Title of the paper

Origin and initiation mechanisms of neuroblastoma

Authors and affiliation

Shoma Tsubota, Kenji Kadomatsu

Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan

Corresponding author: Kenji Kadomatsu, M.D., Ph.D., Department of Biochemistry, Nagoya University Graduate School of Medicine, 65 Tsurumai-Cho, Showa-Ku, Nagoya, 466-8550, Japan. Phone: +81-52-744-2059; Fax: +81-52-744-2065; E-mail: kkadoma@med.nagoya-u.ac.jp

Abstract

Neuroblastoma is an embryonal malignancy that affects normal development of the adrenal medulla and paravertebral sympathetic ganglia in early childhood. Extensive studies have revealed the molecular characteristics of human neuroblastomas, including abnormalities at genome, epigenome, and transcriptome levels. However, neuroblastoma initiation mechanisms and even its origin are longstanding mysteries. In this review article, we summarize the current knowledge about normal development of putative neuroblastoma sources, namely sympathoadrenal lineage of neural crest cells and Schwann cell precursors that were recently identified as the source of adrenal chromaffin cells. A plausible origin of enigmatic stage 4S neuroblastoma is also discussed. With regards to the initiation mechanisms, we review genetic abnormalities in neuroblastomas and their possible association to initiation mechanisms. We also summarize evidences of neuroblastoma initiation observed in genetically engineered animal models, in which epigenetic alterations were involved, including transcriptomic up-regulation by N-Myc and down-regulation by polycomb repressive complex 2. Finally, several *in vitro* experimental methods are proposed

that hopefully will accelerate our comprehension of neuroblastoma initiation. Thus, this review summarizes the state-of-the-art knowledge about the mechanisms of neuroblastoma initiation, which is critical for developing new strategies to cure children with neuroblastoma.

Key words

Neuroblastoma, Neural crest cells, Schwann cell precursors, Sympathoadrenal progenitors, MYCN

Acknowledgments and Funding information

S. Tsubota was supported by a Grant-in-Aid for JSPS Research Fellow (14J00157). K. Kadomatsu was supported by grants for the Practical Research for Innovative Cancer Control from Japan Agency for Medical Research and Development (16ck0106011h0003), JSPS KAKENHI Grant Number JP15k15079, and CREST, JST (15656320).

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

Introduction

Although extensive studies have revealed genomic abnormalities and inter- and intratumoral heterogeneities in neuroblastomas, the mechanisms of its genesis are still largely unknown. Careful observation of spatiotemporal expression profiles and functions of the genes related to the normal development of putative neuroblastoma sources (i.e., adrenal chromaffin cells and sympathetic neurons), derived from the neural crest, is fundamental for understanding the initiation of neuroblatoma tumorigenesis. Sympathoadrenal lineage of the neural crest has been considered to be the plausible cellular origin of neuroblastomas, as summarized in a recent detailed review (Cheung and Dyer 2013). However, the normal development of adrenal chromaffin cells was recently revised by Furlan and colleagues (Furlan et al. 2017) and that study raised a penetrating question about the etiology of neuroblastoma. Meanwhile, proper experimental settings, such as *in vitro* cell culture or genetically engineered animal models, enable functional characterization of oncogenes or tumor suppressor genes in neuroblatsoma, and thus provide a significant insight into the unknown mechanisms of neuroblastoma initiation.

In this review, we will summaize putative cellular origins of neuroblatsoma. Possible mechanisms of neuroblastoma initiation and methodological approaches to functional characterization of the genes of interest will also be discussed.

Inter- and intratumor heterogeneity in neuroblastoma

Neuroblastoma can develop anywhere in the sympathetic nervous system, e.g., in the adrenal gland or in sympathetic ganglia, and it is characterized by significant intertumor heterogeneity. Recently published data from a large international patient cohort showed that neuroblastomas are frequently observed in the adrenal (47%, n = 3966/8369) and abdominal/retroperitoneal regions (24%, n = 1991/8369), as well as observed in the neck (2.7%), thoracic (15%), pelvic (3%), and other regions (7.9%) (Vo et al. 2014). Notably, patients with adrenal tumors showed certain biological and clinical

features that indicated higher malignancy, including *MYCN* amplification (odds ratio: 2.09) and poor prognosis compared to individuals with non-adrenal tumors. An independent cohort also reported distinct genomic profiles and patient outcomes in tumors of different origins (Brisse et al. 2017). Distinct clinical features of the tumors developed in different sites may be explained by the differences in developmental programs of adrenal chromaffin cells and paravertebral sympathetic neurons (Huber 2015).

Two groups recently reported intratumor heterogeneity at the cellular and molecular levels in neuroblastoma (Boeva et al. 2017; van Groningen et al. 2017). There are different types of neuroblastoma cell lines: neuroblastic (N-type), non-neuronal Schwann cell-like (S-type), and morphologically intermediate (I-type) cells, which can be established in an ordinary serum-containing medium (Ciccarone et al. 1989). mRNA expression profiling of neuroblastoma cells from patient-derived fresh tumors cultured using serum-free neural stem cell medium showed that such cells were more related to primary tumors than classical cell lines (Bate-Eya et al. 2014). Furthermore, the same research group established those cell lines from the same patient (van Groningen et al. 2017). They revealed two types of cells within the same tumor, namely undifferentiated mesenchymal cells (MES-type) and committed adrenergic cells (ADRN-type), which showed divergent transcriptomic profiles and super-enhancer-associated transcription factor networks, whereas genetic abnormalities were identical in both types of cells. Importantly, MES and ADRN signature scores based on transcriptomic profiling demonstrated that MES-type cells were similar to human neural crest-derived cell lines, suggesting that MES-type cells are more primitive than ADRN-type cells. In a similar study, super-enhancers and core regulatory circuitries controlling transcriptional program across 25 classical neuroblastoma cell lines and human neural crest cell lines have been recently investigated (Boeva et al. 2017). As in the study by Groningen and colleagues, two distinct cell groups were also observed and one group, including GIMEN, SH-EP, and GICAN neuroblastoma cell lines, resembled human neural crest cell lines. Collectively, the presence of

neuroblastoma cells resembling neural crest cells (NCCs) implies that either NCCs are one of the neuroblastoma cellular sources or ADRN-type cells de-differentiate to MES-type cells, and such transformed cells may generate intratumor heterogeneity in a tumor mass. Considering these inter- and intratumor heterogeneities, the basic knowledge about the development of putative cells that give rise to neuroblastoma, i.e., sympathoadrenal lineage of NCCs and Schwann cell precursors (SCPs, discussed below), is a prerequisite for understanding the mechanisms of neuroblastoma initiation.

Cellular sources of neuroblastoma

Development of sympathoadrenal lineage

The neural crest is a transient cell population in vertebrates that gives rise to various cell populations. Its formation, namely the induction of neural crest and specification of derived cells, is regulated by a complexed gene regulatory network (Sauka-Spengler and Bronner-Fraser 2008). SoxE transcription factors are expressed during neural crest specification in pre-migratory, delaminating, and migrating NCCs, and regulate the differentiation of NCCs into sympathetic neurons, parasympathetic neurons, enteric neurons, sensory neurons, glial cells (Schwann cells and satellite glia), cartilage in the cranial region, and melanocytes (Sauka-Spengler and Bronner-Fraser 2008). NCCs undergo epithelial-to-mesenchymal transition and start migration to their destination. NCCs of the sympathoadrenal lineage migrate ventrolaterally toward the dorsal aorta. Once they reach the dorsal aorta, the sympathetic progenitors stay close it, whereas adrenal chromaffin progenitors migrate further ventrally to associate with adrenal cortical cells that originate from the lateral plate mesoderm (Takahashi et al. 2013). This step-by-step migration is regulated by a cooperative function of extrinsic and intrinsic factors (described below and summarized in the left panel of Fig. 1).

