Homeobox genes in neuroblastoma
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1

Introduction to neuroblastoma and scope of this thesis
1. Introduction

The elucidation of the transcription factor networks that are involved in the normal and carcinogenic processes in cells, may fuel new strategies for cancer treatment. Transcription factors (like homeobox genes) are the key regulators of cellular processes like cell proliferation, cell differentiation, and apoptosis. Consequently, mutation or mis-expression of transcription factor genes brings malfunction of these processes that can result in the oncogenic transformation of cells. The pediatric tumor neuroblastoma arises during embryonic development from elements of the neural crest sometimes even before birth. The involvement of homeobox genes in the normal development of neural crest is well established. Study of the participation of homeobox genes in neuroblastoma etiology is a feasible way to establish important events in neuroblastoma pathogenesis.

In this thesis, we set out to explore the potential role of three homeobox genes in neuroblastoma; PHOX2B, MEIS1, and MSX1. The signaling pathways of these homeobox genes in neuroblastoma are studied and target genes and pathways are further explored.

2. Neuroblastoma

Neuroblastoma is a solid neuro-endocrine tumor that can develop all along the peripheral sympathetic nervous system, in neural tissues in the neck, chest, abdomen, or pelvis, with a major site in the adrenal gland. Neuroblastoma tumors display a wide spectrum of clinical behavior; some tumors regress spontaneously whereas others are highly malignant, with a very poor prognosis despite intensive therapy [1,2]. The majority of neuroblastoma cases are sporadic, but some patients have a familial predisposition to the disease [3-5].

Approximately 5-10% of clinically detected neuroblastoma tumors regress spontaneously [1]. Mass screening studies in Japan, Europe and North America have led to the conclusion that the actual incidence of these regressing tumors is even higher [6-9]. This discrepancy is likely caused by a substantial number of patients who never develop symptomatic disease, as their tumors regress or mature before they become clinically apparent. The reason for spontaneous regression of these tumors, sometimes including their metastases, are still unclear, although delayed activation of normal differentiation or apoptosis pathways has been proposed as a causative mechanism[1]. On the other end of the clinical spectrum of neuroblastoma are aggressive tumors with adverse genetic features like amplification of the MYCN gene. Prognosis for these patients is poor, even with aggressive multimodal treatment. Only 10% of patients diagnosed with metastatic, MCYN-amplified, neuroblastoma have a 3-year event-free survival (EFS), in contrast with to up to 90% for patients without MYCN amplification [1,10].

Neuroblastoma accounts for 8-10% of cancers in children. In the Netherlands, approximately 25 neuroblastoma patients are diagnosed each year. Of these, 50 percent are children younger than two years of age. Neuroblastoma patients that have found to be completely disease-free after treatment often relapse within 2 years, and
then present with tumors that are frequently therapy-resistant [1]. Neuroblastoma thereby accounts for 15% of cancer death in children [1] Additionally, survivors of neuroblastoma have an increased risk of developing complications later in life related to long-term effects of treatment such as chronic health conditions [11], increased rate of mortality [11], occurrence of a second malignant neoplasms [11], decreased emotional health [12], academic and psychosocial problems [13], hearing loss [13] and severe gonadal impairment [14]. Neuroblastoma survivors with multimodal treatment are more than twice as likely to develop a chronic health condition as those who were treated with surgery alone [11]. Given this profound impact on the patient’s quality of life and the disappointing cure rate in high risk patients, further research is needed to better distinguish low-risk from high-risk tumors – thereby preventing excessive treatment -, and to better understand the molecular oncogenic process in the tumor cells, to develop more specific treatment methods.

2.1 Etiology of neuroblastic tumors

Neuroblastic tumors originate from elements of the neural crest, sometimes even before birth. It is believed that normal neural maturation fails, thereby giving rise to cancerous cells that continue to grow and divide. In infants less than 3 months old, small clusters of undifferentiated neuroblasts are often present that can mature into nerve cells or disappear spontaneously [1]. If however these undifferentiated neuroblasts continue to grow, they develop into neuroblastic tumors.

Based on histology (Figure 1), neuroblastic tumors are classified into four categories: ganglioneuroma (Schwannian stroma-dominant); ganglioneuroblastoma, intermixed (Schwannian stroma-rich); ganglioneuroblastoma, nodular (composite Schwannian stroma-rich/stroma-dominant and stroma-poor); and neuroblastoma (Schwannian stroma-poor) [15]. Ganglioneuromas, which are almost invariably benign, are well encapsulated non-cancerous tumors that do not invade or metastasize [15]. Ganglioneuroblastomas are in general benign and usually have a good prognosis [15]. Neuroblastoma is the most aggressive neuroblastic tumor, which can metastasize rapidly to other areas of the body [15].
2.2 Classification of neuroblastoma

Neuroblastoma patients are treated according to a risk classification based on age of the patient at diagnosis, INSS tumor stage (International Neuroblastoma Staging System), and MYCN copy number (Table 1) [1]. The therapy for these different risk categories is diverse. Low risk patients are frequently monitored, and undergo no or only minimal therapy [10], whereas intermediate risk patients are often treated with chemotherapy only. High-risk neuroblastoma patients are treated intensively with a combination of chemotherapy, surgery, radiation therapy, bone marrow/autologous stem cell transplants and/or biology-based therapies with cis-Retinoic acid [16]. Low and intermediate risk groups have a 5 year event-free survival of up to 90%, whereas for high-risk groups this is less than 50% [17].

