Preclinical investigation of novel therapies against neuroblastoma

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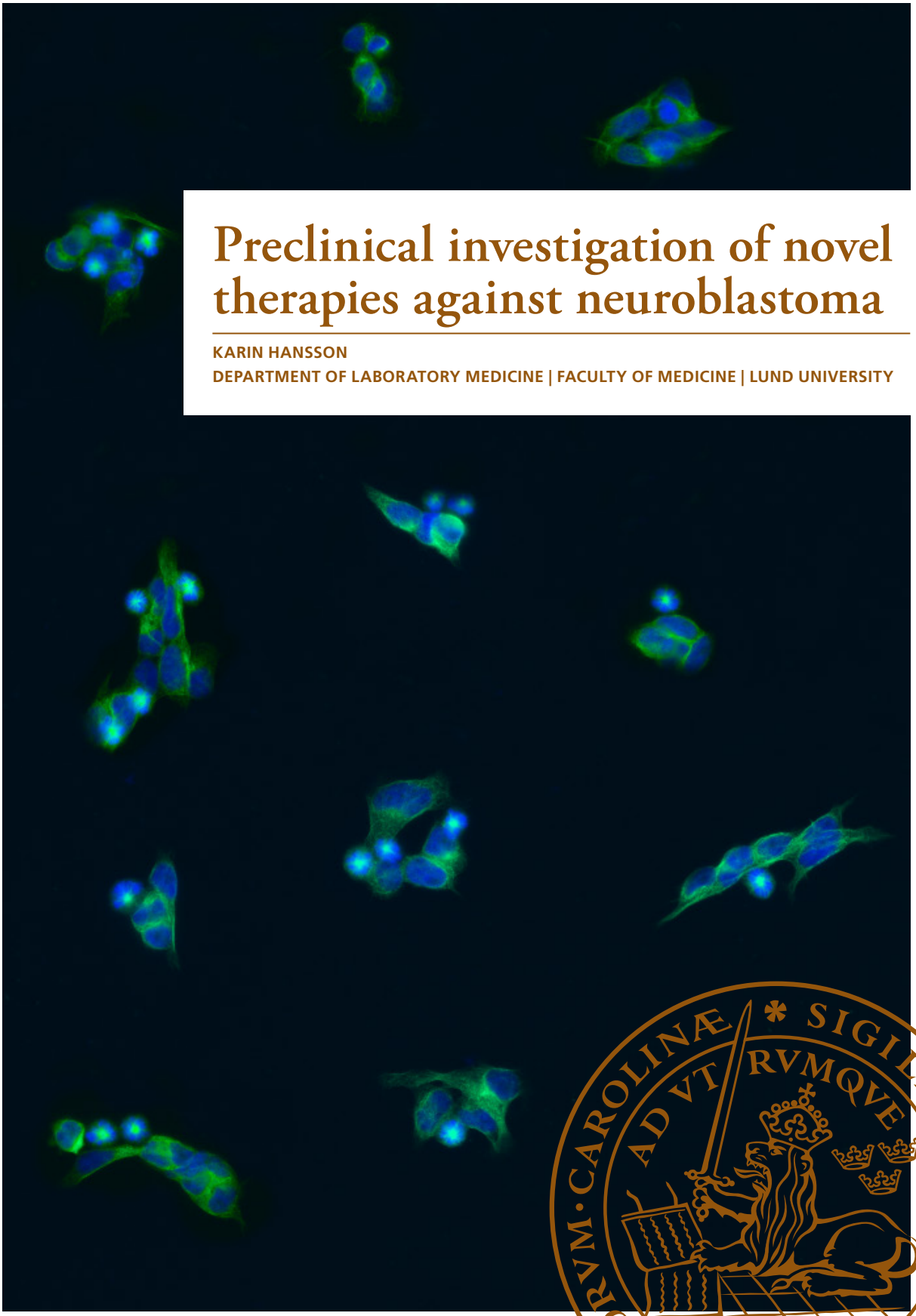

Preclinical investigation of novel therapies against neuroblastoma

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Neuroblastoma is a childhood tumor of the sympathetic nervous system and the most common tumor form diagnosed in infants. Patients with aggressive tumors are treated intensely with multi-modal therapy including chemotherapy, surgery, radiotherapy and immunotherapy. Despite this, many children suffer from incurable relapses and long-term side effects are common. Novel treatments are thus necessary for these patients. The aim of this thesis was to identify and test novel therapies against neuroblastoma. For this work, I have used patient-derived xenograft mouse models and neuroblastoma cells grown as 3D tumor organoids.

In paper I, we identified and tested novel inhibitors targeting the PIM, PI3K, and mTOR pathways. The inhibitors demonstrated promising effects in vitro with reduced viability, increased differentiation and cell death of neuroblastoma cells. Combinatory treatment of the inhibitors with chemotherapeutic agent cisplatin showed synergistic effects. In vivo, combination treatment resulted in slower tumor growth and increased survival of neuroblastoma PDX models.

In paper II, we performed a high-throughput drug screen to identify drugs that target neuroblastoma. We identified several drugs with previously unknown activity in neuroblastoma, among them the KSP inhibitor ARRY-520. Analysis of KSP expression across multiple tumor types demonstrated that neuroblastoma is highly dependent on this protein. Treatment of neuroblastoma PDX cells with ARRY-520 resulted in mitotic arrest and subsequent cell death. KSP inhibition caused tumor regression in vivo and resulted in increased survival of multiple PDX models.

In paper III, we investigated the effects of inhibitor rigosertib against neuroblastoma. Rigosertib reduced cell viability of neuroblastoma cells and caused cell cycle arrest in the G2/M phase. In vivo, rigosertib treatment resulted in decreased tumor growth and increased survival of PDX mice.

In paper IV, we performed a high-throughput combination drug screen with the aim of finding drugs that work synergistically with KSP inhibition. Using in silico synergy prediction tools, we identified drugs with synergistic activity against neuroblastoma. Our results suggest that Ras pathway inhibitors in combination with KSP inhibition represent a promising treatment strategy against neuroblastoma.

In summary, we identified and tested drugs which represent novel therapeutic opportunities against children with high-risk neuroblastoma.

Key words: Cancer, Neuroblastoma, drug testing, preclinical cancer models

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List of papers

This thesis is based on the following papers:

I. Anti-tumor effects of PIM/PI3K/mTOR triple kinase inhibitor IBL-302 in neuroblastoma.
Sofie Mohlin*, **Karin Hansson***, Katarzyna Radke, Sonia Martinez, Carmen Blanco-Aparicio, Cristian Garcia-Ruiz, Charlotte Welinder, Javanshir Esfandyari, Michael O'Neill, Joaquin Pastor, Kristoffer von Stedingk, and Daniel Bexell.
EMBO Molecular Medicine. 2019;11(8):e10058

II. Therapeutic targeting of KSP in preclinical models of high-risk neuroblastoma.
Karin Hansson, Katarzyna Radke, Kristina Aaltonen, Jani Saarela, Adriana Mañas, Jonas Sjölund, Emma M. Smith, Kristian Pietras, Sven Påhlman, Krister Wennerberg, David Gisselsson, and Daniel Bexell.
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III. Preclinical evaluation of rigosertib for the treatment of high-risk neuroblastoma.
Katarzyna Radke, **Karin Hansson**, Jonas Sjölund, Javanshir Esfandyari, Kristian Pietras, Kristina Aaltonen, David Gisselsson, and Daniel Bexell.
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IV. Identification of synergistic drug combinations against high-risk neuroblastoma.
Karin Hansson*, Katarzyna Radke*, Jani Saarela, Aleksandr Ianevski, Philipp Ianevski, and Daniel Bexell.
Manuscript.

* indicates equal contribution

Abbreviations

ADP	Adenosine Diphosphate
AHSCT	Autologous Hematopoietic Stem Cell Transplantation
ALK	Anaplastic Lymphoma Kinase
ATP	Adenosine Triphosphate
BET	Bromodomain and Extraterminal
BMP	Bone Morphogenic Protein
CDK	Cyclin Dependent Kinase
CDX	Cell line-derived Xenograft
CHK1	Checkpoint Kinase 1
CNS	Central Nervous System
COG	Children's Oncology Group
CT	Computerized Tomography
DECREASE	Drug Combination RESponse prEdiction
DSS	Drug Sensitivity Score
EFS	Event-Free Survival
EMT	Epithelial-to-Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
FISH	Fluorescent in situ hybridization
FOXO	Forkhead box O
GAP	GTPase activation proteins
GAP43	Growth Associated Protein 43
GDP	Guanosine diphosphate
GDSC	Genomics of Drug Sensitivity in Cancer
GEF	Guanosine nucleotide exchange factors
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMS	Genomic Medicine Sweden
GPCR	G-protein-coupled receptor
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine triphosphate
INFORM	INdividualized therapy For Relapsed Malignancies in childhood
IDRF	Image-Defined Risk Factor
INRG	International Neuroblastoma Risk group
KSP	Kinesin Spindle protein

MAPK	Mitogen-activated protein kinase
MAX	Myc-Associated Protein
MIBG	Metaiodobenzylguanidine
MRI	Magnetic Resonance Imaging
MSA	Most Synergistic Area
mTOR	mammalian Target of Rapamycin
NCC	Neural Crest Cell
NEPENTHE	NExt Generation PErsonalized Neuroblastoma THErapy
OS	Overall Survival
PDK1	Phosphoinositide-Dependent protein Kinase-1
PDX	Patient-Derived Xenograft
PH	Pleckstrin Homology
PHOX2A	Paired-like homeobox 2A
PHOX2B	Paired-like homeobox 2B
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PIM	Proviral Insertion site in Murine leukemia virus
PNS	Peripheral Nervous System
PPM1D	Protein Phosphatase, Mg^{2+}/Mn^{2+} Dependent 1D
PPTP	The Pediatric Preclinical Testing Program
PRAS40	Proline-Rich Akt Substrate of 40 kDa
PTEN	Phosphatase and Tensin Homolog
RAPTOR	Regulatory-associated protein of mTOR
RBD	Ras-binding domain
RICTOR	Rapamycin-insensitive companion of mTOR
RTK	Receptor Tyrosine Kinase
SCP	Schwann Cell Precursor
SIOPEN	Society of Pediatric Oncology, European Neuroblastoma
TSC2	Tuberous Sclerosis Complex 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Abstract

Neuroblastoma is a childhood tumor of the sympathetic nervous system and the most common tumor form diagnosed in infants. Patients with aggressive tumors are treated intensely with multi-modal therapy including chemotherapy, surgery, radiotherapy and immunotherapy. Despite this, many children suffer from incurable relapses and long-term side effects are common. Novel treatments are thus necessary for these patients. The aim of this thesis was to identify and test novel therapies against neuroblastoma. For this work, I have used patient-derived xenograft mouse models and neuroblastoma cells grown as 3D tumor organoids.

In paper I, we identified and tested novel inhibitors targeting the PIM, PI3K, and mTOR pathways. The inhibitors demonstrated promising effects *in vitro* with reduced viability, increased differentiation and cell death of neuroblastoma cells. Combinatory treatment of the inhibitors with chemotherapeutic agent cisplatin showed synergistic effects. *In vivo*, combination treatment resulted in slower tumor growth and increased survival of neuroblastoma PDX models.

