Oncogenic ALK signaling in neuroblastoma

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Gothenburg 2017

Cover illustration: Oncogenic ALK signaling events

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ISBN 978-91-629-0173-8 (PRINT)
ISBN 978-91-629-0174-5 (PDF)

Printed in Gothenburg, Sweden 2017
Ineko AB

தொட்டனைத் தூறும் மணற்கேணி மாந்தர்க்குக்

கற்றனைத் தூறும் அறிவு

To my Family and Friends

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ABSTRACT

Over the last decade Anaplastic Lymphoma Kinase (ALK), a receptor tyrosine kinase (RTK) has been identified as a translocation partner in diverse cancer types. In tumors, where the full-length ALK RTK itself is mutated, such as neuroblastoma, the picture is less clear regarding ALKs role as an oncogenic driver. Neuroblastoma is a heterogeneous disease of the sympathetic nervous system, accounting for 10-15% of all childhood cancer deaths. A number of small tyrosine kinase inhibitors (TKIs) have been developed to inhibit ALK activity. The data acquired thus far suggests that ALK TKI mono-treatment may not be as effective solution for ALK positive neuroblastoma patients. Therefore, there is a need for combination therapy using drugs towards different targets or signaling pathways to combat the disease. The overall aim of this thesis is to identify targets in signaling pathways that can be inhibited by specific drugs, as a potential poly-therapy treatment strategy in ALK positive neuroblastoma patients.

Using an MS-based phosphor-proteomics approach, we identified STAT3 as a potential downstream target of oncogenic ALK signaling (Paper I). ALK activation of STAT3 results in increased phosphorylation of STAT3 in PC12 cells expressing a gain-of-function ALK mutation. Pharmacologic inhibition of STAT3 using FLLL32 and STATTIC resulted in decreased phosphorylation levels of STAT3 and *MYCN* protein and mRNA levels. This study identified STAT3 as a target of ALK signaling and showed that inhibition of STAT3 using FLLL32 and STATTIC decreases proliferation of neuroblastoma cell lines and regulates the transcription of *MYCN*.

In a subsequent paper, we identified ERK5 as a potential ‘druggable’ target for ALK positive neuroblastoma patients (Paper II). Inhibition of ERK5 activity,

reduced proliferation of ALK positive neuroblastoma cells as well as *MYCN* mRNA levels. Combination of ALK and ERK5 inhibitors abrogated tumor growth and cell proliferation synergistically. Overall, this study showed that ALK activates ERK5 via the PI3K pathway and regulates *MYCN* transcriptionally, suggesting that targeting both ALK and ERK5 might be beneficial for ALK positive neuroblastoma patients.

In paper III, we addressed whether MEK inhibition alone or in combination with ALK inhibitor(s) has therapeutic value in a large panel of neuroblastoma cell lines. MEK inhibition alone in ALK positive neuroblastoma cells or xenografts did not abrogate cell or tumor growth. We showed that pharmacological inhibition of MEK-ERK pathway in ALK-positive neuroblastoma cells results in increased levels of activation/phosphorylation of AKT and ERK5. This feedback response is regulated by the mTOR complex 2 protein SIN1. Our results contraindicate the use of MEK inhibitors as effective therapeutic strategy in ALK-positive neuroblastoma.

Together, this study highlights the importance of full length ALK receptor signaling in neuroblastoma. Further, it shows that combination of ALK inhibitor with PI3K/Akt/mTOR/ERK5 pathway inhibitors might be a potential therapeutic treatment strategy for ALK positive neuroblastoma patients.

Keywords: Neuroblastoma, Anaplastic Lymphoma Kinase, Akt, ERK5, mTOR, MYCN

ISBN: 978-91-629-0173-8 (PRINT)
ISBN: 978-91-629-0174-5 (PDF)

SAMMANFATTNING PÅ SVENSKA

Anaplastiskt Lymfom Kinas (ALK) är en receptor tyrosin kinas (RTK) och har identifierats som en translokationspartner i flera olika cancertyper. Mutationer i fullängds ALK blivit också identifierade i neuroblastom. Neuroblastom är en heterogen sjukdom som uppstår från det sympatiska nervsystemet. Baksidan med neuroblastom är att det står för 10-15% av alla pediatriska dödsfall i väst världen och att ALK är muterat i upp till 10% av dessa fall. Ett antal små hämmare har utvecklats för att inhibera ALK aktiviteten, såkallade tyrosin kinas inhibitorer (TKI). Kliniska prövningar har visat och tyder på att mono-behandling med ALK inhibitorer mot ALK-positiva neuroblastom patienter inte är den optimala behandlingsmetoden. Därför finns det ett behov av att utveckla kombinationer av specifika läkemedel för att behandla ALK positiv neuroblastom. Det övergripande syftet med avhandlingen är att identifiera signalvägar som kan inhiberas parallellt med ALK inhibition för att kunna utveckla en potentiell strategi för kombinations terapi av ALK positiva neuroblastom patienter.

Vi har att använda en MS-baserad fosfor-proteomik strategi, här identifierade vi STAT3 som ett potentiellt effektor av ALK-signalering (Paper I). ALK aktivering av STAT3 resulterade i ökad fosforylering av STAT3 i PC12-celler som uttrycker en aktiverande ALK mutation. Farmakologisk hämning av STAT3 med specifika STAT3 inhibitorer medförde minskad aktivering av STAT3 och reducerad uttrycksnivåerna av nedströms liggande målproteiner, likt MYCN. Denna studie visade ett samband mellan ALK-aktivitet och STAT3 fosforylering, och att inhibition av STAT3 resulterade i minskad tillväxt av neuroblastomcellinjer och reglering av MYCN transkription.

Vi observerade att proteinet ERK5, ett nedströms målprotein efter ALK aktivering, är ett möjligt proteins att inhibera i ALK-positiva neuroblastom patienter (Paper II). Hämning av ERK5 aktivitet med hjälp av specifik inhibitor, XMD8-92, minskade tillväxten av ALK positiva neuroblastomcell linjer och även MYCN mRNA-nivåer, en verifierad onkgen och prognostisk faktor i neuroblastom. En kombination av ALK-hämmare och ERK5-hämmare hindrar både tumör- och celltillväxt synergistiskt. Studien visade att ALK aktiverar ERK5 via PI3K och reglerar MYCN transkription, vilket tyder på att inhibitorer riktade både mot ALK och ERK5 kan vara fördelaktigt för ALK positiv neuroblastom patienter.

Vidare observerade vi att inhibering av proteinet MEK, antingen ensam eller i kombination med ALK inhibitorer, har ett terapeutiskt värde i en

neuroblastomcellinjer med en aktiverad RAS-MAPK signalering. Däremot, farmakologisk inhibering av endast MEK stoppar inte tillväxt av ALK positiva neuroblastomcellinjer eller xenograft transplanterande tumörer. Vi fann att inhibering av MEK-ERK signalleringsvägen i ALK-positiva neuroblastomceller resulterar i ökad aktivering/fosforylering av proteinerna AKT och ERK5. Detta återkopplingssvar regleras av mTOR-komplexet 2 protein SIN1. Våra resultat i preklinisk miljö visar att användning av MEK-hämmare inte är en effektiv terapeutisk behandlingsstrategi i ALK-positiv neuroblastom.

Avhandlingen visar att ERK5 är ett målprotein för ALK aktivering och styr avläsningen/transkriptionen av onkgenen MYCN. Vidare visar avhandlingen att kombinationen av ALK-hämmare med hämmare av PI3K/Akt/mTOR/ERK5 signalvägar kan vara en potentiellt terapeutiskt behandlingsstrategi för ALK positiva neuroblastom patienter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals. Articles are re-printed with permission from publishers.

I. Phosphoproteomic analysis of anaplastic lymphoma kinase (ALK) downstream signaling pathways identifies signal transducer and activator of transcription 3 as a functional target of activated ALK in neuroblastoma cells.
Sattu K, Hochgräfe F, Wu J, **Umapathy G**, Schönherr C, Ruuth K, Chand D, Witek B, Fuchs J, Li PK, Hugosson F, Daly RJ, Palmer RH, Hallberg B. FEBS J. 2013 Nov;280(21):5269-82. doi: 10.1111/febs.12453.

II. The kinase ALK stimulates the kinase ERK5 to promote the expression of the oncogene MYCN in neuroblastoma.
Umapathy G, El Wakil A, Witek B, Chesler L, Danielson L, Deng X, Gray NS, Johansson M, Kvarnbrink S, Ruuth K, Schönherr C, Palmer RH, Hallberg B. Sci Signal. 2014 Oct 28;7(349):ra102. doi: 10.1126/scisignal.2005470.

III. Anaplastic lymphoma kinase addictive neuroblastoma cell lines are associated with growth upon treatment with MEK inhibitor trametinib.
Umapathy G, Gustafsson DE, Javanmardi N, Madrid DC, Martinsson T, Palmer RH, Hallberg B. (Manuscript)

Additional publications not included in this thesis:

FAM150A and FAM150B are activating ligands for anaplastic lymphoma kinase.
Guan J[#], **Umapathy G[#]**, Yamazaki Y, Wolfstetter G, Mendoza P, Pfeifer K, Mohammed A, Hugosson F, Zhang H, Hsu AW, Halenbeck R, Hallberg B, Palmer RH. Elife. 2015 Sep 29;4:e09811. doi: 10.7554/eLife.09811. [#] These authors contributed equally.

The ALK inhibitor PF-06463922 is effective as a single agent in neuroblastoma driven by expression of ALK and MYCN.
Guan J, Tucker ER, Wan H, Chand D, Danielson LS, Ruuth K, El Wakil A, Witek B, Jamin Y, **Umapathy G**, Robinson SP, Johnson TW, Smeal T, Martinsson T, Chesler L, Palmer RH, Hallberg B. Dis Model Mech. 2016 Sep 1;9(9):941-52. doi: 10.1242/dmm.024448.

CONTENTS

ABBREVIATIONS

ALCL= Anaplastic large cell lymphoma

ALK= Anaplastic lymphoma kinase

ALKAL1/2= ALK and LTK ligand 1/2

ATC= Anaplastic thyroid tumor

ATP= Adenosine triphosphate

BRAF= v=raf murine sarcoma viral oncogene homolog= B

CHEK2= checkpoint kinase 2

CML= Chronic myelogenous leukemia

CNS = Central nervous system

Dpp= Decapentaplegic

EGFR= Epidermal growth factor receptor

EML4= echinoderm microtubule=associated protein=like 4

FAM150= Family with sequence similarity 150

FDA= Food and Drug Administration

GISTs= Gastrointestinal tumors

HEN=1= hesitation=1

IGF1R = Insulin=like growth factor=1 receptor

IR = Insulin receptor

Jeb= Jelly Belly

LDLa= Low density lipoprotein class A

LTK= Leukocyte tyrosine kinase

MAM= Meprin A=5 protein and receptor protein tyrosine phosphatse Mu

mTOR= mammalian target of rapamycin

NGF= Nerve growth factor

NPM= Nucleophosmin

NSCLC= Non small cell lung cancer

PC12= Pheochromocytoma 12

RAS= Rat sarcoma

RCC= Renal cell carcinoma

RT=PCR= Reverse transcriptase PCR

SCF= Stem cell factor

TGFβ= Transforming growth factorβ

TKD= Tyrosine kinase domain

TKI= Tyrosine kinase inhibitor

Trk A/B= Tropomyosin receptor kinase A/B

VASP= Vasodilator=stimulated phosphoprotein

1 INTRODUCTION

1.1 General features of cancer

Several centuries of cancer research have generated a complex body of information concerning the cancer disease, revealing it to be a disease associated with aggressive changes in the genome [1, 2]. This led to the discovery of two gene classes: Oncogenes and tumor suppressor genes. In general, oncogenes promote cancer when they have acquired dominant gain of function mutations, while tumor suppressor genes are associated with cancer in a recessive manner when they become non-functional [1, 2].

1.1.1 Oncogenes

Most cells in our body will be subjected to programmed cell death when their normal functions are modified [3]. However, in the presence of activated oncogenic signals, these cells grow and ultimately cause cancer (Figure 1) [4]. The first oncogene was identified more than forty-five years ago by several generations of cell and molecular biologists [5-10]. They discovered a filterable agent in chicken as Rous sarcoma virus (RSV) which has a transforming potential due to acquisition of a normal cellular gene named *c-src* [5-10]. Today, several hundred oncogenes have been discovered.

Proto-oncogenes generally code for different proteins essential for regulation of cellular growth and differentiation [1, 2, 4, 11-14]. A proto-oncogene can assume oncogenic function in one of the following ways:

(i). Point mutations acquired within a proto-oncogene itself, leading to conformational changes in the encoded protein (e.g. NRAS, HRAS, KRAS)

(ii). Gene amplification that leads to increased levels of encoded proteins (e.g. MYCN, EGFR, ERBB)

(iii). Chromosomal translocation, where fusion of a proto-oncogene with another gene to form a fusion protein results in enhanced oncogenic activity (e.g. BCR-ABL, NPM-ALK, EML4-ALK)

A well-known oncogene that can be activated by point mutation is RAS. The RAS oncogene family consists of three members: H-RAS, K-RAS, and N-RAS [15-17]. To date, RAS family members are one of the most mutated oncogenes

found in human tumors and account for around 20 to 30% [18-21]. All RAS proteins are part of the small GTPase class of proteins, which act as molecular switches controlling the intracellular signaling axis [16, 22, 23]. When RAS switched conformation to an active state, it is bound to guanosine triphosphate (GTP), whereas in the inactive state, RAS is bound to guanosine diphosphate (GDP). Switching is mediated through GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF) [24]. In an active conformation, RAS binds to RAF family kinases and signals via its downstream effectors such as MEK and ERK pathway to determine the fate of a cell [23, 25-27]. Altogether, targeting RAS-MAPK would be a potential therapeutic strategy in several cancer types [28-31], although this probably depends on the cancer cell type [32], Paper III.

1.1.2 Tumor Suppressor Genes

Since tumorigenesis is a multistep process, activation of an oncogene alone might not be sufficient for transformation to cancer cells. Combination of several other changes in genome together influence the development of human tumors [23]. Tumor suppressor genes protect the normal cells from transforming into a cancerous cell (Figure 1) [23]. These genes often encode proteins that promote apoptosis and/or regulate the cell cycle [23]. Mutations in these tumor suppressor genes that lead to loss of function promotes tumor development [23].

Retinoblastoma protein (RB or pRB) was the first tumor suppressor gene identified in retinoblastoma, a rare childhood eye tumor [33-35]. In 1971 Alfred Knudson suggested that loss of a single RB copy alone was not sufficient for tumor development, and that loss of both copies of the RB gene is required for the development of retinoblastoma (the 'two-hit hypothesis') [33]. The RB tumor suppressor gene is deregulated indirectly by upstream activators in several cancer types like, lung, breast, melanoma, head, and neck cancers [36, 37]. Research on RB as a tumor suppressor gene led to the discovery of several other tumor suppressor genes.

The tumor protein p53 (TP53 in human and Trp in mice) is the second tumor suppressor gene that was identified [36, 38, 39]. In normal cells, p53 is inactivated by forming a complex with MDM2, an E3 ubiquitin ligase. Upon DNA damage, hypoxia, cell cycle deregulation, oncogene activation, or other stress activators, p53 dissociates from the p53-MDM2 complex and induces apoptosis or cell cycle arrest [36, 38, 39]. p53 has been well studied in colon cancer, where 70 to 80% of the cases follow the 'two-hit hypothesis', resulting in loss of both p53 alleles [38]. Mutations in the p53 tumor suppressor gene

are the most common genetic modification observed in human cancers [38], implying that targeting p53 may be a potential therapeutic strategy in several cancer types. In 1999, Komarov and co-workers identified the first small molecule that inhibits p53-induced transcription and protects the mice from severe damage of ionizing radiation [40]. Restoration of p53 in tumors lacking p53 was challenging, however it has been achieved by genetic as well as pharmacological methods [41, 42]. In 1999, Foster and co-workers identified compounds that reactivate p53 and display an antitumor activity in mice by restoring its transcriptional activity [43]. The current trend of restoring p53 activity includes the targeting of p53 targets such as CDK family members, MDM2, or RAS-MAPK pathway components [42, 44, 45]

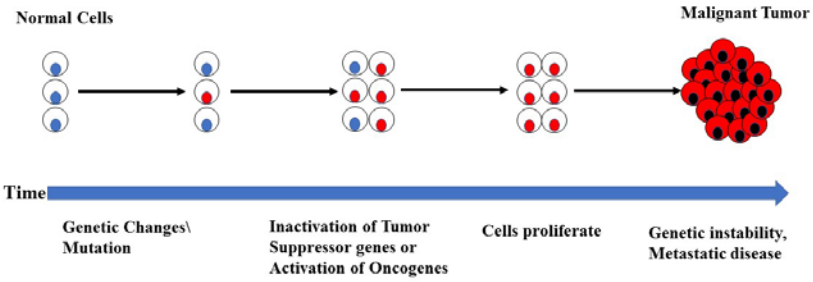


Figure 1- Steps involved in malignant transformation: *Basic steps involved the development of a normal cell into a malignant tumor are shown here.*

1.1.3 Post Translational Modifications (PTMs)

Post Translational Modification (PTMs) plays a central role in cancer progression and as a result, PTMs are of great interest as cancer therapeutic targets [46]. Protein biosynthesis is a multi-stage process, were a cell builds up a protein product. During biosynthesis or after, proteins may undergo several enzymatic modifications to form the mature protein [47] [48]. PTMs can occur on both C- and N- terminal region of the protein and exist in large numbers. The most well studied PTMs are shown in Figure 2, and include:

Methylation- Protein methylation is the addition of a methyl group (CH_3) to a lysine or arginine amino acid residues using specific methyltransferases [49]. Methylation has been widely studied in histone modifications and these modifications repress or activate gene expression.

