Mechanistic Implications and Characterization of Anaplastic Lymphoma Kinase (ALK) mutations in Neuroblastoma

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Akademisk avhandling

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Abstract
Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that was first reported as a fusion partner of nucleophosmin in Anaplastic large cell lymphoma in 1994. ALK is involved in myriad of cancers including neuroblastoma which is the most common extracranial solid tumor affecting young children. It arises in the neural crest cells of sympathetic nervous system origin and is responsible for 12% of all childhood cancer deaths. Several point mutations in ALK have been described in both familial and sporadic neuroblastoma.

With the aim to understand the role of ALK in neuroblastoma further, we investigated the point mutations in ALK reported in patients. Using cell culture based methods and Drosophila as a model organism; we first characterized these mutations under three broad categories: 1) Ligand independent mutations that were constitutively active, 2) Kinase dead mutation and 3) Ligand dependent mutations that behaved as inducible wild type. Further, to understand the activation mechanism of ALK, we constructed mutations that could potentially alter ALK’s conformation based on the available crystal structure. From the data generated, we were able to provide a new perspective to the activation of full length ALK receptor. This was more in line with activation mechanism of insulin receptor and different from that suggested for ALK fusion protein. From a clinical point of view, all the mutations in the study were blocked to different degrees using the ALK inhibitor, crizotinib. Lastly, we identified potential downstream targets of ALK using phosphoproteomics. From the various targets identified, we focused on STAT3 and confirmed its role as a mediator in ALK initiated MYCN transcription. We showed that STAT3 inhibition led to reduction of MYCN levels and thereby identifying it as a potential therapeutic target in neuroblastoma. Overall, our study highlights clinical relevance of ALK mutations in neuroblastoma and from a basic biology viewpoint; it reveals important mechanistic insight into receptor activation.

Keywords  
neuroblastoma, ALK, crizotinib, receptor tyrosine kinase, STAT3, MYCN

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Mechanistic Implications and Characterization of Anaplastic Lymphoma Kinase (ALK) mutations in Neuroblastoma

Damini Chand
To my dearest Dad,

I love you and I miss you even more, even more so now.
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Abstract

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that was first reported as a fusion partner of nucleophosmin in Anaplastic large cell lymphoma in 1994. ALK is involved in myriad of cancers including neuroblastoma which is the most common extracranial solid tumor affecting young children. It arises in the neural crest cells of sympathetic nervous system origin and is responsible for 12% of all childhood cancer deaths. Several point mutations in ALK have been described in both familial and sporadic neuroblastoma.

With the aim to understand the role of ALK in neuroblastoma further, we investigated the point mutations in ALK reported in patients. Using cell culture based methods and Drosophila as a model organism; we first characterized these mutations under three broad categories: 1) Ligand independent mutations that were constitutively active, 2) Kinase dead mutation and 3) Ligand dependent mutations that behaved as inducible wild type. Further, to understand the activation mechanism of ALK, we constructed mutations that could potentially alter ALK’s conformation based on the available crystal structure. From the data generated, we were able to provide a new perspective to the activation of full length ALK receptor. This was more in line with activation mechanism of insulin receptor and different from that suggested for ALK fusion protein. From a clinical point of view, all the mutations in the study were blocked to different degrees using the ALK inhibitor, crizotinib. Lastly, we identified potential downstream targets of ALK using phosphoproteomics. From the various targets identified, we focused on STAT3 and confirmed its role as a mediator in ALK initiated MYCN transcription. We showed that STAT3 inhibition led to reduction of MYCN levels and thereby identifying it as a potential therapeutic target in neuroblastoma. Overall, our study highlights clinical relevance of ALK mutations in neuroblastoma and from a basic biology viewpoint; it reveals important mechanistic insight into receptor activation.
Abbreviations

ABL- Abelson murine leukemia viral oncogene homog 1
ACK- Activated CDC42 kinase
Akt - AKR thyoma
ALCL- Anaplastic large cell lymphoma
ALK- Anaplastic lymphoma kinase
ALO17- ALK lymphoma oligomerization partner on chromosome 17
ARID1A/B- AT rich interactive domain 1A/B
ATC- Anaplastic thyroid tumor
ATIC- 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cydohydrolase
ATP- Adenosine triphosphate
ATRX- Alpha Thalassemia/Mental Retardation Syndrome X-Linked
BARD-1- BRCA-1 associated RING domain protein 1
BDNF- Brain-derived neurotrophic factor
BIM- BCL2-interacting mediator of cell death
BRAF- v-raf murine sarcoma viral oncogene homolog- B
BTD- breakthrough therapy designation
C2orf44- chromosome 2 open reading 44
CADM1- cell adhesion molecule1
CAMTA1- calmodulin binding transcription activator 1
CARS- cysteiny1-tRNA synthetase
CHEK2- checkpoint kinase 2
CLTC-1- clathrin heavy chain-like 1
CML- Chronic myelogenous leukemia
CNS- Central nervous system
CrkL- Crk (CT10 (chicken tumor virus number 10) regulator of kinase) like
DDR1/2- discoidin domain receptors 1/2
DLBCL- Diffuse large B-cell lymphoma
Dok2- Docking protein2
Dpp- Decapentaplegic
Duf- Dumbfounded
EGFR- Epidermal growth factor receptor
EML4- echinoderm microtubule-associated protein-like 4
Ep- Ephrin
ERK- Extracellular signal-regulated kinase
ESCC- Esophageal squamous cell carcinoma
FAM150- Family with sequence similarity 150
FAK- Focal adhesion kinase
FDA- Food and Drug Administration
FGF- Fibroblast growth factor
FGFR- Fibroblast growth factor receptor
FISH- fluorescence in situ hybridization
Flt3- Fms-like tyrosine kinase receptor-3
FN1- Fibronectin 1
FOXO3a- forkhead box O3a
FRS-2- FGF receptor substrate-2
GAK- Cyclin G-associated kinase
GIST- Gastrointestinal tumor
Grb2- Growth factor receptor-bound protein 2
GSK-3α/β- glycogen synthase kinase 3-α/β
HSP- Heat shock protein
HEN-1- hesitation behaviour-1
IGF1R - Insulin-like growth factor-1 receptor
IGSF4- Immunoglobulin superfamily 4
IHC- immunohistochemistry
IMT- Inflammatory myofibroblastic tumor
IR- Insulin receptor
IRK- Insulin receptor kinase
IRS1/2- insulin receptor substrate1/2
JAK- Janus kinase
Jeb- Jelly Belly
KIF5B- kinesin family member 5B
Kirre- k of irregular chiasm
KLC1- kinesin light chain 1
LDLa- Low density lipoprotein class A
LTK- Leukocyte tyrosine kinase
MAM- Meprin A-5 protein and receptor protein tyrosine phosphatase Mu
MAPK- Mitogen-activated protein kinase
MET- Mesenchymal-epithelial transition
MIBG - Meta-iodobenzyl-guanidine
Miple- Midkine and Pleiotrophin
MK- Midkine
MSN - Moesin
mTOR- mammalian target of rapamycin
MuSK- muscle specific kinase
MYH9- non-muscle myosin heavy chain 9
NGF- Nerve growth factor
NPM- Nucleophosmin
NRAS- neuroblastoma RAS viral (v-ras) oncogene homolog
NSCLC- Non small cell lung cancer
Org- Optomotor-blind-related-gene-1
PAGE- Polyacrylamide gel electrophoresis
PC12- Pheochromocytoma 12
PDB- Protein data bank
PDL-1- Programmed death ligand-1
PHOX2B- paired-like homeobox 2b
PI3K- Phosphoinositide-3 kinase
PINK1- Phosphatase and tensin homolog (PTEN)-induced putative kinase 1
PLCγ- phospholipase Cγ
PPF1BP1- protein-tyrosine phosphatase receptor-type F polypeptide-interacting protein-binding protein 1
Papers as part of this thesis

This thesis is based on the given publications which will be referred to as Paper-I, Paper-II and Paper-III in the following chapters. The articles are reprinted with permission from the respective journals.


**PAPER-II Chand D#, Guan J#, Yamazaki Y, Dijk JV, Hugosson F, Ruuth K, Palmer RH and Hallberg B.** *(#shared first authors)*

Novel mechanisms of ALK activation revealed by the analysis of Y1278S neuroblastoma mutation. *(Submitted Manuscript)*


Introduction

1. Protein phosphorylation

About one third of proteins in the human proteome are phosphorylated [1], making protein phosphorylation one of the most prevalent and well-studied post-translational modifications [2, 3]. The first report of phosphate in vitellin (phosvitin) emerged in 1906 by Phoebus Levene and colleagues [4]. In 1950s, Edmond H. Fischer and Edwin G. Krebs described how reversible phosphorylation works to activate proteins through their studies on glycogen phosphorylase [5]. For this remarkable finding, Fischer and Krebs received the coveted Nobel prize in Physiology and Medicine in 1992. Subsequently, the first eukaryotic tyrosine kinases were discovered through studies on polyoma virus middle T-antigen and rous sarcoma virus v-src protein [6-8]. This key discovery introduced tyrosine kinase to the burgeoning field of kinases. The other large sub group of kinases comprises of serine and threonine kinases that phosphorylate serine and threonine residues.

Protein phosphorylation is an important feature of cellular processes such as cell growth, apoptosis, metabolism, transcription, cell movement, cytoskeletal rearrangement and in intercellular communication [9]. It regulates protein function by controlling the catalytic activity via conformational changes that may activate or inactivate the protein. Moreover, phosphorylated proteins act as recruiting ground was made evident through the significant discovery of Src homology 2 (SH2) domain by Tony Pawson in 1990 [10]. Various proteins that have such structurally conserved domains recognize and bind to phosphomotifs. For example, SH2 and phosphotyrosine binding (PTB) domains show specificity for phosphotyrosine (pY). This ability of phosphoproteins determines the extent and longevity of a signal response [1].
1.1 Protein kinases and phosphatases

Phosphorylation is a reversible process that is mediated by kinases and phosphatases, which phosphorylate and dephosphorylate substrates, respectively [11]. Phosphorylation occurs principally on three amino acid residues serine, threonine and tyrosine in eukaryotes. Additionally, phosphorylation at histidine residues is suggested to make up about 6% of the total phosphorylation in eukaryotes [12]. Kinases facilitate the transfer of gamma phosphate group from adenosine triphosphate (ATP) to amino acid side chain. There are a total of 566 protein kinases encoded by the human genome including both typical and the atypical (distantly related or unrelated) eukaryotic protein kinases [13]. While, most are serine/threonine kinases, tyrosine kinases form a modest group of about 90 members [9]. Phosphatases, on the other hand, are enzymes that remove the phosphate group from the amino acid and bring them back to the inactive ‘OFF’ state (Figure-1). Although, that is not always the case, sometimes phosphorylation can lead to an inactive state for e.g. Src when phosphorylated at Tyr527, interacts intramolecularly with SH2 domain that leads to an inactive src [14]. Together, kinases and phosphatases regulate the degree of phosphorylation of proteins in the cell.

