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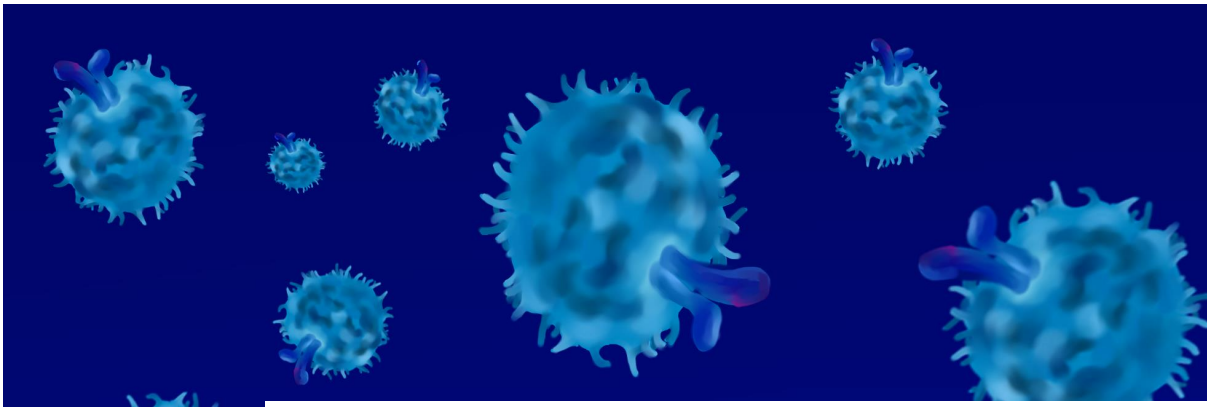

The role of protein kinases in development and disease

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Post-translational modifications are covalent processing events that change the properties, location, and function of proteins. One the most common and well-known modification is protein phosphorylation that is a reversible process where protein kinases add a small phosphate group to their substrate, which in turn can be removed by the action of phosphatases. The overall aim of this thesis is to study the function of protein kinases in various cellular aspects, such as cell signalling, cell development and cancer development. We focus on elucidating the function of Nemo-like kinase and JNK kinase in these biological settings. Nemo-like kinase is an evolutionary conserved atypical MAP kinase-related kinase that is most known as a negative regulator of the Wnt signalling pathway. The JNK kinases are part of a subgroup of the MAPK family and are often activated by stress-related stimuli, such as UV radiation, DNA damage, or oxidative stress. Both protein kinases play important roles in regulating cellular responses including proliferation, differentiation, and migration.

In papers I-III we examine the role of Nemo-like kinase (NLK) in various cellular settings. In paper I we explored the function of NLK in mouse embryonic fibroblast cells and found that deletion of NLK resulted in increased cell proliferation. Furthermore, we could describe a novel way for NLK to negatively regulate Wnt signalling by directly phosphorylating HDAC1. This in turn represses TCF/LEF target gene transcription thereby decreasing cell proliferation. Wnt signalling is known to play an important role in all aspects of the developing immune system. In paper II we conditionally deleted NLK in early T-cell progenitors to study its role in developing T-cells. We found that deletion of NLK resulted in the loss of $CD8^+$ single positive (SP) thymocytes due to increased cell death, without affecting the $CD4^+$ SP thymocyte population. Furthermore, we found that in NLK-deficient mice the phosphorylated levels of HDAC1 and LEF1 were reduced compared to wild-type mice, indicating that NLK is necessary for maintaining the balance of Wnt signalling in developing thymocytes. Previously, we have found that conditional deletion of NLK resulted in a lethal phenotype in mice, where pups died within 12-36 hours after birth due to aberrant lung development. In paper III, we wanted to investigate the role of NLK in non-small cell lung cancer (NSCLC) development. NSCLC is one of the leading causes of cancer related deaths worldwide. It was found that mice harbouring deleted NLK had more lung tumour growth compared to control mice expressing NLK. Additionally, we found increased Ki-67 staining in mice lacking NLK compared to wild-type mice. These preliminary data indicate that NLK under normal circumstances functions as a tumour-suppressor with regards to lung cancer development. In paper IV we examined the role of the JNK signalling pathway in the development of colorectal cancer. One major concern regarding colorectal cancer is that patients are often diagnosed at late stages where tumours have been metastasised. By using small molecules to target the JNK proteins we observed that inhibition of JNK1 resulted in decreased migration and invasion in colorectal cancer cells. Treatment of animals with JNK1 inhibitor reduced tumour metastasis in liver and lung confirming our in vitro data. Taken together, we have shown novel functions of the protein kinases NLK and JNK in various biological circumstances. By further studying the functions of these protein kinases, new roles could be discovered for NLK and JNK kinases to be used as diagnostic tools or new therapeutic treatment strategies.

Key words
Nemo-like kinase, JNK kinases, T-cells, lung cancer, colorectal cancer, and cell signalling

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“I mean, you could claim that anything’s real if the only basis for believing in it is that nobody’s proved it doesn’t exist.”

- Hermione Granger

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List of papers

This thesis is based on the following papers:

I. *NLK-mediated phosphorylation of HDAC1 negatively regulates Wnt signaling*

Chmielarska Masoumi, K., **Daams, R**., Sime, W., Siino, V., Ke, H., Levander, F., and Massoumi, R.

Molecular Biology of the Cell. 2017 Jan 15;28(2):346-355

II. *Deletion of Nemo-like Kinase in T Cells Reduces Single-Positive $CD8^+$ Thymocyte Population*

Daams, R., Sime, W., Leandersson, K., Sitnicka, E., and Massoumi, R.

The Journal of Immunology. 2020 Oct 1;205(7):1830-1841

III. *Deletion of Nemo-like Kinase contributes to increased tumour development in non-small cell lung cancer*

Daams, R., Abassi, Y., Simon Serrano, S., Tas, I., and Massoumi, R.

Manuscript

IV. *Reversine inhibits Colon Carcinoma Cell Migration by Targeting JNK1*

Jemaà, M., Abassi, Y., Kifagi, C., Fezai, M., **Daams, R.,** Lang, F., and Massoumi, R.

Scientific Reports. 2018 Aug 7;8(1):11821

Papers not included in the thesis

I. *Inhibition of mitotic kinase Mps1 promotes cell death in neuroblastoma*

Simon Serrano, S., Sime, W., Abassi, Y., **Daams, R**., Massoumi, R., and Jemaà, M.

Scientific reports. 2020 Jul 20;10(1):11997

II. *Nemo-like Kinase in Development and Diseases: Insights from Mouse Studies*

Daams, R., and Massoumi, R.

International Journal of Molecular Sciences. 2020 Dec 2;21(23):9203

Abbreviations

AC	Adenocarcinoma
AFE	Anterior Foregut Endoderm
AGM	Aorta-Gonad Mesonephros
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
AT1/2	Alveolar Type 1/2 Cell
ATP	Adenosine Triphosphate
BM	Bone Marrow
BSC	Basal stem/progenitor Cell
CAMK	Calcium/Calmodulin-dependent Protein Kinase
CAMKII	Calmodulin-dependent Kinase II
CBFβ	Core Binding Factor Beta
CCSP	Club Cell Secretory Protein
CD1	Cyclin D1
CDK	Cyclin-dependent Kinase
CK1	Casein Kinase 1
CLP	Common Lymphoid Progenitor
CMJ	Cortico-medullary Junction
CMP	Common Myeloid Progenitor
COPD	Chronic Obstructive Pulmonary Disease
CRC	Colorectal Cancer
CSF	Colony Stimulating Factor
cTEC	cortical Thymic Epithelial Cells
CTL	Cytotoxic T Lymphocyte

CXCR4	CXC Chemokine Receptor Type 4
Daam1	Dishevelled Associated Activator of Morphogenesis
DAG	Diacylglycerol
DC	Dendritic Cell
DKK1	Dickkopf 1
DN	Double Negative cell
DP	Double Positive cell
Dsh	Dishevelled
DSK	Dual-specificity Kinase
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
EPO	Erythropoietin
ETP	Early Thymic Progenitor
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration
FLT3	Fms-like Tyrosine Kinase 3
FRP1	Frizzled Related Protein 1
Fz	Frizzled
GATA3	GATA-binding Protein 3
GBC	Gallbladder Cancer
GSK-3β	Glycogen Synthase Kinase-3 Beta
HCC	Hepatocellular Carcinoma
HDAC	Histone Deacetylase
HIPK2	Homeodomain-interacting Protein Kinase 2
HSC	Hematopoietic Stem Cell
HSP27	Heat Shock Protein 27
IASLC	International Association for the Study of Lung Cancer
IFNγ	Interferon Gamma

IkBα	Inhibitor of Nuclear Factor Kappa B Alpha
IKKβ	Inhibitor of Nuclear Factor Kappa B Kinase Beta
IL-6	Interleukin-6
ILD	Interstitial Lung Disease
Int-1	Mouse Mammary Tumour Virus Integration Site 1
IP_3	1, 4, 5-triphosphate
JNK	c-Jun N-terminal Kinase
LCC	Large cell Carcinoma
LEF	Lymphoid Enhancer Factor
LRP	Lipoprotein Receptor Related Protein
LTBP1	Latent Transforming Growth Factor Beta Binding Protein 1
MAPK	Mitogen-activated Protein Kinase
MAVS	Mitochondrial Antiviral-signalling Protein
MEF	Mouse Embryonic Fibroblast
MHC	Major Histocompatibility Complex
MPP	Multipotent Progenitor
mTEC	medullary Thymic Epithelial Cells
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor Kappa B
NLK	Nemo-like Kinase
NotchICD	Notch Intracellular Domain
NSCLC	Non-small Cell Lung Cancer
PCa	Prostate Cancer
PCP	Planar Cell Polarity
PDE	Phosphodiesterase
PD-L1	Programmed Death Ligand 1
PKC	Protein Kinase C
PKG	cGMP-dependent Protein Kinase or Protein Kinase G
PLC	Phospholipase C

PNEC	Pulmonary Neuroendocrine Cell
PTM	Post-translational Modification
RBC	Red Blood Cell
ROCK	Rho-associated Kinase
RUNX	Runt-related Transcription Factor
SAPK	Stress-activated Protein Kinase
SCF	Stem Cell Factor
SCLC	Small Cell Lung Cancer
Ser	Serine
SP	Single Positive cell
SqCC	Squamous Cell Carcinoma
STK	Serine/Threonine Kinase
TAB	Tak1-binding Protein
TAK1	Transforming Growth Factor Beta Activated Kinase 1
TCF	T-cell Factor
TCR	T-cell Receptor
TF	Transcription Factor
TGFβ	Transforming Growth Factor Beta
ThPOK	T-helper-inducing POZ Kruppel-like Factor
Thr	Threonine
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TNFα	Tumour Necrosis Factor Alpha
TNM	Tumour, Node, Metastasis
TPO	Thrombopoietin
T_{reg}	Regulatory T-cell
TSP	Thymic Seeding Progenitor
Tyr	Tyrosine
Wg	Wingless

Abstract

Post-translational modifications are covalent processing events that change the properties, location, and function of proteins. One the most common and well-known modification is protein phosphorylation that is a reversible process where protein kinases add a small phosphate group to their substrate, which in turn can be removed by the action of phosphatases. The overall aim of this thesis is to study the function of protein kinases in various cellular aspects, such as cell signalling, cell development and cancer development. We focus on elucidating the function of Nemo-like kinase and JNK kinase in these biological settings. Nemo-like kinase is an evolutionary conserved atypical MAP kinase-related kinase that is most known as a negative regulator of the Wnt signalling pathway. The JNK kinases are part of a subgroup of the MAPK family and are often activated by stress-related stimuli, such as UV radiation, DNA damage, or oxidative stress. Both protein kinases play important roles in regulating cellular responses including proliferation, differentiation, and migration.

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in mice lacking NLK compared to wild-type mice. These preliminary data indicate that NLK under normal circumstances functions as a tumour-suppressor with regards to lung cancer development. In paper IV we examined the role of the JNK signalling pathway in the development of colorectal cancer. One major concern regarding colorectal cancer is that patients are often diagnosed at late stages where tumours have been metastasised. By using small molecules to target the JNK proteins we observed that inhibition of JNK1 resulted in decreased migration and invasion in colorectal cancer cells. Treatment of animals with JNK1 inhibitor reduced tumour metastasis in liver and lung confirming our in vitro data. Taken together, we have shown novel functions of the protein kinases NLK and JNK in various biological circumstances. By further studying the functions of these protein kinases, new roles could be discovered for NLK and JNK kinases to be used as diagnostic tools or new therapeutic treatment strategies.

Popular Scientific Summary

Protein phosphorylation is a reversible process where protein kinases modify their substrate, which can be undone by phosphatases. Protein kinases are important modulators of proteins for all aspects of cellular biology, including cell proliferation, migration, invasion, cell death, differentiation, as well as regulating functions of proteins within the immune system. Even though protein kinases have been shown to be important regulators of almost all biological functions of proteins, many questions remain regarding the function of these enzymes. This thesis focuses on gaining understanding of how protein kinases function under normal, developmental and disease conditions.

In **papers I-III** the focus lies on the protein kinase: Nemo-like kinase (NLK), which is an evolutionary conserved kinase; meaning it can be found in all organisms from worm, to mouse, to humans. NLK is mostly known to be a negative regulator of the Wnt signalling pathway. A signalling pathway controls and relays the communication between proteins within and between cells. When the Wnt signalling pathway is dysregulated, resulting in the activation or deactivation of proteins, it can cause the development of several diseases such as cancer, metabolic, and neurodegenerative diseases.

In **paper I**, we show that deletion of NLK in mouse embryonic fibroblast (MEF) cells results in increased cell proliferation. However, no differences were found in cell migration, invasion, or survival in MEF cells lacking NLK compared to MEF cells expressing NLK. We found a new way that NLK can negatively regulate the Wnt signalling pathway, which results in decreased cell proliferation when NLK is present in the cells.

The role of NLK within the immune system has not been investigated and in **paper II** our goal was to examine what the function of NLK in early T-cell development is. T-cells are white blood cells that play an important role in adaptive immunity, which is the part of the immune system that creates a memory of what kind of bacteria or viruses our bodies have encountered. The way T-cells develop follows a very strict order, as in cell A develops into cell B that develops into cell C. Eventually, they turn into mature T-cells, of which one type is called CD8 T-cells. These CD8 T-cells are important for killing cells, such as cancer cells, cells infected by bacteria/viruses, or damaged cells. When we remove NLK in T-cells, we found

that the number of CD8 T-cells were reduced by 50% due to them dying. We found that NLK plays an important role in maintaining the normal balance for developing CD8 T-cells.

Dysregulated Wnt signalling has been shown to be involved in the development and progression of many types of cancer, such as lung cancer. In Sweden, lung cancer is the 6th most common type of cancer but the most common type of cancer-related deaths with a relative 5-year survival of approximately 20%. The most common type of lung cancer is called non-small cell lung cancer (NSCLC) that accounts for 80% of all types of lung cancer. The role that NLK plays in the development of NSCLC is still very much debated, where some claim that NLK contributes to NSCLC development and some claim that NLK hampers development of NSCLC. In **paper III** we created various genetically engineered mouse models to study NLK in the development of NSCLC. By removing NLK in the lung we found that these mice developed more lung tumours and cells within these tumours were dividing faster compared to mice that still expressed NLK. Our preliminary data suggest that NLK might protect the lungs from developing tumours, but more research needs to be done to explain how NLK can contribute to less tumour growth.

Finally, in **paper IV** we examined the role that the JNK signalling pathway plays in colorectal cancer (CRC). CRC is the 3rd most common type of cancer in Sweden with a 5-year survival rate of approximately 65%. However, CRC is often diagnosed at late stage disease where the tumours often have metastasised to distant organs. JNK proteins are protein kinases that function by modifying other proteins thereby affecting their function within the cell. By treating CRC cell lines with small molecules that block the action of the JNK signalling pathway we could show that these molecules caused a decrease in cell migration and invasion, which are important for cell metastasis.

In summary, we have shown that protein kinases play important roles in cell signalling, cell development, and disease progression. This makes them interesting targets to study as novel cellular process regulators, but also for the use as prognostic tools and targeted treatment strategies.

Populärvetenskaplig sammanfattning

Proteinfosforylering är en reversibel process där proteinkinaser modifierar sitt substrat vilket kan återställas av fosfataser. Proteinkinaser gör viktiga förändringar på proteiner som påverkar alla aspekter av cellulär biologi, inklusive cellproliferation, migration, invasion, celldöd, differentiering, såväl som reglerande funktioner hos proteiner inom immunsystemet. Även om proteinkinaser har visat sig vara viktiga regulatorer av nästan alla biologiska funktioner hos proteiner, kvarstår många frågor angående funktionen hos dessa enzymer. Denna avhandling fokuserar på att få förståelse för hur proteinkinaser fungerar under normala, utvecklingsmässiga och sjukdomsmässiga förhållanden.