2.3 Genomics of neuroblastoma

Several frequent chromosomal aberrations and abnormal patterns of gene expression are associated with patient prognosis. For some of these aberrations (candidate) oncogenes have been identified, e.g. MYCN (see below). In contrast, no neuroblastoma tumor suppressor gene has been characterized yet. Amplification of the MYCN gene is found in ~20% of neuroblastoma patients and strongly correlates with tumor aggressiveness and poor prognosis. Over-expression of the MYCN transcription factor was the first recurrent gene aberration found in neuroblastoma [18,19]. In these tumors, the region on chromosome 2p24, where the MYCN gene is located, is amplified and usually forms double minute chromosomes or homogeneously staining regions (HSRs). These regions may contain up to 500 additional copies of the gene [20]. Like all Myc family proteins, MYCN contains a Myc box and a basic helix-loop-helix (bHLH) domain which can dimerize with other bHLH proteins to allow binding to DNA target sequences. In this manner, MYCN regulates numerous target genes at the transcriptional level. MYCN functions as an oncogene: MYCN can cause neuroblastoma in a transgenic mouse model [21], whereas MYCN knock-down in neuroblastoma cell lines with MYCN amplification

Table 1: Neuroblastoma risk groups based on clinical and biological features

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tumor location</th>
<th>Low risk</th>
<th>Intermediate risk</th>
<th>High risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Localized tumor confined to the area of origin</td>
<td>All</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2A, 2B</td>
<td>Unilateral tumor with complete or incomplete gross resection</td>
<td>Age &lt;1 year, or Age 1–21 years and MYCN non-AMP, or age 1–21 years and MYCN AMP + FH</td>
<td>None</td>
<td>Age 1–21 years and MYCN AMP + FH</td>
</tr>
<tr>
<td>3</td>
<td>Tumor infiltrating across midline with or without regional lymph node involvement</td>
<td>None</td>
<td>Age &lt;1 year and MYCN non-AMP, or age 1–21 years and MYCN non-AMP + FH</td>
<td>Age 0–21 years and MYCN AMP, or age 1–21 years and MYCN non-AMP + UH</td>
</tr>
<tr>
<td>4</td>
<td>Dissemination of tumor to distant lymph nodes, bone marrow, bone, liver, or other organs</td>
<td>None</td>
<td>Age &lt;1 year and MYCN non-AMP</td>
<td>Age &lt;1 year and MYCN AMP, or age 1–21 years</td>
</tr>
<tr>
<td>4S</td>
<td>Localized primary tumor as defined in Stage 1 or 2, with dissemination limited to liver, skin, or bone marrow</td>
<td>MYCN non-AMP; FH, DI&gt;1</td>
<td>MYCN non-AMP; UH, DI=1</td>
<td>MYCN AMP</td>
</tr>
</tbody>
</table>
decreases their proliferation and/or induces differentiation [22].

Neuroblastomas are known to carry many chromosomal aberrations. Some of the recurrent defects have prognostic significance. In addition, correlations with patient prognosis are known for the following common chromosomal aberrations:

Deletion of one copy of the short arm of chromosome 1 (1p) occurs in 30-35% of all neuroblastoma tumors, the location of the smallest region of overlap (SRO) is at 1p36 [1]. 1p LOH (loss of heterozygosity) is more often observed in patients with advanced stage neuroblastoma. However, since there is a significant correlation between 1p LOH and MYCN amplification, the prognostic significance for 1p LOH alone remains controversial [20]. Several tumor suppressor candidates have been identified in this region such as CHD5 [23], KIF1Bbeta [24] and a tumor suppressing micro RNA, MIR-34A [25,26].

Allelic loss of chromosome 4p is observed in ~20% of neuroblastoma tumors [27]. 4p LOH does not correlate with INSS stage, 1p LOH, or MYCN amplification. An SRO on 4p16 was identified using 8 Italian neuroblastoma patients. [28]. Although the tumor suppressor gene of chromosome 4p has not yet been identified some LOH regions encompass the PHOX2B gene [29] which is found to be mutated in neuroblastoma [30,31]. Approximately one-third of the neuroblastoma patients have LOH of chromosome 11q. There is a significant inverse correlation between MYCN amplification and LOH of 11q [32]. LOH of 11q was found in 44% of cases in a cohort study of 295 neuroblastomas patients, where a common region of deletion mapped to 11q14-23. A correlation between 11q LOH and decreased overall survival probability is predominantly observed in patients with single-copy MYCN tumors [33]. Several putative tumor suppressor genes within 11q such as CADM1, H2AFX, TSLC1, IGSF4, SDHD, MLL, PPP2R1B, and ATM [34-37] have been identified.

