Anaplastic lymphoma kinase activity, a therapeutic target, suppresses neuroblastoma cell differentiation

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Cover illustration: ALK signalling in neuroblastoma

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…So then it is not of him that willeth, nor of him that runneth, but of God that sheweth mercy. Romans 9:16 KJV

*I dedicate this thesis to my wife, Esther Siaw, for her love, support and her tremendous sacrifice which made my PhD journey smooth and successful***.**

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ABSTRACT

Neuroblastoma (NB) is the most common extracranial pediatric solid malignancy caused by the failed differentiation of precursor cells of the developing sympathetic nervous system. NB accounts for about 15% of childhood cancer-related deaths. Treatment failure and relapse are common in NB patients despite intensive chemotherapy and immunotherapy interventions, suggesting the need for new and effective treatment options. Common genetic aberrations associated with NB include *MYCN* amplification, chromosome 11q deletion, 1p deletion, 17q gain, 2p gain, and recurrent mutations in *Anaplastic Lymphoma Kinase* (*ALK*). While treatment of some categories of ALK-positive pediatric cancer patients such as non-Hodgkin lymphoma and inflammatory myofibroblastic tumour (IMT) with the first-generation ALK tyrosine kinase inhibitor (TKI), crizotinib, produced promising results, the outcome for ALK-positive NB patients was less encouraging, hence the need for more potent ALK TKIs for treatment of NB patients. This thesis aimed to further our understanding of ALK signalling and its role in NB differentiation and explore novel ALK TKIs in a neuroblastoma setting.

In the first study, we investigated the therapeutic efficacy of the second-generation ALK TKI, brigatinib, in an NB preclinical setting. Brigatinib was reported to be effective against ALK fusion-positive non-small cell lung tumours. We found that brigatinib potently inhibited both the activity of ALK full-length and growth of ALK-addicted NB cells *in-vitro*, in xenograft and *Drosophila* models. Compared to crizotinib, brigatinib inhibited the activities of different ALK-mutant alleles more effectively and potently inhibited crizotinib resistant ALK mutants in vitro.

In the second study, we characterized a novel *ALK-I1171T* mutant allele which we identified in a tumour from a 16 month old NB patient. We showed that ALK-I1171T is a gain-of-function mutation, which is resistant to crizotinib, but can be effectively inhibited

by second- and third-generation ALK TKIs such as brigatinib, ceritinib and lorlatinib. Based on these results and the severe toxic side effect of the initially administered chemotherapy, ceritinib monotherapy was chosen for this child. After 7.5 months of ceritinib treatment, the primary tumour shrunk in size and was removed surgically. The patient showed complete metastatic remission and remains in remission at 58 months post-treatment.

In the third and last study, we investigated *Disk large homologue 2* (*DLG2*), a gene reported to be uniquely upregulated in transient intermediary cells during Schwann cell precursor (SCP) differentiation to adrenal chromaffin cells. We found that *DLG2*, a gene located on the frequently deleted chromosome 11q in NB, is an NB tumour suppressor gene whose expression is lost in NB cell lines. Restoration of DLG2 expression inhibited NB cell growth and promoted NB cell differentiation. High expression of *DLG2* in NB tumours is associated with good prognosis. Mechanistically we showed that oncogenic ALK maintains an undifferentiated NB cell phenotype by repressing DLG2 expression via the ERK1/2-SP1 signalling cascade.

In summary, these findings highlight the role of ALK in differentiation and therapeutic potential of targeting ALK in ALK-positive NB tumours.

Keywords: Neuroblastoma, ALK, 11q, DLG2, SP1, differentiation, crizotinib, ceritinib, brigatinib.

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SAMMANFATTNING PÅ SVENSKA

Neuroblastom (NB) är en maligniteten hos barn och den troliga orsaken till sjukdomen är misslyckad differentiering av tidiga celler i det sympatiska nervsystemet. NB svarar för cirka 15% av alla barncancerrelaterade dödsfall. Återfall är vanligt förekommande hos NB-patienter, trots förbättrade behandlingsregimer med kemoterapi och immunoterapi, vilket indikerar ett tydligt behov av nya och mer effektiva behandlingsalternativ. Neuroblastom är en heterogen sjukdom med flera och olika genetiska avvikelser vilket inkluderar MYCN-amplifiering, deletion av kromosom 11q och/eller 1p. Upp reglerat uttryck av delar av kromosom 17q och/eller 2p. Få sjukdomsframkallande mutationer av individuella gener har observerats hos NB om man undantar mutationer i *anaplastiskt lymfomkinas* (*ALK*). Behandling av ALK-positiva Non-Hodgkins eller Inflammatorisk Myofibroblastisk tumörer (IMT) med första generationens ALK-tyrosinkinashämmare, crizotinib, gav mycket goda resultat, däremot var behandlingen av ALK-positiva NB-patienter mindre uppmuntrande. Härmed finns det ett behov av förbättrade och mer effektiva ALK hämmare för behandling av NB-patienter. Målet med denna avhandling var främst att öka vår förståelse av ALK-medierad signalering och dess roll i NB-differentiering, samt att utforska nya ALK hämmare för framtiden.

I den första studien i denna avhandling undersökte vi den terapeutiska effekten av en andra generationens ALK hämmare, brigatinib, i preklinisk neuroblastom miljö. Det var redan rapporterat att brigatinib är en effektiv hämmare mot ALK-fusionspositiva icke-små-cellet lungtumörer. I vår studie visar vi att brigatinib blockerar den enzymatiska aktiviteten hos både vildtyps ALK och onkogent ALK. Brigatinib stoppar tillväxten av ALK-positiva NB-cell linjer, mus-xenografter och i ett Drosophila-model system. Jämfört med första generationens hämmare har brigatinib en mer potent aktivitet och hämmar även potentiellt crizotinib-resistenta ALK-mutanter in vitro.

I den andra studien karakteriserade vi en *ALK-I1171T* mutantallel som identifierades i en tumör hos en 16 månader gammal NB-patient. Vi visade att ALK-I1171T mutationen är en konstitutiv aktiv ALK mutation som är resistent mot crizotinib. ALK-I1171T kan effektivt hämmas av andra och tredje generationens ALK TKI såsom brigatinib, ceritinib och lorlatinib. Baserat på resultaten och den allvarliga toxiska bieffekten av den initialt administrerade kemoterapin valdes ceritinib monoterapi för detta barn. Efter 7,5 månaders ceritinib-behandling, minskade primär tumör i storlek, avlägsnades kirurgiskt, och patienten visade fullständig metastaserad remission och är i kontinuerlig remission även efter 34 månader efter behandlingen.

I den tredje och sista studien undersökte genen *Disk large homolog 2* (*DLG2*), en gen som rapporterades vara uppreglerad i övergående fas när celler differentieras från Schwann Cell Precursors (SCP) till binjurekromaffinceller. *DLG2* genen är lokaliserad på den ofta deleterade kromosomen 11q och vars uttryck ofta gått förlorat i NB-cellinjer. Överuttryck av *DLG2*-genen hämmar NB-celltillväxt och främjade NB-celldifferentiering. Högt uttryck av DLG2 i NB-tumörer är associerad med god prognos. Mekaniskt visade vi att onkogen ALK upprätthåller odifferentierad NB-cellfenotyp genom att blockera DLG2-uttryck via ERK1/2-SP1-signalkaskaden.

Sammanfattningsvis visar mina resultat att ALK har en tydlig roll i differentieringsprocessen och att det finns en terapeutisk potential att behandla ALK-positiva NB-tumörer.

Nyckelord: Neuroblastom, ALK, 11q, DLG2, SP1, differentiering, crizotinib, ceritinib, brigatinib.

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals

I. **Joachim T. Siaw***, Haiying Wan*, Kathrin Pfeife, Victor M. Rivera, Jikui Guan, Ruth H. Palmer, Bengt Hallberg. (2016). Brigatinib, an anaplastic lymphoma kinase inhibitor, abrogates activity and growth in ALK-positive neuroblastoma cells, *Drosophila* and mice. *Oncotarget*, 7(20):29011-22. doi: 10.18632/oncotarget.8508.
(* Co-first author)

II. Jikui Guan*, Susanne Fransson*, **Joachim Tetteh Siaw***, Diana Treis*, Jimmy Van den Eynden, Damini Chand, Ganesh Umapathy, Kristina Ruuth, Petter Svenberg, Sandra Wessman, Alia Shamikh, Hans Jacobsson, Lena Gordon, Jakob Stenman, Pär-Johan Svensson, Magnus Hansson, Erik Larsson, Tommy Martinsson, Ruth H Palmer, Per Kogner, Bengt Hallberg. (2018). Clinical response of the novel activating ALK-I1171T mutation in neuroblastoma to the ALK inhibitor ceritinib. *Cold Spring Harb Mol Case Stud,* 4(4):a002550. doi: 10.1101/mcs.a002550.
(* Co-first author)

III. **Joachim Tetteh Siaw***, Niloufar Javanmardi*, Jimmy Van den Eynden*, Dan Emil Lind, Susanne Fransson, Angela Martinez-Monleon, Anna Djos, Rose-Marie Sjöberg, Malin Östensson, Helena Carén, Gunhild Trøen, Klaus Beiske, Ana P Berbegall, Rosa Noguera, Wei-Yun Lai, Per Kogner, Ruth H Palmer, Bengt Hallberg, Tommy Martinsson. (2020). 11q Deletion or ALK Activity Curbs DLG2 Expression to Maintain an Undifferentiated State in Neuroblastoma. *Cell Reports,* 32(12):108171. doi: 10.1016/j.celrep.2020.108171.
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CONTENT

ABBREVIATIONS

ADP	Adenosine diphosphate
ADRN	Adrenergic
ALCL	Anaplastic large cell lymphoma
ALK	Anaplastic lymphoma kinase
ALKAL1	ALK And LTK Ligand 1
ALKAL2	ALK And LTK Ligand 2
ALT	Alternative lengthening of telomere
ARID1A	AT-rich interaction domain 1A
ASCT	Autologous stem cell transplant
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATRA	All trans-retinoic acid
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
BC	Bridge cells
BCL2	B-cell lymphoma 2
BET	Bromodomain and extra-terminal domain
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5
BMP	Bone morphogenetic protein
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
CHD5	Chromodomain helicase DNA binding protein 5
CHEK1	Checkpoint kinase 1
CNS	Central nervous system
CNV	Copy number variation
CRC	Core transcriptional Regulatory Circuitry
CRKL	CRK like proto-oncogene, an adaptor protein
CT	Computed tomography
CXCR4	C-X-C chemokine receptor type 4
DBH	Dopamine beta-hydroxylase

DDR	DNA damage repair
DHFR	Dihydrofolate reductase
DLG2	Disc large homologue 2
DLGAP2	DLG associated protein 2
DDSB	DNA double-strand break
ECD	Extracellular domain
EFS	Event-free survival
EGFR	Epidermal growth factor receptor
EMA	European Medicine Agency
EML4	Echinoderm microtubule-associated protein like 4
EMT	Epithelial-mesenchymal transition
ERK1/2	Extracellular signal-regulated kinase 1/2
FAM150A	Family with sequence similarity 150 member A
FAM150B	Family with sequence similarity 150 member B
FANCA	Fanconi anemia, complementation group A
FDA	Food and drug administration
FGFR1	Fibroblast growth factor receptor 1
FISH	Fluorescence in situ hybridization
FRS2	Fibroblast growth factor receptor substrate 2
GATA3	GATA binding protein 3
GD2	Disialoganglioside 2
GDP	Guanosine diphosphate
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
H2AFX	H2A histone family member X
HAND2	Heart and neural crest derivatives expressed 2
HVA	Homovanillic acid
ID2	inhibitor of DNA-binding 2
IGF	Insulin-like growth factor receptor 1
IMT	Inflammatory myofibroblastic tumour
INRG	International neuroblastoma risk group

INRGSS	International neuroblastoma risk group staging System
INSS	International neuroblastoma staging system
IRS2	Insulin receptor substrate 2
ISL1	ISL LIM homeobox 1
JAK2	Janus kinase 2
Jeb	Jelly belly
LDLa	Low-density lipoprotein class A
LTK	leukocyte tyrosine kinase
MAGUK	Membrane-associated guanylate kinases
MAM	Meprin A5 protein and receptor protein tyrosine phosphatase mu
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homologue
MES	Mesenchymal
MIBG	Metaiodobenzylguanidine
MNA	MYCN amplified
MRE11	Meiotic Recombination 11 Homologue 1
MRI	Magnetic resonance imaging
MYC	Myelocytomatosis Viral Oncogene Homologue
MYCN	Neuroblastoma MYC oncogene
NB	Neuroblastoma
NC	Neural crest
NCA	Numerical chromosome alterations
NCC	Neural crest cell
NDDS	Neuroblastoma new drug development strategy
NEFL	Neurofilament light polypeptide
NEFM	Neurofilament medium polypeptide
NET	Norepinephrine transporter
NGF	Nerve growth factor
NGS	Next-generation sequencing
NPM	Nucleophosmin

NRG1	Neuregulin 1
NSCLC	Non- small cell lung cancer
OS	Overall survival
P13K	Phosphoinositide 3-kinase
PAX2	Paired box gene 2
PC12	pheochromocytoma 12 cells
PDZ	PSD95-Dlg-ZO-1
PFS	Progression-free survival
PHOX2A	Paired mesoderm homeobox protein 2A
PHOX2B	Paired mesoderm homeobox protein 2B
PI3K	Phosphoinositide 3-kinase
PLCγ	Phospholipase Cγ
PSD93	Postsynaptic density protein 93
PTPN11	Protein tyrosine phosphatase non-receptor type 11
RA	Retinoic acid
RAP1	RAS-related protein Rap-1A
RB	Retinoblastoma
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
RTK	Receptor tyrosine kinase
SA	Sympathoadrenal
SAP	Sympathoadrenal precursor
SCA	Segmental chromosomal alteration
SCD2	Suppressor of constitutive dauer formation 2
SCP	Schwann cell precursor
SDF1	Stromal cell-derived factor 1
SH3	Src homology 3 domain
SHANK2	SH3 and multiple ankyrin repeat domains 2
SIOPEN	International Society of Pediatric Oncology, European Neuroblastoma
SMG	Significantly mutated gene
SNAI2	Snail family transcriptional repressor 2

SNP	Single nucleotide polymorphism
SOX1	SRY-box 1
SOX2	SRY-box 2
SP1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
TBX2	T-Box transcription factor 2
TERT	Telomerase reverse transcriptase
TF	Transcription factor
TGFβ	Transforming growth factor-β
TH	Tyrosine hydroxylase
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
mTORC1	Mammalian target of rapamycin complex 1
TRKA	Tropomyosin receptor kinase A
TSG	Tumour suppressor gene
VIM	Vimentin
VMA	Vanillylmandelic acid
WGS	Whole-genome sequencing
WT1	Wilms' Tumour 1

1 INTRODUCTION

1.1 Cancer

Cancer is a genetic disease which involves uncontrolled cell proliferation. It is characterized by genomic instability which manifests through mutations, gene amplifications, deletions or gene translocations, leading to aberrant protein expression and function. Genomic instability gives rise to multiple changes, including inactivation of tumour suppressors and activation of proto-oncogenes (Weinberg, 1989; Yokota and Sugimura, 1993). These result in the disruption of the delicate control and the finely tuned balance of cell growth, differentiation and apoptosis, culminating in unrestrained cell clonal expansion into malignant tumours. The conversion of normal cells into malignant cells involves multiple steps including tumour initiation, promotion and progression (Hanahan and Weinberg, 2011; Pitot et al., 1981). Though cancer has long been considered a genetic disease, there should be caution against such absolute dogma, as the ultimate driver of cancer pathogenesis is aberrant cell signalling, involving abnormal enzymatic activities, in key processes, such as cell cycle, growth, differentiation, survival (Yaffe, 2019). It is projected that cancer could soon rank as the highest cause of death in almost all countries (Bray et al., 2018).

1.1.1 Oncogenes and tumour suppressors

Oncogenes

Normal cell behaviour involves well-regulated cell proliferation, differentiation, programmed cell death, also known as apoptosis, and senescence. Proto-oncogenes mainly stimulate cell division, growth and cell survival. They become oncogenes through the acquisition of gain-of-function point mutations (e.g. in BRAF, RAS, EGFR, ALK), gene amplification (e.g. *MYC*, *MYCN*, *DHFR*, *EGFR*, *RAS*), genomic translocation (e.g. *BCR-ABL*, *EML4-ALK*, *NPM-ALK*,) or epigenetic modifications causing hyperactivation of protein expression and signalling, with concomitant effect of unrestrained cell division, growth and cell survival, the characteristic features of cancer (Croce, 2008; Lee and Muller, 2010; McCormick, 2015; Yaffe, 2019).

Mutations in the *RAS* family genes (*K-RAS*, *H-RAS* and *N-RAS*) are common, and found in 16% of all human cancers, with significant overrepresentation in specific cancers (Prior et al., 2012). For instance, K-*RAS* mutations are found in 95% of all pancreatic cancers and 50% of colon cancers and also represents the most mutated (85%) of all *RAS* gene, with *N-RAS* at 12% and *H-RAS* at 3% (Conti, 1992; Cox and Der, 2010; Miller and Miller, 2011). RAS proteins constitute the founding members of the RAS-related small GTPase

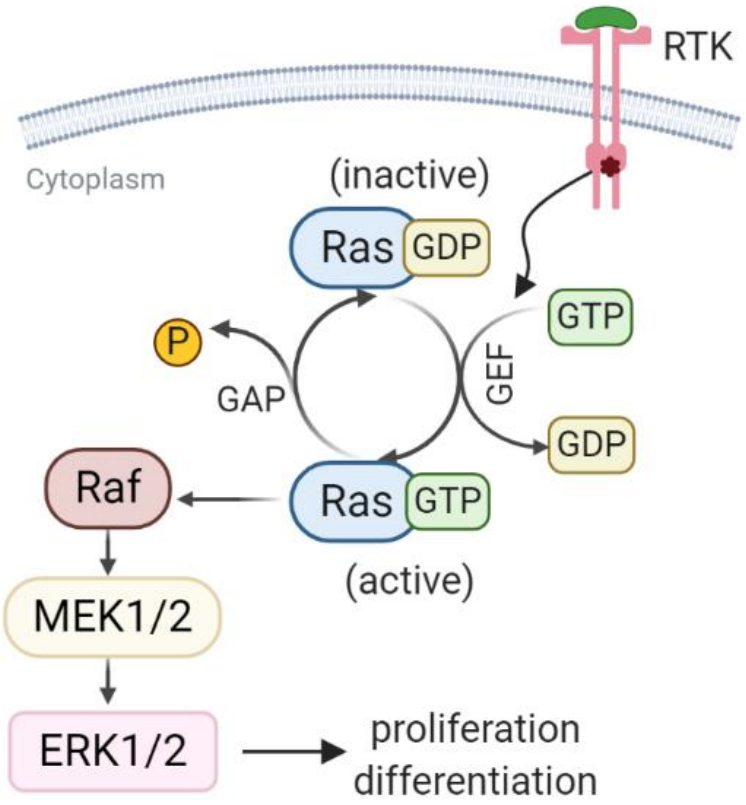

Figure 1. ***Schematic illustration of RAS signalling****. Upon stimulation, eg. from a receptor tyrosine kinase (RTK), guanine nucleotide exchange factors (GEFs) mediate the exchange of GTP for the RAS-bound GDP in a process where GDP-bound inactive RAS is switched to GTP-bound active RAS. Activated RAS then signals to downstream targets. GTPase-activating proteins (GAPs) catalyze the hydrolysis of RAS-bound GTP, leading to the formation of inactive GDP-bound RAS.*

superfamily which act as molecular switches that, when activated through the binding of GTP, elicit a plethora of signalling events that contribute to key cellular processes, including cell proliferation, differentiation, cell division and cell survival (Cox and Der, 2010) (Figure 1). The evolutionary significance of this pathway is highlighted by the increasing number of pathological conditions that have been associated with defects in some of its components (Fernández-Medarde and Santos, 2011). Upstream growth factor receptors like receptor tyrosine kinases (RTKs) can activate RAS/MAPK and PI3K/AKT pathways in normal cells. Therefore, even in the absence of specific mutation in members of the RAS/MAPK axis, constitutive or oncogenic activation of upstream signalling proteins, including RTKs such as ALK and EGFR can potently drive RAS/MAPK signalling (Hallberg and Palmer, 2013; Lemmon and Schlessinger, 2010). These implicate the RAS/MAPK pathway and its components as common oncogenes in cancer and highlights the therapeutic potentials of targeting members of this pathway.

Tumour suppressors

Tumour suppressor genes (TSGs) act to check unrelenting cell growth and promote DNA repair and activation of cell cycle checkpoint (Benedict et al., 1983; Friend et al., 1986; Harris et al., 1969; Lee and Muller, 2010). TSGs may be growth-constraining factors that act to counterbalance growth-promoting proto-oncogenes and oncogenes, thereby making the reduction or loss of tumour suppressor function as essential as oncogene activation in tumourigenesis. Mutations in TSGs are mostly loss-of-function mutations. They are thought to be recessive at the cellular level, necessitating the inactivation of both alleles during tumourigenesis (Knudson, 1971; Payne and Kemp, 2005). This view was based on Knudson's 'two-hit' model of tumourigenesis, in which one mutation (the first 'hit') is usually familial, but could also be sporadic. In contrast, the second hit occurs sporadically and significantly accelerates tumour formation, as famously exemplified in the retinoblastoma gene (*RB*) of hereditary and nonhereditary forms of retinoblastoma (Friend et al., 1986; Knudson, 1971) and *p53* of Li-Fraumeni syndrome (Finlay et al., 1989; Malkin et al., 1990). Knudson's 'second-hit' is frequently in the form of allelic deletion; however, promoter methylation with subsequent loss of gene expression could also occur (Friend et al., 1986).

