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UNDERSTANDING AND TARGETING THE ARCHITECTURE IN CANCER: NOVEL THERAPIES IN NEUROBLASTOMA AND MEDULLOBLASTOMA

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Cover illustration: Illustration of the cytoskeletal differences of blasts treated with vehicle and differentiated cells treated with HA1077. Confocal photo taken of neuroblastoma cells SK-N-BE(2) with Hoechst 33342 (blue) and β3-tubulin (green).

UNDERSTANDING AND TARGETING THE ARCHITECTURE IN CANCER: NOVEL THERAPIES IN NEUROBLASTOMA AND MEDULLOBLASTOMA
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family, dear friends, and everyone else who believed in me.

"Science makes people reach selflessly for truth and objectivity;
it teaches people to accept reality, with wonder and admiration,
not to mention the deep awe and joy that the natural order of things,
brings to the true scientist."
-Lise Meitner

POPULÄRVETENSKAPLIG SAMMANFATTNING AV AVHANDLINGEN PÅ SVENSKA

Efter hjärt- och kärlsjukdomar är cancer den vanligaste dödsorsaken i världen enligt världshälsoorganisationen. I Sverige är barncancer den vanligaste dödsorsaken hos barn i åldrarna 1-14 år, det är ungefär ett barn per dag som får en cancerdiagnos. Forskningen har gett resultat i form av ökad överlevnad, från att bara ett av fem barn överlevde 1960, till att det idag är fler än fyra av fem barn som klarar sig. Den generella barncanceröverlevnaden är nu 85% fem år efter diagnos. Behandlingen består av operation av tumören, kemoterapi och strålning, allt i utprövat behandlingsprotokoll för att optimera överlevnaden. Med teknikens utveckling har man det senaste decenniet ökat kunskapen om cancer genom olika genetiska tekniker. Detta har gjort att man har förstått mer om vad som har gått fel i tumörcellerna och hur man kan behandla cancer med läkemedel riktade mot molekylära mål i tumörcellerna. Dessvärre är överlevnaden inte lika bra för alla cancerformer hos barn, neuroblastom och medulloblastom har bland de lägsta överlevnadssiffrorna.

Neuroblastom är en cancerform som främst drabbar små barn, de flesta insjuknar före två års ålder. Man tror att neuroblastom uppkommer i celler under fosterutvecklingen, celler som sedan kommer bilda det sympatiska nervsystemet och binjuren. Det sympatiska nervsystemet är den del av det autonoma nervsystemet som vi inte kan styra, som reglerar bland annat blodtryck och tarmrörelser.

Medulloblastom är en hjärntumör som uppkommer i lillhjärnan och även den tros utgå från förstadieceller under fosterutvecklingen. Lillhjärnan är den del av hjärnan som är ansvarig för balans och koordination. Åldersfördelningen är lite mer spridd i medulloblastom till skillnad från neuroblastom, då medulloblastom även kan drabba tonåringar och vuxna. 70% insjuknar i medulloblastom innan tio års ålder.

Cancer är en sjukdom som beror på att vanliga celler muterar, det vill säga det uppkommer genetiska avvikelser i cellens kod, DNA. Med hjälp av bilddiagnostik och kunskap om de genetiska avvikelserna kan man gruppera patienter i olika riskgrupper för att ge dem rätt behandling. Bland patienter i högriskgruppen överlever tyvärr endast ungefär hälften, både för neuroblastom och medulloblastom, trots intensiv behandling med kirurgi, strålning och kemoterapi. Även bland patienterna som klarar sig, så medför nuvarande behandlingar risker, många patienter får bestående komplikationer av kemoterapi och strålning. Därför har en stor del av det senaste decenniets forskning fokuserat på att hitta mer skräddarsydda behandlingar med ökad förståelse av tumörbiologin. Man försöker då identifiera specifika förändringar i cancercellerna hos individen, och rikta läkemedel mot dem. Man hoppas på så sätt uppnå mer effektiv behandling av cancern, med mindre bieffekter.

Denna avhandling innehåller tre artiklar, alla handlar om att bättre förstå neuroblastom eller medulloblastom på en molekylär nivå och hur man kan använda denna förståelse för att behandla sjukdomen. I två av artiklarna har vi studerat läkemedel riktade mot enzymet Rho-

kinas (ROCK). Den tredje artikeln handlar om ett protein, teneurin 4, som har visats ha återkommande genetiska avvikelser i neuroblastom.

När nervsystemet mognar i embryot måste en rad signaler slås på och av vid rätt tillfälle för att outvecklade celler ska bli till normalt utvecklade nervceller. Mutationer som uppkommer kan leda till att vissa signaler fortsätter att vara aktiva, och att celler därmed inte mognar ut. En signalväg som är viktig i fosterutvecklingen är Wingless (Wnt). I artikel I visade vi att ungefär var fjärde neuroblastompatient hade minst en mutation i den delen av Wnt-signaleringen som kallas Rho/Rac-signalering (en del av Wnt-signaleringen viktig för cellers utmognad och migration). Vi visade även att denna molekylära signalering är aktiv i både neuroblastom och medulloblastom (artikel I och II), mer specifikt studerade vi ett protein som kallas ROCK som är aktivt i Rho/Rac-signaleringen. Vi visade att med läkemedel som hämmar ROCK förändrades signaleringen så att tumörcellerna mognade ut och växte långsammare, i både försök på tumörceller odlade i laboratoriet och i möss. Molekylärt visade det sig att när enzymet ROCK hämmades så ändrades även annan molekylär signalering i tumörcellen. Exempelvis blockerades det ogynnsamma proteinet MYCN. Läkemedel riktade mot ROCK används kliniskt i vissa delar av världen för att behandla andra sjukdomar än cancer. De två första studierna föreslår att läkemedel som hämmar ROCK kan vara en ny behandling i neuroblastom och medulloblastom.

Teneurin 4 är ett protein som är aktivt under fosterutvecklingen, och har visat sig vara viktigt för den normala utvecklingen av embryon i flugor, möss och människor. I artikel III visade vi att teneurin 4 är högre uttryckt i tumörer från patienter med högrisk-neuroblastom jämfört med andra riskgrupper. Vi visade att när vi tystade genuttrycket av teneurin 4 så växte cancercellerna långsammare och ändrade morfologi och utseende, de såg ut mer som mogna nervceller. Vi såg dessutom att celler utan teneurin 4-uttryck inte bildade tumörer när de injicerades i möss, till skillnad från motsvarande neuroblastomceller med oförändrat teneurin 4-uttryck där tumörer började växa inom fyra veckor efter injektion. Dessa resultat föreslår att teneurin 4 kan vara kan vara ett nytt mål i tumörcellen att rikta behandling mot högrisk-neuroblastom.

Sammanfattningsvis visar vi att Wnt-signaleringen genom Rho/Rac-signalering är aktiv i neuroblastom och medulloblastom, och att hämning med ROCK-hämmande läkemedel gör att tumörceller växer långsammare och mognar ut. Fortsättningsvis är vi de första att visa att teneurin 4 är högre uttryckt i tumörer från patienter med högrisk-neuroblastom än övriga neuroblastom, och att hämning av teneurin 4 kan vara ett nytt sätt att behandla högrisk-neuroblastom.

ABSTRACT

Cancer is the second leading cause of death worldwide after cardiovascular diseases. In Sweden, childhood cancer is the most common cause of death in children 1-14 years of age. Owing to advances in treatment and a better understanding of tumor biology, survival rates have increased to over 80% in most Western countries. However, neuroblastoma and medulloblastoma, two embryonal childhood cancers that arise in neural tissues, do not have equally satisfactory survival rates, especially not in the high-risk patient groups.

Neuroblastoma and medulloblastoma are cancers considered to arise as undifferentiated cells during embryonal development. An orchestra of inductive signals occur during embryonal development that are important to induce cells from totipotent to differentiated normal cells. One of the pathways that is essential during embryogenesis is the Wingless (Wnt) signaling pathway. While Wnt is necessary during early development, dysregulated Wnt signaling may interfere with the differentiation process and participate in the transformation into cancer.

The overall aim of this thesis was to investigate the importance of Rho/Rac signaling (a part of Wnt signaling), in neuroblastoma and medulloblastoma. We especially aimed to gain insights in the function of Rho/Rac signaling in the differentiation process, in search for better understanding of the cancers and new therapies. The first two papers focused on the protein Rho Associated Coiled Coil Kinase proteins (ROCK1 and ROCK2), located downstream of Rho signaling. The teneurin family of proteins have been reported to have reoccurring genetic alterations in neuroblastoma and are suggested to be associated with Rho/Rac signaling. The third paper is exploring the role of teneurins in neuroblastoma tumorigeneses.

In paper I, we investigated mutations in neuroblastoma. We showed that 27.5% of neuroblastoma patients harbor at least one somatic protein changing alteration in a gene involved in neuritogenesis, related to the Rho/Rac signaling cascade. Furthermore, *RhoA* and *ROCK2* were found to be upregulated and more active in high-risk neuroblastoma compared to non-high-risk. In addition, higher expression of *ROCK2* was associated with poor patient survival. Pharmacological or genetic inhibition of *ROCK* caused neuroblastoma cells to differentiate and repressed neuroblastoma cell proliferation, migration, and invasion. Furthermore, downregulation of *ROCK* induced degradation of the MYCN protein. Finally, studies in two different neuroblastoma mouse models demonstrated that ROCK inhibition with the drug HA1077 significantly delayed tumor growth and may hence be a new therapeutic target in neuroblastoma.

In paper II, we continued studying ROCK inhibitors, but selected a more specific and potent pan-ROCK-inhibitor, RKI-1447. We demonstrated that ROCKs are present in medulloblastoma, with higher *ROCK2* mRNA expression in metastatic compared to non-metastatic tumors. Treatment with RKI-1447 inhibited medulloblastoma proliferation as well as repressed cell migration and invasion. Inhibition of ROCK through RKI-1447 also led to downregulation of genes associated with key signaling pathways in proliferation and metastasis e.g., TNFα and epithelial mesenchymal transition according to differential gene expression

analysis. Lastly, we demonstrated that ROCK inhibition by RKI-1447 repressed medulloblastoma growth *in vivo*. Our findings propose that ROCK inhibition is a possible new therapeutic option in medulloblastoma, particularly for children with metastatic disease.

In paper III, we investigated the function of teneurins (TENM1-4). *TENMs* have been found to have genetic alternations in neuroblastoma and are important proteins during the embryonal development in the nervous system of many species. We identified a significant role of TENM4 in neuroblastoma tumorigenicity and differentiation. Silencing *TENM4* with transient knockdown led to an upregulation of genes associated with neuronal differentiation and downregulation of genes associated to pathways related to cancer. Consistent with this, a knockout model of *TENM4* of the *MYCN*-amplified cell line SK-N-BE(2)C induced an evident morphological change consistent with a neuronal like differentiation in the knockout cells. The *TENM4* knockout showed an impaired growth rate and decreased MYCN expression compared to wild type cells. Furthermore, the *TENM4* knockout cells did not form tumors when injected subcutaneously in mice, in contrast to wild type cells that developed tumors within four weeks. Moreover, we detected a significantly higher protein and mRNA expression of TENM4 in high-risk vs. non-high-risk and *MYCN*-amplified vs. non-*MYCN*-amplified human tumors. Our data proposes that a subpopulation of neuroblastomas with *MYCN*-amplification expresses TENM4, and that TENM4 exhibits functions in neuroblastoma development. Consequently, TENM4 may be a potential therapeutic target in neuroblastoma.

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I. Dyberg C, Fransson S, **Andonova T**, Sveinbjörnsson B, Lännerholm-Palm J, Olsen TK, Forsberg D, Herlenius E, Martinsson T, Brodin B, Kogner P, Johnsen JI, Wickström M
Rho-associated kinase is a therapeutic target in neuroblastoma
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II. Dyberg C*, **Andonova T*,** Olsen TK, Brodin B, Kool M, Kogner P, Johnsen JI, Wickström M
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Cancers (Basel). 2019 Dec 26;12(1):73.

III. **Andonova T**, Pepich A, Ajamieh SA, Olsen TK, Tümmler C, Thankaswamy-Kosalai S, Kanduri C, Fransson S, Martinsson T, Baryawno N, Näsman A, Kogner P, Johnsen JI, Wickström M
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*These authors contributed equally to the manuscript and share primary authorship.

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CONTENTS

LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
ALK	Anaplastic Lymphoma Kinase
Dvl	Dishevelled
E	Embryonal day
EMT	Epithelial-to-Mesenchymal-Transitions
FBS	FBS
FDR	False Discovery Rate
FZ	Frizzled
GAPs	GTPase-Activating Proteins
GDIs	Guanosine nucleotide Dissociation Inhibitors
GEFs	Guanine nucleotide Exchange Factors
GSEA	Gene Set Enrichment Analysis
GSK-3β	Glycogen Synthase-3β
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IF	Immunofluorescence
H&E	Hematoxylin-Eosin
HR	High-risk
INSS	International Neuroblastoma Staging System
INRGSS	International Neuroblastoma Risk Group Staging System
KO	Knockout
LCA	Large Cell / Anaplastic
M	Metastatic
MYC	V-Myc Myelocytomatosis Viral Oncogene Homolog
MYCN	V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma
OS	Overall Survival
mTOR	Mechanistic Target of Rapamycin
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PFA	Phosphate-buffered formaldehyde
PHOX2B	Paired-like Homeobox 2b
RNA-seq	RNA-sequencing
ROCK	Rho-associated Coiled-Coil Kinase
SHH	Sonic Hedgehog
TENM	Teneurin
TERT	Telomerase Reverse Transcriptase
Trk	Tropomycin Receptor Kinase
TP53	Tumor Protein 53
WB	Western Blot

WNT	Wingless/Integrated-1
WHO	World Health Organization

1 BACKGROUND

1.1 CANCER

The instant egg and sperm fuse to make the zygote, cells divide at a fast pace for nine months to make up the human body of a baby. At the start of the first week of development, the cells of the embryo are totipotent; they can give rise to any tissue or cell. As they become more specialized during the end of the second week of development, a process called gastrulation occurs. The embryo forms the first three layers of what later will become the entire human body, the germ layers: the ectoderm, endoderm and mesoderm (Ghimire, Mantziou, Moris, & Martinez Arias, 2021). The ectoderm is the origin of the nervous system, skin and a few other cell types, the mesoderm of the heart and other skeletal and smooth muscles, and the endoderm of the lungs, thyroid and other internal organs. From this moment on, as the baby grows the cells become more specialized, and proliferate slower with time (Figure 1) (Martini & Nath, 2009).

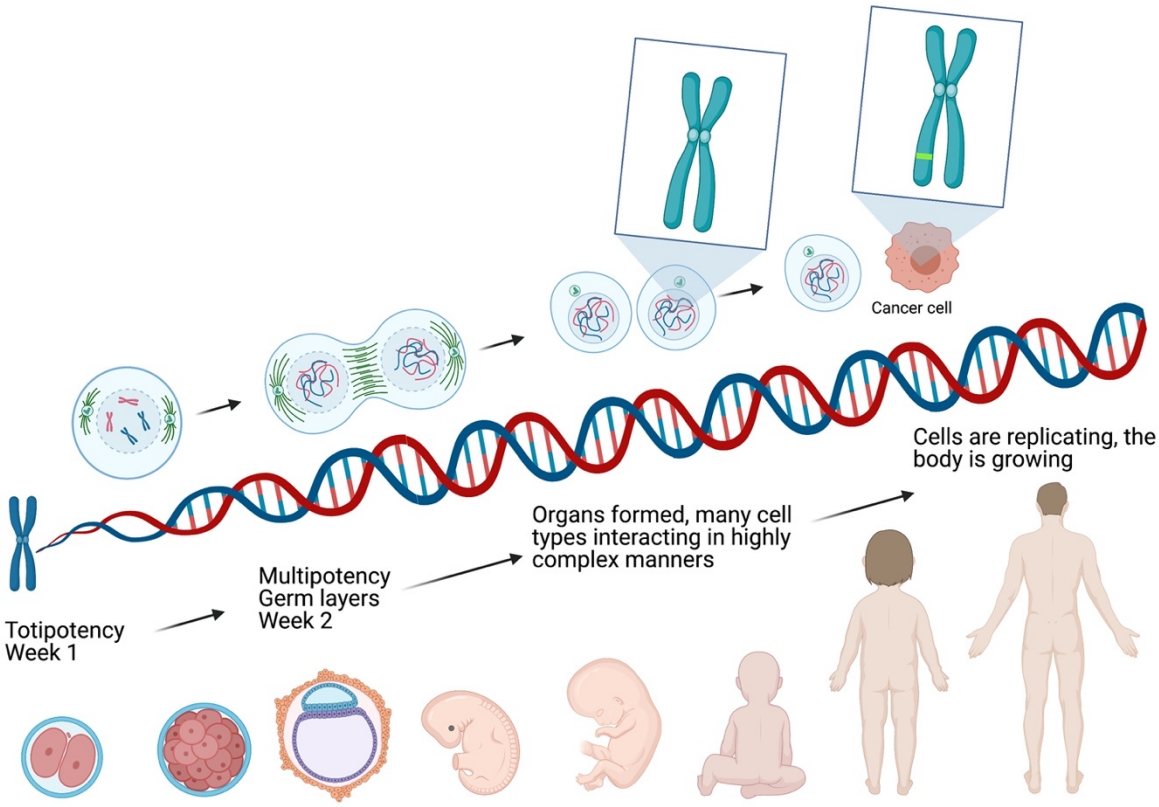

Figure 1. Overview of embryonal development, cell specialization, cell division and cancer initiation. Created with BioRender.com by Teodora Andonova (CC BY 4.0).