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In summary, we identified and tested drugs which represent novel therapeutic opportunities against children with high-risk neuroblastoma.

Populärvetenskaplig sammanfattning

Neuroblastom är en typ av cancer som främst drabbar små barn. Neuroblastom uppstår i det icke-viljestyrda (sympatiska) nervsystemet och den vanligaste platsen för tumörer är i binjuren, ett organ placerat precis ovanför njuren. Patienter med aggressiv neuroblastom behandlas intensivt med en kombination av flera cellgifter, strålning, kirurgi och immunoterapi. Trots denna tuffa behandling är obotliga återfall vanliga och patienter som överlever får ofta komplikationer senare i livet. Detta visar att ny behandling behövs för barn med neuroblastom.

Syftet med denna avhandling var att identifiera och undersöka nya behandlingar mot neuroblastom. Jag har använt mig av cellodlingar och musmodeller som baseras på tumörbitar från patienter med neuroblastom. Dessa modeller bibehåller viktiga egenskaper från originaltumörerna, inklusive deras aggressiva tillväxtsätt och genetiska förändringar. Detta gör dem passande för att studera hur tumörer svarar på behandling. Avhandlingens forskning presenteras i fyra delarbeten och sammanfattas här nedan.

I *delarbete I* undersökte vi effekten av att hämma tre proteiner samtidigt med ett nytt läkemedel. Proteinerna (PIM, PI3K och mTOR), har uppgifter som är viktiga för cancercellers överlevnad och tillväxt. Vi visade att läkemedlet effektivt hämmade proteinerna. Detta resulterade i minskad tillväxt och ökad celldöd av neuroblastomceller. Våra resultat visade även att kombinationsbehandling av läkemedlet tillsammans med cellgifter, som används vid behandling av neuroblastom idag, gav en större effekt än behandlingarna separat.

I *delarbete II* gjorde vi en stor screen där vi undersökte effekten av 500 läkemedel. Vi jämförde effekten av läkemedlen på neuroblastomceller med effekten på friska normala celler och valde ut läkemedel som specifikt påverkade neuroblastomcellerna. Bland de mest verkningsfulla läkemedlen fanns en KSP-hämmare. KSP är ett protein som är viktigt för celldelningen och det finns i större mängder i cancerceller jämfört med vanliga celler. KSP verkar dessutom vara extra viktigt för just neuroblastomceller. Behandling med KSP-hämmaren resulterade i stoppad delning av tumörceller, vilket i sin tur ledde till celldöd. Behandling av möss med neuroblastom resulterade i minskad tumörstorlek och ökad överlevnad av möss, i en modell försvann till och med tumörerna helt.

I *delarbete III* undersökte vi vidare ett annat läkemedel som identifierats från screenen i delarbete II. Vi såg lovande effekter både i neuroblastomceller och i musmodeller, med minskad tumörväxt och ökad överlevnad i möss.

I *delarbete IV* var syftet att identifiera läkemedel som tillsammans med KSP-hämmaren ger en synergistisk effekt. Synergistisk effekt är en samverkade effekt som innebär att den gemensamma effekten är större än summan av de enskilda läkemedlen. Vi testade 500 läkemedelskombinationer och med hjälp av maskininlärning kunde vi förutspå några lovande kombinationer. Preliminära data visar att vi identifierat synergistiska kombinationsbehandlingar som ska testas ytterligare.

Sammanfattningsvis har vi identifierat och testat flera läkemedel med lovande resultat mot neuroblastom. Målet framöver är att vidare undersöka effekten av kombinationsbehandling, för att öka effekten och minska risken för behandlingsresistens hos patienter.

Introduction

Cancer

Cancer is characterized by an abnormal and uncontrolled growth of cells, caused by the acquisition of genetic changes. It is one of the leading causes of death worldwide and currently estimated to 18 million new cases annually (*1*). Although the word cancer covers a broad variety of diseases, ranging from abnormal hematopoiesis in leukemia to large tumor masses in solid cancers, they all share the ability of evading the strictly controlled cell environment and the potential of invading other tissues.

Carcinogenesis, the process of how normal cells become malignant cancerous cells, is a multi-step progression. Accumulated changes result in destabilization of key cellular functions and an abnormal cell phenotype. This makes cancer cells genetically instable, prone to undergo additional changes when pressured, like when being treated with chemotherapy (*2*). One consequence of this instability is that cancers show a high degree of inter- and intratumoral heterogeneity, regarding cellular morphology, mutational landscape, and therapeutic response (*3*). Additionally, aggressive cancers show a high rate of metastasis, the spread of cancer cells from the primary tumor to distant sites. Uncurable metastatic lesions accounts for 90% of all cancer-related deaths (*4*).

In 2000, Hanahan and Weinberg proposed that even though the mechanisms of malignancy varies between different tumor types, they all share six hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, ability of evading apoptosis, limitless replicative potential, sustained angiogenesis, capacity of tissue invasion and metastasis (*5*). These hallmarks aimed at rationalizing the complexity and diversity of cancer. Ten years later, they published an updated version with the addition of two emerging hallmarks, reprogramming the energy metabolism and evading the immune system (*6*). Additionally, they stated that genomic instability and tumor-promoting inflammation are two characteristics of cancer that enable the acquirement of the hallmarks. These publications have been very influential in how researchers regard and target the cancer enigma.

Pediatric cancer

The cancers occurring in children are different from those affecting adults. In children, the most common type of cancer is leukemia, followed by central nervous system (CNS) tumors, while in adults, leukemias are rare and other types of brain tumors are prevalent. Although there are clear similarities regarding the malignant phenotypes, pediatric tumors are distinctly different from the adult (*7*). Pediatric tumors usually harbor fewer mutations compared to adult tumors (*8*). Adult cancers are frequently related to age or lifestyle and some cancers are associated with well-known risk factors, such as smoking or alcohol use (*9*). Cancer can often be cured using chemotherapy, radiotherapy and surgery. However, in children it is important to consider the long-term side effects. Secondary malignancies is common in children, with chemotherapy, iodizing radiation and hematopoietic stem cell transplantation as the main therapeutic risk factors (*10, 11*).

Development of the sympathetic nervous system

The human nervous system

The human nervous system is comprised of two parts, the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous system (PNS). The PNS entails nerves and ganglions throughout the body and is in turn divided into the somatic and autonomic nervous system. The somatic nervous system controls voluntary movements while the autonomic acts unconsciously, regulating bodily functions through the sympathetic "fight-or-flight" and parasympathetic "rest-and-digest" systems.

The adrenal gland

Adrenal glands are hormone producing glands connected to the sympathetic nervous system. They are situated on top of the kidneys and are composed of two parts, the outer cortex and the inner medulla. The medulla produces and secretes catecholamines (adrenaline and noradrenaline) directly into the blood stream. These hormones are related to the fight-or-flight response, affecting heart rate, blood flow and body metabolism. The catecholamine producing cells are called chromaffin cells, or pheochromocytes, and together with sympathetic ganglia, they make up the sympathetic nervous system.

The neural crest

The neural crest is a vertebrate-specific population of cells present only during embryogenesis. It gives rise to a variety of cell types, including peripheral neurons, enteric neurons, glia, melanocytes, Schwann cells, cranial cartilage cells, and chromaffin cells (*12*). The neural crest is situated between the neural plate and the non-neural ectoderm. Upon closure of the neural tube, neural crest cells (NCCs) undergo an

epithelial-to-mesenchymal transition (EMT), allowing them to become less adhesive and to migrate through the embryo to form different types of cells (*13, 14*). The location of the cells along the anterior-posterior axis of the embryo as well as extrinsic and intrinsic signals determine the fate of these multipotent progenitor cells (*15*).

This section will focus on the differentiation of NCCs to sympathetic neurons and chromaffin cells, as these are related to the formation of neuroblastoma. It has long been thought that these cell types originate from a common progenitor, called the sympathoadrenal precursor cell (*16*). However, data from a recent study reevaluating this view indicate that these cell types diverge at an early state during embryogenesis and that most chromaffin cells and some sympathetic neurons arise from Schwann cell precursors (SCPs) (*17, 18*).

Sympathoadrenal precursor cells

Migration of NCCs throughout the embryo is strictly regulated through a complex gene regulatory network. NCCs with a sympathoadrenal fate migrate ventrolaterally towards the dorsal aorta, instructed by several ligand-receptor interactions, where e.g. bone morphogenic proteins (BMPs) secreted from the dorsal aorta are essential. Once the cells reach the dorsal aorta, they aggregate and activate transcription factors important for differentiation to their respective lineages, including HASH-1, PHOX2A, PHOX2B, Hand2, and GATA2/3. HASH-1 and PHOX2B are both thought to promote neuronal and catecholaminergic properties and cause activation of PHOX2A (*19, 20*). After aggregation of NCCs by the dorsal aorta, cells migrate either dorsolaterally, to form sympathetic neurons, or ventrally to form chromaffin cells (*21*) (Figure 1).

Schwann cell precursor cells

Recently, the dogma related to the development of the chromaffin cells of the adrenal gland has been reevaluated (*17, 22*). Through lineage tracing studies in mice, it has been shown that although the sympathetic neurons and a small subset of the chromaffin cells are generated by ventrally migrating NCCs, the majority of the chromaffin cells are derived from SCPs. SCPs are late-migrating NCCs, that reach the developing adrenal gland by migrating along axons of preganglionic neurons (*23*). Upon reaching the adrenal gland, cells differentiate and express lineage specific genes, such as PHOX2B (*24, 25*) (Figure 1). This indicates that neuroblastoma might originate from different lineages during the embryonal development.

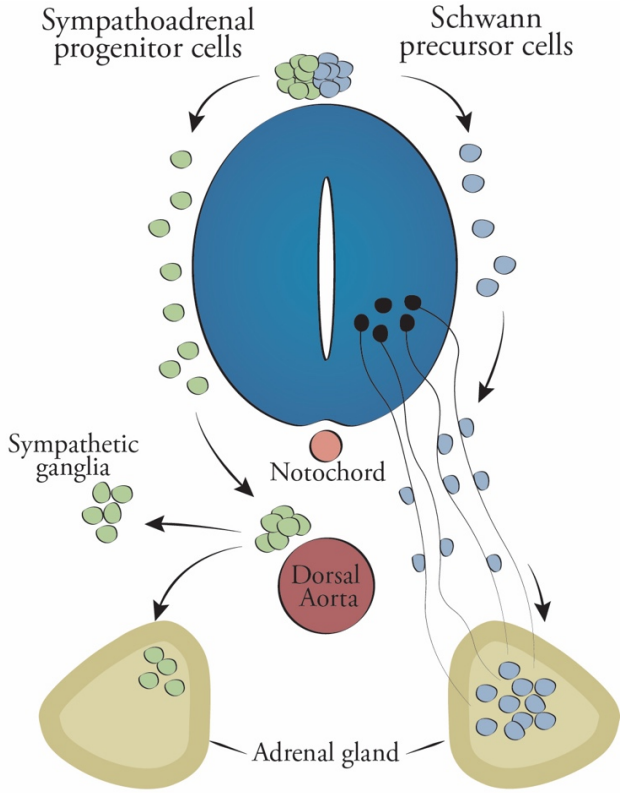

Figure 1. Development of sympathetic ganglia and adrenal medulla.
For simplicity, sympathoadrenal neural crest cells are shown to the left and Schwann cell precursors to the right. Left side: sympathetic neurons and part of the chromaffin cells are developed from sympathoadrenal progenitor cells. Right side: the majority of the chromaffin cells of the adrenal medulla are derived from schwann precursor cells.

