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Oncogenic Pathways and Molecular Prognostics in Neuroblastoma

Kristoffer von Stedingk

LUNDS UNIVERSITET

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Department of Laboratory Medicine Malmö, Translational Cancer Research, Lund University, Sweden.

To be defended at Building 302 Lecture hall, Medicon Village, Lund.

Thursday 10th of October, 2013, at 13:00.

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Abstract

Neuroblastoma is an embryonal malignancy that accounts for 15% of all cancer related deaths amongst children. Although the overall survival of patients has been improving over the last decades, the high-risk neuroblastoma patients have a survival rate of <50%.

Using gene expression microarrays we identify a group of proteins (snoRNPs) whose expression correlates with poor prognosis. We futher show that the snoRNPs are involved in regulation of telomerase activity in neuroblastoma cells. Upon snoRNP knockdown there is an observed increase in anaphase bridge fromation, indicitive of elevated genetic instability. Examination of genes associated with good prognosis revealed genes involved in growth cone formation. Combination of the expressoin of growth cone associated genes with the snoRNPs resulted in a 4-gene prognostic signature. Calculating the ratio (R-score) between the expression of the good and bad prognostic genes removed the need for housekeeper normalization, and provided a means of individual patient analysis. Application of a fixed-value R-score to 3 independent cohorts using standard qPCR revealed its functionality on an individual patient basis, as well as identified a subgroup of ulta-high risk patients who could potentially benefit from new treatment modalities.

Amongst high-risk neuroblastomas is a subgroup of patients harbouring *MYCN*-amplification. Here we show that *MCYN*-amplified tumours have elevated expression of the miR-17-92 cluster of miRNAs. High-throughput proteomic analysis of miR-17-92 overexpressing cells revealed enrichemnt of the TGF-β pathway. Further analyses showed miR-17-92 targeted inhibition of the TGF-β pathway at multiple levels, resulting in increased tumourigenic capacity of the neuroblastoma cells.

Using primarily breast cancer cells, we identified a hypoxia driven induction of the Notch-ligand *JAG2*. Deminished expression of *JAG2* in hypoxic tumour cells resulted in a reduced capacity of neighbouring endothelial cells to form tubes. Evaluation of these results in neuroblastoma revealed a similar pattern of Notch-ligand dependent crosstalk between tumour and endothelial cells, however in this case with via *DLL1*.

Here we have investigated, with a focus on high-risk patients, key signalling patways that are involved in the maintenance and progression of the disease. In addition, we describe a novel prognostic signature that has clinical implications for specifically high-risk patients.

Key words: Neuroblastoma, Prognostics, Telomerase, snoRNP, miR-17-92, TGF-β, Notch, Hypoxia, Angiogenesis

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Oncogenic Pathways and Molecular Prognostics in Neuroblastoma

by

Kristoffer von Stedingk

LUNDS UNIVERSITET

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“The weaknesses include the two investigators”
-Reviewer 2

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I SnoRNPs regulate telomerase activity in neuroblastoma and are associated with poor prognosis.

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II Individual patient risk stratification of high-risk neuroblastomas using a four-gene ratio score.

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Mol Cell. 2010 Dec 10;40(5):762-73.

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List of Abbreviations

ABC	ATP-binding cassette
ADAM	ADAM metallopeptidase
AKT	v-akt murine thymomo viral oncogene homolog 1
ALK	Anaplastic lymphoma kinase
ALT	Alternative lengthening of telomeres
ARNT	Aryl hydrocarbon receptor nuclear
ASCL1	Achaete-scute complex homolog 1
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
BCL2	B-cell CLL/lymphoma 2
BCL6	B-cell CLL/lymphoma 6
bHLH	Basic helix-loop-helix
BIM	BCL2-like 11
BMP	Bone morphogenetic protein
ccRCC	Clear cell renal cell carcinoma
CD133	Prominin 1
CD31	platelet/endothelial cell adhesion molecule 1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
ceRNA	Competitive endogenous RNA
CLL	Chronic lyphocytic leukemia
CNS	Central nervous system
CSL	Recomination signal binding protein for immunoglobulin kappa J region
DBH	Dopamine b-hydroxylase
DICER1	Dicer 1, ribonuclease type III
DKC1	dyskeratosis congenita 1, dyskerin
DLK	Delta homolog-like-1
DLL	Delta-like
DNA	Deoxyribonucleic acid
Drosha	drosha, ribonuclease type III
E-cadherin	Cadherin 1, type 1, E-cadherin (epithelial)
ECM	Extra-cellular matrix
EFS	Event free survival
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
FIH	Factor inhibiting HIF
GAP43	Growth associated rotein 43
GAR1	GAR1 ribonucleoprotein
GAS5	Growth arrest-specific 5 (non-protein coding)
GLUT1	Solute carrier family 2 (facilitated glucose trasnporter), member 1
GLUT3	Solute carrier family 2 (facilitated glucose trasnporter), member 3
GSI	Gamma-secretase inhibitor
HAND2	Heart and neural crest derivatives expressed 2
HES	Hairy and enhancer of split
HEY	hairy/enhancer-of-split related with YRPW motif 1
HIF	Hypoxia-inducible factor
HRE	Hypoxia-response element
ICN	Intracellular notch domain
ID	Inhibitor of differentiation
INRGSS	International neuroblastoma risk group staging system
INSS	International neuroblastoma staging system
JAG	Jagged
MAML	Mastermind-like
MAPK	Mitogen activated protein kinase
MDR	Multi-drug resistance protein

miRNA	Micro ribonucleic acid
MMP	Matix metalloproteinase
MRE	microRNA recognition element
mRNA	messanger ribonucleic acid
mTOR	Mammalian target of rapamycin
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
NAF1	Nuclear assembly factor 1 ribonucleoprotein
ncRNA	non-coding ribonucleic acid
NF	Neurofascin
NGF	Nerve growth factor
NHP2	NHP2 ribonucleoprotein
NOP10	NOP10 ribonucleoprotein
NPY	Neuropeptide Y
NSCLC	Non-small cell lung cancer
nt	Nucleotide
NT3	Neurotrophin 3
NUMB	Numb homolog (Drosophila)
ODD	Oxygen-dependent degradation domain
OS	Overall survival
p53	Tumor protein p53
PDGF	Platelet-derived growth factor
PECAM1	platelet/endothelial cell adhesion molecule 1
PHD	Prolyl hydroxylase
PHOX2A	paired-like homeobox 2a
PHOX2B	paired-like homeobox 2b
PI3K	Phosphotidylinositol 3 kinase
PTEN	phosphatase and tensin homolog
qPCR	Real-time quantitative polymerase chain reaction
RAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RUVBL1	RuvB-like 1 (E. coli)
RUVBL2	RuvB-like 2 (E. coli)
SIF cells	Small intensly fluorescent cells
siRNA	small interfering RNA
SMAD	Small mother against decapentaplegic
SNAIL	Snail family zinc finger
snoRNA	Small nucleolar ribonucleic acid
snoRNP	Small nucleolar ribonucleoprotein
SNS	Sympathetic nervous system
STMN2	Stathmin-like 2
T-ALL	T-cell acute lymphoblastic leukemias
TARBP2	TAR (HIV-1) RNA binding protein 2
TCA cycle	The citric acid cycle
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TGFBR	Transforming growth factor beta receptor
TH	Tyrosine hydroxylase
TRKA	Neurotrophic tyrosine kinase, receptor type 1
TRKC	Neurotrophic tyrosine kinase, receptor type 3
TSG	tumour suppressor gene
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel Lindau
XPO5	Exportin 5
ZEB	Zinc finger E-box binding homeobo

Background

Brief Introduction to Cancer

The word cancer encompasses a wide variety of diseases, which together are one of the leading causes of death worldwide. Recent statistics estimate 7.5 million cancer-related deaths per year. Notably, this number is on the rise, and the number of cancer-related deaths is predicted to almost double by 2030 [1].

Although being a variety of diseases, cancers as a whole are characterized by a number of common features. First of all, cancers have a genetic basis, meaning that they are a result of deregulation of normal gene expression. While the underlying mechanisms behind the genetic abnormalities may vary, including DNA sequence mutations, genomic deletions and amplifications, and epigenetic alterations, the result in often the same; uncontrolled proliferation and immorality. Hanahan and Weinberg provided an updated description of the common characteristics ("hallmarks") of cancer, which in addition to proliferation and immortality included metastatic capacity, angiogenic potential, and genomic instability amongst others [2]. This thesis will address the regulation/deregulation of these cancer-characteristics in the childhood cancer neuroblastoma. In addition, the relevance of these processes in the development, progression and prognostics of neuroblastoma will be discussed.

Development of the sympathetic nervous system

During early embryogenesis, the ectoderm undergoes a folding process giving rise to the neural tube. At the edge of the folding ectoderm a structure known as the neural crest is transiently formed. These neural crest cells migrate throughout the body and are incorporated into almost every organ. During the migration process the neural crest cells undergo differentiation, giving rise to melanocytes, schwann/glial cells and sympatho-adrenal progenitor cells from which the sympathetic nervous system (SNS) is derived. Being one of the multiple pieces of the autonomic nervous system, the SNS is specifically responsible for an organism's "fight or flight" response. The SNS consists of three cell types including chromaffin cells (adrenal medullary cells), small intensely fluorescent (SIF) cells and sympathetic neurons (referred to as neuroblasts

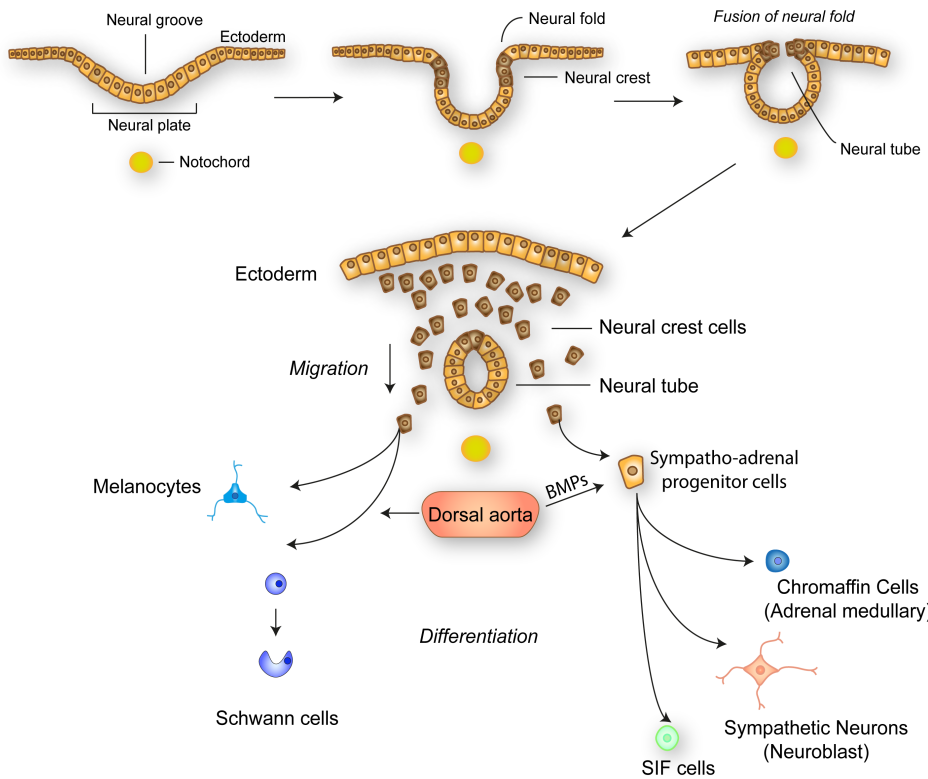

Figure 1: *Schematic of the embryonic development of the sympathetic nervous system (SNS). The cells of the SNS originate from neural crest cells derived from the ectoderm during the formation of the neural fold. Signals (such as BMP) encountered during migration along the neural tube/notochord/dorsal aorta result in commitment of neural crest cells towards a sympatho-adrenal lineage (sympatho-adrenal progenitor cells), which will give rise o the different cell types of the SNS.*

during embryogenesis) [3]. The commitment of neural crest cells towards a sympatho-adrenal lineage is triggered by local secreted factors encountered as they migrate along the neural tube towards the dorsal aorta (Figure 1). Early secreted initiating factors include the bone morphogenetic proteins (BMPs) [4]. This is supported by the observation that neural crest cells expressing a constitutively active BMP-receptor differentiate towards a sympathetic neuron phenotype [5]. Once activated by BMPs, a cascade of events is triggered including migration of the cells to their destined locations (adrenal medulla, paraganglia and sympathetic ganglia) as well as activation of key transcription factors involved in driving neuronal differentiation [6]. Activation of these pro-neural transcription factors eventually results in the expression of neuronal proteins such as STMN2, GAP43 and NF [7] as well as the enzymes TH and DBH involved in the synthesis of catecholamines, a defining characteristic of neuronal cells of the SNS [8,9]. First following BMP stimulation is activation of the basic helix-loop-helix (bHLH) transcription factor *ASCL1* [8]. Knockout experiments of *Ascl1* in mice revealed a loss of sympathetic neurons [10]. Further studies have revealed that *Ascl1*'s impact on neuronal differentiation is at least in part via activation of the transcription factor *Phox2a* [11]. While the BMP, Ascl1, Phox2a cascade does contribute sympatho-adrenal progenitor cell differentiation towards a neuronal lineage; this alone is not sufficient to induce full differentiation [12]. BMP activation has also been shown to drive expression of pro-neuronal markers in an *Ascl1*-independent manner, via *Phox2b* [7]. While Phox2b, like Ascl1, is able to activate Phox2a, Phox2b has also been shown to directly induce the expression of *Hand2*, which when overexpressed is sufficient to drive neuronal lineage differentiation [13]. Through a collaboration of these different BMP driven signalling pathways, the neuronal specific lineage of the sympatho-adrenal progenitors is determined. However, before terminal differentiation of sympathetic neurons is completed, exposure of developing sympatho-adrenal progenitor cells to surrounding bFGF and IGF results in the expression of the neurotrophin receptor Ntrk3 (TrkC). Through the subsequent stimulation of TrkC by the neurotrophin NTF3, Ntrk1 (TrkA) expression is induced. As a result, the cells are then rendered responsive to surrounding NGF [14]. Depending on the availability of NGF the neuroblasts are either stimulated, resulting in survival and differentiation, or undergo apoptosis [14,15]. Disruption of the differentiation/survival and apoptotic signalling in these embryonic neuroblasts is thought to be the cause of neuroblastomas [16,17].

Part I – The Disease

Neuroblastoma

What is Neuroblastoma?