Gain of the entire chromosome 17 or of a long segment of its q-arm is the most frequently observed genomic aberration in neuroblastomas, and occurs in up to 70% of tumors. 17q gain is often correlated with more advanced and aggressive tumors, thereby identifying high risk patients. Candidate tumor driving genes located within this region are NME1, PPM1D and BIRC5 [38-41]. Recent studies use these recurrent aberrations as a part of whole-genome genomic DNA and mRNA profiling to generate neuroblastoma tumor groups for risk stratification [42-45]. Other, less frequent, chromosomal regions have been described in neuroblastoma tumorigenesis: 2q, 3p, 5q, 9, 12, 14q, 16p and 18 [46-52]. For these aberrations, no correlations to clinical data have been found yet. In addition, it is unclear which genes within these regions might be involved in neuroblastoma development.

The best known gene implicated in neuroblastoma pathogenesis is MYCN. Although characterized as a major oncogene in neuroblastoma over 20 years ago, still little is known about its downstream oncogenic pathways. During normal development MYCN is expressed at high levels in the central nervous system, neural crest-derived tissues and several other tissues [53]. MYCN null-mutant mice die before birth and display numerous defects including a reduced amount of neuronal cells
Introduction

[54]. The protein is a major regulator of neuronal cell proliferation, its expression is normally down-regulated upon tissue differentiation during the final stages of nervous system development [55]. Myc oncogenes regulate the expression of several genes involved in cell cycle progression, in accordance with the general pro-proliferative effect of myc family members [56-58]. Recently, direct regulation of the Minichromosome Maintenance Complex (MCM) by MYCN in neuroblastoma was established [57]. This complex is important for initiation and elongation of DNA replication and genome integrity [59]. In addition, MYCN suppresses the expression of the inhibitors of the WNT signaling pathway DKK1 and DKK3 [60,61]. Furthermore, there is a high correlation between DKK3 expression and the differentiation status of neuroblastic tumors [61]. Although no genetic aberrations of WNT pathway members have been linked to neuroblastoma, abnormal nuclear localization of β-catenin in neuroblastoma lines lacking MYCN amplification has been reported [62].

The TRKA and TRKB tyrosine kinase receptor genes are well established prognostic factors for neuroblastoma [1]. Expression of TRKA is highly correlated with low and intermediate risk neuroblastoma tumors and is a favorable indicator for patient survival [63,64]. On the other hand, expression of TRKB causes enhanced proliferate capacity of neuroblastoma and is therefore associated with a more aggressive tumor type [64,65].

Amplifications of the CCND1 cell-cycle oncogene are found in about ~3% of neuroblastoma [66], next to sporadic genomic aberrations in CDK4 and CDK6 [67,68]. Indeed, CCND1 and CDK4 expression are important in maintaining the undifferentiated phenotype of neuroblastoma cells [69]. In addition, silencing CDK2 expression in MYCN-amplified neuroblastoma cells results in a synthetic lethal phenotype [70].

We and others have reported mutations in the PHOX2B homeobox gene in about 3% of sporadic neuroblastoma [30,31]. PHOX2B was also found as the first predisposing gene to hereditary neuroblastic tumours; PHOX2B germline mutations occur in some, but not all, pedigrees with familial neuroblastoma [3,4,31].

Recently the ALK tyrosine kinase receptor gene was found to be mutated in 7% of both sporadic and familial neuroblastoma tumors [5]. Mutations are located within the tyrosine kinase domain of ALK, establishing it as a critical oncogene in neuroblastoma since these mutations resulted in constitutive activation [5].

Overall, only few genes have been identified as implicated in neuroblastoma, providing only limited insight into the molecular pathways involved in neuroblastoma pathogenesis. The identification of additional genes important for neuroblastoma therefore remains essential. We reasoned that, neuroblastoma being an embryonic tumor of the sympathetic nervous system lineage, genes with a key role in its differentiation would be interesting to study.
3. The neural crest

3.1 Origin and development of neural crest cells
Neuroblastoma tumors arise from neural crest-derived cells of the developing sympathetic nervous system. The neural crest is a short-lived structure present in developing vertebrate embryos during neurulation. The neural crest gives rise to a population of migrating cells that can differentiate into a vast array of cell types such as neurons and glia of the autonomic and peripheral nervous systems, pigment cells, the outflow tract of the heart, and the facial skeleton [71]. Neural crest cells become first apparent during the process of neurulation. This process is initiated by the formation of a neural plate by a thickening of the ectoderm (Figure 2). Subsequently, the edges of the plate fold and rise, meeting in the midline to form a tube. The cells at the tips of the neural folds come to lie between the neural tube and the overlying epidermis and give rise to neural crest cells. The neural crest is then situated in between the closed neural tube and the epidermis of the developing embryo. Next, neural crest-derived cells migrate away from the dorsal surface of the neural tube, to give rise to a diverse set of cell types [71]. The spatial position of neural crest cells along the embryonic axis, in combination with signals from their micro-environment, dictates their migration route and cell fate (Figure 3). Based on function, four main categories exist: cranial, vagal and sacral, cardiac and trunk neural crest-derived cells [74]. Cranial neural crest-derived cells

Figure 2: MSX induction and function during neural crest development.
(a) Initial induction of MSX expression in the neural folds (dark blue) is the consequence of a BMP signal from the lateral ectoderm (light blue). (b) After closure of the neural tube, MSX genes are expressed at the dorsal midline. They are necessary to induce the expression of e.g. WNT1 and BMP6; these in turn signal to the dorso-lateral domains, and induce the expression of e.g. Pax3, Pax6, Pax7 and Lim1. In this model, MSX genes are intermediates between BMPs and Wnts (reproduced from [72]).
migrate from the midbrain and hindbrain throughout the head of the developing embryo, giving rise to the cranial bone cells, and neurons of the central nervous system. The cells from the neck and tail form the vagal and sacral neural crest, and differentiate into the parasympathetic neurons that innervate the gut. Both the vagal and cardiac neural crest-derived cells contribute to the development of the heart. Finally, trunk neural crest cells give rise to pigment cells of the skin, the neurons of the sympathetic nervous system, and the epinephrine-producing cells of the adrenal medulla [75].