Neuroblastoma

Introduction

Neuroblastoma is a pediatric tumor of the sympathetic nervous system and it accounts for 15% of all cancer related deaths in children (*26*). It is an embryonal tumor and affects mainly young children, with a mean age at diagnosis of 17 months (*27*). Tumors can arise anywhere along the sympathetic ganglia, but most primary tumors are located in the adrenal gland (Figure 2). Neuroblastoma shows a wide range of clinical behavior, with some tumors that spontaneously regress while others progress and metastasize despite intensive multi-modal therapy. Due to this, great efforts have been made to divide patients into clinical risk groups with the aim of minimizing the side-effects in patient with lower-risk tumors and optimizing the treatment for increased survival in patients with more aggressive tumors.

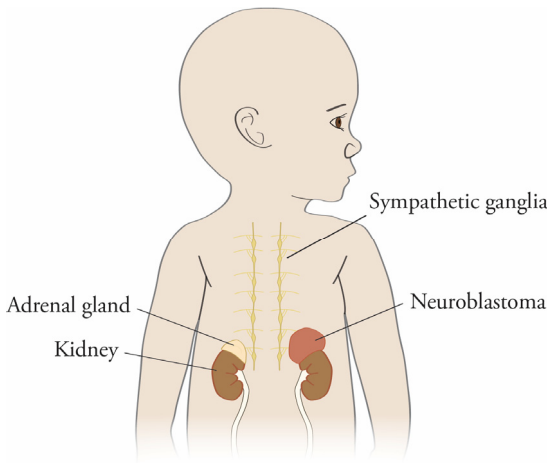

Figure 2. Neuroblastoma.
Neuroblastoma is a childhood tumor of the sympathetic nervous system. The most common primary site for neuroblastoma is the adrenal gland.

Cell of origin

Neuroblastoma is thought to be derived from precursor cells of the sympathetic nervous system, but the exact mechanism of transformation is still unknown. The fact that neuroblastomas can arise along any sympathetic ganglia as well as in the adrenal gland indicates that the cell of origin is either neural crest derived sympathoadrenal cells or Schwann cell precursors, as described in the previous chapter (*28, 29*). Additionally, it has been shown that neuroblastomas at different sites show diverse genomic profiles and that tumors of the adrenal gland generally results in worse prognosis compared to tumors of other primary sites. This also indicates that there could be several initiation mechanisms or cells of origin for neuroblastoma (*30, 31*).

Genetics

Neuroblastoma is a genetically complex disease, with few mutations but frequent larger chromosomal aberrations at the time of diagnosis, indicating that it is a copy-number driven disease. Whole genome sequencing studies show that point mutations overall are rare but those of the *ALK*, *PTPN11*, and *ATRX* genes are among the most common. Chromothripsis, fragmentation of chromosome regions with subsequent random reassembly, and rearrangements of *TERT* are associated with poor outcome. *ATRX* mutations and *TERT* rearrangements both result in telomere lengthening but are mutually exclusive (*32-36*).

The vast majority of neuroblastomas occur sporadically, familial neuroblastoma is found in only 1-2% of all neuroblastoma cases, with mutations in the *ALK* gene being the most common effector in those cases (*37-39*). Mutations in the *PHOX2B* gene have also been linked to familial neuroblastoma (*40, 41*). In sporadic neuroblastoma, amplification of the *MYCN* gene as well as segmental chromosomal aberrations are of importance for the risk stratification of patients.

ALK mutations

The Anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor encoded by the *ALK* gene. It has multiple downstream effectors, including the PI3K and JAK/STAT pathways. Normally it functions during the development of the nervous system but it is a known oncogene in several types of cancer (*42-44*). Mutations results in autophosphorylation and increased kinase activity of the receptor (*45*). Amplification

or mutations of *ALK* have also been found in around 8% of sporadic neuroblastoma and in 14% of all high-risk neuroblastoma and they are associated with poor prognosis (*46, 47*).

MYCN amplification

N-Myc is a homolog of Myc, expressed during neuronal development and encoded by the *MYCN* gene, located on the 2p24 chromosome. N-Myc is a transcription factor and a known oncogene, affecting cell cycle progression and proliferation through several pathways (*48*). N-Myc forms a complex with the Myc associated protein X (MAX) and binds to enhancer-boxes at target genes, thereby regulating the gene expression (*49*). While c-Myc is expressed in all proliferating adult tissues, N-Myc is expressed mainly in developing tissues during the embryogenesis and is almost absent in adult tissues (*50, 51*). Amplification of the *MYCN* gene has been correlated to poor prognosis in neuroblastoma and is one of the most important factors for risk stratification (*52, 53*). It is present in around 20% of all primary tumors and in 50% of high-risk tumors. Even in lower risk tumors without amplification of the gene, increased copy numbers are associated with a worse prognosis (*54*). High activity of the Myc transcriptional targets in non-*MYCN* amplified tumors correlate with a poor prognosis and an undifferentiated histology, even in low or intermediate risk tumors (*55*). Downregulation of N-Myc in neuroblastoma tumors induce differentiation, further indicating a role of N-Myc in maintaining an undifferentiated phenotype in neuroblastoma (*56*).

Segmental chromosomal aberrations

The most common segmental chromosomal copy number alterations in neuroblastoma tumors are gain of 17q, loss of 1p and loss of 11q. They all correlate with worse prognosis, indicating involvement of oncogenes in 17q and tumor suppressor genes in 1p and 11q. Gain of 17q and loss of 1p is correlated with *MYCN* amplification while loss of 11q is inversely correlated with *MYCN* amplification (*57, 58*). Loss of 11q is related to a high amount of segmental aberrations, indicating a chromosomal instability (*59*). Gain of 17q usually arises from unbalanced translocations and it has been shown to be present in more than half of all neuroblastomas (*60*). It has been suggested that Survivin or *PPM1D* are candidate oncogenes related to 17q gain (*61, 62*). For 1p and 11q, loss of heterozygosity of 1p36 and 11q23, respectively, are the most frequently altered regions. However, the exact genes affected are still not clear (*63-65*).

Relapsed tumors

Studies of paired samples from primary and relapsed tumors have shown an increased mutational burden in relapsed samples with both novel single nucleotide variants and copy-number changes previously linked to an aggressive phenotype. Specifically, the mutations acquired were predicted to lead to an increased activity of the Hippo-YAP and Ras-MAPK pathways (*66, 67*). Analysis of *ALK* mutations showed a higher frequency of *ALK* activating mutations in relapsed samples (*68*). This data suggest that targeted therapy may be a feasible option for these patients.

Epigenetics

Epigenetic mechanisms have been described as important for the pathogenesis as well as the intra-tumoral heterogeneity of neuroblastoma. It has been shown that neuroblastoma tumors consist of different cell states, an undifferentiated mesenchymal and a committed adrenergic cell state. These cell states are regulated through distinct super-enhancer-associated transcription factor networks and core-regulatory circuitries, that regulated the transcriptional program of the neuroblastoma cells (*69, 70*).

Diagnosis and staging

The clinical presentation of neuroblastoma varies greatly between patients as it is a very heterogenous disease. The symptoms differ depending on the location of both the primary tumor and the metastases. Tumors in the abdomen can cause hypertension, abdominal swelling or pain while growth or metastases in proximity to the spinal cord may lead to paraplegia.

When neuroblastoma is suspected, a number of tests are conducted. Laboratory testing of urinary catecholamines, such as adrenaline and noradrenaline, can indicate neuroblastoma and is present in 90% of all neuroblastoma patients. Evaluation of tumor spread is done through computerized tomography (CT) imaging or magnetic resonance imaging (MRI). To investigate the presence of both primary tumors and metastases, metaiodobenzylguanidine (MIBG) scanning can be performed. This is done by detecting radiolabeled MIBG in tumor cells through scintigraphy, approximately 90% of neuroblastoma patients have tumors that show an increase of MIBG uptake (*71*). Histological analyses are of great importance for both the diagnosis and risk stratification of neuroblastoma. Neuroblastoma tumors consist of small round blue cells and the level of differentiation correlates to the prognosis (*72, 73*). Finally,

molecular characterization of the tumor is of importance for risk stratification. *MYCN* amplification, diploidy, and chromosomal copy number alterations are all correlated to bad prognosis.

To create international guidelines regarding neuroblastoma risk stratification, the International Neuroblastoma Risk Group (INRG) task force has designed both a tumor staging system and a risk classification system, that determines the treatment used for each patient (*74, 75*).

Neuroblastoma tumor staging

Neuroblastoma tumors are divided into one of four stages (L1, L2, M or MS) based on the tumor location as well as a number of image-defined risk factors (IDRFs). The IDRFs are surgical risk factors based on radiographic images, such as tumors encasing the aorta or other large blood vessels, compression of the trachea or infiltration of adjacent tissues. L1 represent local disease without any additional IDRFs, L2 are locoregional tumors that exhibit one or more IDRFs and M are all metastasized tumors, except for those that meet the criteria of MS (*75*).

The stage MS tumors are a unique group of tumors with metastatic disease but a favorable outcome (*76*). The MS stage encompasses tumors with metastatic lesions in patient under 18 months of age (*77*). When risk factors such as *MYCN* amplification or loss of 11q are present, these patients are considered high-risk but otherwise they have a remarkable prognosis and tumors often regress without treatment (*74, 78*).

Neuroblastoma risk stratification

The four tumor stages are used, together with several other risk factors, to divide patients into pretreatment risk groups, which will determine what treatment protocol the patient will follow. The other factors used for the risk stratification include age at diagnosis, where an age of more than 18 months is correlated to poor prognosis (*79*), tumor histology, including differentiation status, and genetic characteristics, such as cell ploidy, *MYCN* amplification status, loss of 11q, loss of 1p, or gain of 17q (Table 1). The final pretreatment risk groups are: very low-risk, low-risk, intermediate-risk and high-risk and they are determined based on all risk factors. In the lower risk groups, the vast majority of patients show 5-year event-free survival (EFS) while for high-risk patients, the prognosis is still very poor and less than 50% show 5-year EFS (*74*).

Table 1. Neuroblastoma risk stratification factors
Neuroblastomas are divided into risk groups based on a number of risk factors, some are correlated to a favorable outcome and some to poor outcome.

Risk factor	Favorable outcome	Poor outcome
Tumor stage	L1, L2, MS	L2, M
Age at diagnosis	<18 moths	>18 months
Differentiation status	Differentiated	Undifferentiated or Poorly differentiated
MYCN amplification	-	+
Loss of 11q	-	+
Loss of 1p	-	+
Gain of 17q	-	+
Cell ploidy	Triploid, Hyperdiploid	Diploid

Treatment of neuroblastoma

As stated above, neuroblastoma patients are divided into risk groups based on a number of factors, which determines what treatment protocol should be followed. Treatment of neuroblastoma include chemotherapy, surgery, radiotherapy, autologous hematopoietic stem cell transplantation (AHSCT), differentiation therapy and immunotherapy.

