

From the Department of Women's and Children's Health – KBH  
Karolinska Institutet, Stockholm, Sweden

**DELINEATING CELLULAR HETEROGENEITY OF  
NEUROBLASTOMA FOR IDENTIFYING  
THERAPEUTICALLY TARGETABLE VULNERABILITIES**

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**Karolinska  
Institutet**

Stockholm 2025

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Published by Karolinska Institutet.

Printed by Universitetsservice US-AB, 2025

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ISBN 978-91-8017-463-3

DOI <https://doi.org/10.69622/28193366>

Cover illustration: Maess Anand, *Immunofluorescence staining of neuroblastoma*, oil marker and ink on paper, 2025

# Delineating cellular heterogeneity of neuroblastoma for identifying therapeutically targetable vulnerabilities

Thesis for Doctoral Degree (Ph.D.)

By

**Bethel Tesfai Embaie**

The thesis will be defended in public at Karolinska Institutet, BioClinicum, Solnavägen 30, 171 64, J3:06 Ulf von Euler U410033300, May 7, 2025, at 9:00

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### A Physician's Prayer

"From inability to let well alone, from too much zeal for the new and contempt for what is old, from putting knowledge before wisdom, science before art and cleverness before common sense, from treating patients as cases and from making the cure of the disease more grievous than the endurance of the same, good Lord deliver us."

Sir Robert Grieve Hutchinson (1871-1960)

# Popular science summary of the thesis

## Mapping neuroblastoma – from cellular origins to target discovery

### Neuroblastoma complexity

Neuroblastoma is a complex pediatric cancer affecting young children and infants. The cancer develops in the sympathetic nervous system, often arising in the adrenal glands. These tumors are composed of many different cell types, including millions of malignant cancer cells surrounded by supportive tissue and immune cells. Malignant neuroblastoma cells often have structural changes in their chromosomes, known as chromosomal aberrations. In high-risk neuroblastoma, these changes often involve segmental chromosomal aberrations, where parts of chromosomes are either lost or gained. However, in less aggressive cases, neuroblastoma cells only harbor whole-chromosome gains. The most common chromosomal aberrations in neuroblastoma include whole chromosome 17 gain or segmental chromosome 17q gain, and amplification of the *MYCN* gene. These genetic changes affect gene dosage, thereby disrupting cellular functions, leading to uncontrolled cancer cell growth and spread to other parts of the body. Neuroblastoma cells often spread to the bone marrow, making treatment more difficult and reducing survival rates.

### Decoding neuroblastoma development

To decode the complexity of neuroblastoma, we applied a new multi-omics sequencing method in paper I. This technique combines sequencing approaches, allowing us to look at the DNA, RNA and protein at a single-cell level. The majority of the cancer cells were similar to adrenergic cells of the adrenal gland and the sympathetic nervous system. Interestingly, we found a rare cell type in the sequenced tumors, called Schwann cell precursors (SCPs). Normally, during fetal development, SCPs can give rise to many cell types, including cells of the adrenal gland and sympathetic nerves. We discovered pre-malignant SCPs with whole-chromosome 17 gain. These pre-malignant SCPs could be the early seeds of neuroblastoma development.

### Modeling neuroblastoma complexity

One of the biggest barriers in drug discovery is the use of models that fail to accurately reflect human cancer. In paper II, we also used single-cell sequencing techniques to explore the cellular complexity of neuroblastoma models.

Specifically, we focused on the most commonly used genetically engineered mouse model, a *MYCN*-driven model, that spontaneously develops neuroblastoma-like tumors. We cultured these tumor cells and created a new cell-based *ex vivo* model that resembles *MYCN*-driven neuroblastoma in patients. Sequencing and staining of both models confirmed that the tumor cells preserved the adrenergic cell type, similar to human neuroblastoma. Additionally, interaction analysis revealed how the tumor cells communicate with surrounding supportive cells, highlighting potential therapeutic targets. By generating a replica of *MYCN*-driven high-risk neuroblastoma cells, we can study the disease in detail and test new drug candidates with the hope that our findings will translate to clinical outcomes for patients.

### **Bone marrow remodeling during metastasis**

Metastatic disease is notoriously challenging to treat and the major cause of cancer-related death. In paper III, we investigated the changes in the bone marrow microenvironment in metastatic neuroblastoma. Immune cells originate in the bone marrow and normally function to protect the body from pathogens. Their function is impaired when tumor cells invade, as they fail to recognize the cancer cells as harmful. In our study, we identified changes in the proportions of immune cells in metastatic bone marrow samples, which may contribute to a weakened immune response. Immune cells in the metastatic cases expressed inhibitory receptors. Understanding the immunosuppressive microenvironment of neuroblastoma bone marrow metastasis could provide insights for developing new immunotherapies.



## Abstract

Neuroblastoma is a heterogeneous pediatric neuroendocrine tumor and the most common extracranial solid malignancy in children. Although neuroblastoma etiology is elusive, it is thought to originate from the neural crest lineage, which includes multipotent Schwann cell precursors (SCPs) that can give rise to sympatho-adrenal cells, including chromaffin cells and sympathoblasts. Neuroblastoma has a wide range of clinical outcomes. Some tumors regress spontaneously without treatment, while others respond well to chemotherapy. Approximately half of neuroblastoma cases are highly aggressive, leading to refractory and relapsed disease with poor prognosis. High-risk neuroblastoma is often diagnosed with metastases, preferentially spreading to the bone marrow. Common genetic aberrations associated with the high-risk group include *MYCN*-amplification, segmental gain of chromosome arms 1q, 2p, 17q and deletion of chromosome arms 1p and 11q.

To study the cellular identity and clonal development of human neuroblastoma, we performed joint single-cell DNA and RNA sequencing and orthogonal validation by DNA-FISH with immunofluorescence staining in paper I. In addition to malignant adrenergic cells, we discovered aneuploid pre-malignant SCP-like cells in primary tumors. Clonal expansion was evident in both SCP-like and adrenergic subclones. Gain of chromosome 17 was a shared aberration of pre-malignant SCP-like cells across samples. Genetic analysis and phylogeny of tumor subclones suggest that migrating neural crest cells or multipotent SCPs, prone to aneuploidy, may represent putative tumor-initiating events in neuroblastoma. Abnormal SCP-like cells showed upregulated proliferation scores and downregulated antigen presentation via MHC molecule gene expression compared to non-malignant counterparts.

Modeling highly heterogeneous tumors like neuroblastoma is challenging. Since *MYCN* is a potent oncogenic driver in high-risk neuroblastoma, the TH-*MYCN* transgenic mouse model is widely used in preclinical studies. However, the extent to which TH-*MYCN* tumors model the disease has not been explored. In paper II, we comprehensively characterized the single-cell transcriptional landscape of TH-*MYCN* mouse tumors across various ages, both sexes and genotypes. Joint alignment analysis of tumor cells with normal fetal adrenal gland exhibited resemblance with embryonic chromaffin cells and primarily sympathoblasts. Chromaffin to sympathoblast transitions were observed in tumors, consistent

with normal murine developmental trajectories. Comparative analysis with human *MYCN*-amplified neuroblastoma confirmed similarities in the adrenergic tumor cell compartment. Additionally, inferred ligand-receptor analysis revealed potential therapeutic targets in the NCAM and NOTCH signaling pathways.

Existing TH-*MYCN* cell lines lose the adrenergic identity of neuroblastoma and instead acquire a mesenchymal phenotype. To address this issue, we established novel *ex vivo* tumoroids that preserve PHOX2B expression and maintain the adrenergic cellular identity of the originating tumor. While *ex vivo* tumoroid cells demonstrated transcriptional resemblance with embryonic chromaffin cells and sympathoblasts, distinct adrenergic subclusters were enriched in culture. *Ex vivo* enriched gene expression profiles were associated with synaptic signaling, neuronal morphogenesis and metabolic processes. Some of the upregulated genes in the enriched subclusters were correlated with poor neuroblastoma survival.

The major cause of cancer-related death is bone marrow metastasis. To examine the cellular and transcriptional shifts associated with neuroblastoma bone marrow metastasis, we compared the single-cell transcriptomes of non-metastatic and metastatic bone marrow biopsies in paper III. Metastatic tumor cells presented an adrenergic phenotype and acquired a transcriptional signature associated with poor neuroblastoma prognosis. We detected an immunosuppressive microenvironment in the metastatic samples encompassing B cell depletion and enriched regulatory T cell activity. We further identified cytotoxic T cells and CD56<sup>bright</sup> NK cells with upregulated expression of inhibitory receptors in the bone marrow metastatic niche. By flow cytometry analysis, we confirmed the presence of tumor cells and compositional shifts in B cell and T cell populations in matched bone marrow biopsies. Moreover, metastatic samples with enriched macrophages and mature neutrophils contributed to interactions with disseminated tumor cells via NOTCH signaling and other immunoregulatory interactions.

Taken together, this thesis provides an overview of the tumor cell atlas of neuroblastoma and bone marrow metastatic niche remodeling, which may contribute to the identification of new therapeutic approaches. Furthermore, we highlight the translational potential of TH-*MYCN* *in vivo* and novel *ex vivo* models for the therapeutic testing of these new targets.

# List of scientific papers

- I. Olsen TK\*, Otte J\*, Mei S\*, **Embaie BT**, Kameneva P, Cheng H, Gao T, Zachariadis V, Tsea I, Björklund Å, Kryukov E, Hou Z, Johansson A, Sundström E, Martinsson T, Fransson S, Stenman J, Fard SS, Johnsen JI, Kogner P, Adameyko I, Enge M, Kharchenko PV\*, Baryawno N\*.

**Joint single-cell genetic and transcriptomic analysis reveal pre-malignant SCP-like subclones in human neuroblastoma.**

Mol Cancer. 2024 Aug 31;23(1):180.

- II. **Embaie BT\***, Sarkar H\*, Alchahin AM, Otte J, Olsen TK, Tümmler C, Kameneva P, Artemov AV, Akkuratova N, Adameyko I, Stukenborg JB, Wickström M, Kogner P, Johnsen JI, Mei S, Kharchenko PV, Baryawno N.

**Comparative Single-Cell Transcriptomics of Human Neuroblastoma and Preclinical Models Reveals Conservation of an Adrenergic Cell State.**

Cancer Res. 2025 Mar 14;85(6):1015–1034.

- III. Mei S\*, Alchahin AM\*, **Embaie BT**, Gavriluc IM, Verhoeven BM, Zhao T, Li X, Jeffries NE, Pepich A, Sarkar H, Olsen TK, Wickström M, Stenman J, Reina-Bedoya O, Kharchenko PV, Saylor PJ, Johnsen JI, Sykes DB, Kogner P\*, Baryawno N\*.

**Single-cell analyses of metastatic bone marrow in human neuroblastoma reveals microenvironmental remodeling and metastatic signature.**

JCI Insight. 2024 Feb 15;9(6):e173337.

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## List of abbreviations

ADC	Antibody–Drug Conjugate
ADGRB3	Adhesion G Protein–Coupled Receptor B3/ Brain–Specific Angiogenesis Inhibitor 3
AHCY	Adenosylhomocysteinase/ S–Adenosyl–L–Homocysteine Hydrolase
ALK	Anaplastic Lymphoma Kinase
ANAPC11	Anaphase Promoting Complex Subunit 11
B4GALNT1	Beta–1,4–N–Acetyl–Galactosaminyltransferase 1
BDNF	Brain Derived Neurotrophic Factor
BIRC5	Survivin/ Baculoviral IAP Repeat Containing 5
BM	Bone Marrow sample
BMP	Bone Morphogenetic Protein
BRCA1	BRCA1 Cancer gene 1
CART	Cocaine– and Amphetamine–Regulated Transcript
CCNE1	Cyclin E1
cDNA	complementary Deoxyribonucleic Acid
CGH	Comparative Genomic Hybridization
CNS	Central Nervous System
CNV	Copy Number Variation
CRABP1	Cellular Retinoic Acid Binding Protein 1
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T–Lymphocyte Associated Protein 4
DBH	Dopamine Beta–Hydroxylase
DDIT4	DNA Damage Inducible Transcript 4
DDX5	DEAD–Box Helicase 5
DHX36	DEAH–Box Helicase 36

DLK1	Delta Like Non-Canonical Notch Ligand 1
DLL3	Delta Like Canonical Notch Ligand 3
DNA	Deoxyribonucleic Acid
DNTR-seq	Direct Nuclear Tagmentation and RNA sequencing
EGF	Epidermal Growth Factor
EMT	Epithelial-to-Mesenchymal Transition
ERBB3	Erb-B2 Receptor Tyrosine Kinase 3/ Human Epidermal Growth Factor Receptor 3
EU	European Union
FACS	Fluorescence-activated cell sorting
FGFR1	Fibroblast Growth Factor Receptor 1
FISH	Fluorescence In Situ Hybridization
FMO	Fluorescence Minus One
FOXD3	Forkhead Box D3
GCSH	Glycine Cleavage System Protein H
GD2	Disialoganglioside
GEM	Gel Beads-in-Emulsion
GEMM	Genetically Engineered Mouse Model
GFP	Green Fluorescent Protein
GM2	Monosialic Ganglioside 2
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HAND2	Heart And Neural Crest Derivatives Expressed 2
HAVCR2	Hepatitis A Virus Cellular Receptor 2, encoding TIM3
HLA	Human Leukocyte Antigen
IDRF	Image-Defined Risk factor
IGF	Insulin-Like Growth Factor 1
IHC	Immunohistochemistry



INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
ISL1	Insulin gene enhancer binding protein 1
JAG1	Jagged canonical Notch ligand 1
KLRC1	Killer Cell Lectin Like Receptor C1, encoding NKG2A
KPNB1	Karyopherin Subunit Beta 1
KTN1	Kinectin 1
L1CAM	L1 Cell Adhesion Molecule
LAG3	Lymphocyte-Activation Gene 3
LGALS9	Galectin 9
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MDK	Midkine
MDSC	Myeloid-derived suppressor cell
MHC	Major Histocompatibility Complex
MPZ	Myelin Protein Zero
MRCA	Most Recent Common Ancestor cell
mRNA	Messenger Ribonucleic Acid
MSAI	Mirrored Subclonal Allelic Imbalances
MSC	Mesenchymal Stem Cell
MSigDB	Molecular Signatures Database
MYC	MYC Proto-Oncogene, BHLH Transcription Factor
MYCN	N-myc Proto-Oncogene, BHLH Transcription Factor
NAV3	Neuron Navigator 3
NB	Neuroblastoma sample
NCAM1	Neural Cell Adhesion Molecule 1

NCC	Neural Crest Cell
NECTIN2	Nectin Cell Adhesion Molecule 2
NF200	Neurofilament 200
NGFR	Nerve Growth Factor Receptor
NK	Natural Killer cell
NKG2A	Natural Killer Group 2 member A, inhibitory receptor
NME1/2	NME/NM23 Nucleoside Diphosphate Kinase 1/2
NOTCH	Neurogenic Locus Notch Homolog Proteins
NR4A	Nuclear Receptor Subfamily 4 Group A
NRG1	Neuregulin 1
NRXN1	Neurexin 1
NTRK2	Neurotrophic Receptor Tyrosine Kinase 2
NXPH1	Neurexophilin 1
PCR	Polymerase Chain Reaction
PHOX2B	Paired Like Homeobox 2B
PPAT	Phosphoribosyl Pyrophosphate Amidotransferase
PPM1D	Protein Phosphatase, Mg <sup>2+</sup> /Mn <sup>2+</sup> Dependent 1D, WIP1
RA	Retinoic Acid
RNA	Ribonucleic Acid
S100B	S100 Calcium Binding Protein B
SCP	Schwann Cell Precursors
scRNA-seq	single-cell RNA sequencing
SIGLEC10	Sialic Acid Binding Ig Like Lectin 10
SIS3	Specific Inhibitor of Smad3
SIX3	SIX Homeobox 3
SMAD3	SMAD Family Member 3
SNP	Single Nucleotide Polymorphism
SOX10	SRY-box transcription factor 10

SOX11	SRY-box transcription factor 11
SSTR2	Somatostatin Receptor 2
STMN2	Stathmin 2
TAM	Tumor Associated Macrophages
TCE	T-cell Engager
TGF- $\beta$	Transforming Growth Factor beta
TH	Tyrosine Hydroxylase
TH-MYCN	Transgenic model overexpressing <i>MYCN</i> driven by TH promoter
TH-MYCN <sup>CPM32</sup>	Cyclophosphamide resistant TH-MYCN mice
TIGIT	T Cell Immunoreceptor With Ig and ITIM Domains
TIM3	T-cell Immunoglobulin Mucin receptor 3
TNF	Tumor Necrosis Factor
TOX	Thymocyte Selection Associated High Mobility Group Box
UMAP	Uniform Manifold Approximation and Projection
UMI	Unique Molecular Identifier
VAF	Variant Allele Frequencies
WNT3A	Wingless-Type MMTV Integration Site Family, Member 3A







# 1 Background

## 1.1 Cancer

The terms tumor and neoplasm are used interchangeably to describe uncontrolled cell growth. Tumors can be benign (non-cancerous) or malignant (cancerous). The latter denotes an invasive tumor with metastatic potential. Cancer is a malignant tumor, typically depicted as a genetic disease. Malignant tumors have very different genetic and phenotypic properties at both the inter-tumoral and intra-tumoral levels. High inter-tumoral heterogeneity refers to the presence of diverse genetic abnormalities in tumors from different patients. While intra-tumoral heterogeneity describes the distinct cellular populations within the same tumor sample. Intra-tumoral heterogeneity is associated with treatment resistance and poor prognosis (Jamal-Hanjani et al., 2015). Malignant tumors consist of mosaics of cancer cells with varying properties and susceptibility to treatment. Tumor heterogeneity has several layers of complexity (Ramón y Cajal et al., 2020). Given that cancer is heterogeneous and dynamic, the variations in individual patient lesions and the cellular complexity should be thoroughly characterized to achieve the goals of precision medicine. Precision medicine, also referred to as "personalized medicine," is an approach using cutting-edge technologies for tailoring targeted treatment to each patient's unique biological, genetic, and proteomic characteristics. Recent advancements in single-cell RNA sequencing (scRNA-seq) (Olsen & Baryawno, 2018), provide new insight into the relationships between tumor cell heterogeneity, immunological dysregulation and metastatic niches. Moreover, novel bioinformatic tools like *Numbat* and single-cell multi-omics approaches like direct nuclear tagmentation and RNA sequencing (DNTR-seq) can unravel the cellular heterogeneity and clonal evolution of cancers (T. Gao et al., 2022; Zachariadis et al., 2020).