Neuroblastoma is an embryonic malignancy that first presents in children under the age of 15 and accounts for 7% of all childhood malignancies. However, during the first year of life, neuroblastoma is the most commonly diagnosed cancer, with 90% of neuroblastomas occurring before the age of 5. Although being a relatively uncommon disease, neuroblastoma is the cause of up to 15% of cancer-related deaths amongst children [18-20]. With regards to clinical presentation, neuroblastoma is extremely heterogeneous ranging from children presenting with localized disease and extremely good prognoses, to children presenting with widespread metastases and a survival rate of approximately 40-50% [20]. In addition, a subset of patients presenting with metastases are characterized by spontaneous regression accompanied with a good outcome [18]. The principal sites of primary tumour occurrence include the adrenal gland, abdomen and sympathetic ganglia. Based on the location of the primary tumours and their characteristic secretion of catecholamines, neuroblastomas have

long been considered to arise from developing cells of the sympatho-adrenal lineage of the neural crest [18,19,21,22]. Early reports used immunohistochemical approaches to show that neuroblastomas expressed markers observed during development and differentiation of the neuronal lineage of SNS progenitor cells [16,23]. In general, expression of markers that are expressed in later stages of differentiation, such as TRKA and GAP43, is indicative of a better prognosis [24,25]. In 2006, De Preter et al. performed a study where they isolated human foetal neuroblasts as well as chromaffin cells from the developing adrenal gland [17]. Using gene expression array analyses they displayed significant overlaps between neuroblastoma and foetal neuroblast expression patterns, supporting the neuroblast cell of origin theory.

Genetics

Neuroblastomas are characterized by a surprisingly low prevalence of recurrent mutations in comparison to other malignancies [26,27], which makes the task of therapeutic target identification difficult. In addition, heritable neuroblastomas are rare accounting for less than 2% of cases [28-30]. Despite this, a number of genetic events associated with the disease have been identified. Not surprisingly, and in support of the neural crest derived cell of origin, the first identified mutation associated with familial neuroblastoma was in the *PHOX2B* gene [31]. As discussed above, *PHOX2B* is one of the earliest transcription factors involved in the differentiation of the sympatho-adrenal precursors [32]. Although being the first described, *PHOX2B* mutations are not the most common aberration in familial neuroblastomas. Recently, mutations in the anaplastic lymphoma kinase (ALK) gene were reported in approximately 50% of hereditary neuroblastomas [33-35]. Interestingly, *ALK* and *PHOX2B* expression are positively correlated in both neuroblastoma cell lines and primary material. Furthermore, PHOX2B has been shown to induce *ALK* expression [36]. The occurrence of multiple mutations within a common signalling pathway highlights its importance in the development of familial neuroblastoma.

With regards to sporadic (non-familial) neuroblastomas, which account for 98% of the disease, no gene has had as much attention as *MYCN*. In the early 1980's, the *MYCN* gene was reported amplified in neuroblastomas [37-40]. MYCN is member of the MYC-family of basic helix-loop-helix (bHLH) transcription factors. This family of proteins is involved in the regulation of a variety of cellular process including proliferation, apoptosis and differentiation, and it has been estimated that MYC proteins can regulate up to 15% of all protein-coding genes [41]. Today it is estimated that approximately 20-25% of neuroblastomas harbour amplification (greater than 10 copies) of the *MYCN* gene [19]. Much like the genes already discussed with relevance to familial neuroblastoma, MYCN has been implicated to

have roles in the developing neural crest where it is involved in differentiation and proliferation [42]. *In vitro* studies have shown that upon knockdown of MYCN, neuroblastoma cells undergo spontaneous differentiation suggesting a role for maintaining neuroblastomas in an immature neuroblast-like state [43,44]. In addition, ectopic expression of *Mycn* under control of the sympathetic neuronal marker *Th* was used to develop the first mouse model of neuroblastoma, implying that amplification of *Mycn* alone is sufficient to initiate neuroblastoma development [45]. Patients harbouring *MYCN*-amplification are associated with poor prognosis and often present with widespread metastases already at diagnosis. *ALK* mutations, in addition to being present in a large proportion of familial neuroblastomas, are also present in approximately 10% of sporadic cases. Functional studies of *ALK* mutations have revealed a gain of function effect, resulting in increased cell proliferation in neuroblastoma cell lines. In addition, mutation of *ALK*, which often occurs in combination with *MYCN*-amplification, was recently shown to increase the penetrance and accelerate onset of disease in a *MYCN*-driven model of neuroblastoma in zebrafish [46]. This is in agreement with what is observed in clinic where patients presenting with *ALK* mutations in combination with *MYCN*-amplification have an extremely poor prognosis [47]. With regards to older neuroblastoma patients, mutations in the chromatin-modifier *ATRX* are amongst the most common, with 44% of patients over the age of 12 years presenting with *ATRX* mutations [26,48]. Interestingly, *ATRX* mutations are virtually non-existent in patients under the age of 18 months. In line with age at diagnosis, *ATRX* mutations are also associated with a poor prognosis. While specific mechanisms behind the tumourigenic effects of *ATRX* mutations in neuroblastoma patients remain elusive, ATRX has been shown to be involved in telomere regulation in other cell types [49,50]. Potential involvement of *ATRX* mutations in genetic instability through telomere maintenance is discussed in more detail below.

Shifting focus from specific gene involvement in the biology of neuroblastoma to broader genetic aberrations, multiple recurrent events have been described. Firstly, the overall ploidy of a neuroblastoma provides key biological information, with low- and intermediate risk-patients presenting with almost strictly numerical changes (near triploid). On the other hand near diploid or tetraploid tumours often harbour segmental aberrations and are associated with a poor prognosis [51]. Common among these segmental aberrations are gain of 17q (occurring in approximately 50% of sporadic neuroblastomas), as well as losses on 1p and 11q. As is reported with *ATRX* mutations, 11q losses are often observed in *MYCN* non-amplified and genetically complex tumours in older patients [52]. Recently, Molaaner et al. described an additional pattern of genomic abnormalities involving massive chromosomal rearrangements, in approximately 15-20% of high-risk patients [48]. This phenomenon, referred to as chromothripsis has been described in other cancer forms, however at a much lower frequency [53]. The relevance of this aberration in

neuroblastoma biology remains to be investigated, and other sequencing investigations report a much lower frequency in neuroblastomas [27].

Staging and Prognostics

In the 1970's, getting the diagnosis of neuroblastoma, regardless of stage, meant a dismal outcome for the patient with a 5-year survival of only 50%. Thankfully, as with most cancers, the prognosis for neuroblastoma patients has been improving over the years, increasing to an overall survival of 75% during the 1990's and early 2000's. This observed improvement is attributed to the development of better treatment regimens for the low-risk patients. Unfortunately, the same cannot be said for the high-risk patients, who today have an overall survival of 40-50% [18,20].

With regards to determining a patient's risk-group upon diagnosis, there are multiple clinical factors that must be considered. The first, and most reliable factor is the age at diagnosis, with all patients diagnosed at an age of over 18 months having a much shorter life expectancy [54,55]. Metastatic spread at diagnosis is also an important factor with regards to patient prognosis, and serves as the basis for the standard international neuroblastoma staging system (INSS) [19,20]. In brief, the INSS divides patients into 5 main stage groups, stages 1 through 4, and the less-understood stage 4S. Patients presenting with widespread metastases at diagnosis, occurring anywhere along the SNS and in the bone marrow are defined as stage 4 tumours. Patients with stage 4 disease have the worst prognosis of all the stages with an overall survival of 40-50% [18]. Stage 3 patients are identified based on a lesser extent of disease dissemination, however still presenting with metastases beyond the local lymph nodes. These patients in general have a better prognosis than stage 4 patients, however this is dependent on presence of other factors such as age at diagnosis and *MYCN*-amplification status. Localized tumours, with or without lymph node involvement, are classified as stages 2 and 1, respectively. These patients have a good prognosis with an overall survival of over 90%. The fifth group of patients, diagnosed under the age of 12 months and presenting with metastases to the liver and skin, with little bone marrow involvement, are classified as stage 4S. These tumours are characterized by spontaneous regression, a process that to date is not fully understood. As a result, these patients also have a good prognosis with survival rate of over 90% [18,51]. In addition to INSS, a recent additional staging system has been described: the international neuroblastoma risk group staging system (INRGSS) [56,57]. This staging system takes into consideration imaging data that provides information to surgeons on the likelihood of complete tumour resection.

As mentioned above, the genetics of neuroblastomas also contains substantial prognostic information that must be considered by the clinicians. With regards to overall DNA content, early studies revealed that patients presenting with whole

chromosome alterations (near triploid) are generally associated with lower stages of disease and a better prognosis. Patients with segmental aberrations and a near-diploid or -tetraploid genome are associated with higher stages and a poor prognosis [58]. *MYCN*-amplification is also an extremely important factor with regards to determining a patient's prognosis. Patients presenting with >10 copies of the *MYCN*-gene are associated with poor prognosis regardless of stage [19]. As a result, *MYCN*-amplification can be the deciding difference between a low stage and high stage classification with survival rates of >90% and 50%, respectively [51]. Interestingly, recent studies have reported that in addition to *MYCN*-amplification, overall MYC pathway activity provides additional prognostic information [24,59,60]. Fredlund et al. described that patients without *MYCN*-amplification can still have high MYC activity, which is also associated with a poor prognosis. Mechanisms behind this elevated MYC activity in the absence of amplification are still a matter of debate. However it has been suggested that this phenomenon is a result of increased MYC levels, as well as increased MYC/MYCN protein stability [24,60].

In addition to *MYCN*, there are multiple other genetic aberrations that provide prognostic information in the clinic, such as 1p deletion (often co-occurring with *MYCN*-amplification), 11q deletion and gain of 17q. All of these genetic events are observed in tumours with a near-diploid or -tetratploid genome and are associated with a poor prognosis [51].

Recent studies have also focused on a different line of prognostics involving the implementation of expression-based gene signatures. Many signatures have been described using a wide variety of approaches. For example, differential expression based on biological features such as differentiation status, tumour hypoxia and MYC pathway activity, as well as clinical features such as age at diagnosis and overall survival [24,60-65]. Unfortunately, the one feature that all the signatures have in common is their absence from the clinic. The reasons for this are many, including complexity of the assays, lack of additional information to the current stratification schemes, as well as inability to function on an individual patient basis. This topic of discussion is addressed at length in papers I and II.

Therapy

Treating neuroblastoma patients is a difficult task. With regards to developing and testing new therapies, the low number of patients and the sensitivity surrounding testing new approaches in children are issues that are not easy to overcome [51]. In addition, differences in drug metabolism, toxicities and potential for late effects must be considered when transferring therapies from adults to children. However, using the prognostic information described above, it is possible to classify neuroblastoma patients into different risk-groups requiring different approaches and intensities of

Table 1. Phenotypic and Genetic Features of Neuroblastoma, Treatment and Survival According to Prognostic Category				
	Prognostic Category			
Variable	***Low Risk***	***Intermediate Risk***	***High Risk***	***Stage 4S***
Stage*	1,2,3	1,2,3	3,4	4S
Pattern of Disease	Localized tumour; no *MYCN*-amplification	Localized tumour with locoregional lymph-node extension; metastases to bone marrow and bone; age at diagnosis <18 months; no *MYCN*-amplification	Metastases to bone marrow and bone; age at diagnosis > 18 months; *MYCN*-amplification may be present	Metastases to liveer and skin (with minimal bone marrow involvement); age at diagnosis<18 months; no *MYCN*-amplification
Tumour Genomics	Numerical aberrations	Numerical aberrations	Segmental aberrations	Numerical aberations
Treatment	Surgery	Surgery; Moderate-intensity chemotherapy	Dose-intensive chemotherapy, surgery, radiotherapy to primary tumour and resistant metastatic sitesM myeloablative chemotherapy with autologous hematopoietic stem-cell rescue; retinoic acid based therapy	Supportive care
Survival Rate (%)	>98	90 to 95	40 to 50	>90

**Stage categorization is a generalization and different stages could be categorized into different prognostic groups depending on additional clinical features. Table adapted from Maris, 2010* [17]. *Modifications include addition of Stage and MYCN-status.*

treatment regimens (Table 1). Briefly, low-risk patients presenting with localized tumours are usually treated with surgical resection resulting in a survival rate of >98%. Intermediate-risk group patients, including patients under the age of 18 months with lymph node involvement and in some instances metastases to bone/bone marrow are treated with surgery and moderate chemotherapy. With these treatments, intermediate-risk patients have a survival rate of >90%. High-risk patients, on the other hand, include patients over 18 months as well as patients that present with widespread metastases and/or *MYCN*-amplifications, are treated with a combination of surgery, intensive chemotherapy, radiotherapy as well as stem cell transplants. Despite this intense treatment plan, survival rates of high-risk neuroblastomas are <50%. With regards to stage 4S patients, these patients are provided supportive care. This implies a "wait and see" approach, where treatment is withheld unless are life-threatening paraneoplastic effects, such as damage to the liver. In such instances, low-dose chemotherapy and/or local radiation therapy are used, with a survival rate of over 90% [18,51]. In addition to the conventional therapies described above, some high-risk patients (those with a first time remission) are also treated using retinoic acid, with the hopes to induce terminal differentiation of the neuroblastoma cells

[66]. This therapy has had positive effects with a reported reduction in the risk for relapse, however prediction of patients that will respond to this treatment remains to be elucidated. In a recent study by Molenaar et al. they identified mutations amongst genes involved in neuritogenesis and growth cone formation, processes that are affected by retinoic acid [48]. Taking such genetic information into consideration could be of importance in identifying retinoic acid-responding patients.

Part II – Molecular Pathways and Processes

Hypoxia

What is hypoxia?

Hypoxia is a phenomenon that is extremely context and tissue dependent. A general definition of hypoxia is when the oxygen supply to a given cell, tissue or organism is less than that required to maintain a normal biological state. With regards to most human tissues, an oxygen level of approximately 5% is considered to be "normal". When levels drop to around 1%, the tissue is then considered to be hypoxic [67]. It has been reported that oxygen is capable of diffusing up to 150 μm through a tissue [68]. In a cellular context this is equivalent to approximately 10 cell layers from the oxygen source (blood vessel). Due to the growth and expansion that is characteristic of all virtually all solid tumours, recruitment and development of new vasculature (a process referred to as angiogenesis; discussed below) often lags behind, resulting in inadequate oxygen delivery and the formation of hypoxic regions. Starting at

approximately 200 μm from the nearest oxygen source, areas of necrosis can be observed within solid tumours [68]. Depending on the length of exposure to hypoxia, tumours respond in a variety of ways. During the acute phase of hypoxia tumour cells respond by secreting angiogenic factors such as vascular endothelial growth factor A (VEGFA) in order to initiate the recruitment of new blood vessels [69-71]. Although tumours are able to develop new vasculature, often at a higher density than in normal tissues, the vasculature is often immature and lacks supportive cells [72]. As a result the vasculature is only semi-functional and leaky, which over time leads to chronic hypoxic environments within the tumour [71,73]. During longer periods of hypoxia, additional cellular responses occur including a shift of metabolism towards glycolysis [74] as well as altered differentiation states [75,76]. These changes, in combination with the formation of new leaky vasculature, have been described to give rise to the aggressive phenotype associated with hypoxia [77,78].

