Genetic determinants of lung cancer and their application toward therapeutics

The role of Zfp148 transcription factor and anaplastic lymphoma kinase (**ALK**) fusion proteins in non-small cell lung cancer

Abdulmalik Asaadallah A. Bokhari

Department of Medical Biochemistry and Cell Biology,
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: In the middle of the cover is a photo and immunohistochemistry (IHC) of H2228 cells. Detection of EML4-ALK rearrangement by fluorescence (red), filament (green) and nucleus (blue). IHC stain and photo kindly provided by Kathrin Pfeifer. Top left corner; Brigatinib, PBD:5J7H, (teal). Top right corner; Alectinib, PBD:3AOX, (yellow). In the middle left; PBD:4CLI, ALK kinase domain (calcium), ATP-binding pocket (white). In the middle right; Tumor suppressor p53 (warm pink) complexed with DNA (orange). Lower left corner; Lorlatinib, PBD:5AA8 (orange). Lower right corner; Crizotinib, PBD:2XP2 (magenta).

Cover design by Abdulmalik A. Bokhari and Bengt Hallberg.

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abdulmalik.bokhari@gu.se

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“Each journey in our life builds part of our character. Fortunately, we will meet several people who have honesty, sacrifice, and positivity in their thoughts. They always want to encourage our journey and create a blooming environment in our travel paths. Therefore, we prefer to continue moving forward to reach our dreams better than parking our trips.” **Abdulmalik A**. **Bokhari**, **2022**.

The life journey starts with individuals and independence. Next, people take a path to seek out a successful key, such as education. Education is the master key to opening gates to business, increased social status, knowledge, connections with others such as scientists, and jobs. The educational system levels are built on different fields. Selecting a field depends on personal matches or wishes. Fulfilling wishes can be achieved only through persistent work on making that wish come true and transforming that hope into a reality. As a wise person said, desires cannot turn a donkey into a horse, so work hard to reach the goal that allows you to buy your horse. Furthermore, live with high ideals and expectations, and don't give up during your journey. The trip's path is full of dilemmas that need to be solved. On the path, you will meet people who disappoint, discourage, or are jealous, despairing, and pessimistic because you are on your way to flying like an American eagle. Don't listen to others. Focus on your goals. **Abdulmalik A**. **Bokhari**, **2022**.

I dedicate this thesis to my family, friends, and relatives for their unstoppable love and financial and emotional support and for sacrificing their effort and time to create a comfortable journey for my Ph.D.

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Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Lung cancer causes severe morbidity and mortality in millions of patients globally each year. Anaplastic lymphoma kinase (ALK) mutations mediate initiation and progression of lung cancer in adults and young adults. However, the ways in which ALK interacts with genetic factors to trigger a variety of signaling pathways remain poorly understood. We investigated the transcription factor Zfp148, which represses activation of tumor suppressor p53, and downstream signaling targets of ALK fusion proteins in patient-derived non-small-cell-lung-cancer (NSCLC) cell lines. In addition, we investigated the efficacy of the United States Food and Drug Administration (FDA)-approved ALK inhibitors brigatinib, alectinib, lorlatinib, crizotinib, and ceritinib as modes of therapy in patients with lung cancer. The cumulative data reported in this thesis shed light on the interplay between ALK mutations and the signaling pathways triggered by ALK in the process of cancer development. Our goal is to understand underlying molecular mechanisms in patient-derived cell lines and to identify drug combinations to treat ALK-driven NSCLC. To increase our understanding of differences between two clinical variants of ALK fusion proteins (EML4-ALK variant 1 and variant 3) in NSCLC, we investigated how these variants influence the activity of downstream signaling. We showed that triapine inhibitors of the RRM2 have synergistic effects preventing DNA repair, cell cycle regulation, and ALK activation when given in combination with ALK inhibitors in patients with NSCLC expressing EML4-ALK fusion proteins.

Keywords: Zfp148, TP53, ALK, EML4-ALK, crizotinib, ceritinib, alectinib, brigatinib, lorlatinib, RRM2.

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SAMMANFATTNING PÅ SVENSKA

Lungcancer orsakar varje år morbiditet och mortalitet hos miljontals människor värden över. Mutationer i Anaplastic lymphoma kinase (ALK) bidrar bland annat till utveckling av lungcancer hos vuxna och barn. Hur ALK samspelar med andra genetiska faktorer för att stimulera en bred repertoar av signaleringsvägar är fortfarande oklart. Vi undersökte transkriptionsfaktorn Zfp148, som inhiberar aktivering av tumörsuppressorn p53, och signaleringsvägar som aktiveras nedströms om ALK-fusionsproteiner i cellinjer som etablerats från patienter med icke-småcellig lungcancer. Vi testade hur effektiva de av amerikanska läkemedelsverket (United States Food and Drug Administration, FDA) godkända ALK-inhibitorerna brigatiniv, alectiniv, lorlatinin, crizotinib och ceritinib är mot icke-småcellig lungcancer. Sammantaget belyser de data som presenteras i avhandlingen samspelet mellan ALK-mutationer och de signaleringsvägar som aktiveras av ALK vid cancerutveckling. Målet är att förstå underliggande molekylära mekanismer i patient-deriverade cellinjer och att identifiera drogkombinationer för behandling av ALK-driven lungcancer. För att bättre förstå skillnaden mellan två kliniska varianter av ALK-fusionsproteiner (EML4-ALK variant 1 och variant 3) undersökte vi hur varianterna påverkar signaleringsvägar nedströms om mutationen. Vi visade att RRM2-hämmaren triapine blockerar DNA reparation, cell cykel–reglering, och ALK-aktivering på ett synergistiskt sätt när den kombineras med ALK-inhibitorer, hos patienter med ickle-småcellig lungcancer som utrycker EML4-ALK fusionsproteiner.

Nyckelord: Zfp148, TP53, ALK, EML4-ALK, crizotinib, ceritinib, alectinib, brigatinib, lorlatinib, RRM2.

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LIST OF PAPERS

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ABBREVIATIONS

ABCB1	ATP-binding cassette sub-family B member 1.
ABL1	Tyrosine-protein kinase ABL1.
ACK	Activated CDC42 kinase.
AKT/AKT1	Ak strain transforming or RAC (Rho family)-alpha serine/threonine-protein kinase.
ALCL	Anaplastic large cell lymphoma.
ALK	Anaplastic lymphoma kinase.
ALKAL1	ALK And LTK Ligand 1.
ALKAL2	ALK And LTK Ligand 2.
ALT	Alanine aminotransferase.
Apoe	Apolipoprotein E.
A-RAF	A- Rapidly Accelerated Fibrosarcoma.
ARF	ARF tumor suppressor.
ARID1B	AT-rich interactive domain-containing protein 1B.
AST	Aspartate aminotransferase.
ATP	Adenosine triphosphate.
AXL	Tyrosine-protein kinase receptor UFO.
BAX	Bcl-2 Associated X-protein.
BBB	Blood-brain barrier.
BCL11A	B-cell lymphoma/leukemia 11A.
Bcl-2	B-cell lymphoma 2.

bFGF	Basic fibroblast growth factor.
BH3	BCL2-homology domain 3.
BRAF	Proto-oncogene B-Raf.
CAS9	CRISPR associated protein 9.
CASP8	Caspase-8.
c-CBL	Casitas B-lineage Lymphoma.
CCDC6	Coiled-coil domain containing 6 gene.
CD74	Cluster of Differentiation 74.
CDC42	Cell division control protein 42 homolog.
CDK2	Cyclin-dependent kinase 2.
CDKN2A	Cyclin-dependent kinase inhibitor 2A.
CDKs	Cyclin-dependent kinases.
cDNA	Complementary Deoxyribonucleic Acid.
Chks	Checkpoint kinases.
CLTC	Clathrin heavy chain 1.
CMTR1	Cap Methyltransferase 1.
CNS	Central nervous system.
CPS-II	Carbamoyl phosphate synthetase II.
C-RAF	Proto-oncogene c-RAF.
CRC	Colorectal cancers.
CRISPR	Clustered regularly interspaced short palindromic repeat.

CT	Computed tomography.
CTLA-4	Cytotoxic T-lymphocyte–associated antigen 4.
CUX1	Cut like homeobox 1.
Cyclin D2	G1/S-specific cyclin-D2.
CYP3A4	Cytochrome P450 3A4.
CYP3A5	Cytochrome P450 3A5.
DCTN1	Dynactin subunit 1.
DLBCL	Diffuse large B-cell lymphoma.
DNA	Deoxyribonucleic acid.
DNA-PK	DNA-dependent protein kinase.
DR5	Death receptor 5.
Drltk	*Danio rerio* leucocyte tyrosine kinase.
E2F	Group of genes that encodes a family of transcription factors.
EGF	Epidermal growth factor.
EGFR	Epidermal growth factor receptor.
EML4	Echinoderm microtubule-associated protein-like4.
EMT	Epithelial-mesenchymal transition.
ERK	Extracellular signal-regulated kinase.
ERK1	Extracellular signal-regulated kinase 1.
ERK2	Extracellular signal-regulated kinase 2.

EZR	Ezrin.
FDA	Food and Drug Administration.
FGFR3	Fibroblast growth factor receptor 3.
FIG	Fused in glioblastoma gene.
FISH	Fluorescence in situ hybridization.
FOXO3a	Forkhead box O3.
FRS2	Fibroblast growth factor receptor substrate 2.
G1	Growth phase.
GAP	GTP activating protein.
GCC2	GRIP and coiled-coil domain-containing protein 2.
GDNF	Glial-derived neurotrophic factor.
GIST	Gastrointestinal stromal tumors.
GO	Resting phase.
GPX4	Glutathione peroxidase 4.
GRB2	Growth factor receptor-bound protein 2.
GST	Glutathione-S-transferase.
HDAC	Histone deacetylases.
HELP	Hydrophobic echinoderm microtubule-associated protein-like protein.
HER2	Human epidermal growth factor receptor 2.
HER3	Human epidermal growth factor receptor 3.

HGF	Hepatocytic growth factor.
HGFR	Hepatocyte growth factor receptor.
HIP1	Huntingtin-interacting protein 1.
HRAS	Harvey Rat sarcoma.
IGFR1	Insulin growth factor receptor 1.
IHC	Immunohistochemistry.
IRS1	Insulin receptor substrate 1.
JAK	Janus kinase or just another kinase.
JNK	c-Jun N-terminal kinase.
KDM5A	Lysine-specific demethylase 5A.
KEAP1	Kelch-like ECH-associated protein 1.
KIF5B	Kinesin family member 5B.
KIF5B	Kinesin-1 heavy chain.
KIT	KIT is an RTK, found in 7-year-old FeLV infected. male domestic cat with fibrosarcoma, gene named Kit as in kitten.
KLC1	Kinesin light chain 1.
KRAS	Kirsten rat sarcoma.
LB1/CKAP2	Cytoskeleton-associated protein 2.
LDLa	Low-density lipoprotein receptor class A.
LRP1B	Low-density lipoprotein receptor-related protein 1B.
LTK	Lymphocyte tyrosine kinase.

LUAD	Lung adenocarcinomas.
LUSC	Lung squamous cell carcinomas.
M	Mitosis phase.
MAP2K4	Dual-specificity mitogen-activated protein kinase kinase4.
MAPK	Mitogen-activated protein kinase.
McL-1	Myeloid Cell Leukemia.
MDM2	E3-ubiquitin ligase mouse double minute 2.
MDMX	MDM4 Regulator of P53.
MDR1	Multidrug resistance 1.
MEFs	Mouse embryonic fibroblasts.
MEK1	Dual specificity mitogen-activated protein kinase1.
MEK2	Dual specificity mitogen-activated protein kinase2.
MET	Met RTK or hepatocyte growth factor receptor.
MSH6	MutS homolog 6.
mTOR	Mammalian target of rapamycin.
MYC	Myelocytomatosis viral related oncogene.
NB	Neuroblastoma.
NF1	Neurofibromin 1.
NFE2L2	Nuclear factor erythroid 2-related factor 2.

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells.
NGS	Next generation sequencing.
NOTCH	Notch signaling pathway.
NOXA/ PMAIP1	Phorbol-12-myristate-13-acetate-induced protein1.
NPM1	Nucleophosmin 1.
NRAS	Neuroblastoma Rat Sarcoma homolog.
NSCLC	Non-small cell lung cancer.
NTRK	Neurotrophic tyrosine kinase receptor.
ORF	Open Read Frame.
ORR	Overall Response Rate.
OS	Overall survival.
p15	Protien15.
p21	Protien21.
p27	Protien27.
P300	Histone acetyltransferase p300.
P53	Tumor protein P53.
PAI-1	Plasminogen activator inhibitor-1.
PD-1	Programmed death-ligand 1.
PET-CT	Positron emission tomography–computed tomography.
PFS	Progression-free survival.

P-gp	P-glycoprotein.
PI3K	Phosphoinositide 3-kinase.
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic Subunit alpha.
PK	Protein kinases.
PLCγ	Phospholipase C, gamma1.
PML	Progressive multifocal leukoencephalopathy.
PPM1D	Protein phosphatase 1D.
PPP	Pentose phosphate pathway.
PTEN	Phosphatase and tensin homolog.
PTM	Post-translational modification.
PTPN11	Tyrosine-protein phosphatase non-receptor type11.
PTPN3	Tyrosine-protein phosphatase non-receptor type3.
PUMA	P53 upregulated modulator of apoptosis.
RAF	Rapidly Accelerated Fibrosarcoma.
RAS	Rat sarcoma virus.
RB	Retinoblastoma protein.
RET	Rearranged during transfection.
RIP	Receptor-interacting protein.
RNA	Ribonucleic acid.

ROS1	Gene was isolated from a spontaneous chicken sarcoma and has unique sequence with the designated name ros, an RTK.
RSK	Ribosomal S6 kinase.
RTK	Tyrosine kinase receptor.
S	Synthesis phase.
SCF	Stem cell factor.
SCLC	Small cell lung cancer.
Shc	SHC-transforming protein 1 and it is important in the regulation of apoptosis and drug resistance in mammalian cells.
SHP2	Src homology region 2 domain-containing phosphatase-2.
SLC2A	Solute carrier family 2, facilitated glucose transporter member 9.
SLC34A2	Sodium-dependent phosphate transport protein 2B.
SLUG	Snail Family Transcriptional Repressor 2.
SNAIL	Zinc finger protein SNAI1.
SQSTM1	Sequestosome-1.
SRC	Proto-oncogene tyrosine-protein kinase Src: non-receptor tyrosine kinase protein that in humans is encoded by the SRC gene.
STAT	Signal transducer and activator of transcription.
STAT3	Signal transducer and activator of transcription 3.

STK11	Serine/threonine kinase 11.
STRN	Striatin.
TAPE	Tandem atypical β-propeller.
TD	Trimerization domain.
TDM-1	Trastuzumab emtansine.
TFG	Transforming growth factor.
TGF-alpha	Transforming growth factor alpha.
TGFB1	Transforming growth factor beta 1.
TGFBR	Transforming growth factor beta receptor I.
TGF-β	Transforming growth factor-beta.
TIGAR	TP53- inducible glycolysis and apoptosis regulator.
TKI	Tyrosine kinase inhibitor.
TNM	Tumor (T), nodes (N), and metastases (M).
TP53	Tumor protein P53.
TPM-3	Tropomyosin alpha-3 chain.
TPM-4	Tropomyosin alpha-4 chain.
TPR	Translocated promoter region.
TRK-A	Tropomyosin receptor kinase A.
TRK-B	Tropomyosin receptor kinase B.
Trp53	Transformation-related protein 53.
TSGs	Tumor suppressor gene.

TWIST1/2	Twist-related protein 1.
VEGF	Vascular endothelial growth factor.
VIT	Vitrin.
WD	Tryptophan-aspartic acid.
WNT	Signaling pathways.
WWP2	NEDD4-like E3 ubiquitin ligase.
XIAP	X-linked inhibitor of apoptosis protein.
ZEB1	Zinc finger E-box-binding homeobox 1.
Zfp148	Zinc finger protein 148.
ZNF148	Zinc Finger Protein 148.

1 INTRODUCTION

1.1 A glance into cancer

Cancer is the second leading cause of human mortality and morbidity globally, with a tremendous financial and societal burden (1). Cancer arises when normal cellular processes become altered in ways that result in abnormal growth. Most normal cells in the body proliferate, differentiate, and eventually subject themselves to apoptosis (programmed cell death), through modification of their normal physiology (**Figure 1**). Cancer cells evade cell death and instead acquire immortality.

