reduced (more than 33% cells left but statistically significant difference between mutant and wild-type). $p$-values that approach 0.05 significance cut-off are indicated in brackets.
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<th>sox1b</th>
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To determine whether expression of *tal2* is delayed in V2b cells, or whether cells initially express *tal2* but then quickly lose expression in mutants, I examined expression of *tal2* at 22hpf and at 27hpf. In both cases, the phenotype was similar to 24hpf, with only the most ventral row of cells retaining *tal2* expression in *tal1* mutants (Fig. 24). I did not count the cells in embryos of these stages, but it seems that at 22hpf, 24hpf, and 27hpf the *tal1* mutation abolishes *tal2* expression in cells dorsal to KA" cells.

Also, I was intrigued by the very pronounced decrease in the number of GABAergic cells in rows 4 and above. Since there are other GABAergic cells in the zebrafish spinal cord that express *gads*, it was surprising that very few cells persisted in *tal1* mutants. Importantly, the remaining cells express *gads* only very weakly in all rows dorsal to row 1. One possible explanation is that cells initially express *gads*, but expression is then down-regulated either because cells are changing their fates or becoming sick. To test this, I examined expression of *gads* at 22hpf (Fig. 24A, B). No cells dorsal to KA" cells express *gads* at 22hpf in *tal1* mutant. Another explanation could be that V2b cells are delayed in becoming GABAergic, which I tested by investigating expression at 27hpf (Fig. 24C, D). Even though a few sporadic cells express *gads* at 27hpf, the expression is still weaker than in KA" cells and the cells are not positioned in the usual row pattern (Fig. 24C, D). This suggests that these cells are not V2b cells but are instead more dorsal GABAergic cells. In conclusion, V2b cells lose their GABAergic phenotype and expression of *sox1b* and *tal2*, but expression of other markers persists in absence of Tal1.
Figure 24.
Figure 24. Expression of *gads* and *tal2* in *tal1<sup>121384</sup> mutant and sibling embryos at 22hpf and 27hpf. Lateral views of zebrafish spinal cord at indicated stages. Anterior left, dorsal up. Expression of *gads* in *tal1<sup>121384</sup>* sibling embryo at 22hpf (A), *tal1<sup>121384</sup>* sibling embryo at 24hpf (B), *tal1<sup>121384</sup>* sibling embryo at 27hpf (C) and *tal1<sup>121384</sup>* mutant embryo at 27hpf (D). Expression of *tal2* in *tal1<sup>121384</sup>* sibling embryo at 22hpf (E), *tal1<sup>121384</sup>* mutant embryo at 22hpf (F), *tal1<sup>121384</sup>* sibling embryo at 27hpf (G) and *tal1<sup>121384</sup>* mutant embryo at 27hpf (H). In all cases pictures are representative of the phenotype that was observed in appropriate Mendelian ratios in the photographed experiment. Scale bar: 50μm
I also examined expression of *tal1* in *tal1* mutants (Figs 20E, 21E, 22E). I initially found mutants based on their obvious blood phenotype, but confirmed the genotype before counting cells. I observed an expression pattern similar to that seen for other genes. Expression is unchanged in KA" cells, reduced in rows 2 and 3 where KA' cells are found, and only slightly reduced in cells of rows 4 and above where most V2bs are located (Fig. 21C, 21K and 22E). This suggests that *tal1* is not required for its own regulation in KA" cells and V2b cells, and that there is no significant RNA-mediated decay of the *tal1* mutant RNA in zebrafish spinal cord.

Expression of *foxn4* remains unchanged in *tal1* mutants. There is no statistically significant difference in the number of cells in any of the rows that express *foxn4*, or in the total number of cells that express *foxn4* (Figs. 20H and 21H). This suggests that *tal1* is not upstream of *foxn4* in the pathway that leads to formation of V2b cells.

I also examined whether the *tal1* mutation has a heterozygous phenotype. Given that the phenotype in V2b cells is different for different genes in *tal1* mutants, I examined genes that had the most dramatic phenotype in both KA’ and V2b cells (*sox1b*, *tal2* and *gads*), as I reasoned that if there was a heterozygous phenotype it was most likely to be detected with one or more of these genes. However, none of these genes had an expression phenotype in heterozygous *tal1* mutation carriers (Figs. 20A, 20B and 20C). This suggests that the *tal1* mutant does not cause a heterozygous phenotype in zebrafish spinal cord.
3.2.1.3 \textit{gata3}

The \textit{gata3} mutant, as discussed in the introduction, has not been published and was kindly provided by Dr. Steven Harvey in Derek Stemple’s lab at the Wellcome Trust Sanger Institute, UK. This mutant is also likely to be a null allele because the deletion/insertion change in the gene sequence results in a premature stop codon before both of the zinc finger domains (Fig. 4C).

The phenotypes that I observed in \textit{gata3} mutant embryos resemble those seen in \textit{tal1} mutants for KA cells, but there are some notable differences between the two mutants with respect to V2b cells. However, as shown in Fig. 25 and my cell counts in Fig. 26, the lack of \textit{gata3} does not affect the formation of KA” cells, as assessed by expression of all of genes expressed by KA” cells that I investigated in this study. This result is consistent with observations made in morpholino-injected embryos (Yang \textit{et al.}, 2010), and this aspect of the phenotype is similar to the phenotype observed in \textit{tal1} mutant embryos described earlier.

The pattern of KA’-specific gene expression in \textit{gata3} mutants is also similar to \textit{tal1} mutants in several aspects. In \textit{gata3} mutants, KA’ cells in row 2 are lost, as assayed by all of the genes used (Figs 25, 26 and 27). In row 3, there is a reduction in the number of cells expressing all of the genes, but still many cells express \textit{tal1} and \textit{gata2a}, and a smaller number of cells express \textit{gads}. When I recounted cells based on their medial/lateral position, I saw that the cells that remain in row 3 are all lateral (Fig. 27, Table 12). Also, an average of one cell remained labeled in row 3 by \textit{tal2}, \textit{sox1a} and \textit{sox1b} (Fig. 27, Tables 12 and 13). In these cases, instead of re-counting cells in those embryos based on the lateral/medial position, I checked the location of these occasional cells in row 3. The
remaining cells in row 3 were always laterally located, and this is indicated by ‘0m’ in Table 13.
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</tr>
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<td>foxn4</td>
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Figure 25.
Figure 25. Expression of V2b and/or KA markers in the gata3sa0234 mutant and sibling embryos at 24hpf. Lateral views of zebrafish spinal cords at 24hpf. (A-H) show expression of indicated genes in wild-type sibling embryos, and (I-P) show expression in mutant embryos. In most cases (gata3, tal1, sox1a, sox1b, foxn4) embryos shown here were genotyped as wild-type or mutant. Occasional photographs (gata2a, tal2, gads) are of embryos that were not genotyped. In these cases, pictures are representative of the phenotype that was observed in genotyped wild-type and mutant embryos and these phenotypes were observed in appropriate Mendelian ratios in the photographed experiment. Embryos were always genotyped if there was no obvious phenotype. All panels show the merged view of several planes from one side of the spinal cord from the compound microscope. Scale bar: 50μm
Figure 26.
Figure 26. Number of cells labeled in the gata3^sa0234 mutant and sibling embryos at 24hpf. Graphs show average number of cells expressing indicated gene in the spinal cord region adjacent to somites 6-10. Cell counts were performed on genotyped mutant and wild-type embryos, apart from one case (of gata3) where the cell count of mutant embryos is compared to a mixture of heterozygous mutant and wild-type embryos (indicated as ‘sibling’) at 24hpf. At least four embryos were counted in each case. Cell counts from rows 4 are shown as ‘4+’. Statistically significant (p<0.05) differences as assessed by Student’s t-test are indicated by brackets and stars (* p<0.05, **p<0.01, ***p<0.001). Error bars indicate SEM.
Figure 27. Number of cells labeled in the \textit{gata3sa0234} mutant and sibling embryos at 24hpf with distinction between lateral and medial position of the cells. Graphs show average number of cells expressing indicated genes in the spinal cord region adjacent to somites 6-10. Cell counts were performed noting the medial/lateral position of labeled cells. These counts are independent of the previous cell counts that did not address medial/lateral position of the cells. Whenever possible, embryos from the previous cell count were included. If this was not possible, additional embryos were genotyped. Cell counts from rows 4 and above were gathered together and are shown as ‘4+’. Cells in at least four embryos were counted in each case.
The phenotype of V2b cells in gata3 mutants is less clear. The number of cells that are in rows 4 and above labeled by gata2a and tal1 is partially reduced (Fig. 26A, 26F). Only around 2 cells less express gata2a in those rows (reduction from average of 19.2 to 17 cells), which suggests that gata3 might slightly affect gata2a expression in cells of rows 4 and above. tal1-expressing cells display a larger reduction of around half the cells (from average of 22 to 13.25 cells) in mutants compared to wild-type embryos, suggesting that gata3 is required for expression of tal1 in at least a subset of V2b cells of rows 4 and above, or that it is required for tal1 expression to be maintained in V2b cells.

In addition, results differ between expression of sox1a and sox1b in cells located in row 4 and above. The number of sox1a-expressing cells in those rows in mutant embryos was unchanged, but only around a half of cells still express sox1b (Fig. 26C). This suggests that tal1 is required for expression of sox1b in some V2b cells or it is required for maintenance of sox1b expression in those cells, but that tal1 is not required for sox1a expression in V2b cells. This result also suggests that sox1a and sox1b genes are regulated differently in zebrafish spinal cord. However, it should also be noted that the numbers of cells in those rows differ greatly in wild-type siblings from both experiments. As shown later in this results chapter (in section 3.2.3), sox1a-labeled cells coming from gata3 incross are among the highest number out of all three mutant crosses investigated, whereas sox1b-labeled cells coming from gata3 incross are among the lowest. This could partly be due to very small differences in the developmental staging of embryos and/or in how well the staining worked in each case. This results in a difference of almost 10 cells on average labeled in wild-type embryos with sox1a versus sox1b RNA probes. This indicates that careful conclusions should be made when comparing results from different experiments.
However, within an experiment mutant embryos are always compared with wild-type siblings with identical experimental treatment, so these results should still be helpful in understanding the phenotypic change in mutants.

Interestingly, expression of both *tal2* and *gads* in cells in row 4 and above remains unchanged in *gata3* mutants (Fig. 26). This is in striking contrast with the phenotype observed earlier in *tal1* mutants in this domain, where all the cells of row 4 and above were lost in the mutant embryos (Figs. 21-24). Since *tal2* likely represents only a subset of V2b cells, this suggests that *gata3* is not required for *tal2* expression in a sub-population of V2b cells. Given that the GABAergic phenotype also remains unchanged, it appears that *gata3* is not required for V2b cells to acquire their neurotransmitter phenotype.

Expression of *foxn4* remains unchanged in the *gata3* mutant embryos (Figs 25H, 25P and 26G). This suggests that either Foxn4 is upstream of Gata3, which would be consistent with the fact that I did not observe any co-expression of *foxn4* and *gata3* or that the two genes act independently of each other in V2b cells.

I also performed an *in situ* hybridization for *gata3* in *gata3* mutants. In a manner similar to assessing expression of *gata2a* in *gata2a* mutants, I just identified the embryos by their phenotype (this is the only marker that I did this for with this mutant). Therefore, while the mutant cell counts from this experiment are genotyped mutant embryos, the ‘sibling’ cell counts are a mixture of wild-type and heterozygous mutant embryos. (i.e. denoted ‘siblings’). Interestingly, mutant embryos show a weaker expression of *gata3* in V2b cells and KA" cells, but the number of those cells is still similar to the number of cells labeled in sibling embryos (Fig. 25A 25I and Fig. 26A). However, there are no cells
expressing *gata3* in the locations where KA’ cells are normally found in rows 2 and 3, which is consistent with expression of other markers. This suggests that *gata3* is required for correct expression of *gata3* in KA’ cells, and may be required for normal levels of expression in KA” and V2b cells (or there could be some nonsense mediated decay of the *gata3* transcript).

As was the case for other mutants, I also tested whether there is a heterozygous phenotype for this *gata3* mutant allele. Since the phenotype in homozygous mutant embryos was most obvious in KA’ cells, with only slight changes in V2b cells, I decided to look at expression of *gads* in genotyped heterozygous *gata3* mutation carriers. Again, no statistically significant phenotypic difference was found between heterozygous mutants and wild-type embryos (Fig. 20E). This suggests that the *gata3*sa0234 mutation does not have a heterozygous phenotype in zebrafish spinal cord.