HIFs and the hypoxic response

Control of the cellular responses to hypoxia is primarily carried out by the hypoxia-inducible factors (HIFs) [77]. HIFs are heterodimer proteins consisting of alpha and beta subunits. Three HIF-α proteins have been described including HIF1α [79], HIF2α (*EPAS1*) [80] and the less studied HIF3α [81]. HIF1α and HIF2α are basic helix-loop-helix (HLH)-PAS containing transcription factors. The N-terminal basic domain allows for DNA binding and the HLH-PAS domain is responsible for the interaction with the HIF-β subunit (HIF1β/ARNT) [82,83]. While all HIFs are constitutively expressed, in the presence of oxygen the HIF-α subunits are continuously degraded (Figure 2). This process is initiated by the hydroxylation of proline residues present in the N-terminal oxygen degradation domain (ODD) of the HIF-α proteins by prolylhydroxylases (PHDs). Once hydroxylated, the HIF-α proteins are recognized by the E3-ligase complex von Hippel-Lindau (VHL) resulting in their ubiquitination and subsequent proteasomal degradation [84]. However, under hypoxic conditions the lack of oxygen renders the PHDs incapable of hydroxylating the HIFs resulting in HIF-α stabilization. Once stabilized, the HIF-α subunits heterodimerize with ARNT leading to their translocation to the nucleus. Once in the nucleus the HIF/ARNT heterodimer recruits transactivators (CBP/p300) and binds to hypoxic response elements (HREs) in the promoters of their target genes. Binding of the complex to HREs results in transcriptional activation of such prototypical target genes as *VEGFA*, *GLUT1*, as well as genes involved in Notch signalling, all of which are involved in the hypoxic response processes described above [77].

In addition to the PHD-mediated HIF-α degradation, additional levels of HIF regulation exist. The factor inhibiting HIF (FIH) protein is an asparaginyl

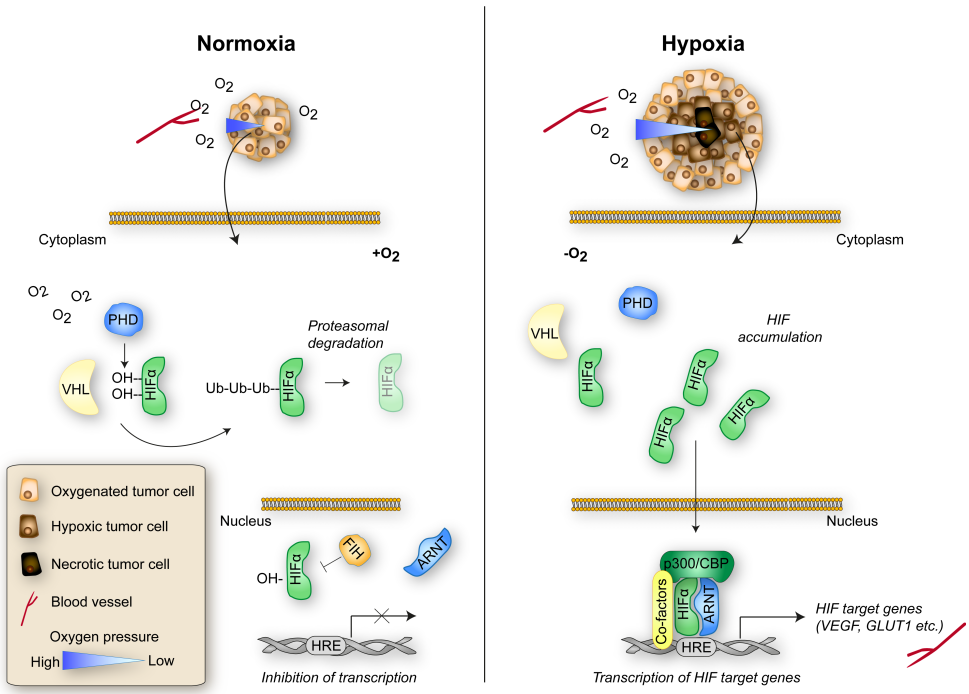

Figure 2. *Schematic of oxygen-regulated HIF activity. HIF proteins are constitutively expressed, however, under normoxic conditions HIF-α proteins are hydroxylated by PHDs leading to recognition by VHL and subsequent degradation. Under hypoxic conditions, the lack of oxygen renders the PHDs incapable of hydroxylation, resulting in HIF-α stabilization and dimerization with ARNT. Once in the nucleus the heterodimer interacts with transacivators p300/CBP as well as other co-factors, leading to HRE-binding and transcriptional activation of HIF target genes.*

hydroxylase that has been shown to hydroxylate aspargine residues in the C-terminal transactivation domain of the HIF-α subunits [85]. Upon transactivation domain hydroxylation, the ability of HIF-α proteins to interact with co-activating proteins is diminished, resulting in inhibition of target gene activation. Much like the PHDs, FIH requires oxygen for hydroxylation and is therefore rendered inactive under hypoxic conditions. Although oxygen-dependent HIF-regulation appears to be major determinant of HIF activity, studies have also reported HIF-regulation (transcriptional activation and/or protein stabilization) by signalling pathways independent of the presence of oxygen. Such pathways include RAS-, EGFR-, and INSR-pathways, all of which have downstream activation of the PI3K/AKT, mTOR and MAPK pathways in common [77]. To add to the complexity, specific target genes for HIF1α and HIF2α have been described, which can result in differential effects in a cell and context dependent manner [71,86]. The divergent effects of HIF1α and HIF2α will be further discussed below.

Hypoxia, cancer and aggressiveness

Hypoxia is a common feature of all solid tumours and has been associated with an aggressive phenotype in a variety of cancers including breast cancer, bladder cancer, colorectal cancer and neuroblastoma amongst others [87,88]. The association to aggressive phenotype has been extensively investigated and multiple underlying mechanisms have been proposed. As described above, the initial hypoxic response involves HIF-mediated activation of *VEGFA* resulting in recruitment and formation of new immature vasculature. The presence of the semi-functional vasculature has been reported to contribute to tumour aggressiveness in multiple ways. For one, due to the less-tight junctions between the endothelial cells (attributed to the reduced presence of supportive cells such as pericytes) the vasculature contains gaps, which provide tumour cells access to the circulation, potentially resulting in metastases formation [89]. In addition, this leaky vasculature results in increased intra-tumoural pressure making drug delivery within the tumour difficult [90]. This is likely a contributor to drug resistance often observed with advanced disease. Hypoxia's effects on drug resistance may also have a more direct route through HIF-dependent activation of multi-drug resistant (MDR) proteins and ATP-binding protein (ABC) transporters. These proteins have been shown to regulate both the cellular influx and efflux of drugs and expression of ABC proteins has been reported to be associated with poor prognosis in multiple tumour types including neuroblastoma [91,92]. Hypoxia-induced alterations to cellular metabolism have also been associated with tumour aggressiveness. A metabolic shift towards glycolysis, in part orchestrated by HIF activation of glucose transporters *GLUT1* and *GLUT3*, provides the cells with a growth advantage under low oxygen conditions [74]. Through the process of glycolysis cells divert from using the citric acid (TCA) cycle, where ATP production is the main objective, and instead focus the use glucose to produce an abundance of building blocks required for the rapid cell division [93]. In addition, glycolysis results in the production and excretion of lactic acid leading to a lowered surrounding PH and elevated degradation of the extra-cellular matrix (ECM) likely contributing to the cancer's metastatic potential [94]. Along with elevated degradation capacity, tumour cells may become more motile and/or less adherent under low oxygen conditions, an observation that has been attributed to HIF-induced dedifferentiation and/or epithelial-to-mesenchymal transition (EMT) [75,95].

With all associations between the hypoxic response and tumour progression, it is not a surprise that mutations affecting the HIF-pathway have been identified as drivers behind multiple malignancies. Such mutations can include activation-mutations along the PI3K, mTOR, MAPK pathways leading to increased *HIF* transcription [77] as well as the well-described inactivation mutations in *VHL* resulting in HIF-protein stabilization [96]. These *VHL* mutations have been reported in approximately 80% of all clear cell renal cell carcinomas (ccRCCs), in strong support of a driving role in the

development of the disease. Interestingly, studies of the HIF-networks in ccRCCs have revealed potentially opposing roles of the two HIF-α proteins. While both HIFs lead to activation of the hypoxic response, RCCs have demonstrated a selective pressure favouring HIF2α [97,98]. In fact, both primary RCCs as well as RCC cell lines often express only HIF2α, while RCCs solely expressing HIF1α have not been described [99]. *In vivo* xenograft studies have revealed that HIF1α expression in RCC cells is associated with diminished tumour growth while HIF2α expression is associated with a stem-like phenotype and increased aggressiveness [98,100]. These studies again highlight the differences between the two HIF-α proteins, an observation that is also described in neuroblastoma [71].

Hypoxia and neuroblastoma

Studies have indeed shown that neuroblastoma cells are capable of initiating a hypoxic response, with observed HIF1α and HIF2α protein expression under low oxygen conditions. Jögi et al. described that upon exposure to hypoxia, neuroblastoma cells appear to shift towards a dedifferentiated state, with lowered expression of sympathetic neuronal markers such as NPY and HAND2 and elevated expression of neural crest markers NOTCH1 and ID2 [75]. Of note, HIF2α has been reported to be expressed and play an important role during SNS development and may therefore be involved in the dedifferentiation process observed in neuroblastoma [75,101-103]. As is observed in RCC, expression of the HIF-α proteins under hypoxia in neuroblastoma cells is a dynamic process. During initial/acute exposure to hypoxia, HIF1α levels rise quickly. However, with onset of chronic hypoxia HIF1α levels decline with an accompanied rise in HIF2α [71]. Considering the often-chronic hypoxic environment observed in solid tumours, this would suggest that hypoxic effects on neuroblastomas might primarily be HIF2α driven. Interestingly, HIF2α stabilization has been reported in in the oxygen rich perivascular niche of neuroblastoma tumours, where its presence was associated with expression of early sympathetic progenitor markers such as *NOTCH1*, *HES-1* and *vimentin* [104]. This supported the notion that HIF2α may be involved in maintaining an undifferentiated phenotype in neuroblastoma. *In vivo* xenograft studies further revealed that upon *HIF2α* knockdown in neuroblastoma cells, there was observed neuronal differentiation accompanied with necrotic regions, again suggestive of a tumour-promoting role for HIF2α in neuroblastoma [105].

Notch

Overview of Notch signalling

The Notch pathway is a highly conserved pathway involved in contact-dependent cell-cell interactions. Notch was originally described in Drosophila where flies displaying "notches" in their wings were found to be heterozygous for the Notch gene. Studies since then have displayed the importance of Notch signalling in a variety of developmental and cellular process including limb development, neurogenesis, stem cell maintenance, proliferation, apoptosis and cell migration. Involvement of Notch in such process can be attributed to its juxtacrine characteristic of inducing differential signalling in neighbouring cells [106,107]. In mammalian cells there are four described trans-membrane Notch receptors (NOTCH1-4) [108]. The Notch receptors are composed of an extracellular domain containing EGF-like repeats required for ligand binding, an ectodomain, a trans-membrane domain and an intracellular domain. Regulation of the Notch receptors occurs at multiple levels [109]. Before transport to the membrane, Notch receptors undergo modification in the Golgi apparatus including a Furine-mediated cleavage leading to the formation of the extra- and intracellular domains of the receptor [110]. In addition, modulation by the Fringe glycotransferases is suggested to regulate differential receptor activation in a ligand-dependent manner [110,111]. Once in the membrane, NUMB and other ubiquitin ligases target Notch receptors, leading to endocytosis and degradation. In doing so, NUMB is involved in the control the number of expressed Notch receptors and therefore regulates the potential activity of the overall pathway [112].

There are 5 described Notch ligands including the delta-like ligands (DLL1, 3 and 4) and the jagged ligands (JAG1 and 2) [109]. These membrane bound ligands contain EGF-like repeats in their extracellular domains that allow for binding and activation of the Notch receptors on adjacent cells [111] (Figure 3). Upon ligand binding to a receptor, conformational changes to the receptor result in multiple protealytic cleavages. The first cleavage is carried out by the extracellular ADAM metalloproteases. The resulting additional conformational changes to the receptor open it for cleavage by the γ-secretase complex. Following cleavage by gamma-secretase, the intracellular domain of Notch (ICN) is released, initiating Notch signal transduction [112,113]. Once released, ICN is translocated to the nucleus where it interacts with the DNA binding proteins CSL and MAML as well as additional co-activators and RNA polymerase II. Although being an extensively studied signalling pathway, identification of Notch target genes remains limited. However, Notch activation is often associated with the expression of two families of bHLH transcriptional repressors, Hair/Enhancer of Split (*HES*) and Hairy-related (*HEY*) [114-116].

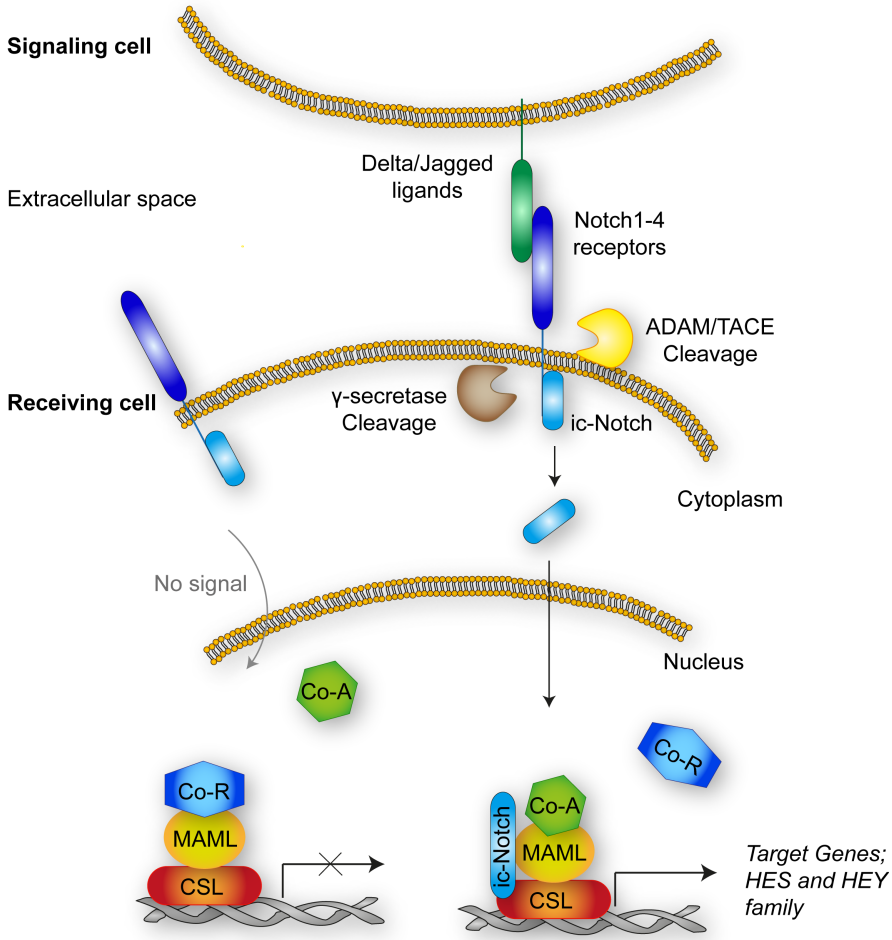

Figure 3. *Schematic of Notch signalling pathway. Juxtacrine signalling between adjacent cells involves expression of a Notch ligand on one cell binding and activating a Notch receptor on a neighbouring cell. Ligand-receptor binding initiates a sequence of protealytic cleavages on the Notch receptor carried out by ADAM and γ-secretase, resulting in the release of the intracellular domain of the receptor (ic-Notch). Once in the nucleus ic-Notch interacts with co-factors MAML and CSL resulting in the replacement of co-repressors (Co-R) with co-activators (Co-A) leading to target gene transcriptional activation.*

In addition to the prototypical Notch signalling described above, many other aspects contribute to the regulation of this complex pathway. For one, atypical ligands exist including the most frequently described Delta homolog-like-1 (DLK1). DLK1, although having differences in its extracellular domain when compared to the typical Notch ligands, does contain EGF-like repeats, which allow for binding and regulation

of Notch receptors [117]. Another layer of complexity arises when discussing activation potential of the different ligands on the different receptors. As mentioned above, modulations to the receptors during maturation result in different activation capacities depending on which ligand is bound [112]. This process is especially relevant in the process of angiogenesis where DLL4 has been shown to be the most potent activator of Notch signalling when compared to the other ligands [118]. This scenario will be discussed in more detail in the angiogenesis section of this introduction. Although Notch is considered a cell-cell juxtacrine signalling pathway, studies have also investigated the effects of same cell ligand-receptor interactions as well as surrounding soluble ligands. In general, such interactions result in inhibition of Notch signalling due to sequestration of the receptors from their typical juxtacrine ligand activation [110,119-121]. Reports on CSL-independent Notch signalling [122] as well as overlaps and interactions with other signalling pathways such as RAS, PI3K and TGF-β all contribute further to the complexity of this central and conserved developmental pathway [123-125].