Delaminated and migrating NCCs express the *Sox10* gene, a member of the SRY-related HMB-box family of transcription factors (Kim et al. 2003; Sauka-Spengler and Bronner-Fraser 2008). SOX10 exerts multiple functions in neural crest derivatives, including sympathoadrenal and glial progenitors,

through the maintenance of the stem cell state (gliogenic and neurogenic potential) and glial lineage identity (Kim et al. 2003). Therefore, $SOX10^+$ migrating NCCs have a potential to differentiate into cells of sympathoadrenal and glial lineage. Expression patterns of SOX10 in sympathoadrenal and glial lineage cells become distinct when their lineages are specified. *Phox2b* gene expression starts right after migrating NCCs reach the dorsal aorta and determines the cells for sympathoadrenal lineage: these cells express both SOX10 and PHOX2B simultaneously at E10.5 mouse embryos (Callahan et al. 2008). However, $PHOX2B^+$ sympathoadrenal progenitors permanently lose SOX10 expression after E11.5, although the latter persists in glial lineage, namely in the satellite glia in sympathetic ganglia (Callahan et al. 2008; Gonsalvez et al. 2013).

Bone morphogenic proteins (BMPs) are the major extrinsic factors secreted from the dorsal aorta that are essential for the generation of sympathoadrenal lineage (Schneider et al. 1999). Schneider and colleagues implanted Noggin-loaded agarose beads into the vicinity of notochord and dorsal aorta to directly inhibit secreted BMPs (BMP-4 and BMP-7), and this manipulation abolished the aggregation of sympathoadrenal progenitors (TH^+/DBH^+/$CASH\text{-}1^+$/$PHOX2A^+$/$PHOX2B^+$) around the dorsal aorta. Thus, BMPs were suggested to act directly on the formation of sympathoadrenal progenitors around the dorsal aorta. Recently, Saito and colleagues used a dominant-negative type of the BMP receptor rather than Noggin to inhibit BMP signaling specifically in migrating NCCs, and this did not inhibit the migration of these cells to the dorsal aorta. Interestingly, the authors found that BMPs secreted from the dorsal aorta induced the expression of SDF1 (the chemokine stromal cell-derived factor-1) and NRG1 (Neuregulin 1) in the para-aortic mesenchyme, which directly regulate the migration of NCCs through CXCR4 (C-X-C motif chemokine receptor 4) and EGF receptors, respectively (Saito et al. 2012). The authors also focused on the late-occurring segregation of adrenal- and sympathetic progenitors. In contrast to the migration of NCCs, direct signaling of BMP-4 and BMP-7 was required for the segregation of adrenal progenitors, and then, NRG1 functioned as an attractant for further migration of adrenal progenitors to the adrenal gland (Saito et

al. 2012). Therefore, BMPs are central extrinsic factors affecting both the migration of NCCs and segregation of adrenal- and sympathetic cell progenitors (Saito and Takahashi 2015; Takahashi et al. 2013). However, this late-occurring segregation was recently revised by Furlan et al., as described in the following section. Therefore, Saito el al. observed either ventrally migrated suprarenal sympathetic progenitors or minor populations of adrenal progenitors.

Several intrinsic/transcription factors, including PHOX2B (Pattyn et al. 1997; Pattyn et al. 1999), ASCL1/MASH-1 (Guillemot et al. 1993), HAND2 (Howard et al. 2000), and GATA2/3 (Tsarovina et al. 2004), control the development of sympathoadrenal lineage. The expression patterns and roles of these transcription factors are well summarized in a review by Huber with a plausible conclusion that PHOX2B is the dominant regulator of sympathoadrenal lineage specification, whereas the contribution of ASCL1/MASH-1 and GATA3 is less significant (Huber 2006).

Development of chromaffin cells in the adrenal medulla

As described above, adrenal chromaffin cells and sympathetic ganglia are considered to share the same origin and step-by-step developmental mode. Numerous studies mostly based this conclusion on the observations of cell differentiation *in vitro* or *in vivo* (e.g., in knockout mice). However, as those studies did not use lineage tracing, whether the putative progenitors of adrenal chromaffin cells and sympathetic ganglia, namely sympathoadrenal lineage cells, indeed generate two distinct cell types has not been directly demonstrated. For example, the migration of adrenal chromaffin progenitors toward the adrenal gland has not been directly observed. In addition, the suprarenal sympathetic ganglion is considered to be derived from sympathoadrenal precursors, and it is still unknown how adrenal chromaffin cells and suprarenal sympathetic ganglion develop from the same set of cells, i.e. sympathoadrenal progenitors (Lumb and Schwarz 2015).

However, a recent study provided convincing evidence that the suprarenal ganglion, similar to other sympathetic ganglia, is generated by migrating NCCs, whereas adrenal chromaffin cells are derived

from SCPs migrating along the nerve to the adrenal (Furlan et al. 2017). The following facts were directly revealed (also summarized in the right panel of Fig. 1).

(1) The majority of TH^+ cells in the adrenal medulla at E17.5 originated from $PLP1^+$ glial precursors as was evidenced by lineage tracing in *Plp1*^{CreERT2}*;R26R*^{YFP} reporter mice at E11.5.

(2) When $SOX10^+$ cells were ablated by diphtheria toxin subunit A in *Sox10*^{CreERT2}*;R26R*^{DTA} mice at E11.5 and E12.5, TH^+ cells were significantly reduced in the adrenal medulla but not in the suprarenal sympathetic ganglion or sympathetic ganglion at E13.5 and E17.5. These results indicate that migrating SCPs ($SOX10^+$/$PLP1^+$) at E11.5 generate most of the adrenal chromaffin cells. Collectively, the authors concluded that approximately 80% of chromaffin cells in the adrenal medulla originated from the migrating SCPs.

(3) Next, Furlan and colleagues focused on the unique developmental mode of peripheral glial progenitors. In parasympathetic outflow, $SOX10^+$/$PHOX2B^+$ SCPs migrate to the site of ganglion formation along their future preganglionic nerves and form parasympathetic ganglia (Espinosa-Medina et al. 2014; Kalcheim and Rohrer 2014). This preganglionic nerve-dependent migration was also observed in the adrenal chromaffin progenitors. When the authors ablated preganglionic sympathetic neurons in the spinal cord that innervated the adrenal gland, the majority of TH^+ cells were reduced in the adrenal medulla but not in the suprarenal sympathetic ganglion. This nerve-dependent migration was distinct from the free migration of NCCs such as sympathoadrenal progenitors.

(4) To characterize the program of SCP differentiation into adrenal chromaffin cells, the authors examined the role of ASCL1, a well-known transcription factor critical for chromaffin cell differentiation (Huber et al. 2002). *Ascl1* knockout and traced cells unusually maintained the expression of glial markers S100β and SOX10. These cells initiated the expression of PHOX2B but failed to acquire catecholaminergic state ($SOX10^-$/$PHOX2B^+$/TH^-), demonstrating an important role of ASCL1 in the proper maturation of SCPs.

(5) To trace the differentiation of sympathoadrenal progenitors, the authors focused on the tyrosine kinase RET, an important regulator of sympathetic neuron development (Enomoto et al. 2001). After RET^{+} cells were traced starting from E10.5 and then examined at E15.5, they were mostly observed in sympathetic neurons of the paravertebral and suprarenal ganglia, whereas a minor population (approximately 20%) was traced in the adrenal medulla, suggesting a small contribution of the sympathoadrenal progenitors in the formation of chromaffin cells in the adrenal medulla.

(6) A subset of TH^{+} cells exhibited cholinergic properties ($CHAT^{+}$) in the sympathetic and suprarenal sympathetic ganglia but not in the adrenal medulla.

(7) Finally, single-cell gene expression analysis of neural crest derivatives in the adrenal gland and suprarenal ganglion clearly revealed distinct gene expression patterns between these two tissues at E12.5 and E13.5. The authors also observed a cascade of pseudo differentiation from SCPs to chromaffin cells, and identified *Ascl1* and *Htr3a* as genes bridging this phenomenon.

