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# EXPLORING INTER- AND INTRA-HETEROGENEITY IN CHILDHOOD NEUROBLASTOMA AND PHEOCHROMOCYTOMA

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# Exploring Inter- and Intra- heterogeneity in childhood neuroblastoma and pheochromocytoma
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By

**Wenyu Li**

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# ABSTRACT

Neuroblastoma is the most common extra-cranial solid tumor of the sympathoadrenal cell lineage, which is a unique pediatric malignancy with remarkable inter- and intra-tumoral heterogeneity. In infants, neuroblastoma can regress spontaneously without treatment, while in older patients, neuroblastoma can develop with lethal progression with less than a 50% survival rate. Age at diagnosis is a clinically relevant prognosis factor. Chemoresistance in neuroblastoma patients has been associated with tumor cell plasticity and intratumoral heterogeneity. However, the causes of tumor cell plasticity and intratumoral heterogeneity are not well understood.

In **Paper I**, we aim to investigate the *in vivo* function of KIF1Bβ in the sympathoadrenal system. *KIF1B*β locates on chromosome 1p36. Loss of heterozygosity at 1p36 strongly correlates with a poor prognosis of neuroblastoma. *KIF1B*β has been suggested to be a 1p36 tumor suppressor gene and demonstrated to be required for neuroblast developmental apoptosis. We showed that the loss of KIF1Bβ impairs neuroblast differentiation during development and causes misexpression of genes required for sympathoadrenal differentiation. We demonstrated that KIF1Bβ mediates neuroblast differentiation by transporting the NGF receptor TRKA. Transcriptomic analysis revealed that *KIF1B*β deficient sympathetic neuroblasts is similar to the profile in non-MYCN amplified high-risk neuroblastoma, independent of the loss of *KIF1B*β neighboring genes on 1p36.

In **Paper II**, we found that loss of NF1 alone or in combination NF1 with KIF1Bβ in mouse sympathoadrenal lineage leads to neuroblastoma, pheochromocytoma, and composite tumors. In addition, mice with NF1 and KIF1Bβ loss have earlier tumor death onset and lower survival probability compared to NF1 loss alone. The single-cell sequencing of tumors and embryonic(E17) adrenal medulla showed a remarkable heterogeneity of chromaffin cells and neuroblasts. Importantly, we observed abundant neuroblasts born in the adrenal medulla in the E17 embryo. Furthermore, computational analysis reveals a cell state transition from chromaffin cells to neuroblasts in embryonic and pre-malignant stages. The chromaffin neuroblasts transition has also been observed in a three-segment structure in which chromaffin cells break through the cortex, suggesting that chromaffin cells acquire neuroblast signature and continue to form neuroblastoma, pheochromocytoma and composite tumors.

In **Paper III**, we explored the inter- and intra-tumoral heterogeneity in human neuroblastoma and why age at diagnosis is one of the important prognostic factors. We analyzed the single-nuclei transcriptomes of human healthy fetal and postnatal adrenal glands and primary neuroblastomas from different stages and risk groups. We found two disease entities with varying signatures of cells in low and high-risk neuroblastoma. Notably, the transcriptome of high-risk neuroblastoma resembles a *TRKB*+ cholinergic progenitor cell population characterized specifically in the human postnatal adrenal glands. Thus, our study reveals two cellular identities reflecting the clinical heterogeneity of neuroblastoma tumors.

# LIST OF SCIENTIFIC PAPERS

I. Neuroblast differentiation during development and in neuroblastoma requires KIF1Bβ-mediated transport of TRKA.
Stuart M. Fell, Shuijie Li, Karin Wallis, Anna Kock, Olga Surova, Vilma Rraklli, Carolin S. Höfig, **Wenyu Li**, Jens Mittag, Marie Arsenian Henriksson, Rajappa S. Kenchappa, Johan Holmberg, Per Kogner, and Susanne Schlisio
*GENES & DEVELOPMENT*, 2017, 31:1036–1053

II. Chromaffin to neuroblast cell state transitions drive tumor plasticity in NF1 and KIF1Bβ deficient neuroblastoma, pheochromocytoma and composite tumors.
**Wenyu Li**, Monika Plescher, Peng Cui, Petra Bullova, Stuart Fell, Igor Ademeyko, Catharina Larsson, Arthur Tischler, C. Christofer Juhlin, Oscar C. Bedoya-Reina and Susanne Schlisio
*(Manuscript)*

III. Single-nuclei transcriptomes from human adrenal gland reveal distinct cellular identities of low and high-risk neuroblastoma tumors.
O. C. Bedoya-Reina*, **Wenyu Li***, M. Arceo, M. Plescher, P. Bullova, H. Pui, M. Kaucka, P. Kharchenko, T. Martinsson, J. Holmberg, I. Adameyko, Q. Deng , C. Larsson, C. C. Juhlin, P. Kogner & S. Schlisio
*NATURE COMMUNICATIONS* (2021)12:5309

# PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. EglN3 hydroxylase stabilizes BIM-EL linking VHL type 2C mutations to pheochromocytoma pathogenesis and chemotherapy resistance.
Shuijie Li, Javier Rodriguez, **Wenyu Li**, Petra Bullova, Stuart M Fell, Olga Surova, Isabelle Westerlund, Danijal Topcic, Maria Bergsland, Adam Stenman, Jonas Muhr, Monica Nistér, Johan Holmberg, C Christofer Juhlin, Catharina Larsson, Alex von Kriegsheim, William G Kaelin Jr, Susanne Schlisio
*PNAS*, August 2, 2019 | 116 (34) 16997-17006

Impaired oxygen-sensitive regulation of mitochondrial biogenesis within the von Hippel-Lindau syndrome.
Shuijie Li, **Wenyu Li***, Juan Yuan*, Petra Bullova, Jieyu Wu, Xuepei Zhang, Yong Liu, Monika Plescher, Javier Rodriguez, Oscar C. Bedoya-Reina, Paulo R. Jannig, Paula Valente-Silva, Meng Yu, Marie Arsenian Henriksson, Roman A. Zubarev, Anna Smed Sörensen, Carolyn K. Suzuki, Jorge L. Ruas, Johan Holmberg, Catharina Larsson, C. Christofer Juhlin, Alex von Kriegsheim and Yihai Cao, Susanne Schlisio
Nature Metabolism volume 4, pages739–758 (2022)

*These authors contributed equally to this work.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADRN | Adrenergic |
| AG | Adrenal gland |
| ALK | Anaplastic lymphoma receptor tyrosine kinase |
| CD133 | CD133 antigen(prominin-1) |
| CHGB | Chromogranin B |
| CHRNA7 | Neuronal acetylcholine receptor subunit alpha-7(nAChRα7) |
| CLDN11 | Claudin-11 |
| DA | Dorsal aorta |
| Dbh | Dopamine beta-hydroxylase |
| DHX9 | DExH-box helicase 9 |
| DRG | Dorsal root ganglion |
| DRP1 | Dynamin 1 like (DNM1L) |
| EMT | EMT, Epithelial-to-mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |
| FACS | Fluorescence-activated cell sorting |
| GDP | Guanosine diphosphate |
| GEMMs | Genetically engineered mouse models |
| GTP | Guanosine triphosphate |
| hNCC | Human neural crest cell |
| IML | Intermediolateral column |
| INSS | The International Neuroblastoma Staging System |
| KIF1Bβ | Kinesin family member 1B isoform beta |
| MAPK | Mitogen-activated protein kinase |
| MAX | Myc-associated factor X |
| MEK | Mitogen-activated protein kinase kinase |
| MES | Mesenchymal |
| MYCN | V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog |
| n | Notochord |
| NC | Neural crest |
| NCC | Neural crest cell |
| NF1 | Neurofibromin 1 |
| NGF | Nerve growth factor |
| NT | Neural tube |

| | |
|---|---|
| OPA1 | OPA1 Mitochondrial Dynamin Like GTPase |
| PDX | Patient-derived xenograft |
| PHD2 | Prolyl hydroxylase domain-containing protein 2 |
| PNMT | Phenylethanolamine N-methyltransferase |
| RA | Retinoic acid |
| RAF | Proto-oncogene c-RAF |
| RAS | Rat sarcoma viral oncogene homologue |
| RET | Ret proto-oncogene |
| RNAi | RNA interference |
| Sc-RNA-seq | Single cell RNA sequencing |
| SCG | Super cervical ganglia |
| SCP | Schwann cell precursor |
| SDHx | Succinate dehydrogenase complex |
| SRG | Suprarenal sympathetic ganglion |
| Th | Tyrosine hydroxylase |
| TMEM127 | Transmembrane protein 127 |
| TRKA | Neurotrophic receptor tyrosine kinase 1 (NTRK1) |
| TRKB | Neurotrophic receptor tyrosine kinase 2 (NTRK2) |
| VHL | Von Hippel-Lindau tumor suppressor |
| XAF1 | X-linked inhibitor of apoptosis-associated factor 1 |
| YME1L1 | YME1 Like 1 ATPase |
| ZNF423 | Zinc finger protein 423 |

