From DEPARTMENT OF ONCOLOGY-PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

EPHRIN AND EPH-RECEPTOR GROWTH FACTOR SIGNALING IN NON SMALL CELL LUNG CANCER –IDENTIFICATION OF BIOMARKERS AND THERAPEUTIC TARGETS

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Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB 2016

ISBN 978-91-7676-285-1

Ephrin and Eph-receptor growth factor signaling in Non small cell lung cancer –identification of biomarkers and therapeutic targets
THESIS FOR DOCTORAL DEGREE (Ph.D.)

Dept of Oncology-Pathology,

Cancer Center Karolinska (CCK) Lecture Hall, R8:00,

Karolinska University Hospital, Stockholm

Friday, the 27th of May, 2016, at 09:00

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“The secret is here in the present. If you pay attention to the present, you can improve upon it. And, if you improve on the present, what comes later will also be better”.

The Alchemist by Paulo Coelho

To my family, husband and daughter

ABSTRACT

Non-small cell lung cancer (NSCLC) is the main subtype of lung cancer (LC) and unfortunately it responds very poorly to conventional chemo- and radiotherapy (RT). Moreover, NSCLC is often diagnosed at a stage where metastases are found and only for a limited number of NSCLC tumors targeted therapies can be used as their oncogenic drivers remains elusive. Thus there is a need of finding novel targets in NSCLC and this thesis focus around these topics. In **Paper I** the aim was to find novel RT targets in NSCLC by global genomic profiling. It was previously shown that NSCLC cells could be sensitized to RT by addition of the staurosporine analogue PKC 412. By global gene expression analyses on this NSCLC system we identified the Eph growth factor receptor ligand Ephrin B3 as a putative RT target as it was downregulated in the combined RT and PKC 412 treated NSCLC cells. Indeed, we demonstrated that Ephrin B3 ablation of NSCLC cells in combination with RT increased cellular senescence, mitotic catastrophe and apoptosis, inhibited the cell survival kinases Akt, MAPKERK, p38MAPK and decreased RT-induced G_2-arrest. Thus we in **Paper I** identified Ephrin B3 as a driver of RT resistance. In **Paper II** the aim was to investigate how Ephrin B3 influences the proliferative "signalome" of NSCLC cells. The phosphoproteome of NSCLC cells with or without Ephrin B3 expression was analyzed using a peptid-based approach in which SCX and TiO_2-based fractionation was used prior to identification by mass spectrometry and Ingenuity pathway analyses. Among the differentially phosphorylated proteins one candidate was the erythropoietin-producing hepatocellular receptor tyrosine kinase class A2 (EphA2), previously shown to control tumor cell signaling. We demonstrated that when Ephrin B3 expression was blocked in NSCLC cells EphA2 lost its phosphorylation on Ser897, a site previously reported to control migration in other tumor types. We also found that inhibition of Ephrin B3 expression suppressed Akt1 Ser129 phosphorylation which was reported to control EphA2 at Ser897. Thus our findings supported a hypothetical mechanism in which NSCLC cell survival signaling was mediated by an Ephrin B3 and EphA2 signaling circuit. In **Paper III** the purpose was to analyze how Ephrin B3 and its putative Ephs mediates their effects on migration and invasion of NSCLC of different histology *in vitro* as well as to reveal as to what extent these signaling components may be operative in NSCLC *in vivo*. Our study identified a novel function of Ephrin B3 where it similar to EphA2 controlled proliferation, migration and invasion of NSCLC cells *in vitro*. We showed for the first time that Ephrin B3 binds EphA2, EphA4, EphA5 and EphA3 indicating a master function of signaling of Ephrin B3 in NSCLC. Moreover, as EphA2 Ser897 and Akt Ser129 both were found in complex with Ephrin B3 in NSCLC cells and given that we observed p38MAPK and Src kinase in such complex our data further add onto how EphA2 may drive NSCLC proliferation and migration. In analyses of NSCLC clinical specimen Ephrin B3 was concomitantly expressed with EphA2 and its known ligand Ephrin A1 but did not correlate to poor survival. Several growth factor receptors, including EphA5, have been shown to control DNA damage response (DDR) signaling and hence to constitute RT sensitizing targets. In **Paper IV** we analyzed if EphA2, EphA4 and Ephrin B3 similarly influenced DDR components and hence could be used combat RT resistance. Our results showed that a combination of RT and ablation of EphA2, EphA4 or Ephrin B3 reduced proliferation and colony forming potential. We also described a novel interaction of EphA2, EphA4 and Ephrin B3 with the DDR proteins pATM (S1981), pDNA-PKcs (S2056) and γH2AX (S139) suggesting that this Ephrin and corresponding Ephs may directly intervene with DDR. Thus this thesis suggests that Ephrin B3 and its associated Ephs may be used as novel therapeutic targets in NSCLC alone or in combination with RT enabling further progress on precision cancer medicine.

LIST OF SCIENTIFIC PAPERS

I. Sara Ståhl, Vitaliy O. Kaminskyy, **GHAZAL EFAZAT**, Alena Hyrslova Vaculova, Salvador Rodriguez-Nieto, Ali Moshfegh, Rolf Lewensohn, Kristina Viktorsson and Boris Zhivotovsky. Inhibition of Ephrin B3-mediated survival signaling contributes to increased cell death response of non-small cell lung carcinoma cells after combined treatment with ionizing radiation and PKC 412. *Cell Death and Disease*, 2013, 4, e454; doi:10.1038/cddis.2012.188.

II. Sara Ståhl, Rui Mm Branca, **GHAZAL EFAZAT**, Maria Ruzzene, Boris Zhivotovsky, Rolf Lewensohn, Kristina Viktorsson and Janne Lethiö. Phosphoproteomic Profiling of NSCLC Cells Reveals that Ephrin B3 Regulates Pro-survival Signaling through Akt1-Mediated Phosphorylation of the EphA2 Receptor. *J. Proteome Res*. 2011, 10, 2566–2578.

III. **GHAZAL EFAZAT**, Metka Novak, Vitaliy O. Kaminskyy, Luigi De Petris, Lena Kanter, Therese Juntti, Per Bergman, Boris Zhivotovsky, Rolf Lewensohn, Petra Hååg and Kristina Viktorsson. Ephrin B3 interacts with multiple EphA receptors and drives migration and invasion in non-small cell lung cancer. *Manuscript*.

IV. **GHAZAL EFAZAT**, Metka Novak, Katarzyna Zielinska-Chomej, Therese Juntti, Teresa Holmlund, Rolf Lewensohn, Petra Hååg and Kristina Viktorsson. EphA2 and EphA4 influences DNA Damage Response (DDR) signaling in Non-small cell lung cancer and alter radiotherapy sensitivity. *Manuscript*.

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LIST OF ABBREVIATIONS

AD	adenocarcinoma
ALK	anaplastic lymphoma kinase
Apaf-1	Apoptotic protease activating factor 1
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BRCA1/2	breast cancer 1/2
CDC25	cell division cycle 25
CHK	checkpoint kinase
CDK	cyclin-dependent kinases
CFSE	carboxyfluorescein diacetate N-succinimidyl ester
DDR	DNA damage response
DR	death receptors
DNA DSB	DNA double strand break
DNA SSB	DNA single strand break
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
Eph	erythropoietin-producing hepatocellular receptor tyrosine kinase
Ephrin	ephrin ligand
GPI	glycosylphosphatidylinositol
H2AX	H2A histone family, member X
HER2	human epidermal growth factor receptor 2
HPLC	high-performance liquid chromatography
HR	homologous recombination (repair)
IGF-1R	insulin growth factor 1 receptor
IPA	ingenuity pathway analysis
IR	ionizing radiation
keV/μm	kiloelectron volt per micrometer
LC	lung cancer
LET	linear energy transfer
LIG4	DNA ligase 4

MRN complex	Mre11/Rad51/Nbs1 complex
Mdm2	mouse double minute 2 homolog
MS	mass spectrometry
NSCLC	non-small cell lung cancer
NHEJ	non-homologous end joining (repair)
PARP-1	Poly(ADP-ribose)polymerase-1
PCR	polymerase chain reaction
PE	plating efficiency
PI3K	phosphatidylinositide 3-kinase
RBE	relative biological effectiveness
RNAi	RNA interference
ROS	reactive oxygen species
RT	radiotherapy
SBRT	stereotactic body radiotherapy
SCLC	small cell lung cancer
SCX	strong cat ion exchange
siRNA	small interfering RNA
TKR	tyrosine kinase receptor
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TNFR	tumor necrosis factor receptor

1 INTRODUCTION

Cancer is a devastating disease which may occur in almost all the sites of our bodies. Cancer development is the result of multiple signaling aberrations of normal cells enabling transformed cells to grow in an uncontrolled way, independently of growth factors and to bypass normal cell death control mechanisms, two of the "hallmarks of tumors" as described by Hanahan & Weinberg [1, 2]. Moreover, it is evident that establishment of a cancer in a human body is a result of the interplay of the tumor cells with the normal surrounding stroma and with the immune system, where the tumor cells turn these normal cellular functions into their favor, enabling the primary tumor cells to migrate, invade and colonize to other sites in the human body, a process called metastasis. Molecular cancer research, which is the topic of the current thesis, aims to understand the underlying mechanisms of such tumor cell behaviors in which the knowledge on how to combat such alterations for therapeutic purposes is central. With respect to the tumor type in point of the current thesis, non-small cell lung cancer (NSCLC), two of the hallmarks of tumors, limited growth potential via aberrant growth factor signaling circuits and escape of immune system control have indeed allowed for molecular targeted approaches [1, 2]. The current thesis focus onto another growth factor receptor family erythropoietin-producing hepatocellular receptor tyrosine kinase (Eph) which show aberrant signaling propensity in multiple tumor types including NSCLC. In particular this thesis focus onto one of the Eph ligands, Ephrin B3 and how it may enable NSCLC cells to proliferate, migrate and invade (**Paper I-III**). Moreover, this thesis also aims to further understand how NSCLC cells respond to radiation therapy (RT) which still is one of the major treatment modalities of NSCLC and which in contrast to targeted agents attack multiple hallmarks of cancer. In this context the present thesis describes a role of Ephrin B3 and associated EphAs to control some of these RT-induced signaling events including DNA damage response (DDR) and apoptosis (**Paper I** and **IV**). On a broader prospective the current thesis is aimed to reveal novel therapeutic targets/strategies and biomarkers for NSCLC enabling a further improvement of precision medicine approach for this tumor malignancy to be taken.

1.1 LUNG CANCER

Lung cancer (LC) is a common cancer diagnose which annually is responsible for 1.6 million deaths worldwide [3]. In males LC is the primary reason of cancer related death and among women it is the second next after breast cancer [4]. In the European Union and United States, smoking stands for more than 90 % of LC in men and between 75-85 % LC in women [5]. In Sweden, LC is the fourth and fifth most common tumor form among women and men respectively [6]. LC incidence differs noticeably due to differences in historical smoking patterns, by sex, age, race/ethnicity, socioeconomic status, and by geography [4]. LC has traditionally based on cellular morphology been divided into two major subtypes derived from epithelial cells, that is Non-small cell lung cancer (NSCLC) and Small-cell lung cancer (SCLC) respectively. The present thesis focuses on NSCLC.

1.1.1 NSCLC and its treatments

Non-small cell lung cancer (NSCLC) is the most common form of LC and accounts for 85% of all LC diagnosed [7]. Unfortunately in about 65% of all patients, NSCLC is detected at a late stage when it is no longer feasible to remove the tumor by surgery [8]. Based on histology, NSCLC is further subdivided into adenocarcinoma (50%), squamous cell carcinoma (40%) and large cell carcinoma (10%), respectively [9]. Adenocarcinomas usually arise in the distal airways and have a glandular histology whereas squamous cell carcinomas have as the name indicate, a squamous differentiation, are found in the more proximal, and is highly associated with chronic inflammation and smoking [10, 11]. The last histological subtype, large cell carcinoma is a description of tumors whose cells neither appears glandular or squamous in shape nor expresses their biomarkers [10]. It has been noted that adenocarcinomas are increasing in women and in never-, light- and former smokers worldwide [12] whereas in current smokers or heavy former smokers squamous cell carcinoma are more common [13]. In 2011, a joint working group consisting of oncologists, radiologists, molecular biologists, surgical oncologists and pathologists made a new histologic classification of NSCLC adenocarcinomas [14]. It was agreed that pathologists need to separate classification of these NSCLC based on molecular aberrations found in the tumor specimen [15]. The reason for this specification is that certain genetic alterations in the NSCLC adenocarcinomas notably mutations in the Epidermal growth factor receptor (EGFR) gene or EML4-ALK translocations render these cases amendable to targeted therapy with small kinase inhibitors towards either aberration i.e. erlotinib/gefitinib or crizotinib. Hence, by this patients will receive a more individualized cancer treatment. Unfortunately, for most NSCLC patients targeted therapy has not yet emerged and given that about 60% of all NSCLC patients present with metastatic disease which has a 5-year survival rate of less than 5% [16] [17] there is a great medical need to find biomarkers and novel therapeutic approaches for NSCLC.

Mutations in EGFR, KRAS, HER2, BRAF and p53 or rearrangements of ALK and ROS1 are all found in various subsets of NSCLC tumors [8] where KRAS mutations are more frequently found in smokers [18]. In addition MET amplifications and RET rearrangements can also be found [19]. Hence, somatic mutations, chromosomal rearrangements and alterations in copy number have all been shown to be increased in NSCLC [20].

