From the Department of Women's and Children's Health

Karolinska Institutet, Stockholm, Sweden

TARGETING ALK AND WIP1 – NEUROBLASTOMA PRECISION MEDICINE UNDER DEVELOPMENT

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Karolinska
Institutet

Stockholm 2022

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Published by Karolinska Institutet.

Printed by Universitetsservice US-AB, 2022

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ISBN 978-91-8016-808-3

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TARGETING ALK AND WIP1 – NEUROBLASTOMA PRECISION MEDICINE UNDER DEVELOPMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Karolinska Institutet, sal Atrium, Wargentinhuset, Nobels väg 12B, 171 77 Stockholm, Nov 18, 2022 at 9:00

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To my family

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer orsakas vanligen av genetiska förändringar, s.k. mutationer, i cellens arvsmassa vilka ibland är medfödda men oftast förvärvade. Cellen börjar ignorera signaler om tillväxtstopp och vila, och börjar dela sig okontrollerat. Cancerceller av cancertyper som är vanliga bland vuxna bär ofta på väldigt många olika mutationer. Kanske har det under människans flera decennier långa liv ansamlats olika mutationer som i summan leder till att cellen till slut tappar kontroll och blir en cancercell. Barncancerceller har i jämförelse långt färre olika mutationer, vilket kan betyda att de mutationer som ändå finns är desto viktigare. En enda mutation kan då till exempel leda till ett felaktigt protein som driver på cancersjukdomen, likt en gaspedal som fastnat (onkogen), eller en trasig broms (tumörsuppressorgen). Målstyrd cancerbehandling syftar till att hitta och bekämpa dessa felaktiga gaspedaler och trasiga bromsar.

Neuroblastom är en av de vanligare formerna av barncancer och drabbar oftast små barn – 90% är yngre än fem år då diagnosen ställs. Tumörerna utgår från det sympatiska nervsystemet och uppstår oftast i binjuren eller längs sympatiska gränssträngen, dvs intill kotpelaren i bröstkorgen eller buken. Det rör sig om en mycket heterogen sjukdom, där tumören hos vissa patienter kan försvinna av sig själv, medan andra har dålig prognos trots intensiv behandling med cellgifter, strålning, kirurgi och stamcellstransplantation. Överlevnaden hos dessa barn med högrisk-neuroblastom är endast omkring 50%.

Olika biologiska undergrupper korrelerar med det kliniska förloppet av neuroblastom, och flera olika cancerdrivande proteiner har identifierats. Denna avhandling befattar sig med två av dessa, ALK och WIP1.

ALK är ett receptorprotein som är viktigt för nervcellers utveckling och normalt skall signalera vidare när en aktiverande molekyl, en s.k. ligand, kopplar på sig. Det muterade ALK-proteinet sänder istället hela tiden utan att vänta på startsignalen, det har alltså omvandlats till ett onkoprotein. Muterat ALK driver neuroblastomsjukdom i ca. 10% av fallen. Flera olika ALK-hämmare finns numera tillgängliga, men erfarenheten av att använda dessa vid just neuroblastom är ännu begränsad. Vi har i två delarbeten tagit avstamp från enskilda barns sjukdom för att lära oss mer om ALK-hämmande neuroblastombehandling.

I **Delarbete I** beskrivs ett barn med metastaserat högrisk-neuroblastom som på grund av en odiagnostiserad underliggande sjukdom fick livshotande biverkningar av cellgift-behandlingen. Då hans tumörvävnad undersöktes med helgenomsekvensering hittades en ALK-mutation av en tidigare okänd sort. Då denna ALK-mutation modellerades i cellkultur kunde dess sjukdomsframkallande egenskaper påvisas, och man kunde även fastställa att denna mutation medför resistens mot den dåförtiden vanligaste ALK-inhibitorn crizotinib, men istället är känslig för den nyare substansen ceritinib. Genom särskild läkemedelslicens kunde patienten erbjudas behandling med ceritinib, vilket ledde till ett lyckosamt behandlingsresultat utan kvarvarande påvisbar cancer.

Den andra patienten, beskriven i **Delarbete II**, insjuknade som spädbarn i metastaserat neuroblastom som inte svarade på någon av cellgiftskombinationerna. Det genomfördes därför en utökad biologisk analys som påvisade aktivering av proteinet ALK utan att någon ALK-mutation kunde påvisas. Istället hittades en mutation i ALK-liganden, vilket aldrig tidigare beskrivits, men man vet numera från djurstudier att överuttryck av ALK-ligand kan predisponera för neuroblastomsjukdom. Då det också fanns extra genmaterial av den kromosombit som innehåller generna för ALK och ALK-liganden, ansågs det möjligt att mutationen i ALK-liganden bidragit till sjukdomsuppkomsten. Eftersom dessutom aktivering av proteinet TRKA påvisades, valdes en ALK-hämmare som också hämmar TRKA, entrectinib, och även visade god effekt mot neuroblastomceller i cellodling. Genom särskild läkemedelslicens och ett s.k. ”compassionate-use program” kunde patienten erhålla behandling med entrectinib, vilket snabbt förbättrade barnets allmäntillstånd och över flera år gav en långsam tillbakagång av hans metastaser.

Den vanligaste ogynnsamma genetiska avvikelsen i neuroblastom är extramaterial av den långa armen av kromosom 17 (17q), vilken bland annat innehåller *PPM1D*, genen för proteinet WIP1 som kontrollerar det viktiga tumörsuppressorproteinet p53. En förlorad funktion av p53 är en vanlig orsak till cancer. WIP1 har visat sig korrelera till aggressiv cancer och dålig prognos för barn med neuroblastom, och överuttryck av WIP1 i möss predisponerar för tumörutveckling.

I **Delarbete III** har vi undersökt effekten av WIP1-hämning på neuroblastom och visat att blockering av genen (knockdown) bromsade neuroblastomcellernas tillväxt både i cellodling och i djurmodell. Farmakologisk hämning av WIP1 undersöktes genom att jämföra olika WIP1-hämmares effekt på olika neuroblastom- och medulloblastomcellinjer. Substansen SL-176 visade sig vara mest effektiv och prövades även framgångsrikt i djurmodell, där tumörerna visades växa långsammare men inte försvinna under behandling med SL-176.

För att identifiera molekylära strategier som lämpar sig för kombination med WIP1-hämning i behandling av neuroblastom, genomförde vi i **Delarbete IV** en kombinations-screening där SL-176 testades ihop med över 500 olika cancermedel. Vi fann att substansen GSK-J4 var en lovande kombinationskandidat, en hämmare av proteinet JMJD3 som medverkar till aktivering och avstängning av olika gener. I fem olika neuroblastomcellinjer kunde vi bekräfta att kombinationen av SL-176 och GSK-J4 verkar starkt synergistiskt och hämmar cellernas tillväxt genom att aktivera cancerhämmande cellulära mekanismer.

Våra studier syftar till att bidra med ny kunskap kring målinriktad precisionsmedicinsk behandling med det övergripande målet att förbättra överlevnad och livskvalité för barn med neuroblastom. Olika ALK-hämmande läkemedel finns redan tillgängliga för kliniskt bruk och utmaningen består i att matcha rätt patient till rätt substans samt att hitta rätt kombination med annan cancerbehandling. Samtidigt återstår mycket arbete innan WIP1-hämning kan komma att användas i kliniken. Dock är det vår övertygelse att olika former av målinriktad behandling i framtiden kommer att göra stor nytta och erbjudas alla barn med högrisk-neuroblastom.

ABSTRACT

Neuroblastoma is the childhood solid tumor accountable for the largest number of deaths, calling for improved treatment. Some genetic alterations are considered crucial in driving initiation and progression of neuroblastoma. These are usually associated with poor prognosis and are viewed as potential therapeutic targets. Anaplastic lymphoma kinase (ALK) is one of the best-established disease drivers in neuroblastoma, harboring oncogenic mutations or amplifications in about 10-15 % of all cases. In 2013, results from the first phase I study of an ALK inhibitor in neuroblastoma, crizotinib, were announced showing disappointing clinical response. Improved later-generation ALK inhibitors have since become available while biological and clinical understanding of ALK mutations in neuroblastoma has increased.

In **Paper I**, a patient with a metastatic high-risk neuroblastoma suffered extreme toxicity due to an underlying condition, Fanconi anemia, prohibiting further conventional treatment. Whole genome sequencing of tumor material revealed a novel ALK variant, *ALK*-I1171T, which was identified as a potent gain-of-function mutant resistant to crizotinib, but sensitive to later-generation ALK inhibitors including ceritinib. It was shown that ceritinib was equally effective as crizotinib in a panel of ALK-driven neuroblastoma cell lines. As a result, the child could be offered ceritinib in monotherapy, achieving complete metastatic remission and enabling resection of the primary tumor, and long-term survival.

In **Paper II**, activation of ALK as well as tropomyosin-related kinase A (TRKA) was observed in the absence of corresponding genomic alterations in an infant with metastasized and treatment-refractory neuroblastoma. A novel germline mutation of the ALK ligand ALKAL-2 was revealed by whole genome sequencing, which in cell culture and drosophila experiments was shown to be functional. Since an inhibitor targeting both ALK and TRKA was available in entrectinib, this drug was tested in neuroblastoma cell lines. Subsequently entrectinib treatment could be offered to the patient, producing a prompt improvement of clinical status and a gradual decline of catecholamine markers and metastases over the course of several years.

The majority of high-risk neuroblastomas contain a gain of chromosome 17q, a feature that correlates with aggressive disease and poor prognosis. The gene *PPM1D* is situated within the gained region and encodes the phosphatase WIP1, an inhibitor of p53 and a negative regulator of DNA damage response. WIP1 is often overexpressed in neuroblastoma, and overexpression in transgenic mice predisposes to tumor formation. In **Paper III** we showed that WIP1 expression correlates to 17q gain in neuroblastoma and medulloblastoma cell lines, which are also highly dependent on WIP1. Knockdown of WIP1 delayed tumor growth. Among different small molecule WIP1 inhibitors evaluated, SL-176 was demonstrated to be effective in all tested cell lines regardless of p53 mutational status. Pharmacologic inhibition of WIP1 with SL-176 in xenograft-bearing mice curbed tumor growth.

To achieve an improved cytotoxic effect, a drug combination screening with the WIP1 inhibitor SL-176 was conducted in **Paper IV**, identifying combination with epigenetic modification by inhibition of the histone demethylase JMJD3 as the most synergistic strategy. Combination of SL-176 and the JMJD3 inhibitor GSK-J4 showed strong synergism in a panel of neuroblastoma cell lines with regard to cell viability, WIP1 downstream targets, cell cycle arrest and apoptosis. Pathway analysis of differentially expressed genes, as identified by RNA sequencing, revealed enrichment of genes involved in pathways associated with DNA damage response.

While ALK inhibitors are already available to a subset of neuroblastoma patients, WIP1 is a promising therapeutic target where much work remains before patients may potentially benefit. It is our conviction that in the future, targeted therapy will be available to all high-risk neuroblastoma patients. This thesis represents a few small steps on the road to accomplish that goal.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THIS THESIS

I. Guan J*, Fransson S*, Siaw JT*, **Treis D***, Van den Eynden J, Chand D, Umapathy G, Ruuth K, Svenberg P, Wessman S, Shamikh A, Jacobsson H, Gordon L, Stenman J, Svensson PJ, Hansson M, Larsson E, Martinsson T, Palmer RH, Kogner P, Hallberg B
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Cold Spring Harbor Molecular Case Studies. 2018 Aug 1;4(4):a002550.

II. **Treis D*,** Umapathy G*, Fransson S*, Guan J, Mendoza-García P, Siaw JT, Wessman S, Gordon Murkes L, Stenman J, Djos A, Elfman LHM, Johnsen JI, Hallberg B, Palmer RH, Martinsson T, Kogner P
Sustained Response to Entrectinib in an Infant With a Germline ALKAL2 Variant and Refractory Metastatic Neuroblastoma With Chromosomal 2p Gain and Anaplastic Lymphoma Kinase and Tropomyosin Receptor Kinase Activation.
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III. Milosevic J, **Treis D**, Fransson S, Gallo-Oller G, Sveinbjörnsson B, Eissler N, Tanino K, Sakaguchi K, Martinsson T, Wickström M, Kogner P, Johnsen JI
PPM1D Is a Therapeutic Target in Childhood Neural Tumors.
Cancers (Basel). 2021 Nov 30;13(23):6042.

IV. **Treis D**, Tümmler C, Åkerlund E, Pepich A, Seashore-Ludlow B, Sakaguchi K, Kogner P, Johnsen JI, Wickström M
Targeted inhibition of PPM1D and histone H3K27 demethylase activity synergistically suppress neuroblastoma growth.
Manuscript

*These authors contributed equally to the manuscript and share primary authorship

RELATED PAPERS NOT INCLUDED IN THIS THESIS

Milosevic J, Fransson S, Gulyas M, Olsen TK, Gallo-Oller G, **Treis D**, Elfman LHM, Wilhelm M, Martinsson T, Baryawno N, Kogner P, Johnsen JI.
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Neuroblastoma Heterogeneity, Plasticity, and Emerging Therapies.
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Djos A, **Treis D**, Fransson S, Gordon Murkes L, Wessman S, Ásmundsson J, Markström A, Kogner P, Martinsson T
Multifocal Neuroblastoma and Central Hypoventilation in An Infant with Germline *ALK* F1174I Mutation.
Diagnostics. 2022 Sep 19, 12(9), 2260.

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LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma kinase
ALKAL1, ALKAL2	ALK ligands
CNS	Central nervous system
ctDNA	Circulating tumor DNA
COG	Children's Oncology Group, clinical trials group based in USA
COX-2	Cyclooxygenase-2
CUP	Compassionate use program
DDR	DNA damage response
DSS	Drug sensitivity score
EZH2	Enhancer of zeste homolog 2, member of PRC2
GSEA	Gene set enrichment assay
H3K27	Lysin 27 of histone 3
HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methyltransferase
IC_{50}	Half-maximal inhibitory concentration
INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
JMJD3	Jumonji domain-containing protein-3, an H3K27 demethylase encoded by KDM6B
KDM6A	Lysin demethylase 6A, encodes UTX
KDM6B	Lysin demethylase 6B, encodes JMJD3
MCTS	Multi-cellular tumor spheroid
MIBG	Meta-iodobenzylguanidine
MYC	Also *c-MYC*, Myelocytomatosis virus related oncogene
MYCN	Neuroblastoma *MYC* oncogene
NF1	Neurofibromin 1, negative regulator of RAS pathway
NRAS	Neuroblastoma RAS viral oncogene homolog
NDDS	Neuroblastoma New Drug Development Strategy

NSCLC	Non-small cell lung cancer
PDX	Patient-derived xenograft
PRC2	Polycomb repressive complex 2
PPM1D	Protein phosphatase magnesium-dependent 1 delta; encodes WIP1
RNA-seq	RNA sequencing
RTK	Receptor tyrosine kinase
shRNA	Short hairpin RNA; artificial RNA used for gene silencing
SIOPEN	International Society of Paediatric Oncology Europe Neuroblastoma Group
TERT	Telomerase reverse transcriptase
TKI	Tyrosine kinase inhibitor
TP53	Tumor protein p53; encodes the tumor suppressor protein p53
TRK	Tropomyosine receptor kinase: TRKA is encoded by *NTRK1*, TRKB by *NTRK2*, TRKC by *NTRK3*
UTX	Ubiquitously-transcribed X chromosome tetratricopeptide repeat protein, H3K27 demethylase encoded by *KDM6A*
WES	Whole exome sequencing
WGS	Whole genome sequencing
WIP1	Wild-type p53-induced phosphatase 1; encoded by *PPM1D*

1 INTRODUCTION AND LITERATURE REVIEW

1.1 CHILDHOOD CANCER

Globally, around 200,000 cases of pediatric cancer are diagnosed every year, but simulation studies accounting for undiagnosed cases suggest the actual number of children with cancer might be as high as 400,000 per year (Ward et al., 2019). Pediatric cancer has been shown to be the ninth leading cause of disease burden in children globally (Force et al., 2019). While the vast majority of childhood death globally can be attributed to infectious diseases and perinatal factors, cancer is one of the main killers for children in high-income countries.

In Sweden, the overall annual incidence of cancer in children under fifteen years is 16/100,000 (Gustafsson et al., 2013), adding up to around 260 cases in this age group each year. These cases divide roughly evenly into the three major groups leukemias/lymphomas, central nervous system (CNS) tumors, and extracranial solid tumors.

When comparing childhood cancers, as a group, with adult cancers, some differences are worth mentioning: Firstly, the influence of life-style factors such as smoking, UV light exposure and diet – paramount in highly prevalent adult cancers like lung cancer, colon cancer and melanoma – is virtually absent in childhood cancer. This may be due to shorter lifetime elapsed for such influences to take effect. In tumor biology, this may be reflected by the observation that childhood tumors, at diagnosis, have accumulated comparatively smaller numbers of mutations (Vogelstein et al., 2013). Moreover, children at the start of cancer treatment usually have perfect liver and kidney functions and thus are able to tolerate relatively higher doses of chemotherapy – a treatment actually developed in the pediatric setting (Farber et al., 1948). On the other hand, their growing organs, especially the developing central nervous system (CNS), are much more sensitive to irradiation than the corresponding adult structures (Duffner and Cohen, 1985; Littman and D'Angio, 1979; Pizzo et al., 1979).

1.2 NEUROBLASTOMA

Neuroblastoma is a tumor derived from the sympathetic nervous system and usually manifests in the adrenal medulla or in sympathetic ganglia of the neck, chest or abdomen. With around 90% of all cases occurring at an age below five years and a median age at diagnosis of about 18 months, neuroblastoma is a tumor of the young child. Around 15 annual cases in Sweden make it the most common solid extracranial tumor of childhood, and it accounts for 5.5% of childhood malignancies and 9% of childhood cancer deaths in Sweden (Gustafsson et al., 2013). The global incidence is estimated to be around 14,000 cases yearly (Ward et al., 2019).

1.2.1 Neuroblastoma etiology

Neuroblastoma arises as a deviant progeny to cells of a temporary embryologic structure termed neural crest. The trunk portion of the neural crest gives rise to sympathoadrenal lineage cells, and these sympathoblasts proceed to differentiate into sympathetic neurons, chromaffin

cells, Schwann cells, mesenchymal stem cells, melanocytes and fibroblasts, among others. The precise neuroblastoma cell of origin within this lineage continues to be a matter of debate, although the past years' technical innovations including single-cell transcriptomics have narrowed down the candidate precursors and added important insight about trans-differentiation between different sympathetic lineage entities (Furlan et al., 2017; Kameneva et al., 2021; Zeineldin et al., 2022).

Single-cell RNA sequencing has also advanced the understanding of the cellular heterogeneity long observed in neuroblastoma cells in culture, with one neuronally differentiated and one more undifferentiated phenotype. Transcriptomic characterization of these differentiation states, now commonly referred to as "adrenergic" and "mesenchymal", has identified unique gene expression signatures for each phenotype which were linked to distinct epigenetic programs (Boeva et al., 2017; van Groningen et al., 2017). It was shown that both differentiation states coexist in patient tumors, that the phenotypes can interconvert and that the mesenchymal differentiation state is more drug-resistant (Boeva et al., 2017; van Groningen et al., 2017).

