From DEPARTMENT OF CELL AND MOLECULAR BIOLOGY
Karolinska Institutet, Stockholm, Sweden

NOVEL ASPECTS OF NEUROBLASTOMA: To Hypoxia and Beyond

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Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet.
Printed by Eprint AB 2019
Cover art by Sofie Önnestam

ISBN 978-91-7831-342-6

Novel aspects of Neuroblastoma: To Hypoxia and Beyond

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The public defence of this thesis will take place at Biomedicum 1 (D0320) (Solnavägen 9, 171 65 Solna) on **Friday the 22th of March 2019 at 9:30.**

One lab accident away from being a supervillain

To my family

Where would I be without you <3

ABSTRACT (ENGLISH)

Neuroblastoma (NB) is a pediatric cancer arising from the neural crest cells forming the sympathetic nervous system. Just as other types of pediatric cancer the driving mutations of neuroblastoma are few and the tumors are instead categorized according to genetic abbreviations such as amplification, loss of heterozygosity, gains and translocations. Neuroblastoma continues to be therapeutically challenging and flabbergasts scientists and doctors with it vast heterogeneity both clinically and biologically. The tumors range from aggressive, fast growing, lethal cancer to metastatic tumors that will spontaneously regress and disappear without any clinical interventions. The survivors of high-risk neuroblastoma struggle with life-long side effects due to the aggressive therapeutic treatment used in these young children. In this thesis I have focused on discovering alternative mechanisms that can contribute to the development and malignancy of neuroblastoma.

Paper I. High-risk neuroblastoma has been shown to have high level of DNA methylation of putative tumor suppressors. We designed a therapeutic strategy where we exploited the reversibility of DNA methylation and combined the DNA-demethylating drug 5-Aza-deoxycytidine (AZA) with the differentiation-promoting activity of retinoic acid (RA) as an alternative strategy to treat high-risk neuroblastoma. In this paper we showed that treatment with AZA restores high-risk neuroblastomas sensitivity to RA. Additionally, the combined systemic distribution of AZA and RA impedes tumor growth and prolongs survival *in vivo*. Genomewide analysis of treated tumors revealed that the combined treatment induced a HIF2α-associated hypoxia-like transcriptional response followed by an increase in neuronal gene expression and a decrease in cell-cycle gene expression. We performed a loss-of-function experiment using a small-molecule inhibitor of HIF2α which resulted in diminished tumor response to AZA+RA treatment. Our study indicated that the increase in HIF2α levels is a key component in the tumor response to AZA+RA and that high levels of HIF2α, but not HIF1α, significantly correlate with expression of neuronal differentiation genes and better prognosis, but negatively correlate with key features of high-risk tumors. Contrary to previous studies, our findings indicate an unanticipated tumor suppressive role for HIF2α in neuroblastoma.

Paper II. We explored the role of hypoxia inducible transcription factor *EPAS1*/HIF2α in neuroblastoma. We analyzed several neuroblastoma tumor expression datasets which showed that *EPAS1* expression is associated with better patient outcome and characteristics of low-risk tumors and did not support an oncogenic role as previously shown in other studies. We continued by treating xenografted mice with HIF2α inhibitors, which did not block in vitro neuroblastoma cell proliferation nor tumor growth. To illuminate the role of *EPAS1* during embryonic development we analyzed single cell data sets from the developing mouse sympathoadrenal lineage, wherein expression of *Epas1* was a strong predictor of the most differentiated cells and negatively correlated with key progenitor characteristics. Additionally, the genes co-expressed with *Epas1* in the sympathoadrenal lineage were associated favorable patient outcome and features of low-risk neuroblastoma.

Paper III. Due to the lack of recurrent mutations in neuroblastoma it has become more important to focus on alternative mechanisms that can influence the development of pediatric cancers. We have studied the role of fusion transcripts and proteins in neuroblastoma to illuminate possible oncogenic properties. Utilizing sequenced neuroblastoma datasets we have been able to identify neuroblastoma specific fusion transcripts. We could identify enrichments of fusions in regions frequently gained or lost in high-risk neuroblastoma as well as fusions of well-known drivers of neuroblastoma. To explore potential oncogenic properties of fusion proteins we focused on the *ZNF451-BAG2* fusion which generates a truncated BAG2 protein, that we call ΔBAG2. When overexpressing ΔBAG2 in neuroblastoma cell lines we see impaired retinoic acid-induced differentiation. Indicating that ΔBAG2 could play a role in neuronal maturation, potentially leading to a less differentiated and more aggressive tumor. Our findings reveal an overlooked mechanism capable of generating altered gene products, which are relevant for neuroblastoma pathogenesis and presents new potential drug targets.

ABSTRAKT (SVENSKA)

Neuroblastom är en pediatrisk cancer som drabbar spädbarn och små barn. Ungefär 20 barn insjuknar per år i neuroblastom i Sverige och är den vanligaste cancern hos barn under ett år. Neuroblastom är en cancertyp som tros uppkomma tidigt under utvecklingen, i celler som utvecklas till nervceller som bildar det sympatiska nervsystemet. Barn med neuroblastom utvecklar tumörer längst ryggraden eller i bukhålan. Vanligaste stället att få neuroblastom på är i binjuren som är placerad strax ovanför njuren.

Neuroblastom är en intressant cancertyp då den skiljer sig mycket från fall till fall både biologiskt och kliniskt. Barn under 18 månaders ålder får ofta en mer lättbehandlad variant av neuroblastom medan äldre barn över 18 månader ofta får en variant som kräver intensivare behandling och som kan vara svårare att bli av med. Dock så finns det en variant som drabbar de allra minsta barnen som ofta redan har bildat metastaser tidigt men som förvånansvärt nog kan växa bort av sig själv utan någon klinisk behandling.

Till skillnad från cancertyper som drabbar vuxna, har barncancer ofta väldigt får mutationer. Det vill säga fel som uppstår i DNA-koden som reglerar och kontrollerar alla våra celler i kroppen. Eftersom man har hittat så få mutationer i neuroblastom, använder man sig istället av genetiska förändringar som skett i tumören för att dela in patienterna i olika subgrupper. Dessa genetiska förändringar är till exempel extra kopior av gener (en del av DNA som bildar ett protein), extra delar eller hela kromosomer (de strukturer som DNA är uppdelat i), att det saknas delar av andra kromosomer eller att en liten del av en kromosom sitter på fel ställe.

Prognosen varierar beroende på vilken ålder barnet är i då det diagnostiseras, om tumören är på ett ställe eller har spridit sig (bildat metastaser) och egenskaper hos den enskilda tumören (genetiska förändringar). Överlevnad hos barn med neuroblastom har ökat från under 20 procent i början av 1990-talet till drygt 60 procent i mitten av 2000-talet. Dock så definieras ungefär hälften som högriskpatienter med hög sannolikhet för återfall och död trots kombinationsbehandling, så det finns fortfarande ett stort behov av att förstå neuroblastom bättre och utveckla nya behandlingsmetoder.

Högrisk-neuroblastom som är svår att operera och som har spridit sig till olika delar av kroppen behandlas med höga doser cytostatika (cellgifter) och operation samt i kombination med stamcellstransplantation. Därefter följer strålning och slutligen behandling med A-vitamin. De barn som överlever den aggressiva formen får ofta komplikationer till följd av den tuffa behandlingen och kan leda till besvär som t.ex. hörselnedsättning, njurskador, hormonrubbningar, infertilitet, neurologiska och psykologiska förändringar samt risk för sekundär cancer.

I denna avhandling har vi fokuserat på att hitta alternativa mekanismer som kan hjälpa till att förklara uppkomsten av neuroblastom och utifrån det har vi designa nya behandlingsmetoder som vi hoppas kan förbättra överlevnaden och livskvalitén hos barn som drabbas av neuroblastom.

LIST OF SCIENTIFIC PAPERS

I. Combined epigenetic and differentiation-based treatment inhibits neuroblastoma tumor growth and links HIF2α to tumor suppression

Isabelle Westerlund, Yao Shi, Konstantinos Toskas, Stuart M. Fell, Shuijie Li, Olga Surova, Erik Södersten, Per Kogner, Ulrika Nyman, Susanne Schlisio and Johan Holmberg

Published in *PNAS* (2017) July 10th, E6137–E6146

II. *EPAS1/*HIF2α correlates with features of low-risk neuroblastoma and with differentiation during sympathoadrenal development

Isabelle Westerlund, Yao Shi and Johan Holmberg

Published in *BBRC* (2019) Jan 22;508(4):1233-1239. Epub:15 Dec 2018

III. Recurrent fusion transcripts associated with key tumor characteristics occur at high frequency in neuroblastoma

Yao Shi, Vilma Rraklli, Eva Maxymovitz, Shuijie Li, **Isabelle Westerlund**, Christofer Juhlin, Adam Stenman, Catharina Larsson, Per Kogner, Maureen J. O'Sullivan, Susanne Schlisio and Johan Holmberg

Manuscript

PAPERS NOT INCLUDED IN THE THESIS

Dynamics of hippocampal neurogenesis in adult humans

Kirsty L. Spalding, Olaf Bergmann, Kanar Alkass, Samuel Bernard, Mehran Salehpour, Hagen B. Huttner, Emil Boström, **Isabelle Westerlund**, Celine Vial, Bruce A. Buchholz, Göran Possnert, Deborah C. Mash, Henrik Druid and Jonas Frisén