![Figure 1 - Interplay between kinases and phosphatases](image)

**Figure 1-Interplay between kinases and phosphatases:** An illustration showing phosphorylation and dephosphorylation of proteins by kinases and phosphatases. ‘ON’ indicates an active state and ‘OFF’ state refers to an inactive protein.
2. Receptor Tyrosine kinase family

The tyrosine kinase family of 90 members is further sub divided into 58 receptor tyrosine kinases (RTK) and 32 non-receptor tyrosine kinases [9]. The RTK family consists of 20 sub families [15]. All RTKs share a common architecture comprising an extracellular ligand binding domain, a transmembrane domain and an intracellular domain that consists of the highly conserved kinase domain [15-20]. Most RTKs with the exception of Insulin receptor (IR) exist as monomers at the cell surface in the absence of a ligand. IR is an inactive heterodimer consisting of two disulfide linked polypeptide chains (α2β2) [21, 22].

The general mechanism of receptor activation involves four key events [15, 23].
1. Ligand binding
2. Receptor dimerization/oligomerization
3. Trans autophosphorylation
4. Assembly and activation of intracellular signaling proteins

2.1 Ligand binding and receptor dimerization

There are four ways in which receptor dimerization may occur. Most of the 58 RTKs will follow one of these ways [15]. Some exceptions are discoidin domain receptors 1/2 (DDR1/2), RTK-like orphan receptor (ROR), muscle-specific kinase (MuSK) and related to receptor tyrosine kinase (Ryk)[15].

A. **Ligand induced dimerization**- As in the case of nerve growth factor (NGF) and Tropomyosin receptor kinase A (TrkA) where the ligand NGF dimer binds to two Trk A molecules simultaneously [24].

B. **Ligand mediated with receptor contact**- Here, although stem cell factor (SCF) homodimer crosslinks two KIT receptor molecules. The two KIT receptors themselves make direct contact with one another [25].

C. **Using accessory molecule**- This type of dimerization involves an accessory molecule which in case of fibroblast growth factor receptor (FGFR) is heparin. Here, two FGF ligands bind two FGFR monomers and two heparin molecules resulting in a complex dimer [26].

D. **Strictly receptor mediated dimerization**- The epidermal growth factor receptor (EGFR) family follows
a different way where the ligand is not directly involved in dimerization. The two receptors exist in intramolecular autoinhibition. Binding of the bivalent ligand leads to conformational changes that stabilize the receptor dimer [27].

2.2 Trans autophosphorylation and release of cis-auto inhibition mode

The activation of RTKs requires the release of autoinhibition mode. Autophosphorylation helps the kinase to adopt an active conformation by phosphorylation of key tyrosine residues that reorients the kinase to release from the autoinhibition state. Even though, the structures of activated forms of tyrosine kinase domains (TKDs) of RTKs are all very similar [28], the inactivated TKDs differ significantly which is implied through the four different autoinhibition modes discussed below.

A. **Activation loop autoinhibition** - In IR, Insulin-like growth factor-1 (IGF1) receptor and FGFR, the activation loop traverses the active site and makes direct contact with it. This blocks access to substrate protein (in FGFR) or both ATP and substrate protein (in insulin and IGF1 receptors) to the active site. Phosphorylation of tyrosines in the activation loop disturbs these interactions and shifts the kinase to an active conformation [28, 29].

B. **Juxtamembrane autoinhibition** - This kind of autoinhibition occurs outside the TKD. The juxtamembrane domain interacts with parts of TKD including the activation loop keeping the autoinhibited conformation stabilized. Examples of this are, Flt3 [30], KIT [31] and Eph family RTKs [32]. Phosphorylation of important tyrosines in juxtamembrane disrupts the autoinhibition and renders the TKD into active state.

C. **C-terminal tail autoinhibition** - In a similar manner, C-terminal tail in the Tie2 receptor blocks the substrate from reaching the active site. Autophosphorylation of tyrosines in the C-terminal tail will release the autoinhibition [33].

D. **Allosteric autoinhibition** - EGFR uses allosteric autoinhibition where in N-lobe of one receptor (activator) interacts with C-lobe of the other (receiver). Activator destabilizes the autoinhibition in the receiver TKD [34].
2.3 Assembly and activation of intracellular signaling proteins

Auto-phosphorylation of the receptor itself is the first phase of phosphorylation. It is followed by a ‘second phase’ autophosphorylation that creates phosphorylated tyrosines binding sites for signaling molecules containing SH2 and PTB domains [15, 35, 36]. These cytoplasmic signaling molecules may also bind indirectly to the receptor via docking proteins. For example, FGF receptor substrate-2 (FRS-2) family members, FRS-2α and FRS-2β behave as docking proteins and mediate signaling for FGF and NGF receptors [16]. Therefore, an activated RTK can influence many different signaling targets via its multiple phosphotyrosine sites and a range of docking proteins that it can phosphorylate.

This is why regulation of RTK signaling ought to be stringent to keep a check on its activity. It can be achieved by either a positive feedback loop, e.g. continued activation of EGFR is reached through transient inhibition of the activity of phosphotyrosine phosphatases (PTPs) [37]. Alternatively negative feedback mechanism by direct activation of PTPs, as an example, SH2 domain containing phosphatases (Shp1/PTPN6) and (Shp2/PTPN11) dephosphorylate EGF receptors, thereby inhibiting activation of the receptor [15].

2.4 RTKs in oncogenic signaling and as drug targets

Despite strict regulation of RTK activity, perturbed activity of kinase has been reported in many diseases including cancer. This can be due to autocrine activation, chromosomal translocation, overexpression or gain-of-function mutations in the kinase [15, 38]. Their involvement in about 50% of oncogenic malignancies makes them a viable druggable target. The first tyrosine kinase inhibitor (TKI) of Abl non-receptor tyrosine kinase, ‘Gleevec’ or imatinib was approved for treatment in chronic myelogenous leukemia (CML) in 2001[39]. It is also potent in gastrointestinal tumors (GISTs) where it blocks the activity of the KIT receptor. Despite the use of several TKIs in cancer therapy, major challenges include side effects due to lack of selectivity towards one target alone and acquired resistance towards the drug. There is therefore a growing need to develop specific TKIs that
can help overcome these problems and improve treatment protocols in patients.

3. Anaplastic lymphoma kinase (ALK)

In 1994, ALK was first discovered as a fusion partner of nucleophosmin (NPM) in Anaplastic large cell lymphoma (ALCL) [40, 41]. The rearrangement fuses NPM on chromosome 5q35 to ALK on 2p23 resulting in a hybrid dimer. In 1997, full length ALK receptor was identified [42, 43]. ALK has highest sequence similarity with IR family. Also, it is very similar (50% amino acid identity) to the leucocyte tyrosine kinase (LTK) [42, 43]. Thus, ALK forms a sub group with LTK under the IR super family.

The function of ALK is still unclear. It is proposed to play a role in development of nervous system, since ALK is expressed in nervous system during embryogenesis. But little or no ALK is detected in adult tissues in mice [43]. Human ALK gene encodes a protein of 1620 amino acids giving rise to a protein with a molecular weight of approximately 180 kD. As a result of post-translational modifications like N-linked glycosylations, ALK is detected at 220 and 140 kD when analyzed by SDS-PAGE [42].

3.1 Domain structure of ALK

Resembling the domain architecture of other RTKs, ALK consists of an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (Figure 2). An N-terminal signal peptide helps in the transport of ALK to the cell membrane. The extracellular portion of ALK comprises of several domains that include two MAM (Meprin A-5 protein and receptor protein tyrosine phosphatase Mu), one LDLα (Low density lipoprotein class A) domain and a glycine rich domain [42-46]. The function of each of these domains is not clearly identified. However, LDLα domain is suggested to play a role in ligand binding owing to its involvement in binding of LDL receptor and LDL [47, 48]. MAM domains are speculated to be involved in cell-cell interactions [49], although their functional significance is unclear as is the case of glycine rich domain. However, it is worth mentioning that in Drosophila ALK, both MAM and glycine rich domains are critical for the activation. Point mutations in MAM and glycine rich (replacing glycine with acidic amino acids) domains, lead to an inactive receptor [50].
3.2 ALK ligand and function in model organisms

A. Mammalian ALK

In mammals, ALK is amongst the very few RTKs that do not have a known ligand and is considered as an ‘orphan receptor’ so far. Pleiotrophin (PTN) and Midkine (MK), which are both heparin binding molecules have been suggested as ligands for ALK [45, 51-55]. However, subsequent studies failed to confirm their role in ALK’s activation [56-59]. A very recent report suggested heparin as a ligand for ALK [60]. This report also proposed that other sulfated proteoglycans might function as ligand or co-ligand for ALK and that the mechanism of binding and activation may be similar to fibroblast growth factor receptors (FGFRs) binding by heparin and FGF [15]. Recently in 2014, Zhang and colleagues showed FAM150A and FAM150B as ligands for LTK [61]. They screened the extracellular proteome...
(3191 extracellular proteins) to find FAM150A and FAM150B that stimulated LTK phosphorylation. Since LTK is a close homologue of ALK, it will be worthwhile to check if these molecules bind and activate ALK. Identification of additional ligands for ALK will enable better understanding of the biological role of the receptor.

Owing to the extensive ALK mRNA expression in the nervous system during mouse embryogenesis, it is postulated to play an important role in normal development and function of the nervous system [42, 43, 62]. However, ALK function is not clearly defined in mammals. This expression pattern is also seen in case of developing central nervous system (CNS) in chick [63] as well as in dorsal root ganglia in rat [64]. Consistent with the expression pattern in mouse, ALK expression is also seen in tissue samples from the adult human CNS [65]. A role for ALK in neuronal differentiation in PC12 cells is shown through studies conducted by several groups [56, 57, 66-69]. ALK mutant (ALK knockout and ALK/LTK knockout) mice are reported as viable without any major altered phenotype [70-73]. However, a recent report on ALK’s role in hypogonadotrophic hypogonadism has shown that ALK knockout males had low levels of serum testosterone with mild disorganization of seminiferous tubules, thereby suggesting a role for ALK in testis development and function [73]. Interestingly, low testosterone level is observed as one of the side effects in patients treated with FDA approved ALK inhibitor, crizotinib [74]. It is important to mention here that apart from the brain, ALK mRNA transcripts of varied sizes are reported in adult human tissues from testis, prostate, ovary, small intestine and colon [40, 43, 62]. However, not many studies have been performed on these transcripts. Additionally, subtle behavioral phenotypes have been described where ALK mutant mice exhibit lower anxiety, increased spatial memory and enhanced performance in novel object-recognition tests [71, 72]. Furthermore, Jurkat cells expressing ALK possess a proapoptotic activity in the absence of a ligand, describing ALK as a dependence receptor [75]. The same group also showed that the peptides derived from the apoptotic domain of ALK lead to apoptosis in ALK expressing ALCL and neuroblastoma cell lines [76].
B. *Drosophila melanogaster Alk*

The ligand for *Drosophila* Alk, Jelly Belly (Jeb) is the most well characterized so far [68]. Jeb is a 61 kD secreted protein comprising a LDLa domain which mediates its binding to Alk [77]. *Drosophila* homologues for MK and PTN, called Miple1 and Miple2 (*Mikdine* and *Pleiotrophin*) have been suggested to be potential ligands for Alk. However, a recent study has reported them dispensable for Alk signaling [78].

