DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH

Karolinska Institutet, Stockholm, Sweden

TARGETING DYSFUNCTIONAL DEVELOPMENTAL SIGNALING CASCADES AS A TOOL FOR TAILORED THERAPY IN EMBRYONAL TUMORS

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To my family

ABSTRACT

Neuroblastoma and medulloblastoma are embryonal malignant tumors of the central and peripheral nervous systems, respectively. The overall survival for both diagnoses is 60-75% despite intense multimodal therapy. This warrants the search for novel therapeutic approaches. Hedgehog (HH) and Wingless (WNT) signaling pathways are important regulators of embryogenesis that have been associated with the development of cancer including medulloblastoma. HH signaling is characterized by Smoothened (SMO)-dependent activation of the GLI transcription factors. WNT signaling is divided into canonical and non-canonical signaling. Canonical signaling involves the key molecule β-catenin while non-canonical signaling includes the planar cell polarity pathway (PCP). PCP signaling including the Rac/Rho cascade are important for proper migration and differentiation of neural crest cells during neuritogenesis. In this thesis we have assessed the role of HH and WNT signaling in neuroblastoma and medulloblastoma, with the aim to gain insights and develop novel therapeutic approaches based on biological understanding of the diseases.

Key molecules within the HH signaling pathway are overexpressed in neuroblastoma. We investigated the effects of inhibiting HH signaling in neuroblastoma and found that inhibition of GLI was more effective in reducing neuroblastoma growth compared to inhibition of SMO located more upstream in the signaling pathway. The GLI inhibitor GANT61 effectively repressed neuroblastoma growth in vitro and in vivo, downregulated c-MYC, GLI1, MYCN and Cyclin D1 expression and augmented the cytotoxic effects when combined with clinically used chemotherapeutic drugs. These findings suggest that inhibition of HH signaling is a highly relevant therapeutic target for high-risk neuroblastoma (Paper I). The DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) is frequently overexpressed in cancers and is coupled to chemoresistance. In a search for new inhibitors for MGMT we investigated cellular regulators of MGMT expression in multiple cancers including neuroblastoma and medulloblastoma. We found a significant correlation between WNT signaling and MGMT expression that was confirmed by bioinformatic analysis and studies of protein expression of MGMT and β-catenin. Pharmacological as well as genetic inhibition of WNT activity downregulated the MGMT expression and restored chemosensitivity of the DNA-alkylating drug temozolomide in vitro and in vivo. These results have possible therapeutic entailments for chemoresistant cancers, especially of brain tumors where temozolomide is commonly used in treatment (Paper II).

Investigating non-canonical WNT signaling in neuroblastoma revealed that overexpression of PCP core genes Prickle1 and Vangl2 led to suppression of neuroblastoma cell growth and reduced Wnt/β-catenin signaling. On the other hand, overexpression of Vangl2 in neural stem cells produced accumulation of active β-catenin and decreased differentiation, suggesting different roles of PCP proteins in tumorigenic cells compared to normal cells (Paper III). Furthermore, genetic analyses demonstrated that neuroblastoma tumors harbor frequent mutations of genes controlling neuritogenesis associated with the Rac/Rho signaling cascade. The majority of these mutations were described to result in inhibition of Rac or activation of Rho. Inhibition of ROCK, a key enzyme downstream of Rho, resulted in differentiation, inhibition of neuroblastoma cell growth and migration and degradation of MYCN protein. Small molecule inhibition of ROCK suppressed MYCN-driven neuroblastoma growth both in a transgenic and in a xenograft model. This study proposes that manipulation of Rho signaling might offer new therapeutic alternatives for neuroblastoma (Paper IV).

Taken together, the work in this thesis demonstrates that the embryonal signaling pathways HH and WNT may offer new therapeutic targets for neuroblastoma and medulloblastoma.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Wickström M*, **Dyberg C***, Shimokawa T, Milosevic J, Baryawno N, Fuskevåg OM, Larsson R, Kogner P, Zaphiropoulos PG, Johnsen JI. 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 signalling prevents chemoresistance. Nature Communications. 2015;6:8904.

III. **Dyberg C**, Papachristou P, Haug BH, Lagercrantz H, Kogner P, Ringstedt T, Wickström M*, Johnsen JI*. Planar cell polarity gene expression correlates with tumor cell viability and prognostic outcome in neuroblastoma. BMC Cancer. 2016;16(1):259

IV. **Dyberg C,** Fransson S, Sveinbjönsson B, Lännerholm-Palm J, Forsberg D, Herlenius E, Martinsson T, Kogner P, Johnsen JI* , Wickström M*. Rho-associated kinase is a potential therapeutic target in neuroblastoma. Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
ALK	Anaplastic lymphoma kinase
BrdU	Bromodeoxyuridine
CamKII	Calmodulin dependent protein kinase II
Celsr	Flamingo
CK1	Casein Kinase 1
CMB	Classic medulloblastomas
CSC	Cancer stem cells
CSF	Cerebrospinal fluid
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DAPI	40,6-diamidino-2-phenylindole
Dgo	Diego
Dhh	Desert Hedgehog
DMB	Desmoplastic/nodular medulloblastomas (DMB)
Dvl	Dishevelled
E	Embryonic days
FZ	Frizzled
GAPs	GTPase-activating proteins
GCP	Granule cell progenitor
GLI	Glioma associated oncogene
GSK-3β	Glycogen Synthase-3β
HH	Hedgehog
Ihh	Indian Hedgehog
INSS	International Neuroblastoma Staging System
INRGSS	International Neuroblastoma Risk Group Staging System
LC/AM	large cell/anaplastic (LC/AMB)
LOH	Loss of heterozygosity
LRP	LDL-receptor related protein
mDia	Mammalian homolog of Drosophila diaphanous

MBEN	Medulloblastoma with extensive nodularity
MGMT	O^6-Methylguanine-DNA methyltransferase
MYCN	V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma
PBS	Phosphate-buffered saline
PFA	Phosphate-buffered formaldehyde
PGE_2	Prostaglandin E_2
Pk	Prickle
PKC	Protein kinase C
PLC	Phospholipase C
PNET	Primitive neuroectodermal tumors
Rho GEFs	Rho GTPase-specific guanine nucleotide exchange factor
ROCK	Rho-associated coiled-coil forming kinase
SHH	Sonic Hedgehog
TVI	Tumor volume index
Vangl2	Van-gogh like 2
WNT	Wingless
WHO	World Health Organization

1 INTRODUCTION

1.1 CANCER

Cancer is a term for diseases in which human cells become abnormal and start to divide without cessation. Cancer development involves several genomic changes, and the transformation of normal cells to cancer cells is a multistep process (Hanahan and Weinberg, 2000). Every day new cells are generated in the human body through cell division. This process is precisely controlled by multiple molecular and cellular mechanisms. However, accidentally cells with acquired capacities to proliferate, migrate and invade other parts of the body develop, that eventually result in malignant cancer (Hanahan and Weinberg, 2000). Defined by Hanahan and Weinberg, a cancer cell needs to have six biological capabilities to develop a human tumor or neoplasm. The six hallmarks are: sustaining proliferative signals, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death, essential for these hallmarks are inflammation, reprogrammed metabolism, evading immune destruction and genomic instability (Hanahan and Weinberg, 2011).

Cancer is still one of the leading causes of death worldwide. Despite significant improvement in cancer therapeutics, cancer is estimated to be the major cause of mortality in the coming decades worldwide, irrespective of income level. The predicted global burden as of 2030 is estimated at 20.3 million new cancer cases, compared to 12.7 million cases 2008. The predicted survival rate in 2030 is estimated to be 65% (Bray et al., 2012).

The development of new, more effective treatments is needed and it is necessary to find new potential targets of these diseases. My hope is that by increasing knowledge about signaling pathways aberrantly expressed in different tumors along with a better understanding of the mechanisms behind cancer development and progression, what will contribute to a higher cure rate for cancer patients in the future.

Pediatric cancers are neoplasms that occur in children and represent <2% of human cancers. The incidence of childhood cancers diverges with age and sex and is the most common reason of death from non-communicable diseases in children >1 year. Embryonal tumors account for roughly 20% of cancers seen in children <15 years of age and the survival for children with these tumors have increased during the last 60 years (Gustafsson et al., 2013) (figure 1). The tumors consist of very undifferentiated cells similar to the cells in a developing embryo. Embryonic tumors include: medulloblastomas, primitive neuroectodermal tumors (PNETs), atypical teratoid/rhabdoid tumor (AT/RT), neuroblastoma, ganglioneuroblastoma, retinoblastoma, nephroblastoma and hepatoblastoma (Tulla et al., 2015).

1.2 NEUROBLASTOMA

1.2.1 Neuroblastoma biology

Neuroblastoma is an embryonal childhood tumor that arises in the tissue of the adrenal medulla, paraspinal ganglia or sympathetic ganglia (Maris, 2010; Ora and Eggert, 2011; Schulte and Eggert, 2015). Neuroblastoma accounts for about 6% of all childhood cancers, but for 9% of all childhood cancer deaths (Gustafsson et al., 2013; Johnsen et al., 2009). It most frequently occurs in the adrenal medulla (Garner and Beierle, 2015) and the median age at diagnosis is 17-18 months (Brodeur, 2003; Maris, 2010). In Sweden, 10-20 children are diagnosed with neuroblastoma each year, with the mean annual incidence rate of 0.7 cases/100 000 children <15 years of age (1984-2010) (Gustafsson et al., 2013).

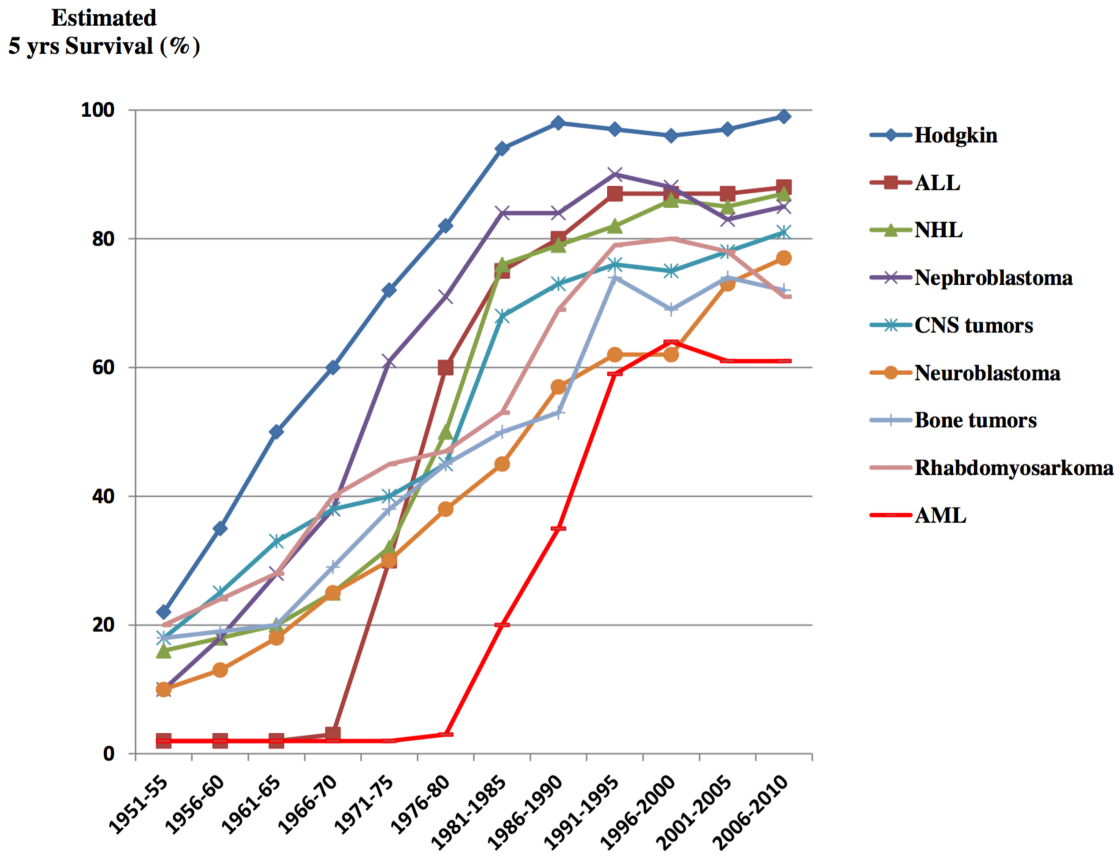

Figure 1. The estimated prognosis (5-years survival) over time for selected diagnostic groups. The prognosis improved considerably during time period 1970-1995. The results during the last decades appear to have reached a plateau, although neuroblastoma and CNS tumors seem to have a continued improved five-year survival. Adapted from (Gustafsson et al., 2013), with permission from the publisher.

