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Neuroblastoma: When Differentiation Goes Awry

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Summary

Neuroblastoma is a leading cause of cancer-related death in children. Accumulated data suggest that differentiation arrest of the neural crest derived sympathoadrenal lineage contributes to neuroblastoma formation. The developmental arrest of these cell types explains many biological features of the disease, including its cellular heterogeneity, mutational spectrum, spontaneous regression, and response to drugs that induce tumor cell differentiation. In this review, we provide evidence that supports the notion that arrested neural crest derived progenitor cells give rise to neuroblastoma and discuss how this concept could be exploited for clinical management of the disease.

Introduction

The link between cancer and arrested differentiation is a concept dating back more than a century, when pathologists first noticed that tumor cells resemble immature cells from developing tissues (Telloni, 2017). For many types of cancer, the inverse relationship between tumor cell differentiation and prognosis has been well established and led to the development of treatment regimens that induce tumor cell differentiation (Sartorelli, 1985). Extensive cancer genomic studies from the past decade have further bolstered the concept that some cancers are a disease of arrested development. For example, somatic mutations that perturb transcription factors or signaling pathways important in development are often found in combination with mutations in oncogenes and tumor-suppressor genes. Similarly,

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This review by Zeinelden et al. discusses the origin of neuroblastoma from the developing sympathoadrenal lineage. The authors argue that neuroblastoma is a disease of arrested differentiation and describe how tumor cell heterogeneity reflects underlying developmental mechanisms.

mutations in epigenetic regulators are highly prevalent and thought to further contribute to changes in the epigenetic landscape that block differentiation and promote tumorigenesis.

The connection between arrested differentiation and oncogenesis is particularly prevalent in pediatric cancer (Chen et al., 2015). Many childhood cancers are not found in adults because they arise during a particular stage of development. For example, retinoblastoma is found only in children younger than 5 years of age because it begins during retinal development in utero (Benavente and Dyer, 2015). Also, the genomic landscape of pediatric cancer is less complex than that of adult cancers (Gröbner et al., 2018), suggesting that differentiation arrest combined with oncogenic mutations in the appropriate developmental milieu underlies tumorigenesis in infants, children, adolescents, and young adults.

One of the best examples of this concept is neuroblastoma, a tumor of the developing sympathoadrenal lineage. Neuroblastoma is the most common extracranial solid tumor of childhood, and most patients are diagnosed under the age of 10 years (Cheung and Dyer, 2013; Matthay et al., 2016). Age at diagnosis inversely correlates with outcome. Infants (< 18 months) have an 88% overall survival rate, and some have spontaneous regression (4S neuroblastoma) without therapeutic intervention (Brodeur, 2018; Kawano et al., 2021). Children between 18 months and 12 years have a 49% overall survival which drops below 10% for adolescents and young adults (>12 years). To date, mutations in *ATRX*, which encodes a chromatin remodeling protein, are the only genetic lesions associated with age at diagnosis in neuroblastoma (Cheung et al., 2012). Other genetic lesions associated with high-risk disease, such as *MYCN* amplification, oncogenic *ALK* and *RAS* pathway mutations and *TRKA* tumor suppressor mutations, are distributed across age groups (Brodeur et al., 2009; Eleveld et al., 2015; Ho et al., 2011; Matthay et al., 2016; Mosse et al., 2008). Though many of these genes play a role in sympathoadrenal development, the biological mechanism underlying highly variable survival outcomes for children with neuroblastoma remains an unanswered question.

In this review, we discuss the molecular, cellular, genetic, and epigenetic evidence in support of neuroblastoma as a disease of arrested differentiation. We review findings from recent single-cell transcriptional profiling of neuroblastoma, which resemble the developmental heterogeneity of neural crest-derived lineages. We also highlight the importance of cellular heterogeneity and arrested differentiation in neuroblastoma biology and treatment. The differentiation arrest of neuroblastoma is relevant for treatment and outcome as shown with retinoic acid (RA) therapy. However, RA is not curative and rare clones of undifferentiated cells may continue to grow and disseminate, emphasizing the importance of further research in this area.

Neuroblastoma arises from neural crest–derived lineages

Neuroblastoma was first reported as a pediatric cancer in the mid-nineteenth century (Dalton, 1885). By 1910, the neuronal origin of the disease was established based on the presence of fibrils resembling those of sympathetic ganglionic neurons (Wright, 1910). The ability of neuroblastoma cells to differentiate into mature neurons was also recognized early (Bailey, 1926). By comparing the neuronal features of neuroblastomas at diagnosis

and recurrence in the same patients, Cushing and Wolbach postulated that environmental factors influence the differentiation of tumor cells (Cushing and Wolbach, 1927). The direct connection between neuroblastoma and sympathetic ganglionic neuronal differentiation was then made by Potter and Parrish, who reported distinct stages of differentiation in a widely disseminated neuroblastoma from a stillborn infant (Potter and Parrish, 1942). These early observations are particularly impressive given our current understanding of neuroblastoma as a cancer of the developing sympathoadrenal lineage.

More than a century and a half of research on the molecular and cellular mechanisms of embryogenesis have provided a strong foundation for contextualizing the developmental origins of neuroblastoma (Bechmann et al., 2021). The sympathoadrenal lineage is derived from neural crest cells that emigrate from the dorsal neural tube and migrate to distant sites during the early stages of embryogenesis (Kerosuo and Bronner-Fraser, 2012). Neural crest cells are proliferating pluripotent progenitor cells whose repertoire of cell fates are determined by their location along the rostro-caudal axis of the developing embryo (Fig. 1A). For example, cranial neural crest gives rise to cartilage and bone whereas sacral neural crest gives rise to enteric neurons. While intrinsic programs may contribute to differentiation of neural crest derived lineages, cell fate specification is regulated primarily by non-cell autonomous cues (Graham, 2003). For example, trunk neural crest will produce mesenchymal cell types (e.g., bone, cartilage) when transplanted into the cranial region even though they do not normally produce those cell types during development (Dupin et al., 2018). Conversely, cranial neural crest cells that never make neurons can produce the entire sympathoadrenal lineage (sympathetic neurons, chromaffin cells, Schwann cells and melanocytes; Box 1) when transplanted into the trunk (Bronner-Fraser and Fraser, 1988). The specification of neural crest-derived neurons into cholinergic (produce acetylcholine) versus adrenergic (produce noradrenaline) can also be regulated by extrinsic cues (Le Douarin, 1975). As mentioned above, cells of the sympathoadrenal lineage are produced by trunk neural crest and the route of migration correlates with cell fate specification (Fig. 1B), further emphasizing the importance of extrinsic cues (Kulesa and Gammill, 2010). For example, the bone morphogenetic protein (BMP) pathway induces the specification of the sympathoadrenal lineage by upregulating the expression of SOX10, ASCL1, HAND2, GATA3, PHOX2B and other transcription factors (Huber, 2006). In addition to the dormant mesenchymal cell fate potential described above from transplantation studies, recent studies have shown that mesenchymal stem cells (MSCs) in the bone marrow niche are derived from trunk neural crest cells (Isern et al., 2014). Taken together, these data suggest that sympathoblasts (Box 1) from the trunk neural crest are bipotential and can generate both neuronal and mesenchymal cell types (Fig. 2).

The migration of the neural crest is completed by E10.5 in mice and post conception day (PCD) 40 in humans (https://www.translatingtime.org/). In the trunk neural crest, there are two overlapping waves of neural crest migration and the sympathoadrenal lineage originates during the first wave (E8.5-E9.5 in mice and PCD 27-30 in humans)(Serbedzija et al., 1990). This is important, because most studies on the specification and differentiation of the sympathoadrenal lineage have focused on the stages of development after sympathoblasts migrate out of the neural crest (Fig. 2). For example, the study by Furlan et al., which showed that Schwann cell precursors (SCPs, Box 1) give rise to chromaffin cells, was

performed at E11.5-E15.5 in mice and an analogous study by Jansky et al. was performed on human fetal adrenal from PCD 50-120 (Furlan et al., 2017; Jansky et al., 2021). While it is tempting to speculate that cellular heterogeneity within neuroblastomas reflects the pluripotency of trunk neural crest cells it is more likely to reflect the that of the post migratory cells of the sympathoadrenal lineage.