*Drosophila* is the most thoroughly studied model with respect to Alk function. Alk has been shown to play a critical role in the development of visceral musculature of the gut during embryogenesis [50]. The absence of Alk results in a gut-less phenotype due to lack of specification of founder cells in the developing visceral mesoderm. Jeb/Alk signaling is crucial for specification of founder cells that fuse with fusion competent myoblasts to give rise to visceral musculature of the gut [50, 77, 79-81]. As a consequence of lack of a functioning gut, the larvae die just after hatching or at 1st instar. The Jeb/Alk mediated ERK activation leads to transcription of various downstream targets such as Duf (dumb-founded)/Kirre (kin of irregular chiasm) [77, 81, 82], Org-1 (Optomotor-blind-related-gene-1) [77], Hand [83] and Dpp (decapentaplegic) [84]. Alk indirectly plays a role in the development of embryonic endoderm via dpp (homologue to mammalian transforming growth factorβ-TGFβ) [84]. Another functional role of Alk lies in the anterograde signaling pathway mediating neuronal circuit assembly in the fly visual system [85]. Further, Jeb and Alk are suggested to be important for the synaptic connectivity in the developing motor circuit [86]. Also, in starvation conditions, Alk helps in brain sparing via the PI3K/Akt pathway [87]. Another study has shown that decreased Alk expression in flies leads to increased resistance to sedating effects of ethanol and this is also seen in case of mice [88]. Alk also controls neurofibromin functions in regulating *Drosophila* body size, memory and learning. Reduced Alk activity leads to an increased body size [89].

C. *Caenorhabditis elegans ALK/SCD-2*

ALK homologue in *C.elegans* is named SCD-2 (suppressor of constitutive dauer formation) after it was initially identified as a suppressor in a genetic screen of TGF-β pathway mutants that led to constitutive dauer formation [90, 91].
The ligand for SCD-2 has been identified as HEN-1 (hesitation behaviour-1) which is a secreted ligand with an LDLa domain similar to the *Drosophila* ligand Jeb [92].

SCD-2 signaling in *C.elegans* is important regulation of presynaptic differentiation [93]. So far it has been shown that Hen-1/SCD-2 genetically mapped signaling includes the adaptor SOC-1 (suppressor of Clr-1) and the MAPK (mitogen-activated protein kinase) SMA-5 (small body size-5) [94].

D. *Danio rerio* alk/ltk

Unlike *D. melanogaster* and *C.elegans*, the zebrafish *Danio rerio* has two members of the ALK family (alk and ltk) [95, 96]. Unusually, zebrafish ltk contains a MAM domain and is expressed in neural crest cells, thus seemingly more related to ALK than to other LTK homologues. There are no reported natural ligand for alk and ltk as yet.

A role for ltk has been identified in the specification of iridophores (mirror-like pigment cells) from the neural crest lineage. Mutations in ltk result in defects in pigmentation patterns and the resulting mutants are called 'shady' [96]. This study showed a function for LTK in vertebrates for the first time as the function of mammalian LTK has not been elucidated as yet. In zebrafish, alk has been shown to play a role in neurogenesis in the developing CNS [95].

3.3 Structure of the ALK kinase domain

Elegant structural studies of ALK in the recent past have encouraged a greater understanding of the receptor's mechanistic. The first X-ray crystal structures of the ALK kinase domain in its inactive unphosphorylated form were reported by Lee et al. and Bossi et al. [97, 98]. The overall structure of ALK kinase domain follows the bilobal kinase fold (N and C lobes) (Figure-3), similar to other tyrosine and serine-threonine kinases [97, 99]. The N-lobe is smaller and comprises of five stranded anti-parallel β-sheets and a single major αC- helix which is responsible for catalysis. The C-lobe is largely helical and contains the activation loop that comprises of the triple tyrosines autophosphorylation motif. The two lobes are
connected by a hinge region which also forms the ATP or substrate binding cleft [97, 98].

Figure 3 - Bilobed kinase domain of ALK: ALK kinase domain (PDB: 3LCT) consists of a smaller N-lobe and a larger C-lobe. The N-lobe (amino acids 1093-1199) includes five anti-parallel β-sheets (shown in blue, numbered 1-5) and a major helix αC (green). The C-lobe (amino acids 1200-1399) on the other hand is largely helical (magenta, denoted as αD-1 and αEF) and has the activation loop αAL (yellow). The three tyrosines (Y1278, Y1282, and Y1283) in the activation loop are indicated as sticks (yellow). Cys1097 is represented as sticks (salmon). Other features highlighted are the hinge region (cyan), glycine-rich 'P-loop' (orange) and catalytic loop in dark grey. Also, the DFG motif (amino acids 1270-1272) at the beginning of αAL is shown in brown. ADP occupying the active site in the hinge region is indicated in red.

An integral part of the structure of kinase domain is the hydrophobic spines. Kornev and colleagues have revealed two hydrophobic structures termed ‘spines’ that contribute to a great extent in the internal dynamics of the kinase [100-102]. The two spines are referred to as regulatory spine (R-spine) and
catalytic spine (C-spine). Both spines are composed of residues from both the N and C lobes. The R-spine directs the positioning of the substrate and the C-spine governs the catalysis by directing ATP binding. Correct alignment of the spines is necessary for the assembly of the active kinase. Although necessary, it is not sufficient to ensure the assembly of active kinase. In ALK, the R-spine consists of the hydrophobic residues namely, C1182 (β4 strand, N-lobe), I1171 (αC-helix, N-lobe), F1271 (DFG motif, C-lobe), H1247 (HRD motif, C-lobe) and D1311 (αF helix, C-lobe). The C-spine comprises of V1130 (β2 strand, N-lobe), A1148 (β3 strand, N-lobe), L1256 (β7 strand, N-lobe), C1255 (β7 strand, N-lobe), L1257 (β7 strand, N-lobe), L1204 (αD helix, C-lobe), L1318 (αF helix, C-lobe), I1322 (αF helix, C-lobe) (Figure-4C). The two spines are anchored firmly through hydrophobic contacts to the αF helix in the C-lobe [100-103].

Since ALK is a member of IR superfamily, comparisons of ALK’s kinase domain with that of the previously determined structure of insulin receptor kinase (IRK) in both active and inactive conformations have been made [104, 105]. The catalytic domains of different kinases adopt similar conformations when active but the inactive conformations are strikingly different [28]. This is also the case of inactive ALK that differs significantly from that of inactive IR. First and foremost, the regulatory spine of inactive ALK adopts an orientation pertaining to an active kinase conformation. As can be seen in Figure 4B and C, the R-spine of inactive ALK resembles that of active IRK, with the F1271 in perfect alignment with the other residues of the regulatory spine. Disassembly of regulatory spine of inactive IRK displays the flipping out of F1151 that then occupies the ATP binding pocket in the catalytic spine (Figure-4A).
Figure 4 - Comparison of regulatory spines of active and inactive IRK with inactive ALK: A. Inactive IRK- Disassembly of regulatory spine (grey) where F1151 of the DFG motif flips out and occupies the ATP binding pocket in the catalytic spine (yellow). This stabilizes the inactive conformation of IRK by blocking ATP binding as well (PDB: 1IRK). B. Active IRK- Active conformation of IRK highlights the assembly of the regulatory spine residues (blue) in perfect alignment with one another (PDB: 1IR3). C. Inactive ALK- Regulatory spine in case of inactive ALK (slate) resembles the active IRK. F1271 can be seen in line with other residues of the regulatory spine (PDB: 3LCT).

Furthermore, inactive ALK adopts the DFG ‘in’ conformation which corresponds to an active state, rather than the inactive DFG ‘out’ conformation (Figure-5) [97, 98, 103]. Moreover, the interlobe closure between N and C lobes of ALK and the positioning of αC helix also differs from that of IRK. Additionally, E1167-K1150 salt bridge, a feature of active kinase conformation, is seen in the inactive ALK structure [97]. In case of gatekeeper residue, ALK differs from other RTKs by having a larger residue as gatekeeper (L1196). Most RTKs have smaller gatekeeper residues (T or V) [102, 106]. All together, these features of ALK kinase domain make it a very distinct receptor type amongst the other RTKs. It highlights the fact that the activation of ALK is a rather unique mechanism which makes it both interesting yet elusive.
Figure 5- DFG ‘out’ and DFG ‘in’ conformations of IRK: A. IRK displaying DFG out- inactive conformation (PDB: 1IRK). On the right, a close up of the dotted region is indicated with F1151 of DFG flipping out. B. DGF in- Active (PDB: 1IR3) conformation of IRK. On the right, close up of F1151 in the active conformation of the kinase is marked by a dotted rectangle. The structure backbone is in green with αC helix (red), activation loop (magenta), glycine rich P-loop (orange). In case of active conformation, ATP analog and peptide substrate are shown in grey and salmon respectively.

Another notable feature of ALK particularly is that its activation loop forms a short helix that packs against the αC-helix [107]. The triple tyrosine motif in the activation loop of ALK (Y-RAS-YY) also differs from that of (Y-XXD-YY) IRK and other RTKs such as insulin like growth factor 1 receptor (IGF1R), tropomyosin kinase receptor A/B (Trk A/B), muscle specific kinase (MuSK), neurotrophic tyrosine kinase receptor-related-2 (Ror2) [108]. In comparison to IRK’s 1158-Y-ETD-YY-
1163, the triple tyrosine motif of ALK is 1278-Y-RAS-YY-1283. ‘RAS’ on one hand is mainly neutral or basic amino acids whereas ‘ETD’ is acidic amino acids. It has been discussed that ALK is exclusively specific to the RAS motif and if the RAS is replaced by ETD, the phosphorylation is reduced dramatically [109]. It is worth mentioning that the sequence of phosphorylation in IR is that the second tyrosine (Y1162) gets initially phosphorylated followed by the first (Y1158) and then the third (Y1163) tyrosine [110]. In case of NPM-ALK fusion protein, it has been proposed that the first tyrosine (Y1278) is preferentially phosphorylated followed by second (Y1282) and the third (Y1283). The first tyrosine in ALK is also postulated to be critical for interaction with STAT3 and transforming ability of NPM-ALK [109, 111]. Additionally, Y1278 has been described as critical tyrosine for maintaining the inactive conformation of ALK by forming a hydrogen bond with cysteine at C1097 from the N-terminal β-turn motif (Figure-3). It is proposed that the loss of this hydrogen bond will result in shift of αC- helix facilitating the activation of ALK [97]. However, in the case of full length ALK receptor, the tyrosine at 1278 does not seem to be as critical. This will be discussed further in Paper-II.