EGFR is frequently overexpressed and/or abnormally activated in NSCLC adenocarcinoma and a small fraction of this NSCLC subtype also displays mutations in the tyrosine kinase domain [21, 22]. Thus, in the USA roughly 10% of patients with NSCLC adenocarcinoma and in East Asia 35% of all such cases have a tumor which harbor EGFR mutation. There are four common mutations identified in EGFR namely exon 18 and 21 point mutations, exon 19 deletions and exon 20 insertions [23, 24].

Around 90% of EGFR mutations which results in a EGFR-driven NSCLC are either deletion of exon 19 (ex19del) or L858R point mutations and NSCLC patients with these mutations are

responsive to treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) e.g. erlotinib or gefitinib [25, 26].

The T790M point mutation in exon 20 is the most common resistance mechanism to EGFR tyrosine kinase inhibitors and can be found in 50-65% of treatment refractory patients that previously were found to have an EGFR mutation of their tumor [19]. The T790M mutation prevents binding of tyrosine kinase inhibitors to EGFR [27] but can also increase the affinity for ATM binding to the kinase, both which lessens the efficacy of tyrosine kinase inhibitor blockade [28]. Identification of oncogenic activation by EGFR mutations or ALK (anaplastic lymphoma kinase) gene rearrangements has changed the standard treatments towards a more molecular targeted approach and genetic analysis for finding the changes in LC is driving treatment to personalized cancer medicine [29].

LC treatment depends on tumor stage. For stage I and stage II the intention with the treatment is curative with first choice being surgical resection of the primary tumor [30]. If surgery is not an option, stereotactic body radiotherapy (SBRT) is a different choice with favorable survival benefit and acceptable toxicity at least for early stage disease (Stage I) [31]. In stage II adjuvant chemotherapy (CT) can be given in which a platinum agent most often is used combined with pemetrexed, vinorelbine or gemcitabine [32]. For stage IIIa surgery is preferred whenever possible and either followed by adjuvant CT, completely resected or by chemoradiotherapy if not. For Stage IIIb chemoradiotherapy is preferred [33]. As for stage IV which is an advanced stage of the disease normally palliative therapy is given using CT with combinations as mentioned above and in second line docetaxel or paclitaxel, but also targeted therapies have emerged such as tyrosine kinase inhibitors against EGFR or ALK [34].

1.2 RADIATION THERAPY AND MOLECULAR SIGNALING

Radiotherapy (RT) is used for loco regional tumor treatment of stage III NSCLC and is given to about 50% of such patients with curative intent [35, 36]. Albeit RT offer a way to control the NSCLC disease at least for some time, two problems with RT is however the intrinsic radiation resistance mechanisms of the NSCLC cells but also and the adverse reactions coming from irradiation of normal tissue surrounding the tumor [36]. In order to circumvent these problems different strategies has been proposed such as radiation protection of the normal tissue cells but more importantly, specific radiation sensitizing of the NSCLC cells [36]. However, in order to do so it is important to understand the underlying molecular mechanisms of the RT resistance such as the targets and cellular pathways that is involved. Molecular pathways or targets suggested used for such purpose are: inhibition of cell cycle control by blockade of CHK1 or CHK2 (Checkpoint Kinase-1 and -2) activity [36] or CDKs (Cyclin-Dependent Kinases) function [37], or blockade of DNA repair by targeting ATM (Ataxia-Telangiectasia Mutated) [38] or DNA-PK [39]. In (**Paper I** and **IV**) of this thesis Ephrin B3, EphA2 and EphA4 are presented as novel molecular targets for RT sensitization. Below the physical and molecular aspects of ionizing radiation (IR) is given and putative RT resistance pathways of relevance to the current thesis is presented.

1.2.1 Basic radiation physical aspects and inflicted cellular damages

Radiotherapy (RT) which can also be referred to as ionizing radiation (IR) and consists of electromagnetic x-rays or γ-rays where the energy of the radiation is deposited in the tissue by photons [36]. The term Linear Energy Transfer (LET) describes the energy which IR deposit per unit of length it cross and is given as keV/µm [32, 40, 41]. Such energy of photons deposited in the matter they cross such as in the cell membrane, cytosol or the DNA causes ionizations of the cellular macromolecules and give rise to the damages of which damages to the DNA is most detrimental for the cell [32, 40, 41].

The main cellular target of IR is the DNA and upon IR, the DNA is passed by an electron or an ion and thereby becomes either directly or indirectly ionized. For conventional RT indirect ionizations of DNA is most common and is a result of ionizations of water molecules resulting in production of highly reactive hydroxyl radicals that diffuse into the DNA and react with the target molecule and cause damages [42]. In addition, reactive oxygen species (ROS) and aqueous free radicals such as reactive hydroxyl radicals and H_2O_2 may also produce such lethal damages to the DNA [42].

1.2.2 DNA damage response (DDR) signaling in response to RT

Upon DNA damage, DNA damage response (DDR) signaling networks become activated which in turn result in cell cycle arrest in G_1 or G_2-phase allowing either DNA repair to take place (**Figure 1**) or different cellular death pathways such as apoptosis, mitotic catastrophe, autophagy and senescence to be triggered (**Figure 2**) [36]. These different cellular events are presented below.

Ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and RAD3-related (ATR) are the main DDR sensors and these kinases phosphorylate and activate downstream proteins upon sensing the DNA damage [43, 44] (**Figure 1**).

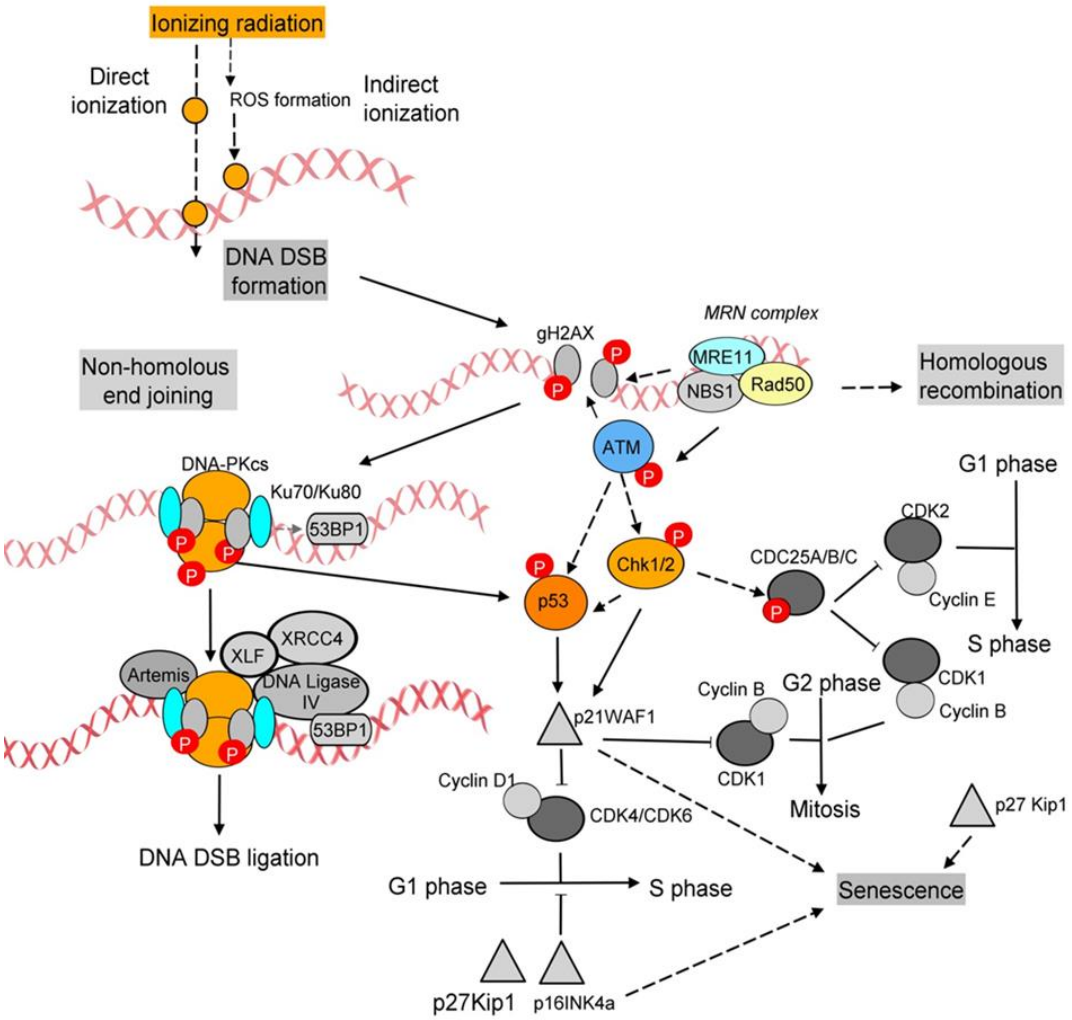

Figure 1: IR-induced DNA damage response signaling and downstream DNA repair and cell cycle signaling events. For details see text.

IR may result in both DNA single- and double strand breaks (DNS SSBs and DNA DSBs respectively) with ATM being the master DDR sensor of DNA-DSBs [45] while ATR is activated in response to DNA SSBs as a result of stalled replication forks [46]. The checkpoint kinase-1 (CHK1) and -2 (CHK2) are the transducers in DDR acting downstream of ATM [47] and together with ATM they phosphorylates the tumor suppressor p53 at various sites [48]. P53 becomes stabilized in the cell nucleus by such phosphorylation and after dissociating from its natural inhibitor Mdm2 (mouse double minute 2 homolog) it can act as a transcription factor for genes involved in IR-induced cell cycle block and/or IR-induced cell death [49]. The stronger the DNA damage level is the more p53 is stabilized [36] and depending on if p53 is becoming also altered by other post translational modifications e.g. acetylated or methylated, it sets the fate of cell survival or cell death [50].

One principle action mechanism of p53 is to cause cell cycle arrest by phosphorylation of p21 (CDKN1A, cyclin-dependent kinase inhibition 1A) which in turn inhibits the cyclin dependent kinases CDK4/CDK6 activity with cyclin D and as a result cells are arrested cells in G_1-phase [50, 53] (**Figure 1**). p21 also blocks the entry of cells from G_2 to M-phase of the cell cycle by binding to the CDK1-cyclinB complex [36]. As p53 is mutated in approximately

50% of all NSCLC cases [51] and since IR-induced G_1 arrest mainly is controlled by the p53/p21 axis in such NSCLC cells, IR-induced G_2-M control is of major importance since it can both carried out independently of p53 [52]. The cell cycle can also be controlled by the phosphorylation of CDC25 (cell division cycle 25) isoforms A, B and C by CHK1 and CHK2. This results in ubiqutination and degradation of CDC25 [36]. These events results in blockage of dephosphorylation and activation of CDK2-cyclin E and CDK1-cyclin B arresting cells in G_1-phase and in G_2-phase of the cell cycle respectively [53] (**Figure 1**).

IR-induced DNA DSBs are repaired by either of two principal pathways namely **non-homologous end joining (NHEJ)** and **homologous recombination (HR)** [53] (**Figure 1**). NHEJ can repair DNA DSBs during the whole cell cycle as it can ligate the DSBs without a need for a correct DNA template. However, NHEJ is error-prone since it during the repair processes may cause short deletions or additions onto the DNA sequences if the DNA ends needs processing before ligation can occur resulting in loss of genetic information [54]. On the contrary HR is an error free DNA repair pathway, but is only available in late S and G_2-phase since it needs an undamaged sister chromatid as a DNA template. It is the Ku70/Ku80 heterodimer that senses the DNA DSBs and decides if it is NHEJ or HR that will become activated [54]. In addition, the Ku70/Ku80 complex may also activate 53BP1 (p53-binding protein 1) which protects the DSB ends against resection [55]. DNA DSB formation also results in chromatin alterations and as a result the histones surrounding the break γH2AX Ser129 stabilize the DNA ends but also to bring together the DSB repair machinery [56, 57] including Artemis and the DNA dependent protein kinase (DNA-PK) [54, 58]. DNA-PK is thus an important DNA DSB sensor which in addition to ATM control phosphorylation of H2AX [58]. Within NHEJ the DNA ends are then subsequently ligated by LIG4 (DNA ligase 4), XRCC (X-ray repair, complementing defective, in Chinese hamster 4) and XLF (XRCC4-like factor) [54].

In HR it is the MRN complex with MRE11/RAD50/NBS1 that starts the DNA DSB repair where the signal is transmitted to ATM and ATR which in turn phosphorylate downstream effectors [36]. RPA also bind to the MRN complex and search for homology between the two sister chromatids [59]. Moreover, the MRN complex in combination with CTIP (CTBP (C-terminal binding protein)-interacting protein) also process the DNA ends in the DNA DSB by resection [60]. By annealing the established single-stranded DNA to the unwound sister chromatid, HR can be initiated. This is done by RAD51 which forms a complex with phosphorylated and activated BRCA2 (breast cancer 2) and subsequently RAD51 can bind single stranded DNA [61]. The cell cycle will be completed if the DNA DSB has been fully repaired while improper DNA repair after IR will start the process of cell death either by mitotic catastrophe, apoptosis or senescence as outlined below [62].

1.2.3 Principal cell death signaling routes and RT-induced signaling effects

IR may induce different cell death routes including **apoptosis** [63], **mitotic catastrophe** [64] and **senescence** [65].

Apoptosis is a cell death mechanism with the characteristics of cell shrinkage, membrane blebbing and condensation/fragmentation of the chromatin in addition to formation of apoptotic bodies [66]. The molecular path of apoptosis resulting in caspase activation and subsequent signaling may be conceived by either of two principal routes, the **intrinsic** or **mitochondria mediated pathway** or via the **extrinsic apoptotic pathway** in which death receptors such as FASR are instrumental [67] (**Figure 2**).