The complexity of cancer development and the acquired biological capabilities needed of cells to become malignant have been summarized by Hanahan and Weinberg as the "hallmarks of cancer" in 2000 and revisited in 2011 and 2022. The six original hallmarks are defined as sustained cell proliferation, insensitivity to growth suppressors, evasion of apoptosis, replicative immortality potential, inducing angiogenesis, as well as invasion and metastasis (Hanahan & Weinberg, 2000). The overwhelming majority of cancer deaths, namely 90% of all cancer patients, are caused by metastasis (Boire et al., 2024). The exact percentage can vary slightly depending on the type of cancer and the population being studied,

but metastases are overwhelmingly the primary cause of cancer mortality. Although metastasis is the main factor in cancer therapy failure and mortality, the mechanism of resistance to treatment is still not well understood.

Additionally, reprogramming energy metabolism and immune evasion were presented as the emerging hallmarks, while genomic instability, mutations, and tumor-promoting inflammation were described as the enabling traits (Hanahan & Weinberg, 2011). Recently, Hanahan incorporated additional prospective emerging hallmarks and enabling characteristics, namely "unlocking phenotypic plasticity," "non-mutational epigenetic reprogramming," "polymorphic microbiomes," and "senescent cells" (Hanahan, 2022). An imperative hallmark of cancer is its phenotypic plasticity, with the ability to transiently change both its morphological and functional characteristics during metastatic spread. The phenotypic plasticity of cancer cells is narrated by the cancer cell of origin, contributing to cancer initiation, progression and response to therapy (Gupta et al., 2019).

Cancer is a leading cause of premature death worldwide. It accounted for 9.7 million deaths in 2022 (Bray et al., 2024). There were up to 19.96 million new cancer cases reported globally that year (Bray et al., 2024). In Sweden, cancer ranks as the second leading cause of death after cardiovascular diseases but is expected to soon be the most common cause of premature death in women (Socialstyrelsen, 2024b, 2024a). In 2023, 73 794 individuals were diagnosed with cancer, and 22 665 people died of cancer in Sweden (Cancerfonden & Socialstyrelsen, 2023). The prevalence and burden of cancer in Sweden and globally emphasize how crucial it is to fund cancer research, national cancer control programs and health care systems to improve treatment and survival.

Tumors can be classified based on the origin of the cells and tissue from which they develop, namely endoderm/ectoderm lineage tumors, mesodermal lineage tumors (which include all sarcomas), and neuroectodermal lineage tumors (Weinberg, 2013). Cells that make up the central and peripheral nervous system originate from the neuroectoderm. Cancers that develop from the neuroectodermal lineage include schwannoma, glioma, glioblastoma, medulloblastoma and neuroblastoma. Pediatric cancers, such as medulloblastoma and neuroblastoma, are common embryonal neuroectodermal malignancies (Tulla et al., 2015).



### **1.1.1 Pediatric cancer**

Cancer has historically been regarded as a disease of old age. Unfortunately, there are obvious exceptions to the late-life pattern of cancers, including early childhood malignancies and young adult lymphomas/sarcomas. The most common forms of pediatric cancer are leukemias and central nervous system (CNS) tumors, primarily brain tumors. Globally, an estimated 400 000 children between the ages of 0–19 are diagnosed with cancer annually (Ward et al., 2019). There is considerable variation in childhood cancer survival between high-, middle- and low-income countries. In high-income countries, five-year survival is over 80%, while survival rates are under 30% in low- and middle-income countries (Lam et al., 2019). In Sweden, pediatric cancer is the primary cause of death for children between the ages of 1 and 14. However, 80% of children who survive cancer endure late complications (Petersen et al., 2023), 30% of whom have severe late effects including second neoplasms, infertility, brain damage, psychological conditions, physical disabilities and most commonly, cognitive impairment (Barncancerfonden, 2021, 2024). These severe life-altering morbidities are often attributed to high dose chemotherapy, radiation or CNS-surgery.

There are important distinctions between pediatric and adult malignancies. Unlike adult cancers, pediatric cancers are rarely associated with lifestyle or environmental factors. This might be because these external risk factors need a longer time to manifest. This is apparent at a genetic level, in which genetic mutations are accumulated in adult cancers over a longer lifetime. In contrast to adult malignancies, pediatric malignancies have a low mutational burden (Gröbner et al., 2018; Ma et al., 2018; Vogelstein et al., 2013) and are, instead, fundamentally disorders of dysregulated development. Since the kidneys and liver of children are typically in excellent condition prior to initial cancer treatment, they may tolerate relatively high doses of chemotherapy. In contrast, developing organs in children, particularly the CNS, are significantly more vulnerable to radiotherapy, with notable susceptibility to cognitive impairment (Duffner & Cohen, 1985; Littman & D'Angio, 1979; Pizzo et al., 1979).

## **1.2 Neuroblastoma**

The German physician Rudolf Virchow, known as the founder of the cellular pathology field, first identified neuroblastoma as abdominal tumors in children and initially referred to the condition as gliomas in 1864 (Virchow, 1865). It was not until

the early 1900s that three principal physicians clarified how neuroblastoma was pathologically defined and the metastatic spread of neuroblastoma. James Homer-Wright was the first to identify the tumor to have originated from primitive neural cells in 1910, describing the cells as undifferentiated nerve cells. He, therefore, proposed the name neuroblastoma, highlighting the bundle of neural cells called rosettes (Wright, 1910). Although Wright's observations focused on the neural nature of neuroblastoma, Robert Grieve Hutchison and William Pepper reported patterns of metastatic spread to the bone and liver, respectively (Hutchison, 1907; Pepper, 1901).

Neuroblastoma is a neuroendocrine tumor that develops from sympathetic nervous system progenitor cells (Hoehner et al., 1996). It is the most common malignancy of infancy, with the highest number of cases during the perinatal period, and the median age at diagnosis is 18 months (London et al., 2005; Matthay et al., 2016; Stiller & Parkin, 1992). Primary tumors often arise in the adrenal medulla, but neuroblastoma can also manifest in the extra-adrenal neural ganglia, retroperitoneum in the abdomen, mediastinum, pelvis and neck (Papaioannou & McHugh, 2005; Rha et al., 2003). A hallmark of neuroblastoma is its high degree of biological and clinical heterogeneity. Tumors can spontaneously regress in some cases, whereas others present aggressive and undifferentiated tumors. The latter indicates an extremely bad prognosis and is frequently resistant to treatment. Approximately half of neuroblastoma cases are in the high-risk group, with less than 50% long-term survival rate due to relapse or treatment resistance (Cohn et al., 2009; Pinto et al., 2015). Thus, there is an urgent need to better understand the biological heterogeneity of neuroblastoma and improve treatment strategies in the high-risk group to improve patient survival.

### **1.2.1 Neuroblastoma staging and treatment**

The two most common staging and risk classification systems of neuroblastoma are INSS (International Neuroblastoma Staging System) developed in 1986 for patients that underwent surgery, and INRG/INRGSS (International Neuroblastoma Risk Group Staging System) a newer system which is based on imaging at diagnosis prior to surgery (Brodeur et al., 1988; Cohn et al., 2009). INRGSS is grouped into four stages: L1 "localized tumors without image-defined risk factors (IDRFs)", L2 "locoregional tumor with at least one IDRFs", M "distant metastatic disease excluding MS", and MS "metastatic disease specifically to skin and/or liver and/or bone marrow in children under 18 months of age" (Cohn et al., 2009; Matthay et al., 2016; Monclair et al., 2009). The earlier INSS has a similar risk

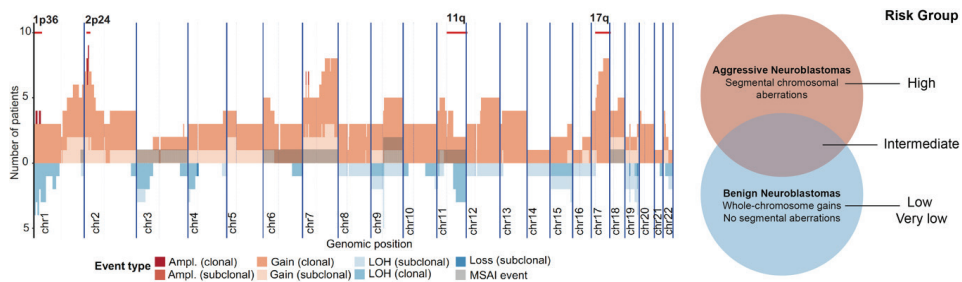
classification with a slightly different name scheme consisting of Stages 1, 2A, 2B, 3, 4, and 4S or "special" neuroblastoma for children under the age of 1 with metastatic dissemination restricted to skin, liver, and/or bone marrow (analogous to MS) (Brodeur et al., 1988).

Depending on the risk group, the most common current treatments of neuroblastoma include high-dose chemotherapy, radiation therapy, surgery, stem cell transplantation therapy, myeloablative therapy, anti-GD2 immunotherapy and cis-retinoic acid (RA) therapy. If the tumor size needs to be reduced before surgery, neoadjuvant chemotherapy can be used, alternatively, adjuvant chemotherapy after surgery can be administered or chemotherapy alone as the main treatment. Treatments for high-risk neuroblastoma typically consist of three phases: induction, consolidation, and maintenance (Pinto et al., 2015).

### **1.2.2 Neuroblastoma genetics**

Different combinations of genetic aberrations are presented in neuroblastoma tumor cells. Amplification of *MYCN* (50- to 100-fold), an oncogenic transcription factor first described in 1983, is a common genetic abnormality in neuroblastoma. *MYCN* amplifications are found in approximately 20% of tumors (variations in different study cohorts) and represent a critical stratifying prognostic marker in neuroblastoma (Kohl et al., 1983; Matthay et al., 2016; Schwab, 2004; Schwab et al., 1983). Amplification of *ALK* (encoding anaplastic lymphoma tyrosine kinase receptor) occurs almost exclusively in *MYCN*-amplified neuroblastomas and correlates with poor clinical outcome (De Brouwer et al., 2010). Tumors possessing *MYCN* amplification have invasive characteristics, which is recognized as a bad prognostic biomarker present in high-risk patient groups (Cohn et al., 2009; Zaizen et al., 1993).

Other genetic aberrations associated with poor prognosis include hemizygous deletions of chromosome arms 1p and 11q; and segmental gain of 17q. It is important to note that whole chromosomal gains (aneuploidy) are often associated with low-risk neuroblastoma, while segmental chromosomal gains or losses are associated with high-risk neuroblastoma (Maris, 2010) (Figure 1).



**Figure 1** *Left:* Overview of somatic copy-number alterations across several neuroblastoma patient samples ( $n=9$ ), with amplifications and gains shown in the upper half, and loss of heterozygosity (LOH) and deep losses in the lower half. Clinically relevant regions (1p36, 11q, 17q and *MYCN* locus: 2p24) are underlined in red, and mirrored subclonal allelic imbalances (MSAI) are marked with gray shading. Reprinted from (Schmelz et al., 2021) with permission from the publisher. *Right:* Genomic differences between aggressive and benign neuroblastoma, with segmental aberrations in high-risk cases and whole-chromosome gains in low-risk group. Reproduced with permission from (Maris, 2010), Copyright Massachusetts Medical Society.

The most frequent genetic aberration associated with high-risk disease is the segmental gain of chromosome 17q (Abel et al., 1999; Bown et al., 1999; Theissen et al., 2014). Genetic studies on large neuroblastoma cohorts by Andersson et al. and Milosevic et al. have suggested that gain of chromosome 17, 17q and/or *MYCN* amplification are early events in neuroblastoma development (Andersson et al., 2020; Gisselsson et al., 2007; Milosevic et al., 2025). Similarly, recent results on neuroblastoma evolution describe whole-chromosome aneuploidy, including chromosome 17 gain, in early MRCA tumors (most recent common ancestor cell) (Körber et al., 2023). The most frequently observed breakpoint is 17q21 (Bown et al., 1999; Lastowska et al., 1997; Meddeb et al., 1996). Several cancer-associated genes have been identified on chromosome 17q including *BIRC5*, *BRCA1*, *BRIP1*, *EME1*, *ERBB2*, *IGF2BP1*, *ncRAN*, *NME1*, *NFI*, *PPM1D*, *RAD51C*, *TBX2* and *TRIM37* (Mlakar et al., 2024).

Additional somatic genomic variations that contribute to neuroblastoma tumorigenesis and progression are inactivating *ATRX* mutations and *TERT* rearrangements (Peifer et al., 2015; Valentijn et al., 2015). Although familial neuroblastoma is rare, accounting for <2% of cases, gain-of-function *ALK* mutation is the major cause of hereditary neuroblastoma (75% of familial neuroblastoma cases) (Janoueix-Lerosey et al., 2008; Mossé et al., 2008), followed by *PHOX2B* mutations, both vital factors in neural crest development (Mosse et al., 2004; Trochet et al., 2004).

Overall, the mutation frequencies within the genome of neuroblastoma and most childhood cancers are low relative to adult tumors (Gröbner et al., 2018; Vogelstein

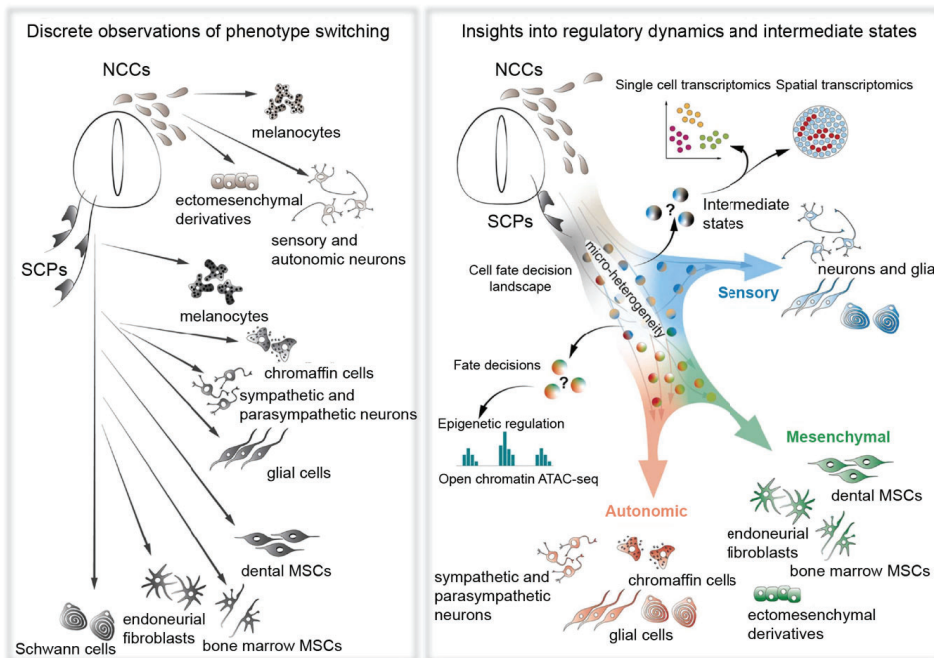
et al., 2013). This implies that many mechanisms of cancer progression described in adult cancers do not necessarily hold true for neuroblastoma.

### **1.3 Neural crest development**

Pediatric cancers have been described as a deregulation of normal development (Grimmer & Weiss, 2006). The combination of disease onset and clinical presentation suggests that neuroblastoma arises as a consequence of dysregulation in the developing neural crest. However, the precise cell of origin for neuroblastoma has yet to be defined. Neural crest cells are a population of multipotent stem cells that emerge from the ectoderm at the neural plate border after neural tube closure. During embryogenesis, epithelial-to-mesenchymal (EMT) transition drives neural crest cell delamination and subsequent migration away from the neural tube. Neural crest cells migrate extensively throughout the embryo, giving rise to a wide variety of differentiated cell types distinguished by spatiotemporal differences in gene expression (Gandhi & Bronner, 2018; Green et al., 2015; Martik & Bronner, 2017).

