Functional studies of two forkhead genes

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Abstract

Forkhead genes are functionally diverse and several have been linked to human disease. A previous screen for forkhead genes identified the family member *FOXS1*. To characterize the function of this gene, we produced a mouse model in which the *Foxs1* gene was replaced by a *lacZ* marker allele. During embryogenesis, *Foxs1* was most prominently expressed in peripheral sensory neurons and cerebellum, while a more widespread expression was seen in adult animals. Mutant animals displayed a complex phenotype, which included an enhanced coordinated sensorimotor performance and, in male mice, a lowered weight gain on a high-fat diet. We speculate that the relatively mild phenotype may be due to compensatory effects exerted by other forkhead genes.

Genetic tracing of cells of the boundary cap had shown that they contribute to both sensory neurons and glial cells of the peripheral nervous system, suggesting that they could be multipotent stem cells. We investigated their stem cell properties by culturing cells of the dorsal root ganglia and associated boundary caps. This resulted in the formation of neural crest stem cell clones that were shown to be derived from the boundary cap cells. In vitro differentiation of the stem cell clones gave rise to functional sensory neurons of different subclasses. Our results suggest that cells of the boundary cap are multipotent, sensory-specified stem cells that persist throughout embryogenesis.

A second forkhead gene, *Foxi1*, had previously been shown to be of importance in the regulation of the proton-secreting capacity in kidney collecting ducts, endolymphatic sac and epididymis. To gain further knowledge of the mechanisms involved, we investigated the role of Foxi1 in the regulation of V-ATPase subunits B1, a4, A1 and E2. Our results support a direct role of Foxi1 in the regulation of both the specifically expressed B1 and a4 subunits and the ubiquitously expressed subunits A1 and E2 in all of the tissues studied.

Keywords: forkhead genes, Foxs1, Foxi1, vacuolar type H^+-ATPase, boundary cap, neural crest stem cells

List of publications

This thesis is based on the following three articles, which are referred to in the text by their roman numerals (I-III). The articles are included at the end of the thesis.

I. Heglind M, Cederberg A, Aquino J, Lucas G, Ernfors P, Enerback S (2005) Lack of the Central Nervous System- and Neural Crest-Expressed Forkhead Gene *Foxs1* Affects Motor Function and Body Weight. Mol Cell Biol 25:5616-5625.

II. Hjerling-Leffler J, Marmigere F, Heglind M, Cederberg A, Koltzenburg M, Enerback S, Ernfors P (2005) The boundary cap: a source of neural crest stem cells that generate multiple sensory neuron subtypes. Development 132:2623-2632.

III. Vidarsson H, Westergren R, Heglind M, Blomqvist SR, Breton S, Enerback S (2009) The Forkhead Transcription Factor Foxi1 Is a Master Regulator of Vacuolar H^+-ATPase Proton Pump Subunits in the Inner Ear, Kidney and Epididymis. PLoS ONE 4:e4471.

Publication not used in this thesis

- Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto N-J, Enerback S, Nuutila P (2009) Functional Brown Adipose Tissue in Healthy Adults. N Engl J Med 360:1518-1525.

Abbreviations

BMP – Bone morphogenetic protein
E – Embryonic day/stage
FGF – Fibroblast growth factor
FORE – Forkhead-related
Fox – Forkhead box
Maob – Monoamine oxidase B
NCSC – Neural crest stem cell
bNCSC – Boundary cap neural crest stem cell
sNCSC – Sciatic nerve neural crest stem cell
P – Postnatal day
Pax – Paired box
Snail – Snail homolog
Sox – SRY-box containing gene
Trk – Neurotrophic tyrosine kinase receptor
Trp – Transient receptor potential channel
V-ATPase – Vacuolar type H^+-ATPase
Wnt – Wingless-related MMTV integration site

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Background

Forkhead transcription factors

Transcription factors

Several mechanisms for regulation of eukaryotic gene expression are known, the most important of which is initiation of transcription (Lodish et al., 1995). Regulation of transcription is central both to tissue-specific gene expression and to the regulation of gene activity in response to specific stimuli (Latchman, 1997). Transcription is controlled by the action of transcription factors that interact with regulatory cis-elements in DNA. Transcription factors display a modular structure and generally consist of a DNA-binding domain, which confers the specificity of DNA-binding, and one or more activating or inhibitory domains, which interact with other factors to either promote or repress initiation of transcription (Brent and Ptashne, 1985; Frankel and Kim, 1991; Latchman, 1997). Transcription factors are usually classified into families based on sequence similarities in the DNA-binding domains. It has been estimated that as many as 7% of mouse genes encode transcription factors (Gray et al., 2004).

The forkhead family of transcription factors

The forkhead domain was identified 20 years ago as a novel, highly conserved DNA-binding domain common to both the drosophila fkh protein (the factor responsible for the drosophila forkhead mutation) and the then newly isolated rat transcription factor HNF-3A (Weigel et al., 1989; Lai et al., 1990; Weigel and Jäckle, 1990). This domain has since been found in over a hundred genes in a wide variety of species (Wijchers et al., 2006). Together these genes constitute the forkhead family of transcription factors.

Based on sequence similarities in the forkhead domain, family members are organized into 19 different classes denoted by the letters A-S (Kaestner et al., 2000; Wijchers et al., 2006). Apart from the highly conserved DNA-binding domain, forkhead genes share little sequence homology and, accordingly, they display a wide functional diversity. In mammals, more than 40 forkhead genes have been identified, and many of them have been targeted in mouse models. Studies of mouse models carrying mutations in forkhead genes have shown that several of these genes are indispensable for proper embryonic development and survival beyond the neonatal period (reviewed in Carlsson and Mahlapuu, 2002). Furthermore, important functions of forkhead genes have also been demonstrated in processes as diverse as cell-cycle regulation (Ye et al., 1999; Seoane et al., 2004), metabolism (Cederberg et al., 2001; Wolfrum et al., 2004) and regulation of the immune system (Hori et al., 2003; Johansson et al., 2003). Mutations in forkhead genes have also been linked to human disease (reviewed in Hannenhalli and Kaestner, 2009).

In Paper I, we characterized a new mouse model in which the forkhead gene *forkhead box S1* (*Foxs1)* had been inactivated by replacement of its coding sequence with a *lacZ* marker gene.

Neural crest and cranial placodes

The neural crest is a transient, multipotent population of cells that originate at the dorsal neural tube and migrate extensively throughout the embryo to give rise to a wide variety of tissues, including bone and cartilage of the head, pigment cells of the skin and neurons of the peripheral sensory, autonomic and enteric nervous systems (Le Douarin and Kalcheim, 1999). It has been suggested that the acquisition of the neural crest was a key event in the evolution of vertebrates because its contribution to neomorphic head structures, such as jaws, allowed a switch from passive to active feeding (Northcutt, 2005). Formation of the neural crest is a complex process that involves coordinated changes in gene expression, developmental potential, cell-cell adhesion and an epithelial-mesenchymal transition (Cheung et al., 2005).

Neurulation and formation of the neural crest

In vertebrate embryos, formation of the neural tube, which ultimately develops into the central nervous system, is the result of neurulation. Neurulation begins when dorsally located epithelial cells of embryonic ectoderm undergo a change in morphology, becoming columnar and elongated in shape, and start to express unique molecular markers. These events are induced by signals from the adjacent mesoderm and non-neural ectoderm and result in a regionalized thickening of the dorsal ectoderm, referred to as the neural plate (Smith and Schoenwolf, 1997; Wolpert et al., 1998; Scott, 2000; Sauka-Spengler and Bronner-Fraser, 2008). As the neural plate extends anteroposteriorly and narrows mediolaterally, its lateral borders, induced by bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wingless-related MMTV integration site (Wnt), are elevated and begin to fold towards each other, leaving between them a neural groove along the dorsal midline of the embryo (Smith and Schoenwolf, 1997; Scott, 2000; Sauka-Spengler and Bronner-Fraser, 2008). At this stage, the neural folds contain precursor cells of the neural crest that express characteristic factors such as members of the SRY-box containing gene (Sox), snail homolog (Snail), Fox and paired box (Pax) families (LaBonne and Bronner-Fraser, 1999; Dottori et al., 2001; Aybar et al., 2003; Cheung and Briscoe, 2003).

Fusion of the neural folds along the dorsal midline results in neural tube closure and its separation from the overlying epidermal ectoderm (Scott, 2000; Colas and Schoenwolf, 2001). Around the time of neural tube closure, neural crest precursor cells undergo an epithelial-mesenchymal transition to become migratory neural crest cells that start to delaminate from the dorsal neural tube. The epithelial-mesenchymal transition involves profound changes in cytoskeletal organization and adhesive

properties and requires repression of adhesion molecules, such as cadherins, by factors of the Snail family (Nakagawa and Takeichi, 1998; Thiery, 2003; Taneyhill et al., 2007; Kalluri and Weinberg, 2009). In vertebrates, neurulation generally occurs in waves extending anteriorly and posteriorly from sites of neural fold fusion. Consequently, events of neural tube closure and neural crest emigration at different levels along the anteroposterior axis will be separate in time (Bronner-Fraser, 1993).

Pathways of neural crest cell migration in the trunk

Upon delamination, streams of neural crest cells migrate from the dorsal neural tube along different pathways. The path that will be used by an individual cell depends on the timing of migration as well as on its position along the anteroposterior axis. Based on differences in migratory patterns and the tissues to which the migratory cells contribute, the neural crest can be divided into several regions, usually designated cranial, vagal, cardiac, trunk and lumbosacral neural crest (Bronner-Fraser, 1993).

In the trunk region, neural crest cells migrate via two main pathways, the dorsolateral and the ventral pathways (Figure 1) (Le Douarin and Teillet, 1974; Le Douarin and Kalcheim, 1999). In mice, migration along both pathways is initiated at embryonic day/stage 8.5 (E8.5) (Serbedzija et al., 1990). Neural crest cells in the dorsolateral pathway migrate between the ectoderm and the somites and, after invading the ectoderm, they give rise to the melanocytes of the skin (Erickson et al., 1992). In the ventral pathway, two overlapping phases have been described (Serbedzija et al., 1990). Neural crest cells of the first phase migrate ventrally, through the anterior portion of adjacent somites, to form the sympathetic ganglia and the chromaffin cells of the adrenal glands (Le Douarin and Teillet, 1974; Serbedzija et al., 1990). In a second phase, cells enter the anterior portions of the somites where they remain to form the dorsal root ganglia (Figure 1) (Rickmann et al., 1985; Bronner-Fraser, 1986; Serbedzija et al., 1990).

The migration of neural crest cells is strictly confined to the anterior half of each somite, a migratory behavior that provides the basis for segmentation along the anteroposterior axis, leading to formation of distinct peripheral sensory and autonomic ganglia (Loring and Erickson, 1987; Teillet et al., 1987). The secreted factors F-spondin and semaphorin 3f, the latter by acting as a repellant towards the neuropilin 2a1 receptor present in neural crest cell membranes, have been suggested to be important in inhibiting migration through the posterior halves of the somites (Debby-Brafman et al., 1999; Gammill et al., 2006).

Migratory pathways of the cranial neural crest

The cranial neural crest can be subdivided into forebrain, midbrain, and hindbrain regions (Bronner-Fraser, 1993). Cranial neural crest cells populate the head and pharyngeal arches and are responsible for the formation of a large part of the skeletal, cartilaginous and connective tissues of the face and neck (Le Douarin and Kalcheim,

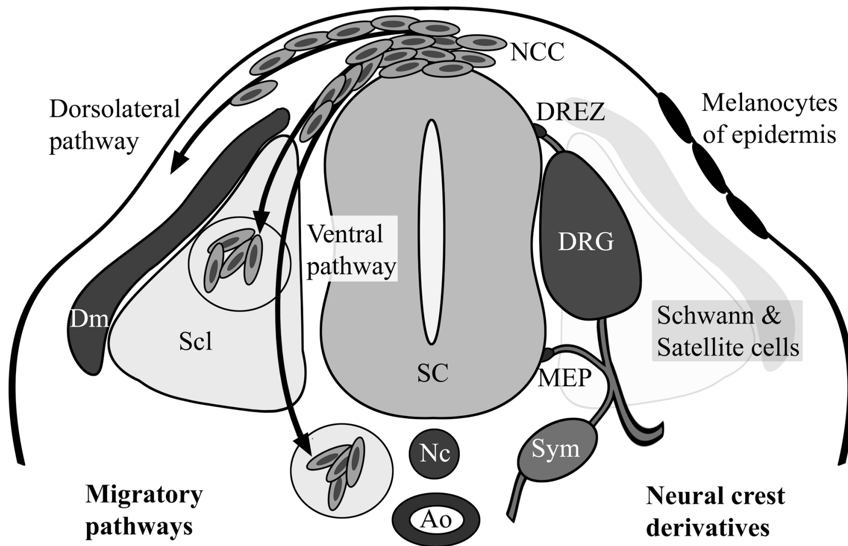

Figure 1 Schematic representation of trunk neural crest migratory pathways and derivatives. Neural crest cells (NCC) delaminate from the dorsal aspect of the forming spinal cord (SC) and enter the dorsolateral or ventral pathways (left). Upon reaching their target areas neural crest cells of the dorsolateral pathway give rise to melanocytes of the epidermis while those of the ventral pathway give rise to neurons of the dorsal root ganglia (DRG), sympathetic ganglia (Sym) and also all Schwann and satellite cells of the peripheral nervous system (right). The approximate positions of the boundary caps of the dorsal root entry zone (DREZ) and motor exit points (MEP) are indicated (right). Abbreviations: Dm, dermamyotome; Scl, sclerotome; Nc, notochord; Ao, dorsal aorta.

1999). In unsegmented broad streams, cells from diencephalic and mesencephalic levels of the neural crest migrate ventrally to contribute to the frontonasal process and the periocular area of the face region and to the first pharyngeal arch, where they will give rise to structures such as meninges, facial skeleton and the incus and malleus of the middle ear (Serbedzija et al., 1992; Bronner-Fraser, 1994; Le Douarin and Kalcheim, 1999). The segmented nature of the hindbrain causes a separation of the migrating neural crest cells at this level into three distinct streams. The streams are formed by cells migrating from the neural tube at the levels of rhombomeres 1/2, 4 and 6, while rhombomeres 3 and 5 are free of neural crest cells (Lumsden and Guthrie, 1991; Serbedzija et al., 1992). Hindbrain neural crest cells enter the pharyngeal arches from where they contribute to the formation of tissues such as the hyoid bone (Le Douarin and Kalcheim, 1999). The cranial neural crest also provides neurons and glial cells to the sensory ganglia of cranial nerves V, VII, IX and X. The formation of distinct cranial sensory ganglia is, like in the trunk, a result of the segmentation of the neural crest migratory streams (Lumsden and Guthrie, 1991).