Very low-risk and low-risk tumors

The aim for patients with very low- or low-risk tumors have long been to minimize the therapy to reduce the toxicity and side-effects of treatments. These patients have localized tumors and surgical resection is often curative and show excellent 5-year overall survival (OS) (*80, 81*). Infants with smaller tumor masses show excellent survival even with observation only, eliminating the risks of surgery for these patients (*82*). Currently, investigations from the Children's Oncology Group (COG) and Society of Pediatric Oncology, European Neuroblastoma (SIOPEN) are being done to determine if this observational strategy is feasible in older patients as well (NCT01728155 and NCT02176967).

Intermediate-risk tumors

The treatment for patients with intermediate-risk tumors includes surgery and chemotherapy treatment. In recent years, the doses of chemotherapy have been reduced, with maintained high survival rates. The intensity and length of the treatments

is dependent on the response of each patient (*83, 84*). Even in these patients, current investigations of how to minimize the treatment while still keeping a good survival is conducted (NCT02176967).

High-risk tumors

For high-risk patients, the therapy is intense but the survival is still poor. In the last 30 years, the 5-year OS of high-risk neuroblastoma patients have increased from around 30% to 50% (*85*). Current treatment for high-risk neuroblastoma is divided into three parts: induction, consolidation and maintenance therapy.

Induction therapy

The aim of induction therapy is to reduce the tumor burden and to eliminate metastatic lesions before surgical resection. Patients are given a combination of chemotherapeutic agents, but the specific regimen differs between cooperative groups. European protocols currently follow the 'rapid COJEC' regimen, comprised of 8 cycles of vincristine, cisplatin, etoposide, cyclophosphamide, and carboplatin over the course of 70 days (*86*). Patients that respond to the induction therapy has significantly better survival while older patients usually have tumors less responsive to chemotherapy (*87*).

Upon surgery, complete resection of the tumor should be attempted if deemed safe, although this is often difficult due to the high amount of infiltration of large vessels or neuronal tissues in neuroblastoma. For localized high-risk tumors, total resection has shown to improve OS but for metastasized tumors this is still unclear (*88-90*). The risks of surgery should therefore be taken into consideration.

Consolidation therapy

Consolidation therapy through myeloablative chemotherapy with AHSCT has shown to increase the survival of neuroblastoma patients (*91, 92*). However, studies are ongoing to establish the optimal chemotherapy regimen. A combination of busulfan and melphalan has shown promising results in increasing survival compared to other regimens (*93, 94*). For AHSCT, blood stem cells are collected from the peripheral blood and transplanted back after end of chemotherapy (*95, 96*). Additionally, it has been shown that radiation of the tumor bed increases the survival of patients (*97, 98*).

Maintenance therapy

After completion of induction and consolidation therapy, many patients show complete remission. However, relapse is common and patients often benefit from maintenance therapy where they are treated with isotretinoin and anti-GD2

immunotherapy. Isotretinoin (or 13-cis-retinoic acid), a vitamin A derivate that is active during embryonic development, has shown to induce neuroblastoma cell differentiation (*99*). GD2 is a cell surface marker expressed on neuroblastoma cells and treatment with antibodies against GD2 together with GM-CSF and interleukin-2 results in increased survival of patients (*100*).

Relapsed neuroblastoma

Despite the aggressive treatment, many high-risk neuroblastoma patients will have tumors that do not respond or will develop relapses after end of treatment. Relapsed patients are commonly treated with additional chemotherapy or ^{131}I-MIBG therapy (*101-104*). While this has shown an increased survival of patients, the 5-year OS for patients with relapsed tumors is only 20% (*104*). New treatment strategies are needed for these patients.

As described previously, relapsed patients show a higher mutational burden and an activation of the Ras-MAPK pathway, indicating that these patients might benefit from personalized targeted therapy. Several clinical trials are ongoing for treatment of relapsed or refractory neuroblastoma is currently being conducted (*105*). However, the mutational burden is still very low and neuroblastoma patients have a high degree of intratumoral heterogeneity, complicating the use of targeted therapy.

Therapeutic targets and treatments

Introduction

The current treatment against high-risk neuroblastoma is based mainly on chemotherapy and surgery with the addition of radiotherapy, immunotherapy and AHSCT. In the last decades, the survival for neuroblastoma patients has increased remarkably (*106-108*). This is largely due to advances in treatments using chemotherapy as well as major international collaborations (*109*). However, it is primarily within the low- or intermediate-risk group patients that this increase in survival is observed and high-risk patients still only have a survival rate at about 50%. Additionally, many patients relapse after completion of therapy, demonstrating the need for novel treatment strategies (*80, 86, 110, 111*).

The development of high-throughput techniques, including genomic and transcriptomic analyses, has given the opportunity for detailed classification of tumors and opened the possibility for targeted therapy against neuroblastoma. Several studies aiming at molecularly categorizing pediatric diseases, including neuroblastoma, to identify targets are currently in progress. This includes the INdividualized therapy For Relapsed Malignancies in childhood (INFORM) study in Germany (*112*), NExt Generation PErsonalized Neuroblastoma THErapy (NEPENTHE) study in USA (NCT02780128), and the Genomic Medicine Sweden (GMS) studies in Sweden. Nevertheless, as described above for neuroblastoma, pediatric tumors harbor relatively few genomic aberrations, complicating the use of targeted therapy for these tumors.

Recent studies demonstrate that neuroblastomas show a high degree of intratumoral heterogeneity, both at the genetic and molecular level. Even though neuroblastoma overall has a low mutational burden, there is a significant amount of genetic heterogeneity caused by temporal evolution (*113*). Analysis of cell lines and patient samples show that most neuroblastomas include two distinct subpopulations, or cell states, with different transcriptomic profiles, an undifferentiated mesenchymal and a committed adrenergic population (*69, 70*). Data show that the cell populations can interconvert, suggesting plasticity between the cell states. Results indicate that the mesenchymal subpopulation is more resistant to chemotherapy and enriched post-

therapy (*69*). The exact mechanism of this interconversion is not known but *in vitro* studies suggest that it is enabled by activation of the notch signaling pathway (*114*). Results of single-cell profiling studies in neuroblastoma cells also indicate that there is an interconversion of these states and further, this seems to be mediated by a third SCP-like cell state (*29*).

The fact that neuroblastomas show few genomic aberrations but high intra-tumoral heterogeneity needs to be taken into account when treating patients. Personalized medicine as well as general chemotherapy is often followed by treatment resistance due to genetic changes or surviving subclones. It is also challenging to find targeted therapies that can affect all cells within a tumor.

Numerous drugs and pathways are currently being investigated as treatment strategies for neuroblastoma. Several ALK inhibitors, alone or in combination, have shown promising effects in preclinical studies (*115-117*) but initial clinical studies have had more disappointing effects (*118*). This might be due to acquired resistance (*119*). However, a case report of complete remission after ALK inhibition in an ALK mutated patient has been presented, demonstrating that it could be a viable option for a subgroup of patients (*120*). Targeting N-Myc has been a goal for decades and the most established way of achieving this is by indirect inactivation. Bromodomain and extraterminal (BET) domain proteins affect the transcriptional activity of *MYCN (121)* and inhibition affects neuroblastoma growth through downregulation of *MYCN* expression (*122*). Cyclin dependent kinase 1 (CDK1), Aurora A, and phosphatidylinositol 3-kinase (PI3K) have also shown to stabilize N-Myc and have been subjected to therapeutic inhibition in preclinical models of neuroblastoma (*123-125*).

This thesis aimed at investigating novel treatments against neuroblastoma. This includes compounds targeting the PI3K pathway, the Ras pathway and mitotic inhibitors. An introduction to these mechanisms is presented here.

The PI3K signaling pathway

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases activated by receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs). As responders of growth factors and insulin, they are related to several key cellular pathways including cell proliferation, growth and metabolism. They act downstream by phosphorylating the third hydroxyl group of phosphatidylinositol (PI). Mutations or activation of the PI3K signaling pathway is one of the most common alterations in

cancer, making the PI3K pathway a promising target for cancer therapy. PI3Ks are divided into three classes based on their structure and activity (*126*). They act downstream through many different pathways related to oncogenesis, including involving downstream effectors Akt and mammalian target of rapamycin (mTOR).

Classes of PI3K

Class I PI3Ks phosphorylates PI(4,5)P_2 to PI(3,4,5)P_3. They are further divided into two subclasses, Class IA and Class 1B, based on how they are regulated. Class IA is activated by RTKs, GPCRs and certain oncogenes while Class IB is activated by GPCRs only. They consist of two domains, a catalytic and a regulatory subunit. Class IA contains one of three isoforms of p110 catalytic subunits (p110α, p110β, or p110δ) and one of five p85 regulatory subunits, (p85α, p55α, p50α, p85β, or p55γ). Class IB contains a p110γ catalytic subunit and one of the regulatory isoforms p101 or p87. p110α and p110β are expressed ubiquitously while p110γ and p110δ are expressed mainly in leukocytes (*127*). The p85 regulatory units contain a p110-binding domain, flanked by two Src-homology 2 (SH2) domains that binds to activated receptors. In the absence of pathway activating signaling, the regulatory unit of PI3K inhibits the p110 catalytic subunit (*128, 129*). Hence, the regulatory subunit mediates localization and activation of the catalytic subunit that in turn phosphorylates PI. Alternations in the signaling pathway of Class I PI3Ks have been related to cancer and mutations in *PIK3CA*, encoding p110α, and *PTEN* are common in several types of cancer (*130*).

Class II PI3Ks phosphorylates PI to PI(3)P and PI(4)P to PI(3,4)P_2 and consists of only one unit, a p110-like catalytic unit. There are three isoforms (PI3K-C2α, PI3K-C2β and PI3K-C2γ) that are all activated by RTKs, cytokine receptors, and integrins. PI3K-C2α and PI3K-C2β are ubiquitously expressed but PI3K-C2γ expression is mainly restricted to the liver (*131*). The specific functions of the isoforms is still being unraveled but data suggest that the isoforms have distinct, non-overlapping roles related to cell migration, mitosis, and glycogen storage as well as functions related to the endocytic system (*132*).

There is only one known class III PI3K (also called VPS34). It is conserved in all eukaryotes and ubiquitously expressed in mammals. It forms a heterodimer with VPS15 and acts by phosphorylating PI to PI(3)P. It has an important role in vesicle trafficking and autophagy (*133*).