Recently crystal structures of two hot spot mutations of ALK i.e. F1174L and R1275Q in neuroblastoma have been reported [107]. F1174 sits at the base of αC- helix from where it makes interactions with F1098 in the β-turn, F1271 in the activation loop and F1245 in the C-terminal kinase domain. These phenylalanine interactions form the hydrophobic pocket in the kinase domain. Mutations in these residues have been reported as gain-of-function mutations in neuroblastoma. R1275 is located in the activation loop of the kinase domain where it interacts with the neighbouring D1276 and also forms hydrogen bond with the carbonyl group of D1163 in the αC-helix. Both these interactions help to keep the helical conformation of the activation loop, a feature that is exclusive to ALK [97, 98, 107]. Epstein et al. discussed interesting structural features of both F1174L and R1275Q mutations. They observed that structure of F1174L was strikingly similar to the wildtype and showed features pertaining to an inactive conformation of ALK. In contrast, structure of R1275Q was dramatically different from the wildtype and showed loss of short helical segment at the beginning of the activation loop [107]. Only in case of R1275Q, an alternate activation loop conformation was observed.
3.4 ALK signaling

Although, there have been several reports on signaling of full-length ALK in the recent years, most available information about ALK signaling today comes from studies on fusion forms of NPM-ALK and echinoderm microtubule-associated protein-like 4 (EML4)-ALK. Therefore, care must be taken while applying the present knowledge to different forms of activated ALK as fusion protein and mutated or amplified full length receptor. Also, it is important to add that since most studies are based on oncogenic ALK, there is not much known about physiological ALK signal transduction in mammals.

By and large, ALK is involved in signaling pathways such as PLCγ and Ras/Erk1/2 pathways that result in cell proliferation and PI3K/Akt and JAK/STAT pathways that mediate cell survival.

**Phospholipase Cγ (PLCγ)** - PLCγ via its SH2-domain binds NPM-ALK at amino acid position 664, which results in activation and phosphorylation of PLCγ [112].

**Ras/Raf/Mek/Erk1/2** - Adaptor proteins like insulin receptor substrate-1 (IRS-1) (binds at Y1096), src homology 2 containing (Shc) (binds at Y1507) and growth factor receptor-bound protein 2 (Grb2) help in stimulation of MAPK signaling by NPM-ALK [113-115].

**PI3K/Akt (or PKB)** - Activation of PI3K-Akt pathway by NPM-ALK occurs through interaction with p85 subunit of PI3K resulting in tumor growth and reduced apoptosis [116-118]. This results in phosphorylation of mammalian target of rapamycin (mTOR) [119, 120] and also of glycogen synthase kinase 3-β (GSK3β) at serine 9, leading to a reduction in GSK3β activity [121]. NPM-ALK directed activation of PI3K/Akt also regulates transcription of forkhead box O3a (FOXO3a) target genes [122]. Moreover, sonic hedgehog (shh) pathway has been described as functioning downstream of Akt in ALK+ ALCL Karpas-299 cells [123].

**Janus kinase (JAK)/signal transducer and activator of transcription (STAT)** - Many studies have reported activation of STAT3 by NPM-ALK but the exact mechanism of STAT3 activation remains unclear to date [124-127]. Some have suggested that JAK3 associates with ALK and leads to STAT3 activation [126, 128]. While others have reported direct
interaction between the first tyrosine of Y-RAS-YY motif of ALK and STAT3 [111]. Also, since STAT1 is suggested to inhibit STAT3, a recent report showed downregulation of STAT1 in ALK+ ALCL [129]. There have also been studies that report NPM-ALK mediated activation of STAT5B that leads to apoptosis and cell cycle arrest. Although, some groups have not detected STAT5 activation by NPM-ALK [126, 127, 130].

Various adaptor proteins are involved in ALK signaling, such as Suc1-associated neurotrophic factor target 2 (SNT2), fibroblast growth factor receptor substrate 2 (FRS2), insulin receptor substrate 2 (IRS2), SHC and growth factor receptor-bound protein 2 (GRB2). ALK signaling further effects more downstream targets, such as BCL-2-interacting mediator of cell death (BIM), p27 and cyclin D2, which are important for cell survival and growth [46, 131-134]. Proteomics approach has helped in identification of more targets of ALK, namely Dok2, IRS1, SHC, Crk, CrkL, STAT3, VASP and ATIC [113, 135, 136].

In neuroblastoma cell lines using phosphoproteomics approach, protein tyrosine phosphatase non-receptor type 11 (PTPN11) and STAT3 were identified as two phosphoproteins with increased phosphorylation upon ALK induction. Other targets identified were, MAPK1, MAPK3, GSK-3α, STAT3, FAK and CrkL [137]. STAT3 as a downstream target of ALK will be further discussed in Paper-III. Additionally, MYCN has been identified as a transcriptional target of full length ALK receptor [138]. A recent report by Umapathy et al. showed stimulation of ERK5 by ALK in neuroblastoma. Also the report added that, ERK5 mediates ALK induced transcription of MYCN. This is important in terms of potential therapy in neuroblastoma patients [139].

Although, there is a certain level of commonality between signaling by ALK fusions and full length receptor, there can be stark differences based on the fusion partner and the tumor type. Therefore, a better understanding of ALK signaling and its downstream targets in both fusions and point mutations is essential in order to design therapies in various ALK-positive cancers.
4. ALK in cancer

4.1 ALK Translocations

Since the original discovery of ALK as fusion partner of NPM in ALCL in 1994 [40], a number of translocations involving the ALK locus have been reported in a myriad of cancers (Figure-5). Around 22 different genes have been described to be translocated to ALK including EML4-ALK in NSCLC (Non-small cell lung cancer), NPM-ALK in DLBCL (Diffuse large B-cell lymphoma) and ALCL, TPM3/4 (Tropomyosin 3/4) in IMT (Inflammatory myofibroblastic tumor). Some common features between the various fusion partners include 1) Initiation of transcription of fusion proteins is determined by the promoter of the partner protein. 2) The partner protein controls the subcellular localization of the fusion protein, which means that fusion proteins can be in the nucleus and/or in the cytosol. 3) Dimerization of ALK fusions occurs via the partner protein and involves trans-autophosphorylation, thereby leading to activation of the ALK kinase domain.
Figure 6 - ALK in cancer: A pictorial representation of various different ALK positive cancers. ALK fusion proteins are reported in varied number of cancers. Different fusion partners are indicated in blue under the cancer type they are involved in. The kinase domain of ALK rearranges with the N-terminal of these partner proteins resulting in a fusion protein. ALK amplification is described in many cancers shown in magenta. Point mutations in ALK are seen in both primary tumors as in the case of neuroblastoma and as secondary mutations in crizotinib-resistant patients (shown in green). Abbreviations for fusion partners are explained in sections 4.1.1-4.1.6.

4.1.1 Anaplastic large cell lymphoma (ALCL)

ALCL is a rare type of Non-Hodgkin’s lymphoma involving aberrant T-cells. It mostly occurs in children and young adults. The most well studied ALK translocation i.e. NPM-ALK is seen in 60-80% of the ALCL cases [140-142]. Numerous other ALK translocation partners have been reported in ALCL like, Tropomyosin 3/4 (TPM3/4) [143-145], TRK-fused gene (TFG) [146, 147], ring finger protein 213 (RNF213; also known as ALO17-ALK lymphoma oligomerization partner on chromosome 17) [148], Moesin (MSN) [149, 150], non-muscle myosin heavy chain 9 (MYH9) [151], clathrin heavy chain-like 1 (CLTC-1) [152], 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC) [153-155].

4.1.2 Non-small cell lung cancer (NSCLC)

Lung cancer is the most common cause of cancer death worldwide, with around 1.6 million deaths from lung cancer in 2012 (19.4% of the total 8.2 million deaths from cancer) [156]. Lung cancer is divided into two subtypes namely: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). About 80% of lung cancers are of NSCLC type. EML4-ALK fusion in NSCLC was first described in 2007 and this translocation is observed in about 5% of NSCLC [157, 158]. Although the percentage of ALK fusions in NSCLC is less than that of ALCL, the high incidence of lung cancer makes NSCLC the largest ALK positive patient population. Other ALK fusion partners in NSCLC apart from EML4 are TFG [157], kinesin light chain 1 (KLC1)
[159], kinesin family member 5B (KIF5B) [160, 161], protein tyrosine phosphate non-receptor type 3 (PTPN3) [162] and striatin (STRN) [163].

4.1.3 Inflammatory myofibroblastic tumour (IMT)

IMT was the first solid tumour to be associated with ALK rearrangements [164]. These tumours mostly effect young individuals [165] and arise most commonly in lung, abdomen and retro peritoneum [166]. Around 50% of IMTs have ALK fusions and some of the fusion partners are TPM-3 and 4 [167], ATIC [168], CLTC1 [169, 170], Ras-related nuclear protein-binding protein 2 (RAN-BP2) [171], cytoeinyl-tRNA synthetase (CARS) [148, 172], SEC31 homologue A (SEC31L1) [173] and protein-tyrosine phosphatase receptor-type F polypeptide-interacting protein-binding protein 1 (PPF1BP1) [174]. As in the case of ALCL where ALK positive patients have a higher 5 year survival than ALK negative patients [142, 175-178]. In IMT also, ALK fusions are indicative of better prognosis[179]. Furthermore, a very recent report described ALK positive cutaneous IMT for the first time [180].

4.1.4 Diffuse large B-cell lymphoma (DLBCL)

ALK positive DLBCL is a rare variant of DLBCL, which is a cancer of B-cells. The most frequently observed ALK rearrangement in this cancer type is the t(2; 17) (p23; q23) resulting in CLTC-ALK [152, 176, 181-184]. Other ALK fusions in DLBCL include NPM-ALK, SEC31A-ALK [185], sequestosome -1 (SQSTM1) - ALK [186-188]. DLBCL is associated with poor clinical outcome and does not respond well to chemotherapy [189, 190]. Targeted therapy against ALK might then prove promising in this patient group.

