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NOVEL THERAPEUTIC TARGETS IN PEDIATRIC NEURAL TUMORS

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Novel therapeutic targets in pediatric neural tumors

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To my dear family: my mother, my twin-sister and to my late beloved father, who passed away from cancer.

"I do not think there is any thrill that can go through human heart like that felt by the inventor as he sees some creation of the brain unfolding to success… Such emotions make a man forget food, sleep, friends, love, everything."

- Nikola Tesla

ABSTRACT

The embryonal tumors neuroblastoma and medulloblastoma are two of the most common and deadliest tumors in childhood. Both are heterogeneous tumors that arise in the peripheral and central nervous system, respectively. The observed heterogeneity in these tumors is reflected by patient outcomes, where patients with favorable, low-risk tumors, have survival rates exciding over 90%. In contrast, patients classified as high-risk show an aggressive tumor behavior with survival rates of less than 40%. Because of aforementioned reason, there is a great need of finding novel and better therapeutic approaches for patients with high-risk disease.

Cancer is a disease where normal cells divide in an uncontrolled fashion, propagating and invading nearby and distant tissues, shrewdly circumventing cell intrinsic, and external defense mechanisms against oncogenic transformation. Cancer cells hijack and deregulate signaling networks that in normal cells regulate fundamental processes. In this thesis, several cellular networks are investigated, namely Hedgehog (HH), Wingless (Wnt), DNA repair and the DNA damage response (DDR) pathway, with the aim to identify novel therapeutic targets in neuroblastoma and medulloblastoma.

Neuroblastoma has been linked to aberrant HH signaling. Here we demonstrate that the GLI oncogene is highly vulnerable in non-*MYCN* amplified high-risk neuroblastomas tumors. The GLI antagonist GANT61 inhibited neuroblastoma growth in preclinical models, and potentiated the cytotoxic effects of conventional chemotherapeutic drugs. Our findings suggest that targeting the HH signaling in neuroblastoma is a highly attractive therapeutic target for high-risk neuroblastomas (Paper I). The DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) is associated with chemoresistance and is frequently overexpressed in cancer. In search for modulating MGMT activity to restore chemosensitivity in neuroblastoma and medulloblastoma, we found a novel link between Wnt signaling and MGMT gene regulation. By breaking this interaction through pharmacological and genetic inhibition we demonstrate that MGMT expression is suppressed and sensitivity to chemotherapy is restored. These results provide a basis of combining Wnt inhibitors with chemotherapy in patients with high MGMT expression (Paper II). One of the most common chromosomal abnormalities found in neuroblastoma and medulloblastoma and predictor of adverse outcome is gain of the q-arm of chromosome 17 and isochromosome 17q. The p53 protein phosphatase magnesium-dependent 1 delta *(PPMID)*/Wild-type p53 induced phosphatase 1 (WIP1) is suggested by several reports to be one of the putative oncogenes located on 17q. Our study demonstrates that *PPM1D*/WIP1 can be activated through several mechanisms, including copy number gain, gene amplification, alternative splicing and oncogenic mutations. Moreover, *PPM1D*/WIP1-transgenic mice develop a variety of cancers following external DNA stress, thus confirming the oncogenic role in cancer development. Our preclinical genetic, molecular, and pharmacological findings propose WIP1 as a novel therapeutic target in neuroblastoma and medulloblastoma (Paper III).

LIST OF SCIENTIFIC PAPERS

The following papers and manuscript are included in this thesis and will be referred to by their Roman numerals:

I. Wickström M*, Dyberg C*, Shimokawa T, **Milosevic J**, Baryawno N, Fuskevåg OM, Larsson R, Kogner P, Zaphiropoulos PG, Johnsen J. *Targeting the hedgehog signal transduction pathway at the level of GLI inhibits neuroblastoma cell growth in vitro and in vivo.* International Journal of Cancer. 2013;132(7):1516-24.

II. Wickström M*, Dyberg C*, **Milosevic J***, Einvik C, Calero R, Sveinbjörnsson B, Sandén E, Darabi A, Siesjö P, Kool M, Kogner P, Baryawno N*, Johnsen JI*. *Wnt/β-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signaling prevents chemoresistance.* Nature Communications. 2015;6:8904.

III. **Milosevic J**, Treis D, Gallo G, Fransson S, Wickström M, Gulyas M, Elfman L, Sveinbjörnsson B, Hertwig F, Bartenhagen C, Wilhelm M, Abel F, Javanmardi N, Thankaswamy-Kosalai S, Eissler N, Tanino K, Matthias Fischer, Kool M, Sakaguchi K, Kanduri C, Baryawno N, Martinsson T, Johnsen JI*, Kogner P*. *The PPM1D encoded WIP1 phosphatase is significant for cancer development and tumor progression and provides a therapeutic target in neuroblastoma and medulloblastoma.* Manuscript.

*These authors contributed equally to the manuscript and share primary and senior authorship, respectively.

CONTENTS

LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma kinase
APC	Adenomatous poliposis coli protein
ARF	Alternative reading frame
ARID1A/B	AT-rich interactive domain-containing protein 1A/B
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia and rad3 related
ATRX	Alpha thalassemia/mental retardatein syndrome X linked
BARD	BRCA1 associated RING domain 1
Bcl-2	B-cell lymphoma 2
BIRC5	Baculoviral IAP repeat-containing protein 5
BRCA1	Breast cancer 1
BDNF	Brain-derived neurotrophic factor
CAMTA1	Calmodulin binding transcription activator 1
CASC1	Cancer susceptibility candidate 1
CHD5	Chromodomail helicase DNA binding protein 5
Chk1	Checkpoint 1
Chk2	Checkpoint 2
COS2	Costal-2
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protrin
CTNNB1	Beta-catenin
DDR	DNA damage response
Dhh	Desert hedgehog
EFS	Event-free survival
ERα	Estrogen receptor alpha
FRZ	Frizzled
GANT-61	GLI antagonist 61
GLI	Glioma-associated oncogene
GSK3-β.	Glycogen synthase kinase-3β
H2AX	H2A histone family, member X

γH2AX	Gamma H2A histone family, member X
HH	Hedgehog
i17q	Isochromosome 17q
IDRF	Image-defined risk factors
Ihh	Indian hedgehog
INK4	Inhibitors of CDK4
INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
LGR	Leucin-rich repeat-containing G-protein coupled receptor
Lin28b	Lin-28 homolog B
LMO1	LIM domain only 1
LOH	Loss of heterozygosity
LRP	Low-density lipoprotein receptor-related protein
MDM2	Murine double minute 2
MGMT	O6-methylguanine DNA methyltransferaa
Mir34a	MicroRNA 34a
MMTV	Mouse mammary tumor virus
MNA	MYCN amplification
MYCN	V-Myc avian myelocytomatosis viral oncogene neuroblastoma
MYC	V-Myc Myelocytomatosis Viral Oncogene Homolog
NBAT1	Neuroblastoma associated transcript 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated Bcells
NF1	Neurofibromatosis
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
OS	Overall survival
OTX2	Orthodenticle homeobox2
p38MAPK	p38 Mitogen-activated protein kinas
PHOX2B	Paired-like Homeobox 2b
PNET	Primitive neuroectodermal tumors

PPM1D	Protein phosphatase magnesium-dependet 1 delta
PFS	Progression free survival
PTCH	Patched
PTPN11	Tyrosine-protein phosphatase non-receptor type 11
RITA	Reactivation of p53and inductionof tumor cell apoptosis
SHH	Sonic hedgehog
SMO	Smoothened
SUFU	Suppressor of fused homolog
TCF/LEF1	T-cell factor/lymphoid enhancer-binding factor 1
TERT	Telomerase reverse transcriptase
TP53	Tumor Protein 53
Trk	Tropomyocin receptor kinase
UNG2	Uracil-DNA glycosylase
VGEF	Vascular endothelial growth factor c
WHO	World health organization
WIP1	Wild-type p53 induced phosphatase 1
WNT	Wingless

1 INTRODUCTION AND BACKGROUND

1.1 CANCER

Cancer is a condition where the body´s own cells have "gone wild", and lost their own fine-tuned control over growth and acquired the potential to invade and propagate in an unrestraint manner, causing life-threatening consequences to the organism…

Karkinos (greek word for crab*)*, was the first word for cancer used by Hippocrates (460-375 BC) that appeared in the medical literature (Mukherjee, 2011).*"The tumor, with its clutch of swollen blood vessels around it reminded Hippocrates of a crab dug in the sand with its legs spread in a circle"* (Mukherjee, 2011). However, cancer as a disease was originally described much earlier. Around 3000 BC the description of "a bulging tumor of the breast" was found in the Edwin Smith Papyrus, describing what appeared to be a breast cancer (Hajdu, 2011). Cancer is not exclusive to man, it is an entity that has existed long before human kind, as revealed by paleopathologic findings found in animals from prehistoric times (Hajdu, 2011).

Cancer is a complex disease that we are still far from eradicating. This complexity is demonstrated by the different biological ingenuities that a normal cell acquires on its road of transformation to become a cancer cell. These "hallmarks" are mainly comprised by six biologic skills, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastases (Hanahan and Weinberg, 2000). Later, in 2011 additional four hallmarks complementing the previous six were added, namely inflammation, genome instability, avoidance of immune destruction and deregulation of cellular energetics (Hanahan and Weinberg, 2011).
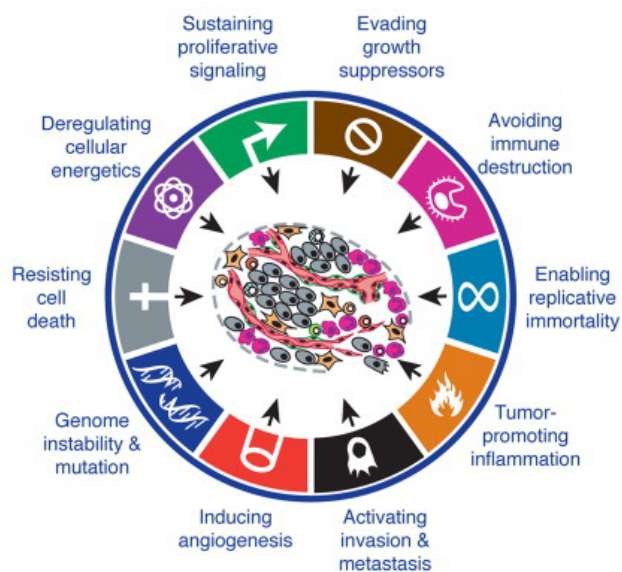

Figure 1. The ten hallmarks of cancer. Adapted from Hanahan & Weinberg, in compliance with the conditions of the Elsevier user license. Copyright © 2011 Elsevier, Inc.

Cancer accounts for one in six deaths world wide, with 8.8 million victims in 2015, which makes it the second leading cause of death in man after cardiovascular diseases (http://www.who.int/Topics/cancer/en; *Accessed* February 2018).

In Sweden, approximately 65.000 individuals were diagnosed with cancer in 2015 and the overall 10-year survival is about 65% (https://res.cloudinary.com/cancerfonden/image/ upload/v1422262211/documents/cancer-i-siffror.pdf; *Accessed* January 2018). There are close to 500.000 individuals living in Sweden that have been diagnosed with cancer at some point during their life and two thirds of these are diagnosed above 65 years of age. The most common malignancies in Sweden are breast cancer in women (constitutes 30.3% of all cancers in women) and prostate cancer in men (constitutes 32.3% of all cancers in men). The second most common cancers in both men and women are different types of skin cancer (malignant melanoma, basal-cell carcinoma and squamous-cell carcinoma), which constitute about 10% of all cancers (https://res.cloudinary.com/cancerfonden/image/upload/v14222622 11/ documents/cancer-i-siffror.pdf Accessed: January 2018).

Depending of the origin of the cell and tissue from which the tumors arise, they are classified into four main groups, namely epithelial, mesenchymal, hematopoietic and neuroectodermal (Weinberg, 2014). Neuroectodermal tumors originate from the outer cell layer of the early embryo, from cells that form the central and peripheral nervous system. Cancers arising in the neuroectoderm include neuroblastomas, medulloblastomas, gliomas, glioblastomas and schwannomas (Tulla et al., 2015; Weinberg, 2014). The embryonal childhood cancers neuroblastoma and medulloblastoma are further described in subsection 1.3 and 1.4, respectively.

1.2   PEDIATRIC CANCER

Tumors in childhood differ from tumors that arise in adults. Unlike adult tumors, malignancies in children are rear events (15 per 1000,000 children per year), accounting for less than 2% of human cancers (Friedman and Gillespie, 2011). In Sweden, around 300 children are diagnosed with cancer each year, and cancer is the primary cause of death to disease in children under the age of 15 years (Gustafsson et al., 2013). The etiology of most childhood cancers is unknown. This is unlike adult cancers, where the specific risk factors are known to a much higher degree. Also, adult cancers usually need several decades to develop, and have therefore much higher mutational load than pediatric cancers that have a very short latency period (Johnsen et al., 2009; Scotting et al., 2005; Zhang et al., 2015). Furthermore, tumors in adults are most commonly carcinomas with epithelial origin, whereas pediatric cancers are mainly embryonic, arising due to disordered development (Friedman and Gillespie, 2011; Johnsen et al., 2009; Scotting et al., 2005). The most common childhood cancers include leukemia, CNS tumors, lymphoma, neuroblastoma, Wilms tumor, sarcomas (rhabdomyo-, osteo- and Ewing sarcoma) and retinoblastoma (Friedman and Gillespie, 2011; Gustafsson et al., 2013; Tulla et al., 2015; Zhang et al., 2015).
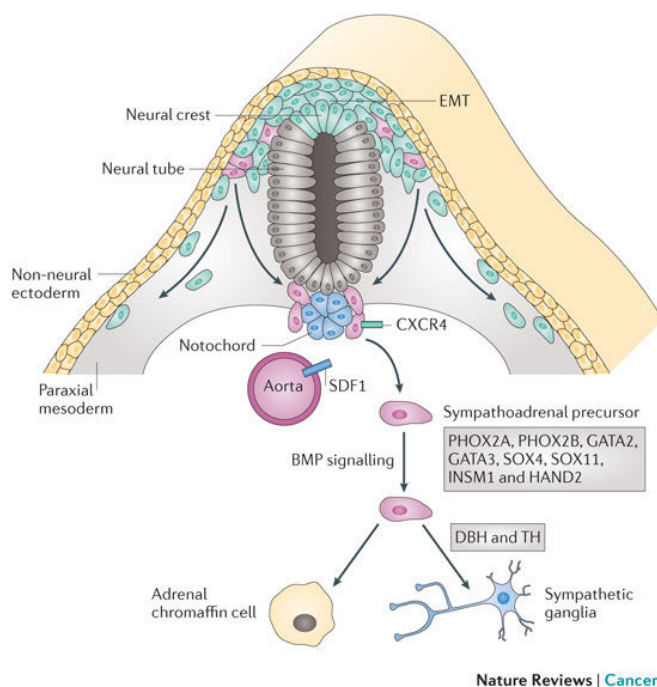
1.3   NEUROBLASTOMA

1.3.1   Historical overview and Epidemiology

As early as 1864 neuroblastoma was first described as "glioma" by the German pathologist Rudolf Virchow, "the father of modern pathology" (Virchow, 1865) . However, the name neuroblastoma was first suggested by James Homer-Wright in 1910 (Wright, 1910). He described the cells of the tumor to be more or less undifferentiated nerve cells or neuroblasts, hence the name neuroblastoma (Wright, 1910).

Neuroblastoma is an embryonal tumor of the sympathetic nervous system. It arises from cells of neural crest stem cell lineage, and is the most common malignancy diagnosed in infancy (<1 year of age), with 25-50 cases per million individuals (Brodeur, 2003; Maris, 2010; Matthay et al., 2016; Tulla et al., 2015) and 10 cases per million children under 15 years of age (Gustafsson et al., 2013; Heck et al., 2009; Irwin and Park, 2015; Träger, 2009; Whittle et al., 2017). Most neuroblastoma patients are diagnosed before five years of age, with a median age of 18 months, and with a slight male dominance (~1.2/1 male/female ratio) (Gustafsson et al., 2013; Irwin and Park, 2015; Träger, 2009; Whittle et al., 2017). Neuroblastoma is responsible for 6 -10% of all childhood malignancies in Europe and USA (Brodeur and Bagatell, 2014; Gustafsson et al., 2013; Howlader N et al., 2012; Park et al., 2013). Approximately 650 children are diagnosed with neuroblastoma each year in the United States (Howlader N et al., 2012) and in Sweden, this number is approximately 15 (Gustafsson et al., 2013). Although the survival of children diagnosed with neuroblastoma has improved over the last decades this childhood cancer still accounts for 10% of cancer related deaths of young children (Brodeur, 2003; Gustafsson et al., 2013; Park et al., 2013).