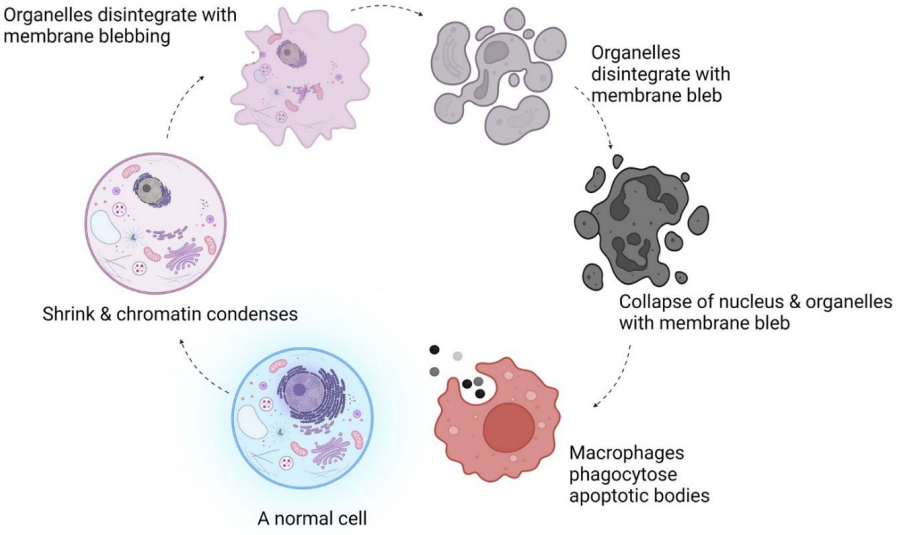

Figure 1. ***Apoptosis of normal cells****.*

1.2 Characteristics of cancer cells

In 2000, Hanahan and Weinberg defined eight types of malfunction as hallmarks of cancer. These hallmarks reflect the multi-step process by which normal cells are transformed into neoplastic, tumorigenic, and eventually malignant cells. The eight hallmarks are summarized below and in **Figure 2** (2).

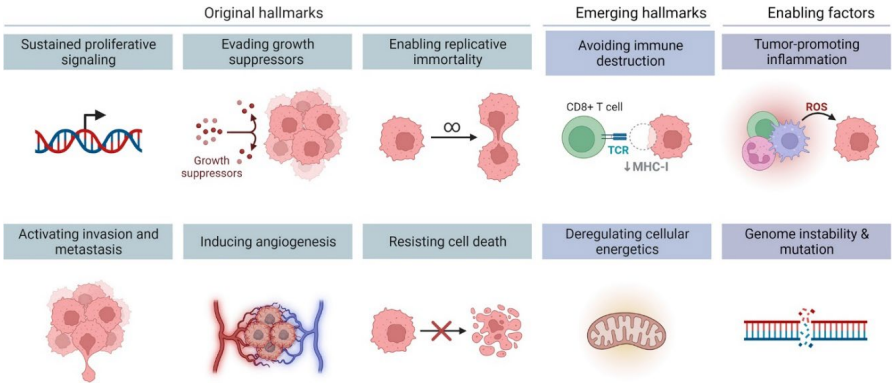

Figure 2. ***Hallmarks of Cancer****.*

Sustaining proliferative signaling: Proliferation of normal cells is rigorously controlled by the release of growth promoting signals from the tissue to maintain homeostasis. By various mechanisms, cancer cells reduce their dependence on paracrine signals and acquire the capability to sustain perpetual proliferation.

Evading growth suppressors: Retinoblastoma-associated (Rb) and tumor suppressor P53 (P53) are canonical growth suppressors that arrest progression through the cell cycle in response to various stressors. Cancer cells evade these signals with the help of Rb and P53 mutations that inactivate the proteins. An alternative way for cancer cells to evade growth suppression is to inactivate the transforming growth factor-beta (TGF-β) pathway, which normally suppresses cell cycle progression and promotes apoptosis.

Resisting cell death: Regulated cell death prevents neoplastic transformation of normal cells. Apoptosis in particular is a barrier against tumor formation. Tumor cells must evade activation of apoptosis in order to sustain periods of nutrient deficiency, hypoxia and other stressors.

Enabling replicative immortality: Telomeres are regions of repetitive DNA at the ends of chromosomes that protect the ends of the chromosomes and are essential for cellular replication. In normal cells, telomeres are limited and degrade over time, resulting in senescence (nonproliferation with the viable state) and eventual crisis (cell death). To maintain constant proliferation, cancer cells repair and maintain their telomeres, making them limitless and thus avoiding senescence and crisis.

Inducing angiogenesis: Tumor development requires enhanced angiogenesis to provide tumor cells with sufficient supplies of nutrients and oxygen. Tumor cells

releases angiogenic growth factors such as vascular endothelial growth factor (VEGF) to stimulate growth of new blood vessels into the tumor.

Activating invasion and metastasis: The processes of invasion and metastasis start with local invasion, which is followed by distant metastasis as the cancer cells spread throughout the body. Cancer cells penetrate neighboring cells and expand in a local invasion. Metastasis spreads local malignant cells via lengthy migration procedures that result in colonization of distant sites.

Deregulating cellular energetics: Normal cells acquire most of their energy from glycolysis and pyruvate oxidation in mitochondria. By contrast, cancer cells have increased energy requirements and depend on elevated rates of glycolysis and lactic acid fermentation in the cytosol.

Avoiding immune destruction: The immune system identifies and destroys abnormal cells in the body to prevent malignancies. Cancer cells develop ways to evade the immune system, which allows them to rapidly proliferate and invade tissues.

In addition, tumorigenesis is fueled by *genome instability* that increases the emergence of oncogenic mutations, fusions, and copy number alterations, and by *tumor-promoting inflammation* that creates a micro environment that promotes tumor progression, invasion and metastasis.

1.3 Summary of the cell cycle and oncogenesis

The cell cycle is the common fundamental mechanism by which all mammalian cells proliferate, grow, and develop. To ensure identical cell division, the cell cycle is divided into different regulatory phases, including resting phase (GO), growth phase (G1), synthesis phase (S), growth phase (G2), and mitosis phase (M). Each phase is characterized by specific cellular processes such as DNA synthesis, duplication of organelles, growth, DNA damage repair, and gamete formation for sexual reproduction. If abnormal conditions, such as DNA damage, arise at any point during the cell cycle, the cell cycle is arrested, and the cell does not continue to the next phase. This cell cycle regulation is enabled in part by tumor suppressors that halt the cell cycle at certain “checkpoints” when there are aberrant conditions (**Figure 3A**). Control of the cell cycle thus depends on checkpoints and cascades of protein phosphorylation that are activated when certain conditions are met. One vital mechanism that drives cell cycle regulation at checkpoints is the binding of cyclin-dependent kinases (CDKs) with cyclin proteins to form active complexes (**Figure 3B**).

Genetic abnormalities such as point mutations, DNA rearrangements, DNA amplifications, and epigenetic modifications can cause functional shifts of the cell cycle that transform normal cells into cancer cells (**Figure 3C**). The EMAL4–ALK fusion is a DNA rearrangement that creates an oncoprotein with the ability to continuously activate downstream signaling via dimerization of proteins such as Rat sarcoma (RAS) protein, which is essential for cellular growth, proliferation, and migration (**Figure 3D**). This allows cancer cells to bypass the need for external growth factors and sustain chronic proliferative signaling.

Tumor suppressor 53 (p53) is another protein involved in cell cycle checkpoints. Before the cell enters the S-phase, the p53 protein checks that the cell's DNA is intact. If the DNA is damaged, the p53 protein will activate cell cycle inhibitors such as CDK-interacting protein 1 (p21), which prevents cyclin E binding to CDK2, resulting in stoppage of G1 phase activity, cell cycle arrest, and apoptosis (**Figure 3E**). Inactivation of tumor suppressor genes such as p53, allow cancer cells to bypass cell cycle checkpoints, resulting in uncontrolled cell division (**Figure 3F**).

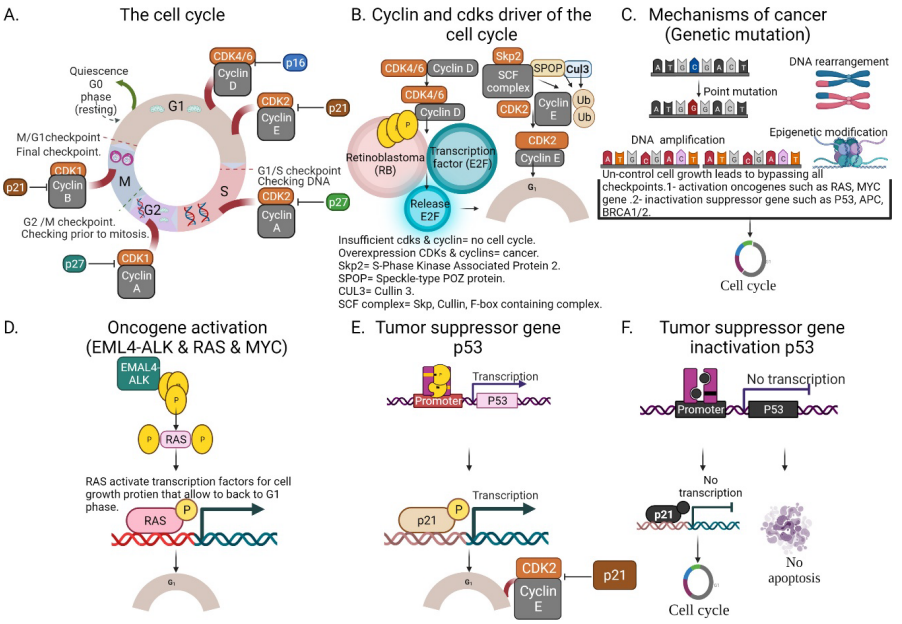

Figure 3. ***Mechanisms of cell cycle and oncogenesis****.*

1.4 Tumor suppressors

Tumor suppressor genes (TSGs) and proto-oncogenes play essential roles in cancer development. TSGs, also known as anti-oncogenes, have functions that

help to control cell growth and prevent cancer, such as the regulation of cell division and DNA repair (3). Proto-oncogenes are genes that normally function in development but can become oncogenes when they are mutated. Oncogenes often result from the continual activation of proto-oncogenes. By contrast, TSGs lead to cancer when they are inactivated. The most commonly mutated TSGs include p53, CDKN2A and retinoblastoma protein (RB1) (3).

1.4.1 The p53 protein

Discovered more than 40 years ago, the tumor suppressor p53, also known as phosphoprotein p53, cellular tumor antigen p53, or transformation-related protein 53 (Trp53), is the guardian of the genome and the most frequently altered gene in cancers (4). The p53 protein was long thought to prevent cancer by either inducing apoptosis or senescence in response to various cellular stressors. However, modern research demonstrates that p53 plays a broader role and suppresses tumor formation in a comprehensive manner.

In humans, p53 is encoded by the *TP53* gene (4). The homolog of *TP53* in mice is the *Trp53* gene (4). The human p53 protein contains 393 amino acids and includes two N-terminal trans-activation domains, one proline-rich domain, a specific DNA-binding domain, a C-terminal domain containing oligomerization, and a regulatory domain (5). *TP53* is mutated in approximately 50% of cancers, making it the most commonly mutated gene in cancer, whereas germline *TP53* mutations are rare (6).

Inactivation of tumor suppressor p53 promotes tumorigenesis by altering multiple biochemical, pharmacological, and physiological processes in human cancers (7). To activate p53 may therefore be an effective therapeutic strategy. In tumors with p53 mutations the goal is to reactivate misfolded p53 proteins (7). In tumors where p53 is suppressed by other mechanisms, the goal is to unleash p53 from the influence of negative regulators. To achieve this, we need to better understand how p53 is regulated under physiological and pathological conditions.

1.4.2 p53 suppresses tumorigenesis by different mechanisms

The main function of p53 is to prevent the formation of tumors. Accordingly, inactivation of p53 leads to tumor formation. The p53 protein is a transcription factor that operates by activating the transcription of other genes. The target genes of p53 in turn regulates DNA repair, removal of damaged cells, cell cycle arrest, senescence, apoptosis, and metabolic adaptations. Some of the p53 outputs are discussed briefly below.

Cell cycle arrest: The cyclin-dependent kinase (CDK) inhibitor p21 plays an essential role in promoting cell cycle arrest (8). Nuclear localization correlates with

the inhibitory effect of p21 on cell cycle progression (8). P21 can be induced via p53-dependent or p53-independent pathways (8). Multiple functions such as DNA repair, modulation of apoptosis, and transcriptional regulation are all attributed to p21 (8).

Senescence: Senescence causes cells to lose the capability to divide and proliferate in response to mitogens or growth factors. Senescent cells have decreased replicative capacity and increased expression of genes including p16, p53, and CDK inhibitors such as p15, p27, and p21 (9).

Apoptosis: Pro-apoptotic proteins such as BAX, PUMA, and NOXA are members of the Bcl-2 protein family. The BH3 interacting-domain death agonist is a robust pro-apoptotic inducer of apoptosis. P53 regulates NOXA, and expression of NOXA can activate several death mechanisms by inhibiting the anti-apoptotic proteins Bcl-2 and Mcl-1, leading to BAX activation. PUMA is induced by either p53-dependent or p53-independent pathways (10).

Metabolic adaptations: TP53- inducible glycolysis and apoptosis regulator (TIGAR) is highly expressed in cancer, and its regulation by p53 plays essential diverse roles in glycolysis and redox balance. The fructose-2,6-bisphosphatase TIGAR represses glycolysis and increases reductive biosynthesis through the pentose phosphate pathway (PPP) (11), thus suppressing tumor formation. However, TIGAR can also promote tumor formation (11).

1.4.3 P53-activity is controlled by MDM2 and MDMX

The regulation and activation of p53 are affected by several factors including post-translational modification (PTM) of p53, heterogeneity in the spatial composition of response elements (REs) of p53 target genes, the temporal dynamics of p53 activities, and interaction between p53 and cofactors (4). The regulation of p53-stability by MDM2 and MDMX is of particular importance to control p53-activity.

MDM2 and MDMX (also known as HDMX and MDM4) are potent negative regulators of p53 (7). MDM2 and MDMX both consist of an amino-terminal p53 binding domain, a carboxyl-terminal RING domain, and sequence motifs for nuclear import and export (7). MDM2 functions as a E3 ubiquitin ligase that poly-ubiquitinates p53 for proteasomal degradation, thus keeping the level of p53-activity low in unstressed cells (7). The negative regulation of p53 by MDM2 is balanced by interactions with an alternate open reading frame (ARF), which directly inhibits MDM2 and thus activates p53 function (7). The ARF protein (also known as p14 in humans and p19 in mice) is encoded by the CDKN2A gene and will be further discussed in later sections. MDMX does not have E3 ubiquitin ligase activity, but

the relative abundance of MDMX can increase or decrease the ubiquitin ligase activity of MDM2 (7). Finally, both MDM2 and MDMX can inhibit the p53 transactivation process by involving the p53 amino-terminal transactivation domain via connected N-terminal hydrophobic pockets (7).

Both the MDM2 promoter and the MDMX promotor harbors p53-responsive elements and are transcriptionally activated by p53, thus forming a negative feedback loop. However, MDM2 is more responsive to p53 activation, whereas MDMX is induced by p53 only under particular conditions (7).

The significance of MDM2 and MDMX regulation of p53 is evident from cancer genetic studies: MDM2 or MDMX genes are frequently amplified in human cancers, thus contributing to tumorigenesis by destabilizing p53 and blocking p53-dependent transcription (7). Amplification of MDM2 and MDM4 is mutually exclusive with p53-mutations (7).

1.4.4 Pathways regulating cell death and survival converge on MDM2 and MDMX

Several kinases regulate cell death or survival by phosphorylating MDM2 and MDMX proteins. For example, AKT kinases promote cell growth and survival by phosphorylating MDM2 (at Ser166 and Ser186) and MDMX (at Ser367), thus increasing MDM2's destabilization of p53 (7). Similarly, phosphorylation of MDMX by casein kinase 1 alpha (CK1α) reduces p53 activity.

In instances of DNA damage, the ataxia-telangiectasia mutated (ATM) and checkpoint kinases (Chks) can also phosphorylate MDM2 or MDMX, leading to dissociation or destabilization of MDM2 or MDMX that prevent their destabilization of p53 (7). Other kinases that can phosphorylate MDM2 and MDMX in response to DNA damage are ABL (also known as ABL1), which phosphorylate MDMX, and DNA-dependent protein kinase (DNA-PK), which phosphorylates MDM2 (7). Moreover, dephosphorylation of MDM2 and MDMX by protein phosphatase 1D (PPM1D) impair their function and contribute to oncogenesis (7).

1.4.5 P53 reactivation in cancer therapy

Inactivation of tumor suppressor p53 promotes tumorigenesis by altering multiple biochemical, pharmacological, and physiological processes in human cancers (7). Therefore, activating p53 may be an effective therapeutic strategy. In tumors with p53 mutations, the goal is to reactivate misfolded p53 proteins (7). In tumors where p53 is suppressed by other mechanisms, the goal is to unleash p53 from the

influence of negative regulators. To achieve this, we need to better understand how p53 is regulated, under physiological and pathological conditions (7).

Approximately 50% of patients with cancer have lost tumor suppressor function because of mutations in the TP53 gene (7). In addition, MDM2, MDMX, and other signaling modules can downregulate p53 function. Selective targeting of either MDM2 or MDMX is a viable strategy for cancer therapy because deactivation or deletion of MDM2 can cause p53 activation in tumor cells (7).

There are several therapeutic strategies to reactive p53 by targeting MDM2 or MDMX. One strategy is to modulate protein expression with molecules such as NSC207895 (inhibits MDMX transcription) or 17-AAG (a HSP90 inhibitor). Another strategy is to target protein–protein interactions between p53 and MDM2 or MDMX using molecules such as nutlin3a, MI-219, and AM-8553, which target the MDM2 N-terminal p53-binding pocket; SJ-172550 and WK298, which target the MDMX N-terminal p53-binding pocket; RO-5963, SAH-p53-8, PMI peptide, and pDI peptide, which target the N-terminal p53-binding pockets of both MDM2 and MDMX; and RITA, which targets the p53 terminal domain. It is also possible to target MDM2 E3 ubiquitin ligase activity with molecules such as HLI98, MPD (targets the MDM2 RING domain), MEL23, and MEL24. Another strategy is to activate p53 by inhibiting p53–MDM2–proteasome interaction with JNJ-26854165 (target: MDM2) (7). Finally, heat shock protein 90 (HSP90) and RITA can reactivate p53 and induce tumor cell apoptosis (7).