#### **1.3.1 Nerve-associated Schwann cell precursors**

Schwann cell precursors (SCPs) are multipotent stem cells of the neural crest lineage and were first described by Jessen and Mirsky in 1991 (Jessen & Mirsky, 1991). Nerve-associated migratory neural crest cells gradually transform into early SCPs. Peripheral nerves serve as a stem cell niche for SCPs, promoting their migration, development, survival and lineage progression (Heermann et al., 2012). SCPs are primarily known for their role in generating myelinating and non-myelinating Schwann cells, however, several studies have shed light on their broad range of non-glial derivatives. SCPs migrate along nerve axons to various sites, detach, and give rise to many cell types including chromaffin cells of adrenal medulla and Organ of Zuckerkandl (Akkuratova et al., 2022; Furlan et al., 2017), parasympathetic neurons (Dyachuk et al., 2014), enteric neurons (Espinosa-Medina et al., 2018; Uesaka et al., 2015), carotid body glomus cells (Furlan & Adameyko, 2018; Hockman et al., 2018; Kastriti & Adameyko, 2017), melanocytes (Adameyko et al., 2009; Kameneva, Artemov, et al., 2021; Kastriti et al., 2022), mesenchymal progenitor giving rise to dental populations (Kaukua et al., 2014) and chondrocytes (Xie et al., 2019). The large variety of SCP-derived progeny highlights a multipotency comparable to their parental population of neural crest cells (Furlan & Adameyko, 2018; Solovieva & Bronner, 2021) (Figure 2).



**Figure 2** Single-cell transcriptomics and multi-omics of neural crest cell lineages and nerve-associated SCPs reveal meta-stable states, micro-heterogeneity, and pre-bifurcation dynamics, offering new insights into fate specification and transcriptional programs that drive differentiation into distinct cell types. NCCs – neural crest cells, SCPs – Schwann cell precursors, MSCs – mesenchymal stem cells. Reprinted with permission from (Erickson et al., 2023) the publisher.

Nerve-associated SCPs are transcriptionally similar to migratory neural crest cells, as they both express *Sox10* and *FoxD3* (Nitzan et al., 2013; Paratore et al., 2001; Soldatov et al., 2019). Kastriti et al. used single-cell transcriptomics of the entire neural crest lineage from embryonal to postnatal stages to examine the transcriptional similarities and distinctions between neural crest cells and SCPs (Kastriti et al., 2022). The fundamental findings demonstrated that both neural crest cells and SCPs express the core neural crest transcription factors, including *Sox10*, *FoxD3*, *Tfap2a/b* and *Ets1*. Additionally, they identified a shared transcriptional “hub”/SCP state, characterized by the expression of *Sox8*, *Itga4*, *Dlx1/2*, *Ednrb* and *Serpine2*. Prior to commitment and further differentiation, the individual cells appear to be micro-heterogeneous and biased toward distinct fates (Kastriti et al., 2022) (Figure 2). High expression of *Sox8* and *Itga4* distinguish “hub”/SCP state from early delaminating neural crest cells. The majority of the “hub” cells are SCPs from embryonic stages E10.5 and older. Together, this suggests that SCPs are transcriptionally distinct from early migratory neural crest cells.

In mouse embryos, the earliest SCPs form at embryonic day (E)9.5–E11.5 and SCPs cease to generate neuroendocrine cells at E15.5 (Dyachuk et al., 2014; Furlan et al., 2017). The survival, development and lineage progression of SCPs is critically dependent on axoglial signaling involving nerve-derived neuregulin-1 (NRG1) (Jessen & Mirsky, 2005). Axons produce neuregulins that bind to and activate the receptor tyrosine kinases of the ErbB family on the membranes of SCPs (Jessen & Mirsky, 2005). Neuregulins are a subclass of ligands in the epidermal growth factor (EGF) family. There are four neuregulin genes (NRG1–4) described in mammals, of which a multitude of various isoforms are produced using distinct promoters and alternative exon splicing (Falls, 2003). At least 20 distinct neuregulin isoforms can be generated that differ in structure and are classified as types I, II, III, IV, V and VI. The major classes of isoforms have differential spatiotemporal expression, exhibiting tissue-specific functions (Britsch et al., 1998; Hayes et al., 2007; Meyer et al., 1997). NRG1 types I and III are produced by sensory neurons, of which NRG1 type III is the most important type that is presented by axons to Schwann cells during early development (Meyer et al., 1997; Taveggia et al., 2005). The NRG1 ligand binds to the extracellular domain of the ERBB3 receptor, which in turn promotes ERBB2/3 dimerization, necessary to initiate intracellular tyrosine kinase activity (Citri et al., 2003). Embryonal NRG1/ERBB axoglial signaling stimulates SCP survival, proliferation and migration along the axon (Britsch et al., 1998; Garratt et al., 2000). In later developmental stages, NRG1/ERBB signaling controls myelination, and neuronal overexpression of NRG1 type III induces hypermyelination (Michailov et al., 2004). SCPs need to maintain their contact with nerves to give rise to mature Schwann cells, whereas they generate other cell types if they detach from the nerve fibers (Nitzan et al., 2013). Thus, SCPs respond differently to NRG1/ERBB signaling based on the developmental stage and environmental factors.

### **1.3.2 Sympathoadrenal development**

The most common primary site of neuroblastoma is the adrenal gland. It is an endocrine organ located on top of each kidney. The adrenal gland is made up of the outer cortex, which arises from coelomic mesoderm, and inner medulla, which is derived from neural crest cells (Yates et al., 2013). The adrenal medulla primarily consists of neuroendocrine chromaffin cells with oxygen-sensitive ion channels and responds to stress by producing catecholamines, including epinephrine (adrenaline) and norepinephrine (noradrenaline), a neurotransmitter discovered by Ulf von Euler. In addition to the chromaffin cells, the adrenal medulla also

contains a smaller population of sustentacular cells and sympathoblasts, both of which are derivatives of nerve-associated SCPs (Kameneva, Artemov, et al., 2021; Kastriti et al., 2020; Santambrogio et al., 2025).

The Organ of Zuckerkandl was initially described by Emil Zuckerkandl in 1901, and later in 1903 Alfred Kohn defined the cellular composition. It is an extra-adrenal chromaffin organ located adjacent to the dorsal aorta between the kidneys during development (Ehrhart-Bornstein et al., 2010; Schober et al., 2013). The Organ of Zuckerkandl consists of chromaffin cells that lack phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine, resulting in the exclusive production of norepinephrine (Kastriti et al., 2020). Normally, this transient organ regresses after birth, however, it can give rise to tumors, including neuroblastomas and paragangliomas (Subramanian & Maker, 2006).

Lineage tracing in mice has shown that chromaffin cells of the adrenal medulla arise from SCPs, via an intermediate progenitor (bridge) cell type, expressing both glial and sympathoadrenal lineage markers including *Htr3a*, *Dll3*, *Ascl1* and *Sox11* (Furlan et al., 2017; Hanemaaijer et al., 2021; Kameneva, Artemov, et al., 2021). Murine immature chromaffin cells can, in turn, transition into intra-adrenal sympathoblasts (Kameneva, Artemov, et al., 2021) (Figure 3). SCPs also give rise to chromaffin cells of the Organ of Zuckerkandl and some sympathoblasts of the sympathetic paraganglia (Kastriti et al., 2019). Correspondingly, single-cell transcriptomics of human adrenal development confirm that SCPs give rise to intra-adrenal sympathoblasts and chromaffin cells via a “fork-like” transition (Jansky et al., 2021; Kameneva, Artemov, et al., 2021).

#### **1.4 Single-cell transcriptomics of neuroblastoma**

The cellular and transcriptional heterogeneity of neuroblastoma has been associated with different cell states along the sympathoadrenal development. Multiple single-cell or single-nuclear RNA-sequencing studies of neuroblastoma patient tumors have demonstrated transcriptional similarities with normal fetal adrenal sympathoblasts/neuroblasts and chromaffin cells (Bedoya-Reina et al., 2021; Dong et al., 2020; Jansky et al., 2021; Kameneva, Artemov, et al., 2021; Kildisiute, Kholosy, et al., 2021; Olsen et al., 2024; Slyper et al., 2020).



### 1.4.1 Tumor cell atlas

Matched pre- and post-treated biopsies from a patient with intermediate-risk neuroblastoma were initially profiled with scRNA-seq by Slyper et al. (A. G. Patel et al., 2024; Slyper et al., 2020). The pre-treated biopsy predominantly comprised of malignant neuroendocrine cells (expressing neuroblastoma markers *TH*, *PHOX2B* and *B4GALNT1*), but the cellular composition shifted post-treatment, resulting in higher proportions of endothelial cells and fibroblasts, corresponding to fibrosis after treatment. The remaining neuroendocrine cells presented similar copy number aberrations before and after treatment, but no aberrations were detected in the fibroblast cell cluster (Slyper et al., 2020). Single-nucleus RNA-sequencing (snRNA-seq) of two other neuroblastoma primary samples (intermediate- and low-risk) revealed varying proportions of cell types, including detections of neural crest cells, expressing SCP-related genes *S100B*, *FOXD3*, *SOX10* and *ERBB3* (Slyper et al., 2020).

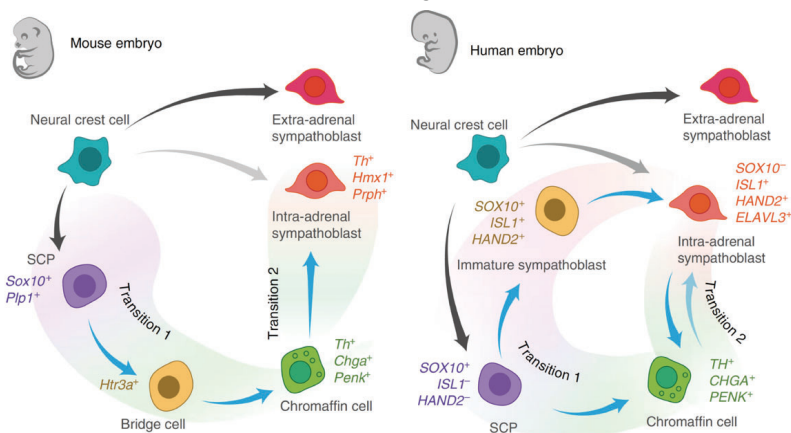
Single-cell transcriptomic profiling of two human embryos and four fetal adrenal glands were generated by Dong et al. for comparative analysis with sixteen primary neuroblastoma samples (Dong et al., 2020). Cytotoxic treatments before surgical resection of neuroblastoma often result in largely necrotic or fibrotic tissue. Unlike other scRNA-seq studies, the data generated by Dong et al. encompasses treatment-naïve adrenal neuroblastoma samples across diverse genetic subsets, risk groups, age and sex, yielding the most high-quality viable cells (Bonine et al., 2024; Dong et al., 2020). Similar to the previous study, neuroendocrine malignant cells were detected by inference of large-scale copy number variations (inferCNV) (Dong et al., 2020; Slyper et al., 2020). The most common aberration in this dataset was segmental chromosome 17q gain, and notably, two *MYCN*-amplified cases presented genetic subclones (Dong et al., 2020). The transcriptional profiles of the malignant cells resembled the noradrenergic/adrenergic phenotype (Boeva et al., 2017; Van Groningen et al., 2017) and were predominantly described as “chromaffin-cell-like” (Dong et al., 2020). This sparked a discussion, regarding the ambiguity in the gene expression markers used to annotate sympathoblast and chromaffin cells, which was clarified by inter-species variations between mouse and human adrenal medulla development (Bedoya-Reina & Schlisio, 2021; Kameneva, V.Artemov, et al., 2021; Kildisiute, Young, et al., 2021; Z. Liu & Thiele, 2020; Yang et al., 2021).

In a subsequent scRNA-seq study, Kildisiute et al. compared human fetal adrenal transcriptomes with tumors revealing a pan-neuroblastoma cell state, resembling

fetal sympathoblasts (Kildisiute, Kholosy, et al., 2021). Tumor cells demonstrate transcriptional similarities with sympathoblasts independent of risk group (Kildisiute, Kholosy, et al., 2021). Of note, a population of Schwannian stroma, with low probability of copy number changes, was detected in mainly pre-treated tumors. Moreover, differential expression analysis between tumor cells and normal adrenal medullary cells revealed genes associated with high-risk neuroblastoma, including *SIX3* and *SSTR2* (Kildisiute, Kholosy, et al., 2021). The latter is a somatostatin receptor expressed in the majority of neuroblastomas, including relapsed and refractory disease (Alexander et al., 2019). Hence, targeting somatostatin receptors with somatostatin analogue lutetium-177 octreotate ( $^{177}\text{Lu}$ -DOTATATE) represents a promising therapeutic approach and is currently in clinical trial (LuDO-N trial) (Park et al., 2024; Sundquist et al., 2022).

#### 1.4.2 Fetal adrenal gland cell atlas

The cell type heterogeneity and lineage trajectories of human and mouse adrenal gland development have been comprehensively characterized to shed light on normal development and gain insights into the origins of neuroblastoma (Bedoya-Reina et al., 2021; Jansky et al., 2021; Kameneva, Artemov, et al., 2021). During normal human sympathoadrenal development, SCPs exhibit the highest stemness and bifurcate into sympathoblasts/neuroblasts and chromaffin cells (Jansky et al., 2021; Kameneva, Artemov, et al., 2021) (Figure 3). Kameneva et al. also identified a second transition from sympathoblasts to chromaffin cells of the human adrenal medulla, which is the opposite direction from the transitions identified in mice (Kameneva, Artemov, et al., 2021) (Figure 3).



**Figure 3** Order of transitions within developing human and mouse sympathoadrenal systems, highlighting the predominant intra-adrenal sympathoblast differentiation (blue arrows) and the conventional extra-adrenal sympathoblast differentiation, along with alternative sympathoadrenal differentiation pathways in humans (gray arrows). © 2021 Kameneva et al. Reprinted from (Kameneva, Artemov, et al., 2021) with permission from the publisher.

Furthermore, neuroblastoma cell transcriptomes have been mapped onto normal developing adrenal cells, which shows the highest resemblance with fetal sympathoblasts/neuroblasts (Jansky et al., 2021; Kameneva, Artemov, et al., 2021). Schwann cells expressing *CDH19*, *PMP22*, *PLP1*, and *S100B* were detected in tumor samples, although these cells present low inferred CNVs (Jansky et al., 2021). Jansky et al. further showed correlations between neuroblastoma risk groups and transcriptional resemblance with developing cell types. High proportions of late neuroblasts are associated with the low-risk group, whereas cycling neuroblasts and immature tumor cells resembling bridge cells are associated with high-risk neuroblastoma (Jansky et al., 2021). Kameneva et al. performed a joint alignment analysis of healthy adrenal tissue and neuroblastoma transcriptomes from Dong et al. and data from our research group (Paper I: Olsen et al., 2024). In consensus with previous studies, the majority of tumor cells align with fetal sympathoblasts. Additionally, Schwann- and mesenchymal-cell clusters in neuroblastoma samples align with fetal SCPs and abdominal mesenchymal cells, respectively (Kameneva, Artemov, et al., 2021). Proliferating sympathoblasts gene expression signature is significantly associated with poor survival rates, independent of *MYCN*-status. In contrast, high SCP signatures show a favorable prognosis, although non-significant (Kameneva, Artemov, et al., 2021). Taken together, there is an overall consensus across these back-to-back studies that neuroblastoma cells correspond to various stages along the sympathoadrenal developmental trajectories. Tumors with early, cycling sympathoblasts/neuroblasts are associated with a dire prognosis.

A snRNA-seq study of neuroblastoma and human adrenal gland has shown a correlation between low-risk-enriched cell clusters, resembling sympathoblasts and chromaffin cells, with younger age at diagnosis (Bedoya-Reina et al., 2021). Whereas, high-risk neuroblastomas comprise an undifferentiated cluster (expressing *MYCN*, *ALK*, and *BRCA1*) reminiscent of *NTRK2*-positive postnatal cholinergic progenitors, which are correlated with age at diagnosis and worse survival (Bedoya-Reina et al., 2021). This study substantiates the age-dependent risk stratification of the disease (Bedoya-Reina et al., 2021; Brodeur, 2003; Moroz et al., 2011).

### **1.4.3 Immune cell atlas**

Although tumor cell populations were the main focus of the aforementioned scRNA-seq studies, subsequent efforts were dedicated to deciphering the complex neuroblastoma immune microenvironment (Costa et al., 2022;

Verhoeven et al., 2022; Wienke et al., 2024) and metastatic bone marrow microenvironment (Fetahu et al., 2023; Lazic et al., 2021; Mei et al., 2024). Infiltration of myeloid cells including monocytes, tumor associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs), have been described to have pro-tumorigenic and -metastatic features in neuroblastoma (Asgharzadeh et al., 2012; Costa et al., 2022; Fetahu et al., 2023; Hashimoto et al., 2016; Wienke et al., 2024). Furthermore, intratumor T cells express elevated levels of inhibitory receptors (*LAG3*, *TIGIT*, *CTLA4*, *HAVCR2*/TIM-3 and *PDCD1*/PD-1) associated with T cell exhaustion (Costa et al., 2022; Wienke et al., 2024). Examination of cellular interactions between tumor, stromal and immune cells has enabled the identification of immunosuppressive ligand-receptor interactions. Immunoregulatory interactions *CD24*—*SIGLEC10*, *NECTIN2*—*TIGIT* and *LGALS9*—*HAVCR2* (encoding TIM-3) are present in the neuroblastoma tumor microenvironment, which may signify potential targets for immunotherapy (Verhoeven et al., 2022; Wienke et al., 2024).