Acetylation- Protein acetylation is the addition of an acetyl group (CH_3 CO) to a lysine amino acid residue or to the N-terminal region of the protein [50]. N-terminal acetylation plays a key role in protein stability, localization, protein metabolism and biosynthesis. Whereas, histone lysine acetylation plays a vital role in regulation of gene expression. Acetylation of non-histone proteins like STAT3 and p53 has been implicated in several cellular processes such as DNA repair, cell cycle regulation, mRNA stability, and apoptosis [51-53]. Deregulation of these cellular processes plays a vital role in cancer progression.

Glycosylation- Protein glycosylation is the addition of carbohydrate groups to serine, threonine or asparagine amino acid residues, forming a glycoprotein. N-linked glycosylation is the most common form of glycosylation and is important in protein folding and cellular attachment. Several studies have indicated that modifications in cell surface glycosylation can promote tumorigenesis [54-59].

Ubiquitination- Protein ubiquitination is the addition of ubiquitin to lysine amino acid residues of a substrate protein. Ubiquitination controls the substrate protein function, for example by preventing or inducing protein-protein interactions or affecting protein activity by regulating their cellular localization and degradation [60-62].

Phosphorylation- Protein phosphorylation is the addition of a phosphate group (PO_4) to serine, threonine, tyrosine or histidine amino acid residues. Addition of phosphate groups (phosphorylation) to proteins is facilitated by kinases, and removal of phosphate groups (dephosphorylation) from the proteins is facilitated by phosphatases. Phosphorylation and dephosphorylation play important roles in several cellular processes like metabolism, cell movement, cell growth, apoptosis, and signal transduction [46, 63-66]. Thus, any deregulation in protein phosphorylation process is likely to drive oncogenesis. Therefore, targeting receptor tyrosine kinases has become popular in recent years and several tyrosine kinase inhibitors or serine/threonine kinase inhibitors are now approved by FDA for treatment of different cancer types [66-72].

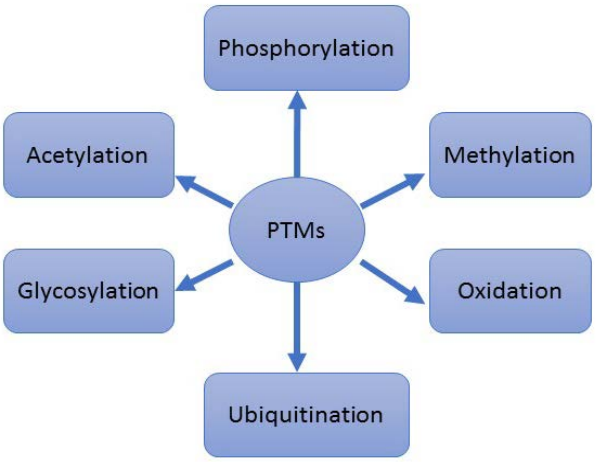


Figure 2- Post translational modification: *A pictorial representation of the most important post translational modifications involved in cancer.*

1.2 Receptor Tyrosine Kinase superfamily

Protein tyrosine kinases are enzymes that facilitate phosphoryl transfer from a high-energy donor molecule to tyrosine residues of a substrate protein [73, 74]. The tyrosine kinase superfamily of ninety members is subdivided into two classes: (i) 58 Receptor tyrosine kinases (RTKs) and (ii) 32 Non-receptor tyrosine kinases [65, 75]. The RTK superfamily is further subdivided into 20 sub-families [76]. RTKs generally share a common domain architecture: an extracellular domain that contains a ligand binding region, a transmembrane domain and an intracellular kinase domain [76-80]. The general paradigm of receptor activation includes four main events: (1) ligand binding, (2) ligand-induced receptor dimerization, (3) tyrosine auto phosphorylation and (4) activation of signaling proteins (Figure 3) [76, 79, 81]. RTKs are the key regulators of numerous critical cellular processes such as proliferation, survival, differentiation, migration, and metabolism (Figure 3) [82, 83]. As deregulation of RTK activity- due to chromosomal translocation, overexpression or gain-of-function mutations in the kinases contributes to tumorigenesis [82], targeting oncogenic kinase signaling is an attractive option in the field of cancer- targeted therapy.

Gleevec/Imatinib was the first tyrosine kinase inhibitor (TKI) approved by FDA in 2001 for the treatment of chronic myelogenous leukemia (CML), where it blocks the activity of Abl non-receptor tyrosine kinase [84]. Seven

years later, Gleevec was approved by FDA for use in patients with KIT receptor positive gastrointestinal stromal tumors (GISTs) [85]. Based on this, several other TKIs entered the pharmaceutical market, including gefitinib, erlotinib, lapatinib crizotinib, semaxinib, afatinib and sunitinib [86-94]. The modes of action of these TKIs are based on four different mechanisms. They either 1) compete with high-energy donor molecules such as ATP, 2) compete with the kinase substrate, 3) compete with both or 4) act in an allosteric manner [95]. Overall, TKIs are an important class of drugs for targeted therapy to inhibit specific malignancies.

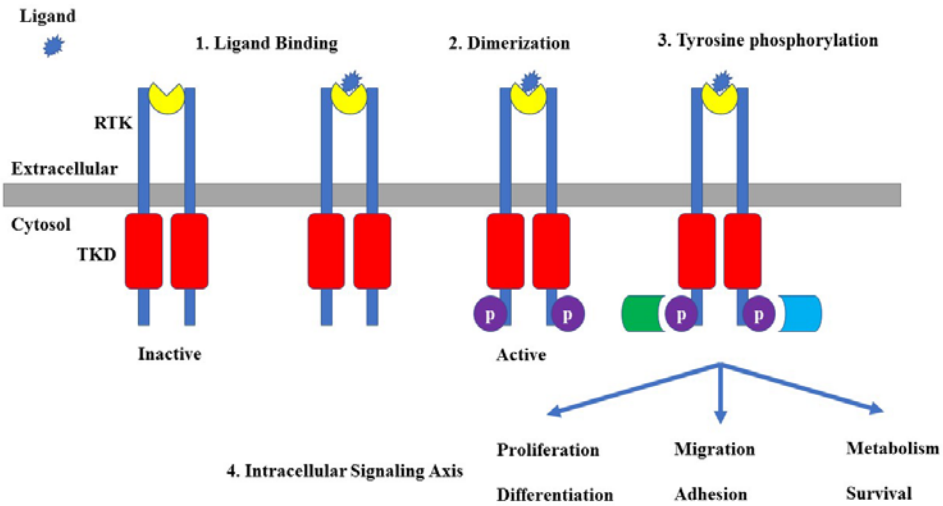


Figure 3- Activation of receptor tyrosine kinases: *The inactive receptor tyrosine kinase encounters a signaling molecule (ligand). Upon ligand binding the receptor dimerizes (active state) which leads to tyrosine auto- phosphorylation. In turn, tyrosine phosphorylation results in the recruitment of other signaling molecules that determine the fate of the cell.*

1.3 The RTK- Anaplastic Lymphoma Kinase (ALK)

Anaplastic Lymphoma Kinase (ALK) was first described in 1994 as a fusion partner with nucleophosmin (NPM) in Anaplastic large cell lymphoma (ALCL), from which the ALK name was derived [96]. The chromosomal rearrangements occur between the chromosomes 2p23 ALK: 5q35 NPM, where the region encoding the kinase domain of ALK is linked to the N-terminal coding region of NPM [96, 97]. The full length ALK receptor was first described in 1997 by two independent groups. It consists of an extracellular domain, a transmembrane domain, and an intracellular kinase domain [98, 99]. ALK shares a high sequence similarity with the Insulin receptor (IR) super family and it also shares almost 50% protein sequence similarity with leucocyte tyrosine kinase (LTK). Together, ALK/LTK form a unique subgroup under the IR superfamily [98, 99]. Human ALK is 1620 amino acids, encoding a protein of approximately 180kDa. However, post translational modifications like N-linked glycosylation results in the full length ALK being detected at 220kDa in SDS-PAGE [99].

1.3.1 Structure of ALK

Like other RTKs, ALK consists of an extracellular ligand binding domain, a transmembrane domain and an intracellular kinase domain (Figure 4, 5).

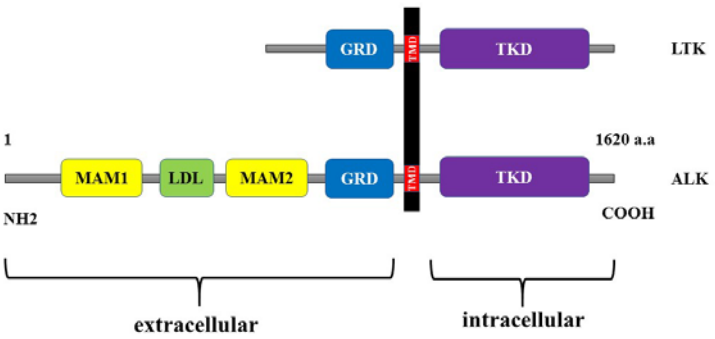


Figure 4- Domain structure of human LTK and ALK: *The extracellular region of human ALK contains two MAM domains (264-427 a.a and 480-626 a.a), an LDLa domain (453-471 a.a) and a glycine rich domain (816-940 a.a). A transmembrane domain (1031-1057 a.a) links the extracellular region with intercellular region containing the tyrosine kinase domain (1116-1383 a.a). On the top, leucocyte tyrosine kinase (LTK) domain structure is shown.*

ALK extracellular domain

The extracellular region of ALK consists of two MAM (Meprin, A-5 protein and receptor protein tyrosine phosphatase Mu) domains, an LDLa (Low density lipoprotein class A) domain, and a glycine rich domain (GRD) [98-101]. The functions of the ALK extracellular domain are still unclear. However, it has been speculated that these domains might be involved in ligand assembly, interaction with substrates (co-receptors), dimerization and proteolytic cleavage [102],[103]. Future studies should uncover the importance of these domains for keeping the ALK receptor in a stable or quiescent form.

ALK intracellular domain

Like other kinases, the kinase domain of ALK consists of a conserved small N-terminal lobe and a large C-terminal lobe [104-106]. The N-terminal lobe consists of five stranded antiparallel β-sheets and a regulatory αC-helix which is important for catalysis. The large C- terminal lobe is mainly helical and contains the activation loop (A-loop). The N- and C-terminal lobes are linked by a hinge region which forms a cleft for ATP or substrate binding (Figure 5) [104, 106]. Furthermore, Kornev and colleagues showed that protein kinases contain two hydrophobic motifs, termed regulatory (R-spine) and catalytic (C-spine) spines [105]. Both spines are conserved across all kinases and contain residues from both the N- and C- lobes. The R-spine is vital in determining the active and inactive conformations of the ALK kinase. The regulatory-spine consists of the hydrophobic residues namely I1171, C1182, H1247, F1271, and D1311 in ALK. The C-spine regulates catalysis by governing ATP binding. The C-spine consists of residues V1130, A1148, L1256, C1255, L1257, L1204, L1318, I1322 in ALK [102, 105, 107, 108]. The A-loop in the C-terminal lobe contains an autophosphorylation motif YxxxYY similar to that of the IR super family (Figure 5). However, in the IR the second tyrosine is first phosphorylated followed by the first and third. In contrast, in the case of ALK fusion oncogenes it has been suggested that Y1278 in ALK is the first tyrosine to be phosphorylated followed by the second (Y1282) and third (Y1283) [104, 106]. Furthermore, it has been described that Y1278 is vital for maintaining the quiescent form of ALK by hydrogen bonding with the C1097 residue in the N-terminal β-turn motif [104, 106]. Thus, conformational changes in this inhibitory structural feature can potentially release ALK from its quiescent conformation [104, 106].

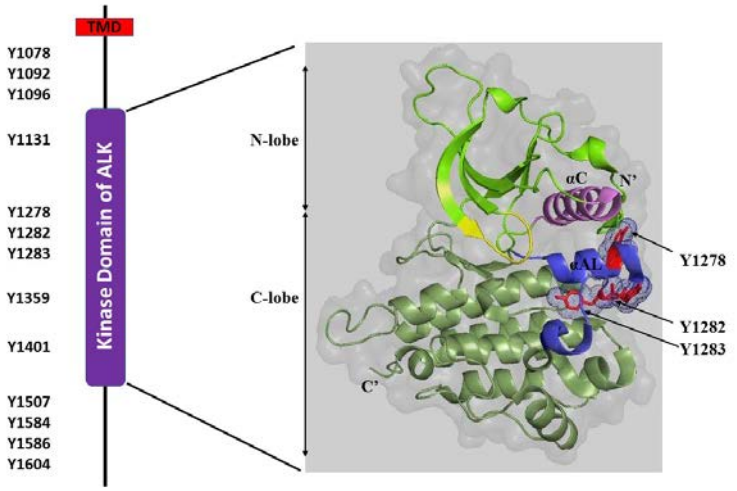


Figure 5- Kinase domain of ALK: *On the left, possible tyrosine phosphorylation sites in the tyrosine kinase domain of ALK are indicated. On the right, crystal structure of ALK kinase domain (PDB: 3LCT) is shown. The kinase domain of ALK contains a smaller N-terminal lobe and a larger C-terminal lobe. The smaller N-terminal lobe (1093-1199 a.a) contains a major αC helix (magenta), the glycine loop (yellow) and five anti-parallel β-sheets (green). The larger C-terminal lobe (1200-1399 a.a) is largely helical which consists of activation loop (αAL- shown in blue). The activation loop includes three auto-phosphorylation sites (Y1278, Y1282, and Y1283- shown in red).*

1.3.2 ALK in model organisms

Drosophila melanogaster DAlk

The physiological function of ALK has been thoroughly studied in the *Drosophila* model system. Like mammalian ALK, DAlk also contains several putative domains, an extracellular ligand binding domain, a transmembrane domain and an intracellular kinase domain, of which kinase domain of DAlk shares high sequence similarity with IR superfamily [100]. Lorén and colleagues have shown that ALK mRNA is mainly distributed in CNS and visceral muscles of *Drosophila melanogaster* [100]. Further they have shown that loss of function *Drosophila Alk* mutants resulted in gut-less phenotype [109]. Overall DAlk plays an important role in formation of visceral musculature of the gut during early embryogenesis [100] [109]. Jelly Belly

(Jeb) is the ligand for *Drosophila* Alk. The *Jeb* gene encodes a secreted protein containing a LDL receptor motif which mediates its binding to DAlk and activates DAlk in the visceral muscle founder cells, leading to the activation of ERK signaling [109-112]. ERK activation mediated by Jeb/DAlk regulates Duf (dumb-founded)/ Kirre (kin of irregular chiasm), Dpp (decapentalegic), Hand and Org-1 (Optomotor-blind-related-gene-1) transcriptionally [110-114]. DAlk also plays a role in the development of embryonic endoderm by regulating the transcription of Dpp (homolog of mammalian TGF-β) [114]. Two independent studies have shown that Jeb/DAlk signaling plays an important role in the visual system of the fruit fly and synaptic connectivity in developing motor circuits [115, 116]. Further in 2011, Cheng and colleagues reported that DAlk protects neuroblast growth in starvation conditions via the PI3K/Akt pathway [117]. In the same year Gouzi and colleagues showed that DAlk signaling plays a vital role in body weight determination and associative learning in *Drosophila* by controlling neurofibromin 1 [118]. Recently, it has been shown that DAlk signaling acts in the *Drosophila* mushroom body and negatively regulates sleep [119].

Caenorhabditis elegans SCD-2

In *C.elegans* SCD-2 (suppressor of constitutive dauer formation) is an ALK homologue which plays a critical role in dauer formation, which is a developmentally arrested third larval stage in *C.elegans* [120-122]. SCD-2 was first described as a suppressor in a screen of TGF-β signaling mutants which led to constitutive dauer pattern, from which SCD name was derived [121]. Hen-1 is the ligand for ALK homologue SCD-2 in *C-elegans*, which lacks a mammalian ortholog. Hen-1 gene encodes a secreted protein with similarities to *Drosophila* Jeb containing an LDL receptor repeat [123]. Hen-1 plays a vital role in sensory integration and behavioural plasticity [123]. To show that SCD-2 and Hen-1 function in same genetic pathway, Shinkai and colleagues created a double mutants *scd-2;hen-1* and showed that double mutants exhibit a similar phenotype as each of the single mutants [124].