The autonomic nervous system consists of central nervous system (CNS)-derived cholinergic preganglionic neurons and neural crest derive adrenergic or cholinergic postganglionic neurons (Fig. 3A). The chromaffin cells of the adrenal medulla are also neural crest derived adrenergic cells, but they do not extend dendrites or axons like other autonomic neurons. Rather, they directly secrete neurotransmitters (adrenaline and noradrenaline) into the circulation (Fig. 3B). Chromaffin cells are stimulated by acute exposure to acetylcholine or chronic exposure to pituitary adenylate-cyclase-activating polypeptide PACAP encoded by the *ADCYAP1* gene. While many neuroblastomas express high levels of catecholamine biosynthetic enzymes, this is not universally true and a subset of neuroblastomas express PACAP neuropeptide and acetylcholine biosynthetic enzymes (Fig. 3B). As such, it remains an open question whether this variability in neurotransmitter secretion within neuroblastomas is reflective of a sympathoadrenal or chromaffin origin.

In addition to these peripheral autonomic neurons, neural crest-derived sympathoblasts also produce SCPs (Fig. 2) (Bechmann et al., 2021; Furlan et al., 2017). SCPs are multipotent progenitor cells that can differentiate into Schwann cells, as well as melanocytes and mesenchymal cells (fibroblasts and myofibroblasts) (Fig. 2B) (Solovieva and Bronner, 2021). Thus, sympathoblasts have the potential to produce both neuronal and mesenchymal cell types during normal development, and recent studies suggest that SCPs can cross the mesenchymal-neuronal developmental boundary (Furlan et al., 2017). Genetic lineage tracing studies in mice have shown that the major source of chromaffin cells in the adrenal medulla is from SCPs, and that this process occurs late in development (E11.5-E14.5). Single cell RNA-seq analysis of human fetal adrenal at similar stages of development was consistent with that model (Jansky et al., 2021; Kildisiute et al., 2021a). Thus, there is an early source of chromaffin cells from the sympathoblast and a later source of chromaffin cells from SCPs (Bechmann et al., 2021; Furlan et al., 2017). For the remainder of this review, we will refer to the specification and differentiation of chromaffin cells and sympathetic neurons from sympathoblasts as “early” sympathoadrenal development (Fig. 2). The specification of SCPs from sympathoblasts and subsequent differentiation into chromaffin cells will be referred to as the “late” sympathoadrenal development (Fig. 2). Differentiation of chromaffin cells from SCPs is accompanied by progressive downregulation of SCP genes (*SOX10, FOXD3*) and upregulation of chromaffin cell genes (*PHOX2B, ASCL1, TH*) in a “bridge” cell state (Furlan et al., 2017). While the process of sympathoadrenal development started with epithelial to mesenchymal transition (EMT), it ends with a mesenchymal to epithelial transition (MET) as cells re-establish their polarity and cellular contacts (Kerosuo and Bronner-Fraser, 2012). This is particularly relevant for neuroblastoma, as EMT and MET associated with the normal development of the sympathoadrenal lineage may play a role in intra- and inter-tumor heterogeneity, metastasis, and prognosis. Collectively, decades of intensive study dissecting neural crest differentiation have combined multiple lines of investigation to discern the developmental hierarchy of

neural crest differentiation. These findings have invigorated efforts to map and compare intratumoral heterogeneity within neuroblastoma to normal neural crest developmental states.

Neuronal and mesenchymal features of neuroblastoma

The first indication that neuroblastoma tumors exhibited cellular heterogeneity reflecting sympathoadrenal developmental heterogeneity came from studies in neuroblastoma cell lines. In the early 1980s, methods were developed to grow patient tumor samples as primary cultures and to generate immortalized cell lines (Thiele, 1998). Even with the limited availability of molecular probes at that time, it was quickly established that there were at least 2 types of cellular phenotypes in neuroblastoma cultures. The neuronal (N-type) neuroblastoma cells have features of neurons of the sympathoadrenal lineage ranging from expression of neuron specific neurofilaments to morphological differentiation and secretion of neurotransmitters (Fig. 2,3) (Thiele, 1998). The substrate adhesive (S-type) neuroblastoma cells were originally defined based on their flat, adherent cellular morphology as they spread out over the substrate in culture. These cells were later found to express genes and proteins (e.g., vimentin) found in non-neuronal (mesenchymal) neural crest derivatives (Thiele, 1998).

Over subsequent decades of research on neuroblastoma cell lines, it has been reported that S-type cells have molecular features of Schwann cells, melanocytes, ectomesenchymal derivatives and smooth muscle (Fig. 2) (Ciccarone et al., 1989; Rettig et al., 1987; Sadée et al., 1987; Sugimoto et al., 1991). It was not known if those different cellular phenotypes reflected inter- or intra-tumor developmental heterogeneity, or if it was an artifact of changes in the extrinsic cues in culture relative to the tumor microenvironment in patients. While there was evidence for S-type cells in patient tumors, it has been difficult to definitively demonstrate that S-type cells within patient tumors were malignant. Specifically, NB tumors often have non-malignant Schwann cells and mesenchymal cells in the tumor microenvironment and discriminating between cells of the tumor microenvironment from malignant cells with mesenchymal phenotypes remains a challenge.

Advances in molecular biology techniques have enabled more precise exploration of neuroblastoma heterogeneity. Boeva et al. identified super-enhancers (SEs) with neuroblastoma cell lines using H3K27Ac ChIP-seq data (Boeva et al., 2017). This in-silico analysis identified two major core regulatory circuits (CRCs) and provided a transcriptional signature of the neuronal and mesenchymal neuroblastoma cells in patient tumors. *MYCN* amplified tumors had stronger neuronal signatures, but there was no absolute correlation between any known genetic or clinical feature to the cell state-specific gene expression signatures within patient tumors (Boeva et al., 2017).

In another study, van Groningen et al. analyzed super-enhancers in primary cultures of 3 different isogenic patient neuroblastoma tumors that had gene expression signatures of both neuronal- and mesenchymal- cell types which they referred to as adrenergic (ADR) and mesenchymal (MES), respectively (van Groningen et al., 2017). Using CD133 cell surface expression as a marker for MES-type cells, they separated ADR-type and MES-type cells

from mixed primary patient samples. Purified MES-type and ADR-type cells were able to interconvert between cell states, and the authors were able to classify ADR and MES neuroblastoma cells using 369 gene and 485 gene signatures respectively. As shown in Boeva et al., patient tumors had mixed gene expression signatures and the MES-type cells were more resistant to chemotherapy.

Taken together, decades of research on neuroblastoma cell lines have shown that there are at least two types of neuroblastoma cells. There are cells with neuronal features referred to as N-type, group I, or ADR-type; hereafter, we will refer to these cells as ADR cells (Box 1). The other major cell type is non-neuronal and has been referred to as S-type, group II, or MES-type. Their precise cellular identity has remained elusive, but they share similarities with a variety of non-neuronal neural crest derivatives including Schwann cells, melanocytes, smooth muscle and ectomesenchyme cells. This cell type will be referred to as MES cells for the remainder of this review (Box 1). Individual neuroblastoma cells may be biased toward the ADR- or MEScellular phenotypes but they can interconvert in culture and in vivo (Ross et al., 1983; van Groningen et al., 2017). The interconversion may reflect the formation of chromaffin cells or post-ganglionic sympathetic neurons from SCPs late in sympathoadrenal development or it may reflect the bipotentiality of the sympathoblast to produce both cell types (Fig. 1C). There are also reports of an intermediate-type cell that may be a precursor to both mesenchymal and neuronal cell fates during normal development (Fig. 2) (Thiele, 1998).

Mapping neuroblastoma cellular heterogeneity to sympathoadrenal development

Despite these important advances in our understanding of cellular heterogeneity in neuroblastoma, it is possible that there are additional neuroblastoma cell populations yet to be identified or that ADR and MES cells can be further subdivided. While bulk RNA analyses of patient tumors suggest that most neuroblastomas have a mixture of cell populations, it is not clear how much of that apparent heterogeneity is from the non-malignant tumor microenvironment. Similarly, we cannot distinguish between separate cell populations with ADR and MES gene expression signatures and a single cell population with a hybrid ADR/MES gene expression signature. That is, while neuronal (ADR) and non-neuronal (MES) cell fate specification programs are mutually exclusive during normal development, that may not be the case in tumors. Finally, it is not known if all the cell populations are intermixed or if there are geometrical partitions within the tumor and how these developmental aspects of intra- and intertumor cellular heterogeneity relates to patient outcome.