Neuroblastoma originates from the sympatho-adrenal progenitor cells which develop from the trunk neural crest cells. These sympatho-adrenal progenitors further differentiate under influence of signals derived from e.g. the somites, the ventral neural tube, and the notochord, to form the secondary sympathetic ganglia, the pre-vertebral ganglia, the adrenal medulla and extra-adrenal chromaffin tissues, e.g. the organ of Zuckerkandl [76]. For several genes which are involved in the differentiation of the sympatho-adrenal progenitor cells a role in neuroblastoma has
been established. The PHOX2B and Notch signaling pathway are among these and will be discussed in more detail below and in chapters 2 and 5 of this thesis.

3.2 Neural crest cell differentiation

Two mechanisms have been proposed to drive neural crest differentiation. Both models imply that the early neural crest population is composed of multi-potent progenitor cells which undergo progressive lineage restrictions [71,74,77,78]. The first model predicts that the final specification and colonization of neural crest cells depends on the signals they receive from their micro-environment. Support for an environmentally induced differentiation of neural crest cells is derived from studies with metastatic melanoma cells. These cells resemble a multi-potent, dedifferentiated cell type like those of the neural crest [79]. Transplantation studies of GFP-labeled human metastatic melanoma cells in embryonic chicken showed that these tumor cells were able to invade and migrate along neural crest migratory routes [80]. The metastatic melanoma cells did not form tumors and some expressed melanocyte and neuronal markers undetected at the time of transplantation [80]. This indicates that the micro-environment influences the behavior of the otherwise highly malignant melanoma cells. Similar results were obtained using mouse melanoma cells transplanted into the neural tube of chicken [81]. These cells successfully migrated to the neural crest micro-environment. However, these melanoma cells underwent apoptosis during migration along the neural crest routes [81]. In contrast, injection of the same cell suspension into the eye cup of the chicken embryo did result in invasive melanomas. This suggests that the micro-environment of the neural trunk is able to control the highly malignant melanoma cells, whereas the micro-environment of the eye could not. In summary, these results suggest that exposure to specific micro-environments results in differentiation of the multi-potent neural crest cells.

The second model assumes a pre-programmed migratory routing and differentiation program potential of neural crest cells that is acquired before leaving the neural tube. In support of this, the TRKC-expressing cells emerging from the neural tube in quail never give rise to melanocytes, whereas the c-Kit expressing cells solely produce melanocytes [82]. This suggests that neural crest cells are pre-programmed to a specific cell faith. A similar fate restriction was observed for a specific subpopulation of the neural crest cells in chicken, which solely give rise to the nociceptor sensory neuron population [83]. In a different study, two distinct neural crest-derived cell populations were isolated and subsequently transplanted into the developing gut wall of chicken. Only the neural crest-derived cells isolated from the gut were able to migrate and form neurons, whereas sciatic nerve neural crest-derived cells could not [84]. In summary, these studies suggest that the neural crest cells fate is predetermined and the micro-environment is not able to influence this preset differentiation pattern.

Although the above two models appear mutually exclusive, there is increasing evidence that neural crest cells differentiation is driven by interactions of extrinsic as well as intrinsic programs. Many studies demonstrate heterogeneity in the neural crest population. For example, a labeling study using single migrating neural crest cells in the trunk of the chicken embryo resulted in either multiple or single neural crest derivates [85]. Another study in Axolotl showed that the dorso-laterally migrating
neural crest cells are committed to form pigment cells, whereas those from the anterior trunk neural folds can also contribute to glia and neuron cells [86]. The presence of both multi-potent and restricted progenitor cells co-existing within the population supports both models. It seems that genes induced by the micro-environment as well as genes expressed by the specific neural crest cells themselves are responsible for the proper activation and silencing of signaling pathways. Triggering neural crest cells to differentiate could therefore be accomplished by both changing the micro-environment or the multi-potent cells themselves.

3.3 Pathways and genes in neural crest development
There are three main regulatory pathways involved in the closure of the neural folds and subsequent rise of the neural crest cells: the bone morphogenetic protein (BMP) pathway, the fibroblast growth factor (FGF) pathway, and the WNT signaling pathway [71,74,87]. The crucial role for BMP, FGF and WNT signaling in neural crest formation has been established in various model organisms [71,88]. In general, BMP, FGF and WNT molecules are secreted from the underlying mesoderm and adjacent non-neural ectoderm (Figure 2) and function simultaneously to segregate neural from non-neural ectoderm during neurulation.