# 1 INTRODUCTION

## 1.1 PHEOCHROMOCYTOMA

### 1.1.1 Introduction to Pheochromocytoma

Pheochromocytoma is a rare neuroendocrine tumor arising from chromaffin cells in the adrenal medulla that derive from the neural crest. Tumors arising from extra-adrenal chromaffin cells are referred to as extra-adrenal pheochromocytoma or paraganglioma (Figure 1). Pheochromocytoma and paraganglioma affect 2-8 per million people, with a prevalence between 1:2500 and 1:6500(H. Chen et al., 2010). It has most frequently been diagnosed in adults between 40 and 50, with approximately equal sex distribution (Mannelli et al., 2009; Young, 2007). Although rare, pheochromocytoma and paraganglioma carry the highest degree of heritability (around 40%) of all human tumors (Dahia, 2014), they are usually benign tumors, with approximately a quarter of malignant cases. The World Health Organization (WHO) defines malignant pheochromocytoma and paraganglioma as the presence of distance metastasis of chromaffin cells to the sites where it´s not typically found, most commonly in bone, lymph nodes, liver, and lung.

### 1.1.2 Clinical presentations of Pheochromocytoma and Paraganglioma

Pheochromocytoma and paraganglioma are tumors arising from chromaffin cells, which can synthesize, metabolize and secrete adrenaline (epinephrine), noradrenaline (norepinephrine), and some dopamine into the bloodstream. The releasing of these hormones will produce various responses to different tissue. In patients, the secretion of adrenaline and noradrenaline is disrupted, thus contributing to the variable clinical presentations, including hypertension, tachyarrhythmia, sweating, pallor, fever, headaches, and weight loss.

### 1.1.3 Mutations in Pheochromocytoma and Paraganglioma

Around 40% of pheochromocytoma and paraganglioma patients were detected with a germline mutation in susceptibility genes(Favier, Amar, & Gimenez-Roqueplo, 2015). Based on the transcriptional profile, pheochromocytoma and paraganglioma can be separated into two clusters: the kinase receptor-signaling gene cluster and the pseudo-hypoxic gene cluster. The first cluster involves the somatic or germline mutation of the genes encoding the *RET* (rearranged-during-transfection) proto-oncogene, the *NF1* (neurofibromin 1) tumor

suppressor, *TMEM127* (transmembrane protein 127), *MAX* (Myc-associated factor X), and *KIF1B*β (Kinesin Family Member 1B) and results in abnormal stimulation of kinase signaling pathways and lead to the uncontrolled proliferation, growth, and survival of cells (Nolting & Grossman, 2012). The pseudo-hypoxic gene cluster is related to the mutation of genes in the *SDHx/PHD2/VHL* (Nolting & Grossman, 2012) that mimics cellular hypoxia, resulting in decreased apoptosis, proliferation, and angiogenesis (Jochmanova, Zelinka, Widimsky, & Pacak, 2014). A study from Favier´s lab also observed a hypermethylator phenotype associated with the downregulation of key genes involved in neuroendocrine differentiation in *SDHx*-related tumors (Letouze et al., 2013).

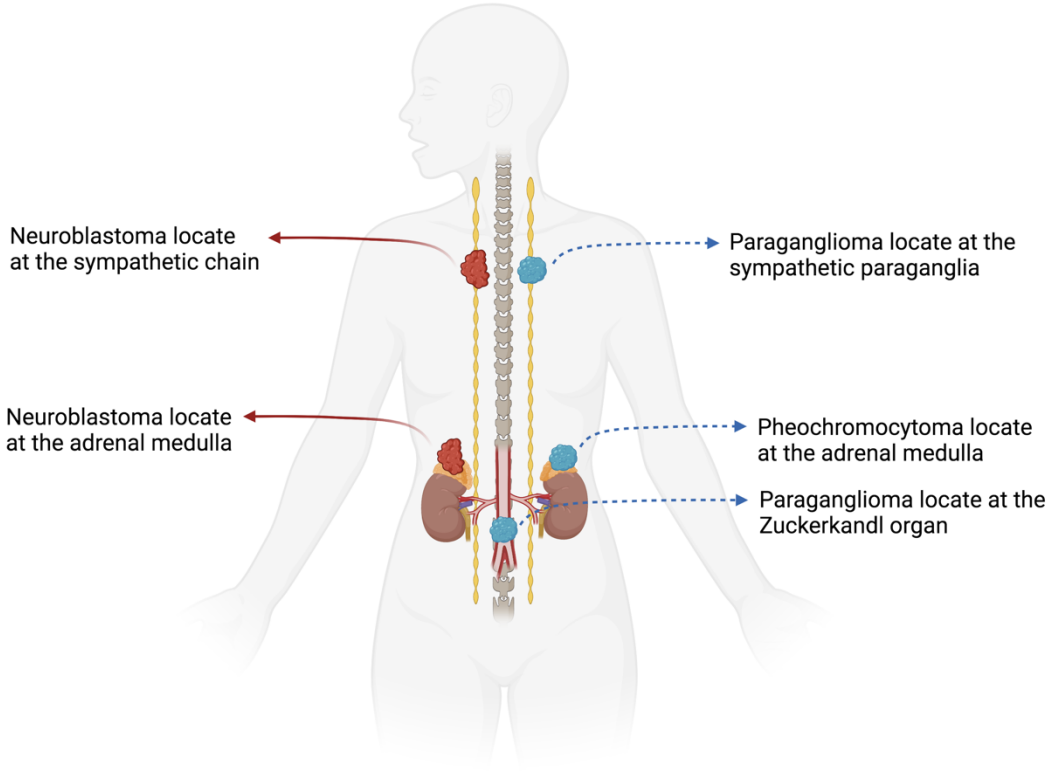


**Figure 1. Anatomic overview of neuroblastoma, pheochromocytoma and paraganglioma.** *Created with Biorender.com.*

## 1.2 NEUROBLASTOMA

### 1.2.1 Introduction to neuroblastoma

Neuroblastoma is a solid tumor of the sympathoadrenal system that is derived from the neural crest (Cheung & Dyer, 2013). It can be found anywhere along with the sympathetic nervous system, e.g., in the superior cervical, celiac ganglia, the paraspinal, or the adrenal gland, where the majority is localized (Figure 1), thus by displayed a significant intertumoral

heterogeneity. A large-scale analysis of the international patient cohort showed that 47% of neuroblastoma is observed in the adrenal and 24% in abdominal/retroperitoneal regions, 2.7% in the neck, 15% in the thoracic, 3% in the pelvic, and 7.9% in other sites (Vo et al., 2014). Unlike pheochromocytoma and paraganglioma, which mainly affect adults, neuroblastoma is the most common and deadly extracranial malignancy of childhood, with a median age at diagnosis of 18 months. Neuroblastoma was detected in 7% of pediatric cancer patients younger than 15 years old and accounted for 12%-15% of all childhood cancer-related death (Brodeur, 2003; Maris, 2010).

The unique feature of neuroblastoma is its complexity and heterogeneity, resulting in various clinical presentations. Neuroblastoma can spontaneously differentiate and regress in some patients, leading to complete recovery without treatment; some other neuroblastomas, however, could develop to widespread metastasis and resistance to treatment and result in poor survival (Whittle et al., 2017). According to The International Neuroblastoma Staging System (INSS), neuroblastoma can be classified into different stages: 1, 2A, 2B, 3, 4, and 4S. Besides the stage of the disease, age, tumor histology, DNA ploidy, *MYCN* amplification, chromosome arrangements, and neurotrophin receptors are important prognostic factors for patients. Compared to Pheochromocytoma and paraganglioma, neuroblastoma is more aggressive, and patients with high-risk neuroblastoma have only a 50% survival rate. The treatment for neuroblastoma varies between patients and includes surgery, chemotherapy, radiation, and bone marrow transplantation (Tolbert & Matthay, 2018).