Neuroblastoma is an undifferentiated tumor, consisting of small, round blue cells. These cells are called neuroblasts and rarely differentiate spontaneously. However, some tumors show some differentiation and are called ganglioneuroblastomas. The furthermost differentiated form is called ganglioneuroma (Brodeur, 2003).

Some neuroblastomas have the unique ability to regress spontaneously or differentiate into a benign ganglioneuroma. This spontaneous regression is particularly seen in infants. Older children more often have widespread or metastatic disease at the time of diagnosis and therefore an overall poorer prognosis (Brodeur, 2003). Neuroblastomas mostly metastasize to lymph nodes, bone and bone marrow (figure 2) (Johnsen et al., 2009). The tumor is believed to originate from primitive precursor cells present in the neural crest and is the most common and deadly extracranial solid tumor in children (Brodeur, 2003; Louis and Shohet, 2015; Ratner et al., 2016; Schulte and Eggert, 2015). The neural crest is a transient structure present during embryonal development and neural crest cells migrate during mid-gestation throughout the body along two defined routes, one ventral pathway giving rise to glial cells and neurons and one lateral pathway giving rise to melanocytes (Green et al., 2015; Mohlin et al., 2011; Takahashi et al., 2013). These migrating progenitor cells are extremely proliferative and are regulated through interplay between different signaling pathways (Green et al., 2015; Jacob, 2015; Newbern, 2015; Szabo and Mayor, 2016). Cells in the embryo are exposed and guided by different signaling factors and strong proliferative signals drive the growth of the embryo and limit the cells to differentiate and exit the cell cycle (Green et al., 2015; Maguire et al., 2015; Munoz and Trainor, 2015). Therefore, changes in the guiding signals and the inability of progenitor cells to differentiate and exit the proliferative state could promote transformation and potentially drive tumor formation and development of neuroblastoma (Grimmer and Weiss, 2006; Johnsen et al., 2009; Louis and Shohet, 2015; Maguire et al., 2015).

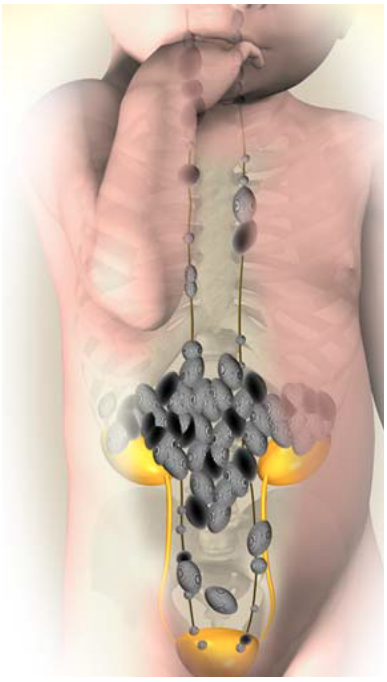

Figure 2. Illustration of neuroblastoma and potential locations. Neuroblastoma primary tumors derived from the neural crest arise in the sympathetic nervous system including the adrenal medulla, sympathetic ganglia and paraganglia. Neuroblastomas mainly metastasize to lymph nodes, bone and bone marrow, and in infants also spread to liver and subcutaneous tissue.

Reprinted from (Johnsen et al., 2009) with permission of Springer.

1.2.2 Neuroblastoma genetics and prognostic factors

Neuroblastoma shows heterogeneous biological and clinical features extending from natural regression to extremely malignant disease with metastatic spread (Brodeur, 2003). Spontaneous regression was described as early as 1927 in a paper by Cushing and Wohlbach (Cushing and Wolbach, 1927). The prognosis for the patient prominently depends on two factors: the age of the patient at diagnosis and whether the tumor has metastasized. Younger children tend to respond better to treatment, especially children with less widespread disease (Breslow and McCann, 1971). Aside from age and tumor burden, there are some genetic, molecular and histological patterns that are of importance for the prognosis and can be useful in the choice of therapy.

Somatic genetic changes, for example activation of oncogenes, gain or loss of alleles, or changes in tumor cell ploidy are of importance in the development of neuroblastoma (Brodeur, 2003). The v-myc avian myelocytomatosis viral oncogene neuroblastoma (MYCN) proto-oncogene located at the 2p24 locus is frequently amplified (22%) or overexpressed and several studies have revealed that it characterizes a subset of very aggressive neuroblastomas correlated with poor prognosis. MYCN is a transcription factor that forms heterodimers with the MAX protein, and this complex activates transcription of several target genes. These target genes are widely involved in metabolism, apoptosis and cell growth (Brodeur, 2003; Johnsen et al., 2009; Mohlin et al., 2011). In neuroblastoma MYCN can be amplified five to several hundred-fold and the amplification leads to increased proliferation, decreased apoptosis and may also cause a differentiation arrest (Lu et al., 2003; Schwab, 1993).

Other genetic changes identified in neuroblastomas are complete or partial gain of chromosome 17q and deletions of the short arm of chromosome 1 (1p). The most frequent

genetic abnormality is gain of chromosome 17q, with an incidence rate of 63-83% (Caren et al., 2010). This gain is associated with an advanced disease and more often found in tumors of children older than one year (Caren et al., 2010). Some of the 17q gain tumors also show loss of 1p and amplification of MYCN (Abel et al., 1999; Bown et al., 1999). Loss of heterozygosity (LOH) on chromosome 11q is another genetic abnormality in neuroblastoma. The LOH is rarely coupled with *MYCN* amplification but despite *MYCN* amplification, the LOH on chromosome 11q is associated with an aggressive disease and decreased probability of survival (Attiyeh et al., 2005; Caren et al., 2010).

About 1-2% of neuroblastomas are inherited in an autosomal dominant manner. The most commonly affected gene in these neuroblastomas is activating mutations of the anaplastic lymphoma kinase (*ALK*) oncogene (Mosse et al., 2008). Also, somatic mutations of the *ALK* gene have been found in 7-10% of neuroblastoma making this tyrosine kinase transmembrane receptor an attractive target for treatment for this group of neuroblastoma patients (Caren et al., 2008; Chen et al., 2008; George et al., 2008; Hallberg and Palmer, 2013; Janoueix-Lerosey et al., 2008; Molenaar et al., 2012; Pugh et al., 2013).

1.2.3 Neuroblastoma staging and treatment

The clinical staging criteria are used as a measure of the tumor burden in the patient. All of the above should be taken into account when appropriate treatment is chosen for the patient. There have been different staging systems over the years. In 1988, a new revised neuroblastoma staging system known as the International Neuroblastoma Staging System (INSS) was presented. This was based on new research and a better understanding of the disease (Brodeur et al., 1993). However, later it was shown that this staging system had some weaknesses and treatments among different cooperative groups were not based on the same prognostically genetic features. Therefore, a new International Neuroblastoma Risk Group Staging System (INRGSS) was developed that will ensure that children, irrespective of nationality are stratified into identical treatment groups. This system was designed to stratify patients pre-treatment, together with prognostic factors including histology, *MYCN* status, age, 11q status, and DNA ploidy (Cohn et al., 2009). Locoregional tumors are staged as L1 or L2 based on the presence or absence of Image defined risk factors (IDRF). Metastatic spread tumors are staged as M, except when the metastases are restricted to liver, skin and/or bone marrow in children below 18 months of age. They are staged as MS (Monclair et al., 2009).

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

Table 1. International Neuroblastoma Risk Group Staging System. Adapted from (Monclair et al., 2009), with permission from American Society of Clinical Oncology.

The current treatment for children with neuroblastoma, stratified into the high-risk group, includes chemotherapy, surgical resection, myeloablation, local radiation and treatment with 13-cis retinoic acid (Ora and Eggert, 2011; Pearson et al., 2008). The common European induction chemotherapy includes dose cycles of etoposide and cisplatin alternating with vincristine, doxorubicin and cyclophosphamide (Pearson et al., 2008). For tumors that are stratified into lower risk groups, the current trend has been to reduce the intensity of the therapy without myeloablation and avoiding radiotherapy and chemotherapy in many children (Maris, 2010; Ora and Eggert, 2011).

1.3 MEDULLOBLASTOMA

1.3.1 Medulloblastoma biology

Brain tumors are the second most common cancer diagnosis in children below 15 years of age. Medulloblastoma is the most common malignant pediatric brain tumor. In Sweden, 10-15 children are diagnosed with medulloblastomas every year with a peak incidence at seven years of age (Gustafsson et al., 2013; Lannering et al., 2009; Polkinghorn and Tarbell, 2007). The male/female ratio of medulloblastoma is 1.5:1 (Coluccia et al., 2016) and in Sweden, the overall survival rate is 58.8 % (Gustafsson et al., 2013). Symptoms of medulloblastoma include morning vomiting, headache and truncal ataxia, which usually exist for 1.5-3 months before diagnosis (Polkinghorn and Tarbell, 2007). The tumor most often arises in the cerebellum, and is believed to originate from primitive neuronal cells (Northcott et al., 2011). It has been shown by gene-array data, that medulloblastomas are molecularly different from other brain tumors including PNETs (figure 3) (Guessous et al., 2008; Pomeroy et al., 2002). There are two major germinal zones involved in the development of the cerebellum: the ventricular zone (VZ) and the external granule layer (EGL). The VZ gives rise to neurons and glia cells and is found at the top of the fourth ventricle. The EGL, on the other hand, gives rise to glutaminergic neurons and is found on the posterior external surface of the cerebellum

(Coluccia et al., 2016). Medulloblastoma is, according to the WHO, classified as a grade IV tumor and histopathologically classified into different subgroups: classic medulloblastomas (CMB), desmoplastic/nodular medulloblastomas (DMB), extensive nodularity (MBEN) and large cell/anaplastic (LC/AMB) (Guessous et al., 2008; Polkinghorn and Tarbell, 2007). The histological grading assists in predicting the biological behavior and is a key factor influencing the choice of tumor treatment (Louis et al., 2007).

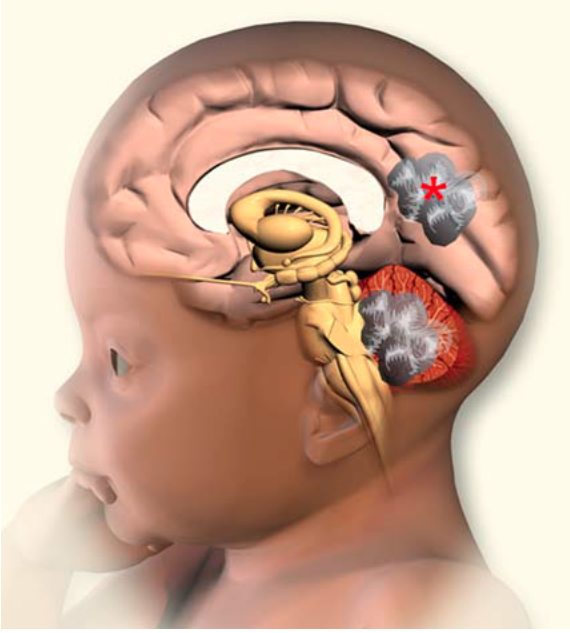

Figure 3. Illustration of medulloblastomas localization. Medulloblastoma primary tumors arise in the infratentorial posterior fossa of the CNS whereas supratentorial primitive neuroectodermal tumours (sPNET) arise in the superior fossa (indicated by asterisk, *).

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1.3.2 Molecular subgroups of Medulloblastoma

The development and growth of medulloblastoma has been associated with several molecular dysfunctions. These molecular aberrations include deregulation of developmental signaling pathways, important for normal embryonal development, such as hedgehog (HH), wingless (WNT) and deregulation of other oncogenic pathways including c-MYC and MYCN (Guessous et al., 2008). In recent years, tremendous improvements have been made in understanding the biology and pathogenesis of medulloblastoma. Large-scale genetic, transcriptome and epigenetic analysis of medulloblastomas worldwide has revealed four different molecular subgroups. The four principal subgroups have been named: Sonic Hedgehog (SHH), WNT, group 3 and group 4 (Taylor et al., 2012) (figure 4). The four subgroups have different demographic parameters, tumor appearance and clinical behavior (Coluccia et al., 2016).