PTEN

The most important regulator of PI3K signaling is the tumor suppressor PTEN. PTEN is a phosphatase whose principal function is to dephosphorylate PI(3,4,5)P_3. Loss of

PTEN results in excessive PI(3,4,5)P_3, driving downstream signaling to promote cell growth and proliferation (*134, 135*).

mTOR

mTOR is a serine/threonine kinase present in one of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of mTOR, the regulatory-associated protein of mTOR (raptor), the proline-rich Akt substrate of 40 kDa (PRAS40), and mammalian lethal with SEC13 protein 8 (mLST8). It is activated by the PI3K mediator Akt through phosphorylation of PRAS40 and Tuberous Sclerosis Complex 2 (TSC2). Phosphorylation of PRAS40 and TSC2 leads to their inactivation and prevents them from inhibiting mTOR (*136-141*). Activated mTORC1 phosphorylates downstream targets ribosomal protein S6 kinase (S6K) (*142*).

mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated protein kinase interacting protein 1 (MSIN1), and mLST8. Far less is known about this complex and how it is regulated and it was first described as an mTOR complex regulating actin of the cytoskeleton (*143, 144*). In the PI3K pathway, it is known to phosphorylate and activate Akt (*145*). In neuroblastoma, it is shown that mTORC2, but not mTORC1, regulate *HIF2A* expression through activation of the PI3K pathway (*146*).

PI3K/Akt/mTOR signaling

Upon growth factor signaling, the SH2 domains of p85 regulatory PI3K units bind to phosphorylated tyrosine on growth factor receptors or proteins. This results in the disinhibition of the p110 catalytic unit and recruitment of the enzyme to the cell membrane. Another way of activating PI3K signaling is by direct binding of p110 to the Ras protein (*147*). Activated PI3K produces PI(3,4,5)P_3, leading to recruitment of signaling proteins with a pleckstrin homology (PH) domain, such as Akt and phosphoinositide-dependent protein kinase-1 (PDK1). The recruitment of Akt and PDK1 enables PDK1 to phosphorylate Akt at T308, followed by phosphorylation at S473 by mTOR complex 2 (mTORC2) (*145*). The phosphorylation and consequent activation of Akt results in further phosphorylation of several downstream targets, including glycogen synthase kinase 3 (GSK3), forkhead box O (FoxO) transcription factors, PRAS40 and tuberous sclerosis complex 2 (TSC2). Phosphorylation of PRAS40 and TSC2 leads to activation of mTOR complex 1 (mTORC1) (*148, 149*). Akt also phosphorylates the cell death protein BCL2-associated agonist of cell death (BAD) (*150*) (Figure 3).

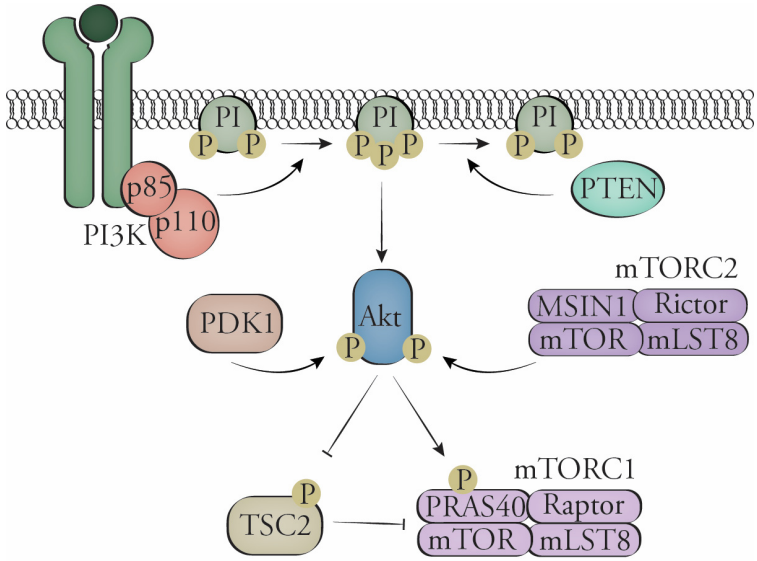

Figure 3. The PI3K pathway.
Extracellular signaling results in activation of PI3K via the p85 regulatory unit. The p110 catalytic unit phosphorylates PI(4,5)P2 to PI(3,4,5)P3. This phosphorylation leads to the recruitment of Akt and PDK1 to the cell membrane, allowing PDK1 to phosphorylate Akt. mTORC2 further phosphorylates Akt to fully activate it. Activated Akt phosphorylates several downstream targets, including TSC2 and PRAS40, both leading to the activation of mTORC1. PTEN inhibits the PI3K pathway by dephosphorylating PI(3,4,5)P3 to PI(4,5)P2.

Targeting the PI3K pathway

PI3K signaling affects several hallmarks for oncogenesis, such as proliferation, cell growth, angiogenesis, and motility, making it a well-studied target for cancer therapy. The pathway have several targetable proteins and inhibitors targeting PI3K, Akt, mTOR, or a combination of them have shown promising preclinical results (*151*). However, these have often had disappointing effects in clinical trials and despite extensive testing only a few have reached the clinic. The rapamycin-derivatives temsirolimus and everolimus are approved for clinical use for several cancer types, including renal cell carcinoma and pancreatic neuroendocrine tumors (*152-156*). PI3K inhibitors idelalisib and copanlisib have been approved for use in leukemia and lymphoma (*157-160*). There are several possible explanations for the relatively low success rate. The PI3K pathway has extensive roles in cell signaling and inhibition often results in resistance due to compensatory mechanisms or activation of alternate pathways (*161, 162*). Inhibition of the pathway have also shown dose-limiting toxicities (*163*). This can be overcome by targeting several parts of the pathway, but it might be difficult to predict what compensatory mechanisms to target. In a clinical setting,

targeted therapy towards the PI3K pathway will probably have to be combined with chemotherapy, immunotherapy, or other targeted therapies (*148*).

Proviral Insertion site in Murine leukemia virus (PIM) kinases are a family of three (PIM1, PIM2, and PIM3) serine/threonine kinases. They are constitutively active, meaning that their activity is directly correlated to expression (*164*). They phosphorylate several target proteins related to survival and growth signaling, including the pro-apoptotic protein Bad and substrates of the PI3K pathway. PIM kinases are oncogenes and can stabilize Myc proteins, promoting Myc-dependent transcription (*165*). PIM activity has been shown to be related to PI3K inhibitor resistance in breast cancer, through activation of PI3K downstream effectors independent of Akt (*166*). PIM inhibition has shown to reduce growth of PDX models of breast cancer with elevated levels of Myc (*167*).

PI3K signaling in neuroblastoma

Mutations in *PIK3CA*, encoding p110α of PI3K, are frequent in several types of cancer but rarely found in neuroblastoma (*168, 169*). However, p110α has been shown to be highly expressed in aggressive neuroblastoma while p110δ expression is more prominent in favorable tumors (*170, 171*). Further, activated Akt correlates with a poor prognosis in neuroblastoma (*172*). Preclinical studies show that PI3K signaling stabilize N-Myc through the phosphorylation and inactivation of GSK3β (*125*). Therapeutic inhibition of PI3K results in downregulation of N-Myc, decreased growth of neuroblastoma cells *in vitro* and *in vivo* and reduced angiogenesis. *MYCN* dependent models were shown to be more sensitive to this inhibition compared to other models. Lastly, the effects were dependent on complete blockage of mTOR and is a result of destabilization of N-Myc (*125, 146, 173-176*).

The Ras signaling pathway

Ras is a family of GTPases that activate signaling related to cell survival and proliferation. Ras responds to extracellular signaling and is activated by RTKs, GPCRs and integrins (*177*). There are three main Ras isoforms in human; H-Ras, K-Ras and N-Ras, and 20% of all cancers carry mutations in at least one of these isoforms (*178*). *KRAS* mutations cover more than 80% of all *RAS* mutations. Expression analyses have revealed distinct locations and functions for the different Ras proteins. For example, *HRAS* and *NRAS* are not essential for mouse development while mutations in *KRAS* cause mouse embryonic lethality at day E12-E14 (*179*). Additionally, the different

isoforms are mutated in different types of cancer, *KRAS* mutations are often prevalent in pancreatic cancer while *NRAS* is found is some hematological cancers (*180, 181*).

Ras is active when bound to guanosine triphosphate (GTP) and becomes inactive when it hydrolyzes GTP to guanosine diphosphate (GDP). This switch between active and inactive states is regulated by guanosine nucleotide exchange factors (GEFs) and GTPase activation proteins (GAPs). GEFs stimulate the dissociation of GDP and uptake of GTP, consequently activating Ras, while GAPs inactivate Ras by increasing the hydrolysis of GTP to GDP. GTP binding to Ras results in conformational changes of the enzyme, allowing it to bind to and activate its effectors (*182*) (Figure 4). Furthermore, post-translational modifications of the protein are essential for its function. Farnesylation by farnesyltransferase is necessary for the activation of all isoforms of Ras (*183, 184*).

Ras activates several pathways downstream by binding to its effectors via a Ras-binding domain (RBD), of which the MAPK pathway is the most extensively studied (*185*). The ability of Ras to activate the PI3K pathway is also of importance for the oncogenic functions of Ras (*147, 186*).

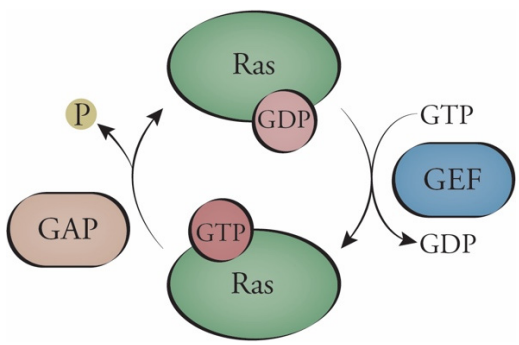

Figure 4. Activation of the Ras protein.
Guanosine nucleotide exchange factors (GEFs) activate Ras by stimulating dissociation of GDP and uptake of GTP. Guanosine activation proteins (GAPs) inactivate Ras by increasing the hydrolysis of GTP to GDP.

The MAPK pathway

The most reviewed effector of Ras is the serine/threonine kinase Raf (or MAPKKK). There are three Raf isoforms; A-Raf-1, B-Raf, and C-Raf. Mutations in B-Raf have been shown to be present in several tumor types, particularly in melanoma and thyroid cancer (*187*). Raf phosphorylates and activates MEK1 and MEK2 (MAPKK), that in turn phosphorylate and activate ERK1/2 (MAPK) (*188*). ERK regulates a diverse

spectrum of targets related to cell growth and proliferation, including the cytoskeleton (*189*) and transcription factors N-Myc and c-Jun (*190-192*).

Targeting the Ras pathway

Ras is the most frequently mutated oncogene in cancer and has long been investigated for possible pharmacological inhibition, but few FDA approved inhibitors exist. Compounds targeting Ras-binding, Ras-activation or its downstream targets Raf, MEK and ERK have all been subject to inhibition. Directly targeting Ras has proven challenging and even though compounds that prevent activation through inhibition of GEF has given promising preclinical results, they have not reached the clinic yet (*193, 194*). K-Ras inhibitor AMG 510 was the first direct Ras inhibitor to reach clinical trials in 2019 and have shown encouraging effects (*195*). MEK inhibitors trametinib, cobimetinib, and binimetinib as well as B-Raf inhibitors vemurafenib, dabrafenib and encorafenib are all approved for use in melanoma (*196-198*).