### 1.2.2 Genetic abnormalities in neuroblastoma

Compared to adult cancer, somatic mutation is rarely observed in neuroblastoma. The most frequently detected genetic abnormalities are *ALK* (8-12% of patients) mutation, *MYCN* (25–33% of patients) amplification, and chromosomal deletion/rearrangements, which include the loss of 1p36, 11q23, and 14q23, as well as unbalanced gain of 17q22 (Molenaar, Koster, et al., 2012). The alteration of chromosomes is a prognosis marker and could be relevant to the patient's clinical outcome. Hemizygous loss of chromosome 1 (1p36) is significantly associated with bad outcomes in neuroblastoma patients. Numerous studies revealed the potential candidate tumor suppressor genes from this region. However, there is still no bona fide tumor suppressor identified in chromosomal 1p36.

## 1.3 KIF1B BETA

### 1.3.1 Introduction to KIF1Bβ

KIF1Bβ is a kinesin-3 family member that can transport essential cellular cargos to specific destinations. The gene was first identified as a potential tumor suppressor on chromosome 1p36 by Akira Nakagawara et al. in 2000 (Miki Ohira et al., 2000). Akira Nakagawara and his colleagues screened 29 neuroblastoma cell lines. They identified a 500-kb homozygous deletion located at the distal region of 1p in neuroblastoma cell line(s) that harbored at least six genes (Miki Ohira et al., 2000). Among these genes, *KIF1B*β is the only gene observed with tumor suppression activity and low expression in unfavorable subsets of primary neuroblastoma.

### 1.3.2 KIF1Bβ and neuroblastoma

In 2008, a three-generation cancer-prone family with a *KIF1B*β germline variant was detected with neural and nonneural tumors (Schlisio et al., 2008; Yeh et al., 2008). The grandfather with *KIF1B*β germline mutation presented pheochromocytoma while his granddaughter not only developed bilateral pheochromocytoma but also had neuroblastoma in her early childhood, which suggests *KIF1B*β could be the potential 1p36 tumor suppressor gene.

Studies revealed that loss of KIF 1Bβ protects neuroblasts from apoptosis in response to NGF withdrawal (Schlisio et al., 2008). During the development of the normal embryo, the growth factors such as nerve growth factors (NGFs) are limited. Neuronal progenitor cells compete for the NGF, and only the winner cells which obtain enough NGFs can survive and undergo differentiation. Cells that fail to compete for NGFs undergo developmental apoptosis. In addition, Chen et al. demonstrated that KIF1Bβ interacts with the RNA helicase A (DHX9), thus leading to the nuclear accumulation of DHX9 and subsequent the induction of proapoptotic X-linked inhibitor of apoptosis-associated factor 1 (XAF1), ultimately causing apoptosis (Z. X. Chen et al., 2014). Thus, the cells failing to cull properly during development appear to be predisposed to oncogenic transformation. The genetic aberrations disturbing NGF-dependent neuronal survival/apoptosis have been associated with neuroblastoma and other neural crest-derived origin malignancies, such as paraganglioma and pheochromocytoma.

Another study from our group provided an additional mechanism to elucidate the effects of KIF1Bβ on tumorigenesis. They observed that KIF1Bβ and DRP1 are silenced in 1p36 hemizygous-deleted neuroblastomas. Furthermore, they demonstrated that the KIF1Bβ activates $Ca^{2+}$-dependent phosphatase calcineurin (CN). KIF1Bβ binding to CN mediates the dephosphorylation of Dynamin-related protein 1 (DRP1), causing mitochondrial fission and apoptosis (Li et al., 2016). Further, Ando et al. observed that KIF1Bβ, in collaboration with YME1L1, is vital in intrinsic mitochondria-mediated apoptosis by regulating mitochondria structure and function. YME1L1 is a mitochondrial metalloprotease that functions in the cleavage of the mitochondrial GTPase OPA1, thus resulting in mitochondrial fragmentation, suggesting the disrupted KIF1Bβ/YME1L1/OPA1 mechanism may contribute to the development of neural crest-derived tumors, including neuroblastoma and pheochromocytoma.

Yet, another study from our lab identified pathogenic *KIF1B*β mutation that is defective in developmental apoptosis and sympathetic system tumors. Furthermore, they showed that KIF1Bβ deletion in the mouse sympathetic nervous system impaired neuron differentiation and function. In addition, they discovered that KIF1Bβ is required for the anterograde transportation of the nerve growth factor (NGF) receptor tropomyosin receptor kinase A (TrkA), further leading to NGF-dependent neuronal differentiation. Moreover, the analysis of neuroblastoma tumors suggests the contribution of KIF1Bβ loss to a less differentiated and more aggressive disease regardless of *MYCN* amplification and the loss of other 1p36 candidates (Fell et al., 2017).

## 1.4 NF1

### 1.4.1 Introduction to Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is one of the most common autosomal-dominant genetic diseases that affect 1 in 3000 individuals worldwide. The affected individuals express various clinical presentations, including café-au-lait macules (hyperpigmented spots on their skin), autism spectrum disorder, learning difficulties, neuroendocrine tumors, neurofibroma, and breast cancer (Evans et al., 2010; Uusitalo et al., 2015). Furthermore, glioma, pheochromocytoma, and neuroblastoma are also associated with NF1 (Lodish & Stratakis, 2010; Varan et al., 2016).

In the 1990s, Dr. Francis S. Collins and Dr. Ray White identified the *NF1* gene on chromosome 17q11.2 as the genetic basis for these presentations. This gene contains 61 exons and encodes a large protein of 2818 amino acids called neurofibromin. Although neurofibromin is ubiquitously expressed, its highest level is observed in cells of the nervous system, including neurons, Schwann cells, and oligodendrocytes (Daston et al., 1992; Gutmann, Wood, & Collins, 1991).

### 1.4.2 The function of Neurofibromin

Neurofibromin acts as the negative regulator of the rat sarcoma viral oncogene homolog (RAS)–mitogen-activated protein kinase (MAPK) pathway. Cellular RAS proteins present two forms; the majority form is its inactivated GDP-bound form, with a small part present in the active GTP-bound form. Once RAS proteins are in their GTP-bound form, they can upregulate the downstream RAS/RAF/MAPK signaling pathway, allowing uncontrolled cell growth. The critical function of neurofibromin is to promote the transformation from the active RAS-GTP into its inactive RAS-GDP state. Therefore, the loss of neurofibromin function by *NF1* gene mutation will lead to the sustained activation of RAS-GTP, leading to the prolonged activation of the RAS-MAPK signaling pathway and resulting in increased proliferation and uncontrolled cell growth (Bergoug et al., 2020; Cichowski & Jacks, 2001; Karen Cichowski, 2001).

### 1.4.3 NF1 and Pheochromocytoma

Although, in general, pheochromocytoma and paraganglioma are rare tumors, *NF1* is one of the many susceptibility genes. The constitutive mutation of *NF1* is responsible for inherited tumor syndromes neurofibromatosis 1(NF1). Around 50% of pheochromocytoma in NF1 are familial cases, and the rest half cases occur due to de novo mutation. Burnichon et al. analyzed 202 pheochromocytomas and paragangliomas and showed that 41% (25/61) of the sporadic cases carried *NF1* inactivating mutation, of which 21/25 cases were identified with the loss of the wild-type *NF1* allele. Besides, 56% of the identified somatic mutations are carried by the *NF1* gene, suggesting that this gene is frequently mutated in sporadic pheochromocytoma and paraganglioma (Burnichon et al., 2012).

### 1.4.4 NF1 loss in Neuroblastoma

The association between neuroblastoma and NF1 is not common. In an early study of 10 neuroblastoma cell lines, 4/10 showed reduced, or loss of neurofibromin, and *NF1* mutations

were identified in 2/4 of these cell lines. Furthermore, in neuroblastoma patients with somatic *NF1* mutation, the reduced expression of neurofibromin is associated with poor prognosis, while higher expression has been correlated with longer progression-free survival. In the same study, genomic aberrations of *NF1* were only detected in 6% of primary neuroblastomas. (Han, Spengler, & Ross, 2011; Holzel et al., 2010).

Hölzel and colleagues performed an unbiased large-scale RNAi genetic screen in *NF1*-deficient neuroblastoma cell lines and identified an association between NF1 with retinoic acid (RA) treatment resistance. Loss of NF1 leads to the activation of RAS-MEK signaling, then represses ZNF423, a crucial transcriptional coactivator of the retinoic acid receptors. They also observed that high levels of ZNF423 were significantly associated with the sensitivity of the cells to RA treatment. Significantly, neuroblastomas with low expression of both NF1 and ZNF423 correlated with poor outcomes. Furthermore, the inhibition of NF1 downstream ERK signaling restores cell sensitivity to RA, suggesting a potential treatment that can sensitize NF1-deficient neuroblastomas patients to RA treatment (Holzel et al., 2010).