1.4.6 The CDKN2A locus

CDKN2A is a tumor suppressor controlling tumor proliferation in cell cycle regulation. The most commonly mutated TSGs include *CDKN2A, p53*, and retinoblastoma protein (*RB1*). *RB1* inhibits *E2F* transcription factors to avoid cell cycle progression (3). *P53* integrates cell signaling related to DNA integrity and replication and acts as a transcription factor for p21, which in turn halts the cell cycle in the G1 phase.

CDKN2A encodes both tumor suppressor p16 and *p14*/*p14ARF*/*ARF* and mutations in *CDKN2A* lead to inactivation of both *p16* and *ARF* proteins. *P16* plays a diverse role in the G1-to-S-phase cell cycle and acts as a checkpoint mechanism, and *p16* prevents cells from entering the cell cycle (G1 phase), inhibits cyclin Ds, and CDK4/6, and maintains *E2F* suppression by *RB1* by preventing the phosphorylation of RB1 and non-phosphorylated Rb1 remains attached to the *E2F* (12).

ARF is the most frequently mutated tumor suppressor protein in human cancer and acts as a tumor suppressor via the p53, p21, and MDM2 pathways. ARF can

stabilize and activate p53 by directly inhibiting MDM2 and activating p21, which leads to inactivating the cell cycle mechanism. In response to oncogenic stress, ARF-mediated p53 activation plays an essential role in protection against cancer (13). P53 and *CDKN2A* are the first and second most frequently inactivated TSGs in human cancers, respectively.

2 Zinc finger protein 148

Zfp148 (also known as ZBP-89, BFCOL1, or BERF-1) plays an essential role in the transcriptional regulation of various genes and functions, including cell growth and death (14). Zfp148 is a transcription factor that binds to promoters of target genes and either activates or suppresses their transcription. Zfp148 is a member of the Krüppel-type zinc-finger family (Cys2-His2-type), which also includes GATA-1, Egr1, WT-1, and Sp1 (14). It is located on chromosome 3q21 (794 residues) and is proximal to a breakpoint for translocations that cause leukemia (14). DNA binding is mediated by Krüppel-type zinc finger domains that bind to GC-rich DNA sequences (14). The intron/exon structure of Zfp148 is highly conserved (14). Both human and mouse Zfp148 comprise nine exons and four Krüppel-type zinc fingers, which reside within the amino-terminus of the protein (14). The zinc finger region contains a glutamic acid-rich domain located within the first 100 residues followed by two basic domains flanking the zinc-finger DNA-binding region (14). A third basic domain plus serine-rich and PEST (proline, glutamic acid, serine, threonine) domains lie in the distal 250 residues, which interact with p53 and p300 (**Figure 4**) (14).

2.1 Zfp148 suppresses cell proliferation by activating the cyclin dependent kinase inhibitor p21

Accumulating evidence indicate that Zfp148 physically and functionally interacts with important cell cycle regulators such as p21 and p53. However, its role in cell cycle regulation is not clear. Zfp148 and the co-activator p300 are required for proliferation arrest of HT29 cells in response to butyrate. Zfp148 contains consensus binding elements to transcriptionally activate p21 expression, and its amino-terminal domain can cooperate with p300 as a transcriptional coactivator to enhance p21 transcription. The interaction between Zfp148, p300 and the p21 promoter appears to be regulated by ataxia-telangiectasia mutated (ATM) in response to histone deacetylase inhibitors (HDACi) such as butyrate. ATM phosphorylates Zfp148 by binding with the zinc-finger and N-terminal domains of Zfp148 in an HDACi-dependent manner (15), thus causing p300 binding to the amino-terminal of Zfp148 (15). The depletion of Zfp148 or ATM prevents histone deacetylase inhibitors (HDACi) from inducing p21 expression.

Zfp148 causes arrest of cell proliferation and is indirectly involved in the cell cycle through its physical interaction with p53 (15). Investigations are ongoing to uncover the essential physiological role of Zfp148 (15).

2.2 Zfp148 interacts physically with the tumor suppressor p53

The transcriptional activation of p21 by Zfp148 may be further accentuated by physical interaction between Zfp148 and p53, a major regulator of p21 transcription.

Three pieces of evidence suggest that Zfp148 physically interacts with p53 proteins. First, co-immunoprecipitation (co-IP) analysis found that Zfp148 and p53 form a complex in AGS cells. A complex with endogenous p53 and Zfp148 was co-precipitated from crude extracts of AGS cells using p53 as bait (16). However, since the complex was pulled down with only one antibody against p53, it is possible that the antibody bound to additional proteins and that Zfp148 was pulled down by one of those proteins. To confirm the physical interaction with p53, the complex should be precipitated with antibodies against both proteins and with extracts of Zfp148- and p53-knockout cells as negative controls. Second, the G245D point mutation in p53 prevents binding of p53 with Zfp148 in co-IP experiments in Hep3B cells and thus omitted the nuclear translocation of Zfp148. The evidence of a physical interaction between overexpressed Zfp148 and mutant $p53^{G2445D}$ in Hep3B cells is more compelling (17). Complex formation was demonstrated using antibodies against both proteins, and with FLAG and HA-tagged proteins to further exclude the possibility of nonspecific binding of antibodies. Third, recombinant Zfp148 bound to p53 in cell-free glutathione-S-transferase (GST) pull-down assays. Binding domains were mapped by using smaller GST-fragments of the proteins as baits. Bindings was mediated by the zinc finger domains of Zfp148 and the DNA binding and C-terminal domains of p53. However, these domains are broad and further mapping is required to identify residues that are critical for the interaction.

Finally, strong evidence supports a physical interaction between Zfp148 and p53 but some controls and experiments are missing. In particular, complex formation between the endogenous proteins should be demonstrated by additional antibodies and in a wider range of cell lines, and protein-protein colocalization should be demonstrated with sophisticated methods.

2.3 Evidence from Zfp148 knockout experiments suggests that Zfp148 sustain cell proliferation by suppressing p53-activation under oxidative conditions

Several studies investigate the effect of targeting Zfp148 in mice. The majority of these studies report that homozygous deletion of Zfp148 causes perinatal lethality

and transient growth defects in pups that survive the perinatal period (18,19). However, one study demonstrated defective germ cell development and infertility in heterozygous mice that was not reported in other knockout studies (20), and one reported neural tube defects and anemia that was later attributed to an off-target effect (18,21). In addition, conditional targeting strategies have revealed roles of Zfp148 in specific organ systems: conditional deletion of Zfp148 in hematopoietic cells indicates that Zfp148 plays a role in erythroid development (21,22). Moreover, targeting of a splice variant of Zfp148 showed increased intestinal inflammation after dextran sulfate treatment, indicating a potential role of Zfp148 for maintaining the integrity of the intestinal epithelium (23), a finding that was later corroborated in mice with conditional deletion of Zfp148 in gut epithelium showing defective defense against Salmonella typhimurium (24).

The perinatal lethality of Zfp148 knockout mice has been attributed to ectopic activation of p53 (18). Newborn Zfp148 knockout mice showed signs of cyanosis, leading to an investigation of their lung status. Zfp148-deficient lungs showed defective sacculation and glycogen accumulation, indicating a maturation defect that could be traced back to arrested cell proliferation in the prenatal lung parenchyma (18). Mechanistic studies revealed that mouse embryonic fibroblasts (MEFs) generated from Zfp148 knockout embryos ceased to proliferate after a few passages in culture, a phenotype caused by oxidative stress and activation of p53 (18). Strikingly, the lung maturation defect and perinatal lethality of Zfp148 knockout mice were rescued by deletion of one or both copies of Trp53, or by supplementing the drinking water of pregnant females of heterozygous intercrosses with the antioxidant n-acetyl cysteine. The result raises the possibility that Zfp148 sustains cell proliferation under oxidative conditions by suppressing p53-activation, a mechanism that could potentially be mediated by the previously demonstrated physical interaction between Zfp148 and p53.

The idea that Zfp148 regulates p53-activity gained further support in a study of atherosclerosis development in $Apoe^{-/-}$ mice (25). Inactivation of p53 accelerates atherosclerosis development in various mouse models, opening up for the possibility that p53-activation may protect against the disease. It was therefore tested whether heterozygous deletion of Zfp148 could protect $Apoe^{-/-}$ mice against atherosclerosis by unleashing p53-activity. Indeed, deletion of one copy of Zfp148 reduced atherosclerosis development in $Apoe^{-/-}$ by arresting macrophage proliferation in a p53-dependent manner (25). Zfp148 deficiency did not affect lipid metabolism and the protective effect was intrinsic to lesional macrophages, as demonstrated by bone marrow transplantation. The study shows that heterozygous inactivation of Zfp148 is sufficient to cause therapeutically meaningful activation of p53.

2.4 The role of Zfp148 in cancer

ZNF148 (the human ortholog of Zfp148) is overexpressed in human colorectal cancers (CRC), hepatocellular carcinomas, gastric cancer, esophageal squamous cell cancer, and pancreatic cancer compared to adjacent normal tissue (26–29). High expression of ZNF148 is associated with decreased survival in colorectal cancer, esophageal squamous cell cancer and clear cell renal cell carcinoma (28,30), but not in hepatocellular carcinomas type (26). Thus, it is possible that ZNF148 promotes tumor formation in gastrointestinal cancers, especially given that Zfp148 is required for maintaining the integrity of intestinal epithelium (24,31–34). The functional importance of Zfp148 has been investigated in mouse models of colorectal cancer driven by APC mutations (35,36). Mutations in the adenomatous polyposis coli *(APC)* gene are common in colorectal cancer. $APC^{Min/+}$ mice, which are considered an authentic model of sporadic and inherited colorectal cancer, develop multiple adenomatous polyps in the small intestine with hemorrhage, anemia, and death after 30 weeks (37,38).

Deletion of one copy of Zfp148 markedly reduced the number of adenomas and extended the survival of $APC^{Min/+}$ mice compared to controls (35). Deletion of Zfp148 had no effect on the size of tumors or the rate of tumor cell proliferation or apoptosis. Thus, Zfp148 may not affect the progression of existing tumors but the initiation of new tumors. In line with this, intestinal explants from Zfp148-deficient $APC^{Min/+}$ mice showed increased expression of phosphorylated p53, p53-target genes, and apoptosis after treatment with a glycogen synthase kinase-3 inhibitor that mimics the tumor initiation event in $APC^{Min/+}$ mice. There was no increase in p53-activity or apoptosis in explants from control mice. The result indicate that Zfp148-deficiency prevents tumor initiation by unleashing p53-activity. As such, there was no reduction of tumor numbers in Zfp148-deficient $APC^{Min/+}$ mice on $Trp53^{+/-}$ or $Trp53^{-/-}$ genetic backgrounds.

Other studies investigated the impact of deleting Zfp148 in certain intestinal cell populations in $APC^{FL/+}$ mice (39). Deletion of one copy or two alleles of Zfp148 in the gut epithelial cells by using the *villin-cre* transgene markedly reduced tumor development by diminishing expression of β-catenin target genes, the key mechanism downstream of APC-mutations. (36). Moreover, deleting Zfp148 in colonic epithelial cells by using the $Cdx2NLSCre^{ERT2}$ transgene reduced polyp formation by enhancing p16-dependent senescence (39). Thus, three independent studies demonstrate that Zfp148-deficiency can protect against colorectal tumors, although the suggested mechanisms differed (p53-driven apoptosis versus p16-driven senescence versus reduced expression of β-catenin target genes).

Can therapeutic targeting of Zfp148 reduce tumor development by increasing p53-activity?

As mentioned before, re-activation of p53 is an attractive novel therapeutic strategy to fight cancer. However, knockouts of MDM2 or MDMX in mice demonstrate that unrestricted p53-activity damages normal tissues and may be lethal (40). It is therefore desirable to find ways to activate p53 that are not associated with severe side effects. The studies on Zfp148 mentioned above suggest that deletion of a single copy of Zfp148 is sufficient to induce therapeutically relevant activation of p53 (25,35). Importantly, mice lacking one copy of Zfp148 do not show any obvious health problems, are fertile and exhibit a normal life span (18). Thus, therapeutic targeting of ZNF148 (the human ortholog) may protect against cancer without causing unacceptable side effects. However, for this to become a reality, the mechanism linking Zfp148 deficiency to increased p53-activity needs to be thoroughly investigated. We will address this in paper I.

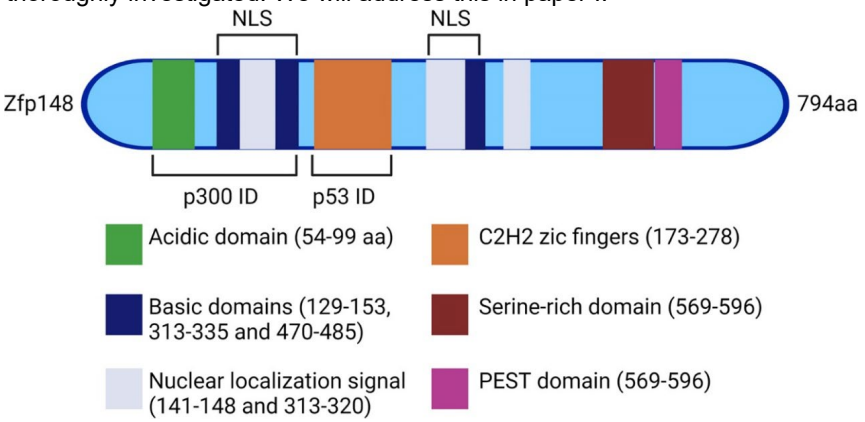

Figure 4. ***Zinc finger protein 148 structure****.*

3 Oncogenes

Genes in the nucleus carry vital information necessary for cells to function, grow, and survive. Proto-oncogenes are genes that are normally involved in cell growth, division, proliferation, and survival (41). However, a proto-oncogene can turn into an oncogene when mutation causes a conformational change in the translated protein that leads to enhanced or constitutive activation. In this case, the cell loses its proper regulation which can lead to cancer growth. There are several ways a proto-oncogene can become an oncogene:

1- Point mutation. A point mutation can change a proto-oncogene into an oncogene with constitutive protein activity. Well-known examples of point mutations in cancer are the G12, G13, and Q61 mutations in the KRAS protein, which is a small GTP-binding protein. The KRAS protein is activated when GTP-bound and inactivated when GDP bound. KRAS activation depends on specific guanine nucleotide-exchange factors such as Son of Sevenless (SOS), which exchanges GDP for GTP. Inactivation of GTP-bound KRAS is mediated by hydrolysis from both intrinsic activity and GTP-activating proteins such as GAP. The G12, G13, and Q61 mutations change the ability of the KRAS protein to hydrolyze GTP to GDP, causing oncogenic KRAS to become more or less constitutively active, leading to further activation of downstream signaling pathways of RAS (42).

2- Gene amplification. Gene amplification is a common genetic change in cancer. It is believed to occur in some instances during the progression of non-invasive-to-invasive breast carcinoma (43). In this case, cells overexpress certain parts of the chromosome, and each part can express from one to several hundred genes. Gene amplification promotes cancer by creating imbalanced expression of proteins within the cell. The first evidence of gene amplification in cancer was EFGR overexpression in breast cancer (44).

3- Genetic rearrangement or chromosomal translocation. Chromosomal translocation can take place when two chromosome breaks occur in the same time and space and one chromosome part attaches to another part of the same or another chromosome (45). The fusion of an end-breakpoint of one chromosome with the end-breakpoint of a different chromosome can lead to the formation of a new fusion genes, which can act as an oncogene (45). Chronic Myeloid leukemia (CML) was described as a type of cancer of the white blood cells by David A. Hungerford and Peter C. Nowell (46–49). The disease is associated with a chromosomal rearrangement, called the Philadelphia chromosome or the breakpoint cluster region-Abelson (BCR-ABL) rearrangement, which causes a fusion of two genes, the a tyrosine kinase ABL and BCR (47–49) (**Figure 5**).

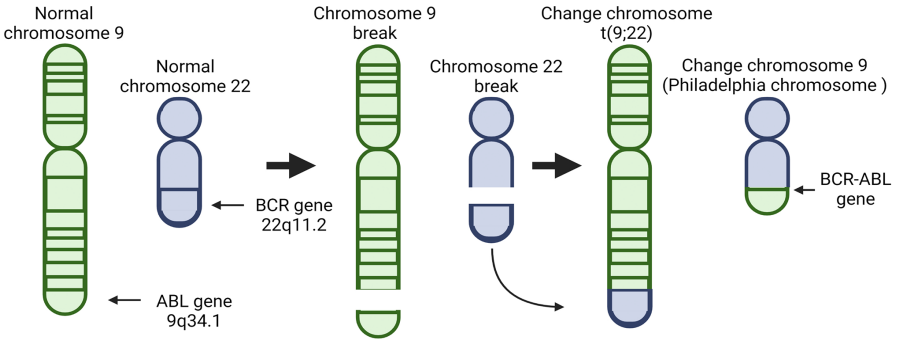

Figure 5. ***Schematic of Philadelphia chromosome****. The BCR-ABL fusion-protein is a constitutively activated tyrosine kinase oncoprotein that can trigger activation of multiple downstream pathways?* (50,51).