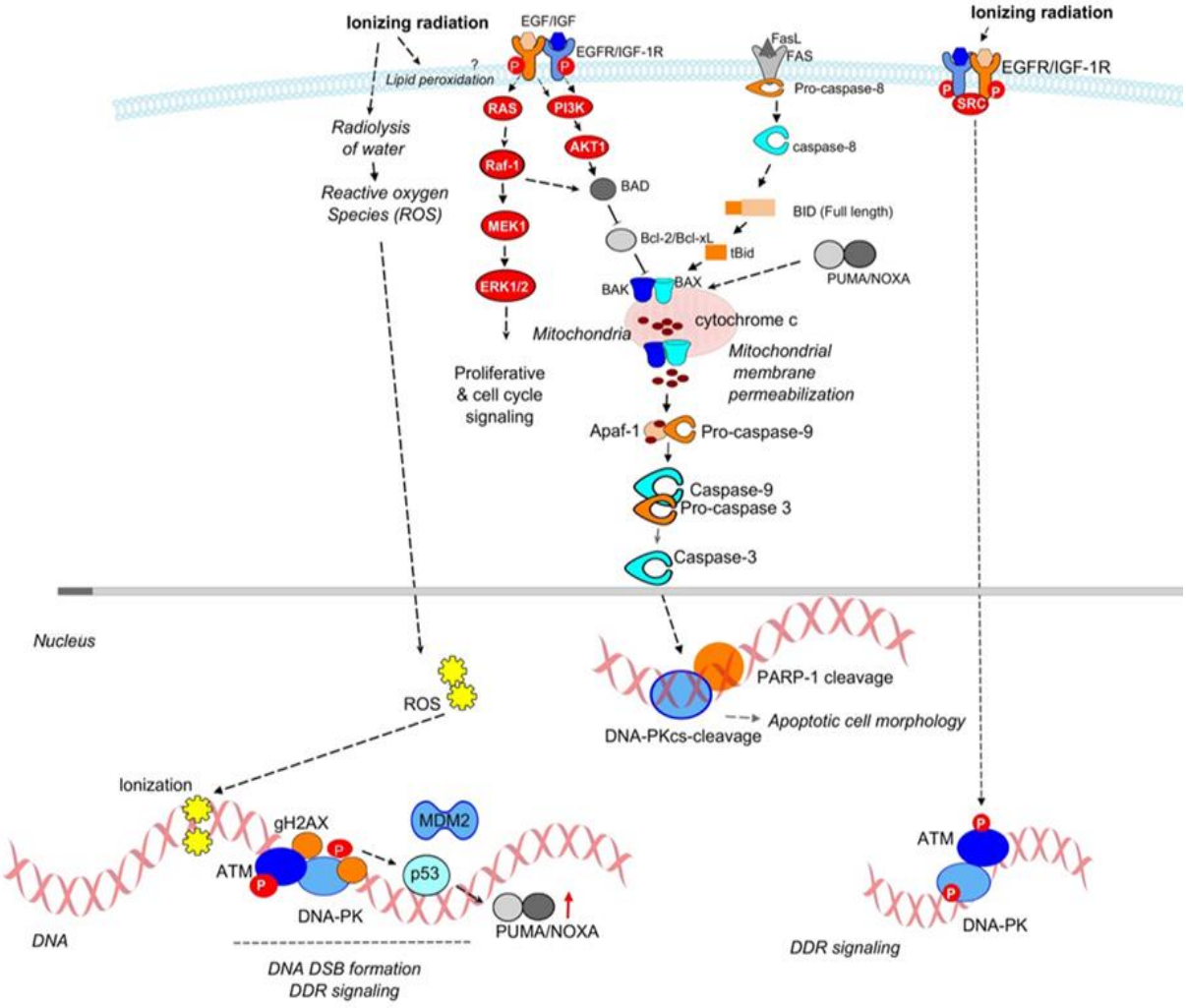

Figure 2: **IR-induced apoptotic signaling.** Ionizing radiation (IR) causes formation of ROS which trigger DDR signaling. DDR may activate apoptotic signaling via mitochondria, the apoptosome and caspase-3 resulting in apoptotic morphology in which signal via the p53/NOXA/PUMA axis is one path by which the DDR signal is transmitted. Apoptosis may also be initiated via Fas/FasL/caspase-8/Bid. IR also activates EGFR/IGF-1R with subsequent MAPK/ERK signaling. Such signaling may alter Bad/Bcl-xL function and block apoptosis**.**

In the **intrinsic apoptosis signaling cascade** which is activated in response to IR, release of cytochrome c from the mitochondria is important [68] and is controlled by members of the BCL2 (B-cell lymphoma 2) family proteins [69]. BCL2 proteins may either be anti-apoptotic

where BCL-XL and BCL-2 is operative, or pro-apoptotic where Bak and Bax (BCL2-associated X protein) as well as the BH3-only proteins Bid, Bim, Bad, PUMA (p53-upregulated modulator of apoptosis) and NOXA are members [70].

Upon nuclear accumulation of p53, the pro-apoptotic BCL-2 genes BAX, PUMA and NOXA are activated by transcription and may transmit a pro-apoptotic signal onto mitochondria [71-73]. Subsequently, complex of the pro-apoptotic Bax or Bak and the anti-apoptotic BCL2 proteins are dissolved resulting in Bak/Bax oligomeric pore formation [71, 74] which may cause inner mitochondrial membrane (IMM) permeability transition [75]. The mitochondrial protein cytochrome c is released to the cytosol [75] and forms a complex with APAF1 (apoptotic protease activating factor 1) and pro-caspase-9 [76]. As a result, caspase-9 is cleaved and activated [76] and will further activate the effector caspases caspase-3 and -7, causing the cleavage of signaling and structural proteins resulting in the above described morphological features of apoptosis [77]. In addition Akt is a proliferation and anti-apoptotic factor which in response to IR is activated by PDK1 and PDK 2 [36] which in turn are activated by the EGFR-ERBB2 heterodimers [78, 79].

The **extrinsic apoptotic pathway** is on the other hand dependent on signaling through death receptors (DRs) which belong to the TNFR (tumor necrosis factor receptor) family [80]. Here, cell surface DR such as FASR binds to its ligand FasL and as a result a complex is formed in which the Fas-associated protein with death domain (FADD) is bringing pro-caspases together resulting in cleavage and activation [81]. This causes cleavage of pro-caspase-8 into caspase-8 which may subsequently activate caspase-3 and result in the apoptotic morphological characteristics [81].

The principal pathway of apoptosis activated in response to IR in which DNA DSB or SSB repair has been un-successful is the intrinsic route [63, 68, 82, 83]. The complete picture on how IR may trigger apoptosis is not clear but one important player is p53 which in response to DNA damage activate transcription of Bcl-2 family proteins resulting in the molecular path outlined above [63, 68, 82]. In addition, production of free radicals by IR can trigger cytochrome c release and may also initiate mitochondrial Ca2+ release which in turn also may influence pro-apoptotic responses [84].

Mitotic catastrophe is a form of cell death induced as a consequence of dysfunctional cell division resulting in micro- or multi nuclei formation [36, 85]. **Senescence** occurs mainly either in the G_1 or G_2 phase in response to IR where p53 activation results in p21 accumulation and cell cycle arrest [65].

1.2.4 IR resistance signaling networks

The intrinsic resistance to RT displayed in NSCLC cells is reported to be a result of several aberrations such as deregulated growth factor signaling, decreased function of cell death signaling pathway and increased DNA-repair. Some of these aspects in light of the current thesis are described below.

The IGF-1R (Insulin growth factor 1 receptor) is involved in RT resistance in numerous ways [86-88]. Thus inhibition of IGF-1R and IR resulted in an increase accumulation of NSCLC cells in G_2-phase of the cell cycle [88]. Interestingly, IR was shown to directly activate IGF-1R early after IR [87] and it was reported that such activated IGF-1R may increase binding of the NHEJ protein Ku70/Ku80 to DNA and in this way promote DNA repair [87]. Moreover, IGF-1R was also shown to activate p38MAPK [87], a MAPK kinase which regulates the balance between apoptosis and autophagy [89] and was found to control IR resistance in NSCLC cells [90]. Indeed inhibition of IGF-1R with small molecule kinase inhibitor disrupted the IGF-1R and p38MAPK complex, inhibited the p38MAPK activity and sensitized cells to RT-induced cell death [87]. Similarly it has been reported that upon IR EGFR shuttle into the nucleus, increases phosphorylation and activation of DNA-PK and promotes DNA repair capacity [78].

In addition downstream targets of IGF-1R or EGFR such as K-RAS, PI3K and Akt signaling pathway are also reported to be involved in RT resistance [91]. Thus inhibition of K-RAS was shown to increase the RT sensitivity of NSCLC cells and K-RAS mutations was reported to be important for PI3K- and Akt-mediated RT resistance [92]. Moreover, with respect to NSCLC cells it has been shown that they often display high phosphorylation of Akt Ser473 and RT resistance [93]. It was also demonstrated that inhibition of the upstream Akt1 kinase PI3K resulted in additive effect when used in combination with IR in part as a result of increased apoptotic signaling [93]. Furthermore, targeting either Akt1 or the MAPK ERK1 also sensitized several NSCLC cell lines to DNA damage induced cell death [94].

All in all this suggest that growth factor signaling may in multiple ways influence cellular RT response and this is also further illustrated in **Paper I** and **Paper IV** with respect to Ephrin B3, EphA2 and to some extent EphA4 signaling in NSCLC cells.

1.3 MOLECULAR TARGETING OF GROWTH FACTOR SIGNALING IN NSCLC

The hallmarks of cancer are described to be self-sufficiency in growth factors, limitless replicative potential, anti-apoptotic capacity, neo angiogenesis and ability to invade and metastasize [1, 2]. These capabilities of tumor cells are a result of oncogene activation or loss of tumor suppressive gene function coming from point mutations, gene amplifications/ rearrangements, epigenetic silencing of transcription or loss of heterozygosity respectively [95]. Hence it becomes important to find the “driver oncogene” responsible for tumor cell proliferation/survival where EGFR, p53, K-RAS, HER2, MYC, MET, ALK and BCL2 is the common activated driver oncogenes in NSCLC [96, 97].

Constitutively active or overexpressed EGFR has been associated with poor prognosis and is common in several cancer types [98]. EGFR can activate two major pathways involved in tumor cell growth, protein translation, angiogenesis, cell metabolism and invasion [99] namely the PI3K/Akt/mTOR and the RAS/RAF/MEK/MAPK pathway [100] (**Figure 3**).

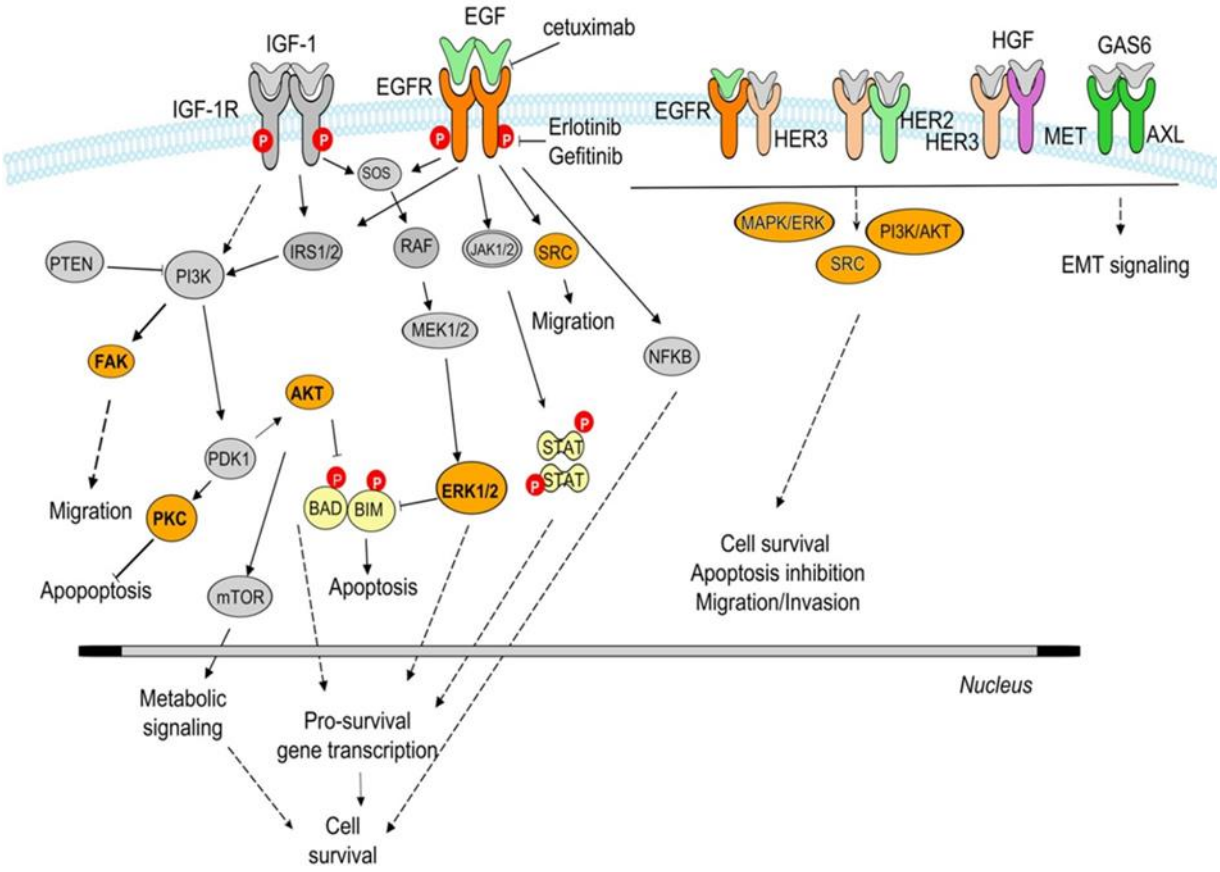

Figure 3: Growth factor receptor signaling in NSCLC. Multiple growth factors (IGF-1R, EGFR, HER2, HER3, MET, AXL) are concomitantly activate at the plasma membrane as homo-or heterodimers in NSCLC cells. Upon ligand binding the tyrosine kinase domains of these growth factor receptors are phosphorylated and they initiate multiple kinase cascades (PI3K/Akt, MAPK/ERK, JAK/STAT, SRC and NFKβ) which promote proliferation, migration, invasion and metabolic signaling but blocks apoptosis. The yellow marked kinases are those studied in the present thesis. The action points of small kinase inhibitors (erlotinib/gefitinib) and inhibitory antibody (cetuximab) is shown.