Evidence accumulates to consider neuroblastoma as a disease caused by failed differentiation of precursor cells, leaving them vulnerable to additional oncogenic events (Chen et al., 2015; Zeineldin et al., 2022). These oncogenic events include chromosomal rearrangements, where some recurrent patterns are observed, as well as gene alterations and epigenetic changes, some of which will be discussed further on in this thesis.

However, awareness has grown that tumors cannot persist without a network of non-malignant neighboring cells, which may be reprogrammed by the tumor cells to serve their needs. These are, among others, immune cells, stroma cells and endothelial cells. This tumor microenvironment has become another focus of research interest in the neuroblastoma field (Sherif et al., 2022).

1.2.2 Clinical characteristics and risk stratification

Typically, neuroblastoma presents as an abdominal mass in an ill-appearing child; other symptoms include diarrhea, fatigue, fever, anemia, weight loss, the autoimmune opsoclonus-myoclonus syndrome which may occur as a paraneoplastic manifestation, as well as Horner's syndrome, paralysis through spinal cord compression, and other local symptoms caused by primary tumor or metastases (Figure 1-1). Neuroblastoma metastasizes primarily to regional lymph nodes, bone and bone marrow; further to liver and skin, especially in young infants, while lung and CNS metastases occur more rarely (Matthay et al., 2016). Some neuroblastomas secrete catecholamines that cause hypertension and whose metabolites can be quantified in the urine. Diagnosis relies on radiology, most importantly magnetic resonance imaging (MRI), ^{123}I-meta-iodobenzylguanidine (MIBG) scintigraphy, bone marrow aspiration and biopsy of the primary tumor with histopathologic workup.

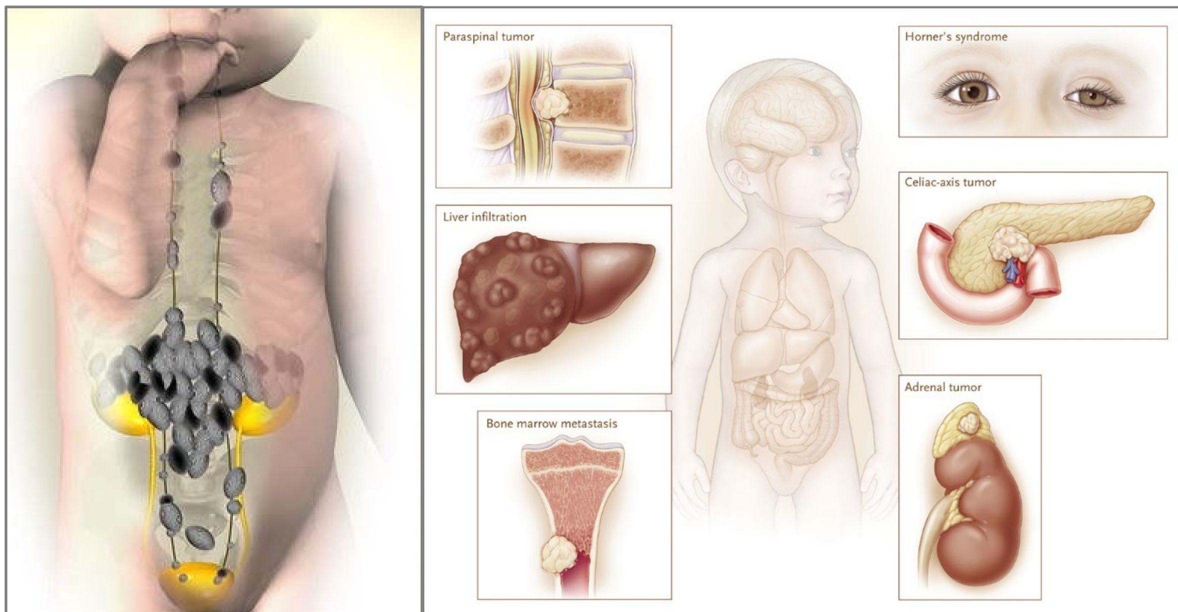

Figure 1-1: ***Left***: *The neuroblastoma primary tumor most commonly originates from the adrenal medulla, followed by paraspinal ganglia of the sympathetic trunk in the abdomen or thorax. From (Johnsen et al., 2009), reprinted in accordance with the Creative Common license.* ***Right***: *Common clinical manifestations of neuroblastoma. Reproduced with permission from (Maris, 2010), Copyright Massachusetts Medical Society.*

The disease of neuroblastoma displays striking heterogeneity, with cases of spontaneously regressing or differentiating tumors on the one hand, and on the other hand high-risk neuroblastoma cases with metastatic spread at diagnosis and fatal outcome in spite of intensive multimodal treatment. While the mechanisms behind this diversity are only partly understood and represent an important focus of current research activity, a number of robust risk factors based on tumor biology have been identified, some of which have also entered clinical risk group assessment.

Amplification of the *MYCN* oncogene has long been recognized as the most powerful biologic marker for unfavorable prognosis, and carries a hazard ratio around 4 (Cohn et al., 2009). Segmental chromosomal aberrations such as 1p or 11q deletion and 17q gain are associated with high-risk neuroblastoma (Bown et al., 1999; Brodeur, 2003; Carén et al., 2010; Maris, 2010). Triploid or near-triploid tumors are associated with a favorable outcome (Spitz et al., 2006). However, large sequencing studies have more recently added insight that a defining molecular risk marker is the presence of telomere maintenance mechanisms, as brought about by a variety of genetic alterations including *MYCN* and *TERT* (Ackermann et al., 2018).

The heterogeneous behavior of the disease has produced a gamut of diverse treatment approaches stretching from mere observation all the way to multimodal treatment including intensive multi-drug induction chemotherapy, surgery, autologous stem cell transplantation, irradiation and maintenance medication. To help with treatment stratification, different classifications have been suggested.

The International Neuroblastoma Staging System (INSS) was developed in 1986 as a post-surgical staging system (Brodeur et al., 1988). It discerns stages 1, 2A, 2B, 3, 4, and 4S, the

latter (S for "special") denoting a condition in children under the age of one year with metastasis limited to skin, liver and bone marrow, which has a tendency to regress spontaneously.

The newer International Neuroblastoma Risk (INRG) classification (Figure 1-2) was conceived in 2005 and represents a pretreatment staging and risk classification system based on tumor imaging and some biologic properties (Cohn et al., 2009; Monclair et al., 2009). The presence of image-defined risk factors – usually interference with vital structures – discriminates between stages L1 and L2. Stage M denotes distant metastatic disease, and MS – in analogy to stage 4S of the INSS classification – "metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow" (Monclair et al., 2009). Further, histologic category, grade of tumor differentiation, and the tumor biologic characteristics of *MYCN* status, 11q aberration and ploidy are assessed to finally assign one of the pretreatment risk groups A-C (very low), D-F (low), G-J (intermediate), or K-R (high) (Cohn et al., 2009).

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

Figure 1-2*: The INRG classification. From (Cohn et al., 2009), reprinted with permission from the publisher.*

Recently, the INRG classification underwent a revision which, among others, adds chromosome 1p aberrations as an additional biologic risk marker, introduces an age-associated risk divider at five years and reallocates some risk factor constellations to the pretreatment groups Low risk, Intermediate risk and High risk (Irwin et al., 2021).

1.2.3 Current treatment strategies

Full-scale neuroblastoma treatment is tainted with side effects and even a certain degree of mortality. To keep the risk of intervention proportionate to the risk of the disease, treatment is stratified according to the INRG risk groups described above.

Patients who do not fulfil high-risk criteria are in most European countries treated within the Low and Intermediate Risk Neuroblastoma European Study (LINES) (EudraCT 2010-021396-81, *clinicaltrials.org* identifier NCT01728155). According to INRG stage, age and biologic risk factors, the patients are allocated to ten risk groups, where groups 1-6 include low risk patients while groups 7-10 encompass intermediate risk patients. In the lowest risk groups of infants where spontaneous tumor regression has a certain probability, observation – sometimes in conjunction with surgical resection – is the management of choice. Remaining groups are treated with two to eight courses of chemotherapy including etoposide/carboplatin (VP/Carbo) and cyclophosphamide, doxorubicin and vincristine (CADO).

High-risk neuroblastoma therapy is complex and includes a host of different treatment modalities. Different treatment protocols evaluate new variations, amendments and innovations, but the therapeutic backbone commonly consists of three phases: Induction aims at inducing remission through intense chemotherapy courses as well as surgery of the primary tumor. The consolidation includes myeloablative chemotherapy followed by autologous stem cell transplantation and irradiation. This is followed by the post-consolidation phase aiming at minimal residual disease, in which differentiating agents such as isotretinoin and/or immunotherapy are administered. An overview of different high-risk neuroblastoma treatment protocols is found in Figure 1-3 (Chung et al., 2021). Treatment response is evaluated according to the revised International Neuroblastoma Response Criteria (Park et al., 2017).

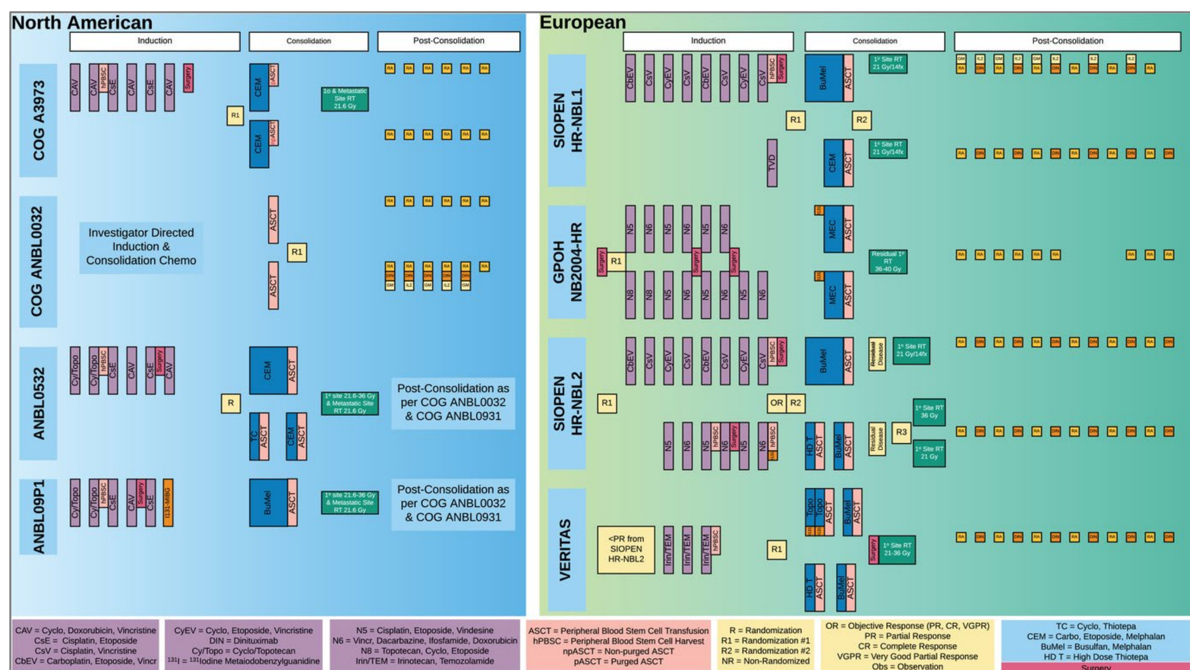

__Figure 1-3__: An overview of current North American and European neuroblastoma treatment protocols illustrates the complexity of high-risk neuroblastoma therapy. From (Chung et al., 2021), reprinted with permission from the publisher.

Through these intense treatment protocols, survival of neuroblastoma patients has increased throughout the past five decades. Yet, overall mortality remains high at about 20-35%, and among high-risk patients, comprising about half the children with neuroblastoma, the survival

rate is only about 40-60% (Gustafsson et al., 2013; Irwin et al., 2021; Ladenstein et al., 2017). For those INSS stage 4 patients who experience a relapse, 4-year overall survival is only 26%, despite significant improvement through the addition of anti-ganglioside immunotherapy (London et al., 2017). Furthermore, survivors often suffer long-term sequelae from disease and treatment.

Therefore, alternative treatment strategies for patients with neuroblastoma are warranted to improve the prognosis. During the last decade, multiple clinical phase I and II studies have been initiated to evaluate novel therapeutic strategies. These include enhanced implementation of immunotherapy, targeted irradiation with ^{131}I-MIBG and ^{177}Lutetium DOTATATE (DuBois et al., 2021; Sundquist et al., 2022), and drugs specifically targeting the initiating and perpetuating mechanisms in neuroblastoma. The latter represent the focus of this thesis.

1.3 TARGETED THERAPY IN CANCER

The introduction of conventional chemotherapy was a true breakthrough in the care of children and adults with cancer. For the first time, diseases like leukemia and lymphoma could be cured. As chemotherapy was further refined through combination of different drugs and risk-adjusted protocols, survival and long-term outcome improved. However, eventually this development reached a plateau for many types of cancer.

Conventional chemotherapeutic agents have in common that they interfere with cell cycle progression and proliferation, therefore hitting hardest on cells with high proliferation rates like tumor cells and epithelia. However, as biologic understanding of initiating events in tumor development increased, so did the hope to target these events, or hallmarks, selectively, thereby avoiding toxicity and achieving better treatment results.

The first and most famous proof of concept for targeted therapy in cancer was the drug imatinib (Gleevec®, Glivec®), which was specifically developed to inhibit the over-active bcr-abl tyrosine kinase arising in cases of Philadelphia-chromosome-positive chronic myelogenous leukemia (CML) (Deininger et al., 2005). Imatinib was approved in 2001 and became a story of success, turning a fatal diagnosis into a manageable chronic condition. Its indication has since been extended to other malignant diseases harboring the same chromosome translocation, for example acute lymphoblastic leukemia (ALL), illustrating the trend to choose treatment according to biologic features rather than only diagnosis.

Since imatinib, biological understanding of malignant diseases has snowballed, numerous potential therapeutic targets have been identified and scores of potential substances have been screened, tested and sometimes pushed through to clinical approval. Lung cancer, melanoma and breast cancer, among others, have been fields of especially intense research regarding new therapeutic targets. Targeted cancer therapy in clinical use includes, among others, the group of monoclonal antibodies frequently targeting growth receptors, such as trastuzumab (Herceptin®, targets HER2) and bevacizumab (Avastin®, targets VEGF), and the group of

small molecules like imatinib and vemurafinib (Zelboraf®, targets B-Raf), usually targeting intracellular kinases.

However, especially cancers affecting adult patients frequently accumulate a great number of mutations, illustrating the readiness to circumvent successful targeted therapy by mutation and the arousal of resistant clones (Gröbner et al., 2018; Vogelstein et al., 2013). This often results in initial treatment response which is lost after some months of medication, necessitating a switch to another fitting therapeutic agent, if available.

In contrast, pediatric cancers and embryonal malignancies have fewer mutations, and these might signify developmental aberrations rather than results of environmental influences (Gröbner et al., 2018). Moreover, the overlap with the mutational spectrum of adult cancers seems to be limited (Ma et al., 2018). A smaller number of mutations might imply greater disease-driving importance of the mutations present. Based on a number of pediatric oncology sequencing studies at different centers, it has been estimated that around 50% of pediatric cancers contain an event accessible to targeted therapy (Forrest et al., 2018; Gröbner et al., 2018). Some of these recurrent alterations are presented in Figure 1-4.

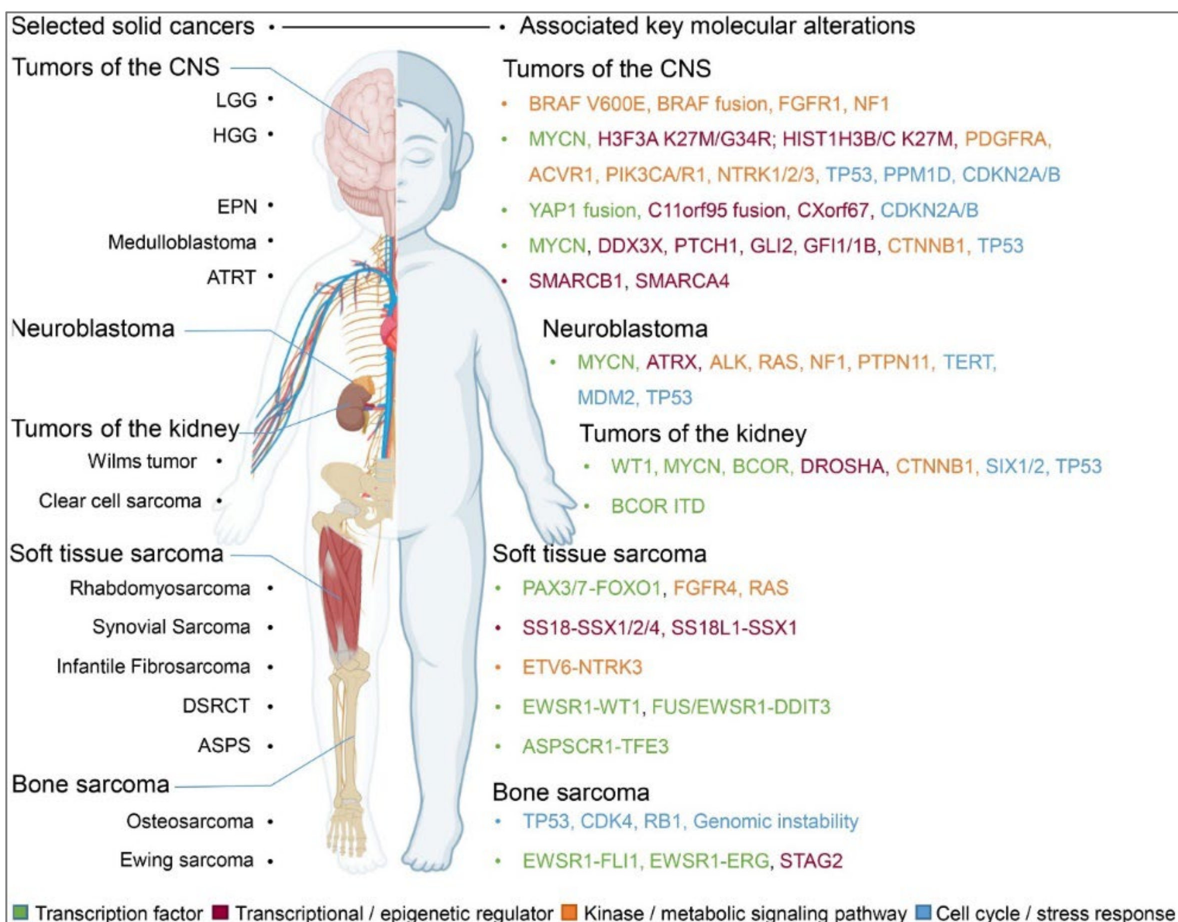

Figure 1-4*: Some recurrent molecular alterations in pediatric solid tumors. LGG, low grade glioma; HGG, high grade glioma; EPN, ependymoma; ATRT, atypical teratoid/rhabdoid tumor; DSRCT, desmoplastic small round cell tumor; ASPS, alveolar soft part sarcoma. From (Blattner-Johnson et al., 2022), reprinted with permission from the publisher.*

1.4 TARGETED THERAPY IN NEUROBLASTOMA

Established recurrent genetic alterations in neuroblastoma, many of which represent important potential therapeutic targets, affect a long list of genes including *MYCN*, *ALK*, *MDM2*, *TP53*, *RAS*, *NF1*, *BRAF*, *TERT*, *ATRX* and *PTPN11*, and others (Jones et al., 2019). The Neuroblastoma New Drug Development Strategy (NDDS) initiative assembled experts from North America and Europe to prioritize among neuroblastoma targets at different stages of development (Moreno et al., 2020). Some of these are of special interest to this thesis and will be reviewed below, along with selected emerging neuroblastoma targets.