## 1.5 DEVELOPMENT OF THE SYMPATHOADRENAL LINEAGE

Neural crest cells (NCC) are a population of transient cells unique to vertebrates and develop from the delaminated cells after the neural tube closure. The neural crest cells migrate extensively through the developing embryo before differentiating into diverse cell types that contribute to forming different structures. Delamination of neural crest cells undergoes epithelial-to-mesenchymal transition(EMT), a process where cells lose polarity and become less adhesive before cells start the migration to their destination. These cells migrate along the stereotyped paths individually or collectively. Eventually, they differentiate into various cell types, including much of the craniofacial skeleton, sympathetic and peripheral nervous system, melanocytes, and adrenal chromaffin cells after reaching numerous parts of the embryo (Bronner & Simoes-Costa, 2016).

It was believed that chromaffin cells and sympatoblasts have common progenitors and share the same developmental mode. Various research mainly draws this conclusion according to the studies of *in vivo* or *in vitro* cell differentiation while lack of lineage tracing. Thus, the putative progenitors of sympathoadrenal lineage (sympathetic neuroblasts and adrenal chromaffin cells) have not yet been directly revealed. It remained unclear how adrenal chromaffin progenitors migrate and develop into the adrenal medulla. Similarly, it is still

unknown if suprarenal sympathetic ganglion and adrenal chromaffin cells both are derived from a common sympathoadrenal precursor or differ in their origin, such as sympathetic cells are mainly derived from neural crest cells, whereas paraganglia and chromaffin cells of Zuckerkandl organ and adrenal gland mainly derived from Schwann cell precursors(SCP), a population of multi-potent nerve-associated neural crest-like cells (Lumb & Schwarz, 2015).

Until recently, emerging findings suggest that there are two valves of multipotent neural crest cells migration toward the dorsal aorta during early embryogenesis and contribute to the differentiation of sympathetic and chromaffin cells (Furlan et al., 2017; Huber, Kalcheim, & Unsicker, 2009; Saito, Takase, Murai, & Takahashi, 2012). The early migration of neural crest cells migrates freely towards the dorsal aorta ventrolateral. This process is finely regulated by the cooperation of diverse intrinsic and extrinsic factors. A study from Adamyko's lab in 2017 demonstrated the sympathetic and adrenergic lineages split at an unexpectedly early stage at E11.5 during mouse embryo development, which challenged the idea of a common sympathoadrenal lineage close to the dorsal aorta. Free neural crest cells from the early migration directly contribute to sympathetic neurons and only around 20% of chromaffin cells in the adrenal medulla, while a considerable proportion of adrenal chromaffin cells differentiated from SCP of the late migration (Furlan et al., 2017) (Figure 2).

Interestingly, a recent study from the same lab indicates that the immature chromaffin cells mainly derived from SCP contribute to the intra-adrenal neuroblast population in mice. However, in the human adrenal medulla, they observed SCP populates both the chromaffin cells and neuroblasts; importantly, neuroblasts also contribute to the formation of chromaffin cells (Kameneva et al., 2021). Jansky et al. sequenced neuroblastomas and normal human developing adrenal glands and identified SCP differentiated into neuroblasts and chromaffin cells (Jansky et al., 2021). Another study from Behjati´s lab also observed that SCP gives rise to both neuroblasts and chromaffin cells in the human fetal adrenal gland (Gerda Kildisiute & Kholosy, 2021). Knowledge of the highly dynamic transitory states within the developing adrenal gland reconstructed the cellular differentiation routes is not well understood but is crucial for understanding the heterogeneity and plasticity of neuroblastoma and pheochromocytoma, which originate during sympathoadrenal differentiation.

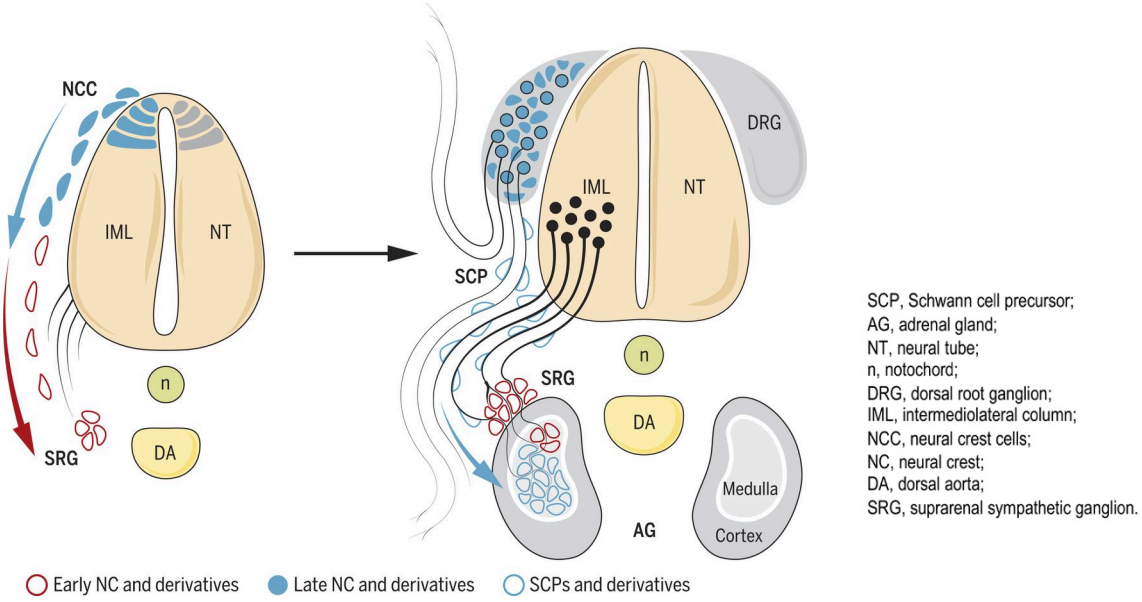


**Figure 2. "Adrenal medulla largely originates from Schwann cell precursors.** Overview of adrenal medulla development by lineage tracing of mouse neural crest derivatives and nerve ablation analysis. Red encodes early NCCs and their derivatives. Blue encodes late neural crest and SCP-derived cell types." Reprinted from Myltipotent peripheral glial cells generate neuroendocrine cells of t(von Stedingk, Gisselsson, & Bexell, 2019)he adrenal medulla. *Science, 7 Jul 2017, Vol 357, Issue 6346, with permission from AAAS.*

## 1.6 HETEROGENEITY IN NEUROBLASTOMA

The heterogeneity of neuroblastoma is characterized by the diverse clinical presentations and possible outcomes (inter-tumoral), the different phenotypes and various properties of neuroblastoma cells (intra-tumoral). The inter-tumoral heterogeneity commonly presents genotype variations between patients, while intra-tumoral involves the phenotypic and functional heterogeneity in cancer cells within the same tumor (Bedoya-Reina et al., 2021; von Stedingk et al., 2019).

In 2017, two critical studies demonstrated the intratumor heterogeneity of neuroblastoma at the cellular and molecular levels (Boeva et al., 2017; van Groningen et al., 2017). First, neuroblastoma cell lines were initially established by an ordinary serum-containing medium, and three different cell types were observed: neuroblastic (N-type), non-neuronal Schwann cell-like (S-type), and intermediate (I-type) cells (Ciccarone, Spengler, Meyers, Biedler, & Ross, 1989). Then, three decades later, Van Groningen established new neuroblastoma cell lines by culturing them with a neural stem cell medium. They identified two types of cells showing divergent expression of CD133 within the same tumor. The two types of cells were

named undifferentiated mesenchymal cells (MES-type) and committed adrenergic cells (ADRN-type). The MES-type and ADRN-type cells showed identical genetic abnormalities but different transcriptomic profiles. Importantly, they observed the similarity of the transcriptomic profiling between MES cells to human neural crest-derived cell lines, indicating that MES cells correspond to precursors of the adrenergic lineage (van Groningen et al., 2017).

After studying 25 neuroblastoma cell lines and two human neural crest cell (hNCC) lines, Boeva, V. et al. identified two distinct groups of neuroblastoma cell lines. Group I included 18 neuroblastoma cell lines, and group II comprises the GIMEN, SH-EP, and GICAN neuroblastoma cell lines which resemble hNCC lines. The discovery of neuroblastoma cells resembling hNCCs support that neural crest cells could be one of the cellular sources of neuroblastoma (Boeva et al., 2017).