4 Receptor Tyrosine Kinase superfamily

One of the largest protein families in the human genome is the protein kinase family. There are 518 protein kinases identified and they are broken down into different families and subclasses (52). One such family is the protein tyrosine kinase (PTK) family, which facilitate the transfer of the gamma-phosphate group from ATP to the tyrosine residues of a substrate protein (53–55). The PTK superfamily is divided into two groups: 58 different Receptor Tyrosine Kinases (RTKs) and 32 different cytoplasmic Protein Tyrosine Kinases (PTKs) (52,56).

The 58 RTKs are further grouped into 20 subfamilies, which are classified according to their sequence and molecular architecture. The RTK extracellular domains differ between subfamilies and contain a diversity of structural domains (57–60). Receptor tyrosine kinases (RTKs) play essential roles in receiving signals and relaying the information to intracellular pathways to regulate numerous fundamental cellular processes, serving as important signals for cellular hemostasis or changes in cellular task (53–55). Termination of RTK activation is controlled very thoroughly through activation of downstream tyrosine phosphatases and receptor-mediated endocytosis followed by receptor degradation. Dysregulation of RTK signaling can cause disorders in cellular functions (61) and gain-of-function mutations can lead to the development of cancer (53). Furthermore, numerous studies of RTK's importance in mammalian development have been investigated in genetically modified mice where dysregulation can cause severe developmental abnormalities or embryonic lethality (62).

Structurally, RTKS have three domains: an extracellular domain for binding to ligands, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase

domain (63–65). Receptor activation can be summarized by four main processes: ligand binding to the extracellular domain, extracellular ligand-induced receptor dimerization, auto-trans-phosphorylation of the tyrosine receptor, and activation/phosphorylation of downstream signaling (**Figure 6**) (57,66,67). The phosphorylated tyrosine on the receptor serves as docking positions for a number of different intracellular adaptor and effector enzymes that transfer signals to the cytoplasm and nucleus, resulting in changes in cell function or fate (64). The transmembrane domain plays an important role in RTK signaling, not only by contributing to the stability of the receptor within the membrane but also by maintaining the signaling competent dimeric receptor shape (68).

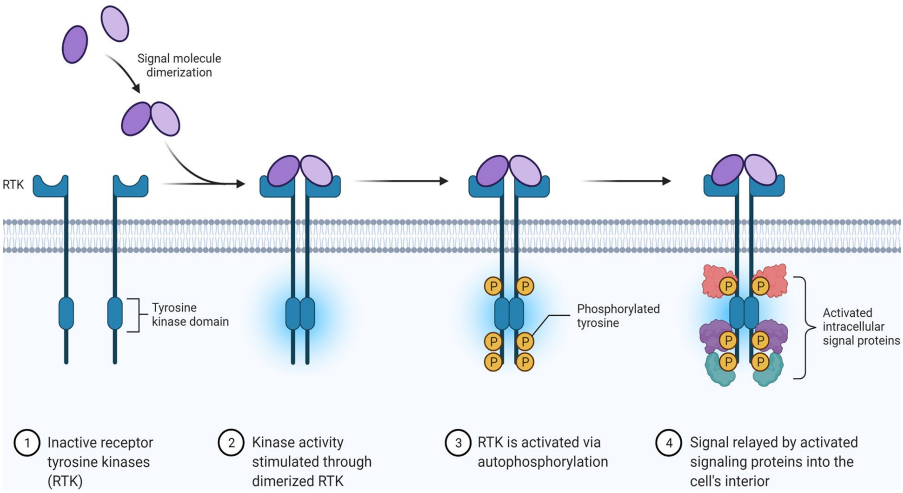

Figure 6. ***Schematic of activation of tyrosine kinase receptors****.*

4.1 ATP binding and Phosphorylation in Tyrosine Kinase Domain

The intracellular receptor kinase domain comprises two conserved lobes, the amino-terminal lobe and the carboxy-terminal lobe (64). The two lobes are connected through a hinge region to create a cleft or channel for ATP binding, the so called ATP-binding pocket, allowing the catalytic kinase reaction to proceed (64). The flexibility and dynamic infrastructure of the kinase domain is essential in allowing allosteric regulation to control the active and inactive conformations of the amino-terminal and carboxy-terminal lobes (64). The kinase domain also includes internal hydrophobic non-contiguous motifs that make up the conserved regulatory spine and catalytic spine (64). The catalytic spine is in a position where it can bind the adenine base of ATP, and the regulatory spine, can fold and unfold to switch between active and inactive conformations (64). Included in the regulatory spine is

the DFG motif (which can be active or inactive) that contains a phenylalanine packed into the hydrophobic pocket between the amino-terminal lobe and the carboxy-terminal lobe, creating the hydrophobic regulatory spine (69). In the inactive (DFG-out) conformation, the ATP-binding site in the catalytic spine is blocked by an aspartate residue, whereas the active (DFG-in) conformation allows ATP binding and catalysis (69).

After the receptor dimerization and conformational change the enzymatic activity of the tyrosine kinase domain starts when the adenine base of the ATP molecule binds to the pocket of the catalytic spine (60). The adenine base and the phosphate groups (tail) of the ATP then create multiple hydrogen bonds in the binding hinge (pocket) region, starting catalysis (60,70). This allows the enzyme to convey the γ-phosphate (phosphate number three in the ATP tail) to the substrate tyrosine enzyme (60). The next step involves the DFG-in conformation, where two asparagines can bind an Mg^{2+} ion to the catalytic spine and the regulatory spine (60). The Mg^{2+} ion stabilizes the negative charge of the phosphate groups (alpha (α), beta (β), and gamma (γ)) of ATP (60). In addition, it recruits H_2O molecules into the binding pocket, anchoring the ATP in the kinase domain and creating enough space for the phosphoryl to be conveyed onto a tyrosine residue (60,71,72). Hydrolysis of ATP requires only one molecule of water (60). To stabilize the tail of the ATP, the beta- and gamma phosphates interact with the glycine-rich loop and the alpha-C-helix of the enzyme to coordinate their locations (60). Finally, the ATP stabilizes and connects with the hinge region, the alpha-C-helix, the glycine-rich loop in between the amino-terminal lobe and catalytic loop in the carboxy-terminal lobe (60,72) (**Figure 7**).

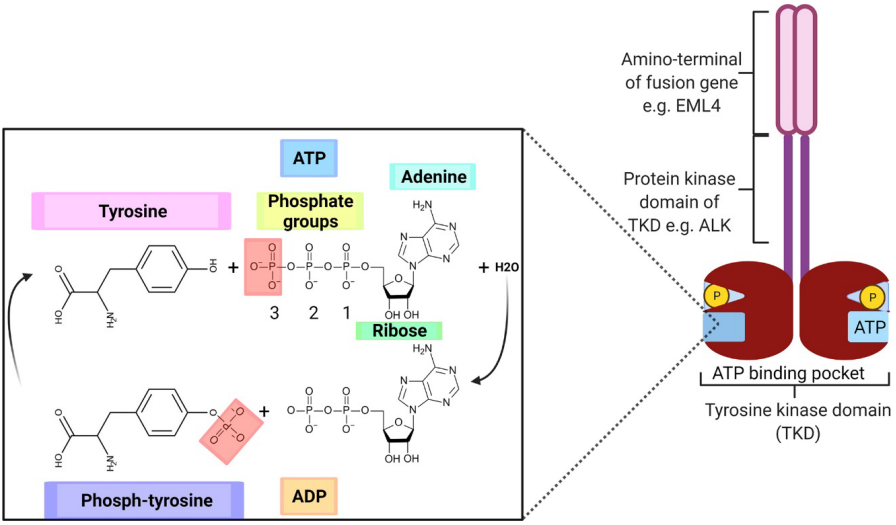

Figure 7. ***Schematic of ATP binding and tyrosine kinase phosphorylation****: The tyrosine executes nucleophilic attack on the gamma phosphate of ATP (highlighted in red), which conveys a phosphoryl group onto the tyrosine to form phospho-tyrosine and ADP.*

4.2 Anaplastic lymphoma kinase (ALK)

ALK was discovered in 1994 in anaplastic large cell lymphoma (ALCL) cell lines caused by a recurrent chromosomal rearrangement t(2;5)(p23;q35) that fuses the nucleophosmin 1 (*NPM1*) gene with a portion of the kinase domain of *ALK* (73). A couple of years later ALK gene was sequenced and has shown that ALK is a receptor tyrosine kinase (RTK), which shares 50% homology with leucocyte tyrosine kinase (LTK) protein sequence. (74–76). LTK and ALK receptors together are a subfamily within the insulin receptor superfamily based upon kinase domain similarities. In humans, the protein encoded by the *ALK* gene consists of 1620 amino acids and has a molecular weight of 180 kDa. However, due to post-transcriptional modifications, such as N-glycosylation, the molecular weight of ALK increases to 220 kDa (77). Structurally, ALK comprises three domains: a unique extracellular domain for ligand-binding (e.g., ALKAL1 and ALKAL2 (78–80)), a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain(64).

The extracellular ligand-binding domain consists of two MAM (meprin/A5 protein and receptor tyrosine phosphatase mu) domains which may function in cell/cell interactions (81). In addition to the MAM domains, the extracellular domain contains a glycine-rich domain, a membrane-proximal EGF-like module and a low-density

lipoprotein receptor class A (LDLa) domain (78). Recently, several groups revealed the structure of the interaction between ALK and its ligands, ALKALs, which shows a novel ligand-mediated dimerization mechanisms for RTK's (82–84). Briefly, the structure of ALK shows a unique novel array of TNF-like modules, which act together with the membrane proximal EGF-like model and the glycine-rich domain of the extracellular part of ALK. These three parts regulate receptor activity by binding to ALKALs, thereby the ligand enables the dimerization and activation of the receptor complex (**Figure 8**).

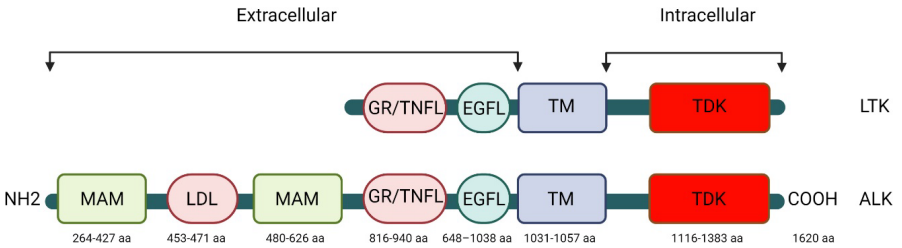

Figure 8. ***ALK and LTK structure****: The extracellular domain is essential for ligand binding, dimerization, and interaction with other receptors, leading to intracellular activation. The extracellular domain of LTK contains the glycine (GR) embedded with TNF-like (TNFL) domain. The extracellular domain of ALK contains the glycine (GR) domain, the membrane-proximal EGF-like module (EGFL), two domains of meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu (MAM) with different amino acids length and a low-density lipoprotein class A (LDLa) domain. In addition, LTK and ALK contain transmembrane (TM) domain located in links between the extracellular domain and the intracellular domain. The intracellular domain harbors tyrosine kinase domains (TDK), which are responsible for activating other receptors.*

The structure of the ALK kinase domain was revealed 2010 by several groups, providing important insight into the regulatory mechanisms of ALK activation (85,86). The ALK kinase domain is very similar to that of other RTKs. As mentioned previously, the kinase domain contains two lobes, N and C-terminal lobes, and two spines, the catalytic and regulatory spines, which are conserved among kinases (86). Similar to the insulin receptor, ALK has a Y XXX YY autophosphorylation motif. The three tyrosine residues Tyr1278, Tyr1282, and Tyr1283 (87) are essential for kinase activity within the intracellular kinase domain and thus the activation of the receptor (**Figure 9**). It has been proposed that the third tyrosine, Y1283, in the ALK kinase domain appears to be the most important for activation of the full-length receptor (88).

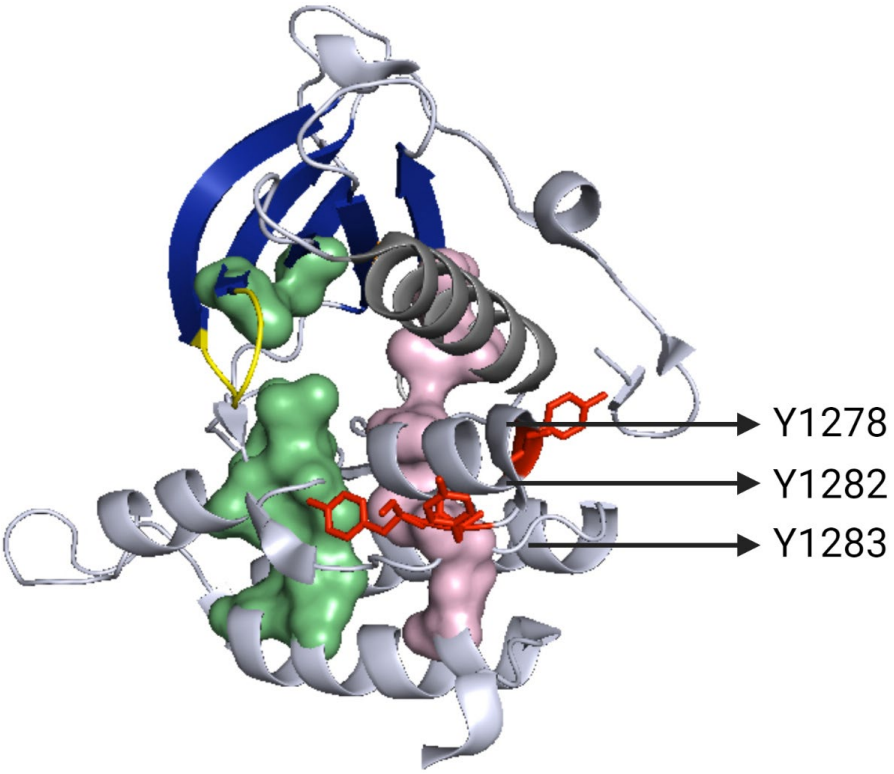

Figure 9. ***Three dimensions of the anaplastic (ALK) domain****. ALK kinase domain consists of the small N-lobe and a large C-terminal lobe. The two lobes are connected by a flexible hinge region which contains the ATP binding site and the substrate binding site. The N-terminal lobes consist of alpha (αC) helix (dark gray), glycine loop (yellow), and five beta-sheets (blue). The C -terminal lobe contain an activation loop including the three important tyrosine residues for activation of the kinase (Y1278, Y1282, and Y1283 (shown in red, sticks). Regulation within and between N-and C-lobes is flexible, through the hinge region and intramolecular changes, which allows to active and inactive the kinase conformation. The regulatory (pink) and catalytic (green) spines consist of two hydrophobic non-contiguous motifs that span both the N- and C-terminal lobes, which is conserved across all kinases. The adenine ring of bound ATP can complement the hydrophobic residues of the catalytic spine. The hydrophobic regulatory spine contains the residues, F1271, I1171, D1311, C1182, and H1247 amino acids (not shown)* (64). *The catalytic spine (green) contains the residues, V1130, A1148, L1256, C1255, L1256, L1257, L1318, and I1322 (not shown). Both spines are anchored in the most distal α helix from the ATP binding pocket of the C-lobe (PDB:3LCS)* (64).

5 ALK RTK in other model systems

Homologs of ALK have been identified in non-human species, such as the fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), zebrafish (*Danio rerio*), mouse, rat and chicken (89,90).

5.1 Drosophila melanogaster

During *Drosophila melanogaster* embryogenesis, the ALK gene plays an essential role in the formation of the visceral mesoderm and, later in development, it affects body size and the nervous system (91). A complete lack of ALK expression in the fly causes undeveloped founder cells in the embryonic visceral muscle, leading to a non-functional gut and embryonic lethality (91). Activation of ALK signaling in *Drosophila* requires Jelly belly (Jeb) and the *Drosophila* ALK ligand which define the ligand receptor-pair that functions *in vivo* to form mature visceral mesoderm. The ligand-receptor pair stimulates downstream signaling through the MAPK-ERK1/2 pathway and regulates transcription of targets, such as dumbfounded/kirre and org-1 (92,93). In the nervous system, the ligand-receptor pair mediates neuronal circuit assembly in the optic lobe/visual system (93–95). Additionally, *Drosophila* ALK is suggested to play a role in growth, body-size, and learning (93–95).

5.2 Zebrafish

In zebrafish (*Danio rerio*), both LTK and ALK genes are expressed. The zebrafish LTK contains a MAM domain and is expressed in the neural crest. In this manner the leucocyte tyrosine kinase (Drltk) is similar to ALK. Furthermore, it has been reported that Drltk plays an essential role in controlling iridophore proliferation, development of neural crest-derived pigment cells, and neurogenesis (96). Gain-of-function mutations in Drltk can lead to the development of ectopic iridophores and excess iridophore production, whereas loss-of-function mutations in Drltk are lethal in zebrafish larvae and adults (96). The ligands of zebrafish Drltk are AlKal1, AlKal2a, and AlKal2b, which play a role in the development and control of crest-derived iridophores (96).