The DNA present at the beginning of life, remains the same throughout life, except for minor changes that occur. Every time a cell divides, the DNA is copied. With every cell division there is a probability that the DNA is not perfectly copied and that aberrations may occur. Changes include missing or wrongly copied nucleotides, so called deletions or point mutations, parts of the DNA can be produced in multiple copies, so called amplifications or pieces of chromosomes may also change positions, termed translocations. The possible changes in the

DNA that can occur with every division are numerous. Furthermore, external factors may also change the DNA such as sun exposure (UV light), smoking and radiation. Most of these changes will be corrected in the cell by DNA repairing mechanisms, or the cells internal system will inform it to die (apoptosis). However, when the genetic aberrations cannot be corrected and occur in genes that regulate cell death or proliferation, it can lead to cancer. Such genetic changes can, for example, cause the cell to gain the ability to continuously proliferate. This is a hallmark of all cancers, as they need to have the basic capacity to replicate excessively and sustain this characteristic (Hanahan & Weinberg, 2000). To be able to sustain chronic proliferation the cells have to evade growth suppressors, resist cell death and multiply by sustaining proliferative signaling (Figure 2). Cancer cells can also deregulate signals that stop them from enabling replicative immortality. Furthermore, cancer cells need to have other characteristics to be able to survive in the body and spread. In order for a solid tumor to continue to grow beyond a certain size, it needs to grow blood vessels, this is accomplished by the ability to induce angiogenesis to ensure supply with nutrients. In order to avoid destruction, successful cancer cells must evade attacks from the immune cells. A devastating point in many cancers, is the cancer cells' ability to free themselves from their current location, enter and move in the bloodstream or lymph, resettle at a new location, and start growing a new tumor. This process is called metastasis, and means that the cancer cells have gained even more features to be able to relocate, making them more difficult to eliminate (Hanahan & Weinberg, 2011). Nonetheless, cancer commences with DNA alterations (mutations), in a part of the genome that encodes for genes that regulate growth, either by inducing proliferation (oncogenes), or in genes controlling cell death and DNA repair mechanisms (tumor suppressor genes). This leads to a cascade of changes with the accumulation of more mutations as the cells divide. The faster the cells replicate, the greater is the risk that the DNA changes further, and in this way cells acquire new characteristics (Hanahan & Weinberg, 2000, 2011).

It is estimated that 9.8 million people died of cancer worldwide in 2018. One in every sixth deaths is due to cancer, making cancer the second leading cause of death globally (https://www.who.int/news-room/fact-sheets/detail/cancer; accessed 23rd of Dec 2020)

In Sweden 65 956 people were diagnosed with cancer in 2019. The most common cancer diagnoses are breast cancer for women and prostate cancer for men. Most women died of lung cancer, while most men died of prostate cancer. (https://www.socialstyrelsen.se/globalassets/sharepoint-dokument/artikelkatalog/statistik/2020-12-7132.pdf, accessed 25th Dec 2020)

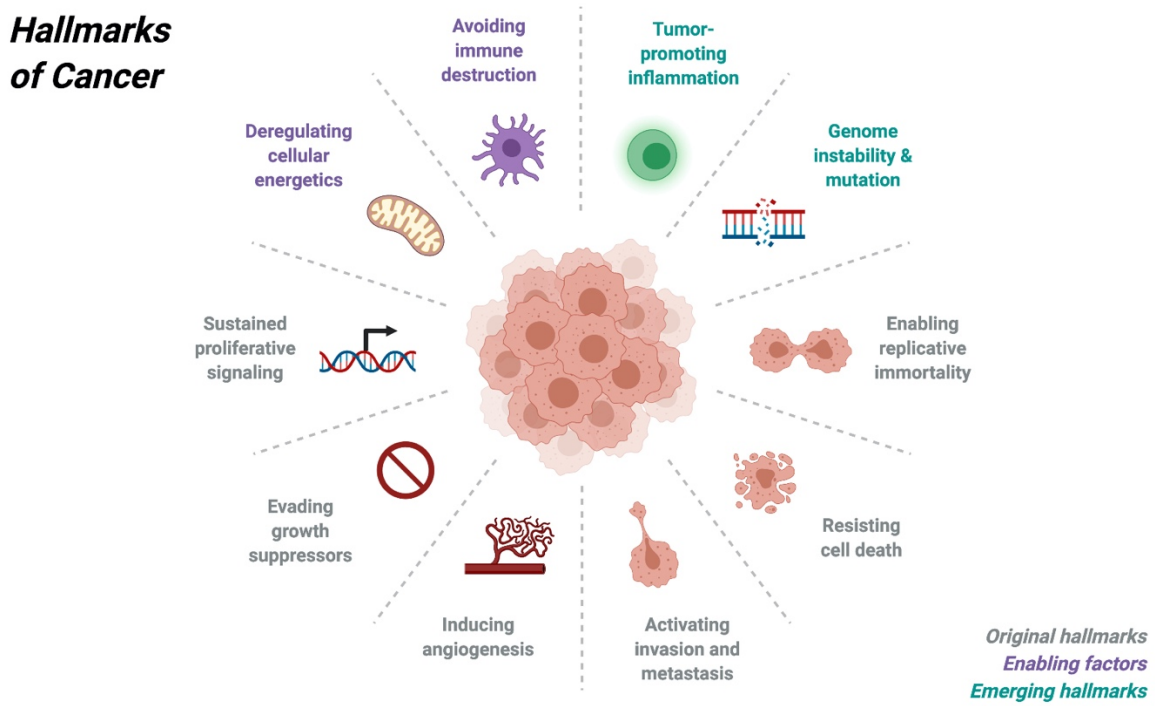

Figure 2. The hallmarks of cancer, figure based on (Hanahan & Weinberg, 2000, 2011). Created with BioRender.com template Hallmarks of Cancer (CC BY 4.0).

1.2 PEDIATRIC CANCERS

In comparison to adult cancers, pediatric cancers are rarer, and the disease etiology is different. In general, pediatric cancers have a lower mutational load while adult cancers exhibit more genetic aberrations accumulated over time. This is partly caused by the factors discussed above, such as random mutations over time where age is a factor i.e., accumulated cell divisions, UV exposure or smoking (Alexandrov et al., 2013). In childhood cancers, many of the mutated genes are found to affect signaling pathways important during embryonic development and may hence lead to disrupted differentiation of cells. The theory is that the cells continue to grow as they did in the early stages of development, instead of slowing down and specializing as differentiating cells do (Baryawno, Sveinbjornsson, Kogner, & Johnsen, 2010; Johnsen, Dyberg, & Wickstrom, 2019; Marshall et al., 2014). Interestingly, this is also true for many of the adult cancers, however, the difference is a lower mutational load in childhood cancers compared to adult cancers, and so the penetrance of a few genes in these pathways is stronger.

About 350 children under the age of 18 are diagnosed with cancer in Sweden each year, and cancer is the number one cause of disease-related deaths in children under the age of 15 years in Sweden. About one third of these children are diagnosed with different forms of leukemia, another third is diagnosed with brain tumors, and the remaining third is diagnosed with other types of cancer (Gustafsson, Kogner, & Heyman, 2013; Lähteenmäki, 2020). The most frequently diagnosed childhood cancers include leukemia, neuroblastoma, lymphoma, tumors of the central nervous system including medulloblastoma, retinoblastoma, sarcomas (osteo-, rhabdomyo-, and Ewing- sarcoma) and Wilms tumor (Friedman & Gillespie, 2011; Gustafsson et al., 2013; Tulla et al., 2015). Both medulloblastoma and neuroblastoma are childhood cancers that develop in the nervous system. Medulloblastoma is one of the most common

childhood brain tumors and is found in the central nervous system, more specifically, in the brainstem and the cerebellum (Figure 6) (Northcott et al., 2019). Neuroblastoma is a cancer of the peripheral nervous system, where it most often manifests in one of the adrenal glands, but it can also occur in the neural ganglia in the abdomen, chest or neck (Figure 3) (Johnsen, Kogner, Albihn, & Henriksson, 2009; Katherine K. Matthay et al., 2016). While neuroblastoma has been shown to be a copy number driven-disease (Ma et al., 2018), medulloblastoma is even more genetically heterogeneous and is both point mutation-driven and has many different chromosomal aberrations (Northcott et al., 2017).

The estimated 5-year survival rates for childhood cancer patients in Sweden dramatically increased from the 1950s until today due to introduction of chemotherapy and radiotherapy, and improvements in treatment protocols and surgical procedures with an overall 85% survival rate for all childhood cancers (Gustafsson et al., 2013; Lähteenmäki, 2020; Turup, 2018). Chemotherapy is an essential contributor to the improved overall survival, nonetheless it is not expected that more intensified treatment regimens and/or combinations of existing chemotherapeutics will lead to a substantial increase in the survival of childhood cancer patients (Pui, Gajjar, Kane, Qaddoumi, & Pappo, 2011). Understanding the genetic alterations in cancer is of fundamental importance to further understand cancer biology, thus approaching treatment of cancer with precision medicine to improve survival and decrease chronic toxicity (Downing et al., 2012; Katherine K. Matthay et al., 2016; Northcott et al., 2019). Reducing treatment-related toxicity is of special importance for children as they are still developing and have their whole life ahead of them. Improved understanding of dysregulated signaling pathways is of necessity in order to find new druggable targets to improve survival and quality of life (Pui et al., 2011).

1.3 NEUROBLASTOMA

1.3.1 Historical overview and epidemiology

Neuroblastoma was first described by the "father of modern pathology" the German pathologist Rudolf Virchow in 1864 when he termed it "glioma" (Virchow, 1865). However, it was in 1910 that James Homer-Wright introduced the term "neuroblastoma". In his paper he wrote; "The essential cells of the tumor are considered to be more or less undifferentiated nerve cells or neurocytes or neuroblasts, and hence the names neurocytoma and neuroblastoma." He further described why he thought this name was fitting; "…have the same morphology as the cells from which the sympathetic nervous system and the medulla of the adrenal develop, and which are regarded by embryologists as arising from migrated primitive nerve cells" (Wright, 1910). 110 years later, it is still assumed that neuroblastoma arises from the embryonal cells of the neural crest (Figure 4).

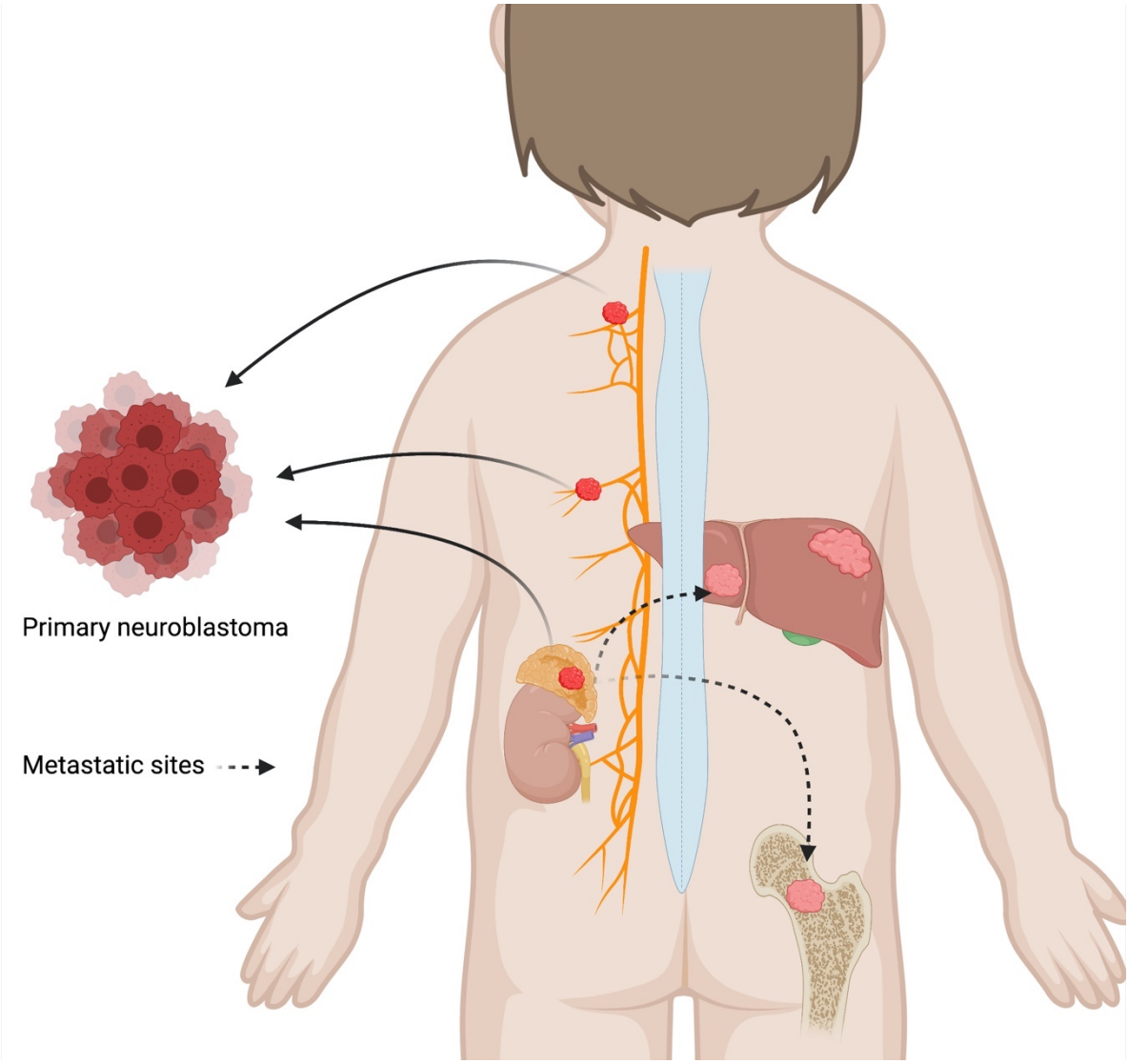

Figure 3. Locations of neuroblastoma growth in the peripheral nervous system and adrenal gland. Created with BioRender.com by Teodora Andonova (CC BY 4.0).

Neuroblastoma is the most common extracranial solid tumor of childhood, affecting about 15-20 children per year in Sweden (Gustafsson et al., 2013). Neuroblastoma occurs in small children; the median age of onset is 18 months and it is rarely found in children above the age of seven (Katherine K. Matthay et al., 2016). Neuroblastoma accounts for 6% of the childhood cancers but for 15% of cancer related deaths of young children (Park et al., 2013).

1.3.2 Biology

The neural crest is a transient structure that only exists for a brief time in the developing embryo and arises from the ectoderm layer. It is a structure specific to vertebrates and the cells that differentiate from this structure eventually contribute to the majority of tissues or organs in the body. The neural crest is composed of relatively few cells relative to the many tissue types it yields. This highly pluripotent structure forms neurons, glial cells, melanocytes, endocrine cells, connective and adipose tissues. The neural crest develops according to a rostrocaudal gradient along the body that stalk definitive migration paths at particular stages of development, finally reaching target locations where the cells settle and differentiate (Purves, 2008). During

the beginning of the 20th century great efforts were made to understand the development of the embryo. Sven Hörstadius, a Swedish biologist, presented in his monograph the early studies he had performed that demonstrated the development of the neural crest in amphibians, but also work of others in birds and other animals (Bronner & Simoes-Costa, 2016). These experiments were performed by surgically removing the neural crest structure from growing embryos of different animal species and observing the development (Bronner & Simoes-Costa, 2016; Mayor & Theveneau, 2013). Later, more advanced dyes were used to label neural crest cells and follow their migration and differentiation into pigment cells, dorsal root ganglia, sympathetic ganglia and cells around the dorsal aorta (Bronner & Simoes-Costa, 2016; Serbedzija, Bronner-Fraser, & Fraser, 1989). Eventually it was hypothesized that neuroblastoma develops from neuroblasts, and therefore from the neural crest. Recent data with RNA sequencing have shown that neuroblastoma indeed may have more similarities with the neural crest structure, than a proximally close adrenal gland cortex (De Preter et al., 2006). Furthermore, other studies have shown that neuroblastoma cells resemble adrenal sympathoblasts (Kildisiute et al., 2021) and that even the heterogeneity of neuroblastoma may be associated to different precursor structures of the neural crest (Hoehner et al., 1996). More recently, data have revealed that the neural crest cells position themselves on top of growing nerves and transform into a cell type termed "Schwann cell precursors (SCPs)". These cells are present for a substantial amount of time during development where they retain their neural-crest multipotency and give rise to chromaffin and sympatho-adrenal cells of the adrenal gland region, which is specifically the location where neuroblastoma most commonly appears (Furlan et al., 2017). Hence, one theory for the development of neuroblastoma as a disease, is that neuroblastic cells, that have been unable to differentiate, and that still retain their SCP characteristics make up a possible neuroblastoma stem cell.

1.3.3 Genetic alterations in neuroblastoma

The underlying causes for the development of neuroblastoma are connected to genetic alterations in the neuroblastoma tumor. It is a remarkably heterogeneous cancer, and some of its heterogeneity in growth and well as response to treatment is correlated to genetic alterations. The alterations can be sporadic or familial.