Danio rerio DrAlk/DrLtk

The zebrafish *Danio rerio* has two members of the ALK family (DrAlk and DrLtk) [125-127]. In zebrafish, DrAlk is found to be highly expressed in the developing central nervous system [125]. Inhibition of DrAlk in this organism resulted in severe complications in neuronal differentiation and neuron survival in the CNS without affecting the neuron progenitor formation [125]. However, inhibition of DrLtk resulted in a failure to establish iridophores (pigment cells that arise from the neural crest) [127]. As yet no ligand has been

reported to activate ALK signaling in zebrafish. Recently, zebrafish has been employed as a transgenic model system for neuroblastoma pathogenesis, a solid extracranial childhood cancer [128].

Mammalian ALK

In mammals, the biological function of ALK is not well known. However, by comparing with other model organisms, it has been suggested that ALK might play a role in development of the mammalian nervous system. In 1997, Iwahara and colleagues showed that ALK mRNA is expressed mainly in the brain and spinal cord of the mouse [98]. Furthermore, using RNA *in situ* hybridization, they indicated that ALK mRNA is expressed in different parts of nervous system, such as the olfactory bulb, thalamus, and ganglia of embryonic and neonatal mice [98]. Vernersson and colleagues have shown that mALK mRNA and protein expression overlap in specific regions of central and peripheral nervous systems [129]. Pulford and colleagues have shown that human ALK is expressed in tissue samples of adult human CNS, consistent with expression patterns of mouse and *Drosophila melanogaster* [130]. However, the role of ALK in mammals is still unclear. Several studies have indicated that ALK or ALK/LTK knockout mice are viable and do not show any major altered phenotype [131-133]. However, a recent study reported that ALK knockout males had low testosterone levels in serum and a mild disorder of seminiferous tubules, indicating a role of ALK in testis development and function [134]. Interestingly, treatment with crizotinib an FDA approved ALK inhibitor in metastatic NSCLC patients resulted in low testosterone levels [135, 136].

The ligand for human ALK had remained a mystery until 2015. Two small basic proteins, FAM150A and FAM150B, were recently identified as potential ligands for LTK [137]. Further, they have shown that binding of both FAM150A and FAM150B in the ECD of LTK, stimulated the receptor activation, and activated ERK downstream signaling [137]. LTK shares high sequence similarity with ALK and like ALK, it has a unique structural region in the membrane proximal region called the glycine-rich domain (GRD). It was also reported that mutations in the glycine-rich domain, led to an inactive receptor *in vivo* [110]. Given these similarities, in 2015 Guan and colleagues were the first to report that FAM150A and FAM150B are potential ligands for human ALK [138]. This was further supported by Reshetnyak and colleagues studies which showed that FAM150A (AUGβ) and FAM150B (AUGα) are potential ligands for human ALK [139]. Recently, the HUGO gene nomenclature committee (HGNC) have designated the ligands as ALKAL1 (FAM150A) and ALKAL2 (FAM150B) [140]. Several previous studies have reported that the heparin binding molecules Pleiotrophin (PTN), Midkine

(MK) and heparin itself are ligands for mammalian ALK [101, 141-145]. However, subsequent studies have reported that these molecules might not have a role in ALK activation [103, 146-148]. Guan and colleagues also reported that both FAM150A and FAM150B stimulate ALK signaling in neuroblastoma cells and that co-expression of both FAM150 proteins with human ALK, was able to drive human ALK activation in *Drosophila melanogaster*. Furthermore, they showed that these molecules bind to the ECD of ALK and enhance ALK activity in ALK positive neuroblastoma cells [138]. Taken together, the recently described potent ligands of human ALK might have significance in human cancers with ALK overexpression, however their role in other ALK positive cancers remains a crucial question.

1.4 Oncogenic ALK signaling core

Several ALK downstream signaling have been described, however most studies are from ALK fusion forms like NPM-ALK and EML4-ALK rather than from the mutated full length receptor. Understanding both types of signaling events will be beneficial for ALK positive targeted therapies.

RAS-MAPK

The RAS-family is the small GTPase class of proteins that control the activity of many signaling pathways of which the Mitogen Activated Protein Kinase (MAPK) plays a vital role in tumorigenesis [149-153]. Activated RAS translocate Raf-1 to the plasma membrane (active form) and activates MAPK kinase (MEK) to activate the 41kDa and 43kDa (ERK1/2) MAP kinases (Figure 6) [150-154].

In ALK fusion proteins such as NPM-ALK, MAPK signaling is mediated by binding of adaptor proteins like insulin receptor substrate-1 (IRS-1), growth factor receptor-bound protein 2 (Grb2) and src homology 2 containing (Shc) to the activated/phosphorylated ALK tyrosine residues. Furthermore, it has been shown that Grb2 binds to Y1507 and IRS-1 binds to Y1096 residue site [155-157]. In 2007, Degoutin and colleagues showed that adaptor proteins like Shc and Fibroblast Receptor substrate-2 (FRS-2) are recruited also upon ALK full-length receptor activation [158]. Pharmacological inhibition of the MAPK pathway induces apoptosis and reduces cell growth in ALK-positive ALCL [159-161]. Recently, it has been suggested that the EML4-ALK fusion protein is primarily dependent on RAS-MAPK pathway signaling, which can be used as a polytherapy strategy to treat EML4-ALK positive lung cancers [162, 163]. Following this study, Eleveld and colleagues suggested that targeting the RAS-MAPK pathway would be beneficial also for ALK-positive neuroblastoma

patients, where ALK is a full-length receptor [164]. However, ALK positive neuroblastoma cells are not primarily dependent on RAS-MAPK pathway signaling as will be discussed in Paper III.

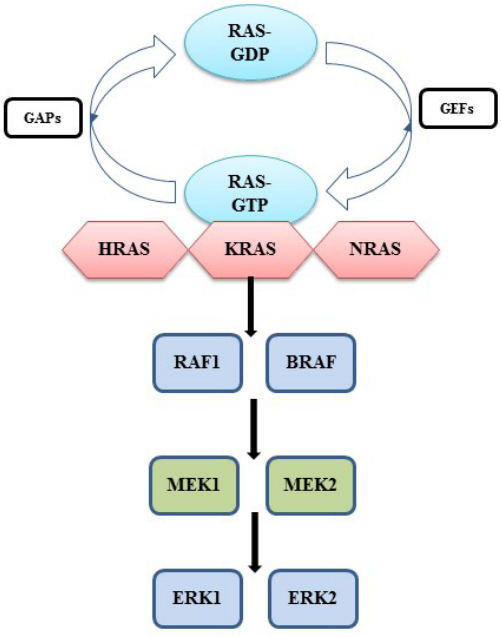


Figure 6- Activation of RAS-MAPK pathway: *The classical RAS-RAF-MEK-ERK signaling pathway is shown here.*

JAK/STAT

The JAK/STAT pathway plays an important role in several cellular processes like proliferation, survival, apoptosis, differentiation, and oncogenesis. Upon receptor activation, recruited JAKs are activated and create a binding site for STATs [165-171]. Tyrosine phosphorylated STATs form dimers and translocate to the nucleus [167-171]. The dimerized STATs activate or repress several transcription factors (Figure 7) [167-171].

Activation of STAT3 by ALK has been mostly studied for the NPM-ALK fusion protein; however, the mechanism of activation is still unclear [172-174]. Several studies have shown that NPM-ALK activates STAT3 in a JAK3 dependent manner [172, 174]. However, Marzec and colleagues have shown that NPM-ALK phosphorylates STAT3 independent of JAK3 [173]. Inhibition of PP2A activity, a serine/threonine kinase phosphatase positively regulates STAT3 in ALK positive ALCL [175]. ALK positive ALCL possesses enhanced STAT3 activity due to the absence of its inhibitor PIAS3 in these cells [175]. Furthermore, selective inhibition of STAT3 results in induction of apoptosis and suppression of proliferation in ALK-positive ALCL [172, 173]. In EML4-ALK positive lung cancer, the STAT3 signaling pathway is also important for the transforming activity of EML4-ALK [176]. Taken together, STAT3 may play an important role in ALK positive cancers. The role of STAT3 as a downstream target of full length ALK receptor will be discussed in Paper I.

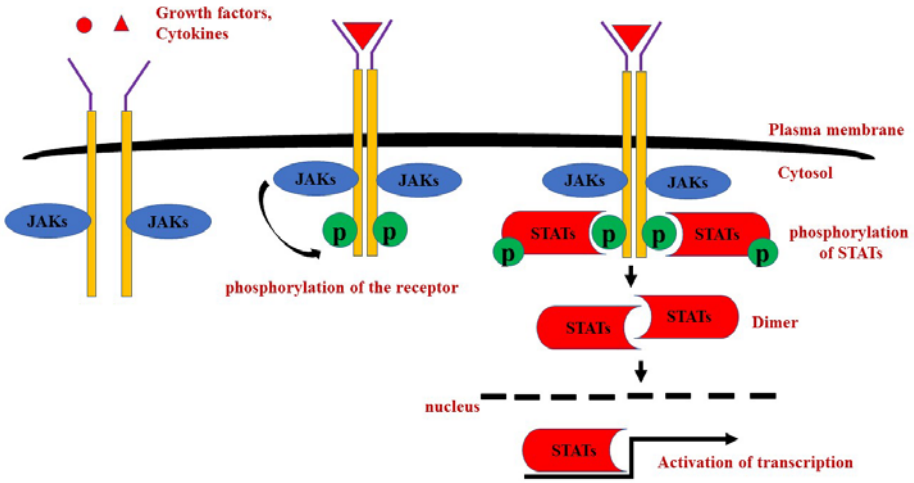


Figure 7- Activation of JAK/STAT pathway: *Basic steps involving the activation of JAK/STATs upon receptor activation is shown here.*

PI3K-AKT

The PI3K/AKT signaling cascade plays an important role in carcinogenesis. PI3K consists of two subunits: (i) a regulatory subunit (p85), and (ii) a catalytic subunit (p110) [177, 178]. The PI3K complex activates number of proteins, of which Akt plays a vital role in several cellular processes [177, 178]. The

mTOR complexes are important in PI3K/Akt signaling cascade where they act both downstream and upstream of Akt (Figure 8) [179].

PI3K/Akt signaling cascade activation is important for the transforming activity of NPM-ALK in ALK-positive ALCL [180-182]. In 2005, Polgar and colleagues showed that PI3K activation is mediated by an interaction of NPM-ALK with the regulatory subunit p85, resulting in decreased apoptosis [181]. Further, it has been shown that activated PI3K/Akt signaling activates mTOR complexes and glycogen synthase kinase 3beta (GSK3β) to promote oncogenesis [183-185]. The NPM-ALK/PI3K/Akt signaling cascade regulates survival and proliferation signals through activation of the FOXO3a transcription factor [186]. It has also been shown that the PI3K signaling cascade regulates the Sonic hedgehog (Shh) signaling pathway in ALK-positive ALCL [187]. Pharmacological inhibition of PI3K activity using PI3K inhibitors resulted in reduced cell proliferation and induction of apoptosis in ALK-positive ALCL [180, 182]. The PI3K signaling cascade also plays an important role in EML4-ALK positive lung cancers and in ALK-positive neuroblastoma [188, 189]. Inhibition of PI3K activity using PI3K inhibitors led to reduced tumor growth in mice xenografts and cell growth *in vitro* in EML4-ALK positive lung cancers and in ALK-positive neuroblastoma [188, 189]. Taken together, the PI3K signaling cascade provides a bonafide target in cancers that express ALK fusion proteins or mutated full length ALK receptor.

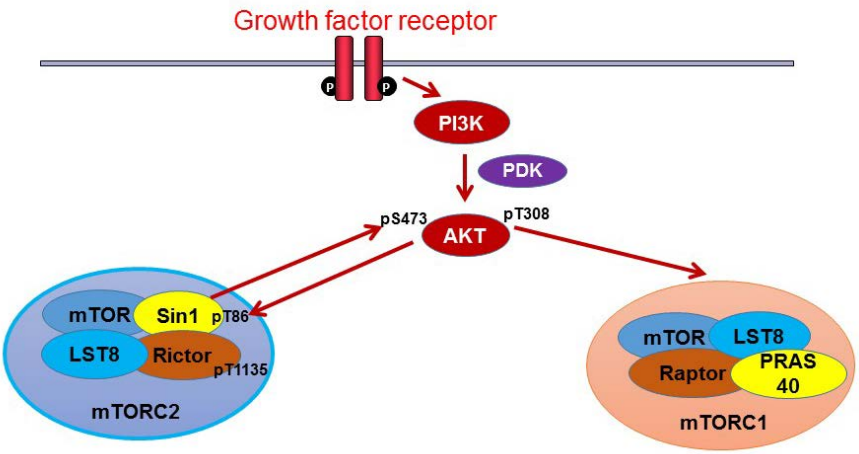


Figure 8- PI3K-AKT-mTOR signaling pathway: *The canonical PI3K-AKT-mTOR signaling pathway is shown here.*

ERK5/BMK1

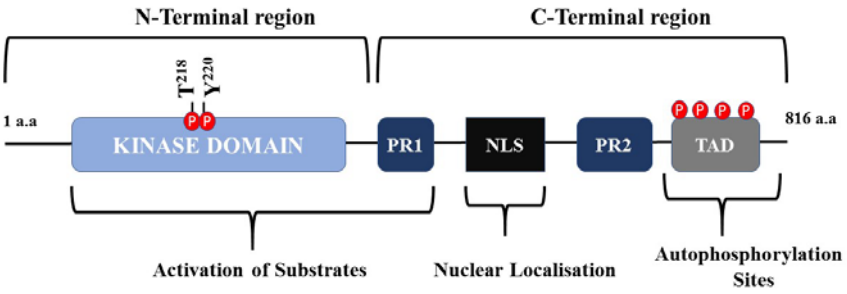
The *MAPK7* gene encodes the ERK5 protein, identified by two independent groups in 1995, as a member of Mitogen activated protein kinases (MAPKs) [190, 191]. ERK5 is found to be expressed in many tissue types, particularly in lung, brain, heart, kidney, skeletal muscles, and placenta [190, 191]. Depending on its activation ERK5 localizes to the cytoplasm or nucleus [192]. ERK5 is also called Big MAP kinase 1 (BMK1) due to its unique C-terminal region (Figure 9) [192]. The ERK5 protein consists of a kinase domain in the N-terminal region and a nuclear localization domain, two proline-rich-domains (PR1 and PR2) and a transactivation domain in the C-terminal region (Figure 9) [190, 193-195]. Similar to ERK1/2, ERK5 also has a dual phosphorylation site (TEY) in the kinase domain which is important for activation [192, 194, 195]. Upon activation ERK5 autophosphorylates in the transactivation domain to enhance its transcriptional activity [196]. In the cytosol, the N- and C-terminal domains of ERK5 are connected together when ERK5 is in an unphosphorylated state (inactive form) and when ERK5 is activated/phosphorylated it translocate to the nucleus [192, 197]. ERK5 responds to several mitogenic signals, such as cytokines and growth factors (EGF, VEGF, FGF, and NGF) and to cellular stress [198-202]. ERK5 is believed to be activated by a linear signaling cascade, where MAPK kinase kinase 2/3 (MEKK2/3) activates MAPK kinase 5 (MEK5), which then activates Big MAP kinase (BMK1) (Figure 9) [203, 204]. Upon activation by MEK5, ERK5 regulates several transcription factors like the MEF2 family of transcription factors, c-MYC, SRF accessory protein 1 (SAP1) and cyclic adenosine monophosphate (cAMP) [194, 195].

ERK5 plays a vital role in the regulation of several cellular processes like proliferation, survival, angiogenesis, and differentiation [195, 205]. To understand the physiological role of ERK5, several ERK5 knockout mice have been generated [201, 206, 207]. Similar to MEKK2/3 or MEK5, ERK5 targeted deletion results in embryonic lethality in mice due to cardiovascular defects and vascular integrity [201, 206, 207]. Hayashi and colleagues also showed that ablation of ERK5 in an inducible knockout mouse model leads to endothelial cell apoptosis [206]. Kato and colleagues have shown that BMK1 is required for cell cycle regulation and proliferation stimulated by epidermal growth factor (EGF) in HeLa cells [199]. Other studies have also indicated the participation of ERK5 signaling in the regulation of cell proliferation of breast cancer and prostate cancer cell lines [208, 209]. However, the activation of the ERK5 signaling pathway (AKT-MEKK3-ERK5) via oncogenic receptor activation in a solid tumor was first described by Umapathy and colleagues in 2014 [210]. Yang and colleagues had shown that ERK5 interacts with

promyelocytic leukemia protein (PML) and controls its antitumor effect [211]. Furthermore, they showed that pharmacological inhibition of ERK5 suppresses tumor growth and cells overcome G_1-S transition checkpoint [211]. Recently, it has been shown that ERK5 plays an important role in maintaining the 'stemness' of cancer stem cells (CSCs) and also for maintaining the embryonic stem cell identity [212, 213]. Overall, ERK5 marks itself as a vital signaling event in several different cancer types and also maintains stem cell identity. ERK5 as a downstream target in mutated full length ALK receptor will be further discussed in Paper II. Investigation of its role in ALK fusion cancers should provide a more complete picture of regulation of ERK5 by ALK and a better understanding of ERK5 as a therapeutic target for ALK-positive cancers.