4.1.5 Renal cell carcinoma (RCC)

Translocations involving ALK and Vinculin (VCL) have been detected in RCC. This translocation is mostly seen in young patients [191]. In adult RCC, EML4 and TPM3 have been detected as fusion partners to ALK [192]. ALK rearrangements are not common in RCC.
4.1.6 Other cancers

Colon carcinomas harbor ALK rearrangements with EML4 and chromosome 2 open reading 44 (C2orf44). In breast cancer, EML4–ALK has been reported [193, 194]. A novel ALK fusion involving fibronectin 1 (FN1)–ALK in patients with serous ovarian carcinoma has also been detected [195]. TPM4–ALK has been described in Esophageal squamous cell carcinoma (ESCC) though in a low frequency [196, 197].

4.2 Overexpression of ALK

Many cancer forms like NSCLC, breast cancer, melanoma, neuroblastoma, glioblastoma, astrocytoma, retinoblastoma, Ewing’s sarcoma and rhabdomyosarcoma have been associated with ALK overexpression [58, 198, 199]. Amplification of ALK in neuroblastoma has been described by Carén et al. [200] and will be discussed in the section 4.3.2-Other genetic anomalies in Neuroblastoma.

4.3 Point mutations in ALK

Activating point mutations in ALK have been described in neuroblastoma, NSCLC, Anaplastic thyroid tumor (ATC) and in crizotinib resistant fusions in NSCLC and IMT [201-206].

In ATC, L1198F and G1201E ALK point mutations have been described as constitutively active [203].

Many ALK point mutations in crizotinib resistant NSCLC have been reported in the recent past [207]. Mostly, the secondary mutations are localized at the ATP binding pocket of ALK. These include the gatekeeper mutation L1196M and C1156Y that were found in tumor cells from a single crizotinib resistant patient [202]. Other secondary mutations reported are 1151Tins, S1206Y, L1196M and G1202R in four NSCLC patients [208, 209]. L1196M and G1269A were reported in patients with acquired resistance to crizotinib [210]. L1152R was reported in tumor cells derived from a NSCLC patient [211]. Studies from Heuckmann et al. identified L1196M, F1174L and G1269S crizotinib resistant mutations in NSCLC cell lines. They showed that the mutations were sensitive to TAE-684. They also identified two additional mutations namely L1198P and D1203N [212]. Furthermore, a recent report showed L1196M, G1269A, I1171T, S1206Y, G1202R and F1174C in cell lines from biopsies.
of patients with crizotinib resistant NSCLC [213]. Two other mutations, V1180L from a NSCLC cell line (H3122) treated with alectinib and I1171T from a crizotinib resistant patient treated with alectinib were described as alectinib resistance mutations. However, V1180L retained sensitivity to ceritinib and AP26113 while I1171T was sensitive to ceritinib and showed partial response to AP26113 [214]. I1171N was also reported as a common mutation in ALK-positive NSCLC patients who show disease progression while on alectinib but are sensitive to ceritinib [215]. Overall, about 30% of ALK secondary mutations contribute to crizotinib-resistant mechanisms in ALK positive lung cancer [216].

4.3.1 Neuroblastoma (NB)

Neuroblastoma is a pediatric cancer that arises in the neural crest cells of sympathetic nervous system origin. It is the most common extra cranial solid tumor that is responsible for 12% of all childhood cancer deaths [217-219]. As in the case of diseases of developing tissue, neuroblastoma affects very young children with the median age of 17 months at diagnosis [217]. Most tumors arise in the adrenals but can also originate in abdomen, pelvis, neck and chest region [217]. Even though the first report of ALK expression in neuroblastoma was in 2000 [220], it was only eight years later in 2008 that ALK point mutations were described in both familial [221, 222] and sporadic neuroblastoma [200, 223, 224]. Most of these reported mutations are located within or adjacent to the kinase domain. The two hot spots in the kinase domain are F1174 (mutated to C, I, L, S or V) and R1275 (mutated to Q or L). These mutations occur in about 85% of all cases with mutant ALK [200, 221-225]. ALK mutants such as K1062M, F1174L and R1275Q have been shown as oncogenic when expressed in nude mice as well as in NIH3T3 cells [131, 223, 224]. Additionally, F1174L mutation is suggested to potentiate oncogenic activity of MYCN in neuroblastoma mouse model over expressing ALK F1174L [226]. ALK mutations (F1174L/S, Y1278S, R1275Q, L1196M and T1151R) in relapsed neuroblastoma have also been reported [227]. Abrogation of ALK expression using siRNA led to decrease in proliferation in neuroblastoma cell lines [222].
Figure 7- Three classes of ALK point mutations in neuroblastoma:
Some of the ALK point mutations in the kinase domain are indicated 1) **Class-I**- Ligand independent mutations in red, 2) **Class-II**- Kinase dead mutation in magenta and 3) **Class-III**- Ligand dependent mutations in blue. Other highlighted features include αC helix (orange), activation loop αAL (cyan), DFG motif (grey), P-loop (salmon) and catalytic loop in yellow. The structure was generated using Pymol and PDB coordinates: 3LCT.

Several of these mutations have been experimentally tested to determine their role as driver or passenger mutations. Studies from our group on characterization of these mutations, broadly classifies them into three categories (Figure-6): **1) Class-I-Ligand independent mutations** (F1174I/L/S) [223, 224, 228-230] , **2) Class-II- Kinase dead mutation** (such as I1250T) [231]. I1250T mutation has been seen in two patients. It is proposed that this mutation acts in a dominant negative manner as in the case of kinase dead BRAF and oncogenic RAS that work together in melanoma progression [232] and **3) Class-III-Ligand dependent mutations** [228]. These mutations are not constitutively active and depend on ligand/agonist antibody for their activation. Although the role of ligand independent and kinase dead mutation can be predicted, the role of this group of mutations as driver or passenger in the oncogenesis is rather unclear at this point. These classes will be further discussed in Paper-I.
4.3.2 Other genetic anomalies in NB

Apart from ALK point mutations several other genetic aberrations in neuroblastoma include deletion of parts of chromosomes 1p and 11q, 17q gain, ALK amplification, MYCN amplification and triploidy [200, 221, 225, 233-235] and several others that will be discussed below. Taken together, these genetic aberrations are indicative of massive heterogeneity that exists within these tumors which makes it all the more challenging to treat them.

1p36 deletion correlates with MYCN amplification and advanced neuroblastoma stage [233]. Conversely, 11q23 deletion is rarely seen in MYCN amplified tumors [233]. Putative tumor suppressor genes that are deleted in these regions include calmodulin binding transcription activator 1 (CAMTA1) on chromosome 1 and tumor suppressor in lung cancer 1/Immunoglobulin superfamily 4/cell adhesion molecule1 (TSLC1/IGSF4/CADM1) on chromosome 11 [236, 237]. CAMTA1 induced differentiation and TSCL1 reduced proliferation in neuroblastoma cell lines, suggesting their role as tumor suppressors in neuroblastoma [236, 237].

Gain of parts of 17q is associated with poor prognosis and enhanced survival of neuroblastoma due to over expression of survivin (an inhibitor of apoptosis) [238, 239].

DNA content (near diploid or hyper diploid/triploid) is yet another genetic marker in neuroblastoma. Triploidy is associated with favorable outcome of the disease whereas near diploid state is associated with malignant neuroblastoma [233, 240].

ALK over expression in neuroblastoma has been described both in cell lines and patient samples and correlates with poor disease prognosis [241-245]. Mutation independent ALK over expression in patients also results in poor prognosis of the disease, reinforcing that ALK ought to play an important role in neuroblastoma [244]. Further, ALK amplification is most often seen together with MYCN amplification in neuroblastoma [200, 246, 247]. It has also been shown that neuroblastoma with increased levels of wildtype ALK resembles neuroblastomas with mutated ALK and that the highly expressed wildtype ALK
contributes to oncogenic progression [245]. Additionally, it has been reported that mechanisms other than mutation and amplification may regulate ALK expression in neuroblastoma since the ALK mRNA and protein levels do not necessarily correlate [244]. A recent report described translocations as a novel mechanism of ALK activation in neuroblastoma [248].

MYCN amplification on chromosome 2p24 (next to ALK on 2p23) is seen in about 22% of overall neuroblastoma cases and is indicative of poor survival in neuroblastoma patients [233, 249, 250]. As MYCN is involved in key processes such as cell proliferation, growth, apoptosis and differentiation, it is often seen over expressed in many cancers other than neuroblastoma like retinoblastoma, glioblastoma, SCLC [251-253]. MYCN amplification alone does not initiate tumor formation [254]. In neuroblastoma, ALK is described as regulator of transcription of MYCN and that they both together cooperate in transformation of NIH3T3 cells [138].

Furthermore, paired-like homeobox 2B (PHOX2B) mutations have been reported in hereditary neuroblastoma [255, 256]. Genome or exome analysis of neuroblastoma tumors has led to identification of some more genes that are mutated in addition to ALK, these are Alpha Thalassemia/Mental Retardation Syndrome X-Linked (ATRX) - a chromosome remodeling gene [257, 258], T-cell lymphoma invasion and metastasis 1 (TIAM1) - a regulator of cytoskeleton and neuritogenesis [258], regulators of Rac/Rho pathway [258], AT rich interactive domain 1A and 1B (ARID1A and 1B)- chromatin remodeling genes [259]. In addition to these, the Maris group examined 240 stage-4 neuroblastoma tumors at diagnosis. Their study reported PTPN11, ATRX, MYCN and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) in addition to ALK as genes that were mutated somatically. In germline tumors, they found enrichment of ALK, checkpoint kinase 2 (CHEK2), Phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and BRCA-1 associated RING domain protein 1 (BARD1) [219]. Recently, another study from their group has reported frequent mutations in RAS-MAPK pathway in relapsed neuroblastoma [260]. Loss of cyclin dependent kinase inhibitor 2A (CDKN2A) gene in neuroblastoma is suggested to result in poor prognosis of the disease [261]. High expression of mouse double minute 2 homolog (MDM2) at mRNA levels has also been shown to be associated with poor event free survival and thus can be used as
Long noncoding RNA, NBAT-1 (neuroblastoma associated transcript-1) was recently reported as a biomarker in neuroblastoma wherein loss of NBAT-1 increased cellular proliferation and invasion. [263]