1.3.2   Biology

The cell of origin in neuroblastoma is thought to be from the sympathoadrenal lineage of the neural crest during development (Figure 2) (Anderson and Axel, 1986; Anderson et al., 1991; Hoehner et al., 1996) and the tumors arise in the adrenal glands or sympathetic ganglia (Matthay et al., 2016; Shohet and Foster, 2017). Neuroblastoma, a term that usually encompasses all peripheral neuroblastic tumors derived from the ectoderm, is a heterogeneous disease with tumors that either regress

Figure 2. **Development of the sympathoadrenal lineage.**
of the neural crest. Reprinted from Cheung & Dyer, 2013
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spontaneously or differentiate into a benign ganglioneuroma. These tumors mainly consist of neuroblastic cells and Schwannian cells in various proportions (Brodeur, 2003; Luksch et al., 2016; Shimada et al., 1999).

The most common metastatic sites of neuroblastoma include bone, bone marrow and lymph nodes (Brodeur, 2003; Johnsen et al., 2009; Shohet and Foster, 2017). Children of more than 1 year of age that get diagnosed, usually have extensive or metastatic disease with much worse prognosis (Brodeur, 2003). In contrast, infants with stage 4S disease have neuroblastoma tumors that preferably metastasize to the liver and skin. These children have excellent prognosis as their tumors usually spontaneously regress (Brodeur, 2018; Brodeur and Bagatell, 2014; D'Angio et al., 1971).

1.3.3 Genetic predisposition

Hereditary neuroblastoma accounts for <2% of all neuroblastomas with autosomal dominant inheritance (Cheung and Dyer, 2013) and are usually diagnosed at nine months of age. Approximately 80% of familial neuroblastomas are linked to genetic mutations in either *ALK* (75% of familial neuroblastoma) or *PHOX2B* (5% of familial neuroblastoma) genes, which are both involved in neural crest development (Mosse et al., 2004; Trochet et al., 2004). Familial neuroblastoma is also associated with neural cristopathies, including neurofibromatosis type 1 (NF1), Hirschsprung disease and congenital central hypoventilation syndrome (CCHS) (Martinsson et al., 1997; Morgenstern and Irwin, 2014). Moreover, neuroblastoma is found in other cancer predisposition syndromes like the Li-Fraumeni syndrome (p53-germ line mutation), RAS-MAPK syndrome (*PTPN11* gene mutation) and Noonan and Beckwith-Wiedemann syndrome (aberrations on at the 11p15 region, in *CDKN1C* and /or *IGF-2)* (Morgenstern and Irwin, 2014).

Several genome-wide association studies (GWAS) have identified germline predisposing genetic variants associated with susceptibility to sporadic high-risk disease; *BARD1, NBAT1, CASC15, CASC14, LIN28B* and *LMO1,* among others (Brodeur and Bagatell, 2014; Capasso et al., 2009; Diskin et al., 2009; Newman and Nuchtern, 2016; Pandey et al., 2014; Wang et al., 2011a). These single nucleotide polymorphisms (SNPs) are more common than the *ALK* and *PHOX2B* germline mutations, however they don´t have as powerful effect on the individual neuroblastoma risk as do *ALK* and *PHOX2B* (Brodeur and Bagatell, 2014; Irwin and Park, 2015).

1.3.4 Sporadic neuroblastoma

1.3.4.1 Mutations

Neuroblastoma is a disease with low mutational frequency, compared to adult tumors (Pugh et al., 2013). Unlike *ALK*, which is mutated (8-10%) and amplified (3%) in over 10% of sporadic neuroblastoma tumors, *PHOX2B* sporadic mutations are not commonly found (Brodeur, 2003; Morgenstern and Irwin, 2014). In recent years, with the contribution of exome- and whole genome sequencing studies, additional mutations have been discovered in

neuroblastoma tumors associated with high-risk disease, including mutations in *ATRX* (2.5% inactivating mutations), *ARID1A/1B* (2-3% inactivating mutations), V-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived (*MYCN*) (1.7% activating mutations), *PTPN11* (2.9% activating mutations) and *NRAS* (0.83% activating mutations), among others less frequently mutated genes (Brodeur and Bagatell, 2014; Pugh et al., 2013; Sausen et al., 2013). More recent data indicate a remarkable accumulation of mutations and other aberrations in relapsed tumors (Eleveld et al., 2015; Schleiermacher et al., 2014). When analyzing mutations it is obvious that uncommon mutations accumulate in specific pathways like the one controlling neuritogenesis (Dyberg et al., 2017; Molenaar et al., 2012) among others.

1.3.4.2 MYCN Amplification

V-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived, is a member of a family of highly conserved oncogenes that include *MYC* (*c-myc*) and *MYCL* (Schwab et al., 1983). *The MYCN gene* was first described in 1983 to be an amplified gene (50-100 fold) in neuroblastoma, hence the "N" (Kohl et al., 1983; Schwab, 2004; Schwab et al., 1983). Together with its partner in crime Max (Myc-associated factor X), the MYC protein binds to E-box sequences resulting in a massive regulation of its target genes that are involved in fundamental cellular processes, including proliferation, growth, migration, self-renewal, apoptosis, protein synthesis, angiogenesis, metabolism and differentiation (Huang and Weiss, 2013). *MYCN*-status is used as a prognostic factor in risk stratification (Brodeur et al., 1984; Cohn et al., 2009). Approximately 20-25% of neuroblastoma and near 40% of the high-risk tumors have *MYCN* amplification (MNA), defined as having more than 10 copies of the gene (Ambros et al., 2009).

Furthermore, high *MYCN* expression has shown to be associated with invasive and metastatic behavior (Zaizen et al., 1993). In fact, the 3-year event-free survival (EFS) in infants that have metastases and *MYCN* amplification is only 10% compared to 93% of patients with non-MNA tumors (Schmidt et al., 2000). Also, the oncogenic role of *MYCN* was further demonstrated in the transgenic mouse model with targeted overexpression of *MYCN* to the peripheral neural crest (Weiss et al., 1997). Interestingly, a *MYCN* 157-gene target signature showed poor prognosis for patients with and without MNA, suggesting that the oncogenic role of *MYCN* is significant in non-MNA tumors as well (Valentijn et al., 2012).
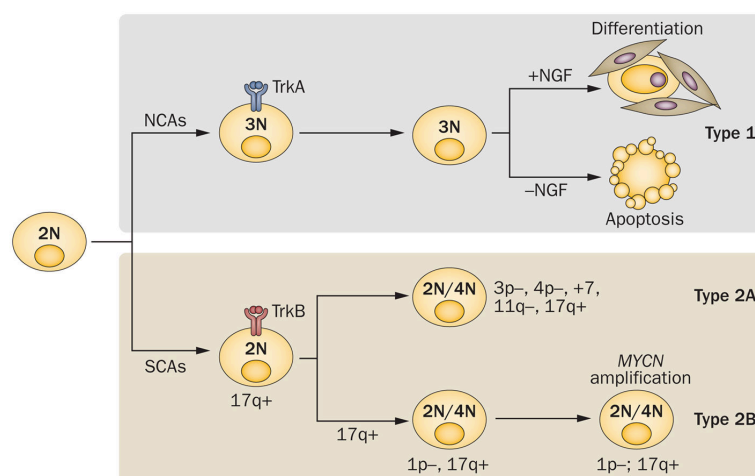
1.3.4.3 Prognostic Chromosomal Abnormalities

Neuroblastoma is a heterogeneous disease harboring many chromosomal aberrations that could be divided into two categories; numerical only, with whole chromosome gains and tumors with segmental chromosomal aberrations, that affect only a part of the chromosome (Caren et al., 2010; Irwin and Park, 2015; Matthay et al., 2016; Morgenstern and Irwin, 2014). The loss of 1p, -11q, and gain of are the most well established abnormalities associated with poor patient outcome (Abel et al., 1999; Brodeur et al., 1977; Caren et al., 2010; Caron, 1995; Gilbert et al., 1984; Irwin and Park, 2015; Lastowska et al., 1997a;

Matthay et al., 2016). Considerable effort has been made to search for potential oncogenes and tumor suppressors located in regions that are gained, and lost, respectively. Gain of the long arm of chromosome 17 (17q-gain) affects half of all neuroblastoma cases, (described more in detail in subsection 2.3.1) and 30% of all tumors have lost the short arm of chromosome 1 (1p loss) (Abel et al., 1999; Bown et al., 1999; Brodeur, 1989; Brodeur et al., 1977; Lastowska et al., 1997a; Pugh et al., 2013). Unbalanced 1;17 translocations (loss of 1p and gain of 17q) have previously been reported (Caron et al., 1994; Lastowska et al., 1997c; Van Roy et al., 1994). In addition, both of these aberrations correlate with MNA. Another group of tumors that have shown to be associated with high-risk disease are tumors that have lost the q-arm of chromosome 11 (11q loss). While MNA and 11q-deletions are almost always mutually exclusive (Caren et al., 2010), the majority of these tumors have 17q-gain. Patients with 11q-deleted tumors are generally older (median age at diagnosis; 42 months) and have substantially more aberrations than MNA tumors (median age at diagnosis; 21 months) (Caren et al., 2010), which could partly be explained by the fact that the 11q-deleted tumors have had longer time to acquire further aberrations. However, it is more plausible that chromosomal instability in 11q-deleted tumors is related to loss of genes involved in the control of genomic integrity, namely *H2AFX, ATM* and *CHEK1* (Caren et al., 2010; Takagi et al., 2017) among other genes (Morgenstern and Irwin, 2014). Candidate genes that have been proposed to have tumor suppressive roles on 1p include *CHD5, CAMTA1, KIF1B* and *mir-34A* among many others that have been suggested (Matthay et al., 2016; Morgenstern and Irwin, 2014).

1.3.5 Neuroblastoma subtypes

While there are different ways to classify neuroblastomas in subtypes, most often three subtypes are considered, according to their genetic profiles. Type 1 neuroblastoma, which consist of low-risk tumors with hyperdiploid or near-triploid DNA (numerical chromosome alterations but no segmental aberrations) and they often have high expression of the favorable nerve growth factor receptor TrkA (Figure 3). Patients are usually less than 1 year of age, and tumors belonging to this subtype may differentiate or undergo apoptosis, to the great benefit of these patients who have excellent prognosis (Brodeur, 2003). The other two subtypes are Type 2A and Type 2B. They have more aggressive natures, with chromosomal aberrations, commonly including 17q-gain in addition to other subtype-specific chromosomal alterations, such as Type 2A 14q- and

Figure 3. Genomic model of neuroblastoma development.
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6

11q deletion in Type 2A and 1p LOH and MNA in Type 2B (Figure 3). Patients are generally older, their tumors take longer time to develop and are consequently more advanced. These patients have much worse survival than patients who have type1 tumors. Type 2B subtype tumors are the most aggressive, often expressing TrkB and the TrkB ligand BDNF in an autocrine fashion, giving them a survival advantage (Brodeur, 2003; Brodeur et al., 1981).

1.3.6 Staging and risk classification

There have been several staging systems for neuroblastoma over the years. The one most widely accepted and used over the last thirty years is the International Neuroblastoma Staging System (INSS) that was first proposed in 1988 to replace the previous Evan's staging system (Brodeur et al., 1988; Evans et al., 1971), and revised in 1993 (Brodeur et al., 1993) (Table 1). The INSS is a post-surgical staging system, which is limited by the surgical approach used. Also, groups from different parts of the world used different criteria to classify and treat patients, which made it difficult to compare different clinical trials. To address these concerns, in 2009 the International Neuroblastoma Risk Group Staging System (INRGSS) was developed based on clinical criteria and tumor imaging for the International Neuroblastoma Risk Group (INRG) Classification System. Patients were stratified before treatment into identical riskgroups (very-low-risk, low-risk, intermediate-risk or high-risk groups), irrespective of nationality, facilitating comparison of risk-based clinical trials globally (Table 2) (Cohn et al., 2009; Monclair et al., 2009). The INRGSS was tailored to comprise one of seven prognostic factors in the INRG pre-treatment classification system (Figure 4) (Cohn et al., 2009). The other prognostic factors included are age, histologic category, grade of tumor differentiation, the status of the *MYCN* oncogene, chromosome 11q status, and DNA ploidy (Cohn et al., 2009). Briefly, localized tumors are staged L1 and L2 based on the presence or absence of image-defined risk factors (IDRF). Metastatic tumors are denoted as stage M, excluding stage MS tumors, in which metastases are restricted to the bone marrow, liver, and skin in children under the age of eighteen months (Table 2) (Monclair et al., 2009). The classification of patients into different risk groups dictates the treatment plan. Status of *MYCN*, together with stage, age at diagnosis and histology are the strongest prognostic factors in neuroblastoma to date (Irwin and Park, 2015).

Table 1. International Neuroblastom StagingSystem (INSS)

Stage	Definition
1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive)
2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically
2B	Localized tumor with or without complete gross excision; with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically
3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvemen
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S)
4S	Localized primary tumor (as defined for stage 1, 2A or 2B), with dissemination limited to skin, liver, and/or bone marrow (limited to infants <1 year of age and bone marrow with <10% tumor cell involvement)

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Stage	Definition
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Localized tumor with the presence of one or more image-defined risk factors (IDRFs)
M	Metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months of age at diagnosis with metastases limited to the skin, liver, and/or bone marrow

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1.3.7 Survival and treatment according to risk-stratification

Forty per cent of neuroblastoma patients have low-risk disease with tumors often spontaneously regressing. Low-risk neuroblastoma is defined as disease that is curable with no or minimal cytotoxic therapy (Kushner and Cohn, 2005). Together with intermediate-risk disease it constitutes approximately half of newly diagnosed cases (Whittle et al., 2017). Patients belonging to these risk groups have very favorable outcome (80-100% are cured) with treatment strategies including observation alone, surgical resection or surgery with moderate-dose chemotherapy (Kushner and Cohn, 2005; Strother et al., 2012). In contrast, patient in the high-risk group constitute around half of all neuroblastoma patients (80% are >18 months of age at diagnosis). These patients have long-term survival rates of 40-50%, despite intensive multi-modal therapy. The treatment of high-risk neuroblastoma patients consist of mainly four phases: induction chemotherapy (cycles of topotecan, etoposide, cisplatin, vincristine, doxorubicin and cyclophosphamide), local control (surgical resection and radiotherapy), consolidation (high-dose bone marrow ablative therapy followed by autologous bone marrow stem cell rescue) and maintenance phase to eradicate residual disease (treatment with differentiating agents and immunotherapy) (Kushner and Cohn, 2005; Ladenstein et al., 2017; Maris, 2010; Matthay and Cheung, 2005; Whittle et al., 2017). High-risk patients who do not respond to aggressive induction therapy have survival rates of less than 20% (Matthay and Cheung, 2005; Whittle et al., 2017).

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			H	Intermediate
				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 4. International Neuroblastoma Risk Group (INRG) Consensus Pretretment Classification schema. Adapted from Cohn et al., 2009 with permission from American Society of Clinical Oncology.

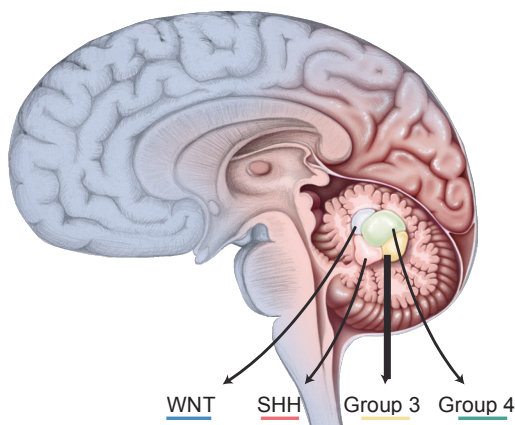## 1.4 MEDULLOBLASTOMA

1.4.1 Epidemiology and biology

Brain tumors are the second most common malignancy in children, and medulloblastoma accounts for 12-15% of all childhood brain tumors (Lannering et al., 2009; Ostrom et al., 2014; Ostrom et al., 2017). In Sweden, approximately 10-15 children are diagnosed with medulloblastoma each year (Gustafsson et al., 2013; Lannering et al., 2009). The majority of medulloblastomas (80%) are diagnosed in children under 15 years of age and only 1-2% are diagnosed in adults (Ostrom et al., 2017).