Published in *Cell* (2013) June 6th, 153(6): 1219–1227

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LIST OF ABBREVIATIONS

ALK	Anaplastic Lymphoma Kinase
AZA	5-aza-2´-deoxycytodine
DNMT	DNA- Methyltransferase
EFS	Event free survival
EMT	Epithelial to Mesenchymal Transition
EP	Endpoint
EPAS1	Endothelial PAS domain-containing protein 1
ES	Embryonic stem
GO	Gene ontology analysis
HIF2a	Hypoxia-inducible-factor-2alpha
HIFs	Hypoxia Inducable Factors
HREs	Hypoxia Response Elements
HSC70	Heat shock cognate 70
i.p.	Intraperitoneal
INRG	International Neuroblastoma Risk Group
INSS	International Neuroblastoma Staging System
LOH	Loss of Heterozygosity
NB	Neuroblastoma
NCCs	Neural crest cells
OS	Overall survival
PHDs	Prolyl-hydroxylases
pTAU	Phosphorylated TAU
RA	Retinoic acid
RAREs	Retinoic Acid Response Element
RARs	Retinoic Acid Receptors
ROS	Reactive Oxygen Species
RXRs	Retinoid X Receptor
SCPs	Schwann cell precursors
TSSs	Transcription start sites
VHL	Von Hippel-Lindau

1 INTRODUCTION

1.1 Cancer

Cancer is not one disease it is a collection of related diseases defined by uncontrolled cell proliferation in a tissue. Caused by changes that alter gene expression and provide a growth benefit in cancer cells compared to normal cells. These changes can be caused by many different factors; mutagenic, viruses, epigenetics, stress, radiation or simply age [1, 2].

Cancer is caused by dynamic changes in the genome often by mutations producing oncogenes or alter tumor suppressor genes which transform normal cells in to malignant cancer cells. Cancer development is multi-step process in which cells progressively accumulate genetic change which may contribute to a growth advantage, similar to Darwinian evolution, and as changes accumulate it can dive the development and progression of cancer. For a cancer tumor to develop a cell needs to survive, proliferate and often also spread to different parts of the body causing metastases [1, 2]. To do so the cell has to elude anti-cancer defense mechanisms built in to cells and tissues.

Mutation frequency vary a lot depending on the type of cancer and the tissue it originates from. Pediatric cancers have very low mutation rate (as low as 0.1 mutations/Mb) compared to cancer types in the adult. There are also cancers with extremely high mutation rate, such as melanoma and lung cancer which can exceed 100 mutations/Mb. Cancers with extremely high mutation rate are thought to be due to exposure to well known carcinogens such as UV-light and tobacco smoke [3]. When looking at a specific cancer type the amount of mutations may still vary a lot, this could be due to underlying key biological features. In head and neck cancer the tumors can have a viral or non-viral origin which may affect the mutation frequency [4]. However, the majority of mutations are “passenger” mutations rather the “drivers” and are in many cancers correlated with age [5].

1.2 Adult vs Pediatric Cancer

Taken in to account that for a normal cell to become a malignant tumor cell it has to sustain proliferative signaling, resist cell death, evade growth suppressor, induce angiogenesis, enable replicative immortality and activate invasion and metastasis [1, 2]. It´s not surprising that cancer is a disease of the elder, simply because of the time it would take to accumulated genetic damage to be able to do all of that. The question remains however; how can a disease that takes decades to develop in an adult, become a full blown metastatic cancer in a child less then a year of age, as in the pediatric cancer Neuroblastoma?

While cancer cells and embryonic cells are not identical it is clear that cancer cells have retained and/or regained embryonic features which gives a selective advantage contributing to cancer development. Due to the early onset in pediatric cancers it may indicate that the first oncogenic event happened early in life or already prenatally. Could it be that young children

provide an environment that may provide favorable conditions for cancer development? Developmental process from a fertilized egg to a complex multicellular organism as humans is long and complicated process. Genes have to be turned off and on in a certain order for specialized tissues to develop. Once turned of lineage specific genes has to stay off for a cell to continue developing in the right track [6]. It is easy to imagine that a mistake can halter a cell in an undifferentiated stage and can eventually potentially lead to development of cancer. Neuroblastoma has been suggested to develop due to abnormal or complications during differentiation. During embryonic, prenatal as well as postnatal development many stem cells are still present, cell populations are still expanding and differentiating to give rise to specialized tissues.

Hereditary disease is believed to contribute to around 10% of adult cancers. In neuroblastoma it has been estimated that less than 1-2% are familial [7-10] and around 9% overall in pediatric cancer [11], suggesting that germ line mutations does not contribute to the neuroblastoma cancer evolution. Although it cannot be excluded that these numbers are effected by the fertility of childhood cancer survivors [12].

One of the major differences comparing adult and pediatric/childhood cancer is the mutational load. Adult cancer such as colon, brain, pancreas and breast cancer have an average of 33-66 somatic mutations causing alterations in protein products. Pediatric and liquid (leukemia) cancers harbor on average 9.6 mutations per cancer. Although, if a cancer relapse the tumor or metastasis has often acquired new mutations. Cancer caused by mutagens (such as lung cancer and melanoma) harbor shockingly 200 mutations per tumor [5]. Giving an indication that pediatric cancer is probably not caused by mutagens. The involvement of environmental factors during pregnancy is hard to exclude, but there is clearly a lower impact of exogenic toxic effect in children then in adults. Smoking, sun exposure, overweight and alcohol consumption can be excluded [13]. Certainly, the low number of mutations suggests that only a few genetic alterations are required to drive pediatric oncogenesis. The lack of driving mutations can be limiting as the mutations may provide more possible therapeutic targets, making pediatric cancer a therapeutic challenge.

In certain tumors in self-renewing tissue the number of mutations are directly correlated with age [14]. Additionally, more than half of the somatic mutations are often “passenger” occurring during, or even before, tumor development. This may be one explanation why pediatric cancer has fewer mutations, since pediatric cancer often is thought to arise from non-renewing tissues, which hasn’t had time to accumulate as many mutations [5].

1.3 Neuroblastoma

Neuroblastoma (NB) is the most common extra cranial solid tumor in children and the most frequently diagnosed neoplasm in infants. NB accounts for around 10% of all childhood cancers [15, 16]. Unproportionally, NB accounts for around 15% of all oncologic deaths in children under 15 years of age. NB is a pediatric cancer thought to arising in the neural crest

cells of the sympathoadrenal lineage and tumors can form anywhere in the sympathetic nervous system. Most commonly as an abdominal mass in the adrenal medulla (around 50%) or in the para spinal ganglia, posterior mediastinum, pelvis and neck. Around half of the NB are metastatic and metastatic sites often include bone marrow, cortical bone, liver and lymph nodes [15, 17, 18].

Neuroblastoma is a fascinating disease with immense heterogeneity both biologically and clinically. Ranging from fast growth tumors with poor patient survival to being one of the cancers with the highest reported cases of spontaneous regression without any clinical intervention [19, 20]. Like other pediatric cancers NB harbors few somatic mutations and is instead characterized by genomic changes identified, which allows tumors to be divided into subgroups. Even though a lot of progress has been made in understanding the pathogenesis behind NB as well as developmental biology of the sympathetic nervous system, NB remains a therapeutically challenge. The prognosis of high-risk NB is still bad and the patients that survive treatment are left with life long side effects.

The mean age at diagnosis of familial NB is 9 month compared to the mean age of 18 months in the general population. About 40% of children with NB are diagnosed before one year of age, 75% before 4 years and 98% before they reach 10 years [10]. Neuroblastoma detection in early life or in utero suggests that early disruption of normal developmental processes may play a part in tumor initiation.

Staging of NB is important to be able to select the right course of treatment. Age, histology, DNA ploidy, MYCN amplification, chromosomal changes, neurotropin receptors and metastatic status, is taken into account when staging NB. NB is classified using two different systems; one pre-surgical risk classification system which is based on image tests usually CT or MRI and is classified into four groups International Neuroblastoma Risk Group (INRG)[21] or post-surgical International Neuroblastoma Staging System (INSS)[22].

Table 1. Summary of staging systems for neuroblastoma

INRG staging system	INSS
L1: Localized tumor, not involving vital structures and confined to one body compartment	Stage 1: Low risk, no MYCN amplification, localized tumor that can be removed by surgery.
L2: Locoregional tumor with presence of one or more image-defined risk factors	Stage 2: Relative low risk, no MYCN amplification, localized tumor, tumor cells may have some spread to local lymph nodes.
M: Distant metastatic disease	
MS: Metastatic disease in children younger than 18 months with metastases confirmed to skin, liver and/or bone marrow	Stage 3: Intermediate risk, unable to resect the full tumor during surgery, tumor extending beyond the midline and lymph node involvement.
	Stage 4: High risk, can have MYCN amplification, older age at diagnosis and metastatic disease.
	Stage S4: Spontaneous regression, tumors that appear metastatic but undergo regression without the intervention of therapies.

Histopathologically are NB undifferentiated with lots of small round cells (neuroblasts). The tumors are classified as favorable or unfavorable depending on the degree of neuroblast differentiation, Schwannian and stroma content, mitosis-karyorrhexis index and age at diagnosis [23].