Familial neuroblastoma

Familial neuroblastoma accounts for 1-2% of the neuroblastoma cases. The majority of the familial neuroblastoma cases are caused by mutations in *Anaplastic Lymphoma Kinase* (*ALK*). ALK is a tyrosine kinase receptor. Germline gain-of-function by amplification of *ALK* has been identified as the main predisposing factor for familial neuroblastoma (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). Germline loss-of-function mutations in the *paired like homebox 2B* (*PHOX2B),* a master regulator of neural crest development, can also be the cause of familial neuroblastoma (Trochet et al., 2004). There are currently around twelve identified genes that may influence neuroblastoma disease initiation as a factor in familial neuroblastoma, however their penetrance is not evident. Each gene has a relatively modest individual effect on disease initiation. Multiple genes can however cooperate in an individual

patient to promote malignant transformation during neurodevelopment (Katherine K. Matthay et al., 2016).

Several somatic genetic alterations have been identified in neuroblastomas, including point mutations, gene amplifications and chromosomal alterations (Figure 4).

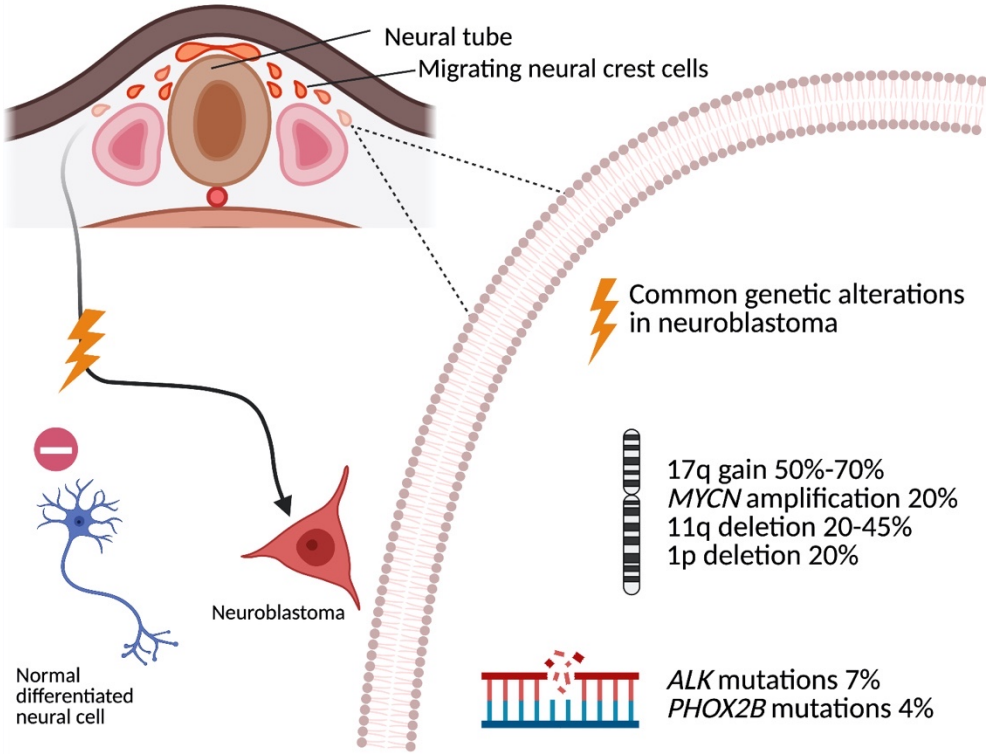

Figure 4. Overview of neuroblastoma development from the neural crest and most common genetic alterations in neuroblastoma. Created with BioRender.com by Teodora Andonova (CC BY 4.0).

Gene Amplifications

V-Myc avian myelocytomatosis viral oncogene neuroblastoma (*MYCN*) amplification occurs in about 20% of patients (Cohn et al., 2009) and is one of the strongest predictors of poor prognosis in neuroblastoma (Morgenstern et al., 2016). It is associated with advanced tumor stage and disease progression, independent of local or metastatic stage of disease and age at diagnosis (Brodeur, Seeger, Schwab, Varmus, & Bishop, 1984; Seeger et al., 1985) and it is used as a biomarker for risk stratification (Cohn et al., 2009). MYCN is a member of the MYC family of proteins, the N stands for neuroblastoma as it was first found in neuroblastoma (Kohl et al., 1983; Schwab et al., 1983). *MYC* is a transcription factor and master regulator activating 15% of the genome and, furthermore, upregulating cancer hallmarks (J. H. Patel, Loboda, Showe, Showe, & McMahon, 2004) As *ALK* and *MYCN* are both located on chromosome 2p, they can also be co-amplified (Katherine K. Matthay et al., 2016).

Somatic mutations

Similar to other pediatric cancers, neuroblastomas bear a low mutational burden (12-18, median 15 mutations) (Alexandrov et al., 2013; Ma et al., 2018; Pugh et al., 2013). *ALK* gene alterations, as mentioned previously, are associated with hereditary neuroblastoma, but also occur sporadically in approximately 14% of high-risk neuroblastomas (10% activating mutations, 4% amplifications) (Bresler et al., 2014; Chen et al., 2008; Janoueix-Lerosey et al., 2008). Likewise, mutations in *PHOX2B* occur in approximately 4% of spontaneous high-risk neuroblastomas. Genomic analysis of neuroblastomas using whole-genome sequencing have recognized loss-of-function genomic modifications in *ATRX* (coding Transcriptional regulator ATRX that belongs to the family of chromatin remodeling proteins) in approximately 10% of patients with 2.5% inactivating mutations and additional 7% with deletions (Cheung et al., 2012; Molenaar et al., 2012; Pugh et al., 2013). Other genes involved in chromatin remodeling with reported recurrent mutations in neuroblastoma include the Polycomb complex genes *ARID1A* and *ARID1B*. Recurrent events in high-risk neuroblastoma (2-3% inactivating mutations) are haploinsufficiency for *ARID1A* and *ARID1B*, but the effects on chromatin structure have not been defined yet (Sausen et al., 2013). Furthermore, mutations have been identified in the tumor suppressor p53 gene (*TP53*; 1-2% in primary tumors, 10% in relapsed and recurrent tumors) and *MYCN* (1.7% activating mutations) (Molenaar et al., 2012; Pugh et al., 2013).

Chromosomal rearrangements

Low-risk neuroblastoma frequently presents with whole chromosomal gains, the tumor cells are commonly hyperdiploid (Ambros et al., 2009). Almost all high-risk neuroblastomas also show recurrent segmental chromosomal copy number alterations, however, aberrations often only affect one part of a specific chromosome (Caren et al., 2010; Irwin & Park, 2015). Unbalanced gain of parts of chromosome 17q occurs in over half of neuroblastoma cases (Abel, Ejeskar, Kogner, & Martinsson, 1999; Bown et al., 1999; Bown et al., 2001) and loss of 1p is observed in about one-third of cases (Attiyeh et al., 2005). Loss of 1p and gain of 17q both correlate with *MYCN*-amplification and poor prognosis. Additionally, deletion in chromosome 11q has been found in about 30% of high-risk cases but is inversely correlated with *MYCN*-amplification (Attiyeh et al., 2005). Other typical segmental chromosomal alterations in neuroblastoma include gain of 2p and 1q as well as loss of 14q, 4p and 3p, but correlation to prognosis is less established for these copy number alterations compared to 1p, 11q and 17q (Huang & Weiss, 2013; Pugh et al., 2013). Loss-of-function in *TERT* (telomerase reverse transcriptase) promoter rearrangements, initiating enhancer hijacking has been detected in approximately 25% of patients (Peifer et al., 2015).

1.3.4 Staging and risk classification

Due to the heterogeneous character of neuroblastoma, some patients only need to be observed or undergo surgery to recover, while other patient groups do not survive even with the most exhaustive treatment program. It is consequently important to appropriately risk-stratify

patients to ensure that they receive the optimal treatment regimen. Risk stratification and treatment of neuroblastoma patients have varied between nations and groups worldwide, which has made comparisons between clinical trials difficult. To address these matters, the International Neuroblastoma Risk Group Staging System (INRGSS) was developed in 2009, founded on clinical information and tumor imaging for the International Neuroblastoma Risk Group (INRG) Classification System. Stratification of patients before treatment into defined risk groups (very-low-risk, low-risk, intermediate-risk or high-risk groups), was enabled, and so possible to compare risk-based clinical trials globally (Figure 5) (Cohn et al., 2009; Monclair et al., 2009)

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

Figure 5. International Neuroblastoma Risk Group (INRG) Consensus Pretreatment Classification schema. (Cohn et al., 2009) published in American Society of Clinical Oncology (CC BY 4.0).

The INRGSS (Monclair et al., 2009) stratifies by way of imaging into the stages of L1, L2, M and MS. L1; the tumor is local and restricted to one body compartment without any image defining risk factors (IDRFs), L2; the tumor is local, however with one or more IDRFs. M is defined as metastatic disease, meaning, the metastatic site is located distantly away from the primary site (another organ or body compartment) and MS; metastatic disease in infants (< 18 months of age) with metastases restricted to skin, bone marrow and the liver. The most updated prognostic assessment, INRG (Figure 5) (Cohn et al., 2009) combines the imaging stage by INRGSS, together with histology, genetic features of the tumor and age at diagnosis to divide subsets of children at diagnosis into different risk groups, from very low to high. Age at diagnosis has been established as a risk factor in neuroblastoma, as patients above the age of 18 months have been associated with a worse prognosis. A more differentiated histology towards a ganglioneuroma rather than neuroblastoma is also a better prognostic factor. As

described previously, *MYCN* status is a defined factor for high-risk disease, independent of age or localization of the tumor (Cohn et al., 2009).

Since 2009 there have been updates to the INRG, even though there has not been an official update yet. Previously bone scans were used to evaluate for metastatic disease in the bone. This has been replaced by the use of I-123-metaiodobenzylguanidine (I-123-MIBG) scans (Katherine K. Matthay et al., 2016) which is a radiotracer absorbed by a majority of neuroblastoma cells allowing a more precise detection of metastatic cells. In patients with MIBG non-avid tumors, fluorodexoyglucose (FDG)-positron emission topography (PET) may be used (Sokol & Desai, 2019).

Currently *MYCN*-amplification and 11q-deletion are the only genetic prognostic factors included in the INRG prognostic risk assessment, however, some groups do utilize 17q gain and 1p loss as additional prognostic factors. As more novel prognostic tools will arise as well as new treatment options such as immunotherapy, more molecular prognostic factors will probably be used to risk-stratify patients (Katherine K. Matthay et al., 2016).

1.3.5 Survival and treatment according to risk-stratification

Treatment of neuroblastoma patients differs widely between risk groups and can consist of observation only, surgery, chemotherapy, radiotherapy, autologous hemopoietic stem cell transplantation (AHSCT), differentiation therapy and immunotherapy (Katherine K. Matthay et al., 2016).

40 % of neuroblastoma patients present with low-risk disease with often spontaneously regressing tumors. Low-risk neuroblastoma is identified as curable with no or minimal cytotoxic therapy (Kushner and Cohn, 2005). For the very-low risk group, observation is often sufficient for patients <18 months of age, with imaging stage L1, L2 or MS without genomic factors, as the tumor often regresses. For patients past infancy (above one year of age), in stage L1, the tumor should be resected (Katherine K. Matthay et al., 2016). Low-risk and intermediate-risk disease combined comprises approximately half of newly diagnosed cases (Whittle et al., 2017). Intermediate-risk patients are non-*MYCN*-amplified, and have an INRG stage L2, or an INRG stage M in patients less than 18 months, or stage MS with unfavorable genomic features. Estimated overall survival of these patients is more than 90% for infants with a stage M disease, however only 70% of children older than 18 months with an INRG stage L2 in this group survive (Baker et al., 2010; Kohler et al., 2013). Patients with low- or intermediate-risk neuroblastoma have exceptional outcomes; the SIOPEN LNESG1 study (International Society of Pediatric Oncology European Neuroblastoma Research Network Localized Neuroblastoma European Study) established that solely surgery, was curative in almost all patients (De Bernardi et al., 2008). Moreover, observational studies have proven that infant patients with localized tumors can be cured without treatment, counting surgery (Hero et al., 2008).

More than 80% of patients that are older than 18 months are found in the high-risk group with metastatic disease. Long-term survival rates are 40-50% for these patients, despite intensive multi-modal therapy. In addition, patients 12-18 months of age with metastatic disease and unfavorable biological features are found in this group. The remaining 15-20% of high risk patients are of any biological feature, with *MYCN* amplification (Cohn et al., 2009). Current high-risk treatment regimens include surgery, five to six cycles of induction chemotherapy, consolidation therapy, therapy with AHSCT and irradiation, as well as postconsolidation therapy to treat minimal residual disease (Pinto et al., 2015). Despite improvements in event-free survival in high-risk patients, 50% of this patient group relapses (Katherine K. Matthay et al., 2016; K. K. Matthay et al., 1999).

Nearly all of neuroblastoma patients treated according to the high-risk protocol experience considerable treatment-associated acute toxicities, such as myelosuppression, renal dysfunction and poor weight gain. However, chronic treatment-related toxicity can also be seen in intermediate- and low-risk neuroblastoma survivors, including hearing loss, impaired growth, infertility and hypothyroidism (Matthay et al., 2016). Hence, improved treatments are especially necessary for high-risk patients, but may also help intermediate and low-risk patients.

1.4 MEDULLOBLASTOMA

1.4.1 Epidemiology and biology

Medulloblastoma is one of the most common brain tumors of childhood, responsible for about 20% of all childhood brain tumors (A. J. Gajjar & Robinson, 2014). Unlike neuroblastoma, medulloblastoma varies in age at onset, from early childhood to adulthood. A meta-analysis of seven studies of medulloblastoma occurrence showed that 21% of the patients were infants (age <4), 67% children (age 4–16) and 12% adults (age >16) (Kool et al., 2012). With current therapy protocols that include resection, craniospinal irradiation and chemotherapy, about 70-75% among children above the age of three are cured (A. J. Gajjar & Robinson, 2014). Medulloblastoma is a heterogeneous disease in regard to age, genetic alterations and prognostic factors. It is presumed to originate from diverse distinct neuronal stem or progenitor cell populations during early life. Genetic analyses based on transcriptional and epigenetic profiles have shown that medulloblastoma consists of at least four subgroups with specific genetic, transcriptional, clinical and prognostic characteristics with different clinical outcomes. In 2010 a group of experts from around the world met in Boston and divided medulloblastoma in four different groups, these are named: Wingless (WNT), Sonic Hedgehog (SHH), Group 3 and Group 4 (A. Gajjar et al., 2015; Ramaswamy, Remke, Bouffet, et al., 2016).

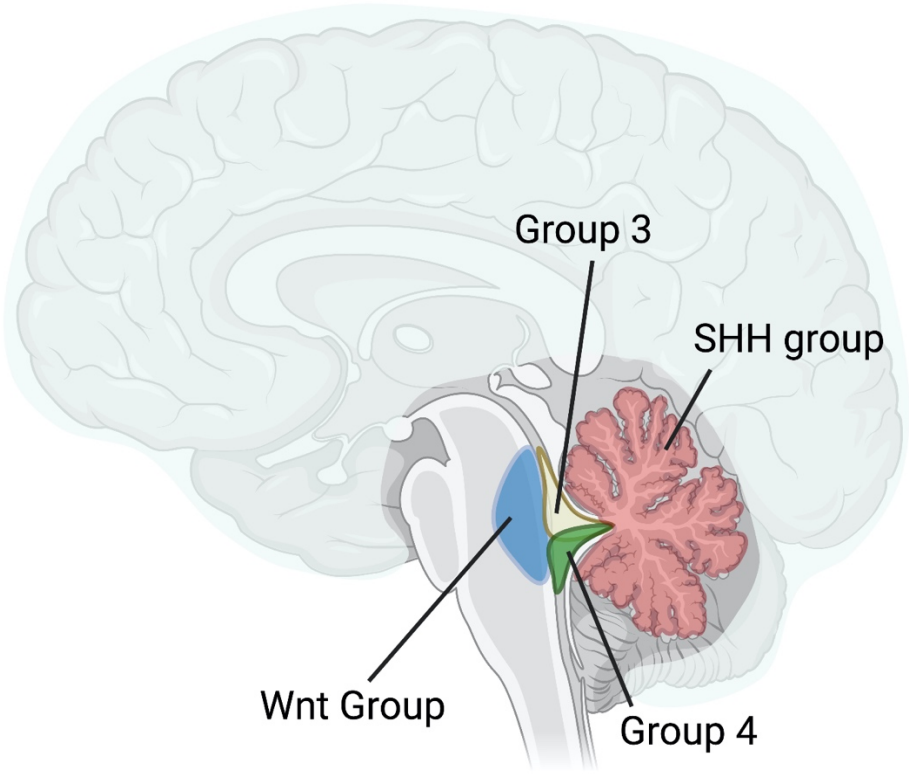

Figure 6. Common location of the different groups of medulloblastoma based on magnetic resonance imaging scans. Sagittal section of brain and brainstem. Groups are color-coded accordingly: Wnt (blue), SHH (red), Group 3 (yellow), Group 4 (green). Figure was inspired by (Northcott et al., 2019) but created by Teodora Andonova with BioRender (CC BY 4.0).

Wingless (Wnt) group

The Wnt-subtype of medulloblastoma is the rarest subtype accounting for about 10% of all medulloblastoma cases and has the best prognosis of all the subtypes. WNT group medulloblastoma tumors are characterized by nuclear accumulation of β-catenin. Mutations in *CTNNB1* (the β-catenin encoding gene) is found in more than 90% of WNT group tumors, leading to a constitutively active Wnt signaling. These tumors are characteristically situated engaging the fourth ventricle and infiltrating the brain stem, in the midline of the brain (Figure 6) (A. J. Gajjar & Robinson, 2014; Northcott et al., 2019).