5. Clinical detection of ALK

The United States (US) Food and Drug Administration (FDA) approved Crizotinib in 2011 for treatment of ALK positive NSCLC patients. Concurrently, the Vysis ALK Break-Apart FISH probe Kit (Abbott Molecular, Inc.) was approved to detect ALK rearrangements in NSCLC [103, 264]. This companion kit was designed for diagnosis of the patients that most likely would benefit from crizotinib treatment. Although, FISH is thus far the gold standard for detecting ALK rearrangement in NSCLC, it is technically challenging and expensive. This has led to development of other diagnostic methods such as immunohistochemistry (IHC) and PCR based techniques like Reverse transcriptase PCR (RT-PCR), 5’ rapid amplification of cDNA ends (RACE). Since, ALK mutation testing is recommended for all NSCLCs, it is important to explore other molecular diagnostic methods and in some cases use a combination of two methods. Some groups have studied the different detection methods and drawn comparisons between them which are discussed below [265-267].

i. **FISH**- In the Vysis ALK FISH analysis procedure, unstained tissue is hybridized overnight with the ALK probe that is examined by fluorescence microscopy [267, 268]. The green fluorophore binds ALK at 5’ region and red binds to the 3’ of ALK. The ALK probe shows separated red and green fluorophores or loss of the green signal when ALK translocation is present in the sample. A few disadvantages of the technique are: 1) it is expensive, 2) it needs accurate interpretation, 3) does not identify specific translocation types. The advantages of FISH on the other hand are it is reliable and detects all ALK rearrangements irrespective of the fusion partner.

ii. **IHC**- The challenge in performing IHC in NSCLC is the low protein levels of ALK [269]. In contrast, IHC identifies ALK in ALCL readily [65, 270]. There is no
standard protocol for IHC, some antibodies that are used are D5F3 (Cell signaling technology, Danvers, MA, USA), 5A4 (Novocastra, Newcastle, UK) and ALK clone ZAL4 (Invitrogen, Carlsbad, CA, USA) [196, 271, 272]. Challenges in using IHC include: 1) tissue preparation, 2) choice of antibody, 3) signal enhancement systems. There are advantages to IHC such as low cost, easy, less time consuming, easy to interpret. However, there is a need to validate its clinical application in ALK rearranged NSCLC [267, 269, 273].

iii. PCR based methods

a. **RT-PCR**- In this protocol, mRNA is converted to cDNA by reverse transcriptase. cDNA is then amplified using specific primers. Commercially available kits come with primer sets for most or all EML4-ALK transcripts [270, 274-276]. The amplicons can be further identified using sequencing, electrophoresis, fluorescent probe degradation, Nanostring and counter capture technology [267, 268, 275, 277, 278]. RT-PCR is a sensitive and reproducible technique, although expensive.

b. **RACE**- EML4-ALK fusions harbour the kinase domain of ALK encoded by exon 20 and follows distal 3’ exons [279]. RACE analysis was used to quantify relative 5’ and 3’ ALK mRNA levels in NSCLCs [195]. EML4-ALK mRNA was first reverse transcribed into cDNA and then different portions of the cDNA were amplified using primers specific to exons between E13-E18 and E22-E27. The E22-E27 domains showed increased expression when normalized to 5’ ALK mRNA expression. PCR based methods in NSCLC have advantages and disadvantages. Advantages: 1) high specificity, 2) highly sensitive detection- can detect EML4-ALK fusion transcript diluted in over 90% wild-type RNA, and 3) is less expensive than FISH. The disadvantages: 1) it can miss rare or novel translocations, 2) it can have contamination problems, and 3) RNA degradation or poor sample quality can hinder detection [267, 268, 275, 277,
Other techniques for detection of EML4-ALK in NSCLC;

iv. **Deep sequencing** - Second-generation sequencing has been used to identify complex ALK rearrangement in lung adenocarcinoma that was undetected by Vysis FISH assay [280]. Sequencing results exposed a complex ALK rearrangement that involved at least five different genomic loci, which included the canonical EML4-ALK breakpoint. EML4 and ALK genes were separated by small rearrangements that made their detection by FISH difficult. IHC results further showed that the tumor was ALK positive and the patient responded to crizotinib therapy. Second generation sequencing may therefore be useful for NSCLC patients that carry driver mutations that are not detected or are difficult to detect by other methods.

v. **Exon Array profiling** - ALK rearrangements in breast, colorectal and NSCLCs have been detected by exon array profiling (Affymetrix Human Exon 1.0 Arrays) [193]. Bioinformatics analysis revealed differences between 5’ and 3’ ALK exon expression among some tumors. Investigation of these samples showed EML4-ALK fusions in 2.4% of breast and colorectal cancers, and in 11.3% of NSCLCs. Even though, this method is able to detect EML4-ALK fusions, it is complex, expensive, and technically challenging method.

6. ALK inhibition- current and future therapy

Targeted therapy has been a successful strategy in cancer treatment. With TKI imatinib’s approval by FDA in 2001, the field of targeted therapy gained more impetus and several other inhibitors came under clinical trials. In case of ALK, the small molecule inhibitor, crizotinib, has proven to be very effective. Although, it was first developed to work against Mesenchymal-epithelial transition (MET) factor receptor [216], crizotinib’s effectiveness in ALK positive NSCLC patients
led to its expedited approval in 2011 by FDA. Unfortunately, despite the great results, drug resistance in case of targeted therapy is inevitable and such is the case of crizotinib too. This has led to the development of several next generation inhibitors against ALK.

1. **Crizotinib**: Pfizer’s crizotinib (PF-02341066) is an orally bioavailable, ATP competitive, small molecule inhibitor. It targets ALK, MET and ROS-1 (Table-1). The remarkable success of crizotinib in Phase I and II trials in ALK-positive NSCLC, led to its accelerated approval by FDA [264, 281-284]. It does not have serious side effects and is generally well tolerated with mild gastrointestinal symptoms [285]. Other adverse effects reported for crizotinib are visual disturbances and increased aminotransferase levels [286]. The efficacy of crizotinib has also been tested in tumor types other than NSCLC. Partial response in patients with ALK rearranged IMT has been reported [287-289]. In both adult and pediatric ALCL patients, the response to crizotinib has been promising [289-291]. In preclinical models and Phase-I trial of neuroblastoma, crizotinib has proven to be effective [230, 289]. Although, the response to the crizotinib has been remarkable, there are two major limitations: 1) Poor activity of crizotinib in the brain makes it difficult to use it for CNS metastasis. There are a number of patients reported with CNS relapse after crizotinib treatment [292-294]. 2) Acquired resistance to crizotinib has been the biggest challenge since there can be multiple resistance mechanisms. Median progression free survival with crizotinib in ALK-positive NSCLC is 8-10 months [282], after which ALK secondary mutations ensue and the response to crizotinib significantly drops leading to crizotinib resistance. Several such secondary mutations have been reported as discussed previously in section 4.3. There are other causes for resistance such as target gene modification by amplification or secondary mutation [208, 210, 295], using bypass signaling pathway other than the oncogenic driver [208] and an increased affinity to ATP binding as in case of F1174L ALK mutation [296]. To combat resistance, development of next generation inhibitors that are more potent against ALK is the need of the hour. Phase-I trials of some of ALK’s second generation inhibitors (ceritinib, alectinib, brigatinib) have shown efficacy against crizotinib resistance and also in CNS [297-299].
Next generation ALK inhibitors;

2. Ceritinib- Recently in 2014, FDA approved the use of ceritinib (by Novartis) for treatment of ALK-positive NSCLC patients that have shown resistance to crizotinib treatment. Ceritinib, like crizotinib is an ATP competitive inhibitor that binds to the ATP binding cleft (Figure-8). Ceritinib is a derivative of Novartis’ previous compound NVP-TAE684 [300] and is effective against ALK, IGF-1R, INSR and STK22D [208, 213, 287]. Its efficacy has been reported in both crizotinib naïve and crizotinib treated patients [216, 301]. In a preclinical study, it was able to inhibit ALK-crizotinib resistant mutations (L1196M, I1171T, S1206Y and G1269A) but was ineffective against G1202R and F1174C mutations [213]. In a preclinical setting, ceritinib was able to overcome alectinib resistance in tumors with secondary mutations I1171T and V1180L [214]. Conversely, there has been a report suggesting that alectinib can overcome ceritinib resistance induced by L1196M and G1269A mutations [302, 303].

Common side effects upon ceritinib treatment include nausea, vomiting, fatigue, diarrhea and increased levels of transaminases. Six percent of patients had to discontinue ceritinib due to adverse side effects [301].

**Figure 8- Different ALK inhibitors occupying the active site:** Structures of different ALK inhibitors occupying the active site is shown: Crizotinib (magenta, 2XP2), Ceritinib (green, 4MKC), Alectinib (blue, 3AOX) and PF-06463922 (red, 4CLI). The ATP binding pocket is shown as grey surface. The structure of alectinib extends further more into the active site compared to other three inhibitors.
3. **Alectinib**- Next generation inhibitor, Alectinib (CH5424802) is a selective and potent ALK inhibitor, which is being developed by Roche [302]. It received breakthrough therapy designation (BTD) by FDA in 2013 and was approved in Japan in 2014. Apart from ALK, it has activity against LTK and RET and GAK but not INSR, IGF-1R, MET and ROS1 [302, 304, 305]. It has shown remarkable efficacy in intracranial metastases in crizotinib refractory patients [306-309]. It is potent against secondary ALK mutations like L1196M, 1151ins, L1152R, C1156Y, F1174L and G1269A but shows low potency in case of G1202R mutation [303, 310]. Also, it is effective against two neuroblastoma hotspot mutations F1174L and R1275Q, which mean that it may be of clinical use in neuroblastoma [302].

Two novel ALK mutations, V1180L and I1171T have been reported as mutations conferring resistance to alectinib [214]. Additionally, a study by Isozaki et al. described I1171T mutation in a patient with resistance to alectinib [311]. Ceritinib and AP26113 showed efficacy against V1180L and I1171T mutations. Also, ASP3026 showed effectiveness against V1180L but not I1171T mutation in BaF3 cells [214].

MET amplification in alectinib-resistant tumors has been detected but no tumor samples before treatment could be tested [312]. Also, an alectinib resistant cell line showed MET activation induced by hepatocyte growth factor in an autocrine manner [311]. However, the cells were sensitive to crizotinib both in vitro and in vivo. This could mean that in crizotinib naïve patients treated with alectinib and showing MET activation, a possible treatment with crizotinib may be a viable option.

4. **AP26113**- AP26113 is a second-generation inhibitor in development by Ariad. It is a potent inhibitor of ALK, EGFR and ROS1 [313]. Initial results from an ongoing phase I/II trial (NCT01449461) of AP26113 exhibited overall response rate of 67% in ALK positive NSCLC patients with crizotinib resistance. Also, tumor regression was reported in case of CNS metastatic lesions in 4 of 5 patients [314]. It also showed activity in a patient with ALK positive IMT [287].
5. **PF-06463922**- Pfizer’s PF-06463922 is a novel, selective, highly potent, small molecule, ATP competitive inhibitor that can cross blood-brain barrier. It shows high activity against all known ALK and ROS1 mutations identified in crizotinib resistant patients [287]. It is highly effective in crizotinib resistant cells [315]. Preclinical studies in mice with EML4-ALK driven brain tumors treated with PF-06463922 displayed tumor regression and increased overall survival [315-317]. These results seem very promising and indicate that PF-06463922 may be a very effective therapeutic agent in patients with crizotinib resistance and/or in patients with CNS tumors with either crizotinib resistance or crizotinib naive background [315-317]. A phase I/II trial (NCT01970865) of PF-06463922 in ALK positive and ROS1 positive NSCLC is ongoing.