What is the biological explanation for metastatic tumors that spontaneously regress and disappear? Could that information benefit the children with high-risk NB or are the high-risk tumors different? Tumors stage 4s (s=special) occur in young children which gets small localized primary tumors, although highly metastatic, but with a unique pattern of metastasis mainly in the liver and skin. The tumors will regress and disappear without any or little clinical intervention [24]. Infants under 1 year of age are at risk for both anesthetic and surgical complications associated with resection of adrenal tumors [25] and should not be subjected to surgery if not necessary. A study showed that 80% of patients where an adrenal mass was detected during the perinatal period, who did not get surgical intervention had 100% survival [26]. The exact mechanism through which NB tumors spontaneously regress and differentiate is still unclear, several alternatives have been suggested; delayed activation of developmentally programmed cell death by neurotrophin, epigenetic regulation and loss of telomerase activity.

Screening studies in Japan, Europe and Canada have been performed measuring tumor-derived catecholamines in urine samples of six month infants. The incidence of NB in the screened population was substantially increased (1:2000 vs 1:7000) but the NB had favorable biological features and did not improve survival, resulting in the termination of screening studies worldwide [27, 28]. The increased prevalence of NB in the screening studies indicates that spontaneous regression occurs at least as frequently as NB detected clinically. Additionally, this indicated that favorable tumors rarely evolve into biologically unfavorable NB tumors and neuroblastoma tumors likely have established features of either low- or high-risk disease at the time of disease onset.

NB is a hard disease to treat without causing excessive harm. Due to the young age of the patients, treatment with chemotherapy will affect many cell types and tissues. Treatment strategies depend on risk stratification and treatment range from observation (only), surgery and/or chemotherapy to high-risk NB high-dose chemotherapy, surgery, autologous stem cell transplantation, radiotherapy, immunotherapy and differentiation therapy (with Retinoic acid). Treatment with retinoids have shown to increase event free survival (EFS) in high risk NB after chemotherapy and autologous stem cell transplantation [29]. Survivors of aggressive muli-modal therapy face long-term consequences such as poor growth, hearing loss, developmental, renal and lung impairment, endocrine disturbances, neurological deficits and secondary cancers [30].

Retinoic acid (RA) is a vitamin A metabolite, which has been shown to be important during embryonic development for organogenesis, the development of the sympathetic nervous

system and can differentiate neuronal cells in vitro [31]. RA regulation of specific target genes goes through the retinoic acid receptors (RARs), which functions as a ligand-dependent transcription factor. RARs forms heterodimers with retinoid X receptors (RXRs) and bind to retinoic acid response elements (RAREs) in the promoter region of target genes. In absence of a ligand it actively represses transcription together with co-repressors [32]. Treatment with all-*trans*-retinoic acid (tretinoin) and 13-*cis*-retinoic acid (isotretinoin) has been used in a randomized control study to treat high risk neuroblastoma in combination with myeloablastive chemotherapy, total body irradiation and transplanted autologous bone marrow and improved treatment outcome [29].

Neuroblastoma is thought to originate in neural crest derived cells, where defects in neural crest cell migration, maturation or differentiation might contribute to neuroblastoma development. Neural crest cells (NCCs) are a transient collection of multipotent embryogenic progenitors, which arise between the neuroepithelium and the developing epidermis and can give rise to a variety of cells. The neural crest cells develop from the ectoderm, specifically between the neural plate and the epidermis at the neural plate border. During neurulation the neural plate invaginates at the dorsal midline and create a neural grove, the neural folds will eventually meet and fuse, creating the neural tube. The neural crest is located on the dorsal tip of the neural tube. To be able to migrate the cells undergo epithelial to mesenchymal transition (EMT). The cells that will form the sympathoadrenal lineage will migrate out around the neuronal tube to the region laterally to the notochord and the dorsal aorta. The NCCs migrate extensively during embryogenesis and give rise to a vast variety of cells; the peripheral nervous system, the enteric nervous system, melanocytes, Schwann cells, adrenal medullary cells and cells of the craniofacial skeleton [33].

Postsynaptic neuroendocrine chromaffin cells are located in the adrenal medulla and produce catecholeamines which are released into the blood stream, where they effect stress response and metabolism. A recent study by the Adameyko lab looking at the cellular origin and development of the adrenal medulla, show that a large number of chromaffin cells actually arise from Schwann cell precursors (SCPs). The study shows that NCCs split into two lineages after the first migration, one which will from the sympathetic neurons and one lineage that will from SCPs, which in turn will later form the chromaffin cells of the adrenal medulla [34].

1.4 Alterations in Neuroblastoma

Although some patients with Neuroblastoma have predisposition to the disease, most NB occur spontaneously. Activating mutations of anaplastic lymphoma kinase (ALK) are one of the few hereditary mutations which predispose to neuroblastoma [7]. A single-base substitution in the ALK gene causes a constitutive activation of the receptor tyrosine kinase [7, 35]. ALK alterations occurs both as gene-activating mutations and gene amplification. Around 10% of sporadic neuroblastoma have an ALK activating mutation and additionally

5% of neuroblastoma has ALK amplification, both which is associated with poor prognosis [35, 36]. ALK has been shown to protect against *in utero* nutrition deprivation during the neuroblast growth in early sympathoadrenal development. Knockdown of ALK lead to a decrease of proliferation [35]. ALK is involved in the regulation of MYCN transcription and is suggested to work synergistically in neuroblastoma to drive tumor development [37, 38]. ALK stimulates the transcription of a number of genes for example HIF1/2, VEGF, NFkb and SHH [39].

The 1-2% of NB which is familial, two genes have been identified ALK and PHOX2B accounting for 80% of hereditary NB. Inactivating mutation of PHOX2B accounts for 5% of these. PHOX2B is expressed in NCCs and is required for normal development of the sympathetic ganglia and aberrations can have varying effects on differentiation [40, 41].

Amplifications of genes are common in many types of cancer and often occur in oncogenes. In NB the amplification of MYCN, which occurs in around 20% of NB tumors, is strongly correlated with poor out come and strongly correlated with worse prognosis, aggressive tumors and decreased overall survival [42]. MYCN is normally located at the short arm for chromosome 2 and when expressed form a heterodimer together with MAX. Together they acts as a transcriptional activator promoting to cell cycle progression [43, 44]. MYCN is highly expressed in early post-migratory neural crest cells during normal sympathoadrenal development, regulating the ventral migration and expanding neural crest population. MYCN levels are gradually reduced as the cell differentiate into sympathetic neurons [45]. High levels of MYCN reactivates cell cycle and prevents cell death caused by NGF withdrawal in rats [46, 47]. In zebrafish overexpression of MYCN blocks chromaffin cell differentiation and causes hyperplasia in adrenal sympathetic neuroblast [38]. MYCN overexpression in transgenic mice forms neuroblastoma tumors [48], however the effect varies depending on genetic background.

Large chromosomal rearrangement is a common feature in many types of cancers. When a large part of the chromosome is duplicated or deleted it is hard to identify specific genes that are important for pathogenesis. Homozygous deletion often involves just a few genes and targets tumor suppressor genes. Deletion in one of the homologous chromosomes resulting in a loss of heterozygosity (LOH) and is a common feature in NB. One of the most common LOH occurs in the short arm of chromosome 1 (1p). The smallest region, the 1p36 locus is lost in around 35% of the NB and is often correlated with advanced disease stage and MYCN amplification. The gene or genes located at the 1p36 locus responsible for the pathogenesis is not yet clear. Several genes have been proposed to be the 1p36-encoded tumor suppressor. CHD5 is a chromatin remodeler involved in neuronal differentiation, suggested to have tumor suppressive properties [49-52]. KIF1Bß, another gene located at the 1p36 locus is involved in developmental apoptosis and mutation have shown to impaired apoptosis in sympathetic neurons during developmental culling [53, 54]. However, no bona fide tumors suppressor function has yet to be defined at the 1p36 locus and so the quest to find the 1p36 tumor

suppressor continues [55]. Although it cannot be excluded that the collective loss of genes in this region contributes to the pathogenies of NB.

Another region exhibiting LOH is the long arm of chromosome 11 (11q), deletion of 11q23 are found in around 40% of NB tumors, making it the most common deletion in NB. Interestingly it is inversely associated with MYCN amplification, but still remains correlated with high-risk features. LOH of 11q is associated with decreased event-free survival, but only in patients lacking MYCN amplification [56].

High expression of TrkA is a favorable indicator, thought to mediated apoptosis or differentiation. High expression levels of TrkA are correlated with young age, absence of MYCN and low grade tumors [57]. Conversely, high expression of TrkB is associated with bad prognosis particularly in those harboring MYCN amplifications [58].

Trisomy of chromosome 17 or gain of the long arm (17q) occurs in more than half of neuroblastoma and is therefore the most prevalent genetic abnormality identified in NB [59-61]. *Survivin,* an inhibitor of apoptosis has been suggested as the gene located in this region providing a survival benefit [62]. The partial gain of 17q due to unbalanced translocation is associated with 1p- and MYCN+ and worse prognosis. The common site for translocation of 17q gain is to chromosome 1 (1p) [60].

When looking at the karyotypes of tumors in NB patients; polyploidy (often close to triploid) is associated with less aggressive NB and is thought to be due to defects in mitosis causing the whole chromosome gains or losses. Diploid has worse prognosis in patients younger than 18 months with metastatic disease [63]. After 2 years of age the karyotype loses its prognostic significance, this is thought to be due to the hole chromosome gain without structural rearrangements in infants and after 2 years the hyperploidy is associated with possible oncogenic rearrangements [16, 64].