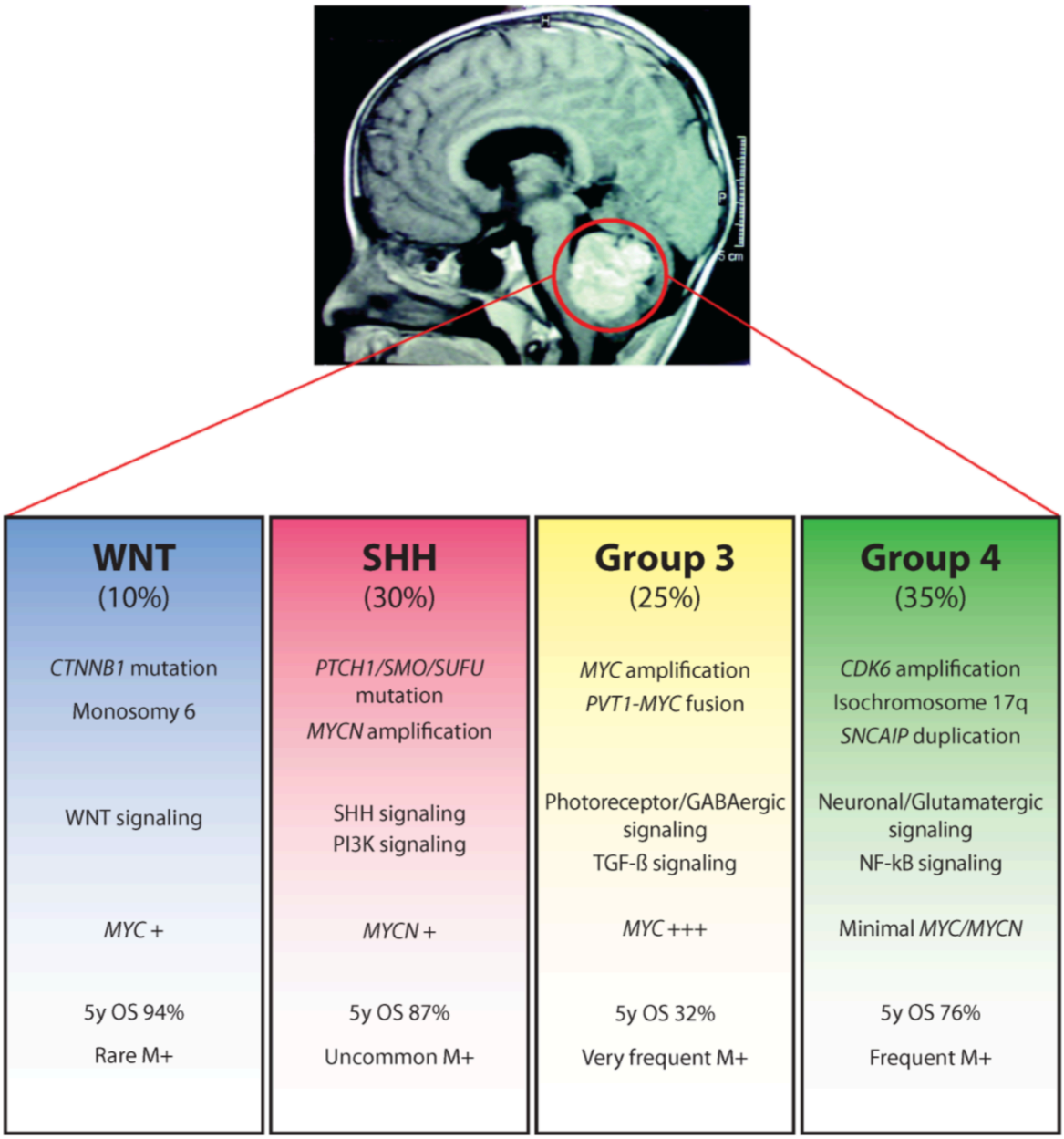

Figure 4. Features of the four medulloblastoma subgroups. Claudia C. Faria, Christian A. Smith and James T. Rutka (2013). New Molecular Targets and Treatments for Pediatric Brain Tumors, Evolution of the Molecular Biology of Brain Tumors and the Therapeutic Implications, Dr. Terry Lichtor (Ed.), InTech, DOI: 10.5772/53300. Available from: http://www.intechopen.com/books/evolution-of-the-molecular-biology-of-brain-tumors-and-the-therapeutic-implications/new-molecular-targets-and-treatments-for-pediatric-brain-tumors. Reproduced with permission from the authors.

The SHH group is named after the Sonic Hedgehog signaling pathway. This signal transduction pathway is important for granule cell progenitor (GCP) proliferation and purkinje cells provide the signal by secreting the SHH glycoprotein. Aberrant development of granule cells can result in medulloblastomas and includes both excess and absence of signals for GCPs to proliferate (Polkinghorn and Tarbell, 2007). The SHH medulloblastomas are

primarily found in infants (0-3 years) and adults (>16 years). Most of the nodular/desmoplastic medulloblastomas are classified into the SHH group (Taylor et al., 2012). SHH tumors frequently express high levels of MYCN as well as loss off chromosome 9q (Northcott et al., 2011). The prognoses for medulloblastoma patients with SHH tumors, are intermediate and similar to group 4 patients.

Germline mutations in the receptor protein patched homologue 1 (*PTCH1*) that result in constitutive activation of the pathway cause a condition termed Gorlin syndrome (described on page 16). These individuals have a predisposition to develop medulloblastomas (Taylor et al., 2012).

The WNT group is named after the WNT signal transduction pathway and arises from cells of the lower rhombic lip (Phoenix et al., 2016). This group has a very good prognosis compared to the other three subgroups and the long-term survival for patients in this group is >90%. The WNT pathway is important for cell proliferation and WNT medulloblastomas are commonly somatic mutated in *CTNNB1* encoding β-catenin. These tumors also show chromosome 6 monosomy (Taylor et al., 2012). Tumors in the WNT subgroup are distributed across all age groups but are more common in older children. The tumors have a classic histopathology (Northcott et al., 2011). One new hypothesis for why WNT patients have an excellent survival rate is that WNT medulloblastomas secrete WNT antagonists that silence WNT signaling in neighboring endothelial cells. As a result, a formation of a highly aberrant and hemorrhagic vasculature occurs that lacks an intact blood brain barrier and therefore, these tumors are more susceptible to chemotherapy and more curable (Phoenix et al., 2016).

Group 3 medulloblastomas are believed to arise from cerebellar progenitor cells (Kawauchi et al., 2012). The tumors usually express high levels of c-MYC and *c-MYC* gene amplification seems to be restricted to this group. Amplification and over expression of the *OTX2* gene is also limited to group 3 and group 4 tumors. OTX2 is a transcription factor that plays an important role in brain morphogenesis and when dysregulated may be involved in neoplasmic transformation (Di et al., 2005). Some of the tumors in group 3 display gain of chromosome 1q, or/and loss of chromosome 10q and 5q. Tumors in this group are seen in infants and children and more frequently observed in males than females. Many group 3 tumors belong to LC/AMB histology, and are commonly metastatic. This group has a poor prognosis (Taylor et al., 2012).

Group 4 tumors are more similar to group 3 tumors than to SHH or WNT tumors and 66% of the tumors in this group have an isochromosome 17q change. Loss of chromosome X is also seen in this group. There are reports that show that 80% of females in group 4 have a loss of chromosome X. The male/female ratio in this group is 2:1, and most of the tumors belong to classic medulloblastomas histology (Taylor et al., 2012). The peak incidence is 5-13 years of age. Group 4 patients have an intermediate prognosis (Kool et al., 2012).

1.3.3 Therapy for Medulloblastoma

Current treatment often includes surgery, radiation and chemotherapy. Even though the survival rates have increased during the last few years and the Nordic countries have a high cure rate compared to other international population studies, there are still improvements to be made (Lannering et al., 2009). The treatment depends upon age, metastatic status, subgroup classification and extent of surgical resection. Surgery is performed to collect tissue, reduce mass effect and recreate changed or obstructed cerebrospinal fluid. This is followed by radiotherapy. Radiotherapy is only given to children older than 3-5 years of age. This is due to the higher sensitivity for radiation correlated to cognitive impairments in children younger than 3 to 5 years (von Hoff and Rutkowski, 2012). Following radiotherapy, the children are treated with chemotherapy. The addition of chemotherapy after radiation has increased survival rates and allowed a decrease of radiotherapy doses, especially for standard risk patients. Commonly, a combination of either cisplatin, N-(2-chloroethyl)-N-cyclo-hexyl-N-nitrosurea (CCNU) and vincristine are given for eight cycles or a combination of cisplatin, cyclophosphamide and vincristine (von Hoff and Rutkowski, 2012). Depending on the dose and volume of used radiotherapy and age at diagnosis, survivors often suffer from life-altering side effects of treatment that increase over time. These life-altering side effects include neurological, endocrinological and social sequelae (Northcott et al., 2011). Because of this, it is important to investigate candidates for targeted therapy with fewer side effects with the potential to directly target underlying abnormal signaling pathways included in the different molecular subgroups (von Hoff and Rutkowski, 2012).

1.3.4 Resistance to therapy

Despite progress in improving survival over the past decades, there is still a reduced quality of life for survivors and some patients will still relapse and have a negative prognosis (Gottardo et al., 2014). These relapses can occur many years after the diagnosis of the primary tumor. Development of resistance to chemotherapeutic drugs is another major problem. There is increasing interest in the possibility that resistance to chemotherapeutic drugs is caused by cancer stem cells (CSC). Therefore, stem cell specific pathways may provide desirable targets to sensitize CSC to therapy. Experiments have shown that the most aggressive medulloblastomas have a high proportion of cells expressing CD133. CD133 itself seems to be a marker of both normal neural stem cells and CSC. Moreover, cells expressing CD133 preferentially activate DNA repair proteins more effectively than CD133-negative cells. Ultimately this population of cells have evolved a more effective system for repairing DNA damage, causing them to become resistant to radiation treatment (Visvader and Lindeman, 2008).

One enzyme that repairs DNA damage is O^6-Methylguanine-DNA methyltransferase (MGMT). MGMT is the main enzyme in the defense against mutation-driven carcinogenesis caused by O^6 alkylators. Alkylating agents are extremely reactive molecules that cause cell

death by binding to DNA and are used in the therapy of many different types of cancer, for example brain tumors as medulloblastoma. There can be many alkylation lesions initiated in the DNA, but the most mutagenic is on the O^6 position of the DNA base guanine. MGMT repairs these O^6MeG by transferring the alkyl group to a cysteine residue. After the transfer, the MGMT protein becomes inactivated and marked for degradation. As a result of this, the amount of lesions that can be repaired are equal to the amount of MGMT (Christmann et al., 2011). The promoter that controls the expression and activity of MGMT can be methylated. The methylation of the promoter silences the gene and the cells stop producing MGMT. Patients with lower expression of MGMT have been shown to have a better overall and disease-free survival and respond better to alkylating agents than patients with high MGMT levels (Esteller et al., 2000). Furthermore, it is of great interest to increase the sensitivity to alkylators in resistant tumors, by inhibiting the MGMT enzyme. There are several MGMT inhibitors on the market that have been shown to inactivate MGMT activity but still, this depletion also depresses basal levels of MGMT in normal cells leading to myelosuppression (Esteller et al., 2000).

2 DEVELOPMENTAL WNT AND HH SIGNALING IN CANCER

2.1 WNT SIGNALING

Cell signaling is a key factor in the development of all multicellular organisms. WNTs are secreted lipid-modified signaling proteins that seem to predominantly function over short distances and are important for normal development of the embryo (Clevers et al., 2014). The WNTs form a large family consisting of 19 WNT proteins, which share 27% to 83% similarity in the amino acid sequences (Miller, 2002). The first WNT gene, *Wnt-1*, was discovered in 1982 and was found to be a proto-oncogene that causes mammary tumors in mice (Nusse and Varmus, 1982). WNTs are involved in many cellular activities and influence multiple processes during development, including differentiation, proliferation, survival, movement and polarity. Several reports show that inappropriate WNT activity is linked to developmental defects and to a variety of cancer, including medulloblastomas and neuroblastomas (Gibson et al., 2010; Liu et al., 2008; Miller, 2002; Northcott et al., 2012; Northcott et al., 2011).