The effect of BMPs is determined by the expression level and timing of these secreted proteins during neurulation [89]. High levels in the neural plate induce epidermis formation, intermediate levels results in neural crest cells, and low levels give rise to neural tissue [74]. Up-regulation of BMP in non-neural ectoderm triggers the epidermal differentiation program. This results in an increase of MSX1 protein levels at the border of the neural plate [90,91], which on its turn stimulates BMP expression further [91-93]. Although BMP is well established as an inductive signal for the formation of neural crest, several studies have shown that BMP alone is not sufficient [87,89,94-96].

In addition to BMP signaling, an FGF signaling cascade is necessary to induce neural crest [97,98]. FGF is secreted by the mesoderm [97,98]. The importance of an FGF signal for neural crest formation is demonstrated by the loss of expression of neural crest markers after over-expression of a dominant negative FGF receptor [99]. Even in the absence of mesoderm induction, FGFs are able to stimulate neural crest induction [100]. Co-expression of a WNT inhibitor in Xenopus inhibits the ability of FGF to induce neural crest markers [95], suggesting that the neural crest induction by FGF is a secondary effect of its ability to induce WNT expression [74].

WNT proteins are sufficient to induce neural crest markers in isolated neural tissue, whereas BMPs require additional factors [87,88,95]. The neural crest-inducing signal derived from the non-neural ectoderm is widely believed to be a WNT protein [71,101]. Since WNT1 expression is lost in MSX1/MSX2 double null-mutant mice, BMP-induced MSX1 expression is believed to be necessary for WNT1 induction [90]. In agreement with this hypothesis, ectopic expression of MSX1 induces WNT1 expression in the chicken brain and lateral ectoderm [90]. On its turn, MSX1 expression levels are positively regulated by both BMP and WNT proteins [102,103]. Hence, MSX genes play a central role acting as intermediate signaling molecules between the BMP and WNT signaling pathways in early neural crest development. While the WNT, FGF and BMP signaling pathways induce neural crest formation
transcription factor genes such as ASCL1, PHOX2B, MSX1, NOTCH, and the proto-oncogenes MYC and MEIS1 are important for neural crest specification. These pre-migratory neural crest marker genes are necessary for the initiation of neural crest migration, maintenance and specification. For instance, while over-expression of MEIS1 induces neural crest markers [105], over-expressing of activated Notch abrogates neural crest marker expression resulting in a loss of neural crest derivatives [106]. Expression of ASCL1 commits cells to a neuronal fate, and subsequent PHOX2B expression then specifies these cells as neurons of the autonomous nervous system, upon which they become migratory [107]. Sympathetic neurons of the peripheral nervous system achieve their final identity by the expression of the tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), both key enzymes for catecholamine synthesis [76].

Since the list of genes expressed by pre-migratory neural crest cells is still growing, the relationship between the genes involved in neural crest differentiation could not yet be fully characterized. Nevertheless, it is apparent that a delicate balance between gene repression and activation in pre-migratory neural crest cells is needed for a proper development of neural crest-derived cells and tissues. Disturbance of this balance has been shown to result in aberrant differentiation in model organisms like *Xenopus* and chicken. The pathways (like WNT and NOTCH) and genes involved are therefore extremely interesting candidates to study in neuroblastoma.

### 3.3.1 The WNT pathway

The WNT signaling pathway involves a complex network of proteins and plays an important role in embryogenesis and cancer. WNT signaling can act along at least three different pathways: the canonical WNT/β-catenin cascade, and the non-canonical planar cell polarity (PCP) and WNT/Ca²⁺ pathways.

WNT proteins such as WNT1, -3a and -8, bind a receptor complex consisting of a member of the Frizzled family of trans-membrane receptors (Fz), and a low-density lipoprotein receptor-related protein family member (LRP-5 or LRP-6) [108,109]. Formation of this ternary complex is necessary to activate the canonical WNT pathway (Figure 4), in which Fz interacts with Dishevelled (Dvl) resulting in Dvl phosphorylation by WNT. WNT also promotes LRP phosphorylation which subsequently recruits Axin [110]. Next, Dvl forms a complex with Axin resulting in disruption of the cytoplasmic protein complex that normally targets β-catenin for destruction by the proteasome. This stabilization of β-catenin enables its nuclear translocation where it activates TCF-mediated transcription of target genes [109], such as the CCND1 [111] and MYC [112] genes (see also 2.3).

Besides the canonical WNT/β-catenin pathway, several β-catenin independent ‘non-canonical’ WNT signaling pathways are known: Protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CamK) can be activated by several WNT and Fz proteins [108,109], in the WNT-Ca²⁺ pathway. Another β-catenin independent WNT pathway is the planar cell polarity pathway which activates the Jun-N-terminal kinase (JNK) via an Fz receptor [108]. This signaling cascade controls cell movement and polarity, partly via activation of the JNK signaling pathway.