Accordingly, blocking EGFR signaling is becoming more and more important. EGFR activity can be inhibited by either of two principal ways: by using blocking antibodies e.g. cetuximab or panitumab that binds to the EGFR extracellular domain thus inhibiting its dimerization or by blocking the intracellular kinase domain of mutated EGFR by using small molecules e.g. gefitinib or erlotinib [101]. EGFR expression is known to increase upon RT-induced tissue damage and monoclonal antibodies against EGFR are effective when EGFR is overexpressed hence rationalizing their use in combination with RT [95]. Thus cetuximab is used together with RT for advanced head and neck cancer [102]. Moreover, with respect to NSCLC tumors with EGFR activating mutations, the use of EGFR TKIs may lead to a rapid tumor regression and is also reported to improve RT sensitivity [24, 95]. However some challenges remains such as the activity of nuclear EGFR which may due to its localization, not be targeted by the current approaches [103].

1.4 EPH GROWTH FACTOR RECEPTORS AND THEIR LIGANDS EPHRINS

Receptor tyrosine kinases are known to function as proto-oncogenes and have a role in tumorigenesis. Mutated EGFR is indeed a target in certain NSCLCs, which small molecules

have been developed towards [104]. Yet other growth factor signaling circuits should be explored as targets and one such potential growth factor signaling circuit is the **erythropoietin-producing hepatocellular (Eph) receptors** and their ligands **Ephrins** [105] which is in focus of the current thesis.

1.4.1 The Eph and Ephrin signaling network

The Eph kinases were identified about 30 years ago and today they represent the largest transmembrane receptor tyrosine kinase (RTK) family [106]. In normal cells Ephs have been demonstrated to influence the cell position, cell migration but also in a pronounced way regulate cell-cell interaction [107-110] (**Figure 4**). Thus Ephs and Ephrins control several developmental processes including tissue homeostasis, formation of tissue boundaries, axon guidance, remodeling of blood vessels and organ size [123].

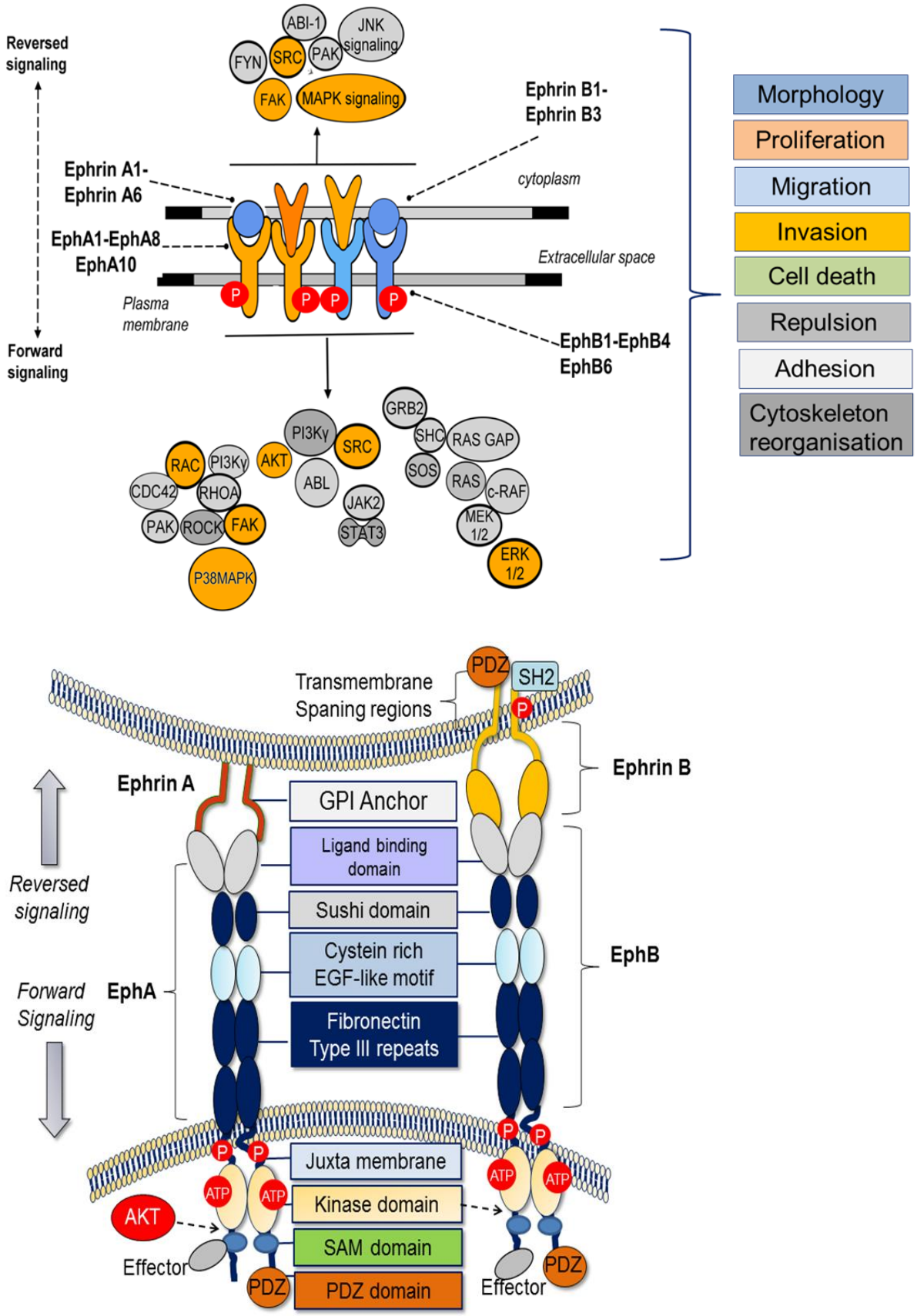

Figure 4: The Ephrin and Eph signaling circuit in tumor cells. For details see text.

There are 14 Ephs described within the human genome and they are based on sequence homology, divided into class A (EphA1-8 and EphA10) and class B receptors (EphB1-4 and EphB6) [111]. The extracellular part of the Ephs contains the N-terminal ligand-binding domain which has a cysteine-rich region with an EGF-like motif, immunoglobulin-like motifs, and two fibronectin type III repeats [106]. The extracellular motif of the principal Eph also has a membrane-spanning region and a cytoplasmic region including a juxtamembrane region with a tyrosine kinase domain which acts as the active kinase site of the receptor [112, 113]. However EphA10 and EphB6 do not have kinase activity due to modifications of their kinase domain and in addition several of the Ephs have alternative spliced forms that differ from the prototypical structure, hence resulting in different functions [114].

The eight Eph ligands Ephrins, are divided into two classes based on their structure and sequence namely class A or B. The Ephrin A ligands are linked to the membrane via a GPI (glycosylphosphatidyl-inositol) anchor containing a signal peptide whereas the Ephrin B ligands contain a transmembrane region that spans the entire membrane [105]. The nomenclature of the Ephrins and Ephs is based on the fact that it was assumed that Ephrin A ligands bind to members of the Eph class A whereas Ephrin B ligands should bind to Eph class B [115]. However this is not the case and several promiscuous bindings between Ephrins and Ephs of the opposite class such has been identified [105]. One example is the binding of Ephrin B1-3 to EphA4 and Ephrin A5 binding to EphB2 and EphB4, the later which also interact with Ephrin B2 [105]. However the complete picture of Ephrin and Eph interaction pattern remain to be solved as illustrated in the **Paper II-III** of this thesis. One interesting feature of the Eph-Ephrin signaling axis is its bidirectional capacity meaning that it causes both a forward and a reverse reaction in the Eph or Ephrin expressing cell respectively [116-118] (**Figure 4**). Thus, upon ligand-receptor interaction the Eph kinase domain gets activated through phosphorylation and dimerization. Subsequently this leads to the transduction of the typical forward signal in the Eph-expressing cell with a subsequent activation of downstream signaling cascades [119]. Additionally, the engagement of Ephrins to Eph also triggers signaling in the ligand-bearing cells [106]. For instance, in the cytoplasmic region of Ephrin B ligands, phosphorylation of tyrosine residues results in the recruitment of signaling effectors and activation of signal transduction cascades [106]. Hence, Eph kinase activity triggers forward signals whereas the reverse signaling is in part controlled by the Src family kinases [105].

Another way of regulating Eph activity is via the action of soluble Ephrin As that are released from the cell and also can bind Ephs such as Ephrin A1 binding to EphA2 [120]. In addition kinase-independent Eph signals and Ephrin-dependent signals can occur [121, 122]. The bidirectional signals via Eph/Ephrins may also result in the elimination of adhesive Ephrin-Eph complexes from sites of cell-cell contact through mechanisms of endocytosis resulting in their internalization in either the Eph- or the Ephrin- expressing cell [113]. Such signaling results in Eph-repulsive responses between the cells [123]. In addition another mechanism is operative in which protease-mediated cleavage of the extracellular domain of Eph or Ephrin allows cell separation [124-126]. Yet other mechanisms may stabilize the Eph levels. E-

cadherin promotes Ephrin A1 and EphA2 to be localized to the epithelial cell junctions [127, 128] and Ephrin A5 binding to EphA4 is reported to be stabilized by the proteolytic actions of the metalloproteinase ADAM19 at neuromuscular junctions [129].

All in all, a combination of Eph-dependent adhesive or repulsive forces may drive the individual cell populations that express different combinations of Ephrins and Ephs, and in tumors such may allow oncogenic signaling to be executed [123]. Both Ephrins and Ephs are expressed in most tissues with different expression patterns and can be co-expressed in the same cells [113]. Recently it was shown that beside the regular in *trans* signaling where Ephrin and Eph are expressed on the opposite cells result in a signal, co-expression of Eph and Ephrins on the same cell can exhibit a signal in lateral cis resulting in inhibition of the Eph activation in *trans*. Interestingly such cis signaling may also be operative in NSCLC cells as it was shown that co-expression of Ephrin A3 with EphA2 and EphA3 can inhibit their ability to become activated by binding Ephrins in *trans* [130]. Moreover it was also demonstrated that such cis interaction of Ephrin A3 and EphA3 was enhanced by a specific EphA3 mutation [130].

1.5 EPH AND EPHRIN DYSREGULATION IN CANCER

Both Ephs and Ephrins are reported to play a role in almost all tumor malignances and in breast, glioma, prostate, leukemias, melanomas and LC, Ephrin/Eph signaling has been studied in depth [106]. The deregulated expression of Ephs and Ephrins are found in the tumor cell *per se* and in the tumor microenvironment i.e. in the tumor stroma [116, 131]. Altered Ephrin and Eph signaling are indeed reported to influence several signaling pathways that are involved in tumor cell behavior regulation, e.g. the MAPK/ERK and PI3K/Akt, both shared with EGFR and IGF-1R signaling cascades, controlling proliferation, positioning and migration capacity [123].

With respect to NSCLC, Ephrin B3 mRNA expression was reported to be increased in NSCLC tumor specimen and was found to be associated with a higher risk of relapse [132]. EphA3, EphA2, EphA7 and EphB3 expression were similarly reported to be up-regulated in NSCLC [106, 134]. Moreover, NSCLC cell migration and invasion *in vitro* was shown to be prevented by forced overexpression of EphA4 [132] or EphB3 [133]. Not only is Eph expression deregulated in NSCLC, global analyses of mutations in the genome of NSCLC adenocarcinomas revealed that EphA3 and EphA5 were among the top five most frequently mutated genes with mutations found in both the ligand binding as well as in the kinase domain of the receptor [135-137]. On the contrary the expression of Ephrins and Ephs can also be downregulated in tumors. In metastatic NSCLC cases EphB6 was shown to have decreased expression as compared to non-metastatic cases [138]. Moreover, EphB6 mutations were linked to metastasis in a subset of NSCLC patients [139]. A role of EphA2 in cancer in general and in NSCLC in particular is evident and is in focus of this thesis. It will therefore be discussed in depth below.

1.5.1 EphA2 and tumor cell signaling

EphA1 was as the first member of Eph family cloned in the late 1980:ies [140] and following this EphA2 was identified by screening a cDNA library for sequence homology to EphA1 [141]. Approximately 25-30% of EphA2 show sequence homology with other Ephs [112]. Ephrin A1 is a well described ligand of EphA2 [142] which was found based on its binding to the extracellular region of EphA2 [143]. Based on the crystal structure of the extracellular domain of EphA2 it has been postulated that a high concentration of the EphA2 clustering independent of Ephrin could impart a typical cancerous cell phenotype [144]. Accordingly, overexpression of EphA2 has been found in many tumor forms such as in lung adenocarcinoma, glioma, breast, colorectal, ovarian and prostate cancer where it is reported to drive proliferation and invasion [131, 145-152]. The overexpression of EphA2 has been linked to a poor prognosis in several tumors including LC [147]. An EphA2 mutation at G391R in NSCLC has also been identified which result in a constitutive active EphA2 that trigger activation of Src [153]. Thus, by activating focal adhesions, actin cytoskeletal regulatory proteins and mTOR, tumor survival and invasiveness is increased [153].