Notch and cancer

Being central in cell fate and developmental processes, defects in Notch signalling have been reported in multiple malignancies including blood, breast, brain and skin cancers, to name a few [126-129]. In T-cell acute lymphoblastic leukemias (T-ALLs) it was discovered that *NOTCH1* was involved in a chromosomal translocation (7;9), resulting in constitutive activation of ICN1 [126], although these translocations were found in only 1% of T-ALLs. This discovery lead to further studies that identified mutations in the *NOTCH1* gene leading to increased receptor cleavage, in 55% of all T-ALLs [130]. In breast cancers, elevated Notch signalling has been associated to a lower differentiation state and more aggressive disease [131]. Furthermore, elevated JAG1 expression and ICN1 staining have also been associated with poor prognosis and increased risk for recurrence [132]. In addition, lowered NUMB expression, involved in the degradation of Notch receptors, has been reported in up to half of all breast cancers, and was associated with an increased sensitivity to Notch inhibiting drugs including γ-secretase inhibitors (GSIs) [133]. In mouse models of breast cancer, expression of intracellular domains of the notch receptors in mammary epithelial cells resulted in a developmental block and eventual onset of tumour formation [134]. Similarly to breast cancer, reduced levels of NUMB expression have also been reported in medulloblastomas potentially resulting in elevated Notch activity [135]. In support of the relevance of Notch signalling in medulloblastoma, xenograft studies on Notch inhibition revealed decreased tumour growth and increased apoptosis upon treatment with GSIs. In addition, GSI treatment also lead to a reduction of CD133-expressing cells, indicating a potential role Notch signalling in the maintenance of medulloblastoma stem cells [136]. As opposed to the cancers described above, Notch

signalling in skin cancers has been associated with tumour-suppressive effects. *Notch1* knockout mice have an increased susceptibility for both basal cell and squamous cell carcinomas [137]. *In vitro* studies have also reported reduced proliferation and increased differentiation of mouse keratinocytes upon *Notch1* activation [138]. Taken together, these studies highlight the importance of Notch signalling in a variety of malignancies, however also show the extreme tissue-specific effects that this pathway may have. This is important when discussing the use of Notch inhibitors as a cancer therapy. Although GSIs have shown promising effects in clinic, severe side effects may also occur such as gastro-intestinal problems arising from deregulation of intestinal crypt stem cells [139]. Studies have however described reduced side effects to Notch inhibiting treatments when the GSIs are administered intermittently and in combination with corticosteroids [140,141]. In addition, recent studies have described the development of NOTCH1 decoys consisting of the NOTCH1 extracellular EGF-repeat domains functioning as competitive inhibitors for the Notch ligands [142]. It was suggested that due to the size of the molecules, they are more readily available within the vasculature than in the surrounding tissues such as the intestinal crypt cells mentioned above. Administration of such molecules would therefore allow for the anti-angiogenic effects of Notch-inhibition on tumour growth (discussed below) while avoiding off-target effects in other tissues.

Notch signalling in SNS and neuroblastoma

Both Notch ligands and receptors are expressed in the developing SNS, although little is known about their specific roles [143]. As has been described within the CNS, it has been proposed that Notch signalling is involved in the maintenance of sympatho-adrenal progenitor cells [144]. In support of this, mouse knockout studies targeting Notch components result in premature neuronal differentiation and a reduction in neuronal progenitors. In addition, overexpression of ICN1 in a chick embryo model of neuronal development resulted in an increased number of sympathetic ganglia progenitors [144]. Molecular studies have also displayed a role for Hes1 in the inhibition of *Ascl1* transcription, thereby preventing differentiation of neural crest cells towards sympathetic neuronal progenitors (described above) [145]. Taking into consideration the relevance of Notch signalling in the developing SNS, it seems reasonable to expect a role for Notch signalling in neuroblastoma. Indeed, treatment of neuroblastoma cell lines using the GSI DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) resulted in differentiation and neuritogenesis, suggesting a Notch-mediated maintenance of a dedifferentiated state [146]. In addition, overexpression of ICN1 renders neuroblastoma cells incapable of differentiating [147]. As previously mentioned, exposure of neuroblastoma cells to hypoxia results in a loss of neuronal markers (NPY and HAND2) with an increase in neural crest makers (NOTCH1, HES1 and HEY1) [75]. Furthermore, HIF2α

positive stem-like cells located in the perivascular niche of neuroblastoma tumours co-express *NOTCH1* and *HES1* [104]. Taking all into consideration, it seems possible that a combination of Hypoxia, HIF and Notch activation contribute to the maintenance of immature stem-like cells in neuroblastoma and may play a role in the aggressiveness of the disease.

TGF-β

Overview of TGF-β

With their name originating from their ability to transform normal cells into cells capable of growth in soft agar, the transforming growth factor-beta (TGF-β) family includes a variety of proteins including the TGF-β isoforms, bone morphogenic proteins (BMPs), amongst others [148]. Functioning as a typical ligand-receptor activated signalling pathway, the variety of members within the TGF-β family allows for its involvement in plethora of cellular processes including cell growth, differentiation, apoptosis, adhesion, migration, and EMT. The TGF-β ligands include TGFβ1-3, which are secreted and held inactive in the surrounding ECM. The TGF-β family of receptors are heterodimers and can be divided into three subtypes: TβRI, TβRII and TβRIII. Upon matrix metalloproteinase (MMP)-mediated release of TGF-β from the ECM, TGF-β binds a TβRII-family receptor (TGFBR2) resulting in heterodimerization with and transactivation/phosphorylation of a TβRI-family receptor (TGFBR1) (Figure 4). Once activated, the TGFBR1/2 complex relays the signal via the small mother against decapentaplegic (SMAD) proteins. The receptor associated SMAD2 and SMAD3 upon phosphorylation form a complex with SMAD4. The SMAD activated complex is then translocated to the nucleus where it binds cofactors (co-activators/co-repressors) leading to DNA binding and regulation of target gene transcription [149,150]. With regards to the TβRIII receptor family, these proteins lack a kinase domain however have been shown to have ligand binding capacity. By binding ligands TβRIIIs act as ligand presenters to TβRIIs thereby potentiating TGF-β signalling [150].

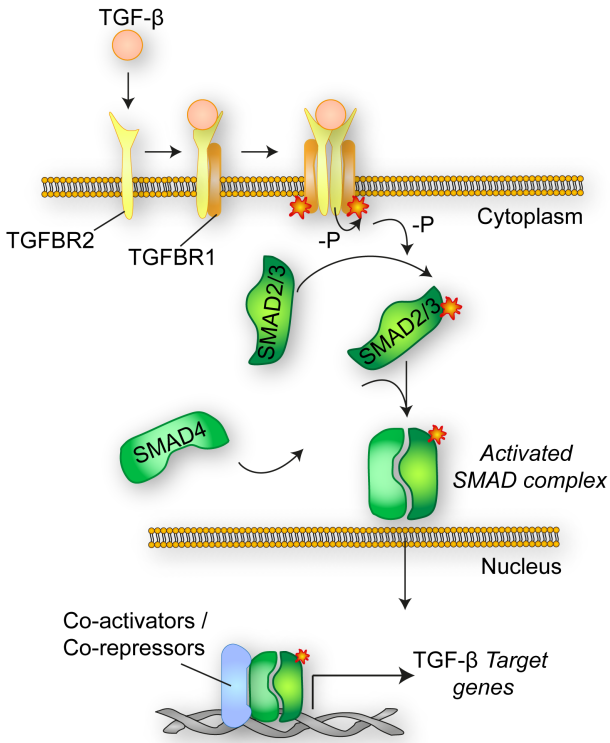

Figure 4. *Schematic of canonical TGF-β signalling. TGF-β binding to TGFBR2 results in TGFB2-TGFBR1 heterodimerization. Activated TGFBR complex phosphorylates SMAD2/3 leading to interaction with SMAD4 and translocation to the nucleus. In the nucleus the SMAD complex interacts with Co-activators/Co-repressors (context-dependent) resulting in transcriptional regulation of TGF-β target genes*

TGF-β and cancer

The discussion around the role of TGF-β signalling in cancer is complex and extremely cell type and context dependent. Studies have reported loss-of-function mutations within the TGF-β pathway in a variety of cancers including breast, colon and pancreatic cancers [151-153]. These mutations would be suggestive of a tumour suppressor gene (TSG) role for TGF-β signalling. Molecular studies into the mechanisms behind TGF-β's cellular effects have revealed that TGF-β signalling can result in up-regulation of cell cycle inhibitors such as p15 and p21, while at the same time repressing the expression of cell cycle drivers such as *MYC* [149]. In addition, reports have also described TGF-β-dependent expression of pro-apoptotic genes such

as *BIM*, as well as repression of the ID gene family, resulting in increased differentiation [154,155]. Taken together, TGF-β activity has been implicated in preventing proliferation, activating apoptosis and inducing differentiation, all characteristics of a prototypical TSG.

Interestingly, and in contrast to the studies discussed above, reports have described oncogenic roles of TGF-β signalling in multiple cancer types [156-158]. This is commonly attributed to TGF-β's capacity to induce EMT, resulting in a more motile and mesenchymal-like cell phenotype [159]. TGF-β-mediated SMAD activation has been shown to elevate the expression of transcriptional repressors such as *SNAIL* and *ZEB*. These genes in return repress the expression of E-cadherin (*CDH1*), which is a hallmark EMT. In addition to promoting EMT, TGF-β signalling has also been shown to induce *MMP* expression and thereby aid in the degradation of the ECM surrounding both the primary and metastatic sites [160]. The degradation of ECM is not only important for the migration of tumour cells, but also for the recruitment of new blood vessels to the growing tumour. Thus, through elevated of *MMP* expression, as well as TGF-β-dependent induction of *VEGFA*, TGF-β likely also plays a role in tumour angiogenesis [161]. In addition to providing the nutritional support to the tumour, the newly formed vessels also provide a potential "highway" for tumour cells to metastasize, once again implicating TGF-β in the metastatic process. Although TGF-β signalling is implicated in oncogenic processes, the cytostatic effects must be overcome in order for TGF-β to contribute to tumour progression. This provides reasoning to the idea that during early tumour formation TGF-β acts as a TSG, where as during progression of the disease these TSG effects are circumvented, turning TGF-β into a *bona fide* oncogene [162].

TGF-β in neuroblastoma

When investing retinoic acid (RA)-induced differentiation of neuroblastoma cells, it was noted that there was an up-regulation of TGF-β pathway components including TGF-β1 as well as the TGF-β receptors (TGFBR1-3) [163]. Following this observation it was reported in multiple studies that stimulation of neuroblastoma cells with TGF-β1 also resulted in induction of differentiation [164-166]. In addition, overexpression of TGFBR2 resulted in a differentiated phenotype and growth inhibition both *in vitro* and *in vivo* [165]. These results are corroborated by the observations presented in paper III of this thesis where we describe a MYCN-dependent inhibition of TGF-β signalling via induction of the miR-17-92 cluster of microRNAs (miRNAs) resulting in a more aggressive phenotype [167]. Confusingly, in recent work by Lynch et al. they also describe a MYCN-dependent regulation of TGF-β signalling via miRNA, however in a positive fashion [168]. In this case MYCN inhibits miR-335 expression, resulting in an up-regulation of non-canonical

TGF-β signalling through MAPK and Rho-associated coiled-coil containing protein (ROCK1). This non-canonical TGF-β activity was shown to have pro-migratory and invasive effects neuroblastoma cells, suggesting an oncogenic role for TGF-β. Although this may seem contradictory to the previous results described in neuroblastoma, these differential effects could potentially be explained by differences between canonical (SMAD-dependent) and non-canonical (SMAD-independent) TGF-β signalling. However, together these results indicate an intricate interplay between MYCN and TGF-β, potentially contributing to the aggressive behaviour of *MYCN*-amplified neuroblastomas.

Angiogenesis

What is angiogenesis?

Angiogenesis can be described as the process of producing new vessels from pre-existing vasculature. In the case of solid tumours, this process is continuously ongoing and is often referred to as neoangiogenesis. In brief, signals from the growing tumour interact with the surrounding vasculature resulting in stimulation and activation of the endothelial cells [73]. The activated endothelial cells undergo a cell-fate determination process (discussed at length below) becoming one of two types: a "tip cell", responsible for guiding the newly forming vessel, or a "stalk cell" responsible for vessel elongation and tubulogenesis (lumen formation) [169,170]. With regards to tubulogenesis, the mechanisms behind this process are still a matter of debate. What can be said is that lumen formation is a result of cytoskeletal reorganization in response to polarity establishment, most likely under the control of interactions with the surround ECM. Two models of how this process occurs have been proposed. The first model, referred to as "cell hollowing" involves an intracellular accumulation of pinocytotic vesicles within a given stalk cell, which when joined together form a central vacuole [171,172]. Fusion of vacuoles across adjoining cells then results in the formation of the vascular lumen. Cell hollowing works upon the assumption that the invading vessel is composed of a single polarized-layer of endothelial stalk cells. In contrast, the second proposed model of tubulogenesis, referred to as "chord hollowing", assumes a depolarization of stalk cells during the process of vessel elongation, resulting in a multicell-layered vessel [173,174]. Upon cues from the surrounding ECM, endothelial cell polarity can be restored with the basal membrane facing the ECM and the apical membrane forming at cell-cell junctions. It is at these

cell-cell junctions that intracellular vacuoles cluster, which when fused across multiple cells forms the new vascular lumen. In a review by Tung et al. the molecular mechanisms behind the different models of tubulogenesis are discussed in more detail [175]. Following vessel elongation and lumen formation, the final step of angiogenesis involves the recruitment of supportive perivascular cells such as pericytes. As discussed above, this final maturation step is often deregulated in tumours resulting in the "leaky-vessel" phenotype associated with neoangiogenesis [73,89,175].