5.3 Mammals

In mice, ALK plays an essential role in cellular communication and central nervous system (CNS) development (91,93,97–99). *In vitro* and *in vivo* data have established roles for ALK in the development of synapses, axonal regeneration, targeted axonal growth, neuromuscular junctions, behavioral responses and determination of sleep patterns, mood, and susceptibility to depression (100). Furthermore, *in situ* hybridization data confirmed the presence of *ALK* in the thalamus, hypothalamus, dorsal root ganglia and olfactory bulb of the developing mouse brain (101). In the human fetal brain, the distribution of *ALK* was found to be more limited and mainly scattered in the neural and endothelial cells and the pericytes (102). Although ALK is present in fetal brains, its expression decreases to almost nil immediately after birth (103).

ALK and LTK are expressed in neural tissues during mammalian development and in adult neurogenesis (104). Analyzed gene expression, behavioral phenotype, and an indicator of adult neurogenesis within knockout of ALK has a variable effect such as learning performance. Adult neurogenesis in mice can be affected and decreased up to 50% in the newborn neurons in both younger and older adult males due to loss or inhibition of ALK and ALK/LTK function (104). Finally, ALK appears to be involved in hypogonadotropic hypogonadism by regulating the timing of pubertal onset and testis function at the upper levels of the hypothalamic-pituitary gonadal axis (99).

5.4 Wild type activation and signaling

In general, the function of the ALK extracellular domain is to receive and interact with specific ligands. The ALK extracellular domain interacts with ligands ALKAL1 and ALKAL2, which induces a conformational change in the kinase domain, from inactive to active conformation (57,105,106). The dimerization of the receptor allows transphosphorylation of tyrosine residues in the juxtamembrane domain to suspend the autoinhibitory machinery, which promotes the receptor's activation, and phosphorylation of tyrosine residues (57).

5.5 Oncogenic signaling

In ALK gain-of-function (ligand-independent) can be caused by, a mutation in the intracellular kinase domain that leads to continuous ALK activation (57). Another scenario occurs with ALK fusion oncoproteins that dimerize and are continuously active within the cell. (57). The NPM-ALK fusion protein activates the PI3K/Akt kinases and downstream signaling cascades, leading to phosphorylation of FOXO3a transcription factor that is essential for cell survival, and inhibition of FOXO3a phosphorylation leads to induced caspase-3 activity (57,80,107–110).

Other downstream signaling targets of ALK, which are activated or phosphorylated by ALK, are various cellular proteins, such as GRB2, IRS1, Shc, FRS2, PLCγ, NF1, cyclin D2, NIPA and CDC42. These proteins initiate several other signaling cascades, including the MAPK-ERK, PI3K/AKT/mTOR, JAK-STAT, and phospholipase C pathways (111). Activating downstream signaling pathways such as JAK-STAT, PI3K-AKT, PLCY-PKC, and RAS-MAPK can stimulate several biological hallmarks like cell survival, differentiation, proliferation, metabolism, migration, and adhesion (57) (**Figure 10**).

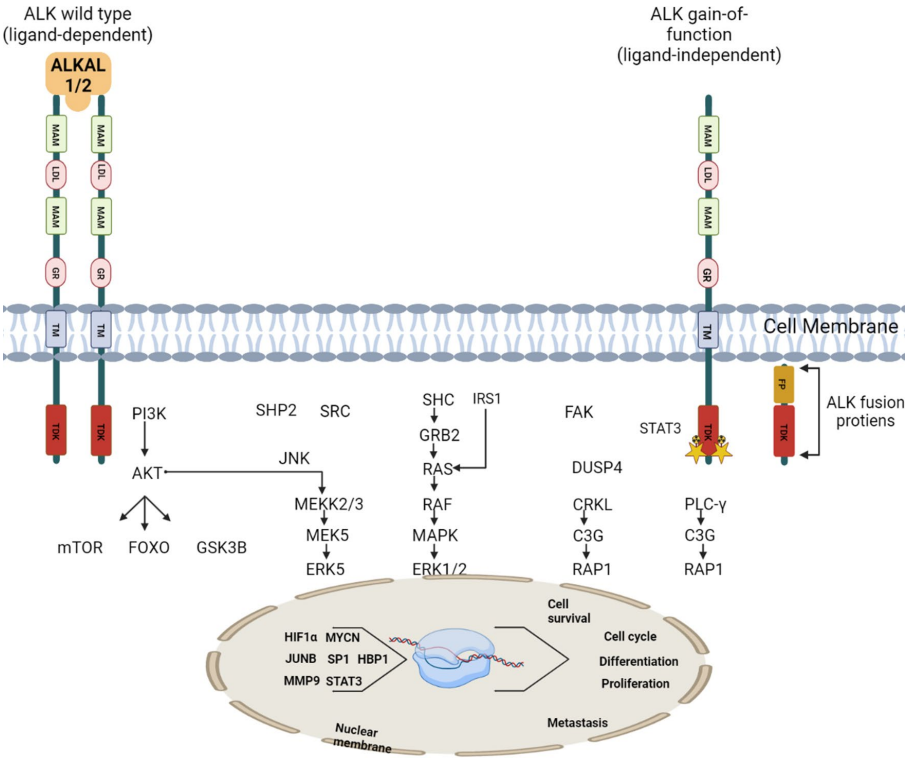

Figure 10. ***Schematic representation of ALK downstream signaling****. ALK wild type receptor is activated in a ligand-dependent manner. The oncogenic ALK signaling could either occur through 1) translocation and fusion with a partner gene generating a ALK fusion proteins that causes dimerization to activate downstream signaling, 2) gain-of-function mutation in the full length ALK kinase domain, or 3) chromosomal ALK receptor amplification* (111)*. ALK activate several downstream signaling cascade such as PI3K and RAS/MAPK pathway which activate further downstream target such as AKT/mTOR and ERK1/2, respectively. Both pathways participate in cellular read outs such as regulation of cell cycle, survival, cell mobility, adhesion, and metabolism* (112,113)

6 ALK in disease

6.1 Neuroblastoma

Neuroblastoma (NB) is a childhood cancer contributing to 15% of all cancer-related deaths in this age group (114). The incidence of NB is one out of 7,000 children (115) and is rarely encountered in children above 10 years. Almost 90% of the cases are diagnosed in children earlier than five years of age, and a hereditary linkage can be found in 1–2% of the cases (116). NB is a heterogeneous tumor related to the nervous system and is thought to arise from neural crest cells of the

sympaticoadrenal lineage. Although not confirmed, neuroblastoma is considered to originate from undifferantiated neural crest cells through a block of differentiation (117). The tumor is commonly encountered in the adrenal (suprarenal) glands, but can be noticed also in the chest, abdomen, neck, and spine (118,119). The overall survival rate is <40% at five years of age (120).

Neuroblastoma is associated with a number of genetic aberrations, for example deletion of chromosome 1p and gain of parts of 17q. Furthermore, aneuploidy and amplification of MYCN and deletions of whole or parts of chromosome 11q are commonly observed. Recent studies also suggest that aberrant regulation of the ALKAL2, ALK and MYCN in chromosome 2p-gain/amplified may drive ALK signaling activity (117). In year 2008, point mutations were reported by a number of independent laboratories in both familial and somatic neuroblastoma (121–125) and around 5-8% of all cases have a mutation in ALK. Today, more than 35 recurrent *ALK* mutations have been identified in neuroblastomas so far; however, most of these are passenger mutations (126). The “hot-spot” point mutations, which are found in 85% of all ALK-mutated (ALK^+) neuroblastomas, are at the F1174, F1245, and R1275 residues of the ALK kinase domain (44).

Overexpression

Overexpression of ALK in cancer: Overexpression of ALK has been noted in multiple human cancers (44). Apart from neuroblastoma, it has been observed in cancers such as thyroid carcinoma, NSCLC, breast cancer melanoma, glioblastoma, astrocytoma and retinoblastoma (127). Overexpression of ALK in cancers may have importance for tumor initiation and progression. However, this is not clarified at a molecular level and the significance of overexpression in a clinical situation is also unclear.

ALK as a fusion protein, ALK-positive translocations

Formation of ALK fusion proteins: Genetic rearrangements, can produce various fusion genes involving an intracellular protein kinase domain (128). These fusion genes are often oncogenic and harbor an activated tyrosine kinase domain upon dimerization. Such oncogenic fusion proteins have been identified in a multitude of cancers (129). Advanced techniques, such as *in situ* hybridization, immunohistochemistry, and reverse-transcriptase polymerase chain reaction (RT-PCR) are tools employed in the clinic to uncover fusion proteins. (130). The first discovered ALK-fusion protein was NPM-ALK (131), and that discovery was followed by numerous observations of other ALK-positive translocations, such as variants of ALK-positive fusion proteins in Anaplastic Large Cell Lymphoma (ALCL)

and a number of ALK-fusions in Inflammatory Myofebroblastic Tumors (IMT), Non-Small-Cell-Lung-Cancer (NSCLC), Diffuse B-Cell lymphoma (DBCL), Squamous Cell Carcinom of the eospheagus (SCC), breast cancer, colon cancer, renal medullary carcinom and renal cell carcinom.

6.2 Anaplastic Large Cell Lymphoma (ALCL)

ALCL refers to a type of aberrant T-cell growth, non-Hodgkin lymphoma, which was described for the first time in 1985. Genetic rearrangements including 60–80% of ALCLs, where a t(2;5)(p23;q35) translocation generates a fusion between *NPM* and *ALK* (129). The ALK rearrangement is most common in systemic ALCL cases and approximately 80% of ALCL harbours, a NPM-ALK fusion partners, which is associated with a favorable prognosis (132). A number of other fusion partners for ALK have been reported in ALCL, such as ALK lymphoma oligomerization partner on chromosome 17 (ALO17) (133), 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleotide (ATIC) (134), tropomyosin 3 (*TPM3)* (134), tropomyosin 4 (TMP4) (135), TRK fused gene (TFG) (136), factor receptor-associated factor 1 (TRAF1) (137), myosin heavy-Chain 9 (MYH9) (138), and Clathrin heavy chain 1 (CLTC) (139).

6.3 Inflammatory myofibroblast tumors (IMT)

IMT is most frequent among younger persons but can occur with patient of higher age. Tumors, which falls in the category “inflammatory pseudotumors, are located at many different locations in the body, such as head and neck as well as upper respiratory tract, but also pelvis and retroperitoneum (REF). 50% of IMT carrying ALK rearrangements (140), and the first ALK+ IMT fusion was discovered in 1996 (141). Similar to ALCL other ALK fusion partners exist, such as cysteinyl-tRNA synthetase (CARS-ALK) (142), 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleotide (ATIC-ALK), SEC31L-ALK (143), Liprin-beta-1 (PPFIBP1-ALK) (144), tropomyosin 3 (TPM3-ALK) and tropomyosin 4 (TPM4-ALK).

6.4 DLBCL

Diffuse large B-cell lymphoma (DLBCL) is abnormal cell growth leading to cancer of B cells, a type of non-Hodgkin lymphoma. The B-lymphocytes are essential for production of antibodies that generally help fight infections (145). Approximately 7-8 cases per 100,000 adult patients have diffuse large B-cell lymphoma (146,147) It occurs in young adults but rarely in children (148). It is rare and aggressive when patients harbor ALK-rearranged diffuse large B-cell lymphoma (149).

7 Lung cancer

Lung cancer is the most common cause of cancer-related death in males and the second-most common cause in females (1). In Europe in 2020, the incidence of lung cancer among males was 100 cases per 100 000 individuals, while that among females was 45 cases per 100 000 individuals (150). In 2020, there were 2.2 million new cases of lung cancer and more than 1.8 million deaths due to lung cancer globally (151). Almost 99% of lung cancers are carcinomas, in which neoplastic cells develop from epithelial cells, whereas the remaining 1% are sarcomas (152). Lung cancer lesions are classified as either small cell lung cancer (SCLC), which comprises 10–15% of all cases (153), or non-small cell lung cancer (NSCLC), which accounts for the rest (154). NSCLC is a histological subtype of epithelial lung cancer that metastasize more slowly than SCLC (155). Of the NSCLCs, 40% are lung adenocarcinomas (LUADs), 25–30% are lung squamous cell carcinomas (LUSCs), and 10–15% are large cell undifferentiated carcinomas (**Figure 11**).

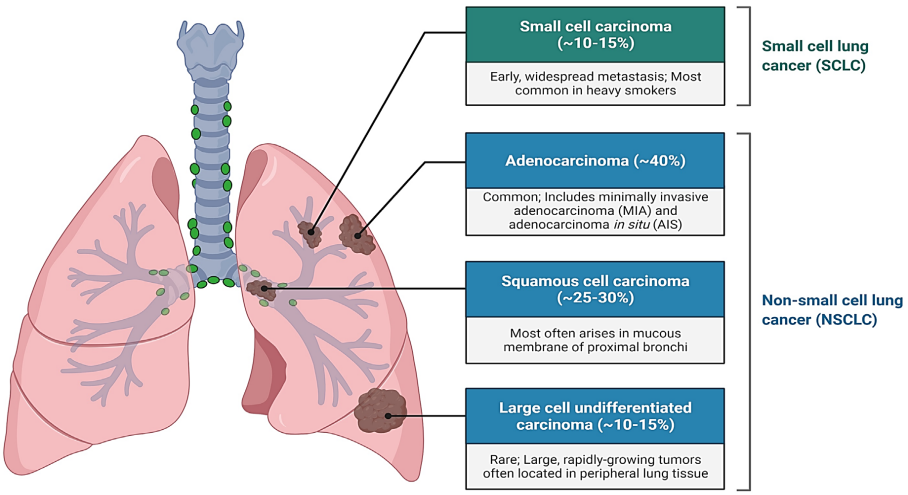

Figure 11. ***Types of lung cancer****.*

Tobacco smoking has been identified as the most common cause (83%) of lung cancer (156), although 8.3% of patients with lung cancer claim to have never smoked (156). Other factors contributing to lung cancer etiology are genetic predisposition and exposures to radiation, asbestosis, and air pollution (157). The common symptoms of lung cancer are anorexia, weight loss, cough, hematemesis, shortness of breath, and chest pain (158). Lung cancer is typically diagnosed by chest X-ray and computed tomography (CT) with further confirmation by

bronchoscopy under the guidance of CT. The average age at diagnosis is around 70 years. Lung cancer is notorious for its ability to metastasize to distant sites of the body (155). Treatment modalities typically include chemotherapy, radiotherapy, and surgical intervention. Despite recent advancements in cancer treatment, in 2021, the five-year survival rate among patients with lung cancer in the United States is currently only 23.7% (159).

7.1 ALK-positive non-small-cell-lung-cancer

Fifteen years ago, Mano and his colleagues employed an old-fashioned foci analysis to gain information about putative oncogenes from a retrovirus cDNA expression library created from male NSCLC patient (160). The same year Rikova and colleagues executed the first global phospho-profiling of NCLC cell lines (161). Both groups were successful in identifying the echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion protein formed by the inv(2)(p21;p23) inversion, which is found in 3–5% of NSCLCs (130,160,161). Rikova and coworkers also found another ALK fusion protein, TFG-ALK, in their study (161).

7.2 The genetic landscape of lung cancer

Investigations of the genetic landscape of NSCLC indicate that various subtypes exist and are dependent on environmental cues. For instance, tobacco smokers develop mostly lung squamous cell carcinoma (LUSC) but also a considerable part of lung adenocarcinoma (LUAD) (162) . Non-smokers develop LUAD due to other mutagens, such as air pollution and toxic substances. Genetically NSCLC is regarded as very heterogenetic disease, which complicate the treatment regimes in the clinic. Although, tumor growth is due initially to a specific genetic mutation, but progression of tumor growth depends on more mutations in pro-oncogenes or tumor suppressor genes (163) which probably increase the genetic and epigenetic instability (164). The accumulated defects on genes as well as on chromosomal level mediate an imbalanced gene expression (164). The initial tumor clone will expand and by time develop separate populations of cells with different clonal genotypes, resulting in intratumor heterogeneity also known as tumor evolution (164,165).

Identification of chromosomal aberrations and mutations in individual patients, by various means, such as sequencing with next-generations sequencing (NGS), comparative genomic hybridization (CGH), SNAPhot, PCR, and Fish, it is possible to identify putative driver mutation/s involved in the initiation and progression of the disease. For instance, CGH array is molecular cytogenetic analysis to detect chromosomal aberration and copy number alteration profiles of the tumor. (166,167). Investigation of copy number alterations from patients show that it's possible to distinguish differences between LUSS and LUAD of NSCLC. These

analyses provide an outline of the genomic alterations in NSCLC, clarifying differences but also similarities between subgroups of the disease (168,169).

To clarify and understand the background of the diseases, such as NSCLC and other cancer types, NGS has made a big impact in patient care and improved the treatment options. With this technique, it is possible to reveal both common and rare genetic mutations. With this in mind, researchers and clinicians have taken major steps into targeted therapies against constitutively active proteins and signaling pathway that can be therapeutically exploited. For example, NGS has become a standard of care diagnostic tool in Swedish children diagnosed with cancer with the possibility to make faster, more detailed and targeted treatment (170). NGS can also be used as biomarkers for other clinical related questions.