#### **1.4.4 Bone marrow metastasis**

Metastatic spread is the major cause of cancer-related death in children and adults (Dillekås et al., 2019). At the time of neuroblastoma diagnosis, distant metastases are evident in approximately half of the cases (Maris et al., 2007; Swift et al., 2018). The most common sites of neuroblastoma metastasis are bone and bone marrow, accounting for over 90% of metastatic neuroblastoma cases (S. Liu et al., 2023; Méhes et al., 2001; Merugu et al., 2020). The first single-cell transcriptomic study of neuroblastoma bone marrow metastasis revealed an immunosuppressive microenvironment including enriched neutrophils, MDSC-like cells and CD8-positive T cells, and a decrease in progenitor B cells (Lazic et al., 2021). Multiplexed imaging analysis identified biomarkers for the detection of disseminated tumor cells, including GD2, B7-H3/CD276, CD56, CD24 and FAIM2 (Lazic et al., 2021). A subsequent study also used scRNA-seq to characterize eleven neuroblastoma bone marrow metastatic samples and five non-metastatic samples (control) (Fetahu et al., 2023). Metastatic neuroblastoma cells were transcriptionally similar to noradrenergic cells, predominantly resembling neuroblasts/sympathoblasts (Fetahu et al., 2023; Mei et al., 2024), comparable to previous studies on primary tumors (Bedoya-Reina et al., 2021; Dong et al., 2020; Jansky et al., 2021; Kameneva, Artemov, et al., 2021; Kildisiute, Kholosy, et al., 2021; Slyper et al., 2020). Shifts in cell type composition included increased T and NK cells, but a B cell depletion in metastatic bone marrow compared to control

samples (Fetahu et al., 2023; Mei et al., 2024), similar to their first scRNA-seq study (Lazic et al., 2021). Additionally, Fetahu et al. described bone marrow metastasis-associated monocytes with pro-tumorigenic/metastatic features, akin to TAMs (Fetahu et al., 2023).

Recent work has been dedicated to evaluating paired primary tumors and bone marrow metastases, controlling for inter-individual differences (Hochheuser et al., 2024; Nian et al., 2024). Consistent with previous reports, metastatic tumor cells expressed neuroendocrine markers, including *PHOX2B*, *GATA3*, and *HAND2* (Hochheuser et al., 2024; Nian et al., 2024) all of which are part of the neuroblastoma adrenergic core regulatory circuitry (Boeva et al., 2017; Van Groningen et al., 2017). Evolutionary trajectory analysis revealed a “starter” subpopulation of tumor cells defined by its early stage in the developmental trajectory (Nian et al., 2024). Both studies detected increased cell cycle activity in metastatic tumor cells and “starter” cells (Hochheuser et al., 2024; Nian et al., 2024). The malignancy of these cells was confirmed with inferCNV, which revealed clonal differences between primary and metastatic tumor cells in most of the cases (Hochheuser et al., 2024). Gene set enrichment analysis (GSEA) of these clones revealed biological processes related to metastasis, including tumor invasiveness, migration and TGF- $\beta$  signaling pathway (Hochheuser et al., 2024). The latter was also identified as the strongest signaling pathway associated with interactions between “starter” tumor cells and the microenvironment (Nian et al., 2024). The “starter” cell exhibited higher expression of SMAD2, SMAD3, and TGF- $\beta$  receptors (Nian et al., 2024), potentially targetable with a specific inhibitor of Smad3 (SIS3) (Louault et al., 2024).

## 1.5 Modeling neuroblastoma

Given the complexity of neuroblastoma, it is important to use appropriate models that recapitulate the disease. Although well-established model systems have yielded considerable insights into neuroblastoma biology and drug discovery, some discrepancies between the primary tumors and preclinical models need to be considered.

### 1.5.1 Cell lines and xenografts

*In vitro* cell lines are widely used in preclinical drug testing and for basic research. Neuroblastoma cell lines have distinct phenotypes and were previously categorized as N-type (neuroblast) or S-type (substrate adherent) (Biedler et al., 1973). Their ability to interconvert between these phenotypes was later described

as intermediate I-type cells (Ciccarone et al., 1989). In 2017, two studies explored the molecular features of these two phenotypes by transcriptomic and epigenetic profiling of neuroblastoma and neural crest-derived cell lines (Boeva et al., 2017; Van Groningen et al., 2017). Super-enhancer-associated networks of transcription factors distinguish each phenotype. The two phenotypes were described as adrenergic/noradrenergic (expressing PHOX2B, TH, DBH and DLK1) and mesenchymal/neural crest cell-like cells (expressing PRRX1, SNAI2, VIM, FN1 and YAP1) (Boeva et al., 2017; Van Groningen et al., 2017). Transitions between these phenotypes can be genetically induced by overexpression of mesenchymal transcription factor *PRRX1* or NOTCH3 intracellular domain, which reprograms the super-enhancer landscape from adrenergic to a mesenchymal state (Van Groningen et al., 2017; van Groningen et al., 2019). They proposed the involvement of NOTCH signaling in the trans-differentiation between these cell states (van Groningen et al., 2019).

While previous studies on super-enhancers focused on cell lines, recent analysis on neuroblastoma patient samples have revealed super-enhancer-derived epigenetic subtypes specific to clinical groups of neuroblastoma (*MYCN*-amplified, *MYCN* non-amplified high-risk and *MYCN* non-amplified low-risk) (Gartlgruber et al., 2021). The mesenchymal phenotype is often associated with therapy resistance and enriched during relapse (Avitabile et al., 2022; Gartlgruber et al., 2021; Van Groningen et al., 2017; Westerhout et al., 2022). Moreover, scRNA-seq of ten peripheral neuroblastic tumors has shown the presence of malignant adrenergic and mesenchymal cells interconnected by a newly identified transitional cell state expressing *SOX11*, *MYCN*, *EZH2* and *NEUROD6* (X. Yuan et al., 2022). The expression of the transitional signature is associated with poor neuroblastoma patient survival (X. Yuan et al., 2022).

The cellular plasticity of neuroblastoma cells was recently investigated in cell lines, cell line-derived xenografts, and patient-derived xenografts (PDX) (Thirant et al., 2023). CD44 is a mesenchymal-specific surface marker which can be used to detect and sort mesenchymal cells by flow cytometry/FACS. In line with previous studies, CD44-positive mesenchymal cells exhibit chemoresistance compared to their adrenergic counterpart. *In vitro*, adrenergic to mesenchymal transitions are promoted by extrinsic factors EGF and TNF $\alpha$ . In contrast, *in vivo* models reversely reprogram mesenchymal cells towards adrenergic cells. scRNA-seq of primary neuroblastoma and PDX tumors exhibit heterogeneity encompassing adrenergic

tumor cells with a subset concurrently expressing mesenchymal markers, similar to the cell lines and xenografts (Thirant et al., 2023).

According to a recent study, these neuroblastoma phenotypes are indicative of distinct cell types (Bukkuri et al., 2024). What distinguishes the cell type abundance or susceptibility to treatment is the proliferation and survival rates, rather than phenotypic switching.

### 1.5.2 TH-MYCN transgenic mice

Given that the *MYCN* oncogene plays a key role in neuroblastoma tumorigenesis, aggressiveness and prognosis, Weiss et al. generated the TH-MYCN transgenic mouse model in 1997 (W. A. Weiss et al., 1997). Human *MYCN* oncogene is overexpressed under the rat tyrosine hydroxylase (TH) promoter, which gives rise to neuroblastoma-like abdominal or thoracic tumors. These tumors often arise in the paraspinal ganglia and adrenal gland, reminiscent of the primary sites of human neuroblastoma development. This transgenic model is widely used in neuroblastoma research as it accurately reflects human neuroblastoma features, including microRNA expression profiles (Terrile et al., 2011), genetic modifications (Hackett et al., 2003; W. A. Weiss et al., 2000) and pathology (Moore et al., 2008).

A study characterizing homozygous (TH-MYCN+/+) and hemizygous (TH-MYCN+/-) TH-MYCN mice has revealed significant differences in neuroblastoma incidence, tumor latency, tumor growth and treatment windows (Rasmuson et al., 2012). The tumor growth and incidence were shown to be in significant correlation to the transgene status. Homozygous tumors are highly aggressive with 100% tumor penetrance, whereas penetrance in hemizygous mice is ~50%. Homozygous mice developed tumors at 4.0–6.9 weeks of age, whereas hemizygous mice developed tumors at 5.6–19 weeks of age. The average time from palpable tumor until sacrifice (treatment window) was 5.2 days for homozygous mice and 15 days for hemizygous mice. These clear phenotypic distinctions may be a result of *MYCN* gene dosage, as higher *MYCN* expression induces rapid tumor growth (Rasmuson et al., 2012).

A recent study utilized scRNA-seq to evaluate the tumor cell composition of three TH-MYCN mice and further compared it with biopsies from neuroblastoma patients, exhibiting a shared immunosuppressive tumor microenvironment (Costa et al., 2022). TH-MYCN tumor cell clusters express adrenergic transcription factor *Phox2b* (Costa et al., 2022; Embaie et al., 2025). A subset of cancer-associated fibroblasts express chemokines involved in immunosuppression, including *Cxcl1*,

*Cxcl12* and *Ccl2*, which is also evident in human neuroblastoma. Myeloid cells are the most abundant in the mouse tumor microenvironment, and subcluster analysis revealed heterogeneous macrophages and distinct PMN-MDSCs (Costa et al., 2022).

Expression of adrenergic transcription factor PHOX2B and disialoganglioside GD2 is lost in cell lines derived from TH-MYCN tumors (McNerney et al., 2022). The TH-MYCN cell lines acquire a mesenchymal phenotype expressing YAP1 (McNerney et al., 2022). *In vitro* lineage switching poses a major challenge in drug discovery due to significant discrepancies in treatment sensitivity or resistance. Hence there is a need for *ex vivo* TH-MYCN models that recapitulate the primary tumors (Embaie et al., 2025).

Although TH-MYCN mice exhibit a limited capacity to metastasize to the bone marrow (<5% incidence) (W. A. Weiss et al., 1997), efforts have been devoted to the development of metastatic TH-MYCN models. The first bone marrow metastatic neuroblastoma model in immunocompetent mice was established in caspase-8-deficient TH-MYCN mice (Teitz et al., 2013). Subsequently, a chemoresistant model TH-MYCN<sup>CPM32</sup> was generated, exhibiting enhanced spontaneous bone metastases, which is reminiscent of refractory neuroblastoma (Yogev et al., 2019). Another approach to model metastasis is the intravenous injection of luciferase- and GFP-tagged TH-MYCN tumor cells into immunocompetent mice, which were shown to disseminate to bone marrow, lung, kidney, brain, and skin (Rahavi et al., 2023).

### 1.5.3 Organoids and tumoroids

Organoid models are novel *ex vivo* cell culture systems which can facilitate the development of individualized precision medicine. From the 1950s, the term "organoid" was initially used to describe the ability of primary dissociated cells to self-organize and form structures *in vitro* (Clevers, 2016; Moscona & Moscona, 1952; P. B. Weiss & Taylor, 1960). These findings served as the foundation for our knowledge of cellular organization during development. In recent years, organoids have instead been used to nominate three-dimensional cellular organizations that mimic the structure and functionality of a particular organ. This modeling system enables self-renewing stem cells to grow, self-organize into organotypic structures within the support of three-dimensional artificial extracellular matrix proteins and under the guidance of tissue-specific essential morphogens and growth factors. Organoid size is often limited to a few millimeters, but their



continued development into more intricate and mature organ-like structures is constrained by the absence of a working circulatory system or lack of vascularization (Lancaster & Knoblich, 2014; Takebe et al., 2013). Organoids have been developed to represent a variety of organs, including the brain (Lancaster et al., 2013; Quadrato et al., 2017), the gut (Sato et al., 2009), the liver (Takebe et al., 2013), and the prostate (Chua et al., 2014; Drost et al., 2016). These organotypic structures emulate the cellular composition and architecture of the tissue from which they originated, hence can serve as a promising tool for investigating various stages of development and disease.

Tumoroids are defined as *ex vivo* organotypic cultures derived from neoplastic tissue. Both organoids and tumoroids were initially established from epithelial tissues from the intestine and epithelial tumors, respectively. Toshiro Sato, Hans Clevers and colleagues in 2009 first developed long-term organoid culture techniques recapitulating mouse intestine crypt-villus units. They demonstrated that single Lgr5+ stem cells can give rise to multiple cell types that make up the crypt-villus organoids, independent of mesenchymal cellular niches (Sato et al., 2009). A comprehensive culturing procedure for mouse and human intestinal tissue of normal and malignant origin was reported by the same group in 2011. Supplementing an artificial extracellular matrix, such as Matrigel or xeno-free hydrogel matrices, with a combination of essential growth factors, such as WNT3A, R-spondin, and EGF, as well as the BMP inhibitor Noggin and, particularly for culturing human cells, A83-01 inhibitor of TGF- $\beta$  type I receptor and SB202190 inhibitor of p38 MAP kinases, represent the fundamental basis of the methodology of establishing organoids (Sato et al., 2011). Great advancements have been made to develop novel hydrogel systems that mimic tissue-like matrices to support the growth of three-dimensional culture systems (De Santis et al., 2021).

There are vital aspects of adult and pediatric malignancies that vary. The frequency of recurring mutations and the overall mutational burden in pediatric malignancies are very low in contrast to adult cancers, which often take several years to develop by accumulating genetic alterations over time (Gröbner et al., 2018). Organoids are promising models and analytical platforms to study the oncogene-independent features of childhood cancers, as this technique can easily be incorporated with other technologies, including live cell imaging, single-cell genomics and genome editing.

Although emerging neuroblastoma 3D cultures have been described in literature, these often represent free-floating spheroid models, which lack extracellular matrices (Kock et al., 2020). PDX-derived “organoids”, namely PDXOs, are 3D *in vitro* models produced from patient tumors after passaging and expansion in animal models. Neuroblastoma PDXOs have been used for drug screening studies (Hansson et al., 2020; Mañas et al., 2022; Radke et al., 2021).

Organoids and tumoroids are crucial for applying modern precision medicine on pediatric cancer and neuroblastoma in an ethical, xeno-free and resource-efficient manner. New insights regarding the cellular and clonal heterogeneity, and phenotypic plasticity in neuroblastoma enable us to predict the level of complexity required to adequately model neuroblastoma development and biology. Recently, drug sensitivity profiling of fifteen neuroblastoma patient-derived organoids was established by high throughput screening (Langenberg et al., 2025). Integration of whole genome sequencing, bulk mRNA sequencing, and drug response profiling of the novel patient organoids and cell lines provides a comprehensive resource for precision medicine (Langenberg et al., 2025).

## 2 Research aims

In the era of single-cell multi-omics, the characterization of cellular and molecular complexity of cancer and metastasis have become inevitable. The heterogeneity of neuroblastoma attributes to therapy resistance and relapse. Hence, the overarching aim of this thesis was to explore the transcriptional landscape of neuroblastoma tumors, disseminated tumor cells and the metastatic bone marrow niche. Harnessing these single-cell transcriptomic profiles, we propose potential prognostic biomarkers and therapeutic targets. The specific research aims were to:

**Paper I:** Examine the cellular ancestry and clonal development of neuroblastoma by joint single-cell DNA and RNA sequencing of sorted cells and spatial validation.

**Paper II:** Characterize the tumor cell atlas of neuroblastoma transgenic mouse model and *ex vivo* cultures by comparative transcriptomic analysis with fetal adrenal gland and human neuroblastoma to decipher the heterogeneity, cell-cell interactions, and clinical relevance of these models.

**Paper III:** Investigate the compositional and transcriptional alterations in the niche associated with neuroblastoma bone marrow metastasis by scRNA-seq and protein validation of bone marrow biopsies from non-metastatic and metastatic cases.



### 3 Materials and methods

It is critical to select appropriate methods and model systems to address specific research questions. The rationale behind utilizing the methods relevant to this thesis is outlined in this section. Detailed descriptions of the materials and methods used in each study are fully described in the corresponding papers.

#### 3.1 Clinical sampling

Diagnosis and treatment of patients with neuroblastoma adhered to national and international guidelines and protocols. Tumor biopsies, surgical resections (Paper I) and bone marrow biopsies (Paper III) were collected from patients with neuroblastoma after verbal and written consent was provided by their parents or legal guardians. We collected samples according to Etikprövningsmyndigheten (The Swedish Ethical Review Authority) approved permits with the following reference numbers: 03-736, 2009/1369-31/1, 2022-07254-01. We collected relevant clinical data from hospital records in accordance with the ethical permits. For both studies, a broad range of clinical samples were collected, including different genetic features, risk stratifications and age groups.

After pathological evaluation of the collected samples, primary tumors were dissected for array comparative genomic hybridization (CGH) with single nucleotide polymorphism (SNP) analysis or whole genome sequencing, cryopreservation, formaldehyde fixation for immunostaining/DNA-FISH, enzymatic dissociation for Fluorescence-activated cell sorting (FACS) and scRNA-seq or DNTR-seq. Tumor pieces were enzymatically dissociated into single-cell suspension using collagenases I-IV and dispase II dissolved in medium containing 2% serum to digest the connective tissue, then filtered to remove cell aggregates and debris. Bone marrow biopsies were cryopreserved for subsequent flow cytometry analysis or FACS and scRNA-seq.