Hypoxia, HIFS and angiogenesis

As described above in the discussions surrounding hypoxia, low oxygen levels within solid tumours often results in the initiation of neoangiogenesis. This initiation step is primarily under the control of hypoxia-induced HIF stabilization and activation of its target genes [70]. The most commonly described HIF target with regards to angiogenesis is *VEGFA*, which is transcriptionally induced through direct HIF-binding to an HRE in its promoter [69]. Production and secretion of VEGFA is a key event in the initial activation of resting endothelial cells, as well as the formation of the growth-factor gradient that provides direction to the newly forming vessel. In addition to their role in VEGFA production, HIFs have also been shown to elevate levels of other angiogenic factors including *VEGFR1*, *PDGFB*, *EPO* and *FGF2* [77,176,177]. Interplay between HIFs and other pathways can also have effects on neoangiogenesis, including HIF-Notch and HIF-TGF-β pathway interactions. Hypoxia is reported to have an elevating effect on Notch signalling at multiple levels, including up-regulation of Notch pathway components and its target genes [75,178-183]. As will be discussed below and in paper IV, regulation of the Notch pathway is central to the process of angiogenesis, and HIF-dependent Notch regulation results in observable effects in developing vasculature. With regards to HIF-TGF-β interplay, previous reports have indicated that hypoxia can induce elevated levels of TGF-β signalling in tumour cells [184]. As mentioned above, TGF-β activation can lead to *MMP* induction resulting in ECM degradation, a process required for invasion of the developing vessels [160].

Notch and angiogenesis

Upon initial VEGFA stimulation of endothelial cells, a cascade of events occurs including endothelial cell-fate determination resulting in tip and stalk cell formation. The Notch pathway tightly regulates this essential cell-fate determination in a process referred to as lateral inhibition [175]. Breaking this incredibly complex and dynamic process down into the key steps leading to cell fate determination, the initial

activation of VEGFR2 by VEGFA results in formation of filopodia and a non-proliferative cell state [185,186]. In addition, there is a rapid up-regulation of the Notch ligand *DLL4*. Upon localization to the membrane, DLL4 binds and activates NOTCH1 on the adjacent endothelial cell(s). Activation of NOTCH1 in return results in the down-regulation of VEGFR2 altering the cell's response to VEGFA stimulation. As apposed to the DLL4 expressing cells, these Notch-activated cells instead respond to VEGFA stimulation by proliferating [185,186]. Once these steps have been completed, the once homogenous population of luminal endothelial cells has now given rise to two cell populations; Firstly, a non-proliferative, VEGFA-sensitive and DLL4 expressing tip cell responsible for the guidance of the new vessel along the VEGFA gradient towards the hypoxic source. Secondly, proliferative, VEGFA-insensitive stalk cells responsible for vessel elongation. Taking all into consideration, Notch-activation results in a stalk cell phenotype, whereas low levels of Notch activation results in a tip cell phenotype. In support of this notion, multiple studies have shown that inhibition of Notch in endothelial cells results in an overrepresentation of tip cells leading to hyper-branching and an increase in vascular density [187-189].

In addition to the essential steps described above, other levels of angiogenic "fine-tuning" have been described. With focus on the NOTCH-activated stalk cells, up-regulation of *VEGFR1* has been reported. VEGFR1, although having a high affinity for VEGFA, only produces a low level of activity upon stimulation when compared to VEGFR2 stimulation. As a result, VEGFR1 acts in competitive manner to bind VEGFA and, in doing so, further prevents VEGFR2 stimulation in the stalk cells [190]. Considering the different effects observed upon VEGFA stimulation of the two VEGF-receptors, stimulation of VEGFR1 could also provide an explanation for the proliferative phenotype observed in stalk cells when exposed to VEGFA. This hypothesis however remains to be investigated. Benedito et al. described an additional level of angiogenic fine-tuning where the role of the Notch-ligand JAG1 was investigated [118]. In a mouse model of retinal angiogenesis, *JAG1* deletion in endothelial cells resulted in the presence of fewer tip cells and reduced vascular branching. Overexpression of *JAG1* on the other hand resulted in a hyper-branching phenotype and an overrepresentation of tip cells, a scenario often associated with Notch inhibition. Interestingly, it was shown that in comparison to DLL4, JAG1-induced NOTCH1 activation produced a substantially weaker Notch signal. As a result, although being an activator of Notch signalling, JAG1 stimulation resulted in an overall reduction in Notch activity in endothelial cells resulting in a hyper-branching phenotype. In an earlier study in head and neck squamous cell carcinoma, it was found that JAG1 expression on tumour cells resulted in extensive branching of the surrounding vasculature [191]. Although this hyper-branching was attributed to JAG1-dependent Notch receptor activation in the endothelial cells, the study by Benedito et al. [118] may provide insight into the phenotypic response that was

observed. Moreover, these studies together suggest that ligand-dependent fine-tuning of Notch signalling in the process of angiogenesis involves interaction of multiple cell types.

HIF-Notch interplay and potential effects on angiogenesis

As described above, studies have observed elevated Notch activity under hypoxic conditions, however a consensus on the underlying mechanisms behind these observations has yet to be reached. Some of the observations can be explained by the existence of target genes common to both pathways, such as *HEY1* that contains both HREs as well as CSL binding sites within its promoter [182]. On the other hand, Gustafsson et al. described an increase in ICN1 levels in addition to elevated CSL-reporter signal when cells were exposed to hypoxia. Such effects are most likely not a result of shared target genes [178]. This observation was attributed to a HIF-ICN1 interaction, where HIF contributed both the ICN1 stability as well as increased the CSL-complex transcriptional proficiency. Other studies have reported elevated levels of Notch ligands upon tumour cell exposure to hypoxia [182,183]; however the effects this may have on Notch activity was not addressed. Elevation of Notch ligands in hypoxic tumour cells may not only be relevant within the tumour cells themselves, but may also have an effect on neoangiogenesis. Although the VEGFA-gradient effects on endothelial cell Notch signalling may, in part, account for the hyper-branching associated with neoangiogenesis, hyper-branching in mouse xenograft models does not occur in a gradient manner. What is observed in these mouse models is an even more exaggerated branching upon contact of the recruited vasculature with the implanted tumour cells [192]. This may be indicative of a tumour cell contact-dependent Notch inhibition within the endothelial cells. As discussed above, expression of JAG1 within endothelial cells (and potentially on tumour cells) has an overall Notch inhibitory effect resulting in increased vascular branching [118,191]. With this in consideration, hypoxia induced Notch ligand expression on tumour cells could result in similar effects. This notion is addressed in paper IV.

Anti-angiogenic therapy

As has been discussed at length, recruitment of new vasculature is considered essential for the development and progression of solid tumours. With this knowledge, many efforts have been made with regards to developing anti-angiogenic therapies. The approach that has gained the most attention is the use of VEGF-inhibitors such as Avastin/bevacizumab. This drug is used clinically in the treatment of a variety of malignancies including colon cancer and RCC. However, acquired resistance as well

as links to increased metastatic potential limit Avastin's anti-tumour potential [193-196]. On a promising note, recent studies using mouse models have elucidated that the increased metastatic potential of cancer cells following Vegf-inhibition can be attributed to an increase in c-Met expression. In addition, combined inhibition of both Vegf and c-Met resulted in a decrease in tumour growth without any observed increase in metastases [197,198].

In order to address anti-angiogenic drug development from a different angle, other studies have focused on targeting the Notch pathway. DLL4-specific inhibitors in murine tumour models have displayed inhibitory effects on tumour growth attributed to excessive hyper-branching of non-functional vasculature [187,188]. Unfortunately DLL4 inhibitors have also been associated with adverse side effects including liver haemorrhaging following prolonged use [199]. More broad-spectrum Notch inhibitors such as GSIs have also been investigated with similar anti-tumour effects to those observed with the aforementioned therapies [141]. Unfortunately, Notch inhibition using GSIs has detrimental effects on intestinal crypt stem cells, resulting in gastro-intestinal problems for the patients [139]. As with studies addressing the adverse affects of VEGF-inhibition, further studies have revealed that an intermittent GSI treatment plan in combination with glucocorticoids can prevent the gastrointestinal problems associated with GSI treatment [140]. In addition, a study by Wu et al. addressed the possibility of inhibiting Notch signalling using NOTCH1 and NOTCH2 specific antibodies. This approach resulted in disrupted angiogenesis and reduced tumour growth, without any observed intestinal toxicity that is associated with broad-spectrum Notch inhibitors [200]. Taking all into consideration, the development of anti-angiogenic drugs is a promising approach to cancer therapy; however, efforts to address the adverse effects associated with the treatments could increase their clinical potential.

Part III – Non-coding RNAs

Brief description

It is estimated that approximately 90% of the human genome is transcribed. To date, protein-coding genes (mRNAs) have received the majority of interest with regards to tumour biology, however account for a mere 2% of the human genome [201]. In recent years, studies have shed light on what has been hidden within the remaining 88%, leading to the discovery of a variety non-coding RNAs (ncRNAs). Based primarily on size and function, ncRNAs are divided into a number of subgroups including long non-coding RNA (lncRNA), short interfering RNA (siRNA), piwi-associated RNA (piRNA), small nucleolar RNA (snoRNA) and microRNA (miRNA) [202]. These varieties of RNAs can range from 18-300 nucleotides (nts) in length and are primarily implicated in modification and modulation of protein-coding genes [201]. Although there are many RNA subtypes that can be discussed, this thesis will focus specifically on the biogenesis and oncogenic roles of miRNAs and snoRNAs.

microRNAs

miRNA biogenesis and function

MicroRNAs are a group of small ncRNAs of approximately 22 nts in length. Typically, miRNAs are derived from the introns of both expressed protein-coding genes as well as other larger ncRNAs [203]. Originating from expressed regions of the genome, miRNAs are transcribed in a polymerase II-dependent manner, resulting in primary miRNAs (pri-miRNAs) with sizes between 500-3000 nts (Figure 5). These pri-miRNAs then undergo a number of endonuclease-dependent processing steps before giving rise to the functional 22-nucleotide miRNAs. The first step occurs in the nucleus where the pri-miRNAs are cleaved by the endonuclease DROSHA, resulting in the formation of 60-70 nt hairpin precursor miRNAs (pre-miRNAs). The pre-miRNAs are then transported from the nucleus to the cytoplasm via Exportin (*XPO5*). Here the pre-miRNAs are bound and processed by another endonuclease DICER1, which removes the hairpin structures and produces double stranded RNAs (miRNA-miRNA*) consisting of the functional miRNA and its complementary strand (miRNA*) [204]. Through interaction between the miRNA containing DICER1, TRBP as well as Argonaut proteins, the double-stranded miRNA is incorporated into the RNA-induced silencing (RISC) complex [205]. At this point, the RISC complex expels the complementary miRNA while retaining the functional miRNA, which now acts as a guide for the RISC complex to its targets. Guidance of the complex is a result of seed regions consisting of 2-7 nts located in the 5' end of miRNAs that are complementary to miRNA recognition elements (MREs), primarily located in the 3' UTRs of target genes [203]. Binding of the miRNA/RISC complex results in inhibition of translation or degradation of the target gene. Recent estimates indicate that approximately 60% of protein-coding genes are targeted by miRNAs [206]. In addition, individual miRNAs have been reported to have multiple targets, while at the same time individual genes can be targeted by different miRNAs [203]. The combined binding of different miRNAs may result in an additive effect on target inhibition [167,207], as is discussed in paper III.

Being a relatively new field of molecular biology, studies are emerging that change the way one must think about the function of miRNAs. Originally, miRNAs were described as fine-tuners of gene expression however a recent study has indicated that miRNAs can also function in an "all-or-nothing" manner [208]. Expression of miR-34a in colon cancer stem cells was shown to regulate a Notch activity threshold, where the presence or absence of the miRNA determined a cell's fate to differentiate or maintain its stem cell phenotype. In a study by Karreth et al. the ability for individual miRNAs to target common MREs present in different mRNAs was investigated. This led to the description of competitive endogenous RNAs (ceRNAs)

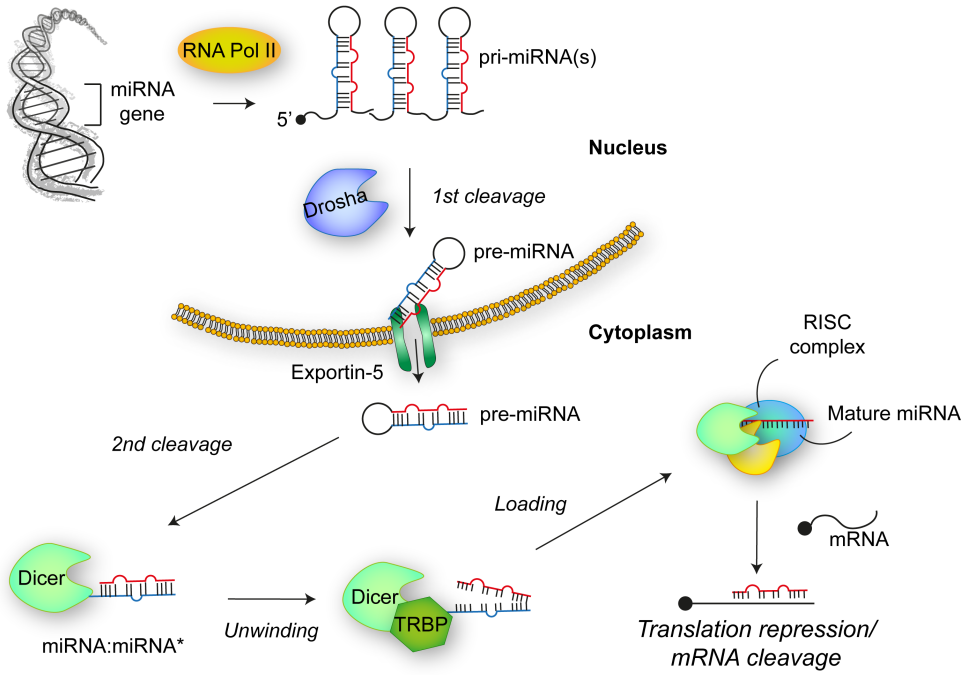

Figure 5. *Simplified schematic of miRNA biogenesis and function. Upon transcription and splicing from introns the pri-miRNAs are produced. The pri-miRNAs are cleaved into pre-miRNAs by Drosha before being expelled from the nucleus via Exportin-5. Once in the cytoplasm the pre-miRNAs are bound and cleaved by Dicer, producing a double stranded miRNA-miRNA* RNA (miRNA* is complementary to the functional miRNA). Through further interaction with DICER and TRBP (amongst other proteins such as Argonaut), the double-stranded miRNA is unwound, expelling the miRNA* for degradation. The new mature miRNA-containing protein complex (RISC) is then guided to target mRNAs via seed sequences in the miRNA, resulting in translational repression and/or cleavage of the target mRNA.*

adding yet another layer of complexity surrounding functional roles of miRNAs [209]. In this study Karreth and colleagues discovered common MRE sequences expressed in both *ZEB2* and *PTEN* indicating regulation by a common miRNA. Upon *ZEB2* depletion in a mouse model of melanoma, miRNA targeting of *PTEN* was elevated resulting in PI3K/AKT activation and an increase in tumourigenicity. In this case *ZEB2* acted as a *PTEN* ceRNA with miRNAs being the limiting factor in the system. Another study by Helwak et al. has brought into question the rigidness of the seed sequence described to be in the 5' end of miRNAs [210]. Intriguingly, in a subset of approximately 20% of miRNA-mRNA interactions it was found that the binding was dependent on a seed sequence in miRNA's 3' end. With this in mind, previous descriptions of miRNA-target prediction systems may require updating.