Cranial placodes

Cranial placodes are discrete ectodermal thickenings that form in characteristic positions in the head region of vertebrate embryos. The placodes, like the neural crest, are transient and give rise to migratory cell populations that contribute to the formation of cranial structures by providing a variety of different cell types. The mammalian set of placodes includes the dorsolaterally positioned trigeminal and otic placodes as well as the epibranchial placodes which arise at the dorsal margin of the pharyngeal clefts (Begbie et al., 1999). These placodes all contribute sensory neurons to the peripheral ganglia of the cranial nerves (D'amico-Martel and Noden, 1983). It is not clear whether the placodes arise by subdivisions of a single common panplacodal primordium (Streit, 2004; Schlosser, 2006) or if they arise as more or less separate entities at their specific locations in the embryonic head (Graham and Begbie, 2000; Begbie and Graham, 2001a). However, their induction appears to be dependent on signals from surrounding tissues acting via the BMP, FGF and Wnt pathways (reviewed in Baker and Bronner-Fraser, 2001). When migrating away from their sites of origin, cells of the epibranchial placodes form distinct streams that spatially overlap with streams of migrating neural crest cells (Begbie and Graham, 2001b). Upon removal of the neural crest, the streams of placodal cells are perturbed, which results in abnormal ganglia formation and aberrant axonal connections. It has thus been suggested that epibranchial placodal cells are guided to their correct positions by cells of the neural crest (Begbie and Graham, 2001b).

Peripheral sensory ganglia

The peripheral nervous system

The peripheral nervous system consists of all the neurons and glial cells that are positioned outside of the brain and spinal cord. The somata of its neurons are located in peripheral ganglia and the axons are gathered into spinal and cranial nerves which connect peripheral targets throughout the body with the spinal cord and brain stem, respectively. The peripheral nervous system can be divided into autonomic and somatic divisions.

The autonomic nervous system is, in its entirety, of neural crest origin. It consists of sympathetic and parasympathetic subdivisions and it provides motor innervation to smooth muscle, cardiac muscle and glandular epithelia. In addition, autonomic sensory nerve endings can be found in most visceral organs from where myelinated and unmyelinated projections are sent off to the spinal cord and brain (Le Douarin and Kalcheim, 1999).

The somatic nervous system consists of somatic motor and somatic sensory divisions. Neurons of the motor division are located in the spinal cord and innervate striated muscle, which is responsible for voluntary movements. The sensory division of the somatic nervous system is responsible for transmission of somatic sensory

information from the periphery to the central nervous system. The somata of sensory neurons are located in bilateral dorsal root ganglia along the length of the spinal cord and also in the peripheral ganglia of cranial nerves V, VII, VIII, IX and X. Neurons in sensory ganglia send peripheral projections to specialized receptors that detect sensory stimuli such as touch, pain and taste. The sensations are transduced into action potentials which are then propagated towards the central nervous system. Developmentally, sensory neurons originate from precursors migrating from the neural crest and a subset of the ectodermal placodes to the sites of forming ganglia where they settle and differentiate (Le Douarin and Kalcheim, 1999).

Formation of the sensory ganglia

The neural crest provides the glial component of the peripheral nervous system and also all the neurons of the dorsal root ganglia (Le Douarin and Teillet, 1974). In addition, neurons of the proximal part of the trigeminal ganglion as well as the proximal root, superior and jugular ganglia of cranial nerves VII, IX and X, respectively, are of neural crest origin (Figure 2) (D'amico-Martel and Noden, 1983). This is in contrast to the distal parts of the trigeminal ganglion and the distal geniculate, petrose and nodose ganglia in which the neurons are derived from the trigeminal and epibranchial placodes. The neurons of the vestibulocochlear ganglion of cranial nerve VIII are also of placodal origin (Figure 2) (D'amico-Martel and Noden, 1983).

As migration ceases, neural crest and placodal cells aggregate at the sites of ganglia formation and start to proliferate (Lawson and Biscoe, 1979; D'amico-Martel, 1982). In both sensory cranial and dorsal root ganglia, distal divisions are populated by large cells whereas smaller cells are located in the more proximal divisions. At cranial levels, the large neurons originate in the placodes while in dorsal root ganglia, both large and small cells derive from the neural crest. Generally, the distally located large cells, which display a limited proliferative capacity, initiate and finish cell divisions earlier than the more extensively proliferating population of small proximal cells (Lawson and Biscoe, 1979; D'amico-Martel, 1982; Fontaine-Perus et al., 1985; Frank and Sanes, 1991).

The proliferative phase is followed by differentiation of cells into different subtypes of sensory neurons and to satellite and Schwann cells of the glial lineage. During differentiation, neurons of the sensory ganglia send out central and peripheral axonal projections and gradually acquire a typical pseudo-unipolar morphology (Matsuda and Uehara, 1984). The growing neuronal projections are guided towards their target tissues by complex patterns of environmental cues that either attract or repel the extended growth cones (reviewed in Huber et al., 2003; Masuda and Shiga, 2005). At various time points during differentiation, the sensory neurons become dependent upon target and environmentally derived survival factors. When such factors are absent or at concentrations below certain thresholds, the developing neurons are degraded by

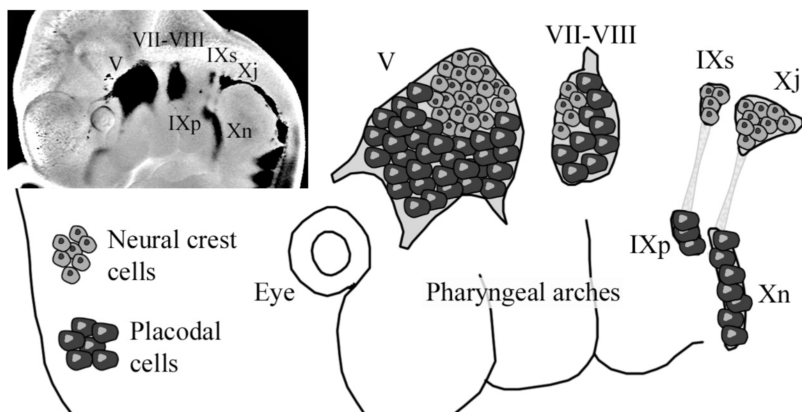

Figure 2 Schematic representation of neural crest and placodal contributions to sensory cranial ganglia. The photograph shows the head region of an X-gal stained E11.5 Foxs1$^{+/\beta\text{-gal}}$ embryo with clearly distinguishable sensory cranial ganglia. The drawing part of the figure represents an enlargement of the region of the eye, pharyngeal arches and cranial ganglia. The relative positions of neural crest and placodal cells within the forming ganglia are indicated. Abbreviations: V, trigeminal ganglion; VII-VIII, combined facial and vestibulocochlear ganglia; IXs, superior ganglion; IXp, petrosal ganglion; Xj, jugular ganglion; Xn, nodose ganglion.

apoptosis (Hamburger et al., 1981; Hamburger and Yip, 1984; Kalcheim and Le Douarin, 1986; Buchman and Davies, 1993; Hory-Lee et al., 1993).

The neurotrophins constitute a group of well-characterized factors that function both as guidance molecules and survival factors for differentiating sensory neurons. The neurotrophins exert their effects by interacting with their respective tyrosine kinase receptors which are expressed in various combinations, depending on sensory subtype and developmental stage, in the plasma membranes of differentiating neurons (reviewed in for example Snider and Silos-Santiago, 1996; Paves and Saarma, 1997; Ernfors, 2001; Fariñas et al., 2002; Kirstein and Fariñas, 2002; Lewin and Barde, 2003). In individual ganglia, competition for neurotrophins and other survival promoting factors can lead to apoptotic degeneration of up to 50% of the neuroblasts produced during proliferation (Hamburger et al., 1981; Le Douarin and Kalcheim, 1999).

Specification of sensory neurons

Sensory neurons in the dorsal root ganglia are born in two distinct waves of neurogenesis (Lawson and Biscoe, 1979; Frank and Sanes, 1991; Ma et al., 1999). The first wave is initiated by expression of neurogenin 2 in a subset of migratory cells that give rise to a distal cell population of generally large neurons in the ventrolateral portion of the dorsal root ganglion (Ma et al., 1999). Upon arrival at the site of ganglion formation, later migrating neural crest cells start to express neurogenin 1, initiating a second wave of neurogenesis which contributes both large and small diameter neurons to the more proximal, dorsomedial portion of the ganglion (Ma et al., 1999; Zirlinger et

al., 2002). In mice, the spatial separation of large and small cells into ventrolateral and dorsomedial divisions, respectively, is not as pronounced as in the chick (Montelius et al., 2007). Expression of high levels of neurogenins largely coincides with the loss of expression of Sox10, a marker of multipotency, and with the initiation of expression of Brn3a, a transcription factor expressed in sensory precursor cells and neurons (Fedtsova and Turner, 1995; Kim et al., 2003; Montelius et al., 2007). The neurogenins are required for neurogenesis to proceed normally and in double knock-outs, no dorsal root ganglia are formed (Ma et al., 1999).

Specification into sensory or autonomic lineages has been suggested to take place before segregation into neuronal or glial lineages and to be mediated by factors produced by the dorsal neural tube (Anderson, 2000; Zirlinger et al., 2002). It appears that Wnt-signaling has the capacity to induce a sensory neuronal fate in neural crest cells (Lee et al., 2004). The Wnt-signaling can, however, be counteracted by BMP which is known to be a potent inducer of neurogenesis and to promote autonomic lineage segregation (Morrison et al., 1999; Kleber et al., 2005). Accordingly, the balance between Wnt and BMP signaling has been suggested to be of key importance in directing the fate of neural crest cells towards either the sensory or autonomic lineage (Kleber et al., 2005). Wnt and BMP signals can also act synergistically to suppress differentiation and thereby preserve multipotency and expression of neural crest stem cell (NCSC) markers (Kleber et al., 2005).

Neuronal subtypes of dorsal root ganglia

Further development of the sensory neurons in the dorsal root ganglia includes acquisition of subtype-specific properties. The neurons of the dorsal root ganglia are quite heterogeneous and can be classified into subtypes based on characteristics such as cell size, axon myelination, conduction velocity, expression of neurotrophin receptors and cell type-specific transcription factors as well as functional properties (Le Douarin and Kalcheim, 1999).

Functionally, sensory neurons can be divided, somewhat roughly, into nociceptive, mechanoreceptive and proprioceptive subtypes. The neuronal expression of the three high-affinity neurotrophin receptors (TrkA, TrkB and TrkC) has been widely used to delineate these functional subtypes. TrkA is expressed by small diameter, late born, unmyelinated or thinly myelinated nociceptive neurons (Mu et al., 1993; Snider and Wright, 1996; Rifkin et al., 2000) that innervate skin and viscera. They respond to noxious stimuli and mediate sensations of pain. TrkC-expressing cells are large, thickly myelinated neurons that display high conduction rates. These neurons convey proprioceptive information from muscle spindles and Golgi tendon organs, which are involved in sensing limb movement and position, and they are thus important for maintenance of body posture (Mu et al., 1993; Bibel and Barde, 2000; Rifkin et al., 2000). The large-to-medium-sized mechanoreceptive neurons convey mechanical sensations, such as touch and vibration, from various dermal receptor organs including

hair follicles and Ruffini, Pacinian and Meissner corpuscles. Neurons of this functional class cannot be as clearly distinguished by any single Trk. Instead these neurons have been found to often express TrkB and/or TrkC (Mu et al., 1993; Snider and Wright, 1996; Marmigere and Ernfors, 2007).

Recently, a family of ion channels that have the potential to more specifically mark functionally different sensory neurons have been identified (Caterina et al., 1997). This is the family of transient receptor potential (Trp) ion channels and its members may be the principal cellular components determining the specific receptive properties of sensory neurons. The Trp superfamily participates in a variety of functions in both excitable and non-excitable cells and has been shown to have critical roles in sensory modalities such as touch, hearing, taste, olfaction, vision, and thermal sensation in species ranging from flies to mice and humans (reviewed in O'Neil and Heller, 2005; Venkatachalam and Montell, 2007; Tsunozaki and Bautista, 2009).

The boundary cap

At trunk levels, neural crest cells of the ventral migratory stream give rise to the autonomic and dorsal root ganglia (Le Douarin and Kalcheim, 1999). In addition, a subpopulation of late emigrating neural crest cells aggregates in distinct clusters at the surface of the spinal cord (Altman and Bayer, 1984; Niederlander and Lumsden, 1996). These cell clusters are known as boundary caps. In mice, the neural crest-derived boundary cap cells arrive at the spinal cord around E10.5. At this time, the cells start to express the zinc-finger transcription factor Krox-20 and monoamine oxidase B (Maob) (Topilko et al., 1994; Vitalis et al., 2003). The boundary caps appear transiently at prospective motor exit points and dorsal root entry zones (Figure 1) and they disappear within the first postnatal week (Altman and Bayer, 1984). Distinct gaps in the basal lamina allow direct contact between boundary cap cells and the neuroepithelium and could serve to facilitate in- and outgrowth of axonal projections (Niederlander and Lumsden, 1996; Vitalis et al., 2003).

Although it has been speculated that boundary caps have important functions in controlling the entry of primary afferents to the spinal cord (Golding and Cohen, 1997), their ablation appears not to disturb axonal outgrowth at the motor exit points (Vermeren et al., 2003). However, genetic as well as surgical ablation of boundary cap cells lead to migration of motor neurons out of the spinal cord. Thus, an important function of the boundary cap cells could be to maintain the boundary between the central nervous system and the periphery, allowing passage of axons while blocking neuronal migration (Vermeren et al., 2003).

Despite continuous proliferation of boundary cap cells, their number in the boundary cap gradually decreases by a mechanism other than apoptosis (Altman and Bayer, 1984; Golding and Cohen, 1997). Instead, at approximately E11, boundary cap cells start to leave the motor exit points and dorsal root entry zones, from where they migrate away along the spinal nerve roots towards the dorsal root ganglia (Maro et al.,

2004). These migrating cells have been shown to contribute to the Schwann cells ensheathing the spinal nerve roots and they also contribute to approximately 5% of the dorsal root ganglia neurons. The boundary cap cells give rise mainly to small and medium-sized neurons characterized by expression of nociceptive-specific markers, although occasional proprioceptive neurons are also formed (Maro et al., 2004). Based on the contribution of boundary cap cells to both neural and glial cell lineages and their continued proliferation in the dorsal root ganglia after cessation of migration, it has been speculated that boundary cap cells could constitute a late-surviving multipotent stem cell population (Maro et al., 2004; Mosher and Morrison, 2004). The stem cell properties of boundary cap cells were investigated in Paper II.