Transduction and response of WNT signals involves binding of WNT proteins to two different classes of cell surface receptors, the Frizzled (Fz) receptors and the LDL-receptor related protein (LRP) (Bejsovec, 2000). There are ten different known types of Fz receptors in humans, which bind different WNTs with varying specificity (Miller, 2002). The mechanism of LRP signaling is uncertain but the suggestion is a binding between the cytoplasmic domain of LRP and the WNT antagonist Axin (Mao et al., 2001). After binding to the receptors, the WNT signals are transduced through at least three different intracellular signaling pathways. The most studied pathway is referred to as the canonical WNT/β-catenin

pathway. The two other pathways are both denoted non-canonical pathways. They are divided into the WNT/planar cell polarity (PCP) pathway and the WNT/Ca^{2+} pathway. Dependent of the combination of WNTs and Fz, one of these pathways is activated and this will lead to unique cellular responses (Miller, 2002).

2.1.1 Canonical WNT signaling

The central player in this pathway is β-catenin. In the absence of a WNT ligand, the level of β-catenin is kept low. This is done by continually targeting β-catenin for ubiquitination and degradation in the proteasome by phosphorylation through glycogen synthase-3β (GSK-3β) and casein kinase 1 (CK1) attached to a scaffolding complex consisting of adenomatous polyposis coli (APC) and Axin. This complex of proteins is called the destruction box (Nelson and Nusse, 2004). The free levels of β-catenin remain subsequently low, which lets the DNA binding T-cell factor/lymphoid enhancer factor (Tcf/Lef) proteins to bind to transcriptional co-repressors, such as Groucho and block the transcription of target genes (Barker and Clevers, 2006; Clevers and Nusse, 2012). In the presence of WNT proteins, they act on target cells, by binding to FZ/LRP complex at the cell surface and induce the connection of Axin with the phosphorylated tail of LRP. These receptors send a signal to numerous intracellular proteins including Dishevelled (Dvl), Axin, APC, GSK3-β and β-catenin (Logan and Nusse, 2004). The destruction box ceases to function and newly synthesized β-catenin is accumulated and translocates to the nucleus. In the nucleus β-catenin binds to Tcf/Lef DNA-binding proteins and the repressing effect of Groucho is inhibited. Instead β-catenin converts the Tcf/Lef complex into a transcriptional activator complex. Upon this binding, several transcriptional co-activators are recruited to further induce expression of target genes, which play a direct role in tumorigenesis (Clevers and Nusse, 2012; Logan and Nusse, 2004) (figure 5). Loss of function of APC caused by mutation stabilizes β-catenin and activates transcription of target genes. Mutation in APC increases the risk of colorectal and other cancers (Taipale and Beachy, 2001). Other mutations in the pathway can also form cancer, for example the already mentioned mutations activating β-catenin in medulloblastomas (Northcott et al., 2011).

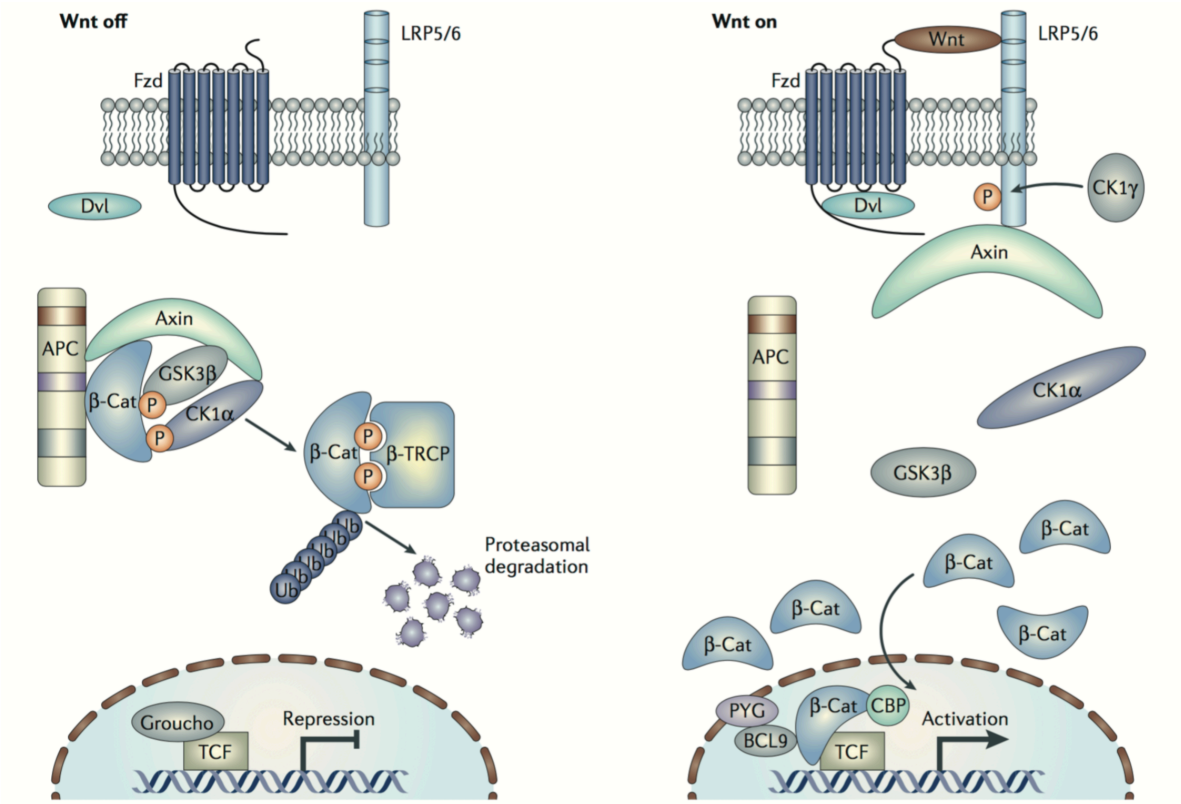

Figure 5. An overview of the canonical WNT signaling (Barker and Clevers, 2006). Reprinted by permission from Macmillan Publishers Ltd.

2.1.2 Non-canonical WNT signaling

In recent years, more and more is becoming known about the non-canonical WNT signaling pathways, which mediate the signals independently of β-catenin. The non-canonical WNT signaling can be further divided into two distinct pathways: The planar cell polarity signaling and the Ca^{2+} signaling (Komiya and Habas, 2008).

Planar cell polarity signaling

The planar cell polarity proteins create polarity within a tissue plane by asymmetric expression and cell morphology. Cells are organized along two axes: the PCP axis (i.e., from front to back) and the apical-basal axis (i.e., from top to bottom) and this is an important hallmark of embryonic development. Proteins involved in core PCP signaling include Flamingo (Celsr), Fz, Dvl, Van Gogh like 2 (Vangl2), Diego (Dgo) and Prickle. Recent studies have shown a surprisingly wide array of roles for PCP proteins. In the developing nervous system, PCP signaling is involved in many processes for example gastrulation, neurulation, neuronal polarity, axon guidance and tangential neuronal migration (Goodrich, 2008; Gray et al., 2011). The canonical WNT pathway and PCP pathway have been shown to

crosstalk and antagonize each other, although the mechanism is still unclear (Chan et al., 2006). After stimulation by WNT ligands to the Fz receptor, the signal is transduced to Dvl, leading to activation of the small GTPases Rho and Rac (Komiya and Habas, 2008). Activation of Rho stimulates axon retraction and keeps the cell undifferentiated, whereas stimulation of Rac promotes axon extension and neuritogenesis (Katoh et al., 2000). The Rho subgroup consists of RhoA, RhoB and RhoC. They are involved in the actin cytoskeleton function and act as a molecular switch in several processes such as adhesion, migration of neural crest cells and cell cycle progression. They can be found in a GDP-bound inactive form or a GTP-bound active form. This transformation is made by specific guanine-nucleotide exchange factors (GEFs) (Narumiya et al., 2009). The activation of GEFs is controlled by GTPase-activating proteins (GAPs) (Katoh et al., 2000). There are two main downstream effectors of Rho; one is mammalian homolog of Drosophila diaphanous (mDia) and the other is Rho-associated coiled-coil forming kinase (ROCK) (Narumiya et al., 2009) (figure 6).

ROCKs are protein serine/threonine kinases that phosphorylate various substrates involved in regulating actin-myosin cytoskeleton and contractility via myosin light chain (MLC) phosphatase and LIM kinases. Two isoforms of ROCK exist: ROCK1 and ROCK2, sharing 65% homology in the amino acid sequence (Riento and Ridley, 2003). ROCK1 is most highly expressed in lung, liver, spleen and testes, while ROCK2 is most highly expressed in brain and muscles (Hahmann and Schroeter, 2010). Several studies have shed light on how Rho proteins contribute to tumorigenesis and overexpression of ROCK has been associated with numerous cancers including melanoma, breast, prostate and colon cancer (Liu, 2011; Routhier et al., 2010; Sahai and Marshall, 2002; Zhang et al., 2014).

The fact that embryonic development shares many similarities with cancer development and that these conserved pathways are deregulated in tumorigenesis, support the theory that certain cancers are result of development gone wrong. Accumulating evidence confirms a role of PCP signaling in cancer development (Wang, 2009). Molenaar et al published a paper in Nature 2012, identifying genes mutated in neuroblastoma tumors related to the non-canonical WNT signaling pathway. These mutated genes are involved in growth cone stabilization and neurite outgrowth, important developmental signals of differentiated neuronal cells and neuritogenesis. Most tumors with mutations in these genes were aggressive high-stage neuroblastomas (Molenaar et al., 2012). In recent years, other groups have also reported recurrent mutations of genes involved in this signaling pathway in neuroblastoma (Pugh et al., 2013; Sausen et al., 2013).

Since Rho proteins are involved in the process of tumorigenesis and seem to be important for cancer progression, targeting the pathway might be particularly attractive and a potential new target for the treatment of neuroblastoma and other types of cancer. There are several ways in which pharmacological agents could target this pathway: either by targeting Rho proteins or by targeting Rho protein effectors such as ROCK (Sahai and Marshall, 2002).

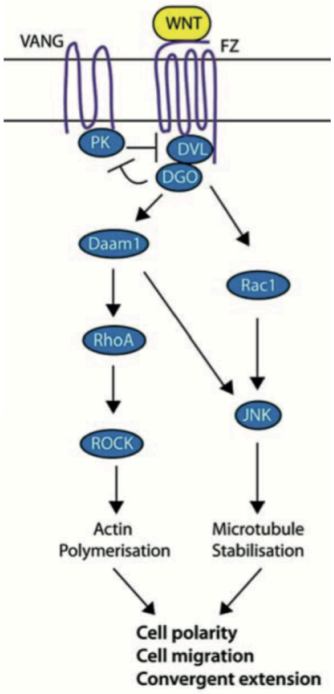

Figure 6. Non-canonical PCP signaling involves interactions between Fz, Vang, Pk and Dgo with activation of RhoA and ROCK via Daam1, which leads to actin polymerisation, and Rac1 and JNK, which leads to microtubule stabilization. This regulation of cytoskeletal dynamics controls cell polarity, cell migration and convergent extension (Clark et al., 2012). Reprinted with the permission from S. Karger AG.

Ca^{2+} signaling

Activation of WNT/Ca^{2+} pathway leads to higher levels of intracellular Ca^{2+} and stimulation of calmodulin dependent protein kinase II (CamKII) and protein kinase C (PKC) via phospholipase C (PLC). This is mediated via G-proteins and Dvl. In this pathway calcium acts as a second messenger and activates different downstream effectors (Kuhl et al., 2000; Wang, 2009). The pathway is important for normal development and several reports have shown that intracellular calcium release is essential for body plan specification and interferes with gastrulation (Kohn and Moon, 2005; Kuhl et al., 2000).

2.2 SONIC HEDGEHOG SIGNALING

The Hedgehog family of proteins has long been known to regulate cell growth, migration of cells in the neural crest and patterning during embryonal development. There are three identified HH subgroups: the *Desert Hedgehog* (Dhh), *Sonic Hedgehog* (Shh) and *Indian Hedgehog* (Ihh) (Echelard et al., 1993).

HH is able to affect distal tissue, operating over a long range in a concentration dependent manner. In the absence of HH, the 12-span transmembrane protein Patched (Ptch) is enriched in primary cilia, and acts to catalytically inhibit Smoothened (Smo). Activation of the HH signaling is started by the binding of HH ligand to Ptch receptor that moves Ptch out of the cilia resulting in loss of inhibition by Ptch and activation of Smo. Smo is a seven transmembrane-receptor resembling the FZ family of WNT receptors. Smo is stabilized and activated and affects a large complex of proteins containing Cos2 (Costal-2), Fu (Fused) and

Sufu (Suppressor of Fused). The activation of Smo leads to triggering of the glioma-associated oncogene (GLI) family of transcription factors, activating expression of specific HH target genes (Taipale and Beachy, 2001; Varjosalo and Taipale, 2008). In vertebrates, three forms of GLI transcription factors (GLI1-3) exist. The GLIs are zinc finger proteins that bind to DNA through five Zn-finger domains (Sasaki et al., 1999). It seems that GLI1 functions as transcriptional activators of HH target genes, whereas GLI2 and GLI3 can function as both activators and repressors (Rahnama et al., 2006). GLIs start transcription of target genes including *PTCH1*, *GLI1*, *GLI2*, *Cyclin D1*, *c-MYC* and *MYCN* (Ng and Curran, 2011; Wickstrom et al., 2013) (figure 7).