To begin to answer some of these questions, multiple groups have carried out single-cell gene expression profiling of neuroblastomas and the developing sympathoadrenal lineages in embryos (Bedoya-Reina et al., 2021; Dong et al., 2020; Jansky et al., 2021; Kameneva et al., 2021; Kildisiute et al., 2021a). To date, 64 tumors have been profiled along with human embryos, fetal adrenal and postnatal adrenal. In the first study by Dong et al., researchers performed single cell RNA-seq (scRNA-seq) on human embryos (4 weeks post-conception),

fetal adrenal glands (8-14 weeks post-conception) and neuroblastoma tumors located near the adrenal gland. In the fetal adrenal gland, they manually annotated 3 cell types as SCPs, sympathoblasts and chromaffin cells. The researchers then analyzed 14 neuroblastomas and 2 ganglioneuroblastomas and concluded that most of the tumor cells resembled developing chromaffin cells with fewer sympathoblasts (Dong et al., 2020). However, independent reanalysis of the data showed that the authors had mistakenly switched chromaffin and sympathoblast assignments because of their manual annotation of cell types and cross species differences (Bedoya-Reina and Schlisio, 2021; Kildisiute et al., 2021b; Yang et al., 2021). Specifically, they used *CARTPT* as a marker of sympathoblasts based on data from murine adrenal development. While *Cartpt* is expressed in murine sympathoblasts, *CARTPT* is expressed in chromaffin cells in humans (Kameneva et al., 2021). This experience reinforces the importance of using well-validated and robust species-specific gene signatures to annotate cell types before applying them to tumor datasets.

In a subsequent study comparing scRNA-seq of neuroblastoma to human fetal adrenal medullae, the Behjati lab used multiple unbiased approaches to assign cell-type identity to clusters (Kildisiute et al., 2021a). In human fetal adrenal medullae, the authors identified SCPs, bridge cells, chromaffin cells and sympathoblasts. Tumors were then analyzed using either the 10x Genomics (5 tumors) or CEL-Seq2 (16 tumors) platform. Tumor cells (3,396 cells), identified using inferred copy number analysis, were found to most closely resembled ADR cells. Analysis of bulk RNA-seq from 650 tumors in the TARGET dataset confirmed the presence of their ADR gene expression signature in all tumors and suggested that patients with less adrenergic neuronal differentiation had worse outcomes.

A third study published by the Schlisio lab analyzed 11 neuroblastomas (3,212 cells) using SMART-seq2 (Bedoya-Reina et al., 2021). In their neuroblastoma cohort, they identified two distinct groups of tumor cells. Cells in the first were undifferentiated, expressed *PDGFRA*, and resembled MES neuroblastoma cell lines; the second group resembled ADR neuroblastoma cell lines. In agreement with prior studies, high-risk neuroblastomas had less of a neuronal differentiation cell signature. Though the authors found cells resembling MES cell lines, they concluded that there were no tumor cells mimicking fetal SCPs. This highlights the challenge in the field regarding the precise identity of the MES cells.

The most recent study from the Westermann lab performed single-cell transcriptomic analysis of normal human fetal adrenal identified SCPs, chromaffin cells, a bridge population, and sympathoblasts (Jansky et al., 2021). The most proliferative cells were either SCPs or sympathoblasts. All 14 neuroblastomas analyzed in this study had malignant cells with the ADR signature and 3 had a subpopulation of cells with the MES signature. The authors proposed that the MES neuroblastoma tumor cells most closely resembled bridge cells (Jansky et al., 2021).

There are several themes that have emerged from these independent scRNA-seq studies of neuroblastoma and developing sympathoadrenal lineage. First, ADR cells were identified in every patient tumor and there is agreement that tumor cells have features of neuronal differentiation. Second, there is general agreement that patients with more differentiated ADR gene expression signatures have better prognoses. Some studies identified tumor

cells with the MES signature, but the precise cell-type identity (SCPs, Schwann cells, melanocytes, smooth muscle, MSCs, fibroblasts, myofibroblasts) remains elusive. Third, all studies used inferred copy number analysis to identify tumor cells; this method is imprecise, and it is possible that the presence or absence of MES cells reflect differences in the thresholds used for copy number inference, differences in data quality or variation in computational methods to annotate tumor cells from the microenvironment. Fourth, cell-type assignments can also by imprecise based on whether clusters are manually annotated using a small number of genes or if cell type identities were assigned using unbiased methods. For example, hybrid gene expression signatures of multiple cell fates in a single cell type were not evaluated. Finally, there may be differences in sympathoadrenal development between mice and humans and this must be considered when assigning cell types within scRNA-seq data.

Despite these important advances in our understanding of the cellular heterogeneity of neuroblastoma and the developing sympathoadrenal lineage, there are several challenges to consider when mapping neuroblastoma gene expression signatures to the sympathoadrenal lineage. The first challenge is the role of non-cell-autonomous cues in mesenchymal cell fate specification along the rostral caudal axis of the developing neural tube. We know from cell transplantation studies that trunk neural crest cells that normally give rise to the sympathoadrenal lineage have the dormant potential to produce mesenchymal cell fates if exposed to different extrinsic cues. Moreover, they also produce mesenchymal stem cells of the bone marrow during normal development (Isern et al., 2014). Furthermore, the discovery that SCPs can produce both chromaffin cells and mesenchymal cells further complicates our ability to interpret the heterogeneity within neuroblastoma tumors in relation to developmental heterogeneity. Future studies should focus on discerning the role of extrinsic signals and intrinsic programs in the ADR and MES cellular heterogeneity in neuroblastomas and refining the gene expression signatures of each normal cell population.

Another challenge is the interpretation of the directionality of the developmental transitions as it relates to neuroblastoma interconversion. During normal development, the sympathoblast and SCPs can give rise to both neuronal and mesenchymal fates (Fig. 2). Therefore, it is not known if the interconversion between ADR and MES neuroblastoma cell states reflects that normal developmental potential, or a consequence of different extrinsic cues. In either case, we cannot infer cellular origin from the observation of ADR and MES neuroblastoma cell interconversion. As the study by Wang et al. showed, definitive identification of cell types is challenging for the sympathoadrenal lineage, and this is even more complicated for neuroblastoma tumors (Wang et al., 2019). While there are two major types of tumor cell lines (ADR and MES), and those cell populations are present in patients' tumors at different ratios, identifying their correlates in normal development is not straightforward. The ADR cells have many similarities with adrenal chromaffin cells in that they produce catecholamines. However, they form neuronal processes in culture even though chromaffin cells in the adrenal medulla lack any neuronal processes. The post-ganglionic sympathetic neurons with dendrites and axons can be cholinergic or adrenergic and there is evidence for acetylcholine production in a subset of neuroblastomas. The MES cells have features of SCPs and their non-neuronal (mesenchymal) derivatives (Furlan et al., 2017), but unambiguous cell-type assignments remain elusive. This may reflect

the dormant mesenchymal potential of trunk neural crest, the ability of sympathoblasts to produce MSCs or presence of a hybrid cell state that doesn't normally exist during development. It is possible that our expectation for tumor cells to accurately recapitulate the normal gene expression programs is misguided. We know in other tumors with neuronal features that the cells can express hybrid signatures that never exist normally during development due to deregulation of the normal process (Norrie et al., 2021). Rather than trying to precisely align developmental and cell heterogeneity, it may be more informative to study the lineage relationships in tumors with molecular barcoding or other methods. This could help us gain a deeper understanding of the clonal dynamics in tumors and how developmental heterogeneity contributes to clonal evolution and selection during treatment. Indeed, emerging evidence suggests that MES cells preferentially survive treatment and contribute to neuroblastoma recurrence (van Groningen et al., 2017).

Mutational spectrum of neuroblastoma

The landscape of genomic mutations provides further evidence for neuroblastoma as a disease of arrested differentiation (Brady et al., 2020). One of the best examples is the homeobox transcription factor, PHOX2B. Sympathoadrenal development requires PHOX2B to activate transcription factors (HAND2, GATA2/3, ASCL1), which are required for cell fate specification within the sympathoadrenal lineage (Tomolonis et al., 2018). For example, ASCL1 activates PHOX2A which in turn activates genes required for catecholamine biosynthesis (Tomolonis et al., 2018). Due to this prominent role in sympathoadrenal development, it is not surprising that reduced expression of PHOX2B in familial neuroblastoma can promote malignant transformation (Raabe et al., 2008).