7.3 Oncogenic drivers in lung cancer

We know that cancer develops due to the accumulation of specific somatic mutations in the genome. One of the first steps that revealed the existence of mutations happened during early 1970 when scientists performed calcium-DNA transfection into cultures of NIH3T3 cells to detect putative oncogenes in the DNA of cancer cells from patients (171). Cells transfected with putative oncogenic DNA converted a non-cancerous NIH3T3 cell into a cell that was strongly tumorigenic without contact-inhibited and anchorage-independent growth. Later in the 2000's, advancements in NGS aided researchers and clinicians to whole genome-, and whole exome sequencing (WGS and WES) for the discovery of mutations (drivers and passengers) and polymorphisms, transcriptome sequencing for quantification of gene expression, deoxyribonucleic acid (DNA) methylation analyses and chromatin immunoprecipitation mapping of DNA-protein interaction (172). These new technologies have clearly guided the development of molecularly targeted therapy and is useful in treatment and care. Today, we have a list of known gain-of-function mutations that are associated with FDA-approved targeted therapies and the list is growing every year (173). Oncogenic genes can be divided into different groups, such as tyrosine kinases, downstream signaling effectors, chromatin modifiers and small GTP-binding proteins.

7.3.1 Epidermal growth factor receptor (**EGFR**)

EGFR is a member of the ErbB family of tyrosine kinases. The ligand of the extracellular receptor domain of EGFR can activate the EGFR tyrosine kinase. Mutations affecting the active site of the kinase (e.g., the L858R substitution in exon 21, deletions in exon 19, and other mutations in exons 18 and 20) cause kinase activity through dimerization and auto transphosphorylation of the kinase domain of EGFR (105,174). First-generation approved EGFR tyrosine kinase inhibitors employed in the clinic to treat lung cancer patients include erlotinib (FDA-approved

(175)), gefitinib (FDA-approved (176)), and icotinib. The second-generation EGFR inhibitor afatinib (FDA-approved (177)) showed benefit to progression-free survival in patients with lung cancer (178–182). The third-generation EGFR inhibitor osimertinib was developed to treat patients that experience relapse after tumors acquire mutations such as the T790M substitution or an insertion in exon 20 of EGFR that render the tumor resistant to first-generation and second-generation EFGR inhibitors (183–185). In general, EGFR inhibitors are valuable therapies for patients harboring EGFR-mutated lung cancer (186). For instance, osimertinib show a robust overall response rate and progression free survival in treatment naïve patients with EGFR mutation (187).

7.3.2 Human epidermal growth factor receptor 2

HER2 is another member of the ErbB family of RTKs. HER2 amplification is found in approximately 25% and 1.2% of all breast cancers and lung cancers, respectively (188). About 3–4% of patients with metastatic LUAD have insertions or duplications in exon 20 of the kinase domain of HER2. A common insertion in exon 20 that modifies the drug-binding pocket of HER2 renders lung cancer cells resistant to HER2 inhibitors such as neratinib, lapatinib, dacomitinib, and afatinib (189).

7.3.3 Mesenchymal–Epithelial Transition (MET)

MET is a proto-oncogene member of the RTK family. Hepatocytic growth factor (HGF) can bind to the extracellular portion of MET as a ligand (190). The carboxy-terminal multifunctional docking site of MET consists of an intracellular juxtamembrane portion (encoded in exon 14) and a kinase domain that engages tyrosine Y1003 with the casitas B-lineage lymphoma (c-CBL) E3 ubiquitin ligase (191). Recurrent MET mutations in lung cancer are typically point mutations or amplifications in splicing-regulatory sites of exon 14 or mutations that cause skipping of exon 14. Deletions in the splicing-regulatory sites of exon 14 result in degradation of the MET receptor and extended receptor signaling, loss of the juxtamembrane domain, and impairment of receptor ubiquitination (192). MET exon 14 mutations are present in approximately 3% of advanced NSCLCs, whereas MET amplifications are less frequent in lung cancer. MET tyrosine kinase inhibitors that are developed or under development include crizotinib, capmatinib, savolitinib, tepotinib, and merestinib (193). At this point, there is no clear evidence that supports a specific treatment modality; however, several trials are investigating MET inhibitors in patients (194,195).

7.3.4 Rearranged during transfection (RET)

RET (“rearranged during transfection”) encodes an RTK for the glial cell line-derived neurotrophic factor family of ligands (GDNF, NRTN, ARTN, PSPN, and GD15)

(196). RET fusions and loss of function mutations were identified in thyroid carcinoma (197,198). In lung adenocarcinoma tumors, oncogenic RET rearrangements occur at a frequency of 1-2% (199,200). The kinesin family member, 5B gene (KIF5B), is a common RET fusion partner (201). RET fusions carry an intracellular tyrosine kinase domain that can homodimerize by interacting with a fusion partner coiled coil, causing autophosphorylation that activates downstream signaling like JNK, JAK/STAT, PI3K, and RAS/MAPK (202). To detect RET fusion, FISH and NGS can be used for clinical diagnosis and RET inhibitors like lenvatinib, cabozantinib, and vandetanib can be used in patients lung cancer (201). Furthermore, retrospective data showed alectinib is an effective inhibitor of RET (203,204).

7.3.5 Neurotrophin kinase (**NTRK**)

NTRK (TRKA encoded by *NTRK1*, TRKB encoded by *NTRK2*, and TRKC encoded by *NTRK3*) rearrangement is a rare oncogenic driver in lung cancer, appearing in approximately 1% of patients (0.2% to 2.4% colorectal cancers (205)) (206–208). Gene fusions, overexpression, and point mutations of TRK proteins induce receptor dimerization to activation of the kinase domain (208). The first-generation TRK inhibitors, larotrectinib and entrectinib show clinical antitumor activity in TRK fusion-positive cancers and should be considered first-line therapeutic options for TRK fusion-positive lung cancers (209). Next generations TRK inhibitors, such as selitrectinib, repotrectinib (NCT04094610) and taletrectinib are in ongoing clinical trials.

7.3.6 Proto-oncogene 1 receptor tyrosine kinase (**ROS1**)

ROS1 is another member of the RTK family. ROS1 mutations occur in approximately 1–2% of LUADs (210). ROS1 mutations are often gene fusions between the 3' tyrosine domain of ROS1 and a diversity of 5' fusion partners such as sodium-dependent phosphate transport protein 2B SLC34A2, ezrin EZR, HLA class II histocompatibility antigen gamma chain CD74 (most common), ROS1 inhibitors such as crizotinib — approved by the FDA is highly effective against *ROS1*-rearranged lung cancer and is used in clinic (211,212). Next-generation ROS1 inhibitors such as ceritinib, brigatinib, entrectinib, lorlatinib, and repotrectinib (TPX-0005) have shown potent anti-tumor effects in preclinical trials (213–217).

7.3.7 Phosphoinositide 3-kinases (**PI3K**)

The phosphoinositide 3-kinase cascade regulates many biological functions to maintain cell survival during stress, such as proliferation, protein synthesis, differentiation, genomic stability, and angiogenesis (218). PI3K mutations are

common in breast cancer, (approximately 40% of cases). The majority of mutations, including three hot spot mutations (H1047R, E542K, and E545K), constitutively activate PI3K, which phosphorylates downstream signaling pathways such as AKT/mTOR (112,218–221). PI3KCA mutations were seen in 2–5% of patients with NSCLC (113) in a Phase I clinical trial study of the potent oral panPI3K inhibitor, (Pictilisib) leading to the shrinkage of tumor between 13-18% (113,222). Another oral pan-PI3K inhibitor, BKM120, studied in several xenograft lung cancer models, had significant as antitumor effects similar to BKM120 inhibitor used in a Phase I trial of patients with lung cancer (113,223,224). The intravenous pan-PI3K inhibitor is BAY 806964, and the potent oral irreversible pan-PI3K is PX866 (113,225,226).

7.3.8 Phosphatase and tensin homolog (PTEN)

Phosphatase and tensin homologs are phosphatases involved in cellular biological functions such as cell survival, energy metabolism, proliferation, and motility. Genomic PTEN mutations are associated with cancer in organs including prostate, breast cancer, glioblastoma, and lung cancer (227). Approximately 46.1% of NSCLC patients with poor prognosis factors harbor inactivation mutations and loss expression of PTEN tumors (228). PTEN also acts as a tumor-suppressor to regulate PI3K/AKT, and its mutation can cause loss of PTEN function and dysregulation of the PI3K signaling network (227).

7.3.9 BRAF

Proto-oncogene B-Raf (BRAF) is a member of the RAF family of tyrosine kinases activated by GTP-bound RAS proteins (153). The most common mutation in BRAF is the Class I mutation V600E, which occurs in about 3% of LUADs and causes constitutive activation of BRAF, leading to increased ERK phosphorylation (229). Class II and III BRAF mutations include G469, G466, G596, and K601. BRAF inhibitors can only inhibit V600X-mutated BRAF. The BRAF inhibitor dabrafenib is used in combination with trametinib, a MEK inhibitor approved by the FDA for patients with BRAF V600E-mutated lung cancer associated with effectively tolerated outcome, potent response, and improved survival compared with a single treatment (230,231).

7.3.10 KRAS

KRAS is a member of the GTPase family of small intracellular guanine nucleotide-binding proteins. Most mutations in KRAS occur in exons 2 and 3 (232).KRAS is mutated in approximately 25–30% of lung cancers, making it the most common oncogenic driver of lung cancer (233–235). The most common mutations in KRAS are G12V and G12C (236). The G12D mutation is often found in smokers and co-occurs with the TP53 mutation in approximately 40% of cases and the STK11/LB1

mutation in approximately 32% of cases(237). Recently a new type of small inhibitors have seen the daily light, which include AMG-510 (sotorasib; FDA approved in 2021), and adagrasib (MRTX849) against G12C-mutated KRAS in non-small cell lung cancer (238,239). It was reported that in patients with previously treated with chemotherapy and checkpoint inhibitors KRASG12C-mutated NSCLC, adagrasib showed clinical efficacy (240). Recently, in December 2021, another small inhibitor, MTRX1133, was presented, which showed eminent pre-clinical activity against another KRAS mutation, the G12D-mutated KRAS (241,242). The MRTX1133 has shown to be efficacious against $KRAS^{G12D}$ mutation in mouse tumor (243).

8 Distribution of oncogenic drivers between NSCLC smokers and non-smoker patients

A recent comprehensive molecular characterization of NSCLC patients shows that EGFR mutations are very common in non-smokers and that KRAS mutations are most common mutation found in the smoking patient group. Another major observation is that ALK mutations are more common in the non-smoking patient group (244). It's been reported that these patients are also younger in ALK^{+} non-small cell lung cancer (244). Initial evaluation of several mutations, including EGFR, KRAS, BRAF, HER2, and PIK3CA, together with ALK fusion, across-examine 28 testing centers in France (235) (**Figure 12**).

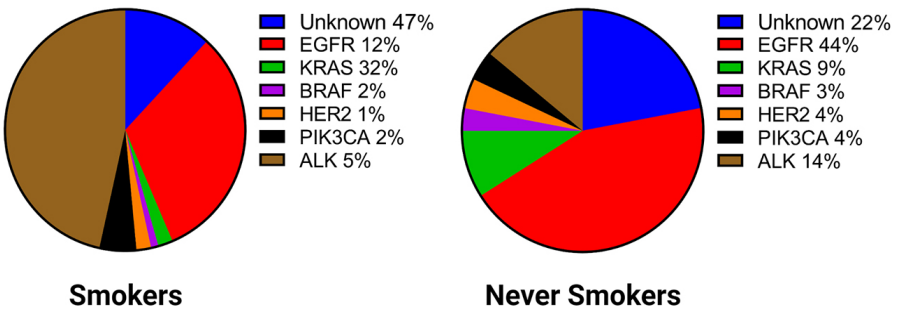

Figure 12. ***The pie chart shows 18.679 patients' samples of NSCLC compared with smokers with never smokers****: A. 17.664 patient samples diagnostic with LUAD, EGFR 12%, KRAS 32%, BRAF 2%, HER2 1%, PIK3CA 2%, ALK 5%, unknown 32% and full wild-type 15% (patients' profile without mutations or ALK rearrangement). B. 1619 patient samples diagnostic with LUAD and have never smoked, EFGR 44%, KRAS 9%, BRAF3%, HER2 4%, PIK3CA 4%, ALK 14%, unknown 13%, and full wild-type 9%* (235).

9 Treatment and clinical stages of lung cancer

Cancer staging (from stage I for primary tumors to stage IV for advanced metastatic disease) reflects cancer progression and the manner in which the malignant cells grow and spread, which in turn helps to determine the type of treatment required (245). Pierre Denoix developed a global classification system to identify the extent of cancer spread called the Tumor-Nodes-Metastasis (TNM) Classification of Malignant Tumors (245). T describe the tumor site and size, N describes the regional lymph node involvement, and M describes the presence or otherwise of distant metastatic spread (246).

Decisions about lung cancer treatment can depend on the tumor stage, the histological subtype of the tumor, and the molecular aberrations present in the tumor cells. Primary treatment modalities can include surgical intervention, chemotherapy, radiotherapy, targeted molecular therapy, and immunotherapy.

9.1 Surgery

Surgery is most effective strategy in preventing the spread of the tumor and might include the use of noninvasive equipment (245,247). In addition, techniques such as transthoracic needle aspiration, transbronchial needle aspiration, endoscopic ultrasound with needle aspiration, and thoracoscopy can be used can be used to obtain biopsy specimens to assist in clinical staging and diagnosis (246,247).

9.2 Chemotherapy

In lung cancer, the tumor type can determine the most suitable chemotherapy regimen (248). The limitation of applying chemotherapies depends on tumor stage and tumor type when cancer can not be removed surgically and has already spread to more than one area in the patient's body (249). Furthermore, chemotherapy combined with radiation can be used to treat early-stage lung cancer (250). Cisplatin and etoposide are standard chemotherapy treatments for SCLC and can be combined with other drugs such as carboplatin, gemcitabine, paclitaxel, vinorelbine, topotecan, and irinotecan (251,252). Cisplatin and derivative of cisplatin crosslink double-stranded DNA to inhibit replication, transcription, and induction of damage to the DNA that mediate apoptotic signals (249). Cisplatin or carboplatin is the standard first-line chemotherapy for NSCLC and can improve survival even in patients with advanced NSCLC (253).

9.3 Radiotherapy

The application of radiotherapy is recommended to be pre-operative care to decrease the cancerous tumor and used after surgical operation to prevent the

growth of tumor cells with chemotherapies combination to destroy any remaining cancer cells (254). Chemotherapy combined with radiotherapy can be curative in patients with NSCLC who are not eligible for surgery because of high risk of comorbidities (255). Radiation applied at a high dose over a short period can accelerate treatment regimens (256). In some patients with lymph node involvement, radiotherapy after resection of the affected lymph nodes improves survival. Likewise, post-surgical radiotherapy with chemotherapy can enhance survival in patients with NSCLC and radiation kills cells by apoptosis or interphase death, which can be used with surgery to clean the tumor area (257).

9.4 Immunotherapy

Immunotherapy can be part of curative treatment regimens to fight lung cancer (258). The concept of immunotherapy is to increase the number of tumor cells killed by the body's immune system by manipulating "checkpoint" proteins to express such as anti-programmed death-ligand 1 (PD-L1), and/or anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which interacts with receptors expressed on the T cell surface (259,260). Tumors can inhibit the immune response by acquiring mutations that either prevent immune cells from penetrating the tumor or down regulate signaling pathways that stimulate T cells (261,262). In addition, the tumor microenvironment has functions that can affect T cell activation, so that instead of cytotoxic T cells the tumor contains suppressive immune cells that protect against immune activity (261,262).

10 Prognostic factors in lung cancer

A prognostic factor is a situation, biological condition, or clinical characteristic that can help determine the likelihood a patient will recover from disease. It is helpful to consider prognostic factors before starting cancer therapeutics to avoid unnecessary discomfort, complexity, and even potential harm due to inappropriate treatment (263). Prognostic factors may include patient age, sex, performance status, ethnicity, and smoking status; disease characteristics such as TNM stage and histological status; and molecular markers such as HER2 amplification, KRAS mutation, and differential expression of certain genes or proteins (264,265). In NSCLC, the essential prognostic factors used in clinical care are tumor stage, patient age, and patient performance status (266–268). In addition, molecular markers associated with patient and disease characteristics help predict tumor aggressiveness (269). For the majority of molecular markers, immunohistochemistry is used to evaluate the levels of relevant proteins (270).

11 Anaplastic lymphoma kinase in NSCLC

ALK chromosomal rearrangements occur in approximately 3 to 6 % of LUADs. These rearrangements result in fusions of ALK with multiple partners including EML4 (most common), kinesin light chain 1 (KLC1), tyrosine-protein phosphatase non-receptor type 3 (PTPN3), kinesin family member 5B (KIF5B), Striatin (STRN), solute carrier family 2A (SLC2A), cap methyltransferase 1 (CMTR1), vitrin (VIT), GRIP and coiled-coil domain-containing protein 2 (GCC2), Cut like homeobox 1 (CUX1), and B-cell lymphoma/leukemia 11A (BCL11A). Other ALK fusion proteins share different breakpoints but similar in ALK breakpoints, including transforming growth factor TFG-ALK (T3; A20), kinesin family member 5B KIF5B-ALK (K24; A20 and K15; A20), clathrin heavy chain 1 CLTC-ALK (C31; A20), translocated promoter region TPR-ALK (T15; A20), Sequestosome-1 SQSTM1-ALK (S5; A20), Dynactin subunit 1 DCTN1-ALK (D26; A20), Huntingtin-interacting protein 1 HIP1-ALK (H21; A20), and Striatin STRN-ALK (S3; A20) (271). Soda *et al.* made the first discovery of ALK fusion in lung cancer patient when they profiled EML4–ALK rearrangement in lung cancer in 2007 (160). The identified fusion protein was recognized as variant 1, in which a portion of exon 20 of ALK was fused with exon 13 of EML4 (160) (**Table 1**).