Neural crest stem cells

General properties of stem cells

Stem cells are undifferentiated cells that are characterized by their ability to self-renew and give rise to a diverse range of different cell types. The concept of self-renewal refers to the capacity of stem cells to undergo numerous cycles of mitotic cell division that repeatedly generate at least one daughter cell with properties equivalent to the mother cell (Smith, 2006). Two different types of divisions are thus possible. In symmetric divisions, two identical daughter cells that both display stem cell properties are produced. Asymmetric divisions, on the other hand, give rise to one stem cell and a somewhat more fate-restricted progenitor cell (Morrison et al., 1997). The potency of a stem cell is reflected by the range of cell types it can generate (Smith, 2006). Pluripotent stem cells are often described as capable of giving rise to most or all cell types of the body. Multipotent stem cells are more restricted in the range of cells they can generate but are generally considered to be able to produce several or all of the different cell types of, at least, the tissue in which they reside. Numerous different types of stem cells of varying potencies have been isolated from both embryonic and adult tissues and it has recently been reported that differentiated somatic cells can be reprogrammed into an embryonic-like pluripotent state (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). Such induced pluripotent stem cells may prove to have the potential to alleviate the need for sparsely occurring natural stem cells for use in research and therapy (Kim et al., 2009; Woltjen et al., 2009).

Properties of neural crest stem cells

Delaminating and migrating neural crest cells display a large heterogeneity in their proliferative and developmental potentials, ranging from multipotent stem cells to committed unipotent progenitor cells (Baroffio et al., 1988; Baroffio et al., 1991; Henion and Weston, 1997). During migration and post-migratory stages, neural crest cells gradually become more restricted in their potential to generate different cell types (Henion and Weston, 1997). However, rare multipotent stem cells do persist in

embryonic as well as adult neural crest-derived tissues (Morrison et al., 1999; Sieber-Blum et al., 2004).

NCSCs with a capacity to self-renew were originally isolated from the pre-migratory neural crest population contained in rat neural tube explants (Stemple and Anderson, 1992). These NCSCs were shown to have the capacity to differentiate into peripheral neurons, glial cells and smooth muscle cells (Stemple and Anderson, 1992; Shah et al., 1996). NCSCs with similar properties were later isolated from post-migratory neural crest populations located in the embryonic sciatic nerve and gut (Morrison et al., 1999; Bixby et al., 2002). Although autonomic as well as sparse sensory neurons are formed in cultures of neural tube explants, NCSCs isolated from this tissue or the sciatic nerve produce only autonomic neurons and have never been observed to give rise to sensory neurons, either in vitro or in vivo (Greenwood et al., 1999; Morrison et al., 1999; White et al., 2001). Even under forced overexpression of neurogenins, cultured sciatic nerve NCSCs (sNCSCs) fail to give rise to sensory neurons (Lo et al., 2002). In Paper II, we investigated whether NCSCs isolated from the boundary cap, in contrast to sNCSCs and neural tube-derived NCSCs, were capable of giving rise to sensory neurons.

NCSCs with varying potencies have also been isolated from other tissues such as hair follicles of adult mouse skin and cardiac neural crest derivatives of adult mice (Delfino-Machín et al., 2007). As yet, no marker common to all NCSCs is known. However, p75, nestin and Sox10 have been found in several types of NCSCs and may therefore be involved in some kind of general mechanism of importance to these cells (Delfino-Machín et al., 2007).

V-ATPases in the kidney, epididymis and inner ear

Structure, function and cellular localization of V-ATPases

Vacuolar type H^+-ATPases (V-ATPases) are ubiquitous multisubunit ATP-driven proton pumps that couple the energy released upon hydrolysis of ATP to active transport of protons across cellular membranes (Wagner et al., 2004). V-ATPases were originally identified in intracellular chromaffin granules (Bashford et al., 1975) but were later found to be also present in the plasma membranes of certain cell types specialized in acid-base regulation (Brown et al., 1988; Brown et al., 1992; Stankovic et al., 1997). These cells, sometimes referred to as mitochondria-rich cells, secrete protons or bicarbonate to the extracellular environment and regulate the pH in tissues such as the kidney, epididymis and inner ear (Brown et al., 1992; Brown and Breton, 1996; Stankovic et al., 1997; Dou et al., 2004; Pastor-Soler et al., 2005). V-ATPases also reside in the membranes of organelles such as lysosomes, secretory vesicles and early endosomes, where they regulate the intraluminal acidification required for proper function (Nelson and Harvey, 1999; Futai et al., 2000; Nishi and Forgac, 2002).

The V-ATPases are composed of two distinct functional domains, the cytosolic V1 domain and the membrane-bound V0 domain (Figure 3). The V1 domain consists of eight subunits denoted A-H. Alternating A and B subunits form the hexagonal head piece that is responsible for ATP hydrolysis. The V0 domain is composed of six subunits denoted a, c, c' and c", d and e, and it is responsible for the translocation of protons across the membrane. The two domains are connected to each other via a stalk-like structure composed of the subunits C-H of the V1 domain (reviewed in Forgac, 1999; Nishi and Forgac, 2002; Wagner et al., 2004; Breton and Brown, 2007; Jefferies et al., 2008).

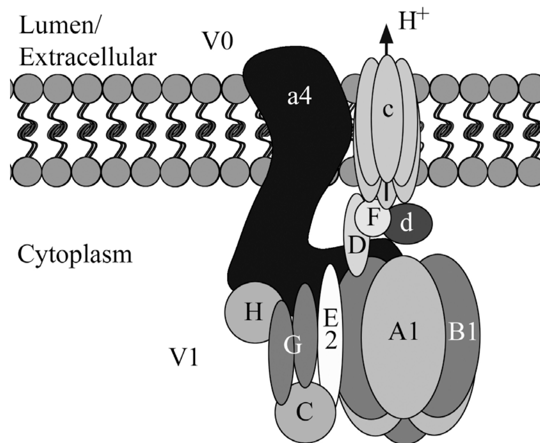

Figure 3 Schematic representation of the V-ATPase complex. Note that the a and B subunits are represented by the specifically expressed a4 and B1 isoforms while the E subunit is represented by the ubiquitously expressed E2 isoform. This reflects the subunit composition of V-ATPases located in the plasma membranes of kidney intercalated cells, inner ear FORE cells and epididymal narrow and clear cells. (Figure redrawn from Fig. 9 in Paper III).

The kidney

Maintenance of volume and composition of the body fluids is essential for homeostasis, and every cell in the body is critically dependent on systemic pH for its proper function. In the short term, acid-base balance is controlled mainly by changes in the rate of respiration. The kidney displays a somewhat slower response to changes in systemic pH and controls acid-base balance by excreting either acidic or basic urine, as regulated mainly by the balance between reabsorption of HCO_3^- and secretion of H^+ (Guyton and Hall, 1996). An average human produces approximately 70 millimoles of non-volatile acids per day that have to be secreted via the kidneys (Gluck et al., 1996). Hydrogen ion secretion coupled to bicarbonate reabsorption takes place in most parts of the renal tubules but primary active secretion, resulting in net excretion of protons, occurs only in late distal tubules and collecting ducts (Gluck et al., 1996). The cell type responsible for primary active secretion, referred to as the intercalated cell, achieves this by means of

the V-ATPase (Gluck et al., 1996; Wagner et al., 2004). Although the secretion of hydrogen ions in the distal tubules and collecting ducts accounts for only a small portion of the total amount of hydrogen ions secreted, this process is important in forming maximally acidic urine (Finberg et al., 2005). Failure of this mechanism to secrete sufficient amounts of protons results in metabolic acidosis (Karet et al., 1999; Smith et al., 2000).

The epididymis

The epididymis is a single highly convoluted tubule that receives spermatozoa from the testis (Cosentino and Cockett, 1986; Robaire and Viger, 1995). Based on morphological features and patterns of gene expression, the epididymis can be divided into four regions, the initial segment, caput, corpus and cauda (Stoffel and Friess, 1994; Jervis and Robaire, 2001). When the spermatozoa reach the epididymis, they are immature and incapable of progressive movement and of fertilizing an egg (Lacham and Trounson, 1991; Yeung et al., 1993). During their passage through the epididymal lumen, they face an environment optimally suited to their maturation and subsequent storage in the epididymal cauda. The luminal fluid provides a complex microenvironment rich in secreted factors and, in going from proximal to distal regions, a progressively more acidic pH (Levine and Marsh, 1971; Levine and Kelly, 1978; Turner, 1991). The acidification is of importance for maintaining the sperm in a quiescent state during maturation and storage, allowing full functionality to be reached only after ejaculation, upon mixing with the alkaline prostatic fluids (Acott and Carr, 1984; Carr et al., 1985). The V-ATPase, located in the apical membranes of narrow and clear cells scattered along the length of the epididymal epithelium, is an important factor for the secretion of protons into the lumen and thus for acidifying its contents (Brown et al., 1992; Herak-Kramberger et al., 2001; Pietrement et al., 2006). Failure to achieve a proper intraluminal acidification of the epididymal duct has been shown to cause male infertility in mice (Blomqvist et al., 2006).

The endolymphatic sac

The inner ear develops from the otic placode which, in the mouse, forms at around E8.5. It consists of the cochlea, the vestibular system and the endolymphatic sac. The cochlea provides the basis for hearing as it converts the mechanical motions of the auditory ossicles into action potentials that mediate auditory information to the brain. The ampullae of the semicircular canals, the saccule and the utricle constitute the vestibular system of the inner ear. This system is responsible for transduction of mechanical stimuli associated with head position and head motion (Lang et al., 2007). The cochlea, vestibular system and endolymphatic sac are fluid-filled compartments that are interconnected via a series of ducts. Referred to as endolymph, this fluid surrounds the sensory hair cells of these organs and its composition and homeostasis is critical for hair cell function (Juhn et al., 1991; Karet et al., 1999; Hulander et al., 2003). The pH of the

endolymph is lower in the endolymphatic sac than in the cochlea and vestibular ducts, although the functional basis for this remains unclear (Tsujikawa et al., 1992; Couloigner et al., 1999). It has been suggested that the main function of the endolymphatic sac is regulation of endolymph volume (Rask-Andersen et al., 1987; Rask-Andersen et al., 1999; Salt and DeMott, 2000; Salt, 2001). V-ATPase proton pumps have been detected in the apical membranes of mitochondria-rich forkhead-related (FORE) cells in the endolymphatic epithelium. Here the V-ATPase has been implicated in luminal acidification (Stankovic et al., 1997; Dou et al., 2003; Dou et al., 2004). Mutations in V-ATPase subunits have been shown to result in progressive hearing loss in combination with distal renal tubular acidosis (Karet et al., 1999; Smith et al., 2000).

Ubiquitous and tissue-specific subunits of the V-ATPase

The various subunits of the V-ATPase are generally classified as being either ubiquitously or specifically expressed. While there is a generic structure of the V-ATPase complex based on the assemblage of ubiquitous subunits, some specialized cell types, including renal intercalated cells, present a different holoenzyme composition based partially on specifically expressed subunits (Smith et al., 2002; Breton and Brown, 2007). In the kidney, the ubiquitously expressed subunits are generally found in the membranes of intracellular organelles where they are involved in intraluminal acidification. Specifically expressed subunits, on the other hand, are to a larger extent distributed to V-ATPases in the plasma membrane where they are important for acidification of the extracellular environment (Sun-Wada et al., 2005). Some of the subunits have several isoforms encoded by different genes. Included in this group are the B and E subunits of the V1 domain and the a subunit of the V0 domain. The subunits discussed in this thesis are the B1, a4 and E2 subunits and also the A subunit, represented by the single A1 isoform (van Hille et al., 1993; van Hille et al., 1995). B1 and a4 are specific subunits and both of them are expressed in intercalated cells of the kidney (Nelson et al., 1992; Smith et al., 2000; Finberg et al., 2003; Stehberger et al., 2003), narrow and clear cells of the epididymis (Brown et al., 1992; Breton et al., 1996; Smith et al., 2001) and FORE cells of the endolymphatic duct and sac (Karet et al., 1999; Dou et al., 2004). In contrast, the A1 and E2 subunits are considered to be ubiquitously expressed (van Hille et al., 1993; van Hille et al., 1995; Imai-Senga et al., 2002; Sun-Wada et al., 2002). In the epididymis, the E2 subunit has been detected in the apical membrane of narrow and clear cells where it co-localizes with the B1 and A1 subunits (Breton et al., 1996; Herak-Kramberger et al., 2001; Paunescu et al., 2004; Pietrement et al., 2006). In the kidney, E2 has been found in intercalated cells of the connecting segment and collecting ducts (Paunescu et al., 2004).

ATP6V1B1 and *ATP6V0A4* in distal renal tubular acidosis

Distal renal tubular acidosis represents a failure of α-intercalated cells of the renal collecting ducts to acidify the urine. The condition, in its inherited form, has three variants, autosomal dominant and autosomal recessive with or without deafness (Fry and Karet, 2007). Two genome-wide linkage analyses identified mutations in *ATP6V1B1* and *ATP6V0A4* as being responsible for several cases of recessive distal renal tubular acidosis. While the mutations in *ATP6V1B1* in most cases were accompanied by early onset hearing loss, mutations in *ATP6V0A4* were initially claimed to only rarely result in deafness (Karet et al., 1999; Smith et al., 2000). In subsequent studies, additional disease-causing mutations were identified and a higher frequency of hearing loss was also detected for *ATP6V0A4*, although generally with a later onset (Stover et al., 2002; Vargas-Poussou et al., 2006). After identification of the disease-causing genes, their expression in renal intercalated cells and proton-secreting cells of the inner ear could be confirmed (Karet et al., 1999; Smith et al., 2000; Smith et al., 2001; Dou et al., 2003; Dou et al., 2004).

Atp6v1b1 null mutant mice, in contrast to affected humans, do not display hearing loss and their inner ears appear normal on histological examination (Dou et al., 2003). They do, however, display a slight urinary acidification defect. Upon acid challenge, the *Atp6v1b1* null mice were found to produce a more alkaline urine and they had a more severe acidosis than controls, demonstrating that the B1 subunit in mice is required for production of maximally acidic urine (Finberg et al., 2005). In the absence of B1, a compensatory increase of the ubiquitous B2 subunit was detected in renal tubular cells. Under these conditions, B2 was found to be located in the apical membrane indicating that it might be involved in a mechanism partially compensating for the loss of B1. Such a mechanism could to some degree explain the milder phenotype in mice compared with humans (Finberg et al., 2005).