Overall, these results show that *gata3* is not required for correct formation of KA” cells, but is required for correct formation of KA’ cells, which is in agreement with previous descriptions of *gata3* morpholino knock-down phenotypes (Yang *et al.*, 2010). The mutation in *gata3* appears to have only a partial effect on expression of markers in the V2b domain. Slight, statistically significant reductions were found in expression of *gata2a* and *sox1b*, while larger reductions of around half of the cells were observed for *tal1*. No statistically significant difference between expression patterns in V2b cells of mutant embryos compared to wild-type was found in the expression of *gata3*, *sox1a*, *tal2*, *foxn4*, or *gad*. Also, *gata3* appears to be expressed more weakly in mutant embryos but in a similar number of KA” and V2b cells compared to the sibling embryos. Taken together this
suggests that \textit{gata3} is required for the formation of KA' cells and for expression of a small subset of genes in V2b cells. While the KA'' and KA' phenotype in \textit{gata3} mutants is comparable to that in \textit{tal1} mutants, the V2b phenotypes are different as in \textit{tal1} mutants. V2b cells are no longer GABAergic and they also have a more dramatic loss of expression of several other genes, most markedly \textit{tal2} and \textit{sox1b} (Fig. 26F, 26G). The lateral/medial position of cells in each row is shown in Table 12, and all above results are also summarized in Table 13.
Table 12. Lateral/medial cells counts in *gata3*<sup>sa0234</sup> mutant embryos and their siblings. Values represent average numbers of cells expressed in each row of the spinal cord for any given marker. Columns in yellow color show mutant values, and no color shows wild-type values. First value in each cell represents number of medial cells, and second value represents total number of cells in a specific row. Number of embryos counted is indicated at the bottom of each column.

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</table>
**Table 13. Summary of the phenotype observed in gata3sa0234 mutant embryos and their siblings.** Values represent average numbers of cells expressed in each row of the spinal cord for any given marker. First value corresponds to genotyped mutant embryos, and second value represents number of cells in genotyped wild-type embryos, apart from gata3 expressing cells, where values are an average from heterozygote and wild-type embryos. Values of rows 1-8 show cell numbers in individual spinal cord rows. Row ‘4+’ represents values from rows 4-8 grouped together. Total number of cells from rows 1-8 is shown in the last row, but no colors were used in this case. In other cases, colors indicate whether the differences are statistically significant. Red and grey colors show no statistically significant difference between wild-type and mutant cell counts using Student’s t-test. Also, grey color indicates values where very few cells were present in either wild-type or mutant embryos (less than 2 cells). Other colors denote a statistically significant difference ($p \leq 0.05$) between the numbers of cells in wild-type and mutant embryos. Dark green = cells almost completely lost in mutants (less than 15% cells left in mutants), light green = cells significantly reduced (less than 33% cells left in mutants), blue = cells reduced (more than 33% cells left but statistically significant difference between mutant and wild-type), yellow = statistically significant increase between mutant and wild-type. $p$-values that approach 0.05 significance cut-off are indicated in brackets.
Table 13.

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3.2.2 There is no obvious developmental delay in *gata2a*, *gata3* or *tal1* mutants

Sometimes mutations can cause an overall delay in development. Given that KA and V2b cells form gradually over time, differences in the developmental stage of embryos could result in different numbers of KA and V2b cells being present. Therefore, to exclude the possibility that some of the phenotypes described above could be the result of a developmental delay specifically in mutant embryos, I examined the position of lateral line primordium in identified wild-type and mutant embryos. The lateral line primordium is a structure that moves along the embryonic trunk at both sides of the embryo at a well characterized pace (Kimmel *et al.*, 1992), and its position is one of the most precise ways to assess developmental stage of zebrafish embryos between 24 and 30hpf. Conveniently, one of the probes that I used as a marker of V2b and KA cells, *sox1a*, also labels the lateral line primordium (Fig. 28A). When I examined the position of this structure on both sides of spinal cord, I saw no statistically significant difference between the stages of wild-type and mutant embryos in either *gata2a*, *tal1* or *gata3* mutant lines (Fig. 28B, 28C and 28D, respectively). This shows that mutations in those genes do not cause a developmental delay in zebrafish embryos.
Figure 28. Position of lateral line primordium in wild-type and mutant zebrafish embryos at 24hpf. (A) sox1a expression in 24hpf zebrafish trunk with arrow indicating lateral line primordium. Anterior left, dorsal up. The dots in the upper half of the embryo are spinal cord cells. (B-D) Graphs representing position of lateral line primordium in genotyped wild-type and mutant embryos from incrosses of gata2a\textsuperscript{um27} (B), tal1\textsuperscript{t21384} (C) or gata3\textsuperscript{sa0234} (D) heterozygous parents. The average was calculated using single values that represent position of primordium on each side of the embryo. At least 4 embryos were analyzed in each case, resulting in at least 8 single values used for calculations. Error bars represent SEM. Scale bar = 50\textmu m
3.2.3 Comparison of cell count numbers of wild-type embryos from different experiments

All of my interpretation of results from mutant embryos assumes that the number of cells labeled by certain RNA probes is consistent between different in situ hybridization experiments. To test this, I assessed the reproducibility of my in situ hybridization experiments by comparing the number of cells labeled with each marker gene used in wild-type sibling embryos from the different experiments described above (Figs 29, 30).

For gata3-labeled cells, I compared the expression pattern in genotyped wild-type embryos from both an incross of heterozygous tal1 mutants and an incross of heterozygous gata2a mutants (Fig. 29A). I found no statistically significant differences between the overall number of cells labeled or the number of cells labeled that were located specifically in row 1 ($p=1$, Appendix Table 2), row 2 ($p=0.099$, Appendix Table 2) or row 3 ($p=0.423$, Appendix Table 2). However, in row 4, there was a statistically significant difference between number of cells labeled in both experiments (average of 10 cells labeled in tal1 incross experiment, and 12.75 cells in gata2a incross experiment, $p=0.048$). This is likely to be because it becomes more difficult to accurate assign cells to a particular row, in more dorsally located positions. Cell counts are more difficult to perform consistently when cells are further away from the notochord, partly because of the cuboidal shape of cells. Cells are not always arranged in clear/neat rows, especially in more dorsal positions where their dorso-ventral position depends on more ventrally located cells. Consistent with this hypothesis – if I compare the number of cells in row 4 and above (identified as ‘4+’ in figures), these numbers (19 for wild-type embryos from the tal1 mutant incross, and 19.5
for wild-type embryos from the *gata2a* incross) are not statistically significantly different (Fig. 29E; *p*=0.94, Appendix Table 2).

In a similar way, I compared the number of *gata2a*-labeled cells in genotyped wild-type embryos from *gata3* and *tal1* incross experiments. In rows 1-5, no significant difference was found between experiments, although in row 1 the *p* value was approaching significance (Fig. 29B, *p*=0.053, Appendix Table 2). In row 6, there is a statistically significant difference between *gata2a*-expressing cells in both experiments (*p*=0.017; Appendix Table 2). Again, when the values from rows 4 and above are gathered together, no significant difference can be found between wild-type embryos from both experiments (Fig. 28F), which is consistent with hypothesis presented earlier that slight differences in cell counts in these more dorsal rows reflect a difficulty with precisely identifying rows far away from notochord. Taken together, these results indicate that similar numbers of both KA cells and V2b cells are labeled in both experiments.

For *tal1* I compared expression in genotyped wild-type embryos from *gata2a*, *gata3* and *tal1* incrosses of heterozygous parents. Pairwise comparison with Student’s t-test showed no significant differences between average values in rows 1-3, but the *gata2a* versus *gata3* incross comparison for row 2 approached significance (*p*=0.055; Appendix Table 2). Small significant differences were found between various experiments in rows 4, 5 and 6 (Fig. 29C; Appendix Table 2). In row 4 the difference in the number of cells labeled in *tal1* wild-type siblings versus *gata3* wild-type siblings were statistically significantly different (*p*=0.12, Appendix Table 2). Also, in row 5 there were statistically significant differences found. *gata2a* wild-type siblings had significantly more cells expressing *tal1* in
row 5 (average of 10.25 cells, Fig. 29C), than in gata3 wild-type siblings (average of 6.75 cells, \( p=0.01 \)), or tal1 wild-type siblings (average of 4.75 cells, \( p=0.07 \)). Also, in row 6 significant differences were found between all three of the pairwise comparisons (Appendix Table 2). In addition, my comparison of cell counts from row 4 and above shows that the number of cells in tal1 wild-type siblings is significantly lower than in the other two experiments (16.5 cells in the tal1 incross, versus 25.75 cells in gata2a incross (\( p=0.09 \)) and 22 cells in gata3 incross (\( p=0.006 \)) (Fig. 29G, Appendix Table 2). Since cells are usually specified from the ventral to dorsal, with older cells located more dorsally, this suggests to me that potentially the embryos from the tal1 incross experiment might have been slightly younger than in the other two experiments. This hypothesis would be supported by the fact that the numbers of more dorsal (rows 5 and 6), as well as to a smaller extent the number of KA" cells labeled by tal1 is smaller in these wild-type siblings.

Analysis of tal2-labeled wild-type mutants shows a similar trend. The only statistically significant difference that could be found in the cell counts by row from three individual experiments was in row 6, where tal1 wild-type siblings have more cells labeled than gata3 wild-type siblings (Fig. 29D). However, when the values from row 4 and above were combined, there was no statistically significant difference between any of the experiments (Fig. 29H, Appendix Table 2).

Therefore, given that the differences within individual cell populations do not differ to a large extent, and that I am comparing embryos of the same stage within each experiment (by comparing mutants to their wild-type siblings from the same cross), I
believe that we can still draw conclusions about Tal1 function from the *tal1* mutant experiments.
Wild-type expression patterns of \textit{gata3, gata2a, tal1} and \textit{tal2}

\textbf{A} \textit{gata3} \hspace{1cm} \textbf{E} \textit{gata3}

\textbf{B} \textit{gata2a} \hspace{1cm} \textbf{F} \textit{gata2a}

\textbf{C} \textit{tal1} \hspace{1cm} \textbf{G} \textit{tal1}

\textbf{D} \textit{tal2} \hspace{1cm} \textbf{H} \textit{tal2}

Figure 29.
Figure 29. Expression of *gata3*, *gata2a*, *scl* and *tal2* in wild-type embryos from different experiments. Graphs show average number of cells that express indicated genes in a 5 somite length of spinal cord adjacent to somites 6-10. Counts are for both sides of the spinal cord and at least 4 embryos were counted in each case. Cells were counted in individual rows along the dorsal-ventral axis with row 1 being the most ventral row, which is located just above the notochord. Embryos are from incrosses of *tal1*^21384*, *gata2a*^um27* and *gata3*^sa0234* heterozygous mutants and were either genotyped (wild-type), or assessed by the clearly visible phenotype in approx. 25% of embryos (sibling). (A-D) show cell counts in each individual row. (E-H) show the same values for rows 1, 2 and 3, with values from row 4 and above grouped together. Statistically significant (*p*<0.05) differences as assessed by Student’s t-test are indicated by brackets and stars (* *p*<0.05, ** *p*<0.01, *** *p*<0.001). Error bars represent SEM.
I also compared sox1a expression in genotyped wild-type embryos from incrosses of heterozygous parents of all three mutants. In rows 1, 2, 3 and 4, no significant differences were found between the wild-type expression in all three experiments (Fig. 30A, Appendix Table 2). However, small significant differences could be seen in number of cells labeled in rows 5 and 6 (Fig. 30A). In row 5, less cells were stained in tal1 wild-type siblings than in gata2a wild-type siblings (p=0.046), although none of the other pairwise comparisons were statistically significant. In row 6, less cells are labeled in tal1 wild-type siblings, with statistically significant differences between these embryos and both gata2a (p=0.027) and gata3 wild-type siblings (Fig. 30A; p=0.021; Appendix Table 2). In row 7, no statistically significant difference was detected, most likely due to the fact that very few cells are labeled in this row in any of the experiments. The lower number of cells in dorsal rows labeled with sox1a in tal1 wild-type siblings is also seen when the values are combined from rows 4 and above (Fig. 30E). It appears that tal1 wild-type siblings have less cells than both gata2a and gata3 wild-type siblings, but a statistically significant difference could be detected only between tal1 wild-type siblings and gata3 wild-type siblings in this case (Fig. 30E, Appendix Table 2). This suggests that the tal1 embryos might have been slightly younger and/or the probe did not stain as well in this particular tal1 experiment as in the other experiments.
Similarly, no differences are observed between expression of *sox1b* in wild-type sibling embryos that come from incrosses of all three mutants in individual rows 1-4 (Fig. 30B, Appendix Table 2). However, there is a small variation in cell counts in rows 5, and 7, with no statistically significant difference in the number of cells of row 6. *gata2a* wild-type siblings appear to have most cells labeled in row 5, with statistically significant differences between *gata2a* wild-type siblings and both *tal1* (*p*=0.045), and *gata3* wild-type siblings (*p*=0.031) (Fig. 30B, Appendix Table 2). However, in row 7, the *tal1* wild-type siblings have most cells labeled, with statistically significant differences only between *tal1* wild-type siblings and *gata3* wild-type siblings (Fig. 30B; *p*=0.040; Appendix Table 2). When the values from row 4 and above are combined, no statistically significant difference can be found between the number of cells labeled in either of the experiments (Fig. 30F). Again, the differences in individual rows most likely occurred due to difficulties identifying the exact position of cells far away from the notochord in the individual embryos.

The number of GABAergic cells also differs only within occasional individual rows between all three experiments. In rows 1-3, and in row 5 there is no statistically significant difference between numbers of cells labeled by *gads* in wild-type sibling embryos from either experiment (Fig. 30C). However, in row 4, the least cells are labeled in *tal1* wild-type siblings, with a statistically significant difference between this experiment and the *gata3* wild-type siblings (Fig. 30C). At the same time, in row 6 the *tal1* wild-type siblings appear to have the most cells labeled with *gads*, with statistically significant differences found between this experiment and both *gata2a* (*p*=0.007), and *gata3* wild-type siblings (*p*=0.016). No statistically significant difference was detected between the more dorsal rows. The difference in individual row counts between the *tal1* wild-type siblings and other
experiments is not apparent when cells from row 4 and above are combined, as no statistically significant difference was detected in either comparison (Fig. 30G, Appendix Table 2).