Another example is the ALK receptor tyrosine kinase, which is the most common familial neuroblastoma predisposition gene and is somatically mutated in sporadic cases (Mosse et al., 2008). Mutations in *ALK* are oncogenic by promoting ligand-independent activation of the kinase (Bresler et al., 2014). In mouse embryos, *Alk* is expressed in the developing neural crest (Gonzalez Malagon et al., 2018) and *Alk*-deficient mice have defects in neuronal differentiation (Weiss et al., 2012). While the precise molecular and cellular function of ALK in sympathoadrenal development has not yet been elucidated, it is possible that ALK plays a role in regulating the proliferation of sympathoblasts and deregulation of ALK signaling through oncogenic mutations leads to hyperplasia and malignant transformation.

Oncogenic pathways and developmental pathways are often integrated because proliferation and differentiation must be precisely coordinated during development. Indeed, some genetic lesions in neuroblastoma play a dual role by blocking differentiating and promoting tumorigenesis. For example, *MYCN* amplification leads to persistent high levels of expression and is a strong indicator of poor prognosis in neuroblastoma patients (Cheung and Dyer, 2013). During normal development, transient low-level expression of MYCN is important for promoting neural crest cell migration and neuronal cell fate specification (Wakamatsu et al., 1997). However, when MYCN is persistently expressed at high levels in the sympathoadrenal lineage, this leads to sympathoblast hyperplasia and neuroblastoma formation (Cohen et al., 2020; Weiss et al., 1997). Thus, MYCN amplification blocks

differentiation and promotes deregulated proliferation which can ultimately leads to tumor formation in patients.

Disruption of the normal epigenetic mechanisms during neural crest development may result in differentiation arrest and neuroblastoma formation. Somatic mutations in chromatin remodelers, including *CHD5* (Koyama et al., 2012), *ARID1A/ARID1B* (Sausen et al., 2013), *BRG1* (Jubierre et al., 2016), and *ATRX* (Cheung et al., 2012) have been found in neuroblastoma. As for transcription factors like PHOX2B and MYCN, a major question in the field is whether these mutations block sympathoadrenal cell differentiation, promote tumorigenesis or both.

Among the recurrent somatic mutations in chromatin remodelers, *ATRX* mutations are the most common and are associated with high-risk disease, older age at diagnosis, and poor prognosis (Cheung et al., 2012; Zeineldin et al., 2020). ATRX is crucial for the development of the nervous system, and germline mutations of *ATRX* cause developmental defects and neuronal cell death (Berube et al., 2005). Unlike *PHOX2B*, germline *ATRX* mutations do not increase neuroblastoma susceptibility, suggesting that additional mutations are required for tumorigenesis (Cheung and Dyer, 2013).

ATRX is involved in the deposition of histone H3.3 at repetitive regions across the genome, including telomeres (Wong et al., 2010). Altered H3.3 deposition at DNA repeats can cause accumulation of secondary DNA structures, particularly at G-rich regions (G-quadruplex structures) that can interfere with DNA replication and transcription (Law et al., 2010). As a result of defects in H3.3 deposition at telomeres, *ATRX* mutant neuroblastomas undergo homologous recombination and have the alternative lengthening of telomeres (ALT) phenotype (Clynes et al., 2015). This allows *ATRX* mutant neuroblastomas to maintain long telomeres and prevent replication-induced telomere attrition and crisis (Cheung et al., 2012). The defects in H3.3 deposition also disrupt expression of genes important for neuronal differentiation including the retinoic acid responsive genes (Zeineldin et al., 2020). Together, these findings suggest that *ATRX* mutations promote tumorigenesis through the ALT telomere maintenance program and block neuronal differentiation. Interestingly, *ATRX* mutations are mutually exclusive with *MYCN* amplification (Zeineldin et al., 2020). This is unusual because in most tumors, potent oncogenic lesions (e.g., *MYCN*) and tumor suppressor mutations (e.g., *ATRX*) cooperate in the aggressive forms of disease. Our work showed that *MYCN* amplification and *ATRX* mutations are incompatible in neuroblastoma because each lead to DNA replicative stress and the combination is synthetic lethal (Zeineldin et al., 2020). Ongoing efforts are focused on exploiting this synthetic lethality with drug combinations that induce DNA replicative stress to improve outcomes for patients.

Spontaneous regression of neuroblastoma

Despite an overall poor outcome, neuroblastoma has one of the highest rates of spontaneous regression without therapeutic intervention (Kawano et al., 2021; Matthay et al., 2016). Most cases of spontaneous regression are seen in patients younger than 18 months at diagnosis. Brodeur has proposed candidate mechanisms for the spontaneous regression including loss of paracrine signaling, failure to maintain telomeres, immune rejection,

and epigenetic activation of the sympathoadrenal-differentiation program (Brodeur, 2018). Signaling through the neurotrophin receptors (NTRK1,2,3) and their ligands (NGF, BDNF, NTF3) is important for neural crest and sympathoadrenal development and in neuroblastoma cells (Anderson, 1993). For example, neuroblastoma cells with high NTRK1 expression can undergo apoptosis in the absence of its ligand (NGF) and when NTRK1 is stimulated by its ligand, they differentiate (Nakagawara et al., 1993). Thus, a 4S neuroblastoma tumor with high NTRK1 in an infant may grow for a period when NGF is present but spontaneously regress when the ligand dissipates as part of normal development. In contrast, BDNF signaling through NTRK2 in neuroblastoma is often found in *MYCN* amplified neuroblastomas with poor prognosis (Nakagawara et al., 1994). Thus, developmental signaling through the NTRK family of receptors is perturbed in neuroblastoma and may contribute to the heterogeneity of patient response based on age of diagnosis.

The rate of spontaneous regression of neuroblastoma is also thought to be higher than that reported for clinical cases, because some tumors probably regress without ever being diagnosed. Large-scale screening of young children for elevated catecholamine levels in urine led to a higher rate of neuroblastoma diagnoses but no improvement in overall survival, because most of those additional diagnoses spontaneously regress without intervention (Yamamoto et al., 1998). In addition, hyperplastic foci that histologically resemble neuroblastoma have been identified at autopsy in 1 of every 40 infants younger than 3 months who died of non-neoplastic causes (Beckwith and Perrin, 1963). The authors called these foci “in situ neuroblastoma” and speculated that they are like neuroblastoma that undergoes spontaneous regression. They reasoned, that as the incidence of in situ neuroblastoma is 200 times greater than that of malignant neuroblastoma, most neuroblastomas undergo spontaneous regression. Together, these data suggest that in situ neuroblastoma and rests represent a form of normal hyperplasia involving progenitors of sympathoadrenal lineage during development. When those hyperplastic cells sustain an oncogenic mutation, they may progress to neuroblastoma.

It is possible that the switch between “early” and “late” modes of sympathoadrenal development may play a role in the spontaneous regression of 4S NB in infants (Kastriti et al., 2020). That is, early in development when SCPs do not contribute to the sympathoadrenal lineage, the rests may lack SCPs or SCP-derived cells and then spontaneously regress. However, at later stages of development when SCPs contribute to sympathoadrenal development, the SCPs may interfere with this spontaneous regression. Thus, further studies on the signaling between SCPs and their derivatives and the different populations of tumor cells, immune cells and the remainder of the tumor microenvironment are needed to determine if any aspect of the “early” and “late” mechanisms of development contribute to spontaneous regression of 4S NB in infants.

Inducing neuronal differentiation as a therapeutic approach in neuroblastoma

To date, the most effective agent for inducing neuroblastoma differentiation in culture and in vivo is retinoic acid (RA). Sidel treated a neuroblastoma cell line with RA and showed

an irreversible reduction in proliferation and an increase in neurite formation across several neuroblastoma cell lines (Seeger et al., 1982). The RA derivative, 13-cis RA, moved quickly into clinical testing for neuroblastoma and is now part of the standard of care (Matthay et al., 1999). The rationale is that 13-cis RA induces differentiation of residual tumor cells after patients receive chemotherapy, surgical resection, and bone marrow transplantation.

Another strategy for exploiting the differentiated features of neuroblastoma cells for therapeutic benefit incorporates immunotherapy. Two groups identified the GD2 ganglioside on the surface of neuroblastomas and other tumors of neuroectodermal origin (Cahan et al., 1982; Schulz et al., 1984). Research on myelin sheath composition in relation to multiple sclerosis showed that GD2 is a minor proportion (approximately 2%) of the gangliosides expressed on cells in the brain and spinal cord but makes up a higher proportion (5%-8%) of those expressed on peripheral nerves (Svennerholm et al., 1994). Therefore, researchers incorporated anti-GD2 immunotherapy in the treatment of neuroblastoma and demonstrated efficacy in preclinical models and patients (Nguyen et al., 2018; Yu et al., 2010). Anti-GD2 immunotherapy is now standard of care for patients with high-risk neuroblastoma, and efforts are ongoing to reduce the side effects and improve efficacy of anti-GD2 immunotherapy (Furman et al., 2019; Furman et al., 2022).