A different mode of TSG inactivation is through the phenomenon of dominant-negative mutation. Contrary to the Knudson's two-hit hypothesis, the remaining wild-type allele does not require inactivation since the dominant-negative mutant protein plays the role of inactivating the wild-type protein by binding to the latter to form a non-functional protein complex (Kern et al., 1992; Unger et al., 1992). This category of TSG inhibition is well illustrated in some p53 mutants. Certain p53 mutant proteins form heterotetramer complex with wild-type p53 resulting in the inactivation of the latter (Kern et al., 1992; Unger et al., 1992). These indicate that a dominant-negative mutation in one p53 allele is enough to inactive the function of p53 in a cell. Certain mutations in Wilms' tumour gene (*WT1*) are also considered to act in a dominant-negative way (Haber et al., 1992; Reddy et al., 1995).

Some TSGs however, have haploinsufficient phenotypes. In this scenario, a mutation in, or deletion of, one allele of a tumour suppressor gene results in the manifestation of extreme sensitivity to reduced gene dosage. In other words, a single functional copy of this gene is inadequate to maintain normal function in cell growth and development. Earlier haploinsufficient TSGs that were identified include the cyclin-dependent kinase inhibitor $p27^{kip1}$, *p53* and ***TGF-β*** (Fero et al., 1998; Tang et al., 1998; Venkatachalam et al., 1998). Over 40 TSGs have since been shown to exhibit evidence of haploinsufficiency between the period of 1998 and 2005 (Payne and Kemp, 2005).

Another way of inhibiting TSG function is through the transcriptional repression of the TSG expression by oncogene mediated signalling, which might involve a direct binding/action of transcription repressors or via epigenetic silencing (Kazanets et al., 2016; Mirmohammadsadegh et al., 2006; Sasahara et al., 1999; Yan et al., 2006). Oncogenic RAS signalling, through SP1, was found to inhibit the expression of RECK, a tumour suppressor which inhibits tumour invasion and metastasis (Sasahara et al., 1999). Furthermore, oncogenic NPM-ALK signalling in *ALK*-positive T cell lymphoma cells facilitates epigenetic silencing of the context-dependent TSG, STAT5A (Zhang et al., 2007). Therefore, identifying instances and unravelling the mechanisms of oncogene mediated suppression of TSGs may help develop therapeutic strategies to restore their (TSGs) expression in tumour cells. This approach was explored in Paper III of this thesis.

Genetic lesions, frequently in the form of mutation, cause gain-of-function activation of oncogenes and loss-of-function in TSGs. However, considering the low spontaneous mutation rate, i.e. about one mutation in every 10^7 cell divisions, for any given gene in cells, and the requirement of multiple mutations in tumourigenesis, one could expect that a considerable length of time may be needed to achieve specific mutation permutations vital to transform normal cells to malignant tumours (Kumar and Subramanian, 2002). There is a higher cancer incidence in ageing populations (Smetana et al., 2016), confirming that cancer in general is an age-related disease. There are nevertheless childhood cancer types in humans, which require special consideration.

1.1.2 Adult cancers and childhood cancers

As mentioned above, cancer is often regarded as an age-related disease, most frequently diagnosed in adults. The incidence of most cancers increases with age, with a rapid rise starting in midlife (White et al., 2014). Despite its paucity, pediatric cancer is the second most common cause of death in children below the age of 14 years (CDC, 2020; Saletta et al., 2014). Adult cancers tend to arise from a multistep process which progresses over several years or decades with simultaneous accumulation of several mutations (Scotting et al., 2005). On the contrary, pediatric cancers develop over a much shorter time, with some even occurring *in-situ* (Beckwith and Perrin, 1963; Scotting et al., 2005), suggesting that much fewer events may drive their initiation and progression.

Adult cancers generally tend to have higher mutation burden compared to pediatric cancers (Gröbner et al., 2018; Kandoth et al., 2013). In a study which looked at the mutational landscape and significance across different cancer types, Kandoth and colleagues coined the phrase “significantly mutated genes” (SMGs) to describe genes under positive selection either in individual or multiple cancer types that tend to exhibit higher mutation frequencies (Kandoth et al., 2013). These SMGs play roles in a vast range of cellular processes. The authors showed that 47% of pediatric tumours contain

at least one SMG mutation, with the majority (57%) harbouring only one (Gröbner et al., 2018). On the contrary, 93% of adult tumours exhibit a minimum of one mutation in an adult-related SMG mutation, and 76% of these harbour multiple SMG mutations (Gröbner et al., 2018; Kandoth et al., 2013). The frequent mutual exclusivity of most SMGs in different childhood cancer types highlights the specificity of single putative driver genes in pediatric cancers, in contrast to more recurrent co-mutation in adult cancer types (Gröbner et al., 2018; Kandoth et al., 2013). Evidence from this and many other reports strongly point to a comparatively higher mutational burden, frequently in the form of point mutations, in adult cancers. This fact could stem from the chronic exposure of adults to mutagenic processes such as smoking and ultraviolet radiation during their lifetime (Carpenter and Bushkin-Bedient, 2013). This difference in mutational burden impacts the patient tumour's response to targeted therapy, resistance and relapse.

About 90% of human cancers arise from epithelial tissues and are hence referred to as carcinomas, which include, for instance, tumours of the gastrointestinal tract, genitourinary tract, skin, breast, prostate and lung (Frank, 2007). These epithelial tissues self-renew continuously throughout life and constitute the source of most adult cancers (Frank, 2007; Tomasetti et al., 2013). The renewing cells, called stem cells, of these epithelial tissues, have a higher risk for accumulating mutations (Frank, 2007). Cancer incidence in these renewing tissues has been found to rise sharply with age (Frank, 2007; Tomasetti et al., 2013). By contrast, pediatric cancers often originate from rapidly dividing progenitor cells of developing organs and tissues, where cell division is comparatively little later in life (Rahal et al., 2018; Tomasetti et al., 2013). In general, pediatric cancers tend to favour a developmental model of cancer initiation. Here, block of terminal differentiation of precursor cells may underline the mechanism of origin of the disease. The precursor cells are immature cells of the developing organ from which these tumours arise. For instance, genes that are overexpressed in Wilms' tumour (pediatric kidney tumour) are mostly similar to those expressed at an early stage of kidney development, such as *PAX2*, *EYA1*, and *HBF2*. In contrast, those downregulated in the tumour are similar to genes expressed at the late stages of kidney development, such as *WT1* (*Wilms' tumour 1*) (Dekel, 2003; Hastie, 2017). Furthermore, gene expression analysis has shown that different clinical stages of neuroblastoma (NB) reflect differentiation arrest at different stages of the sympathoadrenal (NB cellular source) developmental trajectory (Hoehner et al., 1996; Nakagawara and Ohira, 2004). These spurred the motivation to explore the therapeutic use of differentiation agents to induce terminal differentiation of pediatric tumours like NB and embryonal rhabdomyosarcoma (Svalina and Keller, 2014). Understanding the molecular mechanisms involved in the suppression of differentiation of precursor cells of tumour origin will enable identification of biomarkers and targets for therapeutics.

1.2 Neuroblastoma

NB is the most common extracranial childhood solid malignancy. This cancer is suggested to arise from neural crest (NC)-derived cells, of the developing sympathetic nervous system (SNS), where tumours are located in the adrenal gland or sympathetic ganglia and account for approximately 15% of childhood cancer-related deaths (Gatta et al., 2014; Maris et al., 2007; Matthay et al., 2016; Park et al., 2010). NB patients show striking variability in clinical outcome when the disease is classified by age, stage, ploidy, histology, and biologic characteristics such as *MYCN* amplification status and TRKA expression (Combaret et al., 1997; Goto et al., 2001; Schmidt et al., 2000; Tanaka et al., 1995). The International Neuroblastoma Staging System (INSS) has classified NB into five main stages based on the above-mentioned parameters (Brodeur et al., 1993). Stage 1 and 2 generally represent localized, non-metastatic, completely resectable tumours or tumours with incomplete excision. Stage 3 represents unresectable tumour with not very distant metastatic tumour. Stage 4 tumours are advanced with distant metastatic disease. Stage 4S is the last category with localized primary tumour as defined by stage 1 or 2 in patients under 12 months with dissemination limited to the liver, skin, and/or bone marrow. Generally, stage 4S NB represents a more favourable group with tumours which undergo spontaneous regression with little or no therapy (Brodeur, 2018; Matthay, 1998; Nickerson et al., 2000). NB is also classified, by the International Neuroblastoma Risk Group Staging System (INRGSS), as low, intermediate and high-risk based on INSS stage, age, DNA ploidy, histology, grade of tumour differentiation, *MYCN* amplification status and chromosome 11q status (Cohn et al., 2009; Monclair et al., 2009; Sokol et al., 2020).

Imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) are used to diagnose NB tumours. Metaiodobenzylguanine (MIBG) scanning is also used to diagnose both primary and metastatic NBs (Vik et al., 2009; Yang et al., 2012). Urine catecholamine metabolites such as vanillylmandelic acid (VMA) and homovanillic acid (HVA), are used for diagnostic and follow-up purposes (Barontini de et al., 1971; Matthay et al., 1999). The biopsy from a tumour is used to diagnose and obtain genetic data needed for risk-group assignment and treatment stratification. Today genomic profiling of NB tumours is performed using different technologies/methods, including (i) whole-genome sequencing by next-generation sequencing (NGS), (ii) targeted sequencing by sanger sequencing and NGS, (iii) single nucleotide polymorphism (SNP) arrays for detection of structural copy number variations, (iv) comparative genomic hybridization (aCGH) for detection of whole and structural chromosomal copy number variation and (v) Fluorescent *in-situ* hybridization (FISH) for detection of structural alterations (Bignell et al., 2004; Moreno et al., 2020; Savelyeva and Schwab, 2001; Zhao et al., 2004)

1.2.1 Genetic abnormalities in neuroblastoma aetiology

Though the exact aetiology and initiation (origin) of the NB are not clear and very enigmatic, certain genetic factors have consistently been associated with NB pathogenesis. These genetic factors can be constitutional or somatic aberrations or both. However, while only 1-2% of NBs occur in the familial context, an overwhelming 98% of all NB cases occur sporadically (Deyell and Attiyeh, 2011). Genetic abnormalities found in NB tumour cells occur in the form of mutations, segmental chromosomal alterations (SCAs) and numerical chromosome alterations (NCAs). Common somatic mutations are found in genes such as *ALK* (10%), *PTPN11* (2.9%) and *ATRX* (2.5%), whereas the latter, i.e. mutation, is even higher in NB patients older than five years (Cheung et al., 2012; De Brouwer et al., 2010; Pugh et al., 2013). SCAs include alterations such as *MYCN* amplification (25%), chromosome *17q* gain (65%), *11q* (20-45%), deletion, *1p* deletions and 2p gain (Abel et al., 1999; Carén et al., 2008; Carén et al., 2010; De Brouwer et al., 2010; Hallberg and Palmer, 2013; Javanmardi et al., 2019; Mlakar et al., 2017; Pugh et al., 2013).

1.2.1.1 *MYCN* amplification

MYCN is a member of the MYC family of TFs with basic helix-loop-helix motifs, which complexes with other proteins and binds directly or indirectly to target gene regulatory elements, in an E-box-dependent or independent manner, to activate or repress gene expression (Corvetta et al., 2013; Wenzel et al., 1991). MYCN controls the expression of thousands of genes that regulate cell proliferation or cell cycle progression, maintenance of pluripotency of cells and is involved in organogenesis during embryonic development (Higashi et al., 2019; Hsu et al., 2016; Sawai et al., 1993; Stanton et al., 1992). During embryogenesis, MYCN plays a role in the regulation of NC cell (NCC) fate in the aspects of ventral migration of NCCs and neural differentiation (Wakamatsu et al., 1997). Mouse embryos deficient in *MYCN* expression exhibited a tremendous decline in the number of mature neurons of the sympathetic ganglia and dorsal root ganglia, thereby highlighting the essential role of MYCN in the formation of NC-derived neurons (Stanton et al., 1992). Furthermore, MYCN expression is lost or significantly reduced in adult tissues and differentiated NC-derived neurons (Higashi et al., 2019; Zimmerman et al., 1986). Together, these findings imply a spatio-temporal mechanism of the regulation of MYCN expression.

MYCN is located on chromosome 2p24, and its amplification is associated with 20-25% of NB, (Brodeur et al., 1984; Carén et al., 2010; Mathew et al., 2001; Muñoz et al., 2006). In all NB disease stages, *MYCN* amplification represents the essential genetic alteration which strongly predicts worst prognosis, aggressive phenotype and poorer survival, and

is mostly associated with an advanced stage of disease (Brodeur et al., 1984; Campbell et al., 2017; Cohn et al., 2009). Deregulated *MYCN* expression drives NB cell proliferation and promotes undifferentiated NB cell phenotypes and poor prognosis (Goto et al., 2001). Paradoxically, some NB cells lines, such as SK-N-AS, with a unique genetic setup, exhibit very low *MYCN* expression, and rather unresponsive to differentiation agents such as retinoic acid (RA). In contrast, NB cells with moderate to high *MYCN* expression such as SH-SY5Y and SK-N-BE(2) respectively, differentiate in the presence of RA (Guglielmi et al., 2014). Restoring *MYCN* expression in SK-N-AS induced and further potentiated RA-mediated neuronal differentiation (Guglielmi et al., 2014). These findings suggest that even though high MYCN expression blocks NB differentiation, MYCN nevertheless plays in RA-induced differentiation (Guglielmi et al., 2014). Indeed, it has been shown that *MYCN* expression is rapidly downregulated in the presence of RA prior to biochemical and morphological differentiation of NB cells (Thiele et al., 1985).

NB tumours have been shown to consist of two unique cell types, namely mesenchymal (MES) and adrenergic (ADRN) cell types (van Groningen et al., 2017). ADRN NB cells are described as $CD133^-$ cells that express genes such as *PHOX2A*, *PHOX2B* and *DBH* that drive adrenergic lineage differentiation (van Groningen et al., 2017). In contrast, MES NB cells express the stem cell marker CD133 and show high expression of mesenchymal markers such as *SNAI2*, *VIM* (vimentin) and *FN1* (fibronectin) (van Groningen et al., 2017). Non-MYCN or very low-MYCN expressing NB cell lines are more mesenchymal with a striking genetic resemblance to undifferentiated NCCs. They are resistant to differentiation by RA, whereas, those with moderate to high *MYCN* expression resemble committed adrenergic lineage precursors and are responsive to RA (Masserot et al., 2016; Messi et al., 2008; van Groningen et al., 2017). MYCN has been suggested to cooperate with and amplify the physiological output of a special class of transcription factors that include PHOX2B, ASCL1, GATA3 and HAND2, which form Core transcriptional Regulatory Circuitry (CRC) that promote and maintain ADRN identity of NB cells (Wang et al., 2019). ADRN NB cells are generally sensitive to chemotherapy and responsive to RA induced differentiation (van Groningen et al., 2017). These properties of NB cells suggest that despite the poor prognosis associated with *MYCN* amplification, the latter offers a therapeutic vulnerability to explore with differentiation-inducing agents such as RA since differentiated NB tumour histology predicts good prognosis.

1.2.1.2 Chromosome 1p deletion and 17q gain

Another unbalanced chromosomal aberration in NB cells is the deletion of chromosome 1p arm, which was first identified as a recurrent chromosomal aberration during cytogenic analyses of primary NB tumours and NB cell lines (Brodeur et al., 1977; Gilbert et al.,

1982). Chromosome 1p deletion (1p-del) occurs in about 30-35% of NB cases and has a strong association with *MYCN* amplification, and predicts poor prognosis in a univariant analysis but is not an independent predictor of overall survival (OS) (Jensen et al., 1997; Maris et al., 2001; Maris et al., 1995; Martinsson et al., 1995). Two 1p del critical regions have been reported, one that is distally located in a shorter 1p36.2-3 del, which is frequently associated with *MYCN* single-copy NB, and a more proximal one located in a longer 1p35-36.1 del, having a major association with about 70% of *MYCN*-amplified (MNA) NB tumours (Maris et al., 2001; Schleiermacher et al., 1994; Takeda et al., 1994). *CHD5* (*chromodomain helicase DNA binding protein 5*), *CAMTA1*, *KIF1B* and *ARID1A* are examples of TSGs that are located on chromosome 1p, and lost in 1p del (Bagchi et al., 2007; Fujita et al., 2008; García-López et al., 2020). CHD5 overexpression inhibited tumour cell growth in NB xenograft models (García-López et al., 2020). Recently, the loss of *ARID1A* in 1p-del has been shown to potentiate *MYCN*- mediated oncogenesis in NB models (García-López et al., 2020; Shi et al., 2020), and causes the conversion of NB cells from adrenergic to mesenchymal cell state (Shi et al., 2020). It is worth noting that while the majority of 1p-del occur in the form of somatic aberrations, rare cases of constitutional 1p-del have been identified (Maris et al., 2001; White et al., 1997).

The most recurrent segmental chromosomal alteration in NB is the unbalanced gain of the long arm of chromosome 17 (i.e. 17q-gain), occurring in about 54-62% cases, and is associated with poor prognosis (Bown et al., 1999; Gilbert et al., 1984; Vandesompele et al., 2005). The gain of 17q is a common feature of advanced NB disease, tumours in children who are less than one year of age, and frequently occurs with MYCN amplification or 1p-del (Bown et al., 1999; Lastowska et al., 1997). The fundamental mechanism of 17q-gain is an unbalanced translocation, with different partner chromosomes, of which the most frequent site of the translocation is 1p, resulting in a gain of the distal arm of 17q and a simultaneous loss of 1p (Lastowska et al., 1997; Savelyeva et al., 1994; Van Roy et al., 1994).

PPM1D and *BIRC5* (*survivin*) located on chromosome 17q, are highly expressed in NB tumours and strongly associated with poor prognosis (Islam et al., 2000; Saito-Ohara et al., 2003). Knockdown of *PPM1D* or pharmacological inhibition of *BIRC5* significantly suppresses growth and induces apoptosis in NB cells, making these genes possible therapeutic targets in NB (Islam et al., 2000; Lamers et al., 2011; Moreno et al., 2020; Saito-Ohara et al., 2003).

1.2.1.3 Chromosome 11q deletion

Chromosome 11q alteration is one of the most recurrent SCAs, which occurs in about 20-45% of all NB cases and is associated with worst prognosis (Carén et al., 2010; Mlakar et al., 2017; Spitz et al., 2003; Spitz et al., 2006). 11q-deletions (11q-del) are significantly associated with advanced stages of NB (Juan Ribelles et al., 2019; Mosse et al., 2007). While NB patients with stage 4S tumours generally tend have better prognosis, stage 4S tumours harbouring 11q-del tend to have increased relapse susceptibility (Juan Ribelles et al., 2019; Spitz et al., 2006). These observations suggest 11q deletion as a potential prognostic marker of NB patient outcome, hence, the inclusion of 11q-del as an independent risk factor in the International Neuroblastoma Risk Group (INRG) pretreatment risk classification (Cohn et al., 2009). 11q-alteration is mostly associated with older NB patients, with a median age at diagnosis for NB tumours with 11q-del around 36-42 months, while that of MNA tumours is about 21-24 months (Carén et al., 2010; Juan Ribelles et al., 2019). 11q-del tumours also exhibit a characteristic propensity of high chromosome instability phenotype (Carén et al., 2010; Spitz et al., 2003).

The high frequency of chromosomal breakage associated with 11q-del suggests chromosomal instability and shows that certain genes on the q-arm of chromosome 11 might play key roles in the associated genomic instability phenotype (Carén et al., 2010; Spitz et al., 2003). Unbalanced 11q-del occurs in an almost exclusively hemizygous manner, suggesting that the recurrent genomic instability phenotype could be due to haploinsufficiency, epigenetic modification, or inactivation of second allele by mutation (Juan Ribelles et al., 2019; Mlakar et al., 2017). Genes such as *ATM* (11q22.3), *MRE11A* (11q21), *CHEK1* (11q24.2) and *H2AFX* (11q23.3), located on chromosome 11q, are involved in the maintenance of genomic stability (Ditch and Paull, 2012; Mandriota et al., 2015; Ward and Chen, 2001).

Ataxia-telangiectasia mutated (ATM) regulates cell cycle checkpoints and plays a role in the coordination of cellular response to DNA double-strand breaks (DDSBs) by activating specific DNA repair and signalling pathways, thereby contributing to maintaining genomic stability (Ditch and Paull, 2012; Mandriota et al., 2015). Mandriota et al. described *ATM* as a potential haploinsufficient NB tumour suppressor gene, which when inactivated mimics 11q-del related aggressive phenotype in NB (Mandriota et al., 2015). During DNA damage repair, MRE11 participates in the formation of a trio-protein complex called MRN complex (MRE11-RAD50-NBS1), which identifies DNA damage sites, recruits ATM to the DNA damage site and aids ATM to initiate DNA damage repair by the phosphorylation and activation of its respective substrates (Dupré et al., 2006; Podhorecka et al., 2010). At the DNA damage site, a histone variant H2AX, coded by *H2AFX* gene, is phosphorylated on serine 139 by ATM and ATR in response to DDSBs and single-strand

breaks respectively (Rogakou et al., 1998; Ward and Chen, 2001). Phosphorylation of H2AX to γH2AX at DDSB sites has been suggested to be critical in the co-localization and assembly of DNA damage repair (DDR) proteins for nuclei foci formation and promotion of DDSB repair and genome stability (Podhorecka et al., 2010). Hence, loss of H2AX is associated with impairment of recruitment of DDR proteins such as BRCA1 and NBS1 to DDSB foci, repair defects and increased chromosomal instability in human cells (Bassing et al., 2002; Celeste et al., 2002). CHEK1 acts to relay checkpoint signals when it is phosphorylated by the ATR or ATM (Bartek and Lukas, 2003; Walworth et al., 1993). In response to DNA damage, upstream checkpoint kinases, particularly ATR, rapidly phosphorylate CHEK1 at serine-317 and serine-345, leading to CHEK1 activation (Zhao and Piwnica-Worms, 2001). Activated CHEK1 in turn relay checkpoint signals by phosphorylating numerous downstream targets resulting in cell cycle checkpoint activation, cell cycle arrest, DNA repair or cell death when DNA damage is severe to stop damaged cells from continuing through the cell cycle (Carr et al., 1995; Patil et al., 2013; Walworth et al., 1993). The apparent cluster of some vital DDR genes on 11q suggests the importance of the chromosome 11q arm in maintaining genome stability and, therefore, might explain the observed increase in genomic instability associated with 11q-del NB tumours. This condition also implies that a homozygous 11q deletion could result in unsustainable or deleterious genomic instability, with no potential growth advantage to tumour cells, hence the frequently observed hemizygous 11q-del instead in NB. it is therefore reasonable to assert that hemizygous 11q aberrations create a tolerable genomic instability that could be beneficial in tumour progression and or perhaps contribute to providing the genetic aberration milieu needed for tumour initiation.