In 1925 Bailey and Cushing were the first to describe medulloblastoma, as a "small blue cell tumor of the cerebellum" (Bailey and Cushing, 1925). Back then, intracranial small, round blue-cell tumors were usually considered as similar entities, grouped under primitive neuroectodermal tumors (PNETs) (Northcott et al., 2012b; Rorke, 1983). Thanks to molecular profiling, medulloblastoma was recognized as a distinct pediatric brain tumor (Pomeroy et al., 2002).

Medulloblastoma is a heterogeneous tumor of the cerebellum and is histopathologically classified into different histologic subtypes; classic, anaplastic-, large-cell-, extensively nodular- and desmoplastic/nodular medulloblastoma (Gilbertson and Ellison, 2008; Northcott et al., 2012b). The survival of children with medulloblastoma has significantly improved over the last decades. The survival rates mirror the heterogeneity and pathobiological behavior of medulloblastoma tumors. In the 1980s the average 5-year survival was 50-60%, today the 5-year survival of children with low, average, high-risk and very high-risk disease is >90%, 80%, 60-65% and <50% respectively (Gajjar et al., 2013; Packer, 2008; Ramaswamy et al., 2016b; Schwalbe et al., 2017).

1.4.2 Molecular subgroups

In 2010 at a consensus conference in Boston, the Medulloblastoma Working Group came to recognize four distinct molecular subgroups of medulloblastoma supported by evidence, based on clinical, genetic, transcriptional and demographic differences (Taylor et al., 2012). The subgroups were named Wingless (WNT), Sonic Hedgehog (SHH), Group 3 and Group 4, respectively (Figure 5) (Northcott et al., 2011). However, the intertumoral heterogeneity within each subgroup and the observed differences in patient survival warranted further stratification within each major subgroup. Currently the revised WHO classification of medulloblastoma

Figure 5. Medulloblastoma subgroups.
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includes following genetical subgroups; WNT, SHH-TP53 wild-type, SHH-TP53 mutant and the Non-WNT/Non-SHH subgroup (Group3 and Group 4) (Figure 6) (Cavalli et al., 2017; Louis et al., 2016; Sengupta et al., 2017) . Since medulloblastomas belonging to Group 3 and Group 4 are more related to each other than WNT and SHH, they are grouped under Non-WNT/Non-SHH tumors. Expression and epigenetic analysis revealed that the SHH group contains two distinct entities and was therefore recognized as SHH-TP53 wild-type and SHH-TP53 mutant (Louis et al., 2016)

1.4.2.1 WNT medulloblastoma

The medulloblastoma WNT subgroup consists of tumors that have aberrant Wnt/β-catenin signaling, hence the name. Approximately 10-15% of all medulloblastoma tumors belong to this group and 85-90% of WNT-tumors have mutations in the *CTNNB1* gene that encodes the β-catenin protein, leading to constitutively active WNT signaling (further described in subsection 2.2.1) (Clevers and Nusse, 2012; Gibson et al., 2010; Sengupta et al., 2017). Tumors belonging to this group rarely metastasize and often have chromosome monosomy 6 (80-85%), mutations in *DDX3X* (50%), SMARCA4 and TERT (31%) (Jones et al., 2012; Pugh et al., 2012; Remke et al., 2013; Robinson et al., 2012). Patients with tumors belonging to the WNT medulloblastoma subgroup have excellent prognosis: 5-year survival for patients under 16 years is >95% while older patients have intermediate outcomes (Clifford et al., 2015; Kool et al., 2012; Zhao et al., 2016). TP53 mutations found in the WNT subgroup have no prognostic significance (Chiang and Ellison, 2017). WNT tumors are usually located in the lateral recess of the brainstem, near the rhombic limp (Ramaswamy and Taylor, 2017). These tumors are usually hemorrhagic causing disruption of the blood-brain barrier, which makes WNT medulloblastomas vulnerable to chemotherapy (Phoenix et al., 2016).

Recently, a study by Cavalli et al., identified two WNT subtypes, α and β (Cavalli et al., 2017). WNTα is found in children with monosomy 6 tumors while WNTβ tumors are diploid for chromosome 6 and found in older patients. Both subtypes have similar survival (Cavalli et al., 2017). Previous studies have reported differences in biology and prognosis between WNT medulloblastoma found in children compared to WNT tumors in adults (Zhao et al., 2016).

1.4.2.2 SHH medulloblastoma

Medulloblastoma tumors belonging to the SHH subgroup are heterogeneous tumors that are genetically and transcriptionally characterized as having a high activation of the SHH signaling pathway (described further in subsection 2.1.1) (Kool et al., 2014; Northcott et al., 2011; Taylor et al., 2012). Commonly found mutated genes involved in the SHH pathway are age-dependent and include Protein patched homolog 1 (*PTCH1*), Smoothened (*SMO*) and Suppressor of fused homolog *(SUFU)* (Kool et al., 2014). *SUFU* mutations are usually observed in SHH tumors from infants and children between 0-4 years of age and 42% of tumors have mutations in *PTCH1*. Thirty-six per cent of tumors found in older children (4-17 years of age) have somatic or germline *PTCH1* mutations, along with glioma-associated oncogene 2 (*GLI2*) and *MNAs* (Kool et al., 2014). In addition, majority of *TP53* mutations

10

occur in this age group and were recently genetically renamed "SHH-activated, *TP53* mutant medulloblastoma" (Louis et al., 2016). There are several tumor predisposition syndromes linked to SHH tumors, namely Gorlin syndrome (germline mutations in *PTCH1*) and Li-Fraumeni syndrome (germline mutations in *TP53*) (Gururangan et al., 2015; Zhukova et al., 2013). Thirteen per cent of medulloblastoma tumors are *TP53* mutated and patients having mutant p53 have one of the worst outcomes of all medulloblastoma patients (Ramaswamy et al., 2016b). Finally, SHH tumors from adults (<17 years) frequently have *SMO* and *PTCH1* and *TERT* promoter mutations (Kool et al., 2014; Remke et al., 2013).
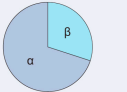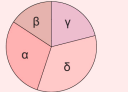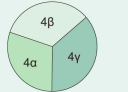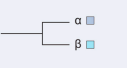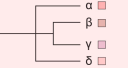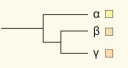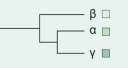
Sonic hedgehog tumors are more difficult to treat, because of an intact blood-brain barrier (Phoenix et al., 2016) and the outcome of patients is age specific/dependent. Infants and young children have excellent prognosis while patients with TP53 mutations do much worse (Kool et al., 2012; Zhukova et al., 2013).

1.4.2.3   *Group 3 (Non-WNT/non-SHH) medulloblastoma*

Patients belonging to Group 3 are commonly observed in infants and males. They have the worst survival (5-year survival ~50%) of all medulloblastoma patients, usually with small large-cell anaplastic primary tumors (located in the forth ventricle near the brainstem) that frequently have metastasized (40-50%) at the time of diagnosis (Kool et al., 2012; Northcott et al., 2012a). Group 3 medulloblastoma tumors have activated GABAergic pathway (overexpression of *GABRA5*) (Cho et al., 2011; Northcott et al., 2017; Northcott et al., 2011; Taylor et al., 2012) and overexpression of *MYC* with 17-20% *MYC* amplified tumors (Cho et al., 2011; Kool et al., 2012). Other genetic- and chromosomal aberrations manifested in Group 3 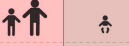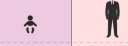tumors, usually involve *OTX2* amplifications (Northcott et al., 2012c), GFI1A/B activation (Northcott et al., 2014), and isochromosome 17q (i17q), predictor of bad prognosis (Shih et al., 2014). In addition, these tumors often have gain of 1q and loss of 10q, 11, 16q and 17p (Northcott et al., 2012a).

1.4.2.4   *Group 4 (Non-WNT/non-SHH) medulloblastoma*

The majority of medulloblastoma tumors (>40%) belong to Group 4 subtype, and are much more prevalent in males, with peak incidence 5-13 years of age (Kool et al., 2012; Northcott et al., 2012a). Group 4 tumors have mainly classic histology, predominately glutaminergic activated signaling, and are considered to be copy number driven tumors (Ramaswamy and Taylor, 2017; Sengupta et al., 2017). Isochromosome 17q is the most common chromosomal aberration (80%) observed in Group 4 (Shih et al., 2014). In addition, amplification of *MYCN* (10%) and *CDK6* (5%), duplications of *SNCAIP* (10%), loss of choromosome 11 and 8, mutations that inactivate histone demethylase gene *KDM6A* (10%) and copy number changes of genes involved in NF-κβ signaling are frequently observed in Group 4 subtype (Cavalli et al., 2017; Cho et al., 2011; Jones et al., 2012; Northcott et al., 2011; Northcott et al., 2012c; Pugh et al., 2012; Robinson et al., 2012). Patient with Group 4 tumors have intermediate 5-year survival similar to SHH subtype patients, and 30% of tumors have metastasized at time of diagnosis (Northcott et al., 2012a).

Figure 6 table:

Subgroup	WNT		SHH				Group 3			Group 4		
Subtype	WNT α	WNT β	SHH α	SHH β	SHH γ	SHH δ	Group 3α	Group 3β	Group 3γ	Group 4α	Group 4β	Group 4γ
Subtype proportion	(pie: α, β)		(pie: α, β, γ, δ)				(pie: 3α, 3β, 3γ)			(pie: 4α, 4β, 4γ)		
Subtype relationship	(tree: α, β)		(tree: α, β, γ, δ)				(tree: α, β, γ)			(tree: β, α, γ)		
Age												
Histology			LCA Desmoplastic	Desmoplastic	MBEN Desmoplastic	Desmoplastic						
Metastases	8.6%	21.4%	20%	33%	8.9%	9.4%	43.4%	20%	39.4%	40%	40.7%	38.7%
Survival at 5 years	97%	100%	69.8%	67.3%	88%	88.5%	66.2%	55.8%	41.9%	66.8%	75.4%	82.5%
Broad	6^-		$9q^-$, $10q^-$, $17p^-$		Balanced genome		7^+, 8^-, 10^-, 11^-, i17q		8^+, i17q	$7q^+$, $8p^-$, i17q	i17q	$7q^+$, $8p^-$, i17q (less)
Focal			MYCN amp, GLI2 amp, YAP1 amp	PTEN loss		$10q22^-$, $11q23.3^-$		OTX2 gain, DDX31 loss	MYC amp	MYCN amp, CDK6 amp	SNCAIP dup	CDK6 amp
Other events			TP53 mutations			TERT promoter mutations	High GFI1/1B expression					

Age (years): 0-3, >3-10, >10-17, >17

Figure 6. Graphical summary of the 12 Medulloblastoma Subtypes. Schematic representation of clinical data, copy-number events, and relationship between the subtypes inside each of the four medulloblastoma subgroups. Reprinted from Cavali et al., 2017 with permission from the publisher Copyright © Elsevier Inc.

1.4.3 Risk-stratification, treatment and survival

Medulloblastoma patients are stratified into average/standard-risk and high-risk groups (Table 3) (Ramaswamy and Taylor, 2017; Sengupta et al., 2017). Standard-treatment of medulloblastoma tumors includes surgery, radiation and chemotherapy (Ramaswamy and Taylor, 2017; Sengupta et al., 2017; von Hoff and Rutkowski, 2012). The first step of treatment is surgery (maximal resection) after which tissue diagnosis is established.

High-risk disease includes patients with residual disease receive high dose of craniospinal radiotherapy (36-39 Gy in 30 fractions and posterior fossa boost to 54-56 Gy) and chemotherapy (cisplatin, cyclophosphamide and vincristine) (Packer, 2008; Sengupta et al., 2017). Currently, the 5-year survival rate for patients treated with high-risk treatment regimens is 60-65% (Taylor et al., 2003). Patients that have average/standard-risk disease, receive lower dose of radiotherapy (23.4 Gy in 30 fractions and posterior fossa boost to 54-56 Gy) and chemotherapy (children >3 years of age, with non-disseminated disease) including vincristine, cisplatin, cyclophosphamide and lomustine (Packer and Vezina, 2008; Sengupta et al., 2017). Average/standard-risk patients have a 5-year survival of 80% (Ramaswamy and Taylor, 2017; Taylor et al., 2003). Adult medulloblastoma patients are in most cases treated with radiotherapy (craniospinal) and chemotherapy (Lassaletta and Ramaswamy, 2016; Sengupta et al., 2017). Because of the severe and devastating long-term effects of radiotherapy on infants, radiation is preferably avoided until the patient is 3 years of age. Instead a more intense high-dose chemotherapy is applied together with stem cell rescue regimen (Cohen et al., 2015; Duffner et al., 1993; Packer and Vezina, 2008; Rutkowski et al., 2010).

Historically, stratification was solely based on clinical variables, including age, metastasis and residual tumor (Ramaswamy and Taylor, 2017; von Hoff and Rutkowski, 2012). Today, histological subtypes and molecular subtypes are important prognostic factors (Ramaswamy et al., 2016b; von Hoff and Rutkowski, 2012). Patients with non-metastatic WNT medulloblastomas are considered to have low-risk disease. These patients have excellent survival (>90%), which includes treatment with surgery, radiation, with or without chemotherapy (Cho et al., 2011; Clifford et al., 2015; Ellison et al., 2011; Northcott et al., 2011). In addition, patients with Group 4 non-metastatic tumors with loss of chromosome 11 are also considered as having low-risk tumors (Shih et al., 2014). Non-metastatic tumors that qualify into the average/standard-risk category (75-90% survival rate) (Gajjar et al., 2006; Lannering et al., 2012) belong to patients with the following medulloblastoma subtype characteristics: SHH (wild-type TP53 and non-*MYCN* amplified), Group 3 (non-*MYC* amplified) and Group 4 tumors (Ramaswamy et al., 2016a; Shih et al., 2014). High-risk disease with a patient survival rate of 50-75% include SHH-*MYCN* amplified tumors, metastatic non-infant SHH tumors with wild-type TP53, and metastatic Group 4 tumors (Kool et al., 2012; Shih et al., 2014). Lastly, the very high-risk group with the worst survival (<50%) consist of SHH tumors with TP53 mutations (anaplastic morphology) and metastatic Group 3, *MYC* amplified tumors (Cho et al., 2011; Shih et al., 2014; Zhukova et al., 2013). The vast majority of TP53 mutations found in the SHH subgroup are germline mutations (Li-Fraumeni syndrome), and are as such, very hard do treat and tend to develop secondary malignancies (Kool et al., 2014; Ramaswamy et al., 2015; Zhukova et al., 2013).

Table 3. Staging and risk stratification of medulloblastoma.

Modified Chang Staging			
T stage		**M stage**	
T1	Tumor <3 cm in diameter	M0	No evidence of gross subarachnoid or hematogenous metastasis
T2	Tumor ≥3 cm in diameter	M1	Microscopic tumor cells found in CSF
T3a	Tumor >3 cm and with extension into aqueduct of Sylvius or foramen of Luschka	M2	Gross nodular seeding intracranially beyond the primary site (in cerebellar/cerebral subarachnoid space or in third or lateral ventricle)
T3b	Tumor >3 cm and with unequivocal extension into brainstem	M3	Gross nodular seeding in spinal subarachnoid space
T4	Tumor >3 cm with extension past aqueduct of Sylvius or down past foramen magnum	M4	Metastasis outside cerebrospinal axis
Risk Stratification			
Standard (Average) Risk (66%)		**High Risk (34%)**	
>3 years old		<3 years old	
<1.5 cm^2 residual disease after resection		Subtotal resection, >1.5 cm^2 residual tumor	
M0 by craniospinal MRI and CSF		M+, leptomeningeal seeding, and location outside of the posterior fossa	

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2 PAPER-SPECIFIC BACKGROUND

2.1 PAPER I

2.1.1 Hedgehog signaling

In 1980, Nusslein-Volhard and Wieschaus were first to discovered the Hedgehog (HH) gene with the help of the fruit fly *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus, 1980). Genetic analysis revealed fifteen loci that were detrimental for development of the segmental pattern of the larva. Since then, a lot of effort and research has been made to investigate the significance of the Hedgehog signaling pathway in embryonic development and disease.