miRNAs in cancer

With their ability to influence the expression and/or translation of protein-coding genes, miRNAs have been implicated, in a target-dependent manner, to have both TSG and oncogenic roles in tumour biology. In an early study of chronic lymphocytic leukemia (CLL) a deletion at 13q14 was identified. Investigation of this deleted region revealed no potential protein-encoding TSGs. The deletion did however span over two miRNAs, miR-15a and miR-16-1 [211]. This observation was one of the first indications of miRNA involvement in tumour development. Later reports also discovered mutations in both miR-15a and miR-16-1 is a subset of CLL patients, as well as an overall reduction in expression of both miRNAs in approximately 70% of all CLL patients [212]. Molecular investigation into the functional roles of miR-15a and miR-16-1 revealed their involvement in the negative regulation of *BCL2* [213,214]. The combination of these studies lead to the description of the first bona fide miRNA TSGs. Since then, a multitude of miRNAs have been identified with key roles in tumour development. In general, descriptions of the tumourigenic functions miRNAs implicate their involvement in the regulation of previously identified signalling pathways with known cancer-related roles. One such example is the identification of the let-7 family of TSG miRNAs which were shown to be negative regulators of the oncogene *RAS* in lung, breast and prostate cancers amongst others [215,216]. In MYC-pathway dependent tumours including Burkitt's lymphoma and neuroblastoma, the miR-34 family was found to inhibit cell growth and proliferation via negative regulation of *MYC/MYCN* as well as *E2F* transcription factors [215]. In addition, in multiple malignancies including neuroblastoma, miR-34 has been shown to be involved in a positive feedback-loop with p53 [217,218]. All considered, the miR-34 family represents an additional example of miRNAs with tumour-suppressive roles. In 2006, Costinean et al. reported the development of the first solely miRNA-based transgenic mouse using overexpression of miR-155 [219]. This mouse model was a result of studies indicating elevated levels of miR-155 in a variety of B-cell lymphomas. The introduction of miR-155 transgene resulted in elevated proliferation rates of pre-B cells leading to the development of B-cell malignancy. To date, one of the most investigated families of miRNAs with oncogenic potential is the miR-17-92 cluster on chromosome 13 [220]. The miR-17-92 cluster consists of 6 miRNAs including miR-17, miR-18a, miR19a, miR19b, miR-20a and miR-92a. Early studies identified amplifications of the miR-17-92 region in B-cell lymphomas [221], which were later identified in a variety of malignancies including medulloblastoma, colon cancer, lung cancer and neuroblastoma [222]. He et al. reported contribution of the miR-17-92 cluster to the tumourigenicity of a MYC-driven mouse model of B-cell lymphoma and termed the miR-17-92 as a potential oncogene [221]. Further studies identified miR-17-92 as a direct target of MYC and MYCN where induction of miR-17-92 lead to increased tumour growth and proliferation [223,224]. Proposed mechanisms behind these

effects included miR-17-92-dependent inhibition of cell cycle inhibitors as well as inhibition of pro-apoptotic genes such as *BIM* [215].

In addition to the emerging research into the molecular biology surrounding miRNAs, studies have also addressed their diagnostic and prognostic potential. In a variety of malignancies including CLL, prostate cancer, lung cancer, and neuroblastoma, prognostic miRNA expression signatures have been developed, showing similar, if not better, results than previously reported mRNA-based signatures [212,225-228]. On the diagnostic front, Barker et al. reported the use of miRNA expression profiles to identify the primary tumour site in metastatic cases of head and neck carcinomas [229]. In addition, studies have provided evidence that circulating miRNAs are more stable than circulating mRNAs which has initiated investigation into the use of serum derived miRNA expression analysis as a diagnostic tool in prostate and gastric cancer [230,231].

snoRNAs

snoRNA biogenesis and function

The snoRNAs are a subclass of small ncRNAs with sizes varying from 60-300 nts. The majority of snoRNAs are derived from introns of protein-coding genes and to date approximately 200 have been identified [232]. Two subtypes of snoRNAs have been described; the box C/D and the box H/ACA containing snoRNAs, involved in methylation and pseudouridylation of target RNAs, respectively [201]. Upon transcription and splicing from introns, snoRNAs recruit a number of supporting proteins known as small nucleolar ribonucleoproteins (snoRNPs), with which they form a complex and are stabilized. With respect to box C/D snoRNAs, they interact with the four proteins NOP56, NOP58 SNU13 and the methyltransferase Fibrillarin (NOP1). The C/D box snoRNP complex is beyond the scope of this thesis, however more details regarding their biogenesis and functions can be found in the review by Mannoor et al [201]. The box H/ACA snoRNAs initially interact with the proteins NAF1, NHP2, NOP10 and the pseudouridine synthase DKC1 (Figure 6) [233]. NAF1 acts as a RNA-binding protein, which binds the newly synthesized snoRNA to aid in the recruitment of the other snoRNP subunits. During a maturation process however, NAF1 is replaced by GAR1 resulting in a functional snoRNP complex [234]. It has also been reported that the protein SHQ1 plays an essential role in stabilizing an otherwise unstable DKC1 during the early stages of snoRNP complex formation [234,235]. Once the mature snoRNP complex is formed, the H/ACA

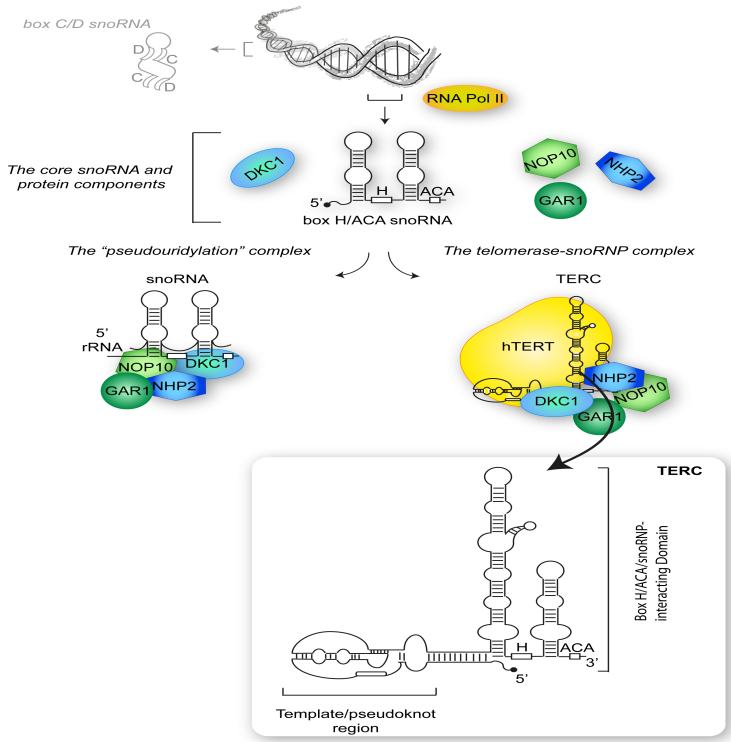

Figure 6. *Schematic of box H/ACA snoRNA-snoRNP complex formation. Upon transcription (and in most cases splicing from introns) of snoRNAs, the two-hairpin RNA structure interacts and is stabilized by snoRNP proteins. For typical snoRNAs, the complex consists of DKC1, GAR1, NOP10 and NHP2. The snoRNA then acts as a template for target recognition leading to pseudouridylation. In the case of the H/ACA containing TERC-snoRNP complex formation, the enzymatic protein TERT (telomerase) is also recruited. The TERC template/pseudoknot region then serves to direct the telomerase complex to telomeres on chromosome ends.*

snoRNA acquires a double-hairpin configuration, with the H-box acting as the hinge between the hairpins and the ACA-box present at the 3' tail [201]. Much like seed sequences in the context of miRNAs, loops within the snoRNA-hairpin domains act as guiding sequences to position the snoRNP complex correctly on its target RNA or DNA (in the case of telomerase, discussed below) [233]. In the majority of cases the snoRNP complex targets maturing ribosomal RNA (rRNA) resulting in isomeric modifications of specific uridines in a process called pseudouridylation. Interestingly this has been reported to be the most common of all RNA modifications [236].

snoRNAs in cancer

Although many studies have focused on the biogenesis and normal functions of snoRNAs, investigations into their functional roles in cancer are limited. Multiple "guilt-by-association" studies have however reported differential expression, mutations and translocations of snoRNAs in a variety of different cancers. Examples include sequence mutations of snoRNA U50 in approximately 10% of prostate cancers and 25% of breast cancers [237,238]. Reduced expression of *GAS5* in breast cancer, which encodes 9 C/D box snoRNAs has also been reported [239]. In addition, translocations involving the *GAS5* gene were described in a subset of B-cell lymphomas [240]. In a study by Gee et al. it was found that low expression of three snoRNAs, RNU43, RNU44 and RNU48 was associated with poor prognosis in both breast cancer and head and neck squamous cell carcinoma, displaying the potential prognostic relevance of snoRNAs in the cancer setting [241].

Recently, studies have started to emerge addressing the functional roles of snoRNAs in cancer. In a paper by Mei et al., they describe a potential oncogenic role for the H/ACA box snoRNA42 where they report reduced NSCLC tumour growth both *in vitro* and *in vivo* upon snoRNA42 knockdown [242]. In addition, overexpression of snoRNA42 in human bronchial epithelial cells resulted in increased proliferation and colony formation. With the newfound interest in the roles of non-coding RNAs in tumourigenesis, it is likely that studies into the roles of specific snoRNAs will become more common in the coming years.

In addition to investigating the snoRNAs themselves, other works have investigated the roles of the supportive machinery: the snoRNPs. In a recent study, elevated levels of the C/D box associated snoRNP Fibrillarin was noted in breast cancers [243]. Upon Fibrillarin inhibition a p53-dependent cell cycle arrest was observed, which was attributed to defective ribosomal biogenesis. In addition they reported that elevated snoRNP levels interfere with stress-induced p53 activation, suggesting an anti-apoptotic and pro-tumourigenic role for snoRNPs in breast cancer. With regards to the H/ACA box snoRNPs, it has been shown that mutations in *DKC1* result in a disease known as dyskerin-congenita, which is characterized by early aging as well as an increased risk for developing cancer [244]. Interestingly, these effects have been attributed to a decrease in telomerase activity resulting in increased genetic instability. This snoRNP-telomerase is discussed in more detail below and is the main subject of paper I.

Telomerase complex

With each cell division, chromosome ends become progressively shorter, a result of imperfect DNA duplication. Blackburn and colleagues in 1978 discovered that long

repeats of the nucleotides TTAGGG existed at the ends of chromosomes, preventing the loss of important genetic information during cell divisions [245,246]. Later studies revealed that these telomeric repeats, in combination with bound Shelterin proteins, protect the chromosomes from end-to-end joining and from being recognized as double strand breaks [247]. In normal cycling cells the length of telomeres predetermines the life span of the cell, with the gradual loss of the telomere leading to senescence and/or cell death. Stem and progenitor cell populations, however, circumvent this aging process by activating a telomere maintenance program through the expression of a protein complex known as telomerase [248]. In the case of malignant cells however, both telomerase as well as a homologous-recombination based alternative lengthening of telomeres (ALT) can maintain telomere length, accounting for their limitless proliferative potential [249,250]. The telomerase complex is comprised of a protein-enzymatic subunit TERT, a telomerase RNA component (*TERC*) as well as the H/ACA box associated snoRNP complex including DKC1, NHP2, GAR1 and NOP10 [251]. Unlike most snoRNAs, *TERC* is not derived from introns of other genes, but contains its own regulatory regions [233]. *TERC* does however contain a box H/ACA motif that is responsible for the recruitment and binding of the snoRNP complex. In addition to the box H/ACA motif, *TERC* also contains an 11 nt sequence that is complimentary to the TTAGGG repeats present in telomeres. Much like the hairpin-loop sequences present in snoRNAs, this 11 nt template guides the telomerase complex to the telomere [201]. The enzymatic subunit TERT has reverse transcriptase activity and is responsible for the elongation process of the telomeres, while the snoRNPs are responsible for stabilizing *TERC* as well as assisting in proper docking of *TERC* in TERT. As mentioned above, other supportive proteins are also involved in the maintenance of telomeres including the Shelterin proteins, which induce conformational changes of the telomeres thereby protecting them from being recognized as DNA-damage. In addition, the ATPases Pontin (RUVBL1) and Reptin (RUVBL2) have been shown to facilitate in interactions between DKC1 and *TERC* as well as the subsequent docking with TERT. Studies have suggested that the presence of all the subunits mentioned above is required for a functional telomerase complex [252]. Deletions or mutations in primarily, but not exclusively, *TERT*, *TERC* and *DKC1* have been shown to result in the dyskerin-congenita [244]. As noted previously, these patients display premature shortening of telomeres attributed to a dysfunctional telomerase complex. In addition to premature aging, patients also have an increased risk for developing a variety of malignancies [253]. This observation has been described to be a result of increased genetic instability due to a lack of telomeres. At first, a lack of telomerase activity resulting in cancer formation may seem counterintuitive considering the dependence of cancers on telomerase activity for their immortality. Recent studies from the lab of DePinho have shed light on this issue [254,255]. Using a mouse model of T-cell lymphoma they displayed that a loss of telomerase activity did indeed promote early tumour development. Due to the genetic instability of these tumours and

accompanying rapid increase of amplifications and deletions, these tumours eventually experienced cellular crisis leading to tumour regression. However, upon late re-activation of telomerase via conditional expression of *TERT*, the complex genome was re-stabilized resulting in the avoidance of cellular crisis and instead dissemination of the disease. With this in consideration, telomere maintenance can have both a tumour-suppressive role in tumour initiation as well as a tumour-promoting role in tumour progression. In the case of cancers resulting from mutations or deletions in the telomerase complex, it is possible that other mechanisms of telomere maintenance, such as ALT, are acquired to allow for disease progression [255].

Telomeres and neuroblastoma

The role of telomere maintenance in neuroblastoma is still a matter of debate. While the earliest study addressing the issue described an association between short telomeres and poor prognosis [256], more recent studies have reported a worse prognosis for patients with longer telomeres. In addition, elevated telomerase activity was observed in approximately 30% of neuroblastomas, which again was associated with shorter EFS and OS [257,258]. Another topic of controversy surrounding telomeres in neuroblastoma is the mechanism that controls telomere maintenance. Multiple studies have indicated *TERT* as a MYC target and correlation between *MYCN* and *TERT* expression have been reported in neuroblastoma [24,60,259-262]. In addition, TERT expression in neuroblastoma has been associated with high-stage disease [262,263]. Considering these observations and the relevance of MYC pathway activity in neuroblastoma, it seems plausible that TERT may be responsible for telomere maintenance. On the other hand, a study by Onitake et al. reports that TERT expression/telomerase activity did not correlated with telomere length in neuroblastoma and suggested that telomere length could be maintained through alternative mechanisms [250]. We report in paper I that snoRNP expression correlates with telomerase activity in neuroblastoma cell lines and primary tumours. In addition, knockdown of the enzymatic snoRNP *DKC1* resulted in reduced telomerase activity and increased anaphase-bridge frequency, a phenomenon associated with loss of telomeres [264]. However, another study by Lundberg et al. observed a correlation between the presence of ALT and longer telomeres in *MYCN* non-amplified tumours [249]. With so many contradicting results, it is hard to draw any definitive conclusions, however studies have reported that ALT and telomerase activity are not necessarily mutually exclusive in tumour cells [265-267]. Talking all into consideration, it could be possible that multiple mechanisms of telomere maintenance are active simultaneously in neuroblastoma, or that different mechanisms could be active in a patient-subgroup dependent manner. One interesting idea comes from the newly described *ATRX* mutations in neuroblastoma,

which define a subgroup of older patients with a poor prognosis [26,48]. These patients, despite their poor prognosis, do not harbour *MYCN*-amplifications. In addition, *ATRX* mutations were associated with elongated telomeres [26]. Previous reports have indicated that *ATRX* mutations are commonly associated with ALT [49,50], which may provide insight into the relevance of ALT in *MYCN* non-amplified tumours reported by Lundberg and colleagues [249]. In support of this notion, the average age at diagnosis of patients defined as having long telomeres in this study was 75 months, compared to <20 months in the remaining patients, indicative of *ATRX* mutations. On the other hand, in the younger patients without *ATRX* mutations, including those with *MYCN*-amplification, mechanisms of telomere maintenance such as snoRNP regulation may be of more relevance. Future investigations into the different mechanisms of telomere maintenance in a patient subgroup-dependent manner may help clarify this debated area of neuroblastoma biology.