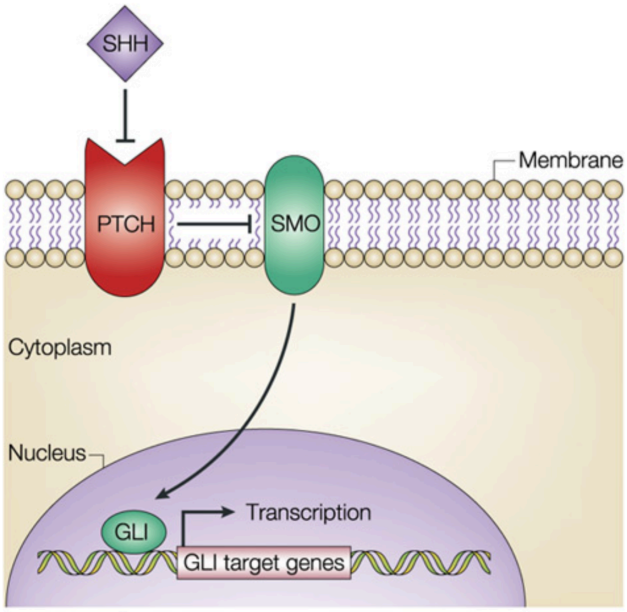

Figure 7. Sonic Hedgehog (SHH) acts on the membrane–receptor complex that is formed by Patched (PTCH) and Smoothened (SMO) to inhibit the repression of SMO by PTCH. SMO then signals intracellularly to activate GLI, and hence transcription of its target genes. (Owens and Watt, 2003) Reprinted by permission from Macmillan Publishers.

Germline mutations in this pathway that affect HH signaling are associated with developmental deficits and malformations. One of the most noticeable is cyclopia associated with loss of SHH signaling. In this defect, the eye fields are fused in the midline causing one big eye (Murdoch and Copp, 2010; Taipale and Beachy, 2001). The involvement of SHH signaling in cancer was first discovered in nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin syndrome. Gorlin syndrome is an autosomal dominant disorder linked to an increased risk of cancer, including medulloblastomas and basal cell carcinoma. 1-2% of medulloblastomas and 0.5% of basal cell carcinoma have Gorlin syndrome. In tumors, the mutations lock the pathway into a ligand independent state of constitutive activity (Hahn et al., 1996; Taipale and Beachy, 2001; Varjosalo and Taipale, 2008).

The HH pathway is closely connected to many other signaling pathways, such as the canonical WNT pathway (Teglund and Toftgard, 2010). Many synthetic small molecules can inhibit Smo activation. There are several Smo inhibitors that entered clinical trials in both pediatric and adult cancers (Varjosalo and Taipale, 2008). The major drawback with these inhibitors is that they may be ineffective against tumors that have molecular lesions downstream of Smo. Therefore, attempts to develop agents that target more downstream in

the signaling pathway have been done (Ng and Curran, 2011). In a cell-based screen one effective small-molecule targeted GLI was found. The molecule was named GANT61 (for Gli-ANTagonist) and was capable of reducing GLI-mediated transcription (Lauth et al., 2007). Several studies show that GANT61 can reduce cancer growth and may be a novel treatment for cancer with aberrant activation of SHH signaling (Lauth et al., 2007; Wickstrom et al., 2013).

3 AIMS OF THE THESIS

The general aim of my thesis was to investigate the possibility to develop novel therapeutic approaches based on biological understanding of the two most common and deadly solid tumors of childhood, medulloblastoma and neuroblastoma. Hopefully, this knowledge will lead to better survival, fewer side effects, and ultimately a better quality of life for these children.

The specific aims of my thesis were:

- To study the role of SHH signaling in neuroblastoma, and to investigate the effects of SHH inhibition on neuroblastoma growth.
- To investigate PCP signaling in neuroblastoma cells and its effect on tumor viability.
- To evaluate the effect of ROCK2 inhibition in neuroblastoma.
- To gain further insight into resistance mechanisms protecting cancer cells from chemotherapeutic drugs.

4 MATERIALS AND METHODS

The materials and methods in this thesis are here described briefly, but are also described in detail in papers I-IV.

4.1 PATIENT MATERIAL

All neuroblastoma tissue samples were collected at the Astrid Lindgren Hospital, Karolinska University Hospital. Human medulloblastoma and glioma samples were obtained from our cooperating partners Dr. Peter Siesjö and Dr. Anna Darabi, University of Lund whereas tissue samples from colon carcinoma patients were supplied by Dr. Baldur Sveinbjörnsson, University of Tromsö, Norway. Ethical approvals were obtained from the Karolinska University Hospital and Lund University Hospital. Medulloblastoma and glioma: LU1028-03, ETIK642/2008, neuroblastoma: 2009/1369-31/1, 03–736, colon: http://www.biomax.us/tissue-arrays/Colon/BC05002

4.2 IN VITRO

Cell lines

Human tumor cell lines from different tumor types were established from high-risk patients. For control purposes, the mouse neural cell line C17.2 and the MRC-5 lung fibroblast cell line were used. Different cell lines were used in this thesis depending on the specific purpose of the experiment. All cell lines were cultured in complete medium at 37°C in a humidified 5% CO_2 atmosphere. For all treatments in vitro, OptiMEM was used supplemented with 100 μg/mL penicillin/streptomycin.

Viability assay

To measure cytotoxic effects of drugs in the different papers, the colometric formazan cell proliferation reagent WST-1 (Roche) was used. The method shows the metabolic activity of the mitochondria in the cells as a marker for vital status. In viable cells, mitochondrial enzymes cleave tetrazolium salt to form formazan (Guertler et al., 2011). Therefore, cell survival can be quantified indirectly by WST-1. Cells were plated in OptiMEM into 96-well plates (density 5000-10 000 cells/well) and allowed to attach overnight. The following day, cells were incubated with drugs in triple combinations. Absorbance at 450 nm was measured with a microplate reader and the survival ratios of the controls compared to treated cells were calculated. In the papers, cell survival is presented as survival index (SI). The IC_{50} value (inhibitory concentration 50%) was stated as the concentration giving an SI of 50%.

In paper I and II we used the fluorometric microculture cytotoxicity assay (FMCA), as described in detail previously (Lindhagen et al., 2008). Briefly, the assay is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate to fluorescein by cells with intact cell membrane. Results from FMCA have been shown to correlate with cell viability methods based on tetrazolium dyes (Lindhagen et al., 2008) .

Immunohistochemistry/ Immunocytochemistry

Formalin-fixed and paraffin-embedded tissue sections were processed according to the specific protocols (see respective paper) and used for detection and distribution of proteins in xenograft and human materials. Mouse embryos were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) and cryoprotected overnight in 30% sucrose in PBS (see Paper III). Primary antibody was incubated 24-48 h at 4°C. Thereafter, sections were incubated with secondary antibody for 30 min at room temperature and mounted with Dapi-flouromount-G (Biotech) mounting medium. Matched isotype control was used as a control for non-specific staining. Pictures were taken using a Zeiss microscope. Quantification was performed either by counting positive stained cells or by measuring the stainings automatically using Leica Qwin IM500 software.

Immunocytochemistry staining was performed on fixed cell cultures to determine the relationship between proteins and differentiation status in cells. Anti-β-catenin, anti-MGMT and mouse anti-beta-tubulin III/Tuj1 were used as primary antibodies followed by incubation with Alexa flour-conjugated secondary antibody and mounted with mounting medium. Pictures were taken on a microscope (see respective paper).

Clonogenic assay

To measure tumorigenic capacity, clonogenic assay was used. Cells were seeded in 6-well plates cell^{+} at a concentration of 150-200 cells per well, in complete medium, and allowed to attach before drug treatment or transfection with siRNA. After attachment, the medium was changed to OptiMEM containing the drug(s) of interest for 48-96 h. After 7-14 days of incubation in drug-free medium, cell cultures were rinsed with PBS, fixed in formaldehyde and stained with Giemsa (Gibco). Colonies (<75 cells/well) were counted manually. For each treatment, the clonogenic forming ability of treated/transfected cells compared to untreated controls was calculated.

Western blotting

For the analysis of proteins from tissue and cells, western blots were performed. Proteins were extracted from cells and tissue using RIPA buffer (25mM Tris (pH 7.8), 2mM EDTA,

20% glycerol, 0.1% Nonidet P-40 (NP-40), 1mM dithiothreitol) added with a MiniComplete protease inhibitor cocktail (Roche Diagnostic). For detection of phospho-proteins a phosphatase inhibitor cocktail 1 (Sigma-Aldrich) was added. Equal amounts of proteins were loaded into each well and separated by SDS-polyacrylamide 10% (Bio-Rad) and nylon or PVDF nitrocellulose membranes, probed with primary antibody (see respective paper) and diluted in 5% BSA or 5% dry milk in TBS-T. After washing the secondary antibody, conjugated with horseradish peroxidase (Cell signaling), diluted in 5% dry milk in TBS-T was added. Pierce super signal (Pierce, Rockford) was used for detection on Kodak films. ImageJ was used for pixel density analysis and relative protein expression.

Measurement of cell cycle distribution

Cells were seeded in flasks, left to attach, and treated with the drug. After treatment, cells were pulsed with Bromodeoxyuridine (BrdU) for 15 min, at 37°C, harvested and washed in PBS. Fixation of cells was performed in 4% phosphate-buffered formaldehyde (PFA), 12 minutes at 90 °C and suspended in 95% ethanol, 4°C until analysis. Prior to FACS analysis, cells were stained with 40,6-diamidino-2-phenylindole (DAPI) and Anti-BrdU monoclonal, phycoerythrin labeled antibody (Biosite). Samples were analyzed using BD LSR II flow cytometry and analysis was undertaken with the FACS Diva software (BD Bioscience).

Flow cytometry

To measure sub-G0 cell population, cells were trypsinized, washed with ice-cold PBS, and fixed in 70% ethanol at -20°C, followed by incubation with RNase and propidium iodide (Sigma-Aldrich). All analyses were performed with BD LSR II Flow Cytometer and analysis was done with FACS Diva software (BD Biosciences). Isotype controls were used as negative controls.

Real-time RT–PCR analyses

Total RNA was prepared from cells with the RNeasy mini-kit (Qiagen) or TRIzol reagent (Life technologies) according to the manufacturer's instructions. One hundred to 500 ng of total RNA was used for cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems) or High capacity cDNA reverse transcription kit (Life technologies). The PCR experiments were performed with TaqMan primers or SYBR Green primers (see respective paper) and TaqMan Universal PCR Master Mix (Applied Biosystems) or Power SYBR Green master mix (Life technologies) in 96-well plates. The PCR reaction was performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems) or 7300 Real-Time PCR system (Life technologies). All samples were performed in triplicate and all experiments

included a no template control. Relative expression was determined with a standard curve or calculated with the $2^{-\Delta\Delta Ct}$ method.

Luciferase assays and transfections

Cells were plated in 24-well plates and left to attach overnight. The following day, cells were transfected with a TCF/LEF reporter plasmid (Super 8x TOPflash) together with a Renilla-Luc plasmid and siRNA or cDNA (see paper II and III) (Veeman et al., 2003). Alternatively, cells were transfected with the TCF/LEF reporter plasmid and the Renilla-Luc, and 24 h later, drug treated. Super 8x TOPflash is a β-catenin reporter with TCF/LEF sites upstream of the luciferase reporter. It measures β-catenin mediated transcriptional activation.

In paper I, cells were transfected with a GLI reporter plasmid 12xGLIBS-Luc together with Renilla-Luc and 24 h later, drug treated (Shimokawa et al., 2008). A Dual Luciferase Assay Kit (Promega) and a luminometer (Perkin Elmer) were used to measure luminescence. Renilla-Luc was used as a transfection control and the values were normalized to the Renilla-Luc before calculating relative levels.