## 1.7 PLASTICITY IN NEUROBLASTOMA

Cancer cell plasticity refers to the collectively molecular and phenotypic changes during cancer progression. “Unlocking phenotypic plasticity” was proposed by Douglas Hanahan as one of the new dimensions of the hallmarks of cancer (Hanahan, 2022). During organogenesis, cells undergo determination and terminal differentiation to perform homeostatic functions; meanwhile, progenitor cells stop growing during these processes. The cellular differentiation accompanied by antiproliferative creates a barrier to the continuing proliferation, which is necessary for neoplasia (Figure 3). Another study by Yuan et al. suggests that unlocking the typically restricted capability for phenotypic plasticity enables the cells to escape from terminal differentiation, which is believed to be critical for tumor pathogenesis (Yuan, Norgard, & Stanger, 2019).

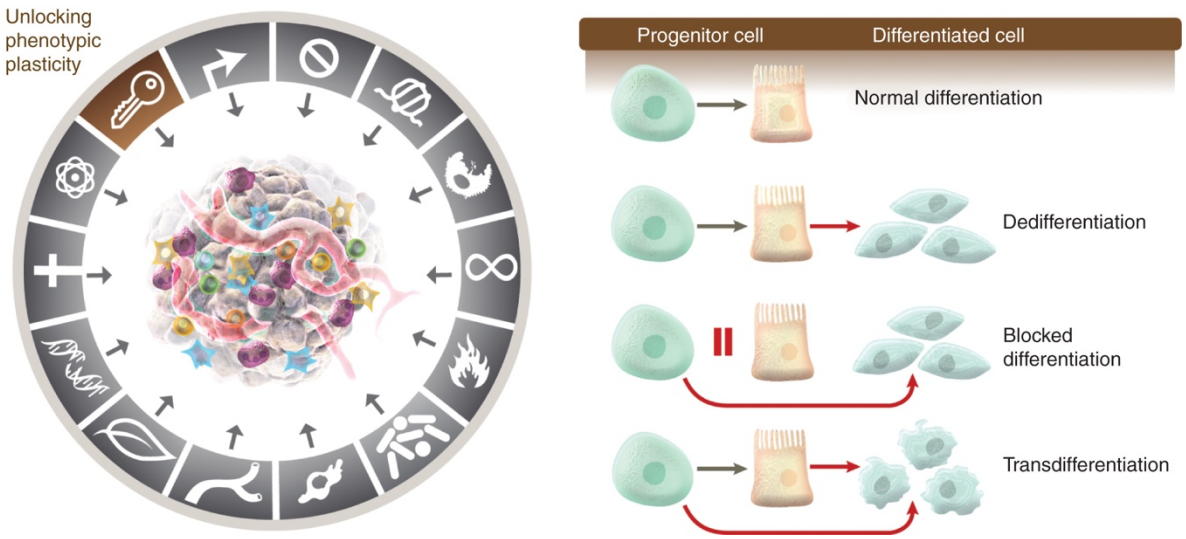


**Figure 3. Unlocking phenotypic plasticity.** Reprinted from *Hallmarks of Cancer: New dimensions, Cancer Discov (2022) 12 (1): 31–46,* Douglas Hanahan, with permission from AACR.

The intra-tumor heterogeneity is associated with tumor progression and drug resistance. Cancer cell plasticity, genetic and epigenetic alterations, gene regulation variations, cellular state transitions, or environmental disturbances have been believed to contribute to intra-tumor heterogeneity and promote cancer cell diversity. The best well-known case of tumor cell plasticity is epithelial-mesenchymal-transition (EMT). This process is commonly seen during embryonic development when cell identities are regularly shifted, enabling cells to migrate and differentiate to the destined site. Multiple finely regulated steps are involved in EMT, including the loss of epithelial features (cell polarity and cell-cell junctions) and the induction of genes that regulate the mesenchymal state. It is also crucial for the development of neural crest during vertebrate embryogenesis, where neural crest cells detach from the neuroepithelium and migrate throughout the embryo to drive tissue morphogenesis (Bardot & Hadjantonakis, 2020).

Schwann cell precursors (SCPs) were believed to be committed to Schwann cell differentiation. However, recent research indicates that SCPs may give rise to several other cell types, including chromaffin cells, neuroblasts, melanocytes, parasympathetic neurons, etc (Adameyko et al., 2009; Vyacheslav Dyachuk et al., 2014; Zhu, Ghosh, Charnay, Burns, & Parada, 2002). These researches indicate that the plasticity of SCP is more widely contributed to tissue morphogenesis and regeneration than we learned before. These findings provide another dimension to understanding sympathoadrenal development and neuroblastoma heterogeneity.

In recent years, the transition between different neuroblastoma cell states has also been invested *in vitro* and these studies strongly suggest the plasticity of these cells. Though adrenergic (ADRN) and mesenchymal cells (MES) differed in transcriptome profile and super-enhancer expression, undifferentiated MES and committed ADRN cells can interconvert and resemble cells during lineage differentiation stages. Single-cell RNA-sequencing (scRNA-seq) confirmed the existence of both cell types (MES cells and ADRN cells) in SK-N-SH cells (Jansky et al., 2021). The dedifferentiation from MES cells to ADRN cells may contribute to intratumor heterogeneity during tumor progression (van Groningen et al., 2017). In addition, MES cells have been shown to have similarities with neural crest precursors and are more resistant to chemotherapy. These differences in migration/invasion, chemoresistance, and possible interconversion between ADRN and MES cells may have significant clinical relevance.

## 1.8 NEUROBLASTOMA MOUSE MODELS

The new sights regarding tumor heterogeneity and plasticity facilitated our understanding of neuroblastoma initiation and progression. However, these new findings lack the proper models to validate the extensively functional studies and monitor the effects of potential anti-cancer drugs. Patient-derived xenografts (PDX) models *in vivo* and patient-derived organoids closely resemble the human patient tumor. However, The lack of consistency and standardization between pre-clinical studies limited the utilization of PDX models (Meehan et al., 2017).

Neuroblastoma genetically engineered mouse models (GEMMs), especially the *Th-MYCN* mouse model, have been applied extensively to validate the role of *MYCN* amplification experimentally. In the *MYCN*; *Dbh*-iCre model, tumor incidence is more than 75% in different mouse strains; however, the time to develop a tumor is relatively long (80 days on average), which renders it to be a proper childhood tumor model (Althoff et al., 2015). The GEMMs of *ALK*-mutant under *Dbh*- or *Th*- promoter develop neuroblastoma in penetrance less than 50% and very long latency. Interestingly, crossing *DBH*-iCre-*ALK* mice with *TH-MYCN* mice results in much higher tumor incidence, shorter latency, fewer genomic arrangements (Bram De Wilde et al., 2018; Lukas C. Heukamp et al., 2012), and lower somatic mutations (Bram De Wilde et al., 2018). However, the transgene integration site is not well defined, potentially leading to less robust MYCN expression. Notably, the tumor

penetrance of heterozygous *TH-MYCN* mice is strain-dependent, which reduces the potential for studying the combination with other cancer-relevant genes (William A.Weiss, Ken Aldape, Gayatry Mohapatra, G.Feuerstein, & Bishop, 1997). The overexpression of LIN28B has been demonstrated to upregulate MYCN expression in mouse sympathoadrenal lineage. Conditional *LIN28B* transgenic mouse model under *DBH* expression develops neuroblastoma with a short latency but only 25% penetrance (Molenaar, Domingo-Fernandez, et al., 2012).

GEMMs have been widely utilized for validating drug resistance genes, potential drug targets, and novel therapy pre-clinical testing, thus providing a new dimension for dissecting complex recurrent aberrations and having an increasingly important role in understanding neuroblastoma tumorigenesis.

# 2 RESEARCH AIMS

In **Paper I**, we aim to identify the *in vivo* function of KIF1Bβ in the sympathoadrenal system.

In **Paper II**, we aim to understand the sympathoadrenal tumor cell plasticity and heterogeneity. Specifically, we established a mouse model with the conditional knockout of KIF1Bβ and another tumor suppressor, NF1, to monitor the role of KIF1Bβ and NF1 combination in tumor cell plasticity and intra-tumor heterogeneity.

**Paper III** presents the inter-heterogeneity of childhood neuroblastoma. We aimed to understand how age at diagnosis is important for the prognosis of neuroblastoma.

# 3 RESULTS AND DISCUSSION

## 3.1 Paper I

In this paper, we investigated the mechanistic role of KIF1Bβ in the sympathetic system and its potential neuroblastoma tumor suppression function. We generated conditional gene targeting of KIF1Bβ in the mouse sympathetic lineage and crossed with mice expressing Cre-recombinase under the control of the gene for dopamine beta-hydroxylase (*DBH*-Cre). Based on the previous results from our lab (Schlisio et al., 2008), we expected that the KIF1Bβ knock-out mice would develop sympathetic system hyperplasia due to the impaired NGF-induced apoptosis of the neurons.