Based on a phosphor-proteomics approach several other targets of ALK have been identified, including Crk, CrkL, Dok2, ATIC, VASP, MAPK1, MAPK3, FASP and PTPN11 [155, 214-216]. However, future research will tell us more about their role in regulating cell proliferation and survival in ALK positive cancers. Both ALK fusions and the full-length receptor share common downstream targets, however they might regulate different signaling cascades based on the tumor type. Therefore, understanding the oncogenic ALK signaling axis will be essential to develop new polytherapy strategies in ALK positive cancers

A.



B.

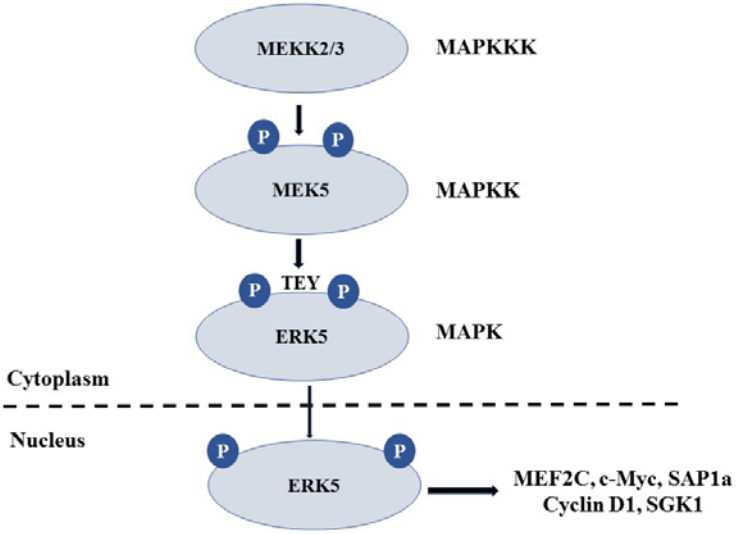


Figure 9- ERK5 structure and signaling: *(A) Domain structure of ERK5 is shown. ERK5 structure (816 a.a) consists of smaller N-terminal region and a larger C-terminal region. The N-terminal region consists of a kinase domain (78-406 a.a), which comprises dual TEY (T218/Y220) phosphorylation site. The larger C-terminal region consists of two proline rich domains (PR1- 434-485 a.a and PR2- 578-701 a.a), a nuclear localization signal domain (NLS- 505-539 a.a) and a transactivation domain (TAD- 664-789 a.a). (B) Linear signaling cascade of ERK5 is shown.*

1.5 ALK positive cancers

ALK can be oncogenic in three ways (Figure 10),

1. Chromosomal translocation
2. Overexpression
3. Point mutations

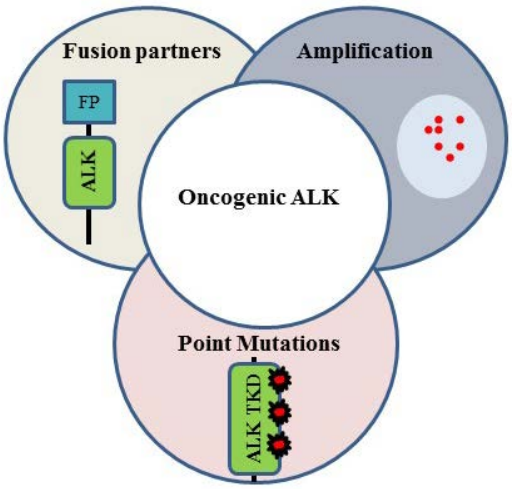


Figure 10- Oncogenic ALK in cancer: *Figure represents various ALK positive cancer types.*

1.5.1 ALK chromosomal translocations

ALK was originally discovered as a fusion protein partner with nuclear protein NPM in 1994 in ALCL [96], since then almost 30 different ALK fusion partners have been identified (Figure 11), suggesting that the *ALK* locus is a 'hot spot' for translocation, although the reasons are not clearly understood. Almost all ALK fusion proteins share common features, including: (i) the promotor of the fusion partner will initiate the transcription, (ii) subcellular localization is also facilitated by the fusion partner, (iii) ALK fusion dimerization/oligomerization is determined by the fusion partner, which leads to trans-auto phosphorylation and by which it signals to its downstream targets [102, 217-220].

Anaplastic large cell lymphoma (ALCL)

In 1985, Stein and colleagues are the first to describe ALCL as a neoplasm, which possess Ki-1 antigen in the abundant cytoplasm [221]. ALCL commonly occurs in children and young adults, a rare type of Non-Hodgkin's lymphoma involving T-cell receptor rearrangement [222] [223]. In ALCL, the well-studied ALK translocation fusion partner is NPM-ALK, which occurs almost in 80% ALCL cases [220, 222, 224, 225]. NPM-ALK was first discovered in 1994 in ALCL, since then several other ALK translocation fusion partners have been reported in ALCL like, Moesin (MSN), ALK lymphoma oligomerization partner on chromosome 17 (ALO17), TRK-fused gene (TFG), Tropomyosin 3 (TPM3), Tropomyosin 4 (TPM4), non-muscle myosin heavy chain 9 (MHY9), ATIC, CLTC-1 and TRAF-1 [96, 102, 220, 226-234].

Inflammatory myofibroblastic tumor (IMT)

IMT are rare mesenchymal neoplasms that frequently originate in the lung, abdomen, and retroperitoneal region and mostly affect young adults [235]. Almost 50% of IMT cases have rearrangement involving the *ALK* locus (2p23) of which TPM3-ALK fusion protein is present in the half of the cases [236, 237]. Similar to ALCL, ALK possess several other fusion partners like, TPM4, SEC31 homologue A (SEC31L1), protein-tyrosine phosphatase receptor-type F polypeptide-interacting protein-binding protein 1 (PPFIBP1), Ras-related nuclear protein-binding protein 2 (RANBP2), cysteinyl-tRNA synthetase (CARS), ATIC, CLTC [102, 220, 238-244]. ALK translocations in both ALCL and IMT are associated with better prognosis [225, 245, 246].

Diffuse large B-cell lymphoma (DLBCL)

DLBCL is the most common type of lymphoma, which accounts almost around 30 to 40% lymphoma cases [247]. In which ALK positive DLBCL is very rare, however ALK rearrangement in DLBCL is associated with poor prognosis and response to chemotherapy treatment is ineffective [248, 249]. This rare type of DLBCL ALK positive group might benefit from ALK target therapies. The common ALK translocation rearrangements observed in DLBCL are sequestosome-1 (SQSTM1), NPM-ALK, CLTC-ALK, SEC31A [233, 250-255].

Non-small cell lung cancer (NSCLC)

Lung cancer is the leading cause of cancer death worldwide, which is classified into two subgroups: (1. Small cell lung cancer (SCLC) and (2) Non-small cell

lung cancer (NSCLC) [256-258]. Almost 80% of lung carcinoma belongs to NSCLC subgroup. EML4-ALK fusion protein was first described in the year 2007 by two independent groups, which accounts for around 7 to 9% of all NSCLC cases [218, 259]. The fusion is linked by an inversion in 2p chromosome locus, which results in the fusion of N-terminal region containing coiled coil domain of EML4 gene with tyrosine kinase domain of ALK gene [218]. Thirteen different EML4-ALK variants have been described to date [102, 260]. Almost all EML4-ALK variants contain exons 20-29 of ALK, whereas it contains different exons of EML4, which might play role in stability or activity of the fusion protein [102, 261, 262]. Interestingly, Doebele and colleagues demonstrated a new ALK fusion variant involving exon 6 of EML4 to exon 19 of ALK (E6;A19), however significance of these exon 19 of ALK fusion variants are currently unknown [263]. ALK targeted therapies shows promising results towards ALK positive NSCLC, however understanding the resistance mechanisms that arise in response to ALK inhibitor therapy will be a challenging in coming years [263-266]. ALK translocation other than EML4-ALK reported in NSCLC are, HIP1-ALK, STRN-ALK, PTPN3-ALK, TFG-ALK, KLC1-ALK, KIF5B-ALK, and TPR-ALK [102, 220, 259, 267-271].

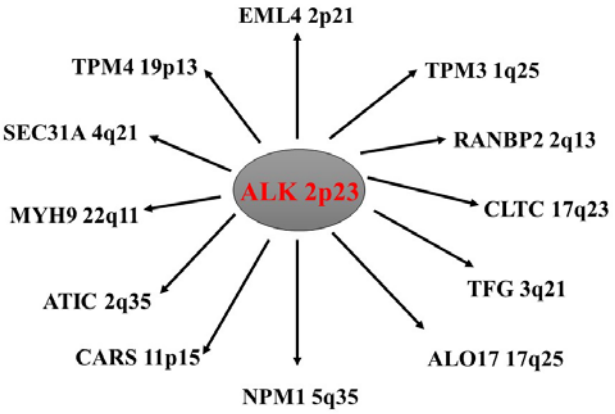


Figure 11- ALK fusion proteins in cancer: *Figure showing various different ALK fusion partners.*

1.5.2 ALK overexpression

Overexpression of ALK protein has been described in many cancer forms including retinoblastoma, astrocytoma, glioblastoma, melanoma, breast cancer, NSCLC, Ewing's sarcoma, rhabdomyosarcoma, and neuroblastoma (Figure 12) [147, 272-274].The importance of these events in the progression of disease is not known.

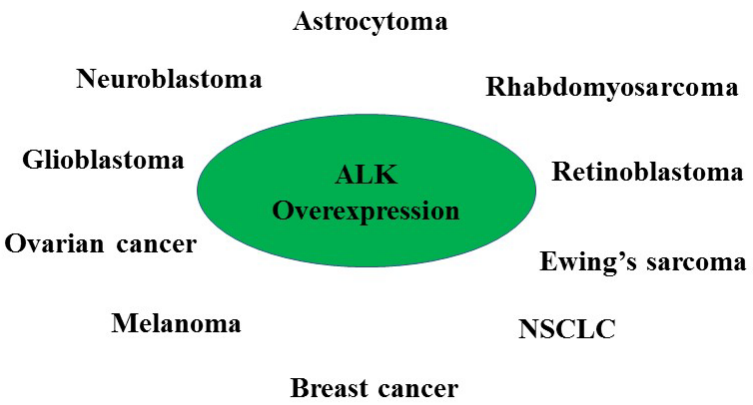


Figure 12- ALK overexpression in cancer: *A pictorial representation of ALK overexpression in different cancer types.*

1.5.3 ALK point mutations

Cancer forms like Anaplastic thyroid tumor (ATC), NSCLC, and neuroblastoma have been associated with activated ALK point mutations (Figure 13) [275-280].

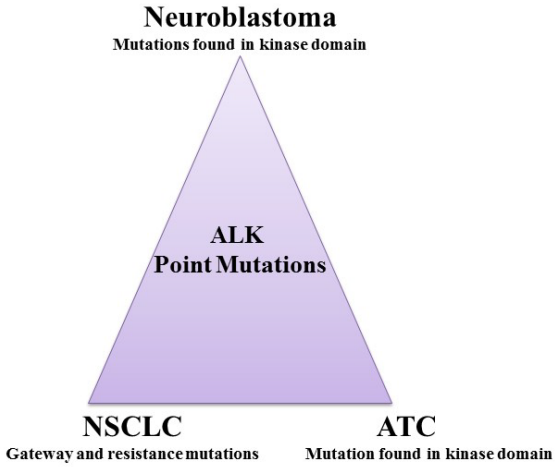


Figure 13- ALK point mutations in cancer: *A pictorial representation of ALK point mutations in various cancer types.*

ALK-L1198F and ALK-G1201E were described as gain-of-function activating point mutations in Anaplastic thyroid tumor (ATC) [280]. However, Guan and colleagues have recently shown that neither ALK-L1198F nor ALK-G1201E are constitutively active [281].

ALK point mutations observed in NSCLC are mostly secondary mutations occurred after crizotinib treatment, the first ALK inhibitor approved by the FDA for treatment of ALK positive NSCLC patients [282]. However, a few oncogenic ALK point mutations have also been described in lung adenocarcinoma [279]. These include mutations in the MAM domains (S413N, V597A), glycine rich domain (G881D), between MAM2, and glycine rich domain (H694R) and in the kinase domain (Y1239H, E1384K) [279]. Of these V597A, G881D, H694R, E1384K showed high kinase activity and activated downstream signaling components such as STAT3, AKT, and ERK [279]. The secondary mutations observed after crizotinib treatment are mainly confined around ATP binding site of ALK (Figure 14) [276]. These include two

gatekeeper mutations found in crizotinib resistance patients namely L1196M and C1156Y [276]. ALK-G1202R, S1206Y, G1269A, L1152R, L1198P, D1203N, I1171T/N/S, F1174C/V, L1198F, E1210K, and V1180L are also secondary mutations observed in NSCLC patients after ALK TKI treatment (Figure 14) [263, 266, 283-291]. Currently, all described secondary mutations can be overcome by current ALK TKI, however understanding the resistance mechanisms, and implementing other treatment strategies represent significant challenges.

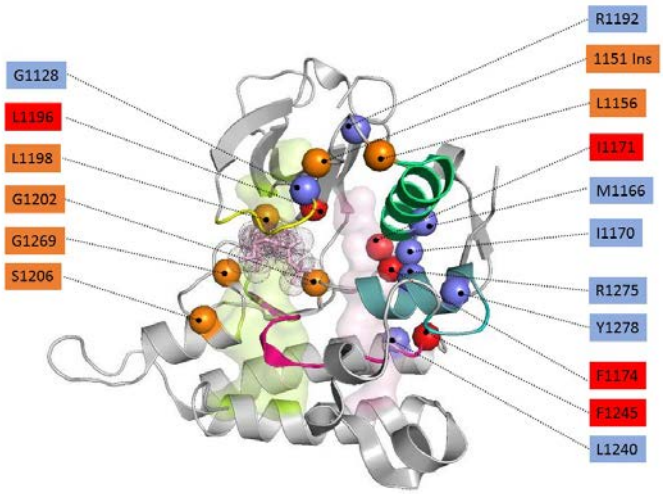


Figure 14- Mutations in ALK kinase domain: *ALK tyrosine kinase inhibitor resistance mutations in ALK fusions are shown in orange. Activating ALK points found in neuroblastoma are shown in blue. Mutations found in both ALK fusions and neuroblastoma are shown in red.*

1.6 Neuroblastoma

Neuroblastoma (NB) is a childhood cancer that gives rise to undifferentiated neural crest precursor cells of the sympathetic nervous system. It accounts almost 8-10% of all childhood cancer deaths, which makes it a most common extra cranial solid tumor [292-297]. NB is a very complex disease which affects very young children with median age of 22 months at diagnosis [298, 299]. Children can develop tumors at any point along the sympathetic chain, however it most frequently originates in the adrenal medulla, then originates

to nerve tissues of abdomen, chest, pelvis and neck region [292, 300, 301]. NB is classified into five stages (stage1-4 and 4S) clinically according to International Neuroblastoma Staging System (INSS) [293, 295, 302]. Stage 1and 2 are designated as Early stage NB tumors which usually do not metastasize to bone marrow and usually respond to chemotherapy and radiation. Stage 3 and 4 are designated as advanced stage NB tumors that usually metastasize and also become resistance to chemotherapy treatment. Stage 4S is designated as fifth stage of NB tumors where children go through spontaneous regression without treatment [293, 295, 302, 303]. In addition to stage classification, NB tumors are divided into three risk groups (low, intermediate and high risk) based on age, histology and MYCN status [303]. Chromosomal aberrations, age, disease stage, and genetic abnormalities are all contributing factors in NB tumorigenesis.

1.6.1 Chromosomal aberrations and genetic lesions in NB

The most common genetic anomalies seen in NB are deletion of parts of chromosome arms 1p and 11q, 17q gain, triploidy, MYCN and ALK amplifications [295, 304-308].

Loss of parts of chromosome arm 1 (1p36) usually accounts for 25-35% NB tumors [292, 293, 295]. 1p loss of heterozygosity (LOH) correlates with amplification of MYCN and leads to unfavorable conditions in NB clinical groups [303]. To determine the importance of chromosome 1 in NB, Bader and colleagues transferred the normal portions of chromosome 1 short arm into the neuroblastoma cell line [309]. Transfer of chromosome led to differentiation of neuroblastoma cells and suppression of tumorigenicity [309]. Several potential tumor suppressor genes reside in this region which includes chromo-domain helicase DNA-binding domain 5 (CHD5), kinesin superfamily protein 1B beta (KIF1Bβ), microRNA-34a (mir-34a) calmodulin binding transcription activator 1 (CAMTA1) and p73. Introduction of CHD5, KIF1B, mir-34a or CAMTA1 decreased cell proliferation and leads to apoptosis [303, 310-313]. However, further studies indicate that there is no correlation between tumor suppressor gene p73 and NB development [314].