The ability of neuroblastoma cells to reuptake catecholamines and catecholamine metabolites also has been exploited clinically for neuroblastoma diagnosis and treatment. Metaiodobenzylguanidine (MIBG) is a catecholamine analog that is taken up by catecholaminergic cells. There are two forms of MIBG based on the half-life of their iodine radiolabel: ^{131}I-MIBG has a half-life of 8 days and is being tested for treatment, and ^{123}I-MIBG has a half-life of 13 hours and is used for diagnostic imaging (Matthay et al., 2007). MIBG was first used to treat pheochromocytoma, another tumor that arises from adrenal medullary cells (Wieland et al., 1980). Currently, MIBG is used for recurrent neuroblastoma and is being tested in newly diagnosed patients with high-risk disease.

While RA, anti-GD2 immunotherapy, and MIBG are three examples of basic developmental neurobiology research being integrated with cancer biology to improve outcomes, it is important to emphasize that current chemotherapeutic agents can eliminate neuroblastoma in a significant fraction of patients. However, there are limitations due to the cellular heterogeneity of neuroblastoma. Specifically, anti-GD2 immunotherapy and MIBG approaches are focused on targeting the tumor cell population(s) in neuroblastoma with the ADR phenotype while RA is used to induce differentiation in neuroblastoma precursors that underwent differentiation arrest. While data are still emerging regarding the lineage relationships between the MES and ADR cells, our current understanding is that the MES cells are not responsive to RA, do not display GD2 on their cell surface and are not likely to take up MIBG because they are not catecholaminergic. Thus, future efforts should focus on understanding the implications of neuroblastoma heterogeneity in responding to different therapeutic strategies.

Multiple groups have started to explore the biological basis underlying the differential response of ADR versus MES neuroblastoma cells to therapy. In one study, knocking down ASCL1, an ADR TF, disrupted the neuroblastoma CRC and made these cells more

responsive to RA alone or in combination with JQ1, an epigenetic agent (Alleboina et al., 2021). Disrupting MES CRCs by targeting *SNAI2* was also shown to induce RA-sensitivity in neuroblastoma cells (Vrenken et al., 2020). Zimmerman et al. treated two *MYCN*-amplified neuroblastoma cells lines with all-trans retinoic acid (ATRA), the active metabolite of RA, and assessed changes in CRCs (Zimmerman et al., 2021). ATRA reduced the expression level of several ADR CRC-associated TFs including MYCN, GATA3, PHOX2B and ASCL1. Moreover, ATRA induced the emergence of a novel CRC characterized by the binding of the transcription factors MEIS1, SOX4, and RARA to novel super-enhancers. The overall result was a mixed picture, with preservation of a fraction of the classical ADR CRC-associated TFs, suggesting that some ADR-associated CRCs are important in RA-inducing differentiation (retino-sympatho CRCs). This is intriguing because the absence of these retino-sympatho CRCs in MES neuroblastoma cells may explain the lack of MES neuroblastoma response to RA-inducing differentiation. This discrepancy in RA response between MES and ADR cells highlights the importance of understanding neuroblastoma transcriptional and cellular heterogeneity for better treatment of the disease.

The differential response of MES and ADR cells is not limited to RA. Recently, Westerhout et al. tested the sensitivity of two pairs of isogenic MES/ADR cell lines with activating *ALK* mutations to two ALK inhibitors (Westerhout et al., 2022). They found, that unlike ADR cells, MES cells with *ALK* mutations were resistant to ALK inhibition. The authors showed that ADR cells had an enrichment for H3K27Ac near the *ALK* locus with a possible physical interaction between the site of the peak of this chromatin mark and the *ALK* transcription start site, suggesting a functional super-enhancer. This super-enhancer was absent in MES cells with no detectable expression of mutant or wildtype *ALK*. Using previously published scRNA-seq data, the authors showed that *ALK* is expressed in committed sympathoblasts but not SCP cells in mouse developing adrenal medullae, supporting the model that MES cells represent more immature forms of cells relative to ADR cells. Taken together, these examples highlight the importance of studying the normal sympathoadrenal differentiation program in neuroblastoma and elucidating the lineage relationships before, during and after treatment.

Developmental heterogeneity and clonal evolution

Extensive genomic studies have demonstrated evidence of parallel clonal evolution of primary and metastatic sites and treatment resistant clones that contribute to clonal selection in refractory/recurrent neuroblastoma (Abbasi et al., 2017; Chicard et al., 2018). While those studies were useful in elucidating the clonal architecture of tumors before and after treatment, they provided little insight into the underlying phenotypes of the cells that survive treatment and contribute to clonal selection and disease recurrence (Fig. 4). However, when combined with sc/sn RNA-seq analyses or other methods, researchers can begin to understand the properties of the cells that survive treatment and how they may change during clonal selection and evolution (Patel et al., 2022). This is particularly relevant for neuroblastoma because of the observation that ADR and MES cells can interconvert and have differential sensitivity to chemotherapy (Fig. 4A,B). For example, it is possible that MES cells represent a minor population in a patient's tumor at diagnosis because they are

dividing slowly or are quiescent. During treatment, the more rapidly dividing ADR cells may be eliminated, and a subset of the MES cells survive. This would be consistent with the clonal selection from genomic studies (Fig. 4C,D). Those residual MES cells may then become re-activated and interconvert to ADR cells to re-establish the cellular heterogeneity of the original tumor. Indeed, it was shown that clonal expansion of neuroblastoma cells in vitro usually results in a mixed population of cells with both adrenergic and mesenchymal phenotypes (Ross et al., 1983). Moreover, patient-derived neuroblastoma cells sorted into ALDH1A3-positive (MES), or ALDH1A3-negative (ADR) cells grew in mouse adrenals to form heterogenous population that contains both ALDH1A3 positive and negative cells (van Groningen et al., 2021). Recently, NOTCH signaling was shown to switch the transcriptional profile of ADR cells to that of MES cells emphasizing the complex intersection of cell intrinsic and extrinsic signaling (van Groningen et al., 2019). While this is just one possible model (Fig. 4B–D), it highlights the complexity of developmental heterogeneity and clonal selection in the context of treatment and recurrence (Fig. 4).

There are now tools available to unambiguously label individual clones of cells with unique barcodes and profile their cellular identity at single cell resolution to gain a deeper understanding of developmental heterogeneity and clonal evolution during treatment (Patel et al., 2022). This type of research has the potential to transform our understanding of disease recurrence and develop biomarkers that could predict tumor behavior and/or therapeutic response. It can also help adopting better therapeutic combinations that eliminate all the clones (major and minor) during initial treatment to prevent disease recurrence in pediatric solid tumor. Indeed, this is particularly important for neuroblastoma, because patients with recurrent neuroblastoma have survival rates below 10%.

Conclusions and future directions

Neuroblastomas arise because of differentiation arrest of the developing sympathoadrenal lineage and includes adrenergic (ADR) and non-neuronal mesenchymal (MES) cell populations. Studies on neuroblastoma cell lines dating back more than 3 decades suggested that tumor cells are heterogeneous with cellular phenotypes that may resemble some aspects of normal developmental heterogeneity. Recent sc/sn-RNA-seq studies have further refined this developmental heterogeneity and provide a foundation for understanding their lineage relationships and their ability to interconvert. A detailed understanding of those relationships is essential to understand clonal evolution and selection with treatment, with the goal of preventing recurrence by effectively targeting residual tumor cells. The efficacy of targeting neuroblastoma with chemotherapeutic agents have improved by targeting the neuronal cell types (anti-GD2 and MIBG) and by inducing differentiation in precursor cells (RA). However, this regimen is not effective against the mesenchymal (MES) cells and these tumor cell population might contribute to disease recurrence so new agents to target the mesenchymal population are needed. This will help researchers design more effective treatment regimens that achieve the goal of total clonal elimination to achieve cure.