In NSCLC a higher EphA2 expression compared to normal non-tumor tissue is reported and EphA2 expression correlated to poorer prognosis in addition to a history of smoking [145]. Moreover, a high EphA2 expression was found in advanced stage of the disease. In addition, patients displaying brain metastasis exhibited high EphA2 levels [145]. Interestingly, in embryonic fibroblasts EphA2 were shown to be an important p53-independent and caspase-8-dependent pro-apoptotic factor [154]. In addition downregulation of Ephrin A1 in breast cancer cells was shown to increase EphA2 tumor invasiveness [155]. Moreover, in both prostate cancer and glioma cells association of Ephrin A1 and EphA2 was reported to inhibit EphA2 Ser897 as well as Akt Ser129 phosphorylation resulting in inhibition of proliferation- and invasion signaling mediated by EphA2 [150, 156]. However, the results regarding EphA2 phosphorylation status and tumor malignancy are contradictory and suggest that certain sites indeed may block EphA2 growth and invasion controlling capacity [146, 157-160]. Thus it was reported that NSCLC treatment with an Ephrin A1-Fc resulted in a transient increase of EphA2 phosphorylation contributing to a decrease of total EphA2 expression due to rapid internalization and degradation [146].

In prostate and breast cancer EphA2 phosphorylation was shown to be necessary to confer the oncogenic potential of EphA2 [157-159]. Moreover other studies suggest that EphA2 phosphorylation is not needed in order to impart tumorigenicity [128, 160] or that EphA2 phosphorylation causes tumor suppression [161]. When screening the literature it becomes evident that the function of EphA2 depends on the conditions and available ligands as illustrated in **Paper III** of the current thesis. For instance EphA2 activation was reported to inhibit chemotactic migration of glioma and prostate cancer cells upon interaction with Ephrin A1 whereas overexpression of EphA2 triggered migration in a Ephrin A1 independent manner [150]. In NSCLC stage I higher expression of EphA2 and Ephrin A1 was shown to correlate to good clinicopathological features [162]. Thereby indicating that in presence of Ephrin A1, EphA2 has a tumor suppressive role [162].

1.5.2 EphA2 as a therapeutic target

Several tyrosine kinase receptors have been targeted for their critical roles in tumorigenesis [163] and an interest in EphA2 as a therapeutic target has emerged since EphA2 is overexpressed in various cancers while expressed at rather low levels in normal cells. EphA2 has indeed been evaluated as a drug target using several approaches such as RNA interference (RNAi), Ephrin A1 mimicking agonistic antibodies, virus vector-mediated gene transfer that target deregulated Ephrin A1 and EphA2 signaling in tumor cells, immunoconjugate approaches but also small-molecule inhibitors which block kinase domain and nanoparticles loaded with CT and with EphA2 as targeting moiety [164].

Monoclonal antibodies have been designed against the extracellular domain of EphA2 [165]. Indeed treatment with these EphA2 agonist monoclonal antibodies alone or in combination with the mitosis inhibitor paclitaxel was reported to reduce tumor growth in mice, and it is believed this is a result of EphA2 internalization and degradation causing inhibition of the Ras/MAPK pathway [164]. Hence, monoclonal antibodies specific to EphA2 could function similarly as Ephrin A1 and reduce the oncogenic potential [164]. By using monoclonal antibodies to deliver CT agents, immunoconjugates will induce cytotoxicity in tumor cells and it is believed that since EphA2 is less expressed in normal than in tumor cells, the normal cells will be spared [164]. Immunotherapy is also a way to target EphA2 since epitopes on EphA2 are differentially displayed in cancer versus normal cells [166]. In breast- and NSCLC cells some EphA2 antibodies is reported to react strongly but not to normal immortalized breast cells indicating that EphA2 epitopes indeed can be used as therapeutic targets [164].

In prostate cancer cells, a small molecule against EphA2 inhibited EphA2 phosphorylation [164]. In addition dasatinib which is an FDA approved small-molecule tyrosine kinase inhibitor was shown to prevent EphA2 activity [167] and to cause decreased expression of EphA2 in breast cancer cells [168]. RNA interference (RNAi) approaches have also been used in order to suppress EphA2 overexpression. Hence EphA2 expression was inhibited by RNAi in pancreatic adenocarcinoma-derived MIA PaCA2 cells and blocked tumor growth in a nude mice xenograft model concomitantly with increased apoptotic signaling [169]. Inhibition of EphA2 in human glioma-derived U-251 cells was similarly reported to increase caspase-3 activity and apoptosis in addition to a reduction in tumor cell proliferation [170]. Suppression of EphA2 by siRNA in malignant mesothelioma derived cells decreased cell proliferation and downregulated migration as EphA2 overexpression increased cell proliferation [173]. Moreover siRNA against EphA2 in human glioma cells induced apoptosis and inhibited proliferation [170].

EGF or EGFR signaling has also been reported to regulate EphA2 activity and expression in NSCLC [145] and in head and neck carcinoma-derived cell lines [171, 172]. EphA2 suppression in such cells decreased EGF-induced migration indicating that there is a cross-talk between EGFR and EphA2 signaling that could be used for therapeutic purposes [171, 172]. Recently it was also shown that a small molecule against the EphA2 kinase domain

could revert erlotinib resistance *in vivo* in mice in which a decreased EphA2 expression level was evident [213, 214].

All in all these studies indicate that EphA2 inhibition is a feasible approach for targeting different tumors including NSCLC. Moreover, inhibition of EphA2 in combination with targeting of other oncogenic signaling molecules e.g. mutated EGFR is thus an important strategy for targeted cancer therapies approaches.

2 AIMS

The overall aim of this thesis was to analyze mechanisms of Ephrin ligand and Eph receptor signaling in NSCLC cells alone or in combination with radiotherapy (RT). The specific aims of the PhD project were:

- To reveal novel RT sensitizing targets in NSCLC cells by using global gene expression profiling and to validate Ephrin B3 as such novel candidate (**Paper I**).

- To understand how Ephrin B3 influences the proliferative signalome of NSCLC cells by application of a global phosphoproteomic profiling and subsequent validation of signaling components (**Paper II**).

- To reveal if and how Ephrin B3 mediate effects on NSCLC cell migration and invasion and delineate putative Ephs such as EphA2 involved in its action mechanism *in vitro* and *in vivo* (**Paper III**).

- To address the impact of EphA2, EphA4 and Ephrin B3 on RT sensitivity in NSCLC cells and analyze their effect on DDR (DNA Damage Response) signaling components DNA-PK and ATM (**Paper IV**).

3 MATERIAL AND METHODS

For **Paper I-IV** all corresponding material and methods are described in brief below.

3.1 CELL LINES AND MODEL SYSTEMS

To cover the different histological subtypes that NSCLC is classified into, a panel of NSCLC cell lines of adenocarcinoma, mixed large cell/adenocarcinoma, squamous cell carcinoma and adenosquamous cell carcinoma origin was used (**Paper I-IV**). The cell lines alongside their histology, radiosensitivity, mutation status of Eph and K-Ras are presented in **Table 1**. The intrinsic radiotherapy sensitivity of the cell lines measured as surviving fraction 2 Gray (SF2) in colony formation assay has been published [174-177] and these values were used in **Paper IV** to correlate basal Ephrin B3, Ephrin A1, EphA2 or EphA4 expression to RT sensitivity. Clonogenic survival assay is commonly used to describe the RT sensitivity of a given cell line and is most often described as survival fraction 2 Gray (SF2). In a clonogenic survival assay the potential of the cells to form clones is described as a function of a given radiation. Thus the surviving fraction 2 Gy is the amount of cells that survive after being irradiated with the dose 2 Gy. An SF2 0.8 means that 80% of the cells treated with 2 Gy still had their clonogenic capacity and SF2 1.0 means that all of the cells has survived. The surviving fraction of each absorbed dose is calculated as the ratio of the mean PE (plating efficiency) of irradiated cells over the PE in dishes with non-irradiated cells used as control [40]. In **Paper IV** cells were allowed to form colonies for 9 days in order to be able to measure reproductive cell death. The colonies were subsequently stained with Giemsa and the total colony number/dish was counted.

Table 1. Histology, SF2 values and mutation status of the cell lines used.

Histology	Cell line	Mutation status/Variant type	SF2
Adenocarcinoma	H23	EPHA6/Insertion, KRAS/SNP	0.2
	H1299	EPHA6/Insertion, EPHA7/SNP	0.3
	H157		0.6
	A549	EPHA1/SNP, EPHA6/Insertion, EPHB6/Deletion, KRAS/SNP	0.7
	H661	EPHA6/SNP,Insertion	0.9
Adensquamous cell carcinoma	H125		0.4
Squamous cell carcinoma	U-1752		0.9
Mixed large cell and adenocarcinoma	U-1810		0.8

Abbreviations: SF2 = surviving fraction 2 Gray, SNP = single nucleutide polymorphism

https://cansar.icr.ac.uk/cansar/cell-lines/A549/mutations/

http://www.broadinstitute.org/ccle/home

The mixed large cell and adenocarcinoma U-1810 cell line has been used as a model system in all the papers of this thesis. Berg et al., at the department of Oncology at Uppsala Academic hospital in Sweden isolated the U-1810 cells from a patient with undifferentiated large cell carcinoma/adenocarcinoma and hence the ``U´´ stands for Uppsala. Similarly the

U-1752 cell line was also a kind gift from Uppsala University whereas the rest of the cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

With respect to radiation, the U-1810 cell line has over the years been extensively studied and found to be highly radiation resistant [94, 178, 179]. Moreover, it was previously demonstrated that U-1810 cells could be sensitized to RT by the staurosporin analogue PKC412 in part as a result of increased pro-apoptotic signaling but also as a consequence of mitotic catastrophe [94, 178]. In **Paper I** and **IV** this cell line was therefore chosen as a model system to reveal drivers of RT resistance and to understand Ephrin B3 and EphA2, EphA4 and EphA5 in this context. In **Paper II**, U-1810 was used to further investigate the signaling pathways driven by Ephrin B3 and in **Paper III** it was chosen alongside U-1752 and H23 to get a deeper understanding of the role of EphA2 and Ephrin B3 in pro-survival signaling, proliferation and migration in relation to histology.

In order to analyze if inhibition of Ephrin B3 or EphA2 expression could decrease invasive capacity of NSCLC cells (**Paper III**) CL1-5 adenocarcinoma cells, with high invasive potential, kindly given by Dr Pan-Chyr Yang (Institute of Biomedical Sciences, Academina Sinica, Taiwan) was used. The CL1-5 cells have been generated from adenocarcinoma CL1-0 cells by selecting for clones with increased invasion potential in transwell invasion chamber assay [180,181].

3.2 IRRADIATION

In **Paper I** and **Paper IV** conventional radiation was delivered as photons of gamma rays using a ^{60}CO source (absorbed dose 2 Gy, 4 Gy or 8 Gy) with the monthly dose rate <0.5Gy/min determined according to decay of the source. In **Paper IV** for some experiments irradiation was carried out on ice in order to inhibit DNA DSB repair during the irradiation procedure while all other IR procedures was carried out at room temperature.

3.3 RNA INTERFERENCE

In **Paper I-IV** short interference RNA (siRNA) was used to block expression of Ephrin B3, EphA2 or EphA4. A siRNA consists of short double stranded RNA with 20-22 nucleotides which upon cleavage in the cell bind to specific sequences of mRNA and after transcription results in mRNA digestion and subsequently inhibition of mRNA expression [182]. The challenges with this method are that for each single siRNA in each model system the experimental conditions (e.g. transfection time and amount of siRNA) need to be optimized in order to have a good knockout of the target gene. In addition off-target effects resulting in non-specific RNA degradation by the siRNA remains a challenge as it may blur the interpretation of results. To avoid this, a non-targeted siRNA which was designed and tested for minimal targeting of different genes was applied in **Paper I-IV**.

The siRNA of Ephrin B3 (Qiagen, Maryland, USA) applied in **Paper I-IV** was custom made and previously described to be unique towards Ephrin B3 [183]. In addition, in **Paper I** a

second Ephrin B3 sequences was used to confirm the results [183]. For siRNA targeting of EphA2 in **Paper II-IV** and EphA4 in **Paper IV** four different sequences was used to improve on both efficiency and specificity of the siRNA towards its target. In all experiments approximately 500 000 cells were seeded in 10 cm dishes followed by siRNA transfection using 100nM siRNA. Cells were seeded 24h prior to siRNA transfections which were carried out for 24h-48h with different post incubation times. Knock-down was confirmed by western blot or Real-time quantitative PCR as describe in **section 3.4**.

3.4 CELL BASED ASSAYS

To assess the different outcomes of NSCLC cells in response to EphrinB3, EphA2 and EphA4 siRNA and/or irradiation treatments different cell based assays was used and are described in brief alongside their rationale below.

3.4.1 Analysis of proliferation and cell death

Apoptotic morphology of the cell nuclei was in **Paper I** analyzed by staining the nuclei with mounting media containing 4,6´diamino-2phenylindole (DAPI) and examined in a fluorescent microscope. Cells were determined to be apoptotic if a fragmented nuclei was evident and the number of such cells was counted. Apoptosis was also biochemically examined in **Paper I** by analyzing cytokeratin 18 cleavage by caspase 3 using an antibody, M30, which specifically recognize this caspase-released neo epitope of cytokeratin 18. By using Fluorescence Associated Cell Sorting (FACS) the percentage of cells with M30 CytoDeath-FITC antibody (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) positivity was detected. In addition analysis of PARP-cleavage as a result of caspase-3 activity was analyzed by western blotting as a way to demonstrate apoptosis in **Paper I**.