Overall, the study by Furlan et al. provided fundamental knowledge to better understand the mechanisms of neuroblastoma biology, assuming that the findings in mice are indeed applicable to humans. Thus far, it has been presumed that sympathoadrenal progenitors are the source of neuroblastoma (Cheung and Dyer 2013). Given that neuroblastomas predominantly occur at the adrenal gland (approximately 50%), SCPs are considered to be one of the origins of neuroblastoma. Because primary neuroblastomas occur at the adrenal gland and paravertebral sympathetic ganglia, the cells of neuroblastoma origin are likely either sympathoadrenal progenitors or SCPs. Furthermore, the difference of the cell of origin might explain distinctly worse clinical presentations of adrenal neuroblastomas compared to those of non-adrenal ones, as mentioned above (Vo et al. 2014).

The origin of stage 4S neuroblastoma

Stage 4S neuroblastoma is a unique and special type of neuroblastoma (D'Angio et al. 1971; Evans

et al. 1971). Patients with stage 4S neuroblastomas show clinical presentations similar to those of stage 1 and stage 2 neuroblastomas but have metastatic tumors restricted to the skin, the liver, or the bone marrow (Evans et al. 1971). Stage 4S neuroblastomas spontaneously regress with minimal or no treatment (D'Angio et al. 1971) and show different molecular transcriptome (Benard et al. 2008) and DNA methylome (Decock et al. 2016) signatures compared to those of stage 4 neuroblastomas. This unique neuroblastoma has been thought to be a metastatic tumor of genetically identical cells (Brodeur and Bagatell 2014). However, it is more likely a multifocal disease of NCCs (van Noesel 2012). Dissemination sites of stage 4S neuroblastomas, namely the skin, the liver, and the bone marrow, are one of the destinations for migrating NCCs. Indeed, multipotent neural crest-derived cells, which can be isolated from the trunk skin, express neural crest stem cell marker p75 and SOX10, and represent glial or melanocyte lineages (Wong et al. 2006). Nagoshi and colleagues focused on the NCC marker *Mpz* (myelin protein zero; also known as P0) and used *P0-Cre/Floxed-EGFP* mice to trace migration of NCCs in the bone marrow and aorta-gonad-mesonephros region (Nagoshi et al. 2008). $EGFP^+$ cells were observed in the dorsal root ganglia, whisker follicles, and tibia bone marrow. In the latter tissue, $EGFP^+$ cells were detected along blood vessels near the inner surface of the bone cortex. Importantly, $EGFP^+/SOX10^+/p75^+$ NCCs invaded the dorsal aorta and were observed in the peripheral blood and fetal liver during embryonic stages, suggesting that NCCs migrate from the aorta-gonad-mesonephros region to the bone marrow through blood vessels (Nagoshi et al. 2008). Therefore, dissemination sites of stage 4S neuroblastoma and destinations of $SOX10^+/p75^+$ NCCs coincide (Fig. 2). These observations support the hypothesis that stage 4S neuroblastoma is a multifocal cancer rather than a metastatic one, influencing migrating NCCs. As discussed earlier, there are at least two distinct neuroblastoma cells types, i.e., MES-type and ADRN-type. Therefore, investigating the “cell types” of stage 4S neuroblastoma at various locations may provide an insight into the genesis of stage 4S neuroblastoma. However, the etiology of this tumor and its unique ability to undergo “spontaneous

regression” remain largely enigmatic.

Mechanisms of neuroblastoma initiation

Genetic abnormalities in neuroblastoma

Several recent genetic analyses by high-throughput sequencing technologies have revealed the landscape of genetic variation in human neuroblastoma. In 2013, genomic DNA samples of 240 high-risk neuroblastomas have been sequenced, and it was found that recurrent somatic mutations in genes such as *ALK* (9.2%), *PTPN11* (2.9%), *ATRX* (2.5% and 7.1% focal deletions) were rare (less than 25% in total), whereas chromosomal alterations, such as *MYCN* amplification (32.1%), *17q* gain (80.4%), and *11q* loss (47.5%), were frequently observed in that cohort (around 90% in total) (Pugh et al. 2013). In 2015, two research groups reported recurrent genomic rearrangements affecting chromosomal regions close to the telomerase reverse transcriptase (*TERT*) gene locus that led to significant transcriptional up-regulation of *TERT* (Peifer et al. 2015; Valentijn et al. 2015). Importantly, *TERT* rearrangements were found only in high-risk neuroblastomas (Peifer et al.: 31%, 12/39; Valentijn et al.: 23%, 17/75) in an almost mutually exclusive manner with *MYCN* amplifications and *ATRX* mutations. However, both groups also observed a set of neuroblastomas without any of those aberrations, which left open the question of how these tumors (around 30%) developed. With regard to the chromosomal alterations, a close association between *MYCN* and *let-7* microRNA family has been reported recently (Powers et al. 2016). Notably, in neuroblastomas with amplified *MYCN*, abundant *MYCN* mRNA works as a competing endogenous RNA to sponge *let-7* miRNAs, attenuating their tumor-suppressive functions. In addition, a genetic loss of *let-7* family members, including *let-7a2* and *let-7g*, was observed in chromosome *11q* and *3p*, respectively, where chromosomal losses were frequently observed in neuroblastomas without amplified *MYCN*. Therefore, Powers and colleagues concluded that *let-7* disruptions by multiple mechanisms are a common event in the majority of neuroblastomas.

Although chromosomal gains and losses affecting oncogenic and tumor suppressive genes definitely occur in neuroblastomas, as discussed above, whether these events initiated neuroblastomas is still largely unsolved. Few recurrent genetic alterations indicate that epigenetic abnormalities during normal development are likely involved in the initiation of neuroblastoma tumorigenesis, which then trigger genomic instabilities leading to the chromosomal alterations found in primary tumors at diagnosis.

Neuroblastoma initiation in animal models

Neuroblastoma initiation can be reproduced using genetically engineered animal models. Th-MYCN mouse is the most widely used mouse model, in which rat tyrosine hydroxylase (*TH*) promoter drives human *MYCN* expression (Chesler and Weiss 2011; Weiss et al. 1997). Ectopic *MYCN* expression in sympathoadrenal lineage initiated neuroblastoma formation that mirrored clinico-pathological features of human neuroblastomas, including chromosomal changes (Hackett et al. 2003). In physiological settings, proliferating neuroblasts undergo either differentiation or apoptosis upon the withdrawal of nerve growth factor; however, ectopic *MYCN* expression around the perinatal stages altered this normal process and resulted in aberrant proliferation of these cells in Th-MYCN mice (Hansford et al. 2004). Although ectopic *MYCN* overexpression is unlikely to be the initial event in the development of neuroblastoma, it triggered the sequence of events leading to neuroblastoma in mice.

In 2008, four groups reported germline and somatic mutations in the *ALK* gene in neuroblastoma: point mutations, including R1275Q and F1174L, converged on ALK tyrosine kinase domain and resulted in constitutive activation of the protein (Chen et al. 2008; George et al. 2008; Janoueix-Lerosey et al. 2008; Mosse et al. 2008). Mutant ALK (F1174L), which was found only in sporadic neuroblastomas, exhibited transformative ability in transgenic mice (Berry et al. 2012; Heukamp et al. 2012). Importantly, mutant *ALK (F1174L)* driven by the rat *TH* promoter did not

trigger neuroblastoma in *ALK*^{F1174L} transgenic mice, but it accelerated Th-MYCN-driven tumorigenesis (Berry et al. 2012). This was also observed in a zebrafish model, in which the dopamine-β-hydroxylase promoter was used to express the mutant *ALK (F1174L)* (Zhu et al. 2012). In contrast, mutant *ALK (F1174L)* driven by the chicken β-actin promoter caused tumors in mice (Heukamp et al. 2012). More recently, Cazes and colleagues produced *Alk*^{R1279Q} and *Alk*^{F1178L} knock-in mouse models to assess the tumorigenic potential of ALK mutants (Cazes et al. 2014). Both Alk knock-in models promoted MYCN-driven neuroblastomas when the mice were crossed with MYCN transgenic mice; however, they never developed tumors by themselves, suggesting that activated Alk itself is not sufficient to induce neuroblastoma *in vivo*. Overall, these studies indicate that expression levels of mutant *ALK* are critical for triggering neuroblastoma *in vivo*.