#### 3.2 TH-MYCN transgenic mice

TH-MYCN transgenic mice are characterized by the overexpression of human MYCN under the TH promoter. This MYCN-driven model spontaneously develops tumors in the sympathoadrenal system, predominantly in paraspinal ganglia and adrenal glands, resembling the primary sites of human neuroblastoma development. This is the most commonly used transgenic *in vivo* model in neuroblastoma preclinical studies since it accurately reflects the human disease, in terms of microRNA expression profiles (Terrile et al., 2011), genetic modifications

(Hackett et al., 2003; W. A. Weiss et al., 2000) and pathology (Moore et al., 2008). Furthermore, these mice are immunocompetent, and the intra-tumoral immunosuppressive landscape is comparable to human neuroblastoma (Costa et al., 2022). This allows us to study the complex interactions within the tumor microenvironment in paper II.

The following ethical permits for the use of research animals were approved by the regional ethics committee for animal research which is appointed under the Swedish Court and Swedish Board of Agriculture: 5163-2019 and 4356-2024. All efforts were made to optimize the welfare of the animals, and both the EU and national regulations were followed: EU directive 2010/63/EU, SFS 2007:488, SFS 2018:1192 and SFS 2019:66.

Similar to clinical tumors and biopsies, tumors from TH-MYCN mice were collected and dissected for cryopreservation, formaldehyde fixation for immunostaining, enzymatic dissociation for FACS and scRNA-seq, as well as *ex vivo* tumoroid cultures.

### 3.3 Cell lines

Relevant models are important to study cancer and test new drugs. 2D cell lines have been widely used for cancer research, drug discovery and target validation (Weinstein, 2012). Cell lines have the benefit of being stable in culture for manipulating gene expression, thereby enabling mechanisms of action studies. In paper III we used a cell line for this purpose, to knockdown *AHCY*, *PPAT*, or *GCSH* genes, and evaluate cell growth. Yet, more cell lines with a broad representation of genetic and biological aspects of neuroblastoma are needed to determine the role of these genes in this disease.

The degree to which these cell lines accurately depict the disease has been debated. Cell line culture medium is often supplemented with serum, which can cause genetic and phenotypic alterations, as well as terminal differentiation (Lee et al., 2006). Gene expression analyses reveal that cancer cell lines are more like one another than the primary patient tumor from which they are derived (Gillet et al., 2011). In contrast, cell lines cultured in serum-free media preserve the traits of the primary patient tumors (Bate-Eya et al., 2014), hence, we established *ex vivo* cultures in serum-free conditions.

### 3.4 Tumoroids

We have established and characterized TH-MYCN-derived *ex vivo* tumoroids in paper II. The tumor cells were embedded in Matrigel and cultured in a serum-free medium. The culture medium contained supplements that promote neuronal growth such as N2, B27, BDNF, IGF and NRG1. Moreover, the medium was also supplemented with A83-01 (inhibitor of TGF- $\beta$  type I receptor), which disrupts EMT through inhibition of the SMAD signaling pathway (Tojo et al., 2005), which may prevent transition to mesenchymal phenotypes in TH-MYCN tumor cell cultures. Tumoroid cells were expanded by passaging, and cells were harvested for cryopreservation, scRNA-seq and formaldehyde fixation for immunostaining. The tumor cells self-organized into tight spheroid-like structures and preserved the gene and protein expression of their originating tumor. Since these tumoroid cultures resembled the disease to a larger extent than 2D cell lines, they may be applicable for drug testing.

### 3.5 Immunostaining and DNA-FISH

Orthogonal approaches to validate the spatial context of protein expression and copy number changes of cells of interest include immunohistochemistry (IHC), immunofluorescence protein staining and fluorescence in situ hybridization (FISH). In papers I and II, these methods were used to study abnormal SCPs and N-MYC-positive tumor cells, respectively. To detect SCPs, we stained for SOX10, ERBB3, MPZ and PLP1. The following adrenergic markers were used: PHOX2B, ISL1, TH, and CART. Since 17q gain is the most common aberration and the neuroblastoma samples with predicted abnormal SCPs harbored copy number changes on chromosome 17, we applied DNA-FISH targeting chromosome 17q (PPM1D locus, 17q23.2).

### 3.6 FACS and flow cytometry

FACS was used to sort viable non-erythroid cells prior to scRNA-seq in all the papers included in this thesis. In papers I and II, we also utilized FACS to enrich for SCP-like cells for DNTR-seq and scRNA-seq, respectively. ERBB3-positive SCP-like cells and CD24-positive adrenergic cells were enriched. Unfortunately, due to the enzymatic dissociation of the tumor, mesenchymal marker epitopes were cleaved and could not be selected.

In paper III, flow cytometry was used to validate the abundance of metastatic tumor cells, B cells, cytotoxic T cells (CTLs) and NK cells. The first transcriptomics

study on neuroblastoma bone marrow metastasis identified CD276, GD2, CD24 and CD56 as tumor surface markers (Lazic et al., 2021), which we used to detect and quantify the proportion of tumor cells in bone marrow biopsies from samples BM1 and BM3. The dump channel for this staining panel included the following immune markers: CD235ab erythrocytes, CD45 pan-immune cells, CD19 B cells, CD94 NK and T cells.

Moreover, we conducted flow cytometric validation of immune cell panels, highlighting significant shifts in CD19-positive B cell abundance. Additionally, we stained for the quantification of PD1-positive CTLs and CD56<sup>bright</sup> CD16<sup>negative</sup> NK cells in the bone marrow metastatic niche. Importantly, these analyses were performed to compare neuroblastoma metastatic (BM1, BM3, BM11a, BM12, BM13) and non-metastatic (BM8, BM5, BM9, BM14, BM7, BM6) bone marrow samples that were matched with the scRNA-seq data. The use of matched sampling allows for a direct comparison and validation of scRNA-seq data at the protein level, thereby controlling for inter-individual variabilities. Furthermore, we utilized UltraComp eBeads™ Plus Compensation Beads (Invitrogen™ 01-3333-42) for the compensation of each antibody. Additionally, gating strategies were based on unstained cells and Fluorescence Minus One (FMO) controls.

### **3.7 Single-cell RNA sequencing**

To comprehensively map the cellular and transcriptional heterogeneity of neuroblastoma, bone marrow metastasis, TH-MYCN transgenic mouse tumors and *ex vivo* tumoroids, we performed scRNA-seq (10x Genomics Chromium). The Chromium Next GEM Single Cell 3' technology was chosen for its ability to efficiently capture transcriptomic profiles from heterogeneous neuroblastoma samples, including rare cell populations within tumors and metastatic niches. After FACS, viable single cells were resuspended in a reverse transcription master mix, and loaded onto Chromium Next GEM Chip G with barcoded Gel Beads and Partitioning Oil, to generate Gel Beads-in-Emulsion (GEMs). Within each GEM, single cells were lysed, and by reverse transcription with oligonucleotides, each transcript was tagged with a unique 10x Barcode and Unique Molecular Identifier (UMI). Then we performed amplification of barcoded cDNA, followed by library construction including fragmentation, adapter ligation and sample index PCR. Libraries were sequenced on the Illumina NextSeq 550 and 2000 platforms. The sequenced data was processed using the Cell Ranger pipeline (10X Genomics) and aligned to respective human or mouse reference genomes to obtain gene-cell count matrices. Then the count matrices were filtered to remove low-quality



cells based on the number of UMIs per cell, mitochondrial transcripts per cell and doublet removal. For papers I and III, *Conos* and *Pagoda2* were primarily applied for integration, and *Numbat* for subclonal CNV predictions in paper I. In paper II, *Seurat* was used for downstream analysis including clustering, differential gene expression, cell cycle scoring, and pathway enrichment. Furthermore, TSCAN was used for trajectory analysis, CellChat for intercellular interaction analysis, inferCNV to detect malignant cells, R2 database and survminer for survival analysis.

### **3.8 Direct nuclear tagmentation and RNA sequencing**

Fresh tumor samples THA004 and THA016 were enzymatically dissociated and stained for FACS. The staining panel included immune marker CD45, SCP marker ERBB3, and adrenergic cell marker CD24. Cell viability was confirmed with both Calcein AM and Viability Dye eFluor™ 450. Four populations of viable single cells were sorted: CD45-positive, CD45-negative, CD45-negative-CD24-positive and CD45-negative-ERBB3-positive. Single cells of each enriched population were sorted into 384-well plates containing lysis buffer for effective cell membrane lysis and then stored at -80°C to keep the nuclei intact. DNTR-sequencing was performed by nuclear separation followed by whole genome sequencing and mRNA library preparation as described by Zachariadis et al. (Zachariadis et al., 2020).

### **3.9 Ethical considerations**

Primary tumors, bone marrow biopsies, transgenic mice and ex vivo cultures were used to study neuroblastoma in this thesis. Using these samples and models raises ethical considerations regarding their application in research.

Neuroblastoma remains a very deadly and highly metastatic disease in young children and infants. Therefore, we urgently need to identify more effective drug targets. Thus, these studies would be considered beneficial to society, particularly for childhood cancer patients and their families. Comprehensively characterizing the transcriptional and genomic profiles of clinical samples, including primary tumors and bone marrow biopsies at a single-cell resolution, is arguably an efficient use of rare material. One could argue that the research question regarding characterizing the relevance of the currently used neuroblastoma models, or the aim to establish tumoroid models that recapitulate the parent tumor, is a justifiable use of animal models.

### **3.9.1 Ethical considerations regarding clinical sampling**

In papers I and III, we collected patient-derived tumor and bone marrow samples. Clinical sample collection, research application and handling of personal data are approved and included in O3-736, 2009/1369-31/1, 2022-07254-01 ethical permits. Patient tumor samples were obtained from surgery or fine/middle needle biopsy and did not cause any more unnecessary suffering or harm to the patient. The patients' well-being is a priority to the physicians with the oath to act in the best interest of the patients' health when providing medical care. Researchers and clinicians have a moral obligation not to perform research on vulnerable groups, with the aim to help other groups. This thesis aims to specifically help the vulnerable group at hand, so it is morally justifiable to perform research on material that is obtained from patients with neuroblastoma.

Their vulnerability also brings us to the issue of autonomous and voluntary informed consent or decision-making. Since the patients with neuroblastoma are young children and infants in a vulnerable group, they are not eligible for autonomous informed consent, hence, their parents or legal guardians are given the responsibility of giving consent. The parents are informed about the research study early on and given the opportunity to give informed consent. The information that is given to the parents is clear and understandable so that they can make an informed decision. It is crucial that they are informed that the study is completely voluntary, with no form of manipulation in the decision-making.

With regard to confidentiality and the handling of sensitive personal data, all the patient information is confidential in these studies. Any identifiable information in connection with sampling is processed to restrict any unauthorized access. When the contact is complete, the patients' name and personal numbers are coded to replace the information about the individuals and kept separate. Only the data controller for each study has access to the coded information.

The four central principles of biomedical ethics by Beauchamp and Childress are autonomy, non-maleficence, beneficence, and justice. In these studies, we have utmost respect for patient autonomy, with the intention of giving adequate and understandable information to the children's parents so that they make an informed and voluntary decision on behalf of their children. Regarding beneficence, we aim to identify therapeutically targetable vulnerabilities by examining ancestral clones of neuroblastoma and transcriptomic profiles that define metastatic spread to the bone marrow. The samples obtained for paper I

are surgical tumors, in cases where the surgery is performed to remove a tumor as a form of ongoing cancer treatment or diagnosis. The fine/middle needle bone marrow biopsies for paper III were collected for diagnostic purposes. Hence there is no additional harm done to the patients for these research studies, implying non-maleficence. Lastly, with the social benefits and potential medical improvements for cancer patients implicated in these studies, it is reasonable to argue that the obligation of justice is met.

### **3.9.2 Ethical considerations regarding preclinical models**

The regional ethics committee for animal research has critically reviewed and approved the use of TH-MYCN transgenic mice as experimental animals for paper II. This committee is appointed by the Swedish Board of Agriculture and the Swedish Court, which approved the following ethical permits: 5163-2019 and 4356-2024. For the use of this animal model, we have thoroughly considered and implemented the principle of the 3R's of Russell and Burch – Replacement, Reduction and Refinement. The animal-based research has been performed humanely. To replace, we primarily examined the *in vitro* cell line 9464D. We also established *ex vivo* tumoroid models to replace or complement the use of *in vivo* models. With regard to reduction, we have investigated TH-MYCN mice that were part of an untreated control group from other research studies. The tumors that were obtained from these mice were further dissected to be used for multiple investigations, including qPCR, RNA sequencing, immunohistochemistry, multiplexed immunofluorescence staining or tumoroid culturing for drug screening. Using this strategy, more data can be attained per animal in the experiment. Hence, the experimental outcomes from each animal are maximized to reduce the number of research animals used. To refine the procedures, we aimed to improve the welfare of research animals, and ensured to minimize suffering and distress. The mice were kept in a maximum of eight per cage, where they were given sterile water and food *ad libitum*. TH-MYCN mice were palpated weekly for tumors and frequently monitored for signs of welfare issues.

*Ex vivo* and *in vivo* tumor models are more complex and better represent the human disease when compared to *in vitro* tumor cells. The tumor cell composition, tumor microenvironment, and stem cell niche components are recapitulated in *ex vivo* and *in vivo* models. The limitations of 2D cell lines grown on plastic and cell line-derived xenograft models are apparent since they only represent artificially grown end-stage tumor cells, which present very different phenotypes from that of neuroblastoma patient tumors. It is, therefore, justifiable to use neuroblastoma

animal models, such as TH-*MYCN* and the establishment of *ex vivo* models, to maintain the complexity of the disease. This gives us the potential to reveal novel therapeutic targets for pediatric cancers like neuroblastoma. Furthermore, *ex vivo* tumoroids reduce the reliance on experimental animals for the identification of potential therapeutic targets and pharmacological testing.

## 4 Results

### 4.1 Paper I: Discovery of premalignant SCP-like subclones in neuroblastoma

The etiology of neuroblastoma is unclear, but it is thought to arise from the neural crest lineage. Malignant neuroblastomas often harbor chromosomal aberrations, and consequent genomic instability probably plays a key role in tumor initiation and progression. To identify the cellular ancestry of neuroblastoma, we used scRNA-seq to study eighteen human neuroblastoma samples with diverse clinical characteristics. We applied the *Conos* algorithm to integrate the single-cell transcriptomes of the neuroblastoma samples. Eighteen cell populations were identified, spanning tumor, stromal and immune cells, of which the latter has been comprehensively characterized by our lab (Verhoeven et al., 2022). Hence, we focus on the tumor and stromal compartments in paper I. The stromal cell clusters comprised endothelial cells (*CALCRL*, *CDH5*, *CLDN5*, *EGFL7*, *KDR*, *PECAM1*), pericytes (*ACTA2*, *CALD1*, *MYH11*, *MYL9*, *PLN*, *SPARCL1*, *TAGLN*, *TINAGL1*), and myofibroblasts (*ANGPT2*, *BGN*, *COL1A2*, *COX4I2*, *PDGFRB*, *SPARC*). Adjacent to the myofibroblast cluster, we identified a mesenchymal cluster expressing *DCN*, *MGP*, *LUM*, *OGN* and *COL6A3*.

#### 4.1.1 Identification of abnormal SCP-like cells

The majority of the tumor cell compartment expressed adrenergic markers *PHOX2B*, *TH*, *DBH*, and *CD24*. Strikingly, the adrenergic tumor cells were connected to the mesenchymal cluster by cells expressing neural crest or Schwann cell markers, including *SOX10*, *ERBB3*, *STOOB*, *FOXD3* and *PLP1*. To confirm the cell state identity of the tumor cells in relation to normal fetal adrenal development, we performed joint alignment analysis of non-immune cell clusters. Adrenergic tumor cells aligned with normal fetal sympathoblasts and partially chromaffin cells. The stromal compartment aligned with normal fetal mesenchymal cells. Importantly, the newly identified cells connecting the tumor and mesenchymal compartments, expressed *SOX10* and aligned with human fetal SCPs.

*Numbat* is a computational method which predicts genomic CNVs and subclonal structure from scRNA-seq data (T. Gao et al., 2022). We used CNV profiles from array CGH data from matched samples to validate the predicted *Numbat* pseudobulk CNV profiles. Inferred CNVs showed that adrenergic tumor cells were indeed malignant. In contrast, no CNVs were detected in pericytes,

myofibroblasts, mesenchymal and endothelial cells. Surprisingly, clonal CNVs were identified in a fraction of the neuroblastoma cells that aligned with the fetal SCPs, specifically in an untreated sample NB26 (primary adrenal tumor; INRGSS: L1). *Numbat* detected four subclones in this sample, of which clone 2 comprised SCP-like neuroblastoma cells with gain in chromosomes chr6, chr8, chr10 and chr17. The daughter clones 3 and 4 presented distinct UMAP clustering of adrenergic tumor cells and maintained whole-chromosome 17 gain.

Allele-specific CNVs were analyzed to determine whether these SCP-like neuroblastoma cells are ancestral cells to the adrenergic tumor cells in this sample NB26. We investigated the shared whole-chromosome gains in chr6, chr8 and chr10, which all had allelic imbalance. But chr17 was excluded since there was no allelic imbalance. To evaluate chromosomal imbalance, alleles were phased into major and minor haplotypes and quantified the number of these in single cells. The SCP-like and adrenergic cells shared gains of chr8 and chr10, which suggests that the clones share a common ancestor, with chr8, chr10, and chr17 gains as putative tumor-initiating events (Figure 4). In contrast, the gain of chr6 was "mirrored," meaning that the opposite chromosomes were gained in the two cell types at a later stage. Therefore, in this sample, the SCP-like clone shares CNVs and a direct common ancestor with the adrenergic tumor cells (Figure 4).