Ras signaling in neuroblastoma

Ras was first associated with neuroblastoma in 1983 when N-Ras was discovered to be present in SK-N-SH cells (*199*). However, subsequent analyses detected virtually no Ras mutations is neuroblastoma samples (*200, 201*). More recent studies using whole genome and exome sequencing show that mutations related to the Ras pathway, including *NRAS*, *BRAF* and *PTPN11*, are present in neuroblastoma, but at very low frequencies (*32, 202*). A genetic screen has identified loss of tumor suppressor *NF1* to activate the Ras-MAPK pathway and to be related to poor outcome in neuroblastoma (*203*). Comparison of primary and relapsed neuroblastoma tumors show that recurrent mutations in *KRAS* and *HRAS* are present in relapsed samples (*66*), as well as other mutations predicated to activate the Ras-MAPK pathway (*67*). Activation of the pathway in patient tumors has been shown to correlate with a worse prognosis (*204*). These data indicate that targeting the MAPK pathway might be beneficial for high-risk neuroblastoma patients.

The cell cycle

All cells are required to divide to create functional organs and organisms. This division is tightly regulated by a complex control system, ensuring accurate DNA replication and appropriate cell division. However, this control system is often disturbed in cancer

cells, resulting in genomic instability and uncontrolled proliferation. The cell cycle is divided into four phases: The S phase, where DNA replication occurs, the M phase, were cells are divided into two daughter cells, and two gap phases (G1 and G2) that separate these phases. The S, G1, and G2 phases make up the interphase, the time between the mitotic cell divisions.

The progression of cells through the cell cycle is regulated by several checkpoints, the major being the G1, the G2/M and the spindle assembly checkpoints. During the G1 checkpoint, also called the restriction checkpoint, cells with DNA damage are constrained from entering the S-phase. The G2/M checkpoint similarly restricts cells from entering the M-phase prematurely. The spindle assembly checkpoint, also called the mitotic checkpoint, ensures that cells undergo proper segregation of chromosomes. The fact that these checkpoints often are disturbed in cancer makes the cell cycle and the mitosis promising targets for cancer treatment.

Cyclin-dependent kinases

The cell cycle checkpoints are largely controlled by cyclin-dependent kinases (CDKs) and their activator cyclins (*205*). CDKs are serine/threonine kinases and their activity are regulated by binding of cyclins at different stages during the cell cycle. Signals promoting cell cycle entry induce the expression of cyclin D (D1, D2, and D3), that bind and activate CDK 4 and 6, promoting entry into the cell cycle. Next, CDK2 is activated by cyclin E and cyclin A to promote DNA replication during the S phase. At the end of interphase, CDK1 is activated by cyclin B to stimulate the onset of mitosis (*206*). Inhibitory proteins such as p27, p57, and ink4 proteins, block the interaction of CDKs with their respective cyclin. Aberrant signaling of CDKs has been correlated to oncogenesis in several types of cancer (*207-209*). However, the concept that each phase of the cell cycle is regulated by a different CDK have been challenged. For example, genetic studies in mice show that only CDK1 is essential for execution of the cell cycle (*210, 211*).

Mitosis

The M-phase consists of mitosis and cytokinesis. Mitosis is divided into the prophase, prometaphase, metaphase, anaphase, and telophase. During the prophase, chromosomes are condensed and the forming of the mitotic spindle starts. In the prometaphase, the nuclear envelope breaks down and chromosomes can attach to the mitotic spindles. In metaphase the chromosomes have aligned at the equator with microtubules attached to opposite sides of sister chromatids. During anaphase, the sister chromatids are separated to form two chromosomes. The microtubules get shorter

and spindle poles move apart, both contributing to the segregation of chromosomes. Finally, the chromosomes arrive at the spindles to decondense during the telophase. New envelopes form around each chromosome set, making two nuclei and marking the end of mitosis (Figure 5). After mitosis, the cell is divided into two daughter cells through cytokinesis. Actin and myosin filaments make up the contractile ring that divides the cell into two.

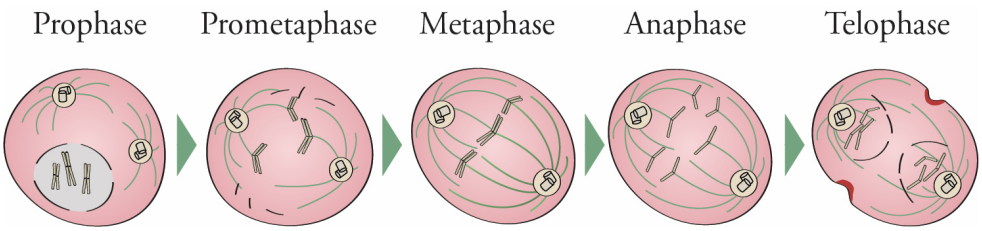

Figure 5. The phases of mitosis.
Prophase: mitosis starts with condensation of chromosomes and formation of the mitotic spindles. Prometaphase: chromosomes attach to the mitotic spindles after breakdown of the nuclear envelope. Metaphase: the chromosomes are aligned with microtubules from each pole attached. Anaphase: sister chromatids are separated and move apart with the spindle microtubules. Telophase: Chromosomes arrive at each respective pole and decondense. New nuclear envelopes are formed and the cell is divided into two.

The mitotic spindle and kinesins

The equal division of sister chromatids to two daughter cells is reliant on the microtubule-based mitotic spindles (*212, 213*). The mitotic spindles are bipolar, with the slow-growing minus end attached to the centrosomes and the fast-growing plus end directed towards the other pole (*214*). The spindles consist of three types of microtubules; interpolar, kinetochore, and astral microtubules. Interpolar microtubules can attach to microtubules from the other pole while kinetochore microtubules attach to the kinetochore of the chromatids. Astral microtubules are directed outward to stabilize the spindles in the dividing cell (*215, 216*). Recently, a fourth class of spindle-associated microtubule have been described, the so-called bridging fibers that are also associated with the segregation of chromatids (*217*). The assembly and function of the mitotic spindles are dependent on several motor proteins, including kinesin.

Kinesins are a family of microtubule-associated motor proteins with diverse functions, including an important role in mitosis. The kinesins bind to microtubules and move across these through hydrolysis of Adenosine triphosphate (ATP) to adenosine diphosphate (ADP). There are more than 40 kinesins identified in mammals, divided into 14 subfamilies (*218*). Mainly three kinesins (kinesin 5, 10, and 14) are responsible for separation of microtubules and chromatids during the mitosis. Kinesin 5 (KIF11, kinesin spindle protein, Eg5) is essential for separation of the centrosomes to create and

maintain bipolar spindles. It binds to antiparallel microtubules and slide these apart by moving towards the plus-end of the microtubules (*219*) (Figure 6).

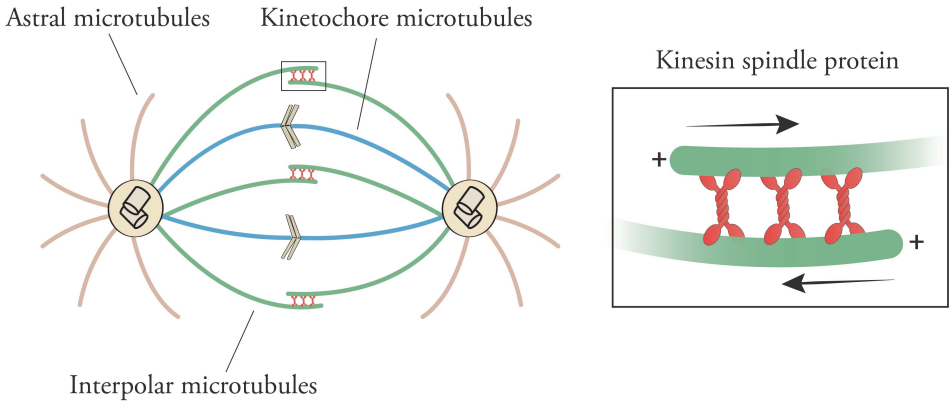

Figure 6. The role of kinesin spindle protein.
There are three types of microtubules of the mitotic spindle; astral, interpolar and kinetochore microtubules. Kinesin spindle protein binds to antiparallel interpolar microtubules and slides these apart, creating bipolar spindles.

Targeting mitosis

Inhibitors targeting microtubules are used in several tumor types, including neuroblastoma. The microtubule-destabilizing chemotherapy agent vincristine is used in the standard treatment for high-risk neuroblastoma and cause apoptosis through inhibition of microtubules in the mitosis. Although effective, microtubule targeting agents result in toxic side-effects, partly because they also target microtubules of non-mitotic cells (*220*). Therefore, inhibitors that specifically target mitotic proteins have been developed, with the indication that they have several advantages over the classical chemotherapeutic drugs (*221*). First, they only target mitotic cells, reducing the side effects related to post-mitotic tissues. Secondly, they do not cause DNA damage, an effect of chemotherapies that has shown to induce resistance. However, they have shown to be less effective in clinical studies compared to common chemotherapies and they might not differentiate between cancer and normal cells, causing dose-limiting toxicities.

Mitotic inhibitors in neuroblastoma

Overexpression of the mitotic protein Aurora A have shown to be correlated to poor prognosis in neuroblastoma and inhibition results in decreased growth of cell lines (*222*). Phase I and II studies of combination treatment using an Aurora kinase inhibitor

(alisertib) with temozolomide and irinotecan show promising results (*223, 224*). A combination of CDK4/6 inhibitor ribociclib and ALK inhibitor ceritinib show synergistic and anti-tumor activity in several *in vivo* models (*225*). Checkpoint kinase 1 (CHK1) is important for DNA damage response in the cell cycle checkpoints and inhibition show anti-tumor effects in neuroblastoma cell lines and cell line-derived xenograft models (*226*). The KSP inhibitor ispinesib has been tested on neuroblastoma cell lines and xenografts through the pediatric preclinical testing program (PPTP). These studies show an effective anti-tumor activity but with a high degree of toxicity in mice (*227*).

Preclinical models of cancer

Cancer research is dependent on relevant models for both studying the disease and testing novel drugs. *In vitro* cultured cancer cells have historically been a keystone of cancer research but they do not recapitulate all characteristics of a tumor. Since many new therapeutic strategies focus on molecularly specific targeting, relevant models that properly represent the disease are more important than ever. Many *in vitro* and *in vivo* models have been developed for studying cancer, including genetically engineered models and models based on human tumor samples, each with their own advantages and limitations. Cancer is a complex and heterogeneous disease that cannot be completely represented in one model and each research field benefit from different models. Here, I present some models currently used in neuroblastoma research with focus on those used in this thesis.

2D *in vitro* models

Cancer cell lines derived from patient tumors grown as 2D cultures, propagated in culture for decades, have been essential for the characterization of cancer and development of therapies (*228, 229*). The advantage of these models include that they are stable to culture and manipulate, facilitating the investigation of specific mechanisms. However, the extent to which these cell lines represent the disease has been extensively discussed. Cell lines in culture undergo genetic changes that affect their morphology, proliferation rate and drug response as a result of clonal selection of properties that benefit expansion in culture (*230, 231*). Additionally, cell lines are commonly grown in serum-supplemented medium. It has been shown that addition of serum to cell lines can cause genetic changes and phenotypical aberrations, including terminal differentiation (*232*). Analyses using gene expression profiling show that cancer cell lines resemble each other rather than the patient tumor they originate from (*233*). In contrast, cell lines grown in serum-free media retain the characteristics of the original patient tumor (*234*).