6. **ASP3026**- Astellas Pharma’s ASP3026 is a potent inhibitor of ALK, ROS1 and ACK. Preliminary results from a phase I study of ASP3026 in patients with advanced solid tumors showed a favorable safety profile with an average dose of 525 mg daily (NCT01401504). The clinical activity of ASP3026 has not been reported as yet [318]. Common side effects include gastrointestinal symptoms.

Other ways to inhibit ALK;

**Heat shock protein 90 (HSP90) inhibitors**-
Protein chaperones such as HSP90 are important in maintaining the stability of proteins involved in normal cellular functions and in tumorigenesis [319]. Fusion proteins rely heavily on HSP90 for their folding, transport and stability [320]. Three HSP90 inhibitors namely retaspimycin hydrochloride (IPI-504), AUY922 and ganetespib (STA-9090) have been tested in patients with ALK rearrangements [287]. Preclinical data showed that crizotinib resistant ALK positive cell lines were sensitive to HSP 90 inhibitor, 17-AAG (IPI-504 is a derivative of 17-AAG) [321, 322]. Ganetespib demonstrated efficacy in NSCLC cell lines with ALK, ROS1 or RET rearrangements[287]. Phase II study of AUY922 in NSCLC patients showed partial responses in 6 of 21 (29%) patients with ALK rearrangements.

Synergistic anti-tumor effects of TKI and HSP90 in ALK or MET driven cancers in a preclinical setting have led to a
number of clinical trials testing this combination therapy [323, 324]. This is particularly important for patients that cannot tolerate TKI because of TKI-associated pneumonitis.

**Table 1- ALK inhibitors in clinical trials**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Company</th>
<th>Targets other than ALK</th>
<th>Phase</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crizotinib (PF-02341066)</td>
<td>Pfizer</td>
<td>MET, ROS1</td>
<td>I,II,III</td>
<td>Approved in 2011</td>
</tr>
<tr>
<td>Ceritinib (LDK378)</td>
<td>Novartis</td>
<td>IGF-1R, INSR</td>
<td>I,II,III</td>
<td>Approved in 2014</td>
</tr>
<tr>
<td>Alectinib (CH5424802)</td>
<td>Roche</td>
<td>LTK, GAK, RET</td>
<td>I,II,II/III</td>
<td>BTD in 2013, Approved in Japan (2014). NCT01588028</td>
</tr>
<tr>
<td>Brigatinib (AP26113)</td>
<td>Ariad</td>
<td>EGFR, ROS1</td>
<td>I/II</td>
<td>BTD in 2014. NCT01449461</td>
</tr>
<tr>
<td>ASP3026</td>
<td>Astellas</td>
<td>ROS1, ACK</td>
<td>I</td>
<td>NCT01401504</td>
</tr>
<tr>
<td>X-376, X-396</td>
<td>Xcovery</td>
<td>MET</td>
<td>I (X-396)</td>
<td>NCT01625234</td>
</tr>
<tr>
<td>TSR-011</td>
<td>Tesaro</td>
<td>TRK-A,B,C</td>
<td>I/IIa</td>
<td>NCT02048488</td>
</tr>
<tr>
<td>PF-06463922</td>
<td>Pfizer</td>
<td>ROS1</td>
<td>I/II</td>
<td>NCT01970865</td>
</tr>
<tr>
<td>RXDX-101, NMS-E628</td>
<td>Ignyta</td>
<td>ROS1, TRK's</td>
<td>I</td>
<td>N/A</td>
</tr>
<tr>
<td>CEP-28122</td>
<td>Teva</td>
<td>RSK-2, 3,4</td>
<td>Not clinical</td>
<td>N/A</td>
</tr>
<tr>
<td>CEP-37440</td>
<td>Teva</td>
<td>RSK-2,3,4</td>
<td>I</td>
<td>NCT01922752</td>
</tr>
</tbody>
</table>

**Immunotherapy**-
Anti-PDL-1 agent, MPDL3280A received break through therapy designation by FDA in February, 2015 for treatment in PDL-1 positive NSCLC who have had platinum based chemotherapy and an EGFR or ALK inhibitor [216]. This indicates that immunotherapy could be a treatment option for ALK positive patients who were treated with or without ALK TKIs. In March 2015, FDA granted approval to anti-PD-1 agent, nivolumab for treatment of patients with metastatic squamous NSCLC.
7. Therapeutic approaches in neuroblastoma

**Retinoic acid treatment**-
Inducing differentiation using retinoic acid derivatives slowed growth of neuroblastoma cells in culture [325-327]. The first clinical trial using 13-cis retinoic acid for treatment of high-risk neuroblastoma patients indicated survival advantage with minimal toxicity [328]. This approach is now a standard protocol in high-risk neuroblastoma patients [233].

**Immunotherapy**-
Preclinical studies using inhibitory antibodies against ALK in neuroblastoma cell lines inhibited downstream signaling and caused cytotoxicity [56, 329]. However, these antibodies cannot be used in ALK fusion proteins but may be useful in cancers such as neuroblastoma that harbor ALK amplification and point mutations. Neuroblastoma cells express high levels of gangliosides and sialic acid on their surface [330], which are required in migration, metastasis and adhesion [331]. Several antibodies against neuroblastoma surface antigens, disialoganglioside GD2 have been raised and tested as therapeutic agents [332-334].

**Induction of apoptosis**-
A novel synthetic retinoid- N-(4-hydroxyphenyl)retinamide (fenretinide) is under clinical trials in neuroblastoma patients [335-337]. This agent leads to apoptosis instead of differentiation. High risk neuroblastomas mainly those with MYCN amplification express brain-derived neurotrophic factor (BDNF) and TrkB. This may be representative of a survival pathway that overcomes treatment and induces resistance [338-340]. Inhibition of this pathway using Trk-specific inhibitors promotes apoptosis when used alone or in combination with conventional therapy [341, 342].

**Inhibition of angiogenesis**-
Due to the high vascularity of neuroblastoma tumors, inhibition of angiogenesis is a favorable approach [343]. TNP-470 was efficacious in treating neuroblastoma in
animal models [344-346]. However, it is too toxic to use as therapeutic agent. Another approach uses metronomic therapy (chronic administration of low-dose chemotherapy) as another way to inhibit angiogenesis [347]. The development of several other agents is under way to block angiogenesis [348-351].

**Radiation therapy**

Meta-iodobenzyl-guanidine (MIBG) compound is rapidly taken up by neuroblastoma cells and concentrated in secretory granules. Radioactive MIBG is useful in diagnosis as well as in treatment of neuroblastomas [352-355]. In the coming years, proton-beam therapy to treat children with neuroblastoma and other cancers will become more available [356-358].
AIM

- **Aim of the project** - To characterize different ALK point mutations found in neuroblastoma using cell culture and *Drosophila* as model organism and to check if they are responsive to the ALK inhibitor, crizotinib. Further, to study mechanistic implications of some of these mutations on the activation of full length ALK receptor.

- **Specific aim**

  **Paper-I** To classify different ALK point mutations in neuroblastoma into ligand independent, kinase dead and ligand dependent categories, using cell culture and *Drosophila* as model organism.

  **Paper-II** To investigate the importance of tyrosine 1278 (Y1278) in the activation mechanism of full length ALK receptor.

  **Paper-III** To conduct a search to identify potential downstream targets of ALK in neuroblastoma using mass spectrometry based phosphoproteomic analysis.
Results and Discussion

Paper-I- Cell culture and *Drosophila* model systems define three classes of anaplastic lymphoma kinase mutations in neuroblastoma. *(Chand et al., 2013)*

In this study, we investigated two novel ALK mutations (A1099T and R1464STOP) along with the previously reported but uncharacterized mutations T1087I, D1091N, T1151M, M1166R, F1174I and A1234T [222-224]. A1099T mutation was identified in a Japanese infant diagnosed with neuroblastoma and R1464STOP was detected in a patient as a part of TARGET (therapeutically applicable research to generate effective treatments) initiative.

1. Investigating the role of ALK mutants as driver or passenger in neuroblastoma progression using cell culture based system

Both downstream signaling potential and differentiation ability of all the eight ALK mutants was assayed using PC12 cells. The ALKF1174S was included as a positive control [229]. The agonist antibody mAb31 was used to stimulate the receptor as previously described [230]. ALKF1174I and ALKM1166R displayed robust activation of ERK and STAT3 indicating their constitutively active nature. The remaining mutant receptors showed wildtype like characteristics with increased activation of ERK upon stimulation with mAb31.

In line with signaling results, ALKF1174I and ALKM1166R displayed neurite outgrowth in a ligand independent manner. On the other hand, the rest of the mutants in the study did not give rise to neurites in the absence of stimulating antibody. Upon addition of mAb31, neurite outgrowth levels significantly increased, similar to wildtype. Further in all mutants tested, crizotinib effectively blocked the neurite outgrowth.

All the previously described mutants were studied for their transforming ability in NIH3T3 cells. Similar to the results from the previous assay, ALKF1174I showed robust foci formation. However, ALKM1166R showed weak foci formation. The remaining mutants did not yield any foci.

Likewise, in BaF3 transformation assay, only ALKF1174I resulted in IL-3 independent lines. The remaining ALK mutants including ALKM1166R were not able to transform BaF3 cells.
Crizotinib blocked phosphorylation of ALK (Y1278 and Y1604) as well as phosphorylation of ERK in case of both ALKF1174L and ALKF1174I. Furthermore, they displayed differential sensitivity to crizotinib indicative from their different IC50 values. This could be important clinically in terms of designing treatment protocols and patient stratification based on different ALK mutations that they may carry.

2. Confirming the status of ALK mutants by ectopic expression in Drosophila eye

*Drosophila* was used as a model system in this study to further exemplify the mutations. Transgenic flies that ectopically expressed ALKA1234T, ALKR1464STOP, ALKF1174L (as positive control) and ALKWT were generated. ALKF1174L gave rise to a rough eye phenotype whereas ALKA1234T and ALKR1464STOP did not give rise to any obvious phenotype. This further confirmed their role as ligand dependent mutations.