EML4 is a family of proteins that play a role in the regulation of microtube division. There are six different EML4 subtypes expressed in humans (272). EML4 consists of an N-terminal coiled-coil (AKA TD; trimeric dimerization) required for oligomerization and self-association, a basic region required for microtubule association (272,273), and a C-terminal region containing repeats of a hydrophobic echinoderm microtubule-associated protein-like protein (HELP) motif and WD (tryptophan-aspartic acid) (272,273). The core HELP-WD region creates a novel tandem atypical beta-propeller, referred to as the TAPE structure of EML4 (272,273). In EML4–ALK fusion proteins, the TAPE domain is truncated in many variants, resulting in an incomplete structure that makes EML4–ALK fusion proteins unstable (272,273).

EML4-ALK translocation occurs through an inversion rearrangement accident on chromosome (**Figure 13**). Several of different rearrangements have been found and they all include the CC domain which is dimerization domain for the kinase EML4-ALK variants 1 (E13; A20) and 3a/b (E6; A20) together constitute the majority (70–80%) of EML4-ALK variants in ALK^+ NSCLC, followed by EML4-ALK variants 2 and 5 (**Figure 14**) (274).

Cancer type	ALK	ALK rearrangements	References
NSCLC	3-7%	*TPR, Cysteine-rich motor neuron 1 protein (CRIM1), EML4, STRN, transforming growth factor (TFG), huntingtin-interacting protein 1 (HIP1), PTPN3, KIF5B, KLC1, CLTC, CMTR1, VIT, GCC2, CUX1, BCL11A,* dynactin *subunit 1 (DCTN1), SQSTM1,* BReast *CAncer (*BIRC 6), and *SLC2A.*	(275–283)

Table 1. ***Schematic representation of the different categories of ALK-positive cancers****.*

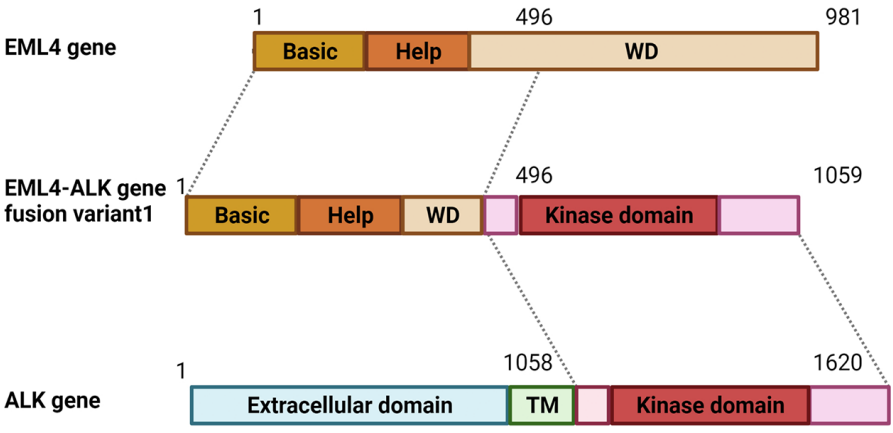

Figure 13. ***ELM4-ALK rearrangement****. The fusion gene of the amino-terminal fragment of EML4 includes the basic region, the help domain, and a portion of the WD-repeat region) with the intracellular part of ALK containing the tyrosine kinase domain. TM, transmembrane domain.*

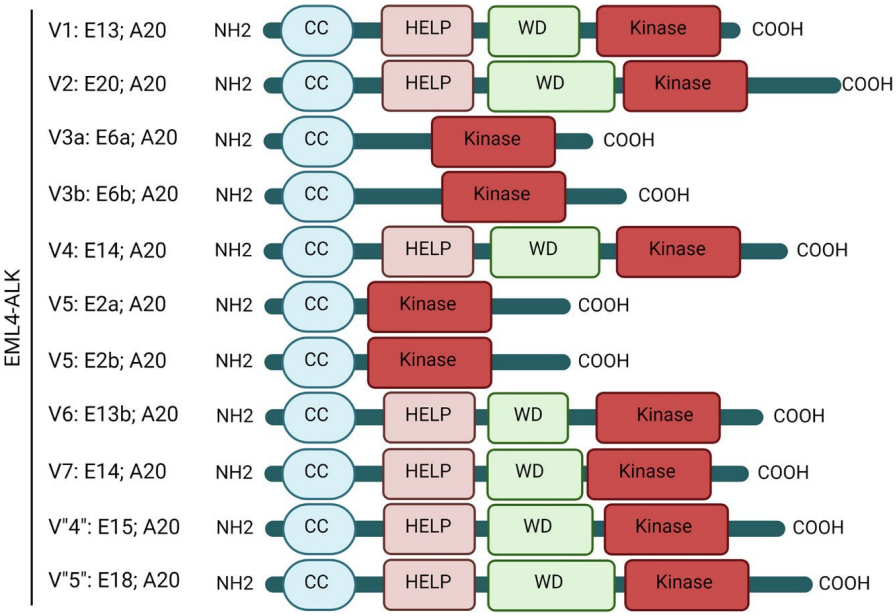

Figure 14. ***Schematic of the structural composition of ALK fusion proteins*** (271): *Most EML4-ALK fusion proteins have an ALK breakpoint at exon 20 (or rarely exon 19), whereas the EML4 breakpoint occurs frequently at different exons. For example, EML4-ALK variant 1 (EML4 exon13 with ALK exon 20) contains a fusion from EML4 exon 1 to exon 13 and EML4-ALK variant 2 has a similar breakpoint in exon 20 of EML4 and ALK (EML4 exon 20 with ALK exon 20), with a fusion from EML4 exon 1 to exon 20. Thus, the breakpoint in exon 20 of EML4 can produce EML4-ALK fusion proteins of different sizes. The structural parts of EML4 fusion proteins may include a C-terminal coiled-coil (CC) domain, a HELP domain, and a whole or partial WD repeat domain. Variant 3a/b (E6a/b; A20) does not include the WD repeat and consist of exons 1–6 of EML4; exon 6b includes 188 base pairs downstream of exon 6a and is fused with exon 20 of ALK. Variant 4 (E14; A20) contains exons 1–14 of EML4 with an insertion of four extra amino acids of unknown origin, and the ALK fusion part lacks 49 base pairs at the N-terminus. In variant 5 (E2a; A20), EML4 exon 2 is fused of to ALK exon 20, whereas in variant 5 (E2b; A20), EML4 exon 2 is linked to ALK at a breakpoint 117 base pairs upstream of exon 20. In variant 6 (E13b; A20), EML4 exon 13 is fused to ALK exon 20, which contains an insertion of 19 amino acids derived from the upstream intron 19. In variant 7 (E14; A20), EML4 exon 14 is fused with a version of exon 20 of ALK lacking the first four amino acids. In variant "4" (E15; A20), EML4 exon 15 is fused with exon 20 of ALK. Variant "5" (E18; A20) consists of EML4 exon 18 fused with exon 20 of ALK.*

It's been reported that the size and subcellular localization of different types of EML4-ALK fusion proteins may play an important role in determining which ALK TKIs can be effective since the fusion partner decide where and when the fusion protein going to be expressed. For example, it been suggested that EML4-ALK fusion variant 1 localizes in the cytoplasm, whereas variant 3 is observed to

localizes in microtubules and the nucleus (284). These differences, as well as differences in fusion protein stability, can determine how patients with lung cancer respond to ALK inhibitors and what resistance mechanisms may come into play (241).

12 ALK targeted therapy

To treat advanced disease, agents targeting specific molecules are used to inhibit the drivers of cancer growth, proliferation, and metastasis (285). Targeted inhibitors are molecularly designed to interfere with abnormalities in biochemical pathways, such as tyrosine kinase inhibitors that target specific RTK's (285).

12.1 Molecular inhibitors of ALK

The RTK TKI's currently approved by the FDA are as follows (**Figure 15**):

Crizotinib (Xalkori®, Pfizer): James Christensen and coworkers presented crizotinib in 2007 as ATP-competitive and selective inhibitor of c-Met and ALK positive rearrangements, which inhibited ALK phosphorylation and downstream signal transduction in a potent manner (286). Initial phase I clinical trial showed anti-tumoral effect against ALK positive NSCLC cancer (287) and crizotinib is superior to chemotherapy treatment in patient with ALK positive NSCLC (288). Crizotinib was approved by the FDA in 2011 for the treatment of ALK^+ NSCLC.

Ceritinib (Zykadia®, Novartis): This second-generation ALK inhibitor was approved by the FDA in 2014 for the treatment of metastatic ALK^+ NSCLC, especially in cases that are refractory to treatment with crizotinib (289). Ceritinib treatment selectively inhibits the ALK enzyme activity in the single digit nM range, resulting in restriction of cellular proliferation and metastasis.

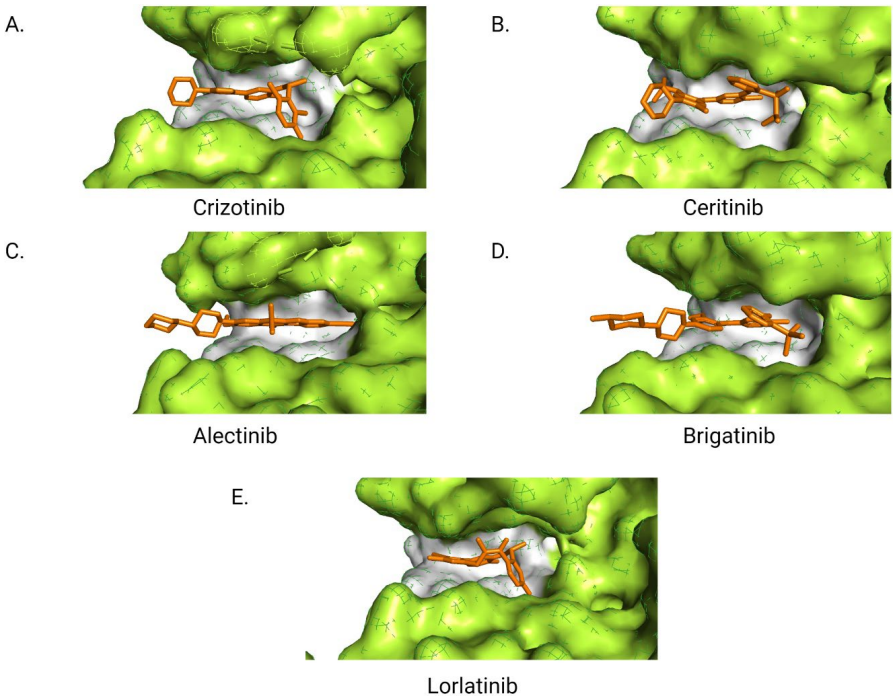

Figure 15. ***The molecular structures of the different anaplastic lymphoma kinase (ALK) inhibitors approved by the FDA****. The ATP-binding pocket of the anaplastic ALK domain (lemon) is a recipient site for the molecular structure of different tyrosine kinase inhibitors (TKIs) (orange). Visualize the recipient site of the ATP-binding pocket of the wild-type ALK kinase domain model with different suitable ALK TKIs, exhibiting the diversity in binding sites between different ALK TKIs. (A) Crizotinib (PF2341066), PDB: 2XP2). (B) Ceritinib (LDK378, PDB: 4MKC). (C) Alectinib (CH5424802, PDB: 3AOX). (D) Brigatinib (PDB: 5J7H). (E) Lorlatinib (PF06463922, PDB: 4CLI)* (64).

Alectinib (Alecensa®, Chugai Pharmaceutical): Alectinib was developed to be highly potent against ALK positive rearrangement, with an IC50 1.9 nanomolar in cell-viability assays (290). This second-generation ALK inhibitor was approved by the FDA in 2015 for patients with ALK^+ NSCLC who could not tolerate or did not benefit from crizotinib treatment (291). In 2017, the FDA approved alectinib as a first-defense line drug for patients with ALK-positive non-small cell lung cancer. The result from the phase III ALUR study showed highly significant improvement in progression-free survival (PFS), objective response rate (ORR), and central nervous system (CNS) with efficacy and safety of alectinib versus chemotherapy in

advanced/metastatic anaplastic lymphoma kinase ALK-positive non-small-cell lung cancer (292).

Brigatinib (Alunbrig®, Ariad Pharmaceuticals): The FDA approved this second-generation molecular inhibitor of ALK in 2017 for patients with ALK positive NSCLC (293). Brigatinib is effective against many resistance mutations such as ALK-G1202R, ALK-F1174L, ALK-C1156Y, and also show activity against the EGFR-T790M mutation (294).
Final analysis outcomes in the ALTA-1 with longer-follow-up patients' treatment have assessed brigatinib as more strongly tolerable than crizotinib 35-month vas 8 months for crizotinib (295).

Lorlatinib (Lorbrena®, Pfizer): This third-generation inhibitor of ALK and ROS1 is indicated for treatment of ALK^+ NSCLC (210). Lorlatinib can cross the blood-brain barrier and can therefore be used to treat brain cancers (296). Common side-effects are hypercholesterolemia, hypertriglyceridemia, edema, peripheral neuropathy, neurocognitive dysfunction, mood swings, and fatigue (263). Co-administration of lorlatinib with rifampicin has been reported to cause hepatotoxicity (267). Lorlatinib is highly potent in cells with resistance mutations, such as the solvent-front G1202R mutation (297). The patients who were suffering from NSCLC and treated with lorlatinib showed highly significant progression-free survival (PFS) and intracranial ORR (IC-ORR) response with safety outcomes of adverse events (298).

Entrectinib was FDA approved August 2019. (RXDX-101) inhibits ALK, ROS1, and TRK-A/B/C and promotes tumor regression in mouse models of ALK^+ NSCLC (299). It has also shown activity against some crizotinib-resistant cancers (300). The molecule can cross the BBB and can therefore inhibit CNS tumors. Activity against ROS1 has been demonstrated both *in vitro* and *in vivo* (300).

13 Mechanisms resistance and relapse of first and second generation ALK inhibitors in lung cancers

It rapidly became clear that long-term treatment with crizotinib in ALK positive NSCLC patients leads to drug resistance and relapse mediated by mutations acquired by tumor cells (301). The resistance could occur in three different ways. Firstly, ALK kinase domain mutation (around 30%), secondly, amplification of the ALK locus (around 30%) and, finally, activation of ‘bypass’ signaling pathways (64).

Mutations of the ALK kinase domain occurs in close proximity to the ATP binding pocket of the kinase domain, where they all vary slightly in their contact point with ALK inhibitor employed for treatment. The resistance mutations mediate a steric

interference for the inhibitor binding that allows continuation of ALK fusion kinase activity again even in the presence of the TKI (64). A common resistance mutation is the gatekeeper residue, R1196M also observed in other RTKs (64,302,303). Other common resistance mutations are the “solvent front mutations”. Similar to the R1196M mutation, solvent-front mutations mediate steric hindrance of kinase inhibitor to bind in the ATP binding pocket of the kinase domain (304).

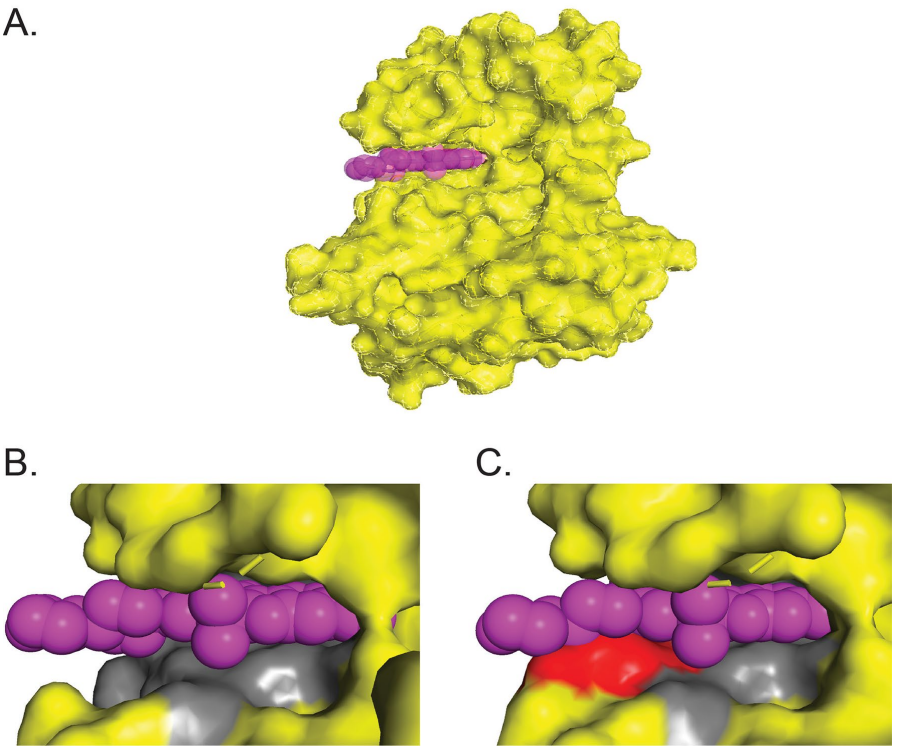

Figure 16. ***Three dimensions of anaplastic lymphoma kinase (ALK) tyrosine kinase domain*** *(yellow): A. Zoom out showing the general interaction of ALK inhibitors (alectinib-purple) in the ATP-binding site of ALK kinase domain (gray). B. Zoom in showing the molecular interaction of alectinib binding in the ATP-binding site of the ALK kinase domain (gray). C. Steric hindrance of alectinib binding in the pocket with a G1202R mutation (red).*

One common solvent front mutation is the G1202R. It was recently shown that 37% of patients harbouring the EML4-ALK variant 3 rearrangement came up with the resistance mutation G1202R but not in patients with EML4-ALK variant 1 (241). Furthermore, other known “solvent front mutations” are the G1202del, D1203N, S1206Y/C, and G1202R mutations (**Figure 16**) (304–309).