All transfections were performed using Lipofectamin 2000, according to the manufacturer's instructions (Invitrogen).

Wound assay

Wound assay or "in vitro scratch" assay is a method for studying cell migration *in vitro.* The method is based on the fact that cells tend to migrate and replace the lost cells into a formerly colonized area (Liang et al., 2007). Cells were plated into 6-well plates in serum-reduced medium and cultured until confluent. The cells were then transfected with siRNA or treated with drugs. A scratch was made using a 200μm pipette tip in the cell monolayer and images were captured directly and after 18 h. A line was drawn underneath the well in order to ensure that the identical area was photographed. Images were captured just above or below the line. Three independent experiments were made with at least three images per treatment. The results were analyzed using automated analysis in TScratch software (Geback et al., 2009).

4.3 IN VIVO

Three animal models were used: a human xenograft model in nude mice, a transgenic mouse model that carries the human MYCN oncogene (TH-MYCN) and a model with transgenic mouse embryos.

Human xenografts model

For the xenografts studies, four-six weeks immunodeficient female NMRI nu/nu mice (Scanbur) were used. Mice were housed and maintained under special pathogen-free conditions with access to sterile water and food ad libitum. Tumor cells were injected subcutaneously into the flank under general anesthesia (2-4% Isofluran). At the appearance of palpable tumors, the mice were randomized into treatment or control groups. Tumor growth was measured daily by using a digital caliper, and the volume was calculated by the formula length x width^2 x 0.44. Each mouse was treated for 10-12 days and then euthanized in carbon dioxide. Tumors were weighed and dissected in smaller parts. The parts were either snap-frozen in liquid nitrogen or fixed in 4% PFA. Tumor volume index (TVI) was calculated using the measured volume divided by the volume measured at treatment start.

The TH-MYCN transgenic model

We used the transgenic TH-MYCN mouse model that overexpresses MYCN in neuroectodermal cells (Weiss et al., 1997). The TH-MYCN mouse model predominantly develops neuroblastoma-like tumors in the abdomen, but thoracic tumors might also occur. The animals were obtained from the Mouse Model of Human Cancer Consortium Repository as a N16 backcross to the 129X1/SvJ background. The animals were housed at a maximum of six per cage in an enriched environment with access to food and water *ad libitum*. Pups were biopsied at two weeks of age for genotyping and further randomized. In our hands, 100% of homozygous mice developed tumors compared to 50% of heterozygous mice (Carlson et al., 2013; Rasmuson et al., 2012). At four and a half weeks of age, homozygous mice started to receive HA-1077 treatment by intraperitoneal injections for 10 days. The animals were closely monitored for weight loss and signs of toxicity. At sacrifice, the animals were euthanized using CO_2 and the tumor was excised, weighed and divided for fixation in 4% PFA or frozen at -80°C for PCR and western blot analyses.

Transgenic mouse embryos

For paper III, transgenic mouse embryos overexpressing Vangl2, coupled to a nestin promotor, were generated by Karolinska Center for Transgene Technologies. In the production of transgenic mice, Construct was injected into 1-cell stage mouse embryos by pronuclear injection (Lindqvist et al., 2010). By using an appropriate plasmid/enhancer, it is possible to drive the expression of the gene to a specific cell population during a defined developmental time interval. A construct with nestin as a promotor/enhancer results in overexpression in neural stem cells between embryonic days (E) 7.5 and 16.5 (Johansson et al., 2002). Pregnant mice were sacrificed at E 8.5 or 9.5 by spinal dislocation and the embryos were rapidly dissected out and fixed in ice-cold 4% PFA. By PCR, transgenic

embryos were identified and only embryos unequivocally overexpressing the gene were included in the transgenic group.

All animal experiments were approved by the regional ethics committee for animal research appointed and under the control of the Swedish Board of Agriculture and the Swedish Court (N304/08, N391/11, N163/03, N142/06, N391/11, N26/11).

4.4 STATISTICAL ANALYSIS

The IC_{50} values were determined from log concentrations-effect curves in GraphPad Prism (GraphPad Software) using non-linear regression analysis. For drug combination data, the Chou-Talalay method was used with the CalcuSyn (Biosoft) software. Analysis was performed as previously described and combination index (CI) at different effect levels were reported (Chou and Talalay, 1984; Ponthan et al., 2007). CI at 70% effect was chosen for presentation (IC_{70}). Values of CI equal to 1 indicate additive, less than 0.8 indicate synergistic and greater than 1.2 indicate antagonistic interactions, respectively. Analysis was performed using one-sample t-test.

For the in vitro and in vivo studies, parametric tests were used to determine statistically significant differences. Comparisons between two independent groups were analyzed by t-test. Three or more independent groups were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Treatment groups with repeated measurements over time were compared with two-way ANOVA followed by Bonferroni multiple comparisons post-test. Correlations were assessed with Spearman nonparametric test to determine if a correlation existed. Survival analysis was evaluated with log-rank test. All tests were two-sided, carried out in GraphPad and $p<0.05$ was considered significant.

Kaplan-Meier survival estimates and gene correlation graphs were extracted from the R2 database (R2: microarray analysis and visualization platform (http://r2.amc.nl)).

5 RESULTS AND DISCUSSION

5.1 SHH SIGNALING IN NEUROBLASTOMA (PAPER I)

Activation of the SHH signaling pathway results in rendered expression of a number of different genes important for cancer development including induced expression of MYCN (Teglund and Toftgard, 2010; Varjosalo and Taipale, 2008). Also both neuroblastoma cell lines and neuroblastoma tissue samples exhibit high expression of key molecules in the SHH signalling pathway (Mao et al., 2009; Schiapparelli et al., 2011). Therefore we investigated the effects of suppressing SHH signaling on neuroblastoma growth. Since all small molecule inhibitors of SHH signaling that currently are in clinical trials target SMO (Ng and Curran, 2011; Scales and de Sauvage, 2009), we decided to investigate the effect of a compound specifically targeting GLI1 further downstream in the SHH signaling cascade. This was done to include molecular lesions located downstream of SMO. Initially we used seven human neuroblastoma cell lines with different biology (table 2) for evaluation of the Gli antagonist GANT-61 cytotoxicity compared to two Smo inhibitors: SANT1 and cyclopamine, using FMCA. All tested cell lines were more sensitive to GANT61, with IC_{50} values in the range of 5.8-12.4 µM, compared to cyclopamine and SANT1 (figure 8). An inverse correlation was seen between *GLI-1* and *MYCN* mRNA expression. High expression of *GLI-1* was associated with increased drug sensitivity to GANT61, while cells with high expression of *MYCN* were more resistant to GANT61.

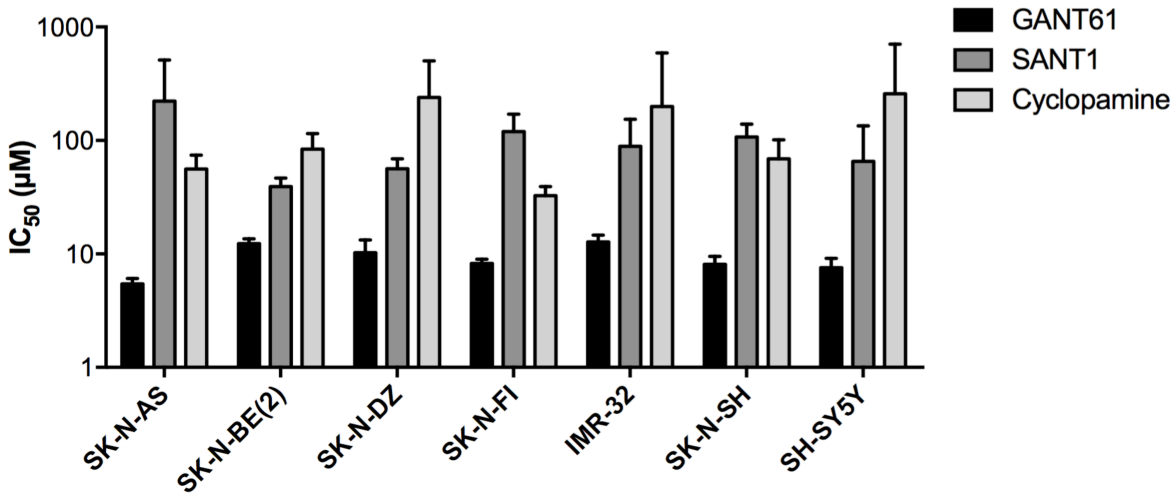

Figure 8. IC_{50} (µM) for GANT 61, SANT1 and cyclopamine in seven neuroblastoma cell lines. Mean and S.D. are shown.

Cell line	*MYCN* amp	*MYCN* single copy	Multidrug resistance
SK-N-AS		+	
SH-SY5Y			
SK-N-FI			
SK-N-SH		+	+
SK-N-DZ	+		Moderate
IMR-32	+		
SK-N-BE (2)	+		+

Table 2. Neuroblastoma cell line characterization

We also investigated the potential synergistic or additive effects of GANT-61 in combination with the chemotherapeutic drugs cisplatin, doxorubicin, irinotecan and vincristine that are commonly used in the treatment of neuroblastoma. Three neuroblastoma cell lines with different GANT-61 sensitivity were selected. The drug combination screening with GANT-61 demonstrated synergistic or additive effects in all three tested cell lines. This finding is interesting since cancer treatment often uses a combination of different drugs, and new substances that synergize with existing drugs for treatment have a higher possibility to be included as a treatment option. One major obstacle in cancer treatment is acquired or intrinsic multi-drug resistance in cancer cells. One mechanism by which cancer cells acquire resistance to chemotherapeutic drugs is by upregulation of membrane drug proteins. The activated proteins eject the chemotherapeutic drugs by acting as drug efflux pumps. There are speculations that SHH pathway activation can be involved in resistance to chemotherapeutic drugs. Significant upregulation of SHH and GLI-1 expression was detected in a majority of remaining tumors after therapy (Sims-Mourtada et al., 2007). Furthermore, the differentiation status of the cell can be changed when treated with SHH in culture (Okano-Uchida et al., 2004). Therefore, inhibiting SHH pathway may not only affect tumor proliferation, but also increase the effects of chemotherapy, resulting in improved cancer treatment responses.

Since GLI-1 and GLI-2 are transcription factors, we wanted to confirm that their transcriptional activity was suppressed upon treatment of neuroblastoma cells with GANT-61. This was performed with a GLI-dependent luciferase reporter plasmid (Shimokawa et al., 2008). The transcriptional activity was suppressed by 36-80% dependent on the cell line investigated. In addition, a downregulation on both c-MYC and MYCN was observed on protein level. This was expected, since both *c-MYC* and *MYCN* are reported to be regulated by SHH signaling (Kenney et al., 2003; Teglund and Toftgard, 2010). GANT61 also induced apoptosis in all tested cell lines and reduced the number of S-phase cells.

To see if GANT-61 activity was translatable into the *in vivo* situation, SK-N-AS cells were xenografted into the flanks of nude mice. When a tumor had been established (>0.15 ml) the mice were randomized to treatment or vehicle treatments by oral gavage (50 mg/kg GANT-61) for 12 days. Tumor growth was monitored daily for 12 days. *In vivo,* GANT-61

significantly inhibited tumor growth compared to vehicle-treated mice. At day 12, tumors treated with GANT-61 were 63% of vehicle treated tumors (p=0.03).

Our results highlight that neuroblastoma cells are more sensitive to GLI inhibition compared to SMO inhibition. Based on our findings, we can suggest that neuroblastoma cells may have molecular lesions located downstream of SMO and additionally, only siRNA knockdown of *GLI* had an impact on neuroblastoma cells. siRNA knockdown of *SMO* had no significant effect on neuroblastoma cell survival. Our results also demonstrate a correlation between GLI1 expression and sensitivity to GANT-61 and an inverse correlation between MYCN and GLI1. This inverse correlation was also seen when analyzing the publicly available R2 microarray database available at (http://r2.amc.nl) (figure 9). These findings may suggest a shift from SHH dependent signaling to MYCN-driven signaling, causing a tumor to become resistant to SHH inhibition.

In summary, the results in Paper I indicate that inhibition of SHH signaling at the level of GLI transcription factors is a novel treatment option to target high-risk neuroblastoma without MYCN amplification.