Sonic hedgehog (SHH) group

The Sonic Hedgehog group is named after the Sonic Hedgehog signaling pathway. This group makes up 30% of all medulloblastoma patients, however, in contrast to the Wnt group, activation of SHH signaling is associated with a range of different genetic aberrations and clinical appearances. The association between SHH signaling and medulloblastoma was first found after patients with Gorlin syndrome with germline mutations in the *PTCH1* tumor suppressor gene showed an elevated risk to develop medulloblastoma. The *PTCH1* gene encodes for the PTCH1 protein which is a receptor for the SHH protein ligand and other homologues. Approximately 40% of SHH group tumors present with *PTCH1* loss of function

mutations, making it the most prevalent mutation in the subgroup. Additional common mutations are loss of function mutation in *SUFU* (13%) and activating mutations in *SMO* (9%). Other genetic abnormalities in the SHH pathways have also been identified, such as amplification of the transcription factors *MYCN* and *GLI2* as well as loss off chromosome 9q. Chromothripsis occurs particularly in *TP53* mutant SHH patients. SHH medulloblastoma occurs most often in children under the age of 3 or adolescents and adults above the age of 16 (A. J. Gajjar & Robinson, 2014; Northcott et al., 2019).

Group 3

Group 3 accounts for approximately 25% of all medulloblastoma patients and have the worst outcome among all the medulloblastoma subgroups due to factors such as younger age at diagnosis which does not allow for radiotherapy, high frequency of metastatic spread at diagnosis, *MYC* amplification and LCA (Large cell/Anaplastic) histology. Germline mutations have not been identified as a predisposition for group 3 medulloblastoma. Recurrent somatic genomic aberrations have been defined, perhaps most notably amplification of MYC which occurs in about 17% of patients. Less than 10% of patients have mutations in genes regulating chromatin remodeling such as *SMARCA4* and *KMT2D*. However, more than 50% of group 3 tumors do not harbor any of these genetic aberrations, instead these tumors carry extensive chromosomal structural alterations such as abberrations in 17q where 40-50% of patients have an isochrome aberration, (the q-arm is duplicated while the p-arm is lost). Other chromosomal structural aberrations are copy-number gain of chromosome 1q and 7, and losses in 10q and 16q. Group 3 tumors are present almost only in infants and children. The frequency of metastatic spread at diagnosis is about 40-45%, giving rise to particularly poor prognosis in group 3 medulloblastoma (A. J. Gajjar & Robinson, 2014; Northcott et al., 2019).

Group 4

Group 4 is the most common of the four groups of medulloblastoma and is responsible for 35% of medulloblastoma cases. This subtype is found in all age groups and has an intermediate prognosis when treated with standard therapy. This is a highly copy number dependent group, large chromosomal anomalies are common. Particularly gains of 17q (more than 80% of patients) and chromosome 7 (40–50%). Deletions of chromosomes 17p (>75%) and 8 (40–50%) are also common. Somatic mutations are very few in Group 4 medulloblastomas, no specific gene is found to be mutated in more than 10% of patients. The most established driver event is enhancer-hijacking-mediated overexpression of *PRDM6* (PR/SET Domain 6), which is identified in ~17% of patients. *MYCN* is also amplified in this patient group (6%). The most frequent mutation seen in group 4 medulloblastomas occurs in the *KDM6A* gene with 9% of patients in group 4. Mutations in various genes regulating epigenetic events are also affected in medulloblastoma, such as *KDM6A* mutated in 9% of patients, a demethylase enzyme that controls the methylation of lysine-27 of histone H3 (H3K27) (A. J. Gajjar & Robinson, 2014; Northcott et al., 2019).

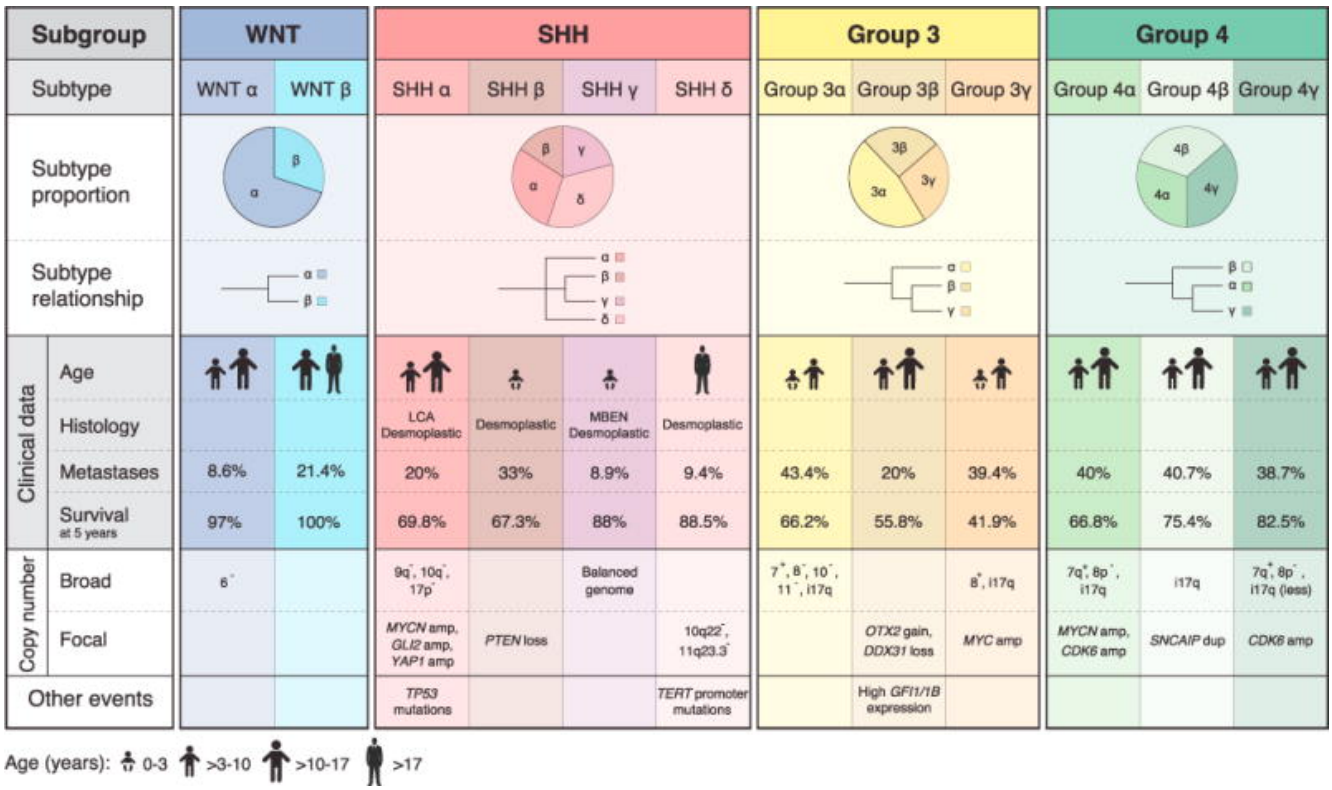

Figure 7. Key clinical characteristics of medulloblastoma groups and subgroup proportions including common genetic aberrations. (Cavalli et al., 2017) published in Cancer Cell with (CC BY 4.0)

1.4.2 Risk-stratification, treatment and survival

With the new stratification of medulloblastoma by genomic groups, epigenetics, RNA expression and histology, an understanding has been shaped on a genomic rather than solely image-dependent treatment regimen. Surgery is the first step of the treatment where maximal resection is performed, from which diagnosis from tissue is defined (Figure 7) (Ramaswamy & Taylor, 2017).

Risk Category	Low Risk	Standard Risk	High Risk	Very High Risk
Survival (%)	>90	75-90	50-75	<50
Subgroup, clinical and molecular characteristics	Non-metastatic	Non-metastatic, *TP53* WT and no *MYCN* amplification	One or both: • Metastatic • *MYCN* amplification	*TP53* Mutation
	Non-metastatic and Chromosome 11 loss	Non-metastatic and no *MYC* amplification	Metastatic	Metastatic
		Non-metastatic and no chromosome 11 loss		

Figure 8. Risk stratification for non-infant medulloblastoma according to genetic and clinical characteristics. (Juraschka & Taylor, 2019) published in Journal of Neurosurgery with (CC BY 4.0).

Medulloblastoma patients are divided into low risk, standard risk, high-risk and very high-risk groups (Figure 8). (Juraschka & Taylor, 2019; Ramaswamy, Remke, Bouffet, et al., 2016)

Low-risk patients are Wnt-group patients without signs of metastasis, as they have a survival rate of over 90%. Treatment regimen includes surgery and radiation, with or without chemotherapy (Clifford et al., 2015). Non-metastatic and chromosome 11 loss tumors in group

4 patients are also regarded low risk due to better survival (Clifford et al., 2015; Juraschka & Taylor, 2019).

Average/standard risk treatment of medulloblastoma tumors comprises radiation, surgery and chemotherapy. Adult medulloblastoma patients are in majority of instances treated with chemotherapy and craniospinal radiotherapy (Sengupta, Pomeranz Krummel, & Pomeroy, 2017). Radiotherapy is avoided until the patient is 3 years old due to the devastating long-term effects of radiation on infants. High-dose chemotherapy is applied instead, as well as a stem cell rescue regimen (Cohen et al., 2015; Packer & Vezina, 2008). The 5-year survival of these patients is 80% (Ramaswamy & Taylor, 2017). Average/standard-risk category patients include patients with non-metastatic tumors, non-*MYCN*-amplified and SHH *TP53* wild-type, Group 3 which are non-*MYC* amplified and Group 4 tumors (figure 8) (Juraschka & Taylor, 2019; Ramaswamy, Remke, Adamski, et al., 2016; Shih et al., 2014).

The patients with worst survival rates are the high-risk disease patients with a survival rate of 50-75%. This group comprises subgroups SHH-*MYCN*-amplified tumors, metastatic Group 4 tumors and non-infant metastatic SHH tumors with wild-type *TP53* (Kool et al., 2012). The poorest survival (<50%) in the very high-risk group consists of SHH tumors with *TP53* mutations (LCA morphology) and metastatic Group 3 *MYC*-amplified tumors (Cho et al., 2011; Shih et al., 2014; Zhukova et al., 2013). The majority of *TP53* mutations discovered in the SHH subgroup are germline mutations (Li-Fraumeni syndrome). They are very hard to treat as they have a tendency develop secondary malignancies with the treatment (Kool et al., 2014; Ramaswamy, Nor, & Taylor, 2015; Zhukova et al., 2013). Patients with residual medulloblastoma are also included in the high-risk disease, these patients receive high doses of craniospinal radiotherapy and chemotherapy (cyclophosphamide, cisplatin and vincristine) (Sengupta et al., 2017).

2 RESEARCH SPECIFIC LITERATURE REVIEW

2.1 THE DEVELOPMENT OF THE NERVOUS SYSTEM

Cells undergo a transition in embryogenesis from totipotent to differentiated cells in a healthy organism. There are several signaling pathways that govern the differentiation of cells at specific times in the developing embryo. If this signaling is incorrectly tuned it may result in improper differentiation of cells and the consequence may be development of cancer. Indeed, Julius Cohnheim, a student of Virchov (the father of pathology), suggested that cancer possessed embryonic characteristics due to its morphological features (Capp, 2019).

Early during human embryogenesis at around day 15, the blastocyst is starting to change with a thickened line forming of differentiating cells called the primitive streak. This is the commencement of gastrulation. The primitive streak then creates a primitive node, that invaginates and migrates inwards to create a new layer. When this process is complete, the embryo consists of the three distinct layers mentioned earlier; the ectoderm, mesoderm and endoderm (Figure 9, step 1). At this point the embryo has a defined midline, as well as cranial and caudal direction (Martini & Nath, 2009; Purves, 2008).

From the primitive streak, another indentation is formed called the primitive pit, from which the notochord is formed (Figure 9, step 1). The notochord is crucial for the development of the nervous system. The notochord sends signals to its surface layer, the ectoderm, to differentiate to neural precursor cells. This process is called neurulation, where the midline ectoderm due to the signals from the notochord becomes a distinct columnar epithelium called the neural plate. The neural plate (ectoderm layer) starts folding inwards, towards the notochord (Figure 9, step 2).

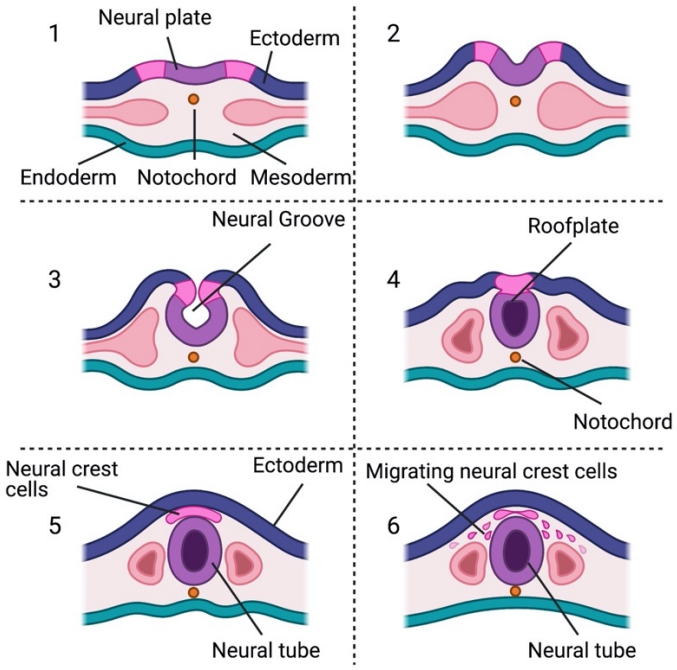

Figure 9. Neurulation in the human embryo. Created by Teodora Andonova with BioRender.com (CC BY 4.0)

It does this to make the neural tube, there are however cells left of the ectoderm that do not make up the tube that will be the neural crest cells, and the leftover top layer of the ectoderm that will make up the skin. The neural tube is the early embryonic structure that gives rise to the brain and spinal cord (Martini & Nath, 2009; Purves, 2008). When the invagination is deeper, it is called the neural groove (Figure 9, step 3). Until the top of the neural groove has closed with the other side and is fused together, thus forming the neural tube (Figure 9, step 4).

The early structure of the ectoderm has now been divided in three places, between forming the neural tube which will make up the central nervous system, the leftover cells close to the roofplate called the neural crest cells and the leftover top layer of the ectoderm that will make up the skin (Purves, 2008) (Figure 9, step 5).

The neural crest cells, in difference to the neural tube, do not only make up neuronal cell types, but also non-neuronal cell types. To differentiate into diverse cell types, they have to migrate through the loosely packed mesenchymal cells in order to reach their final destinations (Figure 9, step 6). They follow specific pathways where they are further exposed to inductive signals to differentiate into the correct type of cells, including the neurons and glia of the sensory and autonomic ganglia, the medulla of the adrenal gland, as well as many non-neuronal related cells such as pigment cells, cartilage, and bone (Purves, 2008).

There is especially one mechanism in the development of the neural crest that is also common for cancer. This process is called epithelial- to mesenchymal-transition (EMT), and occurs when the neural crest cells migrate. This is the same process that is occurring during metastasis in cancer. Epithelial cells (as well as many other cell types) have an apicobasal axis polarity, with adherence and tight junctions between cells and a well-defined top-part and bottom-part of a cell. Epithelial cells are in general tightly spaced with other cells through cell-cell adhesion molecules. Mesenchymal cells on the other hand do not have an apicobasal axis, and are loosely organized in a three-dimensional extracellular matrix. The conversion of epithelial cells to mesenchymal type of cells is central for embryonic development and involves distinctive phenotypic changes that comprise the loss of cell-cell adhesion, the loss of cell polarity, and the attainment of migratory and invasive properties (Figure 10) (Thiery, Acloque, Huang, & Nieto, 2009).

The ectoderm is the common structure during early embryogenesis where mutations can occur for both medulloblastoma and neuroblastoma. However, eventually there is a division between the two organs, the cerebellum and brain stem that develops from the neural tube (from where medulloblastoma occurs) and the adrenal medulla and the sympathetic neurons that develop from the neural crest (from where neuroblastoma occurs)(Purves, 2008).

The key signaling pathways that are involved in early embryogenesis are Wnt, Hedgehog, Notch, Protease-activated receptors (PAR) and the Tumor Growth Factor β (TGFβ) signaling pathways (Purves, 2008). Wnt and Hedgehog have been mentioned while describing neuroblastoma and medulloblastoma. However, the focus of this PhD thesis is on the Wnt signaling pathway, and signaling of the connected teneurin family of proteins.

2.2 WNT SIGNALING

2.2.1 The role of Wnt signaling in embryogenesis and differentiation

The Wnt signaling pathway was first described in 1980, when Christiane Nüsslein-Volhard and Eric Wieschaus did a systematical search of lethal embryogenic mutations in *Drosophila Melanogaster.* One of the 15 genes that caused a lethal embryological mutation was the Wg gene. Wg stands for Wingless, since the flies that had the mutation did not develop wings (Nusslein-Volhard & Wieschaus, 1980). Two years later, Roel Nusse and Harold Varmus detected a gene called Integrated1 (Int1) that could cause mammary glandular cancer in mice (Nusse & Varmus, 1982). It was later understood that the two genes were in fact the same gene and it received the combined gene name from Int1 and Wg to Wnt (Wingless/Intergrated 1) (Nusse et al., 1991). The Wnt family of proteins are lipid-modified proteins that are secreted from cells in an autocrine and paracrine fashion and operate over short distances (Clevers, Loh, & Nusse, 2014). Wnt proteins are essential during embryonal development of many different species, and dysfunctional Wnt signaling leads to a distorted anterior/posterior positioning (Brafman & Willert, 2017; Croce & McClay, 2008).