Amplification of ALK occurs in approximately 8% of patients exposed to crizotinib, and it does not overlap with secondary crizotinib-resistance mutation of the kinase domain. ALK amplification in response to crizotinib exposure leads to an increase in the expression of the target protein, which can affect the effectiveness of TKIs (304).

13.1 Bypass resistance mechanisms

Bypass mechanisms of resistance involve parallel activation of downstream signaling pathways even during inhibition of the target protein. Thus, downstream oncogenic signaling pathways remain highly activated even if the TKI successfully inhibits ALK (**Figure 17**) (310).

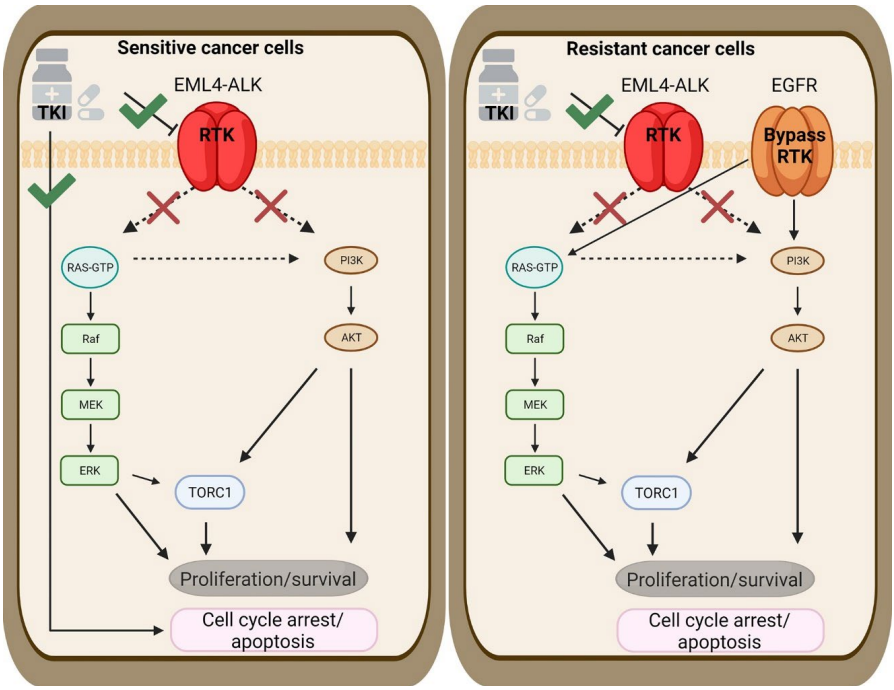

Figure 17. ***Mechanisms of therapeutic resistance to tyrosine kinase inhibitors****. Activation of a secondary RTK (EGFR) through the creation of bypass pathways. For example, in sensitive cancer cells (left), the mutation of oncoproteins (EML4-ALK) exerts control over RAS, MEK/ERK, and PI3K/AKT downstream signaling pathways. Exposure to tyrosine kinase inhibitors suppresses the biochemical cascade and leads to cell cycle arrest and apoptosis. On the other hand, in resistant cancer cells (right), a bypass pathway reactivates a secondary RTK (EGFR) and activates at least one downstream signaling pathway, leading to cell proliferation and survival even in the presence of the tyrosine kinase inhibitor.*

Bypass resistance mechanisms can involve several tyrosine kinase receptors, such as SRC, Pi3K, MEK, EGFR, MET, HER3, AXL and insulin growth factor receptor 1 (IGFR1) (310–312). However, the biological mechanisms by which these genes are activated are not clear and cannot easily be determined using next-generation sequencing. This suggests that some bypass resistance mechanisms might be mediated by overexpression or overactivation of tyrosine kinase receptors/intracellular phosphokinase or mutation in tumor suppressor gene (310). Bypass resistance might be overcome with drug combinations targeting multiple signaling pathways, such as a combination treatment with lorlatinib and SHP2 inhibitor, which is an ongoing clinical trial (NCI number) (280,281).

13.2 Retrospective analysis of treatment responses to ALK TKIs

A retrospective analysis of 35 patients with ALK$^+$ NSCLC was reported by Yoshida and colleagues in 2016 in Japan (313). The overall response rate (ORR) to crizotinib was higher (74%) in patients harboring variant 1 than in the patients with other variants (63%) (274). The median progression-free survival (PFS) was 11 months for patients with variant 1 and 4.2 months for patients with other variants (P-value<0.05) (274). Several subsequent retrospective analyses conducted in other countries have supported the findings of Yoshida et al. (314), which been summarized by Ou et al. (Ignatius Ou) (315).

14 Synchronous TP53 mutations

TP53 mutations are one of the most frequently found genomic alterations in several types of cancer, including ALK$^+$ NSCLC, (274). TP53 mutations together with EML4-ALK rearrangement mutations mediate poor prognose for both PFS and overall survival (OS) (274,316). Under most treatment conditions, such as crizotinib treatment, chemotherapy, or next-generation ALK TKI treatment, TP53 mutation confers shorter median PFS (274,314,317–323).

15 EML4-ALK variant 3 versus TP53 positive in NSCLC

Patients with harboring EML4-ALK variant 3 or TP53 mutations were reported to have poor prognosis (274). The presence of both EML4-ALK variant 3 and TP53 mutation confers and even worse prognosis according to retrospective studies by Christopoulos et al., that included 102 patients (274,324). Furthermore, increased numbers of metastases was associated with the presence of EML4-ALK variant 3 and TP53 mutations (274). The report showed that the median PFS was 16 months in patients carrying EML4-ALK variant 1/2 versus 7 months for patients harboring EML4-ALK variant 3 (274). Additionally, Tanimoto et al. reported that the presence of EML4-ALK variant 1 together with TP53 mutation was associated with shortened

PFS so TP53 positive patients with EML4-ALK may need a combination of ALK TKIs and chemotherapy (274,314).

16 AIMS

Specific aims

Paper I

To investigate the role of the Zfp148 transcription factor in the regulation of tumor suppressor 53 (p53) and the potential for therapeutic p53 activation to protect against cancer.

Paper II

Initial characterization of four novel ALK positive patient-derived NSCLC cell lines.

17 RESULTS and DISCUSSION

17.1 Paper I

We investigated mechanisms behind the activation of tumor suppressor p53 and proliferation arrest in Zfp148-deficient mouse embryonic fibroblasts (MEFs). Global gene expression analysis revealed that Zfp148-deficiency downregulated E2F-dependent cell cycle related genes, in a p53-dependent and p53-independent manner. This expression pattern suggested that the Cdkn2a transcripts ARF and p16 may be involved. ARF and p16 mRNA was markedly upregulated in *Zfp148*$^{gt/gt}$ MEFs compared to controls, and CRISPR-CAS9 mediated knockout of ARF, but not p16, rescued proliferation arrest of *Zfp148*$^{gt/gt}$ MEFs. Because Zfp148 regulates transcription of target genes by interacting with GC-rich DNA sequences, we performed genome wide chromatin immunoprecipitation to identify target genes. Zfp148 bound to GC-rich sequences in the ARF promoter, raising the possibility that Zfp148 suppresses p53-activity by repressing ARF-transcription. However, Zfp148 preferentially interacted with promoters of other transcription factors indicating that Zfp148-deficiency may have pleiotropic effects. In line with this, CRISPR-CAS9 mediated inactivation of the human homologue ZNF148 in hundreds of human cancer cell lines did not have any impact on the growth or survival of these cells. The data suggest that Zfp148 is not a major regulator of p53-activity in human cancer cells and that therapeutic targeting of Zfp148 will not be effective against human cancer.

17.2 Paper II

We defined patient-derived EML4-ALK variant cell lines, including CUTO8 and CUTO9 (variant 1) and CUTO29 and YU1077 (variant 3). EML4-ALK fusion was validated in each cell line by genetic characterization. We show that EML4-ALK-variant 1 is less stable than EML4-ALK-variant 3. We treated the cell lines with different tyrosine kinase inhibitors to define their sensitivities to the ALK specific inhibitors. In addition, we verified the effects of the inhibitors on the cell cycle and correlated the sensitivities of the cell lines with the activities of genes downstream in the EML4-ALK oncogenic signaling cascade. We performed RNA sequencing to investigate the genome-wide effects of ALK tyrosine kinase inhibitors on transcription and validate cofactors involved in ALK activation. The RNA sequencing results validated several targets, such as RRM2, and determined their importance in cancer cell growth and migration. The results also defined PARP-induced apoptosis using TKIs and RRM2-inhibitors via western blot, indicating its role in DNA damage. siRNA was shown to inhibit RRM2 in vitro cell cycle upon treatment with TKIs with decreased cell proliferation and DNA damage due to apoptosis by inhibiting RRM2.

18 CONCLUSION

18.1 Paper I

Summary of Zfp148 involvement in cancer

Zfp148 is a transcription factor that binds to GC-rich domains of DNA and suppresses the p53 pathway-dependent arrest of cellular proliferation in mice (35) and, presumably, humans. Zfp148 deletion leads to ectopic p53-dependent arrest of proliferation in mouse embryonic fibroblasts (MEFs) and pulmonary cells in mouse embryos; however, the underlying mechanisms are not clear (325). Lessons from mouse experiments suggest that the human ortholog *ZNF148* can be a therapeutic target for treating lung and colorectal cancers (36). However, the mechanism behind ZNF148–p53 interaction(s) is still not understood; although, physical interaction between the proteins has been demonstrated (326).
As part of this thesis, we investigated the mechanisms behind proliferation arrest in Zfp148-deficient MEFs. Comprehensive genomic analyses of MEFs with Zfp148 and p53 deficiencies showed that Zfp148 binds to the ARF promoter and inhibits ARF transcription (327). ARF is a potent activator of the p53 pathway, which can explain why Zfp148 deficiency arrests cellular proliferation (328). However, chromatin immunoprecipitation demonstrated that Zfp148 preferentially binds to the promoters of other transcription factors as well, indicating that deletion of Zfp148 might cause pleiotropic effects (36). Moreover, deletion of ZNF148 had no impact on the growth or survival of hundreds of human cancer cell lines (36) suggesting against its use as a therapeutic target. Overall, the integrated data failed to distil out a clear mechanism of ZNF148–p53 interaction or highlight any clear benefit of targeting ZNF148 in humans as an effective oncotherapeutic strategy. ZNF148 was therefore not investigated further in this thesis.

18.2 Paper II

In this study we have generated and investigated three novel EML4-ALK-positive patient–derived Non-Small-Cell-Lung-Cancer (NSCLC) cancer cell lines, CUTO8 (variant 1), CUTO9 (variant 1) and CUTO29 (variant 3) and included a fourth ALK-positive cell line YU1077 (EML4-ALK variant 3), which has not been characterized previously to study ALK-positive signaling and responses. Variants 1 and 3 are the most common EML4-ALK variants expressed in ALK-positive NSCLC, and currently cell lines representing these EML4-ALK variants are limited.

We have established expression and correct molecular weight of the corresponding ALK fusion protein and assessed their sensitivity to a range of ALK tyrosine kinase

inhibitors. As predicted, these patient derived cell lines show differential sensitivity to lorlatinib, brigatinib and alectinib, with EML4-ALK variant 3 containing cell lines exhibiting increased sensitivity to lorlatinib and brigatinib as compared to alectinib. Furthermore, we employed whole genome analysis and RNA-seq that identified the ribonucleotide reductase regulatory subunit 2 (RRM2) as one downstream and potential therapeutic target in ALK-positive NSCLC. Here, we provide a characterization of four novel EML4-ALK-positive NSCLC cell lines, highlighting genomic heterogeneity and differential responses to ALK TKI treatment.

19 MATERIALS AND METHOD

This section provides a summary of the main materials and methods used in this thesis. More detailed methodological descriptions can be found in the Material and Methods section of the published manuscript.

19.1 Cell culture

The patient-derived ALK^+ NSCLC cell lines CUTO8, CUTO9, CUTO29, and YU1077 were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in T75 flasks incubated at 37°C with 5% CO_2 and 95% humidity.

19.2 Proliferation assay using tyrosine kinase inhibitors

Patient-derived ALK^+ NSCLC cell lines were seeded in triplicate on 48-well plates. Starting the next day, the cells were treated with alectinib, brigatinib, and lorlatinib for 6 days, with new media and inhibitors added within 3 days. After 6 days, the cells were analyzed by resazurin assay.

19.3 Inhibition of ALK activity in lung cancer cell lines

Cell lines were seeded and treated with the inhibitors alectinib, brigatinib, and lorlatinib. Cells were collected after treatment, lysed with RIPA buffer (25nM Tris PH7.5, 150nM NaCl, 100µL of 1mM DTT, 100µL of EDTA, and two protein inhibitor tablets), and proteins were analyzed by immunoblotting.

19.4 Immunoblotting

Cells were lysed with RIPA buffer and mixed with 1X SDS sample loading buffer (for 4X=Tris-HCl: 0.2 M, DTT: 0.4 M, SDS: 277 mM, 8.0%, bromophenol blue: 6 mM, and glycerol: 4.3 M) sample buffer. Proteins were separated on 8% bis-acryl-tris gel, blocked with 3% milk or bovine serum albumin, depending on each primary antibody recommendation, and transferred to a nitrocellulose membrane. The membrane was then incubated with primary antibodies overnight on a shaker device at 4°C. The next day, the membrane was incubated with secondary antibodies (diluted 1:5,000) at room temperature for 1 h on a shaker device. Chemiluminescence substrates were then added to detect the proteins.

19.5 Sample extractions for RNA sequencing

Three independent biological replicates of each cell line were treated for 6 h and 24 h with three different TKIs (alectinib, brigatinib, and lorlatinib). Each replicate included negative controls treated with DMSO. Each drug was administered at the IC50 determined in cell proliferation assays. Total RNA was extracted from the cells

using a Total RNA Isolation Kit (Promega). The RNA samples were then transported to a service contractor (Novogene) for RNA analysis.

19.6 Software for data analysis

Western blot membranes were visualized with prime western blot detection reagent substrate on an Odyssey Fc machine. Band intensities were determined with Image Studio Lite software. Images were generated using Adobe Photoshop and Illustrator 2022. GraphPad Prism 9 was used to create growth curves to determine IC50 values and for statistical analysis of cell cycle and combination treatments.

19.7 Proliferation assay using triapine

Cell lines were seeded in triplicate onto 48-well plates and exposed to triapine for 3 days. The cells were then analyzed by resazurin assay.

19.8 Small-interfering RNA

Following the typical RNAiMAX transfection protocol, cell lines were seeded on 6-well plates for western blot and 24-well plates for resazurin assay. We added siRNA to knockout RRM2 and interfere with RRM2 expression, leading to degradation of RRM2 mRNA after transcription and preventing translation.

19.9 Cycloheximide-chase method

Cell lines were seeded at a concentration of 500,000 cells/well on a 6-well plate. The next day, the cells were treated with cycloheximide for 1 h, 3 h, 6 h,12 h, or 24 h, followed by western blot to detect band intensity using image studio software version 5.2.5 (LI.COR).

19.10 Cell cycle analysis

Cell lines were seeded at one million cells per 35mm petri dish. The next day, cells were exposed to the respective IC50 concentrations of three different TKIs for 48 h. Cells were then harvested, rinsed once with 0.5 mL phosphate buffered saline (PBS), centrifuged for 5 min at 500g at room temperature. PBS was then discarded and cells were fixed with 70% ethanol for 3 h at 4°C. After 3 hours, cells were centrifuged for 5 min. at 500g at room temperature. Ethanol was then discarded and the remaining pellet was rinsed with PBS and centrifuged for 5 min. at 500g at room temperature. PBS was then discarded and 0.5mL of DAPI solution was added and incubated for 5 min. For cell cycle analysis, Fixed-Cell-Cycle-DAPI-Assay was performed using a NucleoCounter NC-3000 slide (Chemometec, Denmark) according to the manufacturer's protocol. The plot manager in the NucleoView NC-3000 software was used to analyze the cell cycle data.

19.11 Combination treatment

Cell lines were seeded at a concentration of 500,000 cells/well on a 6-well plate. The next day, the cells were exposed to 100 nM lorlatinib and 3 mM triapine for 24 h. Western blot was then performed to detect the inhibition of band intensity.

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