LIN28B and its homolog LIN28A are RNA binding proteins that selectively block the processing of *pri-let-7* miRNAs (Viswanathan et al. 2008). Genomic amplifications and overexpression of *LIN28B* have been observed in most high-risk neuroblastomas (Molenaar et al. 2012). Suppression of *let-7* miRNAs production by excessive LIN28B results in the up-regulation of N-Myc (encoded by *MYCN*) protein levels. Importantly, ectopic expression of *LIN28B,* restricted to sympathoadrenal lineage, initiated neuroblastoma formation in transgenic mice, demonstrating oncogenic potential of *LIN28B* (Molenaar et al. 2012). Interestingly, ectopic LIN28B expression did not affect the proliferation of neuroblasts around the perinatal stages (up to postnatal day 20), suggesting that LIN28B-driven neuroblastoma requires additional signals or genetic abnormalities at late postnatal stages (Hennchen et al. 2015). Because the majority of neuroblastomas exhibit *LIN28B* overexpression without genomic aberrations, the upstream signaling proteins, such as epigenetic regulators, might trigger the cascade of *LIN28B*-driven neuroblastoma tumorigenesis.

These lines of evidence demonstrated oncogenic potentials of several genes; however, the nature of neuroblastoma initiation mechanisms and epigenetic abnormalities during embryonic development are still largely uncertain.

Epigenetic abnormalities in early neuroblastoma tumorigenesis

Recently, we reported the evidence of possible epigenetic abnormalities critical to the initial stage of neuroblastoma tumorigenesis (Tsubota et al. 2017). We investigated the earliest events of neuroblastoma tumorigenesis in Th-MYCN mice. Using the newly established sphere culture condition containing chick embryo extract, we were able to isolate sympathoadrenal progenitors from E13.5 embryos. Importantly, initiation of *MYCN* expression was confirmed at E13.5 in a small subset of $PHOX2B^+$ sympathoadrenal progenitors. Both wild-type (WT) and Th-MYCN cells formed primary spheres, but only Th-MYCN spheres were passageable, indicating that $N\text{-}Myc^+$ cells were enriched by long-term culture. We compared transcriptomes of E13.5 WT and Th-MYCN spheres and investigated gene sets contributing to transcriptomic differences between them. MYC target genes were up-regulated in Th-MYCN spheres as expected. Importantly, we found significant down-regulation of polycomb repressive complex 2 (PRC2) target genes, which were defined in embryonic stem (ES) cells (Ben-Porath et al. 2008). We observed chromosomal alterations in tumor tissues but not in E13.5 Th-MYCN spheres, as well as specific promoter-associated DNA methylations in E13.5 Th-MYCN spheres. However, the latter barely contributed to transcriptomic differences. These multiple omics analyses suggested that transcriptomic alterations regulated by N-Myc and PRC2 initiated neuroblastoma tumorigenesis in Th-MYCN mice (Fig. 3). In addition, chromatin immunoprecipitation sequencing for H3K27me3, a repressive histone mark modified by PRC2 (Margueron and Reinberg 2011), revealed an increase in H3K27me3 levels around the transcription start sites of PRC2 target genes in E13.5 Th-MYCN spheres, indicating that target genes were epigenetically suppressed by PRC2. Remarkably, as previously reported (Corvetta et al. 2013), we observed a physical interaction between N-Myc and PRC2 in E13.5 Th-MYCN spheres, implying that N-Myc recruits PRC2 at certain genomic loci to epigenetically suppress their target genes. Inhibition of the EZH2 protein, an essential component of PRC2 complex, by knockdown or a

histone methyltransferase inhibitor de-repressed PRC2 target genes, including cyclin-dependent kinase inhibitors (Mills 2010) and known PRC2 targets in neuroblastomas (Wang et al. 2012), and resulted in growth suppression of *in vitro* sphere and *in vivo* tumor formation in Th-MYCN mice. These results indicate that PRC2 is required for oncogenic potential of MYCN. Furthermore, dysregulation of *MYCN* signaling in sympathoadrenal progenitors, in which EZH2 is expressed during embryonic development, initiates neuroblastoma tumorigenesis (Fig. 3).

However, several issues remain uninvestigated. First, because our spheroid culture method selectively isolates neuroblastoma cells from tissues, the effects of the environment *in vivo* are not considered. Second, although we observed a downregulation of PRC2 targets in Th-MYCN spheres, whether these targets are actually downregulated in neuroblastoma cells *in vivo* is not clearly known. Third, although the array CGH analysis revealed no genomic alterations in E13.5 Th-MYCN spheres, whether subcutaneous tumors derived by injection of such cells acquire chromosomal changes remains unclear. Further investigation of these points will provide more convincing conclusions on the pathogenesis of neuroblastoma.

In vitro culture studies

To investigate neuroblastoma initiation mechanisms, genetic manipulations of the putative neuroblastoma source cells, such as SCPs and sympathoadrenal progenitors, are needed. Classical neuroblastoma cell lines cultured in serum-containing medium or newly established neuroblastoma cells generated with serum-free neural stem cell medium (Bate-Eya et al. 2014) are useful for general characterization of neuroblastoma and therapeutic target search. However, they are not appropriate for investigations of neuroblastoma initiation mechanisms. Therefore, genetically engineered animal models or *in vitro* cell culture approaches are utilized to study initial events directly. *In vitro* cell culture method is particularly useful because researchers can easily manipulate and analyze functions of the genes of interest. For example, putative sources of neuroblastoma, such as SCPs and

sympathoadrenal progenitors, can be directly isolated from embryonic mice. Another strategy is *in vitro* induction of these cells from NCCs or pluripotent stem cells, such as induced pluripotent stem (iPS) or ES cells. So far, only the induction of multipotent NCCs from ES cells using SOX10 and other factors was reported (Kim et al. 2014). However, efficient induction of SCPs or sympathoadrenal progenitors has not yet been achieved.

JoMa1 is a multipotent neural crest cell line expressing *Sox10* and *p75*, which is kept in immortalized and undifferentiated state by a tamoxifen-activated c-Myc transgene (Maurer et al. 2007). It has been reported that *MYCN* and mutant *ALK (F1174L)* could transform JoMa1 cells, which could then cause neuroblastoma formation *in vivo* (Schulte et al. 2013). In addition, a new neuroblastoma model system based on NCCs isolated from E9.5 neural tube explants has been described recently (Olsen et al. 2017). The authors isolated migrating $SOX10^{+}$/$p75^{+}$/$ASCL1^{+}$ NCCs that were negative for the sympathoadrenal marker TH and PHOX2B. These cells were then ectopically transduced with *MYCN* and subsequently transplanted subcutaneously into WT mice to evaluate transformation potential of *MYCN*. Notably, these cells triggered the formation of poorly differentiated neuroblastoma, which exhibited gene expression profile and chromosomal alterations similar to those of human neuroblastomas.

As mentioned above, we also established spheroid culture method using chick embryo extract that can enrich $PHOX2B^{+}$ sympathoadrenal progenitors from the anlage of the adrenal medulla or sympathetic ganglia (Tsubota et al. 2017). Real cellular sources of neuroblastoma are still ambiguous: NCCs, SCPs, or sympathoadrenal progenitors can be the cells that give rise to neuroblastoma. However, the various methods outlined above can be utilized to study further the factors determining susceptibility to neuroblastoma transformation and oncogenic potentials of oncogenes or tumor-suppressors in neuroblastoma.