Loss of parts of chromosome arm 11 (11q23) usually accounts for 30-40% of NB tumors and inversely correlated with MYCN amplification [303]. 11q LOH is usually correlated with unfavorable clinical outcome in NB patients [303]. Similar to chromosome 1, transfer of chromosome 11 also induced NB

cell line differentiation [309]. Potential tumor suppressor genes that localize to this region are immunoglobulin superfamily 4 (IGFSF4) and Tumor suppressor in lung cancer/cell adhesion molecule 1 (TSLC1/CADM1) [303, 315, 316]. Transfer of TSLC1 gene into NB cell lines expressing low TSLC1, led to decreased NB cell proliferation [316].

Gain of parts of chromosome arm 17 (17q22) usually accounts for 40-50% NB cases and associated with poor prognosis [303]. Gain of 17q correlates with MYCN amplification and leads unfavorable conditions in NB patients [293, 295, 303, 317]. Genes localized in this region include survivin, NM23A, PPM1D [303]. Survivin (inhibitor of apoptosis) is associated with poor prognosis and is frequently overexpressed in NB tumors [303, 318].

Another important prognostic marker in NB is near diploidy or hyper diploidy (triploidy) state. Triploidy is correlated with less aggressive tumors and malignant NB is associated with near diploidy [293, 295].

Amplification of MYCN gene on chromosome 2p24 is the one of the main hallmarks of NB. Amplification of MYCN gene is usually accounts between 20-30% of all NB cases and associated with poor survival [293, 295, 319]. MYCN is involved in several cellular process like cell proliferation, apoptosis, survival, and differentiation [320]. As in NB, MYCN has been reported to be overexpressed in several other cancer forms like glioblastoma, retinoblastoma, and SCLC [321-323]. To study NB tumorigenesis several transgenic mice have been developed. In this system, overexpression of MYCN acts as an initiator of NB tumor progression, however several studies have indicated that MYCN cooperates with other oncogenes to drive NB tumorigenesis [324, 325]. Targeting MYCN in these cancer forms might improve clinical outcome.

Amplification of the *ALK* gene or overexpression of ALK protein has also been described for the development of NB [326, 327]. Amplification of *ALK* gene can lead to ALK activation which correlates with poor survival in NB patients [326, 327]. Other than amplification of the *ALK* gene or overexpression, ALK point mutations were also been reported in both familial and sporadic NB [304, 305, 328-330]. Most of these described mutations are confined within the ALK kinase domain and are reported to be around 7-9% of all NB cases [331]. Mutations in ALK-F1174 (V, L, S, I, C) and ALK-R1275 (L or Q) are the two most frequently observed hot spot mutations in the kinase domain, accounting for 70-80% of all ALK mutant cases [304, 305, 328-330]. These two hot spot mutations or ALK-K1062M mutations resulted in transforming phenotypes when expressed in nude mice or NIH3T3 cells [328, 329]. Also, in co-operation with MYCN, ALK-F1174L mutation enhances the tumorigenic

activity in NB mouse models [325]. Further in 2011, Schonherr and colleagues reported a kinase dead mutant (I1250T) which potentially acts in a dominant-negative manner [332]. Based on the activation of the receptor ALK mutations can be characterized into three groups: (i) Ligand independent mutations (F1174L, Y1278S, R1275Q), (ii) Kinase dead mutations (I1250T) and (iii) Ligand dependent mutations (A1234, A1099, T1151) [333]. Recently, it has been reported that activating ALK point mutations (F1174L/S, Y1278S, L1196M and T1151R) are observed in 30-40% relapsed NB cases [164, 334-336]. Pharmacological inhibition or siRNA knockdown of ALK in NB cells results in decreased cell proliferation [330, 337]. Taken together, targeting ALK and its downstream target might benefit ALK positive NB patients.

Other factors which also contribute NB tumorigenesis are, LOH of 14q, amplification of DDX1 gene at 2p24, Neurotrophin receptors, ganglioside GD2, polycomb complex protein Bmi-1, micro RNAs (miR-10b, miR-29a/b, miR-335), paired-like homebox 2B (PHOX2B) mutations, Alpha Thalassemia/Mental Retardation Syndrome X-linked (ATRX), checkpoint kinase 2 (CHEK2), BRCA-1 associated RING domain protein 1 (BARD1), loss of cyclin dependent kinase inhibitor 2A (CDKN2A), mouse double minute 2 homolog (MDM2) and glycosyltransferase (B4GALT3) [303, 338-344]. Recently, Pandey and colleagues reported that long noncoding RNA, NBAT-1 regulates NB tumorigenesis via cell proliferation and neuronal differentiation [345].

1.6.2 Treatment strategies in neuroblastoma

Chemotherapy

Chemotherapy is preferred based on NB risk group. For intermediate NB risk group carboplatin, cyclophosamide, doxorubicin, and etoposide are preferred [346, 347]. For high NB risk group cisplastin, cyclophosamide, topotectan, vincristine and etoposide are preferred [346, 347]. However the cure rates has not been changed significantly in recent years [347].

Retinoids

NB is characterized as poorly differentiated cells, therefore induction of differentiation in these cells should reduce the proliferation of NB cells. Several studies have shown that 13-cis retinoic acid (RA) induces differentiation in NB cells in culture [348, 349]. RA has been preferred in children with high risk NB due to increases in survival rate and reduced

toxicity in those patients [350]. However, combinatorial treatment with RA produces even more better survival rates [351].

Immunotherapy

In 1977, Shochat and colleagues reported that NB cells express high levels of sialic acid and gangliosides on their surface [352]. However, sialic acid did not correlate with the prognosis of NB when compared to gangliosides [352]. These agents are required in cell migration, adhesion and metastasis [353]. Immunotherapy with the anti-GD2 (disialoganglioside) monoclonal antibody dinutuximab, a tumor-associated surface antigen has been tested in several clinical trials alone or in combination with differentiation therapy (13-cis retinoic acid) or with granulocyte macrophage colony-stimulating factor GM-CSF or with IL-2 [351]. Dinutuximab (Unituxin) was approved by FDA in 2015 as a first-line therapy for treating high risk NB patients [354].

Radionuclide therapy

^{131}I-metaiodobenzylguanidine (^{131}I-MIBG), a radionuclide has also been implicated as a therapeutic agent in NB. NB cells actively take up ^{131}I-MIBG and improve the response of NB patients [355]. However, long-term toxicity can be severe in these cases [355].

Programmed cell death (Apoptosis)

An alternative way to reduce the proliferation of NB cells is by inducing apoptosis. Fenretinide, is a retinoid which induces apoptosis in a caspase dependent manner in NB cells [356]. Combination of fenretinide with chemotherapeutic drugs had a synergistic induction of apoptosis in NB cells [357]. Targeting neurotrophin receptors also induce apoptosis in NB cells [358]. Expression of TrkB, a neurotrophin receptor, correlates with MYCN amplification and together leads to clinically unfavourable NB cases [303]. The FDA has provided Orphan Drug designation to a Trk inhibitor (Entrectinib) for treating NB patients [359].

Targeting MYCN

MYCN status has been a bonafide prognostic marker in NB [293, 295]. Targeting of MYCN would be beneficial for high risk NB cases. Similar to other Myc proteins, MYCN lacks appropriate motifs for drugs to bind to its DNA binding domain [360]. Therefore, targeting MYCN indirectly to regulate its activity has been a widely accepted approach in recent times. There are few

indirect MYCN targeting approaches including Aurora kinase A/B inhibitors, BET bromodomain family members inhibitors, inhibitors of the MYCN/MAX interaction, ornithine decarboxylase (ODC1) inhibitors, PI3K/AKT/mTOR inhibitors, ERK5 inhibitors and ALK inhibitors [210, 351, 361]. Recent studies have indicated that ALK regulates MYCN transcriptionally via AKT/ERK5 pathway [210, 325, 361], suggesting that targeting ALK and its downstream targets (AKT/ERK5) in ALK positive NB cells might be a potential therapeutic target.

Other possible NB therapies include inhibition of Heat shock protein 90 (Hsp90), targeting non coding RNAs, DNA methylation, checkpoint inhibitors and also protein glycosylation [299, 351]. Altogether, following the 'triangle theory' would benefit the NB patients in the near future (Figure 15).

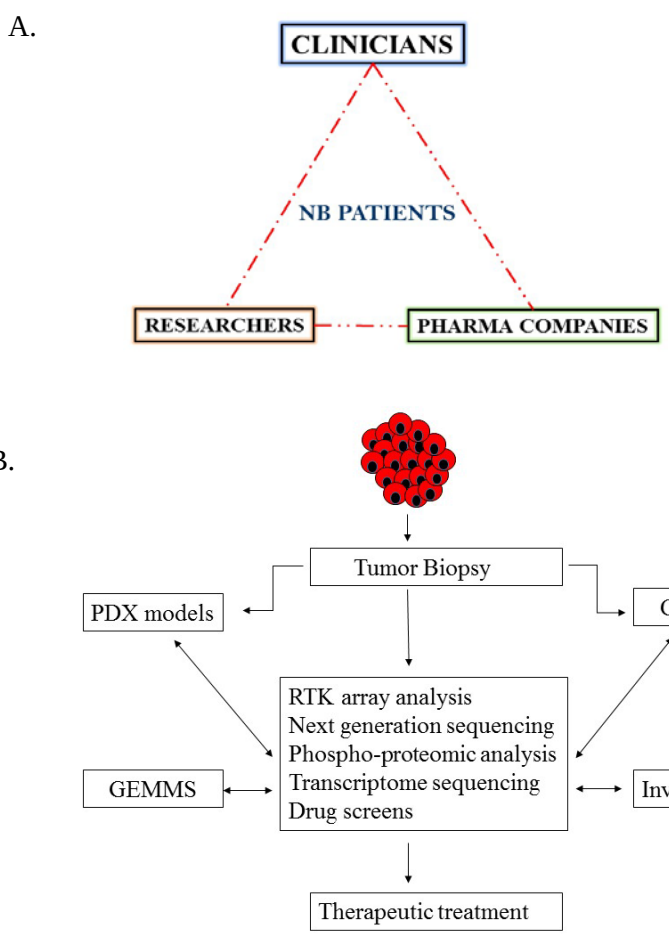


Figure 15- Triangle Theory: *(A). A pictorial representation of triangle theory. (B). Experimental platforms to treat NB patients in near future. Patient derived xenograft (PDX). Genetically modified mouse models (GEMMS). Figure adapted from (363).*

1.7 Targeting ALK: Treatment of ALK positive cancers

Since oncogenic ALK signaling is involved in several cancer forms, targeting ALK and its downstream partners should be therapeutically beneficial in ALK positive cancer patients. NVP-TAE684 was one of the first ALK specific inhibitors identified to target the ATP binding site of ALK. Several studies have shown that treatment with ALK specific inhibitor NVP-TAE684 reduced cell proliferation of ALK positive ALCL, NSCLC, and NB cell lines [337, 362]. However, treatment with NVP-TAE684 is toxic over time [337]. To date, several other ALK inhibitors have been developed and explored in clinical trials with ALK positive patients.

Crizotinib

Crizotinib was the first ALK targeted TKI to enter into clinics, even though it was initially described as a potent MET kinase inhibitor (Figure 16) [363]. In 2011, the FDA approved crizotinib for treatment of ALK positive NSCLC patients based on the remarkable results of phase I/II clinical studies [363]. Further, in phase III clinical studies, crizotinib was superior to conventional chemotherapy in advanced ALK positive NSCLC [363]. The common adverse effects described for crizotinib treatment are diarrhoea, visual disturbances, nausea, vomiting, constipation, dizziness and peripheral edema [265]. Similar to NSCLC, efficacy of crizotinib has been tested in other ALK positive cancer forms. The response to crizotinib has been encouraging in both paediatric and adult ALCL patients [364, 365]. However, it had a partial response in patients with ALK positive NB and ALK rearranged IMT [364]. Across all clinical trials, crizotinib responds with 8 to 11 months of median progression free survival (PFS) [265]. However, response to crizotinib is transient due to acquisition of secondary mutations or by ALK copy number gain or bypass survival signaling via alternative oncogenes (Table 1) [263, 265]. Several studies have also indicated the ineffectiveness of crizotinib on brain metastasis in ALK rearranged NSCLC cases [366]. Due to poor activity of crizotinib in brain and acquiring several secondary mutations, second generation ALK TKIs have been developed.

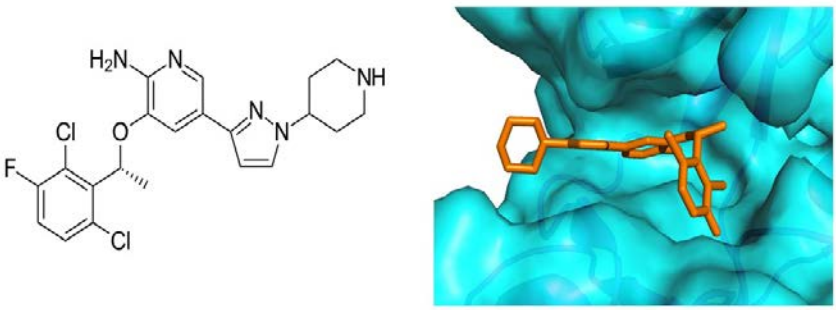


Figure 16- ALK TKI-Crizotinib: *On the left, the chemical structure of crizotinib is shown. On the right, binding of crizotinib into the ATP- binding pocket of the ALK kinase domain is shown.*

Ceritinib

In 2014, FDA approved the second generation ALK TKI ceritinib for crizotinib resistance ALK positive NSCLC patients. However, as with other second generation ALK TKI, ceritinib has not yet been approved as a first line therapy for ALK positive NSCLC patients [265]. Similar to crizotinib, ceritinib is an ATP competitive inhibitor which binds to the ATP binding pocket (Figure 17). Ceritinib is a derivative of the ALK specific inhibitor NVP-TAE684 and in addition to inhibiting ALK is effective against the activity of IGF-1R, STKK22D, and INSR [283, 286]. Since the expression of IGF-1R correlates with NB tumorigenicity [367], ceritinib might offer therapeutic advantages in ALK positive NB patients. The common adverse effects seen with ceritinib are, diarrhoea, vomiting, fatigue, abdominal pain, rash, arthralgia, transaminases, and dyspnoea [265]. Several studies have indicated that ceritinib is able to overcome both ALK-crizotinib resistance mutations (G1269A, L1196M, I1171T/N, and S1206C/Y) and ALK-alectinib resistance mutations (I1171T/N/S and V1180L) (Table 1) [285, 286]. Similar to ALK positive NSCLC, ceritinib is effective in ALK rearranged ALCL [368]. The median PFS with ceritinib in ALK positive NSCLC is 7 to 8 months, after which ALK secondary mutations arise and response to ceritinib significantly decreases [287].

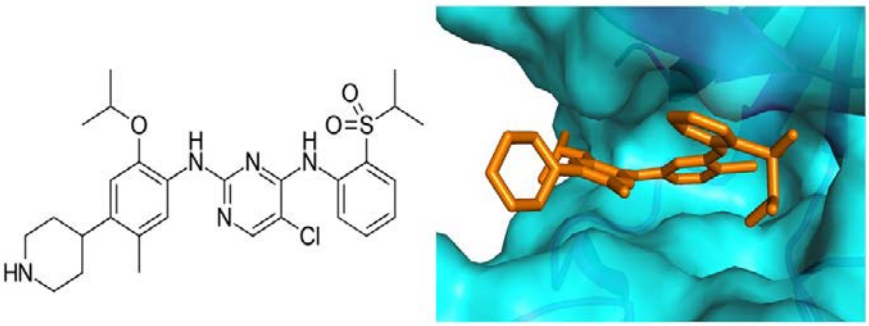


Figure 17- ALK TKI-Ceritinib: *On the left, the chemical structure of ceritinib is shown. On the right, binding of ceritinib into the ATP- binding pocket of the ALK kinase domain is shown.*

Alectinib

Alectinib is a potent ALK inhibitor with greater specificity towards ALK than crizotinib and also activity against some ALK-crizotinib resistance mutations (Figure 18) [265]. The exceptional success of alectinib in phase I/II trial in Japanese patients with ALK rearranged NSCLC led to its approval in 2014 in Japan [265]. In 2013, FDA granted breakthrough therapy designation (BTD) for alectinib for patients with ALK positive NSCLC who progressed with ALK TKI crizotinib. Alectinib has a high potency towards the gatekeeper mutation ALK-L1196M and also with two NB hot spot mutations ALK-R1275Q and ALK-F1174L (Table 1) [369]. Since the frequency of hot spot mutations in NB is between 70-80%, alectinib might improve the clinical outcome of NB patients. The median PFS with alectinib in crizotinib naive NSCLC is 28 months and 8 to 9 months in crizotinib resistance NSCLC [265]. Similar to other ALK TKIs, alectinib confers resistance with two ALK mutations ALK-V1180L and ALK-I1171T having been reported (Table 1) [285]. In 2014, Isozaki and colleagues established two alectinib-resistance cell lines. The first alectinib-resistance cell line showed increased activity of IGF1R and human epidermal growth factor 3 (HER3) and also neuregulin 1 (HER3 ligand) [370]. The second alectinib-resistance cell line showed stimulation of MET receptor by hepatocyte growth factor (HGF) in an autocrine aspect [370]. Taken together, alectinib acquires resistance in both ALK dependent and independent resistance mechanisms.