In **Paper I**, Senescence Cells Histochemical Staining Kit based on the X-gal staining of cells was used and the percentage of β-galactosidase expressing cells was observed in a light microscope. Briefly, in senescent cells β-galactosidase catalyzes the hydrolysis of β-galactosides into monosaccharides and gives them a distinct blue color.

In **Paper I**, cell division was analyzed by carboxyfluorescein succinimidyl ester (CFSE) staining and subsequent analyses by FACS as described by Quah et al. [184]. CFSE is a cell-permeable agent which labels long-lived intracellular molecules with a carboxyfluorescein which is a fluorescent dye. Hence, when cell division occurs, the progeny of the CFSE-labeled cell are endowed with half of the carboxyfluorescein-tagged molecules [184].

MTS proliferation assay (CellTiter 96 AQueous non-radioactive cell proliferation Assay (Promega, SDS, Falkenberg, Sweden)) was used in **Paper I** as an additional cellular proliferation assay. The MTS assay labels cells with a salt that in viable cells with functional mitochondria is converted to formazan crystals [185]. The resulting formazon crystals are dissolved in a SDS-containing buffer and their absorbance measured at 595 nm in a spectrophotometer is proportional to the number of viable cells.

In **Paper III** and **Paper IV** proliferation post siRNA treatment alone or in combination with IR was also examined by manual counting of trypan blue positive and negative cells in Bürken chambers.

3.4.2 Cellular fractionation and immunoprecipitation

In **Paper IV** the expression level of Ephrin B3 to EphA2, EphA4 and EphA5 and the phosphorylation of the DDR components pDNA-PKcs (S2056), pATM (S1981) and γH2AX (S139) were analyzed in plasma membrane and cell nucleus fractions pre and post IR of NSCLC cells. For that purpose cell extracts were fractionated by using the Qproteome cell compartment kit (#37502, Qiagen, Germany) and analyzed by western blot. The western blot membranes were probed with Caveolin-1 and Histone H3 to reveal membrane or nuclear fraction purity respectively.

In order to determine the binding partners of Ephrin B3, immunoprecipitation was carried out in **Paper II-III**. In **Paper II** a Pierce Direct IP kit (prod #26148 Pierce/Thermo) was used according to manufacturers´ instructions with the modification that UREA buffer (6M urea and 2% SDS in 200mM Ammonuimbicarbonate and proteases inhibitors (Roche, Mannheim, Germany)) was used instead of elution buffer. The reason for the modulation in the protocol was that the samples by this approach also could be used for masspectrometry later on. In **Paper III** 800µg of total cell lysates of U-1810, H23 or U-1752 cells was lysed in buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 and 5% glycerol).Protein G-Sepharose beads (Millipore) was used in order to fish out the immunoprecipitation conjugates and IgG (#12370, Millipore) was applied as a negative control. The immunocomplexes and input was both in **Paper II-III** loaded onto a gel for western blot analysis.

3.4.3 Proximity ligation assay

Proximity ligation assay (PLA) offers a way to study protein-protein interaction [186]. In PLA, two primary antibodies from different species that recognize the antigens of interest are applied followed by secondary antibodies conjugated with PLA probes specific for each of the primary antibodies. Throughout ligation, where oligonucleotides and ligase are added, hybridization of the two PLA probes will start and they will join if the antibodies have bound in close proximity. The amplification part with fluorescently labeled oligonucleotides and polymerase acts as a rolling-cirle amplification (RCA), generating repeated sequences as a products, that can be detected as a fluorescent spots under the microscope [186]. In **Paper IV**, this method was used in order to analyze the interaction of Ephrin B3 with EphA2, EphA4, EphA5, DNA-PKcs (S2056), pATM (S1981) and γH2AX (S139). PLA probes were obtained with the Duolink II assay kit (OlinkBioscience, Uppsala, Sweden), DAPI (Sigma Aldrich) in the mounting medium stained the cell nucleus and an epiflourescent microscope Axioplan 2, Zeiss) with a 100-W mercury lamp, a CCD camera (C474Δ95, Hamamatsu) and emission filters was used for visualization of DAPI (to reveal cell nucleus) and Texas Red (to examine PLA probe labelling) respectively.

3.4.4 Immunoblotting and Real-time quantitative PCR

Western blot (WB) and Quantitative real-time PCR (RT-QPCR) was used as analytical methods in **Paper I-IV** for protein/RNA analysis respectively and was also used to validate siRNA efficiency. In **Paper I-II** proteins were extracted using UREA buffer (6M urea and 2% SDS in 200mM Ammonuimbicarbonate and proteases inhibitors whereas in **Paper III-IV** proteins were extracted using RIPA buffer ((50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Igepal, 5 mM EDTA (pH 8.0), 0.1% SDS supplemented with PMSF (1 mM) and proteases inhibitors. For all experiments in **Paper I-IV** sonication resolved membrane proteins. To determine protein concentration, bicinchoninic acid (BCA) assay (Interchim, MontiuconCedex, France) was used. For smaller proteins (>90kDa) 4-12% Bis-Tris gels was chosen for separation whereas for larger proteins (<90kDa) 3-8% Tris-Acetate NuPAGE® gels (Invitrogen AB, Stockholm, Sweden) was applied.

For RTQ-PCR, total RNA was extracted by Qiagen RNeasy kit (Sollentuna, Sweden) and reversed transcribed (1µg) to cDNA (2.5 µM random hexamer primers, 2 mM dNTPs, 5.5 mM MgCl, 8 U RNAse Inhibitor, 25 U MultiScribe Reverse Transcriptase in reverse transcription buffer (Applied Biosystems)) with heating for 25°C, 10 min, 37°C, 1 h and extension at 95°C for 5 min. 1µl cDNA was mixed with Taqman Fast Advanced Master Mix and primers for EphA2 or EphrinB3, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Relative expression values were calculated using the $2^{-\Delta CT}$ formula and analyses were carried out on ABI Prism 7900HT Sequence detection system (applied Biosystems).

3.4.5 Clonogenic survival assay

The rationale for clonogenic survival assay is given in **section 3.1** and was used in **Paper IV** to examine the difference in clonogenic capacity of Ephrin B3, EphA2 and EphA4 expressing versus non-expressing NSCLC cells in combination with irradiation. NSCLC U-1810 cells were seeded as monolayers and at a confluency of 70-80% transfected with non-targeted or the specific siRNA for 24h. Cells were thereafter either mock-irradiated or irradiated with 4 Gy. At this point cells were counted and 5,000 cells was seeded in 10 cm dishes and allowed to form colonies over 9 days. The clones in the dishes were thereafter stained with Giemsa and quantified with the cell survival relative to Non-target siRNA and irradiated cells determined.

3.4.6 Migration and invasion assay

To measure migration and invasion of tumor cells transwell assay is commonly used [187] and was applied in **Paper III** for that purpose. Thus for migration a transwell insert (Transwell: Millipore, cat.no PIEP15R48, MA) containing a porous filter were applied on which the cells either transfected with non-targeted or Ephrin B3/EphA2 siRNA were applied. In order to synchronize the cell cycle and make the cells more prone to migrate towards the serum, cells were serum starved for 24 h prior to the assessment. The principle of the transwell assay is that cells which have migration capacity will move towards the media

containing serum in the bottom of the insert which act as a chemoattractant and the cells will end up on the bottom of the filter side. The resulting cells on the membrane were fixed in 2.5% glutaraldehyde solution and visualized by staining with 0.5% crystal violet solution and counted by light microscope. In order to examine the invasion potential of NSCLC CL1-5 cells with or without Ephrin B3 or EphA2 expression, a modified version of the transwell assay was applied in which the insert was covered with growth factor-reduced matrigel (2µg, Becton Dickinson). The concept behind this assay is that invasive cells will degrade the matrigel and move towards the bottom of the insert. Cells ending up on the filter was similarly fixed and stained and quantified as for the migration assay.

3.5 OMIC BASED ANALYSIS AND BIOINFORMATICS

Two omics based methods were chosen to explore radiation therapy sensitizing strategies (**Paper I**) and proliferative signaling in response to Ephrin B3 blockade in NSCLC cells (**Paper II**) respectively. These two methods and the principle steps are described in brief below.

3.5.1 Gene array analysis to identify RT sensitizing targets

In **Paper I** Affymetrix-based gene array on the Affymetrix plattform (Affymetrix Inc, CA, USA) was used to identify differentially expressed genes in NSCLC U-1810 cells after treatment with either of the two staurosporine analogues PKC412 or Ro 31-8220 alone or in combination with RT. PKC412 was previously reported to confer RT sensitivity in NSCLC while the opposite was evident for Ro 31-8220 [94, 178]. For the gene expression profiling Affymetrix U133 2.0 plus GeneChip was used on three biological replicates of NSCLC U-1810 cells treated irradiated with 8 Gy and at 24 h post irradiation pulsed for 4 h with either PKC412 or Ro 31-8220. Using RNeasy Mini kit (RNeasy Midi Handbook, Qiagen, KEBO Lab Sweden) according to the manufacturers´ instructions, mRNA of untreated or treated with PKC412 or Ro 31-8220 and/or irradiated U-1810 cells was isolated. cDNA and cRNA synthesis, labeling and hybridization was made in a reaction where cRNA also was biotin-labelled using the In-Vitro Transcription (IVT) kit (Affymetrix Inc). A streptavidin phycoerythrin conjugate was used for the probe hybridization followed by amplification with a biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA) to which a PE-conjugate linked with streptavidin was hybridized. The fluorescent signals from hybridized probes on the chip were imaged by scanning the chip on a fluorometric scanner (Affymetrix Scanner). The obtained data were processed using GeneSpring GX software (AgilentTechnologies) in which the probes for the different gene transcript were summarized. To normalize the gene expression two different methods were used. First the gene expression on each chip was normalized to allow for differences in labelling of the probes by dividing each individual gene of the chip with the 50th percentile of all signals from all probes on that chip. In the next step each gene were normalized against all its values in the different samples in the biological replicate by taking its median expression level across the treatments. These normalized genes were annotated to their different treatments i.e. untreated, PKC412, Ro 31-8220, IR, IR+PKC412 or IR+Ro 31-8220 and ANOVA (parametric test, variances assumed

equal) was used to compare the individual genes in each biological replicate to generate list of significantly up or down regulated genes in IR+PKC 412 vs IR+Ro-318220. By RT-QPCR the expression pattern of some selected genes from the gene array was confirmed.

3.5.2 Phosphoproteomic profiling of Ephrin B3-driven signaling

Phosphorylated peptides are less abundant compared to non-phosphorylated ones and are also because of the poor ioniziation of the phosphopeptide in the MS (mass spectrometry) difficult to analyze in complex cellular sample. Enrichment methods for the phosphpeptides are therefore required. In **Paper II**, phosphorylated peptides from NSCLC U-1810 cells treated with either non-targeting siRNA or siRNA targeting Ephrin B3 were therefore enriched in two different ways. First Strong cation exchange (SCX) chromatography was used in which the SCX column was linked to an Agilent 1200 LC system. In SCX peptides are fractionated based on their net charges where peptides with low positive net charge elute earlier [188]. In order to capture as many peptides as possible in each of the samples, the sample was run three times on SCX, peptides eluted into 12 different fractions and identical fractionation numbers were pooled. Next each of the pooled fractions was subjected to enrichment for phospho-peptides in which TiO_2 magnetic beads (Mag SepharoseTM, GE Healthcare) were used. The principle for enrichment is that the negative phosphate groups on the phosphorylated peptide will bind to the positive titanium charges on the beads while non phosphorylated peptides will not [189, 190]. The peptides that were bound to the beads were after washing eluted in Amonia-based basic buffer (pH>11). In the last step, each sample from the 12 fractions enriched for phosphopeptides was injected into online HPLC-MS performed on a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo) to reveal their corresponding MS spectra. Obtained MS spectra were searched against the Mascot2.2 (Matrix ScienceLimited, London, U.K.) and peptides with annotation of 95% confidence got their MS spectra transferred into Protein center (PROXEON, Thermo Fisher Scientific) in which the phosphorylated sites of the peptides in the different samples were compared and visualized. These analyses generated a list of protein identities corresponding to the phosphopeptides identified in the three MS replicates analyzed. In order to sort out relevant signaling events and generate hypothesis from the achieved phosphoproteomic data in relation to Ephrin B3 siRNA blockade in NSCLC cells, the Ingenuity Pathway Analysis software (IPA) (Ingenuity Systems, Inc., RedwoodCity, CA) was applied. For the IPA analyses protein identities from either treatment were loaded into the software which is based on public and manually curated data and IPA sorted the data into putative signaling networks and processes. From these analyses hypotheses were generated and candidate proteins were thereafter manually evaluated in Protein Center (PROXEON, Thermo Fisher Scientific) for their differential phosphorylation. Validation of the information and hypothesis obtained was done by using siRNA, immunoprecipitation and western blot.