Foxi1 in acid-base homeostasis

Foxi1 expression pattern

The forkhead transcription factor FOXI1 (previously referred to as HFH-3, Fkh10 and freac6) was initially identified as a forkhead gene with a kidney-specific expression (Clevidence et al., 1993; Pierrou et al., 1994). After isolation of the full-length cDNA, FOXI1 was shown be to be a potent transcriptional activator composed of 351 amino acids and its expression was more specifically localized to the distal convoluted tubules of the embryonic (E16.5) and adult mouse kidney (Overdier et al., 1997). At an earlier embryonic stage (E9.5-E10.5), Foxi1 is expressed exclusively in the area of the otic vesicle (Hulander et al., 1998; Ohyama and Groves, 2004). From this time point until E16.5, expression becomes gradually restricted to the endolymphatic duct/sac of the inner ear. Studies performed in Foxi1$^{-/-}$ mice indicated that the cells of the inner ear that expressed Foxi1 were the mitochondria-rich FORE cells of the endolymphatic duct and

sac epithelia (Hulander et al., 2003). In the kidney, *Foxi1* expression is initiated at E15.5 in scattered cells of the medullary collecting ducts and in the newborn, expression is found in a subset of cells within distinct tubules of both cortex and medulla. Co-distribution with *Atp6v1b1* identified these cells as intercalated cells (Jouret et al., 2005), in which Foxi1 expression persists into adulthood (Blomqvist et al., 2004). *Foxi1* expression in the epididymis is initiated at postnatal day 5 (P5) and is localized to epithelial cells of the initial segment, caput, corpus and cauda. Here, like in the kidney, Foxi1 expression co-localized with Atp6v1b1, which identified these cells as narrow and clear cells of the epididymis (Blomqvist et al., 2006).

The phenotype of Foxi1 null mice

By approximately three weeks of age, Foxi1$^{-/-}$ mice display an abnormal circling behavior, a characteristic sign of vestibular dysfunction, and they are also deaf (Hulander et al., 1998). In these mice most of the inner ear compartments, especially the endolymphatic duct and sac, are severely expanded and the otoconia of the utricle and saccule are deformed or absent (Hulander et al., 1998; Hulander et al., 2003). Both these findings have been suggested to be compatible with an altered endolymph composition (Hulander et al., 2003). Electron microscopy analysis of inner ears from Foxi1$^{-/-}$ mice demonstrated a complete absence of the mitochondria-rich FORE-cells in the endolymphatic sac epithelium (Hulander et al., 2003).

An altered setup of cell types has also been demonstrated in the renal collecting ducts (Blomqvist et al., 2004). Normally, the collecting duct epithelium is composed of at least three cell types, the principal cells and the α- and β-intercalated cells. In the collecting ducts of Foxi1$^{-/-}$ mice, only a single cell type, expressing markers of both intercalated and principal cells, can be distinguished (Blomqvist et al., 2004). This malformed epithelium has a decreased capacity for proton secretion and, consequently, Foxi1$^{-/-}$ mice suffer from distal renal tubular acidosis (Blomqvist et al., 2004). Furthermore, Foxi1$^{-/-}$ male mice are infertile and fail to impregnate females. Analysis of sperm morphology revealed an increased frequency of tail angulation, a sign of disturbed sperm maturation caused by insufficient epididymal acidification (Yeung et al., 2002; Blomqvist et al., 2006).

The epithelia of the kidney collecting ducts, the epididymis and the endolymphatic sac are similar in that they all contain Foxi1-expressing mitochondria-rich cells specialized in proton secretion and acidification of the compartments that they surround. These cells use a similar setup of carbonic anhydrases, anion exchangers and V-ATPase proton pumps, and lack of Foxi1 results in the selective loss of one or more of these factors, in combinations that depend on the specific tissue and cell type (Hulander et al., 2003; Blomqvist et al., 2004; Blomqvist et al., 2006). Whether the loss of expression of certain genes in Foxi1$^{-/-}$ animals reflects a failure of direct transcriptional activation by Foxi1, or rather is a result of disturbed differentiation, as suggested by the apparent loss of specific cell types in the kidney and inner ear, or a combination of both, is unclear

(Al-Awqati and Schwartz, 2004). In Paper III, we explore the importance of Foxi1 for expression of subunits of the V-ATPase complex in the kidney, epididymis and inner ear.

Results and discussion

Paper I – Characterization of the Foxs1 mouse model

Genes of the forkhead family of transcription factors play important roles in transcriptional regulation in a variety of species, including drosophila, mouse and human. The approximately 110-amino acid DNA-binding domain is highly conserved and its sequence constitutes the basis for their classification into different groups denoted by the letters A-S (Kaestner et al., 2000). Several mouse models carrying null mutations in forkhead genes have been produced. A large number of these display severe phenotypes that in many cases result in embryonic or perinatal lethality (reviewed in Carlsson and Mahlapuu, 2002). Mutations in forkhead genes have also been shown to cause human disease (Mears et al., 1998; Nishimura et al., 1998; Chatila et al., 2000; Fang et al., 2000; Finegold et al., 2001; Lai et al., 2001; Stankiewicz et al., 2009). The mouse gene *Foxs1* (previously *Fkh3*, *Fkhl18* and *FREAC10*) and the human *FOXS1* (previously *FKHL18* and *FREAC10*) were originally identified in low-stringency screens of lambda phage libraries (Kaestner et al., 1993; Cederberg et al., 1997). Expression pattern analyses of the mouse and human genes indicated differences in tissue localization between the species (Kaestner et al., 1993; Cederberg et al., 1997). To characterize the function and expression pattern of the mouse *Foxs1* gene, we created a β-galactosidase knock-in mouse model targeting the *Foxs1* locus.

The Foxs1 mouse model

Foxs1 is a single exon gene that encodes a protein of 329 amino acids. For construction of the targeting vector, an E. coli β-galactosidase marker gene fitted with a nuclear localization signal was fused in frame with the 16 most N-terminal amino acids of the *Foxs1* coding sequence (Fig. 1 in Paper I). Homologous recombination resulted in an allele in which most of the *Foxs1* coding sequence, including the DNA-binding domain, had been replaced by β-galactosidase while upstream and downstream sequences remained intact. With this type of strategy, the expression of an inserted marker gene can be regulated by the same factors that normally regulate the expression of the replaced gene, and thus reflect its in vivo expression pattern. Breeding of the mouse model resulted in normal-sized litters with the expected Mendelian numbers of wild-type, Foxs1$^{+/\beta\text{-gal}}$ and Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice. Mutant mice, both homozygotes and heterozygotes, appeared fully normal in terms of fertility, life span and general behavior.

Embryonic expression pattern of *Foxs1*

As a first step, we set out to explore the embryonic expression pattern of the inserted marker gene using X-gal staining and immunostaining with β-galactosidase-specific antibodies. By comparing X-gal stained Foxs1$^{+/\beta\text{-gal}}$ E11.5 embryos with wild-type embryos that had been subjected to in situ hybridization with a *Foxs1*-specific probe, we could confirm the fidelity of β-galactosidase as a marker of *Foxs1* expression in vivo (Fig. 2 in Paper I). In Foxs1$^{+/\beta\text{-gal}}$ embryos, marker gene expression was first detected at E9.5 in a subset of the forming dorsal root ganglia and in the trigeminal and facio-acoustic ganglia of cranial nerves V and VII-VIII, respectively. By E11.5, marker gene expression could be detected in all peripheral sensory ganglia along the entire anteroposterior axis, thus including dorsal root ganglia and ganglia of cranial nerves V, VII, VIII, IX and X. Blood vessels of the head stained positive for β-galactosidase from approximately E12.5 (Fig. 2 in Paper I) and by E15.5 *Foxs1* expression had been initiated in the fastigial nucleus and the external granule layer of the forming cerebellum (Fig. 3 in Paper I). Immunostaining of sections of Foxs1$^{+/\beta\text{-gal}}$ E11.5 embryos demonstrated that, at this developmental stage, most or all peripheral sensory neurons, irrespective of their origin in the neural crest or the epibranchial placodes, were positive for the marker gene (Fig. 5 in Paper I) (D'amico-Martel and Noden, 1983; Noden, 1993). The results showed that until at least E11.5, Foxs1 is a rather specific marker of peripheral sensory neurons, a property that was used in Paper II for identification of NCSC clones belonging to a sensory lineage. To assess a potential loss of neurons in sensory ganglia due to a lack of *Foxs1* expression, serial sections of dorsal root ganglia from wild-type and Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ P7 mice were stained for markers of neuronal subtypes together with a general neuronal marker. Cell counts did not reveal any differences.

Expression of *Foxs1* in the postnatal brain

To explore the postnatal expression of *Foxs1*, brains from Foxs1$^{+/\beta\text{-gal}}$ P14 animals were serial sectioned and analyzed for X-gal-positive cells. Positive cells were detected in granule cells of the internal granule layer of the cerebellum and in a small subset of purkinje cells. Staining was also seen in the deep cerebellar fastigial nucleus, the precerebellar external cuneate and lateral reticular nuclei as well as in the cochlear and vestibular nuclei (Fig. 4 and 5 in Paper I). All these structures are formed by streams of migrating, Math1-dependent cells, originating at different anteroposterior levels of the rhombic lip, which constitutes the dorsal-most portion of the hindbrain neuroepithelium (Wingate, 2001; Wang et al., 2005). Functionally, they can be considered as being parts of a sensory network that coordinates proprioceptive, vestibular and auditory input from primary sensory organs (Wang et al., 2005; Wingate, 2005). The input thus handled by this network carries information regarding body position in space.

Other sites of Foxs1 expression in the P14 brain included the entorhinal cortex and nuclei of the anterior thalamus (Fig. 4 in Paper I). These structures are interconnected

via reciprocal projections (Shibata, 1993, 1996), and lesions in either of them have been shown to cause deficits in acquisition of spatial memory (Warburton and Aggleton, 1998; Eijkenboom et al., 2000; Davis et al., 2001; van Groen et al., 2002). In addition, both reduced and elevated numbers of neurons in the anterior thalamus have been demonstrated in psychiatric disorders in humans (Young et al., 2000; Young et al., 2004). Marker gene expression was also detected in the dorsomedial hypothalamic nucleus which is involved in regulation of bodyweight and feeding behavior (reviewed in Bellinger and Bernardis, 2002) and thermoregulation (reviewed in DiMicco and Zaretsky, 2007).

The origin of brain pericytes and vascular smooth muscle cells has not yet been fully established (Majesky, 2007). A recent report claimed a mixed mesodermal and neural crest contribution to these cell populations in the chick (Etchevers et al., 2001). Another study reported that neuroectoderm was capable of providing all perivascular cells of the brain (Korn et al., 2002). Expression of the marker gene in pericytes and vascular smooth muscle cells throughout the brain may, based on the expression of *Foxs1* in neural crest derivatives, indicate a common, possibly neuroectodermal origin of these cells (Fig. 6 in Paper I). The *Foxs1* expression pattern in the P14 brain, with the addition of a later initiated *Foxs1* expression in the ventral hippocampus, persists into adulthood (unpublished data).

Adult expression of *Foxs1*

The expression of Foxs1 was also investigated in non-neuronal tissues of adult mice. Here, Foxs1-positive cells were found in the epithelium of lachrymal glands (Fig. 7 in Paper I) and exocrine glands of the esophagus, ventricle and duodenum. Lymphoid tissue of the large intestine displayed numerous Foxs1-positive lymphocytes. In the spleen, *Foxs1* was expressed in cells lining the sinuses of the marginal zone. Occasional positive cells were also found in renal corpuscles and ducts of the outer medulla. Several epithelia of male reproductive organs, including proximal and distal epididymis, the prostate gland and the spermatic duct and also perivascular cells of the testis stained positive for Foxs1 (unpublished data). Loss of *Foxs1* expression has recently been reported to result in increased apoptosis of periendothelial cells in the embryonic testis and this, in turn, causes structural defects of the underlying endothelium (Sato et al., 2008).

Functional tests of the Foxs1 mouse model

In search of a possible phenotype, the performance of Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice compared with wild-types was evaluated in a series of functional tests that were considered relevant with regard to the *Foxs1* expression pattern. To examine potential memory deficits and hypo/hyperactivity caused by loss of Foxs1 in anterior thalamic nuclei and the entorhinal cortex, mice were tested in a water maze task and in the open field. Furthermore, the strong embryonic expression of Foxs1 in peripheral sensory ganglia

led us to perform tests aimed at detecting a possible sensory phenotype. Mechanosensitivity was evaluated using von Frey filaments while the sensitivity to nociceptive stimuli was tested in a tail-flick assay and by capsaicin injections. However, none of the above tests revealed any significant differences between wild-type and Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice.

Since the β-galactosidase marker was detected in several areas of the brain thought to be involved in the processing of information about body position, we went on to check for differences in coordination and motor function by comparing the performance of wild-type and mutant mice on the rotarod. The rotarod is widely used for assessing sensorimotor coordination and the test is sensitive to damage in the basal ganglia and cerebellum and to drugs that affect motor function (Crawley, 1999; Rustay et al., 2003; Sausbier et al., 2004). Surprisingly, Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice performed significantly better than wild-types on the rotarod (Fig. 8 in Paper I). Although the mechanism behind this is unclear, the loss of Foxs1 in several brain areas claimed to be of importance for coordination of proprioceptive and vestibular information may, in some way, alter the function of this system rendering the mice more capable. There are other reports of mutant mice outperforming wild-types on the rotarod (Rubinstein et al., 1997; Gerlai et al., 2000).

Based on the expression of *Foxs1* in the dorsomedial hypothalamic nucleus, we measured the weight gain of wild-type and Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice on a high-fat diet. Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ male mice gained significantly less weight compared with wild-types while no differences were detected in female mice (Fig. 8 in Paper I). Chemical disinhibition of neurons in the dorsomedial hypothalamic nucleus has been shown to increase sympathetic activation of brown adipose tissue, as reflected by its increased temperature and an increased level of expired CO_2, both of which indicate an elevated metabolic activity (Cao et al., 2004). To investigate a possible activation of brown adipose tissue, we measured mRNA levels of *Ucp1* in brown adipose tissue from wild-type and Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice. A small but significant increase of *Ucp1* expression was detected in male mice while, again, there was no difference in females. Based on these findings, we speculated that loss of Foxs1 in neurons of the dorsomedial hypothalamic nucleus could produce an effect mimicking a chemical disinhibition of these neurons, thereby increasing sympathetic activation of brown adipose tissue. A resulting increase in *Ucp1* expression levels and metabolic activity would then potentially explain the lower weight gain in male Foxs1$^{\beta\text{-gal}/\beta\text{-gal}}$ mice on a high-fat diet.