The HH signaling pathway is fundamental in multiple processes during embryonic development including development of different tissues and organs (patterning and differentiation), cell proliferation and adult tissue homeostasis (Jiang and Hui, 2008; Pasca di Magliano and Hebrok, 2003; Teglund and Toftgard, 2010; Varjosalo and Taipale, 2008). In humans, HH signaling is activated by three ligands: SHH, Indian Hedgehog (IHH) and Desert Hedgehog (DHH) by binding to PTCH. Sonic hedgehog is the most potent and dominant HH ligand implicated in carcinogenesis (Rimkus et al., 2016). The PTCH is a twelve-span transmembrane protein that in a resting state suppresses the activity of the G protein-coupled receptor-like proto-oncogene SMO (Figure 7) (Taipale and Beachy, 2001; Varjosalo and Taipale, 2008; Weinberg, 2014). However, following ligand stimulation, the suppression of SMO by PTCH is released, preventing cleavage of GLI in the cilium. The GLI protein is then free to migrate to the nucleus and act as a transcription factor of HH target genes important for proliferation and survival, including *PTCH1, GLI1, GLI2, CCND1, Bcl-2, VEGF, c-MYC and MYCN*. In the absence of HH ligand, GLI is prevented by the cytoplasmic proteins Costal-2 (COS2) Fused and SUFU to translocate into the cell nucleus (Figure 7). There are three known GLI proteins to date, GLI 1 and GLI 2 that stimulate transcription, and GLI 3, which functions as a transcriptional inhibitor (Ng and Curran, 2011; Pasca di Magliano and Hebrok, 2003).

There are several ways of activating the HH pathway, either through HH ligand-dependent activation or by a ligand-independent mechanism, through inactivating mutations of the *PTCH*, *SUFU* gene or by gain-of-function mutations of *SMO*. Aberrant HH signaling is frequently manifested in various types of cancers. Astonishingly, HH has been implicated in endodermal-driven cancers that account for 25% of human cancer deaths (Lum and Beachy, 2004). In glioblastoma, overexpression of Gli was first discovered (Kinzler et al., 1988), hence the name. Moreover, loss-of-function mutations of the *PTCH1* gene causes Gorlin syndrome, which is a familial cancer syndrome that increases the risk of developing basal cell carcinomas and medulloblastoma (Teglund and Toftgard, 2010). Other tumors with aberrant HH activation include gastric cancer, ovarian cancer, leukemia, lung cancer, pancreatic cancer and colorectal cancer (Chowdhury et al., 2013; Ciucci et al., 2013; Taylor et al., 2002; Wu et al., 2017). Also, in the last decade several studies have shown that proteins involved in

the HH signaling pathway are highly expressed in neuroblastoma, rendering HH-signaling as a compelling therapeutic target in neuroblastoma (Mao et al., 2009; Ruan et al., 2016; Wang et al., 2014).

Overwhelming evidence, implicates the role of aberrant HH signaling in tumor development and progression in many different types of cancers as well as tumor drug resistance. Several inhibitors of the HH pathway have been developed and some are currently in clinical trials (Ng and Curran, 2011; Rimkus et al., 2016). In the past, most efforts have been made to target SMO, however, in recent years several inhibitors have been developed to target GLI, among others. This is because GLI is one of the most characterized oncoproteins in HH signaling and can thus be activated by both ligand-dependent and independent mechanisms (Rimkus et al., 2016).

In paper I in this thesis, we explore GLI as a therapeutic target in neuroblastoma with the small molecule antagonist GANT-61, previously demonstrated having promising effect on tumor inhibition (Gonnissen et al., 2015; Lauth et al., 2007; Schiapparelli et al., 2011).
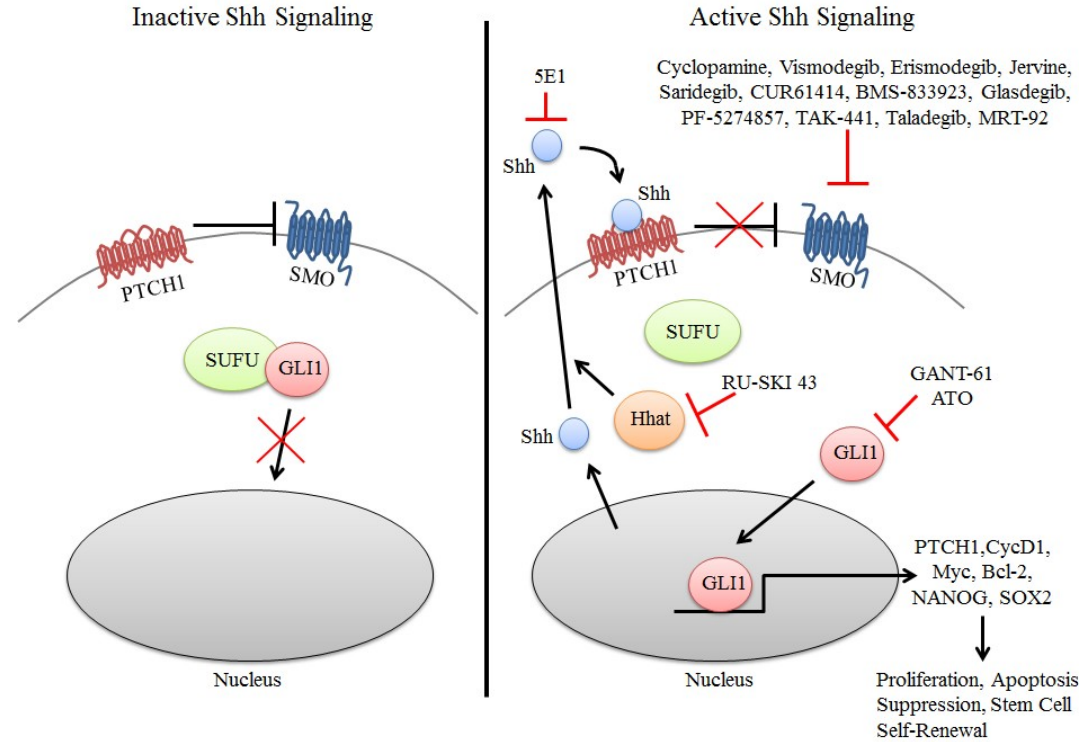

Figure 7. Inhibition of components in the SHH signaling pathway in cancer.
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2.2 PAPER II

2.2.1 Wnt/β-catenin signaling

The *WNT1* (int-1) gene was discovered in 1982 in mammals by Nusse and Varmus while investigating oncogenic transformation in mouse mammary glands by the mouse mammary tumor virus (MMTV) (Nusse and Varmus, 1982). The Wnt/β-catenin pathway is a highly conserved pathway vital in embryogenesis, adult tissue regeneration and homeostasis. It controls many processes including cell proliferation, self-renewal, cell morphology, polarity, migration, cell fate and differentiation (Archbold et al., 2012; Clevers, 2006; Miller, 2002).

The Wnt/β-catenin pathway is controlled by Wnt factors (nineteen factors found in human tissue), which allow cells to remain in a fairly immature state. These factors also mitogen signals through either the canonical Wnt/β-catenin pathway or the non-canonical pathways (β-catenin-independent) that regulate planar cell polarity and intracellular levels of Ca^{2+} (Archbold et al., 2012; Miller, 2002; Weinberg, 2014). In this thesis, we only focus on the canonical Wnt/β-catenin signaling pathway will be described.

Wingless factors act through the seven-pass transmembrane receptor Frizzeled (Fzd) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) receptors to suppress the protein kinase glycogen synthase kinase 3 (GSK3-β). In the absence of ligand-binding, GSK3-β and casein kinase 1 phosphorylate β-catenin that is bound to Axin and the tumor suppressor adenomatous polyposis coli (APC), leading to ubiquitination of β-catenin which is thereby tagged for destruction in the proteasome (Figure 8). This protein complex is called the destruction box. (Archbold et al., 2012; Miller, 2002; Weinberg, 2014).
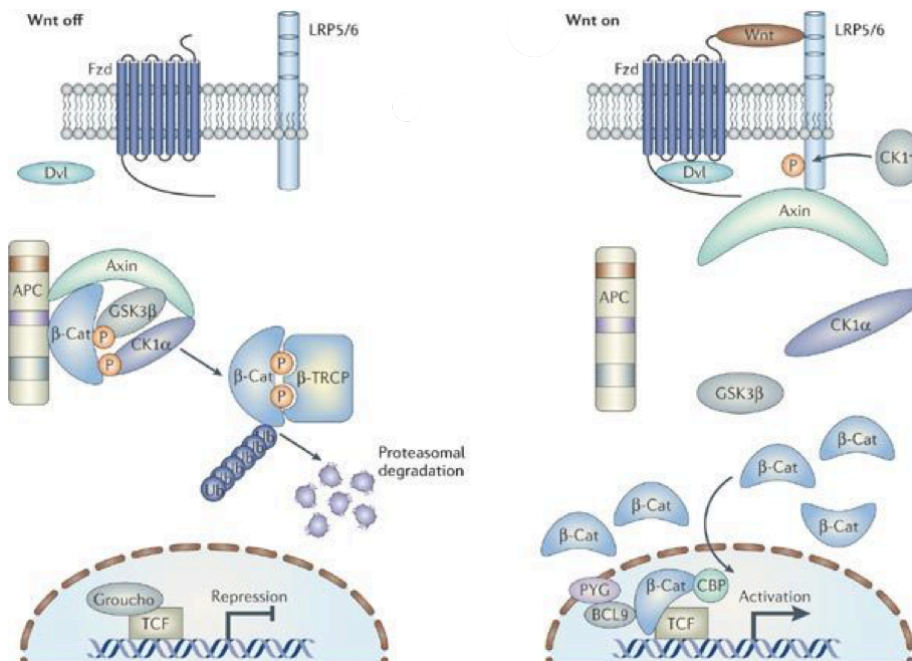

Figure 8. An overview of the Wnt signaling pathway.
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The β-catenin protein can exist in three different states: bound to cell-cell adhesion receptors like E-cadherin, soluble free state in the cytosol where it has a very fast turnover, and finally, functions in the nucleus as a transcription factor.

In a "Wnt-off" state, the destruction complex maintains low levels of β-catenin. However, when Wnt signaling is activated, GSK3-β activity is abrogated, allowing β-catenin to accumulate once escaping degradation. β-catenin is then free to translocate into the nucleus and bind to the Tcf/Lef proteins, resulting in transcription of a number of genes involved in cell growth, proliferation and stem cell maintenance, such as *CCND1, c-Myc, Lgr, Axin 2* (Figure 8) (Barker and Clevers, 2006; Ramakrishnan and Cadigan, 2017).

Aberrant Wnt signaling has been linked to several human diseases, developmental disorders, and most notably to different types of cancers including colorectal-, breast-, lung-, gastric-, prostate cancer, neuroblastoma and medulloblastoma (Gibson et al., 2010; Liu et al., 2008; Lyou et al., 2017; Northcott et al., 2011; Oving and Clevers, 2002).

2.2.2 MGMT

Our cells have sophisticated DNA repair systems that protects the integrity of the genome, guarding against activated oncogenes as well as tumor suppressor genes that have been inactivated, due to several mechanisms resulting from e.g. mutations, amplifications, replication errors, translocations, chromosomal segmental gains, promoter methylation etc. There are more than 160 specific proteins that are involved in cellular DNA repair (Hanahan and Weinberg, 2011; Weinberg, 2014)

The DNA repair system is a double-edged sword; on one end it protects the integrity of the genome in normal cells, rescuing them from malignant lesions. On the other end, DNA repair enzymes also repair the damages caused by alkylating and mutagenic agents, attempting to kill cancer cells.

O6-methylguanine DNA methyltransferase (MGMT) is a cellular DNA repair enzyme that removes mutagenic and cytotoxic (methyl and alkyl-groups) adducts from the O^6 position of guanine of DNA, reversing genetic errors and hence prevent neoplastic transformation (Hegi et al., 2009).

There are several ways cancer cells have found to manipulate MGMT function to circumvent cell cycle arrest and eventually cell death. One mechanism is by epigenetic silencing of the *MGMT* promoter, resulting in decreased levels of MGMT so the mutation-driven tumors are allowed to continue propagating. Another way is by upregulating the expression of MGMT, for the purpose of defending themselves from genomic-attacks caused by alkylating agents, causing resistance to treatment (Baer et al., 1993; Esteller et al., 1999; Hegi et al., 2008). Moreover, MGMT-overexpressing transgenic mice were protected from developing thymic lymphomas after exposure to the chemical carcinogen N-methyl-N-nitrosourea (Dumenco et al., 1993). Conversely, *MGMT* is silenced by promotor methylation in about 40% of gliomas and colorectal tumors, and in 25% of non-small-cell lung tumors, lymphomas and head-and-

18

neck cancers (Esteller et al., 1999). Therefore, treatment strategies to overcome DNA-repair resistance in order to increase sensitivity to alkylating agents have been implemented in treating resistant tumors (Esteller et al., 2000).

In paper II, we explore MGMT-related chemoresistance in different cancers including medulloblastoma, colorectal carcinoma and neuroblastoma applying two treatment strategies.

2.3 PAPER III

2.3.1 17q-gain and PPM1D/WIP1 in neuroblastoma

Gain of 17q is the most common chromosomal aberration in the majority of neuroblastoma tumors (Abel et al., 1999; Bown et al., 1999; Gilbert et al., 1984; Theissen et al., 2014). It predicts unfavorable outcome in patients (Caron, 1995; Lastowska et al., 1997a; Theissen et al., 2014). In fact, 17q-gain is the strongest indicator of adverse outcome compared to any other single clinical or genetic factor, including 1p loss and MNA (Bown et al., 1999; Lastowska et al., 1997a). Approximately 50% of all neuroblastoma tumors and 90% of high-risk tumors have unbalanced 17q-gain (Meddeb et al., 1996). In addition, 75%-84% of neuroblastoma primary tumors were found to have either whole chromosome 17 gain or gain of the q-arm (Bown et al., 1999; Lastowska et al., 1997a; Lastowska et al., 1997b; Meddeb et al., 1996; Plantaz et al., 1997). 17q-gain is associated with other prognostic factors including 1p deletions, stage 4 tumors, age at diagnosis (>1 years of age), diploidy/tetraploidi, MNA, and 11q loss (Caron, 1995; Lastowska et al., 1997a; Meddeb et al., 1996; Morgenstern and Irwin, 2014; Theissen et al., 2014).

The most common breakpoint on 17q was identified as q21 (Bown et al., 1999; Lastowska et al., 1997c; Meddeb et al., 1996) and all extra 17q fragments contain the shortest region of gain (SRG) 17q23.1-17qter, resulting in a 25 Mb long fragment (Meddeb et al., 1996). Considerable effort has been made to search for potential oncogenes on 17q but no specific genes have yet been identified to account for oncogenic transformation or progression in neuroblastoma. Some proposed candidates include *BIRC5* (surviving), *BRCA1, KPNB1, PPM1D* (WIP1) and *NME1/2* (Irwin and Park, 2015; Lastowska et al., 1997a; Meddeb et al., 1996; Morgenstern and Irwin, 2014; Vandesompele et al., 2008). In this theses the 17q-candidate *PPM1D*/WIP1 will be further described and its putative oncogenic role in neuroblastoma development will be investigated in paper III.

The human *PPM1D* gene that encodes the protein WIP1 is located at 17q23.2, included in the region (17q23.1-17qter) of segmental gains of 17q, previously been reported to always be retained (Meddeb et al., 1996).

Saito O-Hara and coworkers were the first to describe the oncogenic role of *PPM1D* in neuroblastoma. By investigating expression of seven candidate oncogenes on 17q in primary neuroblastoma tumors, only *PPM1D* was found to correlate to poor clinical outcome, and *PPM1D* gene silencing resulted in decreased cell proliferation (Saito-Ohara et al., 2003). In a panel of different human cancers, neuroblastoma, medulloblastoma, breast cancer and

leukemia had the highest mRNA levels of WIP1(Richter et al., 2015) and treatment with GSK2830371, a small molecule WIP1 inhibitor, inhibited growth and induced apoptosis of neuroblastoma cell lines *in vitro* (Richter et al., 2015). Other WIP1 inhibitors shown to successfully inhibit growth of a variety of cancer cell lines will be described in subsection 2.3.3.8 WIP1 inhibitors.

2.3.2   17q-gain and PPM1D/WIP1 in medulloblastoma

17q-gain and isochromosome 17q (i17q) are the most common chromosomal aberrations found in medulloblastoma where i17q results in loss of 17p and duplication of 17q (Biegel, 1999; Biegel et al., 1989; Bien-Willner and Mitra, 2014; Bigner et al., 1988; Griffin et al., 1988). Approximately 30% to 50% of medulloblastoma tumors have been reported with extra chromosomal material on 17q (Biegel, 1999; Buss et al., 2012; Ellison, 2002). Interestingly, early cytogenetic studies on medulloblastoma revealed tumors having i17q as a single isolated alteration, suggesting i17q to be an early event, and not a consequence of clonal evolution (Biegel et al., 1989).