A couple of decades ago, Bader and colleagues showed that microcell-mediated transfer of chromosome 11 into an NB cell line, with 11q-del, induced cell differentiation (Bader et al., 1991). This finding suggests that chromosome 11 could harbour pro-differentiation gene(s), even though none had been characterized until recently. Lopez et al. found that *SHANK2,* a gene located on 11q.13, was disrupted by structural variations in non-MNA NB tumours (Lopez et al., 2020). *SHANK2* overexpression in NB cells significantly inhibited growth and potentiated RA-induced differentiation, thereby suggesting a tumour suppressor role of *SHANK2*, a gene frequently disrupted in 11q-del NB tumours (Lopez et al., 2020). These data also suggest that the postsynaptic adapter protein-coding gene, *SHANK2*, could be a pro-differentiation gene on chromosome 11. A more proximal breakpoint cluster mediates *SHANK2* disruption in non-MNA tumours in the 11q13 region. A second distal breakpoint cluster at 11q14 was also identified. The latter leads to disruption of *DLG2* gene (Lopez et al., 2020; Siaw et al., 2020). DLG2 (discussed later in section 1.2.5) has not been functionally characterized in the study by Lopez and colleagues (Lopez et al., 2020).

The paradox of the role of 11q-del in tumour initiation

11q-alteration occurs mostly in older NB patients. Median age at diagnosis for NB tumours with 11q-del is about 36-42 months (Carén et al., 2010; Juan Ribelles et al., 2019). These observations led to the speculation that 11q-del is a late event and hence might not be required for tumour initiation (Juan Ribelles et al., 2019; Mlakar et al., 2017; Spitz et al., 2006). Intriguingly, constitutional 11q alterations have been reported in children and found to be associated with mental and growth retardation and other multiple congenital abnormalities (Mlakar et al., 2017; Passariello et al., 2013). Germline 11-q aberrations in NB are rare and reported germline 11q aberrations in NB so far include six 11q-del, one 11q inversion and two balanced 11q translocations, with associated multiple congenital abnormalities including craniofacial abnormalities (Passariello et al., 2013). One of these patients with NB was diagnosed at three months, with the remainder diagnosed at 18 to 81 months (Koiffmann et al., 1995; Mosse et al., 2003; Passariello et al., 2013; Satgé et al., 2003). Notably, the NB patients with late disease onset were diagnosed as early as three months of age with multiple congenital abnormalities that far preceded the NB onset (Koiffmann et al., 1995; Passariello et al., 2013). In these latter patients, the constitutional 11q-del could strongly be an early event which might have contributed to tumour initiation and/or progression. Several other germline or constitutional 11q-del have been described in children showing mental and growth retardations and craniofacial abnormalities but no neoplasm (Mlakar et al., 2017).

These data suggest that 11q-del alone may not be adequate to cause NB. However, in conjunction with other genetic events, these might sufficiently initiate NB (Mlakar et al., 2017). An interesting result from chromosomal transfer experiment by Bader et al., about three decades ago, showed that transfer of chromosome 11 into an NB cell line induced neuronal differentiation (Bader et al., 1991). More importantly, the gene harbouring the differentiation potential is situated between pter and 11q22-2. This result suggests that certain genes on chromosome 11 may be essential for the differentiation of NC precursors into their fated cell-types such as neurons of the sympathetic ganglia and chromaffin cells of the adrenal gland, the common sites of NB origin. This further indicates that blockade of terminal differentiation of NC-derived precursors, i.e. NB cellular sources, could be an essential early event in perhaps certain subtypes, if not all, of NB. Therefore, it is possible that the mechanism of 11q-del-mediated NB tumour initiation may first involve the blockade of differentiation or terminal differentiation of precursor cells (Bader et al., 1991) (Figure 2). A key feature of undifferentiated multipotent neuronal cell precursors is their rapid progression through the G1 phase of the cell cycle, compared to their differentiated derivatives (Halliwell et al., 2020; Hardwick and Philpott, 2014). This shorter G1 length causes persistent DNA replication stress in undifferentiated neuronal multipotent cell precursors, decreasing upon cell differentiation (Halliwell et al., 2020).

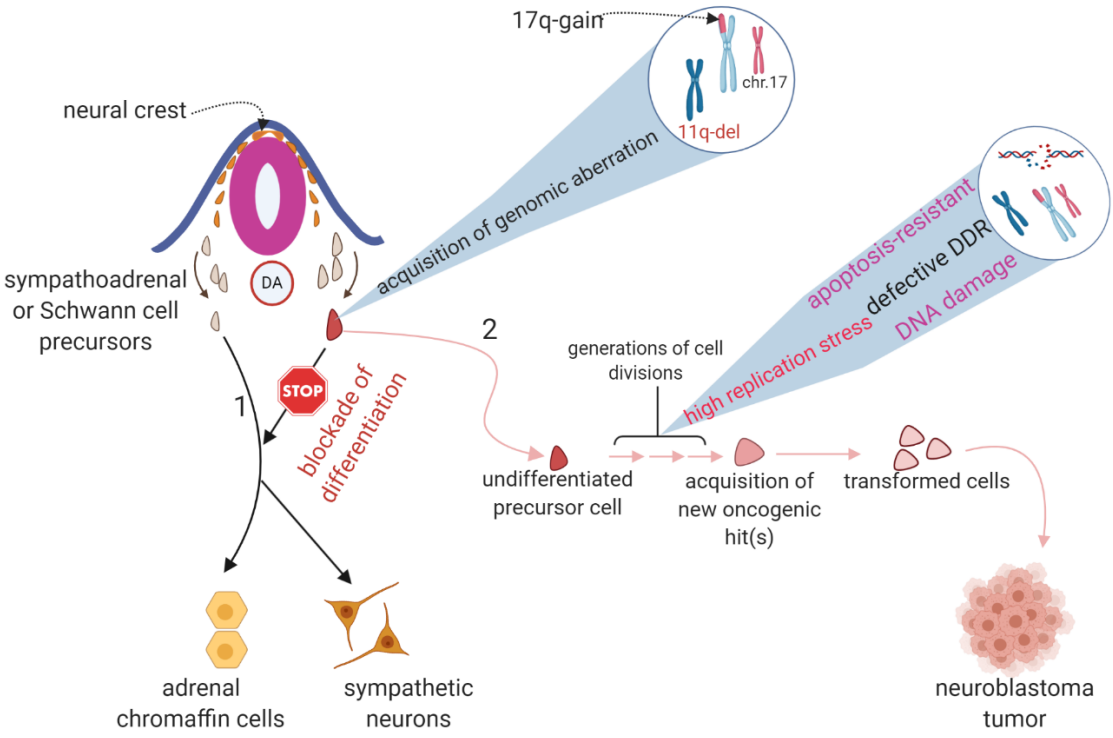

Figure 2. ***A model for the role of 11q-del in NB initiation****. (1) Neural crest-derived sympathoadrenal precursors (SAPs) or Schwann cell precursor (SCPs) normally differentiate into neurons of the sympathetic ganglia and or adrenal chromaffin cells. (2) Early SAPs or SCPs acquire genomic aberrations such as 11q-del, in the 17q-del background, and remain as undifferentiated immature cells. These cells have high replication stress, a G1-related event in multipotent neuronal cells, leading to DNA damages and genomic instability (Halliwell et al., 2020). Cells may be defective in DNA damage repair (DDR) due to 11q del, and resistant to apoptosis due to 17q-gain. High genomic instability may contribute to the acquisition of new oncogenic hits that may promote cell transformation and tumour development. DA denotes dorsal aorta.*

Deletion of the q-arm of chromosome 11 may result in loss of DDR genes such as *ATM*, *MRE11* and *H2AFX* (Ditch and Paull, 2012; Mandriota et al., 2015; Ward and Chen, 2001), which could potentially impair cells ability to repair DNA damages caused by replication stress of shorter G1 length in pluripotent precursor cells (Halliwell et al., 2020), thereby promoting genomic instability.

Upon induction of irreparable DNA damages, cells become inactivated through apoptosis (Kaina, 2003). Cancer cells are able to evade apoptosis generally by inactivation of pro-apoptotic genes such as *P53,* and or by the activation of anti-apoptotic genes (Fernald and Kurokawa, 2013). Unsurprisingly, 11q-del in NB is strongly associated with 17q- gain, and the latter harbours an anti-apoptotic gene called *BIRC5* (*survivin*) which is highly expressed in NB and predicts poor prognosis (Carén et al., 2010; Islam et al., 2000; Lamers et al., 2011). The frequent co-occurrence of 11q-del and 17q-gain in NB tumour

cells may therefore appear to be an important event, where the latter may provide cell survival properties, during NB tumour initiation and progression (Figure 2). It is therefore possible to speculate that 11q-del-mediated genomic instability initiates random genetic events which could take time to attain the right oncogenic milieu necessary for tumour initiation or progression, hence, the late onset of 11q-del NB. In effect, one can suggest that pro-differentiation gene(s) might be located on 11q, and the loss of these genes simultaneously, with the previously mentioned DDR genes during unbalanced 11q deletion, in 17q-gain background, could lead to maintenance of undifferentiated and apoptosis resistant NC-derived precursor cells, with defective DDR machinery (Figure 2). These would appear to create suitable conditions for a journey towards tumour initiation. Pro-differentiation genes of 11q have not be identified until most recently by Lopez et al (discussed in section 1.2.1.3) (Lopez et al., 2020), and us (Siaw et al., 2020) (discussed in section 4.3).

Vulnerabilities of neuroblastoma cells with 11q-del and defective DDR

ATM, MRE11A, H2AFX and *CHEK1* loss or imbalance in 11q was reported in about 21% of NBs, about 90% of which were associated with stage 3 and 4. In addition, 7% of NBs were found to contain rare single nucleotide variants in *ATM* (Takagi et al., 2017). These aberrations in DDR-associated genes could ultimately result in DDR defects in NB cells, making them vulnerable to DNA damage-inducing therapies such as PARP inhibitors. Poly ADP-ribose polymerase (PARP) is involved in repairing single-strand DNA damage. Its inhibition has been reported to exhibit synthetic lethality in 11q-del or ATM defective NB cell lines (Sanmartín et al., 2017; Takagi et al., 2017). Therefore, a combinatorial treatment of 11q-del NB patients with PARP inhibitors and chemotherapy could be an attractive therapeutic strategy.

1.2.2 Stage 4S neuroblastoma and differentiation

NB is clinically heterogeneous and ranges from aggressive disease to spontaneous regression, with little or no therapy. Spontaneous regression is the characteristic feature of stage 4S NB, mostly in children less than 12 months of age (Lavarino et al., 2009). These tumours, capable of spontaneous regression, generally have the following features; no *MYCN* amplification, no chromosome 1p deletion and are near triploid with whole chromosomal gains (Lavarino et al., 2009). They are also characterized by high expression of the tropomyosin receptor kinase A (TRKA) which correlates with differentiated tumour histology and is associated with favourable tumour stage and outcome (Brodeur and Bagatell, 2014; Hoehner et al., 1995). Primary culture of stage 4S tumour-derived cells in the presence of NGF, the cognate ligand for TRKA, induced neuronal differentiation and survival, whereas withdrawal of NGF resulted in apoptotic cell death (Brodeur et al., 1997). These *in vitro* culture behaviours appear to be

reminiscent of the observations that NB tumours expressing high TRKA undergo apoptosis/spontaneous-regression or neuronal differentiation in the absence or presence of NGF in their surroundings, respectively (Brodeur and Bagatell, 2014). Therefore, TRKA/NGF signalling seems to be involved in the underlining mechanism behind the spontaneous regression of stage 4S NB.

The clinical observations of spontaneous regression have evoked great interest in NB differentiation studies *in vitro*. Numerous agents, including RA and nerve growth factor (NGF), have been demonstrated to induce both morphological and molecular changes in NB cell lines that suggest neuronal differentiation (Ponthan et al., 2001; Reynolds et al., 2000). The ability of these agents to induce differentiation of NB cell lines in preclinical models has prompted clinical investigations of these differentiation agents in NB patients (Matthay et al., 1999). Both NB cell lines and tumours exhibit differential responses to RA and other therapies, which may be due to heterogeneity of the NB tumours and cell lines (Sidell et al., 1986). This heterogeneity could reflect differences in the genetic set up of different tumours or differences in gene expression by genetically identical tumour cells (Tsubota and Kadomatsu, 2018; van Groningen et al., 2017; Vo et al., 2014).

1.2.3 Tumour heterogeneity

NB has a remarkable heterogeneous clinical presentation ranging from spontaneous maturation or regression in young infants even with metastatic disease, to unresectable or metastatic unfavourable disease in children >18 months of age at diagnosis. This clinical dichotomy in NB may be reflective of a fundamental biologic intra- or inter-tumour heterogeneity.

Inter-tumour heterogeneity

NB tumours are found in the medulla of the adrenal gland (47%) and the paraspinal or periaortic regions of the sympathetic chain ganglia; subdividing into abdominal/retroperitoneal regions (24%), neck (2.7%), thoracic (15%), pelvic (3%) and other regions (7.9%) (Tsubota and Kadomatsu, 2018; Vo et al., 2014). Different combinations of genomic aberrations characterize primary NB tumours. Inter-tumour heterogeneity defined by anatomical location of primary NB tumour along the sympathetic chain correlates with tumour genomic profile and patient outcome (Brisse et al., 2017). For instance, cervical sympathetic chain NBs have numerical-only chromosome alterations (NCAs) exclusively, compared to adrenal NB, which comprises 16% NCA, 36% segmental chromosome alterations (SCAs) or 48% *MYCN* amplification. Furthermore, 92% of all MNA NBs were found to arise from the adrenal gland, and this location is associated with the worst prognosis (Brisse et al., 2017). These findings reveal a complex inter-tumour heterogeneity in NB defined by differential genetic aberration

profiles with a strong association to tumour anatomical location. Furthermore, intra-tumour heterogeneity is another sphere of complexity with NB tumours.

Intra-tumour heterogeneity

Recent studies have demonstrated that cancer cells with varying driver mutations or chromosomal aberrations can coexist within the same tumour (Gillies et al., 2012). This phenomenon is referred to as intra-tumour heterogeneity and is functionally vital in therapeutic failure and drug resistance (Gillies et al., 2012; McGranahan and Swanton, 2017). NB, like many other cancers, is not exempt from intra-tumour heterogeneity, a critical driver of the fatal outcome of cancer.

Intra-tumoural coexistence of MNA cancer cells, and 11q-del cancer cells, or non-MNA cancer cells in the same tumour has been described in NB (Theissen et al., 2009; Villamón et al., 2013). MNA tumours and 11q-del tumours are thought to represent distinct genetic subtypes of aggressive NB (Carén et al., 2010). Ultra-deep sequencing technologies have also led to the identification of subclonal *ALK* mutation fractions in NB tumours, which is indicative of the presence of both clonal ALK wild-type cancer cells and subclonal ALK mutant cancer cells in the same tumour (Javanmardi et al., 2019). Multiregional whole-genome analyses of tumours have shown that within the same NB tumour, neuroblastic cells characteristically develop along one of four unique evolutionary trajectories as evidenced by the presence of unique tumour genotypes in different areas of the same tumour (Karlsson et al., 2018). This intra-tumour heterogeneity appears to be typically driven by collateral branching evolution, often mixed with linear branching (Andersson et al., 2020). Higher number of clonal branching events occur in a high-risk NB tumour compared to a low-risk tumour (Andersson et al., 2020). Certain mutations or aberrations could exist at subclonal levels within subregions of a tumour and might not be part of the “trunk” of the tumour’s phylogenetic tree (Karlsson et al., 2018; von Stedingk et al., 2019). These findings have implications for treatment failure and relapse in NB.

Another dimension of intra-tumour heterogeneity involves the coexistence of genetically identical but transcriptionally divergent cell types within the same tumour (van Groningen et al., 2017). van Groningen and colleagues described two cell-type compositions of NB tumours, namely undifferentiated mesenchymal cell-type (MES-type); with similar gene expression signature to human NC derived cells, and committed adrenergic cell-type (ADRN-type) (van Groningen et al., 2017). These two cell types are genetically identical but transcriptionally and epigenetically divergent. They are each associated with a unique super-enhancer (SE) landscape, which controls the cell-type-specific gene expression signatures (van Groningen et al., 2017). Similarly, Boeva *et al.* identified the core transcriptional regulatory circuitries (CRCs) that uniquely drive activity at these cell-type-

specific SEs (Boeva et al., 2017), and these include PHOX2B-HAND2-GATA3 CRC in ADRN-type cells and AP-1 transcription factors (TFs) CRC in the NC cell-like MES-type cells (Boeva et al., 2017). CRCs are TFs in an interconnected auto-regulatory feed-forward loop. CRCs and super-enhancers are known as essential elements in defining cell identity (Whyte et al., 2013). Gene expression signature profiling of different primary NB tumours placed these tumours in a continuum between MES and ADRN cells types (Boeva et al., 2017; van Groningen et al., 2017). This observation may reflect tumour cells at different stages of differentiation and further shows that even NB patients with very similar tumour genomic profiles may still be starkly different on the "differentiation scale" (MES ↔ ADRN). Therefore, MES and ADRN cell states in NB tumours could contribute to both intra and inter-tumour heterogeneity in NB. This fact may have a direct implication on the choice of therapy for different NB patients.

Treatment regimens against subclonal mutations or "trunk" mutations may only provide temporary remission in NB. Intra-tumour heterogeneity presents one of the major challenges of precision or personalized therapeutic approaches since this heterogeneity hinders accurate genetic profiling of the tumours (Joung et al., 2016). The presence of subclonal driver mutations in tumours implies routine Sanger sequencing techniques used in tumour profiling could miss these cell populations, leading to incomplete information on the tumour's genome (Javanmardi et al., 2019). Examination of the patient's tumour genome generally involves the use of a single tumour biopsy specimen which could be made obsolete by intra-tumour heterogeneity (Joung et al., 2016). In light of recent studies on NB's intra-tumour heterogeneity, any prudent pipeline for NB treatment planning should aim at first obtaining near-complete information on the tumour's genetic landscape. This approach will involve multiregional tumour biopsies and deep sequencing techniques for more accurate tumour profiling.

1.2.4 The origin of neuroblastoma

NB is an NC-derived malignancy of the SNS. However, the cell(s) of origin of NB is unclear but thought to arise from NC-derived sympathoadrenal lineage precursor cells, which differentiate to adrenal chromaffin cells and neurons of the sympathetic ganglia (Cheung and Dyer, 2013) Figure 3). Recently, Furlan and colleagues found that NC-derived peripheral glia stem cells, referred to as Schwann cell precursors (SCPs), were the main cellular source of adrenal chromaffin cells (Furlan et al., 2017). These SCPs could therefore be a potential cellular source of NB. NB could be described as a tumour of developmental arrest and failed or delayed differentiation (Ratner et al., 2016). Therefore, precisely defining the origin of NB's NC-derived cell(s) and understanding their

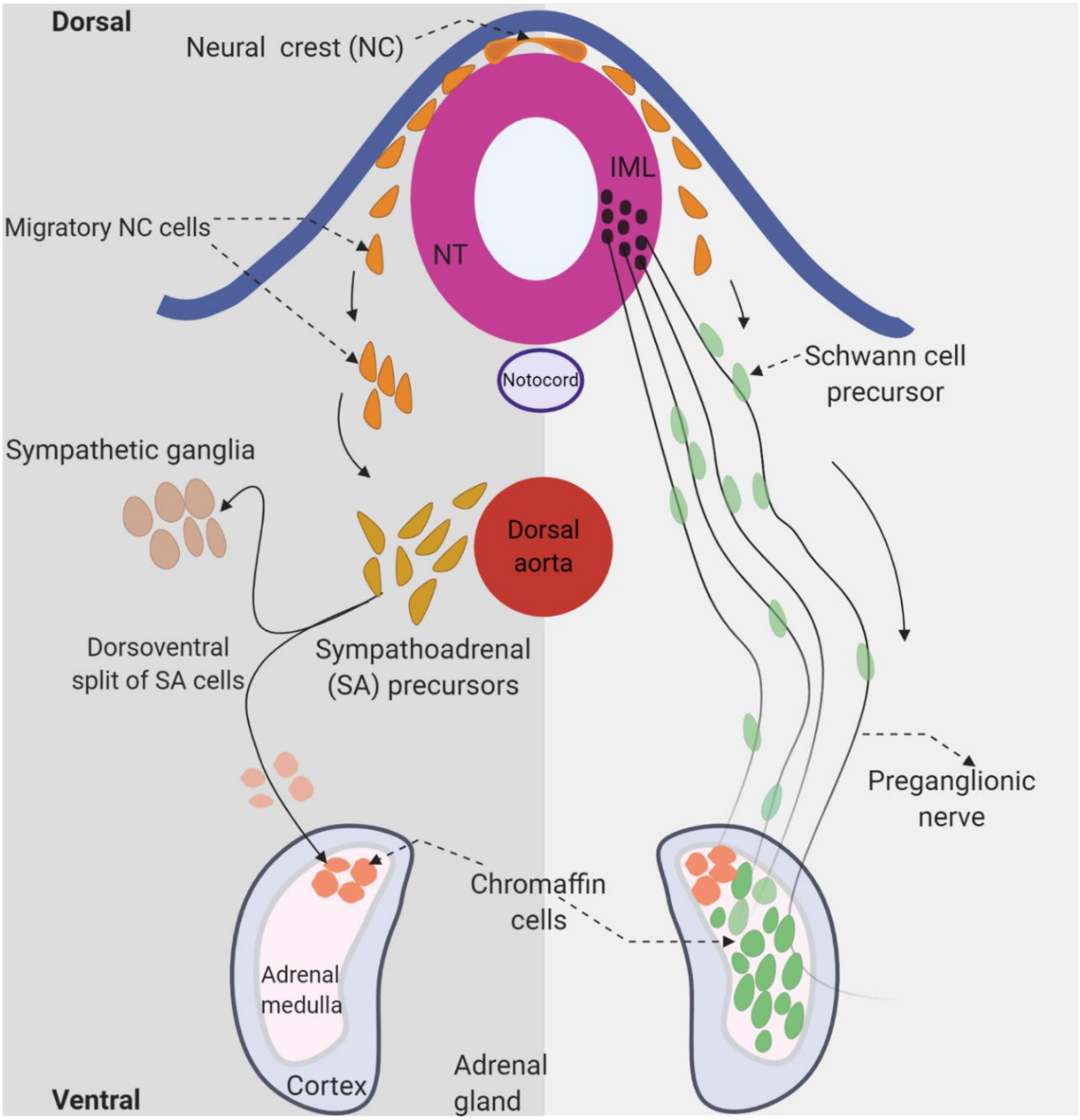

Figure 3. ***Developmental modes of sympathoadrenal (SA) lineage and adrenal chromaffin cells****. (Left panel) Freely migrating SOX10⁺ neural crest cells (NCCs) migrate ventrally towards the dorsal aorta. At the aortic region, PHOX2B expression is induced in these NCCs resulting in their commitment to the Sympathoadrenal (SA) lineage. SA precursors migrate further, in response to dorsal aorta-secreted bone morphogenetic proteins (BMPs), towards the dorsal aorta. At the dorsal aorta, SA precursors split dorsoventrally for differentiation into sympathetic ganglia and adrenal chromaffin cells (20%) respectively. (Right panel) SOX10⁺ nerve-associated Schwann cell precursors (SCPs) migrate on axons of preganglionic neurons of the intermediolateral cell column (IML) that innervate the adrenal gland. When SCPs reach the anlage of the adrenal medulla, they become committed to adrenal chromaffin cells through a transient intermediary cell state called bridge cells. SCP-derived adrenal chromaffin cells make up about 80% of chromaffin cells of the adrenal medulla. Adapted with permission from (Furlan et al., 2017).*

normal development will help ascertain the pathways involved in the blockade of differentiation and the maintenance of undifferentiated NC progenitor tumour initiating clones. This approach will help in identifying drug targets which might provide potential therapeutic benefits to NB patients.