Ideal model

So far, most of the model systems are based on mouse or zebrafish, whereas a human model is lacking. Therefore, *in vitro* induction of adrenal chromaffin cells or sympathetic neurons from pluripotent stem cells, such as human ES or iPS cells, would be an ideal model to investigate mechanisms of neuroblastoma initiation. Paucity of somatic recurrent mutations implies that combinations of multiple risk factors, including reported (Tolbert et al. 2017) and unknown susceptible genes (polymorphisms), trigger epigenetic deregulations during normal development, leading to neuroblastoma initiation. Considering this situation, generation of iPS cells from normal cells (that harbor genetic risk factors) of human neuroblastoma patients and induction of SCPs or sympathoadrenal progenitors from these cells followed by final differentiation to adrenal chromaffin cells or sympathetic neurons may provide an opportunity to investigate genuine mechanisms of neuroblastoma initiation. Using the same approach for tissues from stage 4S neuroblastoma patients or survivors, we could also investigate the enigmatic mechanisms of spontaneous regression of stage 4S neuroblastoma.

Concluding remarks

Although recent studies have described the development of putative cellular sources of neuroblastoma and suggested plausible mechanisms of neuroblastoma initiation, our understanding of neuroblastoma biology is still limited. Because neuroblastoma-like tumors have not been observed in other vertebrates, predisposing risks of neuroblastoma specifically evolved in *Homo sapiens* and closely related genomes during evolution. For example, the *MYCNOS* gene is conserved in the restricted taxonomic group that contains human and chimpanzee (Suenaga et al. 2014). Therefore, as mentioned in the last part, we may not experimentally reproduce the real initiation of neuroblastoma formation without using cells of human origin.

Neuroblastoma is becoming a curable tumor due to the recent progress in treatment strategies, including high-dose myeloablative chemotherapy with autologous hematopoietic stem cell

transplantation and immunotherapy (anti-GD2 antibody and cytokines) (Matthay et al. 2016). However, even after the completion of successive therapy, patients with neuroblastoma typically suffer from disease- and treatment-induced toxicities. Early detection or prediction of the disease followed by low-toxic therapies, including those with molecularly targeted drugs, is required to control the survival and quality of life of neuroblastoma patients. Owing to the paucity of our knowledge about the mechanisms of neuroblastoma initiation, there should be yet undiscovered alternative treatment strategies harnessing the etiology of neuroblastoma.

References

Bate-Eya LT, Ebus ME, Koster J, den Hartog IJ, Zwijnenburg DA, Schild L, van der Ploeg I, Dolman ME, Caron HN, Versteeg R, Molenaar JJ (2014) Newly-derived neuroblastoma cell lines propagated in serum-free media recapitulate the genotype and phenotype of primary neuroblastoma tumours. Eur J Cancer 50:628-637

Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40:499-507

Benard J, Raguenez G, Kauffmann A, Valent A, Ripoche H, Joulin V, Job B, Danglot G, Cantais S, Robert T, Terrier-Lacombe MJ, Chassevent A, Koscielny S, Fischer M, Berthold F, Lipinski M, Tursz T, Dessen P, Lazar V, Valteau-Couanet D (2008) MYCN-non-amplified metastatic neuroblastoma with good prognosis and spontaneous regression: a molecular portrait of stage 4S. Molecular oncology 2:261-271

Berry T, Luther W, Bhatnagar N, Jamin Y, Poon E, Sanda T, Pei D, Sharma B, Vetharoy WR, Hallsworth A, Ahmad Z, Barker K, Moreau L, Webber H, Wang W, Liu Q, Perez-Atayde A, Rodig S, Cheung NK, Raynaud F, Hallberg B, Robinson SP, Gray NS, Pearson AD, Eccles SA, Chesler L, George RE (2012) The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. Cancer cell 22:117-130

Boeva V, Louis-Brennetot C, Peltier A, Durand S, Pierre-Eugene C, Raynal V, Etchevers HC, Thomas S, Lermine A, Daudigeos-Dubus E, Geoerger B, Orth MF, Grunewald TGP, Diaz E, Ducos B, Surdez D, Carcaboso AM, Medvedeva I, Deller T, Combaret V, Lapouble E, Pierron G, Grossetete-Lalami S, Baulande S, Schleiermacher G, Barillot E, Rohrer H, Delattre O, Janoueix-Lerosey I (2017) Heterogeneity of neuroblastoma cell identity defined by transcriptional circuitries. Nat Genet 49:1408-1413

Brisse HJ, Blanc T, Schleiermacher G, Mosseri V, Philippe-Chomette P, Janoueix-Lerosey I, Pierron G,

Lapouble E, Peuchmaur M, Freneaux P, Galmiche L, Algret N, Peycelon M, Michon J, Delattre O, Sarnacki S (2017) Radiogenomics of neuroblastomas: Relationships between imaging phenotypes, tumor genomic profile and survival. PloS one 12:e0185190

Brodeur GM, Bagatell R (2014) Mechanisms of neuroblastoma regression. Nat Rev Clin Oncol 11:704-713

Callahan T, Young HM, Anderson RB, Enomoto H, Anderson CR (2008) Development of satellite glia in mouse sympathetic ganglia: GDNF and GFR alpha 1 are not essential. Glia 56:1428-1437

Cazes A, Lopez-Delisle L, Tsarovina K, Pierre-Eugene C, De Preter K, Peuchmaur M, Nicolas A, Provost C, Louis-Brennetot C, Daveau R, Kumps C, Cascone I, Schleiermacher G, Prignon A, Speleman F, Rohrer H, Delattre O, Janoueix-Lerosey I (2014) Activated Alk triggers prolonged neurogenesis and Ret upregulation providing a therapeutic target in ALK-mutated neuroblastoma. Oncotarget 5:2688-2702

Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, Wang L, Soda M, Kikuchi A, Igarashi T, Nakagawara A, Hayashi Y, Mano H, Ogawa S (2008) Oncogenic mutations of ALK kinase in neuroblastoma. Nature 455:971-974

Chesler L, Weiss WA (2011) Genetically engineered murine models--contribution to our understanding of the genetics, molecular pathology and therapeutic targeting of neuroblastoma. Seminars in cancer biology 21:245-255

Cheung NK, Dyer MA (2013) Neuroblastoma: developmental biology, cancer genomics and immunotherapy. Nat Rev Cancer 13:397-411

Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA (1989) Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. Cancer research 49:219-225

Corvetta D, Chayka O, Gherardi S, D'Acunto CW, Cantilena S, Valli E, Piotrowska I, Perini G, Sala A (2013) Physical interaction between MYCN oncogene and polycomb repressive complex 2 (PRC2) in neuroblastoma: functional and therapeutic implications. The Journal of biological chemistry

288:8332-8341

D'Angio GJ, Evans AE, Koop CE (1971) Special pattern of widespread neuroblastoma with a favourable prognosis. Lancet 1:1046-1049

Decock A, Ongenaert M, De Wilde B, Brichard B, Noguera R, Speleman F, Vandesompele J (2016) Stage 4S neuroblastoma tumors show a characteristic DNA methylation portrait. Epigenetics 0

Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson EM, Jr., Milbrandt J (2001) RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development (Cambridge, England) 128:3963-3974

Espinosa-Medina I, Outin E, Picard CA, Chettouh Z, Dymecki S, Consalez GG, Coppola E, Brunet JF (2014) Neurodevelopment. Parasympathetic ganglia derive from Schwann cell precursors. Science 345:87-90

Evans AE, D'Angio GJ, Randolph J (1971) A proposed staging for children with neuroblastoma. Children's cancer study group A. Cancer 27:374-378

Furlan A, Dyachuk V, Kastriti ME, Calvo-Enrique L, Abdo H, Hadjab S, Chontorotzea T, Akkuratova N, Usoskin D, Kamenev D, Petersen J, Sunadome K, Memic F, Marklund U, Fried K, Topilko P, Lallemend F, Kharchenko PV, Ernfors P, Adameyko I (2017) Multipotent peripheral glial cells generate neuroendocrine cells of the adrenal medulla. Science 357:

George RE, Sanda T, Hanna M, Frohling S, Luther W, 2nd, Zhang J, Ahn Y, Zhou W, London WB, McGrady P, Xue L, Zozulya S, Gregor VE, Webb TR, Gray NS, Gilliland DG, Diller L, Greulich H, Morris SW, Meyerson M, Look AT (2008) Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature 455:975-978

Gonsalvez DG, Cane KN, Landman KA, Enomoto H, Young HM, Anderson CR (2013) Proliferation and cell cycle dynamics in the developing stellate ganglion. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:5969-5979

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian

achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463-476

Hackett CS, Hodgson JG, Law ME, Fridlyand J, Osoegawa K, de Jong PJ, Nowak NJ, Pinkel D, Albertson DG, Jain A, Jenkins R, Gray JW, Weiss WA (2003) Genome-wide array CGH analysis of murine neuroblastoma reveals distinct genomic aberrations which parallel those in human tumors. Cancer research 63:5266-5273

Hansford LM, Thomas WD, Keating JM, Burkhart CA, Peaston AE, Norris MD, Haber M, Armati PJ, Weiss WA, Marshall GM (2004) Mechanisms of embryonal tumor initiation: distinct roles for MycN expression and MYCN amplification. Proceedings of the National Academy of Sciences of the United States of America 101:12664-12669

Hennchen M, Stubbusch J, Abarchan-El Makhfi I, Kramer M, Deller T, Pierre-Eugene C, Janoueix-Lerosey I, Delattre O, Ernsberger U, Schulte JB, Rohrer H (2015) Lin28B and Let-7 in the Control of Sympathetic Neurogenesis and Neuroblastoma Development. The Journal of neuroscience : the official journal of the Society for Neuroscience 35:16531-16544

Heukamp LC, Thor T, Schramm A, De Preter K, Kumps C, De Wilde B, Odersky A, Peifer M, Lindner S, Spruessel A, Pattyn F, Mestdagh P, Menten B, Kuhfittig-Kulle S, Kunkele A, Konig K, Meder L, Chatterjee S, Ullrich RT, Schulte S, Vandesompele J, Speleman F, Buttner R, Eggert A, Schulte JH (2012) Targeted expression of mutated ALK induces neuroblastoma in transgenic mice. Sci Transl Med 4:141ra191

Howard MJ, Stanke M, Schneider C, Wu X, Rohrer H (2000) The transcription factor dHAND is a downstream effector of BMPs in sympathetic neuron specification. Development (Cambridge, England) 127:4073-4081

Huber K (2006) The sympathoadrenal cell lineage: specification, diversification, and new perspectives. Dev Biol 298:335-343

Huber K (2015) Segregation of neuronal and neuroendocrine differentiation in the sympathoadrenal

lineage. Cell Tissue Res 359:333-341

Huber K, Bruhl B, Guillemot F, Olson EN, Ernsberger U, Unsicker K (2002) Development of chromaffin cells depends on MASH1 function. Development (Cambridge, England) 129:4729-4738

Janoueix-Lerosey I, Lequin D, Brugieres L, Ribeiro A, de Pontual L, Combaret V, Raynal V, Puisieux A, Schleiermacher G, Pierron G, Valteau-Couanet D, Frebourg T, Michon J, Lyonnet S, Amiel J, Delattre O (2008) Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature 455:967-970

Kalcheim C, Rohrer H (2014) Neuroscience. Following the same nerve track toward different cell fates. Science 345:32-33

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

Kim YJ, Lim H, Li Z, Oh Y, Kovlyagina I, Choi IY, Dong X, Lee G (2014) Generation of multipotent induced neural crest by direct reprogramming of human postnatal fibroblasts with a single transcription factor. Cell Stem Cell 15:497-506

Lumb R, Schwarz Q (2015) Sympathoadrenal neural crest cells: the known, unknown and forgotten? Dev Growth Differ 57:146-157

Margueron R, Reinberg D (2011) The Polycomb complex PRC2 and its mark in life. Nature 469:343-349

Matthay KK, Maris JM, Schleiermacher G, Nakagawara A, Mackall CL, Diller L, Weiss WA (2016) Neuroblastoma. Nature reviews Disease primers 2:16078

Maurer J, Fuchs S, Jager R, Kurz B, Sommer L, Schorle H (2007) Establishment and controlled differentiation of neural crest stem cell lines using conditional transgenesis. Differentiation; research in biological diversity 75:580-591

Mills AA (2010) Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer 10:669-682

Molenaar JJ, Domingo-Fernandez R, Ebus ME, Lindner S, Koster J, Drabek K, Mestdagh P, van Sluis P, Valentijn LJ, van Nes J, Broekmans M, Haneveld F, Volckmann R, Bray I, Heukamp L, Sprussel A, Thor T, Kieckbusch K, Klein-Hitpass L, Fischer M, Vandesompele J, Schramm A, van Noesel MM, Varesio L, Speleman F, Eggert A, Stallings RL, Caron HN, Versteeg R, Schulte JH (2012) LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. Nat Genet 44:1199-1206

Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkamani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM (2008) Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 455:930-935

Nagoshi N, Shibata S, Kubota Y, Nakamura M, Nagai Y, Satoh E, Morikawa S, Okada Y, Mabuchi Y, Katoh H, Okada S, Fukuda K, Suda T, Matsuzaki Y, Toyama Y, Okano H (2008) Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad. Cell Stem Cell 2:392-403

Olsen RR, Otero JH, Garcia-Lopez J, Wallace K, Finkelstein D, Rehg JE, Yin Z, Wang YD, Freeman KW (2017) MYCN induces neuroblastoma in primary neural crest cells. Oncogene 36:5075-5082

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1997) Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development (Cambridge, England) 124:4065-4075

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399:366-370

Peifer M, Hertwig F, Roels F, Dreidax D, Gartlgruber M, Menon R, Kramer A, Roncaioli JL, Sand F, Heuckmann JM, Ikram F, Schmidt R, Ackermann S, Engesser A, Kahlert Y, Vogel W, Altmuller J, Nurnberg P, Thierry-Mieg J, Thierry-Mieg D, Mariappan A, Heynck S, Mariotti E, Henrich KO, Gloeckner C, Bosco G, Leuschner I, Schweiger MR, Savelyeva L, Watkins SC, Shao C, Bell E, Hofer T, Achter V, Lang U, Theissen J, Volland R, Saadati M, Eggert A, de Wilde B, Berthold F, Peng Z,

Zhao C, Shi L, Ortmann M, Buttner R, Perner S, Hero B, Schramm A, Schulte JH, Herrmann C, O'Sullivan RJ, Westermann F, Thomas RK, Fischer M (2015) Telomerase activation by genomic rearrangements in high-risk neuroblastoma. Nature 526:700-704

Powers JT, Tsanov KM, Pearson DS, Roels F, Spina CS, Ebright R, Seligson M, de Soysa Y, Cahan P, Theissen J, Tu HC, Han A, Kurek KC, LaPier GS, Osborne JK, Ross SJ, Cesana M, Collins JJ, Berthold F, Daley GQ (2016) Multiple mechanisms disrupt the let-7 microRNA family in neuroblastoma. Nature 535:246-251

Pugh TJ, Morozova O, Attiyeh EF, Asgharzadeh S, Wei JS, Auclair D, Carter SL, Cibulskis K, Hanna M, Kiezun A, Kim J, Lawrence MS, Lichenstein L, McKenna A, Pedamallu CS, Ramos AH, Shefler E, Sivachenko A, Sougnez C, Stewart C, Ally A, Birol I, Chiu R, Corbett RD, Hirst M, Jackman SD, Kamoh B, Khodabakshi AH, Krzywinski M, Lo A, Moore RA, Mungall KL, Qian J, Tam A, Thiessen N, Zhao Y, Cole KA, Diamond M, Diskin SJ, Mosse YP, Wood AC, Ji L, Sposto R, Badgett T, London WB, Moyer Y, Gastier-Foster JM, Smith MA, Guidry Auvil JM, Gerhard DS, Hogarty MD, Jones SJ, Lander ES, Gabriel SB, Getz G, Seeger RC, Khan J, Marra MA, Meyerson M, Maris JM (2013) The genetic landscape of high-risk neuroblastoma. Nat Genet 45:279-284