1.4.1 Targeting MYC

MYC proteins are transcription factors important for cell growth and proliferation. The different paralogs c-MYC, MYCN and MYCL, encoded by *c-MYC*, *MYCN* and *MYCL*, respectively, are expressed at different time points of development, but show similar structure and function (Ruiz-Pérez et al., 2017). Regarding neuroblastoma, *MYCN* is sometimes seen as the "master oncogene", held accountable for a majority of high-risk neuroblastoma cases. The generation of the *MYCN*-overexpressing neuroblastoma mouse model was an important milestone in neuroblastoma research, corroborating the paramount importance of this oncogene (Weiss et al., 1997). Approximately 25% of neuroblastomas have *MYCN* amplification and are classified as "high-risk" regardless of other prognostic markers.

Consequently, efforts to find a way to target MYCN specifically have been as intense and enduring as they have been fruitless. As reviewed by Shalaby and Grotzer, difficulties arise both through adverse effects due to the central role of MYC proteins in normal transcription, and on the pharmacologic side due to both the proteins' intranuclear location and their conformations lacking recognizable features (Shalaby and Grotzer, 2015). While attempts of finding new ways to target *MYCN* directly are still being made (Yoda et al., 2019), attention has mostly been redirected toward indirect targeting via inhibition of Aurora A kinase, bromodomain and extra-terminal motif (BET), or mammalian target of rapamycin complex (mTORC1/2). These treatment strategies are designated as prioritized by NDDS and early clinical trials are ongoing (Moreno et al., 2020).

1.4.2 Targeting ALK

ALK (anaplastic lymphoma kinase) is a receptor tyrosine kinase (RTK) of the insulin receptor superfamily, activated by the ligands ALKAL1 and ALKAL2, previously known as FAM150A and FAM150B (Guan et al., 2015). Both ALK and ALKAL2, and also MYCN, are all encoded within a region on chromosome 2p sometimes referred to as the "neuroblastoma cassette" (Javanmardi et al., 2019). ALK has downstream effects on several different signaling pathways including JAK-STAT, PI3K-AKT, mTOR, SHH, and RAS-MAPK, ultimately promoting cell growth, transformation and survival (Figure 1-5) (Hallberg and Palmer, 2013). Physiologically,

ALK is mainly active during embryonic development, especially of the brain (Hallberg and Palmer, 2013).

In cancer, enhanced ALK signaling was first described as a consequence of *ALK-NPM* gene fusion, rendering a truncated protein with active signaling independent of ligand binding, ultimately resulting in anaplastic lymphoma (Morris et al., 1994). In 2007, a similar fusion oncogene, *EML4-ALK*, was recognized to cause ALK activation in around 5% of non-small cell lung cancers (NSCLC) (Rikova et al., 2007; Soda et al., 2007). Shortly after, the role of ALK in hereditary and sporadic neuroblastoma was discovered (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008).

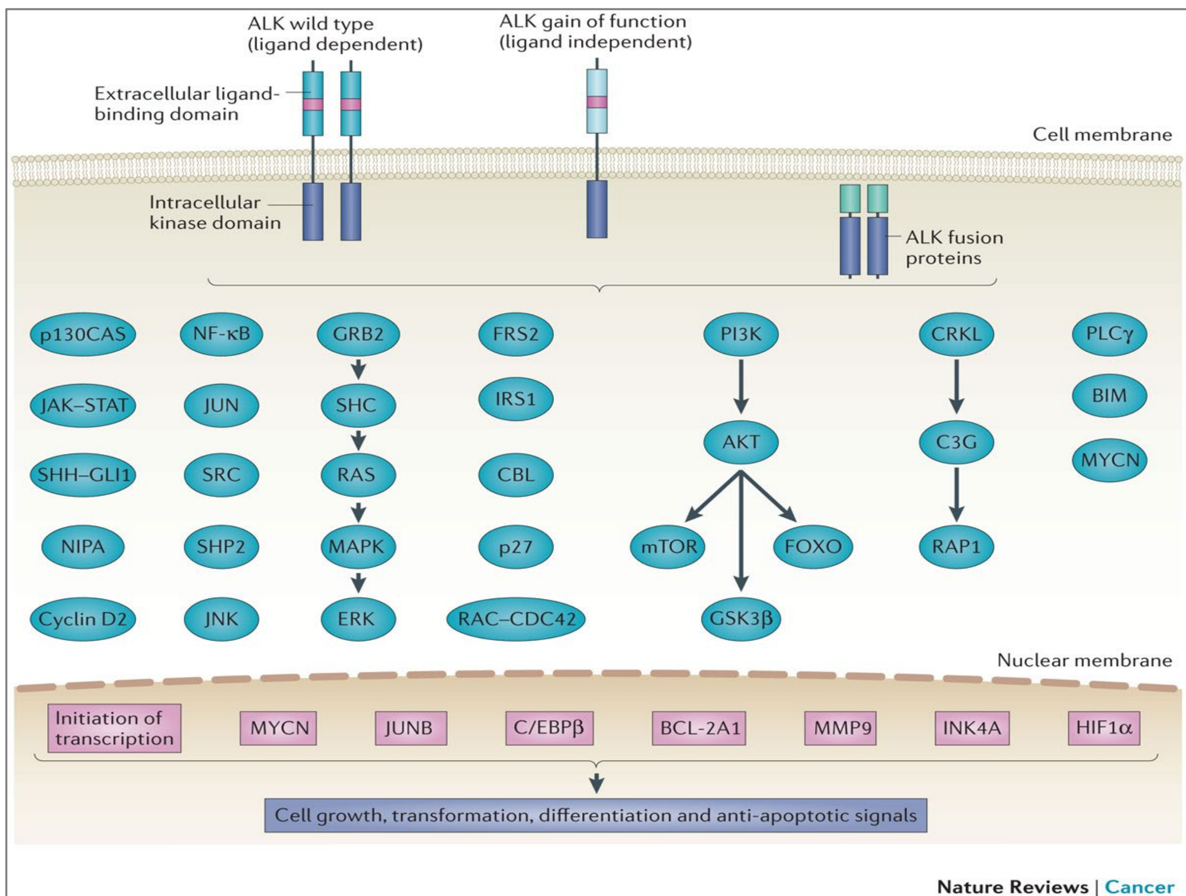

Figure 1-5: *In neuroblastoma, gain-of-function mutations render the ALK receptor independent of ligand binding. In other malignancies including NSCLC, fusions are the most common ALK aberration. Downstream signaling of ALK is complex and involves various pathways and crosstalks. Reprinted from (Hallberg and Palmer, 2013) with permission from the publisher.*

ALK activation in neuroblastoma usually does not happen through the formation of fusion oncogenes, but rather through activating mutations which allow ALK to signal independent of ligand binding (Figure 1-5). These mutations occur with a frequency of up to 50% in the rare hereditary neuroblastomas and 6-10% in sporadic neuroblastomas (Pacenta and Macy, 2018). At relapse, however, this figure is increased to around one third (Eleveld et al., 2015; Schleiermacher et al., 2014). More than 35 different *ALK* mutations have been described in

neuroblastoma, most of which are point mutations resulting in a full-length protein and clustering to the three hotspot positions F1174, F1245 and R1275 (Hallberg and Palmer, 2016). In addition, *ALK* gains and amplifications are seen, predominantly in high-risk neuroblastoma. Since *ALK* and *MYCN* genes are both located on chromosome 2p, amplifications can frequently occur together. The combination of enhanced ALK signaling, through activating mutations or gain, and *MYCN* amplification has proven particularly unfavorable (Hallberg and Palmer, 2013), maybe because ALK activation in neuroblastoma also seems to upregulate MYCN (Claeys et al., 2019; Schönherr et al., 2012).

Crizotinib (Xalkori®) was FDA-approved in 2011 and represented the first ALK inhibitor entering the clinic, initially for the treatment of *ALK*-fusion positive NSCLC (Kwak et al., 2010). Early treatment successes were reassuring, but the majority of NSCLC patients treated with crizotinib progressed because of insufficient penetration to the CNS and because of secondary resistance to the drug. A phase I clinical trial of crizotinib for pediatric patients with refractory solid tumors, including neuroblastoma, showed good tolerability and some treatment effect in patients with malignancies harboring *ALK* aberrations, including one patient from Stockholm with a germline *ALK*-R1275Q mutation (Mossé et al., 2013). In 2019, a phase III trial was started for upfront treatment of high-risk neuroblastoma with crizotinib, among other drugs; however, in an amendment crizotinib was exchanged for the third generation ALK inhibitor lorlatinib (*clinicaltrials.gov* NCT03126916).

Ceritinib, alectinib, entrectinib and lorlatinib are examples of second and third generation ALK tyrosine kinase inhibitors (TKIs), devised to overcome crizotinib resistance. There are slight differences in binding mechanism and in inhibitory effect on additional targets other than ALK (Hallberg and Palmer, 2016). Regarding neuroblastoma, there are completed and ongoing clinical trials for the ALK inhibitors crizotinib, ceritinib, entrectinib, lorlatinib and ensartinib (Table 1). Increasingly, clinical studies are planned as basket trials, tailoring the intervention to the malignancy's biologic characteristics rather than to the diagnosis.

Start	ALK inhibitor	Study phase	Neuroblastoma population	*clinicaltrials.gov*	Status	Reference
2009	crizotinib	I/II	refractory/relapsed NB	NCT00939770	completed	Mossé et al., 2013; Foster et al., 2021
2013	crizotinib	I	refractory/relapsed NB	NCT01606878	completed	Greengard et al., 2020
2013	ceritinib	I	refractory/relapsed NB	NCT01742286	completed	Fischer et al., 2021
2015	ceritinib	II	newly diagnosed high-risk NB	NCT02559778	recruiting	
2016	ceritinib	I	relapsed/refractory NB	NCT02780128	recruiting	
2016	entrectinib	I/II	relapsed/refractory NB	NCT02650401	completed*	Desai et al., 2022
2017	lorlatinib	I	relapsed/refractory NB	NCT03107988	recruiting	
2017	ensartinib	II	refractory/relapsed NB	NCT03213652	recruiting	
2018	lorlatinib#	III	newly diagnosed high-risk NB	NCT03126916	recruiting	
2021	lorlatinib	observational	ALK-aberrant NB	NCT04753658	recruiting	
2022	ceritinib	II	newly diagnosed high-risk NB	NCT05489887	recruiting	

Table 1*. ALK inhibitor studies in neuroblastoma, as registered at clinicaltrials.gov. NB, neuroblastoma; COG, Children's oncology group; NANT, New Approaches to Neuroblastoma Therapy Consortium; NIC, National Institute of Cancer; *ALK study part completed; # initially, crizotinib.*

Though the therapeutic winnings of ALK inhibitors, as a group, initially may have been sobering, some individuals have experienced long response times (Desai et al., 2022; Fischer et al., 2021; Mossé et al., 2013). It becomes increasingly clear that ALK inhibition in

neuroblastoma should be matched to the *ALK* mutation at hand (Bresler et al., 2014; Hallberg and Palmer, 2016; Zou et al., 2015). The third-generation ALK inhibitor lorlatinib unites robust activity against the common *ALK* mutations prevalent in neuroblastoma with favorable pharmacokinetic properties (Guan et al., 2016; Sun et al., 2022). It is now being introduced as an upfront-therapeutic for treatment of ALK-positive high-risk neuroblastoma (NCT03126916, NCT04221035).

However, a strong tendency to develop resistance against ALK inhibitors is known from NSCLC patients, to the extent of physicians anticipating emerging resistance already at the initiation of ALK-inhibiting therapy and planning the treatment as a sequence of different ALK inhibitors (Barrows et al., 2019). *In vitro* and patient data suggest that resistance mechanisms arise both as new, additional *ALK* mutations and as activation of bypass pathways, for example through alterations in the PI3K/AKT and RAS/MAPK signaling cascades (Dagogo-Jack et al., 2019; Redaelli et al., 2018). Recurrently observed resistance mechanisms in neuroblastoma patients include loss of *NF1*, mutations of *NRAS*, and acquisition of additional *ALK* mutations (Berlak et al., 2022; Bosse et al., 2022). A rational way to monitor eventual emergence of resistance mutations is serial assessment of circulating tumor DNA (ctDNA) (Berlak et al., 2022; Bosse et al., 2022; Dagogo-Jack et al., 2019).

1.4.3 Targeting the p53-MDM2 circuit

The protein p53, sometimes called "guardian of the genome", is seen as the most important regulator of DNA damage response (DDR) as induced by e.g., irradiation, hypothermia or cytotoxic chemicals. Within an intricate system of protein crosstalk, it steers the cell towards DNA repair, senescence, cell cycle arrest or apoptosis by acting as a transcription factor crucial to those reactions (Figure 1-6).

P53 is encoded by the tumor suppressor gene *TP53* on chromosome 17p. *TP53* mutations are common in cancers of adult patients, rendering p53 insufficient in its function to protect the genome and resulting in cascading oncogenic mutations; thus, mutant p53 constitutes an important therapeutic target in those cancers (Bykov et al., 2018). However, pediatric cancers less frequently demonstrate mutations in p53. In neuroblastoma, less than 2% display mutated *TP53* at diagnosis, while *TP53* mutations more often eventuate later during the course of treatment (Van Maerken et al., 2009). Instead, tumor cells have developed other strategies to circumvent proliferation control. Overexpression of p53 modulators could be one such strategy.

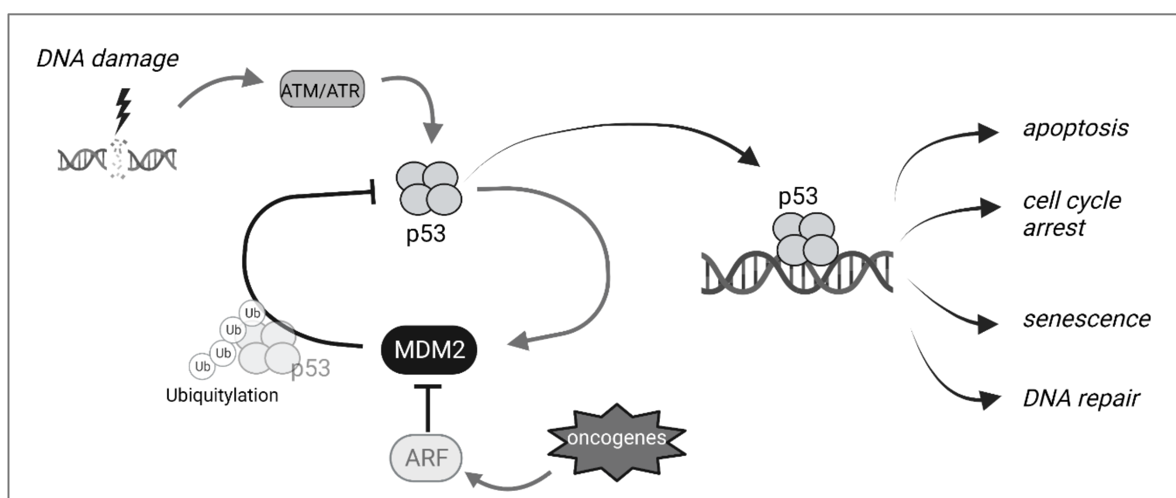

***Figure 1-6**. DNA damage response is orchestrated by p53, which can be activated by DNA damage or oncogene activation. MDM2 is the master regulator of p53 with which it forms a feedback loop. Created with Biorender.com.*

MDM2 (mouse double minute 2, in humans also denoted HDM2), is a key regulator of p53 activity (by ubiquitination), and, if overexpressed, acts as an oncoprotein (Figure 1-6). Various kinases including ATM, Chk1 and Chk2, can change the conformation of p53 in order to prevent MDM2 from binding (Moll and Petrenko, 2003). ARF ($p14^{ARF}$) can inactivate MDM2, thereby increasing p53 levels (Barone et al., 2014). Consequently, inhibition of MDM2 represents an important method to restitute p53 levels, especially in malignancies with fully functional p53. Two important lead substances for inhibition of the MDM2-p53 interaction are RITA and nutlin-3.

RITA is a small molecule whose inhibitory effect on the MDM2-p53 interaction was understood in 2004. RITA binds the N-terminus of p53, leading to a conformational change which seems to block the complex formation with MDM2 (Issaeva et al., 2004). In a manner independent of p53 mutational status, it inhibits neuroblastoma and medulloblastoma cell growth, both *in vitro* and *in vivo* (Burmakin et al., 2013; Gottlieb et al., 2017). It has also been shown effective in many other cancer cell lines (de Bakker et al., 2022; Issaeva et al., 2004). However, findings demonstrating that RITA induces cell death in p53-null cancer cell lines suggest there might be other or additional mechanisms of action than inhibition of the interaction between MDM2 and p53 (Weilbacher et al., 2014).

Nutlin-3 is another small-molecule MDM2 antagonist targeting the MDM2-p53 interaction. Chemically a *cis*-imidazoline derivative, it was identified by screening chemicals to fit into a deep pocket in the MDM2 molecule (Vassilev et al., 2004). Nutlin-3 has been found to inhibit growth of several wild-type p53 neuroblastoma and medulloblastoma cell lines and xenografts (Barone et al., 2014; Künkele et al., 2012). The nutlin-3 derivative idasanutlin and other MDM2 inhibitors are currently being evaluated in clinical trials for various malignancies including neuroblastoma (Fang et al., 2020; Konopleva et al., 2020).

An alternative strategy to target mutated p53, which has also reached clinical testing, is by reactivation of mutant p53. The lead compound in this context is PRIMA-1 which was

identified by a screening for compounds able to restore wild-type p53 properties (Bykov et al., 2002). APR-246 is its most clinically advanced derivative which is currently under investigation for several hematologic and solid malignancies in adults (Duffy et al., 2022).

1.4.4 Targeting WIP1

WIP1 (wildtype-p53-induced phosphatase 1), a serine/threonine phosphatase of the PP2C family encoded by the gene *PPM1D* (protein phosphatase magnesium-dependent 1 delta) on chromosome 17q23.2, is another player that negatively regulates p53 (Fiscella et al., 1997). It does so by directly dephosphorylating p53 at serine 15, but also by inhibiting the p53-phosphorylating kinases ATM, p38/MAPK, CHK1 and CHK2 (Lu et al., 2007) and by stabilizing and enhancing the p53 inhibitor MDM2 (Lu et al., 2008). WIP1 also dephosphorylates γH2AX, thereby stopping DNA double-strand break repair (Cha et al., 2010; Macůrek et al., 2010; Moon et al., 2010b, 2010a). Additionally, WIP1 has been shown to directly dephosphorylate the pro-apoptotic protein BAX (Song et al., 2013). Thus, WIP1 employs various mechanisms to restrain DNA damage response (Figure 1-7). Indeed, a recent mass spectroscopy-based phosphoproteomics study identified as many as 35 putative dephosphorylation targets of WIP1 (Gräf et al., 2022). In the absence of cell stress, WIP1 is thought to help maintain cell and tissue homeostasis and help cells prepare for mitosis (Park et al., 2011).