In case of cell counts performed on wild-type embryos labeled with foxn4 in experiments from all three mutant lines, the difference is visible only in row 3 out of all rows labeled (Fig. 30D, Appendix Table 2). In row 3 of tal1 wild-type siblings, the number of cells is statistically significantly lower than in gata3 wild-type siblings (Fig. 30D). Understandably, the same trend is visible when cells from row 4 and above are pooled together (Fig. 30H). However, foxn4 appears to label only one cell population (most likely early V2b, sometimes probably just after division) that might not be consistently positioned within one specific row, but is found in rows 3-5. Therefore, I decided that a comparison that would better reflect the actual phenotype would be to compare the total number of cells. In this case, there is no significant difference between the number of foxn4-labeled cells in wild-type sibling embryos from either of the experiments.
Figure 30.
Figure 30. Expression of *sox1a*, *sox1b*, *gads* and *foxn4* in wild-type embryos from different experiments. Graphs show average number of cells that express indicated genes in a 5 somite length of spinal cord adjacent to somites 6-10. Counts are for both sides of the spinal cord and at least 4 embryos were counted in each case. Cells were counted in individual rows along the dorsal-ventral axis with row 1 being the most ventral row, which is located just above the notochord. Embryos are from incrosses of *tal1*<sup>t121384</sup>, *gata2a*<sup>um27</sup> and *gata3*<sup>sa0234</sup> heterozygous mutants and were either genotyped (wild-type), or assessed by the clearly visible phenotype in approx. 25% of embryos (sibling). (A-D) show cell counts in each individual row. (E-H) show the same values for rows 1, 2 and 3, with values from row 4 and above grouped together. Statistically significant (*p*<0.05) differences as assessed by Student’s t-test are indicated by brackets and stars (* *p*<0.05, ** *p*<0.01, *** *p*<0.001). Error bars represent SEM.
3.3 Other potential markers of V2b and/or KA cells

Several additional candidate genes that might be expressed in V2b and/or KA cells were identified via either literature searches and/or from another project in the lab that investigated the expression profiles of various zebrafish spinal cord cell types via microarray. In parallel to my examination of gata3, gata2a and tal1 mutants, I also examined the expression of some of these genes in the hope of finding genes that label specifically V2b, KA’ or KA” cells, since most transcription factors described to date are expressed by all three of these cell types. I performed GFP immunohistochemistry and in situ hybridization with these genes in the Tg(gata1:GFP) line that labels KA cells and a subpopulation of V2b cells to investigate whether those candidate genes are expressed by either V2b or KA cells. As shown in Fig. 31, several markers had a partly overlapping expression with Tg(gata1:GFP) cells.
Figure 31.
Figure 31. Genes that are candidates for being expressed by V2b and/or KA cells in zebrafish spinal cord. Lateral views of 24h spinal cords of Tg(gata1:GFP) embryos with GFP expression in green and insm1a (A), mnx1 (B), her6 (C), crb1 (D) and sp8a (E) mRNA expression in red. Each panel represents a merge of multiple projections from one side of the spinal cord followed by a merged image of both green and red channels. White stars indicate cells that are double-labeled. Panels in A’, B’, C’ and D’ show single confocal plane of the area marked with white box in panels A, B, C and D, respectively. Scale bar: 50μm
insm1a was initially identified from the microarray screen by another student in the lab (Paul Campbell). My double labels show that this gene is expressed by a subset of Tg(gata1:GFP) cells (Fig. 31A). The cells that express both insm1a and Tg(gata1:GFP) are located at positions that correspond to all three populations: KA", KA' and V2b cells. Interestingly, these co-labeled cells are sporadic, and overall this gene does not seem to be expressed by all members of any one population. However, because of the significant background in those embryos, it is difficult for me to confidently determine the precise number of co-expressing cells.

mnx1 was also identified by another student in lab (Paul Campbell) from the microarray screen. It does not appear to be expressed by Tg(gata1:GFP) KA" cells (Fig. 31B). However, there are a small number of more dorsal cells (3 cells that I could identify across both sides of the spinal cord of embryo in Fig. 31B) that are co-labeled by both Tg(gata1:GFP) and mnx1. Those cells could potentially be V2b or KA' cells. This would be consistent with the literature that shows that some mnx1-expressing cells have a V2b-like morphology and are GABAergic at 24hpf (Seredick et al., 2012). Interestingly though, in my experiment I could see Tg(gata1:GFP)-positive cells with a V2b morphology that did not express mnx1, which indicates that perhaps only some V2b cells express mnx1. This marker is also expressed in primary motoneurons, together with other members of the mnx family (Seredick et al., 2012). Therefore, despite the fact that the mnx1 appears to be expressed by some V2b cells, it is not a V2b-specific marker.

I identified her6 from the microarray project during my rotation in the lab as a marker that might be expressed by inhibitory neurons. As shown in Fig. 31C, her6 appears
to be expressed by some KA cells, but also by many more cells in the ventral rows where KA cells are located. The ventral expression of *her6* appears to be restricted to rows ventral to the position of V2b cells, and none of the GFP-positive cells with V2b-like morphology co-expressed *her6*. However, this gene seems to be also expressed in cells located in the dorsal spinal cord (Fig. 31C). Interestingly, when Alex Nichitean investigated the expression pattern of this gene in *mindbomb* mutants in which Notch signaling is inactivated, the expression of *her6* was largely abolished. This suggests that *her6* is expressed by cells that depend on Notch signaling for their formation. This group includes KA cells, which is consistent with *her6* being expressed by KA cells. Therefore, *her6* may be expressed by a subset of KA cells, but is not a KA-specific marker due to the more extensive ventral *her6*-expressing domain and the dorsal expression domain.

*crb1* (crumbs family member 1, photoreceptor morphogenesis associated) was also identified by myself during my rotation from the microarray screen as a marker potentially expressed in V2b or KA cells. As opposed to all other genes investigated in this thesis, *crb1* does not encode for a transcription factor but for a transmembrane protein. Interestingly, it appears to be expressed by KA cells, as it is co-expressed with *Tg(gata1:GFP)* (Fig. 31D). Also, it appears that *crb1* might be expressed by additional cells in row 1, which suggests that it might be expressed by other cells in the lateral floor plate region, perhaps p3 or V3 populations. In row 2, all of the *Tg(gata1:GFP)* cells appear to co-express *crb1*, which suggests that all or most of the KA' cells express this gene. None of the cells located more dorsally to this region were found to express *crb1*. This is perhaps one of the most exciting results, as it suggests that only KA cells and not V2b cells express *crb1*. However, this marker is not KA-specific because of the additional *crb1*-expressing cells in the first row
above notochord. Hopefully, future experiments will reveal the identity of those cells and possibly the role of crb1 in KA cells.

sp8a was also identified from the microarray screen as a gene upregulated in inhibitory (versus excitatory) cells of zebrafish spinal cord. However, it does not appear to be expressed by any of the Tg(gata1:GFP) cells (Fig. 31E) although, interestingly, it is expressed by cells that are roughly in the KA’ and/or V2b position. It is likely that not all V2b cells are labeled by this transgenic line based on my other results, and sp8a could be potentially expressed by non-GFP positive V2b cells. However, since none of the sp8a-labeled cells co-label with Tg(gata1:GFP), it is also likely that this gene is not expressed by either V2b or KA cells.

Overall, I identified several genes that are expressed by KA’, KA” and/or V2b cells in zebrafish spinal cord (as summarized in Table 14). crb1 and her6 are expressed by either a subset or all of both of the KA cell populations. insm1a may be expressed by a subset of all three populations, as well as other cells, but this result needs to be repeated with a stronger probe before a final conclusion can be made. mnx1 may be expressed by some V2b cells, but this result needs to be confirmed at a later stage when there are more Tg(gata1:GFP) cells with a V2b morphology, or by co-labeling with a known V2b marker. In all of these cases, the genes are also expressed by additional cell populations and are, therefore, not exclusive to KA”, KA’ and/or V2b cells. I also showed that sp8a is not expressed by KA or V2b cells.
Table 14. Summary of conclusions about novel candidate genes that may be expressed by V2b and/or KA cells. This table shows the general conclusions that may be drawn from experiments that establish the expression pattern of candidate genes at 24hpf in zebrafish embryos. The conclusions are simplifications based on the experiments presented in this thesis and also supported by published literature, as discussed in text. Evidence that comes from this thesis is referenced to the respective figures in the last column.

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</tr>
<tr>
<td><strong>her6</strong></td>
<td>Absent</td>
<td>Most KA cells</td>
<td>Also in some dorsal cells, and in additional cells in KA domain</td>
</tr>
<tr>
<td><strong>sp8a</strong></td>
<td>Absent</td>
<td>Absent</td>
<td>-</td>
</tr>
<tr>
<td><strong>crb1</strong></td>
<td>Absent</td>
<td>All KA cells</td>
<td>Likely in some other cells in KA D-V position</td>
</tr>
</tbody>
</table>
4. Discussion

The primary goal of this study was to better understand the molecular pathways that establish GABAergic V2b and KA cells in zebrafish spinal cord. Previous reports showed that formation of many inhibitory GABAergic spinal cord cells depends on the PAX2 transcription factor in both mouse (Pillai et al., 2007), and zebrafish (Batista and Lewis, 2008). Interestingly, V2b cells in mouse, and both V2b and KA cells in zebrafish, are the only GABAergic spinal cord cells that do not express Pax2 (Pillai et al., 2007; Batista and Lewis, 2008). As would be expected, in the absence of PAX2 and other highly related PAX transcription factors those ventrally located neurons retain their GABAergic phenotype (Pillai et al., 2007; Batista and Lewis, 2008). This indicates that another, pax2-independent mechanism must specify the GABAergic phenotype of those cells. Furthermore, evidence from knock-down studies in zebrafish indicates that there is a difference in how mature KA' and KA" form – expression of at least some genes in KA" cells requires gata2a and not gata3, and KA' cells require gata3 but not gata2a for their correct specification (Yang et al., 2010). This is a very interesting result, but morpholino experiments can sometimes cause non-specific phenotypes due to off-target effects (Eisen and Smith, 2008; Shulte-Merker and Steiner, 2014). Therefore, it was important to confirm this result in mutant fish. Also, this result raised the possibility that transcription factor genes expressed by zebrafish KA", KA' and V2b cells might play distinct roles in formation of those cells. In this thesis I investigate whether tal1, gata2a, or gata3 are required for V2b or KA specification by examining single mutations in these genes. I also investigated whether other transcription factors are expressed by V2b and/or KA cells. In this discussion, first, I will discuss my
analyses of sox1a, sox1b and foxn4 expression in wild-type embryos. Then, I will describe my analyses of KA”, KA’ and V2b cells in tal1, gata2a and gata3 mutants. Finally, I will briefly describe my more preliminary results on other potential markers expressed by V2b and/or KA cells. For reference, the expression patterns of each gene in wild-type embryos are gathered together in Fig. 32.
Figure 32. Expression patterns of V2b and/or KA genes in wild-type embryos at 24hpf. Graph shows average number of cells in each row labeled by in situ hybridization for different genes (represented by different colors). This data is a compilation of all the genotyped wild-type embryo cell counts from incrosses of heterozygous mutants (from at least 2-3 separate experiments). Values in this figure are a combination of values from earlier figures that show expression patterns of these genes presented in the results section. Lines above the bars indicate the expected positions of KA”, KA’ and V2b cells in the zebrafish spinal cord based on current knowledge.
4.1 *sox1a* and *sox1b* are expressed by V2b and KA cells

As explained in the introduction, *sox1a* and *sox1b* are zebrafish orthologs of mouse *Sox1*. There are two zebrafish *sox1* genes because of the additional whole genome duplication event that occurred in teleosts (Amores *et al*., 1998; Taylor *et al*., 2003; Glasauer and Neuhauss, 2014). In amniotes, *Sox1* is broadly expressed in the chicken spinal cord (Okuda *et al*., 2006) and in mouse spinal cord progenitor cells (Pevny *et al*., 1998) and post-mitotic V2c cells (Panayi *et al*., 2010). In zebrafish, *sox1a* and *sox1b* expression had been observed mostly in the retina and brain, but it was reported that it was not detected in the spinal cord at any stage examined (12 and 21 somite, and 25hpf) (Okuda *et al*., 2006). However, I think the authors may have missed expression of *sox1a* and *sox1b* in the spinal cord, as I can see weak expression in their photographed embryos at 25hpf (Okuda *et al*., 2006). Also, direct data submission to the zebrafish expression database by the Thisse lab shows *sox1a* expression in spinal cord at 24hpf (Thisse *et al*., 2004; ZFIN.org). In this thesis, I confirm that both *sox1a* and *sox1b* are expressed at 24hpf in zebrafish spinal cord. In addition, another student in the lab (Alex Nichitean) detected spinal cord expression of *sox1a* at 20hpf, 22hpf and 27hpf (data not shown).