I **artiklarna I-III** ligger fokus på proteinkinaset: Nemo-like kinase (NLK), som är ett evolutionärt bevarat kinas; vilket betyder att det kan hittas i alla organismer från mask, till mus, till människa. NLK är mest känt för att vara en negativ regulator av Wnt-signalvägen. En signalväg styr och vidarebefordrar kommunikationen mellan proteiner inom och mellan celler. När Wnt signalvägen är oreglerad, som resulterar i att proteiner aktiveras eller stängs av kan det resultera i utvecklingen av flera sjukdomar, såsom cancer, metabola sjukdomar och neurodegenerativa sjukdomar.

I **artikel I** visar vi att borttagning av NLK i musembryonala fibroblastceller (MEF) resulterar i ökad celldelning. Emellertid hittades inga skillnader i cellmigrering, invasion eller överlevnad i MEF celler som saknar NLK jämfört med MEF celler som uttrycker NLK. Vi hittade ett nytt sätt hur NLK negativt kan reglera Wnt signalvägen som resulterar i minskad cellproliferation när NLK finns i cellerna.

NLK:s roll inom immunsystemet är inte alls undersökt, så i **artikel II** var vårt mål att undersöka vad NLK har för funktion i tidig T-cellsutveckling. T-celler är vita blodkroppar som spelar en viktig roll i adaptiv immunitet, vilket är den del av immunsystemet som skapar ett minne av vilken typ av bakterier eller virus våra kroppar har mött. Hur T-celler utvecklas följer en mycket strikt ordning, där cell A utvecklas till cell B som utvecklas till cell C. Så småningom förvandlas de till mogna T-celler, varav en typ kallas CD8 T-celler. Dessa CD8 T-celler är viktiga för att döda celler, såsom cancerceller, celler infekterade av bakterier/virus eller skadade celler. När vi tar bort NLK i T-celler fann vi att antalet CD8 T-celler minskade med 50 % på grund av att de dog. Vi fann att NLK spelar en viktig roll för att upprätthålla den normala balansen för utvecklingen av CD8 T-celler.

Avvikande Wnt signalering har visat sig vara involverad i utvecklingen och fortskridningen av många typer av cancer, såsom lungcancer. I Sverige är lungcancer den 6:e vanligaste typen av cancer men den vanligaste typen av cancerrelaterade dödsfall med en relativ 5-årsöverlevnad på cirka 20 %. Den vanligaste typen av lungcancer kallas icke-småcellig lungcancer (NSCLC) som står för 80 % av alla typer av lungcancer. Den roll som NLK spelar i utvecklingen av NSCLC är fortfarande mycket omdiskuterad, där vissa påstår att NLK bidrar till NSCLC-utveckling och vissa påstår att NLK hämmar utvecklingen av NSCLC. I **artikel III** skapade vi olika genetiskt modifierade musmodeller för att studera NLK i utvecklingen av NSCLC. Genom att ta bort NLK i lungan fann vi att dessa möss utvecklade fler lungtumörer och celler i dessa tumörer delades snabbare jämfört med möss som fortfarande uttryckte NLK. Våra preliminära data tyder på att NLK kan skydda lungorna från att utveckla tumörer, men mer forskning behöver göras för att förklara hur och om NLK kan bidra till mindre tumörtillväxt.

Slutligen, i **artikel IV** undersökte vi rollen som JNK signalvägen spelar i kolorektal cancer (CRC). CRC är den 3:e vanligaste cancerformen i Sverige med en 5-årsöverlevnad på cirka 65 %. Men CRC diagnostiseras ofta i ett sent stadium av sjukdomen där tumörerna har metastaserat till avlägsna organ. JNK-proteiner är proteinkinaser som fungerar genom att modifiera andra proteiner och därigenom påverka deras funktion i cellen. Genom att behandla CRC cellinjer med droger som blockerar verkan av JNK signalvägen kunde vi visa att dessa droger orsakade en minskning av cellmigration och invasion, vilket är viktigt för cellmetastaser.

Sammanfattningsvis har vi visat att proteinkinaser spelar viktiga roller i cellsignalering, cellutveckling och sjukdomsutveckling. Detta gör dem till intressanta mål att studera som nya cellulära processregulatorer, men också för användning som prognosverktyg och riktade behandlingsstrategier.

Populairwetenschappelijke samenvatting

Eiwitfosforylering is een omkeerbaar proces waarbij eiwitkinasen hun substraat wijzigen, wat ongedaan kan worden gemaakt door fosfatasen. Eiwitkinasen brengen belangrijke veranderingen aan in eiwitten die alle aspecten van cellulaire biologie beïnvloeden, waaronder celproliferatie, migratie, invasie, celdood, differentiatie, evenals regulerende functies van eiwitten binnen het immuunsysteem. Hoewel is aangetoond dat eiwitkinasen belangrijke regulatoren zijn van bijna alle biologische functies van eiwitten, blijven er veel vragen bestaan over de functie van deze enzymen. Dit proefschrift richt zich op het verkrijgen van inzicht in hoe eiwitkinasen functioneren onder normale, ontwikkelings- en ziekteomstandigheden.

In **papers I-III** ligt de focus op het eiwitkinase: Nemo-like kinase (NLK), een evolutionair geconserveerd kinase; wat betekent dat het kan worden gevonden in alle organismen, van worm, tot muis, tot mensen. NLK is vooral bekend als een negatieve regulator van de Wnt signaalroute. Een signaalroute controleert en stuurt de communicatie tussen eiwitten binnen en tussen cellen. Wanneer de Wnt signaalroute ontregeld is, wat resulteert in het activeren of deactiveren van eiwitten, kan dit de ontwikkeling van verschillende ziekten veroorzaken, zoals kanker, stofwisselingsziekten en neurodegeneratieve ziekten.

In **paper I** laten we zien dat het verwijderen van NLK in embryonale fibroblastcellen (MEF) van muizen resulteert in verhoogde celproliferatie. Er werden echter geen verschillen gevonden in celmigratie, invasie of overleving in MEF-cellen zonder NLK in vergelijking met MEF-cellen die NLK tot expressie brengen. We hebben een nieuwe manier gevonden waarop NLK de Wnt-signaalroute negatief kan sturen, wat resulteert in verminderde celproliferatie wanneer NLK in de cellen aanwezig is.

De rol van NLK binnen het immuunsysteem is helemaal niet onderzocht, dus in **paper II** was ons doel om te onderzoeken wat de functie van NLK in de vroege ontwikkeling van T-cellen is. T-cellen zijn witte bloedcellen die een belangrijke rol spelen bij adaptieve immuniteit, het deel van het immuunsysteem dat een herinnering creëert aan wat voor soort bacteriën en virussen ons lichaam is blootgesteld. De manier waarop T-cellen zich ontwikkelen is heel strikt, aangezien cel A zich ontwikkelt tot cel B die zich ontwikkelt tot cel C. Uiteindelijk worden

het “volwassen” T-cellen, waarvan één type CD8 T-cellen wordt genoemd. Deze CD8 T-cellen zijn belangrijk voor het doden van cellen, zoals kankercellen, door bacteriën/virussen geïnfecteerde cellen of beschadigde cellen. Na het verwijderen van NLK in T-cellen ontdekten we dat het aantal CD8 T-cellen met 50% was verminderd omdat ze stierven. We ontdekten dat NLK een belangrijke rol speelt bij het handhaven van de normale balans voor de ontwikkeling van CD8 T-cellen.

Het is aangetoond dat ontregelde Wnt signalering betrokken is bij de ontwikkeling en progressie van vele soorten kanker, zoals longkanker. In Zweden is longkanker het 6^{e} meest voorkomende type kanker, maar het meest voorkomende type kanker gerelateerde sterfgevallen met een relatieve 5-jaarsoverleving van ongeveer 20%. Het meest voorkomende type longkanker wordt niet-kleincellige longkanker (NSCLC) genoemd en is verantwoordelijk voor 80% van alle soorten longkanker. Over de rol die NLK speelt bij de ontwikkeling van NSCLC wordt nog veel gediscussieerd, waarbij sommigen aantonen dan NLK bijdraagt aan de ontwikkeling van NSCLC en anderen aantonen dat NLK de ontwikkeling van NSCLC belemmert. In **paper III** hebben we verschillende genetisch gemanipuleerde muismodellen gemaakt om NLK bij de ontwikkeling van NSCLC te bestuderen. Door NLK in de long te verwijderen, ontdekten we dat deze muizen meer longtumoren ontwikkelen en dat cellen in deze tumoren sneller deelden in vergelijking met muizen die nog steeds NLK tot expressie brachten. Onze voorlopige gegevens suggereren dat NLK de longen zou kunnen beschermen tegen het ontwikkelen van tumoren, maar er moet meer onderzoek gedaan worden om uit te leggen hoe NLK kan bijdragen aan minder tumorgroei.

Ten slotte onderzochten we in **paper IV** de rol die de JNK-signaalroute speelt in colorectale kanker (CRC). CRC is de 3^{e} meest voorkomende vorm van kanker in Zweden met een 5-jaarsoverleving van ongeveer 65%. CRC wordt echter vaak gediagnosticeerd in een laat stadium van de ziekte, waarbij de tumoren vaak zijn uitgezaaid naar andere organen. JNK-eiwitten zijn eiwitkinasen die functioneren door andere eiwitten te veranderen, waardoor hun functie in de cel wordt beïnvloed. Door CRC-cellijnen te behandelen met medicijnen die de werking van de JNK-signaalroute blokkeren, konden we aantonen dat deze medicijnen een afname in celmigratie en invasie veroorzaakten, wat belangrijk is voor uitzaaiingen.

Samenvattend hebben we aangetoond dat eiwitkinasen een belangrijke rol spelen bij cel signalering, cel ontwikkeling, en ziekteprogressie. Dit maakt ze interessante doelwitten om te bestuderen als nieuwe regulatoren van cellulaire processen, maar ook voor gebruik als prognostische instrumenten en gerichte behandelingsstrategieën.

Background

Post-translational modifications

In eukaryotes, proteins have a widespread range of regulatory, signalling, catalytic, and structural functions. Most proteins are at some point modified through covalent processing events by post-translational modifications (PTMs) that change protein structure, localization, activity, and interactions with other proteins and substrates [1]. There are many types of PTMs including ubiquitination, methylation, acetylation, glycosylation, and phosphorylation [2, 3].

Protein phosphorylation is one of the most common PTMs that was originally described in 1906 by Phoebus Levene who detected phosphate in the protein Vitellin [4], and in 1933 together with F. A. Lipmann identified phosphoserine in the protein Vitellin [5]. However, it wasn't until 1954 that the first enzymatic phosphorylation of proteins was described as an enzyme that catalysed the phosphorylation of a protein substrate by adenosine triphosphate (ATP) [6].

Protein phosphorylation plays an important role in various cellular processes, such as metabolism, transcription, cell growth, cell division, cell death, immunity, and cell motility [7, 8]. Furthermore, deregulation of protein phosphorylation has been implicated to be involved in various diseases, including cancer, inflammatory diseases, and cardiovascular disorders [7, 9, 10].

Protein kinases

Protein kinases are intracellular enzymes that are responsible for protein phosphorylation by transferring gamma phosphate groups to various substrates. This mechanism is reversible by the ability of phosphatases to remove these phosphate groups [3, 10]. Over 500 kinases are encoded in the human genome and make up one of the largest family of genes in eukaryotes [11]. These protein kinases are subdivided into groups depending on the signalling pathway they belong to, such as mitogen-activated protein kinases (MAPK), cyclin-dependent kinases (CDK), calcium/calmodulin-dependent protein kinases (CAMK), casein kinase 1 (Ck1), and tyrosine kinases (TKs), among others [7, 11, 12].

The most common target sites of protein kinases are on Serine (Ser), Threonine (Thr), and Tyrosine (Tyr) residues. It has been shown that based on more than 2000

phosphoproteins the distribution of phosphorylated residue sites are 86.4% pSer, 11.8% pThr, and 1.8% pTyr [13]. Certain protein kinases on only tyrosine, some on both serine and threonine (serine/threonine, STKs), and few on all three, which are dual-specificity kinases (DSKs) [7].

The regulation of most aspects of cellular activity is performed by protein phosphorylation and abnormal phosphorylation is a major contributor of disease development. Protein kinases themselves are regulated by phosphorylation via other kinases, through protein-protein interactions, and gene transcription. Since protein kinases can be regulated by many different actions, they have become a major target for therapy and many protein kinase inhibitors have been developed that either already are used in the clinic or are in clinical trials [14-16].

JNK proteins

The c-Jun N-terminal kinases (JNKs) are serine/threonine kinases, also known as stress-activated protein kinases (SAPK) that belong to a subgroup of the MAPK family of proteins. The JNK pathways are activated by a diverse array of stimuli, such as hormones, cytokines, pathogens, UV radiation, oxidative stress, DNA damage, and viral/bacterial infections [17, 18].

The JNK kinase family is encoded by three highly homologous genes, JNK1, JNK2, and JNK3, with a total of 10 isoforms resulting from alternative splicing of these genes [19]. Furthermore, JNK1 and JNK2 are ubiquitously expressed in tissues, whereas JNK3 is more limited expressed in the brain and to an even lesser extent in the heart and testis [19-21]. JNK1 and JNK2 are important for embryonic development of mice, since dual deletion in mice resulted in hindbrain exencephaly and embryonic lethality [22]. Furthermore, JNK2 is necessary for efficient T-cell activation, but not B-cell activation [23]. JKN3 is highly expressed in the brain and regulates regeneration, differentiation, and programming of neurons [24].

The JNK signalling transduction pathway is a three-part pathway, where extracellular or intracellular stimuli activate JNK kinase kinases, which activate JNK kinases that in turn phosphorylate the threonine and tyrosine residues of the conserved Thr-X-Tyr motif [18, 25, 26]. The JNK phosphoproteins phosphorylate a variety of substrates that affect a range of cellular responses, including cell survival, cell division, immunological responses, and neuronal functions [18, 27]. Aberrant regulation of JNK signalling has been shown to be involved in disease development in animal models, including cancer, birth defects, neurological diseases, and immunological diseases [17, 28]. The JNK signalling pathway is becoming even more complex due to the crosstalk with other signalling pathways including Wnt [29, 30], nuclear factor kappa B (NF-κB) [31], and transforming growth factor beta (TGF-β) [32].

The Wnt signalling pathway

Wnt proteins belong to a major family of conserved, secreted glycoproteins that orchestrate essential roles in various cell biological and developmental processes, contributing to tissue homeostasis, cell fate determination, anterior-posterior axis formation, and neural patterning. [33, 34]. In humans, there have been 19 genes identified that encode Wnts and many are located closely together in the human genome [35, 36]. After translation, Wnt proteins are post-translationally modified and targeted to the endoplasmic reticulum. Following modification, Wnt proteins are transported to the Golgi apparatus by COPII vesicles and further transported to the membrane by Rab8a [37].

Originally, the Wnt protein wingless (*Wg*) was identified in Drosophila during a mutagenesis screen where mutations in *Wg* prevented formation of the wings and halteres [38, 39]. Isolation of the first Wnt gene from mouse mammary tumours, known as *int-1* (mouse mammary tumour virus integration site 1), was found to virally induce mammary tumours [40, 41]. The wingless protein and the murine *int-1* proto-oncogene have shown to be homologous, lending the fusion of these names to become Wnt [42].

The extensively studied Wnt signalling pathway has been shown to be evolutionary conserved in metazoan organisms that regulate many different cellular processes. However, dysregulation of the Wnt pathway signalling has been shown to cause various diseases, such as several types of cancer, neurodegenerative diseases, bone homeostasis, and metabolic disease [33, 43]. Signals from extracellular Wnt proteins stimulate multiple intracellular signal transduction cascades, including the canonical Wnt/β-catenin-dependent pathway and the non-canonical β-catenin-independent pathway [44].

Canonical Wnt signalling

The most well-known Wnt signalling pathway is the canonical or β-catenin-dependent signalling pathway (**Figure 1**). In an inactive state, when Wnt ligands are absent, the transcriptional co-activator β-catenin is phosphorylated by glycogen synthase kinase-3 beta (Gsk-3β), thereby rendering it inactive. The phosphorylation of β-catenin results in the instability and degradation of cytoplasmic β-catenin by the destruction complex consisting of Gsk-3β, Axin, Ck1, and adenomatous polyposis coli (APC) (**Figure 1**) [44, 45].

Inactivation and phosphorylation caused by the destruction complex leads to ubiquitination of β-catenin by the E3 ligase β-TrCP that targets it for proteasomal degradation [46]. Degradation results in the inability of β-catenin to translocate to the nucleus, thereby repressing target gene transcription since β-catenin cannot bind

to the T-cell factor (TCF)/lymphoid enhancer factor (LEF) complex rendering them inactive (**Figure 1**) [33, 44].