In addition to the WNT proteins, several other proteins can bind the Fz and/or LRP receptors and activate the canonical WNT pathway: Norrin binds with high
In the presence of WNT proteins, a protein complex between WNT, Fz and/or LRP is formed on the membrane. This results in activation of DVL protein by phosphorylation. Activated DVL in its turn activates further downstream pathways. In the canonical pathway DVL enables the binding to Axin resulting in a disruption of the APC/Axin/GSK3β destruction complex, and leading to stabilization of the β-catenin protein. This results in accumulation and subsequent translocation of β-catenin to the nucleus. Here, β-catenin binds to TCF, displacing the Groucho-TCF repressor complex. As a result, TCF becomes transcriptionally active inducing expression of target genes. The Ca²⁺ pathway inhibits the canonical Wnt pathway, and controls cell migration. The PCP pathway is involved in cell polarity regulation and also in cell migration. Proteins discussed in this thesis are depicted in grey (reproduced from [113]).
affinity to Fzd4 and subsequently activates the canonical signaling pathway [114], and R-spondins can interact with and induce signals through either LRP6 or Fzd8 [109,115-117]. Finally, several inhibitory Fz/LRP-binding gene families exist: The secreted Dickkopf (Dkk) proteins interfere with the WNT signaling by direct binding to LRP5/6. The human DKK family consists of four members, DKK1-4 [118]. Binding of DKK1 to LRP6 prevents the WNT-Fz interaction and/or LRP6 endocytosis in the presence of the DKK1 co-receptor Kremen [118]. Although binding of DKK2 to LRP6 can sometimes lead to activation of the canonical WNT pathway, in general the effect of DKKs on the WNT signaling pathway is inhibitory [118]. Also the soluble frizzled-related proteins (SFRPs, in humans SFRP1-5 exist) and WNT inhibitory factor (WIF1) can inhibit the WNT signaling pathway by direct binding to WNT proteins [119]. SFRPs share sequence similarity with the cysteine-rich domain (CRD) of the WNT receptor Fz, WIF1 protein resembles the extracellular portion of the derailed/RYL class of trans-membrane WNT receptors (reviewed in [109]). It has been suggested that WIF1 might also promote WNT signaling by WNT stabilization or by facilitating WNT secretion or transport [109].

3.3.2 The Notch Pathway
The Notch signaling pathway plays an important role in the differentiation of the sympathetic nervous system [120,121]. Activation of the Notch signalling pathway in the vertebrate central nervous system inhibits neuronal differentiation, whereas Notch repression causes increased neuronal differentiation [121]. Vertebrates encode four Notch receptors (Notch1-4) and at least 5 ligands (Jagged1 and 2, and Delta-like 1, 3 and 4) [122]. The Notch receptors are single-pass transmembrane proteins. Next to their extracellular, ligand-binding domain, Notch proteins consist of a trans-membrane segment and a cytoplasmic domain containing a nuclear localization signal [122,123].

Interaction of the extracellular domain of Notch on one cell with a ligand receptor on an adjacent cell results in cleavage of the Notch receptor by an ADAM-family metallo-protease just outside the plasma membrane (Figure 5) [124]. The released extracellular portion of Notch continuous to interact with its ligand and is taken up through endocytosis by the ligand-expressing cell. The remaining part of the intracellular domain is cleaved by the γ-secretase protein complex consisting of presenilin, nicastin, PAN2, and APH1 [123]. After cleavage, the Notch intra-cellular domain translocates to the nucleus where it interacts with the transcription factor CSL and causes displacement of bound corepressors and recruitment of co-activators such as mastermind like proteins (MAML) and p300 [122,123]. The now transcriptionally active CSL induces expression of the Enhancer of Split complex genes (in human HES1-7, HEY1-2 and HEYL) [125] by interacting with CSL-binding sequences in their promoters [126,127]. The Enhancer of Split helix-loop-helix transcription factors in turn, repress achaete-scute genes (like ASCL1) which regulate the Delta genes [127,128]. Expression of the ligand on one cell thereby leads to the removal of cell-surface ligand expression on a neighbouring cell. This mechanism of ‘lateral inhibition’ induces the generation of two different cell
Introduction

types from a single precursor cell type, one expressing ligand and the other one expressing the receptor.

A link between the Notch pathway and human cancers has been established in numerous tumor types including cervical and lung cancer, leukemia and neuroblastoma [122,129]. Since the Notch signaling pathway is involved in a wide variety of cellular processes, including stem cell maintenance, cell fate specification, differentiation, proliferation, and apoptosis, it is an important regulatory switch in oncogenic transformation. The role of the Notch pathway in cancer was first described in human T-cell leukemia, where activating mutations within Notch1 have been identified in >50% of tumors [130]. Here, aberrant Notch signaling promotes tumorigenesis. Through interaction with other proteins and signaling pathways (e.g. the WNT pathway) in other tumors, it can also function as a tumor suppressor gene [122].

In the chromaffin cells of the normal adrenal medulla, as well as in several neuroblastoma cell lines, the Delta homologue DLK1 is highly expressed [131]. In addition, NOTCH3 expression shows an inverse correlation with DLK1 in neuroblastoma cell lines. Together, this suggests that neuroblastomas are related to the chromaffin differentiation lineage of the sympatho-adrenal neural crest, and that the Notch signalling pathway is involved in this differentiation. Indeed, over-expression of dominant negative Notch1 in a mouse neuroblastoma cell line induced differentiation, while over-expression of Notch1 resulted in a decreased differentiation [132,133]. Also blocking γ-secretase activity in a human neuroblastoma cell line led to neuronal differentiation [134]. An important conclusion that can be drawn

Figure 5: The Notch pathway
The ligands Jagged or Delta bind to the Notch receptor on an adjacent cell which results in activation of the pathway. Two proteolytic cleavages of the Notch receptor, by ADAM and subsequently by the γ-secretase complex, mediate the release of the intra-cellular Notch (ICN) domain. Nuclear translocation of the ICN enables the interaction with the transcription factor CSL dissociating it from co-repressor molecules (CoR/HDAC). ICN recruits co-activators (CoA) like mastermind (MAML) and histone acetyl transferase (HATs), giving rise to transcriptional activation complex-inducing genes like HEY1 (adapted from [123]).
is that modification of the Notch signalling pathway can induce differentiation of neuroblastoma which will be further discussed in chapter 2. The Notch cascade might be a suitable target for therapy.