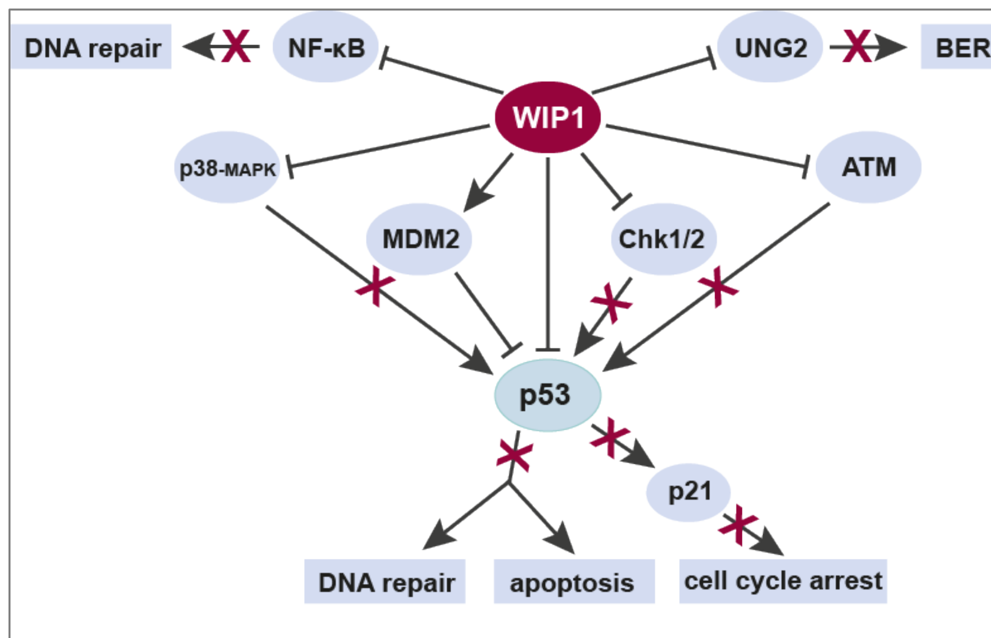

Figure 1-7: *WIP1 is a central inhibitor of DNA damage response. BER, base-excision repair. Adapted from (Oghabi Bakhshaiesh et al., 2017) with permission from the publisher.*

As reviewed by Le Guezennec and Bulavin, knockout mice deficient in *Ppm1d* are viable and show delayed onset of mammary tumors when crossed with transgenic breast cancer mouse models. In turn, transgenic mouse models with overexpression of *PPM1D* along with the breast-cancer-driving oncogene *ErbB2* specifically in the mammary tissue displayed earlier tumor development (Le Guezennec and Bulavin, 2010). Transgenic mice with overexpression

of WIP1 were predisposed to malignancies recapitulating the tumors arising in p53-knockout mice (Milosevic et al., 2021).

In humans, WIP1 is overexpressed in several malignancies including breast and ovarian cancer, medulloblastoma and neuroblastoma (Le Guezennec and Bulavin, 2010; Richter et al., 2015). WIP1 overexpression is predominantly found in p53-wildtype cancers, as an alternative strategy to overcome p53 proliferation control (Pecháčková et al., 2017). Furthermore, gain of 17q, including the locus of *PPM1D*, is a common genetic aberration and marker of poor prognosis in both neuroblastoma and medulloblastoma, and *PPM1D* expression correlates with higher INSS stage and inferior outcome in neuroblastoma (Figure 1-8) (Carén et al., 2010; Gröbner et al., 2018; Northcott et al., 2019; Saito-Ohara et al., 2003). In other malignancies including diffuse midline glioma, truncating mutations of *PPM1D* are recurrent, which have been shown to render the WIP1 protein more stable and produce an oncogenic gain-of-function phenotype (Kahn et al., 2018; Khadka et al., 2022; Kleiblova et al., 2013) As a consequence, WIP1 has been proposed as a therapeutic target in multiple cancers.

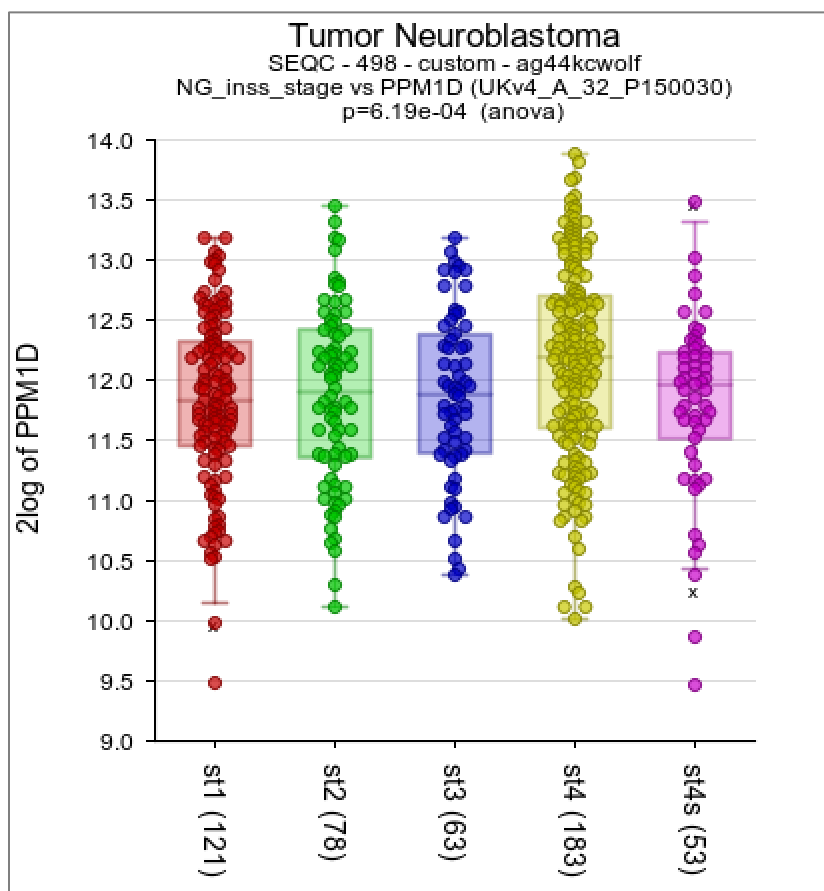

Figure 1-8*: Gene expression of PPM1D correlates to INSS stage and is highest in stage 4 neuroblastomas (yellow). Plot of the publicly available SEQC dataset of 498 neuroblastomas, created using the R2 database 'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)'.*

There are several known WIP1-inhibiting substances (Figure 1-9), none of which have been tested clinically. The first small molecule WIP1 inhibitor presented was CCT007093, identified through a high-throughput screening and shown to selectively impair viability of

the WIP1-overexpressing MCF-7 breast cancer cell line (Rayter et al., 2008). However, CCT007093 has rather low potency and low specificity, as seen by lacking downstream effect on p53-pS15 as well as γH2AX (Lee et al., 2014). Furthermore, the finding that it inhibited growth of U2OS cells in the absence of *PPM1D* while also failing to induce phosphorylation of WIP1 downstream targets H2AX and p53, has made it questionable if CCT007093 is really a WIP1 inhibitor (Nahta and Castellino, 2021; Pecháčková et al., 2016).

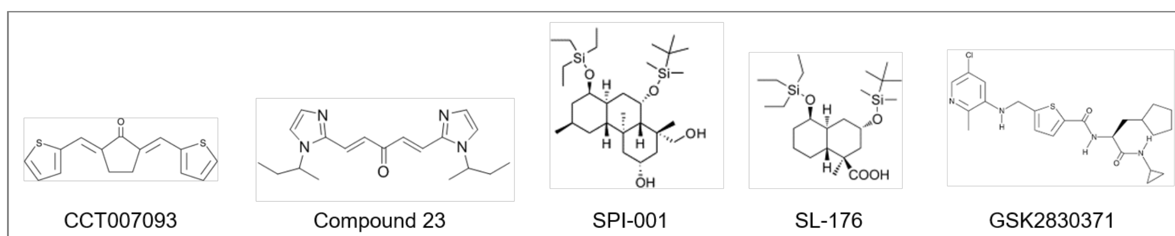

Figure 1-9*: Molecular structures of five WIP1 inhibitors.*

Similar to the above, the WIP1 inhibitor "Compound 23" or C23, was more recently found by screening various curcumin derivates (Wu et al., 2019). An inhibitory concentration 50% (IC_{50}) just below 1 µM was reported for MCF-7 cells. The compound also showed some effect *in vivo* as demonstrated in diabetic mice with established MCF-7 xenografts who received the compound in the form of poly(lactic-co-glycolic acid) nanoparticles (PLGA-NP) (Wu et al., 2019). Improvements in bioavailability and solubility are necessary to make C23 interesting in a clinical context.

GSK2830371, an allosteric inhibitor of WIP1, was synthesized by Gilmartin and colleagues at GlaxoSmithKline and published in 2014 (Gilmartin et al., 2014). The following year, the same group demonstrated the sensitivity of p53-wildtype neuroblastoma cell lines towards GSK2830371, while p53-mutated cell lines were resistant (Richter et al., 2015). Treatment of mice bearing orthotopic neuroblastoma xenografts slowed down tumor growth (Chen et al., 2016). It has recently been shown that GSK2830371 elicits its inhibitory effect by inducing a conformation shift in WIP1 (Miller et al., 2022). The substance is commercially available, but to our knowledge, no clinical studies have been conducted. Notably, *in vitro* studies have demonstrated synergistic effects when combining inhibition of WIP1 and MDM2 (Esfandiari et al., 2016; Pecháčková et al., 2016; Wu et al., 2018).

Based on the lead compound SPI-001 (Yagi et al., 2012), the small molecule SL-176 is the result of a drug-finding effort by the Kazuyasu group (Ogasawara et al., 2015). It has been shown effective in inhibiting p53-S15 dephosphorylation, as demonstrated by immunoblotting, and proliferation of MCF-7 cells (Ogasawara et al., 2015). Further results in neuroblastoma and medulloblastoma cell lines *in vitro* and first *in vivo* experiments are reported in this thesis.

1.4.5 Epigenetic targets in neuroblastoma

The relative paucity of recurrent somatic mutations in neuroblastoma calls for other factors to explain disease development and progression (Ma et al., 2018). DNA methylation and histone

modifications govern accessibility of genes, and disruptions of these mechanisms may promote tumorigenesis for example by silencing of tumor suppressors, while a more permissive epigenetic state allows tumor cells to adapt, evade control mechanisms, activate oncogenes and repurpose enhancers (Flavahan et al., 2017). It is conceivable that the developmental window in which the embryonal cancer's cell of origin arises, a timespan with intense changes in epigenetic states as developing cells commit to their future lineages, confers an increased risk for epigenetic aberrations (Bernstein et al., 2006).

Methylation states of specific genes have been linked to prognosis in neuroblastoma and inhibition of DNA methyltransferases was the first epigenetic modification which was pursued as a therapeutic strategy (Carén et al., 2011; Fetahu and Taschner-Mandl, 2021). Subsequently, insights about interplay between histone deacetylases (HDAC) and MYCN have directed interest toward HDAC inhibitors, and successful preclinical neuroblastoma studies with vorinostat have motivated several phase I and II studies with this drug (Fetahu and Taschner-Mandl, 2021). A phase I study of vorinostat in combination with isotretinoin for post-consolidation maintenance gave some indications of prolonged stable disease (Pinto et al., 2018). In addition, vorinostat has been shown to have radio-sensitizing properties and doubled the response rate to ^{131}MIBG in one phase II trial (DuBois et al., 2021). Other HDAC inhibitors studied in the field of neuroblastoma include panobinostat and romidepsin.

While histone acetylation generally leads to a more open chromatin accessible for transcription, methylation of histones can induce either suppression or activation of genes, depending on the site and the extent of methylation. In the case of lysin 27 of histone 3 (H3K27), an important site in neuroblastoma, methylation leads to a repressive chromatin state and inhibited gene transcription. EZH2 (enhancer of zeste homolog 2), a subunit of polycomb repressive complex 2 (PRC2), is a key histone methyltransferase (HMT) contributing to trimethylation of H3K27, leading to repression of transcription (Figure 1-10). It is often overexpressed in neuroblastoma, partly mediated by MYCN-driven promoter activation (Corvetta et al., 2013). EZH2 is associated with poor prognosis in neuroblastoma and has been suggested as a therapeutic target (Bownes et al., 2021; Duan et al., 2020).

The antagonist of EZH2 and PRC2 is the histone demethylase (HDM) Jumonji domain-containing protein-3 (JMJD3, encoded by *KDM6B*) with the ability to remove methyl groups from di- or trimethylated lysin 27 of histone 3 (H3K27me2/3), thereby rendering DNA more accessible to the transcription machinery (Agger et al., 2007). JMJD3 has been proposed as a tumor suppressor in neuroblastoma, supported by clinical data showing a correlation between high expression and longer event-free survival, while *in vitro* overexpression inhibited neuroblastoma cell proliferation and induced differentiation (Yang et al., 2019).

In contrast, Lochmann et al., when screening a panel of cancer cell lines, found that neuroblastoma cell lines exhibited an increased sensitivity toward the JMJD3/UTX inhibitor GSK-J4, and demonstrated that inhibition of JMJD3 and its homolog UTX (*KDM6A*) achieved

robust reduction of neuroblastoma growth both *in vitro* and in xenograft models. Paradoxically, also in this study, markers of differentiation were increased (Lochmann et al., 2018).

The apparent conundrum that overexpression and inhibition of JMJD3 produce the same effect of constrained cell growth and increased differentiation might just have its resolution in the strive for balance inherent to epigenetic processes. Of note, similar seemingly contrasting findings have been made in the field of leukemia for the effect of JMJD3 and UTX, where the latter appears to take on the role of a tumor suppressor (Abu-Hanna et al., 2022; Ntziachristos et al., 2014).

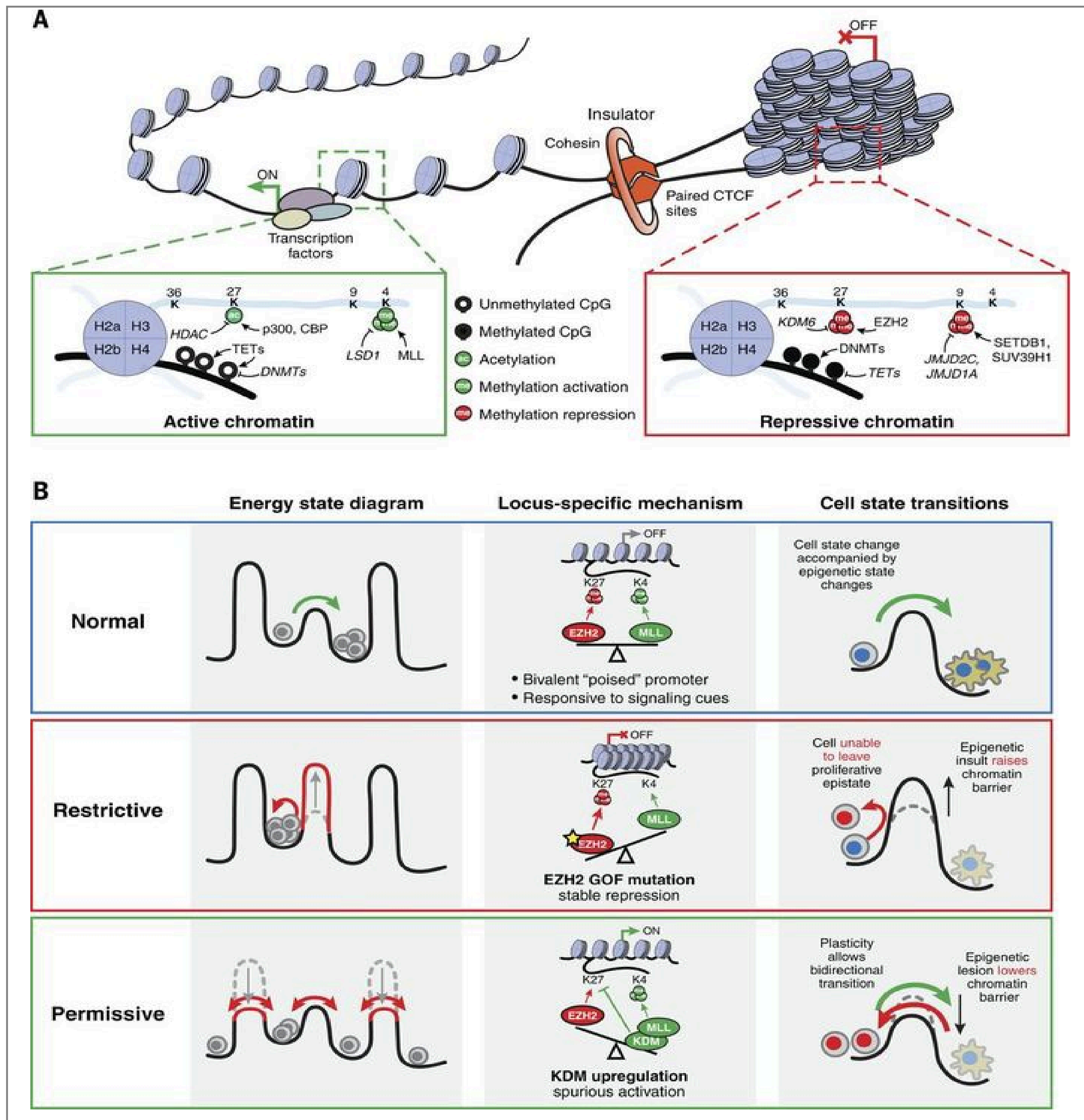

Figure 1-10*: Epigenetic changes at H3K27 alters the chromatin's activity level. Acetylation of H3K27 by HDAC and demethylation by JMJD3 or other histone demethylases (KDM) induces a permissive chromatin state, while EZH2 overweight represses transcription. Epigenetic states influence ease of cell state transitions. Reprinted from (Flavahan et al., 2017) with permission from the publisher.*

1.5 GAPS OF KNOWLEDGE

1.5.1 What is the therapeutic potency of ALK inhibitors in neuroblastoma patients?

ALK has been known as a disease driver in neuroblastoma for less than fifteen years. While ALK inhibitors have been incorporated into neuroblastoma treatment at an exceptionally fast pace, clinical experience still rests on few cases. We still do not know which patients will benefit from ALK inhibition – only those with ALK mutations (and in other malignancies, translocations), or may increased ALK activity caused by other alterations also be targetable by ALK inhibition? Cumulative information is becoming available for considerations regarding the choice of ALK inhibitor, where pharmacokinetic characteristics, the ALK mutation at hand and the possibility to target additional receptor tyrosine kinases (RTKs) with the same tyrosine kinase inhibitor (TKI) must be considered. Open questions also include the timing of initiation of ALK inhibitor treatment, the sequence of ALK inhibitors in case of development of resistance, the management of side effects or long-term consequences, and last not least when and how to discontinue ALK inhibitor treatment.