In group 4 medulloblastomas, Isochromosome 17q together with *MYC* amplification was found to be the only markers associated with poor patient survival (Shih et al., 2014). Moreover, 75 % of group 4 tumors were identified having i17q and *PPM1D*/WIP1 significantly overexpressed, whereas expression of *TP53* was significantly lower in i17q tumors compared to patients with normal 17q. Importantly, *TP53* mutations were not observed in the Group 4 tumors. These observations suggest alternative mechanisms, not involving *TP53* mutations, responsible for the p53 impairment observed in the group 4 medulloblastomas. In these tumors, it seems that there may not be a selective advantage to acquire *TP53* mutations since p53 is already abrogated by other alternative mechanisms.

Castellino and colleagues were the first to propose an oncogenic role of *PPM1D*/WIP1 in medulloblastoma (Castellino et al., 2008), demonstrating highest WIP1 expression in the aggressive Group 3 and Group 4 medulloblastomas and in metastatic tumors (Chang stage M2-3) (Buss et al., 2015), and associated with worse overall survival and progression-free survival (Buss et al., 2015). In addition, several studies have demonstrated that medulloblastoma patients with i17q have early recurrence and worse outcomes (Bien-Willner and Mitra, 2014; Nicholson et al., 2000; Shih et al., 2014).

Finally, several promising *in vivo* studies have been addressing *PPM1D*/WIP1 as a putative oncogene in medulloblastoma development, further suggesting *PPM1D*/WIP1 as an oncogene in medulloblastoma development (described in subsection 2.3.3.6).

2.3.3 PPM1D/WIP1

Wild-type p53-induced phosphatase 1 was first identified by Fiscella and colleagues as a novel nuclear protein phosphatase, induced by ionizing radiation, in a p53-dependent manner (Fiscella et al., 1997). The *PPM1D* gene encodes the WIP1 protein, a type 2C delta protein phosphatase (PP2Cδ) belonging to the PP2C family of phosphatases, responsible for inhibiting cellular stress signaling (Lammers and Lavi, 2007). Together with kinases, phosphatases play crucial roles in regulating fundamental cellular signaling events. But instead of adding phosphate groups to target proteins like protein kinases do, phosphatases remove them by a mechanism called dephosphorylation (thereby inactivating different proteins) (Lammers and Lavi, 2007). The PP2C family of proteins are metal-dependent (Mg^{2+} or Mn^{2+}) phosphatases that dephosphorylate phosphoserine and phosphothreonine residues.

2.3.3.1 Regulation of WIP1

The phosphatase activity of WIP1 is regulated mainly through the level of its expression, and as of yet, no evidence has emerged showing post-translational modifications of the phosphatase activity. The WIP1 protein is ubiquitously expressed, and is transcriptionally activated by exogenous stresses leading to its involvement and regulation of different stress-signaling pathways described in the following section (Chuman et al., 2009; Lowe et al., 2012; Lu et al., 2008a; Lu et al., 2008b). The *PPM1D* gene has several binding motifs for different transcription factors in its promoter region including p53, c-jun, E2F1, ERα, NF-κB and CREB (Lowe et al., 2012). In addition, WIP1 has been shown to be post-transcriptionally regulated by microRNA-16 (Zhang et al., 2010), BRCA1-IRISb (Chock et al., 2010) and by alternative splicing (Chuman et al., 2009), described further in subsection 2.3.3.4.
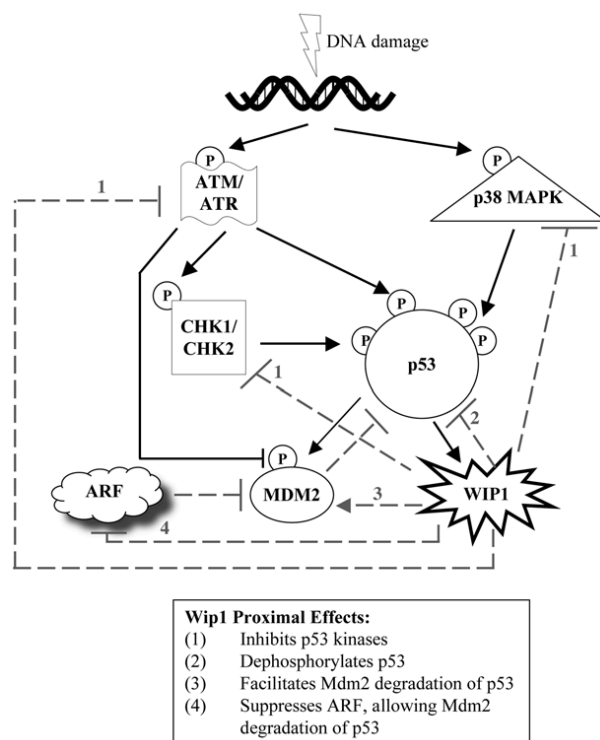
2.3.3.2 WIP1 targets proteins in stress response pathways

WIP1 is involved regulation of multiple cellular processes including DNA repair, cell cycle arrest and apoptosis (Bulavin et al., 2002; Lowe et al., 2012; Zhu and Bulavin, 2012) by inactivating several key proteins involved in these processes. The DNA damage response (DDR) pathway is a multi-step process that facilitates DNA repair following genotoxic stress (e.g. IR- and UV-induced). WIP1 is responsible for maintaining normal cell homeostasis by inhibiting stress signals once the damage is repaired. Several key proteins in the DDR pathway, cell cycle and apoptosis are direct targets of WIP1 phosphatase activity, namely ATM$^{Ser1981/Ser367}$, ATR, Chk^{Ser345}, Chk2^{Thr68}, γH2AX^{Ser139}, p38 MAPK^{Thr180}, p53^{Ser15}, Mdm2^{Ser395}, MdmX^{Ser403}, NF-κB^{Ser536}, p16^{INK4A}, p19^{ARF} and UNG2^{Thr6} among others (Figure 9) (Bernards, 2004; Bulavin et al., 2004; Cha et al., 2010; Chew et al., 2009; Fujimoto et al., 2006; Lowe et al., 2012; Lu et al., 2007; Lu et al., 2005; Lu et al., 2008a; Shreeram et al., 2006a; Shreeram et al., 2006b; Takekawa et al., 2000; Zhang et al., 2009).

Briefly, following genotoxic stress, the DDR pathway is turned on by Ataxia Telangiectasia And Rad3-Related Protein (ATR) (associated with DNA replication stress) or by the sensor kinase ATM (Ataxia telangiectasia mutated) (activated by double-strand DNA breaks), resulting in phosphorylation of p53 through the checkpoint kinases Chk1 and Chk2,

respectively (Lu et al., 2008a; Macurek et al., 2013). Stress-induced phosphorylation of p53 results in its stabilization followed by transactivation of several target genes that temporarily halt the cell cycle (checkpoint arrest) (Lowe et al., 2012; Lu et al., 2008a; Macurek et al., 2013). In parallel, phosphorylation of the DNA-damage marker H2AX^{Ser139} (γH2AX) by ATM/ATR, recruits adaptor proteins and ubiquitin E3 ligases, resulting in ubiquitination of the chromatin at the site of DNA damage (Macurek et al., 2013). This leads to recruitment of BRCA1 and 53BP1 proteins that are key regulators of two DNA repair pathways, homologous recombination and non-homologous end joining, respectively (Chapman et al., 2012; Lukas et al., 2011). In order to avoid incomplete termination of the DDR pathway, WIP1 mRNA levels are kept low early after DNA-damage by the microRNA miR-16 (Zhang et al., 2010). When the damage is repaired, WIP1 levels increase, resulting in inhibition of p53 by a negative feedback loop and termination of the DDR pathway. This mechanism helps the cell to return to its homeostatic state (Lu et al., 2008a; Macurek et al., 2013). WIP1, can inhibit p53 activity directly by dephosphorylating p53^{Ser15}, and indirectly by inactivating numerous proteins upstream of p53, such as ATM/ATR, Chk1/2, p38 MAPK, γH2AX among others, impair p53 function, abrogate apoptosis and as a result promote cell survival (Figure 9) (Lowe et al., 2012). In addition to inactivating several proteins upstream of p53, WIP1 directly dephosphorylates Mdm2^{Ser395}, enabling Mdm2-mediated degradation of p53 (Lu et al., 2008a).

Programmed cell death (apoptosis) is a well-characterized cellular process that prevents unrepaired cells from becoming immortalized cancer cells (Chipuk and Green, 2006; Green and Evan, 2002). Many cancers have defects in proteins that are involved in apoptosis, the most famous one is p53, "The guardian of the genome" (Lane, 1992), which is one of the most frequently mutated genes (50%) in human cancers (Vogelstein et al., 2000). While not all tumors have p53 mutations, the vast majority has impaired p53 function (Mello and Attardi, 2017; Vogelstein et al., 2000). Chronic suppression of p53-guardian function in tumor development can partly be explained by persistent WIP1 phosphatase activity when overexpressed, amplified and/or mutated. Other mechanisms of impairing p53 function, include MDM2/4 amplification or overexpression (Wade et al., 2013) and inactivation of p14^{ARF} (Gallagher et al., 2006), among others.

Wip1 Proximal Effects:
(1) Inhibits p53 kinases
(2) Dephosphorylates p53
(3) Facilitates Mdm2 degradation of p53
(4) Suppresses ARF, allowing Mdm2 degradation of p53

Figure 9. Wip1 inhibits p53 activity by multiple mechanisms.
Reprinted from Lu et al., 2008 with permission from the publisher Taylor & Francis.

Cell cycle arrest is another important process that, similar to apoptosis, prevents cells with damaged genome from becoming transformed and subsequently carcinogenic. Macurek and colleagues showed that the level of WIP1 protein increases from G1 to G2 and then substantially declines in mitosis through proteasomal degradation and phosphorylation-inhibition of its enzymatic activity (Macurek et al., 2013). Furthermore, Wip1-depleted colorectal carcinoma cells were unable to recover from G2 arrest following treatment with doxorubicin (Lindqvist et al., 2009). Through inhibiting pathways involved in the different checkpoints (G2/M, G1/S and intra S phase) WIP1 enables the cell to return to its normal cell cycle progression following stress (Lindqvist et al., 2009; Lu et al., 2008a).

Taken together WIP1 regulates several stress responses, including dampening of the DDR, inhibition of apoptosis and recovery and rescue from cell cycle arrest (Figure 10). In normal healthy cells WIP1 is important for fine-tuning these complex cellular mechanisms. However, during tumorigenesis, over-activation of WIP1 allows cells with oncogenic alterations to propagate thereby circumventing the refined protective mechanisms that have been conserved in different species, throughout evolution.

2.3.3.3   Inflammation

In recent years, emerging evidences have shown that WIP1 plays an important role in the regulation of the immune system. WIP1-knockout mice are viable but show some defects including reduced male fertility (spermatogenesis), small reduction in longevity and higher susceptibility to infections than wild-type mice (Choi et al., 2002). Furthermore, research using WIP1-deficient mice shows that WIP1 is needed for normal development and maturation of T and B lymphocytes by inhibiting the activation of the p53 and p38MAPK pathways (Nannenga et al., 2006; Schito et al., 2006). In contrast, WIP1 deficiency was found to promote differentiation of myeloid lineages resulting in severe neutrophilia due to an overactive p38MAPK-STAT1 pathway (Liu et al., 2013). Moreover, Sun and colleagues showed that WIP1 inhibits neutrophil-mediated acute inflammatory responses, including neutrophil migration and release of inflammatory chemokines (Sun et al., 2014).
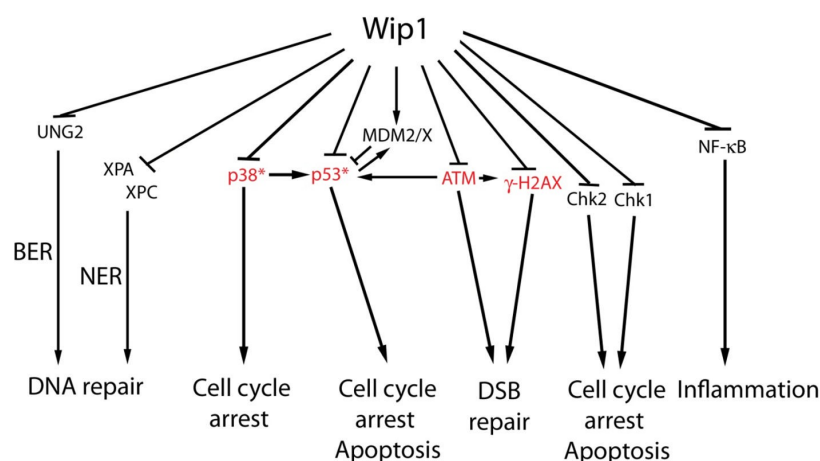

Figure 10. WIP1 targets and functional consequences. Reprinted from Lowe et al., 2012 with permission from the authors.

2.3.3.4 Alternative splicing

A WIP1 oncogenic splice variant was first identified by the cloning of *PPM1D* from the breast cancer cell line MCF-7 (Chuman et al., 2009). There are two known protein variants due to alternative splicing of WIP1, a full length isoform of 605 amino acids (PPM1D605) and a shorter isoform of 430 amino acid long (PPM1D430) variant. PPM1D430 has the first 420 residues in common with the full length protein, and 10 additional amino acids that are PPM1D430-isoform specific (Figure 11). Both splice variants are induced by genotoxic stress and are functional phosphatases (Chuman et al., 2009). They differ in expression pattern and subcellular localization. PPM1D605 is ubiquitously expressed, whereas PPM1D430 is specifically expressed in leukocytes and testis, suggesting a functional difference between the two. This expression pattern is especially interesting, regarding defects in male reproduction and immune response, observed in WIP1-deficient mice (Choi et al., 2002). Furthermore, while the PPM1D605 protein is exclusively nuclear, the shorter splice variant has been found to localize to the nucleus, cytoplasm and at the plasma membrane, analyzed in breast cancer cell lines (Chuman et al., 2009). The functional differences between the two splice variants remain to be further investigated.
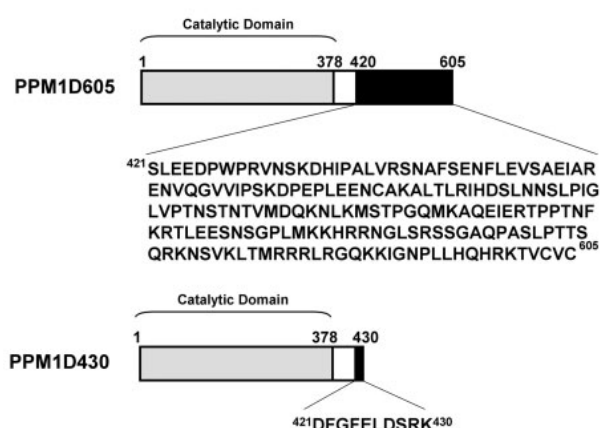

Figure 11. Schematic representation of PPM1D605 and PPM1D430 proteins. The catalytic domain (gray box) and the variant-specific C-terminal residues are represented by black boxes, respectively. Adapted from Chuman et al,. 2008 with permission from the publisher. Copyright © Oxford University Press.

2.3.3.5 PPM1D/WIP1 in human cancers

The role of WIP1 as an oncogene has been implicated in many different human cancers (Lowe et al., 2012). Similar to Mdm2, amplification and/or overexpression of *PPM1D/*WIP1 has been demonstrated in several human cancers that usually harbor wild-type p53 (Momand et al., 1998).

The first evidence of *PPM1D*/WIP1 as an oncogene was shown in two breast cancer studies, where *PPM1D* amplifications were found in 11.3% (Bulavin et al., 2002) and 16% (Li et al., 2002) of primary breast cancers, respectively, and that the *PPM1D* amplification correlated to WIP1 overexpression. Other cancers, besides breast cancer, that have been reported to be *PPM1D*-amplified and/or overexpressed include ovarian adenocarcinoma, adult brainstem glioma, astrocytoma, colorectal carcinoma, gastric carcinoma, glioblastoma multiforme, various hematological malignancies, medulloblastoma, neuroblastoma, pancreatic adenocarcinoma and pediatric brainstem glioma. (Buss et al., 2015; Castellino et al., 2008;

Fuku et al., 2007; Hennika and Becher, 2016; Hirasawa et al., 2003; Hu et al., 2016; Kennedy and Ebert, 2017; Kleiblova et al., 2013; Lambros et al., 2010; Li et al., 2002; Lindsley et al., 2017; Nikbakht et al., 2016; Peng et al., 2014; Saito-Ohara et al., 2003; Tan et al., 2009; Tedaldi et al., 2017; Wang et al., 2011b; Zhang et al., 2014; Zink et al., 2017). Moreover, WIP1 overexpression in different tumors was associated with poor prognosis of patient outcome, and/or higher tumor grade (Bien-Willner and Mitra, 2014; Buss et al., 2015; Hirasawa et al., 2003; Lambros et al., 2010; Liang et al., 2012; Lindsley et al., 2017; Loukopoulos et al., 2007; Peng et al., 2014; Rauta et al., 2006).