3.6 ANALYSIS OF CLINICAL LC MATERIAL

A cohort of 104 NSCLC cases where 92 specimens contained sufficient material, were used in **Paper III** to evaluate Ephrin B3, EphA2 and Ephrin A1 expression. The complete

information on the clinical parameters of the NSCLC cases is given in **Paper III** but is described in brief below. Tumor tissues were obtained from patients operated with curative resection of the tumor at the Department of Cardiothoracic Surgery, Karolinska Hospital, Stockholm, Sweden, between 1988 and 1992. The patient cohort consisted of 43 cases of adenocarcinoma and 49 cases of squamous cell carcinoma. No CT or RT was administered prior to surgery. The tumors used were formalin-fixed and paraffin embedded (FFPE) and a tissue micro array (TMA) was constructed. Slides from this TMA was used and stained with Ephrin B3, Ephrin A1 or EphA2 antibodies. This was followed by incubating the slides with a biotinylated-conjugated secondary antibody and an avidin-biotin-linked peroxidase substrate. A trained pathologist blinded to the study performed a semi quantitative scoring of the immunohistochemistry staining of the specimen where the intensity of immunopositivity was ranked from low score 1 to high score 2-3. The study was ethically approved by the Karolinska Institutet ethical committee (2005/588-31/4).

4 RESULTS AND DISCUSSION

4.1 PAPER I

Inhibition of Ephrin B3-mediated survival signaling contributes to increased cell death response of non-small cell lung carcinoma cells after combined treatment with ionizing radiation and PKC 412.

Intrinsic and acquired resistance impedes the clinical use of RT in treatment of NSCLC. It has previously been shown that adding the PKC inhibitor and staurosporine analogue PKC412 but not Ro 31-8220 sensitized RT resistant NSCLC cells to treatment and that this combination increased apoptotic- and mitotic catastrophe signaling [94, 178].

In this study, we aimed to find novel RT sensitizing targets in NSCLC by using global gene expression profiling. We confirmed previous results [94, 178] and showed that a combination of PKC412 and IR resulted in decreased proliferation but also increased apoptotic signaling via a caspase-3-mediated route. In search for putative targets involved in the sensitization of NSCLC cells to RT by PKC 412, a total gene expression profiling was carried out by Affymetrix gene array. By analyzing genes that showed a 1.5-fold altered expression in the combined treatments of IR and PKC412 or Ro 31-8220, a total of 140 or 179 genes were found to be up-regulated and 253 or 425 genes were down-regulated by either treatment respectively. Some of these genes were, based on public domain data reporting on their relevance to tumor cell signaling in general, selected for validation.

The Eph receptor ligand Ephrin B3 was a gene found to be down-regulated and was validated by RTQ-PCR. A role of Ephrin B3 in IR-induced cellular response and cell death remained at this point elusive but given the described role of Eph signaling in tumors [117] we focused onto Ephrin B3 as a RT sensitizing target. By blocking Ephrin B3 expression using siRNA in combination with IR, NSCLC cells were sensitized to IR as revealed by altered cellular morphology, decreased proliferation and increased apoptosis. It was previously shown that adding PKC412 on top of RT in NSCLC cells may increase cells which commits mitotic catastrophe [94], a form of cell death executed in response of incorrect entry of cells into mitosis [191]. Inhibition of Ephrin B3 expression followed by IR indeed increased mitotic catastrophe. Senescence is characterized by a decreased cellular proliferation, elongated phenotype and an increase in cell size [192]. Such cellular morphology was also observed when combining Ephrin B3 silencing with IR in NSCLC cells. Moreover, quantification of the number of cells with β-galactosidase positivity showed increased number upon ablation of Ephrin B3 expression and IR. It has been reported that $p27^{kip1}$ expression is highest in the G_0 cell cycle phase [193] and in addition to $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ [194] $p27^{kip1}$ is a known biomarker of senescence. Analyses of cell cycle progression by flow cytometry and proliferative signaling by western blotting showed that Ephrin B3 suppression in combination with IR decreased IR-induced G_2 arrest and upregulated $p27^{kip1}$ expression. However, both $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ showed a decreased expression in NSCLC cells where Ephrin B3 blockade was combined with RT. Thus our results suggested that neither $p21^{WAF1/Cip1}$ nor

p16^{INK4a} were responsible for the cell cycle or induction of senescence that was observed upon Ephrin B3 inhibition alone or in combination with IR. Instead obtained results pointed towards a function of p27^{kip1}in the recorded effect on either process.

In NSCLC cells with inhibited Ephrin B3 expression phosphorylation of pAkt (Ser473), pP38 (Thr180/Tyr182) and pERK (Thr202/Thr204) was decreased whereas Ephrin B3 inhibition followed by IR did not alter pERK (Thr202/Thr204) phosphorylation. Eph signaling is known to influence cellular proliferation by altering MAPKs and PI3K/Akt phosphorylation [116, 123] and here we showed for the first time that the Eph ligand Ephrin B3 also is involved in such signaling in NSCLC cells. In summary, we identified Ephrin B3 as a putative driver of RT resistance involving both altered proliferation-and cell death signaling as illustrated in **Figure 5**.

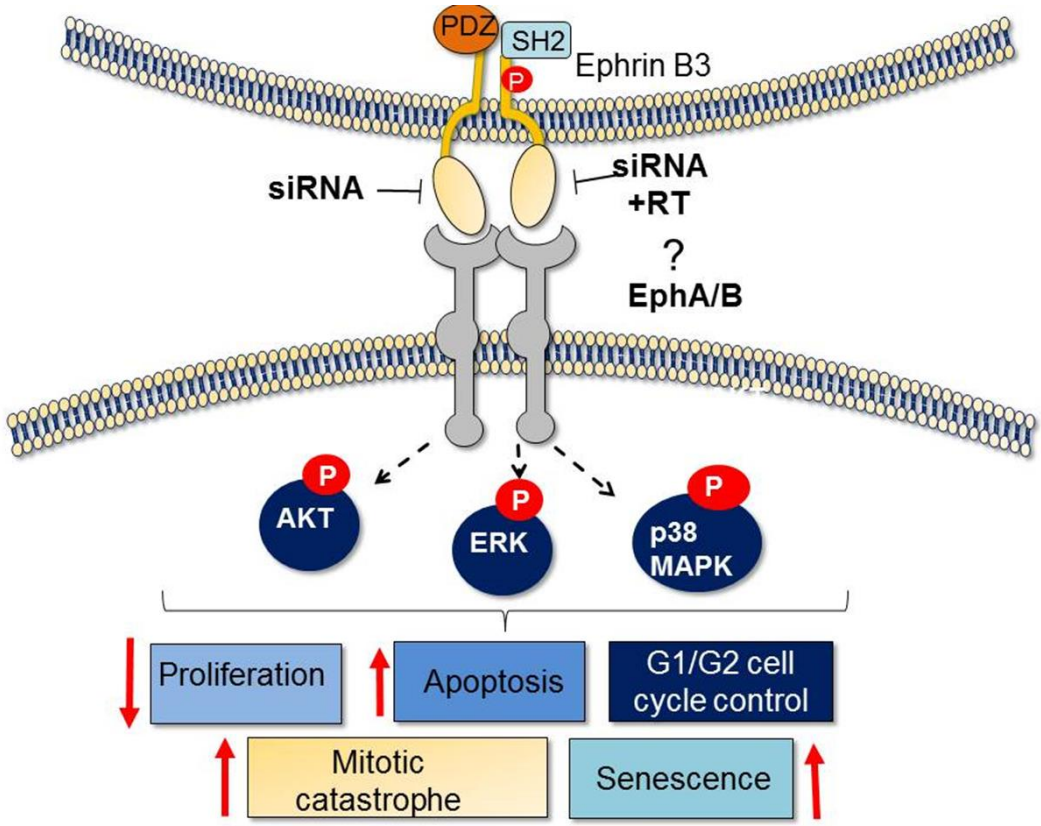

Figure 5: Summary of findings in Paper I.

4.2 PAPER II

Phosphoproteomic profiling of NSCLC cells reveals that Ephrin B3 regulates pro-survival signaling through Akt1-mediated phosphorylation of the EphA2 receptor.

In **Paper I** we identified Ephrin B3 as a RT sensitizer and driver of NSCLC cell survival. In **Paper II** the aim was to reveal signaling networks that Ephrin B3 utilizes to regulate NSCLC proliferative capacity. An unsupervised, global, "bottom up" phosphoproteomic approach was taken in which changes in phospho peptides of NSCLC cells with or without Ephrin B3 expression were analyzed. First, we demonstrated that ablation of Ephrin B3 expression in

NSCLC cells caused a pronounced alteration in cell morphology relative to non-targeting siRNA and ceased proliferation. In order to reveal corresponding alterations in the phosphoproteome of NSCLC cells with or without siRNA-mediated Ephrin B3 suppression, total cell extracts were isolated, lysed after which all cellular proteins were digested into peptides. To enable in depth analyses of phosphorylated peptides and reveal relevant signaling aberrations on a global scale, we here applied SCX fractionation followed by TiO_2-based magnetic beads capture, the former to reduce sample complexity and the later to capture the phosphorylated peptides relative to non-phosphorylated peptides. On the enriched phosphorylated peptides in the different fractions obtained from NSCLC cells with or without Ephrin B3 siRNA treatment nano-LC and mass spectrometry analysis were applied thereby identifying phopsho peptides and corresponding phospho proteins. In total, 1083 unique phosphorylated proteins were identified, 150 in Ephrin B3 expressing and 66 in Ephrin B3 non-expressing NSCLC cells respectively. A much higher number of phospho proteins were found in NSCLC cells with intact Ephrin B3 expression which was not surprising as we found a pronounced block in proliferation upon Ephrin B3 knockout.

On the proteins that consistently were found to be differentially phosphorylated in non-targeted versus Ephrin B3 siRNA treated NSCLC cells, Ingenuity pathway analysis (IPA) was used to select candidates for further analyses. In accordance with the pronounced alteration in cellular morphology upon Ephrin B3 siRNA, IPA also pointed out networks annotated to such cellular functions to be different in Ephrin B3 siRNA expression versus non-target siRNA expressing cells. Given that IPA mainly is based on transcription data we also applied manual evaluation of the phosphoprotein data using the Protein center (PROXEON/Thermo FisherScientific) and the associated information generated for each protein by this program. Results from this analysis indicated a putative action mechanism where active EphA2 and Focal adhesion kinase 1 (FAK1) were involved in EphrinB3-mediated cell survival signaling (**Figure 6**). Thus we found EphA2 to be phosphorylated on Ser897 and FAK1 on Ser538 only in NSCLC cells which expressed Ephrin B3. It was previously reported that in glioma and prostate cancer cells, EphA2 is phosphorylated on Ser897 by Akt1 only in absence of its ligand Ephrin A1 [150]. In line with these findings we found that inhibition of Ephrin B3 expression decreased Akt1 Ser129 phosphorylation in NSCLC cells. Akt1 Ser129 phosphorylation is reported to be activated by CK2 [195] which in turn activate the chaperone HSP90AA1 and rescues EphA2 from proteasome-mediated degradation [196] (**Figure 6**). Indeed analyzing the phosphoproteome of NSCLC cells revealed that Ephrin B3 blockade specifically abolished Akt Ser129 phosphorylation and HSP90AA1 Ser263 phosphorylation whereas CK2 expression was evident regardless if Ephrin B3 expression was blocked. Thus in summary our data suggest a role of Ephrin B3 and EphA2 in driving NSCLC cell survival signaling.

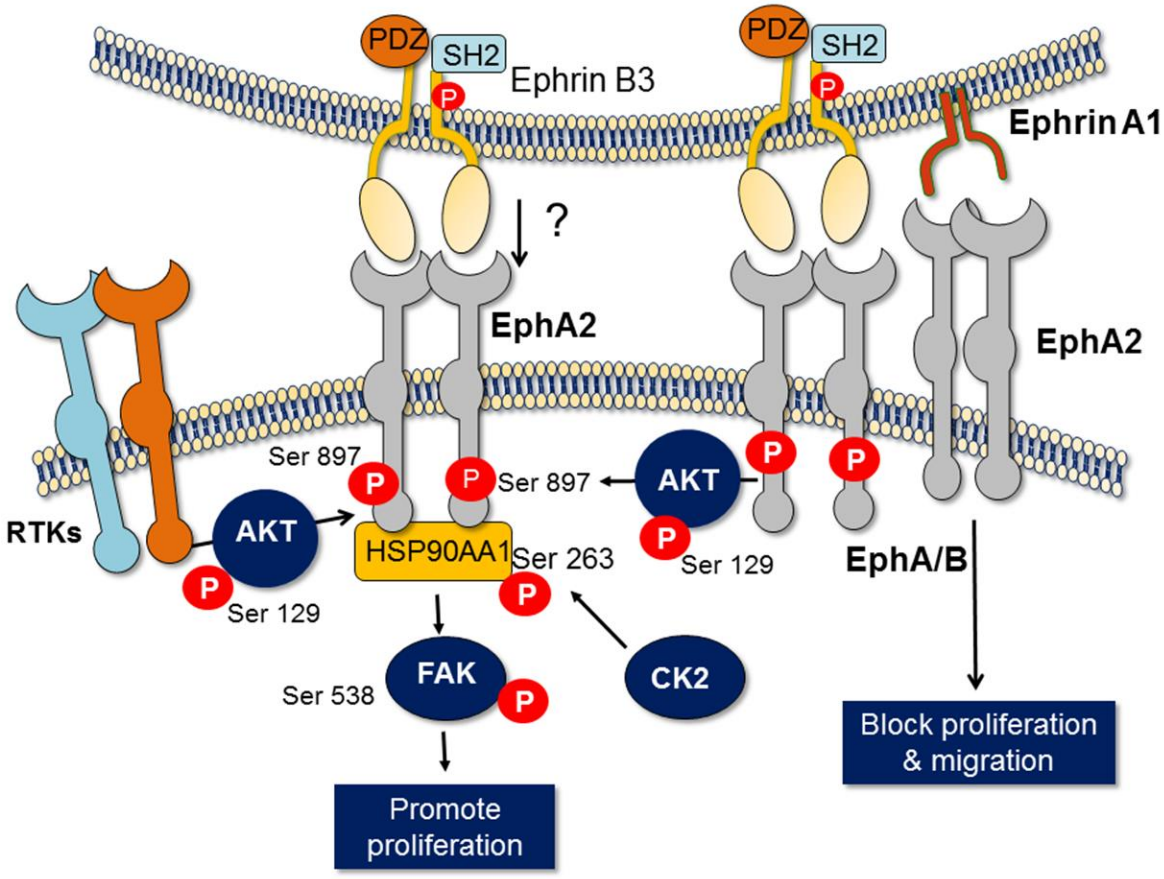

Figure 6: Summary of findings in Paper II.