1.5.2 Is WIP1 a druggable target in neuroblastoma?

At the outset of our studies, the role of WIP1 as an oncogene was not established, even if there were some strong indications. The development of the WIP1-overexpressing mouse model strengthened the importance of WIP1 in cancer (Milosevic et al., 2021). Experiments with the WIP1 inhibitor GSK2830371 showed effects only in a subset of neuroblastoma cell lines with wild-type p53 (Richter et al., 2015), while the observation that 17q is gained in the majority of high-risk neuroblastomas suggests that WIP1 should be of importance across a wider panel of neuroblastomas.

Adjacent questions concern differences in effect and mechanism between various WIP1 inhibitors, and how the approach of WIP1 inhibition might best be used. While there is evidence of some synergism between WIP1 inhibition and MDM2 inhibition (Esfandiari et al., 2016), no wider screening for other beneficial combinations has been undertaken.

2 RESEARCH AIMS

The overall aim of this research is to contribute to the development of specific targeted therapies for childhood cancer, specifically neuroblastoma. Papers I and II report the results of investigations undertaken primarily for the benefit of two individual neuroblastoma patients, while simultaneously generating important insights for the scientific field and the treatment of similar patients. In contrast, papers III and IV relate preclinical investigations into a new therapeutic target that might become clinically relevant in the future.

Specifically, in **Paper I**, we sought to explore the potency of the ALK inhibitor ceritinib in monotherapy for *ALK*-driven neuroblastoma in a situation where conventional chemotherapy and radiotherapy were contraindicated.

Paper II aimed to demonstrate the efficacy of ALK-TRK-ROS1 inhibition in a patient with depleted conventional therapeutic options, where genetic and functional studies had demonstrated a novel ALK ligand mutation and activated ALK and TRKA in conjunction with a chromosome 2p gain.

In **Paper III**, we hypothesized that WIP1 is a therapeutic target in neuroblastoma and medulloblastoma and sought to prove this hypothesis in cell and animal models using gene knockdown and chemical inhibition.

In **Paper IV**, we asked whether WIP1 inhibition using the small molecule compound SL-176 can be enhanced by a drug combination, and discovered an intriguing synergism with GSK-J4, an inhibitor of histone H3K27 demethylase JMJD3.

3 METHODOLOGIC CONSIDERATIONS

In the following section, some reflections on the choice of methods used in the four studies shall be made. For detailed descriptions of the methods, please refer to the respective papers.

3.1 *IN VITRO* METHODS

3.1.1 Considerations regarding the use of cell lines

Cancer cell lines constitute an accessible, cheap and reliable instrument for cancer research and have formed the basis of molecular biology for decades. While a great part of our modern understanding of tumor biology is owed to experiments in cell lines, their use is associated with some well-known risks and disadvantages. Possible problems include contamination with other cancer cell lines, genetic drift, unidentified infections, the unnatural two-dimensional structure and the lack of a tumor microenvironment (Kryh et al., 2011). As a consequence, many *in vitro* results will not be successfully translated to more complex models, nor to the clinic.

On the other hand, it is difficult to altogether abstain from the use of cell lines, since more complicated experiments must always be informed by prior results. Some challenges can be overcome by careful maintenance of cancer cells in culture, avoiding infections, contaminations and too many passages. It is always advisable to include relevant controls and to use a panel of different cell lines, selected by relevant biologic criteria, in the case of neuroblastoma for example *MYCN* status, *ALK* status and *TP53* mutational status.

The use of 3D models, e.g. multi-cellular tumor spheres (MCTS), adds some layers of complexity since the tumor cells will be spatially arranged, sometimes in conjunction with a second cell type, for example fibroblasts. This represents a slight approximation toward a natural tumor growing in a three-dimensional network of other tumor cells as well as a multitude of various cells from different origins. Importantly, MCTSs share several key characteristics with primary tumors, including hypoxic gradients and restrained drug penetration (Kock et al., 2020).

Organoids, a method of increasing popularity, represent the next level of sophistication. If established from tumor tissue or a patient-derived xenograft, these organoids typically contain various cell types in addition to the cancer cells studied, including epithelial and endothelial cells, immune cells, fibroblasts, and others. This makes organoids a clinically more relevant tumor model as compared to cell lines. On the downside, the establishment of an organoid model can be challenging, time-consuming and unreliable, and special resources regarding laboratory plastics and reagents are required.

3.1.2 Cell viability assays to evaluate drug efficiency

Because the main goal of cancer treatment is to kill cancer cells, cell viability assays are an important cornerstone in the *in vitro* evaluation of potential anti-cancer drugs. Since a cell's

viability cannot be determined directly, different surrogate markers are used including metabolic activity, membrane integrity, morphology, and others. In our studies we employed the tetrazolium salts MTT and WST-1 that are cleaved to formazan if NAD(P)H is produced by the mitochondria of a metabolically active cell. Formazan is a dye and can be quantified by measuring absorbance with a spectrophotometer. Two other metabolism-based viability assays used are resazurin, an assay based on redox activity of metabolically active cells, and CellTiter-Glo®, a luminescence-based assay dependent on ATP formation. Importantly, these assays cannot distinguish dead from metabolically inactive cells. In contrast, Sytox™ Green is a nucleic acid stain that develops bright fluorescence when binding to DNA. Since it cannot cross intact cell membranes, the fluorescence will be proportional to the number of dead or dying cells, enabling the assay as a measurement of viability.

Methods based on detection of absorbance are generally less sensitive when compared to fluorescence- or luminescence-based methods, but the sensitivity of WST-1 assays can be improved by optimizing the number of cells seeded and the substrate incubation time. However, the luminescent ATP methods including CellTiter-Glo® have the capacity to detect smaller numbers of cells per well and are therefore superior for high-throughput screening applications as used in Paper IV (Riss and Moravec, 2004).

Regarding the procedure of cell viability assays, one important change was made between Paper III and Paper IV: To comply with the routines established at the drug combination screening facility, we started performing viability assays in cell culture media supplemented with the complete amount of fetal bovine serum. This affects the comparability of results between these two papers.

3.1.3 Gene silencing as a method to gain mechanistic understanding

Gene silencing using small hairpin RNA (shRNA) is an important tool for testing hypotheses regarding a certain protein’s function or importance. It allows for targeted silencing of a selected gene. An engineered short RNA sequence complementarily binds to mRNA transcripts of the gene of interest, thereby initiating their degradation. Depending on the transfection method of choice, gene silencing can be transient or stable. Through lentiviral transfection, integration of the construct into the host genome is achieved and the knockdown will be passed on to daughter cells. Stable knockdown is desirable to achieve reproducible results, and necessary for longer-lasting experiments such as xenograft formation. Though usually specific, efficiency of knock-down differs and usually several clones have to be established, from which the most efficiently silenced clones are selected for further experiments.

3.1.4 Small molecule inhibitors to gain mechanistic understanding

Through our collaboration with the Sakaguchi group at Hokkaido University, we were able to employ a novel WIP1 inhibitor which is not commercially available, SL-176 (Ogasawara et al., 2015). Several additional WIP1 inhibitors were used for comparison. As an advantage over

gene silencing, small molecule inhibitors more closely mimic the therapeutic situation and have the ability to show that a target is, in principle, "druggable". Some small molecule inhibitors later become clinical therapeutics or lead substances for the development of pharmacokinetically better adapted drugs. However, characterization of newer small molecules will often be incomplete, constituting an important disadvantage to their use and increasing the risk of misinterpreting off-target effects as effects mediated by the target under scrutiny.

3.1.5 Drug combination screening

With very few exceptions, pharmacologic treatment of malignancies is always done with drug combinations, fruitful even in the setting of precision medicine as a means to combat drug resistance (Garraway and Jänne, 2012). Efficient drug combinations can sometimes be predicted based on pharmacodynamic knowledge and cancer biology expertise. However, as ever more new small molecule drugs emerge through drug-finding efforts, it becomes increasingly difficult to foresee which drug combinations will be expedient, partly because new substances may be only partially characterized, and drugs designed for a specific target may have unidentified more or less desirable off-target effects.

Online resources like the Cancer Therapeutics Response Portal, where libraries of small molecule substances have been tested in libraries of cancer cell lines, provide ample information and allow some predictions of synergisms (Seashore-Ludlow et al., 2015).

A pragmatic way to study treatment synergism in a given setting is through drug combination screening, facilitated by highly automated high throughput systems, where one drug of particular interest is combined with a large number of different substances in different concentrations, to treat cancer cells *in vitro*. This can be applied to established cancer cell lines in the preclinical setting, or to patient samples *ex vivo* for personalized medicine (Brodin et al., 2019). Useful algorithms have been developed to evaluate drug response profiles beyond the IC_{50} value, yielding a more accurate estimate for clinical response (Fallahi-Sichani et al., 2013; Yadav et al., 2014).

In our approach, the drug of interest (SL-176 or GSK2830371) was combined with 527 individual clinical and experimental cancer drugs (Figure 3-1). A fixed dose of our study drug was added to five different concentrations of each combination drug, yielding a dose-response curve which, by taking into account the half-maximal inhibitory concentration (IC_{50}), slope, area under the curve as well as upper and lower limits, was transformed into a drug sensitivity score (DSS) (Yadav et al., 2014). An important critique of this procedure is that it lacks technical replicates. This represents a trade-off that has been accepted in order to have a tractable number of wells and plates, but it comes with the risk of technically faulty wells causing ill-fitting dose-response curves, which would mainly result in truly synergistic combinations being disregarded.

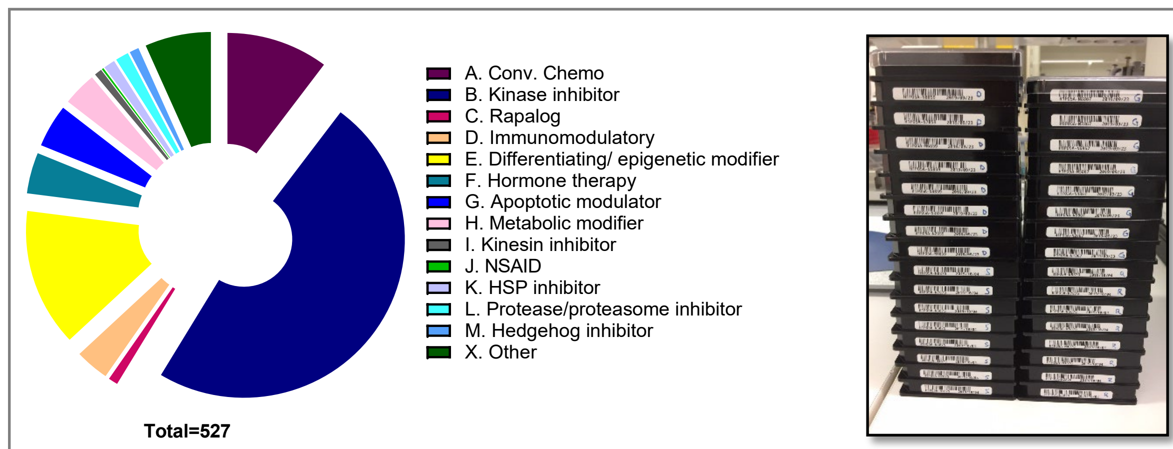

***Figure 3-1**: **Left**: Drug classes of the 527 cancer drugs included in the drug combination screening. **Right**: Stacks of 384-well cell culture plates during drug combination screening.*

3.1.6 Assessment of drug synergy

Once promising combination candidates have been identified through the screening, confirmatory experiments are conducted by combining different concentrations of both drugs in a matrix. Different mathematical methods have been proposed to assess these matrices for potential synergy, antagonism, or merely additive effect, the most well-established being Highest Single Agent (HSA), Bliss independence and Loewe additivity. We have used a newer method termed Zero Interaction Potency (ZIP), in which a delta score of zero indicates that no drug interaction (positive or negative) exists and that the drugs' effects are thus additive (Yadav et al., 2015). A positive delta score indicates synergism, a negative delta score antagonism.

As the level of synergism can vary substantially over different areas of the concentration matrix, a graphical illustration of the drug interaction landscape is useful. The freely available software SynergyFinder provides both calculation of synergy according to the methods presented above, and a three-dimensional representation of the results (Ianevski et al., 2020).

3.1.7 Gene expression analysis using qPCR and RNA sequencing

Treatment effects on transcription can be analyzed by quantitative real-time reverse transcriptase PCR (qPCR) or RNA sequencing (RNA-seq). When qPCR is used, specific targets are selected which will be amplified and quantified using primers and probes specific to this target. qPCR readings are usually reported as cycle threshold (CT) values, denoting the number of amplification cycles needed until a target is detected. The gene of interest is normalized to a housekeeping gene which should be chosen such that it is not affected by the intervention studied. When the difference between the transcript of interest and the housekeeping gene of one treatment group is compared to the difference between transcript of interest and housekeeping gene of another treatment group (e.g. vehicle), this is termed the delta-delta method. Alternatively, quantification can be performed in relation to a standard curve.

In RNA-seq, all mRNAs will be read and identified by using a reference genome. Traditionally, the number of reads for each sample is compared to the number of reads across all samples, which may not be the most intuitive approach when studying drug effects vs. vehicle. Given that the human genome contains roughly 20,000 genes, RNA-seq generates a vast amount of data. This is sometimes best approached using pathway analysis, for example gene set enrichment analysis (GSEA), in which the differentially expressed genes are compared to gene sets corresponding to known biological pathways, thus identifying the biological processes best represented by the detected changes in transcription.

3.1.8 Protein analysis using immunoblot and immunohistochemistry

Immunoblotting, or western blotting, is a standard method in biology well suited to detect proteins within a sample and to achieve a relative quantification. An advantage is that some posttranslational modifications, i.e. phosphorylation, can be detected using specific antibodies, providing information on the activation status of a protein. However, the method consists of a number of different steps – protein extraction, protein quantification, gel electrophoresis, blotting to the membrane, incubation with primary and secondary antibody, signal detection – and therefore is prone to errors. Immunoblotting can at times be performed using antibody arrays, where pre-spotted membranes carry antibodies against multiple proteins, for example different phosphokinases.

Immunohistochemistry also relies on epitope detection by specific antibodies and carries the advantage of assessing the protein of interest within the tissue, adding information on protein distribution within the cell compartment or the extracellular matrix.

3.1.9 Neurite outgrowth assay and focus formation assay

Differentiation of neural cells can be assessed by their formation of neurites, for example in response to a certain stimulus. PC12 cells, derived from rat pheochromocytoma, can differentiate toward a neural phenotype when stimulated with nerve growth factor, resulting in TRKA activation (Marshall, 1995), and toward a chromaffin-like phenotype when exposed to dexamethasone. This attribute has been exploited in constructing the neurite outgrowth assay as readout for ALK pathway activation. *ALK*-transfected PC12 cells differentiate with outgrowth of neurites in the presence of ALK ligand (Degoutin et al., 2007; Guan et al., 2015; Souttou et al., 2001).

The focus formation assay assesses an overexpressed gene's capacity to induce malignant transformation. The gene of interest is inserted into NIH3T3 cells (embryonal mouse fibroblasts), a nontransformed cell line which normally stops growing when a confluent monolayer has formed. Loss of contact inhibition due to oncogenic transformation will result in formation of foci with multilayer growth (Alvarez et al., 2014).

3.2 *IN VIVO* METHODS

3.2.1 Drosophila rough eye model

The "rough eye" model in Drosophila melanogaster represents an *in vivo* method to assess activation of the ALK pathway. Human ALK is ectopically expressed specifically in eye tissue of the fruit fly by guidance of the pGMR-GAL4 driver. ALK activation, either by presence of human ALK ligand or by expression of a ligand-independent mutant ALK, will result in aberrant morphology of the fly eye, termed "rough eye", while wild-type ALK in the absence of human ALK ligand will result in a normal eye (Martinsson et al., 2011).

3.2.2 Xenograft models

A simple vertebrate *in vivo* model for cancer is the xenograft model. This model takes advantage of an immunocompromised host, for example the *nu/nu* mouse that lacks a thymus, consequently does not have a functional adaptive immune system and therefore will allow cells from foreign species to grow within its organism. Cancer cells injected subcutaneously into the flank of these nude mice will grow as a lump, which, thanks to its superficial position and the lack of body hair, will be easily accessible for monitoring e.g. by measuring with a caliper. Different cancer cell lines naturally have different growth characteristics regarding time to first establishment, growth velocity, and composition of the emerging tumor.

This model allows for great flexibility regarding the choice of injected cancer cell line cells, but sufficient numbers and a good condition of the cells are crucial for a successful experiment. While xenograft experiments allow for some important conclusions, for example concerning a drug's pharmacokinetic and pharmacodynamic characteristics and side effects, some important limitations must be kept in mind: The resulting tumor located on the flank lacks its natural tumor niche, the host organism is immunocompromised, and many drawbacks of cancer cell lines are transferred to this *in vivo* model. Orthotopic xenografts are implanted closer to the original anatomical niche, and patient-derived xenografts (PDXs) represent a further refinement of this method where tumor cells from a specific patient are injected into mice after no or minimal passaging (Mañas et al., 2022). Conversely, if engraftment with the desired cells is not accomplished, alternative mouse strains with stronger immunocompromised phenotype are available.

3.3 ONLINE DATA EXPLORATION PLATFORMS

Because today's omics methods generate more data than a single research group can process, data are often made publicly available for other scientists. In fact, etiquette regarding the custody of datasets prompts making data available for further research, sometimes by publication in public domain databases which represent an important repository to the scientific community. The datasets may contain data on gene expression, methylation, mutations, array comparative genomic hybridization (aCGH), drug testing data, microRNA, single nuclear polymorphism data and others, and their main value is in exploration and visualization of data

and hypothesis generation. Examples of these platforms are the R2 Database, the cBioPortal, the Depmap Portal, and the Cancer Cell Line Encyclopedia (Barretina et al., 2012; Boehm and Golub, 2015; Cerami et al., 2012; Gao et al., 2013; "R2 Genomics Analysis and Visualization Platform").

3.4 STATISTICAL ANALYSES

For all statistical analyses in this thesis, GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used. The statistical tests in this thesis were generally performed on measurements made in cancer cell lines, or xenografts derived from these, where normal distribution and relatively little variation is expected. Therefore, parametrical tests are appropriate and were employed according to the following: T-test was used to compare means between two groups, while for comparison of three or more groups, one-way ANOVA followed by Bonferroni multiple-comparisons post hoc test were used. For comparison of three or more groups comprised of technical replicates nested within biological replicates, nested one-way ANOVA followed by Dunnett's multiple-comparisons post-test was used. Paired analysis was performed with repeated measures ANOVA, or, when values were missing, using mixed-effect analysis with Bonferroni multiple-comparisons post hoc test. IC_{50} values were calculated from log concentrations-effect curves using non-linear regression analysis. Survival curves were calculated according to the Kaplan–Meier method and analyzed with log-rank test, while Fisher's test was used to test significance of association between the two categories. Correlations were assessed with Pearson test/Spearman non-parametric test. $p < 0.05$ was considered significant, and all tests were two-sided.