MicroRNA has been shown to play an important regulatory role in cells, targeting mRNA for degradation or translation repression. Additionally, several preclinical and clinical trials have been initiated for microRNA-based therapeutics [65]. LIN28B is an RNA binding protein which regulates the expression of let-7 (microRNA) family and is expressed in developing tissues and stem cells, but gets down regulated during differentiation [66]. LIN28B inhibits let-7 and has been shown to promote a pluripotent state [67]. During embryonic development LIN28B is expressed in the neural tube and regulates developmental timing and cellular growth during neural crest cell lineage commitment [67]. High expression of LIN28B elevates the expression of MYCN and is associated with worse neuroblastoma prognosis. Targeted overexpression of LIN28B in the neural crest can cause the development of neuroblastoma [68].

1.5 Epigenetic Modifications

Epigenetic modifications to DNA are heritable genomic modifications that does not change the DNA sequence but can regulate gene expression through DNA and histone methylation and acetylation that influence chromatin structure. Epigenetic regulation of gene expression is critical for both normal development and to maintain tissue-specific expression [69]. Because of the low mutation load found in NB, scientists have looked at alternative mechanisms such as DNA methylation that have the potential to inactivate tumor suppressors, in the hope of finding more information about the mechanisms effecting and contributing to neuroblastoma. Several studies have looked at DNA methylation in neuroblastoma [70-72] and found several important genes with hypermethylated promoter regions, such as CASP8 [73] and RASSF1A[74].

Epigenetic changes can be inherited through cell divisions and has been suggested as an alternative or additive mechanism to mutations and genetic alterations, in the way of changing gene expression and driving tumor evolution [2]. It has been discovered that the epigenetic landscape is different in cancer versus normal tissue in a variety of cancer [75, 76] and changes were found even in benign and pre-malignant tumors [77, 78]. Several cancer show global hypomethylation and gene promoter hypermethylation [75]. Global hypomethylation is associated with genomic instability [79], loss of imprinting [80] and shows a higher frequency of unbalanced chromosomal translocation causing LOH [76, 81]. Promoter hypermethylation as a way to silence tumor suppressors, has been suggested as a potential therapeutic target.

Epigenetics is thought to be important in generating different phenotypes from identical genotypes. Such as in monozygotic twins which have identical genotype but differ in their epigenetic phenotype [82, 83]. Several diseases have been suggested to develop when the wrong type of epigenetic marks are introduced or are added at the wrong time or at the wrong place. Epigenetic modifications is a non-mutational ways to effect gene regulation which can contribute to genome instability in cancer [2].

Drugs targeting epigenetic modifications have been used as therapy in different cancers. 5-aza-2´deoxycytidine (AZA) is a cytosine analogue which takes the place of the cytosine during DNA replication and prevents DNA methylation by DNA methyltransferease1 (DNMT1) [84]. Treatment with AZA seek to remove methylation and activate genes which have been aberrantly turned off. In cancer for example the hope is to reactivate tumor suppressor genes that have been silenced [84]. There have been studies similar to ours (Paper I.) were the synergy of retinoid with epigenetic modifiers have been efficient in reducing tumors growth [85, 86].

1.6 Hypoxia

Most organisms such as yeast, bacteria, invertebrates and vertebrates require oxygen for survival. Even small changes in oxygen levels can be harmful. However low oxygen (hypoxia) occurs naturally during embryonic development where it coordinates important developmental processes such as the development of blood, placenta, vasculature and the nervous system [87]. During embryonic development the formation of new vasculature is crucial for the developing organism. For new blood vessels to form epithelial cells proliferate and assembly into hollow tubes (vasculogenesis) as well as sprouting (angiogenesis) form already existing ones. After development there is little new formation of blood vessels and the vasculature become mostly quiescent, with exception of wound healing and during the female reproductive cycling [88, 89]. However, fast proliferation in a cancer creates a greater need for oxygen and nutrients as well as a way to remove metabolic waste and carbon dioxide causing a hypoxic environment. Many cancers induce angiogenesis by expressing pro-angiogenetic factors such as VEGF-A. VEGF-A are involved in the organization of new blood vessels during embryonic and postnatal development and survival and endothelial cells. The vasculature in tumors are frequently dysfunctional with leakage, extensive branching and bad flow, probably due to chronic expression of angiogenic factors [87, 90].

Physiological (normoxic) oxygen levels in the human body vary widely (2-9% O_2) depending on the organ. Some organs such as the thymus, kidney medulla and bone marrow can have as low as 1% O_2. Hypoxia is often associated with pathologies such as inflammation, ischemia and cancers with solid tumors. During embryonic development, the first weeks after fertalization before a circulatory system is established, mammalian development occurs in a relatively low oxygen environment (1-2% O_2) [91]. O_2 levels affect placenta development and generation of an utero–placental circulation. As the connection with the maternal vasculature is established the O_2 levels in the placenta increase to around 8% O_2 [87]. It can be concluded that at least part of the peripheral nervous system development occurs during hypoxic conditions.

Physiological hypoxia during embryonic development encountered *in utero* has been shown to be essential for many developmental processes, for example generating the components of the cardiovascular–pulmonary system [87], the development of the neural fold [92], earlier steps in bone formation [93], promoting sympathoadrenal differentiation of NNCs [94]. It is clear that O_2 influences specific cell fates in several developmental processes and can modulate cell fate acquisition in a concentration-dependent manner. Therefore, maybe oxygen concentration can potentially be considered a developmental morphogen, since it influences cell fate in a manner that is similar to for example secreted growth factors.

The atmospheric oxygen (ambient air) that we breathe has an oxygen concentration of 21% O_2. Culturing of cells in different oxygen conditions will have different effects on the cells. In adult humans, stem cells occupy regions with different oxygen concentration. Hematopoietic stem cells reside in the bone marrow where the oxygen concentration is low, other stem cells like spermatogonial stem cells reside in more vascularized areas. Raising the question of how

we should culture our cells *in vitro*; in ambient air or rather at physiological conditions for our experiments to be more clinically relevant? Growing hematopoietic stem cells at 1,5% O_2 instead of in ambient air has shown to promote engraftment and repopularization of the bone marrow [95]. Embryonic stem (ES) cells has been shown to grow more efficiently in lower oxygen conditions and are required to maintain the full pluripotency of mammalian ES cells. ES cells cultured in ambient air differentiate and lose expression of stem cell markers such as OCT-4 [96].

Angiogenesis plays an important role in tumor progression and metastasis formation. Disturbance of the balance of pro-angiogenic and anti-angiogenic factors in cancer is called "the angiogenic switch". Normal endothelial cells of the blood vessels divide every ten years, but tumor endothelial cells can divide as often as every 7-10 days [97]. In cancer some areas of the tumor may be completely deprived of oxygen (anoxia) resulting in necrotic regions. Hypoxia is involved in resistance to radiation and chemotherapy. Hypoxia inducible factors (HIFs) acts as a survival factor by inducing transcription of genes involved in angiogenesis, glycolytic metabolism, oxygen consumption and migration [98]. Tumor hypoxia is often associated with poor prognosis, resistance to radio- and chemotherapy, but the question remains if hypoxia simply is a secondary effect of a fast growing tissue or is it a causative effect that contributed to the malignancy?

Several preclinical and clinical studies have targeted angiogenesis as a therapeutic strategy. By blocking the formation of new blood vessels using anti-VEGF drugs and restricting the tumors access to oxygen and nutrients, as well as increasing the hypoxia in tumors. The treatment with anti-VEGF succeeds in suppressing the formation of new blood vessels, however, some hypoxic cancer cells have shown to instead become more invasive and metastatic [97, 99-101]. Treatment with anti-angiogenic therapy of human glioblastoma showed benefit in treating the primary tumor but caused increased invasion and local metastasis formation [90, 102, 103]. This raises concerns about targeting hypoxia and angiogenesis as a potential therapeutic target in cancer. Antiangiogenic agents have shown promising results in renal cell carcinomas where 30-40% has shown improved progression free survival, however the treatment is associated with vast side effects [104]. In other cancers the agents have shown efficiency in combination with chemotherapy, but is used minutely because of the lack of knowledge on how tumor cells escape the antiangiogenic therapy.

This taken together raises the question whether hypoxia simply is a secondary effect of a tissue with uncontrolled proliferation and underdeveloped vasculature. If so could this be the reason why there are so many contradicting studies when it comes to the oncogenic or tumor suppressive role of HIFs. Or if hypoxia is a primary cause and part of the pathogenesis and drives (or at least contributing) to the cancer development and progression? Whether targeted therapy to block the vascularization, hence increase the hypoxia would be of therapeutic value. Several studies have suggested treatment with HIF2α antagonists in cancer and several

clinical trials are ongoing [105-108]. Thirdly, if the hypoxia response isn't driving the cancer would blockage of HIFs be beneficial as a treatment?

1.7 HIF2α

The hypoxia inducible factors (HIFs) are transcription factors that mediate the primary transcriptional response to hypoxic conditions both in normal and transformed cells. There are three different HIFα:s identified; HIF1α, HIF2α (encoded by EPAS1) and HIF3α. The α subunit is primarily regulated through post-translational modifications (ubiquitination) performed by HIF-specific prolyl-hydroxylases (PHDs) in the presence of O_2. In normoxia proteasomal degradation is mediated in part by von Hippel-Lindau (VHL) tumor suppressor. During hypoxic conditions, the HIFα subunit is instead translocated into the nucleus where it forms a heterodimer with a stable HIFß unit (also known as ARNT). Together the complex binds to hypoxia response elements (HREs) of about 200 genes, such as VEGF, GLUT and EPO, where it activates gene transcription [109]. Oncogenic signaling in cancer cells have been shown to increase transcription and/or translation of HIFαs in an oxygen independent manner [110]. HIF3α mRNA is differentially spliced producing multiple isoforms that either promote or inhibit the activity of HIF complexes. Little is known about the effect of HIF3α in tumor progression in hypoxic conditions [111-113].