Wnt signaling is comprised of three main pathways, the canonical Wnt signaling pathway, the non-canonical Wnt/planar cell polarity (PCP) pathway, and the non-canonical Wnt/calcium pathway. The pathways commence with the Wnt ligands binding to the cell surface receptor Frizzled (Fz). There are many different Wnt ligands and Frizzled receptors, the downstream signaling diverges molecularly in the three different Wnt pathways. The activation of the canonical Wnt signaling pathway leads to a stabilization and nuclear translocation of β-catenin and induction of gene transcription. This is the pathway most clearly associated with cancer, as the adenomatous polyposis coli (*APC*) gene is recognized to be mutated in familial adenomatous polyposis (*FAP)* and known to promote colorectal cancer (Brafman & Willert, 2017; Komiya & Habas, 2008). If APC cannot bind to β-catenin, β-catenin is not tagged for degradation and is henceforth translocated to the nucleus where it acts as a coactivator of transcription factors, leading to cancer. Furthermore, *CTNNB1*, the gene encoding β-catenin, is also the most commonly mutated gene in the Wnt group of medulloblastoma (Northcott et al., 2011). Of note, the non-canonical Wnt/PCP pathway functions independent of β-catenin.

2.3 THE SIGNIFICANCE OF ARCHITECTURE IN CELLS

The simplest way to assess the differentiation status of a cell is by observing its morphology. This may be especially true for neuronal cells as they have a particularly polarized morphology (Hakanen, Ruiz-Reig, & Tissir, 2019). Pathologists use this method of observing the morphological polarity of cells to define various cancers and their grade. It is the organization of the cytoskeleton that defines the morphology of a cell. The regulation of the cytoskeleton controls neuronal cell migration and polarization, neural cone growth, neurite extension and axon guidance (Goodrich, 2008; Hakanen et al., 2019). The non-canonical Wnt/PCP pathway is thought to define the polarity of a cell by controlling its cytoskeletal movement through a highly complex molecular signaling network. Activation of the non-canonical Wnt/PCP signaling pathway leads to the intricate regulation of the cytoskeleton

and orientation of the cell which is important for regulating the migration and differentiation of neuronal cells (Hakanen et al., 2019; Vladar, Antic, & Axelrod, 2009). Furthermore, its importance has also been shown during early embryogenesis as several animal knockout models have demonstrated that defects in PCP signaling lead to defects in neurulation and neural tube closure (Copp, Greene, & Murdoch, 2003; Hakanen et al., 2019).

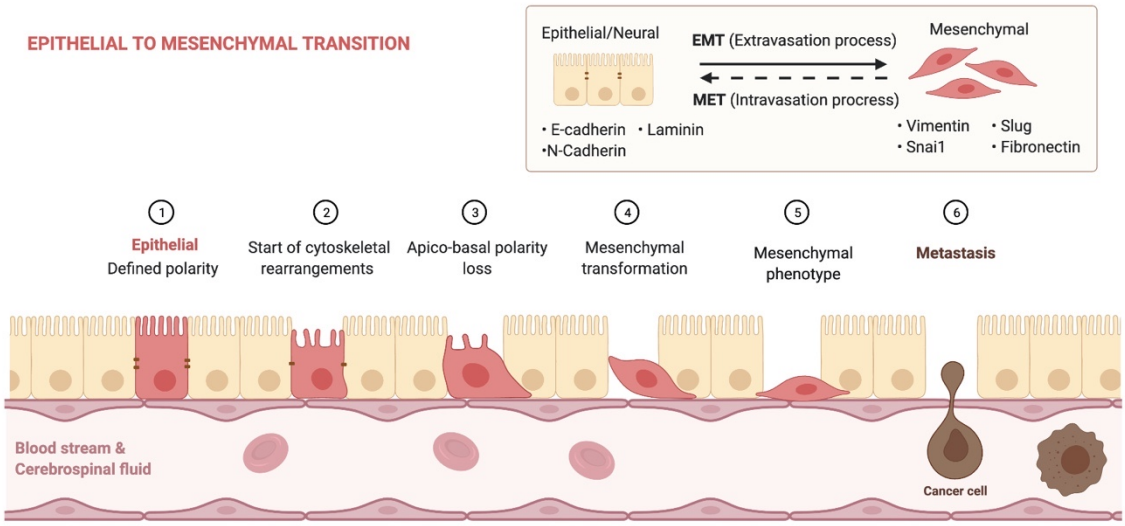

Figure 10. The process of epithelial to mesenchymal transition and loss of cell polarity. Created with BioRender.com (CC BY 4.0). Acknowledgements to illustrator David Camell.

2.3.1 The non-canonical Wnt/Planar Cell Polarity (PCP) signaling pathway

Proteins involved in core PCP signaling include Flamingo (Celsr), Fz, Dishevelled (Dvl), Van Gogh like 2 (Vangl2), Diego (Dgo) and Prickle. Fundamental for the non-canonical Wnt/PCP signaling pathway is the family of Rho GTPase's, with the three classical members Rho, Rac and Cdc42. Rho GTPases are a complex family of proteins regulating cell motility and cell organization. These proteins are guanine-nucleotide-binding enzymes, reaching an active form when bound to guanosine-5'-triphosphate (GTP), and are catalyzed by hydrolysis to guanosine diphosphate (GDP) to become deactivated again. These activation and inactivation processes are regulated by Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanosine nucleotide dissociation inhibitors (GDIs). The GEFs are activators, the GAPs are deactivators, and the GDIs maintain the GDP-bound form of small GTPases and blocks exchange thereby keeping the small GTPase in an off-state (Aspenstrom, 2018; Komiya & Habas, 2008; Mayor & Theveneau, 2014).

The non-canonical Wnt/PCP pathway is initiated by a Wnt ligand (mainly Wnt5 and Wnt11) binding to the Fz receptor that then attracts Dvl, and the pathway continues by activating GEFs, which activate Rho or Rac. Activation of Rho leads to axon retraction, while activation of Rac stimulates axon extension and neuritogenesis (Hakanen et al., 2019; Mayor & Theveneau, 2014). When Rho is activated, it is able to phosphorylate and activate the Rho-Associated Coiled-Coil-Containing Protein Kinases (ROCK1 and ROCK2). Active ROCK molecules phosphorylate a number of substrates, including LIM Kinase and myosin light chain (MLC).

When phosphorylated, these substrates regulate cell organization and contractility by affecting actin filament organization. The two serine and threonine kinases ROCK1 and ROCK2 are the enzymes downstream of Rho. The two kinases share 65% overall identity in the amino-acid sequences in humans, with about 90% identity in the kinase domains (Rath & Olson, 2012). Through its' action on the cytoskeleton, ROCK plays a central role in the regulation of cell migration and has been shown to promote metastasis and increases tumorigenicity in a variety of cancer diagnoses (Aspenstrom, 2018; R. A. Patel et al., 2012; Sadok et al., 2015; Srinivasan et al., 2017; Wei, Surma, Shi, Lambert-Cheatham, & Shi, 2016; Zheng et al., 2017; Zhong et al., 2019).

Furthermore, whole genome sequence analysis of neuroblastoma identified somatic mutations or structural alterations in genes important during neural development, growth cone stabilization, neurite outgrowth and neuritogenesis (Molenaar et al., 2012). Similar findings have subsequently been reported by others (Pugh et al., 2013; Sausen et al., 2013). The majority of identified protein changing mutations in the GEFs and GAPs were predicted to be damaging and occurred in either GEFs activating Rac, or GAPs inactivating Rho. This accumulation of inactivating alterations in the GTPase-regulating proteins would result in more activated Rho or inactivated Rac, which tips the balance towards inhibited neuritogenesis (Molenaar et al., 2012). The dysregulation of Rho/Rac signaling in neuroblastoma towards activated Rho indicates that the downstream kinase ROCK may be a therapeutic target. Furthermore, preclinical studies in different cancer diagnoses have demonstrated therapeutic potential of ROCK inhibition as it impaired tumor cell growth, migration and metastasis in various tumor models (Itoh et al., 1999; R. A. Patel et al., 2012; Sadok et al., 2015; Wei et al., 2016). A large number of different ROCK inhibitors have emerged, most of them ATP competitive, directed against both ROCK 1 and 2, or specifically targeting one of the enzymes. Some ROCK inhibitors are in clinical use for other conditions, such as glaucoma and cerebral vasospasm in Asia and the USA (Feng, LoGrasso, Defert, & Li, 2016; Garnock-Jones, 2014). Other ROCK inhibitors are being evaluated in clinical trials, however only one, the dual ROCK-AKT inhibitor AT13148, as an anti-cancer drug. AT13148 has been evaluated in a phase I dose escalation study and hypotension and headache were identified as dose-limiting toxicities due to increased vasodilation. A narrow therapeutic window, which did not allow significant inhibitory effects on either ROCK or AKT and poor pharmacokinetic profiles led to the recommendation of not proceeding with this compound (McLeod et al., 2020). In this thesis, the effects of four different ROCK inhibitors and one Rho inhibitor have been assessed (**Paper I** and **Paper II**).

2.4 TENEURINS

Another family of proteins that have been identified to be genetically altered in neuroblastoma patients and thought to play a role in non-canonical Wnt/PCP signaling are the teneurin family of proteins (Boeva et al., 2013; Molenaar et al., 2012; Pugh et al., 2013; Sausen et al., 2013). Teneurins (TENM1 to TENM4) are phylogenetically well-conserved type-2 transmembrane proteins (Tucker, 2018; Wides, 2019). The teneurin proteins consists of a smaller N-terminal

intracellular domain, a short transmembrane part, and the vast majority of the protein located extracellularly (Li et al., 2018; Tucker, Kenzelmann, Trzebiatowska, & Chiquet-Ehrismann, 2007). They are cell adhesion molecules with important functions in axon guidance and synapse formation, including directing migration of embryonic neurons (Del Toro et al., 2020; Rubin, Tucker, Martin, & Chiquet-Ehrismann, 1999; Vysokov et al., 2018; Young & Leamey, 2009). Evidence suggests that teneurins can function both as receptors at the cell surface and, after the release of the intracellular domain, as transcriptional regulators (Tucker et al., 2007). The teneurins are found predominantly, but not exclusively, in the nervous system and are highly regulated during development (Tucker & Chiquet-Ehrismann, 2006). They can bind as cis-dimers with each other, or as trans-dimers with other proteins such as latrophilins, which are also important during synapse formation and development of the nervous system (Arac & Li, 2019).

Teneurins were discovered in 1994 in the Drosophila fly, while searching for genes that are important in embryogenesis using agents that mutate the genome. The teneurin protein was considered to function as a pair rule gene since flies with this mutated gene had a deformation in the body segmentation similar to paired gene segmentation, meaning that every odd segment on the drosophila larvae was missing. Hence, the protein was named ODD OZ (Levine et al., 1994). Odd because it was every second, odd segment missing in the fly. The name OZ was given, because the Drosophila fly was lacking, as the authors of the paper described it, a brain, central nervous system and a heart, just like the features that the three characters in the Wizard of OZ, walking with Dorothy, were looking for (Levine et al., 1994). In the previous year, the teneurins had already been discovered by researchers searching for more family members in the tenascin family, and due to similarity between the tenascin and teneurin family of proteins, the name of the protein was finally settled for teneurin (Baumgartner & Chiquet-Ehrismann, 1993; Baumgartner, Martin, Hagios, & Chiquet-Ehrismann, 1994; Rubin et al., 1999). Both names have however still been in use. The first teneurin that was found in *Drosophila Melanogaster* is expressed during early embryogenesis or as the authors described it; “Tena transcripts are detected in the neurogenic ectoderm and possibly in delaminating neuroblasts” (Baumgartner & Chiquet-Ehrismann, 1993). The importance and function of teneurins during embryonic development and in the nervous system has further been confirmed through genetic analysis in chicken and mice (Lossie, Nakamura, Thomas, & Justice, 2005; Tucker, Martin, Kos, & Chiquet-Ehrismann, 2000; Young & Leamey, 2009). One study in *Tenm3* knockout (KO) mice showed that the mice did not form proper neural connections for binocular vision (Leamey et al., 2007). After using genome chemically disrupting elements, mice with strange phenotypes were revealed to be variants of *Tenm4* KO mice. The study demonstrated that a range of mutations in *Tenm4* gave rise to severe defects, with some variation between the mutants, that lead to terminated pregnancy. All embryos were terminated before embryonal day (E), E15 and as early as E6.5. The authors described six different mutants of mice with diverse large structural deletions in *Tenm4* that all aborted the development of the embryo during early embryonal stages, albeit with different ranges. The different ranges in survival and role in development were explained to be due to the long transcript of *Tenm4* and the different

isoforms. Consequently, proper expression of *Tenm4* is critical for gastrulation, mesoderm induction, neurulation and epithelial to mesenchymal transition in the embryos (Lossie et al., 2005; Nakamura, Cook, & Justice, 2013). Another study in mice where a DNA fragment was inserted in the intronic regions of *Tenm4* leading to a frameshift and KO of *Tenm4* succeeded to get through the gastrulation process, development and birth, but had severe tremor and hypomyelination of axons (Suzuki et al., 2012). In humans, mutations in the *TENM* genes have been identified to predispose for different developmental disorders such as colobomatous microthalmia (a severe malformation of the eye), Parkinson's disease, tremor and dysplasia of the hip (Aldahmesh, Mohammed, Al-Hazzaa, & Alkuraya, 2012; Chassaing et al., 2016; Feldman et al., 2019; Hor et al., 2015; Pu et al., 2020).

Molenaar et al. identified structural alterations in genes involved in neuronal growth cone stabilization, including *TENM2*, *TENM3* and *TENM4* (Molenaar et al., 2012). Additional evidence was presented by Pugh et al. (Pugh et al., 2013), that demonstrated that all four *TENM* genes were mutated in a neuroblastoma cohort. Furthermore, translocations of *TENM4* have also been identified in neuroblastoma (Boeva et al., 2013) and teneurin alterations, mutations and chromosomal rearrangements, have been found in other cancer types (Rebolledo-Jaramillo & Ziegler, 2018). The authors, Rebolledo-Jaramillo et al., discuss in their review that it is difficult to predict what effect the different mutations have in such large genes as that of the teneurin family of proteins. As presented and discussed by Lossie et al., (Lossie et al., 2005) TENM4 has a broad expression pattern and is detected in many adult and embryonic tissues. However, some *TENM4* exons are expressed in complicated developmental and tissue-specific patterns. Lossie et al., deduce that their data supports the hypothesis that *TENM4* uses multiple enhancer elements and alternative transcription start sites to direct its complex tissue-specific and developmental expression profiles (Lossie et al., 2005). The authors further suggest that the purpose of these transcripts is to increase the protein diversity of TENM4, including alternative splicing. This is consistent with the importance of TENM4 expression in different species, including that of mice and fly, where a knockout of *TENM4* has led to various detrimental effects in the developing embryo (Levine et al., 1994; Lossie et al., 2005; Nakamura et al., 2013). In humans, there may be four isoforms according to computational analysis of the gene (https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=26011 accessed 25th of February 2021).

Studies on teneurin involvement in cancer, especially on a functional level are few. *TENM4* is the most commonly genetically altered gene of the teneurins in cancer, even though it is unknown what exactly the mutations translate to with respect to protein size and activity (Rebolledo-Jaramillo & Ziegler, 2018). **Paper number III** in this thesis performs functional studies on all teneurins in neuroblastoma using transient knockdown and, furthermore, investigates the role of TENM4 based on the results in the first analysis in greater detail.

3 AIMS OF THE THESIS

The overall aim of my thesis was to increase the biological understanding of Wnt signaling in neuroblastoma and medulloblastoma, and, based on this knowledge, identify novel therapeutic options. Hopefully, this knowledge will be of use to other researchers in the scientific community and will eventually lead to improved survival, fewer side effects, and a better quality of life for these children.

The specific aims of my thesis were:

Paper I: To investigate the role of Wnt/PCP signaling in neuroblastoma, and specifically evaluate ROCK as a therapeutic target.

Paper II: To explore the role of Rho/ROCK signaling in medulloblastoma and evaluate the therapeutic effects of ROCK inhibition.

Paper III: Investigate the role of teneurins in neuroblastoma and, more specifically, functionally assess teneurin 4 in neuroblastoma tumorigenesis.

4 MATERIALS AND METHODS

The materials and methods will be discussed here, as well as the ethical considerations. The methods are however described in detail in their respective papers I, II & III.

4.1 PATIENT MATERIAL

Patient material from Swedish patients were used in paper I for whole genome sequencing and whole exome sequencing, as well as for gene expression analysis (paper III), immunohistochemistry (paper I and III) and ELISA (paper I). The collection of tumors was approved by the Karolinska University Hospital Research ethics committee under ethical approval 2009/1369-31/1 and 2003-736. Informed consent was provided by parents/guardians. In accordance with the approval, the informed consent was either written or verbal, and when verbal or written assent was not obtained the decision was documented in the medical record. The tumor material and blood from patients were obtained by routine surgery or diagnostic biopsy. Hence, tumors in our analysis may already have been treated with chemotherapy. This is ethical in terms of causing the patient minimal pain or harm, where the patient is primarily in focus, not the research. All patient material was handled coded to prevent identification of individuals.