3.3.3 Homeobox genes
The involvement of homeobox genes in proliferation and differentiation of neural crest cells is well established [71,74,78,102,105], however understanding of their downstream target genes is scarce. In general, homeobox proteins regulate the balance between cell proliferation and differentiation [135]. Homeobox proteins regulate the expression of large numbers of target genes by direct binding to their promoters. This interaction is mediated via their homeodomain, a conserved 60 amino acid domain. With the help of additional (often also homeobox-containing) transcription factors, homeobox proteins bind to target sequences, thereby activating or repressing target gene transcription [135]. Their generally broad DNA-binding specificity makes the identification of the downstream target genes challenging. Deregulated homeobox gene expression has been reported in many types of cancer, with activities that can be either tumor-suppressing or -promoting [136-144]. Knowledge of the specific cellular function of homeobox genes will not only elucidate their role in normal development, but may also explain the oncogenic cellular events caused by deregulation of these genes. The involvement of homeobox genes in neuroblastoma pathogenesis is almost completely unknown; below we will discuss three homeobox genes that are the topic of this thesis.

PHOX2B
The PHOX2B transcription factor is exclusively expressed in the nervous system, both in peripheral and central noradrenergic neurons. The protein is important during the differentiation of neural crest cells into chromaffin cells and sympathetic neurons. Both cell types are believed to share a common sympa-tho-adrenal progenitor stem cell [145,146]. PHOX2B null-mutant mice die before birth, but can be rescued from death by the administration of noradrenergic agonists to the mother throughout gestation [147]. In these PHOX2B null-mutant mice, autonomic ganglia are not properly formed [148]. PHOX2B null-mutant mice have aberrant expression of ASCL1, DBH), and TH [148], which are all implicated in chromaffin cell and sympathetic neuron development. The findings suggest that PHOX2B plays a role in the differentiation of noradrenergic cells derived from neural crest in vertebrates [76].

MSX1
The muscle segment homeobox (MSX) gene family is one of the most highly conserved families of the homeobox-containing genes [72]. The human MSX gene family consists of two members, MSX1 and MSX2. During embryogenesis, the MSX1 protein acts as a transcriptional repressor via interactions with other homeoproteins and components of the core transcription complex. MSX1 expression induction by BMP proteins marks one of the earliest events in the neural crest specification [149-152]. MSX1 induces expression of early neural crest cell markers such as snail, slug, and FOXD3 [153]. Inhibition of MSX1 expression causes reduced early neural crest marker expression, whereas its up-regulation induces expansion of the neural
crest cell population [153]. Indeed, MSX1/MSX2 double null-mutant mice showed diminished cell population of the neural crest [154].

**MEIS1**
MEIS1 belongs to the TALE (three amino acid loop extension) family of homeodomain-containing proteins. This homeodomain has a three amino acid loop extension (TALE) between the first and second α-helix of the homeodomain, allowing the binding of other homeodomain-containing proteins for the formation of multi-protein complexes [155].

MEIS1 protein interacts with several HOX proteins [156-159]. In addition, direct interaction of MEIS with PBX, another member of the TALE homeobox family, results in increased nuclear PBX localization [160-163]. Finally, more complex binding was established by the identification of HOX-PBX-MEIS heterotrimeric complexes [164]. In summary, MEIS1 cooperatively binds DNA target sequences in multi-protein complexes containing PBX and HOX (Figure 6).

MEIS1 expression in mice, while present in all stages of embryonic development, is highest in the late stages of nervous system development, suggesting a role in differentiation [105,165]. Ectopic expression of MEIS1 in the *Xenopus* neural tube activates marker genes involved in different stages of neural crest development. Intriguingly, mis-expression of MEIS1 in *Xenopus* also induces oncogenic transformation and can even result in motile, cancer-like masses along the neural tube [165]. Thus, MEIS1 can regulate the differentiation and proliferation of neural crest cells.

### 4. Neuroblastoma genes and pathways in this study

The characterization of genes driving neural crest specification and differentiation can be expected to yield insights in neuroblastoma pathogenesis. In this thesis, we focus on the role of the homeobox transcription factors PHOX2B, MSX1 and MEIS1 which will be discussed below.