Saito D, Takahashi Y (2015) Sympatho-adrenal morphogenesis regulated by the dorsal aorta. Mech Dev 138 Pt 1:2-7

Saito D, Takase Y, Murai H, Takahashi Y (2012) The dorsal aorta initiates a molecular cascade that instructs sympatho-adrenal specification. Science 336:1578-1581

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

Schneider C, Wicht H, Enderich J, Wegner M, Rohrer H (1999) Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. Neuron 24:861-870

Schulte JH, Lindner S, Bohrer A, Maurer J, De Preter K, Lefever S, Heukamp L, Schulte S, Molenaar J, Versteeg R, Thor T, Kunkele A, Vandesompele J, Speleman F, Schorle H, Eggert A, Schramm A

(2013) MYCN and ALKF1174L are sufficient to drive neuroblastoma development from neural crest progenitor cells. Oncogene 32:1059-1065

Suenaga Y, Islam SM, Alagu J, Kaneko Y, Kato M, Tanaka Y, Kawana H, Hossain S, Matsumoto D, Yamamoto M, Shoji W, Itami M, Shibata T, Nakamura Y, Ohira M, Haraguchi S, Takatori A, Nakagawara A (2014) NCYM, a Cis-antisense gene of MYCN, encodes a de novo evolved protein that inhibits GSK3beta resulting in the stabilization of MYCN in human neuroblastomas. PLoS Genet 10:e1003996

Takahashi Y, Sipp D, Enomoto H (2013) Tissue interactions in neural crest cell development and disease. Science 341:860-863

Tolbert VP, Coggins GE, Maris JM (2017) Genetic susceptibility to neuroblastoma. Curr Opin Genet Dev 42:81-90

Tsarovina K, Pattyn A, Stubbusch J, Muller F, van der Wees J, Schneider C, Brunet JF, Rohrer H (2004) Essential role of Gata transcription factors in sympathetic neuron development. Development (Cambridge, England) 131:4775-4786

Tsubota S, Kishida S, Shimamura T, Ohira M, Yamashita S, Cao D, Kiyonari S, Ushijima T, Kadomatsu K (2017) PRC2-mediated transcriptomic alterations at the embryonic stage govern tumorigenesis and clinical outcome in MYCN-driven neuroblastoma. Cancer research

Valentijn LJ, Koster J, Zwijnenburg DA, Hasselt NE, van Sluis P, Volckmann R, van Noesel MM, George RE, Tytgat GA, Molenaar JJ, Versteeg R (2015) TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors. Nat Genet 47:1411-1414

van Groningen T, Koster J, Valentijn LJ, Zwijnenburg DA, Akogul N, Hasselt NE, Broekmans M, Haneveld F, Nowakowska NE, Bras J, van Noesel CJM, Jongejan A, van Kampen AH, Koster L, Baas F, van Dijk-Kerkhoven L, Huizer-Smit M, Lecca MC, Chan A, Lakeman A, Molenaar P, Volckmann R, Westerhout EM, Hamdi M, van Sluis PG, Ebus ME, Molenaar JJ, Tytgat GA, Westerman BA, van Nes J, Versteeg R (2017) Neuroblastoma is composed of two super-enhancer-associated differentiation

states. Nat Genet 49:1261-1266

van Noesel MM (2012) Neuroblastoma stage 4S: a multifocal stem-cell disease of the developing neural crest. Lancet Oncol 13:229-230

Viswanathan SR, Daley GQ, Gregory RI (2008) Selective blockade of microRNA processing by Lin28. Science 320:97-100

Vo KT, Matthay KK, Neuhaus J, London WB, Hero B, Ambros PF, Nakagawara A, Miniati D, Wheeler K, Pearson AD, Cohn SL, DuBois SG (2014) Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: a report from the international neuroblastoma risk group project. J Clin Oncol 32:3169-3176

Wang C, Liu Z, Woo CW, Li Z, Wang L, Wei JS, Marquez VE, Bates SE, Jin Q, Khan J, Ge K, Thiele CJ (2012) EZH2 Mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3, and NGFR. Cancer research 72:315-324

Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop JM (1997) Targeted expression of MYCN causes neuroblastoma in transgenic mice. EMBO J 16:2985-2995

Wong CE, Paratore C, Dours-Zimmermann MT, Rochat A, Pietri T, Suter U, Zimmermann DR, Dufour S, Thiery JP, Meijer D, Beermann F, Barrandon Y, Sommer L (2006) Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. The Journal of cell biology 175:1005-1015

Zhu S, Lee JS, Guo F, Shin J, Perez-Atayde AR, Kutok JL, Rodig SJ, Neuberg DS, Helman D, Feng H, Stewart RA, Wang W, George RE, Kanki JP, Look AT (2012) Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. Cancer cell 21:362-373

Figure 1

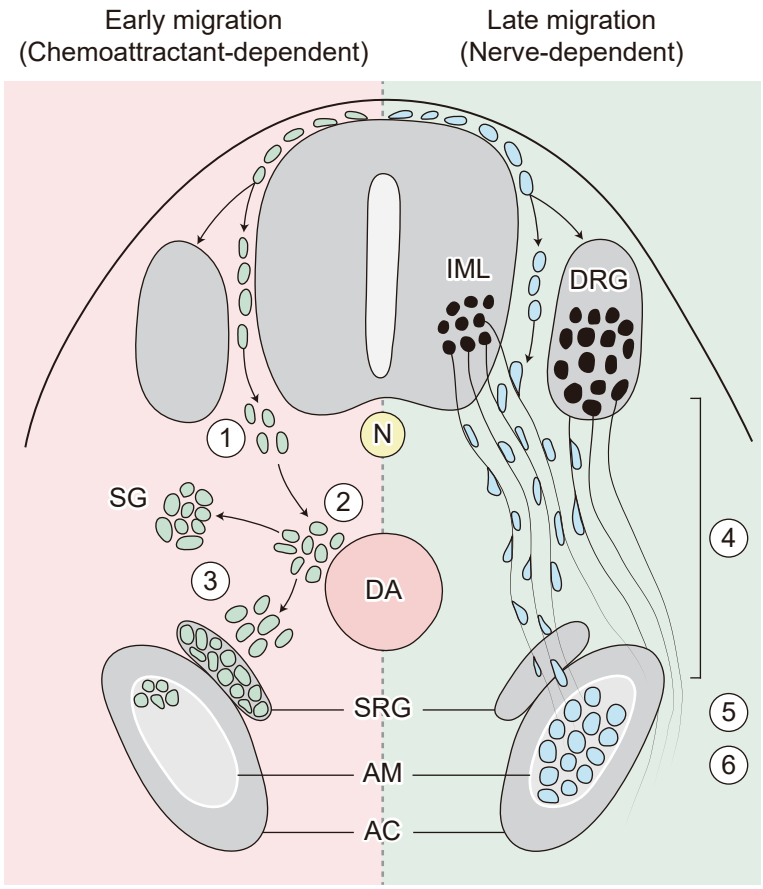
Early migration
(Chemoattractant-dependent)
Late migration
(Nerve-dependent)
IML
DRG
N
1
2
3
4
5
6
SG
DA
SRG
AM
AC