Paper II – Neural crest stem cells of the boundary cap

Just before the arrival of axonal processes of developing peripheral neurons, a late migrating population of neural crest cells forms clusters at presumptive motor exit points and dorsal root entry zones at the surface of the spinal cord (Altman and Bayer, 1984; Niederlander and Lumsden, 1996; Golding and Cohen, 1997). These clusters,

called boundary caps, provide an interphase between the central and peripheral nervous systems and their cells participate in the processes of sensory axon entry and motor axon exit by allowing passage of axonal processes while blocking cellular migration (Niederlander and Lumsden, 1996; Golding and Cohen, 1997; Vermeren et al., 2003). Despite their continuous proliferation, boundary cap cells appear only transiently as they disappear within the first postnatal week by a mechanism other than apoptosis (Altman and Bayer, 1984; Golding and Cohen, 1997). Instead, by approximately E11, the boundary cap cells start to migrate away from the spinal cord along the ventral and dorsal roots and into the dorsal root ganglia (Maro et al., 2004). Here, the capacity of boundary cap cells to contribute both sensory neurons and glial cells suggested that they could be multipotent stem cells (Maro et al., 2004; Mosher and Morrison, 2004). Neural crest stem cells isolated from the neural tube, sciatic nerve and the enteric nervous system had previously been shown to be able to self renew and to be multipotent, giving rise to neurons, glia and smooth muscle-like cells (Morrison et al., 1999; Bixby et al., 2002). However, when differentiated, these cells generated only autonomic neurons and were never, even under forced overexpression of neurogenins, observed to spontaneously give rise to sensory neurons, either in vitro or in vivo (Anderson, 2000; White et al., 2001; Lo et al., 2002). In Paper II, the stem cell properties of boundary cap cells, and specifically, their capacity to differentiate into sensory neurons were investigated.

Clone-forming capacity of dorsal root ganglia

Dissection and dissociation of mouse E11.5 dorsal root ganglia followed by culturing of the dissociated cells resulted, after a few days, in formation of cell clusters that after further growth were transferred to separate wells as individual clones. These could be propagated for several months. Immunostaining showed that the clones expressed p75 and nestin, two markers previously used for identification of NCSCs (Stemple and Anderson, 1992). Reverse transcription PCR demonstrated expression of neurogenin 1, neurogenin 2 and Brn3a, which suggested that the cells were of a sensory lineage. In addition, expression of Krox20 indicated that the clone-forming cells originated in the boundary cap. The overall expression pattern of the cells was similar to that of whole dorsal root ganglia (Fig. 1 in Paper II).

Stem cell properties of boundary cap cells

The cultured cells expressed Krox20 and Maob, both of which had been reported to be specific markers of boundary cap cells (Vitalis et al., 2003; Maro et al., 2004). The location of boundary cap cells in vivo, as defined by these markers, was studied in sections of E10.75 and E11.75 embryos. Overlapping expression was seen in the region of the boundary cap at E10.75. Expression in this region remained in E11.75 embryos but, in addition, cells positive for Maob appeared to be migrating along the dorsal roots, towards and into the dorsal root ganglia (Fig. 2 in Paper II). The lack of migrating

Krox20-positive cells indicated that the boundary cap cells downregulate the expression of this gene as they initiate migration. The migratory route followed by the Maob-positive cells was similar to what had been reported for boundary cap cells (Maro et al., 2004).

The boundary cap origin of the clone-forming cells was confirmed in two separate experiments. The first used the fact that an enrichment step in the culturing protocol resulted in an increased proportion of Maob-positive cells. This increase was shown not to be due to proliferation or induction of the marker. Thus, the Maob-positive boundary cap cells survived and were enriched under the culture conditions that were used (Fig. 2 in Paper II). In the second experiment, dorsal root ganglia were divided into central and proximal parts that were cultured separately. The proximal part, which included the dorsal-most region of the ganglia as well as the nerve root and the boundary cap, generated approximately tenfold more stem cell clones compared with the central part (Fig. 2 in Paper II). These results showed that the clone-forming cells were those derived from the boundary cap.

Defining features of stem cells include a capacity of self-renewal and generation of multiple cell types of the tissues in which they reside (Morrison et al., 1997). The potential of boundary cap cells to give rise to different cell types was investigated in clonal experiments. Single clones were transferred to separate wells and allowed to differentiate. Immunostaining using antibodies against βIII-tubulin, glial fibrillary acidic protein and smooth muscle actin identified clones containing neurons, glial cells and smooth muscle-like cells. In most cases, individual clones generated all three cell types. The capacity of self-renewal was studied by testing the potential of boundary cap cells to give rise to both neurons and glial cells after repeated subclonings. All of the tested clones gave rise to both cell types. Thus, the neural crest cells located in the boundary cap were found to be multipotent stem cells.

Sensory competence of boundary cap stem cells

After 5 days of differentiation, boundary cap neural crest stem cells (bNCSCs) displayed a morphology similar to that of cultured dorsal root ganglion neurons of E11.5 embryos. The capacity of bNCSCs to generate peripheral neurons in these cultures was confirmed by the presence of cells staining positive for both peripherin, a marker of differentiated sensory and autonomic neurons, and βIII-tubulin (Fig. 3 in Paper II). To determine if these neurons were of the sensory lineage, we used the Foxs1 lacZ mouse model in which the early expression of the β-galactosidase marker gene is largely restricted to cells of the sensory lineage (Fig. 4 in Paper II and Fig. 2 in Paper I). bNCSC clones were established from Foxs1$^{+/\beta\text{-gal}}$ E11.5 embryos and upon differentiation almost 70% of the clones expressed the marker gene, which confirmed that bNCSCs gave rise to sensory neurons (Fig. 3 in Paper II). None of the clones expressed autonomic markers.

To investigate if the ability to generate sensory neurons was an intrinsic property of bNCSCs, their competence was compared with that of sNCSCs. The two types of NCSCs were differentiated in the absence and presence of BMP2. BMP2 had previously been shown to be a potent inducer of neurogenesis and autonomic fate in sNCSCs (Morrison et al., 1999). BMP2 stimulated neurogenesis in both bNCSCs and sNCSCs but failed to induce expression of autonomic markers. Staining for Foxs1 expression showed that almost 70% of the differentiated bNCSCs were of the sensory lineage while no sNCSCs expressed the marker gene (Fig. 5 in Paper II). These results suggested that the culture conditions used in this study were not permissive for differentiation of autonomic neurons. They also showed that bNCSC and sNCSCs have different intrinsic properties and that, since sNCSCs did not give rise to sensory neurons, the sensory fate of bNCSCs was not induced by the culture conditions. Rather, in contrast to sNCSCs, the bNCSCs are specified to differentiate into the sensory lineage.

Sensory neuronal subtypes formed by boundary cap stem cells

We used calcium imaging to investigate if the sensory neurons produced by differentiation of bNCSCs were functional and if they were of different sensory subtypes. This method measures the transient changes in neuronal intracellular Ca^{2+} that are produced in response to specific stimuli. It can be used to determine to what type of stimuli individual cells respond and thus to which subtypes they belong. Differentiated bNCSCs were exposed to a battery of different stimuli including capsaicin, an agonist of Trpv1 channels on nociceptive neurons (Caterina et al., 2000). Hypo-osmotic challenge causes swelling of cells which in turn activates stretch receptors present on different types of mechanoreceptive neurons (Viana et al., 2001). Two other members of the Trp-family are thought to be involved in mediating responses to cold. Trpm8 is activated by temperature changes and by the cooling agent menthol while Trpa1 is triggered by cold stimuli (McKemy et al., 2002; Peier et al., 2002; Story et al., 2003). The calcium imaging experiments identified several different cell types that each responded to one or more of the various stimuli applied, demonstrating that bNCSCs give rise to functional sensory neurons of different subclasses (Fig. 6 in Paper II). Using the same strategy, we found that most of the bNCSC clones had the capacity to produce neurons of two or more functional subclasses, suggesting that the bNCSCs had not yet undergone restriction towards specific sensory subtypes (Fig. 7 in Paper II).

Paper III – Foxi1 regulation of V-ATPase subunits

Active proton secretion, and thereby acidification of luminal contents, is an important function of the epithelia of the kidney collecting ducts, the endolymphatic sac of the inner ear and the epididymis. The capacity of these tissues to produce adequate luminal acidification is necessary for maintenance of systemic acid-base homeostasis, endolymph homeostasis and proper post-testicular sperm maturation, respectively. Each

of these epithelia contains a subset of mitochondria-rich cells that abundantly express V-ATPase in their apical plasma membranes, various anion exchangers in their basolateral membranes and cytoplasmic carbonic anhydrase, all typical features of cells specialized in proton secretion (Brown and Breton, 1996; Wagner et al., 2004). Known as intercalated cells, FORE cells and narrow and clear cells, these cell types have all been shown to express Foxi1 and the B1 (Brown et al., 1992; Nelson et al., 1992; Karet et al., 1999) and a4 subunits of the V-ATPase (Smith et al., 2000; Smith et al., 2001; Dou et al., 2004). Mice lacking Foxi1 develop distal renal tubular acidosis (Blomqvist et al., 2004), early onset deafness (Hulander et al., 1998; Hulander et al., 2003) as well as male infertility (Blomqvist et al., 2006), demonstrating the importance of Foxi1 expression for the normal function of these tissues. Lack of Foxi1 expression results in loss of the B1 subunit in mouse kidney and epididymis. In addition, Foxi1 is capable of direct activation of the *Atp6v1b1* promoter as shown in promoter reporter experiments (Blomqvist et al., 2006). Although much is known about the structure, function and cellular localization of the various V-ATPase subunits, the upstream regulators of subunit gene expression are little understood. The aim of Paper III was to further explore the role of Foxi1 as a regulator of V-ATPase subunits.

Foxi1 regulation of *Atp6v1b1*

Foxi1 and *Atp6v1b1* are both expressed in FORE cells of the endolymphatic sac. Deficient expression of Foxi1 in mice as well as mutations in the human *ATP6V1B1* result in sensorineural hearing loss (Karet et al., 1999; Hulander et al., 2003). Because the expression of *Atp6v1b1* in renal intercalated and epididymal narrow and clear cells is dependent on the expression of Foxi1 (Blomqvist et al., 2004; Blomqvist et al., 2006), it thus seemed likely that a similar relationship between *Foxi1* and *Atp6v1b1* would also be present in the inner ear. To explore this possibility, inner ear tissue sections from wild-type and Foxi1$^{-/-}$ E16.5 embryos were immunostained with antibodies against Foxi1 and the B1 subunit (Fig. 1 in Paper III). As expected, Foxi1 and the B1 subunit co-localized to FORE cells in wild-type sections while in Foxi1$^{-/-}$ sections, none of the proteins could be detected. The result shows that in all tissues with a reported expression of both *Foxi1* and *Atp6v1b1*, expression of the latter is dependent on the presence of Foxi1.

Foxi1 regulation of *Atp6v0a4*

Distal renal tubular acidosis with sensorineural hearing loss in humans can also be caused by mutations in *ATP6V0A4*, the gene encoding the a4 subunit of the V-ATPase (Smith et al., 2000; Stover et al., 2002; Vargas-Poussou et al., 2006). Like B1 and Foxi1, the a4 subunit is expressed in renal intercalated cells (Smith et al., 2000; Oka et al., 2001), epididymal narrow and clear cells (Smith et al., 2001; Pietrement et al., 2006) and FORE cells of the endolymphatic duct and sac (Dou et al., 2004). To study a possible role of Foxi1 in the regulation of the a4 subunit, we immunofluorescently

stained tissue sections from the kidney, epididymis and inner ear of wild-type and Foxi1$^{-/-}$ mice (Fig. 1-3 in Paper III). This staining revealed a complete co-localization of Foxi1 and a4 in all three tissues in wild-type mice whereas no expression of the a4 subunit could be detected in Foxi1$^{-/-}$ mice. Thus, in all of the studied tissues, it appeared that the presence of Foxi1 was required for expression of the B1 and a4 subunits of the V-ATPase.

To further explore the relation between *Foxi1* and *Atp6v0a4*, we set out to determine whether FOXI1 was capable of direct transcriptional activation of the *ATP6V0A4* promoter, or if the effect of loss of Foxi1 on the expression of *Atp6v0a4* was mediated by an indirect mechanism. Four potential FOXI1 binding sites were identified within 1 kb of the promoter region directly upstream of *ATP6V0A4*. Three of these, Fk1-3, were clustered tightly together while the fourth site, Fk4, was located some 200 bases downstream of Fk1-3 (Fig. 4 in Paper III). The 1 kb promoter region was cloned into a luciferase reporter vector and used in co-transfections with a FOXI1 expression plasmid. The results showed that FOXI1, in a dose-dependent manner, was capable of direct transcriptional activation of the *ATP6V0A4* promoter (Fig. 4 in Paper III).

To determine the relative importance of Fk1-4 in transcriptional activation, mutations altering the sequence of the four binding sites were introduced. Mutations in Fk1, Fk2 or Fk3 resulted in a significant drop of reporter gene activity, demonstrating their importance for activation by FOXI1. The contribution of Fk4, on the other hand, appeared to be minor (Fig. 5 in Paper III). The relative contributions of Fk1-4 were also studied in electrophoretic mobility shift assays. These confirmed that the clustered Fk1-3 were the major targets of FOXI1 binding (Fig. 5 in Paper III). The in vitro assays were complemented with a chromatin immunoprecipitation (CHIP) analysis that also identified the Fk1-3 cluster as a site of FOXI1 interaction in vivo (Fig. 6 in Paper III). Together, the results strongly support a direct role of FOXI1 as a regulator of *ATP6V0A4* expression in the three tissues studied.