Despite structural similarities, HIF1α and HIF2α have distinct roles during embryonic development. HIF1α depletion is embryonically lethal (E9.5-10.5) in transgenic mice and the embryos dye due to vascular defects [114-116]. HIF2α depleted mice however survive until mid/late gestation or in some cases to birth. Depletion of HIF2α in these pups causes impaired vascular remodeling and cardiac function in embryos [117, 118] and caused neonatal respiratory distress [119] and multi-organ pathology after birth [120]. Suggesting that the two genes regulate overlapping but not identical genes.

Both HIF1α and HIF2α are expressed in hypoxic tumor regions. However, their role in tumorigenesis remains controversial as both have been described as both oncogenes and tumors suppressors in a tumor context dependent manner. HIF1α has been suggested to be involved in the early stages of cancer while HIF2α in the late stages and chronic rather than acute hypoxia [121]. High expression of HIF1α in biopsies of brain, breast, cervical, esophageal, oropharyngeal and ovarian cancers is correlated with treatment failure, mortality and promotes tumor progression [122]. In renal cell carcinoma HIF2α promotes growth, while HIF1α has a tumor suppressive role [123]. In hepatocellular carcinoma [124], colorectal cancer [125] and soft tissue sarcomas [126] has HIF2α been shown to act as a tumor suppressor. Interestingly, in non-small lung cancer has HIF2α been described both as an oncogene [127] and as a tumor suppressor [128]. In glioblastoma the role of HIF2α is not clear since there is contradicting studies published, indicating a role as a tumor suppressor [129] or as an oncogene [130].

In neuroblastoma several studies have published a correlation between high levels of HIF2α and high-risk NB [131-134]. But even in NB there are contradicting studies. In a study by Simon and colleagues they show that HIF1α is highly expressed in MYCN amplified NB tumors, while HIF2α are high in non-MYCN amplified tumors. Additionally, the study shown an interplay between HIF1α and MYCN in NB [135]. The precise role of HIF1α and HIF2α in cancer biology still remain a puzzle.

1.8 Fusion Proteins

Chimeric RNA can be produced by three different mechanisms: Chromosomal rearrangement occurs by translocation, deletion or insertion and can cause the formation of new chimeric RNAs. Chromosome translocation in cancer can cause fusion of genes which creates an oncogene or inactivates a suppressor gene by truncating it or separate it from its promoter [136].

The second mechanism is *cis*-splicing, where read-through transcription produces one long RNA strand. Transcription usually begins at a transcription start site (TSS), guided by a promoter and usually ends at a regulated termination point. Genes are separated by intergenic (non-transcribed) regions. Chimeric transcripts can occur when the RNA-polymerase reads through the termination point and continues to transcribed the consecutive gene and instead stops at the termination point of the downstream gene. The intergenic region of the chimeric transcript is spliced out and the two normally adjacent genes merge into one [136].

Thirdly, *trans*-splicing when two separate RNA strands are spliced and fused into one RNA strand. The RNA can be from two different genes either located on the same chromosome separated by coding gene or in two distal parts of the chromosome, or even on two different chromosomes [136].

Additionally, fusions can be either interchromosomal or intrachromosomal. Interchromosomal fusions occurs between genes located on different chromosomes. Intrachromosomal fusions occurs within the same chromosome. The fusion RNA can have a functional role or can be transcribed into a fusion protein with a different role then their original proteins [136]. Fusions has been detected in tissues in non-pathological condition [137, 138].

Fusion RNAs has been linked to several types of cancer and were first discovered in leukemia and other hematological diseases, but has since then been found in different types of cancer [13]. The BCL-ABL fusion in chronic granulocytic leukemia caused by the "Philadelphia chromosome" [139]. In non-small cell lung cancer EML4-ALK fusion was detected and targeted for treatment using ALK inhibitor [140, 141]. In prostate cancer a large number of tumors harbor the TMPRSS2-ERG fusion [142]. Fusions can be used as biomarkers and as potential therapeutic targets.

Fusion transcripts has been suggested as alternative oncogenic mechanism particularly in pediatric cancer where the mutation load is low and genomic rearrangement are common. Fusion genes has been identified in pediatric cancer such as leukemia (113), lymphomas (41), brain tumors (4), kidney tumors (4), bone and soft-tissue tumors (37) and liver tumors (1) and are associated with varying outcome [13].

1.9 Summary

Neuroblastoma is a pediatric cancer generated in the sympathetic nervous system. It carries a low mutation load and is instead categorized by genetic abbreviations. The prognosis of children with high-risk NB remains poor and those that survive will have long-term side-effects from the toxic treatment. Many pediatric cancers are suggested to have an embryonic origin and NB is thought to originate from cells of the neural crest, however the cell of origin is not yet determined. The growth promoting environment, with lots of stem cells and expanding cell populations during embryonic development together with small changes in the cell might provide favorable conditions for cancer development. Because of the low mutation rate alternative mechanism has been explored, such as failure to differentiate due to DNA methylation silencing of genes important for neuronal differentiation and fusion transcripts caused by genomic translocation, to understand the pathogenesis of NB and find new therapeutic targets.

2 SCIENTIFIC AIMS

The mechanisms driving childhood still remains an unsolved mystery in many ways. Mutations are one of the key drivers of cancer, however, pediatric cancers have a much lower number of mutations compared to adult cancers. In this thesis we have focused on illuminating alternative mechanisms that may be important for the development and progression of pediatric cancer neuroblastoma. Neuroblastoma continues to be therapeutically challenging and flabbergasts scientists and doctors with it vast heterogeneity. The tumors range from aggressive, fast growing, lethal cancer to metastatic tumors that will spontaneously regress and disappear without any clinical interventions.

In **paper I.** we target high-risk neuroblastoma with a new therapeutic strategy. We used a combination treatment with the epigenetic drug AZA and the neuronal promoting drug RA, to inhibit tumor growth by inducing neuronal differentiation.

In **paper II** we continue working with the findings from paper I and explore the role of *EPAS1*/HIF2α in neuroblastoma and evaluate if *EPAS1* has a predictive value of clinical outcome. We also evaluated the expression pattern of *Epas1* in the sympathoadrenal lineage during embryonic development.

In **paper III** we aimed to identify and characterize fusion transcripts in neuroblastoma.

3 RESULTS

3.1 PAPER I.

High levels of DNA methylation of the promoter region of potential tumor suppressor genes has been suggested as predictive factor of poor prognosis in Neuroblastoma [72, 143-145]. This together with the non-responsiveness of many high-risk NBs to neuronal promoting drug RA got us thinking if the failure to differentiate exhibited by high risk neuroblastoma is a consequence of the DNA hypermethylation. To do so we investigated whether targeting the hypermethylated NB genome with DNA demethylating agents could restore the capacity of these cells to respond to differentiation inducing signals and thus counteract tumor growth. In particular, we were interested in understanding the role of DNA methylation and its effect on tumor development and progression. In addition, we aimed to explore if the hypermethylated genome of high risk NB could be exploited as point of entry for treatment of high risk NB with demethylating agents. The need for alternative therapeutic strategies in NB are of importance.

Retinoic acid (RA) is a strong promoter of neuronal differentiation and is used as an adjuvant treatment in high-risk NB patients, however, with limited effect and low overall survival [29, 146]. LOH of at the 1p36 locus (1p36-) is a common feature of NB and is associated with low event free survival and overall survival [147, 148]. To evaluate the effect of RA on NB cells we started by culturing a panel of different cell lines in the presence of RA. Several 1p36+ (with intact 1p36 locus) NB cell lines responded to RA by changing to a more neuronal-like morphology with neurite extension and network formation. Additionally, the cells showed upregulated neuronal markers, accompanied with a cessation of proliferation. To evaluate the response *in vivo* we performed a xenograft experiment, where we transplanted human NB cells subcutaneously into nude mice. A cohort of the mice was subjected to daily i.p. treatment with RA, which results in a distinct reduction of tumor growth.

In our panel of NB cell lines, we found that the more NB cell lines derived from 1p36- tumors tend to be unresponsive to RA and continues growing uninhibited in the presence of high consentrations of RA. We wondered if the RA resistance could be due to a hypermethylated genome. By exploring the reversibility of a hypermethylated genome; we decided to combine the demethylating effect of AZA with the neuronal promoting effect of RA. Pretreatment with AZA for 4 days *in vitro* before combining it with RA induced a similar response as in the 1p36+ cell lines (to RA alone). Our data indicates that the key to the RA responsiveness may lay in the suppression of genes by methylation. However, we cannot exclude a possible secondary effect of the AZA treatment, but it is clear that the combined treatment is pushing the cells to adopt a more mature neuronal-like phenotype.

By using a xenograft mouse model we could study the effect of the combination treatment *in vivo* and evaluate the effect of systemic delivery of AZA+RA could effect tumor progression. We performed two types of xenografts with three different 1p36- cell lines. In the first one

(SK-N-AS) we injected the cells (D0) and started treatment with AZA+RA on D1 (looking at tumor development), which resulted in no difference between the Ctrl (DMSO) and RA (only) cohort. The AZA (only) cohort showed a reduction in tumor growth, and the AZA+RA group showed the biggest difference with tumors four times smaller than the Ctrl group. To evaluate the effect on already formed tumors (tumor progression), we injected cells (LAN-1 and CHP-212) and let the tumors grow for 8 days, to a size around 200mm^3, at which point we started treatment. Resulting in abolished tumor growth in both cell lines after the start of the combined (AZA+RA) treatment.