Unexpectedly, instead of hyperplasia in the sympathetic ganglia, we observed smaller ganglia in the *KIF1B*βcKO mice compared to their wild-type littermates (Figure 4A). We found that the decreased size of sympathetic ganglia in adult *KIF1B*βcKO mice resulted from a reduced cell number and greater cell density. In addition, neurite outgrowth in the primary culture of *KIF1B*βcKO super cervical ganglia (SCG) neurons was reduced compared to wild-type neurons (Figure 4B), suggesting that loss of KIF1Bβ impaired neurons target innervation and leads to aberrant sympathetic nervous system function. We further observed the misexpression of markers associated with sympathetic identity in neurons from P1 sympathetic ganglia, suggesting that the deletion of KIF1Bβ in sympathetic ganglia impairs neuron differentiation and function. Thus, we demonstrated that KIF1Bβ enables NGF-induced neuronal differentiation.

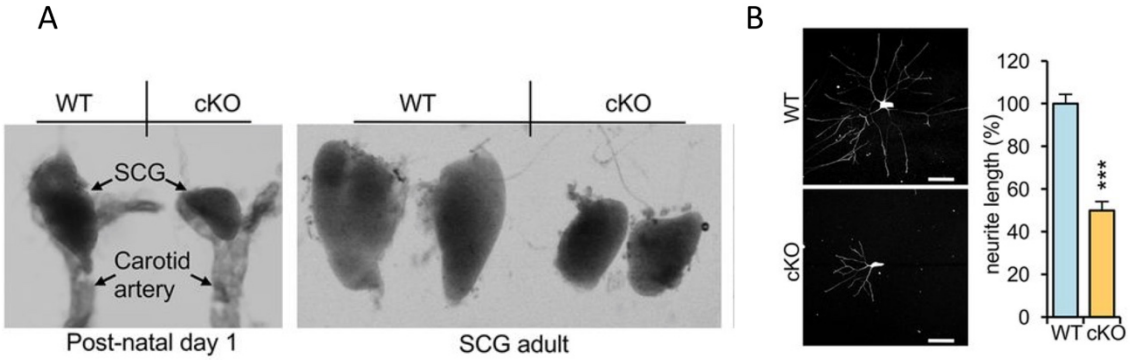


**Figure 4, Loss of KIF1Bβ impairs sympathetic neurons function. A**, Photographs of dissected mouse super cervical ganglia. **B**, Immunofluorescent staining of TUJ1 in neurons from P1 super cervical ganglia and quantification of mean neurite length. *Adapted from paper I.*

During sympathetic axon extension, target tissues secret NGF that binds to TRKA on the neuronal cell membrane, thus activate the neurotrophic signaling pathways (Crowley et al., 1994; Glebova & Ginty, 2004). Therefore, we hypothesize that the ablation of KIF1Bβ impairs the NGF-TRKA neurotrophic signaling and leads to neuronal apoptosis. We observed that TRKA is highly concentrated in the perinuclear region in KIF1Bβ -null cells (NB1 cells, 1p36 -/-), in contrast, TRKA has evenly dispersed in the cytoplasm in KIF1Bβ -expressing SH-SY-5Y (1p36 +/+) cells. Importantly, the reintroduction of wild-type KIF1Bβ into NB1 cells significantly dispersed TRKA away from the perinuclear region; while KIF1Bβ point mutants had mineral effects on TRKA localization.

The binding of NGF to TRKA activates the RAS protein and activates downstream MAP kinases, which are critical for neuronal survival and differentiation. We then introduced the tumor suppressor NF1, which can downregulate the RAS MAP kinase signaling. When we combined the conditional knockout of *KIF1B*β together with *NF1* under *DBH* gene expression, we observed that loss of NF1 rescues the reduced size of SCGs, in support of our hypothesis. The comparison of transcriptome profiles of *KIF1B*βcKO SCGs and non-*MYCN* neuroblastomas shows that unfavorable neuroblastoma resembled *KIF1B*βcKO neurons, suggesting that ablation of KIF1Bβ contributes to the pathogenic development of neuroblastoma.

Thus, in paper I, we demonstrated that KIF1Bβ is necessary for neuronal differentiation through mediating the transport of the NGF receptor TRKA.

### 3.2 PAPER II

This paper aims to understand the sympathoadrenal tumor cell plasticity and heterogeneity. Drug resistance in treatment is a great challenge to cancer management. However, emerging evidence suggests the non-mutational resistance primarily driven by tumor cell plasticity enables tumor cells to be drug insusceptible. In our study, we aim to develop a novel neuroblastoma and pheochromocytoma mouse models to understand the reason that drives tumor cell plasticity and intra-tumor heterogeneity.

As shown in Paper I, We identified a 1p36 tumor suppressor gene *KIF1B*β is required for NGF-dependent sympathoblast differentiation and developmental apoptosis. Although KIF1Bβ loss impairs sympathetic neuron differentiation and function, our *KIF1B*βcKO did

not develop sympathoadrenal malignancies. Therefore, we predicted that loss of KIF1Bβ alone in the sympathoadrenal system might not be sufficient to induce malignancy. Recently, a somatic mutation of *KIF1B*β with an *NF1* germline mutation was identified in a patient with neural crest derived tumors. Loss of NF1 has been associated with sympathoadrenal malignancies. Therefore, we combined the loss of KIF1Bβ with the loss of NF1 in sympathoadrenal and observed neuroblastoma and pheochromocytoma development.

We obtained *KIF1B*βfl/fl;*NF1*fl/fl; *Dbh*Cre mice (referred to as DKO and *NF1*cKO) after two generations at regular Mendelian ratios. As shown in paper II, loss of NF1 or in combination NF1 with KIF1Bβ resulted in super cervical ganglia hyperplasia. Furthermore, after more than one year, we observed in DKO and *NF1*cKO mice neuroblastoma, pheochromocytoma, as well as composite tumors originating at the adrenal gland (Figure 5A), as control mice did not present this tumor-promoting phenotype. The diagnosis of the mouse tumors was performed by the histopathologists Arthur Tischler and Christofer Juhlin based on HE staining and has further been validated via immunohistochemistry according to the specific markers.

Importantly, we observed earlier tumor death onset, significantly lower survival probabilities in DKO mice compared to *NF1*cKO mice (p=0.0053) (Figure 5B), and a shorter median time at 407 days of age compared to that of *NF1*cKO animals at 496 days. One unique observation in these tumor mice is composite tumors consisting of both Pheochromocytoma and neuroblastoma, which also can be observed in some patients (Tran, Fitzpatrick, Cohn, & Pytel, 2017). Dahia and Schlisio identified a *KIF1B*β germline variant S1481N in a 28-yr-old female who developed neuroblastomata at 17 months and a mature ganglioneuroma and bilateral pheochromocytoma in adulthood (Schlisio et al., 2008; Welander et al., 2014). Her paternal grandfather also identified with this allele and was diagnosed with bilateral pheochromocytoma.

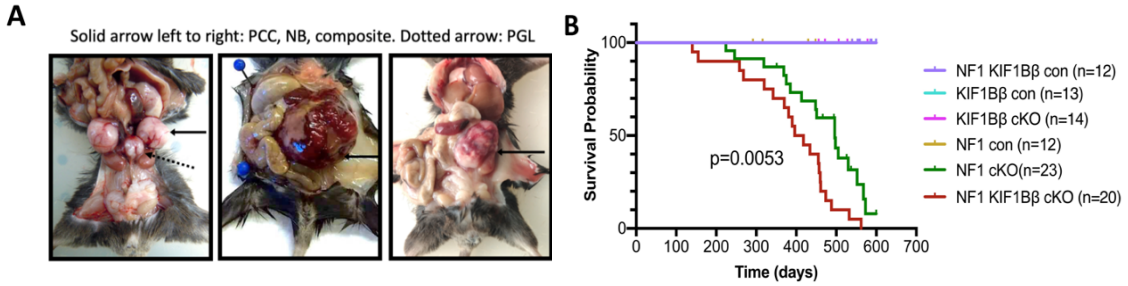


**Figure 5, Mice deleted for KIF1Bβ and NF1 in the sympatho-adrenal lineage develop neuroblastoma (NB) pheochromocytoma (PCC) and composite tumors. A**, Images of autopsies of mice carrying palpable tumors, indicating pheochromocytoma (PCC), paraganglioma (PGL) neuroblastoma (NB) and composite tumor arising from the adrenal gland as indicated. Composite tumor consisting of both, PCC and NB. **B**, Kaplan-Meier analysis of DKO (red), *NF1*cKO (green) and control mice with the endpoint defined as detection of palpable tumors. *Adapted from paper II.*

We further profiled tumor heterogeneity and immaturity on a single-cell resolution of our mouse tumors and embryonic stage(E17). The diverse cell populations observed in *DBH*-positive tumorigenic cells indicate a significant heterogeneity of chromaffin cells, neuroblasts and hybrid cells that expressing both markers. Importantly, we observed more neuroblasts generated in DKO and *NF1*cKO compared to that in wild-type mice in the E17 adrenal gland when we have the conditional knockout events under *Sox10* control at E11.5. RNA *in situ* hybridization has further validated the abundant neuroblasts at E17.