1.2.4.1 Sympathoadrenal lineage

The neural crest is a transient embryonic cell population that is bilaterally organised and arises from the dorsal lip of the developing neural tube (precursor of the spinal cord) during the early stages of embryogenesis in vertebrates (Figure 3). NC cells (NCCs) are multipotent and undergo extensive migration throughout the developing embryo, differentiating into multiple cell types and tissues including neurons and glia of the peripheral and enteric nervous systems, adrenal chromaffin cells, melanocytes and much of the craniofacial skeleton (Tomolonis et al., 2018). NCCs undergo epithelial-mesenchymal transition (EMT) to acquire motile phenotypes. With the inception of EMT, *SOX10*$^{+}$ NCCs delaminate from the neural tube and migrate along specified routes to colonize distant sites throughout the embryo for further specification and differentiation (Theveneau and Mayor, 2012) (Figure 3). The processes of NC induction, delamination and specification of derived progenitors are regulated by a multifaceted gene regulatory network (Sauka-Spengler and Bronner-Fraser, 2008). NCCs concomitantly undergo extensive proliferation to generate enough progenitor cells to populate their target tissues during their migration. *MYCN* is involved in the regulation of NCC fate in the aspects of ventral migration of the cells and neural differentiation (Wakamatsu et al., 1997). The complex nature of NC-derived tissues indicates the presence of multipotent progenitors. NB is believed to result from failed differentiation of sympathoadrenal lineage progenitors of the NC (Ratner et al., 2016). Early-migrating *SOX10*$^{+}$ NCCs fated to become sympathoadrenal lineage progenitors migrate ventrally towards the dorsal aorta, the first blood vessel to form during embryogenesis. The dorsal aorta secretes bone morphogenetic proteins (BMP4 and BMP7) which induce expression of chemoattractants such as stromal-derived factor 1 (SDF1) and neuregulin (NRG1) by para-aortic mesenchymal cells, and through their cognate receptors, CXCR4 and EGFR respectively, act to attract early-migrating NCCs to the dorsal aorta (Saito and Takahashi, 2015; Saito et al., 2012).

In the vicinity of the dorsal aorta, the induction of *PHOX2B* gene expression commits *SOX10*$^{+}$ NCCs to become *PHOX2B*$^{+}$and *SOX10*$^{-}$ sympathoadrenal lineage progenitor cells (Callahan et al., 2008). Sympathoadrenal progenitor cells at the dorsal aorta split in a dorsoventral direction, giving rise to sympathetic ganglionic and adrenomedullary lineages respectively (Figure 3). This segregation is orchestrated directly by differential aortic BMP4 and BMP7 signalling (Saito et al., 2012). After segregation, the sympathetic

ganglionic precursors remain in the aortic region to form the sympathetic chain ganglia. In contrast, the adrenomedullary precursor further migrates ventrally to become associated with adrenal gland to form catecholamine-secreting cells of the adrenal gland, called chromaffin cells (Saito et al., 2012) (Figure 3). Until recently, sympathoadrenal precursors were thought to be the main source of adrenal chromaffin cells and also the main cellular source of NB

1.2.4.2 Schwann cell precursors and adrenal chromaffin cells

Adrenergic chromaffin cells of the adrenal gland were originally considered to arise from fate-committed NC-derived sympathoadrenal lineage progenitors located near the dorsal aorta (Anderson et al., 1991; Huber et al., 2009). These sympathoadrenal precursors later split dorsoventrally forming the sympathetic ganglia and adrenal chromaffin cells respectively, as described above (Saito et al., 2012). However, clear evidence that implicates sympathoadrenal precursors as the direct cellular source of adrenal chromaffin cells is lacking.

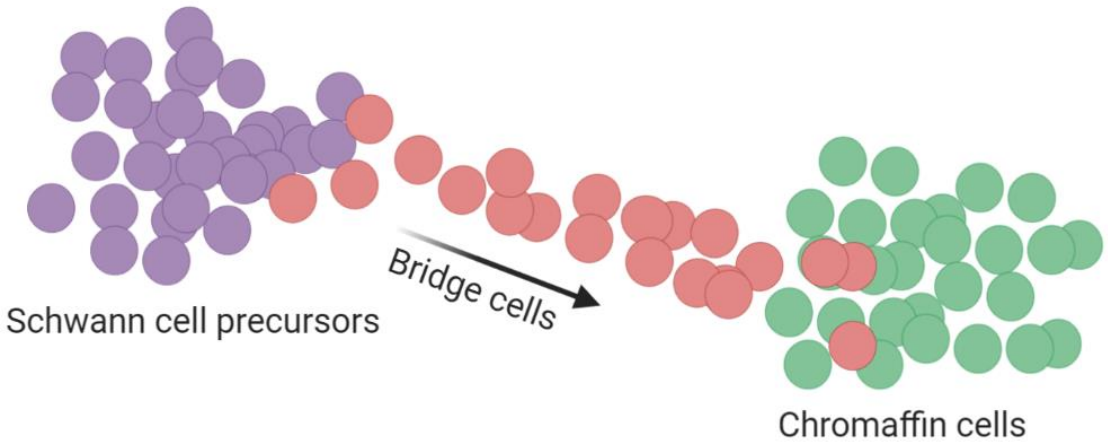

Figure 4. ***Schematic outline of the transcription-based hypothesis outlining the differentiation trajectory of Schwann cell precursors (SCPs) towards adrenal chromaffin fate*** *(Furlan et al., 2017). Neural crest-derived SCPs destined to become adrenal chromaffin cells make the transcriptional transition through a transient cell-state called "bridge cells". Adapted with permission from (Furlan et al., 2017)*

Recent genetic cell lineage tracing experiments by Furlan et al. provided substantial evidence that sympathetic ganglia indeed arise from the early-migrating stream of NCCs committed to sympathoadrenal lineage. In contrast, most of the adrenal chromaffin cells are formed from SCPs (Furlan et al., 2017). SCPs are *SOX10*$^{+}$*/p75*$^{+}$ nerve-associated peripheral glia and multipotent stem cells, which are derived from late-migrating NCCs.

They can differentiate into several cell types, including almost all cells of the parasympathetic nervous systems (Adameyko and Ernfors, 2014). During embryogenesis, late-migrating *SOX10*$^{+}$ NCCs become attached to preganglionic neurons and become SCPs. Preganglionic sympathetic neurons, originating in the intermediolateral cell column (IML) of the spinal cord, are the source of adrenal medulla innervation (Appel and Elde, 1988; Dun et al., 1993) (Figure 3). SCPs destined to become adrenal chromaffin cells migrate along preganglionic neurons towards the anlage of the adrenal medulla. At the adrenal medulla, SCPs initiate the transition to become chromaffin cells. Unique gene expression programs govern SCP-to-chromaffin cell transition. Single-cell RNA sequencing analysis of developing adrenomedullary cells at E12.5 and E13.5, stages at which chromaffin cells start to appear, identified unique gene expression signatures separating three major distinct subpopulations of cells (Furlan et al., 2017). These cell clusters appear to form a continuum where an intermediate cell type spans a gene expression state between SCP-like cells to more chromaffin-like cells as illustrated in Figure 4. This phenomenon reflects a transcriptional transition from SCP to chromaffin cells along a differentiation trajectory and suggested the presence of an intermediate transient cellular state, governed by a complex regulatory network, within the “bridge structure” connecting SCPs and chromaffin cells (Furlan et al., 2017) (Figure 4). The intermediate cells were referred to as “bridge cells”. Some genes were found to be up- or down-regulated at the beginning or end of the SCP-chromaffin route. For instance, SCP genes such as *PHOX2B*, *ASCL1*, *TBX2*, *CHEK1, BUB1, ID2* and *SOX2* were progressively downregulated in the “bridge cells” and in the differentiated chromaffin cells. *MYCN* expression was high in SCPs but appeared to decline upon the commitment of SCPs to the “bridge” structure. Conversely, genes including *DLG2, DLGAP2, NEFM, NEFL*, were uniquely and progressively upregulated later in the “bridge” structure towards chromaffin differentiation (Furlan et al., 2017).

Through genetic fate tracing of nerve-associated SCPs, it was found that the majority (about 80%) of TH^{+} (tyrosine hydroxylase) chromaffin cells of the mouse adrenal medulla originated from SCPs. In contrast, the remaining 20% of adrenal chromaffin cells might be derived from early-migrating NC-derived sympathoadrenal lineage cells (Furlan et al., 2017). These new findings about SCPs as a cellular source of adrenal chromaffin cells may offer new perspectives regarding the cellular origin of NB because it is thought that about half of NB arise from the vicinity of the adrenal gland (Brisse et al., 2011).

Common genes in neural crest and neuroblastoma development

NB is thought to originate from NC-derived progenitor cells, hence, it may share genes and signalling pathways in common with NC-derived progenitors. High expression of, or

mutation in, SCP genes such as *MYCN, PHOX2B*, *ASCL1*, *TBX2*, *CHEK1, ID2,* and *SOX2,* are believed to play vital roles in NB and are associated with poor prognosis (Cole et al., 2011; Decaesteker et al., 2018; Mathew et al., 2001; Mosse et al., 2004; Sauka-Spengler and Bronner-Fraser, 2008; Trochet et al., 2004; Wang et al., 2019). MYCN acts in concert with the NB core regulatory circuitry (CRC) consisting of PHOX2B, TBX2 ASCL1, GATA3, HAND2 and ISL1 in a feed-forward autoregulatory loop to drive NB pathogenesis and the maintenance of adrenergic NB identity (Boeva et al., 2017; Tan et al., 2019; van Groningen et al., 2017). Activated CHEK1 relay checkpoint signals by phosphorylating numerous downstream targets resulting in the activation of cell cycle checkpoints, cell cycle arrest, DNA repair, and cell death when DNA damage is severe in order to stop damaged cells from continuing through the cell cycle (Beckwith and Perrin, 1963; Patil et al., 2013; Schleiermacher et al., 1994; Walworth et al., 1993). CHEK1 is located on chromosome 11q, which is frequently deleted in NB tumour cells and is associated with poor prognosis (Carén et al., 2010). ID2 (inhibitor of DNA-binding 2) and SOX2 maintain self-renewal and undifferentiated state of progenitor cells (Yang et al., 2015; Ying et al., 2003). Inhibition of ID2 in NB resulted in the spontaneous induction of cell differentiation (Ciarapica et al., 2009).

The expression of NB associated genes, including some member genes of NB's CRC, by NC-derived precursors such as SCPs, "bridge cells" and sympathoadrenal precursors potentially substantiates the notion of NC as the cellular source of NB. Additionally, this suggests SCPs as perhaps one of the potential NC-derived progenitor cellular sources of NB. The Furlan "bridge cells" represent a transient developmental cell state, the only route from SCPs to differentiated chromaffin cells, with very dynamic transcriptional profiles that may reflect regulatory activities. Therefore, it is enticing to speculate that perturbation or deregulation of genes and signalling within the "bridge structure" could result in the blockade of differentiation, increase cell proliferation, and potentially contribute to NB pathogenesis. This result implies that the exploration of genes expressed within the bridge structure will help understand the mechanisms involved in the delay or blockade of differentiation, leading to NB (Paper III).

1.2.5 Disks Large Homologue 2 in cancer

Disks large homologue 2 (*DLG2*) is one of the genes which are uniquely upregulated in "bridge cells" during SCP differentiation toward chromaffin cell fate (Furlan et al., 2017). *DLG2* is located on chromosome 11q, which is hemizygously deleted in about 20-45% of NB tumours and is associated with poor prognosis (Mlakar et al., 2017; Spitz et al., 2003; Spitz et al., 2006). Lately, *DLG2* has been suggested to be a tumour suppressor in osteosarcoma (Shao et al., 2019). *DLG2* is located on chromosome 11q14.1 and codes for a protein called postsynaptic density protein 93 (PSD93). DLG2 a.k.a PSD93 protein is a member of the membrane-associated guanylate kinase (MAGUK) family. It acts as a

molecular scaffold for the tethering of membrane structures, clustering of receptors, ion channels and other proteins in signalling (Pan et al., 2011). DLG2, like other MAGUKs, shares a core structural module consisting of a PSD95/Dlg/ZO-1 (PDZ) domain, a Src homology (SH3) domain and a catalytically inert guanylate kinase (GK) domain: These domains are arranged in tandem to form PDZ-SH3-GK structural sequence (Pan et al., 2011) (Figure 5).

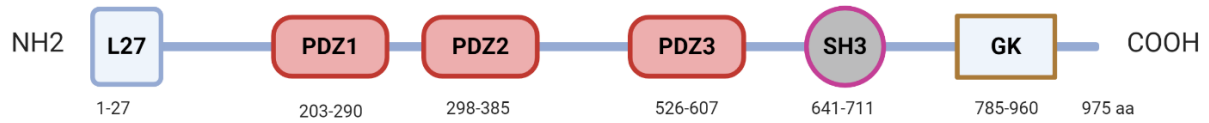

Figure 5. ***Domain organization of DLG2 protein****. DLG2 consists of six domains, namely L27, PDZ1, PDZ2, PDZ3, src homology 3 (SH3) and guanylate kinase (GK). The numbers represent the range of the amino acids (aa) that make up each domain.*

Studies in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* showed that both organisms have *DLG* homologues. *Drosophila DLG* is a vital component of septate junctions and plays a role in sustaining the apicobasal polarity in *Drosophila* epithelium (Woods et al., 1996). The mammalian *Dlg* homologue are a family of four paralogs, namely *DLG1*, *DLG2*, *DLG3* and *DLG4*. Similar to *Drosophila* dlg1, the mammalian homologues localize to the cell membrane and are involved in the formation of different types of cell junctions (Roberts et al., 2012). The proteins of the DLG subfamily of MAGUKs are also expressed in the central nervous system and are mostly restricted to the postsynaptic density of postsynaptic neurons (Oliva et al., 2012). Deletion of *DLG2* (*PSD93*) resulted in disruption of neuronal cholinergic synapse formation and elicited rapid disassembling of synaptic clusters of neuronal nicotinic acetylcholine receptors (nAChRs) in mice (Parker et al., 2004). These findings suggest that DLG2 is required to maintain synaptic stability, and is an integral member of the postsynaptic scaffold at neuronal synapses (Parker et al., 2004).

The *Drosophila* homologue, *Dlg1* is a well-characterized tumour suppressor, which when mutated leads to neoplastic outgrowth in the *Drosophila* imaginal discs (Woods and Bryant, 1991). Expression of Rat homologues of *DLG1* and *DLG3* suppressed tumour formation in *dlg1* mutant *Drosophila* flies (Thomas et al., 1997), indicating a tumour suppressor function by the DLG subfamily of proteins in mammals. This result further suggests a conservation of the tumour suppressor role of DLG homologues across different species. Unlike other members of the DLG subfamily, a tumour suppressor role for DLG2 had not been described until recently. Shao and colleagues have recently

described *DLG2* gene as a tumour suppressor in human and dog osteosarcoma (Shao et al., 2019). However, the role of *DLG2* in NB is unknown. The upregulation of *DLG2* in "bridge cells", which represents a transcriptional trajectory towards chromaffin cell differentiation, may suggest a role for *DLG2* in the differentiation of NC-derived precursor cells. It is worth restating that NB is thought to arise as a result of failed differentiation of NC-derived progenitors, and chromosome 11q which harbours *DLG2* is frequently deleted in NB (Carén et al., 2010; Mlakar et al., 2017; Ratner et al., 2016). Therefore, this result motivates a study to uncover the role of *DLG2* in NB.

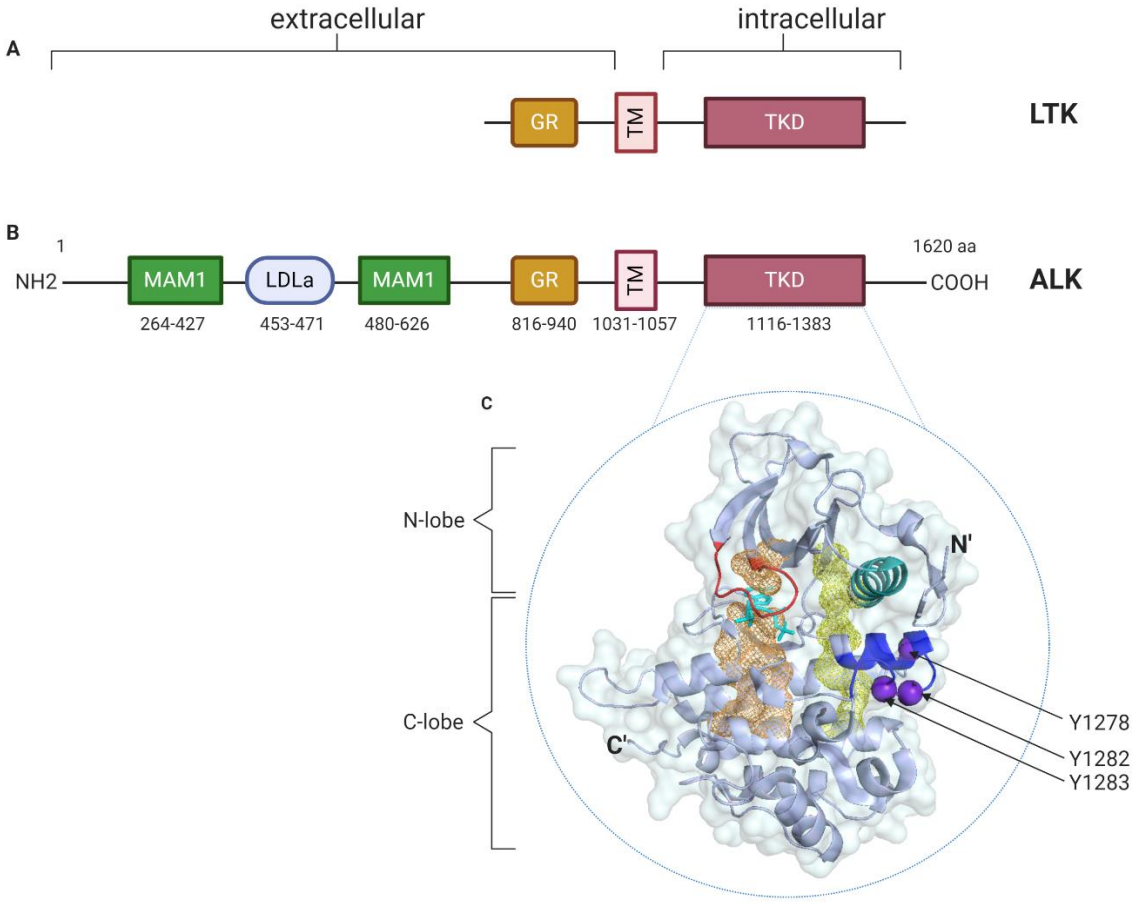

Figure 6. ***Domain structure of human LTK and ALK****. (A, B) The extracellular domain (ECD) of LTK contains only glycine-rich (GR) domain. ALK ECD contains two MAM domains, an LDLa domain and a GR domain. Both receptors have a transmembrane domain (TM) which connects the ECD with the intracellular domain that harbours the tyrosine kinase domain (TKD). (C) The kinase domain of inactive ALK (PDB: 3LCT). The kinase domain consists of a smaller N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe). The N-lobe harbours the αC helix (teal), glycine loop (red), and five β-sheets (grey). The C-lobe consists of the activation loop (blue), which harbours three tyrosine auto-phosphorylation sites (purple balls). ADP (cyan) inside the ATP/inhibitor binding site. Hydrophobic non-contiguous motifs, which are conserved in all kinases, span both the N-lobe and the C-lobe, referred to as regulatory (yellow) and catalytic (brown) spines.*

1.2.6 Anaplastic lymphoma kinase

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK) that together with the related leucocyte tyrosine kinase (LTK) constitutes a subfamily of the insulin receptor superfamily (Figure 6). ALK was originally identified as a fusion partner of nucleophosmin (NPM) in the t(2;5) chromosomal translocation in anaplastic large cell lymphoma (ALCL) (Morris et al., 1994). The *ALK* gene located on chromosome 2p codes for a 1620 amino acid long, 177 kDa polypeptide, which undergoes post-transcriptional N-linked glycosylation to form a mature 200 kDa ALK protein (Morris et al., 1997). Proteolytic cleavage of the full-length ALK yields a tyrosine-phosphorylated 140 kDa truncated receptor (Degoutin et al., 2009; Hallberg and Palmer, 2013).