We further evaluated the gene expression patterns that distinguish abnormal SCP-like cells and non-malignant SCP-like cells from neuroblastoma tumors, and normal fetal SCPs from fetal tissue. While key SCP marker genes were consistently expressed across these cells (*SOX10*, *ERBB3*, *SIOOB*, *PLP1*, *MPZ*), there were some transcriptional differences with possible functional implications. Proliferation scores were significantly higher in abnormal SCP-like cells. In contrast, non-malignant SCP-like cells expressed genes encoding MHC class II molecules (*CD74*, *HLA-DRA*, *HLA-DRB1*, *HLA-DPA1*).

#### **4.1.2 Spatial validation of abnormal SCP-like cells**

Using an orthogonal assay, we spatially validated the abnormal SCP-like cells by combining DNA-FISH targeting chromosome 17 (*PPMID* locus; 17q23.2) with immunofluorescence staining to detect *SOX10*-positive cells. We confirmed a significant number of *SOX10*-positive nuclei with three or more *PPMID*-FISH signals in NB26, as well as in pretreated samples NBO9b (relapsed tumor; INRGSS: M) and NB20 (primary adrenal tumor; INRGSS: M). Similarly, we detected aberrant *PPMID* copy numbers in *PHOX2B*-positive adrenergic tumor cells consistent with

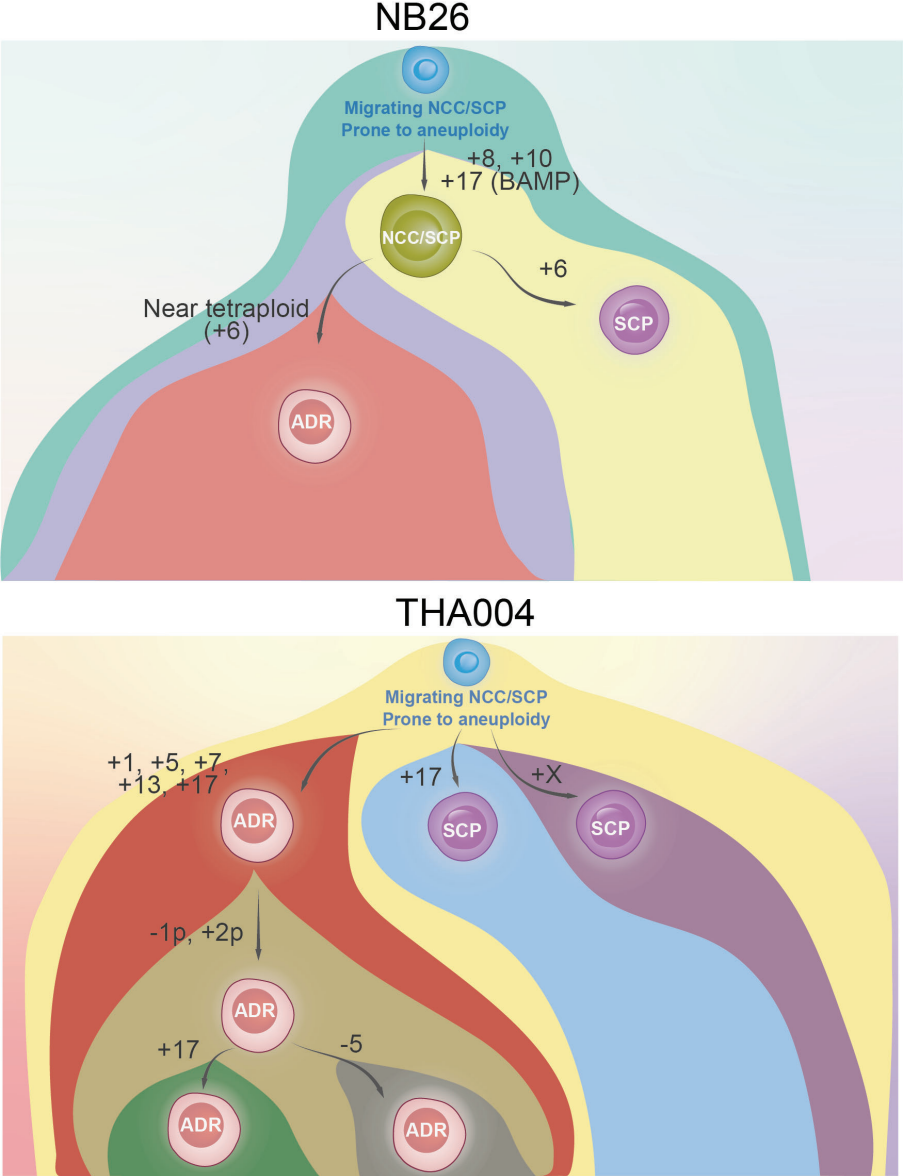
*Numbat* analysis of NB26. During fetal development, SCPs are characterized by their nerve-associated niche. We, therefore, evaluated the proximity of SOX10-positive cells to NF200 neurofilaments. Over 90% of abnormal SOX10-positive cells were located in immediate proximity to NF200 protein in samples NB09, NB20 and NB26. Moreover, neuroblastoma SCP-like cells were shown to express ERBB3 and surrounding adrenergic tumor cells expressed its ligand NRG1, indicating that SOX10-positive cells retain their SCP niche.

#### **4.1.3 Investigation of SCP-like subclones**

For a comprehensive examination of neuroblastoma clonal development, particularly in malignant adrenergic and SCP-like clones, we performed DNTR-sequencing. This technique combines whole-genome and transcriptome sequencing of sorted single cells. We used flow cytometry to enrich for SCP-like cells by sorting ERBB3-positive cells and adrenergic tumor cells by sorting CD24-positive cells. Transcriptional analysis was used to validate the cell state identity of the sorted cells of interest. Gene expression of distinct markers characteristic of adrenergic, SCP-like and mesenchymal cells was confirmed in the DNTR-sequenced data. SCP-like cells were detected in low-risk sample THA004 and high-risk sample THA016.

Whole-genome sequencing data from the single cells was used to determine the abnormality and malignancy of different cell types. As expected, adrenergic cells harbored CNVs that matched the aberrations detected in array CGH data. Of note, SCP-like and mesenchymal cells did not harbor any CNVs in the *MYCN*-amplified sample THA016. Interestingly, a distinct SCP-like subclone with whole-chromosome 17 gain was identified in untreated sample THA004 (primary tumor of the left sympathetic side chain; INRGSS: L1). Gain of chromosome 17 was shared in all malignant clones, including adrenergic tumor cells. Next, we looked at the tumor phylogeny of this sample by evaluating chromosome 17 haplotypes based on common germline SNPs. The chr17 in the SCP-like cells and chr17 from the adrenergic tumor cells were different haplotypes, suggesting that the clone of SCP-like cells was not a direct precursor in this sample; rather, the clones evolved in parallel (Figure 4). Both SCP-like and adrenergic clones were undergoing clonal expansion since the same haplotype was enriched in all cells from the respective clones. Differential gene expression analysis of SCP-like cells with chr17 gain compared to non-malignant SCP-like cells revealed upregulated expression of *ANAPC11*, *DDX5*, *NGFR* and *KPNB1*, all of which are located in the commonly gained chromosome arm 17q.

Taken together, we discovered aneuploid pre-malignant SCP-like cells in neuroblastoma and propose that these cells arise during the early stages of tumor development. Gain of chromosome 17 was a common aberration in pre-malignant SCP-like subclones across samples, suggesting that aneuploidy may be a possible tumor-initiating event in neuroblastoma.





## 4.2 Paper II: TH-MYCN and matched ex vivo tumoroids mimic the adrenergic cell state of human MYCN-amplified neuroblastoma

A major challenge in neuroblastoma research is the discrepancy in cellular and molecular heterogeneity between patient tumors and preclinical models. The shifts in cellular identity from adrenergic tumor cells to mesenchymal phenotypes can affect susceptibility to treatment and impact translatability to clinical studies. In paper II we examined the transcriptional landscape of tumors from homozygous and hemizygous TH-MYCN transgenic mice by scRNA-seq. Unbiased clustering of sequenced cells revealed a variety of tumor, immune and stromal cells. The MYCN<sup>+</sup> tumor cell compartment consisted of chromaffin cells (expressing *Th*, *Cdkn1c*, *Caly* and *Chga*) and sympathoblasts (expressing *Cartpt*, *Isl1*, *Nefl* and *Stmn1*). Pseudo-time trajectory analysis showed transitions from immature chromaffin cells to sympathoblasts, similar to murine adrenal gland development. Cell cycle phases distinguished the remaining sympathoblast subclusters within the tumor compartment. Most of the tumor cells were cycling sympathoblasts and the adrenergic cell state was validated with immunofluorescence analysis.

### 4.2.1 Transcriptional similarities with fetal adrenal gland

Since neuroblastoma often arises in the adrenal gland, we compiled gene signature scores that are characteristic of cell states in normal fetal adrenal medulla. We mapped these gene signatures onto the TH-MYCN tumor cells. Tumor cells exhibited strong expression of normal developing sympathoblast or neuroblast signatures, and a small fraction of tumor cells expressed embryonic chromaffin cell signatures. Joint alignment analysis with fetal adrenal cells (E13.5) (Kameneva, Artemov, et al., 2021) confirmed transcriptional similarities between TH-MYCN tumor cells and embryonic chromaffin cells and sympathoblasts. The proportion of CART-positive sympathoblasts and TH-positive chromaffin cells was validated histologically. Differential expression analysis comparing tumor cells to normal embryonic cells revealed *Fabp5* and *Dlk1* as tumor-associated genes.

Homozygous TH-MYCN tumors exhibited a distinct population of Schwann/SCP-like cells that expressed fetal SCP signatures. Immunofluorescence staining showed that cells expressing Schwann cell markers, SOX10 and PLP1, were not co-expressing N-MYC. MYCN-FISH combined with SOX10-immunostaining in a cohort of MYCN-amplified neuroblastoma and PDX tumors consistently indicated the lack of Schwann cells with aberrant number of MYCN-FISH signals (data not shown). Immunohistochemistry of homozygous TH-MYCN tumors showed that

SOX10-positive cells presented a glial or stromal morphology resembling Schwannian stroma. Similarly, scRNA-seq of ERBB3-enriched tumor cells confirmed that Sox10-positive cells did not express human *MYCN*, which is consistent with recent findings on other *MYCN*-driven GEMM tumors like LSL-*MYCN*;Dbh-iCre (Chapple et al., 2024).

#### **4.2.2 Relevance to human neuroblastoma and inferred interactions**

To evaluate the relevance of this model in relation to human *MYCN*-amplified neuroblastoma, we integrated single-cell transcriptomes from three untreated (Dong et al., 2020) and three treated primary patient samples from paper I (Olsen et al., 2024). The tumor cell compositions of TH-*MYCN* tumors were comparable to human *MYCN*-amplified neuroblastoma. Top differentially expressed genes that distinguished cell clusters in the human tumors were consistently expressed by mouse tumor cell clusters. Further, investigation of cancer-related signaling pathways in the hallmark gene set from MSigDB revealed that both human and mouse tumor cells expressed MYC target genes. Proliferating sympathoblasts in both species expressed genes involved in DNA repair and cycle-related targets of E2F transcription factors. G1 phase sympathoblasts and chromaffin cells expressed genes associated with hedgehog signaling activation. Myeloid cells, in both human and mouse tumors, expressed genes upregulated by activation of multiple pathways, including KRAS, p53, PI3K/AKT/mTOR, MAPK, and TNF signaling via NF- $\kappa$ B.

We further explored intercellular interactions between tumor cells and the microenvironment using a computational prediction tool called CellChat. Interestingly, the outgoing signaling pathways from mouse adrenergic tumor cells included NCAM and NOTCH. Ligand-receptor pairs among these pathways, in human or mouse tumors, revealed NCAM1, FGFR1, L1CAM, DLK1, DLL3 and JAG1, amongst other NOTCH ligands, as potential drug targets.

#### **4.2.3 Ex vivo tumoroids conserve adrenergic cell states**

The reversible plasticity between neuroblastoma adrenergic and mesenchymal cells has been described in cell lines and xenograft models (Thirant et al., 2023). In this study, we have confirmed that TH-*MYCN* tumors predominantly comprise adrenergic sympathoblasts. In contrast, TH-*MYCN*-derived cell lines acquire a mesenchymal phenotype (McNerney et al., 2022). Therefore, we aimed to establish a new *ex vivo* culture system that maintains the cell state identity of the originating tumor. We curated culture conditions to promote self-renewal and

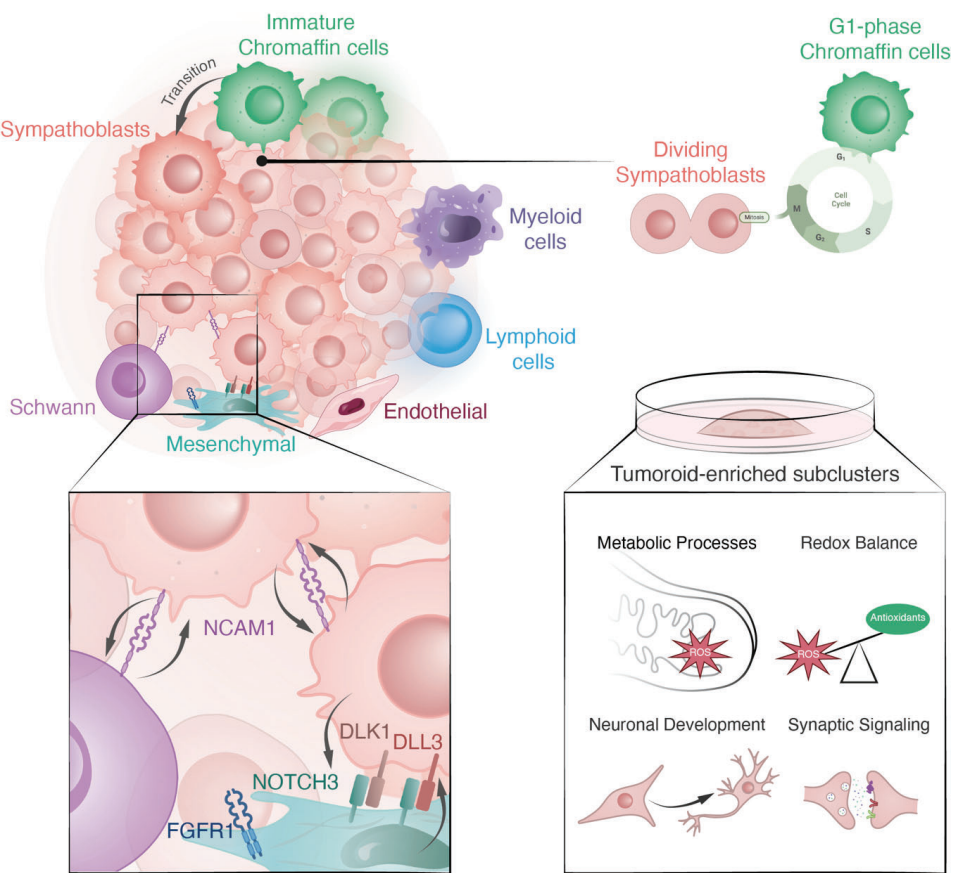
prevent differentiation. TH-MYCN tumor cells were embedded in Matrigel and submerged in a serum-free medium supplemented with essential growth factors. Immunofluorescence staining for adrenergic lineage marker PHOX2B was evident in the majority of N-MYC-positive tumor cells and matched *ex vivo* tumoroid cultures.

Single-cell transcriptomic analysis confirmed that the adrenergic cell state was conserved in *ex vivo* tumoroids. Similar to the primary tumors, cell cycle states shaped the sympathoblast subclusters in the tumoroids. To determine the cell state identity, we evaluated the expression of gene signatures defining developing adrenal medulla cells. Tumoroid cycling sympathoblast clusters expressed embryonic sympathoblast associated genes, while tumoroid non-dividing chromaffin cells resembled embryonic chromaffin cells and committed progenitors.

Intriguingly, four sympathoblast subclusters were enriched in *ex vivo* tumoroid cultures. The first cluster C1 expressed synaptic signaling gene *Nrxn1*, as well as neuronal morphogenesis associated gene *Nav3*, and brain angiogenesis inhibitor *Adgrb3*. Cluster C2 was characterized by the expression of *Sod1* and *Pgk1*, both involved in maintaining redox balance, as well as *Fdps* encoding a metabolic enzyme. S-phase sympathoblasts were transcriptionally similar to cluster C3, however, *Txnip*, *Ddit4*, *Baiap2*, *Cdkn1a*, *Plk2* and *Gadd45g* distinguished these clusters. Cluster C4 gene signature included *Crabp1*, *Ass1*, *Nos1* and *Hmx3*. The biological processes related to *ex vivo* tumoroids were identified using Gene Ontology (GO) term enrichment analysis. Metabolic processes, synapse organization and synaptic signaling were amongst the top significantly enriched pathways (Figure 5). Survival analysis on SEQC-498 (GSE49711) dataset, based on genes expressed by tumoroid enriched clusters C1, C2, C3, and C4, showed that high expression of tumoroid-related genes correlated with poor neuroblastoma patient survival. Additionally, inferCNV analysis predicted that these clusters acquired additional CNVs, indicative of more aggressive features.