3D *in vitro* models

By culturing human-derived cancer cells as 3D cultures in serum-free media, some of the limitations of conventionally grown 2D cell lines can be overcome. 3D cultures have the capacity of retaining the morphology and cell-cell interactions of the original tumor. The cultures can be established with scaffold, such as matrigel, or without a supporting scaffold (*235*). 3D tumor cell models, or tumor organoids, have been established for long-term growth for several tumor types (*236-239*). These studies show that the organoids resemble the original tumor, both genetically and phenotypically. Drug testing in these tumor organoids have given promising results and indicate that the drug response in organoids resemble that in patients (*239*).

Organoids were initially defined as *in vitro* 3D structures derived from stem cells, that can mimic the properties and structure of an organ *(240, 241)*. The term tumor organoid is more vaguely defined and is sometimes interchangeably used with tumor sphere or tumor spheroid. Tumor organoids should resemble the structure of the original tumor, and sometimes the definition include the existence of multiple cell types. Even though they are more representative of the disease compared to conventional cell lines, 3D cultures often lack the complexity that is found in *in vivo* models, including the vascular system and other parts of the tumor microenvironment.

Neuroblastoma 3D models

We have developed and characterized neuroblastoma cells *in vitro*, derived from the PDX models described below (*242*). The cells are grown in serum-free medium as free-floating 3D structures and are here termed tumor organoids. The cells retain the genetic aberrations and protein markers of their respective PDX, even after several passages *in vitro*. The tumor organoids grow as tightly packed cells, similar to the structure found in neuroblastoma patient tumors. Reimplantation of the *in vitro* cultured cells into mice results in tumor growth with distant metastases to lung, liver, and bone marrow. Addition of serum to the growth medium induced differentiation of the cells, indicating that culturing the cells in serum-free medium is essential for retaining the tumorigenic and metastatic capacity (*242*). These cultures mimic the disease more closely than conventional 2D, making them suitable for drug testing.

Genetically engineered mouse models

In contrast to models based on the injection or implantation of tumor tissue, genetically engineered *in vivo* models develop spontaneous tumors due to induced genetic changes. The tumors arise in the relevant tissue and enable the use of immunocompetent mice. However, they do not recapitulate the heterogeneity found in human cancers since they are usually based on the overexpression or elimination of only a few genes. Genetically engineered cancer models can be used to validate oncogenes or to study the addiction of certain genes for various tumor types (*243*).

Neuroblastoma genetically engineered mouse models

The genetically engineered TH-*MYCN* mouse model has been extensively used in neuroblastoma research. This model is based on the overexpression of *MYCN* in migrating neural crest cells through the regulation of a tyrosine hydroxylase (TH) promoter. With *MYCN* overexpression, tumors arise in the sympathetic nervous system, with a histology comparable to neuroblastoma. The mice exhibit distant metastases to liver, lung, lymphatics, kidney, ovary, testes, brain and muscle (*244*). However, they do not develop bone marrow metastases. To solve this issue, the TH-*MYCN* mouse model has been further developed to harbor a loss of Caspase-8. These mice develop tumors in the sympathetic nervous system as well as metastases in the bone marrow (*245*).

Another genetically engineered mouse model of neuroblastoma has been developed through targeted expression of the most frequent *ALK* mutation in neuroblastoma. These mice develop tumors consistent with neuroblastoma and the *ALK* mutation was shown to synergize with N-Myc to induce tumors (*246*).

Xenograft mouse models

One approach for studying cancer mechanisms *in vivo* involves mouse xenografts, which is based on the injection or implantation of human cancer cells or tumor pieces into immunocompromised mice. By injecting cultured cell lines into mice, a cell line-derived xenograft (CDXs) can be created. CDXs share several limitations with their origin, the 2D-grown cancer cell lines. This includes failure to recapitulate original tumor histology and to mimic a representative drug response (*247*). CDXs are frequently established by subcutaneous injection of cells, due to the simplicity of measuring and monitoring the tumors. However, when evaluating the drug response in these models, it does not always accurately predict the response of patients (*230,*

247). Orthotopic injections or implantation into specific organs or tissues result in the formation of tumors surrounded by an appropriate microenvironment, creating a more relevant model. Neuroblastoma orthotopic (adrenal) xenograft models have shown to present a more relevant tumor biology compared to subcutaneous models, including more prominent vasculature and metastatic ability (*248, 249*).

Patient-derived xenograft models

In contrast to CDXs, xenograft models based on injection of tumor pieces directly from patients, patient-derived xenografts (PDXs), retain both genotypical and phenotypical characterizations of their original tumor, even after serial passaging (*250-253*). PDXs have also shown to predict the drug response and resistance profile of patient tumors (*252, 254, 255*). Limitations of PDXs for drug testing includes that it required an extensive use of animals and they exhibit a fairly low engraftment rate, making it both time-consuming and expensive. Additionally, tumors can undergo mouse-specific genetic alterations, different from those found in human (*256*). PDXs require the use of immunodeficient mice, making it impossible to study the immune system. However, the establishment of humanized mice might help circumvent this issue

Neuroblastoma PDX models

We have developed orthotopic neuroblastoma PDX models by implanting tumor pieces into the adrenal gland of mice. The PDX tumors show a neuroblastoma-like histology and retain the chromosomal copy number profile of their original patient tumor (*257*). This is consistent with other extensive studies that, through comprehensive genetic and epigenetic analyses, show stable genetic profiles and a correlation between PDXs and patient tumors (*258, 259*). In addition to being more clinically relevant, implantation of tumor pieces into the adrenal gland gives a higher yield compared to subcutaneous implantations (*260*). When compared to neuroblastoma CDXs, PDXs display a more infiltrative growth into adjacent tissue. The PDXs also metastasize to lung, liver and bone marrow and they show a stable geno- and phenotype through serial passaging (*257, 261*). The neuroblastoma PDXs retain several aspects of the tumor microenvironment found in high-risk neuroblastoma, including tumor-associated macrophages, cancer-associated fibroblasts, pericytes, and endothelial cells. However, the stromal cells were of mouse origin rather than human and the cell types present were dependent on the mouse strain (*262*).

The present investigation

Overview and aims

Neuroblastoma is the most common extracranial solid tumor in children. Half of all neuroblastomas are considered to be high-risk and among these patients, survival is poor and relapses common despite heavy multi-modal treatment. The overall aim of this thesis was to identify and investigate the effect of novel treatments against high-risk neuroblastoma. The results are presented in four separate papers, summarized and discussed below.

The specific aims of this thesis were:

I. To investigate the anti-tumor effects of triple kinase PIM/PI3K/mTOR inhibition in neuroblastoma.

II. To identify and test novel drugs with specific activity towards neuroblastoma from a high-throughput drug screen.

III. To study the tumor inhibiting effects of rigosertib.

IV. To identify drugs that act synergistically with KSP inhibitor filanesib.

Paper I: Anti-tumor effects of PIM/PI3K/mTOR triple kinase inhibitor IBL-302 in neuroblastoma

The fact that PI3K pathway inhibitors have shown promising effects in neuroblastoma (*125, 149, 263*) and that resistance has been linked to PIM activity in other tumor forms (*166*) led us to investigate the effect of simultaneous inhibition of the PI3K, mTOR and PIM pathways. We utilized the dual PIM/PI3K inhibitor IBL-202 and the triple PIM/PI3K/mTOR inhibitors IBL-301 and IBL-302.

Investigation of inhibition in a range of cell lines representing different tumor types demonstrated that neuroblastoma was particularly sensitive to triple PIM/PI3K/mTOR inhibition. Additionally, PIM/PI3K/mTOR inhibition was more effective in reducing neuroblastoma cell viability compared to PI3K inhibition alone. All multi kinase inhibitors affected downstream targets of PI3K, including downregulation of phosphorylated Akt and S6K. The inhibitors also induced morphological differentiation of neuroblastoma cells, in parallel with increased levels of the differentiation linked Growth Associated Protein 43 (Gap43). Importantly, the inhibitors also reduced N-Myc protein levels in *MYCN* amplified neuroblastoma cells. All inhibitors decreased viability and increased cell death through apoptosis in neuroblastoma cells. However, triple kinase inhibition was shown to be more effective at lower concentrations (as determined by EC_{50}) compared to dual inhibition.

Using inhibitors targeting the PIM, PI3K, and mTOR signaling pathways simultaneously, we showed dose-dependent effects in neuroblastoma PDX-derived cells as well as in conventional neuroblastoma cell lines. Further, triple kinase inhibition was shown to be more effective than both single PI3K inhibition and dual PIM/PI3K inhibition. This suggests that targeting these three pathways is a promising approach for the treatment of neuroblastoma. However, although inhibition in xenograft models *in vivo* resulted in decreased tumor growth, these effects were less pronounced. There are several possible explanations for this. *In vivo* settings require a sufficient therapeutic window that allows effect without toxic side effects. This can be influenced by the tumor model as well as the mouse strain used. Additionally, poor pharmacokinetic profiles of the drug or solvent would result in the drug not reaching the tumor in appropriate doses. However, IBL-302 has shown tumor-inhibiting effects in breast cancer xenograft models, although using higher drug concentrations (*264*).

PI3K inhibitors have shown to induce decreased neuroblastoma growth through the destabilization of N-Myc. Consistent with this we showed a downregulation of N-Myc after treatment with IBL-inhibitors in *MYCN* amplified PDX cells. However, when analyzing viability of cells post treatment, the effect of the inhibitors was similar

between *MYCN* amplified and non-*MYCN* amplified cell lines, suggesting that the PIM/PI3K/mTOR inhibitors also have Myc-independent effects.

Currently, a combination of chemotherapeutic agents is used in the treatment of neuroblastoma. While effective for many patients, this leads to long-term side effects, such as heart failure and secondary malignancies. Combination treatment of PIM/PI3K/mTOR inhibition with chemotherapy agent cisplatin, commonly used in standard treatment of neuroblastoma, showed synergistic activity *in vitro*, analyzed by tumor cell viability. *In vivo,* treatment with PIM/PI3K/mTOR inhibition and cisplatin resulted in delayed tumor growth and increased survival of neuroblastoma PDX models. This suggests that adding inhibitors targeting these pathways to current treatment enable lowered chemotherapy doses while still retaining potent effects. This would reduce the risk of both acute and long-term side effects. Further studies elucidating the effect of combining current neuroblastoma treatment with targeted therapy of the PIM, PI3K, and mTOR pathways needs to be conducted.

Paper II: Therapeutic targeting of KSP in preclinical models of high-risk neuroblastoma

We performed a high-throughput drug screen using PDX-derived tumor organoids to find novel treatments against neuroblastoma. The screen was based on a set of 525 approved or emerging oncology drugs and cell viability was used as a readout. The drug activity was calculated using a drug sensitivity score (DSS). Data from a counter screen using healthy bone marrow-derived mononuclear blood cells was used to identify drugs that specifically target neuroblastoma cells. By calculating a selective DSS (DSS(PDXs) – DSS(controls), we identified 56 drugs with selective neuroblastoma activity. We further selected compounds previously not extensively tested in neuroblastoma, among them the KSP inhibitor filanesib (or ARRY-520).