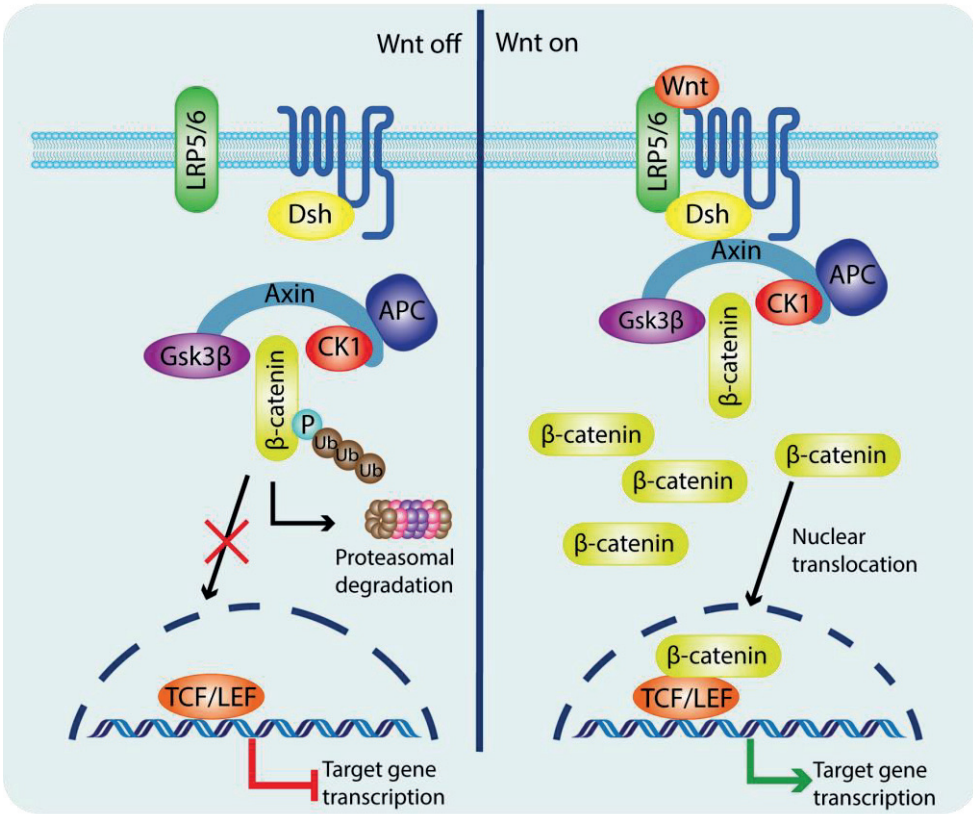

Figure 1. Overview of the canonical Wnt signalling pathway. When Wnt ligands are not present, the destruction complex consisting of Axin, APC, Ck1, and Gsk-3β target β-catenin for proteasomal degradation. However, when Wnt is present, the destruction complex releases β-catenin that accumulates in the cytosol before translocating into the nucleus. Here β-catenin will bind to the transcription factor complex TCF/LEF that start target gene transcription.

The canonical Wnt signalling pathway is activated when Wnt ligands bind to the Frizzled (Fz) receptor and the lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor. Once Wnt has bound to the Fz/LRP5/6 receptor complex, Fz can interact with Dishevelled (Dsh), which is a cytoplasmic protein that functions upstream of Gsk-3β [45, 47].

Axin is a well-known scaffold protein that interacts with the cytoplasmic tail of the LRP5/6 co-receptor through five phosphorylated PPPSP motifs [48, 49]. Once Dsh and Axin are bound to the receptors, downstream signalling is initiated, where Gsk-

3b is sequestered and thereby unable to phosphorylate β-catenin. Stabilized β-catenin can further accumulate in the cytosol before translocating to the nucleus. Inside the nucleus β-catenin will interact with the TCF/LEF DNA-binding transcription factor (TF) complex and promote transcription of target genes, such as Cyclin D1 (Cd1) and c-Myc (**Figure 1**) [44, 50].

Non-canonical Wnt signalling

The β-catenin-independent pathway or the non-canonical Wnt signalling pathway can be divided into two parts: the Wnt/Ca^{2+} pathway or the Planar Cell Polarity (PCP) pathway (**Figure 2**) [44, 51].

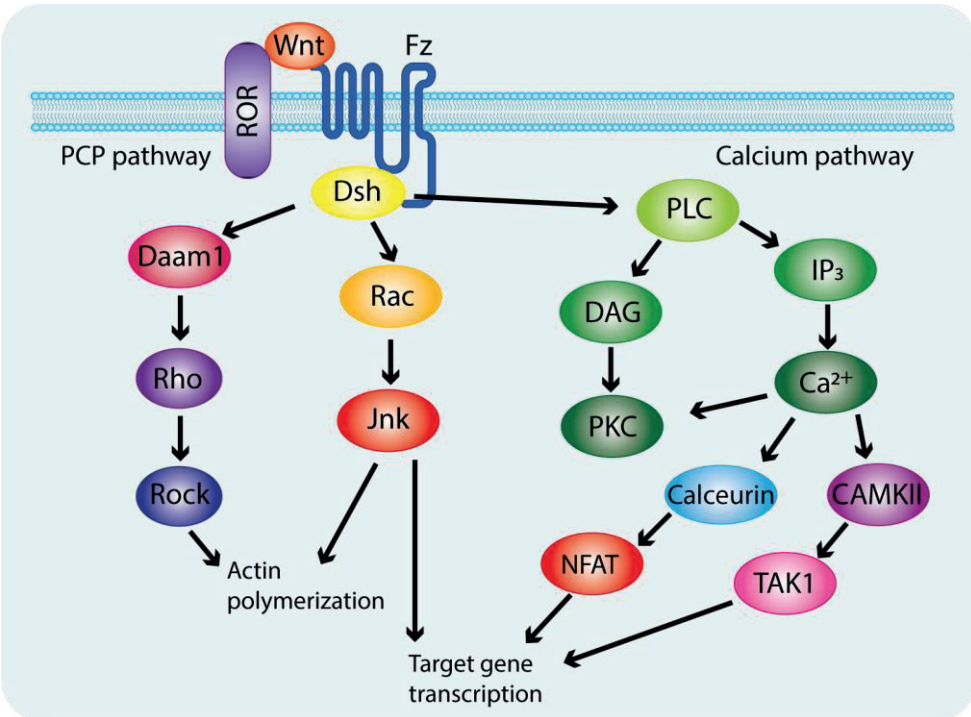

Figure 2. Non-canonical Wnt signalling pathways. Wnt ligands activate Dsh that in turn can activate the small GTPases Rac and Rho, thus activating the planar cell polarity pathway. Furthermore, PLC can become activated which leads to intracellular calcium release, thus activating the Wnt/Ca^{2+} pathway that can signal downstream to start target gene transcription.

Epithelial cells are polarized along the apical-basal axis and are arranged along the plane of the epithelial cell layer to orient themselves. This type of orientation or

polarization of cells is called planar cell polarity [52]. The PCP signalling pathway was identified during genetic studies in Drosophila melanogaster, where mutations in the PCP genes caused disorganized cuticular structures and the compound eye, as well as arrangement defects of photoreceptors in the eye [52, 53].

Once the Fz receptor gets activated by Wnt5 or Wnt11, a signalling cascade starts by activating Dsh that has two domains DEP and DPZ that activate the small GTPases Rac and Rho [54]. First in order to signal through Rho, Dsh activates Dishevelled associated activator of morphogenesis (Daam1) [55], followed by the Dsh-Daam1 complex causing activation of Rho. Once Rho is active, it stimulates Rho-associated kinase (ROCK) signalling, as well as myosin that are necessary for the rearrangement and polymerization of the actin cytoskeleton [44, 45]. The Dsh DEP domain activates Rac independently of Daam1 and in turn activates JNK signalling, resulting in target gene transcription (**Figure 2**) [56].

The second β-catenin-independent signalling pathway is the Wnt/Ca^{2+} pathway. The role of calcium as a second messenger in the Wnt signalling pathway was discovered after injections of Wnt5a or Wnt11 caused increased calcium transients in the enveloping layer of the blastodisc of 1-cell zebrafish embryos [57, 58]. When Wnt ligands bind to the Fz receptor it mediates the activation of Dsh through the activation of G-proteins. After activation of Dsh, multiple signalling cascades can become activated. Inhibition of calcium release can occur after activated phosphodiesterase (PDE) inhibits cGMP-dependent protein kinase or protein kinase G (PKG), which in turn inhibits Ca^{2+} release [44].

Furthermore, the activation of Fz by Wnts leads to stimulation of phospholipase C (PLC) that activates diacylglycerol (DAG) and 1, 4, 5-triphosphate (IP_3). IP_3 in turn causes the release of intracellular Ca^{2+} which triggers activation of protein kinase C (PKC), calmodulin-dependent kinase II (CAMKII), and calcineurin (**Figure 2**). CAMKII has been shown to have multiple effects, such as activate nuclear factor of activated T cells (NFAT) that is involved in the transcription of genes involved in lymphocytes, neurons, and muscle cells [59]. CAMKII is also able to signal through transforming growth factor beta activated kinase 1 (TAK1) and Nemo-like kinase (NLK) that can inhibit β-catenin signalling through inhibition of TCF/LEF [60, 61]. Both non-canonical Wnt signalling pathways have been shown to be important for embryonic development, as well as disease development. Furthermore, the Wnt/Ca^{2+} signalling pathway can affect and modulate both the PCP pathway and the canonical β-catenin-dependent signalling pathway.

The Wnt signalling pathway in cancer

All aspects of developmental biology, as well as at the cellular level, such as cell proliferation, differentiation, survival, motility, and stem cell self-renewal are affected and regulated by the Wnt signalling pathways. However, mutations in the

Wnt signalling genes have resulted in aberrant Wnt signalling, which has led to the development of several types of cancer [51].

In 1991, it was discovered in familial adenomatous polyposis (FAP) – a hereditary form of colorectal carcinoma – that a major underlying cause of this disease development was the mutation of the APC gene [62]. Furthermore, it was shown that APC can directly interact with β-catenin and loss of APC in colon cancer cell lines resulted in overexpression of β-catenin and TCF4, thus indicating that the interaction between TCF4 and β-catenin is regulated by APC [63, 64].

In basal-like breast cancer it was reported that an accumulation of β-catenin in the cytoplasm and nucleus leads to poor prognosis, increased vimentin expression, and worse overall survival [65]. In metastatic breast cancer it was found that tumour-secreted Dickkopf 1 (DKK1) was upregulated in patients with bone metastases but downregulated in patient without bone metastases. In the lungs it was found that DKK1 can suppress latent transforming growth factor beta binding protein 1 (LTBP1)-mediated TGF-β secretion, thereby inhibiting the Wnt/Ca^{2+} –CAMKII–NF-κB signalling cascade, thus affecting breast cancer metastatic growth in the lungs [66].

Mutations in exon 3 of *CTNNB1*, the gene encoding β-catenin, results in stabilization and nuclear translocation of β-catenin and thereby target gene transcription. In hepatocellular carcinoma (HCC), this mutation is observed in 20-35% of all cases [67, 68]. However, using a mouse model of HCC where stabilized β-catenin is expressed, no tumour development 6 months post-induction could be observed in these animals. Indicating that stable β-catenin alone does not cause liver tumour development [69]. Nevertheless, examining Axin1 mutations in HCCs showed that the Axin proteins lack the binding site for β-catenin, resulting in the inability to inhibit β-catenin signalling. Furthermore, addition of Axin1 caused decreased β-catenin/TCF4-mediated transcription, suggesting that induction of wild-type Axin could be considered as a potential therapeutic strategy in HCC [70].

Taken together, the Wnt signalling pathway is not only important for proper development of tissue and organs, but also for keeping the balance among signalling molecules. Above are only a few examples of how aberrant Wnt signalling has been shown to be involved in the initiation, development, and progression of various types of cancers [50, 51, 71, 72].

Nemo-like kinase

The evolutionary conserved, proline-directed serine/threonine atypical MAP kinase-related kinase: Nemo-like kinase (NLK) was originally identified in 1994 in Drosophila. The Drosophila gene *nemo* was shown to be involved in ommatidia morphogenesis to regulate proper photoreceptor cell cluster rotation in the eye [73]. NLK was first cloned in 1998 after it was found to be a murine homolog to the Drosophila *nemo* gene. The amino acid sequence of NLK predicts a protein kinase that has shown to be 41.7% identical to murine Erk-2 and 38.4% identical to human Cdc2. The human form of NLK consists of 515 amino acids, containing a central kinase domain. Furthermore, the NLK sequence encodes a region very rich in histidine, glutamine, proline, and alanine accounting for more than 70% of the amino acid composition of this region [74, 75].

Phylogenetic analysis has divided vertebrate NLK into two types: type-I NLK (Nlk1) and type-II NLK (Nlk2). Zebrafish express both *nlk1* and *nlk2*, where Nlk1 is 73% identical to *Xenopus Laevis* and only 68% identical to human NLK, meanwhile Nlk2 is 97% identical to human NLK [76]. In zebrafish, Nlk1 was shown to be expressed during the gastrulation stages, in the notochord, neural tube, and the eyes. Overexpression of Nlk1 to study its activity during early development, caused embryos with loss of eyes and forebrain [77].

NLK is activated through various mechanisms, one of these is the suggestion that this occurs through autophosphorylation of its activation loop at Thr286, since mutation of Thr286 to aspartic acid or glutamic acid resulted in undetectable autophosphorylation [74]. Furthermore, multiple extracellular ligands have been shown to activate pathways that lead to the activation of NLK, including Wnt1, interleukin-6 (IL-6), and TGF-β [75, 78, 79]. It has also been shown that the non-canonical Wnt/Ca^{2+} signalling pathway can activate NLK. Upon binding of Wnt5a to the Fz-2 receptor, intracellular calcium is released and able to activate CAMKII. Stimulation of CAMKII leads to the activation of TAK1 that in turn causes NLK to become activated (**Figure 3**) [61]. However, it has been shown that TAK1 cannot directly interact with NLK, which has led to the discovery of the scaffold protein TAK1-binding protein (TAB). Both TAB1 and TAB2 have been shown to bridge the interaction between TAK1 and NLK by their scaffolding action [80, 81]. Since TAK1 cannot directly interact with NLK, a mediator must act between the two proteins to propagate the signal. It has been shown that homeodomain-interacting protein kinase 2 (HIPK2) directly interacts with NLK and acts upstream of NLK but downstream of TAK1 (**Figure 3**) [78].

In the literature, NLK is mostly known to be a negative regulator of the Wnt signalling pathway due to its ability to inhibit the TCF/LEF complex, thereby inhibiting target gene transcription by this complex (**Figure 3**) [82]. Initially, it was shown that NLK is able to phosphorylate TCF4, which in turn interferes with the

binding of β-catenin to TCF and the target sites of TCF, causing inhibition of TCF/β-catenin-mediated target gene transcription [83]. Moreover, not only TCF is directly negatively affected by NLK phosphorylation, LEF1 is also a target for NLK phosphorylation. The phosphorylation of LEF1 by NLK occurs at Thr-155 and Ser-166 affecting the recruitment of β-catenin to the NH_2-terminus of LEF1, causing inhibition of the downstream signalling pathway [84]. NLK can phosphorylate TCF4 at Thr-178 and Thr-189, but this phosphorylation does not interrupt the complex building between TCF/LEF and β-catenin but prevents DNA binding by TCF/β-catenin, thereby inhibiting transcription [84]. Another mechanism of negative regulation of the Wnt signalling pathway by NLK is through its direct interaction with histone deacetylase 1 (HDAC1). Previously, we could show that mouse embryonic fibroblast (MEF) cells expressing NLK were able to directly phosphorylate HDAC1, which repressed LEF1 transcriptional activity, resulting in decreased cell proliferation compared to MEF cells lacking NLK expression [85].