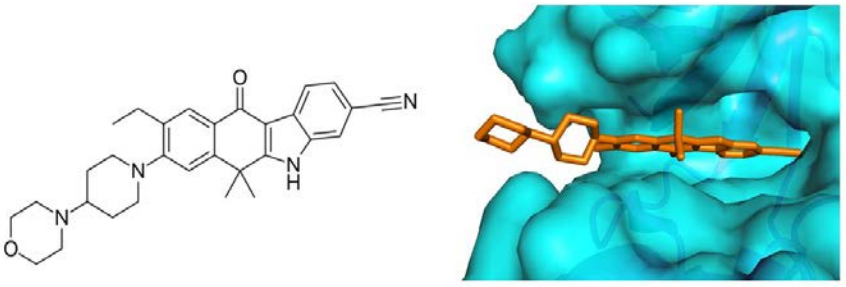


Figure 18- ALK TKI-Alectinib: *On the left, the chemical structure of alectinib is shown. On the right, binding of alectinib into the ATP- binding pocket of the ALK kinase domain is shown.*

Brigatinib

Brigatinib is a potent inhibitor of ALK and other kinases including EGFR and ROS1 (Figure 19). In phase I/II trails, brigatinib showed 72% of overall response with a median PFS of 11 to 13 months in crizotinib resistance ALK NSCLC patients [265]. The common adverse effects seen with brigatinib are, nausea, fatigue, dyspnoea, vomiting, pyrexia, arthralgia and diarrhoea [265]. Recently, it has been shown that brigatinib inhibits ALK receptor activity more effectively than crizotinib in NB cell lines, xenograft models and a *Drosophila* model system [371]. Further, brigatinib confers resistance to E1210K+S1206C, E1210K+D1203N ALK secondary mutations (Table 1) [363].

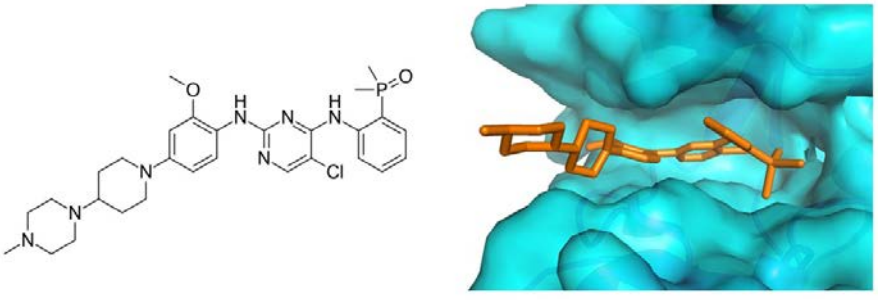


Figure 19- ALK TKI-Brigatinib: *On the left, chemical structure of brigatinib is shown. On the right, the binding of brigatinib into the ATP- binding pocket of the ALK kinase domain is shown.*

Entrecitinib

Entrecitinib is an oral, potent inhibitor of ALK, NTRK, and ROS1 (Figure 20). Entrecitinib is currently being evaluated in phase I/II trials (ALKA-372-001 and STARTRK-1) for patients with ALK, ROS1, NTRK alterations [265]. Recently, Iyer and colleagues have shown that treatment with entrecitinib reduces NB cell proliferation and tumor growth [359]. Entrecitinib has received an orphan drug designation for treating NB patients as well as for NTRK, ALK, ROS1 alterations in NSCLC and metastatic colorectal cancer (mCRC). The most common adverse effects seen with entrecitinib are, diarrhoea, nausea, dizziness, fatigue, constipation, and peripheral neuropathy [265]. Entrecitinib confers resistance to the G1202R ALK secondary mutation (Table 1) [363].

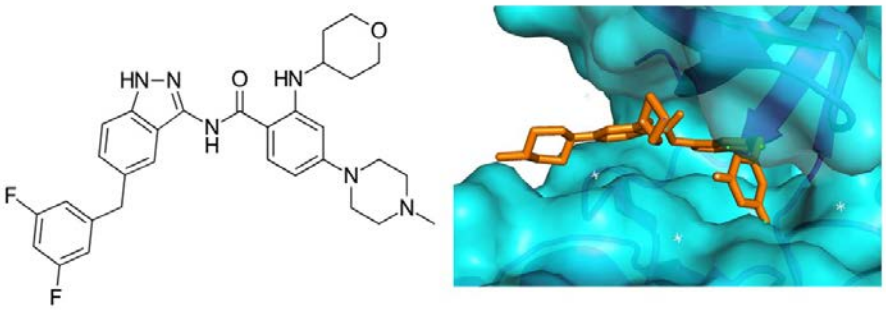


Figure 20- ALK TKI-Entrecitinib: *On the left, the chemical structure of entrecitinib is shown. On the right, binding of entrecitinib into the ATP- binding pocket of the ALK kinase domain is shown*

Lorlatinib

Lorlatinib is a novel, highly potent ALK/ROS1 inhibitor that can pass through the blood-brain barrier (Figure 21).Lorlatinib overcomes almost all known ALK resistance mutations observed with other ALK TKIs [363]. In both *in vitro* and *in vivo* systems, lorlatinib is more potent than other ALK TKIs [372]. Furthermore, it leads to regression of ALK rearranged NSCLC brain metastasis [372]. Recently it has been shown that lorlatinib had superior potency towards ALK activity and also reduced ALK positive NB cell proliferation and tumor growth [373, 374]. A phase I/II trial of lorlatinib is currently being investigated in ALK/ROS1 positive NSCLC [265]. The common adverse effects seen with lorlatinib are, fatigue, slowed speech, hypercholesterolemia, peripheral edema and neuropathy. Recently, Shaw and colleagues reported that therapeutic use of lorlatinib in a patient with crizotinib resistant ALK positive NSCLC led to the appearance of the ALK-L1198F+C1156Y resistance mutation (Table 1) [287]. Interestingly, in the same study it was shown that this lorlatinib resistance mutation is sensitive to crizotinib treatment [287]. Due to its high efficacy, lorlatinib can serve as a perfect partner for combinatorial treatments to overcome the emergence of resistance clones in ALK positive cancers.

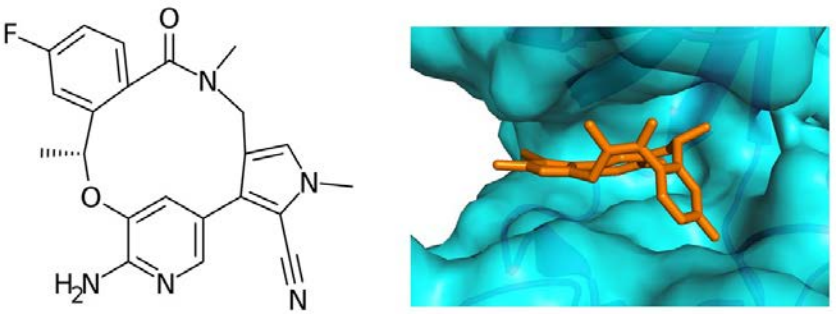


Figure 21- ALK TKI-Lorlatinib: *On the left, the chemical structure of lorlatinib is shown. On the right, binding of lorlatinib into the ATP- binding pocket of the ALK kinase domain is shown.*

1.8 Mechanism of resistance to ALK TKIs in ALK positive cancers

Based on several drug screens, *in vitro*, and *in vivo* models, ALK TKI resistance mechanism can be classified into two major groups [263, 363, 375]: (i) ALK positive mechanism (ALK dependent), which includes ALK secondary resistance mutations or ALK copy number gain, (ii) ALK negative mechanism (ALK independent), which includes the activation of alternative oncogene (EGFR, IGFR, MET, KIT) and lineage alterations [263, 363, 375].

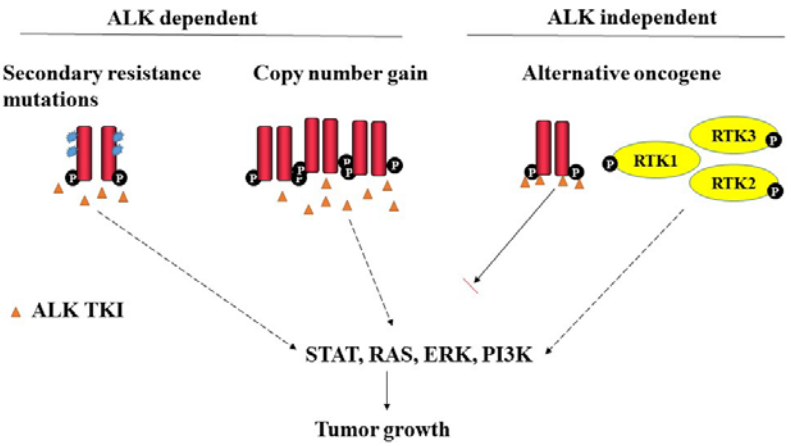


Figure 22- Mechanism of ALK TKI resistance: *A pictorial representation of different classes of acquired ALK TKI resistance.*

ALK positive resistance mechanism

The gatekeeper mutations ALK-L1196M and ALK-C1156Y are the first ALK resistance mutations reported in ALK positive NSCLC [276]. Further this mutation was also identified in a crizotinib resistance cell line which confers resistance to crizotinib by altering the gatekeeper residue and hinders crizotinib binding to the ATP pocket [265, 376]. Similar to gatekeeper mutation, ALK-G1269A mutation also impairs crizotinib binding to the ATP-binding cleft [263]. The other ALK secondary resistance mutations resides around C-terminal end of αC helix (F1174C/V/L) and in N-terminal end (I1151Tins, C1156Y, and L1152R) [263, 276, 285, 286, 363]. The L1152 residue appears on β3 strand which interacts with αC helix of the C-terminal, mutation to

arginine might hinder crizotinib binding to wild type auto-inhibitory conformation (Figure 14, Table 1)[237, 289]. Whereas, the C1156 residue appears to be very far to crizotinib binding, however C1156 residue binds to L1152, and thereby mutation to tyrosine might impair the crizotinib binding [237, 377]. Alterations in I1151 residue might disrupt the bonding with the residue G1129, which might alter the αC helix and hinder the binding of ALK TKIs [237, 285, 363, 378]. The F1174 appears to be in C-terminus end of αC helix, mutations at this site distorts ALK structure by which it impairs the binding of crizotinib [237]. The G1202R, D1203N, and S1206C/Y are the other class of ALK secondary mutations which hinder ALK TKI binding most likely through steric impediment [288, 363]. Several similar mutations have also been described in other cancer forms like neuroblastoma, ALCL and IMT [102, 278]. However, mutations described in NB are primary mutation rather than the ALK TKI resistance mutations. Mostly, all ALK secondary mutations can be overcome by second and third generation ALK TKIs. However, sequential treatment of ALK TKIs in patients leads to dual mutation (C1156Y/I1171N, E1210K/D1203N, and C1156Y/L1198F) which confers resistance to second and third generation ALK TKIs [363]. The dual mutation C1156Y/L1198F was recently found in ALK positive NSCLC patient (Table 1). The patient was sequentially treated with first, second and third generation ALK TKIs (crizotinib, ceritinib and lorlatinib). Interestingly, *in vitro* study demonstrated that ALK mutation L1198F was sensitive to crizotinib, the patient was retreated with first generation ALK TKI crizotinib and had an enduring response [287]. This case serve as a proof for 'triangle theory', which led to the benefit of the patient.

Other than ALK secondary mutations, ALK copy number gain (CNG) can also confer resistance to ALK TKIs. ALK amplification occurs around 8 to 15% in ALK TKI resistance NSCLC cases [263, 283, 363]. In an *in vitro* study, ALK amplification together with gatekeeper mutation ALK-L1196M resulted in high-level resistance to crizotinib [376]. However, ALK CNG have not been observed with second generation ALK TKIs [288].

ALK negative resistance mechanism

The ALK independent resistance mechanism includes the activation of alternative signaling pathway or lineage alterations [263, 363, 375]. There are numerous examples of activation of alternative oncogene, of which EGF receptor activation was the first identified resistance mechanism to ALK TKIs [283, 289, 363]. Analysis of phosphor-RTK array in crizotinib resistance cell line showed increased EGFR tyrosine phosphorylation and also associated with activation of downstream survival signaling like AKT and ERK [289,

379]. EGFR ligand upregulation might have resulted to the activation of the receptor, since mutation or amplification of EGFR was not observed in these cell lines [289, 379]. Neuregulin-1 (NRG1), the ligand for ERBB3/4 RTK was also observed as a vital driver for ALK TKIs resistance [380]. Brigatinib, a TKI inhibitor which inhibits the activity of both ALK and EGFR might be beneficial for EGFR bypass signaling. MET amplification was another bypass signaling recently observed in NSCLC patient treated with second generation inhibitor alectinib. Biopsy did not show any alterations in ALK or EGFR, but showed MET amplification, however patient responded to crizotinib, an ALK/MET inhibitor [381]. KIT amplification, activation of IGF1R and SRC, mutations in PIK3CA, TP53, BRAF, DDR2, FGFR2, NRAS, and MET are the other examples of bypass signaling which confers resistance to ALK TKIs [363]. Recently, it has been reported that activation of another bypass signaling via AXL receptor which also mediates the resistance to ALK inhibitor TAE684 in neuroblastoma [382]. However, the use of next generation inhibitors will provide us the complete picture of ALK resistance mechanism in full length ALK receptor. Reactivation of downstream signaling pathway over time might also contribute to the development of ALK TKI resistance [163]. Recently, it has been reported that KRAS copy number gain or loss of DUSP6 leads to reactivation of MAPK which confers resistance to ALK inhibitors [163].

Epithelial-to-mesenchymal transition (EMT) phenotypic changes might also be responsible for the development of resistance to ALK TKIs [363]. Few studies have reported that ALK inhibitor resistant cell lines and tumor samples express high levels of vimentin (mesenchymal marker) and low levels of E-cadherin (epithelial marker) [363, 383]. However the mechanism of resistance to ALK TKIs with EMT phenotypic changes is still unclear [363, 383]. Recent studies have indicated that drug tolerant persister cells leads to TKI resistance and clinical relapse [384, 385]. However, the role of drug tolerant persister cells in ALK TKI resistance is yet to be resolved. Multidrug resistance 1 (MRD1) gene encodes a protein called P-glycoprotein (P-gp) have also been identified as a potential driver for ALK inhibitor resistance [386].

Table 1: ALK TKIs in clinical testing. Adapted from (363)

ALK TKIs	**Crizotinib (PF-0231066)**	**Ceritinib (LDK378)**	**Alectinib (CH5424802)**	**Brigatinib (AP26113)**	**Entrectinib (RXDX-101)**	**Lorlatinib (PF-06463922)**
Company	Pfizer	Novartis	Genentech	Ariad	Ignyta	Pfizer
Targets other than ALK	ROS1, MET	ROS1,IR IGF1R	RET, LTK, GAK	EGFR, ROS1	NTRKs, ROS1	ROS1
Phase	Phase III	Phase III	Phase III	Phase III	Phase II	Phase III
Approvals	Approved in 2011	Approved in 2014	Approved in Japan (2014), BTD in 2013	BTD in 2014	N/A	N/A
Resistance mutations associated with ALK TKIs	I1151Tins L1152P/R C1156T/Y I1171T/N/S F1174C/L/V V1180L L1196M **G1202R** S1206Y/C E1210K G1269S/A	I1151Tins L1152P/R C1156T/Y F1174C/L/V **G1202R**	I1171T/N/S V1180L **G1202R**	**G1202R** E1210K+ S1206C E1210K+ D1203N	**G1202R**	L1198F+ C1156Y
Resistance mutations targeted by TKIs	L1198F	I1171T/N **L1196M S1206Y/C G1269S/A**	L1152P/R C1156T/Y F1174C/L/V **L1196M S1206Y/C G1269S/A**	I1151Tins L1152P/R C1156T/Y F1174C/L/ V **L1196M S1206Y/C G1269S/A**	C1156T/Y **L1196M**	I1151Tins L1152P/R C1156T/Y I1171T/N/S F1174C/L/V V1180L **L1196M G1202R S1206Y/C** E1210K **G1269S/A**

1.8.1 Combinatorial treatment

Even though all ALK TKIs have high potency towards the ALK activity, each ALK TKI have a unique way of acquiring the ALK resistance mutations. So, combination of ALK TKI with ALK downstream targets or other bypass pathway inhibitors could be beneficial for ALK positive cancer forms and also prevents the ALK resistance mutations. Choosing the right target for poly-therapy is vital, since negative feedback signaling mediate by the target over time might lead to the development of resistance [387] (Paper III). Successful combinatorial treatments should not only show efficacy against mono-treatment, but also in tolerability of dosing levels in patients. Several combinations of ALK TKI with chemotherapy agents, immunotherapy agents and downstream target agents are currently being evaluated (Table 2).