Part IV – Current Investigations

Papers I & II: Biology behind prognostic signatures in neuroblastoma

Background and Aims

In these studies we aimed to address several key issues regarding prognostics in neuroblastoma and the biology behind why prognostic signatures work as they do. In addition we aimed to develop a prognostic signature that could be applicable in a clinical setting on an individual patient basis. Since the development of high-throughput techniques such as gene expression microarrays, much attention has been given to defining prognostic signatures in virtually all known diseases, including neuroblastoma. The methods used to define prognostically relevant genes in neuroblastoma are quite varied across different studies. In general, prognostic genes are identified based on differential expression across clinical (stage, age at diagnosis, *MYCN* status and patient survival) or biological (MYC activity, tumour hypoxia and apoptosis) stratification. Although these methods do produce prognostically significant gene signatures, there are many issues that are left unaddressed. For one, many prognostic signatures that employ clinical stratifications do not address the

underlying biology and processes associated of which the signature is made. Such investigations could shed light on key processes in the progression of neuroblastoma and identify new potential therapeutic targets. The second, and possibly most important issue, is how to employ these prognostic signatures in the clinical setting. The complexity of the signatures with regards to both number of genes to analyze and equipment required to perform such analyses is one hinder that must be overcome before implication in the clinic. In addition, studies to date make use of comparing patients within large cohorts when defining different prognostic groups. Although relevant for identifying clinical groups on which future studies can be performed, such approaches provide little information when dealing with an individual patient in the clinic.

Results: Paper I

In this paper we performed a clinical stratification based on patient survival to identify genes associated with a poor prognosis. Following identification of the most prognostically relevant genes, gene ontology analysis was performed to examine the biological processes enriched in aggressive neuroblastomas. An enrichment of genes involved in the formation and functionality of the snoRNP complex was found. Backwards conditional cox regression analysis of the snoRNP related genes produced a gene signature consisting of only three central genes of the snoRNP complex: *DKC1*, *GAR1*, and *NHP2*. Mean gene expression values of these genes independently predicted patient prognosis when applied to all patients as well as high-risk patients alone. In order to investigate the biological role of these genes in neuroblastoma, the central snoRNP complex gene *DKC1* was knocked down in neuroblastoma cell lines. Knockdown of *DKC1* resulted in reduced telomerase activity accompanied by a reduction in the RNA subunit of telomerase *TERC*. These effects were observed despite stable levels of *TERT* expression. Further investigation of neuroblastoma cells undergoing cell division displayed an increase in the formation of anaphase bridges upon *DKC1* knockdown. In addition, expression of the snoRNP signature correlated with telomerase activity as well as expression of genes associated with genomic complexity in primary neuroblastoma material.

Results: Paper II

Here, using the same methodology as used in Paper I, we instead focused on genes that were associated with a good prognosis. Among genes associated with a good prognosis, an enrichment of genes involved in neuron projection and growth cone formation was found. These growth cone-associated genes as were scrutinized using backwards-conditional cox regression together with the poor prognostic genes identified in Paper I. This analysis lead to the production of a four-gene signature consisting of two genes associated with snoRNPs and two genes associated with growth cone formation. Calculation of the ratio (R-score) between the expression of the two prognostically opposing processes significantly predicted patient survival and

identified an ultra-high risk group of patients amongst patients presenting with *MYCN*-amplification and/or stage 4 disease. Application of the R-score using standard qPCR-based methods of gene expression reproduced the prognostic results obtained from microarray datasets. In addition, we defined an optimal cut-off of the qPCR-based R-score that allowed for prognostic analysis on an individual patient basis.

Discussion

It has previously been described that genomic complexity and presence of segmental aberrations in neuroblastomas is associated with advanced disease and a dismal outcome for the patients [18]. High stage neuroblastomas have also been characterized by having longer telomeres, indicative of activated telomere maintenance systems [26,249,250]. Here we report that expression of genes involved in the formation of the snoRNP complex hold prognostic value in neuroblastoma and are associated with advanced disease. As discussed above in the introduction, the snoRNPs play a central role in the formation of a functional telomerase complex. In recent studies from the DePinho lab, using a T-cell lymphoma model system they describe a mechanism by which telomerase activation in advanced stages of disease allow for tumour maintenance and progression [254,255]. Briefly, the mechanism put forward by the DePinho lab suggests that early and less genetically complex tumours benefit from not having active telomerase. The resulting genetic instability allows for accumulation of genetic aberrations leading to potential increased aggressiveness of the tumour. However, there comes a point at which the genetic instability is no longer beneficial to the tumour, at which point the genome must be stabilized to prevent cellular crisis. Activation of telomerase activity, via *TERT* induction, provided one such method of genomic stability in aggressive tumours and resulted in disease progression *in vivo* [254,255]. In our study we describe an up-regulation of snoRNPs in advanced stages of disease. Interestingly, multivariate analysis including snoRNP and *TERT* expression revealed significant association to prognosis for snoRNPs alone. This could indicate involvement of snoRNPs in genomic stabilization in neuroblastoma via a similar mechanism that in other cancer forms is attributed to TERT. In support of this, knockdown of the central snoRNP protein *DKC1* resulted in diminished telomerase activity and elevated genomic instability as measured by anaphase-bridge frequency and correlation with expression of genes associated with genomic instability.

With regards to the development of prognostic gene expression signatures, there are many issues that have limited their implementation in the clinic. For example, one must minimize the complexity of the assay with regards to both the number of genes that must be analyzed as well as the platform of analysis. Although recent signature studies have made strides with regards to prognostic significance, the average number of genes included in these signatures is approximately 50 [268] and therefore require

the use of high-throughput techniques such as microarrays. In paper II we address these issues and present a prognostic signature consisting of only four genes and requiring standard qPCR analysis. By calculating the ratio of expression of two prognostically opposing processes (R-score), each process acts as an internal normalization factor for the other, therefor eliminating the need for comparison to housekeeping genes. In doing so, we address an additional hinder that must be overcome before a signature can be used in clinic; the ability to perform individual patient analyses. Without the need for housekeeping gene comparison, one no longer needs to compare values across patient cohorts, as is done in the majority of expression-based prognostic analyses. Yet another issue that must be considered is the usefulness of the prognostic signature in clinical practice, i.e. it is important that the signature provides additional information to the current classification systems. Information, such as identification of high-stage patients with a good prognosis, may strictly be indicative that the current treatment regimens are working, but would not warrant treating these patients differently. Identification of a subgroup of patients with a dismal outcome within the patients already defined as high-risk would allow for the definition of a group that, in all likelihood, will not respond to the current therapies. By defining a fixed R-score cut-off, we identified, on an individual patient basis, a subgroup of patients amongst high-risk patients with an overall survival of 20% or less. This survival rate is substantially less than the 40-50% survival rate that is currently observed amongst high-risk patients [18,20,51]. With this in consideration, we can identify a subgroup of patients that could benefit from gaining access to new therapies.

Futures perspectives

Although the R-score appears to have substantial clinical potential, a prospective study will need to be performed to determine its true value. In addition to a prospective study, there are certain standardization issues that must be considered. Although the definition of a fixed R-score allows for individual patient analyses, fluctuations in qPCR-based R-score calculations across labs and across qPCR platforms may be an issue of concern. In order to address this issue one could use a commercially available reference template as a platform control. By calculating the R-score of the reference template on each newly used qPCR platform, one can adjust the R-score cut-off accordingly and thereby minimize misclassification of patients. Such experiments should be performed on the previously examined cohorts at different laboratory centers. If the above studies produce positive results, application of the R-score has the potential to influence the current risk-stratification schemes of neuroblastoma.

Paper III: miRNA contribution to invasiveness of neuroblastoma

Background and Aims

The miR-17-92 cluster of miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-921) is activated in a variety of malignancies including neuroblastoma [269]. The over-expression of miR-17-92 has been attributed to amplification of its genomic region as well as transcriptional activation by MYC and MYCN [221,223,224]. Although an oncogenic role of miR-17-92 has been reported, including regulation of proliferation and apoptosis, identification of specific targets remains limited. In addition, the studies that have addressed this issue have done so with focus on the effects of individual miRNAs of the cluster. As a result, any potential cooperation or additive effects between the miRNAs have been overlooked. In this study we aimed to investigate the global effect of miR-17-92 cluster activation in neuroblastoma and to shed light on specific targets of this family of miRNAs.

Results

We showed that the miR-17-92 cluster was expressed in a series of 95 examined neuroblastoma tumours, with highest expression in the subgroup with *MYCN*-amplification. Quantitative mass spectrometry analysis of SHEP cells with tetracycline-inducible miR-17-92 expression identified 144 significantly down-regulated proteins upon miR-17-92 activation. Among these potential targets there was an observed enrichment of 3'UTR motifs complementary to seed sequences of the miR-17-92 miRNAs (with the exception of miR18a). The number of miRNA recognition elements with target 3'UTRs correlated with the degree of protein down-regulation. This applied with regards to both the number of MREs for an individual miRNA as well as the total number of MREs for members within the miR-17-92 cluster.

In order to investigate the oncogenic pathways affected by miR-17-92 expression, gene set enrichment analysis (GSEA) was employed. Processes found to be down-regulated upon miR-17-92 activation included proliferation, adhesion, TGF-β signalling, oestrogen signalling and RAS signalling. Functional analyses confirmed the effects of miR-17-92 on cellular proliferation and adhesion. In addition, miR-17-92 activation resulted in a prolonged engraftment of the non-tumourigenic SHEP cells in xenograft transplantation experiments. TGF-β pathway activity score displayed a negative correlation with *MYCN*-amplification, MYC pathway activity and miR-17-92 expression in a series of primary neuroblastoma tumours. Tetracycline-induced miR-17-92 expression in SHEP cells resulted in decreased TGF-β pathway activity as analyzed by decreased phosphorylation of SMAD2 and SMAD3 as well as reduced signal from a SMAD-regulated luciferase reporter. In addition, mRNA expression of

TGF-β pathway components (*TGFBR2*, *SMAD2* and *SMAD4*) as well as a panel of TGF-β target genes was down-regulated upon miR-17-92 activation.

Discussion

Considering that miR-17-92 is one of the most frequently activated clusters of miRNAs in cancer, relatively little is known about the specific targets and the cellular effects of the simultaneous activation of all miRNAs within the cluster. Here we identify, using high-throughput proteomics, 144 genes that are specifically down-regulated upon activation of the entire miR-17-92 cluster. Sequence analysis of these gene revealed that the frequency of 3'UTR seed sequences complementary to miR-17-92 miRNAs correlated with degree of mRNA repression. Interestingly, this applied both to the number of seeds from individual miRNAs as well multiple seeds from different miRNAs of the miR-17-92 cluster. This suggests potential cooperation and additive effects between different miRNAs with regards to the repression of their targets. With this in consideration, it is not surprising that only a limited number of miR-17-92 targets have been identified in the studies focusing on individual miRNAs.

MYCN-amplification has long been known to increase the tumourigenicity of neuroblastoma cells. As an example, in xenograft model systems, *MYCN*-amplified cell lines such as SK-N-BE(2)c and KCN readily form subcutaneous tumours, whereas MYCN non-amplified cell lines, such as the SHEP cells used in this study, are mostly non-tumourigenic. Interestingly, we report here that activation of the miR-17-92 cluster in SHEP cells results in longer engraftment periods of the injected tumour cells. Although this could be due to miR-17-92's effects on multiple pathways including previously described proliferation and apoptosis, this suggests a role for miR-17-92 in the oncogenicity of *MYCN*-amplification.

The observed enrichment of genes associated with TGF-β signalling amongst genes down-regulated by miR-17-92 activation was also intriguing. It has previously been described that TGF-β target genes *CDKN1A* and *BIM* are repressed upon miR-17-92 activation in neuroblastoma cells, resulting in heightened proliferation and repression of apoptosis [270]. Here we expand on these previous observations and display that miR-17-92 dampens TGF-beta signalling at multiple levels, both upstream and downstream of SMAD2/SMAD4 activation, including *TGFBR2* inhibition as well as additional TGF-β target genes. The ability of miR-17-92 miRNAs to target a signalling pathway at so many levels likely results in enhanced regulation of a pathway that otherwise would restrict tumour progression. It has previously been shown that activation of TGF-β signalling via TGF-β1 stimulation of TGFBR2 results in terminal neuronal differentiation of neuroblastoma cells resulting in halted growth and loss of tumourigenicity *in vivo* [165]. These observations are corroborated by the results presented in this paper displaying inverse correlations between TGF-β pathway activity and patient prognosis, together solidifying the tumour suppressive role of

TGF-β signalling in neuroblastoma. In summary, miR-17-92 activation provides insight into the mechanism by which aggressive neuroblastomas evade the tumour suppressive effects of TGF-β.

Future perspectives

Considering the tumour suppressive effects of TGF-β in neuroblastoma, miR-17-92 inhibition could be a potential therapeutic approach in aggressive neuroblastomas. As an initial step in this direction, effects of miR-17-92 inhibition/knockdown in mouse models of neuroblastoma such as TH-*MYCN* mice should be performed. In addition, in the current study we addressed the effects of miR-17-92 activation in a cellular system without *MYCN*-amplification. Although this does give insight into the specific targets of miR-17-92, this does not confirm that identical effects would be observed in the presence of *MYCN*-amplification. Considering the complexity of interplay observed between individual miRNAs of the miR-17-92 cluster, investigation of the effects of miR-17-92 inhibition in combination with *MYCN*-amplification could identify additional relevant target pathways/processes that are currently overlooked.

Paper IV: Tumour cell contribution to the angiogenic process

Background and Aims

In this study we aimed to investigate the effects that hypoxia has on the Notch pathway, and how this influences tumour biology. Previous studies have addressed the issue of hypoxia and Notch interplay, with the general consensus suggesting an elevated Notch-pathway activity in areas of low oxygen [75,178]. Although the end result is consistent, the mechanism behind elevated Notch activity remains a matter of debate. In a study by Gustafsson et al., elevated notch signalling was attributed to HIF-α recruitment to CSL-binding sites within the promoters of Notch target genes, and thereby potentiating transcriptional activation [178]. Other studies, however, have attributed the elevated Notch activity to interplays between ICN, FIH and HIFs [180,181].