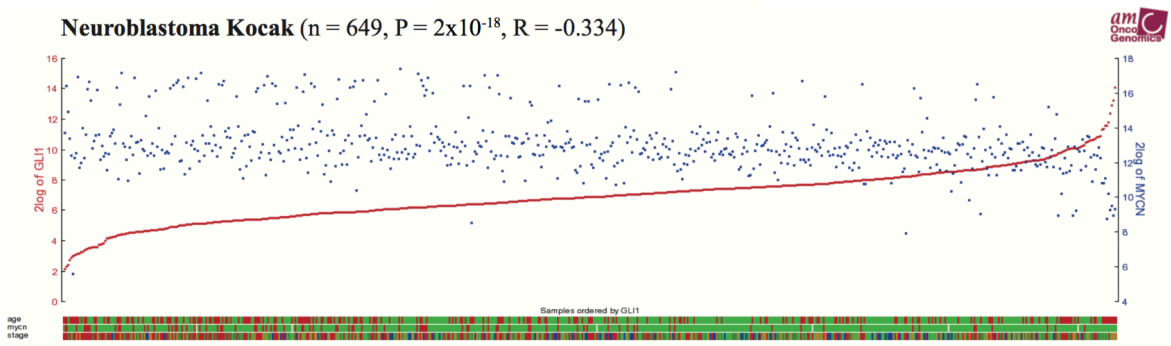

Figure 9. A significant correlation between *GLI1* and *MYCN* expression in neuroblastoma was observed in public available and validated dataset of neuroblastoma samples (Kocak dataset R=-0.334, P=0.2x10^{-18}, n=649).

5.2 TARGETING DRUG RESISTANCE IN CANCER (PAPER II)

The development of cancer cell resistance to chemotherapeutic drugs is one of the major hurdles in the treatment of cancer. It has been estimated that 90% of the treatment failure observed in metastatic cancer is due to the development of mechanisms by the cancer cell to overcome the cytotoxic effects of chemotherapeutic drugs. The majority of chemotherapeutic drugs functions through affecting DNA replication. This strategy has been successful since cancer cells are rapidly proliferating and because of this have inadequate DNA repair mechanisms that in normal cells secure the high fidelity of DNA replication. DNA alkylators are a large group of cytotoxic drugs that are frequently used in cancer treatment. DNA alkylators induce alkylation of DNA which results in base-mispairing and DNA breakage during DNA replication and induction of apoptosis. To overcome the cytotoxic effects induced by DNA alkylators cancer cells have developed mechanisms to avoid the DNA damage induced by these substances. One important mechanism is to overexpress DNA repair enzymes such as O^6-methylguanine-DNA methyltransferase (MGMT). MGMT efficiently removes O^6-guanosine alkylation adducts in a one-step reaction that recovers the guanosine residue in a DNA molecule. During this reaction the double-strand DNA break is restored and the DNA replication functions as normal. MGMT is a so-called suicide enzyme in that the reaction is stoichiometric and MGMT is irreversibly inactivated by proteosomal degradation (Christmann et al., 2011). This makes MGMT an ideal candidate to target in order to restore chemosensitivity to DNA alkylators. Several drugs that inhibit the enzymatic function of MGMT have been developed but none of these substances has been successful in clinical trials mainly because of induction of toxicities in hematopoietic cells.

In an attempt to overcome the toxicity induced by MGMT inhbition, we performed a search for cellular regulators of MGMT expression in tumor cells and tried to determine how to specifically target the regulators to lower the amount of MGMT. In paper II we show that activation of the canonical WNT/β-catenin signaling pathway upregulates MGMT expression in different cancers. Bioinformatic analysis of available expression cohorts identified a correlation between WNT/β-catenin signaling and MGMT expression in cancers of different origin. Immunofluorescence staining on human tumor tissues deriving from colon carcinoma, glioma, medulloblastoma and neuroblastoma revealed cellular co-localization of nuclear β-catenin and MGMT. This correlation was also observed in the majority of investigated cell lines originated from these cancers. Further analyses of the 5´-flanking region of the *hmMGMT* gene for possible Tcf/Lef binding sites, detected eight possible binding sites within the MGMT promoter. Luciferase plasmids with MGMT-5´ regions containing different numbers of Tcf/Lef-binding sites were tested for their activity in cells with genetically or pharmacologically manipulated expression of β-catenin. An interesting finding was that an augmentation of luciferase activity was seen with increased number of Tcf/lef-binding sites when cells were stimulated with LiCl or transfected with cDNA against β-catenin whereas a repression was observed in cells transfected with siRNA against β-catenin or treated with the non-specific WNT signaling inhibitor, celecoxib. To further investigate if WNT signaling plays a role in the regulation of MGMT expression, WNT signaling was

genetically silenced using an inducible β-catenin shRNA. Suppression of β-catenin by shRNA in cells inhibited MGMT expression. These results suggest that the WNT signaling controls the MGMT expression.

To analyze if WNT inhibition could potentiate the effect of the DNA-alkylating drug temozolomide, a panel of cell lines was treated with WNT inhibitors in combination with temozolomide. The inhibitors tested were the non-specific WNT signaling inhibitor celecoxib, the tankyrase/Axin1 inhibitors XAV-939, G007-LK, the Porcupine inhibitors Wnt-C59, LGK974 and salinomycin, which acts by interrupting phosphorylation of the WNT co-receptor lipoprotein receptor related protein 6 (Huang et al., 2009; Kahn, 2014; Lau et al., 2013). Both of the tested porcupine inhibitors as well as salinomycin and celecoxib potentiated the cytotoxic effect of temozolomide in most of the tested cell lines (figure 10).

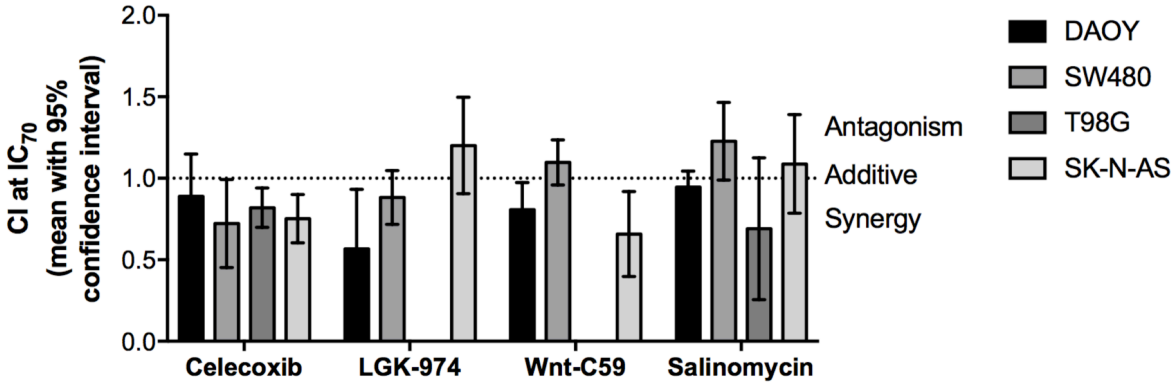

Figure 10: **Mean of combination index (CI) at IC_{70}.** A CI =1 denotes additive effect, CI significantly below 1 is defined as synergy and CI significantly above 1 as antagonism. The combinations with LGK974 and Wnt-C59 in T98G could not be analyzed by the median-effect method since the single drug effect did not achieve a full dose–response curve (as for G007-LK and XAV-939 in all tested cell lines).

There are several reports that show that other signaling cascades affect β-catenin expression. For example, several G protein-coupled receptors can activate β-catenin signaling, including prostaglandin E_2 (PGE_2) via the receptors EP2 and EP4 (Kahn, 2014). PGE_2 is produced from arachidonic acid by a cascade of enzymes where cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are rate-limiting (Smith et al., 2000). Experimental studies have revealed that COX-2 is involved in tumor progression (Wang and Dubois, 2010). Research groups have shown that inflammatory stimuli and hormones might induce COX-2 expression (Wang and Dubois, 2010). Based on the fact that COX-2 is overexpressed in several human tumors including colon carcinoma, glioma, medulloblastoma and neuroblastoma, cancer therapy using a combination of COX-2 inhibition and chemotherapy seems promising (Baryawno et al., 2008; Gasparini et al., 2003; Johnsen et al., 2004; Wang and Dubois, 2010). Moreover, the non-specific WNT inhibitor and COX-2 inhibitor celecoxib was the most

effective compound inducing either synergistic or additive effect when given together with temozolomide in all investigated cell lines *in vitro*. We therefore decided to further investigate the effect of celecoxib. By using western blotting on protein extracts from tumor cells treated with celecoxib on different time points, we demonstrated a clear downregulation on MGMT and β-catenin expression. Moreover, cells expressing different levels of MGMT were treated with temozolomide alone or a combination of temozolomide and celecoxib, to measure clonogenic capacity. MGMT expression levels corresponded to sensitivity to temozolomide, and when celecoxib was used in combination, we saw a significant increase in sensitivity to temozolomide in cell lines with high levels of MGMT. By using the colorectal carcinoma cell line, LS174T, with a Tet-inducible shRNA against β-catenin, we observed a significant decrease in cell growth when cells with silenced β-catenin expression were treated with temozolomide compared to control cells. The effect was depleted when the β-catenin knocked cells were transfected with MGMT cDNA.

We also tested the combination of temozolomide and inhibition of β-catenin *in vivo*. The medulloblastoma cell line D283 with low expression of MGMT and the colorectal cell line LS174T with high expression of MGMT were selected for the *in vivo* studies. Nude mice injected with D283 on the flank were treated with celecoxib alone, temozolomide alone or a combination of temozolomide and celecoxib for 12 days. We observed that the tumor growth inhibiting effect was significantly potentiated when celecoxib and temozolomide were administered together. In the β-catenin inducible cells LS174T, we saw the same effect, i.e. a significant inhibition of tumor growth in mice with β-catenin knocked tumors treated with temozolomide. The expression of MGMT was downregulated in both tumors from D283 mice and LS174T mice.

In summary, the results in Paper II show an indirect strategy for targeting cancer cells with high levels of MGMT by using inhibitors of WNT signaling. WNT inhibitors can increase the sensitivity of tumor cells to alkylating agents such as temozolomide, thereby increasing the effectiveness of the chemotherapeutic treatment. The major advantage of using indirect inhibition of MGMT is the avoidance of some of the toxic effects resulting from the direct inhibition of MGMT.

5.3 PLANAR CELL POLARITY SIGNALING IN NEUROBLASTOMA (PAPER III)

It is important to understand the mechanisms that promote and regulate tumor development, to be able to establish future prognostic markers in neuroblastoma. As described earlier, neuroblastoma originates from cells in the neural crest (Brodeur, 2003; Louis and Shohet, 2015; Ratner et al., 2016; Schulte and Eggert, 2015). Neural crest cells migrate throughout the body and mature into different structures. The migration is dependent on different signals controlling orientation and polarization (Mayor and Theveneau, 2014). In paper III we investigated the importance of the two PCP proteins, Prickle1 and Vangl2, in neuroblastoma growth. *In vitro* manipulation by overexpression of *Prickle1* or *Vangl2* suppressed cell viability and increased differentiation in neuroblastoma cells. Moreover, we observed a reduction of β-catenin activity in neuroblastoma cells. Other studies have reported that the canonical and planar cell polarity pathway can crosstalk and antagonize one another in cancer cells, which is in line with our own findings. It was also reported that high expression of *Prickle1* inhibited β-catenin through degradation of Dvl (Chan et al., 2006). We also observed an upregulation of *Prickle1* and a downregulation of β-catenin when neuroblastoma cells were treated with compounds blocking the non-canonical pathway downstream.

Analysis of *Prickle1* and *Vangl2* in the open-access gene expression database R2 (http://r2.amc.nl) confirmed that high expression of these genes in primary neuroblastoma patient materials is significantly associated with a better clinical prognosis and a low-risk disease.

In contrast to our results in neuroblastoma cells, both the non-tumorigenic cell line C17.2 and transgenic mouse embryos overexpressing *Vangl2* showed increased β-catenin activity and presented reduced differentiation. Our results highlight that expression of *Prickle1* and *Vangl2* have different modes of action in non-tumorigenic cells compared to neuroblastoma cells, but the role of PCP signaling in tumor development is still controversial. Given that the non-canonical PCP signaling antagonizes the canonical signaling, one would presume that PCP signaling can suppress tumor development, but some non-canonical proteins act as both suppressors and oncogenes dependent on tumor stage. At the early stage of cancer they act as suppressors and, as cancer progresses, they promote migration and invasion (Wang, 2009). It was recently shown that the Rictor subunit within the mammalian target of rapamycin complex 2 (mTORC2) binds to Prickle1 and this promotes cytoskeleton changes, cell motility and proliferation of breast cancer cells (Daulat et al., 2016). Also, Vangl2 is overexpressed in endocrine-related tumors (Hatakeyama et al., 2014) and associated with poor prognosis in basal breast cancer (Puvirajesinghe et al., 2016). On the other hand very low levels of Prickle1 and other PCP mediators were detected in pediatric but not adult adrenocortical tumors (Mermejo et al., 2014). Hence, more studies need to be undertaken in order to elucidate the importance of PCP signaling in cancer.