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BOX 1

ADR-type neuroblastoma (neuronal)

N-type neuroblastoma cells appear neuroblastic and can undergo partial neuronal differentiation in the presence of retinoic acid. In classic studies, ADR cells express neurofilaments and do not express vimentin. They have also been shown to express neurotransmitter biosynthetic enzymes. Van Groningen et al. showed ADR are PROM1 (CD133)-negative and had *PHOX2A, PHOX2B, DBH, TH* and 369 other differentially expressed genes relative to MES cells (van Groningen et al., 2017). Additional genes used to identify ADR cells include *CHGA, DLK1* and 18 transcription factors including *ASCL1, EYA1, GATA3, HAND1, SIX3*. Boeva et al. called N-type cells GROUP I and showed that they express many of the same genes as those defined by Goningen et al. with addition of *HAND2* (Boeva et al., 2017). Bedoya-Reina et al. used *ISL1, PHOX2B, CHGB, CHGA, TH, CARTPT, NTRK1* for this cell population (Bedoya-Reina et al., 2021).

MES-type neuroblastoma (substrate adherent)

MES neuroblastoma cells appear flat and adhere to substrate in culture. They do not express neurofilament and they express vimentin and produce collagen and fibronectin. Alpha smooth muscle actin was found only in MES-cells (Sugimoto et al., 1991). Groningen et al. showed that MES cells expressed PROM1 (CD133) along with *VIM, FN1, SNAI2* and 485 other differentially expressed genes relative to N-type cells (van Groningen et al., 2017). Additional genes include *WNT5A, IGFBP2, IL13RAI* and 20 transcription factors including *MEOX1, MEOX2, SIX1, SIX4, SOX9, SMAD3, WWTR1*. The Boeva et al. found that they also express *FOSL1, FOSL2, JUN* (Boeva et al., 2017). Bedoya-Reina et al. used *PRRX1, COL12A1, COL6A3, COL12A1, PDGFRB, PDGFRA* and *YAP1* (Bedoya-Reina et al., 2021). In the Kameneva et al. analysis of normal cells, *VIM* is most highly expressed in SCPs, subepicardial mesenchyme and melanocytes (Kameneva et al., 2021). This is important because in the historical literature, these MES-cells have been attributed to smooth muscle, Schwann cells and melanocytes.

Schwann Cell Precursor (SCP)

SCPs are derived from the developing neural crest and sympathoblasts and migrate along peripheral nerves during development to give rise to glial cells. More recent studies suggest that SCPs may represent a multipotent progenitor cell population that can respond to stress, injury, or disease in a variety of organ sites. There is now evidence that SCPs can produce chromaffin cells of the adrenal medulla and possibly adrenergic post-ganglionic sympathetic neurons. Kildisiute et al. used *SOX10, ERBB3, MPZ,* and *PLP1* as SCP markers (Kildisiute et al., 2021a). Bedoya-Reina et al. also used *S100B, FABP7, FOXD3* (Bedoya-Reina et al., 2021). Jansky et al. used many of the same markers and included *CDH19* (Jansky et al., 2021). In Kameneva et al., they include *EDNRB* and *SPARC* (Kameneva et al., 2021).

Chromaffin cells

Chromaffin cells are found in the medulla of the adrenal gland and secrete epinephrine and norepinephrine. They are essentially adrenergic post-ganglionic neurons that failed to undergo morphological differentiation and form neurites, axons, and dendrites. Bedoya-Reina et al. reported chromaffin cells have *CHGA, CHGB, TH, PHOS2A, PNMT, CARTPT, STMN2* in mature postnatal adrenal glands (Bedoya-Reina et al., 2021). Jansky also used *DDC* for this cell population and *PNMT* as a later marker (Jansky et al., 2021). Kameneva added *PENK, GNAS, ADM* and *DLK1* (Kameneva et al., 2021).

Sympathoblasts

Sympathoblasts are proliferating cells derived from the neural crest that can produce postganglionic sympathetic neurons, chromaffin cells, SCPs, and mesenchymal stem cells of the bone marrow. Jansky et al. used *NEFM, GAP43, STMN2, ISL1, ALK* at early stages to identify sympathoblasts (Jansky et al., 2021). *SYN3* and *IL7* were used for later stage sympathoblasts. Kameneva et al. also added *PRPH, ELAVL3, ELAVL4, PHOX2B, HAND2* (Kameneva et al., 2021).

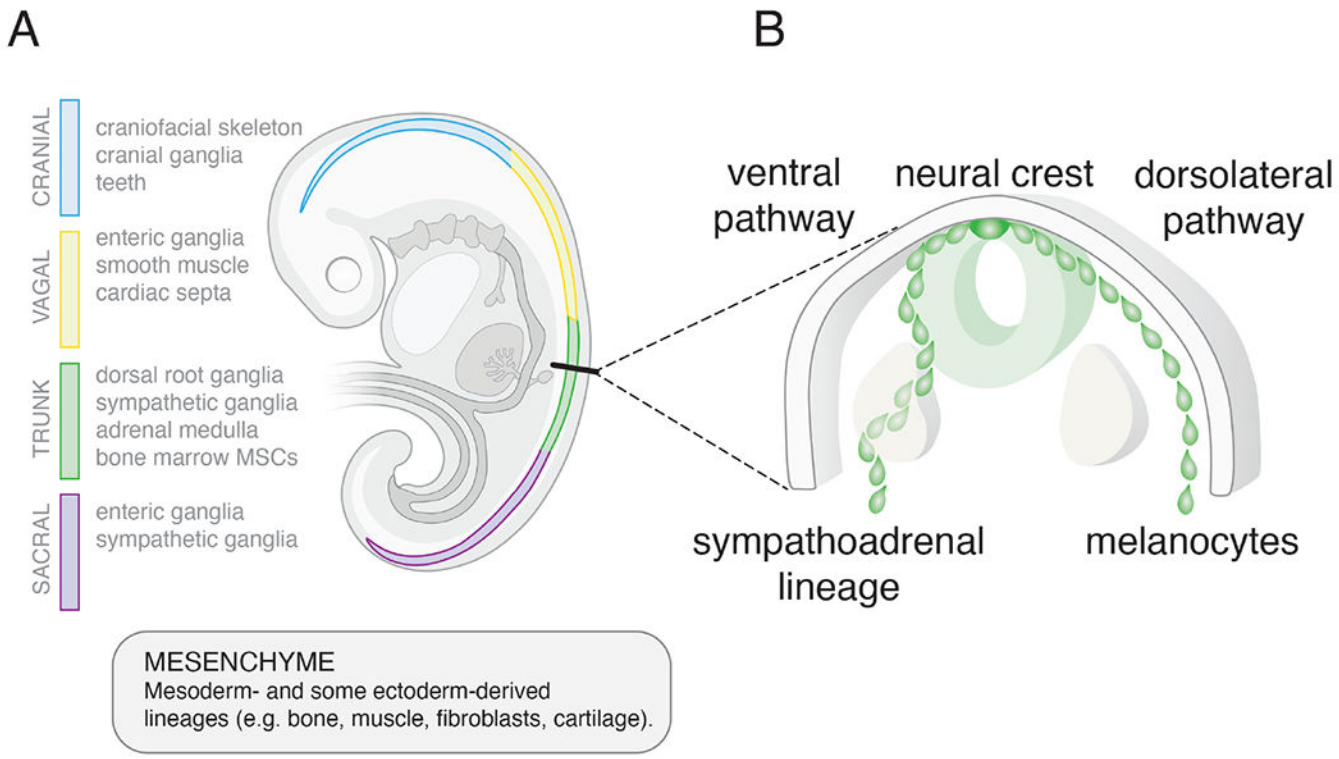

Figure 1. Extrinsic cues regulate neural crest lineage specification.

A) Drawing of a human embryo showing the neural crest (4-color). The cellular lineages derived from the neural crest vary based on their rostral-caudal location as shown (cranial (blue), vagal (yellow), trunk (green), sacral (purple)). Transplantation studies have shown that lineage specification is regulated primarily by the extrinsic cues rather than cell-autonomous factors. Trunk neural crest cells transplanted into the cranial region will produce cranial lineages and vice versa. Neural crest cells are multipotent and can give rise to both neuronal and non-neuronal (mesenchymal) cell types. This is particularly relevant for neuroblastomas that arise from the sympathoadrenal lineage of the trunk neural crest because they have both neuronal (N-type) and mesenchymal (S-type) cell populations. **B)** Drawing of a cross section of the trunk neural crest showing the two paths of migration. Both paths occur bilaterally but for simplicity the ventral pathway is shown on the left side of the embryo and the dorsolateral pathway is shown on the right. Extrinsic cues in these different regions of the embryo contribute to specification of the sympathoadrenal lineage and melanocytes. Thus, not only is the position along the rostral caudal axis important for neural crest derived cell lineage specification but the path of migration (ventral versus dorsolateral) is also important.