Several preclinical studies have indicated the importance of combination of ALK and MEK inhibitors in ALK positive NSCLC, which led to the development of ALK and MEK combination in clinics [163, 363]. In a phase I trial combination of ALK inhibitor, ceritinib is combined with everolimus, an mTOR inhibitor or with LEE011, CDK4/6 inhibitor in NSCLC [363]. Combination of second generation ALK inhibitor alectinib with bevacizumab, an angiogenesis inhibitor is also currently being tested in ALK positive NSCLC patients (NCT02521051) [363]. Other than the targeted agents, ALK TKI are combined with immunotherapy agents like ipilimumab or nivolumab (NCT01998126) or nivolumab (NCT02584634) or pembrolizumab (NCT02511184) or avelumab (NCT02584634), however preclinical data for immunotherapy combinations are limited [363]. Several studies have indicated that use of anti- PD-1 (programmed cell death protein 1), nivolumab can produce durable activity in several cancer forms [363, 388]. However, Gainer and colleagues study shows that ALK rearranged NSCLC have low expression of PD-L1 in the tumor microenvironment [389]. Furthermore, they have shown that ALK positive NSCLC are associated with low response to PD-1 therapy [389]. Preclinical studies associated with combination of ALK TKI and other downstream target inhibitors have also been evaluated in mutated ALK full-length receptor. In 2014, Moore and colleagues have shown that combination of ALK inhibitor with PI3K/mTOR inhibitors reduce the proliferation of NB cells effectively when compared to the mono-treatment [189]. In same year Umapathy and colleagues have shown that combination of crizotinib, an ALK inhibitor with XMD8-92, an ERK5 inhibitor reduce the cell proliferation synergistically in ALK positive NB [210]. A recent study has shown that combination of ALK inhibitor and CDK4/6 inhibitor suppresses the cell growth of ALK positive NB. Similar to ALK positive NSCLC, it has been suggested that combination of ALK inhibitors with MEK inhibitors might be

beneficial for ALK positive NB [163, 164]. However, combination of ALK inhibitor and MEK inhibitor does not synergistically reduce NB cell proliferation (Paper III). Furthermore, MEK inhibition leads to increased AKT pathway activation via mTORC2 (Table 2) (Paper III). Taken together, combination of ALK inhibitor with PI3K/mTOR/ERK5 pathway inhibitors might be beneficial for ALK positive NB patients.

Table 2: Outcome of combinatorial treatment in ALK positive cancers

S.no	Combination	ALK$^+$ cancer types	Outcome
1.	ALK+MEK	EML4-ALK E13;A20 NSCLC	Synergistic inhibition of NSCLC cell proliferation and tumor growth
2.	ALK+MEK	ALK positive neuroblastoma	Increased activation of AKT via SIN1
3.	ALK+IGF-1R	EML4-ALK positive NSCLC	Synergistic inhibition of NSCLC cell proliferation and tumor growth
4.	ALK+mTORC1	NPM-ALK positive ALCL	Synergistic inhibition of ALCL cell proliferation and tumor growth
5.	ALK+AKT/mTOR	ALK positive neuroblastoma	Synergistic inhibition of NB cell proliferation and tumor growth
6.	ALK+mTORC1	ALK positive neuroblastoma	Increased activation of AKT via Rictor
7.	ALK+ERK5	ALK positive neuroblastoma	Synergistic inhibition of NB cell proliferation and tumor growth
8.	ALK+HSP90	EML4-ALK positive NSCLC	Synergistic inhibition of NSCLC cell proliferation and tumor growth
9.	ALK+CDK4/6	ALK positive neuroblastoma	Synergistic inhibition of NB cell proliferation and tumor growth
10.	ALK+CDK4/6	EML4-ALK positive NSCLC	In Phase I trial

2 AIMS

The overall aim of this thesis is to increase our understanding the oncogenic signaling events downstream of the full-length ALK receptor in neuroblastoma. Further, we aimed to identify potential therapeutic target/pathways for poly-therapy treatment for ALK positive neuroblastoma.

Specific aim

Paper I. We aimed to identify putative downstream signaling targets of ALK in neuroblastoma using phosphor-proteomic mass spectrometry analysis. Further, we aimed to investigate whether ALK activates STAT3 in ALK positive NB cells.

Paper II. We aimed to investigate the mechanism underlying ALK activation of ERK5 in ALK positive NB cells. Further, we investigated whether the combination of ERK5 and ALK inhibitors exhibited therapeutic synergy in ALK positive NB cell proliferation and tumor growth.

Paper III. We aimed to investigate the importance of targeting RAS-MEK-MAPK in ALK positive neuroblastoma. Further, we investigated whether MEK inhibitor alone or in combination with ALK inhibitor has therapeutic value in ALK positive NB.

3 RESULTS AND DISCUSSION

This section highlights the main findings of this thesis.

Paper I. Phosphoproteomic analysis of anaplastic lymphoma kinase (ALK) downstream signaling pathways identifies signal transducer and activator of transcription 3 as a functional target of activated ALK in neuroblastoma cells (Sattu K et al.,2013)

Previous studies have indicated the importance of STAT3 activation in ALK fusion cancer forms. In this study we aimed to investigate the importance of STAT3 activation in full length ALK receptor signaling.

Activated ALK phosphorylates STAT3 at Y705 in PC12 cells

Based on the phosphor-tyrosine proteomics screen, we identified STAT3 as significantly tyrosine phosphorylated upon ALK activation. To examine whether STAT3 is activated by full length ALK receptor, we initially employed PC12 cells expressing doxycycline –inducible wild type ALK and the ALK F1174S mutant. Activation of wild type ALK using mAb31 for 24 hours led to visible tyrosine phosphorylation of STAT3, this was not clearly observed after 30 minutes of mAb31 stimulation. In contrast, robust activation of STAT3 was observed upon expression of ALK F1174S mutant. In both the cases, inhibition of ALK activity using crizotinib abrogated STAT3 tyrosine phosphorylation. To further investigate the activation process, we explored whether ALK could interact with STAT3 and regulate its activation. In PC12 cells we were unable to observe an interaction between endogenous STAT3 and doxycycline-induced ALK. However, we were able to show an interaction when FLAG-tagged STAT3 was transiently co-transfected with wild-type ALK or ALK F1174S mutant. Upon stimulation of wild type ALK, interaction between ALK and STAT3 was enhanced and was abrogated by addition of crizotinib.

STAT3 activation is important for initiation of transcription of MYCN in response to ALK activation

Recent studies have indicated that ALK regulates MYCN transcriptionally in neuroblastoma cells and also co-ordinate with MYCN in neuroblastoma pathogenesis. Therefore we investigated a role for STAT3 in the regulation of MYCN. To examine this, we employed several ALK positive neuroblastoma cell lines. These neuroblastoma cell lines contain either constitutively activated ALK mutations (CLB-GE ALKF1174V, CLB-GA ALK R1275Q, and Kelly

ALKF1174L) or overexpressed ALK with an extracellular domain deletion (CLB-BAR Δexon 4-12). RNA interference (siRNA) mediated inhibition of STAT3 in ALK positive neuroblastoma cells led to reduction of MYCN protein expression. Further, pharmacological inhibition of STAT3 activation using FLLL32 and STATTIC also showed reduced MYCN protein expression in ALK positive neuroblastoma cells. To confirm the importance of STAT3 in ALK mediated MYCN transcription, we transfected neuroblastoma cells with a MYCNP-luciferase reporter. Using STAT3 inhibitors to inhibit STAT3, we observed reduced luciferase activity. This was further confirmed with quantitative RT-PCR (qRT-PCR). In keeping with these results, ALK positive neuroblastoma cells treated with STAT3 inhibitors for 24 hours showed a significant decrease in MYCN mRNA levels.

Inhibition of STAT3 activity suppress neuroblastoma cell growth

We next investigated whether STAT3 activity is important for neuroblastoma cell proliferation. To examine this, we treated neuroblastoma cells with STAT3 inhibitors and assessed proliferation over 5 days. Treatment with STAT3 inhibitors reduced neuroblastoma cell proliferation 30-50%. This result was further confirmed using small interference RNA targeting STAT3.

Altogether, our data implicates STAT3 as a potential downstream target of full length ALK. Activated ALK results in activation of STAT3 at Y705 in PC12 cells expressing either wild type ALK or ALK F1174S mutant, as well as in ALK positive neuroblastoma cells. Further, we have shown that STAT3 activity is important for MYCN transcription and that inhibition of STAT3 activity reduces neuroblastoma cell proliferation.

Paper II. The kinase ALK stimulates the kinase ERK5 to promote the expression of the oncogene MYCN in neuroblastoma (Umapathy G et al., 2014)

Previous studies have indicated that treatment with ALK TKI alone is not an effective solution for ALK positive neuroblastoma patients, so combination with ALK downstream targets might be beneficial. In this study we aimed to investigate whether oncogenic ALK activates ERK5 in ALK positive NB cells. Further, we investigated whether the combination of ERK5 inhibitor and ALK inhibitor have synergy effect in ALK positive NB cell proliferation and tumor growth.

ALK activates ERK5 via the PI3K pathway

Based on the phosphor-tyrosine proteomics screen, we identified ERK5 as a putative ALK downstream signaling target. To examine whether ERK5 is activated by full length ALK, we initially employed PC12 cells expressing doxycycline –inducible wild type ALK or the ALK F1174S mutant. Upon ALK activation, ERK5 is phosphorylated at T218/Y220 and phosphorylation of these sites was abrogated by the addition of either ALK or PI3K inhibitors. We next decided to investigate the role of ALK and ERK5 in ALK positive neuroblastoma cell lines, where ALK is constitutively activated. Similar to PC12 cells, ALK activates ERK5 in ALK positive neuroblastoma cell lines and this activation was abrogated in the presence of either ALK inhibitor (crizotinib), PI3K inhibitor (NVP-BEZ235), mTOR complex inhibitor (AZD8055) or ERK5 inhibitor (XMD8-92). Further, activation of ERK5 by ALK was confirmed in IMR-32 cells, where ALK is amplified in exon 3-4. Upon ALK activation in IMR-32 cells using activating monoclonal antibody (mAb46) led to robust activation of ERK5 and the activation was abrogated by ALK/PI3K pathway inhibitors.

Since ERK5 activation by ALK was sensitive to PI3K pathway inhibitors, we next wanted to investigate whether PI3K activity is important for ERK5 activation in ALK neuroblastoma cell lines. To examine the importance of PI3K activity, we transfected constitutively active (p110CAAX) or kinase dead (p110KD) variants of p110 in PC12 cells and ALK positive neuroblastoma cells. The kinase dead variant of p110 significantly reduced the activation of ERK5 in both PC12 cells and ALK positive neuroblastoma cell lines.

AKT phosphorylates MEKK3 to activate ERK5

Previous studies have indicated that ERK5 is activated via the linear signaling cascade (MEKK2/MEKK3 activates MEK5 activates ERK5). The data accumulated thus far suggests that ALK activates ERK5 via PI3K/AKT pathway. To examine whether AKT phosphorylates MEKK3, an upstream activator of ERK5, we used AKT substrate antibody to immunoprecipitate the AKT substrate proteins from ALK positive neuroblastoma cell lines. Immunoprecipitates were subject to immunoblotting with MEKK3 and GSK3β (a known AKT substrate) antibody. Addition of either ALK inhibitor or PI3K inhibitor abrogated the phosphorylation of both GSK3β and MEKK3 in ALK positive neuroblastoma cells, indicating that MEKK3 is a downstream signaling target of AKT. To examine the importance of MEKK3 activity in ERK5 activation by ALK, we used small interference RNA (siRNA) targeting MEKK3. RNA mediated inhibition of MEKK3 in ALK positive neuroblastoma cells led to a reduction in ERK5 phosphorylation.

ERK5 activation is important for initiation of transcription of MYCN

We next investigated the role of ERK5 in the regulation of MYCN expression, since previous work has shown that ALK activity is important for the initiation of MYCN transcription and also that PI3K pathway activity is important for MYCN protein stability. To examine this, we employed SHEP neuroblastoma cell lines, which stably express MYCN under the control of the CMV promotor. We observed that treatment with the PI3K inhibitor (NVP-BEZ235) abrogated the expression of both phosphorylated and total MYCN protein levels. Interestingly, the ERK5 inhibitor (XMD8-92) does not inhibit exogenously expressed MYCN protein levels, indicating that ERK5 activity might not be important for MYCN protein stability. Further, treatment with XMD8-92 significantly reduced MYCN mRNA levels in ALK positive neuroblastoma cell lines. Taken together, our results indicated that ERK activity is important for the initiation of transcription of MYCN, rather than MYCN protein stability.

Combining ERK5 and ALK inhibitors reduces neuroblastoma cell growth synergistically

Having established that ERK5 is activated by ALK in NB cells, we next wanted to investigate whether ERK5 activity plays a vital role in neuroblastoma cell proliferation. Pharmacological inhibition or siRNA mediated inhibition of ERK5 reduced the neuroblastoma cell proliferation up to 20 to 30%. Further, combination of ERK5 (XMD8-92) and ALK (crizotinib) inhibitors suppress

neuroblastoma cell growth synergistically. To examine the effectiveness of XMD8-92 and crizotinib as a combinatorial treatment *in vivo*, we injected human neuroblastoma cells (CLB-BAR) subcutaneously into BalbC/NUDE mice and treated with either crizotinib alone or XMD8-92 alone or in combination of both. Combination of XMD8-92 and crizotinib resulted in greater inhibition of tumor growth than the mono-treatment.

Altogether, our data indicates that ALK activates ERK5 via the PI3K/AKT/MEKK3 pathway and initiates the transcription of MYCN in neuroblastoma cells. Further, we have shown that combination of ALK inhibitor with ERK5 inhibitor was much more effective than the mono-treatment in both cells and xenograft models.

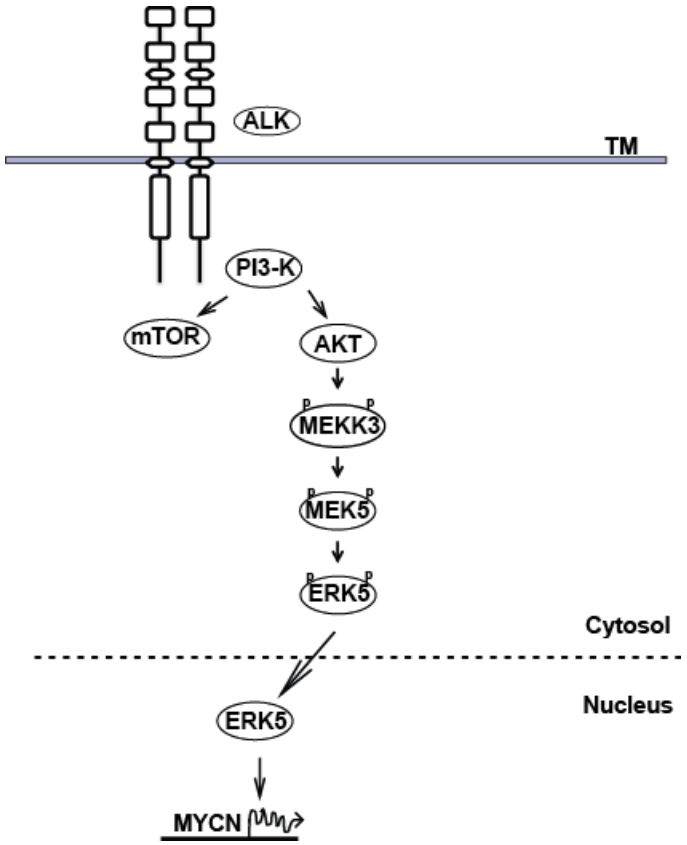


Figure 23- ERK5 is a target of ALK: *Schematic representation of activation of ERK5 by ALK in neuroblastoma.*

Paper III. Anaplastic lymphoma kinase addictive neuroblastoma cell lines are associated with growth upon treatment with MEK inhibitor trametinib (Umapathy G et al., 2014)

Recent studies have indicated that targeting the RAS-MEK-MAPK pathway might benefit the neuroblastoma patients. In this study we aimed to investigate whether MEK inhibitor alone or in combination with ALK inhibitors has therapeutic value in ALK positive neuroblastoma through evaluation of a large panel of NB cell lines.