Analysis of *KIF11*, the KSP encoding gene, in publicly available datasets showed that the expression is high in neuroblastoma, compared to normal tissue as well as other tumor types. Within the neuroblastoma cohorts, high *KIF11* expression was associated with a worse prognosis. *KIF11* gene dependency analysis was performed by using publicly available datasets based on genome-wide viability screens using CRISPR-Cas9 or RNA interference (RNAi). Theses analyses showed that *KIF11* is important for neuroblastoma cell viability.

KSP inhibition of neuroblastoma organoids resulted in dose-dependent decrease of cell viability, mitotic arrest, and increased cell death through apoptosis. RNA sequencing

showed upregulation of mitosis-associated genes in treated cells, further indicating arrest of mitotic cells post treatment. *In vivo*, KSP inhibition caused complete response, i.e. disappearance of measurable tumor, in 6/8 mice of one PDX model. In orthotopic PDX models, treatment resulted in significantly slower tumor growth and increased survival of mice.

We demonstrated that KSP inhibition is a promising treatment strategy for neuroblastoma using several approaches, including analysis of public datasets of neuroblastoma patient samples, gene dependency analysis and treatment of PDX models *in vivo* and *in vitro*. We showed consistent powerful effects across all neuroblastoma models with complete tumor regression in one model, which is rarely seen in preclinical studies of novel treatments.

High-throughput drug screens can be an effective way of discovering novel drugs for the treatment of cancer, but clear limitations also apply. We have used cell viability as a readout to measure the effect of the drugs. This assay does not differentiate between reduced proliferation and cell death, which may lead to overestimation of the drug activity. Additionally, we selected drugs based on a differential response in neuroblastoma cells and control cells. The control cells are primary blood-derived cells and might proliferate at a lower rate than the neuroblastoma cells. This would favor the selection of cell cycle inhibitors as they target dividing cells. This limitation can be applied to all preclinical research that utilizes models to study drug response, as *in vitro* cell cultures and mouse tumor models proliferate faster than patient tumor. However, we show that KSP inhibition is effective in an orthotopic PDX model derived from a treatment-resistant relapse with slow-growing tumors, indicating that the proliferation rate is not the only factor behind the treatment response.

Inhibitors targeting mitotic proteins were developed based on the rationale that microtubule inhibitors, currently used in the treatment of several cancer types, act through inhibiting the mitosis. While microtubule inhibitors, such as vinca alkaloids and taxanes, have been successful strategies for treating cancer, they target both mitotic and non-mitotic cells, resulting in dose-limiting toxicities. Specific mitotic inhibitors could circumvent this issue. In the case of KSP inhibitors, this protein is not expressed in mature neurons and peripheral neuropathy, a common side-effect of microtubule inhibitors, could be avoided. However, although mitotic inhibitors have shown promising results in preclinical models, they have had mostly disappointing effects in clinical trials. This might be due to fact that conventional microtubule inhibitors are not successful due to mitotic inhibition but through other microtubule-associated mechanisms, such as intracellular trafficking (*265*). Others suggest that chemotherapeutic agents are successful due to the fact that they are administered as prodrugs, while specific mitotic inhibitors are not (*266*).

In clinical trials, filanesib has shown a better pharmacokinetic profile compared to previous KSP inhibitors (*267*). High effect of filanesib has been linked to instable Mcl-1, while tumors dependent on other survival signals, such as Bcl-2 or Bcl-Xl, are more resistant to filanesib treatment (*268, 269*). Filanesib has mostly shown clinical effect in hematological malignancies and is currently in clinical trials for use in multiple myeloma. Our results of filanesib sensitivity, based on data from the Genomics of Drug Sensitivity in Cancer (GDSC) screening program, show that *in vitro* models of neuroblastoma are among the most sensitive, together with multiple myeloma and several types of leukemia. This suggests that neuroblastoma is a promising candidate for KSP inhibition.

Resistance to filanesib is expected through activation of alternate pathways that can promote spindle polarity independently of KSP. Is has been shown that KIF15, while not essential for the spindle segregation when KSP is present, can overtake this role when KSP is ablated (*270, 271*). Downregulation of dynein mitotic proteins, that normally counteracts KSP, can also compensate for KSP inhibition (*272*). Resistance towards KSP inhibitors might also occur through point mutations of the inhibitor binding site (*273-275*).

In conclusion, using a high-throughput drug screen we identified KSP inhibitor filanesib as a potent anti-neuroblastoma drug. We show potent effects of KSP inhibition in several preclinical models, including mice with orthotopic PDX tumors. However, there might be a need for combination treatment in patients to avoid resistance.

Paper III: Preclinical evaluation of rigosertib for the treatment of high-risk neuroblastoma

Rigosertib was identified as one of the top selective drugs in the high-throughput screen performed in Paper II. Analysis of publicly available screening data of multiple tumor types revealed that neuroblastoma is the most sensitive to rigosertib. This indicates that rigosertib represents a promising treatment for this tumor type.

Treatment of PDX-derived neuroblastoma organoids with rigosertib resulted in reduced viability, increased cell death, and activation of caspase-3/7. Cell cycle analysis displayed mitotic arrest through an upregulation of cells in the G2/M phase. By western blot analysis, we showed that rigosertib treatment resulted in decreased amounts of phosphorylated Akt and phosphorylated ERK but no effect on phosphorylated C-Raf or N-Myc protein levels. RNA sequencing of treated cells revealed upregulation of

several biological processes after treatment with rigosertib, including metabolic processes, cell cycle and cell death. Gene set enrichment analysis showed that the TP53 pathway, G2M checkpoint and the mitotic spindle were involved in the response to rigosertib treatment. Treatment of mice with subcutaneous PDX tumors resulted in significantly delayed tumor growth and increased survival. TUNEL staining of tumor sections revealed a significant increase of apoptotic cells in treated tumors.

Rigosertib was first described as a PLK1 inhibitor (*276*) but this has been disputed by other groups that has shown effects of rigosertib without affecting PLK1 (*277, 278*). It was later suggested that rigosertib is an inhibitor of the Ras-binding domain, thus affecting several pathways, including the MAPK and PI3K pathways (*279*). However, subsequent studies have not reproduced these results but instead suggested that rigosertib work as a microtubule destabilizing agent (*280-282*).

Our results showed that treatment of neuroblastoma PDX cells with rigosertib induced cell cycle arrest in the G2/M phase at an earlier time point compared to the effects on Ras downstream effectors. This indicates that interference with Ras is not the primary mechanism of action for rigosertib in neuroblastoma. Additionally, our RNA sequencing results were coherent with previous studies that suggest rigosertib to be a microtubule-destabilizing agent.

Despite the ongoing discussion regarding the mechanism of action for rigosertib, this inhibitor is currently in clinical trials for *KRAS* mutated lung adenocarcinoma (NCT04263090) and Myelodysplastic syndromes (NCT01904682). Our studies on neuroblastoma models indicate that rigosertib might be a possible treatment strategy for neuroblastoma patients. However, treatment with rigosertib did not result in regression of PDX tumors in mice, suggesting that rigosertib needs to be combined with other therapeutic strategies for sufficient effects in patients.

Paper IV: Identification of synergistic drug combinations against high-risk neuroblastoma

In paper II, we demonstrated substantial effects of the KSP inhibitor filanesib on neuroblastoma PDX models *in vitro* and *in vivo*. However, combination treatment is likely needed to achieve complete tumor response and to avoid resistance. Using our most resistant PDX-derived neuroblastoma organoid model, we performed a high-throughput combination drug screen. A drug library of 527 drugs was used as a baseline, with the addition of 0 (control), 0.5 or 1 nM filanesib. Neuroblastoma cell viability was used as the readout and DSSs were calculated to assess drug response. The

drug combination screen revealed that the addition of filanesib at these low concentrations resulted in minor overall differences of the drug response in general.

To further analyze the synergy of combining filanesib with other drugs, we utilized DECREASE (Drug Combination RESponse prEdiction), a machine learning tool that predicts complete combination dose-response matrices using a limited set of data. The response of each drug, alone or in combination with 0.5 or 1 nM filanesib, as well as the single response of filanesib at 0.1 – 1000 nM, was used to create dose-response matrices of the predicted combination treatment responses. The predicted matrices were subsequently used to calculate total synergy scores and most synergistic area (MSA) scores using the Bliss independence model. The combinations within the top 20^{th} percentile of total synergy scores and MSA scores were selected, resulting in 85 drug combinations. The matrices of these 85 drugs were examined separately, and 10 drugs with promising matrices were chosen for validation.

Validation of drug combinations was performed by testing them in matrices with 8 concentrations of each drug. The response was evaluated through viability and synergy was calculated using the Bliss independence model. Analysis of drug sensitivity scores and MSA scores revealed that MEK inhibitor trametinib and farnesyltransferase inhibitor lonafarnib act synergistically with KSP inhibitor filanesib to reduce neuroblastoma cell viability.

The use of drug combinations is a promising strategy to avoid acquired resistance to treatments and to elicit greater anti-tumor effects. Synergistic drug combinations, i.e. combinations that elicit a greater effect than the single drugs combined, allows the use of lower doses with a subsequent reduction of adverse side-effects. However, the identification of promising drug combinations experimentally is only feasible to do on a smaller set of drugs. Our results demonstrate the use of high-throughput drug screens in combination with *in silico* prediction for the identification of synergistic drug combinations. By validating predicted synergistic combinations, we show promising results of combining trametinib or lonafarnib with filanesib. However, this data is preliminary and needs to be confirmed in several tumor models. Furthermore, the anti-tumor effects should be verified using other methods, such as various cell death assays and *in vivo* testing.

Overall conclusions

In this thesis, I have identified and investigated the effects of several novel therapies against the childhood tumor neuroblastoma using clinically relevant PDX models and PDX-derived tumor organoids.

In paper I, we showed clear effects of simultaneous inhibition of PIM, PI3K and mTOR using multi kinase inhibitors. The inhibitors induced differentiation, increased neuroblastoma cell death and showed synergistic effects with cisplatin.

In Paper II, we identified several novel anti-neuroblastoma drugs through a high-throughput drug screen. Among these drugs we found filanesib and rigosertib, two inhibitors further investigated in paper II and III, respectively. We showed promising effects of KSP inhibition in several PDX models and inhibition caused complete tumor regression in one PDX model. In paper III, we show that rigosertib caused mitotic arrest and cell death *in vitro* and delayed tumor growth and increased survival *in vivo*. The mechanism of rigosertib is debated but our results suggested that the main mechanism of action in neuroblastoma cells is interference with mitosis.

Paper IV aimed at identifying drugs that work synergistically with filanesib. Using a high-throughput combination drug screen and synergy prediction tools with subsequent validation of identified combinations, we found promising drug combinations that will be further investigated.

In conclusion, we identified several promising treatment strategies for neuroblastoma. In a clinical setting, a combination of several drugs will most likely be used. Further investigation of the identified synergistic combinations as well as combination with currently used treatment of neuroblastoma is warranted.

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