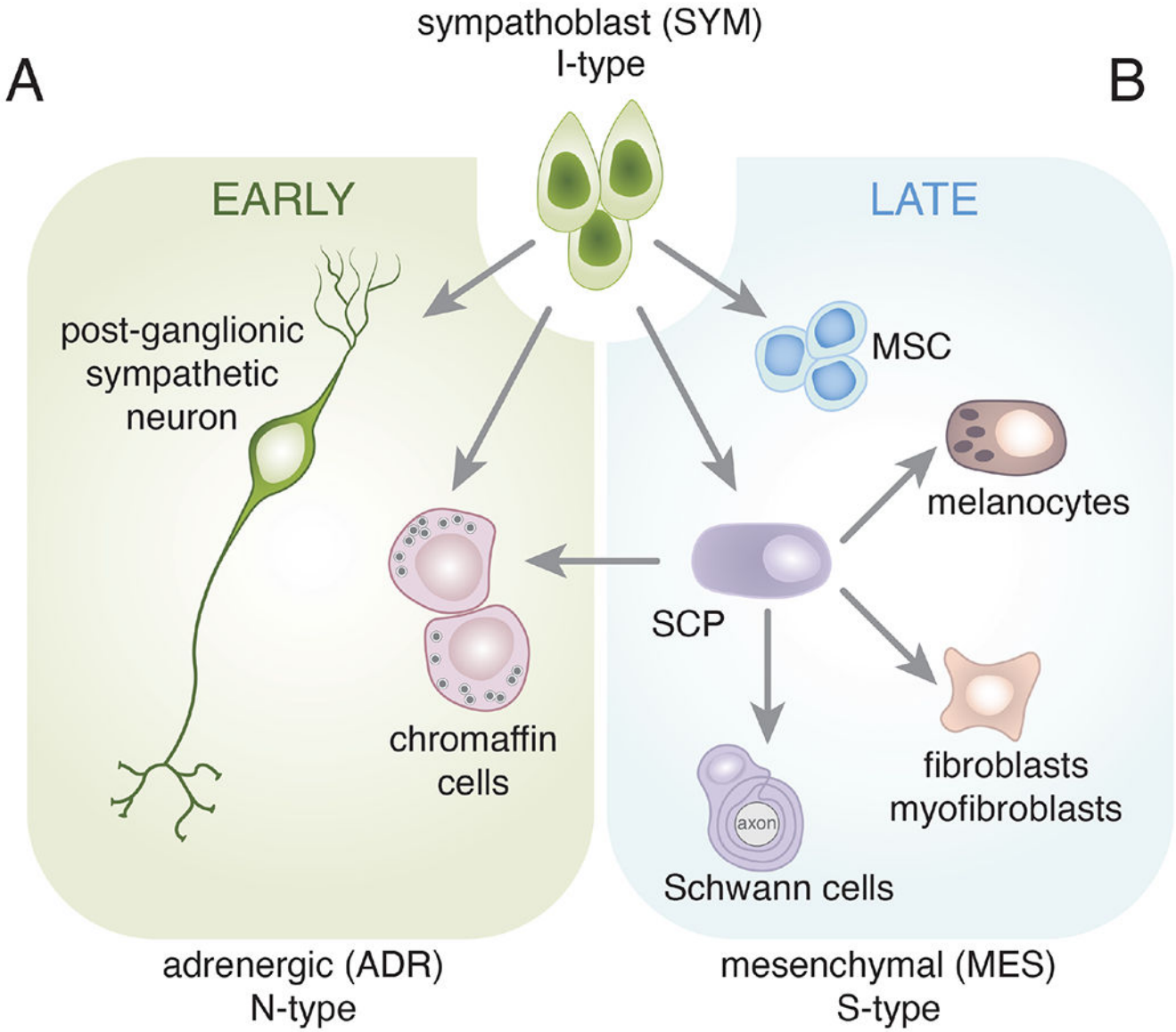

Figure 2. Sympathoblast cell fate specification.

Schematic drawing of major cell fate specification events in the sympathoadrenal lineage and their derivatives. This is not intended to be comprehensive but rather to highlight the multipotency of the sympathoblasts. The neural crest is a transient population and after cells have migrated out of the trunk neural crest along the ventral pathway, a subset of cells commits to become sympathoblasts (I-type). Shortly after the neural crest cells have dispersed and the sympathoblasts have been specified, they form two cell types with neuronal (N-type) features. The post-ganglionic sympathetic neurons may be cholinergic or adrenergic. The chromaffin cells lack traditional dendrites and axons but synthesize catecholamines (epinephrine and norepinephrine) and release those neurotransmitters into the circulation from the adrenal medulla. These are some of the first developmental events of the sympathoadrenal lineage and help to establish the patterning of the sympathetic ganglia and developing adrenal gland. **B)** Later during development, the sympathoblasts

produce Schwann cell precursors (SCPs) and mesenchymal stem cells (MSCs). The SCPs can produce mature Schwann cells that associate with the axons of the sympathetic neurons. They can also produce mesenchymal cell types such as fibroblasts and myofibroblasts. The MSCs migrate to the bone marrow where they contribute to the hematopoietic stem cell niche. Importantly, SCPs have been shown to be the major source of chromaffin cells in the late stages of adrenal development. This resembles the interconversion of N-type and S-type neuroblastoma cells. Therefore, while individual cell types (e.g., SCPs) are multipotent, the sympathoblasts are the most immature cell population in this lineage and may resemble the I-type neuroblastoma cells. This is not intended to be comprehensive, and each arrow may include several distinct stages of cell fate specification and differentiation. For example, bridge cells are thought to be an intermediate state from SCP to chromaffin cells during the later stages of adrenal medulla development.

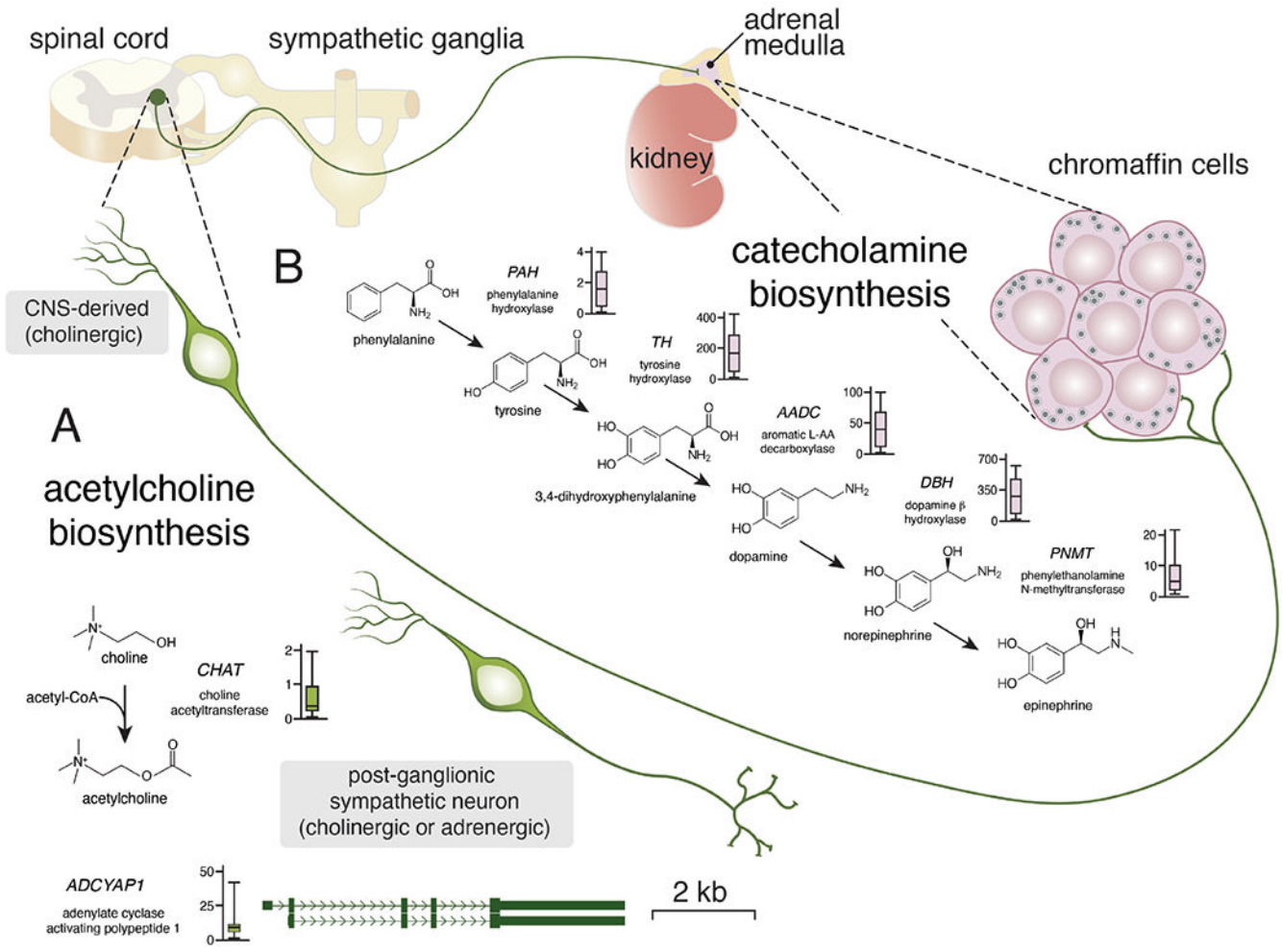

Figure 3. Expression of sympathoadrenal neurotransmitters and neuropeptides in neuroblastoma.