V2b, KA’ and KA” cells in zebrafish all express *tal1, gata2a* and *gata3*, and are GABAergic (Batista *et al*., 2008; Yang *et al*., 2010; Bernhardt *et al*., 1992). All of these cells are also labeled by Tg(*gata1:GFP*), even though they don't express *gata1* (Detrich *et al*., 2004) suggesting that this transgene promoter lacks either a spinal cord repressor element, or acts as an enhancer trap (Batista *et al*., 2008). Based on my results, I propose that both V2b and KA cells also express *sox1a* and *sox1b*, as both of those transcription
factors are co-expressed with GFP in \textit{Tg(gata1:GFP)} embryos (see Fig. 10). However, at 24h the \textit{Tg(gata1:GFP)} line only labels a small number of the V2bs in my experiments (Fig. 10). This suggests that either not all of the V2bs are labeled by \textit{Tg(gata1:GFP)}, or that the expression of the transgene is delayed in most V2b cells. As a result, it is hard to determine whether the \textit{sox1a}- and \textit{sox1b}-positive cells that do not co-express GFP are also V2b cells or might be an additional cell type. However, all of the \textit{Tg(gata1:GFP)} cells express \textit{sox1a} and \textit{sox1b}, which suggests that both of those transcription factors are expressed by all KA cells, and at least the subset of V2bs that was labeled by the transgenic line (Fig. 10 and Table 8).

In mouse, \textit{Sox1} is expressed in V2b cells but also in V2c cells that no longer express the V2b marker \textit{gata3} (Panayi \textit{et al.}, 2010). I was not able to establish whether V2c cells also exist in zebrafish. Given that both \textit{sox1a} and \textit{sox1b} are expressed in a few more cells than \textit{gata3}, \textit{gata2a} and \textit{tal1}, especially in row 6 and 7, it is possible that these more dorsal cells are V2c cells, although they could also be a different cell type (especially given their more dorsal location). The overall similarity of \textit{sox1a} and \textit{sox1b} expression to that of other V2b markers, and the fact that in mouse \textit{Sox1} is transiently expressed by V2b cells (Panayi \textit{et al.}, 2010) suggests that \textit{sox1a} and \textit{sox1b} are expressed by V2b cells. In addition, in all three of the \textit{tal1/gata2a/gata3} mutants, expression of at least one of the \textit{sox} genes is altered in a manner similar to that of other V2b and KA markers, consistent with these genes being expressed by KA and V2b cells in zebrafish. Double in situ hybridization experiments between \textit{sox1a}/\textit{sox1b} and other V2b cell markers (e.g. \textit{tal1}, \textit{gata2a} or \textit{gata3}) could confirm whether \textit{sox1a/b} are only expressed in V2b cells, or whether they also are expressed by additional cells. To see whether V2c cells exist in zebrafish however, transgenic lines that would allow for lineage tracing would be needed. This would allow for
identifying cells that once were V2b cells (e.g. expressed *gata3*), but later express only *sox1a* and/or *sox1b* (become a new subpopulation, V2c). Since degradation time of GFP is quite long after protein is fully folded, use of traditional lines (e.g. *Tg(gata1:GFP)*) would not suffice, as it would be difficult to establish whether cells are still V2b cells or already became V2c cells. However, a transgenic line (e.g. *Tg(gata3:eGFP)*) line could be made to trace the origin of the *sox1a*/*sox1b*-expressing cells to cells that once expressed *gata3*. Such lineage tracing lines are used in mouse and are also available in zebrafish (Hans *et al.*, 2009). However, currently a line that specifically enables tracing of V2b/V2c fate is not available.

In contrast to V2b cells, KA cells are consistently labeled by the *Tg(gata1:GFP)* line: the number of GFP-positive cells is equivalent to the number of KA cells in the first two to three rows of the spinal cord. The co-expression of both *sox1a* and *sox1b* in these GFP-positive cells shows that KA cells express both of those transcription factor genes. Currently there is no evidence from other organisms that indicates expression of *sox1a* and *sox1b* in cells that contact cerebrospinal fluid (cerebrospinal fluid contacting neurons (CSF-cNs) in amniotes; KA cells in zebrafish). It is possible that the Sox1-expressing cells in mouse might be CSF-cNs. More detailed examination by either co-labeling with a CSF-cN specific marker such as *pkd2l1* (Djenoune *et al.*, 2014), or looking at the morphology of the cells that express Sox1 could establish whether expression of Sox1 in CSF-cNs is conserved in zebrafish and amniotes.

In zebrafish, there are temporal and spatial differences in the expression of *sox1* genes in many tissues (Okuda *et al.*, 2006). For example, in retina *sox1a* is expressed a
number of hours before *sox1b* is expressed (Okuda *et al*., 2006). In addition, *sox1b* is expressed only in the forebrain, whereas *sox1a* is expressed in both forebrain and hindbrain (Okuda *et al*., 2006). Therefore, despite the fact that those genes are very closely related and both show high similarity at a sequence level to human and mouse *SOX1* (Okuda *et al*., 2006), their expression and functions in specific cell types might differ. The phenotypes of zebrafish single mutants analyzed in this study support the idea that *sox1a* and *sox1b* are regulated differently, which might also suggest distinct roles for these genes in the development of *V2b* and KA cells. In KA" cells in *gata2a* mutants, I observed that only *sox1b* is lost, while *sox1a* expression is unaffected. In contrast, *sox1b* expression is lost in *V2b* cells in *tal1* mutants, while *sox1a* expression in those cells is only slightly reduced. Similarly, in *V2b* cells in *gata3* mutants, around half of the cells lose *sox1b* expression, while no significant change is observed in expression of *sox1a*. This suggests that *sox1b* may be downstream of *tal1* and *gata3* in *V2b* cells, and *sox1a* downstream of *gata2a* in KA" cells.

Different functions and/or regulation of orthologous genes is often a consequence of additional whole genome duplication events such as the one that occurred at the base of the teleost lineage (Glasauer and Neuhauss, 2014). A study that investigated changes in non-coding elements (CNEs) surrounding all of the *soxB* genes (to which *sox1a* and *sox1b* belong) in the pufferfish showed that about half of the CNEs were split apart after the genome duplication event, suggesting that subfunctionalization may have occurred for those genes (Woolfe and Elgar, 2007). For example, subfunctionalization during evolution occurred in the functions of co-orthologs *sox9a* and *sox9b*, where the craniofacial and pectoral fin phenotypes of single mutants are different from each other but are additive in double mutants (Yan *et al*., 2005). Therefore, it is possible that *sox1a* and *sox1b* in zebrafish
have subdivided more ancestral functions. Alternatively, it is possible that both sox1a and sox1b genes have retained similar functions in zebrafish, but their expression is regulated differently. In case of another zebrafish sox ortholog pair, sox11a and sox11b, the sequence of their enhancers has evolved to varying degrees, but the sequence of the genes has remained similar, and as a result expression of the genes is regulated differently, but the resulting proteins have similar functions (Navralitova et al., 2010). Given the difference between expression of sox1a and sox1b in response to loss of gata2a, gata3 and tal1, it is likely that sox1a and sox1b genes are regulated differently. To test whether they have different functions, mutants of sox1a and sox1b would be needed, which are not available at this time.

4.2 foxn4 is expressed by early V2 cells

foxn4, as explained in the introduction, is probably expressed by zebrafish V2 cells relatively early, as shown by co-expression of foxn4 and vsx1 mRNA in the Tg(vsx1:GFP) line at 18hpf (Kimura et al., 2006). It is interesting, however, that Tg(vsx1:GFP) labeled V2a cells so early, as in my experiments I could not clearly see spinal cord cells at 24hpf, and only sporadic cells were labeled at 27hpf. Also, my double in situ hybridization labeling shows that foxn4 is co-expressed with gata2a, with all the cells that are foxn4-positive expressing also gata2a. However, there are many cells that are not foxn4-positive that express gata2a. This means that either only a subset of gata2a-expressing V2b cells express foxn4, or that most likely foxn4 is expressed early in V2b cells and then downregulated. In this case, the foxn4-negative, gata2a-positive cells would represent more mature V2b cells. Double ISH co-labeling with some of the p2 progenitor/very early V2 cell markers nKx6.1 and nKx6.2 is
consistent with this hypothesis, as many foxn4 cells still express both of those markers. Therefore, I postulate that foxn4 is expressed early by V2b (and V2a) cells, but its expression is transient and quickly downregulated as the cells progress to more mature stages.

As discussed in the introduction, in mouse, Foxn4 is expressed in cells that are common precursors of both V2a and V2b cells, while in chicken there is a significant overlap between expression of Foxn4 and Gata2-expressing V2b cells, but no overlap with Vsx2-expressing V2a cells (delBarrio et al., 2007). In both mouse and chicken Foxn4 plays a crucial role in specification of V2b cell fate (Fig. 3; Li et al., 2005; delBarrio et al., 2007; Misra et al., 2014). Foxn4 is both required for Tal1 expression in mouse and sufficient to induce Tal1 expression in chicken, while loss of Tal1 has no effect on expression of Foxn4 in mouse (delBarrio et al., 2007). Also, Foxn4 is sufficient to induce Gata2 and Gata3 expression in chicken embryos, with Gata2 expression observed before Tal1 and Gata3 expressions (delBarrio et al., 2007). Also, the number of Vsx2-expressing V2a cells decreases in chicken after forced expression of Foxn4 (delBarrio et al., 2007). In mouse, Foxn4 has recently been shown to be responsible for establishing and maintaining the Delta-Notch signaling that is crucial for development of V2a/V2b cells (Misra et al., 2014). This suggests that in amniotes, Foxn4 is upstream of Tal1, Gata2 and Gata3 during V2b cell differentiation, which is in agreement with my observations in zebrafish.

Currently there is no zebrafish foxn4 null mutant available that would help to confirm whether foxn4 is upstream of other V2b genes. However, I investigated whether foxn4 expression is altered by absence of tal1 gata2a, or gata3. The number of foxn4-
expressing cells in each of the three mutants does not change. This supports the idea that 

*foxn4* is upstream of *tal1, gata2a, and gata3* during V2b formation. Also, previous research 

that attempted to investigate the role of *foxn4* in V2b development of zebrafish shows that 

overexpression of full-length transcript in embryos results in roughly a 25% increase in the 

number of GABAergic V2b cells, and a similar decrease in the number of *Tg(Vsx2:GFP)* V2a 

cells (Li Lin, 2008 Master’s Thesis). This would further support the idea that *foxn4* is 

responsible for the specification of V2b versus V2a cell fate in both zebrafish and amniotes. 

In addition, my results indicate that the dorsoventral position at which V2b cells are 

born might change over time. Alternatively, mature V2b cells might migrate in a similar 

way to V2a cells in zebrafish (Kimura *et al.*, 2006). If *foxn4* is expressed only transiently and 

early in differentiation of V2 cells in zebrafish, as it is in amniotes (del Barrio *et al.*, 2007; 

Misra *et al.*, 2014), my results would suggest that the dorsoventral position at which V2b 

cells form changes over time (Fig. 12), as more dorsal cells express *foxn4* at later stages. At 

the same time, V2b cells at 24hpf are found in the same rows but also slightly more dorsally 

than *foxn4*-expressing cells at and/or before 24hpf, which would suggest that cells might 

migrate slightly dorsally to their final positions, after expressing *foxn4*. Also, the total 

number of cells that express *foxn4* changes only slightly, with about 15-17 cells on average 

expressing *foxn4* at any of the stages between 20hpf-30hpf that I examined. Since *foxn4* is 

expressed only in a subset of cells that express V2b markers, but V2b cells are present in all 

rows where *foxn4* is expressed, I think *foxn4* may be transiently expressed in forming V2b 

cells. Also, I think that young V2 cells that express *foxn4* are born more dorsally, but later 

might also migrate slightly more dorsally before becoming fully mature. Given that some 

V2 cells are ventral to *foxn4*-expressing cells at 24hpf (Fig. 12; Fig. 32; Table 7), I propose
that these cells could be older V2b cells born from early foxn4-expressing cells that had already migrated but do not continue to move to even more dorsal positions. This would be partly consistent with observations of Kimura and colleagues which show that more dorsal V2a cells are older than ventral V2a cells (Kimura et al., 2006). As described earlier, the tool used by this team was Tg(vsx1:Kaede) zebrafish line that labels vsx1-expressing cells and enables conversion of the Kaede chromophore from green to red at specific times (Kimura et al., 2006). vsx1 labels V2a cells, but is expressed relatively early in V2 differentiation, together with foxn4 and gata2a (Kimura et al., 2006). Since red (older) cells were found more dorsally, the result was interpreted that V2a cells are born ventrally and then migrate dorsally (Kimura et al., 2006). In addition my results suggest that the position of early V2 cells that express foxn4 becomes more dorsal. It is possible that in previous experiments even some of the Tg(vsx1:Kaede) cells were being born more dorsally (Kimura et al, 2006), but then after conversion of chromophore they migrated even further to their final positions. Even though my interpretation of the results differs from the one presented in the paper describing V2a migration (Kimura et al., 2006), both scenarios are possible given the available data, and my results support the hypothesis that at least part of the migration of V2 cells occurs before the cells become fully differentiated.