Structure of ALK

Wild type ALK is a 1620 amino acid protein comprising of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (Iwahara et al., 1997; Morris et al., 1997) (Figure 6).

Extracellular domain

The extracellular region of ALK contains two MAM (meprin, A-5 protein and receptor protein- tyrosine phosphatase mu) domains, a low-density lipoprotein class A (LDLa) region, and a glycine-rich (GR) domain (Morris et al., 1997) (Figure 6A). The extracellular region of ALK is thought to be required for ligand binding, dimerization and interaction with potential co-receptors, all of which could result in conformational changes leading to activation of the intracellular kinase domain (Hallberg and Palmer, 2013). ALKAL1 and ALKAL2 have recently be identified as the ligands for ALK (Guan et al., 2015; Reshetnyak et al., 2015).

Intracellular domain

Similar to other RTKs, the ALK intracellular region harbours a kinase domain which is made up of a conserved N-terminal lobe (N-lobe) and a C-terminal lobe (C-lobe) (Figure 6C). These lobes are connected by a ‘hinge’ region to form an ATP binding pocket (Bossi et al., 2010; Lee et al., 2010). The C-lobe contains an activation loop which is involved in an inhibitory structural feature that blocks the substrate-binding region of the kinase domain during inactive conformation (Hallberg and Palmer, 2013). A common feature of the insulin receptor (InR) family members is the presence of Y’XXX’YY triple tyrosine motif in the activation loop. Likewise, ALK activation loop contains a 1278-Y’RAS’YY-1283 motif, comparable to 1158-Y’ETD’YY-1163 in the InR activation loop (Wei et al., 1995). The unique ‘RAS’ sequence in ALK activation loop has been shown to be responsible for

phosphoacceptor substrate selectivity of ALK (Donella-Deana et al., 2005). In the fusion protein, NPM-ALK, the initial tyrosine (Y1278) in the triple tyrosine motif (1278-Y'RAS'YY-1283) of ALK has been reported to be the first tyrosine in the motif to be phosphorylated (Donella-Deana et al., 2005; Tartari et al., 2008). It is therefore believed that when ALK is inactive, Y1278 of the activation loop is inaccessible and hence its phosphorylation is crucial for ALK activation by releasing ALK from inactive conformational restraints (Bossi et al., 2010; Lee et al., 2010; Taylor and Kornev, 2011). Guan and colleagues later showed that, contrary to the reported preferential phosphorylation of Y1278 of the triple tyrosine motif in NPM-ALK, the third tyrosine (Y1283) of the motif is rather the critical tyrosine required in the activation of full-length ALK protein (Guan et al., 2017). The authors showed that a single mutation of Y1283 to phenylalanine (Y1283F), but not Y1278F or Y1282F, sufficiently abrogated ligand-mediated phosphorylation of ALK and its downstream target ERKl/2 (Guan et al., 2017). It is worth noting that the activation of the fusion protein, NPM-ALK is preceded by dimerization of the fusion protein, orchestrated by the fusion partner, NPM, through its oligomerization domain. In contrast, activation of the full-length ALK is thought to be initiated by ligand binding of the ECD of the full-length ALK leading to dimerization of the full-length receptor (Bischof et al., 1997; Fujimoto et al., 1996).

Protein kinases, including the RTKs, contain two conserved hydrophobic motifs referred to as regulatory (R) and catalytic (C) spines, and each of these spines consists of residues from both N-lobe and C-lobe (Taylor and Kornev, 2011) (Figure 6C). The R and C spines integrate the core of the kinase domain and regulate its activity (Taylor and Kornev, 2011). The ALK R-spine is made up of I1171, C1182, H1247, F1271 and D1311 residues, and is assembled after phosphorylation of the activation loop, i.e. when the kinase is active (Hallberg and Palmer, 2013; Taylor and Kornev, 2011).

The biological role of ALK in model organisms

Despite the involvement of ALK in numerous cancers, including adult and pediatric cancers, the exact physiological role of ALK in mammals is somehow unclear. *ALK* expression patterns in chickens, mice, rats, and humans suggest a role for ALK in neurogenesis (Hallberg and Palmer, 2013; Iwahara et al., 1997; Vernersson et al., 2006). In the model organism *Drosophila melanogaster, Alk is* essential for the development of visceral mesoderm during embryogenesis, and *Drosophila* embryos lacking Alk expression die due to lack of founder cells (Englund et al., 2003; Lee et al., 2003; Stute et al., 2004). Upon binding of Jelly belly (Jeb), the *Drosophila* Alk ligand, activated Alk signals through the MAPK-ERK1/2 pathway to mediate the specification of visceral muscle precursors called founder cells (Englund et al., 2003; Lee et al., 2003). Further, Alk and Jeb signalling have been reported to be involved in the assembly of the neuronal

circuit in *the Drosophila* visual system and are required for retinal axon targeting and neuromuscular junction function (Bazigou et al., 2007; Rohrbough and Broadie, 2010). Coincidently, two of the common side effects of ALK inhibitors in cancer patients are fatigue and visual disturbances (American Cancer Society, 2020; Camidge et al., 2012). The fatigue phenomena occur at nerve endings and neuromuscular junctions (Boyas and Guével, 2011). Hence, the involvement of ALK signalling in the neuronal circuit in the visual system and at neuromuscular junction imply that the above-mentioned side effects could represent ALK specific effects. *Caenorhabditis elegans'* HEN1 and SCD2, the *C. elegans* Jeb and Alk homologues respectively, are likewise not required for development but also have similar neural functions, play roles in neuromuscular junction formation by regulating presynaptic differentiation, and integration of sensory input and control dauer formation (Liao et al., 2004; Reiner et al., 2008). SCD2/ALK also modulates the transforming growth factor-β (TGFβ) signalling in the regulation of a developmentally arrested diapause state, called dauer stage, in *C. elegans* (Reiner et al., 2008). Both *Drosophila* and *C. elegans* have single orthologous receptors which are similar to ALK, whereas vertebrates genomes harbour the structurally related ALK and LTK receptors.

The structural resemblance between ALK and LTK, particularly in the kinase domain, and their inferred shared ancestral origin, indicate that these receptors may have similar or even overlapping functions in mammals (Weiss et al., 2012) (Figure 6A-B). Both *alk* and *ltk* have been investigated in zebrafish. *Ltk*, previously referred to as *shady* in zebrafish, was found to be uniquely expressed in a subset of zebrafish NCCs, before becoming committed to the iridophore lineage, and *ltk* (*shady*) mutants lack iridophores (pigment cells) (Kelsh et al., 1996; Lopes et al., 2008). Zebrafish ltk rather displays more structural similarities to human ALK, in that both zebrafish ltk and human ALK have two MAM domains in their extracellular domain. In contrast, human LTK has no MAM domain (Fadeev et al., 2018) (Figure 6A-B). Zebrafish *alk* is expressed in the developing central nervous systems and plays a vital role in neural progenitor proliferation, differentiation and survival during embryonic neurogenesis (Yao et al., 2013). *Drosophila* and *C. elegans* Alk ligands have long been known and studied, but vertebrate ALK's have been considered orphan receptors until lately (Ishihara et al., 2002; Weiss et al., 2001). By screening the extracellular proteome, Zhang and colleagues discovered that the two related secreted proteins, FAM150A and FAM150B (family with sequence similarity 150 member A and member B), now known as ALKAL1 and ALKAL2 respectively, are ligands for LTK (Zhang et al., 2014). Shortly after this report, FAM150A/B (ALKAL1/2) were functionally proven to activate human ALK *in vitro* (Guan et al., 2015; Reshetnyak et al., 2015). An *in vivo* study in zebrafish later confirmed zebrafish alkals (Alkal1, Alkal2a and Alkal2b) as physiological ligands for zebrafish alk and ltk, which are required for neural crest-derived iridophores development (Fadeev et al., 2018; Mo et al., 2017).

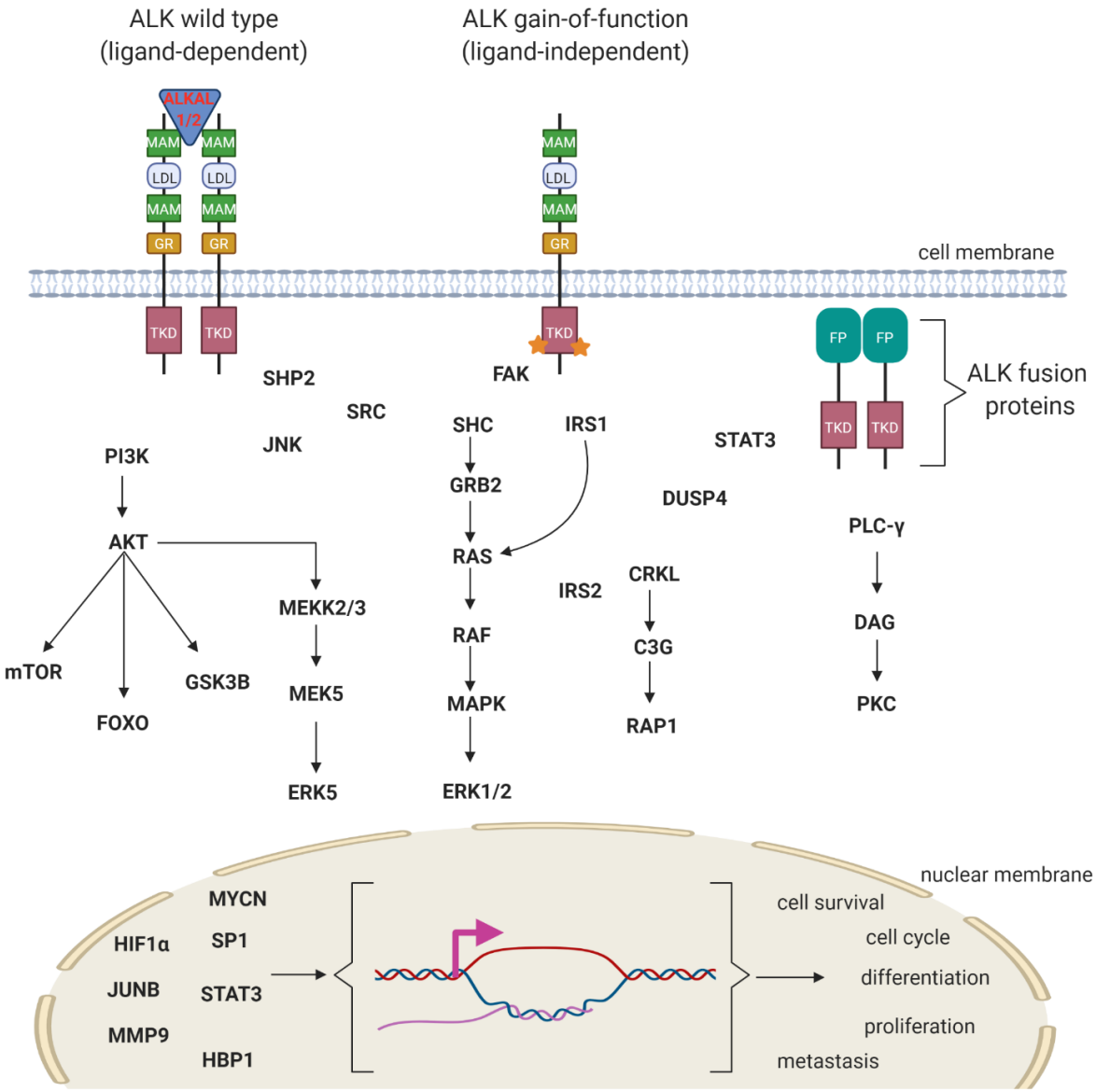

Figure 7. ***Schematic representation of Anaplastic Lymphoma Kinase (ALK) signalling cascades****. Wild type ALK is activated in a ligand-dependent manner, whereas ALK gain-of-function mutant and ALK fusion protein are activated in a ligand-independent manner. Activated ALK signals through numerous signalling axes, such as PI3K-AKT, RAS-MAPK etc., via transcription factors such as MYCN, STAT3, HIF1A etc that mediate different cell responses including cell survival, differentiation, proliferation, and metastasis. Adapted wih permission from (Hallberg and Palmer, 2013).*

ALK loss-of-function including *ALK*/*LTK* double mutant mice were viable, with a reported defect in neurogenesis and testosterone production (Bilsland et al., 2008; Weiss et al., 2012; Witek et al., 2015). *ALK* mRNA is expressed in certain regions of both peripheral and central nervous system, including the sympathetic chain – a common site of NB

origin, thereby suggesting a potential role for *ALK* in nervous system development (Iwahara et al., 1997; Morris et al., 1997; Vernersson et al., 2006). *ALK* is expressed in mouse NCCs at the neural plate border and believed to play a role in the migration of NCCs (Gonzalez Malagon et al., 2018). In the gain-of-function knock-in *ALK* mice, the *ALK* mutant induced increased neuroblast proliferation in ganglia of the sympathetic chain with concomitant enlargement of the ganglia and extended neurogenesis (Borenäs et al., 2020; Cazes et al., 2014; Witek et al., 2015). Considering the NC origin of NB, understanding the function of ALK family RTKs in the development of NC may be crucial in gaining insight into NB pathogenesis, in which a subset has been described to harbour *ALK* mutations (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Javanmardi et al., 2019; Mossé et al., 2008)

1.2.6.1 ALK in cancer and signalling

Several dozens of ALK fusions in diverse cancer types have been described over the years, making *ALK* a hotspot for translocation (Hallberg and Palmer, 2013). *NPM-ALK* accounts for about 80% of all ALK-positive ALCL cases (Amin and Lai, 2007; Umapathy et al., 2019). An example of another notable *ALK* fusion oncoprotein is *EML4-ALK* (Echinoderm microtubule-associated protein-like 4 –ALK). *EML4-ALK* accounts for about 2-9% of non-small cell lung cancer (NSCLC), a subgroup of lung cancer (Kwak et al., 2010; Rikova et al., 2007; Soda et al., 2007; Umapathy et al., 2019). Generally, in ALK fusion proteins, the fusion partners drive the fusion protein's transcriptional activation through their regulatory elements. ALK fusion partners also determine the subcellular localization of the fusion oncoproteins and mediate their dimerization, resulting in constitutive activation (Hallberg and Palmer, 2013).

Furthermore, while point mutations have not been reported in ALK fusion proteins in primary tumours, they commonly arise in relapsed tumour as secondary drug-resistance mutations (Choi et al., 2010; Hallberg and Palmer, 2013). ALK fusions commonly occur in adult cancers. At the same time, in pediatric NBs, ALK aberrations have been frquently reported in the context of full-length ALK (Hallberg and Palmer, 2013).

Both constitutively active ALK fusions and full-length ALK have been reported to activate several downstream signalling pathways, such as RAS/MAPK, PI3K/AKT/mTOR, PLCγ, STAT3/STAT5, CRKL-C3G-RAP1 and MEKK2/3-MEK5-ERK5 (Hallberg and Palmer, 2013; Schönherr et al., 2010; Umapathy et al., 2019)(Figure 7). Activation of ALK also results in successive activation of numerous adaptor proteins such as Shc-GRB2, Src, FRS2, and IRS2 (Hallberg and Palmer, 2013, 2016) (Figure 7). Constitutive activation of ALK has been shown to be an oncogenic driver in many cancers, such as NSCLC, ALCL, IMT and NB thereby making ALK a tractable drug target (Carén et al., 2008; Chen et al.,

2008; Christensen et al., 2007; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008; Rikova et al., 2007; Soda et al., 2008; Wei et al., 1995).

1.2.6.2 ALK in neuroblastoma

The identification of gain-of-function *ALK* mutations in both familial and sporadic NB reported by numerous groups during 2008 have spurred interests to understand the role of ALK-driven signalling, including downstream targets, in NB (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008). ALK-mutants are found in about 8-10% of sporadic NB (De Brouwer et al., 2010; Pugh et al., 2013). ALK point mutations characterised to date are located in the intracellular domain which harbours the ALK kinase domain (Hallberg and Palmer, 2013). In cell culture and in-vitro model systems, ALK mutations in NB have been categorized into three classes using biochemical analysis: gain-of-function ligand-independent mutations, ligand-dependent mutations and kinase-dead mutations (Chand et al., 2013). The gain-of-function mutations result in constitutive or ligand-independent activation of the ALK receptor with concomitant activation of its downstream signalling pathways (Chand et al., 2013; Hallberg and Palmer, 2013)(Figure 7). *ALK* mutation significantly associates with poor survival in intermediate and high-risk NBs (Bresler et al., 2014). In addition, *ALK* mutants occur in about 11% of MNA NB and 7% of non-MNA NB (Bresler et al., 2014; De Brouwer et al., 2010).

Three “hot spot” residues in the ALK kinase domain, namely F1245 (12%), F1174 (30%) and R1275 (45%), account for about 85% of all the *ALK* point mutations (Bresler et al., 2014; De Brouwer et al., 2010). These three residues are involved in autoinhibitory interactions involving αC-helix and the activation loop. These interactions are thought to stabilize the inactive conformation of the non-phosphorylated ALK tyrosine kinase domain (Bossi et al., 2010). Mutations in these residues lead to ligand-independent ALK activation (Chand et al., 2013; George et al., 2008; Schönherr et al., 2011).

As stated earlier, both *ALK* and *MYCN* are located on chromosome 2p.23 and 2p.24, respectively. A low copy number of unbalanced gain of chromosome 2p, which predominantly encompasses *ALK* and *MYCN* loci among others, has been reported in about 19-23% of NB cases, with no focal gain of the *ALK* locus (De Brouwer et al., 2010; Jeison et al., 2010). The unbalanced 2p gain increases *ALK* mRNA and ALK protein expressions and predicts poor patient outcome (De Brouwer et al., 2010; Jeison et al., 2010). Surprisingly, copy number gain of *ALK* locus rarely occurs simultaneously with *ALK* point mutation (De Brouwer et al., 2010), suggesting that *ALK* copy number gain and *ALK* point mutations may represent two distinct modes of *ALK* aberrations. The recently identified ALK ligand gene, *ALKAL2*, is located on chromosome 2p.25 and was found to

be encompassed in most of the unbalanced chromosome 2p gain aberrations, together with *ALK* and *MYCN* (Javanmardi et al., 2019). Although the 2p-gain cassette consisting of *ALK, MYCN* and *ALKAL2* is associated with inferior survival outcome in NB, the presence of *MYCN* on this cassette might overshadow the real significance, if any, of the concurrent presence of *ALK* (a receptor) and *ALKAL2* (ALK ligand) on the same 2p-gain cassette. Therefore, one could ask, what is the biological significance of ALKAL2 to the 2p-gain cassette in NB?

Understanding the biological role of *ALKAL2* with its presence on the 2p-gain cassette could potentially elevate the extent of ALK signalling involvement in NB pathogenesis because unbalanced 2p gain in NB is found in as high as 23% of cases. An infrequent mechanism of *ALK* aberration in NB, occurring in 2-3% of cases, is focal amplification of the *ALK* gene which occurs almost always in parallel with *MYCN* amplification (Brodeur, 2018; Javanmardi et al., 2019; Nakagawara, 1998; Ratner et al., 2016) and are highly associated with aggressive NB clinical phenotype (Bresler et al., 2014; De Brouwer et al., 2010). Another less well characterised mechanism of ALK activation in NB is the deletion of exons leading to truncation of parts of the ALK extracellular region (Cazes et al., 2013; Fransson et al., 2015; Okubo et al., 2012).

Further highlights on the clinical relevance of *ALK* in NB have been demonstrated by studies in which deep sequencing of paired diagnosis-relapse NB samples showed the presence of mutated *ALK* alleles at subclonal levels during diagnosis and a later clonal evolution and expansion of these alleles at relapse (Eleveld et al., 2015; Martinsson et al., 2011; Schleiermacher et al., 2014). Driver *ALK* mutant alleles have been detected in 8-10% of NB samples at diagnosis, increasing to more than 20% in relapse samples (De Brouwer et al., 2010; Pugh et al., 2013; Schleiermacher et al., 2014). In addition, chemotherapy treatment of NB patients also resulted in enrichment of recurrent RAS/MAPK pathway mutations (Eleveld et al., 2015; Padovan-Merhar et al., 2016). These findings suggest a role for ALK as a driver in a subset of primary NB and as a more likely candidate in driving other subtypes of relapse NB (Eleveld et al., 2015; Martinsson et al., 2011; Schleiermacher et al., 2014), thereby making ALK an attractive target for the treatment of ALK-positive primary and relapse NBs.

Notably, *ALK* mRNA and protein expression appear to significantly decrease in all tissues after birth, reaching low levels as early as three weeks of age, and maintained at minimum levels in adult animals (Iwahara et al., 1997). Additionally, *ALK* loss-of-function mutant mice are viable (Bilsland et al., 2008; Witek et al., 2015). These findings imply that the use of ALK inhibitors in the treatment of ALK-positive tumours in children and adults could represent a more tolerable clinical option. Given the potential of ALK-targeted therapy, it is therefore essential to incorporate genomic sequencing or even deep sequencing in

diagnosis and examination of relapse tumour materials for detection of *ALK* mutant alleles, in addition to other targetable oncogenes, and monitoring of their clonal evolution for therapeutic decisions.