Foxi1 regulation of ubiquitous subunits

Some V-ATPase subunits are ubiquitously expressed while others display a more restricted tissue and cell type-specific expression pattern. We have shown above that expression of the specifically expressed subunits B1 and a4 is, at least to some degree, regulated by factors (in this case Foxi1) that also display a restricted, cell-specific expression pattern. But what about the ubiquitously expressed subunits? Is their expression regulated instead by a set of common factors that are expressed in a ubiquitous manner, or are they regulated by distinct and perhaps specifically expressed factors in different tissues? To address this question, we studied the expression of the ubiquitous subunits A1 and E2 in kidney, inner ear and epididymis of wild-type and Foxi1$^{-/-}$ mice. These subunits had previously been shown to be expressed in some of the cell types known to also express Foxi1 (Breton et al., 1996; Herak-Kramberger et al., 2001; Paunescu et al., 2004; Pietrement et al., 2006). Double immunofluorescent

staining of wild-type tissues with antibodies against Foxi1 and A1 and E2 showed that both of the ubiquitously expressed subunits co-localized with Foxi1. No staining for A1 or E2 was detected in Foxi1$^{-/-}$ tissues (Fig. 1-3 in Paper III). This shows that in the kidney, epididymis and endolymphatic sac, the presence of Foxi1 is also necessary for expression of two of the ubiquitously expressed V-ATPase subunits.

Proper assembly of the V-ATPase appears to be important for subunit stability, and it has been speculated that failure to integrate subunits into functional V-ATPase complexes results in their degradation (Leng et al., 1996; Liu et al., 1997). This raised the question whether the lack of immunoreactivity for A1 and E2 subunits was caused by a Foxi1-mediated effect on mRNA levels of these genes, or if the lack of B1 and a4 subunits in some way resulted in destabilization of the V-ATPase complex leading to enhanced degradation of A1 and E2 subunits. To distinguish between these possibilities, we performed combined situ hybridization and immunofluorescent staining on mouse kidney sections using A1- and E2-specific probes and an antibody against carbonic anhydrase II, a marker of intercalated cells. Cells staining positive for carbonic anhydrase II were found in both wild-type and Foxi1$^{-/-}$ sections but A1 and E2 mRNA could only be detected in wild-type sections (Fig. 8 in Paper III), indicating that Foxi1 is required for transcription of these two genes.

Conclusions and concluding remarks

In Paper I, we show that mice lacking *Foxs1* develop and behave normally, are fertile and produce normal-sized litters. In addition, tissues staining positive for the β-galactosidase marker gene appear normal as judged by macro and microscopic examination. Thus, despite an intriguing expression pattern, disruption of this gene results only in a mild phenotype, including an enhanced sensorimotor performance and a slightly lowered weight gain in male mice on a high-fat diet.

Given the often severe and sometimes lethal phenotypes that have been associated with null mutations in other forkhead genes, the mild phenotype resulting from inactivation of Foxs1 may seem surprising. The failure of knock-out mutations to produce obvious phenotypes is, however, not uncommon (Pearson, 2002). The concept of genetic redundancy refers to situations where one gene has the capacity to functionally, either partially or completely, compensate for the loss of another gene. Although it has been claimed that such functional overlap would be evolutionarily unstable, several paralogous genes retaining such compensatory capacities over extended evolutionary periods have been described (Kafri et al., 2006). Examples include the myogenic regulatory factors MyoD and Myf-5 and the paired box genes Pax1 and Pax9 with roles in the control of mammalian organogenesis (Rudnicki et al., 1993; Peters et al., 1999), and a similar relationship may exist between Foxs1 and some other member of the large and diverse family of forkhead genes. It is also possible that a potential survival-promoting function of Foxs1, while remaining masked under standard conditions, would present itself only under certain other conditions, such as disease or injury. Future research may provide further insights into the functions of Foxs1.

In Paper II, we show that the neural crest-derived cells of the boundary cap are capable of self-renewal and of giving rise to different cell types, including neurons, glial cells and smooth muscle-like cells. Boundary cap cells can thus be classified as multipotent NCSCs . Upon differentiation, bNCSCs, independent of extrinsic cues and in contrast to sNCSCs, give rise to sensory neurons. This shows that they are specified to differentiate into the sensory lineage. Furthermore, the sensory neurons formed by bNCSCs are functional and of different sensory subclasses, which suggests that boundary cap cells have not yet undergone restriction towards any specific sensory neuronal subtype. Later studies have shown that bNCSCs, under the influence of neuregulins, are capable of differentiating into mature functional Schwann cells, both in vitro and in vivo. This further demonstrates their multipotent capacity (Aquino et al., 2006).

In Paper III, we show that Foxi1 co-localizes with the B1, a4, A1 and E2 subunits of the V-ATPase in mitochondria-rich FORE cells of the endolymphatic duct and sac. Foxi1 also co-localizes with the a4, A1 and E2 subunits in intercalated cells of the kidney collecting ducts and narrow and clear cells of the epididymis. In these cell types,

the expression of V-ATPase subunits is dependent on the presence of functional Foxi1. Furthermore, FOXI1 is capable of direct transcriptional activation of the *ATP6V0A4* promoter by interacting with cis-elements in position -561/-547 of the a4 promoter. We also show that, at least in the kidney, the loss of the ubiquitous subunits A1 and E2 in Foxi1$^{-/-}$ animals is caused by a failure of mRNA production rather than enhanced subunit degradation. The results presented in Paper III strengthen the role of Foxi1 as an important regulator of V-ATPase subunit availability in the kidney, epididymis and inner ear.

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References

Acott TS, Carr DW (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. Biol Reprod 30:926-935.

Al-Awqati Q, Schwartz GJ (2004) A fork in the road of cell differentiation in the kidney tubule. J Clin Invest 113:1528-1530.

Altman J, Bayer SA (1984) The development of the rat spinal cord. Adv Anat Embryol Cell Biol 85:1-164.

Anderson DJ (2000) Genes, lineages and the neural crest: a speculative review. Philos Trans R Soc Lond B Biol Sci 355:953-964.

Aquino JB, Hjerling-Leffler J, Koltzenburg M, Edlund T, Villar MJ, Ernfors P (2006) In vitro and in vivo differentiation of boundary cap neural crest stem cells into mature Schwann cells. Exp Neurol 198:438-449.

Aybar MJ, Nieto MA, Mayor R (2003) Snail precedes Slug in the genetic cascade required for the specification and migration of the Xenopus neural crest. Development 130:483-494.

Baker CVH, Bronner-Fraser M (2001) Vertebrate Cranial Placodes I. Embryonic Induction. Dev Biol 232:1-61.

Baroffio A, Dupin E, Le Douarin NM (1988) Clone-forming ability and differentiation potential of migratory neural crest cells. Proc Natl Acad Sci U S A 85:5325-5329.

Baroffio A, Dupin E, Le Douarin N (1991) Common precursors for neural and mesectodermal derivatives in the cephalic neural crest. Development 112:301-305.

Bashford CL, Radda GK, Ritchie GA (1975) Energy-linked activities of the chromaffin granule membrane. FEBS Lett 50:21-24.

Begbie J, Graham A (2001a) The ectodermal placodes: a dysfunctional family. Philos Trans R Soc Lond B Biol Sci 356:1655-1660.

Begbie J, Graham A (2001b) Integration Between the Epibranchial Placodes and the Hindbrain. Science 294:595-598.

Begbie J, Brunet J, Rubenstein J, Graham A (1999) Induction of the epibranchial placodes. Development 126:895-902.

Bellinger LL, Bernardis LL (2002) The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: Lessons learned from lesioning studies. Physiol Behav 76:431-442.

Bibel M, Barde Y-A (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 14:2919-2937.

Bixby S, Kruger GM, Mosher JT, Joseph NM, Morrison SJ (2002) Cell-Intrinsic Differences between Stem Cells from Different Regions of the Peripheral Nervous System Regulate the Generation of Neural Diversity. Neuron 35:643-656.

Blomqvist SR, Vidarsson H, Soder O, Enerback S (2006) Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility. EMBO J 25:4131-4141.

Blomqvist SR, Vidarsson H, Fitzgerald S, Johansson BR, Ollerstam A, Brown R, Persson AEG, Bergstrom G, Enerback S (2004) Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxi1. J Clin Invest 113:1560-1570.

Brent R, Ptashne M (1985) A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell 43:729-736.

Breton S, Brown D (2007) New insights into the regulation of V-ATPase-dependent proton secretion. Am J Physiol Renal Physiol 292:F1-10.

Breton S, Smith PJS, Lui B, Brown D (1996) Acidification of the male reproductive tract by a proton pumping(H+)-ATPase. Nat Med 2:470-472.

Bronner-Fraser M (1986) Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev Biol 115:44-55.

Bronner-Fraser M (1993) Mechanisms of neural crest cell migration. Bioessays 15:221-230.

Bronner-Fraser M (1994) Neural crest cell formation and migration in the developing embryo. FASEB J 8:699-706.

Brown D, Breton S (1996) Mitochondria-rich, proton-secreting epithelial cells. J Exp Biol 199:2345-2358.

Brown D, Hirsch S, Gluck S (1988) Localization of a proton-pumping ATPase in rat kidney. J Clin Invest 82:2114-2126.

Brown D, Lui B, Gluck S, Sabolic I (1992) A plasma membrane proton ATPase in specialized cells of rat epididymis. Am J Physiol Cell Physiol 263:C913-916.

Buchman V, Davies A (1993) Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development 118:989-1001.

Cao WH, Fan W, Morrison SF (2004) Medullary pathways mediating specific sympathetic responses to activation of dorsomedial hypothalamus. Neuroscience 126:229-240.

Carlsson P, Mahlapuu M (2002) Forkhead Transcription Factors: Key Players in Development and Metabolism. Dev Biol 250:1-23.

Carr DW, Usselman MC, Acott TS (1985) Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. Biol Reprod 33:588-595.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor. Science 288:306-313.

Cederberg A, Grønning LM, Ahrén B, Taskén K, Carlsson P, Enerbäck S (2001) FOXC2 Is a Winged Helix Gene that Counteracts Obesity, Hypertriglyceridemia, and Diet-Induced Insulin Resistance. Cell 106:563-573.

Cederberg A, Betz R, Lagercrantz S, Larsson C, Hulander M, Carlsson P, Enerbäck S (1997) Chromosome Localization, Sequence Analysis, and Expression Pattern Identify FKHL 18 as a Novel Human Forkhead Gene. Genomics 44:344-346.

Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, Bowcock AM (2000) JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 106:R75-R81.

Cheung M, Briscoe J (2003) Neural crest development is regulated by the transcription factor Sox9. Development 130:5681-5693.

Cheung M, Chaboissier M-C, Mynett A, Hirst E, Schedl A, Briscoe J (2005) The Transcriptional Control of Trunk Neural Crest Induction, Survival, and Delamination. Dev Cell 8:179-192.

Clevidence DE, Overdier DG, Tao W, Qian X, Pani L, Lai E, Costa RH (1993) Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding-domain family. Proc Natl Acad Sci U S A 90:3948-3952.

Colas J-F, Schoenwolf GC (2001) Towards a cellular and molecular understanding of neurulation. Dev Dyn 221:117-145.

Cosentino MJ, Cockett ATK (1986) Review article: Structure and function of the epididymis. Urol Res 14:229-240.

Couloigner V, Teixeira M, Sterkers O, Ferrary E (1999) *In Vivo* Study of the Electrochemical Composition of Luminal Fluid in the Guinea Pig Endolymphatic Sac. Acta Otolaryngol (Stockh) 119:200 - 202.

Crawley JN (1999) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 835:18-26.

D'amico-Martel A (1982) Temporal patterns of neurogenesis in avian cranial sensory and autonomic ganglia. Am J Anat 163:351-372.

D'amico-Martel A, Noden DM (1983) Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. Am J Anat 166:445-468.

Davis AE, Gimenez AM, Therrien B (2001) Effects of Entorhinal Cortex Lesions on Sensory Integration and Spatial Learning. Nurs Res 50:77-85.

Debby-Brafman A, Burstyn-Cohen T, Klar A, Kalcheim C (1999) F-Spondin, Expressed in Somite Regions Avoided by Neural Crest Cells, Mediates Inhibition of Distinct Somite Domains to Neural Crest Migration. Neuron 22:475-488.

Delfino-Machín M, Chipperfield TR, Rodrigues FSLM, Kelsh RN (2007) The proliferating field of neural crest stem cells. Dev Dyn 236:3242-3254.

DiMicco JA, Zaretsky DV (2007) The dorsomedial hypothalamus: a new player in thermoregulation. Am J Physiol Regul Integr Comp Physiol 292:R47-63.

Dottori M, Gross MK, Labosky P, Goulding M (2001) The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. Development 128:4127-4138.

Dou H, Finberg K, Cardell EL, Lifton R, Choo D (2003) Mice lacking the B1 subunit of H+-ATPase have normal hearing. Hearing Res 180:76-84.

Dou H, Xu J, Wang Z, Smith AN, Soleimani M, Karet FE, Greinwald JH, Jr, Choo D (2004) Co-expression of Pendrin, Vacuolar H+-ATPase {alpha}4-Subunit and Carbonic Anhydrase II in Epithelial Cells of the Murine Endolymphatic Sac. J Histochem Cytochem 52:1377-1384.

Eijkenboom M, Blokland A, van der Staay FJ (2000) Modelling cognitive dysfunctions with bilateral injections of ibotenic acid into the rat entorhinal cortex. Neuroscience 101:27-39.

Erickson CA, Duong TD, Tosney KW (1992) Descriptive and experimental analysis of the dispersion of neural crest cells along the dorsolateral path and their entry into ectoderm in the chick embryo. Dev Biol 151:251-272.

Ernfors P (2001) Local and target-derived actions of neurotrophins during peripheral nervous system development. Cell Mol Life Sci 58:1036-1044.

Etchevers H, Vincent C, Le Douarin N, Couly G (2001) The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128:1059-1068.

Fang J, Dagenais SL, Erickson RP, Arlt MF, Glynn MW, Gorski JL, Seaver LH, Glover TW (2000) Mutations in FOXC2 (MFH-1), a Forkhead Family Transcription Factor, Are Responsible for the Hereditary Lymphedema-Distichiasis Syndrome. Am J Hum Genet 67:1382-1388.

Fariñas I, Cano-Jaimez M, Bellmunt E, Soriano M (2002) Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res Bull 57:809-816.

Fedtsova NG, Turner EE (1995) Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. Mech Dev 53:291-304.

Finberg KE, Wagner CA, Stehberger PA, Geibel JP, Lifton RP (2003) Molecular cloning and characterization of Atp6v1b1, the murine vacuolar H+-ATPase B1-subunit. Gene 318:25-34.

Finberg KE, Wagner CA, Bailey MA, Paunescu TG, Breton S, Brown D, Giebisch G, Geibel JP, Lifton RP (2005) The B1-subunit of the H+ ATPase is required for maximal urinary acidification. Proc Natl Acad Sci U S A 102:13616-13621.

Finegold DN, Kimak MA, Lawrence EC, Levinson KL, Cherniske EM, Pober BR, Dunlap JW, Ferrell RE (2001) Truncating mutations in FOXC2 cause multiple lymphedema syndromes. Hum Mol Genet 10:1185-1189.