Furthermore, we collected gene expression data of tumor material from the publicly available dataset platform R2 (http://r2.amc.nl) in all three papers. To retrieve data from the R2 platform there is no requirement to apply for or present an ethical form. Most data are collected recently, and collection is assumed to be performed ethically and accordingly to regulations in each country. There is no profitable opportunity for the database, it is publicly free and available and solely used for accelerating research. Hence, I do not see a reason for there to be any unethical component to our data collection or that of the R2 dataset, it would rather be unethical to not share data freely in order to extend our understanding of cancer and help patients.

4.2 IN VITRO CELL LINE EXPERIMENTS

4.2.1 Cell lines

Many of the cell lines used, both neuroblastoma and medulloblastoma, were collected before the new millennium possibly under other regulations in regard to informed consent than today. However, the use of these cell lines is not hurting anyone further physically, and it is not known who the cells are from. The ethical consideration here is that it is not causing harm and they are anonymous, however, there are industries profiting from selling cancer cell lines, so it could be discussed if possible profits should go to the families.

As a model, cell lines are not perfectly representative of a human body. The benefits are that they are easily accessible, do not cause physical harm to any being, and are usually more of a stable model and easily manageable. They are a good preclinical model as a start and great to build up a general molecular understanding. When robust data have been compiled in cell lines, it is a good moment to continue further with more complex models such as for example *in vivo* models.

4.2.2 Viability assays

WST-1 Assay

To measure cell viability and cytotoxic effects of treatments, the colorimetric formazan cell proliferation reagent WST-1 (Roche) was utilized. The method uses the metabolic activity of the mitochondria as a marker for vitality. In viable cells, mitochondrial enzymes cleave tetrazolium salt to form formazan (Guertler et al., 2011). Cell survival can hence be measured indirectly by WST-1. In the papers, cell survival is measured and presented as cell viability as % of untreated control. Cell viability is used to calculate the inhibitory concentration 50% (IC50) value for a drug, which shows at which concentration of an inhibitor the cell viability is reduced by half. The downside with the WST-1 viability assay is that one cannot differentiate between cytostatic effects, cytotoxic effects or if the cells are just less metabolically active.

xCELLigence RTCA DP

Cell viability and proliferation were also assessed in the xCELLigence Real Time Cell Analysis Dual Purpose (RTCA DP, Agilent). It measures the viability of adherent cells by using electrical resistance on the plate to indirectly measure cell viability/confluency. In this way, cell proliferation is measured in real-time from start to end of the experiment. An electrical signal is sent through the gold covered plate and the resistance of the electrical signal is measured as an indirect cell viability - the greater the resistance, the more viable cells are present. The unit used to measure cell proliferation is the arbitrary unit "cell index". The xCELLigence RTCA DP can also measure invasion in cells. While proliferation is measured with E-view plates, migration and invasion is measured with a different type of plates, so called Cell Invasion and Migration (CIM) plates. The CIM plates have an integrated Boyden chamber, with the gold covered resistance plates on the underside of the Boyden chamber. Addition of a basement membrane matrix (Matrigel, Corning) is not necessary for measuring migration, but invasion is measured with Matrigel on the top side of the chamber where cells are also added. This creates a barrier of matrix proteins, that the cells have to break down before they invade to the other side. In our experimental setup, cells were starved for 6 h before being placed in the upper chamber, while the bottom chamber had Fetal Bovine Serum (FBS) in media as a chemoattractant. Performing invasion experiments requires optimization of cells with cell numbers, chemoattractant and density of Matrigel. Different cells have different capacities to break down Matrigel, and may be attracted to different extent to the chemoattractant of choice. In paper I and II we show that the cells are attracted to chemoattractant as the cells invade faster than without attractant. Also, we purposely keep the experiments at a maximum of 30-40 h as we aimed to minimize the effect of cell division.

IncuCyte

The IncuCyte S3 (Essen Bioscience) is another real-time cell analysis method used in paper III. The IncuCyte performs live cell imaging and analysis on cells seeded in regular plastic plates. It can measure proliferation by analyzing pictures of cells and recognizing the cells by image analysis. Also, one can use dyes to measure different markers. In paper III, we measured

apoptosis by caspase 3 and 7. When caspase 3 and 7 are active inside a cell they can cleave the reagent, giving off a fluorescent dye, which suggests that cells are undergoing apoptosis. The cells are counted by a light microscope, while the apoptosis marker is measured by green fluorescent light (https://eu-shop.essenbioscience.com/products/caspase-3-7-green-apoptosis-assay-reagent accessed 1st of March 2021).

Clonogenic assay

A simple way to measure the tumorigenic capacity of cells is by performing a clonogenic assay. For the clonogenic assay very few cells are seeded, 150-200 cells per well in a 6 well plate, preferably as single cell suspension, as the clone-forming ability of the cells is measured. Cells are let to adhere and then treated with or without drugs. After 7-14 days of incubation, cell cultures are rinsed with phosphate buffered saline (PBS), fixed in paraformaldehyde (PFA) 4% and stained with Giemsa. Colonies with more than 50 cells per colony are counted.

4.2.3 Cell and tissue morphology

Immunohistochemistry (IHC), immunocytochemistry (ICC) and immunofluorescence (IF) are standard methods that have been used in research and clinical settings for many years. Tissue sections were formalin-fixed and when performing IHC, paraffin-embedded according to the specific protocols (see respective paper) and used for detection and distribution of proteins in cells, xenografts and human materials. The benefit of IHC is visualizing where a protein may be expressed in a tissue, and in what type of cells. IF on tissues can also be used in this setting, although more sensitive and the signal may vary a bit more depending on the strength of lasers used on for example a confocal microscope, while evaluation of IHC slides is performed with a regular light microscope and is therefore slightly more stable. Also, the fluorophores are losing their activity faster than the dyes on IHC slides. IF has the benefit of localizing the expression of a protein inside a cell easier, especially if one uses a confocal microscope. Since the methods are antibody based, the method heavily depends on the specificity of the antibody. Hence, optimization experiments to validate the antibody are crucial to understand what it binds to, especially polyclonal antibodies that may have the potential to bind to smaller parts of an antigen and may hence not only be specific for one protein. On the other hand, the sensitivity may increase slightly with a polyclonal antibody as it binds to different parts of the specified protein.

4.2.4 Western Blot

Western blot is another standard laboratory technique where antibodies are used to detect proteins of cells and tissues. With western blot the size of proteins is separated first by electrophoresis, transferred onto a nitrocellulose or polyvinylidene fluoride membrane, and then the primary antibody binds to the protein of interest on the membrane. The benefit of western blot is that it is easier to quantify differences of expression between two samples, compared to using IHC or IF. Different isoforms of a protein may also be detectable by western blot due to separation and visualization of multiple protein sizes.

4.2.5 Small interfering RNA (siRNA)

The discovery of RNA interference (RNAi) has revolutionized the way to study functions of genes and proteins since its discovery during the 1980s. In 2006, Andrew Z. Fire at and Craig C. Mello were awarded the Nobel Prize in Physiology or Medicine for their discovery that double-stranded RNA can suppress gene activity (https://www.nobelprize.org/prizes/medicine/2006/summary accessed 14th March 2021)

Proteins that do not have a small molecule or antibody developed yet to target it, may be studied in a relatively easy way with siRNA. The method is used in all three papers. Especially as we presented in paper III, siRNAs were an easy way to study the effects of downregulated genes where no inhibitors are available, as for the teneurins. It is also often used to confirm effects on a specific target, as it is used in papers I and II.

siRNAs are synthetic double-strand nucleotides of 21-23 base pairs in length. Once inside the cell, the siRNA is processed by the endogenous RNAi machinery. The AGO2-RISC enzyme complex recognizes the double stranded siRNA, where the antisense strand finds target mRNA sequences with the AGO2-RISC complex and the other siRNA strand is degraded. The RISC complex with siRNA bind sequences with perfect or nearly perfect complementarity and induce cleavage of the targeted mRNA. The cleavage in the mRNA is recognized by the cell as a defect mRNA strand and is degraded, hence the gene is not expressed, and no protein is made (Ozcan, Ozpolat, Coleman, Sood, & Lopez-Berestein, 2015).

Using siRNAs preclinically to study genes however does not give a perfect knockdown with no expression at all of the gene, as can be seen in our papers. Also, as it is a transient technology, it is expected the effects are seen from one day up to seven days after transfection. The faster the cells divide the more difficult they may be to transfect. We used the Lipofectamine 2000 or Lipofectamine RNAiMAX from Thermo Fisher Scientific (TFS) as a way to transfect cells. This method is a liposome-based transfection, and works as a cargo to deliver the siRNA molecules through the cellular membrane without any permanent damage to the cell. However, there are still cells dying because of the lipofectamine treatment, hence it is also important to optimize these experiments correctly. To understand the best expected timing and concentration of cells transfected, we used Signal Silence Control with conjugated Fluorophore from CST (#6201) to best adjust the concentration of Lipofectamine 2000/RNAiMAX, amount of siRNA, and when the cells were affected by the siRNA, as the cells give off a fluorescent light when the reagent is processed.

4.2.6 CRISPR/Cas9

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) was the technology that was awarded the Nobel Prize in Chemistry in 2020, received by Emmanuelle Charpentier and Jennifer A. Doudna for their finding and invention of this new genome editing system in 2012 (Jinek et al., 2012). The motivation for this genome editing system's superiority over past gene editing systems is that it is more precise (https://www.nobelprize.org/prizes/chemistry/2020/press-release/ accessed 3rd of March

2021). The CRISPR/Cas system was first identified in bacteria and archaea as an acquired immune system against viruses and phages. The foreign DNA that is invading is handled by Cas nuclease and cleaved into small DNA fragments, which are then integrated into the CRISPR locus of host genome as spacers. The spacers are used as transcriptional templates for producing CRISPR RNA, which is used as a guide of the Cas to find and cleave invading viruses and phages with corresponding DNA (Zhang, Wen, & Guo, 2014).

This mechanism can be used in mammalian and other types of cells to edit the DNA. Instead of the CRISPR RNA, a guide RNA (gRNA) is synthetically created that matches the DNA that should be edited. Importantly, the gRNA also needs to include a protospacer adjacent motif (PAM) that is a complementary sequence in the gRNA and the genome. The DNA helix is unwound and the PAM sequence binds to it, and so does the gRNA. The Cas9 will then break the DNA strand and its attached gRNA by a double strand break. The cell will try to repair this by non-homologous end joining, but since this is not a perfect repair mechanism, the "repair" often ends up adding or deleting extra nucleotides which then often leads to a frameshift mutation. If the goal is to induce a KO with CRISPR Cas9, then the aim is that the frameshift mutation will lead to an early premature stop codon. This means the mRNA will translate a stop codon earlier in its code, and thus not produce a full-length protein, and due to the short protein, it will be terminated by the cell completely by nonsense-mediated decay (Dominguez, Lim, & Qi, 2016; Doudna & Charpentier, 2014).

While the CRISPR/Cas9 system is said to be the most precise and easiest of techniques to use, it is not perfect. To start off, to induce a true KO, cells need to be perfectly in single cell suspension per well which sometimes can be challenging. Both chromosomes need to be knocked out, and there is biological plasticity that may try so save the expression of the gene which can make it hard to KO. The difficulties with CRISPR/Cas9 were demonstrated beautifully by a paper in Nature Methods (Smits et al., 2019) where the authors demonstrated that cells could skip exons by alternative splicing and so jump the early stop codon. Smits et al also demonstrated that even though a gene had an induced mutation on DNA level that should lead to a KO, it was not certain this truly was the case, as some proteins still had a partially active function, although the KO should lead to a premature codon. Hence, even though a protein may illustrate a KO on for example western blot, it may still be active. The authors propose that further clues, such as expected morphology and function of a cell may be a better indicator of a true KO. Other problems with performing CRISPR/Cas9 KO by single cell transfection are that there are some phenotypes that may be clone dependent instead of KO dependent and that it is not possible to get KO clones if the cell is dependent of the gene for survival and proliferation.

4.2.7. Gene expression analyses

Real time polymerase chain reaction (PCR) is a standard laboratory technique to measure gene expression in cells or tissues, with the disadvantage of only measuring up to a handful of genes at a time. It is cheap and can take up to a day to receive data. The last decades' technical advances have made it possible to measure all the genes and their transcript variants expressed

in cells or tissues, so called RNA sequencing. The buildup of data from RNA sequencing of different gene expressions have led to diverse pathway signatures and processes that can be recognized in different cell types and tissues, so called gene set enrichment analysis (GSEA). Thus, a pathway may be enriched in a treated sample compared to a control sample because there are a certain number of genes associated with that signaling pathway that are upregulated compared to the control, for example genes upregulated in neuronal differentiation.

4.3 IN VIVO MOUSE MODELS

Two animal models were used in the papers of this thesis: a human xenograft model in nude mice, and a transgenic mouse model that carries the human *MYCN* oncogene (TH-*MYCN*). The nude mice were of the type NMRI nude/nude in all three papers. The mutation in the mice causes a lack of thymus, and simultaneously the lack of fur. The athymic mice do not produce T-cells, hence they do not develop an adaptive immunity, and allow for growth of xenografts, as well as allografts of normal and malignant tissues. The mice do however retain an innate immune system; hence they are not too susceptible to microbial infections. As a precaution they are in a highly sterile environment with filtered air and sterile food and water.

The TH-*MYCN* mice is the most commonly used transgenic neuroblastoma mouse model. The mice overexpress the human *MYCN* gene by the promotor tyrosine hydroxylase (TH) that is active in neural crest cells during migration. This results in mice that develop tumor masses in the adrenal gland, which resemble neuroblastoma, and have been defined as such. The tumors histologically consist of small, round, blue cells and express specific neuronal markers (Weiss, Aldape, Mohapatra, Feuerstein, & Bishop, 1997). The TH-*MYCN* model has been greatly appreciated for its resemblance to its human counterpart. Hence this is an excellent model to study *MYCN*- overexpressing neuroblastoma.

The transgenic model bears advantages compared the subcutaneous xenograft model, with a spontaneous tumor growing in its typical anatomical site in an immunocompetent mouse. It is considered a more clinically relevant model compared to the xenograft model. However, a great advantage with a subcutaneous xenograft tumor model is that the subcutaneous tumor easily can be assessed in regard to size, without invasive methods or imaging, during the experiment.

The ethical aspects with animal studies are often raised as societal questions. It is right to always weigh the ethical considerations of performing animal studies. However, there is an ethical committee (Jordbruksverket, Swedish Board of Agriculture in Sweden) that assesses the severity levels, the science behind why the animal study is needed, and what can be gained in the animal studies that cannot be gained in other ways. The three Rs (Replace, Reduce and Refine) are kept in mind when planning and performing animal experiments. In the case of the animal studies included in the papers in this thesis, the moment of injecting mice intraperitoneally with treatments has probably been the least comfortable moment, as the mice also need to be stabilized in a firm hold and then injected. The compounds investigated, HA1077 and RKI-1447, induced no apparent side effects. The mice were

closely monitored, and no visible symptoms were evident. In addition, body weights were recorded daily during the treatment periods.

4.4 STATISTICS

In the different papers, various statistical analyses were performed. The rationale behind the statistical methods is briefly explained here. The statistical analysis used specifically for each experiment is written in the papers. IC_{50} values were calculated using "log-concentration-effect curves" from GraphPad Prism (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis for the WST-1 assays. To compare two groups, unpaired t-test was used, assuming normal distribution. A one sample t-test was used to compare the mean in a group with a specific value such as a hypothetical mean. The Holm-Sidak method was used for multiple t-tests, as this is the appropriate method for comparing multiple t-test. When three or more groups were compared, one-way analysis of variance (ANOVA) was used. ANOVA is appropriate to use for the analysis of differences among means. For different treatment categories with repeated measurements over time, two-way ANOVA was used, followed by Bonferroni multiple comparisons post-test. The Bonferroni test is a way to counteract the probability for a false positive significant result even when there is none, due to many comparisons. By adjusting for the number of comparisons made, the Bonferroni is correcting for false positives. Survival analysis was done with log-rank test. All tests were two-sided and carried out in GraphPad Prism.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Mutations and structural alterations in genes associated with neuritogenesis have been reported in about 30% of neuroblastoma patient samples in various large neuroblastoma gene expression cohorts that explore the genetic landscape of neuroblastoma in attempts to understand neuroblastoma pathology better (Molenaar et al., 2012; Pugh et al., 2013; Sausen et al., 2013). These neuritogenesis-related genes belong to the non-canonical Wnt/PCP pathway, and more specifically Rho/Rac signaling. In paper I, we reached a similar conclusion, using whole exome and whole genome sequencing we observed that 27.5% of our neuroblastoma patient samples contained a protein-changing mutation or structural alteration in the Rho/Rac signaling pathway (Figure 11). The Wnt/PCP pathway has been described to be important during neuronal development and differentiation (Hakanen et al., 2019; Kolsch, Seher, Fernandez-Ballester, Serrano, & Leptin, 2007; Vladar et al., 2009; Wallingford & Harland, 2002), further explained in part 2.2 in the literature review. The majority of identified mutations in the study by Molenaar et al., were predicted to be damaging and were predominantly found in either GEFs activating Rac, or GAPs inactivating Rho. This would result in more activated Rho, or inactivated Rac, which both tip the balance towards inhibited neuritogenesis by a more active Rho (Molenaar et al., 2012). Similarly, the majority of the mutations in the GEFs and GAPs identified by us were found in GEFs activating Rac. Moreover, we investigated activation of RhoA and ROCK2 in four neuroblastoma samples and the tumor sample with a mutation in *ARHGEF38* (somatic status not confirmed) demonstrated active RhoA as well as active phosphorylated ROCK2 expression.