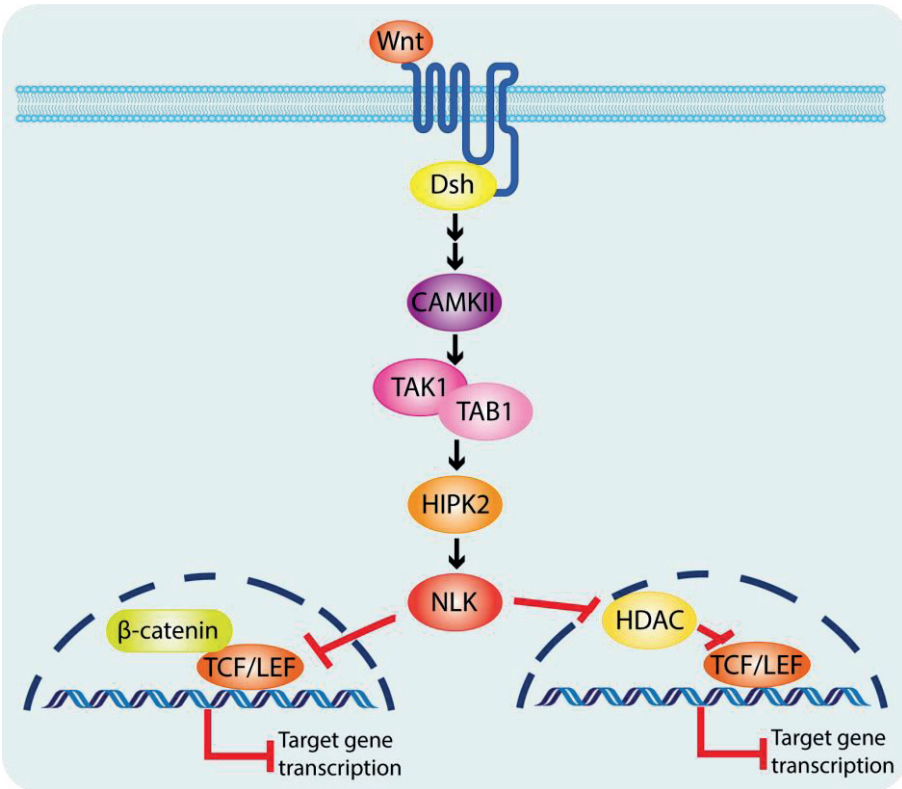

Figure 3. Nemo-like kinase/Wnt signalling pathway. Model showing that NLK is activated through the non-canonical Wnt/Ca^{2+} pathway and can directly interfere with canonical Wnt signalling by inhibiting target gene transcription by negative regulating the TCF/LEF complex either direct or through interaction with HDAC.

Besides being a negative regulator of Wnt signalling, NLK has also been shown to positively regulate this signalling pathway [82]. Nlk2 in zebrafish is necessary for proper function of neural progenitor cells. It was shown that Nlk2 is important for activation, phosphorylation, and transcriptional activity of LEF1. Inhibition of Nlk2 resulted in decreased cell proliferation causing a reduction in midbrain tectum size of zebrafish [76]. These data indicate that the function of NLK is organism and cell type dependent, due to its dual function as both a negative and positive regulator of the Wnt signalling pathway.

In addition to being a regulator of Wnt signalling, NLK can affect other signalling pathways. Through the TAK1-NLK cascade, it was shown that NLK activates STAT3 which is necessary for the proper function of TGF-β-mediated induction of mesoderm in *Xenopus Laevis* [79]. Furthermore, research has provided evidence that IL-6 signalling activates TAK1-NLK signalling, resulting in NLK-mediated phosphorylation of STAT3 at Ser727 [86]. NLK has also been described as a new substrate of Notch signalling. NLK can phosphorylate the Notch intracellular domain (NotchICD), thereby negatively regulating Notch transcriptional activation. Additionally, knockdown of NLK resulted in hyper-activation of the Notch signalling pathway, which caused reduced zebrafish neurogenesis [87]. It was also found that NLK can phosphorylate inhibitor of nuclear factor kappa B kinase beta (IKKβ), thereby inhibiting tumour necrosis factor alpha (TNF-α)-induced NF-κB activity, as well as p65 nuclear localization and degradation of inhibitor of nuclear factor kappa B (IκBα) [88]. Recently, NLK has been shown to interact with YWHAZ (14-3-3ζ), preventing its dimerization which is important for 14-3-3ζ stabilization and function, resulting in decreased migration and growth of NSCLC cell lines [89].

Nemo-like kinase in cancer

Nemo-like kinase has been shown to be an important player in developmental biology, as well as cellular processes, including cell proliferation, cell migration, cell survival, cell differentiation by regulating the expression of several downstream signalling molecules [82]. However, aberrant NLK signalling has been found to affect development, as well as the process of disease initiation and progression, including cancer. Various studies have shown that depending on the type of cancer, NLK can act as an oncogene or a tumour-suppressor gene [90].

In HCC, immunohistochemical analysis of patient data, has shown that NLK was upregulated in these tissues and high expression levels of NLK resulted in significantly worse prognosis, compared to low NLK expression [91]. Additionally, knock-down of NLK in HCC cell lines resulted in decreased cell growth, due to G1/S-phase cell cycle arrest caused by decreased levels of CDK2 and CD1 by NLK [92].

It has been shown that NLK is able to directly bind to c-Myb together with HIPK2, thereby phosphorylating c-Myb and affecting its further signalling [78]. In breast cancer it was shown that high expression levels of NLK were significantly correlated with low expression levels of c-Myb. Furthermore, overexpression of NLK caused cell growth inhibition and cell cycle arrest, as well as increased cell death [93], thus suggesting that NLK functions as a tumour suppressor in breast cancer cells. Moreover, we could establish that NLK is primarily localized in the nucleus of breast cancer cells compared to non-cancerous breast epithelial cells that mainly expressed NLK in the cytosol [94]. The nuclear localization of NLK was mediated by direct interaction with heat shock protein 27 (HSP27) and this interaction prevents NLK-mediated cell death [94].

In gallbladder cancer (GBC), high NLK expression is negatively correlated with overall survival and prognosis of patients. Furthermore, NLK expression is correlated with tumour grade and tumour node metastasis (TNM) grade [95]. These data suggest that NLK might be used as a prognostic marker when diagnosis patients with GBC.

In prostate cancer (PCa) metastases, NLK expression was found to be downregulated compared to normal prostate and primary PCa. Knockdown of NLK causes an increase in androgen receptor (AR)-mediated transcriptional activity and inhibits apoptosis [96].

In colorectal cancer, the expression levels of NLK were increased compared to non-cancerous tissue. Patients with high NLK expression had decreased overall survival and disease-free survival. Moreover, there was a significant correlation between tumour invasion, metastasis, and vascular invasion in samples with positive NLK expression [97]. Expression of NLK in DLD-1 human colon cancer cells resulted in decreased cell growth, but the kinase inactive variant of NLK did not affect cell growth. Furthermore, stable NLK expression increased cell death but did not induce cell cycle arrest [98].

Taken together, the role of NLK in tumour development is widely debated with regards to its oncogenic and tumour suppressive actions due to its tissue-dependent function.

Hematopoiesis

Hematopoiesis is the developmental process in mammalians where all the cells of the blood system are replenished throughout time. It is estimated that approximately 10^{12} new cells are generated each day in the adult human bone marrow (BM) [99]. One of the first times hematopoiesis was studied was in 1961 when bone marrow cells were injected into irradiated mice, which resulted in spleen colonies [100]. In 1966, it was described how an agar *in vitro* culture system was used to quantify the clonal growth of murine bone marrow cells [101]. Later, culture systems were created after the realization that hematopoiesis *in vitro* can be prolonged by seeding bone marrow cells with an adherent cell layer consisting of endothelial cells, fat cells, and macrophages. This created a suitable environment for myeloid cells to differentiate from proliferative stem cells [102]. The use of long-term culture of adherent bone marrow cells, allowed researchers to culture normal murine B cells over time. Between 2-8 weeks of cultures, non-adherent B cells have limited division capacity, however, after 10-14 weeks this particular population of B cells is replaced with cells that are highly replicative [103]. Since blood cells are relatively short-lived, hematopoietic stem cells (HSCs) are necessary to regenerate the various types of blood cell lineages. For instance, after a bone marrow transplant, after injury or infection, hematopoietic cells need to be replenished [100, 104, 105].

The mammalian hematopoietic tree has HSCs at the top, which are defined by two properties. First, they are capable of producing new HSCs – self-renewal capacity, and second, they can differentiate into all types of progenitor cells that can further commit into specific blood cells (multipotency) [106]. The origin of HSCs is still very much debated in the field of hematopoietic research. However, the production of mammalian HSCs occurs in specific places, including the yolk sac, the aorta-gonad mesonephros (AGM) region, which is an area around the dorsal aorta, the placenta, the fetal liver, and the bone marrow [107]. The development of HSCs is often described to occur in waves, where the first wave or "primitive wave" of blood production occurs in the yolk sac, which is necessary to produce red blood cells to oxygenize the developing tissue of the embryo. The second wave or "definitive wave" is the rapid onset of adult-type hematopoiesis occurring in the intraembryonic site later in development [106-108].

The investigation of human HSCs depends on immunohistochemistry and surface antigen staining, where in a simplified view, cells are stained for $CD34^+$. However, CD34 is expressed by both HSCs and vascular endothelial cells, thereby the pan-leukocyte CD45 marker is used to distinguish between these cell types [109, 110]. $CD34^+$ HSCs and early erythroblasts can be found at 18.5 days of embryonic development in the yolk sac and after 22 days red blood cells can be found in the circulation of the embryo [111]. On day 26, the HSCs can be detected in the mesenchyme of the AGM region surrounding the dorsal aorta. Furthermore, it has been shown that preumbilical aortic regions can produce erythroid and granulocyte

progenitor cells [112, 113]. On day 30, the first $CD34^{+}CD45^{+}$ HSCs can be found in the fetal liver and this population proliferates and increases until day 42 [113]. Finally, after 8.5 weeks of embryo gestation, the cartilage is broken down in a process called chondrolysis, meanwhile osteoblasts and osteoclasts are produced and invade the bone marrow cavity. After 10.5 weeks of gestation, hematopoietic stem cells and differentiation progenitor cells can be found in the bone marrow, where further hematopoiesis occurs [114].

The classical view of the hematopoietic cell system is based on a hierarchical tree where the branches consist of the differentiated progenitor cells until they reach their final mature cell type (**Figure 4**).

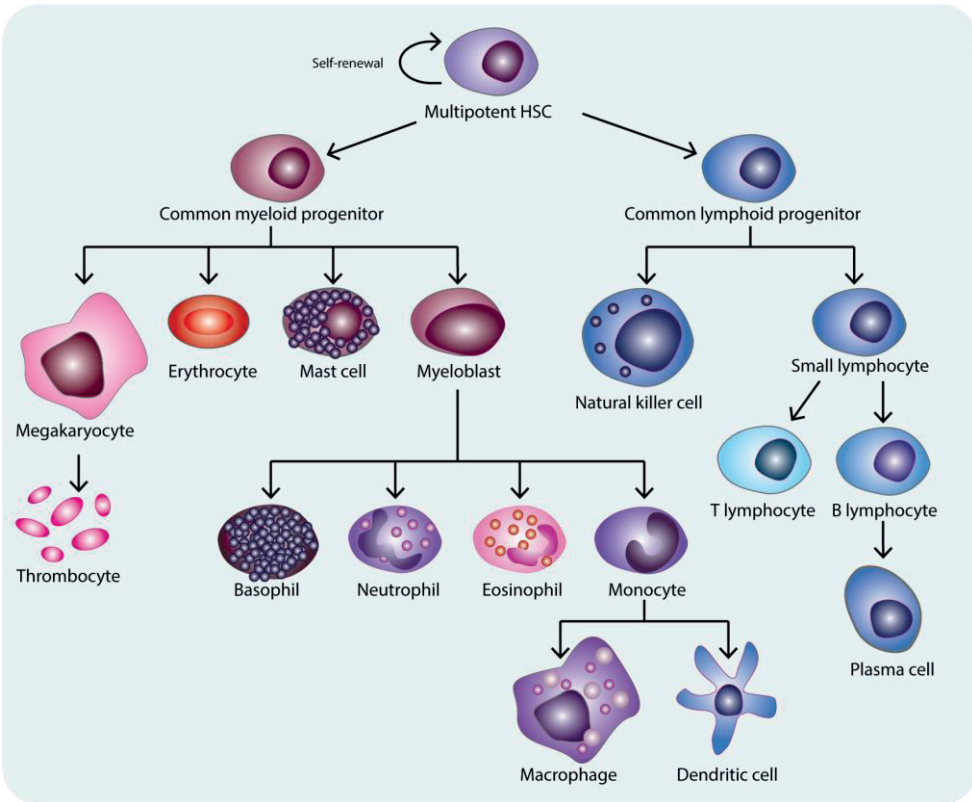

Figure 4. Hematopoiesis. Schematic model showing how hematopoietic stem cells (HSCs) differentiate into the various hematopoietic cell lineages.

This system can be divided into four lineages: first, erythrocytes or red blood cells (RBCs) that regulate oxygenation to and from tissues. Second, the megakaryocyte

lineage gives rise to thrombocytes that contribute to the coagulation of blood during wound healing. Third is the myeloid cell lineage that produces basophils, eosinophils, mast cells, and neutrophils, which belong to the granulocytes. Furthermore, the myeloid lineage gives rise to monocytes, and dendritic cells, all of which are involved in the innate immune system. The fourth group is the lymphoid lineage that produces B- and T-lymphocytes that belong to the adaptive immune system (**Figure 4**) [115-117]. Initially HSCs differentiate in multipotent progenitor (MPP) cells that have lost the ability to self-renew, before the further differentiate into common lymphoid progenitor (CLP) cells and common myeloid progenitor (CMP) cells [118-120]. These oligopotent progenitor cells give rise to all types of blood cells, where CMPs produce erythrocyte, megakaryocyte, granulocyte, and macrophage progenitors, meanwhile CLPs give rise to NK cells, B lymphocytes, T lymphocytes, as well as dendritic cells (DCs) (**Figure 4**) [121].

In order to understand how HSCs differentiate into the various blood cell lineages, researchers stain cell surface molecules on these cells and analyse them by flow cytometry and through the identification of various cytokines necessary to shift differentiation towards a specific cell lineage [121, 122]. Cytokines of hematopoietic cells act at all levels of blood progenitor cell differentiation. These cytokines include the following groups: interferons, interleukins (ILs), erythropoietin (EPO), colony-stimulating factors (CSFs), thrombopoietin (TPO), as well as Fms-like tyrosine kinase 3 (FLT3), and stem cell factor (SCF) [116, 122-124]. Even though the origin of hematopoietic stem cells and the mechanisms involved in the development of blood cell lineages has been extensively studied over the last couple of decades, still many questions arise regarding all aspects of this hierarchical system.

T-cell development in the thymus

The cells of the immune system all stem from HSCs within the bone marrow and once MPPs leave the bone marrow and migrate to the thymus, they commit to their T-lymphocyte cell fate. The microenvironment in the thymus is important for T-cell commitment, selection, expansion, differentiation, and maturation to become self-tolerant and functional T-cells [125]. The thymus can be divided into four areas that have specific functions depending on their microenvironment, which play different roles in T-cell development. 1) The subcapsular zone consist largely of cortical thymic epithelial cells (cTECs). 2) The cortex comprises a mixture of cells, including cTECs, macrophages, and fibroblasts. 3) The medulla contains medullary thymic epithelial cells (mTECs), macrophages, DCs, and Hassal's corpuscles. 4) The cortico-medullary junction comprises a dense network of blood vessels that facilitate the entry of thymocytes to and from the blood [126-128]. Early thymic progenitor cells (ETPs), also known as thymic seeding progenitor cells (TSPs) migrate from the bone marrow and enter the thymus at the cortico-medullary

junction (CMJ) where they undergo T-lymphocyte lineage specification [129]. The Notch signalling pathway plays an important role in early T-cell development and the maturation of ETPs by inhibiting ETPs to differentiate into myeloid cells [130, 131].

The earliest thymocytes are double negative (DN) cells that lack CD4 and CD8 expression, which are further divided into four subsets: DN1-DN4 [132]. DN1 thymocytes express $CD4^{-}CD8^{-}CD3^{-}CD44^{+}CD25^{-}$ and have been shown to be a very heterogeneous population of cells. Within this population there are ETPs that express high levels of cKit ($lin^{low}CD44^{+}cKit^{high}CD25^{-}$), which generate the thymocyte population by rapid expansion (**Figure 5**) [133].