Fontaine-Perus J, Chanconie M, Le Douarin NM (1985) Embryonic origin of substance P containing neurons in cranial and spinal sensory ganglia of the avian embryo. Dev Biol 107:227-238.

Forgac M (1999) Structure and Properties of the Vacuolar (H+)-ATPases. J Biol Chem 274:12951-12954.

Frank E, Sanes J (1991) Lineage of neurons and glia in chick dorsal root ganglia: analysis in vivo with a recombinant retrovirus. Development 111:895-908.

Frankel AD, Kim PS (1991) Modular structure of transcription factors: Implications for gene regulation. Cell 65:717-719.

Fry AC, Karet FE (2007) Inherited Renal Acidoses. Physiology 22:202-211.

Futai M, Oka T, Sun-Wada G, Moriyama Y, Kanazawa H, Wada Y (2000) Luminal acidification of diverse organelles by V-ATPase in animal cells. J Exp Biol 203:107-116.

Gammill LS, Gonzalez C, Gu C, Bronner-Fraser M (2006) Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. Development 133:99-106.

Gerlai R, Pisacane P, Erickson S (2000) Heregulin, but not ErbB2 or ErbB3, heterozygous mutant mice exhibit hyperactivity in multiple behavioral tasks. Behav Brain Res 109:219-227.

Gluck SL, Underhill DM, Iyori M, Holliday LS, Kostrominova TY, Lee BS (1996) Physiology and Biochemistry of the Kidney Vacuolar H+-ATPase. Annu Rev Physiol 58:427-445.

Golding JP, Cohen J (1997) Border Controls at the Mammalian Spinal Cord: Late-Surviving Neural Crest Boundary Cap Cells at Dorsal Root Entry Sites May Regulate Sensory Afferent Ingrowth and Entry Zone Morphogenesis. Mol Cell Neurosci 9:381-396.

Graham A, Begbie J (2000) Neurogenic placodes: a common front. Trends Neurosci 23:313-316.

Gray PA et al. (2004) Mouse Brain Organization Revealed Through Direct Genome-Scale TF Expression Analysis. Science 306:2255-2257.

Greenwood A, Turner E, Anderson D (1999) Identification of dividing, determined sensory neuron precursors in the mammalian neural crest. Development 126:3545-3559.

Guyton AC, Hall JE (1996) Textbook of Medical Physiology, 9th Edition. Philadelphia: W.B. Saunders Company.

Hamburger V, Yip J (1984) Reduction of experimentally induced neuronal death in spinal ganglia of the chick embryo by nerve growth factor. J Neurosci 4:767-774.

Hamburger V, Brunso-Bechtold J, Yip J (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J Neurosci 1:60-71.

Hannenhalli S, Kaestner KH (2009) The evolution of Fox genes and their role in development and disease. Nat Rev Genet 10:233-240.

Henion P, Weston J (1997) Timing and pattern of cell fate restrictions in the neural crest lineage. Development 124:4351-4359.

Herak-Kramberger CM, Breton S, Brown D, Kraus O, Sabolic I (2001) Distribution of the Vacuolar H+ATPase Along the Rat and Human Male Reproductive Tract. Biol Reprod 64:1699-1707.

Hori S, Nomura T, Sakaguchi S (2003) Control of Regulatory T Cell Development by the Transcription Factor Foxp3. Science 299:1057-1061.

Hory-Lee F, Russell M, Lindsay RM, Frank E (1993) Neurotrophin 3 supports the survival of developing muscle sensory neurons in culture. Proc Natl Acad Sci U S A 90:2613-2617.

Huber AB, Kolodkin AL, Ginty DD, Cloutier J-Fo (2003) Signaling at the growth cone: Ligand-Receptor Complexes and the Control of Axon Growth and Guidance. Annu Rev Neurosci 26:509-563.

Hulander M, Wurst W, Carlsson P, Enerback S (1998) The winged helix transcription factor Fkh10 is required for normal development of the inner ear. Nat Genet 20:374-376.

Hulander M, Kiernan AE, Blomqvist SR, Carlsson P, Samuelsson E-J, Johansson BR, Steel KP, Enerback S (2003) Lack of pendrin expression leads to deafness and expansion of the endolymphatic compartment in inner ears of Foxi1 null mutant mice. Development 130:2013-2025.

Imai-Senga Y, Sun-Wada G-H, Wada Y, Futai M (2002) A human gene, ATP6E1, encoding a testis-specific isoform of H+-ATPase subunit E. Gene 289:7-12.

Jefferies KC, Cipriano DJ, Forgac M (2008) Function, structure and regulation of the vacuolar (H+)-ATPases. Arch Biochem Biophys 476:33-42.

Jervis KM, Robaire B (2001) Dynamic Changes in Gene Expression along the Rat Epididymis. Biol Reprod 65:696-703.

Johansson CC, Dahle MK, Blomqvist SR, Gronning LM, Aandahl EM, Enerback S, Tasken K (2003) A Winged Helix Forkhead (FOXD2) Tunes Sensitivity to cAMP in T Lymphocytes through Regulation of cAMP-dependent Protein Kinase RIalpha. J Biol Chem 278:17573-17579.

Jouret F, Auzanneau C, Debaix H, Wada G-HS, Pretto C, Marbaix E, Karet FE, Courtoy PJ, Devuyst O (2005) Ubiquitous and Kidney-Specific Subunits of Vacuolar H+-ATPase Are Differentially Expressed during Nephrogenesis. J Am Soc Nephrol 16:3235-3246.

Juhn SK, Ikeda K, Morizono T, Murphy M (1991) Pathophysiology of Inner Ear Fluid Imbalance. Acta Otolaryngol (Stockh) 111:9 - 14.

Kaestner KH, Knöchel W, Martínez DE (2000) Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev 14:142-146.

Kaestner KH, Lee KH, Schlöndorff J, Hiemisch H, Monaghan AP, Schutz G (1993) Six members of the mouse forkhead gene family are developmentally regulated. Proc Natl Acad Sci U S A 90:7628-7631.

Kafri R, Levy M, Pilpel Y (2006) The regulatory utilization of genetic redundancy through responsive backup circuits. Proc Natl Acad Sci U S A 103:11653-11658.

Kalcheim C, Le Douarin NM (1986) Requirement of a neural tube signal for the differentiation of neural crest cells into dorsal root ganglia. Dev Biol 116:451-466.

Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420-1428.

Karet FE et al. (1999) Mutations in the gene encoding B1 subunit of H+-ATPase cause renal tubular acidosis with sensorineural deafness. Nat Genet 21:84-90.

Kim D, Kim C-H, Moon J-I, Chung Y-G, Chang M-Y, Han B-S, Ko S, Yang E, Cha KY, Lanza R, Kim K-S (2009) Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins. Cell Stem Cell 4:472-476.

Kim J, Lo L, Dormand E, Anderson DJ (2003) SOX10 Maintains Multipotency and Inhibits Neuronal Differentiation of Neural Crest Stem Cells. Neuron 38:17-31.

Kirstein M, Fariñas I (2002) Sensing life: regulation of sensory neuron survival by neurotrophins. Cell Mol Life Sci 59:1787-1802.

Kleber M, Lee H-Y, Wurdak H, Buchstaller J, Riccomagno MM, Ittner LM, Suter U, Epstein DJ, Sommer L (2005) Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. J Cell Biol 169:309-320.

Korn J, Christ B, Kurz H (2002) Neuroectodermal origin of brain pericytes and vascular smooth muscle cells. J Comp Neurol 442:78-88.

LaBonne C, Bronner-Fraser M (1999) Molecular mechanisms of neural crest formation. Annu Rev Cell Dev Biol 15:81-112.
Lacham O, Trounson A (1991) Fertilizing capacity of epididymal and testicular spermatozoa microinjected under the zona pellucida of the mouse oocyte. Mol Reprod Dev 29:85-93.
Lai CSL, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP (2001) A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 413:519-523.
Lai E, Prezioso VR, Smith E, Litvin O, Costa RH, Darnell JE (1990) HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev 4:1427-1436.
Lang F, Vallon V, Knipper M, Wangemann P (2007) Functional significance of channels and transporters expressed in the inner ear and kidney. Am J Physiol Cell Physiol 293:C1187-1208.
Latchman DS (1997) Transcription factors: An overview. Int J Biochem Cell Biol 29:1305-1312.
Lawson SN, Biscoe TJ (1979) Development of mouse dorsal root ganglia: an autoradiographic and quantitative study. J Neurocytol 8:265-274.
Le Douarin NM, Teillet M-AM (1974) Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. Dev Biol 41:162-184.
Le Douarin NM, Kalcheim C (1999) The neural crest, 2nd Edition. Cambridge: Cambridge University Press.
Lee H-Y, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L (2004) Instructive Role of Wnt/{beta}-Catenin in Sensory Fate Specification in Neural Crest Stem Cells. Science 303:1020-1023.
Leng X-H, Manolson MF, Liu Q, Forgac M (1996) Site-directed Mutagenesis of the 100-kDa Subunit (Vph1p) of the Yeast Vacuolar (H+)-ATPase. J Biol Chem 271:22487-22493.
Lewin GR, Barde Y-A (2003) Physiology of the Neurotrophins. Annu Rev Neurosci 19:289-317.
Levine N, Marsh DJ (1971) Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. J Physiol (Lond) 213:557-570.
Levine N, Kelly H (1978) Measurement of pH in the rat epididymis in vivo. J Reprod Fertil 52:333-335.
Liu Q, Leng X-H, Newman PR, Vasilyeva E, Kane PM, Forgac M (1997) Site-directed Mutagenesis of the Yeast V-ATPase A Subunit. J Biol Chem 272:11750-11756.
Lo L, Dormand E, Greenwood A, Anderson DJ (2002) Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development 129:1553-1567.
Lodish H, Baltimore D, Berk A, Zipursky SL, Matsudaira P, Darnell J (1995) Molecular cell biology, 3rd Edition. New York: Scientific american books.
Loring JF, Erickson CA (1987) Neural crest cell migratory pathways in the trunk of the chick embryo. Dev Biol 121:220-236.
Lumsden A, Guthrie S (1991) Alternating patterns of cell surface properties and neural crest cell migration during segmentation of the chick hindbrain. Development 113:9-15.
Ma Q, Fode C, Guillemot F, Anderson DJ (1999) NEUROGENIN1 and NEUROGENIN2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev 13:1717-1728.
Majesky MW (2007) Developmental Basis of Vascular Smooth Muscle Diversity. Arterioscler Thromb Vasc Biol 27:1248-1258.
Marmigere F, Ernfors P (2007) Specification and connectivity of neuronal subtypes in the sensory lineage. Nat Rev Neurosci 8:114-127.

Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P (2004) Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7:930-938.

Masuda T, Shiga T (2005) Chemorepulsion and cell adhesion molecules in patterning initial trajectories of sensory axons. Neurosci Res 51:337-347.

Matsuda S, Uehara Y (1984) Prenatal development of the rat dorsal root ganglia. Cell Tissue Res 235:13-18.

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416:52-58.

Mears AJ, Jordan T, Mirzayans F, Dubois S, Kume T, Parlee M, Ritch R, Koop B, Kuo W-L, Collins C, Marshall J, Gould DB, Pearce W, Carlsson P, Enerbäck S, Morissette J, Bhattacharya S, Hogan B, Raymond V, Walter MA (1998) Mutations of the Forkhead/Winged-Helix Gene, FKHL7, in Patients with Axenfeld-Rieger Anomaly. Am J Hum Genet 63:1316-1328.

Montelius A, Marmigère F, Baudet C, Aquino JB, Enerbäck S, Ernfors P (2007) Emergence of the sensory nervous system as defined by Foxs1 expression. Differentiation 75:404-417.

Morrison SJ, Shah NM, Anderson DJ (1997) Regulatory Mechanisms in Stem Cell Biology. Cell 88:287-298.

Morrison SJ, White PM, Zock C, Anderson DJ (1999) Prospective Identification, Isolation by Flow Cytometry, and In Vivo Self-Renewal of Multipotent Mammalian Neural Crest Stem Cells. Cell 96:737-749.

Mosher JT, Morrison SJ (2004) Crossing the boundaries of sensory neurogenesis. Nat Neurosci 7:900-902.

Mu X, Silos-Santiago I, Carroll S, Snider W (1993) Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. J Neurosci 13:4029-4041.

Nakagawa S, Takeichi M (1998) Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125:2963-2971.

Nelson N, Harvey WR (1999) Vacuolar and Plasma Membrane Proton-Adenosinetriphosphatases. Physiol Rev 79:361-385.

Nelson RD, Guo XL, Masood K, Brown D, Kalkbrenner M, Gluck S (1992) Selectively amplified expression of an isoform of the vacuolar H(+)-ATPase 56-kilodalton subunit in renal intercalated cells. Proc Natl Acad Sci U S A 89:3541-3545.

Niederlander C, Lumsden A (1996) Late emigrating neural crest cells migrate specifically to the exit points of cranial branchiomotor nerves. Development 122:2367-2374.

Nishi T, Forgac M (2002) The vacuolar (H+)-ATPases - nature's most versatile proton pumps. Nat Rev Mol Cell Biol 3:94-103.

Nishimura DY, Swiderski RE, Alward WLM, Searby CC, Patil SR, Bennet SR, Kanis AB, Gastier JM, Stone EM, Sheffield VC (1998) The forkhead transcription factor gene FKHL7 is responsible for glaucoma phenotypes which map to 6p25. Nat Genet 19:140-147.

Noden DM (1993) Spatial integration among cells forming the cranial peripheral nervous system. J Neurobiol 24:248-261.

Northcutt RG (2005) The new head hypothesis revisited. J Exp Zool Part B Mol Dev Evol 304B:274-297.

O'Neil R, Heller S (2005) The mechanosensitive nature of TRPV channels. Pfluegers Arch/Eur J Physiol 451:193-203.

Ohyama T, Groves AK (2004) Expression of mouse Foxi class genes in early craniofacial development. Dev Dyn 231:640-646.

Oka T, Murata Y, Namba M, Yoshimizu T, Toyomura T, Yamamoto A, Sun-Wada G-H, Hamasaki N, Wada Y, Futai M (2001) a4, a Unique Kidney-specific Isoform of Mouse Vacuolar H+-ATPase Subunit a. J Biol Chem 276:40050-40054.

Overdier DG, Ye H, Peterson RS, Clevidence DE, Costa RH (1997) The Winged Helix Transcriptional Activator HFH-3 Is Expressed in the Distal Tubules of Embryonic and Adult Mouse Kidney. J Biol Chem 272:13725-13730.