3.5 ETHICAL CONSIDERATIONS

3.5.1 Ethical considerations regarding patient studies

Children represent a vulnerable group in relation to clinical research. Depending on their age, they might not be able to fully understand the purpose or personal consequences of a study. Even if they do, depending on national legislation, they may not have full authority to make decisions for themselves. In addition, the great variation in body size and composition in children along with the relative infrequency of malignancies renders pediatric patients a more challenging study group. From the viewpoint of the pharma industry, they also constitute a less profitable group.

As a result, availability of data regarding pharmacologic therapy in children has long been limited due to drug approval studies conducted exclusively in adults. Consequently, many medications have been given off-label, presenting the treating physician with difficult ethical deliberations and obstructing the opportunity to gather knowledge from later-phase prescription. Fortunately, these drug investigational shortcomings have been addressed by regulative authorities, who are now mandating that drugs aimed for adult approval must also be studied in relevant pediatric populations (Aurich et al., 2021).

Regarding individual patients who do not meet inclusion criteria and are treated outside clinical studies, ethical permits may still be necessary in some cases and informed consent should be obtained regarding the publishing of case studies. However, the patients´ right to integrity has to be carefully balanced against the ethical need to disperse new knowledge, since other patients might be in urgent need of treatment endorsed by our experiences. Luckily, most families are very much in favor of research and strongly advocate the publishing of research data involving their child.

3.5.2 Ethical considerations regarding the use of animal models

Because cell culture models of all different degrees of complexity still represent a simplification of the multicellular organism and therefore do not allow the study of all pharmacokinetic and pharmacodynamic aspects, animal experiments will continue to be a necessary step in drug development for humans. However, *in vitro* methods should be optimized to inform subsequent animal experiments in a way that reduces the size and number of animal experiments. Animal experiments, in turn, should be refined to yield as much data as possible. Any animal experiment is ethically justifiable only if it is designed in a way that makes it possible to answer the research question, for example by including all relevant control groups. National laws and institutional guidelines regulate animal experimentation to ensure animal welfare. All animal experiments performed within this thesis were approved by the responsible regional ethics committee, and we took care to avoid suffering on behalf of the animals.

3.5.3 Ethical considerations regarding the use of resources

Ethical considerations should not be limited to animal welfare and patient integrity. Since we are all part of a society and inhabitants of the planet, we must ask if the research conducted results in ethical use of the limited resources. A pediatrician must ask herself if the time spent on research should better be spent on patient care. Since research is costly, non-governmental organizations and politicians must ask themselves how much money should be spent on for example childhood cancer research, to the comparative disadvantage of other deserving causes such as vaccination programs or famine relief. History tells us, however, that the sum of research and development has helped humanity forward.

Similar considerations are true for the environmental impact of research. Laboratory work undeniably causes large amounts of garbage; journeys to international conferences cause carbon dioxide emissions. In this respect, replacement, reduction and refinement are of importance even outside animal experimentation. The aspect of refinement maybe has the greatest bearing. Through striving to optimize the research questions asked and the experiments planned, we can nourish a fair hope that advancement through our research contributions will exceed the harm caused by our experiments.

3.6 STRENGTHS AND LIMITATIONS OF THE PRESENTED RESEARCH

3.6.1 Strengths of our research approach

The key strength which fuels the research presented in each of the four papers is collaboration. Our research group already has a broad interdisciplinary buildup including competence in molecular biology, pediatric oncology and pharmacology, and the capacity to perform animal experiments in-house. For the papers presented, further collaborations have been sought out.

In Papers I and II, unique observations in individual patients treated in the clinic spawned research questions which were addressed by geneticists as well as molecular biologists with specific expertise in ALK in their respective laboratories. Important results, quickly taken back to the clinic for the patient's benefit, simultaneously generated scientific insight. This is a resource-intense approach to a clinical problem which nevertheless demonstrates the potency of precision oncology.

In Papers III and IV, our research approach profits from a collaboration with a research group in chemistry who have developed a novel WIP1 inhibitor, SL-176, which is not available commercially. We have utilized SL-176 in cell culture and animal models of neuroblastoma. SL-176 has partly divergent characteristics from other WIP1 inhibitors commonly used and will therefore help nuance the understanding of WIP1 inhibition.

Another important collaboration stands with a Science for Life Laboratory group experienced in, and equipped for, high-throughput drug screening. In partnership with this group, we were able to assess two different WIP1 inhibitors in combination with a library of cancer drugs, generating further insight into WIP1 inhibition and possible synergistic approaches.

3.6.2 Limitations of our research approach

Our molecular case reports rely on individual patients whose situation is very specific and might not be generalized to larger groups of neuroblastoma patients. However, the approach to study individual cases in depth, including molecular and functional studies, fills an important purpose and can provide insight not easily acquired in other ways. Needless to say, generated hypotheses will have to be proven in larger study populations.

All laboratory models are flawed and often it is the combination of different models that strengthens the research approach. The choice of methods is frequently dictated by resources and availability.

3.6.3 Specific limitations of each paper

3.6.3.1 Paper I:

In this study, it would have been advantageous to follow the patient's tumor development more closely before and after initiation of ALK TKI treatment. However, the patient's condition

ordained extreme caution with the use of ionizing radiation, and MRI resources are limited, always requiring anesthesia in young children. Imaging is shown at diagnosis, prior to ALK TKI start, and after 6 and 21 months of treatment.

3.6.3.2 Paper II:

In this study, an ALK-TRK-ROS inhibitor was used for the treatment of neuroblastoma that was both ALK- and TRK-activated at protein level, in the absence of corresponding genetic changes. Instead, a germline mutation of the ALK ligand ALKAL2 was found in the patient. While there is persuasive evidence that entrectinib had a game-changing effect in this patient, we cannot with certainty attribute this effect to inhibition of ALK, even though this is the most likely explanation.

Also, entrectinib therapy was provided in conjunction with cyclooxygenase-2 (COX-2) inhibitor therapy which had been initiated previously. Therefore, a combination effect cannot be excluded. This combination has not been studied *in vitro*.

3.6.3.3 Paper III:

In Paper III, the effect of *PPM1D* knockdown was studied in SK-N-BE(2) cells, a neuroblastoma cell line harboring a loss-of-function *TP53* mutation. Since an important share of WIP1 effects are based on p53 inhibition, it would have been preferable to investigate WIP1 inhibition in a *TP53* wildtype cell line. However, those displayed poor viability after *PPM1D* knockdown, necessitating the choice of SK-N-BE(2) for further experiments. Later findings of approximately equal effects of chemical WIP1 inhibition regardless of *TP53* status endorse the importance of WIP1 beyond inhibition of p53.

Further limitations of Paper III include the use of Western blots with overexposed loading controls which may obscure differences in protein loading. These blots were developed on film and exposure could therefore not be corrected.

3.6.3.4 Paper IV:

The paper describes a newly identified synergism between H3K27 demethylase inhibition and WIP1 inhibition. However, the synergism is only demonstrated by pharmacological inhibition. To rule out off-target effects, it would have been desirable to combine corresponding knockdowns with each other, as well as combining knockdowns with pharmacological inhibition of the respective other target. The latter approach was attempted with sh*PPM1D* SK-N-BE(2) cells, but failed because the transfected cell line, probably by selection, seemed to have lost its knockdown phenotype (data not shown).

4 RESULTS AND DISCUSSION

4.1 PAPER I: TARGETED THERAPY AS THE LESS TOXIC ALTERNATIVE

This study was initiated out of the vital needs of a patient presenting with a therapeutic dilemma. A child under induction therapy for CNS-metastatic neuroblastoma developed extraordinary toxicity which was later understood to be caused by underlying Fanconi anemia. The DNA repair defects associated with this condition precluded further use of chemotherapy and irradiation at conventional doses and also dictated restrictive use of radiologic modalities involving x-rays. Auspiciously, an *ALK* mutation was identified in the child's tumor by exome sequencing, opening up the opportunity of targeted therapy. To date, more than 35 distinct mutations of *ALK* have been reported (Hallberg and Palmer, 2016). Mutations of *ALK* usually occur in the kinase domain and are predominantly found at the three hotspots F1174, F1245, and R1275 (Bresler et al., 2014; Brouwer et al., 2010; Hallberg and Palmer, 2013). Different mutations convey different sensitivities to the various available ALK TKIs, and some mutations are known to be promoted by ALK inhibitor treatment (Hallberg and Palmer, 2016).

4.1.1 *ALK*-I1171T identified as novel gain-of-function ALK mutant

In the present patient, genomic analysis identified a novel heterozygous point mutation at *ALK*-I1171T, in the proximity of *ALK* mutational hotspot F1174. Although this particular mutation had not been reported previously in patients, another mutation of the same amino acid, *ALK*-I1171N, had been observed (Bresler et al., 2014; Mossé et al., 2008), and *ALK*-I1171T had been predicted as a crizotinib resistance mutation following an *in vitro* mutagenesis screening (Zhang et al., 2011). To test whether *ALK*-I1171T constituted a gain-of-function mutant capable of driving the disease, its ability to stimulate neurite outgrowth in PC12 cells (Schönherr et al., 2011) was assessed, demonstrating ALK activity at the same elevated level as the well-characterized variant *ALK*-I1174L. This was further corroborated by this mutant's ability to induce foci of malignantly transformed NIH3T3 cells in a focus formation assay.

4.1.2 ALK-mutated cell lines are sensitive to ceritinib

Phosphorylation assays demonstrated that the *ALK*-I1171T variant, transfected into PC12 pheochromocytoma cells, responded to various ALK inhibitors, with IC_{50} values of later-generation inhibitors ceritinib, lorlatinib and brigatinib one order of magnitude below that of crizotinib. In a panel of characterized gain-of-function *ALK* mutations, all mutations conveyed a somewhat stronger sensitivity towards ceritinib when compared to crizotinib, but for *ALK*-I1171N this difference was most distinct. Conversely, viability experiments were conducted showing that out of a panel of neuroblastoma cell lines, all those with a known *ALK* driver mutation were sensitive to treatment with ceritinib. IC_{50} values were comparable to those of crizotinib. The observation that *ALK* wildtype cell lines were resistant to ALK inhibitors is consistent with the clinical experience that neuroblastomas without *ALK* mutations do not

respond to ALK inhibitor treatment (Mossé et al., 2013). Finally, phosphoproteomic profiling in neuroblastoma cells treated with ceritinib revealed differential phosphorylation patterns in *ALK*-mutant cell lines as compared to *ALK*-wildtype cells.

Taken together, these results rendered ceritinib a suitable TKI for inhibition of ALK-I1171T, well in concordance with *in vitro* experiments conducted by another group already before this mutation had been found in patients (Friboulet et al., 2014).

4.1.3 Ceritinib monotherapy in a patient with a constitutive DNA repair defect

At the time of this intervention, no previous experience was available regarding ceritinib treatment in neuroblastoma patients. When the child was diagnosed with Fanconi anemia, precluding further treatment with chemotherapy or radiation at conventional doses, the finding of an *ALK* mutation presented an option for a more tolerable intervention in the shape of targeted therapy. At the time, few ALK inhibitors were available for clinical use, and crizotinib – the only ALK inhibitor tested in neuroblastoma – had shown disappointing treatment effect in the first trial (Mossé et al., 2013) and appeared less potent *in vitro* toward the ALK mutation at hand. Ceritinib was at the time under clinical investigation for ALK-positive NSCLC as well as anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumour (IMT), and neuroblastoma and could be made available to the patient within a compassionate use program. Treatment was started as soon as the patient had recovered from the hematological toxicities inflicted by the induction treatment.

Ceritinib was tolerated by the child with minor gastrointestinal toxicity. Catecholamine metabolites were normalized after one cycle of four weeks, and the volume of the primary tumor shrunk, allowing for resection after 7.5 months of treatment. Histopathological analyses of the excised tumor revealed signs of differentiation along with calcification and reduced proliferation. Importantly, the patient's CNS metastases – still detectable at start of treatment – responded well to ceritinib therapy as demonstrated by an MRI scan after six months of treatment. After 21 months, the associated skull bone involvement had also cleared up. The patient continued on ceritinib monotherapy for a total of 50 months before the treatment was terminated in the setting of complete remission. Since withdrawal of ceritinib, he has been monitored regularly using circulating cell-free DNA in addition to scheduled MRI scans. Meanwhile, he has received an allogeneic stem cell transplant as a definite treatment for his Fanconi anemia.

Subsequently, a multicenter trial of ceritinib in ALK-positive pediatric malignancies, including 30 neuroblastoma patients, found only partly encouraging results, with 20% of neuroblastoma patients responding to this treatment (Fischer et al., 2021). Interestingly, most of the responding neuroblastoma tumors harbored the mutation *ALK*-R1245Q, while the majority of patients suffering progressive disease despite ceritinib treatment had tumors with mutations at F1174 (Figure 4-1), a site associated with ALK TKI resistance mutations toward crizotinib and ceritinib, as reported in the context of NSCLC (Friboulet et al., 2014; Hallberg and Palmer, 2016).

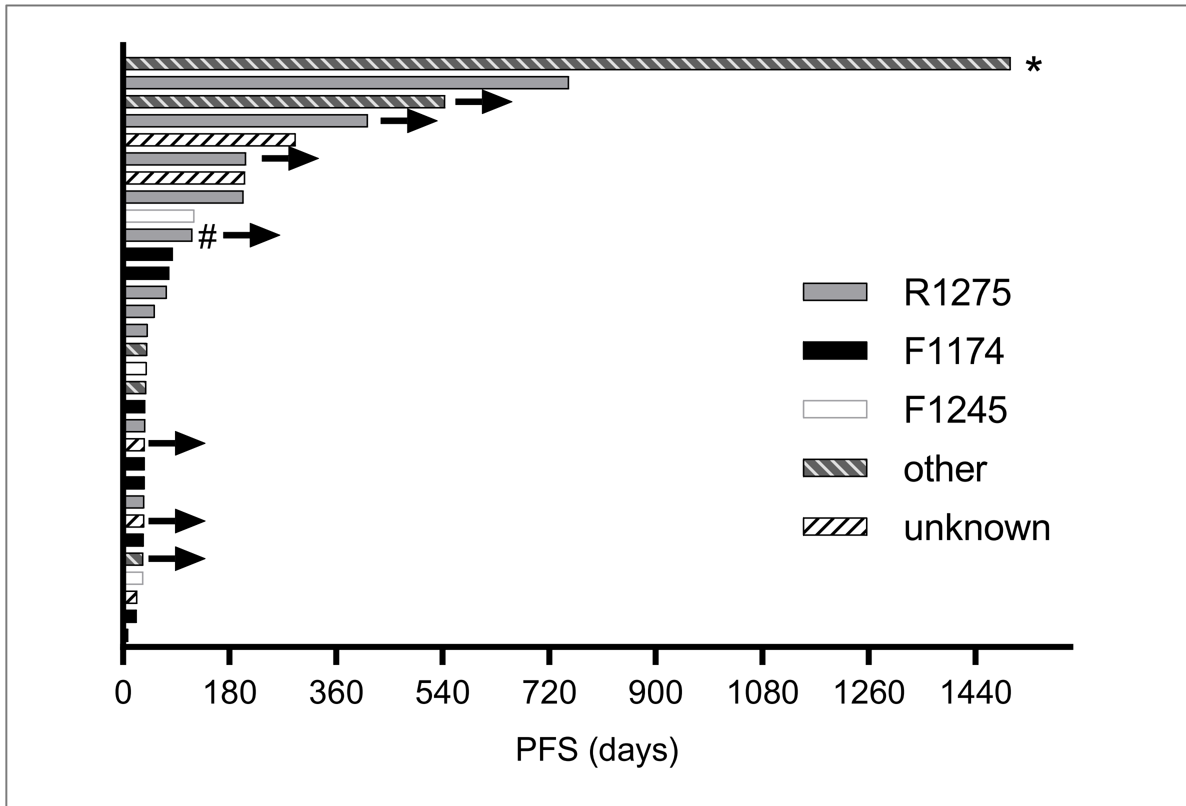

***Figure 4-1**: Progression-free survival (PFS) of neuroblastoma patients treated with ceritinib. Patient described in Paper I (*) and data from patients in (Fischer et al., 2021), fill pattern indicates mutated ALK codons, # indicates germline mutation, arrow indicates PFS longer than depicted. Patients followed for >1 day are included.*

The intracranial drug activity of ceritinib represents a major advancement in comparison to the first-generation ALK inhibitor crizotinib, which displays poor blood-brain barrier penetration (Okimoto et al., 2019). In various trials of ceritinib in NSCLC patients, brain metastatic response rates were around 30-60% (Chow et al., 2022; Mok et al., 2017).

The patient described suffered from Fanconi anemia, one of various cancer predisposition syndromes. Several DNA sequencing studies among pediatric cancer patients congruently found a prevalence of 6-8.5% cancer-predisposing germline mutations (Gröbner et al., 2018; Summers et al., 2022; Zhang et al., 2015). Since knowledge of predisposing syndromes may influence the choice of therapeutic strategy, prognosis, and screening for second malignancies, assessment of both tumor and germline mutations is desirable. To this end, Genomic Medicine Sweden has adapted the strategy of performing whole genome sequencing (WGS) on tumor and normal tissue for all newly diagnosed or relapsed pediatric cancer patients in Sweden (Stenzinger et al., 2022).

4.2 PAPER II: TARGETED THERAPY IN REFRACTORY NEUROBLASTOMA

Study II is based on the case of an infant diagnosed with extensively metastasized and highly metabolically active neuroblastoma. Because the patient was only six months old and the tumor was *MYCN*-nonamplified, he was allocated to the intermediate risk group and treatment was initiated according to LINES. As the patient failed to respond to this protocol, to the ensuing high-risk protocol, and to various rescue chemotherapy treatments, efforts were undertaken to more thoroughly characterize the tumor biology. Genomic reassessment of the tumor after surgery revealed a gain in chromosome 2p, activated ALK and TRKA in the absence of corresponding genomic alterations, and a novel mutation in ALK ligand ALKAL2, which was found to be in the child's germline and inherited from his healthy mother.

4.2.1 The novel ALKAL2 variant ALKAL2-S53L is functional

To assess whether the newly identified variant ALKAL2-S53L retained normal ALK ligand function, the neurite outgrowth assay was employed. By co-transfecting PC12 cells with *ALKAL2*-S53L and wild-type *ALK*, it could be shown that the novel ALKAL2 variant activated ALK equally well as wild-type ALKAL2, achieving neurite outgrowth to the same extent as the constitutionally active ALK mutant ALK-F1174L which was included as positive control.

In addition, *ALKAL2*-S53L was assessed in a Drosophila melanogaster model, which upon ALK activation responds with development of the "rough eye" phenotype. Here, coexpression of human *ALK* with wildtype *ALKAL2* or its variant *ALKAL2*-S53L equally induced this phenotype, confirming that *ALKAL2*-S53L represents a functional *ALKAL2* variant.