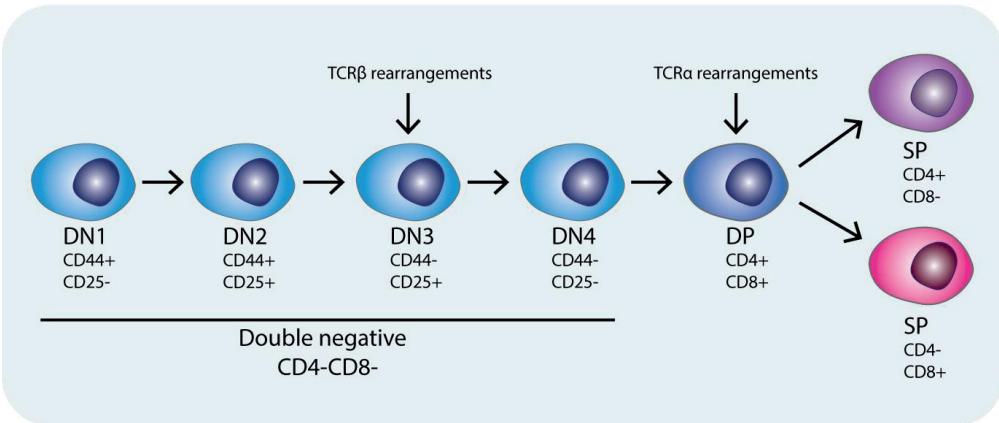

Figure 5. Schematic overview of early thymocyte development. Once thymic progenitor cells reach the thymus they differentiate into double negative thymocytes (DN1-4), followed by differentiation into double positive (CD4+CD8+) thymocytes. The DP thymocytes differentiate and mature into single positive (SP) CD4+ or CD8+ thymocytes.

DN1 cells migrate from the CMJ to the subcapsular zone where they receive signals from fibroblasts and cTECs to differentiate into DN2 thymocytes ($CD44^{+}CD25^{+}$). T-lineage cell fate is further restricted and DN2 cells initiate T-cell receptor (TCR) rearrangement of TCRγ, TCRδ, and TCRβ gene loci [134, 135]. Next, DN2 cells differentiate into DN3 ($CD44^{low}CD25^{+}$) thymocytes within the subcapsular zone of the thymus. Here the specific T-cell fate is decided whether cells become αβ thymocytes or γδ thymocytes due to excessive gene rearrangement of the β, γ, and δ gene loci in order to express functional TCRs. The αβ thymocytes must pass an important checkpoint in their development, known as β-selection, which requires additional signalling from CXC chemokine receptor type 4 (CXCR4) and Notch1 receptor. For thymocytes to pass β-selection they require to have a functional pre-TCR consisting of a TCRβ chain, components of the CD3 molecule, and a pre-Tα

chain [134, 136, 137]. Finally, DN3 thymocytes differentiate into DN4 ($CD44^-$ $CD25^-$) cells who migrate back to the medulla. After thymocytes have passed β-selection and express a functioning pre-TCR, they upregulate CD4 and CD8 expression to become $CD4^+CD8^+$ double positive (DP) cells (**Figure 5**). Next, DP thymocytes undergo positive selection after the initiation of TCRα recombination. During positive selection, the specificity and binding affinity of the αβ-TCR for the major histocompatibility complex (MHC) is tested and DP cells that interact with intermediate affinity survive and continue differentiating. Cells that bind too hard or too unspecific will get pro-apoptotic signals, only DP cells that pass positive selection express self-MHC restricted TCRs [134, 138].

Next, DP thymocytes differentiate in MHC class-I CD8 single positive (SP, $CD4^-$ $CD8^+$) cells or MHC class-II CD4 SP ($CD4^+CD8^-$) cells (**Figure 5**). The committed SP thymocytes migrate to the medulla where SP cells undergo negative selection. During negative selection, SP thymocytes will be eliminated if they bind too strong to self-peptide MHC molecules, thereby eliminating the creation of autoreactive T-cells [139]. Once SP thymocytes express TCRs that bind the proper self-peptide MHC class to become either $CD4^+$ SP T-cells or $CD8^+$ SP T-cells, they are ready to migrate out from the thymic medulla to peripheral lymphoid sites, such as the spleen and lymph nodes [140].

SP thymocytes do not only need to pass positive and negative selection to survive, but their lineage commitment towards $CD4^+$ SP or $CD8^+$ SP cells is strictly regulated by various transcription factors. Lineage choice requires cell specific factors and in $CD8^+$ SP thymocytes, the *Cd4* silencer is upregulated that controls expression of CD4 [141]. Furthermore, the runt-related transcription factor (RUNX) family of transcription factors play a crucial role in driving lineage commitment toward $CD8^+$ SP cells [142]. There are three members of this family: RUNX1, RUNX2, and RUNX3 that form heterodimers with core binding factor beta (Cbfβ), which together activate or inhibit target gene transcription. The Runt homology domain that RUNX transcription factors contain is in direct contact with DNA, something the Cbfβ protein lacks, however DNA binding affinity is significantly increased when RUNX-Cbfβ are complexed [143]. However, the RUNX TFs have different functions during thymopoiesis. RUNX1 is expressed in early thymocyte development, during the DP stage RUNX1 was shown to bind the *Cd4* silencer to assure the survival of CD4 lineage thymocytes [144]. Additionally, RUNX1 has been shown to be important for proper β-selection and positive selection during early thymocyte development [145]. Meanwhile, RUNX3 is upregulated in differentiated $CD8^+$ SP thymocytes and binds to the *Cd4* silencer to remain committed to the CD8 T-cell lineage, even after these cells leave the thymus [146, 147]. The literature has shown that the RUNX TFs favour CD8 T-lineage commitment over CD4. RUNX3 expression contributes to CD8 lineage commitment, not only by binding to the *Cd4* silencer, but also by binding to the *Cd8* enhancer [145, 147]. Moreover, when naïve $CD8^+$ T-cells differentiate in peripheral

effector cytotoxic T lymphocytes (CTLs), it was shown that RUNX3 TFs regulates the expression of interferon-γ (IFNγ), granzyme B, and perforin, which all are specific CTL effector markers [148].

Simultaneously, transcription factors specific to the CD4 T-cell lineage are upregulated to ensure $CD4^+$ SP thymocyte commitment. Opposing the action of RUNX3 in $CD8^+$ SP cells is T-helper-inducing POZ Kruppel-like factor (ThPOK), which is a *Zbtb7b* gene product [149]. ThPOK belongs to the POK family of transcription factors containing a DNA binding domain consisting of multiple zinc fingers and a homodimerization domain [150]. ThPOK is first expressed in $CD4^+$ SP thymocytes where it is upregulated during MHC class-II induced selection, while simultaneously downregulating CD8 expression. Furthermore, it has been shown that upon expression of ThPOK in MHC class-I restricted thymocytes redirects these cells towards CD4 lineage commitment, thereby inhibiting $CD8^+$ SP cell differentiation [151]. Besides ThPOK, the zinc finger transcription factor GATA binding protein 3 (Gata3) is important during multiple steps of T-cell development. Evidence has been provided that Gata3 expression is highest in DP-to-$CD4^+$ SP cell transition and plays an important role in CD4 cell development and acts as a negative regulator of CD8 thymocyte differentiation [152]. Upon deletion of Gata3 in mice, the development of $CD4^+$ SP thymocytes during DP-to-$CD4^+$ SP transition does not result in redirecting cells to become $CD8^+$ SP. However, Gata3 deficient mice did have a partial DN3 differentiation arrest resulting in decreased DN4 and DP cell numbers [153]. These experimental data suggest that Gata3 is important during the early stages of T-cell development, as well as to promote the expression of ThPOK to commit to the $CD4^+$ SP lineage.

In summary, Gata3 can contribute to activation of ThPOK expression which in turn binds to the *Cd4* enhancer, while simultaneously inhibiting RUNX3 expression, thus favouring differentiation of $CD4^+$ SP thymocytes. Meanwhile, RUNX3 proteins bind the *Cd4* silencer and *Cd8* enhancer to repress the expression of ThPOK, to direct cells to differentiate into $CD8^+$ SP thymocytes [142, 149, 154].

Function of Nemo-like kinase in the immune system

The Wnt signalling pathway has been established to play an important role throughout entire early development of T-cells. Wnt signalling is already necessary at the DN stage of T-cell development to contribute to cell proliferation [155]. TCF1 has been shown to be a critical player in T-cell specification, where Notch signals to TCF1 in ETPs to drive T-cell lineage specification within the thymus [156]. Furthermore, deletion of β-catenin in T-cells resulted in decreased splenic T-cells due to a defective β-selection checkpoint. Additionally, TCR-CD3-mediated signalling was affected in these mice resulting in decreased cell proliferation, thus indicating the importance of β-catenin to maintain the T-cell pool [157]. Upon deletion of TCF1 and LEF1 in DP thymocytes, it was found that the differentiation

of $CD4^+$ SP T-cells was significantly impaired and cells were redirected from $CD4^+$ T-cell differentiation to the $CD8^+$ T-cell fate [158].

However, the role that NLK plays in the immune system and more specifically in T-cell development is only newly of interest in the literature, where its function is still not very well understood or elucidated. It was shown that NLK, as well as β-catenin are not necessary for thymus development in mouse embryos [159]. Furthermore, in short, we have generated a mouse model where NLK was deleted in the early stages of T-cell development, which caused a significant reduction in the $CD8^+$ SP thymocyte population due to increased cell death [160] (more regarding this work will be discussed under "The present investigation"). Recently it was shown that TAK1-NLK signalling can regulate the phosphorylation of the Foxp3 in regulatory T-cells (T_{regs}). Additionally, deletion of NLK in T_{regs} resulted in diminished T_{reg}-mediated immunosuppression causing these animals to develop autoimmune encephalomyelitis [161]. NLK was also shown to be a negative regulator of type I IFN signalling and the antiviral immune response by regulating the phosphorylation of mitochondrial antiviral-signalling protein (MAVS) leading to its degradation [162]. Since not much is known about the function of NLK in immune cells and the immune system, it is of interest to look further into the mechanism through which NLK might be able to regulate these cells and systems.

The stages of lung development

The mammalian respiratory system can generally be divided into the upper and lower compartment. The upper respiratory tract consists of the nose, mouth, pharynx, and larynx. Meanwhile the lower tract encompasses the trachea and the lungs [163]. Furthermore, the lower respiratory tract resembles a tree-like structure where the airway tubes branch of from the trachea and the "leaves" of the mammalian airway system are the vascularized alveoli where gas exchange occurs. The lungs, as well as the trachea, oesophagus, liver, and thyroid arise from the anterior foregut endoderm (AFE). Specification of lung development is marked by the expression of transcription factor *Nkx2.1* and decreased expression of *Sox2* in the ventral endodermal cells [164, 165].

Furthermore, human lung development can be divided into five developmental stages: 1) embryonic, 2) pseudo-glandular, 3) canalicular, 4) saccular, and 5) alveolar. During the embryonic stage, two primary lung buds are formed, as well as the trachea that becomes separated from the oesophagus. Branching morphogenesis occurs during the pseudo-glandular stage to generate airways with thousands of terminal branches. Additionally, epithelial cells start to differentiate and assume precise temporospatial placement [165, 166]. Next, lung development reaches the canalicular step, where cell cycle activity decreases, as well as rapid proliferation, so specialization of the airway epithelium can occur in the stalks. Epithelial cells start to express genes specific for the two epithelial cell types of the mature alveoli consisting of: alveolar type I (AT1) and alveolar type II (AT2) cells [167]. After the canalicular stage, the saccular stage takes place, where the canaliculi/terminal branches widen distally and start forming thin-walled epithelial sac clusters, which are the alveolar precursors. Simultaneously the formation of a vast capillary network is formed surrounding the alveolar precursors [164, 168, 169]. Finally, lung development reaches the alveolar stages of maturation, where full maturation of the alveoli occurs. As the alveoli expand, septae grow from the saccular walls to divide the alveoli, thereby increasing their surface area to provide better gas exchange. The capillary network surrounding the alveoli matures and is surrounded by specific gas exchange surfaces [167, 169, 170].

Types of cells residing in the lungs

The mammalian respiratory tract consists of epithelial cells and the trachea contains bands of smooth muscle cells, while in humans also cartilaginous rings are found that provide support and regulate airway flow. In humans the proximal respiratory tract and trachea are lined with pseudostratified epithelium, which consists of a single layer of epithelial cells that make contact with the basement membrane (**Figure 6**). Underneath the basal laminal surface are blood vessels, lymphatic vessels, fibroblasts, nerve cells, smooth muscle cell, as well as cartilage [169, 171].

The pseudostratified epithelium is composed of various cell types, such as goblet and secretory cells that secrete mucus to trap particles during breathing which acts as a first line of defence to protect the body from inhaling unwanted and potentially dangerous particles [172].

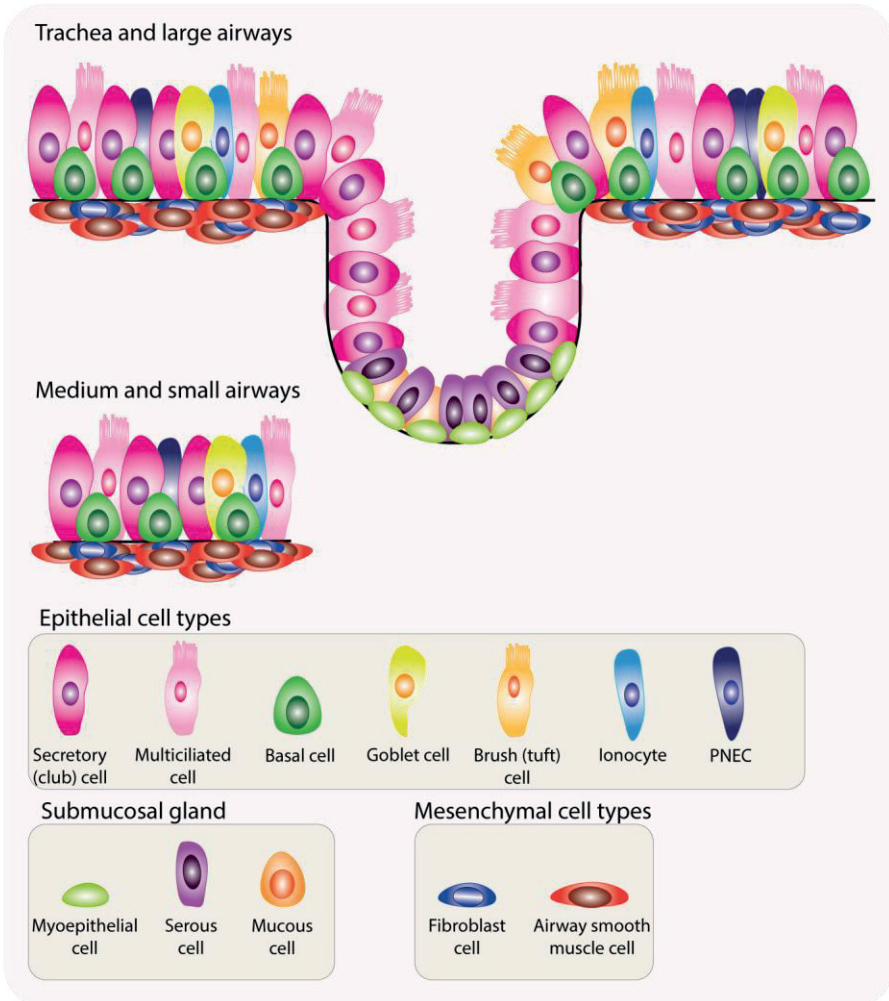

Figure 6. Cellular composition of human airways. A schematic overview of all the distinct cell types found in the human trachea and large, medium, and small airways.

Club cells, also known as Clara cells, are nonciliated, secretory cells that secrete various protective factors, including club cell secretory protein (CCSP) and surfactant proteins A, B, and D. Furthermore, they contribute to remove harmful

substances from the respiratory tract and have immunomodulatory functions [173]. Multiciliated cells are also found in the epithelial lining of the lung and their function is to remove the mucus together with the captured particles. Additionally basal stem/progenitor cells (BSCs) can be found that act as stem cells for the proximal airways and trachea, which are necessary for producing pseudostratified epithelial cells during injury and repair (**Figure 6**) [163].

In the lower respiratory tract and smaller airways pulmonary neuroendocrine cells (PNECs) can be found that prepare the microenvironment of the lungs to affect smooth muscle tone and regulate immune responses (**Figure 6**). Smooth muscle cells and mesenchymal cells such as fibroblasts line the airway tract to provide extracellular matrix (ECM) and secreted factors to modulate the epithelial cells and restrict or expand the airway branches [163, 169, 174].

The alveoli consist mainly of two types of epithelial cells, the AT1 and AT2 epithelial cells that are also known as pneumocytes. The flat, highly elongated AT1 cells are specialized gas exchange cells, since they are involved in more than 95% of the gas exchange surface area and work constitutively together with the underlying capillary blood vessel network surrounding the alveoli [175, 176]. The AT2 cells are cuboidal and produce surfactant that is secreted into the alveolar space to protect the lungs from collapsing and to reduce surface tension and consists of a mixture of proteins and lipids secreted into the alveolar space. Additionally, these cells are more common than AT1 cells and are stem cell like as they can self-renew and differentiate into AT1 cells [176, 177]. The interstitial fibroblasts that support alveolar structure by producing ECM and producing factors necessary for AT1 and AT2 proliferation and differentiation [163]. Moreover, there are alveolar macrophages that play an important role in the innate immune response and regulate homeostasis of surfactant production [178].