Gain-of-function *PPM1D* mutations were first reported by Ruark at al., showing mosaic *PPM1D* mutations in lymphocytes linked to susceptibility to breast and ovarian cancer development (Ruark et al., 2013). Similarly, in the last two years, other studies have reported mosaic *PPM1D* mutations in blood DNA sequence associated with other solid tumors in addition to breast and ovarian cancers, including prostate cancer and head and neck squamous cell carcinoma (Artomov et al., 2017; Cardoso et al., 2016; Tedaldi et al., 2017). A mutation cluster region of *PPM1D* gain-of-function mutations located in exon 6 was identified, causing truncation of the WIP1 protein (Figure 12) (Dudgeon et al., 2013; Kleiblova et al., 2013; Ruark et al., 2013). Since then, several reports have showed gain-of-function mutations in several different cancers (Green and Kieran, 2015; Hennika and Becher, 2016; Hu et al., 2016; Nikbakht et al., 2016; Zhang et al., 2014). The truncated WIP1 protein variant has been extensively investigated in several studies showing an oncogenic phenotype with more stable and consistent phosphatase activity compared to the full length protein (Dudgeon et al., 2013; Kleiblova et al., 2013; Ruark et al., 2013).
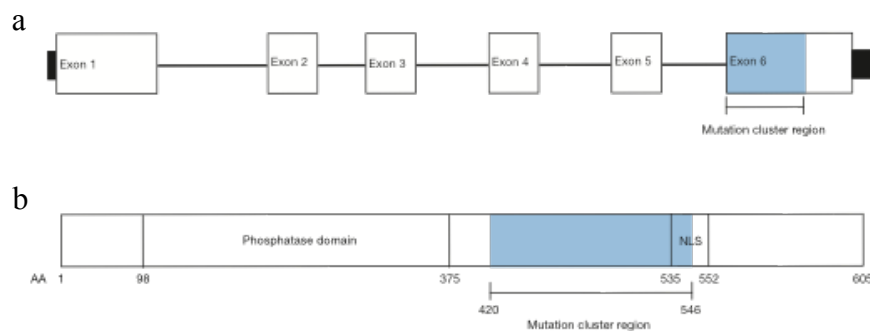

Figure 12. Clustering of cancer predisposition mutations in PPM1D.
a, Mutation cluster region of *PPM1D* (in blue). **b,** *PPM1D*/WIP1 protein displaying site of mutation cluster region downstream of the phosphatase domain and upstream/overlapping the nuclear localization signal (NLS). Adapted from Ruark et al., 2013 with permission from the publisher. Copyright © Springer Nature.

Furthermore, *PPM1D* overexpression, through gene amplification, chromosomal gain, oncogenic mutations and/or alternative splicing, is usually found in tumors with normal (wild-type) p53. This is consistent with the idea that these alterations, give rise to a more persistent p53-inhibitor phosphatase, and abolish the selective pressure to concurrently inactivate p53 by other means (Dudgeon et al., 2013; Kleiblova et al., 2013). Similar findings

have previously been made with oncogenic *MDM2* amplifications observed in tumors with wild-type p53 (Oliner et al., 2016; Wasylishen and Lozano, 2016).

2.3.3.6 PPM1D/WIP1 in different mouse models

Further support proposing *PPM1D*/WIP1 as an oncogene came from *in vivo* experiments demonstrating that WIP1-null mice showed delayed onset of mammary gland tumor development, whereas an increased onset of tumors in the mammary gland was evident in mice overexpressing both PPM1D and ERBB2 in the mammary gland (Bulavin et al., 2004; Demidov et al., 2007a). Similar observations were made in an APC-driven mouse polyposis model where WIP1 deficiency inhibited formation of colorectal cancer (Demidov et al., 2007b). Moreover, PPM1D silencing increased the lifespan of the mice. In a myc-driven lymphoma model, deletion of PPM1D suppressed Eμ-myc-induced B cell lymphomas, and that this delay was ATM- and p53-dependent (Shreeram et al., 2006b). In addition, when crossed with WIP1overexpressing mice, transgenic mice with Sonic hedgehog (SHH)-driven tumors displayed an increased medulloblastoma tumor formation, from 8 to 34% (Doucette et al., 2012). A recent study showed similar observation of the role of WIP1 in modulating hedgehog signaling. This study also showed decreased medulloblastoma incidence in two PPM1D-knockout mouse models (Wen et al., 2016). Interestingly, PPM1D-knockout mice were shown to be resistant to spontaneous tumor development during their lifespan (Nannenga et al., 2006), despite of defects in immune response and spermatogenesis (as described in subsection 2.3.3.3 Inflammation).

2.3.3.7 WIP1 in neurodevelopmental disorders

Recently, emerging scientific publications link *PPM1D* mutations to intellectual disability disorders. Meta-analysis on whole-exome sequencing data on 2,104 patients identified *PPM1D* truncating mutations on exon 6, together with nine other new candidate genes, to be associated with specific intellectual disability phenotypes (Lelieveld et al., 2016). This finding was validated by two other studies showing similar results (Jansen et al., 2017; McRae et al., 2017). Moreover, authors report that *PPM1D* mutations on exon 6 lead to an intellectual disability syndrome associated with hypotonia, small hands and feet, behavioral problems, anxiety, short stature and facial dysmorphisms among others (Jansen et al., 2017). Interestingly, WIP1-deficent mice have also been reported to display symptoms of elevated anxiety and depression-like behavior (Ruan et al., 2016).

Other genes that have been associated with intellectual disability syndrome include *CTNNB1* (β-catenin) and *NF1* (neurofibromatosis 1) (Kuechler et al., 2015; Yap et al., 2014). Germline mutations and sporadic inactivation of the tumor suppressor gene *NF1* have been found in several cancers including neuroblastoma (described in subsection 1.2.3 Genetic predisposition) and is located on chromosome 17q as is *PPM1D*/WIP1 (Brems et al., 2009; Martinsson, 1997; Morgenstern and Irwin, 2014; Yap et al., 2014).

2.3.3.8 WIP1 inhibitors

Several peptide inhibitors and small molecule inhibitors of WIP1 have been shown to be effective in treating different cancer cell lines *in vitro* and *in vivo* in mouse xenograft tumors (Esfandiari et al., 2016; Gilmartin et al., 2014; Kamada et al., 2017; Kozakai et al., 2014; Ogasawara et al., 2015; Pechackova et al., 2016; Rayter et al., 2008; Yagi et al., 2012; Yamaguchi et al., 2006).

The compound GSK2830371, an allosteric WIP1 inhibitor, was shown to effectively inhibit growth of hematopoietic cancer cell lines *in vitro* and in a lymphoma xenograft model. Moreover, GSK2830371 was proven to be effective in *PPM1D*-amplified breast cancer cell lines harboring wild-type p53 (Gilmartin et al., 2014; Pechackova et al., 2016), and was shown to be effective in combination treatment with doxorubicin (Gilmartin et al., 2014) and/or nutlin-3 (Esfandiari et al., 2016; Pechackova et al., 2016)

Since WIP1 can also be overexpressed in p53-mutated cancer cell lines, and the discovery of the oncogenic truncated WIP1 protein variant, small molecule inhibitors SP-001, SL-175 and SL-176 were developed to target WIP1 (Kamada et al., 2017; Ogasawara et al., 2015). Compared to GSK2830371, SP-001 and SL-175, SL-176 was found to be the most potent inhibitor, showing the most potent growth-inhibitory effect in *PPM1D*-amplified breast cancer cell line MCF-7 by inducing G2/M cell cycle arrest and p53-dependent apoptosis (Ogasawara et al., 2015). Furthermore, SL-176 showed high inhibitory effect on p53-null cells overexpressing WIP1, whereas GSK283037 did not (Ogasawara et al., 2015). SP-001, in combination with doxorubicin, suppressed growth of colorectal carcinoma cells that overexpress oncogenic truncated WIP1, whereas doxorubicin alone had limited effect (Kozakai et al., 2014).

In conclusion, there are several putative WIP1 inhibitors, effective in inhibiting cancer cell lines that exhibit different characteristics, such as *PPM1D* amplification, normal *PPM1D*, *PPM1D*-activating mutations, wild-type p53 and mutant p53.

3   AIMS OF THIS THESIS

The general aim of this thesis was to provide new biological understanding and based on this knowledge develop novel therapeutic options against the embryonic neural tumors neuroblastoma and medulloblastoma.

Specific aims:

Paper I. To study the role of Sonic Hedgehog pathway in neuroblastoma development and as a novel therapy target.

Paper II. To elucidate mechanisms behind DNA-repair resistance in cancer treatment.

Paper III. To investigate the neuroblastoma and medulloblastoma 17q-gain candidate-oncogene *PPM1D*/WIP1 in cancer development and progression.

4   MATERIALS AND METHODS

Description of materials and methods included in this thesis are fully described in corresponding papers **I-III**.

5 RESULTS AND DISCUSSION

5.1 TARGETING OF HEDGEHOG PATHWAY INHIBITS NEUROBLASTOMA TUMOR DEVELOPMENT (PAPER I)

The HH signaling pathway is an essential regulator of embryonic development where it directs specific development of tissues and organs, cell proliferation and homeostasis (described in subsection 2.1.1) (Jiang and Hui, 2008; Pasca di Magliano and Hebrok, 2003; Teglund and Toftgard, 2010; Varjosalo and Taipale, 2008). Hedgehog signaling is also implicated in cancers where mutations or aberrant activation of key players of the HH signaling are associated with cancer development in several tissues (Lum and Beachy, 2004; McMillan and Matsui, 2012; Taipale and Beachy, 2001). Neuroblastoma, an embryonic tumor that arises due to disordered development (described in section 1.3), has been linked to having activated HH signaling in several previous studies (Diao et al., 2014; Mao et al., 2009; Ruan et al., 2016; Shahi et al., 2008; Wang et al., 2014; Xu et al., 2012). In paper I, we explored the role of the HH signaling key regulator GLI as a therapeutic target in neuroblastoma.

To investigate HH dependency in neuroblastoma, we used a panel of neuroblastoma cell lines treated with several HH inhibitors, namely the GLI antagonist GANT-61 (Figure 13) (Gonnissen et al., 2015), and two SMO inhibitors, cyclopamine and SANT1 (Rimkus et al., 2016). Here, GANT-61 demonstrated the highest activity against tumor cell viability in all cell lines tested, and showed the greatest cytotoxic effect in the non-*MYCN* amplified and 11 q deleted cell line SK-N-AS. Furthermore, GANT-61 showed a dose-dependent inhibition of colony formation in SK-N-AS, SH-SY5Y and SK-N-BE(2) neuroblastoma cell lines. Encouraged by our *in vitro* findings, we next tested the in vivo efficacy of GANT-61 using the human SK-N-AS cell line. Cells were implanted in immunocompromised mice and treated daily with GANT-61 for 12 days and demonstrated a significant reduction of tumor growth when compared to vehicle treated controls. GANT-61 was further combined with standard neuroblastoma chemotherapy (cisplatin, doxorubicin, irinotecan and vincristine) *in vitro*, and showed to potentiate the cytotoxic effect of chemotherapy in all cell lines tested.

Next, we explored if tumor cell targeting of GANT-61 was directly through GLI inhibition, by measuring mRNA levels of GLI and MYCN after GANT-61 treatment. We used three cell lines with different MYCN expression: low (SK-N-AS), medium (SH-SY5Y) and high (SK-N-BE(2)). Here we found that the mRNA levels of *GLI1* were significantly correlated with increased drug sensitivity to GANT-61, whereas cells overexpressing MYCN were more resistant. To further assess if GANT-61 targeting in neuroblastoma was through HH signaling and GLI specific, we used a GLI-dependent luciferase reporter system. We observed a 80% suppression of *GLI* transcriptional activity following GANT-61 treatment in SK-N-AS cells, whereas the *MYCN*-amplified cell line SK-N-BE(2) had a less noticeable GLI activity suppression (36%). Following GANT-61 treatment, SK-N-AS cells displayed a substantial decrease in both GLI1 and GLI2 mRNA levels, whereas SK-N-BE(2) cells did not. In addition, downregulation of MYCN (in SK-N-AS and SH-SY5Y) and *c*-MYC (in SK-N-

BE(2)) was observed following treatment, both genes previously reported as targets of the HH pathway (Kenney et al., 2003; Oliver et al., 2003; Teglund and Toftgard, 2010). Moreover, all three cell lines exposed to GANT-61 showed induction of apoptosis, and reduced number of cells in S-phase, 72hr after treatment. Furthermore, genetic targeting of GLI1, GLI2, GLI3 and SMO with shRNA in SK-N-AS cells demonstrated a significant reduction in cell viability against all genes except against SMO. Taken together, our data firmly supports that neuroblastoma cells are dependent on HH signaling and GLI function.

Since *MYCN* status is used as a prognostic factor in risk stratification of neuroblastoma and is a strong indicator of aggressive and metastatic tumors (Brodeur et al., 1984; Cohn et al., 2009; Zaizen et al., 1993), we next examined if the observed sensitivity to GLI inhibition in neuroblastoma was related to MYCN expression, and found an inverse correlation between the log IC_{50} values for GANT-61 and mRNA expression levels of *MYCN*. This finding was further validated by expression correlation analyses of GLI and MYCN, using publicly available R2 expression database data, consisting of 88 neuroblastoma patients (http://r2.amc.nl).

In conclusion, this paper supports the notion that HH signaling is crucial in neuroblastoma growth, which is in line with previous findings reported by others (Diao et al., 2014; Mao et al., 2009; Ruan et al., 2016; Schiapparelli et al., 2011). Moreover, our study shows that neuroblastoma cells are more sensitive to GLI inhibitors compared to inhibitors of SMO, suggesting molecular aberrations in key proteins downstream of SMO. Importantly, our observation that GANT-61 is more potent in non-*MYCN* amplified cell lines, proposes GLI as a promising target in high-risk neuroblastoma lacking MYCN overexpression.
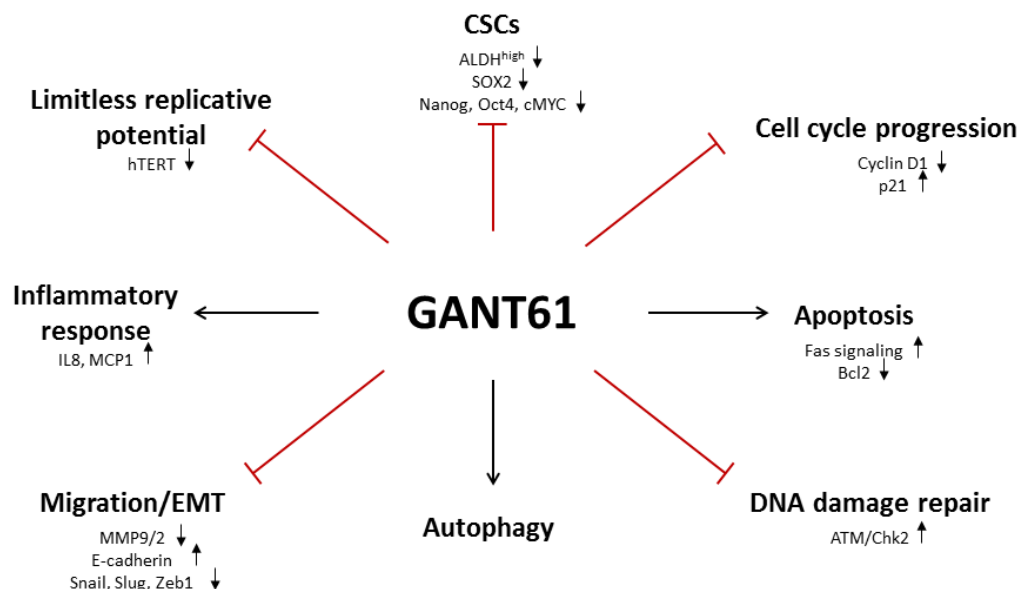

Figure 13. Schematic overview of different GANT61 target sites. Reprinted from Gonnissen et al., 2015 with permission from the publisher. Copyright © 2018 Impact Journals.