Finally, my results suggest that V2b cells are still forming at 30hpf, based on the premise that foxn4 is expressed by early V2 cells. The similarity in the total number of foxn4-expressing cells suggests that the V2 cells differentiate in similar numbers at 20hpf, 22hpf, 24 hpf, 27hpf and 30hpf. The slight increase in total numbers of cells labeled by foxn4 at later stages might suggest that even more V2 cells form at later stages, as compared to earlier stages. This would be in agreement with the evidence for
differentiation of V2a cells at later stages, as vsx2 (also known as vsx2, another V2a marker) mRNA can be detected as late as 48hpf (Kimura et al., 2006). Also, vsx2 expression at late stages (e.g. 32hpf) is found relatively dorsally to that of earlier stages (Kimura et al., 2006). Since V2a and V2b cells are born simultaneously by the Notch-signaling-mediated lateral inhibition mechanism (Kimura et al., 2008; Batista et al., 2008), it is likely that V2b cells are also born at stages past 24hpf. Alternatively, it is possible that at stages other than 24hpf cells other than V2 cells express foxn4 in zebrafish, but currently there is no evidence that would support this. Previous study of foxn4 expression in zebrafish mentions that ‘no staining in neural tube is seen after neuronal differentiation is completed’ (Danilova et al., 2004), it is unclear however what the authors meant by this, and even though later stages were investigated, no spinal cord photographs past 22hpf are provided. Perhaps, investigation of expression pattern at even later stages and/or double ISH experiments with V2b and/or other spinal cord cell markers at later stages could resolve which cells express foxn4 past 24hpf.

4.3 Regulatory network that leads to formation of KA” cells

As described in the introduction, KA” and KA’ cells, contact the central canal and are important for regulation of swimming behaviors in zebrafish (Wyart et al., 2009). Both of these cell types express tal1, gata2a, gata3 (Batista et al., 2008), as well as tal2 (Pinheiro et al., 2004) and sox1a and sox1b (this thesis) and are GABAergic (Bernhardt et al., 1992). Previous knockdown studies in zebrafish suggested that KA” cells depend on gata2a but not on gata3 for their correct specification (Yang et al., 2010). Also, the GABAergic
phenotype of KA” cells may require tal2, but tal2 is not required for expression of other KA” markers such as gata3 or gata2a (Yang et al., 2010).

Interestingly, gata3 seems to be expressed more weakly in KA” cells of wild-type embryos than in more dorsally located cells (Neave et al., 1995; Figs 6A, 18A, 21A, 25A in this thesis), which might potentially reflect its function in KA” cells. However, no similar difference between levels of expression in different types of cells was observed for tal1 or gata2a.

My results using single mutants show that tal1 and gata3 are not required for correct formation of KA” cells, but gata2a is required for expression of most KA” markers. The KA” phenotype in gata3 mutants is consistent with the morpholino knock-down phenotypes described above, but the gata2a mutant phenotype differs in certain aspects (Yang et al., 2010). My data show that gata2a is required for the correct expression of the majority of KA” markers since tal1, gata3, sox1a, as well as the GABAergic phenotype of the cells are all lost in row 1 of gata2a mutant zebrafish. Interestingly though, both tal2 and sox1b expression are retained in gata2a mutant embryos.
Figure 33. Schematic representation of possible genetic hierarchies in KA”, KA’ and V2b cells. Schematics show the potential genetic hierarchies that regulate development of KA” cells (A), KA’ cells (B), and V2b cells (C), based on results presented in this thesis. Arrows indicate genes that appear to be downstream of each other. Black color represents interactions with strong supporting evidence, and grey arrows represent potential interactions that are less strongly supported. Further investigation will be necessary to test the latter and reveal remaining interactions.
The fact that expression of *tal2* is retained in KA” cells in *gata2a* mutants is in sharp contrast to previous knockdown studies, where *tal2* was lost in row 1 in *gata2a* morphants (Yang *et al*., 2010). Yang *et al*., 2010 propose that *tal2* is downstream of *gata2a* in KA” cells, as *gata2a* morpholino injections abolished expression of *tal2* in these cells, but *tal2* morpholino injections did not affect expression of *gata2a* in KA” cells.

The differences between my results and the results of Yang and colleagues could have several explanations. First, it is possible that the morpholino injections exhibited off-target effects that are additional to abolishment of *gata2a* function, and that the effect is, therefore, not seen in *gata2a* mutants. We know that many morpholinos are prone to exhibiting such off-target effects, as the amount of morpholino injected is often in overwhelming excess of target RNA that is available for binding (Bedell *et al*., 2011; Shulte-Merker and Steiner, 2014). Given the difficulty in distinguishing the effects of off-target binding phenotype from the phenotype of knocking down the target mRNA function, it is strongly recommended that the mutant phenotype takes precedence over a morpholino-based phenotype (Schulte-Merker and Steiner, 2014). In case of *tal2* marker, the phenotype of *tal2* expression in KA” cells caused by knocking down *gata2a* with morpholinos is not validated by my observations in the *gata2a* mutant.

In addition, we know that *tal2* is expressed in KA” cells, but that also a subset of *tal2*-expressing cells co-expresses *nkx2.2b*, indicating that *tal2* is also expressed in progenitor p3 cells (Schafer *et al*., 2007). It is possible that if KA” cells do not form in *gata2a* mutant embryos, those cells might be still ‘locked’ in their very late p3 state from which the KA” form. In that case, the cells would express the *tal2* marker, but not have a
mature KA⁺ identity. In this case the tal2-expressing cells in gata2a mutants could be very early KA⁺ cells, that do not express other transcription factors normally expressed by these cells. This scenario would mean that, contrary to the mechanism proposed by Yang et al., 2010, tal2 is either upstream of gata2a in formation of KA⁺ cells, or its expression is independent of gata2a.

tal2 is also expressed by V3 cells in zebrafish that express sim1a (Schafer et al., 2007), and by Sim1-expressing V3 cells in mouse (Zhang et al., 2008). However, since V3 cells do not form until later stages of development (Schafer et al., 2007), the lack of change in tal2 expression at 24hpf is unlikely to be related to V3 cells. It would be interesting to test whether a mutation in gata2a would cause KA⁺ cells to change into V3 cells by examining expression of sim1a. Previous studies suggest that at 48hpf morpholino knock-down of gata2a or of gata3 does not affect vglut2.1 or sim1a expression, however most of these results were ‘data not shown’ (Yang et al., 2010) and I was not that convinced by the data that was shown. The researchers do not show the effect of gata3 morpholino injections, and in gata2a-morpholino injected embryos only vglut2.1 result is shown, which looks to me from photographs like it might be expressed more strongly and/or by more KA⁺ cells (Yang et al., 2010, Supplementary Materials Fig. 2).

A similar mechanism to that proposed above could also explain the retention of sox1b expression in gata2a mutants. If sox1b is also expressed early in KA⁺ cell formation in p3 cells, it might be still expressed in the absence of gata2a. In this case, I would hypothesize that sox1a, in contrast, is expressed only later in KA⁺ cells and not in p3 cells, as this could explain why gata2a mutants lose expression of sox1a but not sox1b in KA⁺
cells. Alternatively, it is possible that the regulation of these sox1a genes differs in KA" cells, and only one copy of Sox1 ortholog (sox1a) is regulated by gata2a in specification of KA" cells. It would be interesting to see whether abolishment of sox1b (but not of sox1a) results in abolishment of KA" formation, to test whether sox1b is upstream of gata2a or acts independently of it. However, as mentioned before, currently there are no mutant strains of sox1a/b are available and only knockdown experiments would be possible.

Finally, it is possible that the loss of Gata2a function in our mutant is not complete and that even though the DNA-binding function is lost, the transcription factor still exerts part of its function by for example binding to other transcription factors. Given that the phenotype seen seems specific to KA" cells and, aside from tal2 marker expression, resembles the morpholino- based studies, I think it is more likely that the mutant we used is a null allele and the phenotype is the consequence of complete loss of gata2a.

Taken together, based on the results obtained in this study I propose that gata2a is required for the correct formation of mature KA" cells and their GABAergic phenotype. Gata2 is upstream of tal1, gata3, and sox1a, and in the absence of Gata2 function KA" cells do not properly differentiate as functional inhibitory cells. However, gata2a is either downstream of tal2 and sox1b, or those markers participate in an alternative, gata2a-independent pathway in formation of KA" cells.

4.4 Regulatory network that leads to formation of KA’ cells

As discussed earlier, KA’ cells are primarily found in row 2 in zebrafish spinal cord, but can also occasionally be found in row 3 where, if located medially, they still access the
central canal with their sensory tuft (Dr. Claire Wyart, personal communication; Djenoune et al., 2014). KA’ cells differ from KA” cells in their dorsoventral position and origin, as they are born from the same progenitor domain as motoneurons (pMN progenitor domain) (Park et al., 2004). Just like KA” cells KA’ cells express gata2a, gata3 and tal1 (Batista et al., 2008), tal2 (Pinheiro et al., 2004; Schafer et al., 2007) as well as sox1a and sox1b (this thesis), and the cells are GABAergic (Bernhardt et al., 1992).

Loss of Gata2a does not have an effect on the expression of the majority of KA’ cells in zebrafish spinal cord, which is consistent with previous morpholino knockdown results (Yange et al., 2010). Interestingly, my cell counts show, however, that there is a small loss in the number of GABAergic cells in row 2 of gata2a mutants, compared to their wild-type siblings (Fig. 19). This suggests that perhaps not all of the KA’ cells are able to become GABAergic in the absence of Gata2a. However, this result is slightly different from the previous knockdown study, which showed that KA’ cells are still GABAergic after injections with gata2a morpholino (Yang et al., 2010). However, since the reduction in KA’ cells that express gads that I observed in gata2a mutants is relatively small, it could have been easily missed by the team of Yang and colleagues as they didn’t count cells (Yang et al., 2010). Also, since only about half of the GABAergic cells are affected in gata2a mutants, future work should probably repeat this experiment to confirm that this phenotype is not due to subtle differences in embryo stages. I took great care during the staging and fixing processes of my experiments to make sure that embryos were always at 24hpf, however it is possible that subtle variabilities in stage may have sometimes occurred due to small changes in the temperature of the incubator and/or other variables.
However, similar phenotypic changes in KA’ cells of gata2a mutant was found with sox1a marker. Since the expression of sox1a is almost completely abolished in row 2 of gata2a mutants, it suggests that gata2a is required for correct expression of both sox1a and GABAergic phenotypes in KA’ cells. Given that sox1b expression remains unaffected in the KA’ cells, it again suggests that those two co-orthologs are differently regulated in specific cell types of the zebrafish spinal cord.

While gata2a is required for sox1a expression and potentially the GABAergic phenotype of KA’ cells, it most likely acts downstream of tal1 and gata3 in these cells, since gata2a expression is abolished in KA’ cells in both tal1 and gata3 mutants. Loss of tal1, causes loss of all markers of KA’ cells (Figs. 21-24), placing it near the top of the genetic hierarchy that leads to formation of KA’ cells. In addition to counting cells in each dorsal/ventral spinal cord row, I repeated my cell counts to distinguish between lateral V2b cells and medial KA’ cells in row 3. These analyses showed that in tal1 mutants only very sporadic cells could be found that might be a KA’ cell. Similarly, loss of gata3 leads to the abolishment of all examined markers that label KA’ cells: no medially located cells in row 2 or 3 express any KA’ markers (Fig. 33). This is consistent with morpholino knockdown results, which show that abolishment of gata3 results in loss of tal2 and GABAergic markers in KA’ cells (Yang et al., 2010). Based on those published results, and on the loss of expression of additional markers in KA’ cells in the gata3 mutants, it appears that gata3 is required for the specification of the global cell fate of KA’ cells. In addition, my results show that tal1 is required for the specification of the global cell fate of KA’ cells, which is the first time that tal1 has been shown to be required for KA/CSF-cN specification in any vertebrate.
Interestingly, loss of *tal1* also results in loss of *tal1* expression in KA’ cells but not in other cells, suggesting that most likely KA’ cells are lost. A similar observation was made in *gata3* mutants in which *gata3* expression seems to be reduced in KA’ cells. However, in *gata3* mutant all the other cells also expressed *gata3* much more weakly when compared to the sibling embryos, which might indicate that *gata3* RNA is subject to nonsense mediated decay in *gata3* mutants. Since expression of both *tal1* and *gata3* as well as all other KA’ markers are lost in KA’ cells of both single mutants, both *tal1* and *gata3* are required on their own for correct formation of KA’ cells. It would be interesting to see whether those cells die, or whether KA’ cells become other cells in absence of *tal1* and *gata3*. Since, as mentioned before, KA’ cells form from the same progenitor domain as motoneurons do (Park et al., 2004), it would be interesting to see whether number of cells that express motoneuron markers (e.g. *islet1/2*) increases in *tal1* and *gata3* mutants.

It would be also interesting to see whether *tal1* and *gata3* act together in specification of KA’ cells by forming a larger complex, or whether they act independently of each other to specify KA’ cells. Studies that involve investigation of protein-protein interactions, such as co-immunoprecipitation experiments, would be needed to answer this question directly.