To investigate the long term effect of the combined treatment we treated the cells *in vitro* with AZA+RA for 10 days after which we terminated the treatment. Even after termination of treatment the cells maintained a pronounced neuronal morphology for at least 18 more days and showed no signs of reentering the cell cycle. In vivo we treated mice for 15 days (D8-D22) after which we terminated the treatment and followed the tumor growth. The tumors did eventually grow to the experimental endpoint (EP) of 1000mm^3, but with an almost doubling for survival 54 days compared to Ctrl (22 days). Showing that the combined treatment with AZA+RA prolongs survival even when the tumor is not removed and the treatment is terminated.

By combining RNA sequencing together with methylation analysis of xenografted tumors at the experimental endpoint of LAN-1 and SK-N-AS tumors, we could get an insight into how the methylome changed after treatment and what the genetic impact was in terms of gene expression. To establish the demethylation effect we performed a bisulfate conversion followed by a genomewide methylation analysis using Infinimum Human Methylation 450 BeadChip array. Unsupervised clustering of differentially methylated points (within 2kb from the transcription startsite (TSS)) showed a clear grouping of tumors treated without AZA (Ctrl or RA alone) and tumors treated with AZA (AZA alone or AZA+RA) and a significant reduction in methylation levels all throughout the genome.

When looking at the expressional changes in the different treatment groups we found very limited effect in the RA (only) treated group. In SK-N-AS a substantial number of genes were differentially regulated in the AZA and AZA+RA groups. However, in the LAN-1 tumors only the combined treatment had a substantial effect on gene expression. Indicating that at least in the LAN-1 cell demethylation alone is not enough to induce expression of suppressed genes. It seems that is not enough to just remove the break, there is a need for some accelerator to get the wheels rolling. The correlation between increased gene expression in genes with demethylated promoters showed weak to no correlation.

Gene ontology (GO) analysis of the treated xenografted tumors at EP when tumors reached 1000mm^3 showed that the MYCN amplified tumors (LAN-1 and CHP-212) showed a response characterized by neuronal differentiation terms while the non-MYN amplified cell line (SK-N-AS) showed categories involving immune response and cell death. Indicating that the cellular response may vary depending on cell type, but that the decreased tumor growth may still occur in tumors with different genetic background.

When looking at the gene list of differentially expressed genes from EP we could not find clear driver genes that we could conclude to be driving the response to AZA+RA. Instead we decided to look earlier when the changes between the treatment groups actually occur. We repeated the xenograft experiment with the LAN-1 cells and decided to harvest half of the tumors at D10 (after 48h of treatment) and the other half at day 14 (six days after treatment). The discovery that there was a decrease in mitotic cells already at D10 when we were still unable to see a difference between the groups in the growth curve, indicated that we were on the right track.

RNA sequencing of the tumors showed a moderate change in gene expression at D10, but a larger change at D14. Unexpectedly, the top hit in the gene set enrichment assay (GSEA) showed "hypoxia". The key regulators of hypoxia are the hypoxia inducible factors (HIFs) shown to increase cell survival and activate hypoxia response elements (HRE). Hypoxia inducible factor 2 alpha (HIF2α) encoded by the gene Endothelial PAS domain-containing protein 1 (*EPAS1)*, was induced by the combined treatment. Contrary to our experiment several studies have suggested *EPAS1* as a neuroblastoma oncogene and have reported high levels of *EPAS1* in high-risk NB [131-134].

HIF´s has been shown to be regulated at a protein level by proteosomal degradation and hypoxia induced stabilization. Western blot at D10 and D14 was performed showing an moderate elevated amount of HIF2α already at D10. This results show that the protein regulation precedes the transcriptional response shown at D14. Expression levels of HIF1α after AZA+ RA treatment remained unchanged. To understand if induction of *EPAS1*/HIF2α is dependent on decreased oxygen levels in the tumors; we decided to treat NB cell *in vitro* in normoxia (21% O_2) or in hypoxia (1% O_2) with AZA+RA. Western blot results show that in contrast to Ctrl, AZA+RA in normoxic conditions upregulate HIF2α equivalent to the levels expressed in hypoxic conditions. Confirming that the response is due to treatment of AZA+RA and not solely dependent on the oxygen level.

To understand the transcriptional changes over time we plotted expression of hypoxia, cell cycle and neuronal genes at our different time points. Compared to untreated cells grown in normoxia there was a clear increase of hypoxia genes in both Ctrl and AZA+RA treated tumors at D10. At D14 there was a significantly higher expression in the AZA+RA tumors compared to Ctrl. At EP the levels of hypoxia genes had decreasing compared to D10, although the tumors were larger, indicating that it might not be oxygen level dependent. Cell cycle genes were significantly lesser expressed in AZA+RA tumors at D14 and EP. Expression of genes associated with neuronal differentiation was slightly increases at D14 and was substantially increased at EP. Concluding that the cells in the AZA+RA group, increase the response to hypoxia at the same time as the cell cycling genes decrease, later leading to an induction of neuronal genes. The first response to the treatment is hypoxia and reduced proliferation, and later neuronal differentiation.

When examining the methylation changes of the promoter of *EPAS1* a significant but minor change in methylation status could be confirmed. Questioning whether the demethylation is

responsible for the increased expression. Although, we cannot rule out that demethylation of the promoter may have occurred earlier, but has then been methylated again.

To understand if the increased *EPAS1* expression after AZA+RA treatment is important for the growth inhibition of the tumors, we decided to perform a loss-of-function experiment. In which we treated the xenografted mice with HIF2α inhibitor PT2385. PT2385 is a small molecule designed to block the interaction between HIF2α and its dimerization partner ARNT, thereby preventing the hypoxia induced stabilization, subsequently preventing the activation of HIF2a target genes and leaving the protein for degradation. Immunoprecipitation was used to confirm the disruption between HIF2α and ARNT. The experiment resulted in a reduction in the anti-tumoral effect of combined therapy in neuroblastoma cells. Tumors treated with AZA+RA+PT2385 lost their responsiveness to the AZA+RA treatment and continued growth in the same pace as the Ctrl group. Indicating that the effect is at least partly dependent on the increase in HIF2α levels. Interestingly, the tumors in the PT2385 (only) group showed an increased growth compared to Ctrl, exhibiting a significant difference at D14 and continued to be larger at EP, however not significantly.

To determine if the genes regulated by the AZA+RA treatment at D14 could be of a clinically predictive value, we utilized a data set of 498 (SEQC) RNA sequenced NB tumors. Genes being significantly upregulated respectively down regulated at least 2-fold after AZA+RA treatment were clustered using a k-mean analysis. Tumors in the cluster expressing up-regulated genes selected from the RNA sequencing showed a better overall survival (OS), as well as lower INSS stage and fewer MYCN amplified tumors. The opposite was shown for the cluster tumors selected by genes down-regulated after AZA+RA treatment.

To clarify the role of *EPAS1* in NB, as our data contradicted previously published data on EPAS1. We plotted *EPAS1* level in the SEQC data (498 sequenced NB tumors) as well as a database (KOCAK) containing 649 microarrayed NB tumors. We found a clear correlation between EPAS1 expression and high event free survival, overall survival (OS), lower risk and tumor grade/stage. The opposite was true when we plotted for HIF1α, indicating that the two hypoxia inducible factors could have opposite functions. Using one of the stronger markers of aggressive NB we could see a negative correlation between MYCN amplified tumors and *EPAS1*expression and a positive correlation with HIF1α. We also looked at protein levels of HIF2α in NB tumors samples and found that 6 out of 8 of the low grade samples (stage 1-3) expressed high levels HIF2α, and in stage 4 only 1 out of 9 samples expressed HIF2α.

Our study suggests that HIF2α may act as a tumor suppressor in NB, potentially due to its capacity to promote neuronal differentiation. Previous studies indicating the HIF2α could be a NB oncogenes were majorly performed using immunohistochemistry as well as with a small number (n=88) of NB arrayed tumors, potentially explaining the differences in our study.

3.2 PAPER II.

The work presented in Paper I showed an upregulation of *EPAS1* after combined treatment with the epigenetic drug AZA and the neuronal differentiation promoting drug RA. We revealed a correlation between expression of the hypoxia inducing factor HIF2α as the tumor growth is impeded and neuronal differentiation induced. Correlating the upregulation of *EPAS1* with better survival and neuronal differentiation in neuroblastoma. However, the role of *EPAS1*/HIF2α tumor biology appears context dependent and several reports has classified HIF2α as an oncogene or as a tumor suppressor in different types of cancer. Additionally, it has been suggesting that recently developed small molecule antagonist specific for HIF2α could be used as a treatment for children with neuroblastoma.

In this article (Paper II) we continued exploring the role of *EPAS1*/HIF2α in neuroblastoma. To establish if expression of *EPAS1* correlates with clinical outcome we decided to start by comparing several datasets of neuroblastoma tumors. Surprisingly, analysis showed that three (n=102, 249 and 88) out of the six available datasets used showed a correlation between high expression levels of *EPAS1* and worse survival, although only one of which was significant. Upon closer inspection, we found that the studies contained an uneven distribution of neuroblastoma tumors. One (n=102) study only contained non-MYCN amp tumors, another (n=249) was biased by the vast majority of tumors being grade IV. Implying that an analysis using these datasets would be less suitable for making conclusions of clinical value.