Results

Using the breast cancer cell lines MCF7 and T47D, we confirmed that hypoxia (1% oxygen) resulted in elevated Notch signalling, as was determined by increased cleavage of NOTCH1 as well as up-regulation of the Notch target *HEY1*. QPCR analysis of the Notch receptors (*NOTCH1-4*) under hypoxia revealed elevated expression of *NOTCH3* alone. Analysis of the Notch ligands, on the other hand, revealed elevated

levels of all ligands, with the exception of *DLL3*. Amongst the expressed ligands, *JAG2* displayed the most consistent up-regulation and was expressed at the highest levels compared to the other ligands, and was therefore investigated further. Hypoxic induction of *JAG2* was confirmed in multiple cellular systems, including mammary epithelial cells, renal proximal tubular cells and a panel of neuroblastoma cell lines. In addition, immunohistochemical analysis of JAG2 and HIF1α expression revealed co-localization in perinecrotic regions of ductal carcinoma *in* situ (DCIS). Chromatin immunoprecipitation (ChIP) analysis of HIF1α revealed a potential HRE at the +1294 position of the of the *JAG2* promoter, suggesting that *JAG2* is a direct HIF target. To investigate the effects of hypoxic JAG2 induction on Notch signalling, *JAG2* knockdown experiments were performed. Upon knockdown of *JAG2* in hypoxic T47D cells, there was an observed reduction in nuclear ICN1 as well as reduced transcription of the Notch target *HEY1*.

GSEA analysis of *JAG2*-correlated genes in two global gene expression studies comprised o 200 breast cancers and 79 renal cell carcinomas, respectively, revealed enrichment of genes involved in vascular development and angiogenesis. Using a co-culture system of hypoxic T47D and MS1 cells (a mouse endothelial cell line), we examined the possible effects that tumour-*JAG2* expression could have on endothelial cell tube formation capacity. Upon knockdown of *JAG2* in T47D cells, we observed a reduced capacity of MS1 cells to form tubes when grown in Matrigel. This effect on MS1 cells was not observed in the presence of T47D (+/- JAG2) conditioned medium alone.

Discussion

As mentioned previously, the mechanisms underlying elevated Notch signalling in hypoxia remains unclear. In addition to interactions involving HIF and ICN, other studies have reported elevation of Notch ligands (*DLL1* and *DLL4*) under hypoxia [182,183]. However, these studies did not address whether the induction of ligand expression contributed to elevated Notch activity. Here we report a HIF-dependent induction of *JAG2* under hypoxia in a variety of cell types. We also demonstrate that induction of JAG2 is a major contributor to the elevated Notch activity observed under hypoxia. Although we do demonstrate the contribution of JAG2 in hypoxic Notch signalling, knockdown of *JAG2* did not result in complete inhibition of the elevated Notch activity. This could be attributed to residual JAG2 levels after the siRNA-mediated knockdowns. However, it could also be attributed to one of the intrinsic mechanisms of hypoxic-induced Notch activity discussed above. In addition, we did observe an elevation of *NOTCH3* under hypoxia, which according to previous studies could also contribute to hypoxic Notch signalling [271,272].

Co-culture experiments with hypoxic T47D and mouse endothelial MS1 cells revealed that knockdown of *JAG2* in the T47D cells reduced the angiogenic capacity of the neighbouring MS1 cells. Interestingly, this effect was not observed when MS1

cells were exposed to T47D conditioned medium, suggesting juxtacrine signalling between tumour and endothelial cells. In the majority of the studies describing the role of Notch in angiogenesis, inhibition of Notch results in hyper-branching and an overall increase in vascular density, attributed to an overrepresentation of tip cells. In this case, the positive correlation between JAG2 expression and endothelial branching/density is indicative of an inhibitory effect on Notch signalling in the adjacent endothelial cells. Although this may seem counterintuitive, a recent study describing the opposing roles of JAG1 and DLL4 provides insight into JAG-induced Notch inhibition [118]. As discussed earlier in the introduction, Benedito et al. put forward an endothelial cell-model of angiogenesis in which DLL4 stimulation of Notch signalling produced a higher level of Notch activity than that produced by JAG1 stimulation. With the assumption that Notch receptors are limiting factors, binding of JAG1 to Notch receptors therefore acts in a competitive manner with DLL4 activation, resulting in an overall reduction in Notch activity. Considering the structural similarities between JAG1 and JAG2, it seems possible that JAG2 expression on the tumour cells in our model may be acting in a similar manner.

Future perspectives

Here we have suggested a mechanism explaining the effects of tumour cell JAG2 expression on endothelial cell branching. In order to provide further support for this model, investigation into the effects on Notch signalling within the endothelial cells must be performed. This could include a variety of approaches including Notch reporter systems as well as analysis of Notch target gene expression within the endothelial cells. The co-culture model system we have employed using human tumour cells in combination with mouse endothelial cells, allows for species-specific qPCR gene expression analysis therefore making Notch target analysis within the different cell types of the co-culture system feasible.

In addition, investigations into the effects of tumour cell JAG2 expression *in vivo* would be of interest. As a first step, stable knockdowns of *JAG2* could be produced and used in xenograft models. With this approach one could examine differences in vascular structure and density in association with JAG2-status. Although one could expect similar results to those observed *in vitro*, with *JAG2*-knockdown resulting in lower vascular density, the effects this may have on tumour growth is hard to predict. As discussed above, previous studies have shown that hyper-branching upon Notch inhibition, although resulting in increased vascular density, produced dysfunctional vessels resulting in hindered tumour growth [89]. With this in consideration, the reduced branching caused by *JAG2*-knockdown could potentially result in the formation of fewer, yet functional vessels and therefore enhance tumour growth. Delineation of such information would be crucial before considering JAG2 as a potential therapeutic target.

Application of the JAG2-model in neuroblastoma

Looking at this study from a different point of view, although hypoxic induction of *JAG2* was reported in multiple cellular systems, the majority of the biological/functional analyses performed were conducted in breast cancer cells. With the focus of this thesis being on neuroblastoma, investigation into the functionality of this model in neuroblastoma cellular systems is of interest. As discussed in the introduction, it has previously been reported that Notch signalling is elevated in hypoxic neuroblastoma cells [75]. Surprisingly, although *JAG2* is induced under hypoxia in neuroblastoma cells (Figure 7A), JAG2 expression did not display correlation with Notch target genes in primary neuroblastomas (Figure 7B). In addition, genes that correlated with *JAG2* expression in a panel of 88 neuroblastomas were not enriched for angiogenic processes. We therefore investigated the other Notch ligands in neuroblastoma cells in order to elucidate if the ligand responsible for the hypoxic effects on Notch signalling could be cell type-dependent. Interestingly, the only other ligand found to be induced by hypoxia in the neuroblastoma cell line SK-N-BE(2)c was the delta-like ligand *DLL1* (Figure 7C). Importantly, *DLL1* displayed correlation with the Notch target gene *HES1* in primary neuroblastomas (Figure 7D). In addition, *DLL1*-correlated gene expression displayed enrichment for genes involved in angiogenic processes, including endothelial cell markers *CD34* and *PECAM1* (*CD31*) (Figure 7E, F & G). These observations lead us to apply the co-culture tube formation model, described in the paper IV, on neuroblastoma cells. Preliminary results revealed that upon *DLL1*-knockdown in hypoxic SK-N-BE(2)c cells, there was a reduced capacity of neighbouring endothelial cells to form tubes (Figure 7H & I). These results suggest that different Notch ligands may contribute to angiogenesis in a tumour cell-type dependent manner. These are however preliminary results, and further investigations (such as those described above for JAG2) must be performed before conclusions can be made regarding the role of DLL1 in neuroblastoma.

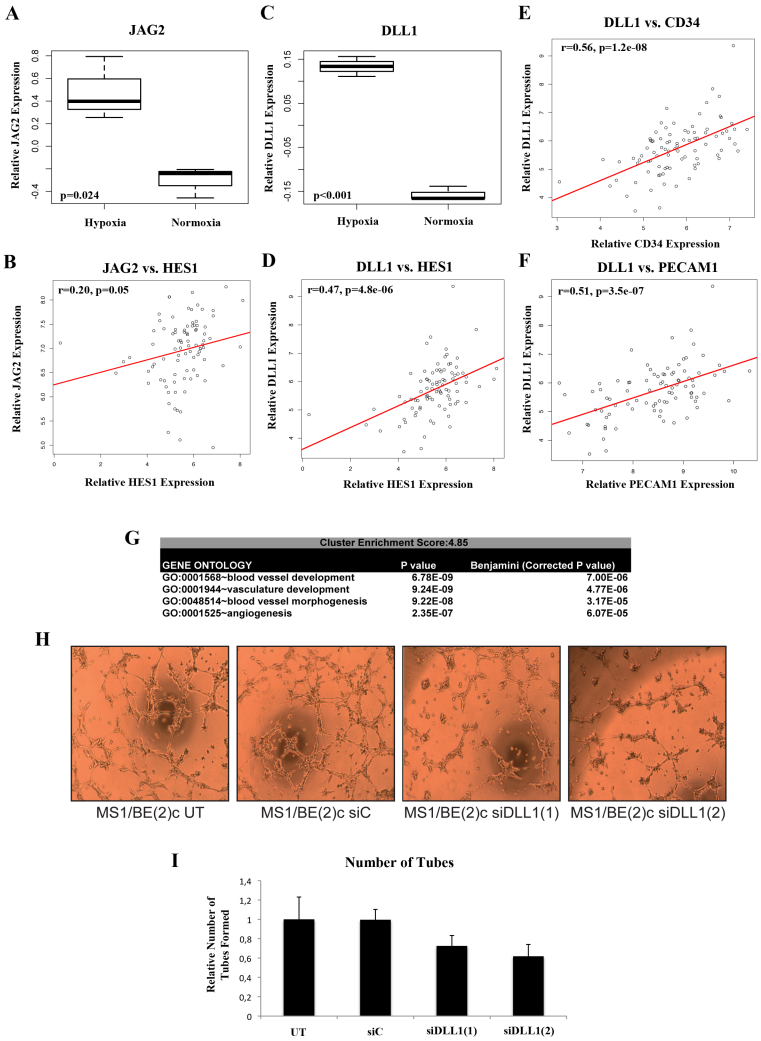

Figure 7 (previous page). ***DLL1 expression in neuroblastoma cells correlates with angiogenic processes and alters endothelial cell tube-forming capacity.*** *(A, C) Boxplot of qPCR based relative JAG2 (A) and DLL1 (C) expression in SK-N-BE(2)c cells cultured in different oxygen conditions. Student's T-test p-values are provided. (B, D) Scatter plots of relative JAG2 (B) and DLL1 (D) expression in relation to HES1 in an expression array of 88 neuroblastoma tumours. (E, F) Scatter plots of relative DLL1 expression in relation to CD34 (E) and PECAM1 (F) in an expression array of 88 neuroblastoma tumours. Pearson correlations (r) and accompanying p-values are provided for all scatter plots. (G) Gene ontology analysis of DLL1-correlated genes in 88 neuroblastoma tumours. p-values and multiple-testing corrected p-values (Benjamini) are provided. (H) Light microscopy images of MS1/SK-N-BE(2)c co-culture experiments. SK-N-BE(2)c cells were cultured in hypoxia for 24 hours and were pre-treated with different siRNAs (Untreated (UT), control (siC) and 2 different DLL1-targeting siRNAs) prior to mixing with MS1 cells. (I) Quantification of the number of tubes formed 8 hours post-cell mixing. Error bars represent standard deviation of 8 replicates. Co-culture experiments were carried out as described in paper IV.*

Popular Science Summary

Cancer is a group of diseases that is responsible for approximately 7.5 million deaths per year, worldwide. In comparison to their surrounding environments, cancer cells typically have a much faster rate of growth, which results in the formation of a tumour. Although uncontrolled growth is sufficient to produce a physical accumulation of cells, other changes must occur in order to allow the fast growing cells to survive as well as to allow the cells to spread, a process known as "metastasis formation". This spreading of the cancer cells throughout the body is considered to be the main cause of death amongst cancer patients. In the works described in this thesis, we focus primarily on a childhood cancer known as neuroblastoma, and investigate a number of changes that happen within the tumour during its development and progression.

One of the first hinders that a tumour encounters as it starts to grow is its supply of nutrients and oxygen. In normal tissues, the present blood vessels are sufficient for delivering the nutrients and oxygen required to keep the cells alive and functioning. In a tumour, on the other hand, the rapid growth of the cancer cells often exceeds the production of new blood vessels, which results in a nutrient/oxygen shortage, a state known as hypoxia. When cancer cells encounter these oxygen shortages they respond in a number of ways to stimulate and speed up the process of blood vessel formation, ensuring oxygen delivery and their survival. In paper IV we describe one such response to low oxygen that involves the production of a protein known as JAG2. By presenting JAG2 on their surface, tumour cells physically interact with the surrounding vascular (endothelial) cells, and trigger them to start forming new vessels.

In addition to delivering oxygen and nutrients to the tumours, blood vessels also provide the tumour cells access to the patients circulation, thereby acting as highways for the tumour cells to spread. However, before the tumour cells can gain entry into the circulation, the tumour cells themselves must become motile. This can be achieved in a number of ways, most commonly of which involves physical changes to the tumour cells appearance. In paper III, we describe a method by which aggressive neuroblastoma cells maintain an "immature" physical state, which resembles that of highly motile cells present during foetal development. By maintaining this immature

state, it is possible that the tumour cells are able to move through tissues and gain access to the patients' circulation, resulting in metastasis formation.

Yet another hurdle that the growing tumour cells must overcome is the fact that all cells have a limited life span. For every cell division that occurs, cells become "older". Within a cell's DNA are structures called telomeres, which essentially are representative of the cell's life span. Each time a cell divides, the telomeres become successively shorter and shorter. Once a critical length is reached, the cells' DNA is unstable, resulting in numerous breaks and degradation leading to cell death. In the case of tumour cells, they acquire methods of maintaining the length of their telomeres, thereby avoiding the aging process and instead reaching a state of limitless growth potential. In paper I, we describe a mechanism by which neuroblastomas maintain their telomeres, and show that this process is active specifically in patients with poor prognoses.

When addressing a patient's prognosis, correct classification of the patient is of utmost importance within the clinical setting. Determining the probability of a patient surviving the disease allows the clinicians to plan the treatment best suited for the patient. In the case of neuroblastoma, patients identified with non-aggressive disease typically undergo routine surgery without the need for harmful therapies such as chemotherapy and radiation therapy. Even with these mild treatment procedures, non-aggressive neuroblastoma patients survive the disease in over 90% of cases. Patients identified with aggressive disease, on the other hand, are subjected to a barrage of treatments including surgery, chemotherapy and radiation therapy amongst others. The use of such therapies, although being beneficial with regards to the patient's survival, can be extremely taxing on the patient's quality of life. In addition, despite such intense treatments, aggressive neuroblastoma patients survive the disease in less than 50% of cases. In papers I and II, we address the issue of current risk-stratification of neuroblastomas and identify a sub-group of patients with an extremely bad prognosis, surviving in less than 20% of cases. In doing so, we identify patients who are in potential need of therapies outside of those currently used to treat neuroblastomas in the clinic today.

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