5.2 MODULATION OF WNT/β-CATENIN SIGNALING AS A STRATEGY TO OVERCOME CHEMORESISTANCE IN CANCER (PAPER II)

Resistance to treatment is one of the most challenging aspects to cure cancer. It is estimated that over 90% of patients with metastatic disease have acquired resistance to chemotherapy by various mechanisms (Longley and Johnston, 2005). Cancer cells, like any other living cell or organism, want to survive, and in order to do so, they have acquired several survival mechanisms to escape the cytotoxic effects caused by chemotherapeutic agents. Two such mechanisms are avoiding cell cycle arrest and apoptosis by activating the inherent DNA repair machinery. In paper II, we explored the therapeutic potential of blocking the activity of the DNA-repair enzyme MGMT (described in subsection 2.2.2) to prevent chemoresistance caused by MGMT. We studied several forms of cancers, such as neuroblastoma, medulloblastoma, colon cancer and glioma, in order to prevent resistance to alkylating agents commonly used in the clinic.

Several MGMT inhibitors has been developed in the last decade as an attempt to block MGMT-mediated cancer treatment resistance to alkylating agents by direct inhibition. However, the efficacy has been poor due to lack of specificity against tumor cells, mainly targeting blood cells that expresses MGMT and hence causing hematologic toxicity in patients (Kaina et al., 2010). In an attempt to avoid MGMT-mediated toxicity, we used an alternative strategy where we investigated the potential of targeting cellular regulators of MGMT. We hypothesized, that targeting signaling pathways that are also regulators of MGMT activity and are overly active in cancer cells but not in normal cells, could both block DNA-repair activity and thus sensitize cancer cells to alkylating agents (Housman et al., 2014), preventing toxicity in normal cells (Kaina et al., 2010).

We first used available patient datasets on pathway-specific gene-expression profiling in medulloblastoma, colon cancer, neuroblastoma and glioma and discovered a correlation between MGMT gene levels and the Wnt signaling pathway (described in subsection 2.2.1). Interestingly, highest expression of MGMT activity was observed in the medulloblastoma WNT subgroup, suggesting a reciprocal liaison between these two. Further, co-localization of nuclear (active) β-catenin and MGMT was found in tumor tissue in patients diagnosed with colon carcinoma, glioma, medulloblastoma and neuroblastoma. This finding was recapitulated in tumor tissues by immunofluorescence and in cell lines derived from similar cancers indicating a strong correlation between MGMT and β-catenin activity as shown by western blot.

To test if β-catenin has a functional role in regulating MGMT activity, we downregulated the expression levels of β-catenin using a conditional shRNA against β-catenin in the colon carcinoma cell line LS174T. We demonstrated that blocking β-catenin and Wnt signaling reduced MGMT mRNA and protein levels. This data indicated that β-catenin might have a regulatory role on MGMT gene activity. We therefore next investigated if the 5′-upstream regulatory region of the *MGMT* gene had binding sites for the Wnt/β-catenin signaling transcriptional activators Tcf/Lef. Strikingly, we found eight sites where Tcf/Lef could bind

in the *MGMT* promoter region, suggesting a direct regulation of Wnt/β-catenin signaling at the gene level. We further substantiated this finding by using luciferase reporter plasmids constructed to recognize different numbers of Tcf/Lef binding sites at the MGMT promoter. Through genetic (shRNA and cDNA overexpression of β-catenin) and pharmacological manipulation (LiCl, PGE_2, Celecoxib) of Wnt signaling we demonstrated an increase of luciferase activity that was dependent on the number of Tcf/Lef binding sites present. These results substantiated that MGMT activity is regulated by the Wnt/ β-catenin signaling pathway.

To test if indirect inhibition of MGMT activity, by targeting Wnt singling, could be a strategy to overcome chemoresistance, we used several Wnt inhibitors in combination with the DNA alkylating agent temozolomide against a panel of different cancer cell lines. Here we tested several Wnt inhibitors, including the Porcupine inhibitors Wnt-C59 and LGK974, the tankyrase/Axin 1 inhibitors XAV-939 and G007-LK, the non-specific Wnt inhibitor celecoxib and salinomycin, which interrupts phosphorylation of the Wnt co-receptor LRP6. Strikingly, all Wnt inhibitors potentiated the cytotoxic effect of temozolomide in the majority of cell lines tested, and celecoxib stood out as the drug with the most promising effect in combination with temozolomide.

Celecoxib is a nonsteroidal anti-inflammatory drug (NSAID) with specific COX-2 (cyclooxygenase-2) enzyme inhibition that results in decreased prostaglandin E_2 (PGE_2) levels (Smith et al., 2000). Prostaglandin E_2 is a fatty acid that has been shown to promote cancer growth in several forms of cancer by activating Wnt signaling through inhibition β-catenin degradation (Barker and Clevers, 2006; Castellone et al., 2005; Kahn, 2014; Shao et al., 2005). In previous studies from our lab and others, celecoxib has been demonstrated to inhibit tumor development and growth in various preclinical models and cancers (Baryawno et al., 2008; Gasparini et al., 2003; Johnsen et al., 2004; Lynch et al., 2016; Steinbach et al., 2000). Because of the aforementioned reasons, and the promising preclinical data, we further investigated if celecoxib could be used to block MGMT-mediated treatment resistance.

Celecoxib was compered with the MGMT inhibitor O6_benzylguanine (O6-BG) in the ability to restore temozolomide sensitivity in cells overexpressing MGMT, and proved to be just as effective as O6-BG. In addition, colony-forming ability of cells with different MGMT expression showed significant dose-dependent restoration of temozolomide sensitivity with celecoxib-temozolomide combination treatment, compared to both treatments alone. The Wnt active colon carcinoma cell line SW480 also showed a striking downregulation of β-catenin and MGMT protein expression following celecoxib treatment, in addition to a significant decrease in transcriptional activity induced by β-catenin. Furthermore, combination treatment with celecoxib and temozolomide successfully inhibited cell viability of SW480, which was revoked following rescue experiments with MGMT cDNA transfection. Similarly, the Tet-inducible LS174T cell line with shRNA against β-catenin, showed a remarkable reduction in cell viability following β-catenin silencing combined with temozolomide treatment. This

inhibitory effect was abrogated when β-catenin-deficient cells were transfected with MGMT cDNA.

Lastly, the anti-tumorigenic effect of celecoxib-temozolomide combination treatment was evaluated in two xenograft mouse models. We used the medulloblastoma cell line D283 MED and the LS174T colon carcinoma cell line. Tumor growth in the D283 MED mice was significantly reduced when the combination of celecoxib and temozolomide was administrated to mice with tumors, compared to single treatment with no observed side-effects in the mice. Moreover, treatment of LS174T xenografts consisted of induced β-catenin depletion in combination with temozolomide. Moreover, tumors treated with either celecoxib or shRNA against β-catenin from showed reduced MGMT protein expression when compared to single treated tumors, further substantiating Wnt signaling as a modulator of MGMT activity.

In conclusion, our findings in paper II, strongly support the use of Wnt inhibitors in combination with the alkylating agent temozolomide (Figure 14). Moreover, we discovered an effective treatment strategy in overcoming MGMT-mediated cancer treatment resistance in various cancer types, successfully avoiding the toxicity commonly observed with inhibitors that directly target MGMT.
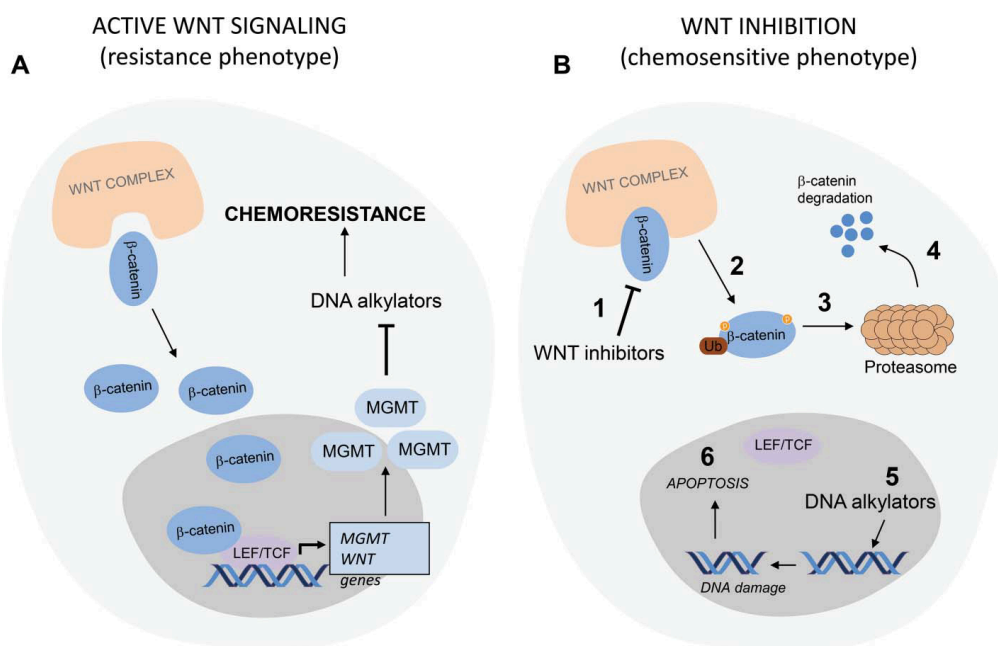

Figure 14. Wnt inhibition resensitizes cancer cells to DNA alkylators through MGMT inhibition.
(A) Active Wnt signalling leads to transcription of Wnt genes including MGMT, which in turn removes alkyl groups on DNA caused by DNA alkylators, ultimately leading to chemoresistance. **(B)** In the presence of Wnt inhibitors (*1*), β-catenin is phosphorylated (*2*), ubiquitinated (*3*) and degraded by the proteasome (*4*). This results in transcriptional stop of MGMT and induction of DNA damage by alkylating agents (*5*), leading to cell cycle arrest and cell death (*6*). Reprinted from Johnsen et al., 2016 with permission from the publisher. Copyright © Taylor & Francis.

5.3 THE P53 PHOSPHATASE PPM1D/WIP1 IS A ONCOGENE IN THE NEURAL TUMORS NEUROBLASTOMA AND MEDULLOBLASTOMA (PAPER III)

Neuroblastoma and medulloblastoma heterogeneity reflects different tumor biology and clinical behavior of distinct genetic subsets (Brodeur, 2003; Cavalli et al., 2017; Louis et al., 2016; Maris, 2010; Northcott et al., 2011). In neuroblastoma, gain of chromosome 17q is the most powerful genetic predictor of adverse clinical outcome (Bown et al., 1999; Lastowska et al., 1997a), and is the most common chromosomal aberration (17q-gain and i17q) found in 30% of medulloblastoma tumors (Biegel, 1999; Ellison, 2002; Schwalbe et al., 2017).

The p53 inhibitor-phosphatase *PPM1D*/WIP1 has been proposed by several studies as an oncogene candidate in17q-gained neuroblastoma (subsections 2.3.1) (Irwin and Park, 2015; Richter et al., 2015; Saito-Ohara et al., 2003) and medulloblastoma (subsections 2.3.2) (Bien-Willner and Mitra, 2014; Buss et al., 2015; Castellino et al., 2008; Doucette et al., 2012; Wen et al., 2016). WIP1 is a serine/threonine phosphatase encoded by the gene *PPM1D,* known to be involved in several cellular processes, including cell cycle control, DNA damage response (DDR) pathways and inflammation, among others, (described in subsection 2.3.3) (Lowe et al., 2012). In paper III, we investigated the proposed oncogenic role of *PPM1D*/WIP1 in these two childhood tumors.

In this paper, we first focused our analysis on clinical neuroblastoma samples. Our analysis of 435 Swedish neuroblastoma tumors revealed that gain of genetic material on chromosome 17q was the most common aberration with either 17q+ or whole chromosome 17 gain in 82% of the tumors investigated. Children with 17q-gain tumors showed a much worse survival than children with normal 17q tumors, 48% and 83%, respectively. Moreover, *PPM1D* was present in at least one extra copy in all 208 tumors showing segmental aberrations of chromosome 17 and was the best oncogene candidate located within the shortest region of overlap and closest to the proximal chromosomal breakpoint site. These data suggest a non-random preference of oncogenic *PPM1D*-gain in tumors with 17q+ but cannot exclude the contributory role of other oncogene candidates located to 17q.

Sequencing analysis using WES and/or WGS of 74 neuroblastoma tumors revealed a truncating gain-of-function *PPM1D* mutation in exon 6 in an aggressive infant *MYCN* amplified neuroblastoma, similar to mutations observed in several other cancers (Kleiblova et al., 2013; Lindsley et al., 2017; Lowe et al., 2012; Ruark et al., 2013; Zhang et al., 2014; Zink et al., 2017).

We next explored the prognostic role and potential association of *PPM1D/*WIP1 with different clinical and biological neuroblastoma risk factors in another clinical cohort consisting of 498 neuroblastoma patients. Here, we observed highest WIP1 expression in stage 4 and high-risk disease, including *MYCN* amplified and 11q-deleted neuroblastomas. The non-*MYCN* amplified high-risk tumors (11q deleted tumors) showed highest *PPM1D*/WIP1 expression in agreement with the finding that 17q-gain is more consistently found in high-risk 11q- tumors than in the MYCN-amplified subset. Here, one could speculate that 11q-deleted tumors can have an increased WIP1-dependecy, as they are

characterized having several genomic alterations, hence an unstable genome compared to other neuroblastomas. Therefore, 11q-deleted tumors would require mechanism that enables this genomic instability during tumor progression, escaping apoptosis and/or cell cycle arrest. This challenging task would be no match for the promiscuous phosphatase WIP1, suggesting WIP1 overexpression/overactivation to be one, among several possible mechanisms, modulating the survival and progression of 11q-deleted tumors. Further, our data also revealed that high *PPM1D*/WIP1 expression was significantly associated with poor overall survival and poor event-free survival. Similar findings were observed when investigating the two *PPM1D*/WIP1 isoforms *PPM1D.aAug10* and *PPM1D.bAug10* as well. In addition, using correlation analysis on gene expression data of 498 neuroblastoma patients from seven countries, focusing on quantitative copy number (CN) information and *PPM1D* expression revealed a remarkable stepwise gene dosage-dependent expression pattern of *PPM1D,* with the highest expression in the tumors with segmental 17q CN gains. The same pattern was observed for the two *PPM1D*/WIP1 isoforms as well. Similar correlations were found when investigating *PPM1D*/WIP1 expression in a different data set, demonstrating significantly higher *PPM1D*/WIP1 expression in high-risk disease and in tumors with unfavorable histology according to the Shimada classification. Moreover, in another tumor cohort high expression of *PPM1D*/WIP1 was found to be associated with Type 2A and Type 2B neuroblastoma subtypes (Brodeur, 2003), further substantiating the association of *PPM1*D/WIP1 with aggressive and unfavorable disease.

To further extend our study we next explored *PPM1D*/WIP1 gene patterns in medulloblastomas. Here, we first confirmed previous reports by Castellino and colleagues, showing highest WIP1 expression in medulloblastomas belonging to Group 3 and Group 4 molecular subgroups (Buss et al., 2015; Castellino et al., 2008). Strikingly, we also detected an oncogenic *PPM1D* mutation in one WNT-medulloblastoma tumor and *PPM1D*-amplifications in three tumors belonging to the specific SHH subset of medulloblastomas in the pediatric age group (non-infant and non-adult ages). This particular subgroup frequently harbor p53 somatic or germline mutations consequently leading to one of the worst outcomes for medulloblastoma patients (Kool et al., 2012; Ramaswamy et al., 2015; Zhukova et al., 2013). Interestingly, the *PPM1D*-amplified tumors in this particular unfavorable subgroup were all p53 wild-type, suggesting an alternative mechanism of impairing p53 function through amplification of *PPM1D*.

Using a panel of preclinical *in vitro* models, we demonstrated that *PPM1D*/WIP1 is crucial for neuroblastoma and medulloblastoma cell growth. For instance, genetic knockdown using shRNA against *PPM1D*/WIP1 severely impaired growth of neuroblastoma and medulloblastoma cell lines. In addition, several WIP1 target proteins exhibited increase in protein phosphorylation following PPM1D knockdown, including γH2AX, ATM, Chk1, Chk2, TP53 and p38, all involved in cell cycle regulation and the DNA-damage response pathway. Further, genetic silencing of *PPM1D*/WIP1 in the neuroblastoma cell line SK-N-BE(2) substantially reduced the colony forming ability, that was even more pronounced following radiation, suggesting a role of WIP1 in radiation resistance. Tumor development of

PPM1D/WIP1 deficient SK-N-BE(2) cells was further evaluated *in vivo* in mouse xenografts, showing a significant delay in tumor development, and tumor growth after *PPM1D*/WIP1 knockdown.