When looking at the remaining three datasets they all had an even distribution of tumors of all stages, a greater number of tumors (n=498, 649 and 251) and were not biased toward a certain genetic alteration. Analysis showed a clear significant correlation between high levels of *EPAS1* and better overall survival (OS). Additionally, one of the studies were RNA-sequence based instead of array-based like the rest. Neuroblastoma tumors with high expression of *EPAS1* have better patient outcomes and are classified as lower grade, indicating that *EPAS1* may have tumor suppressive properties in neuroblastoma.

To further illuminate the role of *EPAS1* we looked at genes involved in neuronal differentiation, proliferation and genes with oncogenic properties already proven in neuroblastoma. When comparing the gene expression of *EPAS1* to these groups of genes, we found a correlation with differentiation associated genes which also correlates with better outcome. Additionally, there was an inverted correlation with proliferation and telomere maintenance genes, which in turn was correlated with worse survival for neuroblastoma patients. We therefore speculate that *EPAS1* may have a role in the neuronal differentiation process during development.

To test the HIF2α antagonist as a potential treatment in neuroblastoma; we grew a panel of neuroblastoma cell lines of varying genetic status in the presents of either HIF2α antagonist #2 or PT2385 at different concentrations. Interestingly we found that in our 1p36+ cell line SK-N-SH there was a small but significant decrease in proliferation in the presence of either inhibitor. On the contrary, in all of the 1p36- cell lines, which are classified as more

aggressive, treatment with one or both of HIF2α inhibitor resulted in an increased proliferation. Complementary we tested the treatment in LAN-1 cells grown in hypoxic conditions and found no effect of the inhibitor.

To investigate the effect of an HIF2α inhibitor in an animal model, we decided to use a xenograft mouse model and proceeded by injecting three of the aggressive cell lines subcutaneously in nude mice. The mice were treating daily with the inhibitor PT2385 and resulted in no significant difference in tumor growth between the treatment groups. Although further experiments are required to verify the clinical effect of a HIF2α inhibitor in neuroblastoma, our results indicated that treatment with the HIF2α inhibitor would not be beneficial for patients with neuroblastoma.

To understand a possible role of HIF2α/*EPAS1* during embryonic development we utilized data from a single cell study of the sympathoadrenal lineage in the developing adrenal gland in mice performed by the Adameyko lab [34]. We utilized the single cell data to study in which cell populations *Epas1* was expressed. We found the *Epas1* expression patterns coincided with the more differentiated cells belonging the chromaffin group. Additionally, we looked at the consensus genes of *Epas1* which in a GO analysis showed a strong neuron/norepinephrine association. This data confirmed our hypothesis that *EPAS1* may play a role in the differentiation process or atleast is correlated with characteristics of more mature cells.

Finally, to evaluate if *EPAS1* and associated genes during development can be of predictive value for clinical outcome in neuroblastoma patients, we went back to the 498 sequenced neuroblastoma tumors. Utilizing the top 100 genes associated with *EPAS1* expression from the single cell data, we could perform a k-means analysis. Resulting in two clusters of tumors, with 58 genes positively associated with *EPAS1* and 10 with an inverse correlation. The first cluster with high expression levels of *EPAS1* associated genes were classified as low-risk, with better overall survival and MYCN non-amplified tumors. Were as the opposite was seen in the cluster of tumors with an inverse expression patters of the *EPAS1* associated genes.

In summary, in paper II we show a correlation between high expression of *EPAS1* in neuroblastoma tumors and better OS survival, lower grade tumors and non-MYCN-amplified tumors. However, we cannot formally exclude the possibility that *EPAS1*/HIF2α might be involved in processes contributing to more malignant neuroblastoma. Judging from the results in this study *EPAS1* cannot be classified as a neuroblastoma oncogene, but might instead be associated with a tumors suppressive role and may also carry a predictive value of clinical outcome in NB.

3.3 PAPER III.

Limiting number of mutations in neuroblastoma and the need for new therapeutic strategies are leading scientists to explore alternative mechanisms underlying the pathogenesis of neuroblastoma. In this study (Paper III) have we used bioinformatic tools to study fusion proteins. Other cancers have shown to have fusion proteins as oncogenic drivers [137, 149]. Using FusionCatcher [150], we analyzed a dataset of 172 paired-end RNA sequenced NB tumors (NB172). Containing 139 high risk, 19 intermediate and 14 low risk neuroblastoma tumors. We found fusions in 163 out of the 172 tumors, with an average of 38 fusions per sample, which is more that the average of mutations found in NB tumors. Additionally, 103 fusions occurred at a frequency higher than 10%. The majority of fusions (786/924) were intrachromosomal fusions. The fusion proteins resulted in truncated proteins, bona fide fusion proteins to deletions of genes. Fusion proteins are capable of generating new gene products with oncogenic potential. Allowing for new unexplored therapeutic targets.

Several of the fusions were enriched in chromosomes which are altered in NB pathology. By looking at fusions in the different risk groups, we found that fusions in the high-risk NB were located on chromosome 17 and 22, while in the low-risk tumors the fusions were enriched on chromosome 11q. Looking at NB statistics aggressive NB gain of 17q is a marker for more aggressive NB [16] while chromosome 22 alterations are associated with metastatic and aggressive tumors [151]. Several of the fusions transcripts comprised of already proven oncogenic genes in NB, such as MYCN, BRCA1, LMO1 and TERT.

To validate the fusion transcripts found in the NB172 data set, we decided to perform our own paired-end RNA sequencing (14 NB tumors + eight NB cell lines) in which we identified 139 fusions out of with 82 (59%) were present in the NB172 tumors. To determine the NB specificity of the fusions, we compared the samples to a dataset containing 161 sequenced healthy human adrenal glands and found very little over lap (6% in the NB172 and 3% on our validation cohort). Comparing our date to another pediatric cancer; rhabdoid cancer where we found more than double the amount of fusions (2055). However, the overlap between the to childhood cancers were only 2%, indicating that the fusions identified are NB specific. Interestingly, contrary to the NB fusions the majority of the fusions found in rhabdoid tumors were interchromosomal fusions.

To validate the fusions, we designed primers spanning the fusion point in selected fusions. Using RT-PCR we analyzed the the chimeric transcript of six different fusions in a panel of ten NB tumor samples, seven NB cell lines and 14 human tissues. Showing that the fusions (5/6) were expressed in an NB specific manner.

One of the top 25 most recurrent fusions in NB is the ZNF415-BAG2 fusion (ΔBAG2), which is present in 31 out of the 172 sequenced NB tumors (18%). BCL2 associated athanogene (BAG2) is a co-chaperone involved in the degradation of miss-folded proteins in an ubiquitin independent manner. BAG2 has also been shown to increase differentiating NB cells and clears phosphorylated TAU from neuronal microtubule, helping to maintain the

stabilization of axons in neurons [152]. Looking at the sequence of ΔBAG2, we could see that this fusion results in a truncated version of BAG2 lacking exon1. When looking at different animal species we found that the truncated part contains a highly conserved N-terminal coiled-coil domain. To understand the role of ΔBAG2 in NB and if it is of clinical significance, we looked at the genes differentially expressed in NB containing ΔBAG2. We found 32 genes with a significantly elevated expression and 34 genes with a decreased expression, which we used in the 498SEQC. The upregulated genes were correlated with high-risk NB, and the inverted correlation with the downregulated genes.

Looking at protein level in a panel of 13 NB tumors and six adrenal glands, blotting for BAG2 showed that the wtBAG2 was expressed in all samples, including both tumors and adrenal glands, however, five of the NB tumors co-expressed ΔBAG2 and none in normal adrenalglands. As BAG2 has been shown to be important for the clearance of phosphorylated TAU (pTAU) [152] and when blotting for TAU we could see that the tumors expressing ΔBAG2 had a much higher expression of pTAU. To validate that this is due to the presence of ΔBAG2, we overexpressed ΔBAG2 in an NB cell line (SK-N-FI) and could clearly show that when ΔBAG2 is over expressed the levels of pTAU increase. Overexpression of wt BAG2 showed a reduction in pTAU and co-expression or wtBAG2 and ΔBAG2 showed that wtBAG2 was unable to clear pTAU, implying a dominant negative relationship with ΔBAG2. BAG2 has been shown to bind to heat shock cognate 70 (HSC70) [153] immunoprecipitation experiment showed that wt BAG2 binds HSC70 but ΔBAG2 does not. Additionally, treatment of SK-N-FI cells with/without overexpression of wtBAG2 or ΔBAG2 showed that cells overexpressing ΔBAG2 showed a weaker response to Retinoic acid (RA) with less neurite formation.

The recurrence of fusion transcripts expressed in a NB specific matter, together with the identification of fusions in already known NB oncogenes, implies that several of the fusion transcripts may have oncogenic properties contributing to the NB pathogenesis. Thus these fusion transcripts could constitute a source of altered transcripts, distinct from DNA-altering mutations, potentially promoting neuroblastoma.