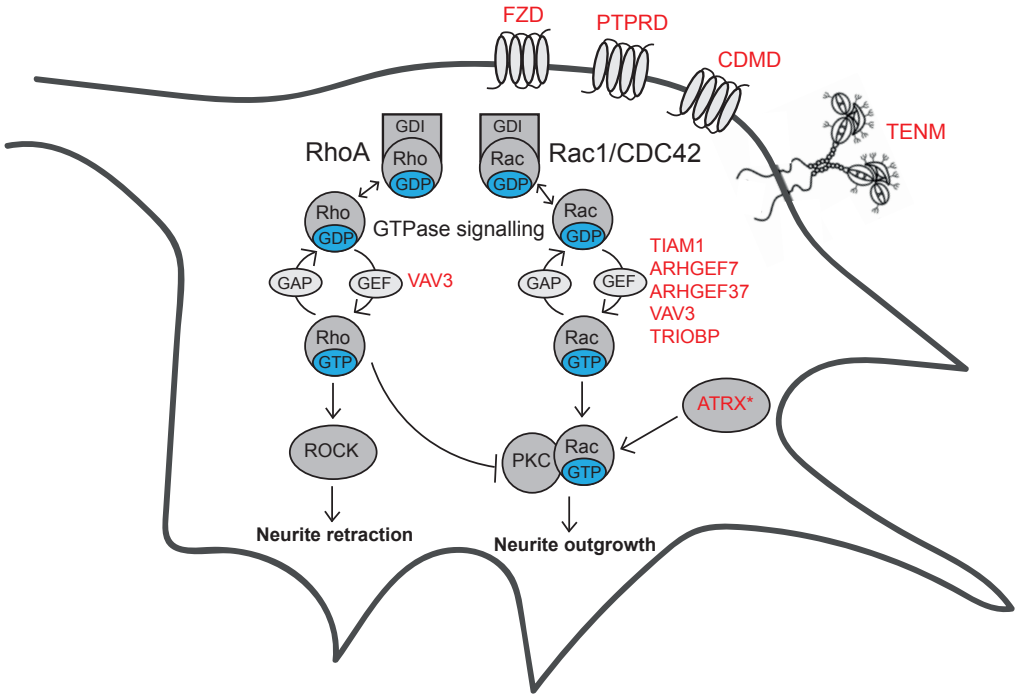

Figure 11. Genes with somatic mutations or structural alterations, in red, in the Rho/Rac signaling pathway, associated with neuritogenesis were identified in neuroblastoma in paper I. Figure was inspired by (Molenaar et al., 2012) but created by Malin Wickström (CC BY 4.0).

As ROCK is activated downstream of Rho, we sought to investigate activation of the Rho/ROCK signaling pathway in neuroblastoma. To explore the association between *ROCK1* and *ROCK2* expression and neuroblastoma survival, we analyzed expression levels in five publicly available neuroblastoma datasets. Both high *ROCK1* and *ROCK2* expression were associated with poor survival. However, the association with high *ROCK1* expression was only significant in three datasets, and one dataset showed a significant correlation in the opposite direction. *ROCK2* expression was more strongly associated with worse survival as four out of five datasets demonstrated a significant correlation between high *ROCK2* and poor survival, while one dataset was not significantly correlated, but showed the same trend.

To further understand the role of active ROCK in neuroblastoma, we investigated two ROCK inhibitors, HA1077 and Y27632, and one Rho inhibitor, Rhosin. A panel of neuroblastoma cell lines were treated with the inhibitors and the effect on cell viability was measured. HA1077 demonstrated the most effective inhibition of cell viability. Y27632 is known to have a stronger affinity for ROCK1, while HA1077 has a stronger affinity for ROCK2 (Feng et al., 2016). HA1077 additionally has affinity for protein kinase A (PKA), protein kinase G (PKG), protein kinase C (PKC) and myosin light chain kinase (MLCK), however, at higher concentrations than needed for inhibition of ROCK (Davies, Reddy, Caivano, & Cohen, 2000; Feng et al., 2016). The stronger effect on cell growth of HA1077 could therefore also be attributed to lower specificity. Thus, we also used siRNA targeting *ROCK2* to confirm the data and observed that specific *ROCK2* suppression induced similar effects on viability as HA1077. Since HA1077 was more potent in inhibiting cell growth and had a described higher preference for ROCK2, it was selected for the subsequent studies. Our *in vitro* studies in the panel of neuroblastoma cell lines revealed that cell lines with *MYCN*-amplification were more sensitive to ROCK2 inhibition by HA1077 in comparison to other cell lines. This led us to investigate the MYCN expression and we could demonstrate that HA0177 led to a downregulation of the MYCN protein, however, we could not detect any changes in the *MYCN* mRNA. Furthermore, we demonstrated that ROCK2 inhibition by HA1077 increased differentiation by assessing morphological changes such as neurite outgrowth and β3 tubulin expression and observed inhibited migration and invasion. MYCN suppression and the effects on differentiation and migration were also confirmed by siRNA-mediated knockdown of *ROCK2*, indicating that indeed these effects were mediated by ROCK2 inhibition. Amplification of *MYCN* is the worst prognostic marker in neuroblastoma (Morgenstern et al., 2016) and it has been evident in the literature that MYCN drives dedifferentiation of cells and proliferation (Huang & Weiss, 2013). Hence, if HA1077 succeeds to suppress MYCN, it is somewhat anticipated that differentiation could result, and furthermore, proliferation would slow down. In line with this, we observed that the SH-EP *MYCN*-inducible Tet21N neuroblastoma cell line was affected differently to HA1077 treatment with *MYCN* transcription on or off (Lutz et al., 1996). Subsequent exclusion of tetracycline, the *MYCN* level increased and the cells were more receptive and responded more to HA1077 treatment compared to tetracycline treated cells with *MYCN* transcription turned off.

The next step was to investigate the therapeutic potential of HA1077 in a more complex and patient close scenario. We therefore studied the effects of ROCK inhibition *in vivo*. Xenografted nude mice with established SK-N-BE(2) tumors were treated with HA1077 (50 mg/kg) or vehicle by daily intraperitoneal injections. SK-N-BE(2) is a *MYCN*-amplified human neuroblastoma cell line that is highly aggressive. Tumor volumes of mice treated with HA1077 were significantly, on average 31% smaller at the end of the experiment, compared to vehicle treated animals; the tumor growth was also significantly slower in HA1077-treated mice.

The TH-*MYCN* transgenic mouse model develops MYCN-driven tumors representative of neuroblastoma within an immunocompetent environment (Weiss et al., 1997). Four-and-a-half-week-old homozygous mice (TH-*MYCN*$^{+/+}$) were treated in the same way as xenografted mice with daily intraperitoneal injections for a total of nine days. The mice were allocated to two treatment groups, one receiving 10 mg/kg or 25 mg/kg HA1077 and compared to mice without treatment. Both treatment groups had a significantly lower tumor burden than the control, and no signs of toxicity were observed throughout the experiment. Similar to the *in vitro* cell lines, mice treated with HA1077 presented a reduction of MYCN protein levels in the tumors, but no alteration in *MYCN* mRNA levels were observed. Our results revealed that ROCK2 inhibition resulted in decreased GSK-3β (Serine9) phosphorylation and increased MYCN (Thr58) phosphorylation. In the canonical Wnt signaling pathway GSK-3β is known to participate in the degradation of β-catenin. However, GSK-3β can also promote MYCN degradation by phosphorylating MYCN, and thus tagging it for proteasomal degradation. An interaction between ROCK and GSK-3β could explain why mRNA levels of *MYCN* remain unaffected, while the MYCN protein appears to be degraded. We propose that ROCK inhibition in neuroblastoma should be further investigated as a novel treatment. Hopefully a combination of current therapy with ROCK inhibition could lead to fewer side effects due to synergism and ROCK inhibition being a potent differentiation therapy.

5.2 PAPER II

Since medulloblastoma also is an embryonal neural tumor and share some molecular similarities with neuroblastoma, we investigated the effects of ROCK inhibition in medulloblastoma. We found that *ROCK2*, but not *ROCK1*, was associated with metastatic disease in medulloblastoma as *ROCK2* expression was significantly higher in samples from medulloblastoma patients with metastasized disease compared to non-metastatic samples. Medulloblastoma patients do generally not die from primary tumors or local recurrence, but rather of metastasized tumors at relapse. A 5-year survival study in medulloblastoma showed that only 6% of patients survive after relapse (Sabel et al., 2016) while overall survival rates are 70% (Northcott et al., 2019). Hence, it is important to identify treatments that can prevent metastatic relapse.

We observed that ROCK1 and ROCK2 protein levels were present in most medulloblastoma cell lines. Interestingly, according to public patient data, group 4 showed a non-significant trend of higher *ROCK2* mRNA expression compared to the other medulloblastoma subgroups. However, the only group 4 cell line included in our study, CHLA-01-MED and CHLA-01R-

MED (01R is from the same patient but collected at relapse) (Ivanov, Coyle, Walker, & Grabowska, 2016) did not express ROCK2 protein at levels detectable with western blot. The two adherent cell lines investigated, DAOY and UW228-3, both considered to belong to SHH subtype (Ivanov et al., 2016), demonstrated the highest expression of ROCK2.

As novel ROCK inhibitors have been introduced, we sought to explore treatment in medulloblastoma with more specific ROCK inhibitors than paper I. Thus, we examined the effect of three ROCK inhibitors, HA1077 that was previously studied in neuroblastoma (Paper I), the newer and specific pan-ROCK inhibitor RKI-1447, and a dual inhibitor of both ROCK 1 and 2, as well as AKT, called AT13148. RKI-1447 has been shown to be a potent inhibitor of ROCK1 and ROCK2 with higher specificity than HA1077 and without affecting AKT (R. A. Patel et al., 2012). AT13148 is a multitarget inhibitor, inhibiting AKT, p70S6K, PKA, ROCK 1 and ROCK 2, and serum- and glucocorticoid-induced kinase (SGK) (Yap et al., 2012). AT13148 may be beneficial in many types of cancers as it is targeting the PI3K-AKT axis, a pathway upregulated is active in many cancers. All three ROCK inhibitors demonstrated dose-dependent effects on cell viability in the medulloblastoma cell lines. Both RKI-1447 and AT13148 showed significantly lower IC_{50} values compared to HA1077 but due to the greater specificity of RKI-1447 for ROCK1 and ROCK2 we decided to continue our study investigating the effect of RKI-1447 in greater detail.

We demonstrated that medulloblastoma cells treated with RKI-1447 had a lower capacity to form clones, and a reduced migration and invasion capability compared to vehicle treated cells. To gain further insights into the molecular effects of RKI-1447 mediated ROCK inhibition, we performed RNA sequencing on RKI-1447 treated medulloblastoma D425 cell and compared to vehicle treated cells. D425 cells belong to group 3 medulloblastoma (Ivanov et al., 2016), which is considered the most aggressive medulloblastoma subgroup, with a high incidence of metastasis (Northcott et al., 2019). D425 has also proven itself as a good model to study metastasis as it has capacity to metastasize in *in vivo* models (Kahn et al., 2018). Gene set enrichment analysis demonstrated that genes linked to several known cancer pathways were downregulated after RKI-1447 treatment such as tumor Necrosis Factor α (TNFα) signaling via Nuclear Factor κβ (NFκβ), TGFβ signaling, Phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian Target of Rapamycin (mTOR) signaling and EMT as compared to cells exposed to vehicle. Surprisingly, genes associated with apoptosis and p53 signaling pathway were also downregulated, even though the cells were fewer following RKI-1447 treatment. One can speculate that this may be the result of a negative feedback loop where the cells try to recover proliferation. The most significantly downregulated genes in RKI-1447-treated cells compared to vehicle cells, were *RHOB, RHOA, vimentin (VIM), JUNB, JUN,* and *JUND.* Vimentin is an intermediate filament, cytoskeletal protein that is highly expressed in the early embryo during gastrulation, during EMT, in normal mesenchymal cells and in cancer cells (Acloque, Adams, Fishwick, Bronner-Fraser, & Nieto, 2009; Liu, Lin, Tang, & Wang, 2015). The role of vimentin in EMT has established it as a marker for metastasis. We also observed decreased protein expression of vimentin after RKI-1447 treatment in D425 and Med8a, another group 3 medulloblastoma cell line, when compared to vehicle treated controls.

Vimentin is considered as a drug target in cancer by different research groups, however, there seem to not exist any specific vimentin inhibitors, yet (Strouhalova et al., 2020).

A strong downregulation of the Jun transcription factor family, including *JUN*, *JUNB* and *JUND*, all components of the transcription factor activator protein-1 (AP-1) signaling, was also observed following RKI-1447 treatment. This discovery proposes crosstalk between ROCK and c-Jun N-terminal kinases (JNK), which primarily proceed downstream of Rac, in counterpart to the Rho/ROCK pathway (Xiao, Chen, Jin, Mao, & Chen, 2017). This interaction may contribute to the effects observed on proliferation since JNKs are important regulators of the cell cycle and are target genes of AP-1 (Shaulian & Karin, 2001).

Finally, we also demonstrated that RKI-1447 treatment suppressed tumor growth *in vivo* compared to the control group. NMRI nu/nu mice were inoculated subcutaneously with the Group 3 D425 cells and treated intraperitoneally with RKI-1447 or with vehicle.

Even though ROCK1 and ROCK2 share 65% homology in their amino acids sequence, the isoforms have been proposed to have different effects in cells (Rath & Olson, 2012). We only observed a higher mRNA expression for *ROCK2* in metastatic compared to non-metastatic samples, whereas no difference in correlation was observed for *ROCK1*. However, it can still be beneficial to target both isoforms as done by RKI-1447 to avoid redundancy. ROCK1 may have compensated if only ROCK2 would have been inhibited. One study investigating the role of Rock1 and Rock2 in embryonal cells from mice, demonstrate that the Rock proteins have complementary roles for each other when either is knocked out, however not fully recovering the effects (Kumper et al., 2016). This has also been observed in a *Rock2* KO mouse model. The mice presented with surprisingly few abnormalities; their paws were deformed with extended shapes of the paws. Furthermore, the mice were tested in a spinal cord trauma experiment. The mice with *Rock2*$^{-/-}$ had a significantly improved axonal growth, in comparison to the control. Axons extended into astroglial scar tissue and displayed a nearly doubled the length of axons compared to control (Duffy et al., 2009).

So far, the only ROCK inhibitor that has entered clinical trials for cancer treatment is AT13148. However, due to a narrow therapeutic window which did not allow significant inhibitory effects on both ROCK and AKT and a poor pharmacokinetic profile the trial led to the recommendation of not proceeding with this compound. Dose-limiting toxicities connected to vasodilation-related side effects such as hypotension and headache were observed (McLeod et al., 2020). There are, however, many different ROCK inhibitors assessed for use in cardiovascular disease, as ROCK inhibition is known to relax the cytoskeleton in cells and so lower the pressure in tissues. Different studies of ROCK inhibition have shown clinical effects such as against vasospastic angina (Masumoto et al., 2002) and cerebral vasospasms in Japan and China where Fasudil (HA1077) is approved. ROCK inhibitors are also used in the area of glaucoma where most of the ROCK inhibitors are administered locally and have been approved clinically by FDA as well, such as Ripasudil which is a modified compound of Fasudil (Feng et al., 2016; Garnock-Jones, 2014). Furthermore, the ROCK2 inhibitor KD-025 (Belumosudil) is presently being evaluated in clinical trials for chronic graft versus host diseases, psoriasis

and sclerosis (https://www.clinicaltrials.gov/ct2/results?term=belumosudil accessed 23rd of March 2021.) However, a more potent inhibitor, like RKI-1447 may lead to more severe side effects by affecting the blood pressure to greater extent than Fasudil. Hence, the safety profile must be carefully evaluated if ROCK inhibitors are going to be introduced in cancer treatment.

In conclusion, ROCK inhibitors should be further investigated as a treatment option in certain cancers including neuroblastoma and medulloblastoma. It may be a valuable addition to current treatment protocols to lower the capacity of migration and invasion, and correspondingly induce differentiation. Consequently, this would lead to new efficient treatment options with fewer side effects.

5.3 PAPER III

As previously discussed, alterations in genes associated with neuritogenesis in neuroblastoma patient samples have been identified, including genes connected to the Rho/Rac signaling pathway (Boeva et al., 2013; Molenaar et al., 2012; Pugh et al., 2013; Sausen et al., 2013). Another neuritogenesis-associated family of proteins that were mutated or structurally altered were the teneurin family of proteins. To date, there are no published studies exploring the function of teneurins in human neuroblastoma, and very few functional studies in cancer in general. With the aim to understand their functions and significance in neuroblastoma, we investigated the effects on cell growth from pooled siRNAs to target the expression of each *TENM* gene in four neuroblastoma cell lines. *TENM4* knockdown resulted in significantly inhibited cell growth, consistent in all four cell lines, compared to the non-targeting control siRNA. Data from publicly available RNA expression arrays also demonstrated that high *TENM4* expression was associated with high-risk disease and *MYCN* amplified tumors, as compared to non-high-risk disease and non-*MYCN* amplified tumors respectively. Furthermore, high *TENM4* expression was associated with worse overall survival. To further evaluate the molecular following downregulation of *TENM4,* we performed RNA sequencing of two cell lines treated with *TENM4* siRNA*;* the *MYCN*-amplified cell line SK-N-BE(2)C and the non-*MYCN* amplified, but 11q deleted *CMYC* overexpressing cell line SK-N-AS. GSEA of siRNA targeting *TENM4* vs non-targeting siRNA demonstrated that genes related to pathways with cancer relevance were downregulated while genes associated with different neurogenesis pathways were upregulated. Genes connected to the unfolded protein response pathway, mTORC1 signaling and E2F targets were downregulated in both cell lines. In regard to cell line-specific results, downregulation of genes in the gene sets MYC targets V1, MYC targets V2 and G2M checkpoint were identified in SK-N-AS. For SK-N-BE(2)C, the cell line specific cancer-associated signaling pathways linked to downregulated genes were in the EMT and TGFβ signaling pathways.