While this patient's ALKAL2 variant by itself was not found to be more potent than wild-type ALKAL2, the tumor did display a chromosome 2p gain which was newly acquired as observed when the surgical specimen was re-assessed. Since this chromosome portion contains both *ALK* and *ALKAL2*, it is conceivable that the sheer amount of ligand and receptor conveyed the ALK activation demonstrated and contributed to drive the disease. This would be in line with findings of Borenäs and colleagues, who showed that downstream activation in response to ALKAL2 exposure was stronger in the neuroblastoma cell line with higher *ALK* expression, and that this signal could be suppressed by use of ALK inhibitor lorlatinib (Borenäs et al., 2021). In addition, overexpression of *ALKAL2* in the Th-*MYCN* neuroblastoma mouse model increased penetrance of and anticipated tumor formation, resulting in neuroblastoma tumors sensitive to ALK inhibitor treatment in the absence of *ALK* mutations (Borenäs et al., 2021). This also supports the claim that the patient's *ALKAL2* mutation may have contributed to the development of his disease.

4.2.2 Entrectinib matches the tumor's biologic properties

Resected tumor material was subjected to immunoblot and phospho-RTK array, revealing increased levels of TRKA and ALK protein as well as activated downstream targets. Even if relative contribution to driving the disease was not possible to establish, the finding of

simultaneous ALK and TRKA activation in the patient's tumor material let a TKI directed at both these targets appear desirable. The ALK-panTRK-ROS1 inhibitor entrectinib matched this profile and was during this period in clinical development for solid tumors. However, only few preclinical studies – and no clinical experiences – were available addressing entrectinib efficiency in neuroblastoma (Aveic et al., 2015; Iyer et al., 2016). Therefore, entrectinib effects on ALK and TRKA were assessed in neuroblastoma cell lines. On the one hand, entrectinib was added to two *ALK*-driven neuroblastoma cell lines, confirming its dose-dependent potency to decrease ALK phosphorylation and inhibit downstream targets. On the other hand, a neurite outgrowth assay based on TRKA stimulation by nerve growth factor was employed (Marshall, 1995), showing that neurite outgrowth of neuroblastoma cells could be abrogated by entrectinib treatment. Thus, entrectinib appeared suitable to target both ALK and TRKA and was recommended for this patient.

Additional inhibitors of both ALK and TRKA have become available at a later point of time, such as repotrectinib (Cervantes-Madrid et al., 2019; Drilon et al., 2018; O'Donohue et al., 2021).

4.2.3 Slow but sustained clinical response to entrectinib

At the time, the phase I/II entrectinib study STARTRK-NG (NCT02650401) was including children and adolescents 2-22 years of age with solid tumors (Desai et al., 2022). Since the patient was too young to enter this study, he was treated according to a compassionate use program. General well-being improved rapidly after onset of treatment, but a biopsy of a liver metastasis after two months still showed viable tumor cells. Subsequently, urine catecholamine markers decreased gradually, normalizing only over the course of several years. Likewise, pulmonary and hepatic metastases diminished very slowly. After almost six years, the patient still continues on entrectinib treatment.

Meanwhile, entrectinib has been studied in additional neuroblastoma patients: STARTRK-NG (*clinicaltrials.org* identifier NCT02650401), a multicenter phase I/II trial of entrectinib in pediatric patients, was primarily designed as a study of *NTRK*, *ALK*, and *ROS1* fusion-positive CNS and extracranial solid tumors; however, neuroblastoma patients were initially included regardless of biologic markers. One neuroblastoma patient included in the trial harbored an *ALK*-F1174 mutation and achieved a complete remission, but because of the overall poor performance in neuroblastoma, inclusion criteria were modified following a protocol amendment to comprise only solid tumors with *NTRK* or *ROS1* gene fusion (Desai et al., 2022).

4.2.4 Bone fractures and other possible side effects

No apparent side effects, apart from mild nausea, were noted during the first months of treatment. After around 6 months of entrectinib treatment, however, from about 2.5 to 3 years of age, the patient developed pathologic fractures in his lower legs, in both tibiae as well as the right fibula. These fractures arose sequentially and without any associated adequate traumata. New metastases were ruled out and after review by tumor-orthopedic surgeons, the fractures

were deemed to be caused by the previous heavy chemotherapy which the patient had undergone. One of the fractures required surgical intervention, but all eventually healed under ongoing entrectinib treatment, and no dose reduction was made since no causal relation was suspected. However, STARTRK-NG subsequently reported bone fractures in 20.9% of patients, raising suspicion of entrectinib as causative (Delgado et al., 2021; Desai et al., 2022). This compares to an estimated background fracture prevalence of 6.7% in high-risk neuroblastoma patients and 3.35% in the general pediatric population, as reported in a recent retrospective study (Scruggs et al., 2022). In contrast, bone fractures in the corresponding adult entrectinib trials were less common at 5.3% (Delgado et al., 2021).

Over the course of several years, it became apparent that the patient's growth catch-up surpassed the desirable level in terms of weight development. At times, even diagnostic criteria for obesity were met. Overweight is a known side effect of both ALK inhibitor treatment and TRK inhibition, and is viewed as an on-site effect regarding both targets, given that both ALK and TRK are thought to play a role in appetite regulation (Liu et al., 2020; Mason et al., 2013; Orthofer et al., 2020; Peled et al., 2020; Yang and Gong, 2019). In STARTRK-NG, weight gain was reported as an adverse event in almost half of all patients, while the adult trials ALKA-372-001, STARTRK-1 and STARTRK-2 reported weight gain in about 10-20% (Delgado et al., 2021; Desai et al., 2022; Doebele et al., 2020; Drilon et al., 2018). For the third-generation ALK inhibitor lorlatinib, weight gain, often associated with hyperlipidemia, appears to be even more common at 90%, as stated in an interim report from the first pediatric phase I study (NCT03107988)(Goldsmith et al., 2020).

4.2.5 Lessons learned

The reported case illustrates how thorough biologic workup can be very beneficial for an individual patient. The decisive findings that opened up for RTK TKI usage in this case were the protein assays demonstrating activation of ALK and TRK in the absence of genomic changes. While efforts to advance precision medicine are flourishing, most programs within pediatric oncology rely on sequencing methods such as whole exome sequencing (WES), whole genome sequencing (WGS), RNA-seq and DNA methylation analysis (Blattner-Johnson et al., 2022). Studies of protein expression are usually too resource- and time-intense to be included on a large scale.

However, since DNA profiling is usually performed in routine clinical workup, the finding of a 2p gain may prompt analysis of ALK protein activation and consideration of ALK inhibitor therapy.

4.3 PAPER III: WIP1, AN IMPORTANT NEW TARGET IN NEUROBLASTOMA

Gain of 17q is the most common genetic aberration in neuroblastoma, and it represents an independent risk factor for poor prognosis (Bown et al., 1999). Mapping of the gained regions

showed a wide variety of breakpoints, indicating that a gene dose effect regarding a gene within the gained region was likely to explain tumor formation, rather than a fusion event (Schleiermacher et al., 2004). The commonly gained region has been localized to chromosome 17q23.1-17qter, a region harboring several genes implicated in human cancers, including *PPM1D*. *PPM1D* encodes the phosphatase WIP1, an inhibitor of p53 and other proteins central to DNA damage response (DDR), providing an attractive mechanistic foundation for this gene's importance. This study addresses the hypothesis that the poor prognosis associated with 17q gain can be explained by increased gene dosage of *PPM1D*, and that its encoded protein WIP1 represents a tractable target in neuroblastoma.

4.3.1 *PPM1D* expression is found in all tested neuroblastoma cell lines and correlates with 17q gain

A panel of neuroblastoma cell lines was assessed with qPCR, showing that *PPM1D* was expressed to different extent in all cell lines, while expression levels similar to the positive control, the breast cancer cell line MCF-7, were approached by neuroblastoma cell lines IMR-32 and SK-N-DZ. Correlation to earlier findings (Kryh et al., 2011; Schleiermacher et al., 2004) demonstrated that *PPM1D* is contained within the gained region of chromosome 17q in all tested cell lines. As expected, *PPM1D* expression in medulloblastoma cell lines was highest in those with 17q gain.

In line with our expectations, assessment of gene dependencies using the open resource Broad Institute Cancer Dependency Map (*https://depmap.org/portal/*) confirmed a higher genetic dependency on *PPM1D* in those neuroblastoma cell lines harboring wild-type *TP53*, as compared to those with mutated *TP53*, illustrating *PPM1D* overexpression as an alternative strategy to overcome the anti-proliferative effects of p53 (Meyers et al., 2017). Proteins involved with p53 degradation – USP7, MDM2 and MDM4 – achieved comparable scores for genetic dependency. It was also demonstrated that neuroblastoma and medulloblastoma cell lines in average are more dependent on *PPM1D* than other cancer cell lines.

4.3.2 *PPM1D* knockdown inhibits neuroblastoma growth

To investigate the role of WIP1 in neuroblastoma, stable shRNA knockdown of *PPM1D* was used. Since most other cell lines were rendered nonviable by *PPM1D* knockdown, the *TP53*-mutated cell line SK-N-BE(2) was selected for further studies. *PPM1D*-knocked SK-N-BE(2) cells displayed poor proliferation rates and reduced clonogenic capacity and were sensitized to irradiation, in comparison to scrambled shRNA controls. Immunoblotting demonstrated a larger degree of phosphorylation in WIP1 targets, consistent with inhibition of the phosphatase WIP1. When SK-N-BE(2) cells harboring *PPM1D* knockdown were subcutaneously injected into nude mice, tumor formation was delayed. Other groups have demonstrated a similar impact of *PPM1D* knockdown on medulloblastoma, lung, colorectal, bladder cancer and renal cell carcinoma cells, strengthening the role of *PPM1D* as an oncogene (Buss et al., 2015; Liu et al., 2014; Peng et al., 2014; Wang et al., 2014; Zhang et al., 2014). Homozygous *PPM1D* knockout

in tumor-prone mammary carcinoma mouse models delayed tumor formation, while overexpression of *PPM1D* in mice that were also irradiated resulted in cancers reminiscent of the phenotype of *TP53*-mutant mice, most commonly thymic lymphoblastic lymphoma, adenocarcinoma and sarcoma (Bulavin et al., 2004; Milosevic et al., 2021).

4.3.3 WIP1 inhibitors mimic effects of *PPM1D* knockdown

Pharmacologic inhibition of WIP1 was assessed using four different small molecule WIP1 inhibitors on six different neuroblastoma cell lines, comparing dose-response curves and IC_{50} values. As expected from literature, the older compound CCT007093 displayed rather low potency, while SPI-001 and especially its derivative SL-176 achieved more potent IC_{50} values across the various cell lines (Ogasawara et al., 2015; Rayter et al., 2008; Yagi et al., 2012). For the allosteric inhibitor GSK2830371 we could confirm the reported pattern that cell lines' sensitivity depends on *TP53* wild-type status (Richter et al., 2015), while the IC_{50} values determined in our study are lodged between those of two previous studies of GSK2830371 in neuroblastoma cells (Chen et al., 2016; Richter et al., 2015). The variation can likely be explained by differences in the treatment protocols regarding number of cells seeded, cell culture medium composition, incubation times, and the assay used. Taken together, SL-176 appeared to be the most potent WIP inhibitor, which, importantly, achieved complete inhibition of viability in all neuroblastoma cell lines.

To further compare WIP1 inhibition with restitution of p53 function through other indirect mechanisms, the MDM2-p53 interaction inhibitors nutlin-3 and RITA were included (Issaeva et al., 2004; Van Maerken et al., 2014; Vassilev et al., 2004). Of these, RITA has also been observed to have WIP1-inhibiting properties (Spinnler et al., 2011). While some neuroblastoma cell lines were very sensitive, there was substantial variation in susceptibility to RITA and nutlin-3. SL-176, on the other hand, efficiently inhibited all eleven neuroblastoma cell lines included in this panel with an IC_{50} range of 0.44 – 1.3 µM.

When SL-176 was further investigated *in vivo*, using nude mice with established xenografts of SK-N-BE(2) cells – or, for the medulloblastoma arm of the study, DAOY cells – no overt side effects were noted and a significant slowdown in tumor growth was demonstrated. Immunohistochemistry of tumors revealed decreased proliferation markers and an increase of apoptosis. However, tumors did not disappear or shrink in response to SL-176 treatment. Similarly, the WIP1 inhibitor GSK2830371 has been investigated in an orthotopic xenograft model of SH-SY5Y neuroblastoma cells, showing that GSK2830371-treated animals developed smaller, but still sizeable, tumors (Chen et al., 2016). No direct comparisons have been made regarding anti-neuroblastoma effects of the two WIP1 inhibitors SL-176 and GSK2830371 *in vivo*.

4.3.4 Significance of WIP1 as a treatment target in neuroblastoma

In synthesis, the role of WIP1 in DNA damage response, the frequent overexpression of WIP1 in neuroblastoma and other cancers, observations in *PPM1D* knockout and overexpression

mouse models, as well as effects of *PPM1D* knockdown in neuroblastoma cell lines argue for the significance of *PPM1D*/WIP1 as an oncogene of importance in neuroblastoma (Richter et al., 2015; Saito-Ohara et al., 2003). *TP53* status appears to influence neuroblastoma cells' vulnerability to WIP1 inhibition, as demonstrated by the Dependency Map findings as well as neuroblastoma cells' divergent sensitivity toward GSK2830371. However, our results of the shRNA *PPM1D* knockdown in *TP53*-mutated cells as well as our experience with the WIP1 inhibitor SL-176 support the oncogenic role of WIP1 in all neuroblastoma cell lines. Altogether, we and others deliver proof-of-principle evidence that WIP1 constitutes a druggable target *in vitro* and *in vivo*. While it is encouraging that neither WIP1 inhibitor investigated in mouse models elicited any overt side effects, changes of the molecular structures will be needed to improve solubility and pharmacokinetic properties before the substances can be tested clinically (Nahta and Castellino, 2021).

4.4 PAPER IV: SYNERGISTIC INHIBITION OF WIP1 AND JMJD3

Owed to cancer cells' outstanding capacity to evolve and adapt, combination pharmacotherapy forms an important cornerstone in virtually all cancer treatment. Neither SL-176 nor GSK2830371 achieved complete anti-neuroblastoma efficiency *in vivo*, making a suitable drug combination desirable (Chen et al., 2016)(Paper III). A drug combination screening was performed in two different neuroblastoma cell lines to identify synergistic molecular strategies for combination with inhibition of WIP1.

4.4.1 Drug combination screening

In IMR-32 cells, the combination screening identified 31 compounds where the ΔDSS – the difference between DSS of the single screening drug and DSS of the combination with SL-176 – surpassed the arbitrarily chosen cut-off of 5 (Figure 4-2). We found the highest ΔDSS, 18.9, for the combination of SL-176 with the substance GSK-J4, an epigenetically active drug which inhibits H3K27 demethylases JMJD3 and UTX (Kruidenier et al., 2012). The corresponding dose-response curves indicated a shift to the left exceeding one order of magnitude when SL-176 was added instead of vehicle. When screening in SK-N-AS cells, we found only two compounds with ΔDSS ≥5. Here, GSK-J4 had the second highest ΔDSS of 5.7, and the combination dose-response curve was shifted to the left. In both cell lines, evaluation of dose-response curves revealed some suspected false-positives among substances other than GSK-J4.

Thus, screening for synergistic combinations with SL-176 in two different cell lines resulted in GSK-J4 as the most promising combination candidate. This was particularly interesting since JMJD3 and UTX inhibition has been implicated previously in neuroblastoma context: Lochmann and colleagues performed a comprehensive study showing that GSK-J4 is effective against a subset of neuroblastoma cell lines *in vitro* and *in vivo* (DuBois and Park, 2018; Lochmann et al., 2018).

In the analogous screening with the commercially available WIP1 inhibitor GSK2830371, we found that 37 drug combinations yielded ΔDSS values ≥5 in IMR-32 cells. Here, carboplatin had the highest ΔDSS of 11.1, when presumed false-positives were excluded. Several MDM2 inhibitors appeared to act synergistically with GSK2830371, which is in concordance with the literature (Chamberlain et al., 2022; Esfandiari et al., 2016; Pecháčková et al., 2016; Wu et al., 2021, 2018).

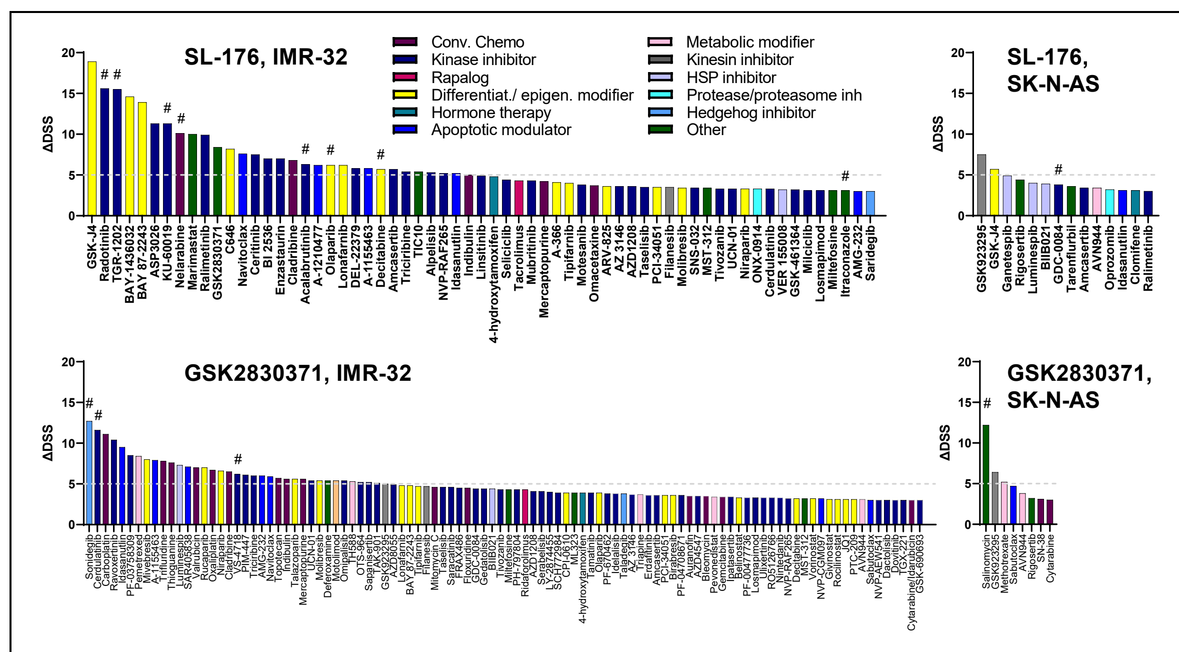

Figure 4-2: Waterfall plots of drug combinations with SL-176 (upper panel) and GSK2830371 (lower panel) resulting in ΔDSS >3. Left, screening in IMR-32 cells. Right, screening in SK-N-AS cells. Dashed line, ΔDSS =5. # indicates substances which are suspected to be false-positives, based on review of the corresponding dose-response curves.