4 DISCUSSION

The role of HIF2α in cancer still remains unclear: is HIF2α a tumor suppressor or an oncogene? Many scientific studies report different roles in different types of cancer, suggesting that the role of HIF2α is context dependent. There has even been contradicting results within the same cancer type, like in glioma [129, 130]. A possibility is that the role of HIF2α in cancer is depending on the cell or origin. In paper I we had the opportunity to analyze *EPAS1* expression in glioma, a cancer type that have both a pediatric version and an adult version of the pathogenesis. When we plotted *EPAS1* expression compared to overall survival (Paper I, S6 A and C), we found a very interesting discovery. In pediatric glioma, high *EPAS1* expression is associated with better prognosis and overall survival. While in adult glioma the opposite is true. In adult glioma high *EPAS1* was instead associated with worse prognosis. The contrast between adult and pediatric glioma with regards to the association between high *EPAS1* levels and overall survival implies that in pediatric tumors of the neural lineage, the role of HIF2α is different from its role in adult tumors HIF2α cancer dependent. In a non-pathogenic condition such as during embryonic development HIF2α plays a particular role when it comes to development of the CNS and heart [118, 154]. Additionally, in Paper II we show that in the sympathoadrenal linage *EPAS1* is expressed in the differentiated cell chromaffin cells (Paper II, Fig4 A).

In neuroblastoma HIF2α has mostly been suggested to be an oncogene [131-134, 155]. However, there are a few studies indicating a tumor suppressor effect before ours in NB (Paper I and II) and other cancer types [124, 125, 135, 156-159]. When looking back at some papers suggesting an oncogenic role for HIF2α, there are some limiting factor in those studies such as the small number of sequenced/arrayed tumors and the fact that immunohistchemical stainings of NB tumors are used to validate the expression of HIF2α. Unfortunately the reliability of HIF2α antibodies are limited, due to the potential unspecificity. In a western blot, however, it is easier to determine the correct band if you use a control or by looking at the ladder. We believe that using a larger set of NB tumors, which is evenly distributed with regards to clinical parameters such as MYCN status and NB staging, will give a more reliable answer to the predicament of the role of *EPAS1* in NB.

Simon et al show an association between high levels of HIF1α in MYCN-amp tumors, and high levels of HIF2α in non-MYCN amp tumors [135]. Recently even the group that has published the majority of the papers on HIF2α as an oncogenes in NB, published a paper [160] in which they show that *EPAS1* is highly expressed in non-MYCN amplified tumors (Fig 2E), however still argue in the paper that HIF2α is oncogenic. Although, it is hard to fully exclude an oncogenic role of HIF2α, however it can be concluded that HIF2α isn't a permanent feature of high-risk NB.

In paper I we saw a week correlation between de methylation of the promoter region and gene expression. Showing that AZA treatment alone is not enough to activate gene

expression, indicating that the mechanisms by which AZA re-sensitize the NB cells to RA might not be due to demethylation alone. Implying that there might be off-target effects. Previous studies has shown that AZA can induce reactive oxygen species (ROS) [161] and DNA damage [162]. However, this was not seen in the tumors treated with AZA+RA, instead they showed a reduction in the response to oxidative phosphorylation and DNA repair (Paper I, Fig 3G).

Our NB cell lines show varying genetic changes SK-N-AS is 1p36-, MYCN wt and p53 mutation, CHP-212 is 1p36-, MYCN amplified and p53 wt and LAN-1 is 1p36-, MYCN amplified and P53 null mutated. Both SK-N-AS and LAN-1 with mutation on P53 exhibit increased apoptosis upon AZA+RA treatment, indicating that the apoptotic response in independent of a normal p53.

Both RA and AZA are FDA approved drugs which are already used for treatment of different cancers types. AZA (decitabine) is used to treat myelodysplasive syndrome [163]. RA (tretinoin and isotretinoin) is used in high-risk NB after myeloablastive chemotherapy, total body irradiation and transplanted autologous bone marrow [29, 146]. There are clinical trails using the combination of AZA+RA to treat acute myeloid leukemia [164, 165]. PT2385 is suggested for clinical trail in different cancer where HIF2α has shown oncogenic properties [108, 166-168]

Many studies start treatment of their xenograft experiments at D1 (the day after cell transplantation), we believe that it is important to let the tumors establish before the treatment start, for the study to be relevant in a clinical perspective. When a patient is diagnosed a tumor has already formed and it is there for of uttermost importance to treat a formed tumor instead of during tumor development. There is a great difference in studying tumor formation or establishment compared to studying the effect of a treatment on an already formed tumors. The xenograft model we used in our study is a good model to study the effect of systemic delivery of a drug on tumor growth. However, there are limitations when using this subcutaneous xenograft model, as the tumors is located in a different organ and therefor environmental factors may influence the results as well as no proper metastasis formation. Another approach that could be beneficial is orthotopic xenografts, where the NB cells instead are injected in the renal capsule [169].

Pediatric neuroblastoma remains a therapeutic challenge and there is a need for new treatment strategies. The low mutation load and the incomplete understanding of what dives neuroblastoma pathogenesis restricts the potential therapeutic targets. In this thesis I have focused on investigating alternative mechanisms such as DNA methylation of promoters of putative tumor suppressors or fusion transcripts and proteins in the quest to further the understanding of neuroblastoma biology and find new potential therapeutic targets.

5 ACKNOWLEDGEMENTS

I would like to start by thank **Karolinska Institutet** and **Ludwig Institute for Cancer Research** for all these years of studies and all these labs that I have had the privilege to work in. Researching adipose tissue, turnover of neurons in the human brain and finally trying to figure out the underlying mechanisms of pediatric cancer. I have really enjoyed my time here and I'm so grateful for all the people from all over the world that I have gotten to know.

I would like to thank my supervisor **Johan Holmberg**, for allowing me to be part of your lab and letting me travel the world and present our work at different courses and conferences. I´m always so impressed with the brilliant questions you ask at every lecture you attend. Also thanks to my co-supervisors **Eva Hedlund** and **András Simon** and all the different founders which make the research possible.

I have spent over half a decade of my life in the **Holmberg lab** and there has been many ups and downs. I hope that you guys will organize ski trips and "lab-day-out" even after I´m gone. **Ulrika;** you have been more important than you think, thank you for being my mentor and friend, listening to me and giving me advise. **Konstantinos;** you are the toughest of all the Tough Vikings, we started our master together and I'm super happy that you decided to join the lab for your PhD. I really enjoyed the Christmas-challenge with you, all the lunches and planning the ski trip. **Yao;** my bench-mate for so many year, thank you and good luck with everything. **Juan, Vilma**, **Anton** and **Erik** it´s been a pleasure, hope to see you guys soon.

Thank you to the **Schiliso group** for all the lab meetings and Christmas dinners we have shared together. **Petra:** I have liked the lunches together, I´m hoping that we will continue with them, you will have to take over the role at the "xenograft-champion" when I´m gone. **Shuijie:** the ping-pong and air-hockey master, I will always remember our "lab-day-out" with the canoes. **Olga, Veronica, Stuart, Karin, Wenyu** and **Oscar** thank you for all the time we have shared in the lab and office.

To everyone that has worked at Ludwig over the years thank you. What an experience it was being at Ludwig. A special thanks to the Ludwig people **Charlotta, Jorge, Erika, Eliza, Soheila** and **Mats**. To the **Muhr** and **Perlmann groups**; **Maria**; you are amazing, thank you for everything, we need to get out to Anki together soon. **Danny** and **Linda:** thank you for always helping out when needed. **Maria P, Lina, Idha, Katarina, Sussie, Cécile, Nigel** and everyone else thank you for everything.

To all the **CMB PUB Crew members** over the years for giving me a reason to paint myself all blue and wearing my leopard underwear on top of my golden leggings… not only at home but also in public ;-) **Giuseppe;** for being an amazing friend, your always there for me and I love our lunches and fika-sessions. **Goncalo;** all our lunches at Könings, I still have a cupong left. **Yildiz, Pedro, Milos, Christina, Steffi, Helena, Anna, Milind, Thibaud, Christina** and **Ingrid**…I have had so much fun with all of you; our beer runs, crazy decorations,

accidentally locking the cleaning lady in the elevator and late night cleaning sessions, I will miss it all.

Tack till all personal på **AFL** för att ni alltid hjälper till när det behövs, alltid ett leende när vi möts och aldrig några problem som inte går att lösa. Jag har spenderat många kvällar och helger hos er på AFL med mina naken möss. Ett speciellt tack till dig **Gun**.

To **Kirsty Spalding** and lab members **Paulina, Maria, Iva** and **Qian** I had a great time in the Spalding lab, our Friday lunches out were the best.

Till **Dfind gänget**; en underbar månad spenderade jag hos er, jag tror aldrig att jag har haft sådan träningsvärk i kinder och käkar efter att ha pratat och skrattat så mycket som jag gjort hos er. Ett speciellt tack till **Patricia, Sofie** (tack för den fina illustrationen) och **Julia** för att ni tagit hand om mig och känt mig så välkommen.

Kristina och **Hannah** tack för allt, jag älskar att jag har kunnat dela denna resa mer er, det har varit tufft. Men att ni är med betyder mycket, i vått och torrt <3

Biobrudarna **Anna, Evelina** och **Ida** min ”Skövde-familj” tänk att vi fortfarande hänger ihop efter alla dessa år. Älskar våra biobrud-helger ihop runt om i Sverige. Till och med när jag bodde i San Francisco var jag med på förfesterna via Skype. Ni är bäst.

Anita och **Svante**; tack för att ni har välkomnat mig in i er familj.

Thomas <3 tack för allt stöd och hjälp under alla dessa år, jag hade inte klarat det utan dig. Ser fram emot fler år och många fler äventyr med dig. Älskar dig.

Farmor och **Farfar** tack för att ni alltid finns där för mig <3
Mormor; jag vet att du är extra stolt just nu.

Mamma, **Pappa** och **Marcus** älskar er! Tack för att ni alltid är där för mig <3 Ni är bäst, vem skulle jag vara utan er.

ÄNTLIGEN!!

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