It is also possible that a larger complex that includes Gata3 and Tal1 forms in KA’ cells, given that in other tissues similar complexes form. For example during blood development, TAL1 and GATA proteins (e.g. GATA-1), are known to form complexes. TAL1 has an ability to bind to E-boxes (consensus sequence CANNTG) (Church et al., 1985), the boxes are however sometimes positioned in a way that also requires both TAL1 and GATA
protein binding to DNA for transcriptional activity (Tripic et al., 2009). In those cases, binding of both TAL1 and GATA proteins together enables formation of a larger complex that may include nuclear non-DNA binding proteins (e.g. lmo2), and only then downstream genes can be expressed (Wadman et al., 1997). Also, sometimes TAL1 is required for transcriptional activity but acts as a cofactor, and does not need to directly bind to DNA. For example, TAL1 acts together with GATA3 (and LMO) in T-cell acute lymphoblastic leukemia, where the full complex binds to DNA through the GATA site, and TAL1 must be present in the GATA3-LMO-TAL1 complex for full transcriptional activity to take place (Ho et al., 1998). Also, GATA protein binding has a large influence on binding specificity and mode of action of TAL1 (repression/activation of target gene expression), and the binding of GATAs appears to regulate the ability of TAL1 to activate or repress transcription depending on the cell type in mouse cell cultures (Tripic et al., 2009; Wu et al., 2014). In fact, binding of GATA proteins appears to be a stronger determinant of specific TAL1 binding than even the presence of E-boxes to which TAL1 binds in mouse blood cell cultures (Ono et al., 1998; Wu et al., 2014). It is therefore possible that in zebrafish spinal cord Gata3 and Tal1 act together in specification of KA’ cells, especially since my results show largely similar phenotypes in KA’ cells resulting from either loss of tal1 or gata3. However, it is not possible to conclude based on my results whether Tal1 acts as a co-factor for Gata3 transcription factors, or whether they bind to DNA independently. Given that both DNA-binding domains are truncated in the mutants, both scenarios are possible and could result in a similar phenotype. More mechanistic studies, such as experiments involving ChIP analysis would enable to better understand this process in KA’s of zebrafish.
Overall, my results are in agreement with the knockdown experiments that suggest that abolishment of *gata3* in zebrafish embryos leads to the loss of KA’ cells (Yang *et al.*, 2010). My research confirms this as all of the genes I examined are lost in KA’ cells in *gata3* mutant embryos. Similarly, my results confirm that *gata2a* is dispensable for KA’ specification, with two major exceptions. First, GABAergic specification, which was unaffected in morpholino-injected embryos (Yang *et al.*, 2010), is abolished in about half of the row 2 cells in *gata2a* mutants (Fig. 19). Also, the expression of *sox1a* is reduced in *gata2a* mutants. These two results, suggest some aspects of KA’ differentiation might be affected by loss of *gata2a*. In addition, loss of *tal1* leads to abolishment of KA' cells in all cases, which is a phenotype not described by any previous studies. I conclude that *tal1* and *gata3* are required for correct KA’ specification, while *gata2a* is dispensable for formation of KA’s but might be required for the expression of *sox1a* and the KA’ GABAergic phenotype.

### 4.5 Regulatory network that leads to formation of V2b cells

To determine the functions of *tal1*, *gata2a* and *gata3* in V2b cell specification I examined expression of genes expressed by these cells in the single mutants for each of these genes. V2b cells are most extensively studied of the three cell types I investigated during this thesis. As mentioned before, they express *Tal1*, *Gata2* and *Gata3* in amniotes (Karunaratne *et al.*, 2002; Peng *et al.*, 2007; delBarrio *et al.*, 2007) and in zebrafish (Batista *et al.*, 2008; Kimura *et al.*, 2008). *Tal1* is expressed in amniotes soon after the beginning of V2b differentiation, after a progenitor cell differentiates into a V2a and V2b cell and the V2a cell downregulates *Gata2* (Smith *et al.*, 2002; Muroyama *et al.*, 2005). In mouse ventricular
zone of the spinal cord, all of the cells that express Tal1 express Gata2, with some cells present that express only Gata2 (Peng et al., 2007). Gata3 in amniotes is expressed by the most mature V2b cells, and is an established marker of V2b cells, marking most laterally located post-mitotic inhibitory V2b neurons (Smith et al., 2002, Karunaratne et al., 2002, Li et al., 2005, Muroyama et al., 20005). In zebrafish V2b cells, tal1 and gata2a begin to be expressed between 16-somite stage and 18-somite stage, while gata3 is expressed later (after 18-somite stage), but at 24hpf the cells express all three of those markers (Batista et al., 2008).

In this thesis, as discussed above – I also show that sox1a and sox1b are expressed by at least some V2b cells although they may also be expressed by additional cells. Also, I confirm that tal2 is expressed in a subset of V2b cells. Therefore, in addition to gata2a, gata3 and tal1 I also examined expression of sox1a, sox1b and tal2 in potential V2b cells in each of the three single mutants.

The changes in expression of known V2b markers (i.e. tal1, gata2a and gata3 markers) in each of the three mutants are always relatively small, with no more than half of the cells being lost in the V2b domain. This suggests that redundancy may exist between these transcription factors. Interestingly, my results also show that the expression of some genes is lost whereas others are retained in V2b cells in mutant embryos, which suggests that neither tal1, gata2a nor gata3 are required for global V2b specification. My single mutant results allow me to draw some conclusions about V2b development but do not fully explain the genetic hierarchy between tal1, gata2a and gata3 in these cells.
In V2b cells in *gata2a* mutants, the only phenotypes observed are small changes in GABAergic phenotype, as well as in *gata3* expression. Given however that only an average of about 3 cells lost expression of either *gads* or *gata3*, these differences may not be very meaningful. For example, *gata2a* mutants show slightly more GABAergic cells in row 3 as compared to the wild-type, which might mean that overall number of V2b cells stays the same and the small differences are due to errors in determining the cell row that a cell is present in. It is however possible that the loss of a few cells might reflect a genuine regulation of *gata3* and/or *gads* by *gata2a*, although in this case the effect is subtle. I counted cells in only 4 embryos in this particular case, so perhaps looking at more embryos or repeating this experiment could re-confirm the conclusion of this result. No significant change was found in expression of other markers (*tal2*, *sox1a*, *sox1b*, *tal1* and *foxn4*) in the V2b domain of *gata2a* mutant.

These results are in sharp contrast to amniotic spinal cord, where *Gata2* is required for specification of both V2a and V2b cells, as at early stage of mouse embryonic development (E10.5) both *Vsx2* and *Gata3* are attenuated in mouse *Gata2* mutant (Fig.3, Zhou *et al.*, 2000; Francius *et al.*, 2014). However, later stage studies in mouse mutant are not possible due to early lethality of embryos right around E10 (Zhou *et al.*, 2000). However, conditional knockout of *gata2a* results in co-expression of both *Vsx2* and *Gata3* markers by the same cells at a later stage, showing that *gata2a* plays an important role in the correct decision between V2a and V2b fate (Francius *et al.*, 2014). Interestingly, cells still express *Gata3* and *Vsx2* in the conditional mice in what appears to be larger numbers than in null *Gata2* mutant, but unfortunately the comparison of those numbers is not provided (Francius *et al.*, 2014). It is possible that at least some of the mutant phenotype
observed in mouse is related to disruptions and/or slight delay in overall mutant development, which does not happen in the conditional knockout mouse. Nevertheless, it seems that in absence of Gata2 V2b cells do not form correctly in mouse (Zhou et al., 2000; Francius et al., 2014). It cannot be ruled out that gata2a plays a similar role in zebrafish, and that even though number cells that express V2b markers does not change, those cells might in addition express V2a markers.

Also, my data suggests that loss of tal1 results in slightly reduced gata3 expression, but gata2a expression is not significantly affected in V2b cells. This indicates that tal1 may be partly required for gata3 expression or maintenance. Since the gata3 mutation also results in a loss of about half of tal1-expressing cells, it is possible that a regulatory feedback loop might exist between those genes. In addition, it is also possible that both Tal1 and Gata3 operate together in a complex that maintains expression of their own genes, as discussed later.

This is again in sharp contrast to the mechanism showed in mouse, where nerve-tissue specific loss of Tal1 results in attenuation of Gata2, and complete loss of Gata3 expression (Muroyama et al., 2005). Also, in chicken, Tal1 is sufficient to induce GATA3 expression, while causing attenuation of V2a marker VSX2 (Muroyama et al., 2005; Peng et al., 2007). My results suggest that tal1 on its own is not required for gata2a expression, while gata3 may only partly depend on tal1 in zebrafish V2b cells. It is however interesting that in the manner similar to mouse described above, I also see a more drastic change in the expression of gata3 than in gata2a expression in tal1 mutant. This suggests that tal1
might be important for maintenance of at least \textit{gata3} expression, in which case experiments at a later stage would show a more prominent change in phenotype.

Interestingly, mutation in \textit{tal1} results in an almost complete loss of \textit{sox1b} expression in V2b cells. At the same time, V2b cells still express \textit{sox1a}. This suggests that \textit{tal1} is upstream of \textit{sox1b} but not of \textit{sox1a} in V2b cells. This is another case where \textit{sox1a} and \textit{sox1b} are regulated differently in specific cell populations. Also, in \textit{tal1} mutants, \textit{tal2} expression is completely abolished in the few cells that would normally belong to the V2b domain at 24hpf, but also earlier at 22hpf and later at 27hpf. In addition, the GABAergic phenotype is lost in most cells dorsal to row 4. Since there are cells that are GABAergic dorsally to row 4 that don’t belong to the V2b population (e.g. V1 cells, where \textit{tal1} is not normally expressed), it is likely that some of those remaining cells are not V2bs. However, as also I explained in the results section, the remaining expression of \textit{gads} is generally weaker in any cells that are not KA"s, which might be a result of embryos being generally a little sicker and/or the other cells not forming correctly in absence of other cells that would normally be in the spinal cord. Since KA" cells still express \textit{gads} normally, as discussed before, it is however unlikely that the \textit{tal1} mutant embryos are sick, unless those cells would form early enough not to be affected. Overall, it appears that \textit{tal1} is required for expression of \textit{sox1b} and \textit{tal2}, and for correct specification of GABAergic phenotype in the V2b cells.

This is interesting, since Tal1 (otherwise known as Scl) and Tal2 are closely related, and at least Tal1 is known to form transcriptional complexes with GATA proteins that specify of many cell types, most notably blood (Bockamp \textit{et al.}, 1994; Tripic \textit{et al.}, 2009).
However, in developing mouse midbrain, a conditional knockout of *Tal1* does not affect the expression of *Tal2* (and other markers of GABAergic neurons of that region, including *Gata2* and *Gata3*) (Achim et al., 2013). However, knockout of *Tal2* abolishes expression of *Tal1* and reduces *Gata2*, *Gata3*, expression and the GABAergic marker *Gad1* (Achim et al., 2013). The double *Tal1/Tal2* knockout does not have a more severe effect on *Gata2/3*, but completely abolishes the GABAergic phenotype of those cells. This suggests that at least in some GABAergic midbrain cells, *Tal2* is responsible for expression of *Tal1*, and that both of those transcription factors are required for correct specification of GABAergic midbrain cells (Achim et al., 2013). It is possible that *Tal2* and *Tal1* play similarly complementary roles in specification of the GABAergic phenotype of V2b cells in spinal cord, but their dependence on each other is reversed. My results indicate that at least in V2b cells, *tal1* (*tal1*) regulates expression of *tal2*. This might mean that the loss of the GABAergic phenotype in those cells is due to lack of both *tal1/tal1* and therefore *tal2*, or *tal1* might be solely responsible for the change in GABAergic phenotype. Experiments that involve abolishment of *tal2* would be necessary to distinguish between those possibilities.

Overall, it seems that in absence of *tal1* V2b cells still form, but lose expression of some genes and their GABAergic phenotype. It is possible that *sox1b* and *tal2* belong to the pathway that specifies the GABAergic phenotype of V2 cells, as their expression in these cells is also lost in *tal1* mutants. This result is in contract to *gata2a* and *gata3* mutants where most V2b cells are still GABAergic. This suggests that *Tal1* but not *Gata2* or *Gata3* is required for the GABAergic phenotype of V2b cells.
My results suggest that gata3 is also not required for expression of most V2b markers. However, subtle changes can be observed in expression of some of the V2b markers. Most strikingly, the number of tal1-expressing cells decreases by half in gata3 mutant embryos (Fig. 26). This is the most drastic change in expression pattern observed in V2b cells in gata3 mutants, and it suggests that gata3 regulates expression of tal1 in V2b cells to some extent. The fact that tal1 expression is not completely lost could be explained by either compensation for lack of gata3 by another transcription factor (which could include Tal1 autoregulation or Gata2a), or it is possible that gata3 is only required for the maintenance of tal1 expression in V2b cells and that more cells would lose tal1 expression at later stages. gata2a expression is also affected in gata3 mutants, but to a much smaller extent (only about 3 cells are lost, which is a statistically significant difference). This might mean that some aspect of gata2a expression is regulated by gata3 in V2b cells, although it may also be just biological noise in my experiments.