Lung cancer: epidemiology, prognosis, and treatment

The respiratory system is continuously exposed to small particles, pathogens, and toxins. Due to its large surface area, respiratory epithelial cells are constantly under pressure by outside factors that affect their proliferation and differentiation. When changes in respiratory cell proliferation and differentiation occur, it can contribute to acute and chronic lung injury, such as asthma, chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), and cancer [179].

In Sweden, lung cancer is the sixth most common type of cancer but is the leading cause of cancer-related death. The relative 5-year survival rate is 16.8% in men and slightly higher at 24% in women. Meanwhile, the 10-year survival rate is only 10.9% respectively 17.4%, due to lung cancer often being diagnosed at a late stage making therapeutic intervention and surgery difficult [180]. However, globally, lung cancer is the second most common type of cancer diagnosed in patients after

female breast cancer. Nonetheless, the 5-year survival rate is globally also between 10% and 20% in both men and women [181].

One of the major risk factors for developing lung cancer during our lifetime is smoking. By increasing the number of cigarettes a person smokes per day, that person also increases the risk of developing lung cancer later in life [182]. Lung cancer is also observed in never-smokers and living with a smoker attribute to lung cancer developed by passive smoking [183, 184]. Occupational exposure to carcinogens has long been described to be a major risk factor for the development of lung cancer. Agents such as asbestos and other hard metals in the industry and mining, radiation and radon exposure, inhaled chemical products, and exposure to outdoor pollution have all been described to contribute to the development of lung cancer [185]. Genetic factors due to a family history of lung cancer also increases the risk of developing lung cancer. For instance, people that carry the TP53 germline mutation are more likely to develop lung cancer, especially in combination with smoking [186].

One of the reasons why lung cancer is such a deadly form of cancer is due to patients being diagnosed in advanced stages of the disease. In most cases lung cancer is diagnosed in symptomatic individuals where the most common symptoms are coughing, fatigue, weight loss, dyspnea, and hemoptysis, which is coughing up blood that originates in the lungs or bronchial tubes. To identify and diagnose lung cancer, the initial diagnostic method is a CT scan followed by sputum analysis if the potential tumour resides in the upper respiratory tract, bronchoscopy, fine-needle biopsy of a lung lesion and lymph node lesion [187].

The International Association for the Study of Lung Cancer (IASLC) has developed a classification system for staging of lung cancer. This classification is based on the TNM-staging system, where T indicates size and extent of the primary tumour, N describes the involvement of regional lymph nodes, and M describes the absence or presence of metastasis [188]. For T1 lung cancer the tumours need to be <3 cm and T1 is further subdivided in T1a-c based on tumour size. In the T2 stage of lung cancer, tumours need to be between <3 and ≤5 cm in size and they have visceral pleural involvement, as well as the main bronchus without touching the carina. The T3 staging, tumours need to be <5 and ≤7 cm in size, as well as involving parietal pleura, chest wall, or have separate nodules within the same lung lobule as the primary tumour. The most advanced stage is T4, where tumours need to be >7 cm in size, involve the diaphragm, recurrent laryngeal nerve, great vessels, separate nodules in the same lung but different lobes as the primary tumour, or be in the visceral pericardium [189-191]. Furthermore, staging can be further and more detailed classified when including lymph node status, where no lymph node involvement obviously gives a better prognosis compared to peripheral lymph node metastasis. The M classification is based on the presence or absence of metastasis, where M0 means no distal metastasis, meanwhile M1c indicates multiple extrathoracic metastases, which results in the worst prognosis [189]. Symptoms and

treatment of metastatic disease depends on the site of metastases, of which the most common are the liver, brain, adrenal glands, and bones [187].

Lung cancer is a heterogeneous disease that can histologically be divided into two main variants: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The most common type is NSCLC that comprises approximately 85% of all cases, meanwhile SCLC accounts for the remaining 15% of lung cancer cases. Based on pathology and histological features, NSCLC can be subdivided into three categories: adenocarcinoma (AC), squamous-cell carcinoma (SqCC), and large-cell carcinoma (LCC) [192]. AC is the most common type of NSCLC and encompasses almost 50% of all cases. It has been found that AC most likely arises in the lung parenchyma epithelium from alveolar type 2 cells or Clara cells [193, 194].

In lung ACs a high rate of genetic alterations, such as somatic mutations and genomic rearrangements can be detected and with new technology enabling high throughput screening of many samples, the most frequent oncogenic and tumour-suppressor genes have been found. Molecular profiling analyses of tumour and matched normal material from 230 AC patients collected by the TCGA was performed to identify the most common genetic mutations. The top five most mutated genes were: *TP53* (46%), *KRAS* (33%), *KEAP1* (17%), *STK11* (17%), and *EGFR* (14%) [195]. KRAS mutations most frequently occur in codon 12, of which the most common variants are *G12V* and *G12D*. The mutational partners for KRAS include TP53, STK11, and CDKN2A [196, 197]. KRAS is a potent driver gene for the development of lung cancer [198, 199]. It has been shown that oncogenic KRAS rapidly produced tumours in mice and resulted in self-renewal and proliferation of AT2 cells without triggering AT2 differentiation in AT1 or Clara cells [200]. It is noteworthy to mention that epidermal growth factor receptor (EGFR) under normal circumstances plays an important role in cell proliferation, cell migration, and cell survival and in NSCLC patients mutated EGFR is found in 10% of American patients [201].

NSCLC treatment depends on the tumour stage, mutational status of the patient, and the patient's condition. Primary treatment plans for resectable and early stage NSCLC is surgery, resulting in the best long-term survival outcome in these patients. In patients where surgery is not feasible, radiotherapy can be used to decrease the size of the tumours [188]. Patients with resected tumours have a chance of tumour relapse and are often treated with adjuvant platinum-based chemotherapy to reduce the risk of relapse and increase the 5-year survival rate [202]. However, almost 40% of NSCLC patients are diagnosed at an advanced stage (stage III and IV), including locally advanced disease or distant metastatic disease where surgical intervention is not a treatment option. In these cases, chemoradiotherapy (chemotherapy and radiation), followed by immunotherapy is advised. Chemotherapeutic treatment for these advanced stage patients consists of a regimen of platinum-based chemotherapy, usually cisplatin or carboplatin in combination with paclitaxel, docetaxel, gemcitabine, or vinorelbine [203, 204]. Patients with

advanced NSCLC might benefit from personalized medicine using targeted therapy if they present any genetic alterations, such as *EGFR, ALK,* or *ROS1* mutations. The most successful targeted therapeutic agent has been against EGFR mutations, where patients harbouring this mutation will receive tyrosine kinase inhibitor (TKI) treatment against EGFR [205]. Osimertinib is a third generation TKI targeting EGFR and is the preferred treatment in patients with metastatic NSCLC bearing an EGFR mutation, since it has shown to improve the overall survival of these patients [206, 207]. Immunotherapy has been shown to be beneficial in patients with locally advanced lung cancer. Programmed death ligand-1 (PD-L1) inhibitor immunotherapy either alone or in combination with standard treatment has become standard of care [206]. Pembrolizumab is the first-line immunotherapeutic agent in NSCLC patients expressing PD-L1, where it has shown to improve progression-free survival and overall survival [208].

The role of the Wnt signalling pathway in lung cancer

The Wnt signalling pathway is highly involved in many biological processes and it is known that aberrant signalling plays an important role in lung disease processes, such as idiopathic pulmonary fibrosis, lung cancer, and COPD [209]. It has been shown that activation of β-catenin alone cannot contribute to lung cancer development, however, in combination with constitutively active KRAS, it causes an increase in tumour number and size in mice [210, 211]. Non-small cell lung cancer cell lines constitutively expressing Wnt signalling by increased β-catenin levels had upregulated TCF reporter activity and increased cell proliferation. When stimulating NSCLC cell lines with Wnt antagonists Frizzled related protein 1 (FRP1) and DKK1, downregulation of β-catenin and TCF was observed, resulting in decreased c-Myc levels [212]. Exposing human primary or immortalized bronchial epithelial cells to cigarette smoke extract resulted in increased cell proliferation, anchorage-independent growth, and the formation of tumours in nude mice. Additionally, data showed that Wnt/β-catenin signalling played a role in initiation, proliferation, and survival of these bronchial epithelial cells [213].

Even though the role of several Wnt signalling molecules in lung cancer development and progression has been described and established in the literature, the specific role of NLK is still debated. Previously, we have engineered a mouse model deficient of NLK and found that these animals had a very short lifespan following birth due to the mice becoming cyanotic. Upon further examination we found that these mice presented defects in lung development, due to hyperthickening of lung mesenchyme and compressed alveoli [214]. Our data indicates that NLK plays an important role in the normal development and maintenance of lung homeostasis. Furthermore, it has been shown that NLK expression is reduced in NSCLC tissue samples compared to non-cancerous tissue. Upon overexpression of NLK, cell migration and invasion were inhibited in lung

cancer cell line A549 [215]. In agreement with the aforementioned study, it was shown that knock-down of NLK resulted in increased cell proliferation due to shorter S-to-G_2/M phase cell cycle transition [216]. These studies indicate that NLK functions as a tumour-suppressor in the development of NSCLC. Contradicting these papers, it has been shown that suppression of NLK caused reduced cell proliferation and tumour sphere formation [217]. Based on these conflicting results, there is an interesting opportunity to elucidate the function of NLK in NSCLC.

Colorectal cancer: epidemiology, prognosis, and treatment

Worldwide, colorectal cancer (CRC) is the third most common type of cancer and is the second most deadly type of cancer after lung cancer. The overall 5-year survival rate for colorectal cancer is approximately 65%, which decreases in higher stage colorectal cancer. CRC is more prevalent in developing countries, where the highest incidence rate are found in European countries, followed by Australia/New Zealand, and North America [181]. Increasing improvements in CRC treatment and screening has led to decreased CRC mortality. This includes new and improved screening tests, removal of polyps, early detection methods, such as colonoscopy, faecal occult blood testing and faecal immunochemistry [218].

A major risk factor to developing colorectal cancer is attributed to a "Westernized lifestyle", which indicates obesity, inactive behaviour, and a high-meat, high-calorie, high-fat diet, often in combination with excessive alcohol consumption [219]. Opposite of the high-fat diet, eating fish and ingesting fish oil has been shown to reduce the incidence of colorectal tumours [220]. Besides lifestyle risk factors, underlying disease also increases the risk of developing CRC over time. It has been shown that patients with chronic inflammatory bowel disease are at higher risk of developing CRC due to chronic inflammation caused by cytokines, increased blood flow, and free radicals predispose the tissue towards tumorigenesis [218, 221].

Mutations of the APC gene is seen in both non-hereditary cases of CRC as well as the cause of FAP due to a germline mutation in the APC gene [222]. As it was mentioned earlier, APC is well-known as a negative regulator of the Wnt signalling pathway. APC is part of the destruction complex that inhibits and degrades β-catenin, thereby inhibiting target gene transcription integral for regulating cell proliferation, migration, and survival [44].

Usually, the development of CRC starts with the proliferation of non-cancerous mucosal epithelial cells that line the large intestine. The uncontrolled proliferation of these epithelial cells grow into a polyp that will gradually grow for many years before turning cancerous [218]. There are two pathways that describe the process of CRC development: the traditional adenoma-carcinoma pathway (70-90% of all CRC cases) and the serrated neoplasia pathway (10-20% of all CRC cases) [223, 224]. Genetic hits play an important role in the development and progression of CRC and at least four sequential genetic changes must occur for this disease to progress. The initial step to forming an adenoma comes through the earliest genetic hit that occurs in the APC gene, resulting in the loss of APC, which seems to be required to initiate clonal evolution within the intestinal epithelium [225]. Next, the acquisition of oncogenic KRAS results in adenoma growth and progression and this mutation can be found in approximately 50% of all sporadic CRCs. The combination of loss of APC and gain of oncogenic KRAS results in a favourable

proliferative environment for cells residing in the adenomas [226]. Subsequent loss of chromosome q18 with SMAD4 that functions downstream of TGF-β, as well as mutations in TP53 result in clonal expansion and malignant transformation [226, 227].

When CRCs remain undetected to the point that they grow into the colon intestinal wall or rectum and can permeate the tissue to reach the blood vessels or lymphatic system, they are able to metastasize. CRC *in situ* indicates that polyps have not grown into the colon or rectal wall, meanwhile local CRCs have grown into the wall but not past it. Once tumours invade regional lymph node the CRCs are referred to as regional cancer, while spreading to distant organs is called metastatic disease [218]. The metastatic process is a well-known stepwise procedure where cells invade locally through the ECM and stromal cells before they invade blood vessels. Cancer cells that survive travelling through the blood stream need to extravasate into the parenchyma of distant organs, where they must settle and survive a new microenvironment. At the distant site, cancer cells proliferate and create micro-metastases before expanding in macroscopic tumours [228, 229].

Despite new and approved screening programs, still approximately 25% of all CRC patients are diagnosed at advanced stage metastatic CRC. Local treatment is only possible when the disease is detected at an early stage, where polyps still can be removed by endoscopy. If the tumour is deemed resectable, surgical resection of the tumour will occur using laparoscopy [224]. Chemotherapy is standard of care in advanced stages and metastatic CRC where fluoropyrimidine-based treatment is most commonly used with or without the combination of oxaliplatin and capecitabine [230]. Depending on the mutational status of CRC patients, targeted therapy might be considered as a treatment option [231, 232]. In 2004, the Food and Drug Administration (FDA) approved the first targeted therapy drug for the treatment of CRC was cetuximab, which is an EGFR inhibitor [232]. Furthermore, VEGF inhibitor bevacizumab is approved for treatment of metastatic CRC and in combination with chemotherapy has been shown to increase progression-free survival but not overall survival [233]. Furthermore, in metastatic CRC immune checkpoint inhibitors are added to the treatment strategy of patients. However, it has been found that only a small proportion of CRC patients respond to this type of treatment [234].

Function of JNK proteins in colorectal cancer

Evidence has shown that the JNK signalling pathway plays an important role in cellular processes, including proliferation, migration, regulation of cell death, and cellular transformation and that aberrant signalling can lead to disease development including cardiac and neurological disorders, and cancer [235, 236]. The role of the JNK signalling pathway in CRC has been well-studied where often activating mutations of this pathway can be found. In hypoxic conditions, JNK signalling was

found to be activated in colorectal cancer cell lines and this activation contributed to increased cell survival. However, inhibition with CC-401 caused increased sensitivity to oxaliplatin under normal and hypoxic cellular conditions [237]. Furthermore, it has been shown that Taurine (an endogenous cell protective agent) can positively regulate the expression of phosphorylated JNK in CRC cells, which led to increased cell death and inhibition of JNK decreased apoptosis [238]. It has been shown that JNK can affect apoptotic signalling by inhibiting the activity of Bcl-2 and Bcl-XL, which are anti-apoptotic proteins, while promoting activation of Bim, Puma, and Bax, resulting in apoptosis [239].

Even though screening for CRC has become much better over recent years, still approximately one fourth of all patients are diagnosed with advanced stage metastatic CRC [218, 219]. Epithelial-to-mesenchymal transition (EMT) is an important step for metastatic tumour progression. Inhibition of JNK promoted pro-epithelial morphological changes in CRC cells under hypoxic conditions. Furthermore, it was shown that JNK inhibition resulted in downregulated cell migration, upregulated E-cadherin, downregulated formation of filopodia and lamellipodia, and inhibition of EMT transcription factors Twist1 and Snail [240].

Treatment strategies of CRC include surgery, chemotherapy, targeted therapy, radiotherapy, and immunotherapy. Thanks to targeted therapies and immune checkpoint inhibitors, new treatment regimens have been established for patients with unusual genetic alterations and metastatic disease. One of the reasons the survival rate of CRC patients with advanced stage cancer is low is due to therapy resistance [231, 241]. PD-L1 expression is found on multiple types of tumour cells, including CRC cells, and it was shown that suppression of PD-L1 contributes to drug resistance in multiple CRC cell lines, as well as in two patient-derived CRC organoids. Furthermore, it was found that drug resistance in PD-L1 deficient cells was a result of increased JNK activity due to degradation of CYLD mRNA by EXOSC10 [242].

The present investigation

Overview and aims

Protein phosphorylation is a common and reversible post-translation modification that has an important function in regulating both developmental and cellular processes [8]. Protein kinases are evolutionary conserved in eukaryotes that alter substrate function or cellular location by adding a phosphate group to a serine, threonine, or tyrosine residue, which in turn can be removed by phosphatases. The catalytic activity of protein kinases is tightly regulated since protein kinases are involved in the regulation and homeostasis of many different cellular pathways. Dysregulation of the function of kinases can results in a diseased state, such as inflammatory disorders and cancer [243-245].