Figure 2

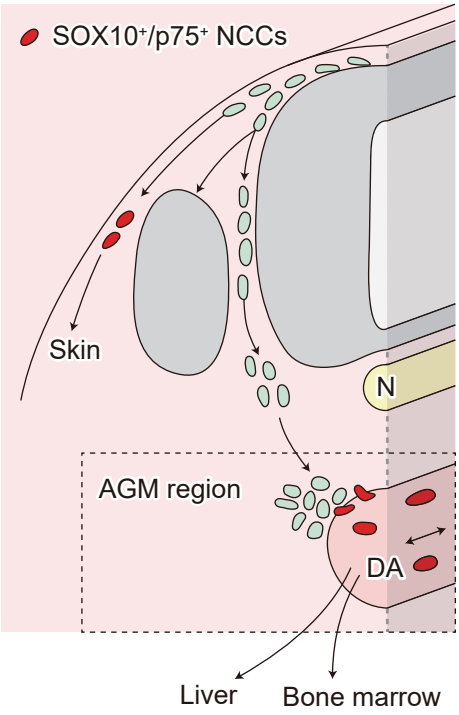

Figure 3

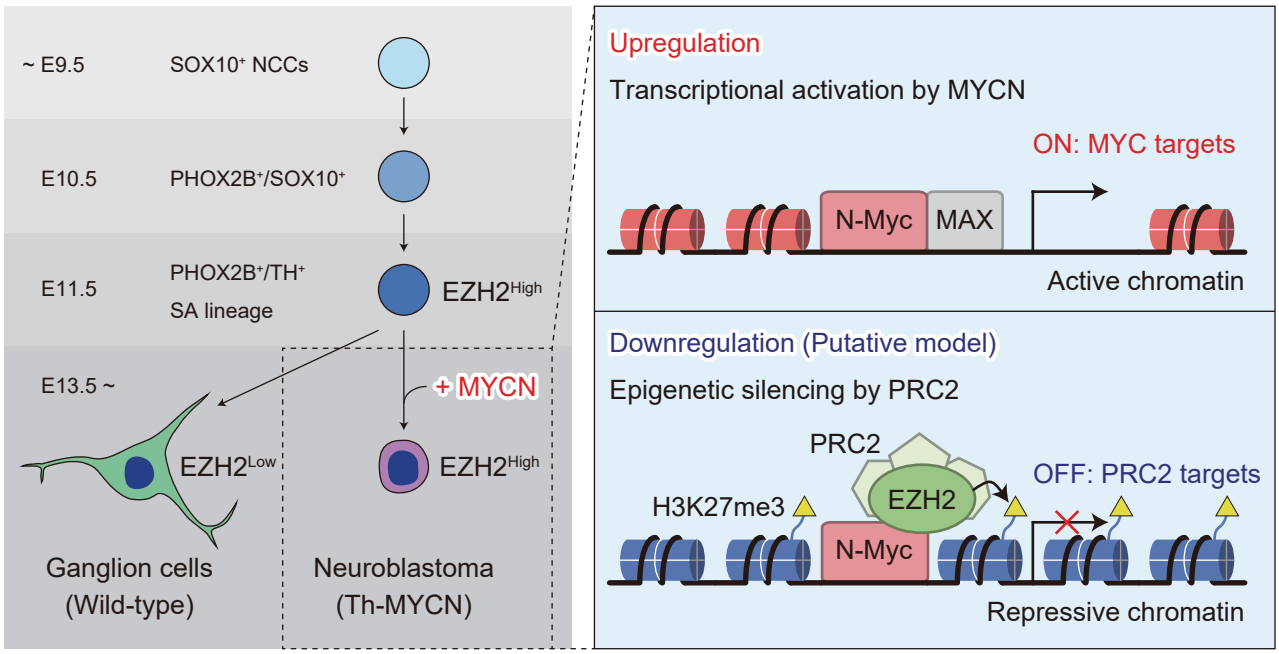
~ E9.5
SOX10⁺ NCCs
E10.5
PHOX2B⁺/SOX10⁺
E11.5
PHOX2B⁺/TH⁺
SA lineage
EZH2High
E13.5 ~
+ MYCN
EZH2Low
EZH2High
Ganglion cells
(Wild-type)
Neuroblastoma
(Th-MYCN)
Upregulation
Transcriptional activation by MYCN
ON: MYC targets
N-Myc
MAX
Active chromatin
Downregulation (Putative model)
Epigenetic silencing by PRC2
PRC2
EZH2
H3K27me3
OFF: PRC2 targets
N-Myc
Repressive chromatin

Figure legends

Fig. 1 Distinct developmental modes of sympathoadrenal lineage and adrenal chromaffin cells. (Left panel) Sympathoadrenal lineage is derived from early-migrating neural crest cells that migrate ventrolaterally and chemoattractant-dependently towards the dorsal aorta. (1) $SOX10^+$ neural crest cells are attracted by SDF1 and NRG1 secreted by the para-aortic mesenchyme through their receptors CXCR4 and EGFR, respectively. (2) Once $SOX10^+$ cells reach the vicinity of the dorsal aorta, they commit to sympathoadrenal lineage and start to express lineage-specifying transcription factor gene *Phox2b* following BMP signaling from the dorsal aorta. This state is transient and the cells simultaneously express both *Phox2b* and *Sox10* genes in mice at around E10.5. (3) These sympathoadrenal progenitors lose *Sox10* expression, become segregated, and migrate to either the anlage of paravertebral sympathetic ganglia or adrenal medulla/suprarenal sympathetic ganglia. BMPs from the dorsal aorta are critical for the migration of sympathoadrenal lineage. A small contribution of these cells in the formation of chromaffin cells in the AM. (Right panel) The majority of adrenal chromaffin cells are derived from late-migrating neural crest cells (termed Schwann cell precursors) that migrate along the preganglionic nerves of the intermediolateral cell column innervating the adrenal gland. (4) $SOX10^+$/$p75^+$ Schwann cell precursors ventrolaterally migrate along the axons of preganglionic neurons in the intermediolateral cell column toward the anlage of the adrenal medulla. (5) Schwann cell precursors commit to adrenal chromaffin cells through a temporal bridging state, when they express genes specific for this state, including *Ascl1* and *Htr3a*. (6) These cells start to express lineage-specific genes, including *Phox2b* and *Th*, for further differentiation to adrenal chromaffin cells. Approximately 80% of the chromaffin cells in the AM is originated from Schwann cell precursors. AC, adrenal cortex; AM, Adrenal medulla; DA, dorsal aorta; DRG, dorsal root ganglion; IML, intermediolateral cell column; N, notochord; SG, sympathetic ganglion; SRG, suprarenal sympathetic ganglion.

Fig. 2 Putative cellular sources of stage 4S neuroblastoma. Neural crest cells (NCCs) migrate dorsolaterally to become pigment cells in the skin, or migrate ventrolaterally toward the aorta-gonad-mesonephros (AGM) region to form sympathoadrenal lineage. A small population of these cells is known to invade the dorsal aorta and travel to the liver or the bone marrow. During embryonic development, $SOX10^{+}/p75^{+}$ cells reside in these destinations, i.e. the skin, the liver and the bone marrow, where multiple tumors are observed in patients with stage 4S neuroblastomas. Malfunction of normal development in these cells might trigger tumorigenic events leading to the multifocal neuroblastoma formation. DA, dorsal aorta; N, notochord.

Fig. 3 Tumor initiation triggered by MYCN and PRC2 in Th-MYCN mice. (Left panel) In wild-type mice, $SOX10^{+}$ neural crest cells (NCCs) commit to $PHOX2B^{+}/TH^{+}$ sympathoadrenal (SA) lineage following the transient state when they express both PHOX2B and SOX10. From embryonic to postnatal stages, these cells differentiate to sympathetic ganglion cells and EZH2 expression becomes gradually down-regulated. In Th-MYCN mice, human MYCN expression, which is driven by rat *Th* promoter, starts in a small population of $PHOX2B^{+}/TH^{+}$ sympathoadrenal progenitors no later than at E13.5. Forced expression of human *MYCN* initiates neuroblastoma formation in mice with high EZH2 expression levels. (Right panel) N-Myc (encoded by *MYCN*) expression alters transcriptome rather than genome or DNA-methylome. Up-regulation of genes including MYC targets is largely mediate by the transcriptional activity of N-Myc with a co-factor such as MAX. In contrast, down-regulation of genes, including polycomb repressive complex 2 (PRC2) targets, is probably mediated by a complex of N-Myc and PRC2 via epigenetic silencing by EZH2, catalyzing repressive tri-methylation mark of histone H3 at lysine 27 (H3K27me3).