1.2.6.3 Synergistic cooperation between ALK and MYCN in neuroblastoma

ALK and *MYCN* are well-established oncogenes in NB and are located on chromosome 2p23 and 2p24 respectively. *ALK* amplification occurs in about 2-3% of NB cases, most frequently with parallel *MYCN* amplification (Azarova et al., 2011; Bresler et al., 2014; De Brouwer et al., 2010; Mossé et al., 2008). Gain-of-function *ALK* mutants, especially *ALK-F1174L*, are generally found to associate with a high proportion of MNA NB cases and this combined occurrence results in a worst outcome (De Brouwer et al., 2010). This result suggests positive pathogenic cooperation between these two aberrations. The first mechanistic link between ALK and MYCN was established by Schönherr *et al.,* where they showed that both wild-type and gain-of-function mutant ALK stimulate the initiation of *MYCN* transcription in NB and neuronal cell lines (Schönherr et al., 2012).

The co-expression of ALK and MYCN synergistically mediated transformation of NIH3T3, mouse fibroblast cells (Schönherr et al., 2012). The pathogenicity of the cooperation between ALK and MYCN was subsequently demonstrated *in-vivo* in mouse and zebrafish NB models (Berry et al., 2012; Zhu et al., 2012). Co-expression of constitutively active ALK and MYCN in mouse and zebrafish models increased tumour penetrance with earlier tumour onset and potentiated lethality (Berry et al., 2012; Zhu et al., 2012) (Figure 8). Overexpression of MYCN drives increased sympathetic neuroblast proliferation and inhibited chromaffin cell differentiation, but eventually elicits a developmentally-programmed apoptotic response in the hyperplastic NC-derived progenitors (Berry et al., 2012; Zhu et al., 2012) (Figure 8). This result gives rise to low penetrance of tumour formation in NB models. On another note, continuous expression of gain-of-function ALK in neuroblasts does not lead to tumour formation (Berry et al., 2012; Zhu et al., 2012). Alk gain-of-function knock-in mice enhanced neuroblast proliferation in the sympathetic ganglia with concomitant enlargement of the ganglia and extended neurogenesis (Cazes et al., 2014). In contrast, expression of gain-of-function *ALK* in neuroblasts or gain-of-function *Alk* knock-in contributes to *MYCN*-driven NB by providing pro-survival signals which enable transformed neuroblasts to escape MYCN-induced apoptosis, thus promoting progression to NB and increases tumour penetrance (Berry et al., 2012; Cazes et al., 2014; Kramer et al., 2016; Zhu et al., 2012) (Figure 8). The dependency of MYCN-transformed-cells on ALK signalling for survival and potentiation further underscore the potential therapeutic benefit of targeting ALK in ALK-positive and MNA NBs.

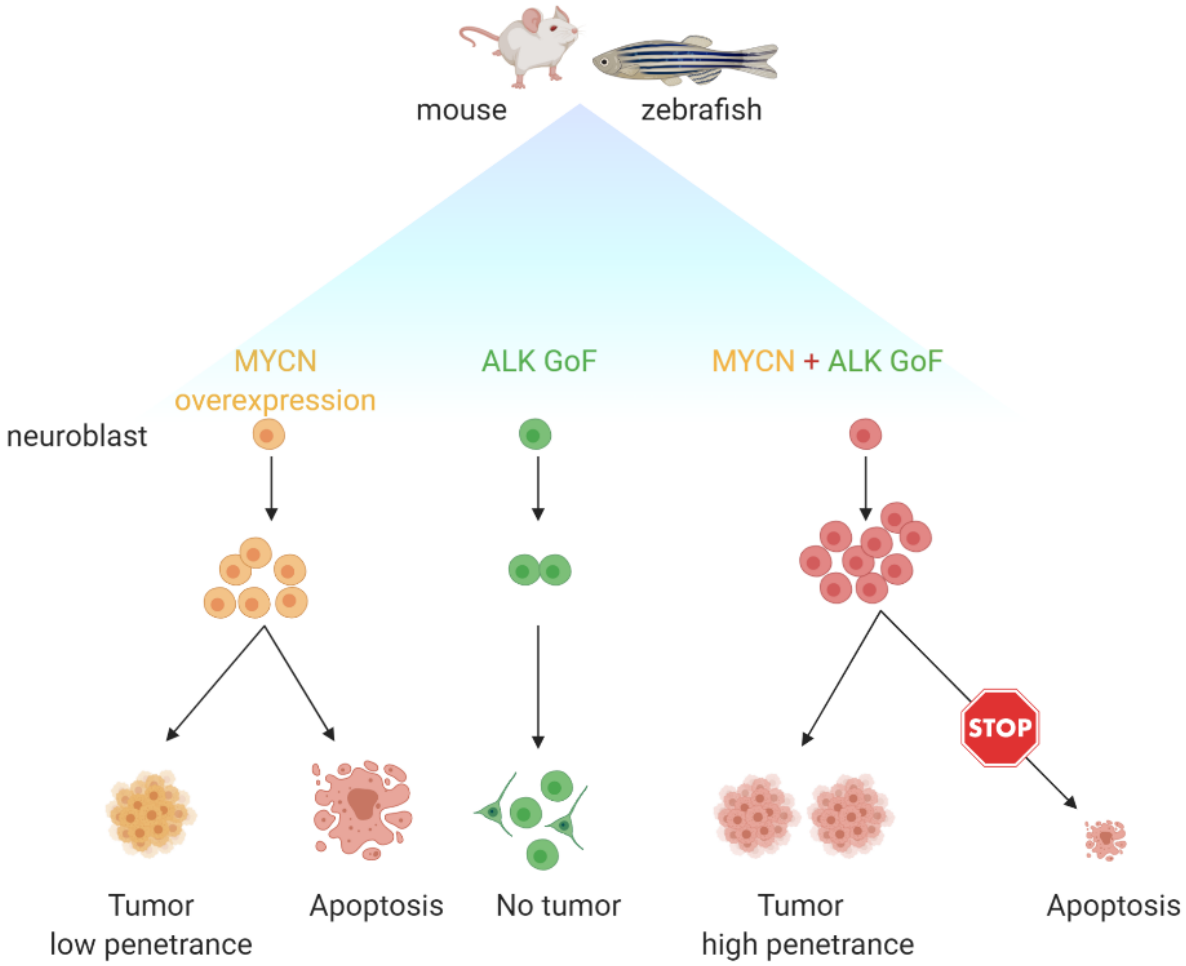

Figure 8. ***A model of the mechanism for cooperation between ALK gain-of-function (GoF) mutant and MYCN in neuroblastoma development in model organisms****. The overexpression of MYCN in neuroblasts drives proliferation and induces developmentally-timed apoptosis of some of the cells, resulting in a low penetrance of tumour formation. Expression of ALK GoF mutant alone in neuroblasts does not lead to tumour formation. ALK GoF signalling enhances the survival of differentiated neuroblasts. Coexpression of MYCN and ALK GoF mutant in neuroblasts promote neuroblast proliferation and blockade of MYCN-induced apoptosis, leading to high penetrance tumour formation. Figure is based on (Berry et al., 2012; Cazes et al., 2014; Zhu et al., 2012).*

1.2.6.4 Role of ALK in neuroblastoma differentiation

While activated ALK has been reported to stimulate neuronal differentiation when expressed in chick sympathetic neuroblasts *in-vitro* (Kramer et al., 2016), SOX10 promoter-driven expression of *ALK-F1174L* in mice NCCs has been found to enhance cell proliferation and block differentiation (Montavon et al., 2014; Vivancos Stalin et al., 2019). Stalin and colleagues reported that ectopic expression of *ALK-F1174L* in migrating

NCCs in mouse resulted in inhibition of differentiation and increased proliferation of early sympathetic progenitor cells but showed no neoplastic transformation (Vivancos Stalin et al., 2019). The expression of ALK-F1174L or ALK- R1275Q in MYC-mediated immortalized NC progenitor cells, MONC-1 or JoMa1, produced undifferentiated non-NB tumours (Montavon et al., 2014). Furthermore, analyses of the prognostic roles of ALK and MYCN protein expressions revealed high ALK and high MYCN protein expression in 41% and 39% NB tumours, respectively (Chang et al., 2020). An overwhelming majority (about 86%) of NB tumours showing high expression of ALK or MYCN protein displayed poorly differentiated or undifferentiated histology (Chang et al., 2020). These suggest a potential role of deregulated ALK activity impairing NC progenitor cells differentiation during neuroblastic tumour initiation and or progression. The mechanism by which gain-of-function ALK impairs NC-derived precursor differentiation is, however, unclear. This result partly motivated our study in Paper III (briefly described in section 4.3).

1.2.6.5 Targeting ALK in cancer

The most common forms of *ALK* aberrations in human cancer manifest as *ALK* chromosomal rearrangements, resulting in the formation of constitutively active oncogenic ALK fusion proteins (Amin and Lai, 2007; Kwak et al., 2010; Lovly et al., 2014). ALK rearrangements account for about 55% of all cases of ALCL, 2-9% of NSCLC and up to 50% of inflammatory myofibroblastic tumours (IMT) (Amin and Lai, 2007; Kwak et al., 2010; Lovly et al., 2014). Point mutations occur in ALK full-length protein, mostly in the kinase domain, and these represent the most recurrent mutations found in NB, up to 10% of the cases (De Brouwer et al., 2010; Hallberg and Palmer, 2013; Janoueix-Lerosey et al., 2008; Mossé et al., 2008). ALK-positive cancers have been shown to be addicted to ALK signalling in preclinical settings, thereby spurring the development of ALK tyrosine kinase inhibitors (TKIs) such as crizotinib, ceritinib, alectinib brigatinib and lorlatinib (George et al., 2008; Soda et al., 2008).

Crizotinib

Crizotinib was initially developed as a potent TKI against MET and became the first ALK TKI to enter clinical trials (Christensen et al., 2007) (Figure 9A). The results of a phase I/II clinical trial in ALK-positive NSCLC patients treated with crizotinib showed remarkable clinical activity resulting in accelerated approval of crizotinib by FDA, in 2011, for treatment of advanced ALK-positive NSCLC (Hallberg and Palmer, 2010; Kwak et al., 2010). Crizotinib was shown to be superior to chemotherapy, as a first-line therapy, in ALK-positive NSCLC patients in phase III clinical trials with mean progression-free survival (PFS) of 8 to 11 months (Shaw et al., 2013; Solomon et al., 2014b). Despite the initial response, crizotinib treated patients eventually develop resistance partly due to

emergence of secondary *ALK* mutations, thereby warranting the search and development of next-generation ALK TKIs (Choi et al., 2010; Lopez et al., 2020; Shaw et al., 2013; Solomon et al., 2014a).

The first clinical trial of crizotinib in pediatric cancer patients, resulted in a rather disappointing outcome for NB patients as only one of 11 NB patients, with known ALK status, showed a complete response (Mossé et al., 2013). A follow-up clinical study which involved crizotinib treatment of recurrent or unresectable ALK-fusion-positive pediatric ALCL and inflammatory myofibroblastic tumours (IMTs) respectively, showed an overall response rate of 83-90% for both cancer types, with a complete response rate of about 80% in ALCL patients and a complete or partial response of about 86% in IMT patients (Mossé et al., 2017). The response rate, type and duration recorded in this study is far superior to those recorded in even most late-phase clinical trial involving crizotinib use in adult ALK-fusion-positive cancer patients (Mossé et al., 2017; Shaw et al., 2013; Solomon et al., 2014a). This difference could be due to the less complex genetic landscape or reduced mutation burden of pediatric cancers and their possible dependency on a single oncogenic driver.

The poor clinical outcome earlier mentioned for NB patients (Mossé et al., 2013) could be due to the heterogeneous clinical nature of NB or could be indicative of a less potent inhibitor due to intrinsic resistance of full-length ALK mutants. Moreover, anecdotal evidence showed that crizotinib treatment of some chemotherapy-refractory NB patients, showing high ALK protein expression, resulted in complete initial response (Verma et al., 2017). This result highlights the potential benefits of exploring and targeting ALK in NB with more potent next-generation ALK TKIs.

Ceritinib

Ceritinib, originally known as LDK378, is an orally administered, ATP-competitive ALK TKI (Marsilje et al., 2013) (Figure 9B). In preclinical studies involving ALK-fusion proteins, ceritinib was 20 times more potent against ALK activity than crizotinib (Shaw et al., 2014). This TKI also exhibited significant antitumour activity in ALK-rearranged NSCLC xenograft models, against both crizotinib-resistant mutations, such as G1269A, S1206Y, I1171T V1180L, and crizotinib-sensitive ALK mutations (Friboulet et al., 2014; Marsilje et al., 2013). Ceritinib displayed antitumour activity against both crizotinib-naïve and crizotinib-resistant tumours in NSCLC patients, and yielded a median PFS of 7 months in phase I clinical trial (Shaw et al., 2014), resulting in the accelerated approval of ceritinib

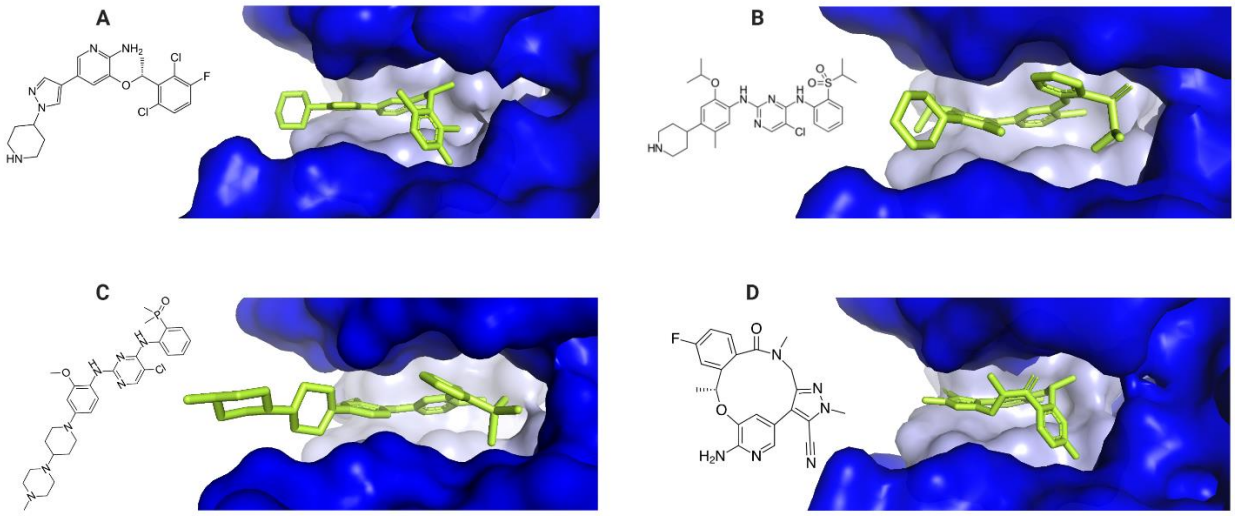

Figure 9. ALK tyrosine kinase inhibitors (TKIs) (lemon green) in the ATP binding pocket of the ALK kinase domain (blue). Visualization of the modelled binding of different ALK TKIs in the ATP binding pocket of wild-type ALK, showing the difference in contact sites between the different ALK TKIs. (A) Crizotinib (PF2341066, PDB: 2XP2). (B) Ceritinib (LDK378, PDB: 4MKC). (C) Brigatinib. (D) Lorlatinib (PF06463922, PDB: 4CLI). Chemical structure (black) of each TKI is located to the left in each subfigure.

by the FDA in 2014. Dose-limiting toxicities associated with ceritinib included diarrhoea, vomiting, dehydration, elevated aminotransferase levels, and hypophosphatemia (Shaw et al., 2014). Subsequently, in a phase III trial, ceritinib was demonstrated to be significantly superior to chemotherapy as a first-line therapy in patients with advanced *ALK*-rearranged NSCLC, with PFS for ceritinib at 16.6 months and that for chemotherapy at 8.1 months (Soria et al., 2017). These demonstrated the potential of ceritinib as a therapeutic option for ALK-positive cancers.

The combination of ceritinib and a dual CDK4/6 inhibitor, ribociclib, synergistically induced tumour regression in NB xenograft model (Wood et al., 2017). The therapeutic benefit of ceritinib in ALK-positive NB patients is unclear and needs further exploration. A phase I trial of ceritinib in NB, IMT, ALCL patients has been completed (NCT01742286) awaiting results, and other trials are currently underway to explore the benefits of combination treatments with ceritinib and other drugs in high-risk NB patients (NCT02559778).

Brigatinib

Brigatinib was developed as a potent TKI of ALK (Figure 9C) but can also inhibit other kinases, including some EGFR mutants, ROS1 and IGF-1R (Zhang et al., 2016). Preclinical studies in *ALK*-positive NSCLC models showed that brigatinib is superior to crizotinib and potently inhibited ALK mutants that confer resistance to crizotinib (G1269A,

S1206Y, I1171T V1180L F1174C/V/L, G1202R), ceritinib (F1174C/V/L, L1198F, G1202R) and alectinib (I1171N/T/S, G1202R) (Zhang et al., 2016). The brain is a common site of disease progression or relapse during treatment failure in cancer patients, and this result could be due to poor penetrance of the blood-brain barrier by many drugs (Gadgeel et al., 2014). Remarkably, brigatinib was found to be superior to crizotinib in improving survival in an orthotopic mouse brain tumour model, which may be indicative of enhanced central nervous system (CNS) penetration by brigatinib compared to crizotinib (Zhang et al., 2016). Clinical trials of brigatinib in ALK TKI-naïve or crizotinib-resistant *ALK*-positive NSCLC patients demonstrated significantly higher systemic and CNS response rates and PFS (Bazhenova et al., 2017; Camidge et al., 2018; Kim et al., 2017). The response rate among patients with measurable brain metastatic lesions was dramatically higher for brigatinib (78%) compared to crizotinib (29%) (Camidge et al., 2018). These reports highlight the potential of brigatinib as a first-line therapy for *ALK*-positive NSCLC patients and more importantly, for patients with baseline brain metastasis.

Recurrent mutations in full-length ALK are found in high-risk NB which is mostly associated with metastatic disease (Carén et al., 2008; Chen et al., 2008; Eleveld et al., 2015; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008). The superior efficacies demonstrated by brigatinib against diverse *ALK*-rearranged mutants and against metastatic brain disease in NSCLC suggest a potential for brigatinib in high-risk *ALK*-positive NB patients. Data on brigatinib in NB is generally lacking, thereby, warranting preclinical studies of brigatinib to ascertain its efficacy in NB setting (Paper I) (Siaw et al., 2016).

Lorlatinib

Lorlatinib is a novel, potent, third-generation macrocyclic TKI of ALK and ROS1 (Figure 9D). Lorlatinib demonstrated enhanced ability to penetrate the blood-brain barrier and exhibited broad-spectrum potency against most ALK kinase domain mutations which are resistant to crizotinib and even second-generation ALK TKIs such as ceritinib, alectinib and brigatinib (Solomon et al., 2018). In general, treatment of ALK-positive NSCLC patients with lorlatinib showed significantly high overall and CNS or intracranial activity in both treatment-naïve patients and those previously treated with other ALK TKIs (Solomon et al., 2018), thereby motivating the accelerated approval of lorlatinib by FDA in 2018, and approval by the European Medicine Agency (EMA) in 2019, for the treatment of crizotinib, alectinib, or ceritinib-refractory ALK-positive NSCLC patients.

Lorlatinib has also been reported to effectively block the growth of ALK-addicted NB in a xenograft model (Guan et al., 2016; Infarinato et al., 2016), which suggests lorlatinib could be a potential therapeutic option for ALK-positive NB patients. The first pediatric phase I

trial of lorlatinib as a single agent and in combination with chemotherapy for treatment of relapsed or refractory NB patients, is currently on-going (NCT03107988).

1.2.7 Treatment strategies in neuroblastoma

The Children's Oncology Group (COG) risk stratification classifies NB as low, intermediate and high-risk groups based on age at diagnosis, tumour stage, and biological factors such as *MYCN* amplification, ploidy, and tumour histology (Davidoff, 2012). This classification and other refined versions are employed in the planning of treatment strategy for NB patients (Davidoff, 2012). The major treatment modalities used in NB include surgery, chemotherapy, radiotherapy, differentiation therapy and immunotherapy (Swift et al., 2018).

1.2.7.1 Surgery

NB patients without metastatic disease undergo initial surgery which aims to resect as much of the primary tumour as is safely possible with minimal residual disease (Luo et al., 2018). This procedure is the most suitable for low- and intermediate-risk NBs. Surgery is also performed to obtain tissues for diagnosis. There are, however, complications that can be associated with surgery. In intermediate-risk NB patients where surgery is considered too risky, tumours are first treated with chemotherapy for debulking and metastatic remission, followed by surgery after tumours response (Hero et al., 2008; Parikh et al., 2015). Surgical resection alone has been shown to be curative in low-risk NBs with 5-year OS at 97% (Luo et al., 2018). In high-risk NBs, with 5-year OS at 40-50%, surgical resection of the tumour after several chemotherapy cycles has been shown to be beneficial (Pinto et al., 2015; Rojas et al., 2016).

1.2.7.2 Radiotherapy and radionuclide therapy

Adrenergic neuroblasts, which are the cellular sources of NB, are typically very sensitive to radiation, thereby motivating the use radiotherapy in protocols for NB treatment, particularly in high-risk NBs (Deacon et al., 1985). Inclusion of radiotherapy in chemotherapy protocols to treat intermediate-risk and high-risk NB patients significantly improves both PFS and OS (Sibley et al., 1995). Targeted radiotherapy involving the use of radio-labelled Iodine (radionuclide) coupled to MIBG (^{131}I-MIBG), has been demonstrated to provide benefits in NB (de Kraker et al., 2008; Swift et al., 2018). Mechanistically, NB cells are MIBG avid, due to the presence of transmembrane norepinephrine transporter (NET) which allows uptake of MIBG, and its later accumulation in neurosecretory granules in the tumour cells (Bomanji et al., 1987; Pandit-Taskar and Modak, 2017). While inside cell granules, radiation from ^{131}I-MIBG results in the efficient

killing of tumour cells (Pandit-Taskar and Modak, 2017). First-line ^{131}I-MIBG targeted therapy in NB achieved a significant response rate of 66%, with myelosuppression, particularly thrombocytopenia, and hypothyroidism as the dose-limiting toxicities (de Kraker et al., 2008; Garaventa et al., 1999; Lashford et al., 1992). This result highlights ^{131}I-MIBG therapy as a valuable option in NB treatment planning.