In summary, we generated scRNA-seq maps of TH-MYCN tumors and *ex vivo* tumoroids. The tumor compartment in these models consistently demonstrated gene signatures associated with proliferating sympathoblasts connected to a subfraction of low-proliferative chromaffin cells (Figure 5). Comprehensive comparative analysis with human MYCN-amplified neuroblastoma revealed shared gene expression profiles with TH-MYCN tumors, suggesting the

translational potential of this model. Moreover, CellChat analysis uncovered potential therapeutic vulnerabilities in the NCAM and NOTCH signaling pathways (Figure 5). Lastly, distinct sympathoblast subclusters were enriched in *ex vivo* tumoroids, with upregulated expression of genes correlated with poor neuroblastoma prognosis.



**Figure 5** Graphical abstract of paper II (Embaie et al., 2025). Single-cell transcriptomics of TH-MYC/N mouse tumors revealed transitions between low-proliferative chromaffin and highly proliferative sympathetic phenotypes. CellChat interaction analysis unraveled potential therapeutic targets including NCAM1, FGFR1, as well as NOTCH-ligands, DLK1 and DLL3. Tumoroid enriched subclusters showed upregulated expression of genes associated with metabolic processes, redox balance, neuronal development and synaptic signaling.

### 4.3 Paper III: Immune cell remodeling and transcriptional reprogramming in neuroblastoma bone marrow metastasis

High-risk neuroblastoma cells often metastasize to the bone and bone marrow (S. Liu et al., 2023). In paper III we systematically characterize the metastatic bone marrow in comparison with non-metastatic bone marrow, using scRNA-seq. Single-cell transcriptomes from seven non-metastatic and eight metastatic bone marrow samples were integrated with *Conos*. Unsupervised clustering of all bone marrow samples resulted in thirteen major cell clusters: progenitor cells (*SPINK2*, *RUNX1*), proerythroblasts (*SOX6*), erythroid cells (*HBB*, *HBD*), plasmacytoid dendritic cells (pDCs) (*IRF8*, *CLEC4C*), myeloid cells (*LYZ*), neutrophils (*LTF*, *LCN2*), pre-pro B cells (*VPREB3*), immature B cells (*MME*), B cells (*CD79A*, *MS4A1*, *BANK1*), plasma cells (*JCHAIN*), T cells (*CD3D*), NK cells (*NKG7*, *KLRB1*) and tumor cells.

#### 4.3.1 Detection of metastatic tumor cells and enriched signature

The presence or absence of tumor cells in the bone marrow biopsies, was initially confirmed by pathologists. We further used inferCNV analysis to validate the malignancy of the tumor cell population. A previous study that combined transcriptomics and spatial deep multiplex imaging analysis found that surface markers CD276, GD2, CD24, and CD56 were highly expressed in neuroblastoma disseminated tumor cells in the metastatic bone marrow (Lazic et al., 2021). Harnessing the same tumor surface markers, we confirmed the gene expression in our scRNA-seq data (data not shown). While *CD276* and *B4GALNT1* (encodes ganglioside GM2/GD2 synthase) were exclusively expressed by tumor cells, *CD24* was also expressed by developing B cells, and *NCAM1* was also expressed by NK cells. Subsequently, we used these surface markers for the flow cytometry staining panel to validate the presence of tumor cells in metastatic bone marrow samples that were matched with the scRNA-seq data, BM1 and BM3.

Notably, the tumor cell population in the metastatic samples expressed adrenergic markers including *PHOX2B*, *STMN2*, *HAND2* and *MDK*. Joint alignment analysis with primary tumors from paper I similarly showed that metastatic tumor cells aligned with the adrenergic tumor cell compartment. We also performed GSEA analysis comparing bone metastatic tumor cells to primary tumor cells. Cell cycle-related target genes of E2F, genes regulated by MYC, mTORC1 complex activation, and oxidative phosphorylation were amongst the enriched pathways of genes upregulated in the metastatic tumor cells. A metastatic signature score was curated by the identification of top differentially expressed genes that were

specific to metastatic tumor cells in the bone marrow. This metastatic signature score was significantly associated with stage 4 neuroblastoma, high-risk patients with poor overall survival.

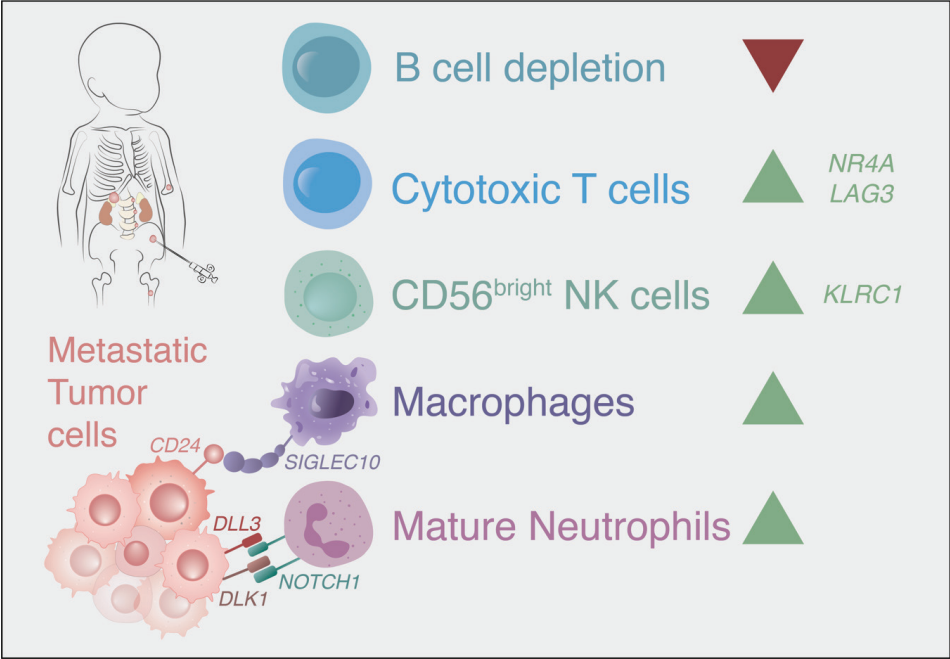
#### **4.3.2 Immunosuppressive metastatic bone marrow niche**

The presence of disseminated tumor cells in the bone marrow significantly shifted the immune cell composition. We observed a significant decrease in B cells and B cell progenitors in the metastatic bone marrow, relative to non-metastatic samples. Correspondingly, we validated a significant reduction in the percentage of CD19-positive B cells in metastatic bone marrow samples by flow cytometry. Through subcluster analysis of the immune cell compartment, we identified an immunosuppressive niche, including upregulated expression of inhibitory receptors in T cell and NK cell subclusters. Metastatic samples showed increased regulatory T cell activity and enrichment of both subpopulations of cytotoxic T cells (CTL-1 and CTL-2). The CTL-1 and CTL-2 subpopulations in metastatic samples exhibited upregulated expression of *LAG3* and *NR4A* genes, which regulate T cell tolerance to maintain homeostasis and inhibit autoimmunity. Flow cytometry analysis confirmed the increased trend of CD8-positive T cells in metastatic cases, with some samples demonstrating PD-1 expression. Subcluster and cell fraction analysis of NK cells showed an increase in CD56<sup>bright</sup> cells in metastatic samples. These immunoregulatory NK cells in the metastatic microenvironment exhibited increased expression of *KLRC1* encoding inhibitory receptor NKG2A and decreased expression of *CD69* and *CD160*, which are involved in NK cell activation. We used flow cytometry to validate the presence of CD56<sup>bright</sup> CD16<sup>negative</sup> cells in matched bone marrow samples (data not shown). However, due to limited sample size, we did not observe marked differences in cell proportions between metastatic and non-metastatic groups.

Within the myeloid lineage, there was an increased proportion of a macrophage cluster in the metastatic cases, which was transcriptionally distinct from primary tumors. Similarly, there was an increased abundance of mature neutrophils (neutrophil-2) in metastatic bone marrow samples. Interestingly, ligand-receptor interaction analysis revealed significant interactions between tumor cells and the myeloid cells. Predicted interactions included CD24-SIGLEC10, expressed by tumor and macrophages, respectively. In concordance with paper II, NOTCH signaling was also prominent in the metastatic tumor microenvironment. Both mature neutrophils and macrophages expressed NOTCH receptors (*NOTCH1*,

*NOTCH4*), while tumor cells expressed ligands *DLL3* and *DLK1*, further signifying their therapeutic potential.

This systematic transcriptional analysis highlights the distinctions between non-metastatic and metastatic bone marrow. Disseminated tumor cells maintain an adrenergic phenotype while acquiring a transcriptional signature associated with proliferation, migration, invasion, and worse prognosis. The presence of tumor cells compromised the number of developing B cells in the bone marrow (Figure 6). We observed an immunosuppressive bone marrow metastatic niche encompassing increased regulatory T cell activity, CTLs with upregulated expression of inhibitory receptor *LAG3* and T cell tolerance related *NR4A* genes, and suppressed *CD56<sup>bright</sup>* NK cells (Figure 6). Enriched macrophages and mature neutrophils in metastatic samples contributed to cellular interactions with metastatic tumor cells via NOTCH ligand-receptor pairs (Figure 6).



**Figure 6** Graphical summary of the immune cell remodeling detected in neuroblastoma bone marrow metastatic samples from Paper III (Mei et al, 2024).





## 5 Discussion

In all studies presented in this thesis, we utilized single-cell transcriptomics to delineate the cellular and transcriptional heterogeneity of neuroblastoma, bone marrow metastasis, transgenic mouse tumors, and *ex vivo* tumoroids. We could explore the tumor cell identity by joint alignment analysis of previously published fetal adrenal gland transcriptomes (Kameneva, Artemov, et al., 2021). Comparative analysis confirmed that the majority of neuroblastoma cells resembled fetal sympathoblasts and a subfraction of tumor cells mimicked fetal chromaffin cells, in papers I and II (Embaie et al., 2025; Olsen et al., 2024). Likewise, metastatic tumor cells in the bone marrow also aligned with adrenergic cells, in paper III (Mei et al., 2024). The co-occurrence of sympathoblasts and chromaffin cells was consistently shown in primary patient tumors, TH-MYCN mouse tumors and *ex vivo* tumoroids, as well as normal adrenal gland development. This fluidity of adrenergic phenotypes offers novel approaches for neuroblastoma differentiation therapy. For example, low-proliferative chromaffin cells in these tumors are less aggressive than proliferating sympathoblasts (Jansky et al., 2021; Kameneva, Artemov, et al., 2021; Kildisiute, Kholosy, et al., 2021). In both tumors and developmental data (Hanemaaijer et al., 2021; Jansky et al., 2021; Kameneva, Artemov, et al., 2021; Kildisiute, Kholosy, et al., 2021), we detect an unbroken continuum of chromaffin and sympathetic transcriptional states, enabling possible transitions between these phenotypes. Therefore, new therapeutic approaches may stem from understanding how to regulate and modify such intra-tumoral transitions.

The cell state identity and heterogeneity of neuroblastoma have been a topic of debate. Neuroblastoma cell line studies have reported the presence of noradrenergic/adrenergic cells and mesenchymal/NCC-like cells (Boeva et al., 2017; Gartlgruber et al., 2021; Van Groningen et al., 2017). In contrast, scRNA-seq studies of patient-derived tumors have predominantly identified malignancy in adrenergic sympathoblasts or neuroblasts (Bedoya-Reina et al., 2021; Dong et al., 2020; Jansky et al., 2021; Kameneva, Artemov, et al., 2021; Kildisiute, Kholosy, et al., 2021). Some studies have detected malignant adrenergic and mesenchymal cells in neuroblastoma, proposing transitions between these states (Thirant et al., 2023; X. Yuan et al., 2022). Although mesenchymal cells were present in both our human- and mouse-derived datasets, malignancy was not evident in these populations. Notably, we discovered previously unidentified pre-malignant SCP-like cells in human neuroblastoma, in paper I (Olsen et al., 2024).

Previous studies have suggested that the mesenchymal phenotype resembles SCPs (Gartlgruber et al., 2021; Jansky et al., 2021; Zeineldin et al., 2022). The mesenchymal subtype is associated with high-risk neuroblastoma, chemotherapy resistance and relapse (Gartlgruber et al., 2021). The clinical presentation and similarities in genomic aberrations between mesenchymal and adrenergic cells suggest that the mesenchymal phenotype is acquired by de-differentiation of adrenergic cells, possibly due to selective treatment pressure. In contrast, aberrant SCP-like cells in paper I harbored few genomic aberrations and were mainly identified in low- and medium-risk cases, indicating that they arise earlier during tumor development rather than due to de-differentiation (Olsen et al., 2024). The putative pre-malignant SCP-like cells' clinical manifestation, particularly in the low-risk group, is in accordance with previously reported association between the SCP gene signature and favorable survival probability, as well as a negative correlation with *MYCN*-status and neuroblastoma stage (Hanemaaijer et al., 2021; Kameneva, Artemov, et al., 2021). It is relevant to note that Körber et al. contend that low-risk tumors exhibit short evolution and less cell death, consequently growing to a detectable size sooner than high-risk tumors (Körber et al., 2023). This phenomenon might elucidate the presence of aberrant SCP-like cells in low-risk cases, whereas in high-risk tumors, SCPs are probably outcompeted by proliferating sympathoblasts.

Schwannian stromal cells have been described in neuroblastoma (Matthay et al., 2016; Mora et al., 2001), and we detected these cells in homozygous TH-*MYCN* tumors in paper II. Recruited Schwann cells have been described to have repair-like functions, promote neuronal differentiation (T. Weiss et al., 2021), and modulate immune response (Zhang et al., 2020), thereby inhibiting tumor progression. Likewise, neuroblastoma cells with mesenchymal features are associated with increased immunogenicity (Sengupta et al., 2022). Comparable to previously reported Schwannian and mesenchymal cells, non-malignant Schwann cells in paper I, also expressed genes encoding MHC class I and II molecules. In contrast, abnormal SCP-like neuroblastoma cells showed enhanced proliferation and downregulated MHC class I and II, thereby reducing immune recognition (Olsen et al., 2024). Beyond the implicated roles, more research is required to clarify the function of abnormal SCP-like cells in neuroblastoma.

Neuroblastoma tumorigenesis may be driven by aneuploidy (Körber et al., 2023). Whole-chromosome aneuploidy is correlated with better neuroblastoma prognosis than segmental chromosomal aberrations, which is a poor prognostic

factor. Akin other pediatric cancers, the somatic mutation burden is relatively low in neuroblastoma. This further suggests that aneuploidy-induced gene dosage imbalance plays a role in neuroblastoma progression. The most common aberration found in neuroblastoma is the gain of chromosome 17(q), and in paper I, we support the notion that chromosome 17(q) gain occurs as an early event, as suggested in other studies (Andersson et al., 2020; Gisselsson et al., 2007; Körber et al., 2023; Saldana-Guerrero et al., 2024).

The multipotency of SCPs during development has been compared to migrating neural crest cells (Furlan & Adameyko, 2018; Solovieva & Bronner, 2021). In paper I, we suggest that the tumor initiating aneuploidy might arise in migrating neural crest cells or SCPs, prior to lineage commitment to the sympathoadrenal lineage, in certain neuroblastoma cases. Pre-malignant SCP-like cells may successively undergo clonal expansion, as the increased dosage of genes in chromosome 17(q) could confer growth advantages. Among the upregulated genes in SCP-like cells with chromosome 17 gain in sample THA004, *ANAPC11*, *DDX5*, *NGFR* and *KPNB1*, have been shown to promote oncogenic signaling pathways, with potential therapeutic implications in cancer (Jiang et al., 2020; F. Li et al., 2023; Shi et al., 2023; X. Zhou et al., 2016; Z. Zhou et al., 2016).

We further identified therapeutically targetable interactions in *MYCN*-driven neuroblastoma and metastatic tumor cells by *in silico* ligand-receptor analysis (Embaie et al., 2025; Mei et al., 2024). Interestingly we detected significant interactions in the NOTCH signaling pathway in paper II and III. Targeting tumor cells that express NOTCH ligands like *DLK1* and *DLL3* with antibody-drug conjugates (ADCs) or bispecific T-cell engager (TCE), respectively, has shown promising results in neuroendocrine tumors (Hamilton et al., 2024; Paz-Ares et al., 2023; Rudin et al., 2023). A similar approach using ADCs can be used to target CD56/NCAM1-positive tumor cells with lorvotuzumab mertansine (IMGN901) or Adcitmer® (Esnault et al., 2022; Geller et al., 2021; Shah et al., 2016; Wood et al., 2013).

Neuroblastoma response to therapy has been associated with adrenergic and mesenchymal phenotypes. Adrenergic tumor cells are often therapy-sensitive, whereas the mesenchymal phenotype has been classified as therapy-resistant. In line with paper II, integrated scRNA-seq analysis of human neuroblastoma and preclinical models confirms that the adrenergic cell type is preserved (Chapple et al., 2024). However, there were discrepancies in the mesenchymal gene

expression program between cell lines and *in vivo* models. A similar inconsistency in the cell types has been reported between TH-MYCN tumors and their cell lines, affecting susceptibility to treatment (McNerney et al., 2022). Hence, in paper II, we established TH-MYCN-derived *ex vivo* cultures that maintain the expression of adrenergic markers (Embaie et al., 2025).