The overall aim of this thesis was to gain understanding of the role protein kinases play under normal, developmental, and diseased states. The results of this thesis are presented in four papers, and these will be summarized and discussed below.

The specific aims of this thesis were:

I. To analyse whether the inhibitory function of NLK on the Wnt signalling pathway is navigated through other regulatory proteins.

II. To investigate the function that NLK has in early T-cell development.

III. To elucidate whether NLK functions as a tumour-suppressor or an oncogene in non-small cell lung cancer.

IV. To examine the effect of JNK1 inhibition on the metastatic capacity of colon cancer cells.

Paper I: NLK-mediated phosphorylation of HDAC1 negatively regulates Wnt signaling

The canonical Wnt signalling pathway is highly involved in many cellular processes, including cell homeostasis, cell proliferation, differentiation, cell survival, and migration. Activation of the Wnt signalling pathway results in accumulation of β-catenin in the cytosol, followed by its nuclear translocation and binding to the TCF/LEF complex that in turn activate target gene transcription [44, 246]. NLK is an evolutionary conserved MAPK-related kinase and a well-known negative regulator of the canonical Wnt signalling pathway by directly phosphorylating the TCF/LEF complex, thereby inhibiting their DNA binding capacity and subsequent transcriptional activity [82].

Previously, we engineered NLK knock-out mice that showed a lethal phenotype where new-born mice died within 12-36 hours after birth [214]. Due to the lethality of this knock-out model, to examine the function of NLK using *in vivo* studies are limited. In **paper I** we isolated mouse embryonic fibroblast (MEF) cells with ($NLK^{+/+}$) or without ($NLK^{-/-}$) NLK expression to characterize the role of NLK on Wnt signalling in these cells. First, we studied cell morphology but could not see any differences between $NLK^{+/+}$ or $NLK^{-/-}$ MEF cells. Next, we performed functional assays investigating the effect of deleted NLK on cell adhesion, cell invasion, and cell migration and could not detect any differences between $NLK^{-/-}$ and $NLK^{+/+}$ cells.

Furthermore, NLK has been implicated to play a role in the regulation of apoptosis, and it has been shown in prostate cancer cells that NLK induces cell death and inhibits AR signalling [96]. Additionally, overexpression of NLK in breast cancer cells resulted in decreased cell proliferation and increased cell death [93]. However, we could not detect any differences in number of apoptotic or necrotic cells between $NLK^{-/-}$ and $NLK^{+/+}$ cells. Neither after inducing apoptosis using TNF-α or doxorubicin, thus indicating that deletion of NLK does not affect cell survival in MEF cells.

Next, we investigated whether NLK influenced cell proliferation using multiple methods, including cell counting and WST-1 cell proliferation assay and found that NLK-deficient cells proliferated at a higher rate compared to $NLK^{+/+}$ cells. After establishing that knock-out of NLK results in increased proliferation, we analysed cyclin D1 levels in these cells and found it increased in $NLK^{-/-}$ cells. Previously, it was shown in HCC cells that downregulation of NLK resulted in decreased cyclin D1 levels [92]. However, knock-down of NLK in NSCLC cell line A549 promoted cell proliferation [216]. These studies together with our data indicate that the effects of NLK on cellular processes are highly dependent in which cell type NLK is expressed.

Previously it has been shown that NLK can directly interact with TCF4 and LEF1, thereby regulating their transcriptional activity [83]. This prompted us to investigate whether the reduced proliferation rate in NLK knock-out cells was regulated through the Wnt signalling pathway and more specifically through regulation of LEF1. NLK deficient cells showed increased TOPFlash reporter assay levels, which is indicative of TCF/LEF activity. Furthermore, elevated levels of interactive LEF1 and β-catenin could be observed in $NLK^{-/-}$ cells compared to $NLK^{+/+}$ cells. One way for NLK to negatively regulate the Wnt signalling pathway is by direct phosphorylation of LEF1 on residue Thr-155 or Ser-166 [84]. Next, we analysed whether phosphorylation of LEF1 was altered in NLK deficient cells but could not observe any differences in phosphorylated LEF1 compared to wild-type NLK cells. These data indicate that another target of NLK must be included in this signalling cascade in order to affect cell proliferation in NLK knock-out cells.

Wnt target gene expression requires the balanced function of repressive and active transcription factor complexes. Evidence has shown that TCF/LEF can be repressors of Wnt signalling when bound to Groucho/TLE. However, β-catenin directly competes with TLE for binding to TCF/LEF and upon replacing TLE, Wnt signalling becomes activated [247]. Moreover, the transcriptional repression of Wnt signalling by LEF1 requires further interaction with HDAC1 [248]. We could show that endogenous immunoprecipitation of LEF1 precipitated HDAC1 in wild-type NLK but not knock-out NLK cells. Previously it has been shown that HDAC1 becomes post-translationally modified through phosphorylation [249], which we could confirm in wild-type NLK cells by performing an *in vitro* kinase assay. Additionally, transfection of $NLK^{-/-}$ cells with full-length or a catalytically inactive mutant of NLK showed HDAC1 phosphorylation in full-length expressing cells, confirming that NLK activity is required for phosphorylation of HDAC1. Earlier it has been shown that HDAC1 is phosphorylated at two serine residues: Ser-421 and Ser-423 and that these sites are necessary for HDAC1 enzymatic activity [250]. To identify phosphorylation sites in NLK expressing cells, we performed mass spectrometry analysis using *in vitro* phosphorylation of HDAC1. One phosphorylated serine peptide corresponding to Ser-421 or Ser-423 was found. The mass spectrometry results were confirmed by observing decreased levels of phosphorylated HDAC1 (Ser-421 or Ser-423) in knock-out cells compared to wild-type cells. These data confirm that Ser-421 or Ser-423 are important phosphorylation sites to regulate HDAC1 activity and that NLK can affect HDAC1 phosphorylation status.

Upon confirming that negative regulation of the Wnt signalling pathway by NLK is through HDAC1, multiple luciferase reporter assays were performed analysing the activity of TCF/LEF. Here we found that β-catenin and LEF1 showed increased luciferase activity, while addition of the combination of NLK and HDAC1 reduced the TCF/LEF1 reporter activity. Furthermore, the inhibitory effect of NLK and HDAC1 required the activation of NLK, since mutated NLK did not affect reporter

activity. When analysing whether this inhibitory effect on TCF/LEF activity is specific to HDAC1, cells were co-transfected with HDAC5, HDAC6, and HDAC7, and found that indeed only HDAC1 together with NLK could reduce the activity in the reporter assay. Taken together, our study has shown a novel signalling cascade, where the presence of NLK results in phosphorylated HDAC1 that in turn can reduce LEF1 and cell proliferation-mediated transcription (**Figure 7**). Meanwhile, in the absence of NLK, cell proliferation is increased since the inhibitory downstream cascade of NLK is not activated.

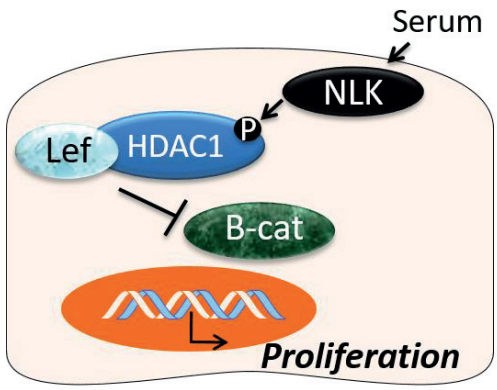

Figure 7. LEF1 activity repressed by NLK and HDAC1. Model of how activation of NLK results in phosphorylation of HDAC1 that in turn can bind to LEF1, thereby inhibiting target gene transcription.

Paper II: Deletion of Nemo-like Kinase in T Cells Reduces Single-Positive CD8$^+$ Thymocyte Population

T-cell development follows a precise sequence of events that is well-characterized by the expression of specific extracellular surface markers [251]. The most immature subset of thymocytes consists of CD4$^-$CD8$^-$ double negative cells that can be divided into 4 subsets (DN1-DN4). Once DN cells reach the DN4 stage they differentiate into CD4$^+$CD8$^+$ double positive (DP) thymocytes. Then double positive thymocytes must go through positive and negative selection before they commit to differentiating in either CD4$^+$ SP or CD8$^+$ SP thymocytes. Finally, CD4$^+$ and CD8$^+$ SP thymocytes migrate out to of the thymus to peripheral lymphoid organs [140].

The Wnt signalling pathway plays an important role during all aspects of T-cell development, from the early stages of T-lineage specification, through all

thymocyte developmental stages, to peripheral T-cell differentiation [155, 252]. Even though the Wnt signalling is such an essential part of developing T-cells, the role of NLK in early T-cell development has so far not yet been addressed. For the purpose of studying if NLK contributes to T-cell development, in **paper II** we crossed *LoxP*-flanked NLK mice [214] with Lck-Cre mice to specifically delete NLK in early T-cells.

Conditional deletion of NLK has previously been shown to have crucial consequences. Depending on genetic background, deletion of NLK was shown to result in birth of mice in the third trimester of pregnancy, or grow to develop neurological deficiencies, as well as abnormal bone marrow stromal cells [253]. Furthermore, in our conditional knock-out mouse model of NLK, pups died within 12-36 hours after birth due to a severe lung phenotype resulting in cyanosis and abnormal alveoli development [214]. Next, we examined whether T-cell specific deletion of NLK affected the general health and development of the mice. We could not observe any differences in animal weight, whole blood counts, thymic size, weight, histological organization, or splenic size and weight when comparing $NLK^{fl/fl}$ animals. Previously reported data showed structurally normal thymi in E15.5 $NLK^{fl/fl}$ embryos [159]. Thus, together with our results confirming that NLK is not involved in thymus development and growth.

When examining the various thymocyte compartments in $NLK^{+/+}$ and $NLK^{fl/fl}$ mice, we found a 50% decrease in $CD8^+$ SP thymocytes in $NLK^{fl/fl}$ mice compared to their control counterpart, without observing any differences in $CD4^+$ SP thymocytes. Additionally, no differences in $CD4^+$ SP and $CD8^+$ SP splenocytes or peripheral blood cells was observed between $NLK^{+/+}$ and $NLK^{fl/fl}$ mice. Upon further examination of $CD4^+$ SP and $CD8^+$ SP T-cells in the spleen and peripheral blood regarding effector versus naïve cells, no differences were found between experimental groups.

T-cells must proceed through various selection processes while differentiating to become fully mature peripheral T-cells. During positive selection, DP thymocyte start expressing TCRβ to generate a functional TCR to differentiate into $CD4^+$ SP and $CD8^+$ SP T-cells. Thymocytes carrying a moderate affinity for self-peptide TCR will receive a survival signal and differentiate into mature T-cells [254]. We examined TCRβ expression in $CD4^+$ SP and $CD8^+$ SP thymocytes and could not find any differences between $NLK^{+/+}$ and $NLK^{fl/fl}$ mice. CD5 is a marker for T-cell activation and a negative regulator of TCR signalling pathways [255]. When examining the expression of CD5 in $CD4^+$ SP and $CD8^+$ SP thymocytes no differences were observed between groups. Neither TCR signalling or activation of T-cells by CD5 were affected by deletion of NLK.

In paper I we could show that NLK could directly affect phosphorylation levels of HDAC1 [85]. Furthermore, previous research has shown that NLK negatively regulates Wnt signalling by phosphorylation TCF and LEF [83, 84]. This prompted

us to investigate whether phosphorylated HDAC1 and LEF1 affects $CD8^+$ SP NLK-deficient thymocytes. Levels of both p-LEF1 and p-HDAC1 were reduced in $CD8^+$ SP $NLK^{fl/fl}$ cells compared to $CD8^+$ SP $NLK^{+/+}$ cells, where the greatest effect was seen on p-HDAC1. These data suggest that phosphorylation levels of HDAC1 and LEF1 in $CD8^+$ SP thymocytes are regulated by NLK, confirming our previously generated data. The function of HDAC1 and HDAC2 during late T-cell development was shown to be important for maintaining CD4 lineage integrity through repression of Runx-Cbfβ, since deletion of HDAC1/2 resulted in MHC class II $CD4^+$ helper T-cells expressing CD8 lineage genes such as *Cd8a* [256]. Furthermore, it was found that TCF1 and LEF1 have intrinsic HDAC activity, which is necessary for regulating $CD8^+$ lineage integrity by repressing $CD4^+$ lineage specific genes, such as *Cd4* and *Foxp3* [257].

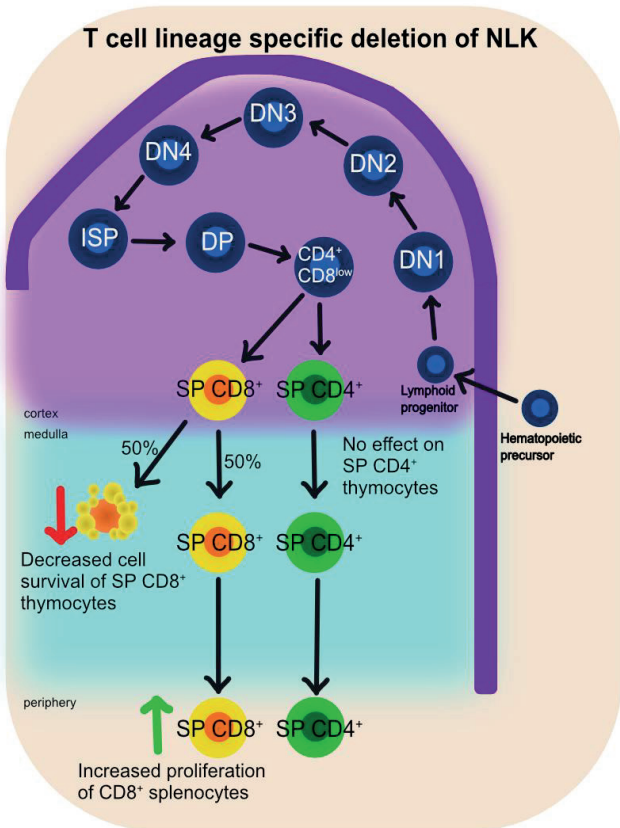

Figure 8. T-cell lineage specific deletion of NLK. Deletion of NLK in early thymocytes results in decreased cell survival of $CD8^+$ thymocytes without affecting the $CD4^+$ SP thymocyte population. Furthermore, NLK-deficient peripheral $CD8^+$ T-cells show increased proliferation to maintain the $CD8^+$ splenocyte pool.

To understand the reason behind the decrease in $CD8^+$ SP thymocytes, we examined whether differentiation from DP-to-SP thymocytes was affected by co-culturing DP thymocytes on OP9DL1 murine stromal cells. After stimulating these cells with various cytokines to direct differentiation into $CD8^+$ SP thymocytes, no differences in differentiation potential were observed between $NLK^{+/+}$ and $NLK^{fl/fl}$ mice. However, when investigating whether cell death caused selective reduction in $CD8^+$ SP $NLK^{fl/fl}$ thymocytes, stained DP, $CD4^+CD8^{low}$, $CD4^+$ SP, and $CD8^+$ SP thymocytes were analysed for Annexin V and DAPI expression. We found an increase in cell death in NLK-deficient $CD8^+$ SP thymocytes compared to NLK wild-type $CD8^+$ SP thymocytes, without observing any effect on the other thymocyte subpopulations. The role NLK plays in cell survival is not well understood, but one recent study could show that TCF1 deletion results in reduced DP T-cell survival due to dysregulated Bcl-XL [258].

Next, we wanted to examine what compensatory mechanism was active in splenic $CD8^+$ SP cells since deletion of NLK resulted in reduced $CD8^+$ SP thymocytes but not splenocytes. $CD8^+$ splenocytes were isolated, CFSE stained, and grown *in vitro* for 24-96 hours before analysing their proliferative capacity using flow cytometry. We could observe increased proliferation after 72 and 96 hours in $NLK^{fl/fl}$ $CD8^+$ splenocytes compared to $NLK^{+/+}$ control cells.

Taken together, in **paper II** we have shown a new light on the function of NLK in developing T-cells. Deletion of NLK resulted in decreased phosphorylation of HDAC1 and LEF1, which in turn results in increased cell death of $CD8^+$ SP thymocytes and induction of compensatory mechanisms to increase the peripheral splenic $CD8^+$ T-cell pool by activating cell proliferation (**Figure 8**).

Paper III: Deletion of Nemo-like kinase contributes to increased tumour development in non-small cell lung cancer (*manuscript*)

Lung cancer is a disease arising from the epithelium lining the respiratory tract and is the second most common diagnosed type of cancer worldwide. Lung cancer is the leading cause of cancer-related deaths and the 5-year survival of patients is estimated to be between 10-20%, since many patients are diagnosed at late stages [181]. Almost 80% of all lung cancer can be ascribed to non-small cell lung cancer (NSCLC), of which adenocarcinoma, accounting for approximately 40% of all NSCLCs is the most frequent subtype [192]. Aberrant Wnt signalling has been shown to be a major contributor to various types of disease development, including lung cancer [51, 259, 260].