Simplified drawing of the neuronal connections in the sympathoadrenal lineage. Central nervous system (CNS) derived pre-ganglionic neurons synapse directly with chromaffin cells of the adrenal medulla where they release acetylcholine. Once stimulated, the chromaffin cells release epinephrine or norepinephrine into the circulation. Some chromaffin cells release epinephrine and some release norepinephrine from their dense core vesicles.

A) Acetylcholine biosynthesis is regulated by choline acetyltransferase and the expression is relatively low (box plot) in the 191 representative neuroblastoma tumors. Acetylcholine is used for acute stimulation of the chromaffin cells. The neuropeptide encoded by *ADCYAP1* is used for more chronic stimulation. While expression of *ADCYAP1* is low in most neuroblastomas, a subset has very high levels of expression suggesting inter-tumor heterogeneity.

B) Drawing of the biosynthetic pathway for catecholamines with relevant gene and abbreviation for each enzyme. Next to each gene name is a boxplot of RNA expression (FPKM) from 191 neuroblastomas tumors (www.stjude.org/pecan). The chromaffin cells of the adrenal medulla secrete catecholamines as do a subset of post-ganglionic sympathetic neurons that are derived from sympathoblasts.

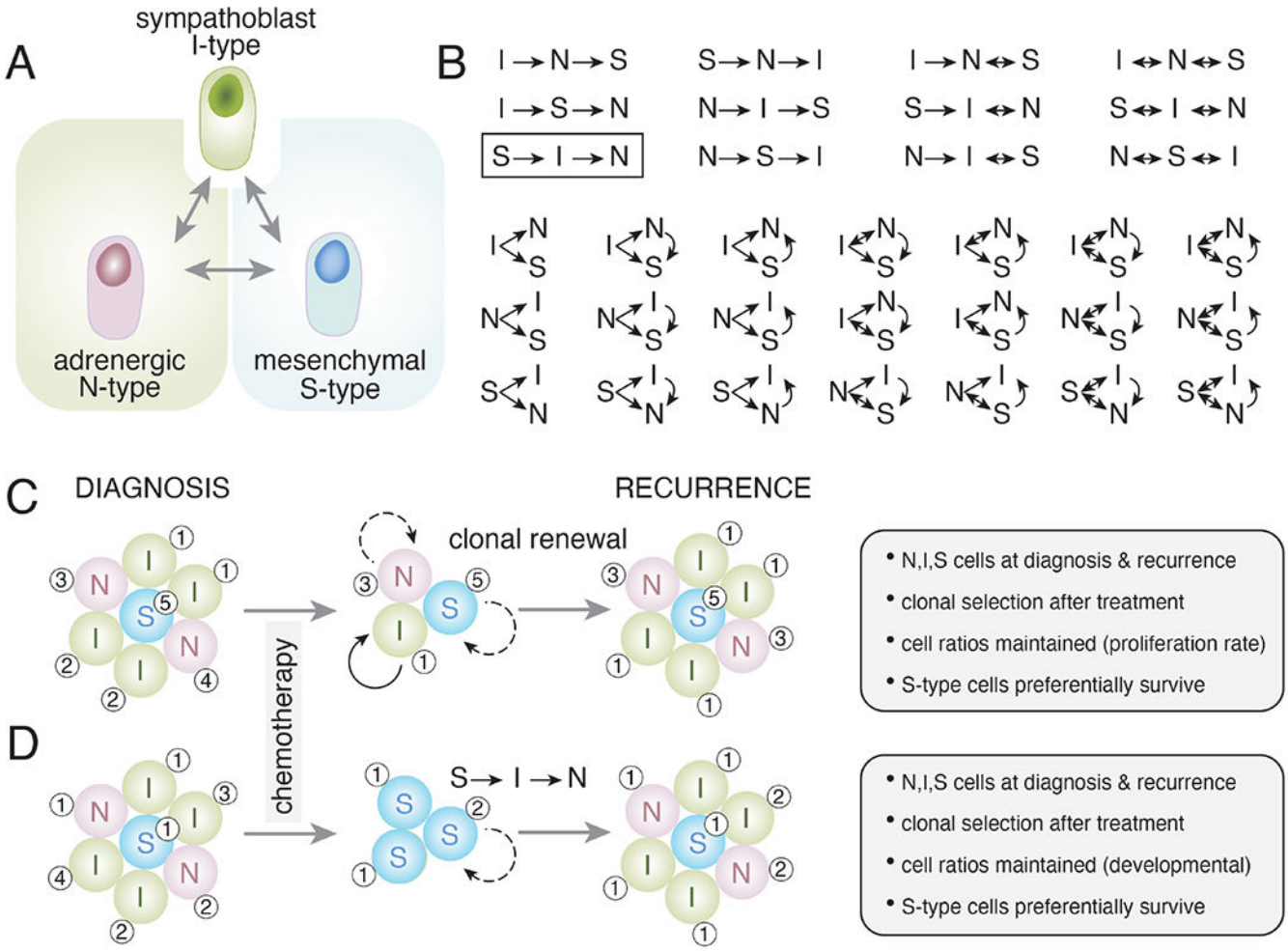

Figure 4. Neuroblastoma differentiation.

A) Drawing of one hypothetical simplified relationship between immature (I-type) neuronal (N-type) and mesenchymal (S-type) neuroblastoma cells. **B)** With just these 3 cell types, there are 34 possible relationships in terms of the ability of cells to produce other cell phenotypes and to interconvert. **C,D)** These 34 different possible relationships are relevant when considering clonal selection and clonal evolution after treatment with chemotherapy. Broad spectrum systemic chemotherapy primarily kills the proliferating cells. In both examples (C,D), the tumor composition is equal with primarily I-type (sympathoblast-like) cells and some with more differentiated neuronal features (N-type) and a small subset of mesenchymal cells (S-type). Chemotherapy eliminates most dividing cells, and it has been shown previously that S-type cells are more likely to survive treatment. This may be due to intrinsic drug resistance or a lower rate of proliferation. In the model in (C), each cell population is clonal and repopulates the same cell phenotype. Some cells may divide more rapidly (solid arrows) than others (dashed arrows). The numbers (1-5) indicate hypothetical genetic clonal relationships. In the model in (D), the S-type cells that survive treatment can produce cells with the other phenotypes (I-type, N-type) according to one of the 34 possible models (box in (B)). The S-type cells produce I-type cells, and they produce N-type cells thereby reconstituting the cellular heterogeneity of the original tumor. Importantly, the

two models give very similar results (gray boxes to right), and the major difference is in the cellular mechanism. Specifically, in both models, N-type, I-type, and S-type cells are present at diagnosis and recurrence. Clonal selection occurs in both models as there are fewer clones in the recurrent tumor than the tumor at diagnosis. The approximate cell rations are maintained in both models either by their proliferation rate, developmental trajectory, or both. In both models, the S-type cells preferentially survive and therapeutic intervention that promotes the differentiation of I-type cells to N-type cells would slow recurrence but may not be curative. Therefore, these two models are consistent with our current understanding of neuroblastoma and the major difference is in the cellular mechanisms that lead to recurrence. These examples are illustrative only and future research should focus on detailed in vivo clonal analysis to discern which of the 34 possible combinations of lineage relationships are relevant in neuroblastoma.