Notably, we identified aggressive sympathoblast subclusters that were enriched in culture. *Neurexin* (*NRXN1*) was amongst the tumoroid-enriched genes, which has been shown to affect neuroblastoma tumor growth and metastasis via binding with its ligand *Neurexophilin-1* (*NXPH1*) (Fanlo et al., 2023). Additionally, *NAV3*, *KTN1*, *PGK1*, *DDIT4*, *CCNE1*, and *CRABP1* were highly expressed by tumoroid-enriched subclusters, in paper II. *Neuron navigator 3* (*NAV3*) copy number changes have been detected in neuroblastoma at high frequencies and implicated in neuronal differentiation (Carlsson et al., 2013). Kinectin 1 (*KTN1*) plays a role in tumor proliferation, invasion and progression of triple-negative breast cancer (L. Gao et al., 2021). Phosphoglycerate kinase 1 (*PGK1*) has been implicated in tumorigenesis, drug resistance, and poor prognosis in renal clear cell carcinoma (He et al., 2022). Similarly, DNA damage-inducible transcript 4 (*DDIT4*) protects cancer cells from hypoxia-induced cell death and facilitates drug resistance in glioblastoma (Foltyn et al., 2019; Ho et al., 2020). In concordance, nuclear *DDIT4* overexpression is significantly correlated with more aggressive and advanced stages of colorectal cancer and pancreatic tumors (Fattahi et al., 2021; Tajik et al., 2023). G1/S-specific cyclin-E1 (*CCNE1*) amplification is common in a variety of tumors, in which high levels of cyclin E is correlated with whole-genome doubling, genome instability, and therapy-resistance (Watkins et al., 2020; J. Yuan et al., 2018; Zack et al., 2013). Interestingly, upregulation of *CRABP1* in neuroblastoma desensitizes cells to retinoic acid differentiation treatment and increases proliferation (Uhrig et al., 2008).

Although many of the aforementioned enriched genes have pro-tumorigenic implications, others have been described to exert tumor suppressive functions, including *ADGRB3* (Bhattacharya et al., 2018) and *DHX36* (Cui et al., 2021), while *PLK2* may have dual roles depending on the cancer type (Zhao et al., 2024). This emphasizes the need for further studies to evaluate the functions of the tumoroid-enriched genes in neuroblastoma.

Lastly, in paper III, we unraveled the immunosuppressive landscape of neuroblastoma bone marrow metastases (Mei et al., 2024). The hematopoietic

cells of the bone marrow exhibited a wide range of cellular and transcriptional alterations in the metastatic cases. Comparable to previous observations, we detected increased fractions of CTLs, neutrophils and a myeloid subcluster (macrophages) (Fetahu et al., 2023; Lazic et al., 2021). Intriguingly, CTLs in the metastatic samples exhibited significantly higher expression of *NR4A* genes, involved in T cell tolerance (X. Liu et al., 2019; Odagiu et al., 2021; Seo et al., 2019). A comprehensive study on pre- and post-treated primary tumors revealed exhausted T cell profiles, including enriched expression of co-inhibitory receptors *LAG3*, *PDCD1* and *HAVCR2* in treated samples (Wienke et al., 2024). We observed similar upregulated expression of these co-inhibitory receptors in CTLs from metastatic cases. We also detected increased CD56<sup>bright</sup> NK cells in metastatic bone marrow, with upregulated expression of *HAVCR2* and *KLRC1* encoding NKG2A (Kamiya et al., 2019). Blocking inhibitory receptors, such as NKG2A with monalizumab, may be a viable immunotherapy strategy in metastatic neuroblastoma (S. P. Patel et al., 2024). Additionally, the macrophage population expressed inhibitory receptor *SIGLEC10*, and metastatic tumor cells expressed its ligand *CD24*, which promotes immune escape (Barkal et al., 2019). This predicted interaction has also been detected in primary neuroblastoma (Verhoeven et al., 2022), signifying that targeting this axis has therapeutic potential in both non-metastatic and metastatic neuroblastoma (X. Li et al., 2024).



## 6 Conclusions

Collectively, the findings from this thesis shed light on key cellular and molecular mechanisms driving neuroblastoma development and progression. In paper I, aneuploid pre-malignant SCP-like cells were identified using *Numbat* allele-specific CNV-prediction tool, DNTR-sequencing and DNA-FISH combined with immunofluorescence protein detection. Chromosome 17 gain was a prevalent aberration in pre-malignant SCP-like cells across a subset of neuroblastoma samples, indicating that aneuploidy may be a putative tumor-initiating event in neuroblastoma.

In paper II, we generated a detailed transcriptomic landscape of TH-MYCN transgenic mouse tumors including comparative analysis with fetal adrenal anlagen and human neuroblastoma. Interactions between tumor cells and their microenvironment unraveled potential therapeutic targets, including NOTCH ligands and NCAM1 signaling molecules. We further developed a novel *ex vivo* culture model that faithfully resembles embryonic sympathoblast signatures and the adrenergic phenotype of primary tumors, underscoring its translational applicability. Aggressive sympathoblast subclusters were enriched in culture, expressing genes associated with poor clinical outcome and pro-tumorigenic implications in other malignancies.

Paper III provides a comprehensive atlas of the cellular composition and transcriptional shifts underlying neuroblastoma bone marrow metastases. Disseminated tumor cells retained an adrenergic identity while acquiring features associated to aggressive behavior and poor prognosis. We also observed immunosuppression within the metastatic niche, characterized by B cell depletion, increased regulatory T cell activity, increased abundance of CTLs and CD56<sup>bright</sup> NK cells with upregulated expression of co-inhibitory receptors. Notably, enriched macrophages and mature neutrophils in metastatic samples contributed to cellular interactions with metastatic tumor cells via the NOTCH signaling pathway.





## 7 Points of perspective

Despite intense multimodal therapy, the clinical outcome for high-risk neuroblastoma remains dire (Matthay et al., 2016). Survival rates drastically drop for patients with bone and bone marrow metastases (S. Liu et al., 2023), emphasizing the need for innovative therapeutic strategies targeting high-risk neuroblastoma and metastatic disease. Our data complements existing single-cell transcriptomics studies while providing new insights into neuroblastoma phylogeny, cellular composition in preclinical models and unraveling new targets in high-risk tumors and metastasis. In this section, we explore potential future research studies that may stem from this thesis.

We thoroughly examined both the cellular heterogeneity and tumor evolution of primary neuroblastoma in paper I (Olsen et al., 2024). We suggest that tumor-initiating events may involve aneuploidy-prone migrating neural crest cells or SCPs. Multipotent SCPs give rise to many different cell lineages and may have implications not only in neuroblastoma tumorigenesis, but also other cancers like melanoma, paraganglioma, Schwannomas, or other pathologies like peripheral neuropathies (Furlan & Adameyko, 2018). Malignant SCP-like cells in human neuroblastoma are rare and even more challenging to detect in preclinical models, including PDXs and patient-derived organoids. We have attempted to evaluate SCP-like cells in cell lines, organoids, PDXs and transgenic models. We propose that SCP-like cells are pre-malignant and may be difficult to propagate in culture due to their neural niche dependency (Kameneva, Artemov, et al., 2021). Another possible explanation is that rare SCP-like cells are outcompeted by proliferating adrenergic cells. Moreover, tissue processing and FACS may introduce selection bias for robust cycling tumor cells. Alternatively, human embryonic stem cells and induced pluripotent stem cells have been employed to study neuroblastoma development (Saldana-Guerrero et al., 2024). This is a promising approach to manipulate early developmental stages of neural crest development and evaluate the potential role of SCPs in tumor initiation by using SOX10- or PLP1-promoters.

While we suggest, initiating whole chromosome aneuploidy may occur in SCPs, studying aneuploidy is notoriously challenging for many reasons. Gene dosage imbalances caused by aneuploidy is difficult to model (Ben-David & Amon, 2020). It is challenging to identify oncogenic drivers in large chromosomal aberrations, like whole chromosome 17 gain, which harbors over a thousand genes, many of which have implications in cancer and other genetic disorders (Zody et al., 2006).

In paper I, we detected upregulated genes in SCP-like cells with gain of chromosome 17, including *ANAPC11*, *DDX5*, *NGFR* and *KPNB1*. Overexpressing or knocking out these genes in neuroblastoma or stem cell models could be the next step to evaluate their oncogenic potential.

In *MYCN*-driven preclinical models we detected adrenergic-predominant tumors, transcriptionally resembling embryonic sympathoblasts and partially chromaffin cells. In paper II, we confirmed the transcriptional similarities between human *MYCN*-amplified neuroblastoma, TH-*MYCN* mouse tumors and *ex vivo* tumoroids, thereby highlighting their translational potential for drug testing (Embaie et al., 2025). *Ex vivo* enriched clusters presented genes with various implications in cancer. While *NRXN1* and *CRABP1* have been studied in neuroblastoma (Fanlo et al., 2023; Uhrig et al., 2008), some enriched genes including *KTN1*, *PGK1*, *DDIT4*, and *CCNE1* have pro-tumorigenic roles in other cancer types (Fattahi et al., 2021; Foltyn et al., 2019; L. Gao et al., 2021; He et al., 2022; Ho et al., 2020; Tajik et al., 2023; Watkins et al., 2020; J. Yuan et al., 2018; Zack et al., 2013). Whereas other tumoroid-enriched genes such as *ADGRB3* and *DHX36*, exert tumor suppressive functions (Bhattacharya et al., 2018; Cui et al., 2021). We provide a list of tumoroid-enriched genes that are associated with poor neuroblastoma prognosis. Although further studies are needed to determine the functions of these genes in neuroblastoma, these signatures may serve as potential prognostic biomarkers or be used for identification of targeted therapies.

Furthermore, our novel tumoroid model system presents a valuable platform for CRISPR knock-out screening and drug screening to identify novel therapeutic targets for neuroblastoma. While mesenchymal cell lines are useful for studying drug resistance, this tumoroid model provides a unique opportunity to test therapeutic agents and perform functional assays while preserving the adrenergic cellular identity, thereby enhancing the clinical relevance of preclinical studies. Intercellular interaction analysis, in papers II and III, revealed potential therapeutic targets enriched in high-risk tumor cells, including NOTCH ligands, NCAM signaling and immunoregulatory interactions (Embaie et al., 2025; Mei et al., 2024). NOTCH ligands highly expressed on primary and metastatic tumor cells include *DLL3* and *DLK1*, which can be targeted with bispecific TCEs or ADCs (Hamilton et al., 2024; Paz-Ares et al., 2023; Rudin et al., 2023). Similarly, NCAM1 can be targeted with ADCs (Esnault et al., 2022; Geller et al., 2021; Shah et al., 2016; Wood et al., 2013). We are currently exploring these therapeutic strategies in preclinical neuroblastoma models.

Additionally, we observed increased expression of genes encoding inhibitory receptors in the immunosuppressive metastatic niche, including *HAVCR2* and *LAG3*. Amongst the upregulated genes in CTLs from metastatic cases were *NR4A1*, *NR4A2*, and *NR4A3*, which can mediate CTL exhaustion via *TOX* and *TOX2* (Seo et al., 2019). Alleviating inhibitory signals by targeting these immunoregulatory networks in neuroblastoma bone marrow metastases presents promising opportunities for immunotherapeutic interventions. However, modeling spontaneous bone marrow metastasis in immunocompetent mice is essential for testing novel immunotherapies. Ongoing studies in the research group are dedicated to developing both *in vivo* and *ex vivo* bone marrow metastatic models. Additionally, we aim to leverage the *ex vivo* tumoroid system described in this thesis to model metastatic neuroblastoma and establish PDX-organoids, enhancing translational research and therapeutic development.



## 8 Acknowledgements

Throughout my time as a PhD student, I have been embraced by such a supportive network of students, researchers and supervisors. I am humbled by the opportunity and possibility to conduct my research studies in the **Kogner-Johnsen-Wickström-Baryawno-Dyberg Group**. I appreciate all our scientific discussions during meetings and all the fun moments in the lab.

I want to start by thanking my main supervisor, **Ninib Baryawno**, who warmly welcomed me to his lab. Thank you, Ninib, for urging us to aim high and be ambitious. You have inspired us by sharing your experiences at Harvard and helped us understand how to navigate the competitive academic milieu. Thank you for paving the way for young academic researchers and for your mentorship! I also want to express how grateful I am for the opportunity to work on interesting research projects and for giving me the freedom to experiment with different approaches and shape my thesis projects. By giving me the freedom to explore different approaches, you have helped me become an independent researcher, and I appreciate that!

**Malin Wickström**, I appreciate your scientific input, attention to detail and continuous support. Thank you for teaching me so much about ethical research, the transgenic model and treatment strategies. Thank you for your dedication to this research group. I am truly grateful for your encouragement throughout my time here!

**John Inge Johnsen**, thank you for always being kind, reassuring and positive. Thank you for sharing your wisdom both in science and in life! You are always optimistic, which has been so meaningful to us in the lab. Thank you for being sincere and open to discussing anything with care.

**Jan-Bernd Stukenborg**, thank you for introducing me to such a fascinating new field of organoid modeling! You have shared such cool techniques, and I appreciate the time you have taken to have fika together, to share your ideas and resources. Thank you for your guidance and generosity!

**Per Kogner**, thank you for your words of wisdom and valuable clinical insights during our group meeting discussions. Your work has saved lives, and your dedication to improving the lives of patients with neuroblastoma is truly inspiring!

I would like to give a special thanks to all the **co-authors** worldwide for their collaborative joint efforts to execute these research studies together.

Particularly, **Hirak Sarkar**, thank you for the countless hours, holidays, weekends, early mornings and late nights that you have dedicated to our research study. Thank you for explaining codes, sharing your honest life and career advice, and our many discussions both during my research visit and via zoom. It has been an honor to work with you!

**Thale Kristin Olsen**, you introduced me to this research group, and I am so grateful for your supervision! Thank you for teaching me everything and letting me be a part of your research projects. I appreciate your kindness and patience when teaching me about the single-cell studies.

**Jörg Otte**, thank you for teaching me how to culture organoids, stainings and more! I am thankful for the time you have taken to guide me through our projects both here and remotely.

I cannot thank you enough, **Adele Alchahin**. Thank you for entrusting me with the TH-MYCN project and welcoming me to collaborate with you on the bone marrow metastasis project. I have learnt so much from working with you, and I appreciate all the advice that you have given me!

I would like to thank the research group leaders who have taught me so much about neuroblastoma research and guided me throughout the years: **Cecilia Dyberg, Oscar Bedoya Reina, Shahrzad Shirazi Fard** and **Jakob Stenman**. I am very grateful for the opportunity to work amongst the wonderful **Lab Buddies** and members of the research group: **Adena Pepich, Alkinoos Polychronopoulos, Celine Hafkesbrink, Conny Tümmeler, Diana Treis, Emmi Puuvuori, Fredrik Sundquist, Gustav Hedberg, Kristina Ihrmark Lundberg, Linda Ljungblad, Lotta Elfman, Manouk Verhoeven, Nicola Bell, Nan Sophia Han, Stefania Aliverti, Teodora Andonova**, and all the **brilliant students** that I had the chance to supervise or work with. It has been an amazing learning experience to work with such knowledgeable medical researchers with the common goal of improving the lives of children with cancer. **Ioanna Tsea** and **Sara Abu Ajamieh**, you have been my classmates, lab-mates and friends for many years! Sara, we have been friends since our Bachelor's studies, and I am thankful for all the things we have in common, like life experiences and shared values. Thank you for being so warmhearted, genuine and generous! Ioanna, you have been my *energizer* and

source of motivation, and it has been my privilege to be your *quencher*. *Betanna* forever! Thank you both for our lovely friendship, and I cherish all our fun moments together!

I want to thank everyone in the **Childhood Cancer Research Unit** for their openness to collaborate, discuss ideas and share resources. I especially want to thank the **KBH department** for their continuous support throughout the years, particularly **Anna Nilsson, Caroline Rådestad, Désirée Gavhed, Rosa Cusato Sörnäs, Sabina Brinkley, Camilla Adle** and **Sandra Brogårde**. I am also very grateful for the **KI-NB board and network** for meetings, conferences and the collaborative opportunities that have fostered both professional connections and lasting friendships.

Thank you to my lovely extended **family**, for asking how I am and about my studies. **Azieb Embaie Norevi**, thank you for being a loving and supportive godmother. **Miriam Fassil**, you are like an older sister to me, and I love you so much! I also want to thank my **friends** for their pep talks and for reminding me to rest and have fun!

My best friend and soulmate, **Carl-Fredrik von Wachenfelt**, thank you for having faith in me and encouraging me to pursue this PhD. It's such a blessing to share a life with you and I am excited for what the future holds for us. ♡ I want to thank your lovely family, who have welcomed me with open arms, and thank them for treating me like a daughter and sister.

Most importantly, I dedicate this thesis to my beloved parents, **Demaris Neamen** and **Tesfai Embaie**, and my sweetest siblings, **Abigail Tesfai Embaie, Makda Tesfai Embaie** and **Daniel Tesfai Embaie**. You mean the world to me, and I love you so much! Mamma, thank you for always prioritizing us, being there for us and for loving us unconditionally. You are my role model. ♡ Pappa, I can talk to you about everything, and I appreciate that you are always so understanding. Thank you for all your advice and help every step of the way. Thank you for telling us: anything is possible if you set your mind to it. ♡ Abby, you are an amazing, successful woman with a beautiful soul! What a blessing it has been to grow up with you and thank you for being there for me! ♡ Makda, I admire how caring, mature and hardworking you are. Thank you for all our long and fun conversations. I love that we are growing closer to each other. ♡ Danny, du är så omtänksam och kärleksfull. Du ser alltid igenom mig när jag är glad eller ledsen, och du är alltid där för att trösta mig. Du bryr dig alltid om andra, och det älskar jag hos dig. Fortsätt vara den du är! ♡





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