The literature is divided regarding the role of NLK in NSCLC, where some have shown that NLK functions as a tumour-suppressor [215, 216], and others that NLK acts as an oncogene [217]. In **paper III** we set out to investigate whether NLK is an oncogene or tumour-suppressor in NSCLC. Previously we have shown that NLK plays a role in lung development in mice, since deletion of NLK resulted in death directly after birth [214]. In the absence of NLK VEGF expression was elevated in isolated pulmonary epithelial cells, promoting cell proliferation of pulmonary epithelial and endothelial cells leading to hyperthickening of lung vasculature that caused compressed alveoli [214].

There are various ways to study the function of specific genes *in vivo* for example by using engineered transgenic animals where the gene of interest is expressed under the control of a tissue-specific promoter. Furthermore, an inducible system can be created by combining Cre recombinase activity with the oestrogen receptor (ER) binding domain (Cre-ER^{T2}), where Cre can be activated by injecting the animals with tamoxifen [261]. In this study we crossed Scgb1-Cre mice with NLK-floxed mice to create NLK specific deletion in Clara cells in the lung. Additionally, it is well established that KRAS and TP53 are frequently mutated in NSCLC [194], and combination of constitutively active KRAS and loss of function TP53 results in lung tumour development [199]. By creating combinations of this model with or without NLK we proceeded to elucidate the role NLK plays in lung cancer development. The Wnt signalling pathway depends completely on the functional activity of β-catenin [44, 246], however its role in NSCLC in combination with NLK has not yet been established. We added floxed-β-catenin (Ctnnb1) mice, which upon Cre recombination will produce constitutively active β-catenin [262], to our study to create various combinations of transgenic animal models to examine the role of Wnt signalling and NLK in NSCLC development.

Injection with tamoxifen on 4 consecutive days results in deleted NLK, oncogenic KRAS, inactivated p53, and stabilized β-catenin in Clara cells. To monitor the health status of our animals, they were weight regularly and kept under observation during the experimental time-frame of 6 months post-injections. No differences in weight over time was observed in any of the experimental and their control groups, thus indicating that deletion of NLK does not affect the overall health of these animals. Which is in stark contrast compared to previous observations where animals had lethal phenotypes when NLK was conditionally deleted [214, 253]. Furthermore, no differences in weight upon sacrifice was observed. However, when weighing the lungs after sacrifice, a significant difference was found between $Ctnnb1^{fl/fl}NLK^{+/+}$ (control) mice and $Ctnnb1^{fl/fl}NLK^{fl/fl}$ mice, where $Ctnnb1^{fl/fl}NLK^{+/+}$ control animals harboured heavier lungs.

Once lungs were resected, we analysed the lungs for macrotumour formation (i.e. tumours visible to the eye without dissecting the tissue). We found no significant differences in macrotumour numbers between animal groups but a tendency to have elevated numbers of macrotumours in $NLK^{fl/fl}$ animal groups. It is important to

mention that this data is still preliminary, and more animals need to be added to each group before final statements and conclusions can be drawn.

Next, H&E staining of lung sections were prepared to analyse the number of microtumours visible in each section. All groups reveal tumour growth except for $Ctnnb1^{fl/fl}NLK^{+/+}$ animals, which confirms previous data stating that stabilized β-catenin under the Clara cell promoter does not develop lung tumours over time in mice [210, 263]. A significant increase in microtumours was found in $Ctnnb1^{fl/fl}NLK^{fl/fl}$ mice compared to control mice. Although not significant, the same trend in increased number of microtumours could be observed in the other groups expressing $NLK^{fl/fl}$ compared to their control counterparts. Predictably, we found the average tumour size to be increased in $Ctnnb1^{fl/fl}NLK^{fl/fl}$ mice compared to $Ctnnb1^{fl/fl}NLK^{+/+}$ mice, however this was not observed in any of the other groups of animals. In summary, our data suggest that NLK has a protective role in the development of NSCLC.

Finally, we wanted to examine what could contribute to the increased tumour growth observed in $NLK^{fl/fl}$ groups. We stained lung sections for Ki-67 to investigate whether cell proliferation was affected by the deletion of NLK. We found that $Ctnnb1^{fl/fl}NLK^{fl/fl}$ mice had a significant increase in Ki-67 positive cells compared to $Ctnnb1^{fl/fl}NLK^{+/+}$ mice. The tendency toward a higher percentage of Ki-67 positive cells was also found in the other groups bearing NLK deletions: $Ctnnb1^{fl/fl}KRAS^{fl/+}NLK^{fl/fl}$ mice and $Ctnnb1^{fl/fl}KRAS^{fl/+}$ $Trp53^{mut}NLK^{fl/fl}$ mice compared to their controls expressing $NLK^{+/+}$. The role of NLK on regulating cell proliferation has been shown and in breast cancer cells NLK expression resulted in decreased cell proliferation and instead induced cell death [93]. Furthermore, in gallbladder cancer cell lines, knock-down of NLK caused decreased cell proliferation and migration [264]. We have been able to show that deletion of NLK in MEF cells results in active Wnt signalling due to the lack of inhibitory phosphorylation of HDAC1, causing increased cell proliferation [85]. In NSCLC cell lines, knock-down of NLK promoted cell proliferation, as well as increased expression of c-Myc and CD1 [216].

Taken together, our data in **paper III** shows that mice deficient in NLK tend to develop more lung tumours, without affecting their overall health in comparison to NLK expressing animals. Furthermore, it seems that deletion of NLK results in active Wnt signalling causing an increase in cell proliferation. However, future research including more animals shall give us the answer to what role NLK plays in lung cancer development, as well as what the underlying mechanism is to the observed increased cell proliferation. Our results suggest that NLK has a protective function in lung homeostasis and might act as tumour-suppressor in the development of lung cancer.

Paper IV: Reversine inhibits Colon Carcinoma Cell Migration by Targeting JNK1

Colorectal cancer (CRC) is one of the most common types of cancer and the second most common sort of cancer-related deaths, due to patients being diagnosed at advanced and metastatic stages of the disease [218].

The JNK kinases are a subgroup of the MAPK family of proteins that have been shown to play a key role in cell proliferation, cell migration, cell differentiation, cell survival, and aberrant JNK signalling can result in disease development, such as cancer [18, 235]. Activating JNK mutations are often found in CRC, and this was found to result in increased cell survival of colorectal cancer cell lines [237]. Inhibition of JNK caused reduced cell migration and inhibition of EMT markers, such as Snail and Twist [240]. Even though treatment regimens are set depending on which stage of CRC a patient is diagnosed with, a major obstacle in treating patients with chemotherapy is multidrug resistance. Drug-resistant colon cancer cells were treated with JNK pathway inhibitor SP600125, which reduced the levels and transport function of ABCC1, thus indicating that JNK signalling contributes to drug resistance [265].

Previously, it was shown that mitotic kinase inhibitors Reversine (2-(4-morpholinoanilino)-6-cyclohexylaminopurine) and SP600125 (anthra[1,9-cd]pyrazol-6-(2H)-one) affect cell invasiveness and migration of soft tissue sarcoma cell lines [266]. Reversine, a small synthetic purine was first discovered as an agent capable of inducing myogenic lineage committed cells to dedifferentiate into multipotent mesenchymal progenitor cells that had the ability to proliferate and redifferentiate into bone and fat cells [267]. Later, studies demonstrated the effect of Reversine on cancer, such as in human lung cancer cells it was shown to cause cell death and growth inhibition [268]. In human breast cancer cells, Reversine was shown to cause growth inhibition through cell cycle arrest, polyploidy, and induction of cell death programs [269]. SP600125 is a reversible ATP-competitive inhibitor, an inhibitor of c-Jun phosphorylation, and an inhibitor of JNK1, JNK2, and JNK3 isoforms [270]. Since JNK has been shown to be able to contribute to CRC development, in this study we wanted to test whether inhibition of JNK signalling using Reversine and SP600125 could affect cell migration and metastatic potential.

In **paper IV** we used the colon cancer cell line RKO and tested whether SP600125 and Reversine could affect wound-healing, by performing a scratch test and using the two-dimensional Oris™ cell migration assay. Both assays showed that Reversine and SP600125 could inhibit cell migration. Furthermore, performing a Boyden chamber assay to study cell invasion, we could show that cells treated with Reversine or SP600125 had reduced cell invasion capacity compared to control cells. Our results are in line with data showing that inhibition of JNK1 resulted in

decreased cell migration in gastric cancer cells [270] and mouse hepatocellular carcinoma cells [271]. When analysing whether Reversine and SP600125 had any toxic effects on our cells using cell viability assays we could not detect any increased cell death parameters when compared to control treated cells. Additionally, treatment of RKO CRC cells with Reversine and SP600125 did not affect cell proliferation or cell cycle progression, since these cells continued to cycle, undergo polyploidy, and underwent histone H3 phosphorylation, which is a tell-tale sign of ongoing mitosis.

Next, we performed a kinase profiler screening to identify common target kinases of these small molecules and our selection criteria for inclusion was kinase inhibition of >90%. We found 22 kinases to be inhibited by both molecules and by using gene ontology analyses we wanted to select signalling pathways involved in migration. The kinase inhibited by both Reversine and SP600125 belonging to the JNK signalling pathway was found to have the highest enrichment score. We found JNK to be highly activated in RKO cells compared to non-cancerous colon mucosal epithelial cells. Treatment with Reversine and SP600125 completely inhibits the phosphorylation of JNK1/2. However, the effect on downstream targets of JNK signalling differed between the two treatments. Reversine negatively affected c-Fos and c-Jun expression, which are direct targets of JNK signalling [272], as well as Akt which also is a major contributor to cell migration [273, 274]. Meanwhile, SP600125 only affected c-Jun and c-Fos but not any of the other downstream components.

Once we established that Reversine and SP600125 could inhibit the JNK signalling pathway and its downstream components, we set out to identify which JNK isoform is affected by treatment with these small molecules. First, we treated cells with a specific JNK2 inhibitor and performed cell migration assays and analysed cell death but could not observe any differences when inhibiting JNK2 alone. To compliment this data we used small interfering RNA (siRNA) against JNK1/2 or JNK2 and performed a scratch assay to investigate the migratory capacity of our transduced cells. We found that knock-down of JNK1/2 inhibited cell migration but knock-down of JNK2 did not influence cell migration. Our data demonstrate that JNK1 but not JNK2 is important in cell migration in colon cancer cells, since inhibition of this pathway using small molecules and siRNA result in decreased cell migratory capacity. This is in line with other studies showing that deleted JNK in mouse embryonic fibroblasts or molecular inhibition of JNK by SP600125 in wild-type fibroblasts caused delayed cell migration [275]. Additionally, inhibition of JNK by SP600125 resulted both in decreased cell migration and proliferation in vascular smooth muscle cells [276].

To study the anti-metastatic capacity of JNK inhibition, we injected RKO cells intravenously into mice and treated them with intraperitoneal injections of SP600125. Upon sacrifice the number of tumour metastases were counted in the liver and lung of drug-treated and vehicle-treated animals. Although not significant,

we could observe that animals treated with SP600125 had fewer lung and liver metastasis compared to control treated animals. Thus, indicating that inhibition of JNK signalling can somewhat inhibit the development of tumour metastasis. In breast cancer cell lines, it has been shown that constitutively active JNK can cause increased cell migration and invasion [277].

In summary, in **paper IV** we have shown that JNK1 but not JNK2 is important for regulating cell migration and invasion of colon cancer cells and the effect could be inhibited by blocking JNK signalling using small molecules Reversine and SP600125. These data suggest that inhibition of the JNK signalling proteins could be an attractive new way to target cancer cells. However, since it seems that JNK1 and JNK2 function differently and have distinct downstream targets, more research must be done to fully understand the mechanism through which these proteins function.

Overall conclusions

- NLK can negatively regulate Wnt signalling by directly phosphorylating HDAC1 that in turn inhibits TCF/LEF target gene transcription.
- Deletion of NLK results in increased $CD8^+$ SP thymocyte cell death due to elevated Wnt signalling.
- NLK seems to have a protective tumour-suppressive role in the development of lung cancer.
- Inhibition of the JNK signalling pathway reduces metastatic spread, invasion, and migration of colon cancer cells.

Future perspectives

The overall aim of this thesis was to study the role of protein kinases in various biological aspects, including cell signalling, immune cell development, and cancer. To further increase the understanding of how protein kinases function, both *in vitro* and *in vivo* approaches were used. Additionally, the obtained information could be further used to establish novel prognostic tools and therapeutic strategies.

In paper I we were able to show that NLK can directly phosphorylate HDAC1 on Ser-421, which in turn inhibits the TCF/LEF complex from binding to DNA and induce target gene transcription. Deletion of NLK in MEF cells resulted in aberrant Wnt signalling causing an increase in cell proliferation due to fast S-to-G2/M phase cell cycle transition. Since we found a novel target of NLK that resulted in the negative regulation of the Wnt signalling pathway, it is of interest to examine other potential targets of NLK. Not only regarding Wnt signalling, but also considering other signalling pathways in which NLK is involved.

Using an in *in vivo* approach we wanted to investigate whether NLK could influence early T-cell development. Recently, it was shown that deletion of NLK affects the function of T_{regs} [161] and myeloid cells [162]. Additionally, we were able to show that deletion of NLK results in decreased $CD8^+$ SP thymocytes. Since the peripheral T-cell pool does not seem affected, it would be interesting to examine if deletion of NLK at later stages of T-cell development or in peripheral CD4/CD8 T-cells might affect their mature functions. In addition, it is necessary to elucidate the molecular mechanism behind the increased cell proliferation seen in NLK-deficient CD8 splenocytes. The role of NLK in immune cell development is not well studied resulting in wondering whether NLK is necessary for the development of other types of immune cells. It was reported that deletion of LEF1 results in defective pro-B cell proliferation and survival [278], creating an interesting question what the role of NLK might be in B-cell development.

We could preliminary show that deleted NLK results in increased lung tumour growth, as well as increased cell proliferation observed through Ki-67 staining. Currently, we are working on including more animals into the study to further examine the role NLK and Wnt/β-catenin signalling play in NSCLC development and progression. By staining sections with trichrome stain and apoptotic markers, we want to gain a greater understanding of how the lung architecture might be altered due to the genetic alterations that we induce in these animals.

Simultaneously, we are establishing 3D organoid cultures from our animal models, where we use lung tissue from healthy animals, as well as $Trp53^{mut}$, $KRAS^{fl/+}$, or $Ctnnb1^{fl/fl}$ animals alone or in combination. The use of patient-derived tumour organoids has shown to be a useful *in vitro* model that retains the original tumour features even over many passages and can be used for precision medicine [279]. These established organoids can contribute to clinical decisions and treatment strategies of the patients. Patients with NSCLC are often diagnosed at late stages of the disease, making treatment difficult and limited. The mutational landscape of lung cancer is getting increasingly more precise, in combination with advances in molecular and imaging techniques, personalized medicine becomes more common [280]. Establishing an organoid-based drug screening platform will lead to the identification of new molecular targeted drugs or combinations of drugs to treat patients with specific gene mutations.

Another emerging way of therapeutic strategy for lung cancer patients is by using immunotherapy to target T-cells and more specifically to treat patients with the immune checkpoint inhibitors PD-L1 and CTLA-4 [281]. Recently, it was shown that the most prominent type of T-cells in the lungs of patients with early stage NSCLC is the $CD4^+$ T cells, however, $CD8^+$ T-cells and T_{regs} were also found [282]. Since we have found that NLK affects $CD8^+$ SP thymocyte viability, it would be of interest to investigate what the immune cell distribution looks like in our lung cancer model. Furthermore, it would be exciting to examine whether deletion of NLK in peripheral T-cells affect the CD8 T-cell population and if so, how it affects tumour development. By using *in vitro* models of NLK-deficient $CD8^+$ T-cells in combination with our organoid system we could analyse whether these T-cells are still functional and affect cell growth *in vitro*.

We were able to show that inhibition of the JNK signalling pathway interferes with colon cancer cell migration and metastatic capacity. Since most colorectal cancer patients are diagnosed with late stage or advanced metastatic cancer, it is important to find or develop novel treatment therapies to treat late stage disease. Current chemo- and targeted therapies only increase the overall survival of patients by a few months and one of the major reasons for this is acquired drug resistance [241]. JNK signalling has also been shown to be involved in multi-drug resistance [265]. It is of importance to study whether the combination of JNK inhibitors and conventional chemotherapy have beneficial effects against late stage colorectal cancer. Furthermore, it is of interest to examine the cellular mechanisms underlying multidrug resistance.

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