1.2.7.3 Chemotherapy

Chemotherapy is generally an essential part of NB treatment protocols. The decision to include chemotherapy in a treatment schedule for NB patient is greatly influenced by the risk group to which the patient belongs (Matthay et al., 2016). For children with low-risk NB and presenting clinical symptoms, a limited number of chemotherapy cycles are applied. In contrast, two to eight chemotherapy cycles are prescribed for intermediate-risk patients (Pearson et al., 1992). The following drugs are used in different combinations for chemotherapy; cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide (Pearson et al., 1992). Current protocols for treating high-risk NB are divided into three main stages – (i) induction chemotherapy involving five to eight cycles of intensive chemotherapy which aims to shrink the primary tumour and reduce metastasis, (ii) consolidation therapy involving a high dose chemotherapy, followed by autologous stem cell transplant (ASCT) and radiation therapy to eliminate the remaining minimal disease, and (iii) maintenance phase by immunotherapy or use of differentiation-inducing agents such as 13-*cis* retinoic acid for treatment of minimal residual disease (RA) (Matthay et al., 2016; Smith and Foster, 2018).

1.2.7.4 Immunotherapy

After two decades of research, patients with high-risk NB can be maintained in continual remission with anti-GD2 monoclonal antibody therapy, and this is becoming one of the standard cares for treating minimal residual disease (Navid et al., 2010). GD2 is disialoganglioside expressed primarily on the surface of NC derived cells and tissues including pain fibres, skin cells and mature neurons (Cheung and Dyer, 2013). NB is a neuroectodermal or NC-derived tumour and specifically expresses GD2, making GD2 an attractive target for immunotherapy. The two frequently explored anti-GD2 monoclonal antibodies in NB are chimeric anti-GD2 antibody (ch14.18) and mouse 3F8 (Sait and Modak, 2017). Ch14.18 has been combined with interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF) and 13-*cis*-RA as a maintenance therapy in treating minimal residual disease after intensive chemotherapy. This combination has significantly improved EFS (Yu et al., 2010). Several other anti-GD2 monoclonal antibodies are currently in clinical trials in NB patients (NCT02650648, NCT00072358, NCT00072358, clinicaltrials.gov).

1.2.7.5 Retinoic acid therapy and differentiation

NB is thought to arise from failed differentiation of NC-derived sympathoadrenal precursors. It has the highest rate of spontaneous regression of any human cancer and can mature spontaneously to a benign ganglioneuroma (Brodeur, 2018; Nakagawara, 1998; Ratner et al., 2016). These observations have aroused an interest in the study NB of differentiation and exploration of differentiation-inducing agents. NGF and vitamin A derivatives such as 13-*cis*-RA, all trans-retinoic acid (ATRA) and fenretinide have been shown to induce neuronal differentiation and growth arrest of NB cell lines *in vitro* (Preis et al., 1988; Påhlman et al., 1984). Incorporation of 13-*cis* RA as a maintenance therapy in high-risk NB patients has significantly improved EFS. This result was particularly dramatic in patients with minimal residual disease prior to RA therapy (Matthay et al., 1999). A recent case report has also corroborated the clinical efficacy of the use of 13-*cis* RA as a maintenance therapy for minimal residual disease in high-risk NB (Sato et al., 2015). Generally, RA has been used in combination with anti-GD2 monoclonal antibodies as a maintenance therapy for high-risk NB (Cheung and Dyer, 2013; Smith and Foster, 2018). Clinical trials are underway to explore further the combination of RA and different anti-GD2 antibodies in high-risk NB settings (clinicaltrials.gov).

High-risk NB represents about 50% of all NBs and has a relapse rate of more than 50% (Cohn et al., 2009). *MYCN* amplification also accounts for 40% to 50% for high-risk NB (Kreissman et al., 2013). Since RA therapy is a component of treatment regimens for high-risk NB patients (Matthay et al., 1999), this implies that a high percentage of RA treated NB patients experience relapse disease. Knowing MYCN expression status could influence NB cell identity, thus, MES versus ADRN, and also responsiveness to RA (Wang et al., 2019), it would be reasonable to ask if high-risk NB patients should be further stratified based on MYCN expression status in deciding who receives RA as a maintenance therapy, hence a study to clarify this possibility and any related therapeutic benefit may be warranted.

1.2.7.6 Targeted therapy in neuroblastoma

While conventional therapies and interventions such as surgery, chemotherapy and radiotherapy have shown great efficacy in treating especially low- and intermediate-risk primary NBs, treatment outcome for high-risk NB patients is poor (Moreno et al., 2020). In relapsed or refractory NBs, the therapies mentioned above fail and result in the death of about 90% of these patients within 5 years (London et al., 2017; London et al., 2011; Moreno et al., 2020). It is evident that treatment options for high-risk NB, and particularly relapsed NB are limited or lacking, making it necessary to explore other novel therapeutic options. Recent investigations which aimed to understand the mechanisms of NB

pathogenesis and relapse have uncovered several novel actionable drug targets such as ALK, CDK4/6, MYCN, CHEK1, BIRC5, MEK etc., which are critical to developing targeted therapies for NB (Moreno et al., 2020; Moreno et al., 2017).

Despite its genetic underpinning, which manifests frequently through SCAs, NCAs and or copy number variations (CNVs), NB is increasingly recognized as a disease of aberrant protein signalling in cells. This result perhaps explains why in both the first and second Neuroblastoma New Drug Development Strategy (NDDS) forums it was recommended that protein products with deregulated or enhanced activities with proven capabilities of driving tumour growth and progression should be prioritized for therapeutic targeting in NB (Moreno et al., 2020; Moreno et al., 2017). The revised NDDS priority targets include ALK, Aurora kinase, CDK4/6, MEK, BIRC5, CHEK1 MDM2, BCL2, PARP, WEE1, mTORC1/2, BET, ATR, CDK7, CDK2/9, BRIP1, RRM2, ATRX, TERT telomerase and ALT (alternative lengthening of telomere lengthening) (Moreno et al., 2020).

Preclinical investigations of some ALK TKIs such as crizotinib, alectinib, ceritinib, lorlatinib and repotrectinib in ALK-positive NB models have yielded encouraging results (Alam et al., 2019; Cervantes-Madrid et al., 2019; George et al., 2008; Guan et al., 2016; Infarinato et al., 2016; Schönherr et al., 2011), thereby motivating the recently completed, and ongoing clinical trials of ceritinib (NCT01742286) and lorlatinib (NCT03107988) respectively in NB patients. MEK inhibitors such as trametinib, selumetinib sulfate, cobimetinib which target activating MAPK pathway mutations, P13K inhibitor SF1126, and CDK4/6 inhibitor ribociclib are currently in clinical trials in NB patients (NCT03434262) (Moreno et al., 2017). Early phase clinical trials for MDM2, CHEK1, and BET bromodomain inhibitors in pediatric cancer patients have just been started (Moreno et al., 2020).

Telomeres are repetitive hexanucleotides DNA sequence at both terminals of a chromosome which protects the chromosome from degradation and interchromosomal fusion (Moyzis et al., 1988). Telomere length undergoes gradual shortening for every DNA replication, and critically short telomeres elicit replicative senescence and apoptosis (Greider, 1990). Immortal cells such as cancer cells bypass this progressive telomere shortening through de novo synthesis and elongation of the telomere, mediated by a ribonucleoprotein called telomerase (Blackburn et al., 1989; Greider and Blackburn, 1987; Kim et al., 1994). The catalytic unit of this enzyme is called telomere reverse transcriptase (TERT) (Blackburn et al., 1989). Telomerase is activated in about 80-90% of human cancers (Bartek and Lukas, 2003; Kim et al., 1994). Additionally, some tumour cells use another homologous recombination mechanism known as alternative lengthening of telomeres (ALT) to maintain or elongate their telomeres in a telomerase independent manner (Bryan et al., 1997; Dunham et al., 2000). TERT-mediated telomere maintenance

and ALT have emerged as important prognostic markers of neuroblastoma (Nozaki et al., 2000; Ohali et al., 2006). Telomerase activation in some high-risk NBs has been found to be due to TERT rearrangement (Peifer et al., 2015; Valentijn et al., 2015). Therapeutic targeting of telomerase and ALT pathways may represent promising strategy for treatment of high-risk NBs (Moreno et al., 2020). Clinical drug candidates for these targets are currently unavailable.

1.2.7.7 Prospects for treatment of relapsed neuroblastoma

About 50% of high-risk NB relapse (Cohn et al., 2009), and the 5-year OS rate for relapse NB stands at 20% (London et al., 2017; London et al., 2011). This discouraging OS rate for relapsed NB may be reflective of a more aggressive relapsed disease or lack of effective treatment options for relapsed disease or perhaps the clinician's inadequate knowledge of the relapsed tumour's new "driver" genetic landscape.

Current treatment options for relapsed NB include chemotherapy and ^{131}I-MIBG radiotherapy, which produce temporary benefits but mostly fail in the long term (Zage, 2018). Clinicians have also been reported to be reluctant to prescribe invasive procedures, including tumour sampling in relapsed high-risk NB patients (Schleiermacher et al., 2014). While treatment naïve NB tumours exhibit paucity of mutations in targetable genes, recent reports have demonstrated that the mutational burden in relapsed NB tumours increase significantly, and have reduced subclonal heterogeneity (Padovan-Merhar et al., 2016; Schramm et al., 2015). Therapeutically relevant target genes such as *ALK*, *ATRX, CDK4* and *FGFR1* are recurrently mutated and enriched in relapsed NB tumours (Padovan-Merhar et al., 2016; Schleiermacher et al., 2014; Schramm et al., 2015). Mutations in reported recurrent RAS/MAPK pathway genes have also been significantly enriched in relapsed NB tumours (Eleveld et al., 2015; Padovan-Merhar et al., 2016; Schramm et al., 2015). These mutated genes consist of druggable targets such as *ALK*, *FGFR1*, *PTPN11*, and difficult-to-drug targets such as K-RAS, H-RAS and N-RAS (Eleveld et al., 2015). Although *ALK* mutations are found in about 10% of primary NBs, this percentage is as high as over 20 - 43% in relapsed tumours, highlighting the clinical and the biological significance of ALK in NB (Eleveld et al., 2015; Schleiermacher et al., 2014). Genomic alterations in genes that are directly engaged in RAS-RAF signalling, such as *PTPN11*, *NF1*, *RAS*, and *RAF*, all together have been found in about 30% of relapsed NB (Eleveld et al., 2015).

While the RAS family genes have proven difficult to target with small molecule inhibitors, the recent identification of a novel pocket called switch-II pocket (S-IIP) in the inactive GDP-bound RAS spurred a breakthrough discovery of a covalent small molecule inhibitor, ARS-1620, that selectively targets and potently inhibits K-RAS(G12C) mutant *in vitro* and

in vivo (Janes et al., 2018; Ostrem et al., 2013). Another covalent inhibitor, MRTX849, inhibits K-RAS(G12C) mutant by binding in the switch-II pocket in K-RAS(G12C) mutant allele and engages in an irreversible covalent bonding with cysteine 12 (Fell et al., 2020). The K-RAS(G12C) inhibitors, ARS-1620 and MRTX849, showed robust antitumour activity in *in vitro, in vivo* and xenograft models, with MRTX849 currently in phase I/II clinical trials in patients with K-RAS(G12C) mutation cancers (KRYSTAL-1 NCT03785249, KRYSTAL-2 NCT04330664) (Fell et al., 2020; Janes et al., 2018; Ostrem et al., 2013). These achievements in targeting K-RAS(G12C) imply that similar strategies could be employed in targeting other K-RAS mutations and other RAS family gene mutations. These results suggest that therapeutic targeting of mutated genes of the RAS signalling cascade may be a viable option for relapsed NB patients.

Altogether, these results suggest that the driver of a relapsed NB is more likely to be a targetable or therapeutically relevant oncogenic pathway. Against this background, the advent of targeted therapies, including ALK TKIs in NSCLC, and their proven success in other cancers, and the recent advances made with RAS inhibitors may be suggestive of a new era and alternative therapeutic option for treatment of relapsed NBs (Fell et al., 2020; Friboulet et al., 2014; Gadgeel et al., 2014; Janes et al., 2018; O'Bryan, 2019; Shaw et al., 2014; Solomon et al., 2014a). It, therefore, implies that the molecular profile of relapsed NB tumours should be a critical reference in treatment decisions, highlighting the need to make genetic profiling of relapsed NB tumours a routine clinical practice.

However, these questions remain: what should be the right time for high-risk NB patients to receive targeted therapy? Should targeted therapy be part of first-line regimens even if the target is present at subclonal level? Or should it be administered as part of maintenance therapy to treat minimal residual disease after conventional therapy sessions? Or should it be applied at relapse? After all, precision medicine is about this issue.

2 AIMS

This thesis aims to further our understanding of ALK signalling and its role in NB differentiation and explore novel ALK TKIs in NB setting.

Specific aims

Paper I

- We aimed to investigate the therapeutic efficacy of the second-generation ALK TKI, brigatinib, in NB cell lines and other preclinical models.
- Further, we aimed to establish inhibitory profiles of brigatinib against major *ALK* gain-of-function mutant alleles identified in NB.

Paper II

- To perform genomic analysis of a tumour biopsy from an NB patient
- To characterize a novel *ALK-I1171T* mutant allele identified in the NB tumour biopsy.
- To perform pharmacological inhibition profile of the novel *ALK-I1171T* mutant with ALK TKIs.

Paper III

- To investigate the role of *DLG2* in NB differentiation.
- To explore the mechanistic relationship between ALK and DLG2 in the regulation of the differentiation state of NB cells.
- To further explore the genetic landscape of the *DLG2* gene in 11q-del NB tumours.

3 MATERIALS AND METHODS

Cell culture

The human NB cell lines used in this study are CLB-BA, CLB-GE, CLB-GA, CLB-PE, IMR-32, SK-N-DZ, SH-SY5Y, SK-N-SH, SK-N-AS and SK-N-BE(2) and a rat pheochromocytoma cell line, PC12. NB cells were cultured in RPM1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin and PC12 cells cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 7% horse serum and 3% FBS (fetal bovine serum) and 1% penicillin and streptomycin, at 37 ˚C with 5% carbon dioxide.

Neurite outgrowth and differentiation assay

PC12 cells (2 x10^6) were co-transfected with pEGFPN1 (0.5 ng) and ALK-mutant (0.75 ng) or ALK-wt (0.75 ng), in 100 µl of Ingenio electroporation solution (Minus Bio LCC). ALK-wild type was stimulated with 1 µg/ml mAb31. Neurite-outgrowth was scored 48 hours post-transfection, by counting the fraction of neurite bearing GFP-positive cell versus all GFP-positive cells visualized under a Zeiss Axiovert 40 CFL microscope. Neurite-carrying cells had neurite(s) that was at least twice the length of a normal cell body diameter.

For differentiation assays, NB cell lines were seeded in collagen-coated 6-well plates, in RPMI-1640 medium supplemented with 10% FBS. After overnight culture, cells were treated with either DMSO or 10µM RA or 100 ng/ml NGF. Differentiation was scored after 24 hrs or more, post-treatment, depending on cell line and differentiation agent used. Cells were considered morphologically differentiated if neurite(s) length was at least 1.5 times that of the cell body.

Drosophila transgenic lines and brigatinib treatment

Transgenic *Drosophila* lines carrying the *UAS-ALK^{F1174L}* or *UAS-ALK^{R1275Q}* gain-of-function mutations were crossed with the *pGMR-Gal4* transgenic driver to drive ectopic expression of ALK mutants in the imaginal discs. The first instar larvae from these crosses were transferred to food containing either 100 µM or 200 µM brigatinib or on food containing 2% ethanol (as control) and allowed to grow at 25 °C.

Immunostaining eye imaginal discs of *Drosophila*

Imaginal discs were obtained from the third instar larvae and fixed for 20 min in 4% formaldehyde diluted in phosphate-buffered saline (PBS). Afterwards, discs were permeabilized for 10 min in PBS, followed by blocking in PBST supplemented with 4% fetal bovine serum. The discs were then incubated with ALK antibody in PBST overnight. Samples were washed with PBS and further incubated for 2 hours in the fluorescently labelled secondary antibody. Discs were fine dissected prior to mounting in flouromount G.

Mouse xenografts

Female BALB/cAnNRj-Foxn1nu mice (Janvier Labs, France) or Female Balbc/nude mice (Charles River, Germany) 4-6 weeks old were housed. After acclimatizing, NB cells, mixed in Matrigel, were subcutaneously injected into the left flank. Tumour size was measured continuously, and then tumours were excised and weighed at the end of the experiment. Following the Regional Animal Ethics Committee approval, all experimental procedures and protocols were performed, Jordbruksverket (230-2014, 01890-2018).

Western blotting and quantification

Whole-cell lysates were collected in RIPA lysis buffer. Protein concentration was determined, and samples were subjected to SDS polyacrylamide gel electrophoresis (PAGE). Samples were transferred from the gel to polyvinylidene difluoride membranes and immunoblotted with primary antibodies overnight at 4°C. After washing in PBST, blots were further incubated with secondary antibodies and subsequently developed with ECL prime western blotting detection reagents. Quantification of western blotting was performed using Image Studio™ Lite software (LI-COR Biotechnology - UK).

Viral transduction and establishing lentiviral stable cell lines

Cell lines were transduced independently with lentiviral particles at MOI of one. Transduced cells were selected with 1 µg/ml of puromycin (Gibco) to establish stable cell lines.

Proliferation assay

Cells were seeded in triplicates into wells of 48-well plates and cultured at 37°C in Incucyte S3 (Essen BioScience, USA). Cell growth/proliferation was recorded by scanning the cells at 12- hour intervals for five days. Cell percentage confluency was

determined for each scan using Incucyte S3 software (Essen BioScience, USA). Cell proliferation was also determined in some cases by resazurin assay.

Cell cycle analysis

Cells were seeded and cultured for 48 hours, harvested and rinsed with PBS, followed by ethanol fixation for 3 hours at 4°C. Cell cycle analysis was carried out using NucleoCounter NC-3000 (Chemometec, Denmark) according to manufacturer's protocol, Fixed-Cell-Cycle-DAPI-Assay. Cell cycle data were analyzed using the plot manager in NucleoView NC-3000 software.

DNA probes and nuclear extract Isolation

The predicted SP1 binding locus (chr11:85628043..85628782) located within one kilobase pair upstream of *DLG2* transcriptional start site was amplified using biotinylated primers in PCR reaction using genomic DNA, extracted from a NB cell line, as template and the control DNA probe (628 bp) was amplified from a random sequence with the *DLG2* gene using a different set of biotinylated primer pair. Nuclear fractions were isolated from the NB cell line using cell fractionation buffers and stored at -80℃.

DNA probe pulldown assay

Nuclear extract (50 µg to 100 µg) was mixed with Dynabeads MyOne streptavidin C1 (Thermo Fisher Scientific, Sweden) and 4 µg of appropriate biotinylated DNA probes and incubated overnight. Dynabeads were washed with ice-cold PBS. After that, beads were heated at 95℃ for 5 minutes in 2X SDS sample buffer. The sample was subjected to PAGE and immunoblotted with SP1 antibody.

SNP microarray analysis and whole-genome sequencing

High-density Affymetrix single nucleotide polymorphism (SNP) microarrays were used for genomic profiling on human NB tumours. The following software packages were used in primary data analysis, the GDAS software (Affymetrix), Chromosome Analysis Suite (ChAS) (Affymetrix) or Copy Number Analyzer for Affymetrix GeneChip Mapping arrays (CNAG 3.0, Genome Laboratory, Tokyo, Japan; www.genome.umin.jp). Whole-genome sequencing (WGS) was performed on tumour DNA and constitutional DNA extracted from the blood of patients with coverage between 30X to 60X using Illumina instrumentation. The Sentieon suite of bioinformatics tools (Sentieon Inc, Mountain View, CA) were used for data processing, including variant calling. Calling of copy number alterations was executed using the Canvas tool (version 1.38.0.1554) (17).

Foci formation Assay

NIH3T3 cells were transfected for 24 hours, with 0.55 µg of indicated plasmids using Lipofectamine 2000 (Invitrogen). After transfection, cells were maintained in DMEM supplemented with 0.5 mg/ml G418 and 10% FBS for few days, and subsequently cultured in DMEM supplemented with 0.25 mg/ml G418 and 5% FBS for 10 – 15 days. Cells were rinsed with PBS and air-dried, followed by methanol fixation and staining with 0.2% crystal violet in 20% ethanol. After this, plates were washed, air-dried and scanned.

Immunohistochemistry

Xylene was used to rehydrate slides, and further with a series of alcohol dilutions and rinsed finally in a buffer (DAKO 8007). For enzyme antigen retrieval PTLINK (DAKO) was used. Slides were treated with EnVision FLEX Peroxidase-Blocking Reagent (DAKO) to block endogenous enzymes. Slides were then incubated with antibodies and rinsed, followed by addition of EnVision FLEX/HRP (DAKO). After rinsing slides, substrate-chromogen was added and incubated for 10 min. Slides were then rinsed and stained in Hematoxylin before mounting.

Statistical analyses

Graphs were generated, and statistical analysis was performed using GraphPad Prism 8.01 or the R statistical package (v. 3.5). Details on the statistical tests used in this study are reported in the respective sections and figure captions of the published papers.

Ethics statement

Written informed consent from the patient's guardians was obtained. The patient was enrolled in the Novartis compassionate use program for ceritinib (CLDK378A2003M). Treatment of patient was done following HR-NBL-1 SIOPEN protocol (permit 02-294). MPA license (LVFS 2008:1) and Clinical Ethical Board meeting supported LDK378 compassionate use. Linkage of clinical information to tumour analyses was performed according to the ethical permit 2009/1369-31/1. For the publication of this research, written informed consent was obtained from the patient's guardians.