Overall, the results from this study and that of previous report by Schönherr et al. classify the ALK mutations in neuroblastoma into: 1. Ligand independent, 2. Kinase dead [231] and 3. Ligand dependent mutations. Importantly, regardless of the mutation characteristic; all mutations studied can be blocked using ALK specific inhibitor, crizotinib. This may have potential clinical relevance with respect to treatment of patients harboring different ALK mutations. It is important to add that although the role of ligand independent and kinase dead mutations can be sought after, the role of ligand dependent mutations is not very clear. Further work will be needed to explain their importance in neuroblastoma progression.
Paper-II- Novel mechanisms of ALK activation revealed by the analysis of Y1278S neuroblastoma mutation. (Chand, Guan et al., 2015)

Structural work by Bossi et al. and Lee et al. on NPM-ALK fusion protein has highlighted the importance of Y1278 in the activation loop and a possible activation mechanism of ALK [97, 98]. Bossi et al. also suggested an interaction between Y1278 and C1097 to keep ALK in inactive conformation [97]. Another study reported that Y1278 is preferentially phosphorylated amongst the triple tyrosine motif (YRASYY) and is important for autoactivation and transforming ability of ALK [111]. However, reports of Y1278S mutations in two neuroblastoma patients [221, 225], drew our attention to the importance of this residue in the activation of full length ALK receptor. To further elucidate the role of Y1278, we constructed mutations in ALK; Y1278A, Y1278D, C1097A, C1097K and C1097S to study their effect on its activity.

1. **Y1278S is ligand independent and constitutively active in cell culture and Drosophila**

To study the effects of the mutations, Y1278A/D/S and C1097A/K/S, we first conducted cell culture based assays to confirm if they were gain-of-function mutations or not. Using PC12 cells, we analyzed the downstream signaling of these mutants. ALK<sub>Y1278S</sub> and ALK<sub>Y1278D</sub> displayed activation of ERK without stimulating antibody, indicating that they were constitutively active. The remaining mutants behaved as inducible wildtype receptor. Results from the neurite outgrowth assay also showed that only ALK<sub>Y1278S</sub> and ALK<sub>Y1278D</sub> were constitutively active and gave rise to neurites without mAb31. All mutants however were sensitive to inhibition by crizotinib.

To further clarify the role of different mutants, their transforming ability was assessed in NIH3T3 and BaF3 cell based assays. In NIH3T3 cells, ALK<sub>Y1278S</sub> and ALK<sub>Y1278D</sub> gave rise to robust foci while the other mutants did not show any foci formation. Further, only ALK<sub>Y1278S</sub> and ALK<sub>Y1278D</sub> mutations rendered BaF3 cells IL-3 independent. Crizotinib blocked phosphorylation of ALK (Y1604) and downstream target ERK in both ALK<sub>Y1278S</sub> and ALK<sub>Y1278D</sub>. Both mutants exhibited differential sensitivity to crizotinib with IC50 values of 50.6 nM and 74.8 nM for ALK<sub>Y1278D</sub> and ALK<sub>Y1278S</sub> respectively.
The above results were confirmed in *Drosophila* where ALKY1278S and ALKY1278D gave rise to rough eye phenotype while the remaining mutants did not show any disorganization of ommatidia in the fly eye. *In vitro* kinase assay results showed ALKY1278S to be very active. ALKY1278D was active but not as much as ALKY1278S while ALKY1278A showed weak activity.

Taken together, the data from the above assays suggested that ALKY1278S and ALKY1278D were constitutively active and ALKY1278A showed wildtype like characteristics. This means that Y1278 is not critical for activation of ALK as had been reported earlier for fusion protein. Additionally, all the mutations at C1097 to alanine, lysine and serine showed wildtype like characteristics. This further suggested that perhaps the interaction between Y1278 and C1097 is not important in regulating the activity of ALK.

1. *In vitro* kinase analysis uncovers Y1283 as the most critical tyrosine in the activation of full length ALK

The above data inferring the constitutively active status of ALKY1278S and ALKY1278D led us to explore the other tyrosines of the motif YRASYY, namely Y1282 and Y1283. Different mutations in ALK were generated: Y1278F, Y1282F, Y1283F, double mutants: ALK-YYY1278, 1282, 1283YFF, ALK-YYY1278, 1282, 1283FYF, ALK-YYY1278, 1282, 1283FFY and the triple mutant ALK-YYY1278, 1282, 1283FFF.

PC12 cell analysis showed that ALKY1278F and ALKY1282F were similar to inducible wildtype. On the contrary, ALKY1283F did not show any phosphorylation of ALK and downstream target ERK. It did not lead to any neurites with and without stimulation, thereby suggesting that Y1283 is critical for activation of full-length receptor. Double and triple mutants gave similar results as that of Y1283F mutation. These results were confirmed in *in vitro* kinase assays where ALKY1283F displayed negligible activity.

Our results indicated that Y1278 is not important for activation of ALK. And the interaction of Y1278 and C1097 is not a contributing factor in the regulation of the kinase. We further propose that Y1283 is the critical tyrosine for activation of full length ALK. Our study adds a new perspective to the activation of ALK, which is different from that of ALK-fusion protein and more in line with insulin receptor.
Paper-III- 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 et al., 2013)

In this study, we used MS-based phosphoproteomics approach to search potential targets of activated ALK. PTPN11 (SHP-2) and STAT3 were identified as two phosphoproteins with increased phosphorylation upon ALK induction. We focused on STAT3 in this study and further investigated its role as a mediator in transcription of MYCN.

1. ALK activation results in phosphorylation of STAT3 at Y705 in PC12 cells

ALK activation of STAT3 was examined using PC12 cells expressing doxycycline inducible wildtype and F1174S mutation. Although, robust activation of STAT3 was seen for F1174S without stimulation, in the case of wildtype prolonged stimulation of about 24 hours led to visible tyrosine phosphorylation of STAT3. Interestingly, STAT3 phosphorylation was not observed at 30 minutes time point, which is when both ALK and ERK are highly phosphorylated upon stimulation. The activation of STAT3 by ALK was abrogated using ALK inhibitor, crizotinib.

To further check for an interaction between ALK and STAT3, immunoprecipitation experiments were conducted. Although, interaction between doxycycline inducible ALK and endogenous STAT3 was not observed in PC12 cells. We were able to detect interaction between ALK and STAT3 by transiently co-transfecting PC12 cells with ALK (wildtype and F1174S) and FLAG-tagged STAT3. This interaction was increased upon stimulation of wildtype and decreased using crizotinib.

2. STAT3 is important for transcription of MYCN in response to ALK activation and in the proliferation of neuroblastoma cell lines

Previous findings have indicated that ALK is involved in regulation of transcription of MYCN and that it cooperates with MYCN in neuroblastoma pathogenesis [138]. Based on these results, we investigated further if STAT3 played a role in this process. Different neuroblastoma cell lines carrying either a gain-of-function ALK mutant (CLB-GEMO ALKF1174V, CLB-GAR
ALKR1275Q and Kelly ALKF1174L) or overexpressed truncated form of ALK (CLB-BAR ALK_{exon4-12}). Small interfering RNAs (siRNA) against STAT3 led to reduction in STAT3 as well as MYCN levels. Pharmacologic inhibition of STAT3 using FLLL32 and STATICT also showed decreased levels of phosphorylated STAT3 and MYCN. Moreover, to confirm that STAT3 was involved in ALK initiated MYCN transcription, we used a MYCNP-luciferase reporter to transfet neuroblastoma cell lines. Upon treatment with STAT3 inhibitors, the luciferase activity was reduced. This was confirmed by quantitative RT-PCR. Furthermore, blocking STAT3 using siRNA and inhibitors led to a decline in growth of neuroblastoma cell lines in a manner similar to that of crizotinib inhibition.

Taken together, our study highlights STAT3 as one of the prominent targets of ALK. ALK activation of STAT3 results in increased phosphorylation of STAT3 at Y705. This activation differs for wildtype and gain-of-function ALK mutation. Further, STAT3 is involved in ALK initiated MYCN transcription. Thereby, suggesting a potential therapeutic role for STAT3 inhibition to regulate MYCN levels in neuroblastoma cells.
Conclusions

Paper-I

- ALK mutants in neuroblastoma can be broadly classified in three categories: Ligand independent, Kinase dead and Ligand dependent.
- All studied ALK mutants, regardless of the type, can be blocked using crizotinib.
- Characterization of mutations may be clinically relevant in patient classification and designing appropriate treatment protocols.

Paper-II

- ALKY1278S and ALKY1278D are constitutively active while ALKY1278A and ALKC1097A/K/S are ligand dependent similar to inducible wildtype.
- This indicates that Y1278 is not critical for activation of full length ALK and that Y1278-C1097 interaction is not important in regulating the activity of ALK.
- ALKY1283F shows very little activity in kinase assay and therefore may be a crucial tyrosine in the activation of ALK.
- All ALK mutants studied are blocked using crizotinib.
- This study sheds light on a novel activation mechanism for full-length receptor which is different from that proposed for ALK fusion protein.

Paper-III

- One of the most prominent downstream targets of ALK identified was STAT3.
- ALK activation in PC12 cells resulted in phosphorylation of STAT3 at Y705.
- Activity of STAT3 is vital for growth and proliferation of neuroblastoma cells.
- STAT3 regulates transcription of MYCN and may therefore be a potential drug target in neuroblastoma.
Acknowledgements

“Isn't it funny how day by day nothing changes, but when you look back, everything is different…” – C.S Lewis

This quote completely sums up my feeling at this point. I think PhD is quite like that, when you look at it from day to day, you don’t think your days are significantly different from one another. But as a whole journey, it is as different as it could be. Start to finish.

I would like to thank a lot of people who have made this journey of mine truly special and memorable. First and foremost, a big thank you to my supervisor, Prof. Bengt Hallberg for this opportunity. You have been a tremendous support and there is so much that I have learned from you. Thank you for always having your door open for discussions, for your enthusiasm and encouragement. Thank you for being forth coming and prompt with all suggestions, corrections/submission of manuscript/abstracts/poster despite your busy schedule. I have always admired that about you. Thank you for your time, energy and trust that you have vested in me over these five years. Thank you! I would like to thank, Prof. Ruth H. Palmer, also my co-supervisor for the most valuable suggestions and inputs on my projects and presentations and for guiding through different stages of my work. Your contribution in my understanding of science has been immense. You are truly inspirational and I am grateful that I had this opportunity to work with you and learn so much from you. I thank you both for the opportunity to move to Gothenburg, it has been a great learning process with moving and starting afresh.

When I started out, I was inexperienced and probably held a pipette twice before in my life. And I cannot thank you enough, Rutan (Kristina Ruuth) for teaching me everything about lab work, from techniques to maintenance of lab notebook and general practices in lab. It has been an absolute delight to work with you. You have rendered me support through all times. It has been great fun to be able to talk to you about anything and everything, academic and personal.

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