Effectiveness of the MEK inhibitor trametinib in neuroblastoma cell lines and xenograft models

Recent studies have suggested that combined inhibition of ALK and MEK-ERK pathway targets may be beneficial as a poly-therapy in ALK positive neuroblastoma patients. To address this, we initially treated a large panel of neuroblastoma cell lines with the MEK inhibitor trametinib and assessed the proliferation over 12 days. The RAS-MAPK pathway activated neuroblastoma cell lines (SKNBE (2) and SKNAS) were sensitive to trametinib treatment. However, ALK-positive neuroblastoma cell lines (CLB-BAR, CLB-GE, CLB-GAR and Kelly) and p53 mutated cell lines (CLB-PE and SKNDZ) continued grow upon treatment with MEK inhibitor trametinib, indicating that trametinib treatment alone might not be beneficial for neuroblastoma patients. Further, to examine the effectiveness of trametinib *in vivo,* we injected human neuroblastoma cells (CLB-BAR, SKNAS) and EML4-ALK positive NSCLC cells (H3122) subcutaneously into BalbC/NUDE mice and treated with trametinib. Similar to *in vitro* assays RAS mutated and EML4-ALK positive NSCLC were sensitive to trametinib treatment, however tumor growth inhibition was not observed upon treatment of ALK addicted NB xenografts with trametinib.

Previous work has suggested that combining MEK inhibitor with ALK inhibitors synergistically reduces EML4-ALK positive NSCLC cell proliferation and tumor growth. Therefore, we investigated whether combination of ALK and MEK inhibitors would synergistically reduce ALK positive neuroblastoma cell proliferation. To examine this, we treated CLB-BAR and CLB-GE ALK positive NB cell lines with either lorlatinib alone or trametinib alone or a combination of both. Combination of lorlatinib and trametinib did not result in synergistic cell growth inhibition in ALK addicted NB cell lines when compared to single agent treatment. Taken together, our data indicates that treatment with trametinib alone or in combination does not offer additional benefit for ALK positive NB patients.

AKT pathway dependence in ALK positive neuroblastoma cell lines

We next set to investigate the mechanisms underlying the lack of effectiveness of trametinib treatment in ALK positive neuroblastoma cell lines. Since previous work has indicated that PI3K/AKT/mTOR/ERK5 pathway is vital for the survival of ALK positive NB cells, we investigated the activity of the AKT signaling pathway in response to trametinib treatment. To address the role of the AKT signaling core, ALK positive neuroblastoma cell lines were treated with either trametinib or lorlatinib. Treatment with trametinib in ALK positive neuroblastoma cells led to a 2-fold increased phosphorylation levels of both AKT (S473) and ERK5, indicating the importance of AKT-ERK5 signaling core in ALK positive neuroblastoma cells. To further evaluate the dependence of the AKT-ERK5 signalling pathway, we treated ALK positive neuroblastoma cells with PI3K (BEZ 235) and ERK5 (XMD8-92) inhibitors and checked for increased activation of MAPK. As expected both PI3K and ERK5 inhibitors reduce the phosphorylation levels of AKT (S473) and ERK5 and increased activation of MAPK was not observed. Altogether, our data reveal the importance of AKT-ERK5 signalling core in ALK positive neuroblastoma cells.

To evaluate the importance of ALK signal in AKT 'super-activation', we employed the IMR-32 neuroblastoma cell line, which does not contain kinase domain mutations of ALK. We observed that stimulation of ALK in IMR-32 cells with FAM150A led to the phosphorylation of both AKT (S473) and ERK5, but treatment with trametinib increased the phosphorylated levels of both AKT (S473) and ERK5 up-to 3- fold; supporting the involvement of ALK full length receptor in the increased activation of AKT signaling core.

mTOR complex 2 drives the super-activation of AKT in ALK positive NB cells upon treatment with trametinib

To examine whether increased activation of AKT is caused by reactivation of Receptor Tyrosine Kinase (RTK) signaling following MEK inhibition, ALK-positive neuroblastoma cell lines were treated with trametinib and reactivation of RTKs was investigated using phospho-RTK array. However, treatment with trametinib in ALK-positive neuroblastoma cells was unable to show any significant reactivation of specific additional RTKs. Since RAS could interact with PI3K and enhance the AKT signaling, we treated ALK positive NB cells with trametinib and investigated the RAS-GTP levels. However, increase in RAS-GTP levels was not detected upon treatment with trametinib. Further, we also examined the lipid ratio of PIP3/PIP2 in ALK positive NB cells treated with trametinib. Similar to the RTK array and RAS-GTP assays, no decrease

or increase of the PIP3/PIP2 lipid ratio was observed, indicating that crosstalk between MAPK and AKT signaling pathways may be responsible for super-activation of AKT signaling axis.

Previously it has been shown that mTOR complexes are involved in many feedback mechanisms, ALK positive NB cells were treated with MEK inhibitor (trametinib), PI3K inhibitor (BEZ 235), mTORC1 & mTORC2 inhibitor (AZD 8055) and mTORC1 inhibitor (everolimus). Both NVP-BEZ 235 and AZD 8055 efficiently blocked the activation of AKT, critically we also found that blocking mTORC1 using everolimus also super-activate AKT similar to the treatment of trametinib, indicating another feedback mechanism via S6K- Rictor (T1135) dependent manner. Interestingly, we also found that super-activation of AKT (S473) following MEK inhibition is Rictor (T1135) independent. Furthermore, combination of trametinib with PI3K or mTOR complex inhibitors abrogated the activation of AKT when compared to the combination of trametinib with mTORC1 inhibitor, suggesting the role of mTOR complex 2 in the increased activation of the AKT signaling axis in ALK positive neuroblastoma cell lines. To confirm the involvement of mTORC2 in super-activation of AKT signaling axis in ALK positive neuroblastoma cell lines, we inhibited mTORC2 activation using small interference RNA (siRNA) targeting Rictor. Compared with scrambled control siRNA, two independent Rictor siRNAs abrogated the phosphorylation of AKT (S473), whereas treatment with trametinib resulted in super-activation of AKT in scrambled siRNA, but not in Rictor siRNA treated ALK addicted NB cells.

Targeting MEK-ERK signaling pathway super-activates AKT via Sin1 T86 phosphorylation

Our data thus far indicates that MEK-ERK pathway inhibition in ALK positive neuroblastoma cells super-activates AKT signaling axis via mTORC2 in a Rictor (T1135) independent manner. Since previous studies have indicated that Sin1 phosphorylation is important for mTORC2 kinase activity, we investigated Sin1 (T86) phosphorylation upon treatment with trametinib or SCH 772984 (ERK1/2 inhibitor) in ALK positive NB cells. We observed that Sin1 T86 phosphorylation was increased following MEK and ERK inhibition, whereas in combination with BEZ 235 super-activation of Sin1 T86 phosphorylation was reduced when compared to MEK or ERK inhibitors alone. We also verified these results with small interference RNA (siRNA) targeting Sin1. We observed that ALK positive neuroblastoma cells in which Sin1 was knocked down showed a significant reduction in super-activation of AKT (S473) phosphorylation following MEK inhibition when compared to scrambled siRNA controls.

Altogether, our data indicate that blocking MEK-ERK signaling pathway in ALK positive NB cells leads to an increased activation of AKT signaling axis via increased Sin1 T86 phosphorylation.

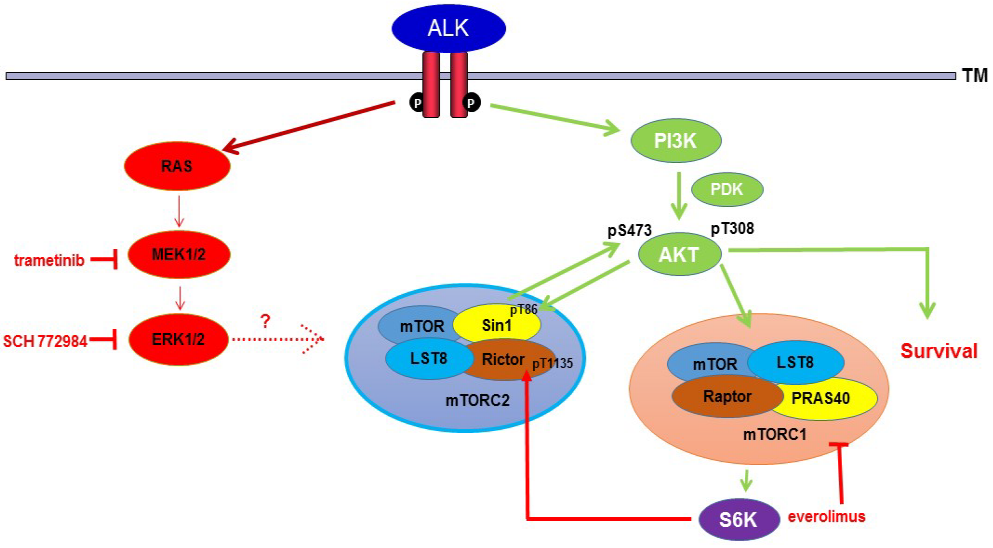


Figure 24- Feedback activation in ALK positive neuroblastoma: *A pictorial representation of different feedback activation in ALK positive neuroblastoma upon inhibition of its downstream targets.*

4 CONCLUSION

Paper I.

- STAT3 was identified as an important target of ALK signaling.
- Activated ALK phosphorylates STAT3 at Y705 in both PC12 cells and neuroblastoma cell lines.
- STAT3 activity is important for neuroblastoma cell proliferation.
- Upon ALK activation STAT3 regulates the initiation of transcription of *MYCN* and may therefore be a potential therapeutic target in NB.

Paper II.

- ERK5 is a target of ALK signaling in neuroblastoma.
- ALK activates ERK5 through a pathway mediated by PI3K, AKT and MEKK3.
- ERK5 activity is important for initiation of transcription of *MYCN* in neuroblastoma.
- Pharmacological or siRNA mediated abrogation of ERK5 suppresses neuroblastoma cell proliferation and may therefore be a potential poly-therapy target in ALK positive NB.
- Combination of ALK and ERK5 inhibitors synergistically reduce neuroblastoma cell proliferation. Therefore targeting ERK5 and ALK may be beneficial in NB patients.

Paper III.

- Inhibition of MEK signaling was not effective in ALK positive neuroblastoma cell lines.

- MEK inhibition in ALK positive NB cell lines leads to increased activation of AKT and ERK5.

- Increased activation of AKT is mediated via the mTOR complex 2 protein SIN1.

- Combination of ALK and MEK inhibitors does not synergistically reduce ALK positive NB cell proliferation.

- Our results contraindicate use of MEK inhibitors as effective single and poly-therapeutic strategy in ALK-positive neuroblastoma.

ACKNOWLEDGEMENTS

Welcome to the most read page of the thesis.

First and foremost, I want to thank my supervisor **Professor Bengt Hallberg** for providing me this opportunity to work with him in this exciting field of ALK. Thanks for your encouraging words and coming along with me through every step of my PhD studies. Thanks for bearing me all these six years. I am not pretty sure that all PhD students will get freedom which you gave me, that made me to think and work independently. Despite of your busy schedule, thanks for having time for discussions, suggestions, manuscript preparation and more importantly giving suggestions for my future carrier. You are an amazing person with lot of energy. Certain quotes from you will remain with me wherever I go, Black is Black (western blotting), for ------- sake, connect with ALK, how is the cellsssssssss, positive and negative controls, Running Gelssss, where is the Fikaaa and finally, love you my love (to Jennifer). Finally, a big thank you for having belief in me over these years. **Professor Ruth H. Palmer,** also my co-supervisor and reviewer. Thanks for your valuable suggestions on my projects/presentation/manuscript and guiding me throughout my PhD studies. Your passion towards science is amazing, I am grateful that I had an opportunity to work in a project with you. I also admire the way of your thinking when it comes to critical experiments. Thanks for your time and sharing your technical knowledge despite your very busy schedule. Bengt and Ruth, your dedication to science and the way you complement each other is your greatest strength, keep on going. THANKS.

When I started out my Master thesis, I hardly had any technical experience in the field. I want to really thank **Christina Schönherr** and **Rutan** for introducing me to molecular biology techniques. Getting nicer western blots was not that easy until I met the 'western guy' **Kamaraj**. Thanks for your suggestions and being patient with me in my ups and downs throughout the stay in Umeå and also now☺. I learned quite a lot of things from you, especially the tasty briyani which you cook. I want to thank you from my

bottom of heart the help you provided me during my worse phases. **Damini**, my colleague and friend, we both started our studies together, when I look back those years we had quite a lot of fun filled memories. Thanks for your time, our chit chat, gossips and our teaching combo will stay with me forever. Thank you **Abeer**, for your critical suggestions and introducing me too the *in vivo* field, you're an amazing person and I wish you all the success in your future endeavors. **Barbara** thanks for your time and suggestion during my ERK5 paper. Thanks, **Jiqui** for sharing your technical experience and knowledge. Thanks for bearing me in the office cabin. I had a great experience working with you in several projects and thanks for your time. I wish you a great success in your future endeavors. Thanks **Yasuo**, when you're around positive energy will be surrounded, thanks for sharing your technical knowledge. I wish you all success in your scientific carrier. Thanks **Joachim**, my student ☺ I have seen your growth in last three years it's amazing, I wish you all success in your PhD. Thanks **Georg**, for sharing your technical experience and your vital inputs during the project presentation and I always admire your passion towards science. Thanks **Kathrin**, for your fun chats and your critical suggestions during JC and project presentation. I wish you both all success in your future endeavors. Thanks **Diana**, for your 'jokes' which will remain in my memory wherever I go. You are the one who I have annoyed the most in the lab, thanks for that freedom which you gave me☺. Thank you, **Asha** my fellow Indian friend ☺ in the lab. You are very kind and nice person, it was always nice talking to you. I wish you all success in your PhD. Thanks **Dan**, for your help during the mice experiments and it always nice talking to you. Also, not allowing us to lift heavy things☺. **Wasi, Ezgi, Martin and Maite** thanks for your suggestions during journal club and project presentations and for the fika☺. Overall, it was an amazing experience in both the labs, once again thank you all☺.

Professor Tommy Martinsson, also my co-supervisor, thanks for your collaboration and critical suggestions throughout my PhD studies

Thanks **Professor Anne Uv,** for your time and helping us to set up the lab in Gothenburg, it was helpful for us when we initially came in. It was always fun when you're around.

I want to thank all my collaborators for their vital inputs in my manuscript, **Mikeal Johansson, Sameul, Louis Chesler, Nathanael S. Gray and Per Kogner.** A special thanks to Per Kogner. I also want to thank my funding agencies- barncancerfonden, Assar Gabrielssons Fond, Albertska Forskningsstiftelsen for supporting my projects.

Edvin and Chinmay, my best buddies back in Umeå and now as well☺. Thanks for the wonderful late evening parties, gossips, food, badminton and also memory game☺. It's always nice speaking to you guys and I wish you all success in your future scientific carriers. **Radha, Geetanjali, Swarupa, Vivek, Saravana, Fazil, Brijesh, Vimal,** thanks for your memories during my stay in Umeå.

Thuccani family (Anna, Gayu, Meenamma, Hari-the ten pack boy, Joey), you are the main reason that we are staying in Gothenburg so long. Your encouraging words and your support to us was vital for our carrier which will remain in our memory forever. Thanks for the lovely time you provided us, especially the late evening chit chat, gossips, games, tasty food, parties and also introducing us to the 'gothenburg nanbargal group' which made us feel like home. **Nanbare** (Netaji -the photographer, komagal,Yuma), I want to thank you for the memories which you gave me was truly amazing. I remember the day we met for the first time, you're the same lovely person till now☺. **Lidl family** (Vaide-Yummy, Rev-always right, chella kuty-coming soon), **Cake family** (Hermus-silent killer, Steffi-pasamana thangachi, Hayren-car boy), **IKEA family** (Arun-my fellow counter, Sasi-one and only universal cop, Advaith-little hero), **Pravin family** (Pravin-buisness kantham, Selva-cinema kantham, Sunil-Phone kantham, Suriya-FB live kantham, Siva-car kantham)thanks for your lovely moments, late night gossips, chit chat, games, cake, food, parties and end of the day we had only fun☺. **Lakshmi,**

Marimuthu Anna, Anand, Grace, Raji, Vettri, Brindha, Ashwin, Kasthuri, Rajesh, Subazini, Ashok, Sravani, Murali, Narthana, thanks for your time and for your wonderful moments. Thanks, **Vignesh ji,** my friend, we had a quiet a lot of funny movie discussions and also some scientific discussions, it was always nice speaking to you. Thanks, **Priya and Taral-**machan, my friends, who shared many fun filled moments during these years.

I want to specially thank **Radhakrishnan, Dhanalakshmi, Santhanakrishnan, Meera Bai,** for their love and affection which they have given me in the past, you are always special. I also want to thank, **Mohanakrishnan family, Chinnikrishnan family, Padhu family, Periya atthai family, Rukumani family, Bommi atthai family, Chandar family, Suresh family, Ashok family, Jaikumar family and Murali Mohan Vittal family** for their memories. I also want to thank **Dr.Srinivasan family and Nandagopal family** for their love and support they shown these years, especially nandagopal sir for your contribution during my school days.

Last but not the least, **my family (Appa dei, Mummy, Papa, Appu, Ramu),** you people are my strength. You people always supported me in critical situations and who kept belief in me all these years. Without your love and support, I would have not reached this stage. Especially Mummy you are amazing, you are such a gem of a person, I am really very lucky. The struggle which you went in my earlier stages was unexplainable. You always supported my decisions and sacrificed lot of things only for me. Finally, love you my love☺.

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APPENDIX