4 RESULT AND DISCUSSION

4.1 Paper I

Brigatinib, an anaplastic lymphoma kinase inhibitor, abrogates activity and growth in ALK-positive neuroblastoma cells, Drosophila and mice.

Crizotinib was the first clinically approved TKI of ALK for the treatment of ALK-positive NSCLC after showing significant efficacy in these patients in a clinical trial (Kwak et al., 2010). However, results from the treatment of ALK-positive NB patients with crizotinib was less encouraging, with limited response observed (Mossé et al., 2013). The use of brigatinib, a second-generation ALK TKI, resulted in about 72% response rate in crizotinib refractory ALK-positive NSCLC patients in a clinical trial (Camidge et al., 2015). As a result of this promising finding, we decided to investigate the therapeutic potential of brigatinib in the NB setting.

We showed that brigatinib inhibited phosphorylation of ALK and its bona fide downstream targets, including ERK1/2, ERK5, AKT and MYCN, in a dose-dependent manner. These effects culminated in the in inhibition, by brigatinib, of growth of ALK-addicted NB cell lines both *in vitro* and in a xenograft model. Brigatinib inhibited NB cells with IC50 values about two-fold less than crizotinib, suggesting a superiority of brigatinib over crizotinib. Brigatinib treatment of ALK-wild type NB cells showed no observable effect on cell growth, demonstrating the specificity of brigatinib against ALK activity. Additionally, we observed similar superior performance of brigatinib *in vitro* using a set of diverse gain-of-function ALK mutant alleles in biochemical and neurite outgrowth assays, in which brigatinib inhibited ALK phosphorylation with IC50 of 5-35 fold less compared to that of crizotinib and robustly abrogated ALK-mediated neurite outgrowth in PC12 cells. These results suggest that brigatinib demonstrates superior inhibition profiles compared to crizotinib in the context of full-length ALK gain-of-function alleles and ALK-addicted NB cell lines.

To test the potency of brigatinib *in-vivo*, we used *Drosophila melanogaster as* a model organism in which ectopic expression of ALK mutant alleles resulted in the disruption of the eyes in all offspring, described as “rough eye” phenotype. Further, growing of fly larvae on brigatinib-containing food led to the dose-dependent rescue of the rough eye phenotype, demonstrating the ability of brigatinib to inhibit ALK activity *in-vivo*.

In summary, we show that brigatinib inhibits the activity of diverse ALK gain-of-function alleles in NB cell lines, in mice xenografts and *in vivo Drosophila melanogaster* models. Together, these results suggest brigatinib is a potent ALK inhibitor, warranting its further investigation in ALK-positive NB setting.

4.2 Paper II

Clinical response of the novel activating ALK-I1171T mutation in neuroblastoma to the ALK inhibitor ceritinib.

The occurrence of *ALK* gain-of-function driver mutations in NB tumours offers a therapeutic target in these tumours to explore with ALK TKIs (Chand et al., 2013; George et al., 2008; Pugh et al., 2013). Ceritinib is an ALK TKI approved for the treatment of ALK-positive metastatic NSCLC patients (Shaw et al., 2014). In this study, we characterized a novel *ALK-I1171T* mutant allele identified in an NB patient. Based on preclinical pharmacological inhibition profiling of the ALK-I1171T gain-of-function mutant against different ALK TKIs, ceritinib was used to treat the patient and showed dramatic response and recovery.

*Patient Presentation***:** A 16-month-old boy was diagnosed with metastatic neuroblastoma stage M (INRGSS). Initial genomic analysis revealed no *MYCN* amplification but showed numeral other unfavourable segmental chromosomal alterations. The patient received different courses of chemotherapy treatment. Chemotherapy was stopped after about three weeks due to severe bone marrow toxicity. Genomic analysis of tumour biopsy and blood sample showed inherited mutations L910F and V230I in the *FANCA* gene. Clinically, the patient displayed Fanconi anaemia features that could be explained by the *FANCA-L910F* mutation. This mutation was thought to cause a defect in DNA repair, advising against the continued use of chemo or radiation therapy. Further genomic analysis also led to the identification of a novel *ALK-I1171T* mutation in the patient tumour. Hence, treatment with ALK TKI was deliberated and preclinical investigation of the ALK-I1171T mutation carried out for the patient.

ALK mutations can be categorized as a gain-of-function ligand-independent (driver) mutation or a passenger mutation (Chand et al., 2013). Since this is the first report of I1171T mutation in full-length ALK in NB, we sought to determine whether it was a gain-of-function or passenger mutation. We observed ligand-independent phosphorylation of ALK-I1171T allele in a biochemical assay and concomitant activation of the downstream target ERK1/2. Furthermore, we found that ALK-I1171T was able to induce neurite outgrowth in the PC12 cells and potently transform NIH3T3 cells leading to a foci formation. These results suggest ALK-I1171T is a gain-of-function mutant capable of ligand-independent activation of bonafide downstream targets, and capable of transforming cells. They further imply that ALK-I1171T could be an oncogenic driver in the NB tumour being investigated.

In order to select suitable ALK TKI for use in this clinical case, we performed a pharmacological inhibition profile of ALK-I1171T mutant against ALK inhibitors ceritinib,

brigatinib, crizotinib and lorlatinib. We showed that the full-length ALK-I1171T was resistant to crizotinib but sensitive to next-generation ALK TKIs as determined in a biochemical assay. The IC_{50} of ceritinib for inhibition ALK-I1171T phosphorylation was 11-fold lower than that of crizotinib, while brigatinib and lorlatinib were about 1/28th the IC_{50} of crizotinib. These results indicate that ceritinib, brigatinib and lorlatinib represent better therapeutic choices for tumours driven by ALK-I1171T mutation. We extended our analysis and showed that ceritinib effectively abrogated the growth of ALK-addicted NB cell lines, with no observable effect on ALK-non-addicted cell lines. The lack of activity against control cell lines, i.e. ALK-non-addicted NB cell lines, by ceritinib, demonstrated the specificity of ceritinib against ALK activity.

Based on the preclinical experimental data outlined here that suggested a possibility of poor response to the first generation ALK TKI, crizotinib, and the indication for Fanconi anaemia preventing chemotherapy or radiotherapy, the patient was recruited unto the compassionate use individual patient program for ceritinib. Treatment of the patient with ceritinib resulted in tumour shrinkage, and the residual primary tumour was removed surgically after 7.5 months of ceritinib treatment. Immunohistochemical analysis of tumour sample revealed a significant reduction in cell proliferation, increased expression of markers of tumour differentiation and the tumour histologically resembled ganglioneuroblastoma which was rich in Schwannian stroma. This result suggests that oncogenic ALK signalling could promote an undifferentiated or poorly differentiated NB tumour histology, which is associated with poor prognosis. One year after surgery and 21 months after the start of ceritinib treatment, clinical evaluation of the patient revealed the absence of residual tumour in the abdomen and complete disappearance of all metastatic disease. The patient remains in complete remission 58 months after continuous treatment with ceritinib.

These results highlight the enormous potential and importance of combined extensive genetic profiling and preclinical investigation in tailoring treatment for NB patients (Figure 10). It also suggests the potential benefit of next-generation ALK TKIs, such as ceritinib, as monotherapy for ALK-positive NB tumours.

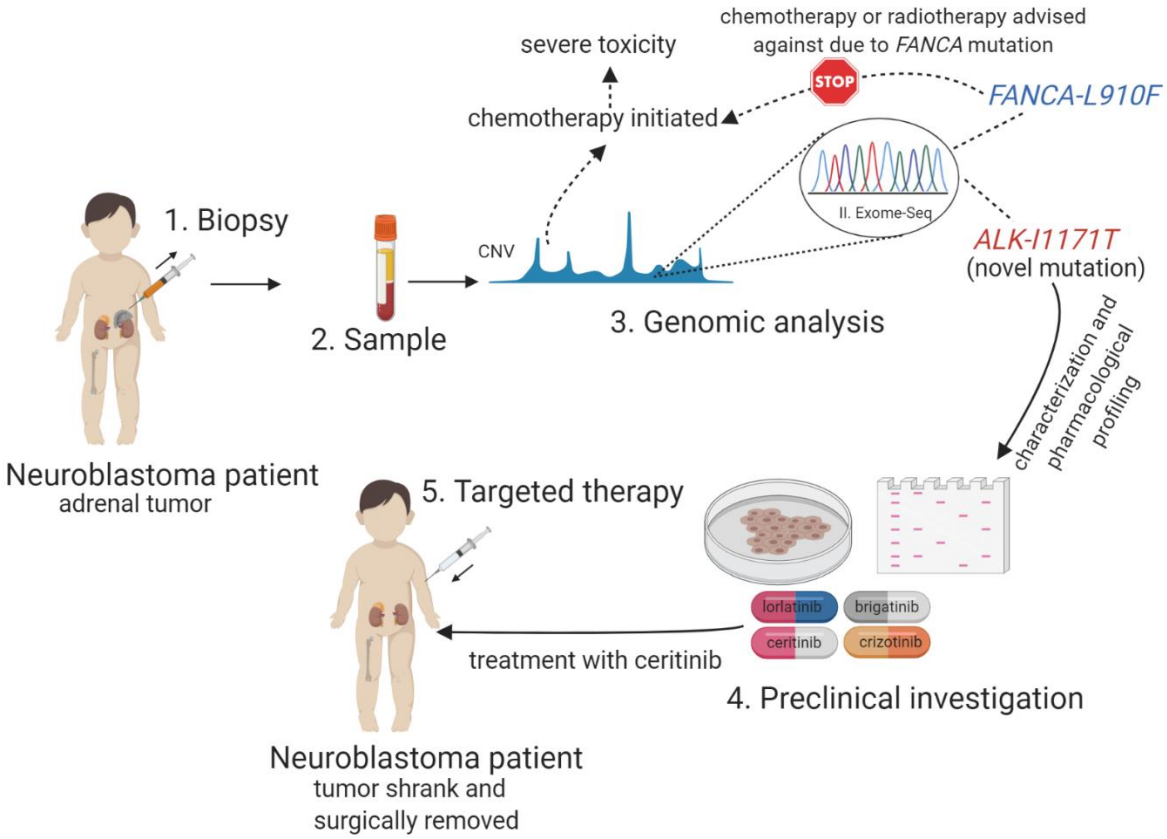

Figure 10. ***An overview of workflow showing steps leading to target identification and targeted therapy administration in neuroblastoma (NB) patient****. A tumour biopsy was taken from the patient for genomic profiling, which indicated several copy number variations (CNVs) indicative of high-risk NB. The patient was put on chemotherapy but later developed severe toxic side effects. Targeted exome sequencing (Exome-Seq) revealed FANCA-L910F and novel ALK-I1171T mutations. Chemotherapy was stopped, and radiotherapy advised against due to germline FANCA mutation. Preclinical investigation of ALK-I1171T revealed a gain-of-function mutation, which is resistant to crizotinib but sensitive to the second and third-generation ALK TKIs, ceritinib, brigatinib and lorlatinib. Treatment with ceritinib resulted in tumour shrinkage and differentiation, followed by subsequent surgical removal of residual primary tumour.*

4.3 Paper III.

11q deletion or ALK activity curbs DLG2 expression to maintain an undifferentiated state in neuroblastoma.

High-risk NB tumours are characteristically undifferentiated or poorly differentiated (Chang et al., 2020). Understanding the mechanism leading to progenitor cell differentiation blockade is essential in discovering new therapeutic approaches against NB. We also showed in Paper II that treatment of ALK-positive NB tumour with ALK TKI promoted tumour differentiation, thereby implicating ALK signalling in NB differentiation (Guan et al., 2018). A recent study by Furlan and colleagues suggested SCPs as one of the NC-derived cellular sources of NB (Furlan et al., 2017). They uncovered a transient intermediate cellular state, consisting of “bridge cells”, identifiable by a unique transcriptional pattern referred to as the “bridge” signature, in the differentiation trajectory of SCPs towards adrenal chromaffin cells (Furlan et al., 2017). We therefore hypothesized that, deregulation of genes in the “bridge cells” could affect differentiation and potentially contribute to NB development. *DLG2* is among the genes that are uniquely upregulated in the bridge cells. Herein, Paper III, we investigated a tumour suppressor role for DLG2 in NB cells.

We showed that high expression, in NB tumours, of genes that are downregulated in bridge cells strongly correlated with poor prognosis, while high expression in NB tumours of upregulated “bridge genes” correlated with better prognosis. More specifically, We found that high expression of *DLG2,* an upregulated “bridge gene”, in NB tumours is associated with better prognosis and event-free survival across four different NB data sets in R2 (http://r2.amc.nl). Importantly, *DLG2* is located on chromosome 11q that is frequently deleted in high-risk NB, and this deletion is associated with poor prognosis (Carén et al., 2010). We showed that DLG2 protein expression is almost lost in a panel of NB cell lines. Overexpression of DLG2 significantly inhibited cell proliferation *in vitro*, induced a G1 cell cycle arrest and displayed robust anti-tumour properties in mice xenograft models. These findings suggest DLG2 is a putative tumour suppressor gene in NB cells.

DLG2 is a “bridge” signature gene that is upregulated in the differentiation trajectory from SCPs to adrenal chromaffin cells (Furlan et al., 2017), suggesting a pro-differentiation role for DLG2. We found that forced expression of DLG2 spontaneously induced both biochemical and morphological differentiation of NB cell lines. Remarkably, DLG2 expression potently enhanced RA induced differentiation of NB cells. One main characteristic feature of NBs capable of spontaneous regression/maturation/differentiation is the high expression of TRKA (Hoehner et al., 1995). We found that DLG2 expression induced high TRKA expression and potentiated

NGF, the ligand of TRKA, induced differentiation of NB. In addition, we showed RA and NGF both positively regulate DLG2 expression. These results suggest that DLG2 promotes differentiation of NC-derived progenitors and might be an integral component of the differentiation machinery in NC progenitors. NB tumours have been described to consist of two cell types, namely ADRN and MES cells, which can coexist and are capable of interconversion (van Groningen et al., 2019; van Wezel et al., 2019). Enrichment of MES cells in NB tumours is highly predictive of relapse (van Groningen et al., 2017; van Wezel et al., 2019). We found that DLG2 overexpression increased ADRN genes and decreased MES genes expression in NB cells.

Mechanistically we showed that oncogenic ALK signalling transcriptionally suppressed DLG2 expression through MAPK/ERK1/2-SP1 signalling axis. In agreement, pharmacological inhibition of ALK, MEK1/2 or SP1 resulted in increased DLG2 expression (Figure 11). Interestingly, similar to DLG2 overexpression, we found that siRNA mediated knockdown of SP1 caused increased DLG2 expression with spontaneous induction of neuronal differentiation and further potentiated RA induced differentiation (Figure 11). These results suggest that ALK-ERK1/2-SP1 signalling promotes undifferentiated NB phenotype through transcriptional repression of *DLG2* expression.

Additionally, we analyzed high-risk 11q-del NB tumours and identified genetic lesions in the *DLG2* gene, located on chromosome 11q, which indicate that the *DLG2* is a target for deletion and disruption in NB tumours, thereby suggesting a tumour suppressor role for DLG2 in NB tumour initiation or progression.

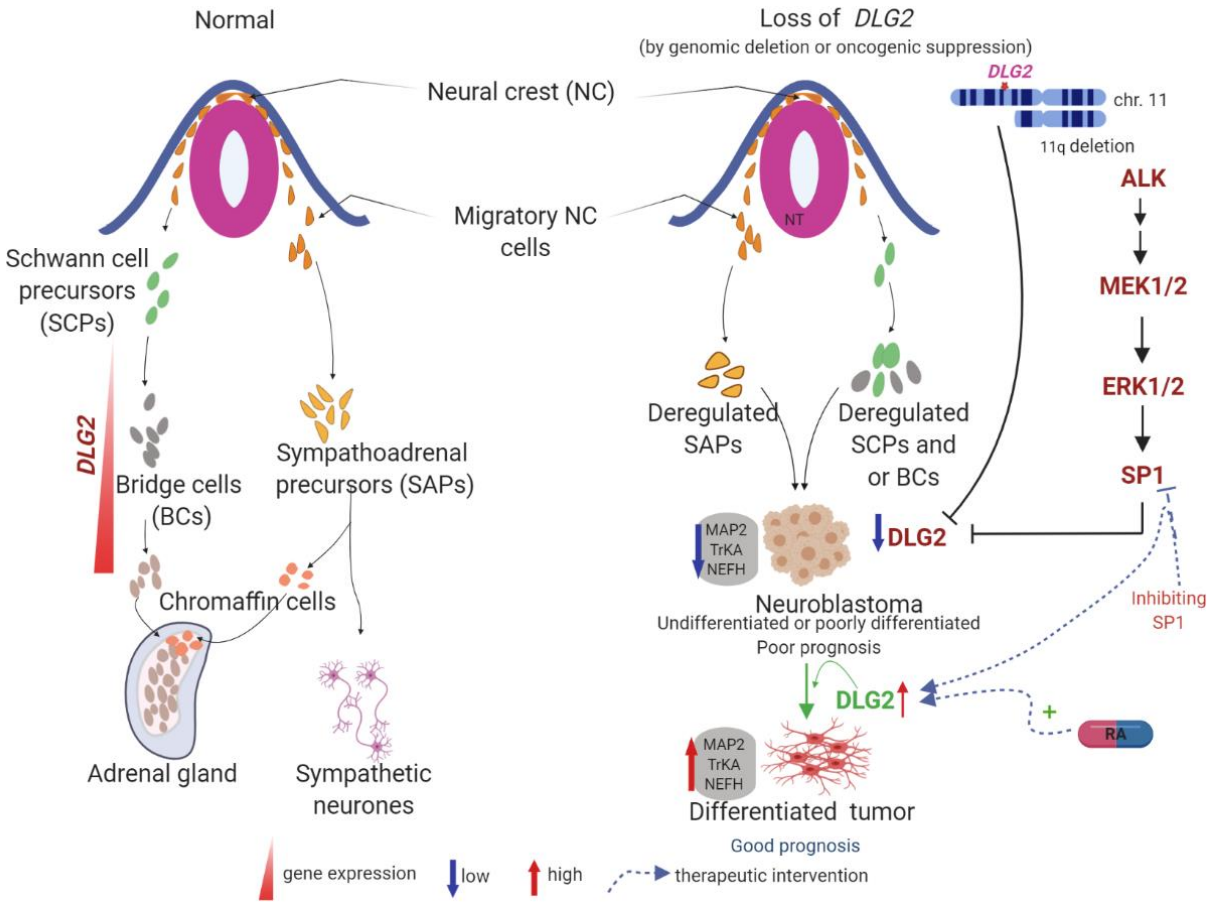

Figure 11. ***A model of the proposed role of DLG2 in embryogenesis and neuroblastoma****. (Left panel) During embryogenesis, neural crest (NC)-derived Schwann cell precursors (SCPs) or sympathoadrenal precursors (SAPs) are committed to differentiation into adrenal chromaffin cells (ChCs) and/or sympathetic neurons through an intermediary cell state called "bridge cells" (BCs), with simultaneous and progressive upregulation of DLG2 (Furlan et al., 2017). (Right panel) In NB, deletion of chromosome 11q (11q-del) leads to, in part, loss or reduction of DLG2 expression in SAPs, BCs or SAPs. These cells become deregulated, leading to the formation of undifferentiated NB tumour. Oncogenic ALK-ERK1/2-SP1 signalling also inhibits DLG2 expression in cells. Treatment of cells with retinoic acid (RA) treatment or inhibition of SP1 restores DLG2 expression and promotes NB cell differentiation. NT denotes neural tube. Figure is adapted from (Siaw et al., 2020)*

5 CONCLUSIONS

Paper I

- Brigatinib abrogated ALK activity in NB cell lines.
- Brigatinib potently inhibited NB cell growth *in vitro* and in NB mice xenografts.
- In general, we showed that brigatinib is a potent inhibitor of ALK, supporting further investigation of brigatinib as a potential therapy for ALK-positive NB.

Paper II

- We uncovered a novel *ALK-I1171T* mutation in the ALK full-length gene and *FANCA* mutations in an NB patient biopsy.
- We characterised ALK-I1171T as a gain-of-function mutation.
- ALK TKIs such brigatinib, ceritinib and lorlatinib potently inhibited ALK-I1171T activity, although it was resistant to crizotinib, a first-generation ALK TKI.
- Monotherapy with ceritinib was well tolerated and produced dramatic patient response involving tumour shrinkage, followed by removal of residual primary tumour by surgery and, ultimately, a complete clinical remission, including all metastatic sites.
- The resected primary tumour showed significant differentiation and resembled ganglioneuroblastoma.
- Our data suggest that ceritinib presents a feasible therapeutic option for ALK-positive NB patients and highlighted the importance of combining preclinical investigations with comprehensive genetic profiling in personalizing therapy.

Paper III

- “Bridge genes” are prognostic of NB patient outcome.
- DLG2 overexpression inhibited NB cell proliferation, induced and potentiated RA mediated cell differentiation, thereby acting as a tumour suppressor in NB.
- Oncogenic ALK signalling suppresses DLG2 expression through the MAPK/ERK1/2-SP1 signalling axis.
- Knockdown of SP1 induced DLG2 mediated differentiation.
- Chromosome 11q harbouring *DLG2* is hemizygously deleted in NB tumours, with genetic lesions such as breakpoints clusters, and rearrangements occurring within or in close proximity to the *DLG2* locus.
- Deletion of *DLG2* and or oncogenic ALK-ERK1/2-SP1 signalling suppression of DLG2 protein expression contributes to promote undifferentiated NB tumour histology.

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"*The journey is never ending. There's always gonna be growth, improvement, adversity; you just gotta take it all in and do what's right, continue to grow, continue to live in the moment".* Antonio Brown.

This PhD journey of mine was adventurous and exciting. One filled mostly with hope, positive expectations and the greatest of all was the experiment of starting a family right at the beginning of my PhD studies. I have no regrets thanks to the great support of my wife, **Esther Siaw**. Thank you Esther for your love and encouragement. I love you.

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