Other studies have demonstrated that mice deficient for PPM1D have a delayed development of mammary tumors in a ERBB2 overexpressing background, or that PPM1D null embryonic mouse fibroblast are resistant to cellular transformation induced by oncogenes such as RAS, ERBB2 and c-MYC (Bulavin et al., 2004). This prompted us to generate a genetically modified mouse model overexpressing WIP1 to further investigate the oncogenic potential of WIP in cancer development. Our transgenic mice developed tumors after cellular stress induced by whole body irradiation, with a striking tumor frequency compared to irradiated control mice in an age- and time of irradiation-dependent manner. The spectrum of observed tumors was similar to tumors obtained in TP53 deficient mice or TP53 deficient mice receiving irradiation (Armstrong et al., 1995; Donehower et al., 1992; Jacks et al., 1994; Liao et al., 1998; Mao et al., 2005; Okazaki and Ootsuyama, 2014; Soussi and Lozano, 2005).

This finding suggests that when *PPM1D*/WIP1 is overexpressed in a cellular stress setting, tumor development is induced by suppressing the activity of p53 and the DDR pathway, allowing genetic errors to accumulate and be passed on to daughter cells, ultimately leading to malignant transformation. Taken together, we propose that *PPM1D* is an oncogene that promotes cell survival through a deficient DNA damage response.

Our observation that external-induced DNA damage in combination with *PPM1D*/WIP1 overexpression induces tumor formation in transgenic mice, and that WIP1 is a strong prognostic factor for survival in neuroblastoma and medulloblastoma, we next sought to confirm *PPM1D*/WIP1 as a potential therapy target in both diseases. We first tested the effects of several specific pharmacological inhibitors against WIP1 (Gilmartin et al., 2014; Ogasawara et al., 2015; Rayter et al., 2008; Yagi et al., 2012). Here, we used a panel of cancer cell lines, including neuroblastoma, medulloblastoma, supratentorial primitive neuroectodermal tumor and breast cancer; WIP1 inhibitors tested were SL-176, SP-001 and CCT007093 (Kozakai et al., 2014; Ogasawara et al., 2015; Rayter et al., 2008; Yagi et al., 2012), and the p53 modulators RITA and Nutlin-3 (Issaeva et al., 2004; Khoo et al., 2014). The WIP1 inhibitor SL-176 was most potent in inducing cytotoxic effects on cell viability compared to the other two WIP1 inhibitors tested, and had similar effects on suppressing cell growth compared to RITA and Nutlin-3. Interestingly, SL-176 displayed the lowest mean of IC_{50}s in the neuroblastoma cell lines of the three tested compounds, including RITA and Nutlin-3. Furthermore, the in vivo efficacy of SL-176 was evaluated in xenograft models for neuroblastoma (SK-N-BE(2) cells) and medulloblastoma (DAOY cells). SL-176 significantly inhibited tumor growth and development with both tumor size and weight at the endpoint of the experiment were significantly smaller in SL-176-treated mice compared to the control mice.

Although mutations in the tumor suppressor gene *TP53* are rarely detected in neuroblastoma and medulloblastoma primary tumors (Ramaswamy et al., 2016a; Tweddle et al., 2003), these

tumors frequently show impairment of p53 activity (Carr-Wilkinson et al., 2010; Ramaswamy et al., 2016a). Our discovery in paper III propose that *PPM1D*/WIP1 is partly responsible for the p53 impairment frequently observed in these pediatric cancers providing a novel insight into the pathogenesis of neuroblastoma and medulloblastoma.
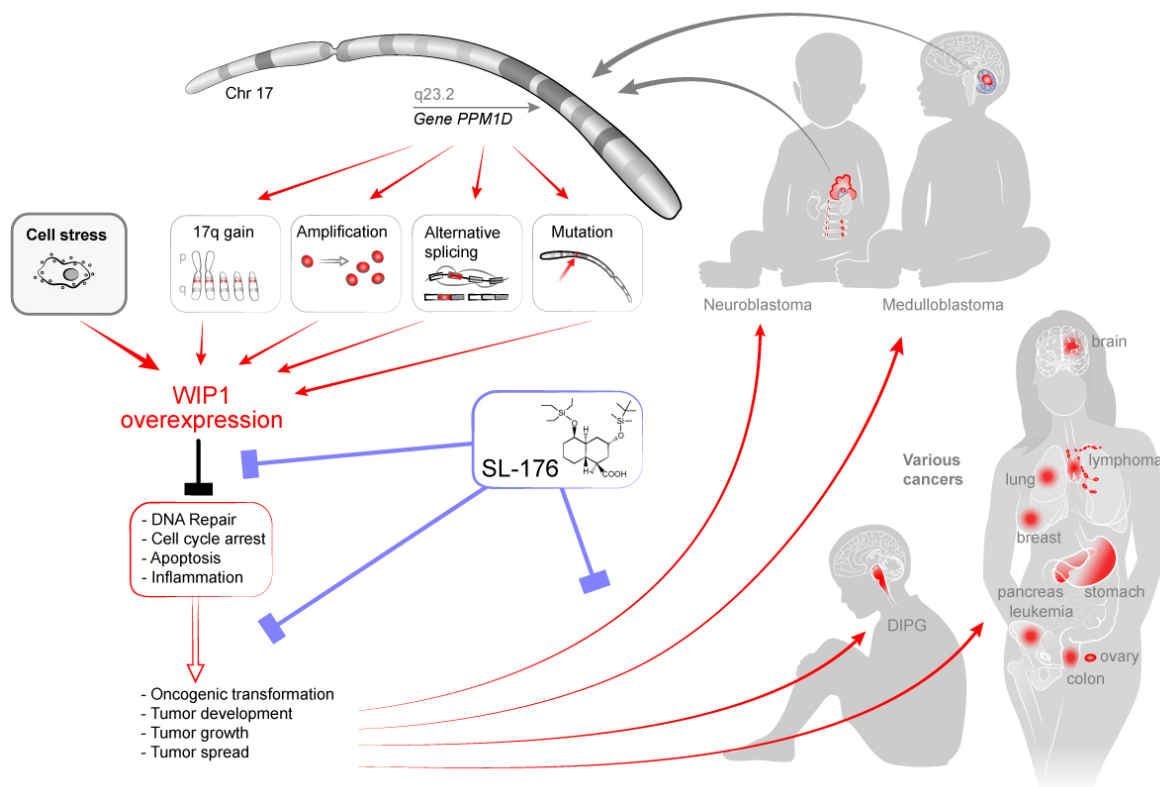

Figure 15. Graphical overview of *PPM1D*/WIP1 in tumorigenesis.

In paper III, we demonstrate that the molecular landscape of *PPM1D* and corresponding WIP1 expression is complex in disease development of neuroblastoma and medulloblastoma. There are several mechanisms that are responsible for the oncogenic switch of WIP1 in tumorigenesis. These mechanisms of oncogenic activation of WIP1 depicted in paper III, include gain-of-function mutations, amplifications, and copy number gains as well as differential expression of *PPM1D* isoforms in human primary tumors (Figure 15). We have also laid evidence that overexpression of WIP1 in *PPM1D*-transgenic mice in conjunction with external DNA stress causes cancer development in an array of different tissues. Finally, we have evaluated the effects of compounds inhibiting WIP1 in neuroblastoma and medulloblastoma cells and in preclinical *in vivo* models suggesting that WIP1 is a significant therapeutic target in these pediatric malignancies and potentially also in other cancers.
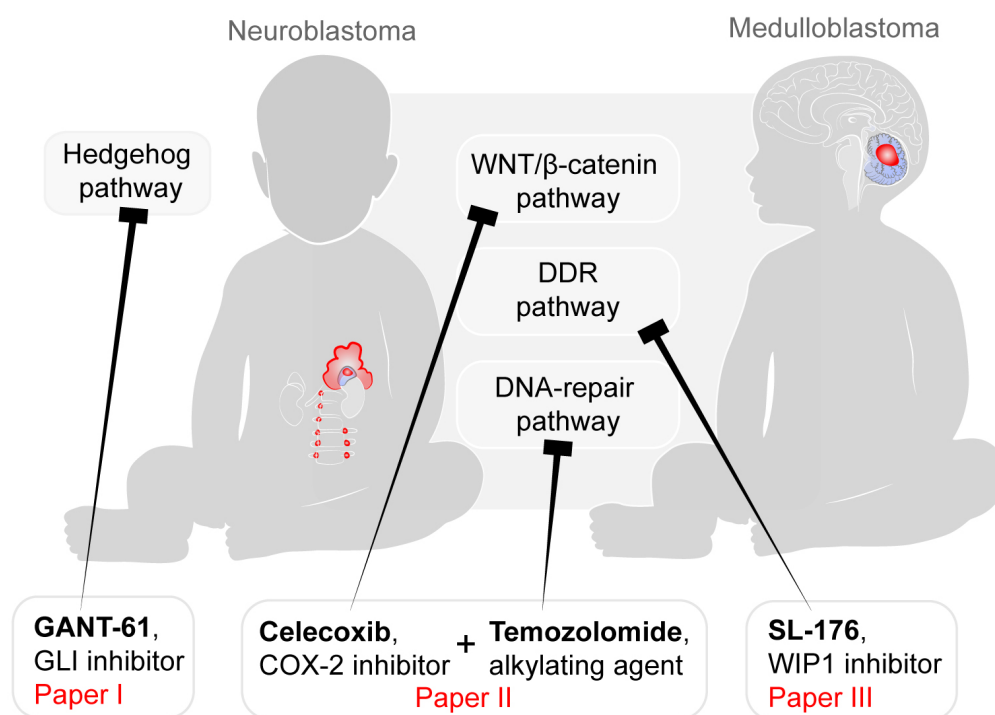
6 CONCLUSION AND FUTURE PERSPECTIVES

Figure 16. Summary of signaling pathways targeted in corresponding papers I-III.

Cancer is one of the leading causes of death worldwide, accounting for 14 million new cases in 2012 and 8.8 million deaths in 2015 (http://www.who.int/Topics/cancer/en; *Accessed* February 2018). Even though cancer in children is a rare event, it is, with the exception of infectious diseases, which are common in developmental countries, the most common cause of death to disease in children under the age of 15 years. The survival among children with cancer has improved dramatically during the last decades thanks to intensive multi-modal therapies, showing a 5-year survival from 30% in the 1960s to 80% in the 2000s in high-income countries (Pritchard-Jones et al., 2013). However, survival is diagnosis-dependent and relates to treatment and clinical and biological risk factors, and children with high-risk neuroblastoma and medulloblastoma still have poor long-term survival rates of less than 40% and often suffer from severe side effects following treatment

The objective of this thesis was to provide the field with novel biological understanding in two of the most common and deadliest childhood tumors, neuroblastoma and medulloblastoma. We hoped to use this new knowledge to develop novel therapeutic strategies against both cancer types (Figure 16).

Research in the cancer field has done tremendous work to provide understanding what makes a normal cell transform into a malignant cell. During this process, cells need to acquire a multitude of survival advantages in order to avoid external attack by the immune system of the organism, and avoid intrinsic cell death programs that prevent cellular transformation. The DNA repair machinery is a process that has been conserved throughout evolution and is one of the primary mechanisms that neoplastic cells have to circumvent to progress into a

malignant state. To acquire this phenotype, cancer cells need at early stages of cancer development, acquire activating mutations promoting oncogenes and/or inactivating mutations affecting tumor suppressor genes, making cells proliferate at uncontrolled rate with the consequence of increased mutation frequency and propagation of mutations to progeny. This gives the transformed cell a survival advantage, and the first crucial steps towards becoming a malignant cell, has been launched. Several oncogenes and tumor suppressor genes that have been described, are found in DNA repair pathways, such as the TP53 gene, or in developmental networks such as the HH signaling and Wnt signaling. These pathways have provided the field fundamental understanding of cancer development and how cancer cells acquire resistance to therapy. The main focus of this thesis was therefore to study these key pathways in mainly two pediatric cancer forms that are diseases that arise during embryonic development and are prone to relapse after conventional cancer therapy, and thus provides good models for studying the aforementioned fundamental questions.

In paper I, I focused on the SHH pathway. This pathways is highly preserved and known to be highly active in normal embryonic development, it is therefore not surprising that the HH pathway is aberrant in embryonic cancers such as neuroblastoma and medulloblastoma. Here, we explored the role of several key regulatory genes of the HH pathway in neuroblastoma and found that the GLI oncogene is essential for neuroblastoma growth. The upstream mediator of HH, the SMO protein, seemed to be less important for the growth of neuroblastoma tumors. Importantly, our data revealed that combining GLI inhibitors with conventional therapeutics could be an attractive treatment strategy in the clinical management of neuroblastoma. HH pathway can crosstalk with other developmental signaling networks including Wnt and the PI3K/Akt pathway (Barker and Clevers, 2006; Lum and Beachy, 2004), it would be interesting to target neuroblastoma tumors by dual targeting against two of the fundamental signaling networks. This has been shown previously by our lab to be efficient in preclinical models of medulloblastoma where Wnt signaling and PI3K/Akt pathway were targeted with specific inhibitors of both pathways (Baryawno et al, Cancer Research, 2010).

Cancer therapy resistance is one of the most challenging questions in the fight against cancer. While the focus of paper I was on the HH pathway, paper II turned the focus to the Wnt signaling pathway, were I studied the role of Wnt in DNA repair and therapy resistance. Here, we provided new insights into the interplay between DNA repair and Wnt signaling, and how cancer cells acquire resistance to chemotherapy through this interaction. In this paper, we discovered a crucial association between Wnt signaling and MGMT, which is a DNA repair protein involved in chemoresistance to DNA alkylators. By blocking this association we could demonstrate sensitivity to chemotherapy in several cancers, including neuroblastoma and medulloblastoma. Although all experiments were performed *in vitro* and *in vivo*, this study provides a new approach of how resistance to cancer treatment can be modulated by upstream signaling of DNA repair proteins, and hence provide a new approach to targeted therapy.

During the last decade, convincing evidence has emerged from several studies proposing *PPM1D*/WIP1 to be a potent regulator of cancer development. In paper III, we provided new evidence suggesting a pivotal role of WIP1 in the tumor growth and progression of neuroblastoma and medulloblastoma. Indeed, we present data proposing that WIP1 is an oncogene contributing to cancer development. We utilized a multitude of preclinical models and genetic data on human tumor material to demonstrate the crucial role of *PPM1D*/WIP1 in both neuroblastoma and medulloblastoma development. Furthermore, we substantiated WIP1 as an oncogene by demonstrating that transgenic mice overexpressing WIP1 subjected to external DNA stress develop a variety of cancer types. This phenotype is normally observed in mice where the tumor suppressor gene p53 is impaired. Although in this study we couldn't detect neuroblastoma or medulloblatoma formation in the *PPM1D*/WIP1 transgenic mice, we believe that this can be explained by the fact that mice were exposed to irradiation post birth while neuroblastoma and medulloblastoma are diseases of aberrant development (Marshall et al., 2014). It would therefore be important to extend this approach and expose transgenic mice to irradiation already during embryonic development, and in fact these experiments are ongoing. Moreover, RNA sequencing and exom seqencing of tumors and control tissues from *PPM1D*/WIP1 transgenic have been performed and the results are currently being analyzed. Lastly, our promising preclinical data using WIP1 inhibitors against neuroblastoma and medulloblastoma provides basis for further development of this novel therapeutic option and perhaps test this further in patients relapsing from their disease.

Cancer is not merely one disease! Cancer exists in more than 200 different types with different biological and clinical characteristics. It would be very presumptuous to believe that one drug or treatment strategy could do all the work and current successful treatments are always variants of combination therapy. The cancer field has further already taken important steps towards personalized medicine where the focus is to target each cancer through its specific biologic vulnerabilities and also taking the specificities of the host into account. In this thesis, we have provided new insights into disease development of neuroblastoma and medulloblastoma, and identified new targets that can be therapeutically exploited in the clinic. This would be of great benefit to each patient that may harbor these genetic alterations either as germline aberrations and/or somatic mutations in cancer cells where a tailored therapy can be implemented. In combination with new and better diagnostic tools that can detect cancers at early stages, and new technologies that can monitor cancer therapy and disease progression with high sensitivity, maybe one day we can eradicate cancer completely, either through prophylactic measures or with delivering more precise and effective treatments.

7 ACKNOWLEDGEMENTS

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