Pastor-Soler N, Pietrement C, Breton S (2005) Role of Acid/Base Transporters in the Male Reproductive Tract and Potential Consequences of Their Malfunction. Physiology 20:417-428.

Paunescu TG, Silva ND, Marshansky V, McKee M, Breton S, Brown D (2004) Expression of the 56-kDa B2 subunit isoform of the vacuolar H+-ATPase in proton-secreting cells of the kidney and epididymis. Am J Physiol Cell Physiol 287:C149-162.

Paves H, Saarma M (1997) Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons. Cell Tissue Res 290:285-297.

Pearson H (2002) Surviving a knockout blow. Nature 415:8-9.

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002) A TRP Channel that Senses Cold Stimuli and Menthol. Cell 108:705-715.

Peters H, Wilm B, Sakai N, Imai K, Maas R, Balling R (1999) Pax1 and Pax9 synergistically regulate vertebral column development. Development 126:5399-5408.

Pierrou S, Hellqvist M, Samuelsson L, Enerback S, Carlsson P (1994) Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. The EMBO Journal 13:5002-5012.

Pietrement C, Sun-Wada G-H, Da Silva N, McKee M, Marshansky V, Brown D, Futai M, Breton S (2006) Distinct Expression Patterns of Different Subunit Isoforms of the V-ATPase in the Rat Epididymis. Biol Reprod 74:185-194.

Rask-Andersen H, Erwall C, Steel KP, Friberg U (1987) The endolymphatic sac in a mouse mutant with cochleo-saccular degeneration: Electrophysiological and ultrastructural correlations. Hearing Res 26:177-190.

Rask-Andersen H, DeMott JE, Bagger-Sjöbäck D, Salt AN (1999) Morphological changes of the endolymphatic sac induced by microinjection of artificial endolymph into the cochlea. Hearing Res 138:81-90.

Rickmann M, Fawcett JW, Keynes RJ (1985) The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. J Embryol Exp Morphol 90:437-455.

Rifkin JT, Todd VJ, Anderson LW, Lefcort F (2000) Dynamic Expression of Neurotrophin Receptors during Sensory Neuron Genesis and Differentiation. Dev Biol 227:465-480.

Robaire B, Viger RS (1995) Regulation of epididymal epithelial cell functions. Biol Reprod 52:226-236.

Rubinstein M, Phillips TJ, Bunzow JR, Falzone TL, Dziewczapolski G, Zhang G, Fang Y, Larson JL, McDougall JA, Chester JA, Saez C, Pugsley TA, Gershanik O, Low MJ, Grandy DK (1997) Mice Lacking Dopamine D4 Receptors Are Supersensitive to Ethanol, Cocaine, and Methamphetamine. Cell 90:991-1001.

Rudnicki MA, Schnegelsberg PNJ, Stead RH, Braun T, Arnold H-H, Jaenisch R (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75:1351-1359.

Rustay NR, Wahlsten D, Crabbe JC (2003) Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. Behav Brain Res 141:237-249.

Salt AN (2001) Regulation of Endolymphatic Fluid Volume. Ann N Y Acad Sci 942:306-312.

Salt AN, DeMott JE (2000) Ionic and potential changes of the endolymphatic sac induced by endolymph volume changes. Hearing Res 149:46-54.

Sato Y, Baba T, Zubair M, Miyabayashi K, Toyama Y, Maekawa M, Owaki A, Mizusaki H, Sawamura T, Toshimori K, Morohashi K-I, Katoh-Fukui Y (2008) Importance of forkhead transcription factor Fkhl18 for development of testicular vasculature. Mol Reprod Dev 75:1361-1371.

Sauka-Spengler T, Bronner-Fraser M (2008) A gene regulatory network orchestrates neural crest formation. Nat Rev Mol Cell Biol 9:557-568.

Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus H-G, Wolfer DP, Pedroarena CM, Storm JF, Ruth P (2004) Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. Proc Natl Acad Sci U S A 101:9474-9478.

Schlosser G (2006) Induction and specification of cranial placodes. Dev Biol 294:303-351.

Scott GF (2000) Developmental biology, 6th Edition. Sunderland, Massachusetts: Sinauer Associates , Inc.

Seoane J, Le H-V, Shen L, Anderson SA, Massagué J (2004) Integration of Smad and Forkhead Pathways in the Control of Neuroepithelial and Glioblastoma Cell Proliferation. Cell 117:211-223.

Serbedzija G, Fraser S, Bronner-Fraser M (1990) Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. Development 108:605-612.

Serbedzija G, Bronner-Fraser M, Fraser S (1992) Vital dye analysis of cranial neural crest cell migration in the mouse embryo. Development 116:297-307.

Shah NM, Groves AK, Anderson DJ (1996) Alternative Neural Crest Cell Fates Are Instructively Promoted by TGF[beta] Superfamily Members. Cell 85:331-343.

Shibata H (1993) Direct projections from the anterior thalamic nuclei to the retrohippocampal region in the rat. J Comp Neurol 337:431-445.

Shibata H (1996) Direct projections from the entorhinal area to the anteroventral and laterodorsal thalamic nuclei in the rat. Neurosci Res 26:83-87.

Sieber-Blum M, Grim M, Hu YF, Szeder V (2004) Pluripotent neural crest stem cells in the adult hair follicle. Dev Dyn 231:258-269.

Smith A (2006) A glossary for stem-cell biology. Nature 441:1060-1060.

Smith AN, Borthwick KJ, Karet FE (2002) Molecular cloning and characterization of novel tissue-specific isoforms of the human vacuolar H+-ATPase C, G and d subunits, and their evaluation in autosomal recessive distal renal tubular acidosis. Gene 297:169-177.

Smith AN, Finberg KE, Wagner CA, Lifton RP, Devonald MAJ, Su Y, Karet FE (2001) Molecular Cloning and Characterization ofAtp6n1b. J Biol Chem 276:42382-42388.

Smith AN, Skaug J, Choate KA, Nayir A, Bakkaloglu A, Ozen S, Hulton SA, Sanjad SA, Al-Sabban EA, Lifton RP, Scherer SW, Karet FE (2000) Mutations in ATP6N1B, encoding a new kidney vacuolar proton pump 116-kD subunit, cause recessive distal renal tubular acidosis with preserved hearing. Nat Genet 26:71-75.

Smith JL, Schoenwolf GC (1997) Neurulation: coming to closure. Trends Neurosci 20:510-517.

Snider WD, Silos-Santiago I (1996) Dorsal Root Ganglion Neurons Require Functional Neurotrophin Receptors for Survival during Development. Philos Trans R Soc Lond B Biol Sci 351:395-403.

Snider WD, Wright DE (1996) Neurotrophins Cause a New Sensation. Neuron 16:229-232.

Stankiewicz P et al. (2009) Genomic and Genic Deletions of the FOX Gene Cluster on 16q24.1 and Inactivating Mutations of FOXF1 Cause Alveolar Capillary Dysplasia and Other Malformations. Am J Hum Genet 84:780-791.

Stankovic KM, Brown D, Alper SL, Adams JC (1997) Localization of pH regulating proteins H+ATPase and Cl-/HCO-3 exchanger in the guinea pig inner ear. Hearing Res 114:21-34.
Stehberger PA, Schulz N, Finberg KE, Karet FE, Giebisch G, Lifton RP, Geibel JP, Wagner CA (2003) Localization and Regulation of the ATP6V0A4 (a4) Vacuolar H+-ATPase Subunit Defective in an Inherited Form of Distal Renal Tubular Acidosis. J Am Soc Nephrol 14:3027-3038.
Stemple DL, Anderson DJ (1992) Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71:973-985.
Stoffel MH, Friess AE (1994) Morphological characteristics of boar efferent ductules and epididymal duct. Microsc Res Tech 29:411-431.
Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like Channel Expressed in Nociceptive Neurons, Is Activated by Cold Temperatures. Cell 112:819-829.
Stover EH et al. (2002) Novel ATP6V1B1 and ATP6V0A4 mutations in autosomal recessive distal renal tubular acidosis with new evidence for hearing loss. J Med Genet 39:796-803.
Streit A (2004) Early development of the cranial sensory nervous system: from a common field to individual placodes. Dev Biol 276:1-15.
Sun-Wada G-H, Tabata H, Kawamura N (2005) Selective Assembly of V-ATPase Subunit Isoforms in Mouse Kidney. J Bioenerg Biomembr 37:415-418.
Sun-Wada G-H, Imai-Senga Y, Yamamoto A, Murata Y, Hirata T, Wada Y, Futai M (2002) A Proton Pump ATPase with Testis-specific E1-Subunit Isoform Required for Acrosome Acidification. J Biol Chem 277:18098-18105.
Takahashi K, Yamanaka S (2006) Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126:663-676.
Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell 131:861-872.
Taneyhill LA, Coles EG, Bronner-Fraser M (2007) Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. Development 134:1481-1490.
Teillet M-A, Kalcheim C, Le Douarin NM (1987) Formation of the dorsal root ganglia in the avian embryo: Segmental origin and migratory behavior of neural crest progenitor cells. Dev Biol 120:329-347.
Thiery JP (2003) Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 15:740-746.
Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi ABY, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 controls myelination in the peripheral nervous system. Nature 371:796-799.
Tsujikawa S, Yamashita T, Amano H, Kumazawa T, Vosteen K-H (1992) Acidity in the endolymphatic sac fluid of guinea pigs. ORL J Otorhinolaryngol Relat Spec 54:198-200.
Tsunozaki M, Bautista DM (2009) Mammalian somatosensory mechanotransduction. Curr Opin Neurobiol 19:362-369.
Turner TT (1991) Spermatozoa Are Exposed to a Complex Microenvironment as They Traverse the Epididymis. Ann N Y Acad Sci 637:364-383.
van Groen T, Kadish I, Michael Wyss J (2002) Role of the anterodorsal and anteroventral nuclei of the thalamus in spatial memory in the rat. Behav Brain Res 132:19-28.
van Hille B, Richener H, Green JR, Bilbe G (1995) The Ubiquitous VA68 Isoform of Subunit-A of the Vacuolar H+-ATPase Is Highly Expressed in Human Osteoclasts. Biochem Biophys Res Commun 214:1108-1113.

van Hille B, Richener H, Evans DB, Green JR, Bilbe G (1993) Identification of two subunit A isoforms of the vacuolar H(+)-ATPase in human osteoclastoma. J Biol Chem 268:7075-7080.

Venkatachalam K, Montell C (2007) TRP Channels. Annu Rev Biochem 76:387-417.

Vermeren M, Maro GS, Bron R, McGonnell IM, Charnay P, Topilko P, Cohen J (2003) Integrity of Developing Spinal Motor Columns Is Regulated by Neural Crest Derivatives at Motor Exit Points. Neuron 37:403-415.

Viana F, Peña Edl, Pecson B, Schmidt RF, Belmonte C (2001) Swelling-activated calcium signalling in cultured mouse primary sensory neurons. Eur J Neurosci 13:722-734.

Vitalis T, Alvarez C, Chen K, Shih JC, Gaspar P, Cases O (2003) Developmental expression pattern of monoamine oxidases in sensory organs and neural crest derivatives. J Comp Neurol 464:392-403.

Wagner CA, Finberg KE, Breton S, Marshansky V, Brown D, Geibel JP (2004) Renal Vacuolar H+-ATPase. Physiol Rev 84:1263-1314.

Wang VY, Rose MF, Zoghbi HY (2005) Math1 Expression Redefines the Rhombic Lip Derivatives and Reveals Novel Lineages within the Brainstem and Cerebellum. Neuron 48:31-43.

Warburton EC, Aggleton JP (1998) Differential deficits in the Morris water maze following cytotoxic lesions of the anterior thalamus and fornix transection. Behav Brain Res 98:27-38.

Vargas-Poussou R et al. (2006) Genetic Investigation of Autosomal Recessive Distal Renal Tubular Acidosis: Evidence for Early Sensorineural Hearing Loss Associated with Mutations in the ATP6V0A4 Gene. J Am Soc Nephrol 17:1437-1443.

Weigel D, Jäckle H (1990) The fork head domain: A novel DNA binding motif of eukaryotic transcription factors? Cell 63:455-456.

Weigel D, Jürgens G, Küttner F, Seifert E, Jäckle H (1989) The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. Cell 57:645-658.

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318-324.

White PM, Morrison SJ, Orimoto K, Kubu CJ, Verdi JM, Anderson DJ (2001) Neural Crest Stem Cells Undergo Cell-Intrinsic Developmental Changes in Sensitivity to Instructive Differentiation Signals. Neuron 29:57-71.

Wijchers PJEC, Burbach JPH, Smidt MP (2006) In control of biology: of mice, men and Foxes. Biochem J 397:233-246.

Wingate R (2005) Math-Map(ic)s. Neuron 48:1-4.

Wingate RJT (2001) The rhombic lip and early cerebellar development. Curr Opin Neurobiol 11:82-88.

Wolfrum C, Asilmaz E, Luca E, Friedman JM, Stoffel M (2004) Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. Nature 432:1027-1032.

Wolpert L, Beddington R, Brockes J, Jessel T, Lawrence P, Meyerowitz E (1998) Principles of development, 1st Edition. London: Current Biology Publications.

Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung H-K, Nagy A (2009) piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458:766-770.

Ye H, Holterman AX, Yoo KW, Franks RR, Costa RH (1999) Premature Expression of the Winged Helix Transcription Factor HFH-11B in Regenerating Mouse Liver Accelerates Hepatocyte Entry into S Phase. Mol Cell Biol 19:8570-8580.

Yeung C-H, Anapolski M, Sipilä P, Wagenfeld A, Poutanen M, Huhtaniemi I, Nieschlag E, Cooper TG (2002) Sperm Volume Regulation: Maturational Changes in Fertile and Infertile Transgenic Mice and Association with Kinematics and Tail Angulation. Biol Reprod 67:269-275.

Yeung CH, Cooper TG, Oberpenning F, Schulze H, Nieschlag E (1993) Changes in movement characteristics of human spermatozoa along the length of the epididymis. Biol Reprod 49:274-280.

Young KA, Manaye KF, Liang C-L, Hicks PB, German DC (2000) Reduced number of mediodorsal and anterior thalamic neurons in schizophrenia. Biol Psychiatry 47:944-953.

Young KA, Holcomb LA, Yazdani U, Hicks PB, German DC (2004) Elevated Neuron Number in the Limbic Thalamus in Major Depression. Am J Psychiatry 161:1270-1277.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. Science 318:1917-1920.

Zirlinger M, Lo L, McMahon J, McMahon AP, Anderson DJ (2002) Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. Proc Natl Acad Sci U S A 99:8084-8089.