Interestingly, overexpression experiments in chicken suggest that GATA3 is sufficient to induce expression of GATA2 (Karunaratne et al., 2002). Also, both Gata2 and Gata3 have 5’ regulatory sequences that can bind other GATA-family proteins (Karunaratne et al., 2002). Despite the fact that Gata3 has so far been considered to be the most terminal V2b marker in amniotes, it is possible that Gata3 regulates some aspects of Tal1 and Gata2 expression in V2b cells. Investigation of the expression of tal1 and gata2a at later stages in gata3 mutant could resolve whether gata3 would be required for maintenance of tal1 and/or gata2a expression.
Interestingly, *gata3* mutants also lose about one-third of *sox1b*-expressing cells in rows 4 and above, while no change in *sox1a* expression is observed. In addition, both of those markers are lost in row 3 cells, where many cells are V2bs. This suggests that at least some V2b cells that express *sox1b* are lost in the *gata3* mutant. If V2c cells exist in zebrafish, it is also possible that some of the lost cells correspond to V2c cells. However, it should also be noted that many more *sox1a*-expressing cells were observed than *sox1b* expressing cells in wild-type embryos from *gata3* incross. Also, since about half of the *tal1*-expressing cells and some of the *sox1b*-positive cells are lost in *gata3* mutants, but almost all *sox1b*-expressing cells are lost in *tal1* mutants in V2b domain, it is possible that *sox1b* expression is lost because *tal1* not being present in the cells. In addition, I observed that some GABAergic cells are lost in V2b domains in *gata3* mutants, and even more GABAergic cells were lost in *tal1* mutants. If *tal1* is required for GABAergic phenotype of V2b cells, and *gata3* is required for correct *tal1* expression in at least a subset of V2b cells, loss of GABAergic phenotype in V2b cells in *gata3* mutants might be mediated by loss of *tal1*. Therefore, one possible interpretation of my results would suggest that *gata3* is required for at least some aspect of *tal1* expression, and loss of *tal1* leads to loss of *sox1b* and GABAergic phenotype of V2b cells.

Overall, I propose here that the mechanism of V2b specification in zebrafish differs from the mechanism in amniotes. Most notably, none of the genes *tal1, gata2a* or *gata3* are required for correct expression of *tal1, gata2a* or *gata3* or global specification of V2b cells on their own. Instead, I propose that in V2b domain of zebrafish spinal cord at 24hpf, *tal1* is required for correct expression of *sox1b, tal2* and the GABAergic phenotype of the V2b cells. Also, *gata2a* might be required for maintenance of *gata3* expression to a small extent,
and \textit{gata3} is required for the correct expression (or maintenance of) about a half of \textit{tal1} and \textit{sox1b} expression in V2b cells. This is summarized in the schematic (Fig. 33C).

In conclusion, my mutant analyses have provided novel insights into the regulatory network that specifies V2b and KA cells. Further research on double mutants, performed by Dr. Banerjee in the Lewis lab, will hopefully help us to even better understand the interactions between \textit{tal1}, \textit{gata2a} and \textit{gata3} and their roles in specification of these cells.

\textbf{4.7 Other candidate genes that may be expressed by V2b and/or KA cells}

The final aspect of this thesis that I will discuss is my analyses of other candidate genes that I thought might be expressed by V2b or KA cells. I will discuss my results for each of these genes in turn.

\textit{4.7.1 crb1}

\textit{crb1} (\textit{crumbs family member 1, photoreceptor morphogenesis associated}) encodes a transmembrane protein that is a member of the Crumbs family. \textit{crb1} was first identified in the apical membranes of fly epithelial cells, and recognized as an essential regulator of the epithelial cell polarity (Tepass \textit{et al.}, 1990). Proteins of the Crumbs family are known to exert their function by assembly of a larger Crumbs complex (Crb) to their intracellular domain, and by binding to other ligands via extracellular domain (Le Bivic, 2011; Pocha and Knust, 2013). Crb inhibits Notch signaling in the fly (Herranz \textit{et al.}, 2006) and during neurogenesis in zebrafish (Ohata \textit{et al.}, 2011). This is exciting, as KA (and V2b
specification) in zebrafish and mammals depends on Notch signaling. Expression and function of key Crumbs proteins, including Crb1, is well conserved between mammals and zebrafish, although in some cases differences exist (Le Bivic, 2011; Bulgakova and Knust, 2009).

In zebrafish, expression of crb1 has been identified in the developing brain and retina (Omori and Malicki, 2006). Crb1 can be first detected in developing brain at 24hpf, with expression in the retina detected only after 48hpf (Omori and Malicki, 2006). Despite experiments being performed at the same developmental stage as my experiments, no expression was detected in the spinal cord (Omori and Malicki, 2006). Here, I show for the first time that crb1 is expressed in the zebrafish spinal cord at 24hpf and identify a subset of crb1-expressing cells as KA" and KA' cells. My results show that expression of crb1 is restricted to the most ventral part of the zebrafish spinal cord, and that at 24hpf crb1 is expressed by all KA" cells and by at least a subset of KA' cells. This is also exciting, as the crb1 appears to be one of the very few genes that are expressed solely by KA cells (and not V2b cells) in zebrafish spinal cord.

In mammals, Crb1 is expressed in mouse developing brain, neural tube, and developing and adult retina cells (den Hollander et al., 2002). In mouse retina, Crb1 is essential for correct polarity and adhesion of specialized photoreceptors (Pellikka et al., 2002) and glia cells (van de Pavert et al., 2007). Also, in humans mutations in the CRB1 are associated with multiple eye dystrophies (den Hollander et al., 2004). Interestingly, in the mouse developing neural tube expression of Crb1 coincides with expression of Nkx2.2, with a slightly broader expression of Nkx2.2 than of Crb1 at E10.5 (den Hollander et al., 2002).
This was interpreted as *Crb1* being expressed by mouse V3 cells (den Hollander *et al*., 2002). My results suggest that in zebrafish, V3 (or their progenitor cells) might also express *crb1*, as cells located between the *Tg(gata1:GFP) KA* cells in the p3/V3 domain express this gene. However, no expression of *Crb1* was so far identified in cerebrospinal fluid contacting neurons (CSF-cNs) of mammals that correspond to zebrafish KA cells. If the expression of this gene is conserved between amniotes and zebrafish, my results suggest that this gene could potentially be expressed by mammalian CSF-cNs. Further functional analysis of this gene is also readily possible, as currently there are several zebrafish mutant strains available, and mouse mutants also exist. Given that this gene is expressed by KA cells it would also be interesting to see whether its expression is affected by mutations in *tal1, gata2a* or *gata3*.

**4.7.2 insm1a**

*insm1a (insulinoma associated 1a)* is a zebrafish ortholog of mammalian *Insm1*. In mammals, *Insm1* encodes a transcription factor with five zinc finger domains, and the protein appears to be highly conserved between humans, mammals, zebrafish, frog and *C. elegans* (Lan and Breslin, 2009). *INSM1* was first identified via screening human genetic libraries as a gene associated with the occurrence of insulinoma (Goto *et al*., 1992). Since then, it was shown to be expressed mainly by the nervous and endocrine tissues (Lan and Breslin, 2009), and in humans has been associated with at least 35 different types of neuroendocrine cancer, including lung, renal, pancreatic carcinomas, as well as neuroblastoma and retinoblastoma (Lan and Breslin, 2009). Previous research efforts have concentrated mostly on elucidating the role of *Insm1* in development of neuroendocrine
cells of the brain and other body tissues, and the function in the spinal cord has not yet been analyzed (Lan and Breslin, 2009, Jia et al., 2015).

In developing mouse, Insm1 is expressed in the endocrine cells of pancreas, the central and peripheral nervous system, as well as in olfactory epithelium, and it appears to be a pan-neuronal marker of developing neurons in the brain (Farkas et al., 2008; Osipovich et al., 2014). In the mouse olfactory epithelium, deletion of Insm1a results in formation of fewer neurogenic basal progenitors and more apical cells that give rise to additional progenitors (Rosenbaum et al., 2011).

Similarly, loss of function experiments show an increase in number of apical progenitor cells in mouse cortex and other neuronal structures and loss of neurons, whereas gain-of-function experiments show that cell cycle progression is inhibited upon forced insm1a expression (Farkas et al., 2008). However, INSM1 is expressed by not only progenitors, but also nascent cells in mouse and human embryonic spinal cords (Duggan et al., 2008; Jacob et al., 2009). However INSM1 is only transiently expressed by post-mitotic neurons, and is not detected in fully differentiated neurons of either mouse or human CNS (Duggan et al., 2008). In addition, in mouse hindbrain, Insm1 acts upstream of Gata2 and is required for correct specification of the serotonergic neurotransmitter phenotype of cells (Jacob et al., 2009).

Insma has two orthologs in zebrafish that arose during the whole genome duplication event in the teleost lineage - insmaa and insmab. In this thesis, I only investigated expression of insmaa. insmaa and insmab are both expressed in similar structures in the zebrafish, but differences can be found between their expression patterns
(Lukowski et al., 2006). At 24hpf, both co-orthologs insm1a and insm1b are expressed in the various structures of the brain including diencephalon and hindbrain, presumptive pancreas tissue and both are expressed by spinal cord cells (Lukowski et al. 2006). In the spinal cord, expression pattern of insm1b is broader than expression of insm1a. insm1a expression is restricted to ventral neurons of the spinal cord, whereas insm1b is expressed in three distinctive stripes across the dorsoventral axis (Lukowski et al., 2006). At this stage, both co-orthologs appear to be expressed specifically by neurons as their expression overlaps with the post-mitotic neuronal marker elavl3, but the expression of insm1b appears to be stronger in the cranial neurons than in the spinal cord (Lukowski et al., 2006). Later in development, expression of only insm1a can be detected in zebrafish retina, where it appears to be transiently expressed between the 24hpf and 72hpf stages (Lukowski et al., 2006) and expressed much later in adult zebrafish photoreceptor cells (Morris et al., 2011). Knockdown studies have shown that insm1a is required for correct development of photoreceptor cells in the zebrafish retina (Forbes-Osborne et al., 2012), but no spinal cord phenotype was reported. Interestingly, insm1a appears to be negatively regulated by Notch signaling (Forbes-Osborne et al., 2012), and the spinal cord KA and V2b cells I investigate in this thesis also depend on Notch signaling for their formation (Batista et al., 2008). It would be interesting to see whether expression of the insm1a in spinal cord also depends on Notch signaling, for example with use of Notch-deficient mindbomb mutant.

My results show that insm1a is expressed in the zebrafish spinal cord in a pattern consisted with previously described expression (Lukowski et al., 2006). My analyses suggest that at least some of the insm1a-expressing cells are V2b and/or KA cells, as they
co-label with $Tg(gata1:GFP)$-positive cells. In future, labeling with markers of other cells would be needed to establish the identity of the remaining $Tg(gata1:GFP)$-negative $insm1a$-positive cells. Since some V2b and KA cells express $insm1a$ though, it would also be interesting to see whether this gene is affected by mutations in $tal1$, $gata2a$ or $gata3$.

4.7.3 her6

her6 ($hairy-related$ 6), a zebrafish ortholog of mouse $Hes1$, encodes for a bHLH transcription factor of the hairy-related Hes/Her family. These proteins often act as transcriptional repressors of neurogenesis and other processes (Kageyama et al., 2007). $Hes1$ appears to be required for the correct formation of neurons from radial glia in the mouse brain, and for formation of optic vesicles (Hatakeyama et al., 2004).

The action of Her/Hes proteins often depends on Notch signaling (Kageyama et al., 2007). For example, in mouse, $Hes1$ is expressed in various structures of the brain, where its expression can be either Notch-independent or Notch-dependent (Kageyama et al., 2007). This is interesting, because as discussed above KA and V2b cell formation in zebrafish also depends on Notch signaling. In zebrafish brain, her6 expression in the brain appears to depend on Notch signaling, as in the diencephalon and hindbrain of $mindbomb$ ($mib$) mutants, her6 expression is reduced (Cunliffe, 2004), but the spinal cord expression in this mutant was not described.

Expression of her6 has been described in several zebrafish tissues, but spinal cord expression has not yet been described. Her6 expression in zebrafish can already be detected at 70% epiboly, with a segmental pattern of expression in the prospective forebrain, hindbrain, and the midline becoming clear near the tailbud stage (Pasini et al.,
Later during development, \textit{her6} is expressed in rhombomeres of the hindbrain, with the expression varying between specific rhombomere segments dependent on the stage (Pasini \textit{et al}., 2001). Also, during somitogenesis, \textit{her6} can be found in the notochord, somites, and in the pre-somatic mesoderm (Pasini \textit{et al}., 2001), where it is required for the expression of the cyclic genes that are involved in somite formation (Pasini \textit{et al}., 2004). Also, in the zebrafish thalamus \textit{her6} appears to be required but not sufficient for the GABAergic phenotype of the neurons, and the cells adopt a glutamatergic phenotype in absence of \textit{her6} (Scholpp \textit{et al}., 2009). In contrast, ectopic GABAergic cells form after forced expression of \textit{her6} via a heatshock mechanism, although this is thought to be achieved via resulting repression of neurog1 by \textit{her6} (Scholpp \textit{et al}., 2009).