Knockdown using siRNA can result in off-target effects. To minimize the probability of off-target effects observed with pooled siRNA against *TENM4*, we investigated three additional single siRNAs targeting *TENM4* together with a non-targeting control. All three single siRNAs significantly impaired cell growth in both neuroblastoma cell lines SK-N-AS and SK-N-BE(2)C respectively. Neither our single, nor pooled siRNA constructs induced a complete

knockdown. To generate a complete knockout of *TENM4* and further investigate its functions in neuroblastoma, we proceeded with CRISPR/Cas9-mediated gene editing to knockout *TENM4*. Out of 13 Sanger sequenced clones, only one clone was identified as a true homozygous KO of *TENM4* with 14 base pair deletions in both alleles. This deletion should lead to an early stop codon and translation termination. The Sanger sequenced clone also displayed phenotypic characteristics in line with the results of GSEA demonstrating upregulated genes associated with neural differentiation with siRNA treatment of *TENM4*. The cells morphology changed to a more neuron-like morphology, with long neurites and what looked like a network of neurons with long dendrites and highly polarized cell bodies. Moreover, we confirmed that *ERBB3* and *CDK6,* genes that were significantly downregulated after siRNA-mediated *TENM4* knockdown, also were downregulated in the *TENM4*$^{-/-}$ clone. TrkA (*NTRK1)* is considered a differentiation marker in neuroblastoma and we observed a significant upregulation in both the siRNA-treated SK-N-AS cell line and in the *TENM4*$^{-/-}$ SK-N-BE(2)C clone when compared to its counterparts. The *TENM4*$^{-/-}$ clone was unable to form clones in a clonogenic assay, compared to an average of 79 clones for the wild type (WT) cells ten days after seeding. In addition, proliferation was significantly delayed in the *TENM4*$^{-/-}$ compared to WT as the *TENM4*$^{-/-}$ cells grow approximately three times slower than WT cells. Apoptosis markers caspase-3 and -7 were also more active in the *TENM4*$^{-/-}$ clone compared to WT cells, indicating enhanced apoptosis in the KO cells. GSEA showed that genes linked to MYC target pathways were downregulated in SK-N-AS. In line with this, we observed reduced MYCN protein expression in the *TENM4* KO cells as compared to WT cells. Furthermore, when the *TENM4*$^{-/-}$ cells were subcutaneously inoculated in mice, no tumor formation was observed after more than 100 days, whereas the median time for tumor take (tumors exceeding 0.2 mL) for *TENM4*$^{+/+}$WT cells was 20 days. Some of the mice injected with *TENM4*$^{-/-}$ presented with a subcutaneous “patch”, too flat to measure with a caliper, that was not increasing in size. It may be remnants of the Matrigel from the injection, or the *TENM4*$^{-/-}$ cells being quiescently present.

To assess TENM4 expression in human neuroblastoma tumor samples, we performed IHC using a polyclonal TENM4 antibody in a small cohort of 22 neuroblastoma patient samples. TENM4 expression was observed in the cytoplasm of tumor cells in about one third of the samples. Furthermore, we observed significant correlation between TENM4 expression and high-risk disease and *MYCN*-amplification. This is in line with with data from publicly validated neuroblastoma cohorts retrieved from the R2 platform which showed higher *TENM4* expression in high-risk disease vs non-high-risk and in *MYCN*-amplified tumors vs non-*MYCN*-amplified tumors.

These data suggest that TENM4 is highly expressed in neuroblastoma high-risk patients, and specifically in *MYCN*-amplified tumors. *TENM4* is situated on the 11q arm, and can be lost with an 11q-deletion, as for example in the 11q-deleted SK-N-AS cell line. Consequently, SK-N-AS only has one copy of *TENM4* (Kryh et al., 2011). Nonetheless, it still seems to be beneficial to inhibit TENM4 in SK-N-AS, as the cells grew slower when *TENM4* was downregulated by siRNA. As a comparison, *TENM4* mRNA expression in SK-N-AS was

about ten times lower than in SK-N-BE(2)C (according to the RNA sequencing of cells treated with non-targeting siRNA). This suggests that it is still relevant to inhibit low levels of *TENM4*, even though the gene is only heterozygously present as it is in, for example, SK-N-AS cells.

TENM4 is required in early development and seems to be important during gastrulation, mesoderm induction and neurulation (Levine et al., 1994; Lossie et al., 2005; Nakamura et al., 2013). This implies that TENM4 is essential during early development and has the capacity to keep cells in a dedifferentiated state when active in embryonal tumors. Even though it was the mutations in the teneurins that lead us to evaluate the role of teneurins in neuroblastoma, we have so far not been able to determine the functional consequences of the genetic aberrations in *TENM4*. Analyzing clinical parameters associated with *TENM4* mutations and structural aberrations will require a larger cohort of patients.

Interestingly, we also observed in SK-N-AS cells that genes in MYC pathways were downregulated following *TENM4* knockdown by siRNA using GSEA and, similarly, MYCN protein expression was downregulated in TENM*4*$^{-/-}$ SK-N-BE(2)C cells. As we have not inhibited *TENM4* in cell lines without a high *MYC* or *MYCN* expression or amplification, we have not examined whether *TENM4* inhibition would have an effect in non-*MYCN* and non-*MYC* neuroblastoma cell lines.

Furthermore, we would have liked to study more *TENM4*$^{-/-}$ clones, but did not identify additional homozygous KO clones except for the one that has been presented. Unfortunately, the investigated *TENM4*$^{-/-}$ clone presented was difficult to grow; the more passages that passed, the slower the cells grew, until they stopped growing and floating dead cells were perceived.

We grew the CRISPR/Cas9 edited cells by single cells in separate wells to form clones. WT SK-N-BE(2)C cells are aggressive and known to have the capability to from clones from single cells. However, out of the 384 cells that were single cell sorted by Fluorescence-activated cell sorting (FACS), and plated in four 96-well plates, only around half succeeded to grow clones that could be transferred to a 24-well plate and further expanded. Several clones that survived a few weeks after the CRISPR/Cas9 editing, and had neuron-like morphology, died shortly after. As the data from the siRNA experiments demonstrated, knocking down *TENM4* led to suppressed growth rates. Consequently, there may have been more *TENM4*$^{-/-}$ cells that that did not succeed to grow and could not be analyzed.

Cells with *TENM4* knockdown or KO were growing slower and showed signs of neural differentiation. Connecting TENM4 to the non-canonical Wnt/PCP pathway, interestingly, we observed that *ROCK2* was downregulated in both cell lines after *TENM4* knockdown as compared to non-targeting control (data not shown in Paper III). Furthermore, genes related to the mTOR signaling pathway and EMT were also downregulated after treatment with the ROCK inhibitor in Paper II, as well as in SK-N-BE(2)C siRNA-mediated *TENM4* knockdown cells. These data suggest that TENM4 may be involved in the non-canonical Wnt/PCP signaling pathways. There are very few reports that have linked the teneurin family of proteins directly to the non-canonical Wnt/PCP signaling pathway. Interestingly, Talamillo et al.

investigated the function of TENM1 in glioblastoma multiforme (GBM) and suggested that the teneurin family of proteins may be involved in the non-canonical Wnt/PCP signaling pathway. The study showed that the intracellular part of TENM1 (called ODZ1 in the article) can be nuclearly translocated by peptidase-like 2a through proteolytic cleavage. The intracellular fragment of TENM1 stimulated cytoskeletal remodeling of GBM cells and invasion was stimulated, both *in vitro* and *in vivo*. Absence of TENM1 following downregulation with siRNA or gene deletion of *TENM1* also greatly reduced the invasive capacity of GBM cells. This action was mediated through an activated transcriptional pathway by TENM1, via the E-box binding Myc protein, that stimulated the expression and activation of RhoA with the subsequent activation of ROCK. In addition, overexpression of *TENM1* in GBM cells reduced survival of xenografted mice (Talamillo et al., 2017). Another connection to Wnt signaling has also been reported in SH-SY5Y neuroblastoma cells, where cells treated with the Wnt ligand Wnt-5 upregulated *TENM3* by gene expression (Bastias-Candia, Martinez, Zolezzi, & Inestrosa, 2019). Contradictive to our data, a study in ovarian cancer also performed a functional experiment on *TENM4* inhibition using siRNA, which resulted in more aggressive tumorigenesis (Graumann et al., 2017). However, the downregulation of *TENM4* gene expression was not demonstrated to successfully reduce *TENM4,* making it difficult to draw any conclusions from those functional data (Graumann et al., 2017). Another recent study demonstrated that TENM4 is highly expressed in tumor sphere forming breast cancer cells grown under stem cell-like conditions compared to cells grown in monolayer cultures. *TENM4* silencing in breast cancer cells by siRNA impaired the tumor sphere-forming ability and migrative capacity. Furthermore, the study also showed that TENM4 was increased in the plasma from mice with aggressive growing xenografts. TENM4 was also significantly increased in the plasma from patients with breast cancer compared to cancer-free patients. The authors of the paper propose TENM4 as a novel biomarker for triple negative breast cancer and a novel therapeutic target (Ruiu et al., 2021).

The literature indicates that the teneurin family of proteins are highly cell-type- and disease-dependent. While TENM1 was associated with worse effects in glioblastoma multiforme (Talamillo et al., 2017), we found that high expression was associated with non-high-risk disease in neuroblastoma (paper III). A recent review article investigated expression profiles of teneurins in different adult cancers vs normal tissue, and concluded that high vs. low expression is associated with varied results, dependent on the cancer type (Peppino et al., 2021).

TENM4 has been detected by proteomics in normal urine samples (Marimuthu et al., 2011), in SH-SY5Y neuroblastoma ectosomes and endosomes (Keerthikumar et al., 2015) and in the SK-N-BE(2) secretome (Gangoda et al., 2015). These data present TENM4 as a measurable marker in healthy and in neuroblastoma samples.

Interestingly, there is a patent application for the use of therapeutics against TENM4 in cancer and the authors present some of the data in the patent application. It is Ruth Chiquet-Ehrismann, one of the first published investigators of teneurins, who has written the patent application together with one co-author. Unfortunately, she is no longer alive and there seems to be no

advances in treatment development for TENM4. The authors stated in the patent application in 2010: “The present inventors have now surprisingly found that expression as well as protein levels of some teneurins, for example teneurin-4 correlate with tumours, for example brain tumours. The present invention hence encompasses a method for treating cancer in a subject by inhibiting a teneurin by administering to said subject a therapeutically effective amount of a modulator of said teneurin…” (patent application number: WO 2010/052288 Al, title: “Teneurins and cancer”).

This data supports what we present in Paper III, that a treatment against TENM4 is of interest in cancer.

6 CONCLUSION AND FUTURE PERSPECTIVES

The aim of this thesis was to increase the knowledge of the non-canonical Wnt/PCP polarity pathway in tumorigenesis and to identify new treatment targets in neuroblastoma and medulloblastoma.

These results could potentially lead to the development of innovative therapeutics for specific subgroups of neuroblastoma and medulloblastoma.

In **paper I** we offer new insights in the non-canonical Wnt/PCP signaling pathway in neuroblastoma and in genetic aberrations that could affect neuroblastoma progression. The exact function of the identified mutations and how they affect the protein activity or expression needs to be further validated. We do however show that inhibited Rho signaling, though ROCK2 more than ROCK1, is a target in neuroblastoma, and that inhibition is associated with neuroblastoma differentiation and repressed growth. Hence, ROCK inhibitors could be a valuable additional treatment for neuroblastoma patients. However more preclinical and clinical studies will be needed to further investigate the effects on the tumor growth, invasion and metastasis, but also possible adverse events, for example in the vascular system. More research needs to be performed in order to implement ROCK inhibition in neuroblastoma patients, with the hope to provide an approach that leads to more effective treatment for high-risk patients with fewer side effects.

In **paper II** we show that the non-canonical Wnt/PCP pathway and ROCK inhibition also is relevant in medulloblastoma, however more specifically with a focus on metastasis and invasion, as patients with recurring metastasized medulloblastoma have a very low survival rate. We demonstrate that medulloblastoma cells treated with ROCK inhibitors suppress invasion as well as proliferation rate, and downregulate the expression of genes involved in several cancer pathways. We also confirm that the ROCK inhibition represses the tumor growth *in vivo.* Using ROCK inhibitors in combination with chemotherapy could potentially be used to limit metastasis and aid in suppressing tumor growth. It could alternatively be used preventively after finalized treatment regimen as post-consolidation therapy to reduce metastasized relapses in group 3 and group 4 patients that have a higher risk for relapse.

In general, there has been more available studies for **paper I** and **II**, while published data for **paper III** about teneurins, specifically studies of teneurins in neuroblastoma have been scarce. Hence, I am content with the information we have succeeded to provide and connect with previous data, both for the neuroblastoma community, but also for the teneurin community. We show that knocking down and knocking out *TENM4* dramatically leads to more differentiated cells and suppressed growth of neuroblastoma cells. We also show that by knocking out *TENM4* in an aggressive neuroblastoma cell line, radically changes the behavior of cells to the point that they do not form tumors, in contrast to their WT counterparts that formed tumors within four weeks. Further analysis is needed of neuroblastoma patients to verify our IHC data of protein expression in neuroblastoma samples. It would be interesting to

try to quantify the TENM4 in urine or blood as an easier way to measure TENM4 presence in patients with neuroblastoma, and further understand the correlation between TENM4 expression and high-risk neuroblastoma patients. Investigations remain to be done to understand what type of inhibitor one would create for TENM4. Perhaps a monoclonal antibody that would bind to the extracellular receptor, or by using gene technology, for example siRNA to reduce the *TENM4* expression.

Cancer is a broad collection of diseases, and highly heterogeneous. One size does not fit all and it has been understood that cancers are highly dependent on tumor genetics. The knowledge about the molecular pathogenesis based on high-throughput omics technologies has led to the opportunity to develop personalized therapies. With the aim for more personalized medicine in cancer, new targeted therapies based on better understanding of the different cancers may increase cancer survival and decrease the risk of complications, ultimately leading to a higher and longer quality of life for patients with a cancer diagnosis.

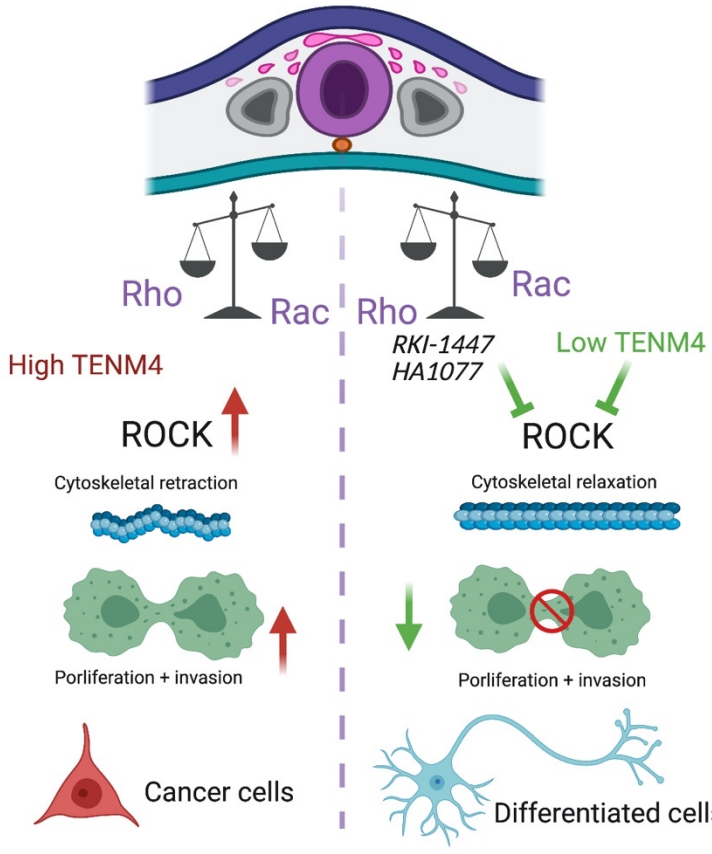

Figure 12. A summary of the pathways in neuroblastoma and medulloblastoma development that were studied in this thesis, and the result when altering those events.

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Баба Наде, ова е и мој и твој сон, твојот успех е цела таа љубов и поддршка што си ја вложила во мене.

Тато, благодарам за сè што си направил за мене. Твоите искрени критики направиле да барам подлабоко и повеќе од себе, за да бидам најдобра верзија што можам.

Мами, љубовта на една мајка стварно се гледа во тебе. Благодарам што секогаш си верувала во мене и што си ми давала бескрајна љубов и поддршка. Благодарам што си ме учела дека можам да успеам во сè додека верувам во себе.

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