We detected hyperplasia in the adrenal medulla in the three-month-old DKO and *NF1* cKO mice. We expect hyperplasia in the adrenal medulla will continue with subsequent tumor development in the DKO and *NF1*cKO mice, subsequently causing tumor development. We observed the medulla breaking through the cortex and adjacent cells termed as "break-through". The cells in the break-through lose *PNMT* and *CHGB* expression and turn on neuroblast signature. Sc-RNA-seq and RNA velocity combining tumor and three-month adrenal gland showed a transition from *PNMT* positive chromaffin cells to neoplastic chromaffin cells and hybrid cells in the three-month adrenal gland (Figure 6). Interestingly, the transition from chromaffin cells to neuroblasts has also been observed in the Sc-RNA-seq of the E17 adrenal gland. Single-cell RNA sequencing and immunohistochemistry in tumors reveal neuroblasts, chromaffin and hybrid signatures which indicate intratumorally heterogeneity and highlight some cells stay in a transiting state.

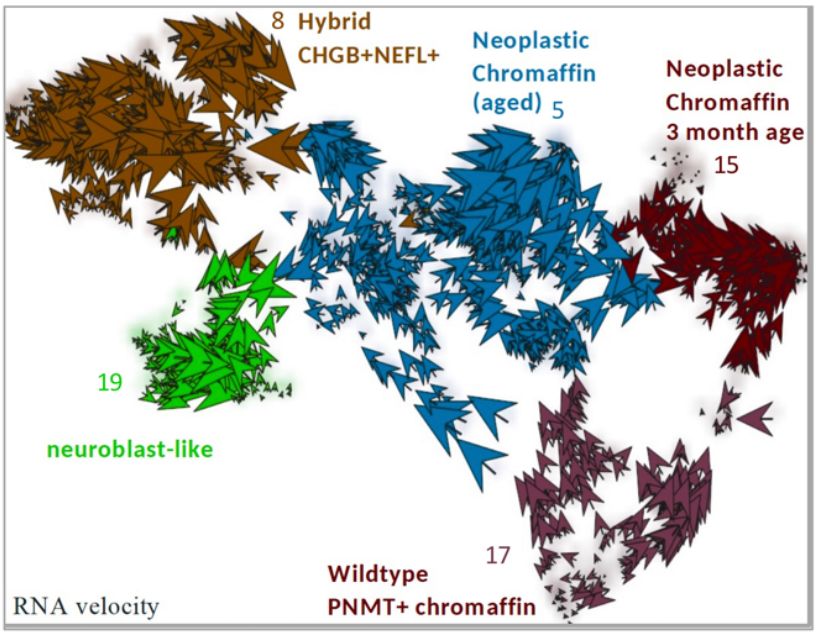


**Figure 6,** A uniform manifold approximation and projection (UMAP) embedding of the *TH*-positive medullary cells from wild-type and neoplastic cells of three-month and aged tumors, overlaid with RNA velocity estimates. *Adapted from paper II.*

To further explore how our mouse model mimics human disease, we compared the RNA *in situ* hybridization result of human pheochromocytoma that harbors the same mutation with the mouse model. Interestingly, we discovered significant hybrid cells in the human tumor, which suggest cell state transition similarly occurs in humans and could be relevant to human sympathoadrenal malignancy.

Thus, by generating and exploring the pheochromocytoma, neuroblastoma, and composite mouse model, our study observed a remarkable heterogeneity and cell state transition in different stages of tumor progression. We hypothesize the cell state transition from chromaffin cells to neuroblasts is responsible for the intra-tumor heterogeneity.

### 3.3 PAPER III

In this paper, we analyzed primary neuroblastoma tumors provided by Per Kogner from Karolinska Hospital and observed inter-tumor heterogeneity. These tumors have been characterized by stage, outcome, *MYCN* expression, and 1p-status. We took advantage of single nuclei RNA sequencing, which allows us to work with any deep-frozen tissue. We isolated nuclei from different tumors and sorted them by FACS. The cDNA library was constructed by SMARTseq2 before RNA sequencing.

To understand why poor prognosis neuroblastomas are usually diagnosed in children older than 18 months, we first analyzed the normal cell populations from the postnatal human and mouse adrenal glands which is the typical location for neuroblastoma. We observed that postnatal human and mouse adrenal glands share common cell populations, but the only chromaffin cell cluster differs from each other. Instead of two clusters with different *PNMT* expressions in mice, human chromaffin cells only come into one cluster. Interestingly, we identified a cluster of cells with high expression of progenitor and migration markers (*CLDN11*) (Figure 7A-B), and importantly, this cluster was observed in the human postnatal adrenal gland only. The RNA velocity analysis indicates that the progenitor cells have the potential to differentiate into chromaffin cells. This has further been validated by RNAscope *in situ* hybridization with the co-localization of the cholinergic nicotinic receptor *CHRNA7* with either progenitor marker *CLDN11* or chromaffin marker *TH* (Figure 7C). RNAscope for the progenitor population in human postnatal adrenal glands at different ages validated the existence, and we observed the population declines in older adrenal glands.

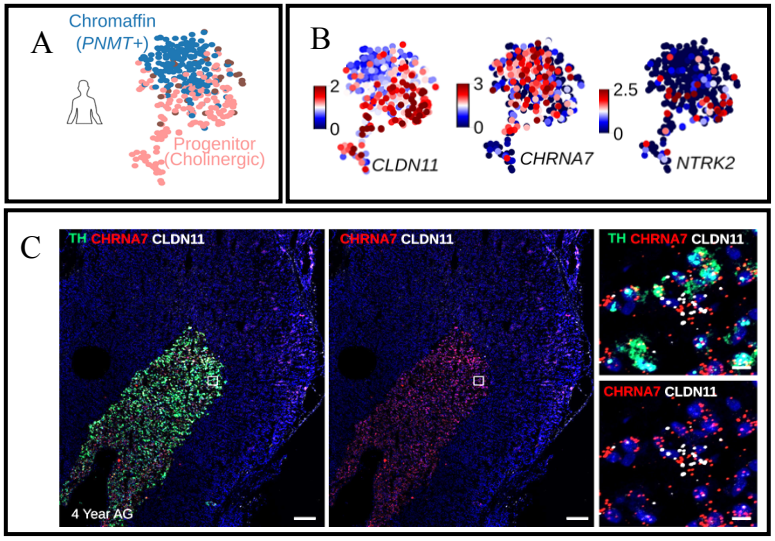


**Figure 7**, **A-B**, Human chromaffin (blue) and cholinergic progenitor (pink) populations with the expression of indicated genes. **C**, RNAscope *in situ* hybridization of a four-year-old human AG that probed for *TH*, *CHRNA7*, and *CLDN11*. Nuclear counter-stained with DAPI. *Adapted from paper III.*

Next, the single nuclei RNA sequencing revealed the intra- and inter-heterogeneity in the human primary neuroblastoma tumors that have been grouped with different stages and genetic subsets. We identified ten different cell clusters, broadly separated into two distinct sections: the first section - favorable neuroblastoma (high *TrkA*, low *TrkB*), and the second section - unfavorable neuroblastoma (low *TRKA*, high *TrkB*) (Figure 8A). The second section also presented a high expression of *MYCN*. RNAscope validated the significantly enriched markers in both favorable and unfavorable neuroblastoma. Interestingly, high-stage neuroblastoma presented a high intra-tumoral heterogeneity by part of tumor staining positive

for *TH* and *MYCN*; while other regions of the tumor show strong signals of *MYCN* and *ALK*, negative for *TH* (Figure 8C).