During my rotation project, I showed that \textit{her6} is also expressed in zebrafish spinal cord. Also, another rotation student, Alex Nichitean, showed that \textit{her6} expression depends on Notch signaling in the spinal cord, as the expression of \textit{her6} in \textit{mib} mutants is significantly reduced (data not shown). Since the expression of \textit{her6} appears to be located in domains where KA" and KA' cells would be found, I further investigated whether this gene is expressed in these cells using double labels. In this thesis I show that \textit{her6} co-labels with \textit{Tg(gata1:GFP)} positive KA cells, demonstrating that \textit{her6} is a novel marker of KA cells. This is exciting, as \textit{her6} appears to be the one of the very few transcription factors that are expressed by KAs but not by the V2bs. Further experiments will hopefully reveal whether \textit{her6} acts downstream of either \textit{tal1}, \textit{gata2a} or \textit{gata3} in KA formation.
4.7.4 *mnx1*

*mnx1* (*motor neuron and pancreas homeobox 1; also known as Hb9*) encodes for a transcription factor with a homeobox-binding domain. Expression of human *MNX1* was first described in pancreas and lymphatic tissues (Najfeld *et al.*, 1992; Harrison *et al.*, 1994). Mutations in this gene were later associated with Currarino syndrome (Ross *et al.*, 1998), in which erroneous secondary neurulation is thought to lead to a series of malformations around the sacral area. More recently, mutations in *MNX1* were also found in association with neonatal diabetes cases (Bonnefond *et al.*, 2013; Flanagan *et al.*, 2014). In mouse, *Mnx1* is required for correct specification of pancreatic β-cells and production of insulin (Harrison *et al.*, 1999; Pan *et al.*, 2015).

Expression of this gene in the spinal cord was first described in frog, and was localized to motoneurons (Saha *et al.*, 1997). Since then, studies have shown that *Mnx1* is required for correct formation of motoneurons in both mouse and zebrafish (Tannabe *et al.*, 1998; Arber *et al.*, 1999; Seredick *et al.*, 2012), and sufficient for specification of somatic motoneurons in chicken (Tannabe *et al.*, 1998). Expression of *Mnx1* in amniote spinal cord depends on Sonic hedgehog (Shh) signaling (Tannabe *et al.*, 1998). Also, in mouse Mnx1 is expressed by excitatory (glutamatergic) interneurons which are involved in locomotor networks (Kwan *et al.*, 2009; Hinckley *et al.*, 2010).

In zebrafish spinal cord, *mnx1* is expressed by both post-mitotic motoneurons and by V2b cells (Seredick *et al.*, 2012). It is first expressed by V2b cells at 16hpf, but its expression persists until at least 24hpf (Seredick *et al.*, 2012). However, *mnx1* alone is not required for formation of primary motoneurons or V2b cells zebrafish (Seredick *et al.*, 2012).
Instead, knockdown analysis showed that \textit{mnx1} together with other \textit{mnx} genes (\textit{mnx2a} and \textit{mnx2b}) are required for correct formation of a specific subtype of motoneurons (MiP) (Seredick \textit{et al.}, 2012). In absence of Mnx proteins, MiPs form V2a interneuron-like characteristics (Seredick \textit{et al.}, 2012).

Since \textit{mnx1} is expressed by zebrafish V2b cells (Seredick \textit{et al.}, 2012), I was interested whether it might also be expressed by KA cells. The experiments in zebrafish show that \textit{mnx1} is not co-expressed with V2a marker \textit{vsx2} or with glutamatergic neurotransmitter markers, and that it is expressed by GABAergic cells (Seredick \textit{et al.}, 2012). Since KA cells are also GABAergic, I was interested to see whether some of those cells express \textit{mnx1}. I hypothesized that especially KA’ cells might express this gene, as both KA’ cells and motoneurons form from the same progenitor domain (Park \textit{et al.}, 2004). Alternatively, it would also be exciting if \textit{mnx1} was expressed by V2b cells but not KA cells, as all genes identified so far as being expressed by V2b cells are also expressed by KA cells. Therefore, \textit{mnx1} could potentially be a V2b-specific marker that distinguishes between those cell types. However, my experiments show that only very few \textit{Tg(gata1:GFP)}-positive cells co-localize with \textit{mnx1}, which could potentially be KA’ or V2b cells. Also, my results indicate that only some V2b cells express \textit{mnx1}, as some cells with V2b-like morphology were observed that did not express \textit{mnx1}. Given that \textit{mnx1} is not expressed by all V2b cells, and is also expressed by other cell types, I conclude that this gene is not a good V2b-specific marker.
4.7.5 \( sp8a \)

\( sp8a \) (\( sp8 \) transcription factor \( a; \) formerly known as \( sp8 \)) is a zebrafish ortholog of mammalian \( Sp8 \) (specificity protein 8) gene. Homologous \( buttonhead \) (\( btd \)) gene was first identified in \( Drosophila \), (Wimmer et al., 1993), and in this species it is responsible for early neurogenesis (Younossi-Hartenstein et al., 1997) and growth of appendages (Estella et al., 2003). More recently, \( Drosophila btd \) was found to maintain the early progenitor state of intermediate progenitor cells and prevent their differentiation into more mature neurons (Xie et al., 2014). Also, in frog (\( X. tropicalis \)), \( sp8 \) controls development of the inner ear (Chung et al., 2014).

In mouse, \( Sp8 \) is expressed in apical ectodermal ridge (AER), where it is required for dorsoventral patterning of the forming limb bud (Bell et al., 2003; Kawakami et al., 2004; Haro et al., 2014). In addition, in mouse brain \( Sp8 \) is required for correct formation of GABAergic neurons of the olfactory bulb (Waclaw et al., 2006). Also, expression of \( Sp8 \) was detected in amniote spinal cord, where it was located to the ventral domain (Bell et al., 2003; Kawakami et al., 2004). Interestingly, in mouse spinal cord expression of \( Sp8 \) was further localized to a subset of \( Lhx3 \)-expressing V2 cells, \( En1 \)-expressing V1 cells and \( Olig2 \) expressing motoneuron progenitor (pMN) cells (Li et al., 2014). Notably, none of the \( Sp8 \)-expressing cells co-expressed \( Gata3 \) V2b marker (Li et al., 2014), which is consistent with my finding in zebrafish that \( sp8a \) is most likely not expressed by V2b cells. Further analysis in amniotes reveals that \( Sp8 \) and \( Nkx2.2 \) co-repress each other, and that \( Sp8 \) acts together with \( Pax6 \) to establish the pMN/p3 boundary (Li et al., 2014).
In zebrafish, *sp8a* is present in the AER (Norton *et al.*, 2005) and in regenerating tail fins (Stoick-Cooper *et al.*, 2007). Also, expression in was described in the anterior neural tube, with especially strong staining in the midbrain/hindbrain boundary (Correa *et al.*, 2005). However, spinal cord expression in zebrafish was documented for the first time by the Lewis lab in a the direct data submission to ZFIN database (England *et al.*, 2014). In this thesis, I show that *sp8a* does not co-localize with *Tg(gata1:GFP)* cells, which shows that this gene is not expressed by KA and most likely by V2b cells. Since it appears to be expressed approximately in a similar dorsoventral position to *Tg(gata1:GFP)*, it is possible that this gene is expressed by earlier V2, V1 cells and/or by pMN cells, as it is in mouse. Further analysis (e.g. by double *in situ* hybridization with markers of those cell types) could reveal whether *sp8a* expression in spinal cord is conserved between zebrafish and amniotes.

### 4.8 Conclusions

In this thesis I show that *sox1a* and *sox1b*, orthologs of mammalian *Sox1*, are expressed by V2b and KA cells in zebrafish spinal cord. My results also suggest that expression of *sox1a* and *sox1b* is regulated differently in KA”, KA’ and V2b cells.

Also, I show that *foxn4* is expressed by early V2 cells that express *gata2a*, and that the position of *foxn4*-expressing cells becomes more dorsal over time, but the total number of cells that express *foxn4* does not drastically increase.

In addition, I demonstrate the importance of *gata2a*, *tal1* and *gata3* for developing V2b and KA cells. My results indicate that *gata2a* is required for correct specification of KA” cells (either their global cell fate or expression of vast majority of markers), and that both
tal1 and gata3 are required for correct specification of global cell fate of KA’ cells. In addition, tal1 is required for the GABAergic phenotype and expression of tal2 and sox1b in V2b cells, but not for expression of many other genes normally expressed by these cells. My results also indicate that in zebrafish neither gata2a nor gata3 are required for correct specification of the V2b GABAergic phenotype or the global cell fate of these cells. Finally, I identify potential novel markers of KA and V2b cells: crb1 and her6 are expressed by KA and not by V2b cells, insm1a is expressed by a few members of both KA or V2b populations, mnx1 may be expressed by very few V2b and/or KA’ cells. All of those markers are also expressed by additional cells, and are, therefore, not specific to one population. However, her6 and crb1 could be good markers to distinguish KA cells from V2b cells. I also show that sp8a is not expressed by either V2b and/or KA cells at 24hpf in zebrafish.

4.9 Future work

Future work building on this research should answer remaining questions about V2b and KA development. For example, even though I show in this thesis that sox1a and sox1b are expressed by KA and at least some V2b cells, I was unable to show whether they are expressed by all V2b cells in zebrafish. Also, it remains to be investigated whether these genes are always co-expressed by the same cells, and whether either of them is expressed by additional cell populations in the zebrafish spinal cord. Hopefully, future double in situ hybridization experiments will answer these questions. In addition, I was not able to test whether V2c cells exist in zebrafish, and further experiments (as discussed in this thesis) would be needed to investigate this. Since sox1a and sox1b appear to be regulated
differently in KA", KA' and V2b cells, it would be also exciting in future to investigate whether those genes play different roles in specification of these cell types.

It would also be interesting to investigate whether the transcription factors Tal1, Gata2 and/or Gata3 act together in specifying KA and/or V2b cells. To this end, investigation of double mutants in these genes are currently being carried by Dr. Santanu Banerjee in the Lewis lab. Since my results show that *gata2a* is required for expression of most KA" markers (but not *tal2* and *sox1b*), it would be interesting to see whether abolishment of either *tal1* and/or *gata3* in addition to *gata2a* results in a loss of *tal2* or *sox1b* expression in KA" cells. Also, since my work shows that only *tal1* is required for the correct specification of the GABAergic phenotype of V2b cells, it would be particularly interesting to see whether double mutant combinations of *tal1, gata2a* and *gata3* affect this and/or other aspects of V2b cell development.

I also hope that the novel markers that I identified for KA but not V2b cells (*her6, crb1*) will help with investigations of the mechanisms that specify KA cells. For a long time, more detailed investigations of KA cells in zebrafish have been hindered by a lack of molecular markers that are expressed specifically by these cells. However, a gene encoding for calcium-permeable polycystic kidney disease 2-like 1 (PKD2L1) channel, first discovered in heart, retina and kidney (Basora *et al.*, 2002), has now been shown to be expressed in CSF-cNs of both brain stem and spinal cord in postnatal P1-P4 (Huang *et al.*, 2006) and adult (Orts-Del'immagine *et al.*, 2012) mouse. *PKD2L1* is conserved across vertebrates, and in the spinal cord it is specifically expressed by CSF-cNs in mouse and
macaque and KA cells in zebrafish (Djenoune et al., 2014). In future, this KA-specific marker may also enable additional investigation of specification of KA cells in zebrafish.
Appendix Tables:

Table 1. *p*-values of Student's t-test comparison of cell counts of *foxn4*-labeled cells in wild-type embryos at 4 different developmental stages. Values correspond to Fig. 12. 4 embryos were counted in each case. Statistically significant differences (*p*<0.05) are indicated in bold. All statistical tests were performed with Student’s t-test.

<table>
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<th>Developmental stage (in hours post-fertilization)</th>
<th><em>p</em>-value (Student’s t-test)</th>
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<td>20vs22</td>
<td>0.700</td>
</tr>
<tr>
<td>20vs24</td>
<td>0.093</td>
</tr>
<tr>
<td>20vs27</td>
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<tr>
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<td>22vs24</td>
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</tr>
<tr>
<td><strong>22vs30</strong></td>
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</tr>
<tr>
<td>24vs27</td>
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<tr>
<td>27vs30</td>
<td>0.266</td>
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</table>

In bold: *p*<0.05
Table 2. *p*-values from pairwise comparisons of wild-type embryos from incrosses of *ntl^t21384*, *gata2a^um27* and *gata3^sa0234* heterozygous mutants. Values correspond to Figs 29 and 30. Statistically significant differences (*p*<0.05) are indicated in bold and are on the pink background. Values that approach significance threshold (*p*<0.065) are italicized and are on the light blue background. All statistical tests were performed with Student’s t-test.

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<th>Marker</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>TOTAL</th>
<th>4+</th>
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<td></td>
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VITA

NAME: Liwia Anna Andrzejczuk (also known as Livia A. Andrzejczuk)

PLACE OF BIRTH: Pila, Poland

DATE OF BIRTH: 27th January, 1988

EDUCATION:

2015
M.S. in Biology
Syracuse University, Syracuse, New York
Thesis
'Specification of V2b and KA neurons in the zebrafish spinal cord.'
Advisor: Dr. Kate Lewis

2012
BSc (Hons) in Biological Sciences (Cell and Molecular Biology), 1st Honors degree
Heriot – Watt University, Scotland, United Kingdom
Thesis
'The influence of ZnO and Ag nanoparticles on human kidney cells.'
Advisor: Dr. Vicki Stone

PUBLICATIONS:


HONORS AND AWARDS

2012 Fitzpatrick Prize (for the best biotechnology-related project at University)
Heriot-Watt University, Edinburgh, UK

2013-2015 Women in Science and Engineering Future Professionals Program Fellow,
Syracuse University, Syracuse, New York

2013-2015 Graduate School Future Professoriate Program Fellow,
Syracuse University, Syracuse, New York