Intriguingly, the strongest combination candidate for combination with SL-176, GSK-J4, did not stand out as a potential synergistic combination partner for the other WIP1 inhibitor, GSK2830371. On the other hand, combining the two distinct WIP1 inhibitors SL-176 and GSK-2830371 resulted in ΔDSS values indicating likely synergy. The potential to combine synergistically with each other and the property to combine differentially with third drugs suggests that SL-176 and GSK2830371 have distinct mechanisms of action in inhibiting WIP1.

For both WIP1 inhibitors, more hits were found in the screening in IMR-32 cells then in SK-N-AS cells, a cell line with mesenchymal characteristics known for its broad drug resistance (van Groningen et al., 2017).

4.4.2 SL-176 and GSK-J4 synergy is confirmed in viability experiments

To verify the putative synergism identified through the screening, we performed viability assays in a panel of neuroblastoma cell lines which were treated with 6-8 different concentrations of SL-176 and GSK-J4 in a combination matrix. Some degree of synergism was found for all five neuroblastoma cell lines tested, including the neuroblastoma cell lines SK-N-

DZ, SK-N-AS and SK-N-BE(2) which by Lochmann et al. had been classified as "resistant" to GSK-J4 (Lochmann et al., 2018). The degree of synergism was highly dependent on drug concentrations chosen, and the highest synergy score was found in IMR-32 cells. Dose levels were identified at which the combination of SL-176 and GSK-J4 achieved nearly complete inhibition, while each single drug at that concentration hardly induced any change in viability.

To further investigate the synergistic effect of SL-176 and GSK-J4 in a three-dimensional tumor model, multi-cellular tumor spheroids (MCTS) consisting of neuroblastoma cells and normal human dermal fibroblasts (nHDFs) were used. Both in IMR-32 and SK-N-AS spheroids, a dose-dependent effect on size and viability was observed after combination treatment, while single drugs at the same doses elicited smaller or no changes. Immunohistochemistry of tumor spheroids six days after addition of drugs showed an increase of tumor cells positive for cleaved caspase-3 in treated MCTSs, and this change appeared stronger after combination treatment, indicating increased apoptosis in cells treated with SL-176 and GSK-J4.

4.4.3 SL-176 and GSK-J4 jointly induce cell cycle arrest and apoptosis

To explore the changes induced by combination treatment on RNA and protein levels, we performed qPCR and immunoblots on neuroblastoma cells treated with vehicle, single drug or the combination of SL-176 and GSK-J4. After 48-72 hours, combination-treated cells showed phosphorylation of WIP1 downstream targets while total p53 and p21 were also upregulated, indicating cell cycle arrest. In addition, an increase of apoptosis markers was observed. Cells treated with only SL-176 or only GSK-J4 showed no such changes. qPCR demonstrated pronounced upregulation of expression for *BBC3* (p53-upregulated modulator of apoptosis; PUMA) and *CDKN1A* (p21) in combination-treated IMR-32, SK-N-BE(2) and SK-N-AS cells, while relatively unchanged *TP53* mRNA levels indicated that the upregulation seen in immunoblots must be of non-transcriptional nature.

Despite minor differences between cell lines, this shows that the synergism between SL-176 and GSK-J4 observed in viability experiments is matched by changes at gene expression and protein level, suggesting that GSK-J4 enhances the molecular effects of SL-176 to an over-additive extent.

4.4.4 RNA-seq indicates upregulation of DDR pathways

In an effort to better understand the mechanisms and the molecular pathways involved in this synergism, RNA sequencing (RNA-seq) was performed. With reference to vehicle treatment, RNA-seq data showed grossly increased numbers of differentially expressed genes in the combination-treated cells, as compared to cells treated with only SL-176 or only GSK-J4.

Both IMR-32 cells and SK-N-BE(2) cells had large numbers of up- and downregulated genes in response to combination treatment, but the overlap of these differentially expressed genes was comparatively small. To understand the signaling pathways involved, we performed gene

set enrichment analysis employing "Hallmark" gene sets (Liberzon et al., 2015), and found that among the top four gene sets indicated for each cell line, the following three were common: TNFα signaling via NFκB, p53 pathway and TGFβ signaling. This confirms that DNA damage response pathways are enriched by the combination of SL-176 and GSK-J4 in neuroblastoma cell lines.

4.4.5 Possible mechanisms underlying the synergism

Inhibition of WIP1 is intended to enhance DNA damage response by releasing p53 and other associated proteins involved with cell cycle arrest and apoptosis, which are dephosphorylated by WIP1. However, a recent study quotes >35 dephosphorylation targets of WIP1, adding layers of complexity (Gräf et al., 2022). The molecular consequences of inhibiting JMJD3 remain even less clear, despite endeavors by several groups to untangle the mechanisms involved.

Even within the field of neuroblastoma, JMJD3 has been ascribed both tumor suppressor and oncogenic properties (D'Oto et al., 2021; Lochmann et al., 2018; Yang et al., 2019). The work by Lochmann et al. showed that JMJD3 inhibition by GSK-J4 induced differentiation, upregulation of *BBC3* and cell death in neuroblastoma cells (Lochmann et al., 2018). At the doses we used, we observed slight upregulation of *BBC3* transcription in IMR-32 cells in response to GSK-J4 treatment, while combination with SL-176 caused a stronger increase both in IMR-32 and, notably, in SK-N-BE(2) cells classified as resistant to GSK-J4 by Lochmann et al. Our experiments did not recapitulate the differentiation effects described by Lochmann et al. after GSK-J4 treatment. This may perhaps be explained by a dose level effect, since the GSK-J4 concentrations in this work were chosen at levels where low doses of GSK-J4 and low doses of SL-176 appeared to cooperate most synergistically.

D'Oto and colleagues' work on neuroblastoma cells presented the model that JMJD3 inhibition causes H3K27me3 accumulation which disturbs the interaction of transcription factors, resulting in downregulation of the CDK4/6-pRB-E2F pathway and of *MYCN* (D'Oto et al., 2021). Regarding effects on *MYCN*, we detected divergent effects in two *MYCN*-amplified cell lines possibly arguing against *MYCN* alteration as a core mechanism. However, the study by d'Oto et al. also demonstrated an increase of p21 after GSK-J4 treatment which was traced back to enhanced chromatin accessibility at the promoter of *CDKN1A*, the gene encoding p21. Thus, the strong increase in p21 that we have observed upon combining SL-176 and GSK-J4 might have its explanation in the combination of higher levels of available p53 and higher accessibility of *CDKN1A*.

Another recent study implicated *SAPCD2* as a central gene for understanding the effects of GSK-J4 in neuroblastoma (Zhang et al., 2022). In our RNA-seq data, this gene was downregulated in SK-N-BE(2) but not in IMR-32 after combination treatment, speaking against SAPCD2 effects as a key concept in this context.

Taken together, the substantial synergism observed between SL-176 and GSK-J4 in neuroblastoma cells appears to have its explanation in convergent effects on several important pathways of DNA damage response, while the precise molecular routes seem to vary according to the respective cell line's prerequisites. Altered chromatin accessibility may also play a role in this synergism, but this has not been studied in the present work. Further investigation including additional cell lines, doses and time points, as well as epigenetically targeted methods, will be needed to fully understand the interplay between these two synergistic substances. Further, since both substances in monotherapy have shown anti-neuroblastoma effect in animal models, it would be highly desirable to conduct an *in vivo* combination experiment.

5 CONCLUSIONS

This thesis aimed to illuminate two therapeutic targets of present or future importance in neuroblastoma precision medicine. We studied ALK inhibitor treatment in specific clinical situations following detailed molecular investigations, and we explored *PPM1D*/WIP1 as a therapeutic target in neuroblastoma by inhibiting WIP1 alone and in combination.

In summary, we draw the following specific conclusions:

- *ALK*-I1171T is a novel gain-of-function *ALK* mutant sensitive to the ALK inihibitor ceritinib.
- Examination of tumor material on protein level may uncover new therapeutic options for the patient.
- ALK activation may possibly be mediated by mutations in ALK ligands.
- The choice of ALK inhibitor should be carefully tailored to the ALK aberration at hand, taking into account possible additional RTK aberrations.
- ALK inhibitors have the potential to induce longstanding remission, but objective treatment effects may be protracted.

- *PPM1D* is overexpressed in neuroblastoma, and many neuroblastoma cell lines are dependent on *PPM1D* for survival.
- Its gene product, the phosphatase WIP1, can be targeted using small molecule inhibitors, leading to enhanced DNA damage response, and constraining neuroblastoma growth *in vitro* and *in vivo.*
- The small molecule WIP1 inhibitors GSK2830371 and SL-176 have distinct cytotoxic profiles, where SL-176 is effective in all tested neuroblastoma cell lines including those with *TP53* mutation.
- There is pronounced synergy between SL-176 and the H3K27 demethylase inhibitor GSK-J4 in activating WIP1 downstream targets and eliciting cell cycle arrest and apoptosis.

6 POINTS OF PERSPECTIVE

6.1 REFINEMENT OF ALK TKI THERAPY

Our studies illustrate the importance of matching ALK inhibitors to the *ALK* aberration at hand and suggest that a wider group of patients may profit from ALK inhibitor treatment, if ALK activation is present in the absence of *ALK* mutations.

Further refinements of ALK inhibition are needed regarding the timing of treatment. Although mutant *ALK* is viewed as a driver mutation in neuroblastoma, ALK TKI studies hitherto have been carried out in refractory or recurrent neuroblastoma. However, in the TITAN study – Transatlantic Integration Targeting ALK in Neuroblastoma – SIOPEN and COG high-risk neuroblastoma protocols will incorporate upfront lorlatinib for patients with ALK-positive tumors (*clinicaltrials.gov* identifiers NCT03126916, NCT04221035). Hopes are that early initiation of ALK inhibition will critically ameliorate outcome for this subset of patients.

Though luckily not observed in our patients, relapse attributable to development of resistance mutations poses a threat to successful ALK inhibitor treatment (Berlak et al., 2022). Furthermore, increased use of ALK inhibitors also necessitates vigilance towards possible side effects caused by long-term ALK inhibition, given that pediatric patients may develop different adverse effects than adults, who are hitherto the foremost source of clinical experience with ALK inhibitors.

6.2 DISCONTINUATION OF ALK INHIBITOR TREATMENT

Since ALK inhibitors have become available to neuroblastoma patients, some responders have remained on this treatment for long times, sometimes exceeding five years. In view of costs and certain side effects, including weight gain, the question arises if and when the ALK inhibitor should be discontinued.

In the field of chronic myelogenous leukemia, the issue of TKI treatment withdrawal has been studied systematically and some guidelines have been proposed, which may in part be adaptable to neuroblastoma. Criteria proposed to be eligible for TKI discontinuation include length of TKI treatment of at least 2-3 years, as well as at least 1-2 years of "deep molecular remission" defined as absence of detectable transcripts in qPCR. Importantly, patients who lost deep molecular remission upon TKI discontinuation and therefore resumed TKI treatment did not experience progressive disease (Inzoli et al., 2022; Shima et al., 2022).

In order to apply a similar approach to solid tumor patients, diagnostic criteria for an equivalent of "deep molecular remission" must be developed. These are currently under discussion within the field and may in the case of neuroblastoma include radiology, MIBG or equivalent, bone marrow sampling and liquid biopsies, which in some cases would need to be tailored to the individual patient (Andersson et al., 2020; Bosse et al., 2022). Systematic studies will be needed to evaluate such an approach.

6.3 EMERGING TARGETS IN NEUROBLASTOMA

In this thesis, we present evidence for the role of *PPM1D*/WIP1 as a therapeutic target in neuroblastoma, supported by epidemiological observations, experimental findings and biologic plausibility. Its inhibition appears to be synergistically augmented by combination with epigenetically active drugs, but further studies are needed to explain the mechanism of this synergism and to confirm its efficacy *in vivo*. However promising these findings, the substances studied are as yet not suited for clinical trials but are in want of pharmacokinetic improvements. Two additional, recently published WIP1 inhibitors might also serve as lead compounds to this end, but have not been tested in neuroblastoma cells so far (Clausse et al., 2022).

Many other neuroblastoma targets have been proposed, and their numbers are increasing steadily. Some developments are driven by new understanding of neuroblastoma biology, other discoveries are serendipitous results of drug screening or drug repurposing programs. Proposed targets span from oncoproteins, tumor suppressor proteins, cancer pathways and RTKs to long non-coding RNA and microRNA, to new epitopes for immunotherapy and targeted radiation (Aravindan et al., 2020). Faced with the dilemma that the number of substances available for clinical testing surpasses the number of patients in the relatively rare malignancy neuroblastoma, the NDDS has agreed upon a ranking of drugs to be prioritized for clinical evaluation (Moreno et al., 2020). However, since one hallmark of high-risk neuroblastoma likely is genetic and epigenetic instability promoting alternating and interplaying oncogenic mechanisms, rational assembly of combination therapeutic backbones will be needed for cure (Aravindan et al., 2020).

6.4 PRECISION MEDICINE IN NEUROBLASTOMA

Contrary to the traditional approach of finding a treatment strategy that fits all or most patients and proving its superiority in large clinical trials, personalized or precision medicine strives to tailor treatment to an individual patient by thoroughly investigating this patient's disease. These investigations aim to identify predictors of response to distinct treatments and can be carried out with varying depth. Numerous national and international programs have been initiated for pediatric oncology, combining different omics approaches including WGS, WES, RNA-seq and methylation assays and always highlighting short turnaround times to allow for timely initiation of tailored targeted therapy or combinations of therapies (Blattner-Johnson et al., 2022). Modeling novel individual mutations in cell lines and fruit flies, as done in our studies, increases complexity and adds predictive value at the cost of a longer time to results. Patient-derived organoid or xenograft models represent an even more sophisticated approach in which a personalized tumor model is exposed to potential treatments, but where a readout can only be expected after several months. In the future, if availability of resources continues to develop favorably, a combination of these approaches might become standard in the care of the child with neuroblastoma.

7 ACKNOWLEDGEMENTS

This inspiring, sometimes frustrating but always enriching time – my PhD journey – is now finally coming to its end. All this would not have been possible, had I not been surrounded and supported by all of you, wonderful colleagues, dear friends, and my beloved family.

My main supervisor **Per Kogner**, thank you for sharing your resources, network and wisdom with me. Your exceptional power to inspire enthusiasm has carried me through a number of challenges, and your ability to make connections based on your wide and deep knowledge of many things, including both science and trivia, never stops astonishing me. You are visionary, you are ambitious, you get things going. Throughout your career, you have had a great impact on the lives of children with neuroblastoma, and it has been an honor to be part of your team.

My supervisor **John Inge Johnsen**, you are solid as a rock. Your deep biological understanding, scientific instinct and laboratory experience has often helped the group find the right path. But your wisdom goes beyond science; you have travelled the world and thought about the big questions in life. I thank you for your kindness, reliability, perseverance and determination to get things finished. This thesis would not have been possible without you.

Dear **Malin Wickström Näsman**, my third supervisor and brilliant friend – thank you so much. Through your sober and systematic approach, you have helped structure my way to this thesis, preferring the feasible over the fantastic. Your scientific knowledge and curiosity, as well as pharmaceutic expertise, have been fundamental to our projects. I wouldn't have been able to finish this work without you, and I certainly wouldn't have had as much fun. I hope for many joint projects in the future.

I thank **Ninib Baryawno**, the fourth P.I. of the group, for encouraging words, interesting discussions and for bringing some momentum and American sting into the group.

Post-docs **Jörg Otte**, **Conny Tümmler**, **Cici Dyberg** and **Thale Kristin Olsen**: Thank you for thoroughly and patiently teaching me new methods, for discussions about life and research, and for ensuring great quality of our scientific output! Extra thanks to Conny for careful editing.

Linda Ljungblad – we haven't had any joint scientific project (yet), but you have been a fantastic friend to lean on especially during our final months of becoming ready for our defenses, having overcome similar challenges during our PhD student time. I am truly grateful for this and looking forward to our PhD celebratory kayaking outing next summer!

I thank former PhD student **Jelena Milošević** for sharing her project with me, introducing me to the field, for many inspiring discussions and for unravelling the importance of WIP1.

All former and present PhD and master students, as well as associated researchers, of the Kogner-Johnsen-Wickström-Baryawno group: **Adele Alchahin, Adena Pepich, Ammelie Boje, Anna Kock, Betty Embaie, Fredrik Sundquist, Gabriel Gallo-Oller, Gustav Hedberg, Hanna Larsson, Ioana Gavriliuc, Ioanna Tsea, Jakob Stenman, Kristina Ihrmark Lundberg, Lena-Maria Carlson, Manouk Verhoeven, Nina Eissler, Quinty Vellema, Sara Abu Ajamieh, Shahrzad Shirazi Fard** and **Teodora Andonova**: Thank you for your comradery, energetic mindset, bright conversations and for creating such a nice work environment.

I'd like to especially thank **Lotta Elfman**, now first mate on M/S Tintomara, for introducing me to many laboratory techniques and filling the laboratory with your friendly spirit. You are much missed in the group.

Thank you, **Désirée Gavhed, Anna Nilsson, Indranil Sinha** and all other dear colleagues and nice people at the **Childhood Cancer Research Unit** and the **KBH administration** for administrative support, commitment, encouragement and inspiring conversations throughout the years.

To cooperation partners near and far, I'd like to extend my gratitude for pleasant and successful collaboration in the past and ongoing, including **Sandra Wessman** and **Lena Gordon Murkes** at Karolinska University Hospital, **Emma Åkerlund** and **Brinton Seashore-Ludlow** at SciLifeLab, **Anna Djos, Susanne Fransson, Ruth Palmer, Bengt Hallberg, Tommy Martinsson** and many others at Göteborg University, and **Keiji Tanino** and **Kazuyasu Sakaguchi** at Hokkaido University.

I would also like to thank my **clinical colleagues at Astrid Lindgren's Children's Hospital, Karolinska Huddinge**, for allowing me time away from the clinic and supporting and encouraging me throughout the years. I thank each and every one of you for being such great friends and doctors.

This research would not have been feasible without the **patients and their families** who participate and engage in childhood cancer research, and the funding agencies, especially **Barncancerfonden**, that make it financially possible. Thank you dearly!

My extended family and all my friends, near and far, old and new – I thank you for being in my life, for support and encouragement, for consolation and celebrations. You mean so much to me!

My dear brothers **Richard Treis**, **Simon Treis** and **Oliver Treis**, my wonderful sisters-in-law **Tanja Treis** and **Catarina Bjelkengren-Treis,** and my spirited nephews and nieces **Johan, Erica, Oscar, Tilda** and **Carl** – I thank you for sharing all sides and tides of life, for giving me perspectives of life outside science and medicine, and for all great family times of the past and the future.

My beloved parents, **Marianne Silverstolpe-Treis** and **Peter Treis**, I cannot thank you enough for all the opportunities you have given me in my life, for bestowing upon me trust, optimism and candor, for supporting and loving me always, and for being wonderful grandparents.

My children **Jakob**, **Rasmus** and **Viktor**: you are three sweet, thoughtful, bright, inquisitive and strong-willed little individuals who teach me so much every day. Being your mom is sometimes challenging, but it is the best part of my life.

Lastly, I thank my husband **Nicky Thilmann**. Living with me has never been easy, and lately, it has certainly changed to the worse. I thank you for putting up with me, for always taking care of me, and for being such a good father to our children. I look forward to the rest of our lives together.

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