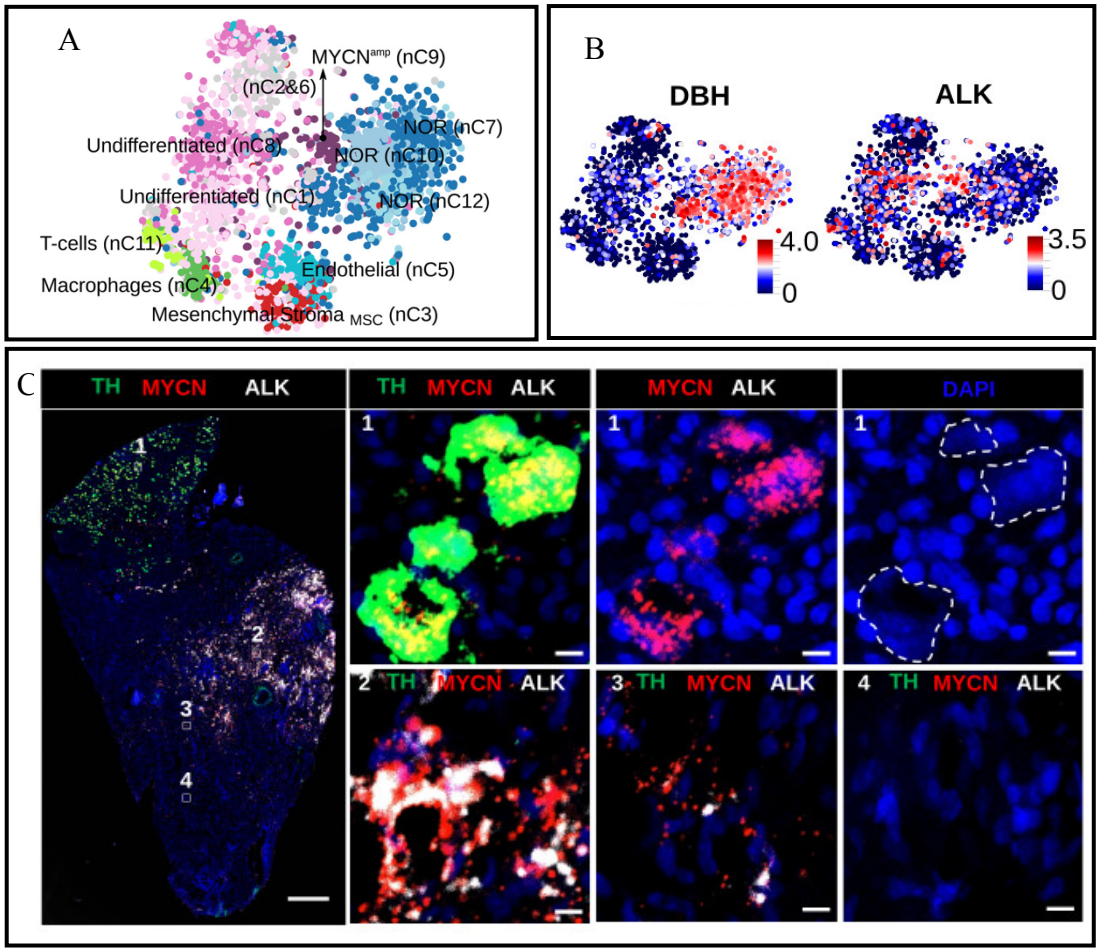


**Figure 8, Intratumoral heterogeneity in neuroblastoma**. **A**, Cells were separated into ten populations. **B**, Illustration of the expression of indicated genes. **C**, RNAscope *in situ* hybridization of high-risk human neuroblastoma (stage 4, *MYCN* amplified) labeled with *TH*, *MYCN*, and *ALK*. Nuclear counter-stained with DAPI. Scalebar of overview: 500μm; boxed image: 10μm. *Adapted from paper III.*

To uncover why high-risk neuroblastoma patients are frequently diagnosed in children older than 18 months, we compared the signatures in low and high-risk neuroblastoma to the embryonic stage and postnatal normal adrenal glands. Low-risk neuroblastoma significantly high express (nor-)adrenergic(NOR) markers are shared with the chromaffin and sympathoblast cluster in mouse and human embryonic adrenal glands. In comparison, the high-risk neuroblastoma characterized by the undifferentiated clusters showed a similar gene signature with the human cholinergic progenitor population that was specifically observed in the human postnatal adrenal gland. Furthermore, we uncovered the transcriptional program in high-risk-associated cell cluster changes with age-at-diagnosis and is correlated with worse prognosis of patients.

Thereby, low-risk neuroblastoma is common in patients younger than 18 months, and high TrkA expression is strongly correlated with a good prognosis, whereas TRKB is associated with poor outcomes. It is possible that the different progenitor populations contribute to neuroblastoma at different ages, thus resulting in various heterogeneity. In this regard, age at diagnosis is an important prognosis predictor.

# 4 CONCLUSION AND PERSPECTIVE

Heterogeneity of tumors represents a great challenge for metastatic tumors. The precise cellular origin of neuroblastoma is not yet well understood. Emerging studies based on transcriptomics analysis suggested the contribution of specific cell types and the maintained developmental cell plasticity in NB oncogenesis. Thus, we took advantage of the neuroblastoma and pheochromocytoma mouse model generated in our lab to investigate the dynamics of tumor formation in a single cell resolution in the different stages of tumor development.

**Impaired differentiation of neuroblasts increases cellular plasticity.**

We recently discovered that KIF1Bβ, a tumor suppressor located on chromosome 1p36, is required for NGF-dependent sympathoblast differentiation and developmental apoptosis. Although our *KIF1B*βfl/fl; *Dbh*Cre mice did not develop any sympathoadrenal malignancy, We demonstrated that KIF1Bβ is required for neuronal differentiation through mediating the transportation of NGF receptor TRKA and observed the impaired TRKA transportation in the pathogenic *KIF1B*β -deficient neuroblastoma. During differentiation, progenitors follow a lineage-specific fate toward tissue-characterized cells with specialized functions. The acquired specific cellular shape and function come with the loss of lineage potential and lead to terminal differentiation, ultimately resulting in the loss of cellular plasticity. In mammalian cells, the normal differentiation process is usually unidirectional, and the natural dedifferentiation is restricted to limited tissues like the liver. Cell differentiation is regulated by a complex tumor suppressors network. For instance, the loss of tumor suppressor KIF1Bβ impairs the sympathetic neuroblast differentiation, and the blocked differentiation potentially susceptible these cells to tumorigenesis.

**Cell state transition causes high cellular plasticity and intra-tumoral heterogeneity.**

To investigate the dynamics of tumor formation in the different stages, we decided to generate a neuroblastoma mouse model that will enable us to monitor the molecular event in tumor progression. In our mouse model, loss of RAS/MAPK signaling tumor suppressor NF1 alone or combined with the loss of KIF1Bβ in sympathoadrenal lineage leads to neuroblastoma, pheochromocytoma, and composite tumors. We observed a remarkable

heterogeneity of chromaffin cells and neuroblasts population in the different stages of tumor progression. Computational analysis of the single-cell sequencing data revealed cell state transitions from chromaffin to neuroblast responsible for the plasticity and heterogeneity. Understanding tumor cell plasticity and heterogeneity will provide insights into novel targeted therapeutic strategies currently missing for neuroblastoma, malignant pheochromocytoma, and paraganglioma. Further analysis is needed to resolve the molecular signals used to control the final fate selection that might be required for phenotypic interconversion within the tumors.

**Inter- and intra-tumoral heterogeneity reveals the cell origin of neuroblastoma.**

Neuroblastoma is a heterogeneous disease with various patient survival. Low and intermediate-risk patients usually have a good prognosis with more than 95% survival rate, while high-risk patients have less than 50% long-term survival even with proper treatment. Age is an important prognosis factor for patients. To understand this, we need to uncover the heterogeneity and "differentiation-like" status of heterogeneous cancer cells compared to their typical origin cell types. We identified a unique progenitor population in human postnatal adrenal glands that resembles high-risk neuroblastoma, while low-risk tumors have a transcriptomic profile similar to the embryonic sympatho- and chromaffin cells.

Timing is a critical factor during development. As shown in paper II, we observed tumors when the genetic event occurs in the mouse sympathoadrenal system at the embryo stage. However, when the knockout happened in postnatal chromaffin cells, we didn't observe any sympathoadrenal tumor (data not shown), suggesting that only the genetic event occurs in the embryonic stage instead of postnatal enable tumorigenesis in our mouse model.

Here we showed inter- and intratumoral heterogeneity of neuroblastoma and the possible different cell origin of high and low-risk tumors. More studies on the molecular biology and pathogenesis of neuroblastoma are needed for the precise treatment of children with this disease.

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