#### 4.1 PHOX2B
The *PHOX2B* gene contains two poly-alanine repeats of 9 and 20 residues located near the C-terminus of the protein. A 5-13 residue expansion of the 20 alanine tract is the major cause of Congenital Central Hypoventilation Syndrome (CCHS), which...
causes respiratory arrests during sleep [166-169]. Besides the expansion of the alanine tract, several patients with CCHS have mutations within the C-terminus of PHOX2B. Since two of these patients were also diagnosed with neuroblastoma, our laboratory determined the PHOX2B sequence in a large series of neuroblastoma tumors and cell lines. These studies resulted in the identification of six different mutations in 237 neuroblastoma tumors and 22 neuroblastoma cell lines [30]. Five mutations were in, or close to, the poly-alanine tract and one mutation was identified near the central homeodomain. Intriguingly, all these mutations result in frame-shifts, differing from the mutations found in CCHS that did not cause PHOX2B translation frame-shifts [167-169]. Additional PHOX2B mutations have been identified in two other neuroblastoma families [3,4]. Furthermore, PHOX2B expression is a specific and sensitive marker for minimal residual disease in neuroblastoma patients [170]. Finally, several studies have linked the PHOX2B transcription factor to TRKA, to the Delta-Notch pathway and to the (nor-) adrenal synthesis route [148,171,172].

4.2 MSX1
MSX gene functions are diverse, ranging from control of cell proliferation to apoptosis [72,173,174]. MSX1/MSX2 double null-mutant mice showed diminished cell population of the neural crest as described above [154], and human and chicken dermis cells lose proliferative capacity upon down-regulation of MSX1 and MSX2 expression [175,176]. Induced expression of MSX1 can even cause de-differentiation and renewed proliferation in terminally differentiated murine myotubes [177]. These results show that MSX genes are important for cell survival. In contrast, over-expression of MSX2 caused apoptosis in cranial neural crest-derived cells in mice [178], and in vitro in cell lines [179]. Depending on their environment MSX genes can either stimulate cell growth or induce differentiation. Since these characteristics are classically associated with either oncogenes or tumor suppressor genes, MSX genes have the potential to behave as both. Not surprisingly therefore, the MSX gene family has been implicated in tumorigenesis, in e.g. breast and cervical cancer [180-182]. Also, MSX1 transgene expression in ovarian cancer cells results in decreased proliferation and induction of G1 cell cycle arrest [174]. The MSX1 gene maps to the short arm of chromosome 4, in a region that is frequently deleted in neuroblastoma tumors [27,28]. In addition, MSX1 is located near an SRO in familial neuroblastoma [28].

4.3 MEIS1
The MEIS1 locus was first isolated as a common site of viral integration for the murine leukemia virus. These integrations caused MEIS1 over-expression and tumor formation in BXH-2 mice [183]. The MEIS1 homeobox transcription factor has since been implicated in tumor development in several human tissues [184-186], although the role of MEIS1 in oncogenesis is best characterized in leukemia. Intriguingly, high MEIS1 expression in bone marrow cells of AML patients is observed in combination with high expression of another homeobox gene HOXA9 [187,188]. We and others have identified MEIS1 amplification in different neuroblastoma cell lines; IMR32 and CHP134 [189-192]. MEIS1 over-expression was established in almost all neuroblastoma tumors and cell lines investigated [189,191-193], but little is known
about a role for MEIS1 in neuroblastoma pathogenesis which will be discussed in chapter 4 of this thesis.

5. Scope of this thesis

In this thesis, we set out to explore the potential role of three homeobox genes in neuroblastoma; PHOX2B, MEIS1, and MSX1. The signaling pathways of these homeobox genes in neuroblastoma are studied and target genes and pathways are further explored.

The homeobox gene PHOX2B can be mutated in neuroblastoma tumors. During normal development, PHOX2B is involved in the differentiation of neural crest cells into chromaffin cells and sympathetic neurons. Failure of the neural crest cells to differentiate into the appropriate cell type might have disastrous consequences for the cell. To shed light on the role of PHOX2B in neuroblastoma, an SJ-NB-8 neuroblastoma cell line with tetracycline-inducible PHOX2B expression was generated, and Affymetrix gene expression profiles were analyzed. These studies are presented in chapter 2, and identified MSX1 as a target of PHOX2B.

To study the role of MSX1 in neuroblastoma, we constructed two neuroblastoma cell lines capable of inducible MSX1 transgene expression. Studies with the SJ-NB-8 MSX1-expressing cell line revealed links of MSX1 with the Delta-Notch and WNT pathways, which are described in chapters 2 and 3.

The transcription factor MEIS1 is amplified or over-expressed in neuroblastoma. The TALE and HOX homeobox genes that form a transcription complex with MEIS1 are highly expressed in neuroblastoma, indicating that the MEIS1 signaling cascade is intact. In chapter 4 we assessed the MEIS1 target gene network by the analysis of Affymetrix gene expression profiles of 110 neuroblastic tumors (83 neuroblastomas, 14 ganglioneuroblastomas and 13 ganglioneuromas). Potential target genes were further analyzed using an IMR-32 neuroblastoma cell line capable of inducible MEIS1 shRNA expression.

The links between MSX1 and the Delta-Notch pathway are further explored in chapter 5. There we find that the MSX gene governs an important part of the downstream network of NOTCH3 transcription factor in the IMR-32 neuroblastoma cell line. Both NOTCH3 and MSX1 have a dramatic effect on the cell cycle. The importance of this link in neuroblastoma tumors is discussed.

Finally, in chapter 6, we try to integrate the findings of the individual chapters of this thesis into a unifying model that explains parts of the transcription factor network underlying the differences between the distinct neuroblastic tumor types.
Introduction


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