In summary, we show that the expression of Prickle1 and Vangl2 has different modes of action in neuroblastoma cells compared to non-tumorigenic cells and that the activity of the non-canonical pathway is of importance for neuroblastoma growth and prognosis.

5.4 RHO-ASSOCIATED KINASE IN NEUROBLASTOMA (PAPER IV)

Compared to adult cancer, neuroblastomas have less reported somatic mutations, with a median of 15 mutations. In order to get further insight into the advanced biology of neuroblastoma, Molenaar et al, published a paper in Nature 2012, identifying multiple recurrent alterations in tumors from 87 untreated neuroblastoma patients (Molenaar et al., 2012). Among the alterations were genes involved in the non-canonical PCP WNT pathway. This pathway is, as previously mentioned, important for the differentiation and maturation of neural crest cells and the failure of neural crest cells to differentiate can result in neuroblastoma (Sebbagh and Borg, 2014). We therefore decided to further investigate this pathway and how it may be manipulated in neuroblastoma. By whole exome sequencing of neuroblastoma patient samples and by analyzing data from research performed by others, we detected frequent mutations in genes involved in neuritogenesis associated with the Rho/Rac signaling. Of the mutations, 58% were detected in GAP and GEF genes. As mentioned before, these mutations could lead to the inhibition of Rac and activation of Rho. Activated Rho further promotes activation of ROCK1 and ROCK2, which phosphorylate downstream targets important for cellular morphology (Molenaar et al., 2012; Sebbagh and Borg, 2014). Previous cell biological studies demonstrated that this pathway is mutated in many cancers, including neuroblastoma (Karlsson et al., 2009).

The first question in this study we sought to examine was the status of Rho activity in neuroblastoma. We first measured the expression levels of downstream ROCK molecules since ROCKs are essential for proper activation of Rho signaling (O'Hayre et al., 2014). ROCK1 and ROCK2 were analyzed in neuroblastoma tumors and cell lines and all tested neuroblastoma cell lines expressed the proteins. Analysis of publically available datasets revealed that high levels of *ROCK1* and *ROCK2* were found in tumors associated with poor prognosis. Given that ROCK proteins are expressed in neuroblastoma, we studied the effect of ROCK inhibition in neuroblastoma cell lines. In Paper IV we used two ROCK inhibitors, HA1077 and Y27632, and one Rho inhibitor, Rhosin. The cytotoxic effects were evaluated in a panel of neuroblastoma cells. HA1077 demonstrated the most effective inhibition of cell growth and was selected for further analysis. HA1077 interacts with the ATP binding site of the kinase domain of ROCK1 and ROCK2 and is approved for clinical use in Japan and China under the name Fasudil (Feng et al., 2016). Treatment with HA1077 induced differentiation of neuroblastoma cells as seen by neurite outgrowth and inhibited cell migration. The ROCKs phosphorylate myosin light chain, which mediates cytoskeletal changes of importance for cell movements (Riento and Ridley, 2003). Therefore, an inhibition of migration was expected. Moreover, expression of MYCN after HA1077 exposure was investigated. MYCN amplification is coupled to patient survival and

neuroblastoma differentiation status; therefore, drugs that inhibit the expression are highly warranted. Treatment of neuroblastoma cells with HA1077 resulted in increased phosphorylated MYCN (T58) and decreased total level of MYCN protein expression compared to untreated control cells. Surprisingly, no differences were found in *MYCN* mRNA levels in HA1077 treated cells compared to untreated control cells. This finding was unexpected, and a possible explanation for this might be that ROCK inhibits MYCN on a post-transcriptional level. Similar results were observed when cells were transfected with *ROCK2* siRNA. Another interesting finding was that the SH-EP *MYCN*-inducible Tet21N neuroblastoma cell line responded differently to HA1077 treatment with and without added tetracyclin (Lutz et al., 1996). Following removal of tetracycline, MYCN level increased and cells were more sensitive to HA1077 treatment compared to tetracycline treated cells.

To investigate the effects of HA1077 *in vivo,* mice with established neuroblastoma SK-N-BE(2) xenografts were treated with HA1077 (50 mg/kg) or vehicle by daily intraperitoneal injections. A reduction in tumor volumes of 31% was observed in mice treated with HA1077 compared to vehicle treated animals; the tumor growth was also significantly slower in HA1077 treated animals. Next we investigated the effect of HA1077 in the TH-MYCN transgenic model that develops MYCN driven neuroblastoma (Weiss 1997). Four and a half week-old homozygous mice (TH-MYCN$^{+/+}$) were treated daily by intraperitoneal injections for nine days, and divided into two groups, 10 mg/kg and 25 mg/kg and compared to mice without treatment. Both treatment groups had a significantly lower tumor burden compared to non-treated animals and no signs of toxicity were seen during the experiment. In TH-MYCN mice treated with HA1077, a reduction on MYCN protein levels was observed as well as inhibited ROCK activity, but no change in *MYCN* mRNA levels.

Treatment with HA1077 or transfection with *ROCK2* siRNA resulted in decreased GSK-3β (Serine9) phosphorylation. GSK-3β is known to phosphorylate β-catenin in the canonical WNT signaling, mark it for degradation and inhibit the WNT pathway, but GSK-3β is also important for normal embryonic development, cell division and apoptosis (Nelson and Nusse, 2004). Interestingly, GSK-3β can be switched on and off by other protein kinases phosphorylating threonine or serine residues. GSK-3β activity is suppressed by phosphorylation of serine 9 (Cohen and Frame, 2001). These results further support the idea of a post-transcriptional inhibition of MYCN by GSK-3β phosphorylation that results in ubiquitin mediated degradation of MYCN (summarized in figure 11). Furthermore, mutations in the pathway that affects the activity of ROCK may affect the expression of MYCN proteins in neuroblastoma patients.

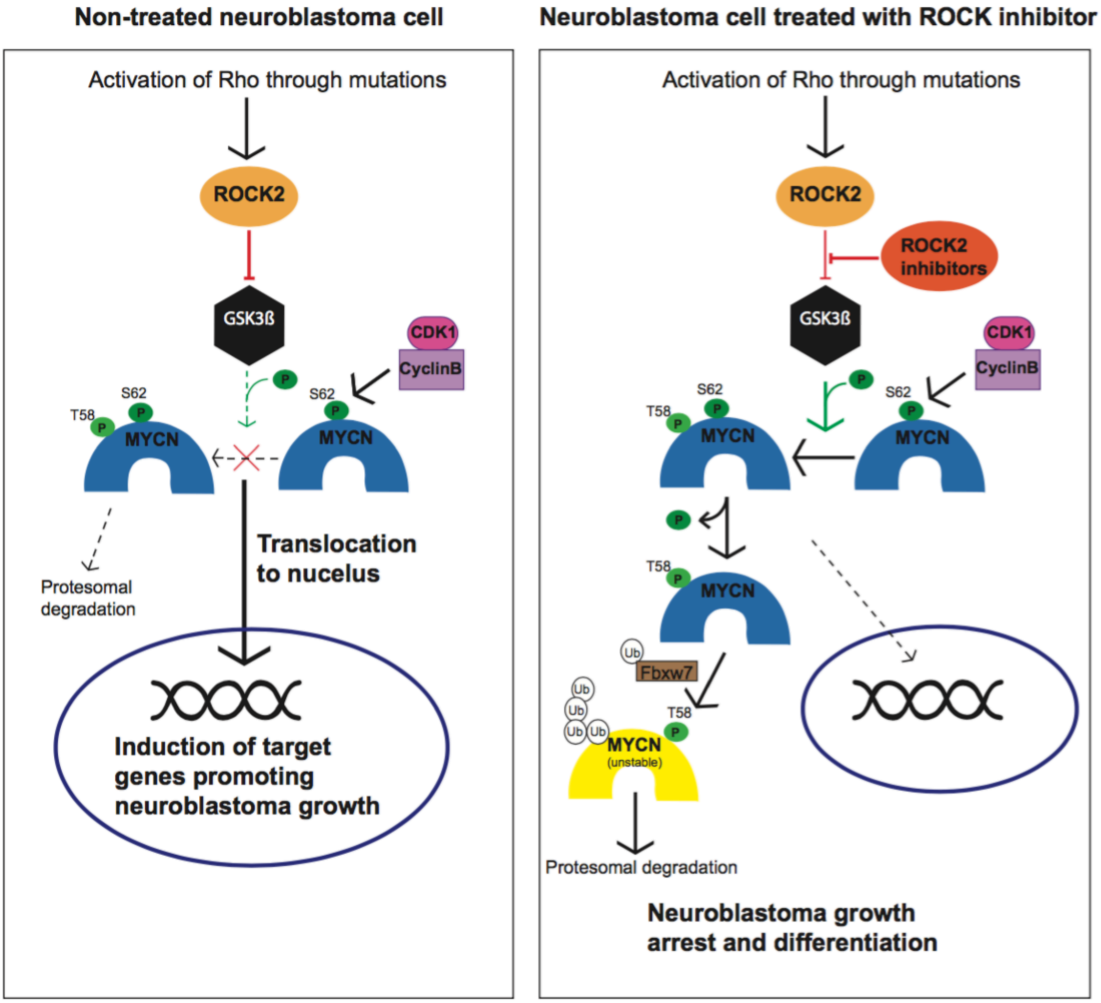

Figure 11. Schematic image over the possible interactions between ROCK2 and MYCN in neuroblastoma.

In summary, the results in Paper IV show promising data that manipulation of the non-canonical PCP WNT pathway through ROCK induced neuroblastoma differentiation as well as MYCN inhibition.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented in this thesis aimed to increase our understanding of signaling pathways important for tumorigenesis and to identify new treatment targets in embryonal tumors. This could potentially lead to development of new novel therapeutic approaches that will lead to better survival, less side effects and ultimately better quality of life for these children.

In Paper I we demonstrated that inhibition of SHH signaling at the level of GLI is a new efficient way to target neuroblastoma without MYCN amplification. This is an important finding since SHH signaling is deregulated in many cancers and approximately 30% of medulloblastoma tumors have activation of the pathway. A further study with more focus on targeting SHH medulloblastomas with GANT-61 would be interesting since SHH medulloblastomas Smo inhibitors-non responders often have mutations downstream of SMO or in the phosphoinositide 3-kinase (PI3K) pathway. Therefore, inhibition of SHH signaling at the level of GLI can be effective (Ransohoff et al., 2015). Also a combination therapy with SHH inhibitors plus PI3K inhibitors may represent a new promising way to target medulloblastomas SHH tumors. Future studies will reveal if this is a mode to avoid resistance to conventional chemotherapy in this group of patients.

In Paper II we showed that a combination therapy using the WNT inhibitor celecoxib together with temozolomide is more effective than temozolomide given alone in cancer treatment in several cancer forms. Therefore this should be further evaluated in a clinical study as a treatment option for both pediatric brain tumors and adult cancers since therapy resistance is one of the biggest drawbacks in cancer therapy in general. In further research, the use of this mechanism could lead to development of more effective WNT inhibitors that can be used in combination with alkylating agents in cancer therapy.

In Paper III and IV we provide knowledge about the non-canonical PCP pathway in neuroblastoma. The results of these studies propose that the pathway is important in tumorigenesis. The results in Paper III were mainly conducted *in vitro.* Therefore it is of great importance to go further with an *in vivo* experiment to investigate our findings, pointing at a tumor suppressive role of Prickle1 and Vangl2 in neuroblastoma. Overall, Paper III strengthens the idea that tumor cells and non-tumor cells have different mode of action but several questions remain unanswered at present. A further study with more focus on these differences is therefore suggested. The results in Paper IV further support the hypothesis that the non-canonical pathway is important to investigate in neuroblastoma. There are still many unanswered question about which mutations that affect tumor progression and the consequences and importance of the mutations. Since ROCK inhibition induces differentiation in a similar way as retinoic acid, a future study with more focus on combination therapies is therefore suggested.

In conclusion, there is abundant room for further progress to identify new targets in cancer therapy, but the findings in this thesis have provided us with new knowledge of possible targets that appear to be effective. I hope that the work presented in this thesis will provide us with new tools for the treatment of embryonal tumors.

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