4.3 PAPER III

Ephrin B3 interacts with multiple EphA receptors and drives migration and invasion in non-small cell lung cancer.

In Paper III the aim was to analyze Ephrin B3 and EphA2 for their role in controlling proliferation, migration and invasion by analyzing in NSCLC cells of different histology *in vitro* and to understand the relevance of Ephrin B3 signaling *in vivo* using clinical NSCLC specimens. In **Paper I-II** we found Ephrin B3 to be expressed and be of importance for proliferation and RT response of NSCLC LC/AC U-1810 cells and here we therefore analyzed the expression of Ephrin B3, different Ephs i.e. EphA2, EphA4, EphA3 and EphA5 but also the EphA2 ligand Ephrin A1 in a panel of NSCLC cell lines of different histology i.e. adenocarcinoma (AC), squamous cell carcinoma (SQ) and mixed large cell adenocarcinoma carcinoma (LC/AC). Analyses demonstrated that Ephrin B3, EphA2 and EphA3 were expressed in the majority of the NSCLC cell lines examined but to various magnitudes and with no clear correlation to histology. EphA2 has in NSCLC and other tumor types been shown to control proliferation and migration [150] and Ephrin B3 has been reported to regulate migration [183]. Here we for the first time demonstrate such an effect of Ephrin B3 in NSCLC cells by using siRNA. Thus in both AC and LC/AC NSCLC cells Ephrin B3 reduced proliferation whereas in SQ NSCLC cells no effect was evident. The decrease in

proliferation was about the same magnitude as was achieved by a blockade of EphA2 in either cell type.

Importantly by using matrix gel coated transwell assay we found that both endogenous Ephrin B3 and EphA2 controlled the invasive potential of NSCLC cells. Moreover, migration capacity in AC, LC/AC and SQ NSCLC cells was also reduced to a magnitude similar or even more pronounced than was seen upon EphA2 siRNA blockade in the same cells. Eph signaling has previously been linked to EMT (epithelial to mesenchymal transition) signaling and in particular E-cadherin was demonstrated to influence EphA2 stability [127]. Indeed western blot profiling of E-cadherin, and two other EMT regulating proteins vimentin [197] and Rac [198] revealed an increase in E-cadherin and vimentin expression while no major changes in Rac expression were found in NSCLC cells deprived of either Ephrin B3 or EphA2 expression (**Figure 7**).

In **Paper II** we found that Ephrin B3 expression controls EphA2 Ser897 phosphorylation, however the putative Ephs by which Ephrin B3 may exerts its effect in NSCLC cells remained elusive. In **Paper III** we therefore applied immunoprecipitation and demonstrate for the first time that Ephrin B3 binds EphA2, EphA4, EphA3 and EphA5 in NSCLC cells of different histology. Moreover, Ephrin B3 immunoprecipitation revealed that it was in complex with EphA2 Ser897 in which also Akt Ser129 and p38MAPK were found, a feature observed in all the three NSCLC cell lines examined.

The expression of Ephrin B3 in NSCLC clinical specimen has not previously been studied and given our *in vitro* analyses of a role of Ephrin B3 in control of NSCLC proliferation, migration and invasion we therefore analyzed its expression in a NSCLC patient cohort in relation to survival. By immunohistochemistry we demonstrated that Ephrin B3 is concomitantly expressed with EphA2 and Ephrin A1 in the majority of the NSCLC clinical specimen but in our material neither Ephrin B3 nor EphA2 could be linked to patient survival in the cohort as a hole. A higher level of Ephrin B3 expression was yet found in NSCLC AC relative to SQ ($P=0.047$) but the size of the clinical cohort precluded survival analyses to be performed. All in all, we found a novel role of Ephrin B3 in controlling NSCLC cell migration/invasion propensity and to be a partner of multiple Ephs in this tumor type.

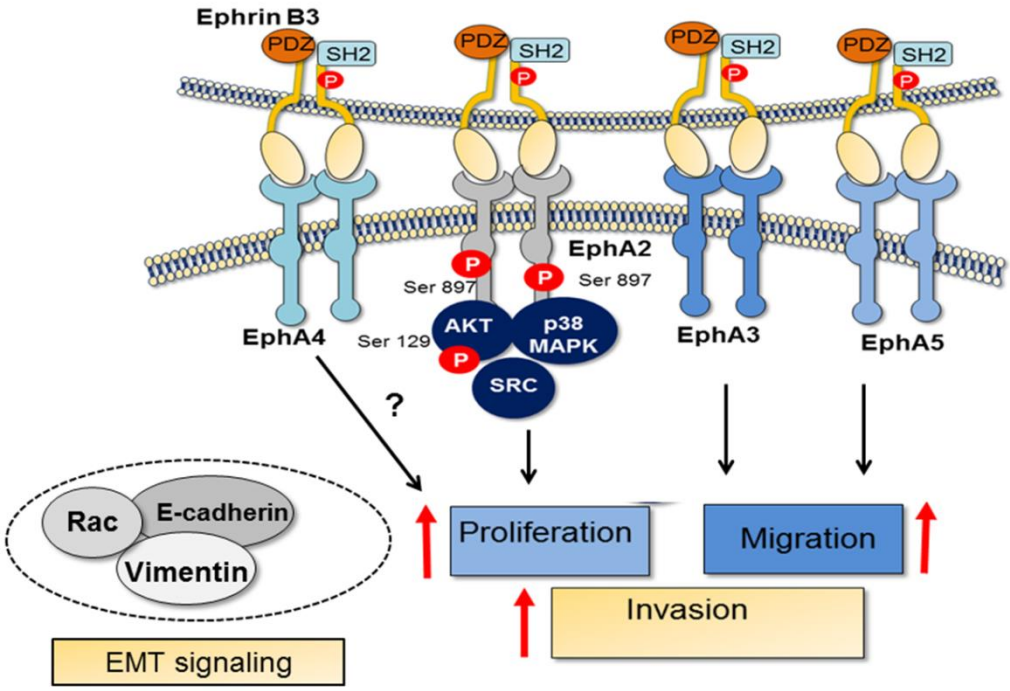

Figure 7: Summary of findings in Paper I-III.

4.4 PAPER IV

EphA2 and EphA4 influences DNA Damage Response (DDR) signaling in Non-small cell lung cancer and alter radiotherapy sensitivity.

A recent report showed that EphA5 controlled NSCLC RT response and directly interfered with DNA damage response (DDR) signaling via ATM [199]. In **Paper IV** we therefore intended to study if EphA2 and EphA4 influenced basal or RT-induced DDR signaling and if this could be used for RT sensitization of NSCLC cells.

Ephrin B3, Ephrin A1, EphA2 and EphA4 expression were first analyzed in a NSCLC cell line panel in relation to their radiation sensitivity measured as Surviving fraction 2 Gray (SF2) in clonogenic survival assay. No correlation between SF2 and protein expression levels was observed across the entire NSCLC cell line panel however a higher EphA2 expression was found in some of the most RT resistant NSCLC cell lines. Nevertheless, we found that inhibition of EphA4, Ephrin B3 and to some degree EphA2 expression in such RT resistant NSCLC cells decreased proliferation and colony forming potential upon IR.

By cellular fraction of RT resistant or- sensitive NSCLC cells into membrane and nuclear segments and by proximity ligation assay (PLA), the localization of EphA2, EphA4 and Ephrin B3 and their interaction with the DDR components pATM (S1981), pDNA-PKcs (S2056) and γH2AX (S139) were studied pre- and post IR. In both RT responsive and refractory NSCLC cells western blot analysis demonstrated Ephrin B3, EphA2, EphA4 and

EphA5 to be expressed in both the membrane and nuclear fractions already prior to IR (**Figure 8**). Accordingly PLA showed EphA2, EphA5 and to some degree EphA4 to interact with Ephrin B3 in the nucleus but with no differences pre- and post IR. Importantly, we for the first time demonstrated that EphA2, EphA4, and Ephrin B3 interacted with pATM (S1981), pDNA-PKcs (S2056) and γH2AX (S139) in NSCLC cells and we observed alterations in these interactions upon RT (**Figure 8**). In line with the previous report on the engagement between EphA5 and pATM (S1981) [199], we confirmed this interaction in the present study. In conclusion, **Paper IV** reveals that in addition to Ephrin B3, EphA2 and EphA4 are likely also molecular targets that can be used for RT sensitization of NSCLC cells. Moreover, our data thus suggests that interfering with these targets may beyond influencing RT-induced cell death propensity and cell cycle progression (**Paper I**) also intervene with DDR as part of their action mechanism.

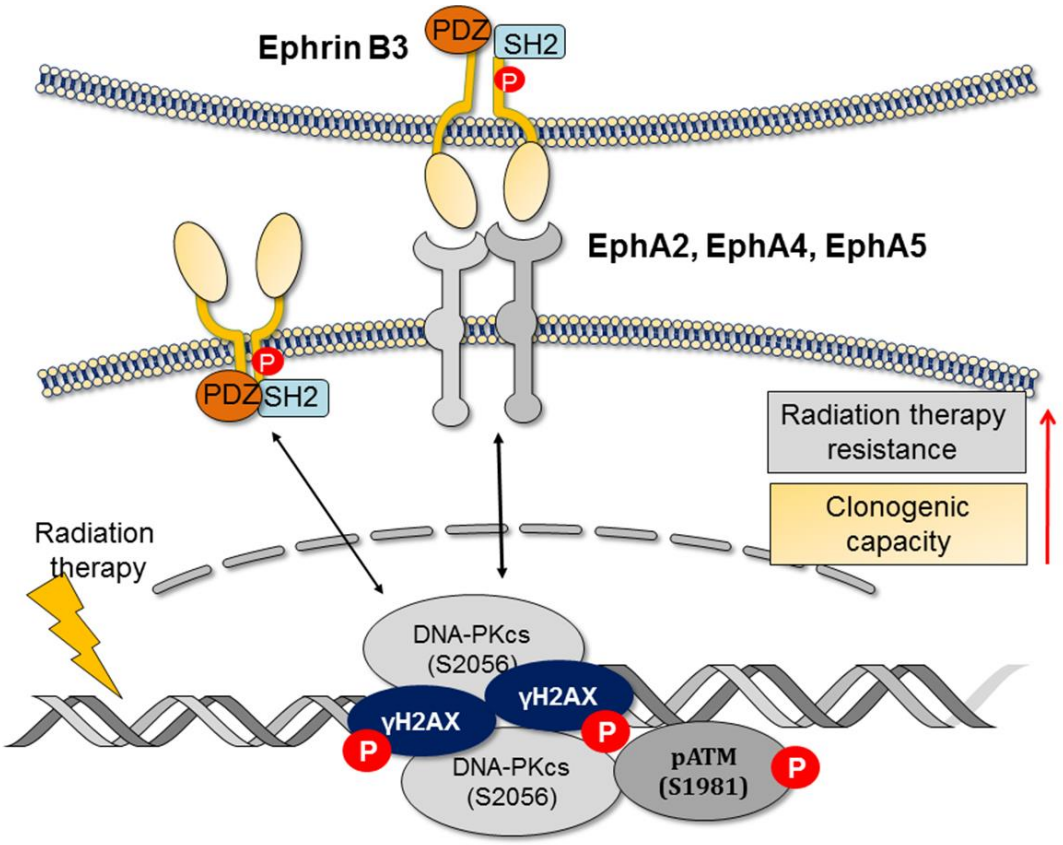

Figure 8: Summary of findings in Paper IV.

4.5 EPHRIN AND EPH SIGNALING IN NSCLC: LESSONS LEARNED IN CONTEXT OF CURRENT KNOWLEDGE OF THE FIELD

In **Paper I**, Ephrin B3 was identified as a RT sensitizing target in NSCLC and in **Paper IV** also EphA2 and EphA4 were identified as molecular switches that enable RT sensitization in NSCLC cells. Ephrin B3 was in **Paper I** demonstrated to control proliferation, cell cycle progression and to protect from RT-induced cell death via multiple routes. Results from **Paper IV** indicated that in addition to Ephrin B3 also EphA2 and EphA4 also intervened with DNA damage response (DDR). Interestingly, our results of EphA2 and EphA4 as RT

sensitizing targets are novel but in line with the report on EphA5 by Staquinici et al. who described EphA5 as a RT response modulator in NSCLC cells by interacting with the DDR component ATM [199].

The interest of targeting EphA2 which is overexpressed in various tumors has emerged and multiple strategies for such targeting have been described. This therapeutic avenue on EphA2 is further emphasized since we in this thesis show that EphA2 also interact with DDR signaling upon RT.

The interconnection between growth factor receptors and DDR signaling is most well characterized for EGFR and IGF-1R [200, 201]. Thus both EGFR and IGF-1R are reported to interact with DNA-PK when cells are subjected to RT [87, 202, 203]. RT not only activates DNA repair but also EGFR [202, 203] and IGF-1R [87] which subsequently impacts ERK and Akt signaling and as a result IR-mediated apoptosis is inhibited. Moreover, EGFR triggered Akt has been shown to directly control DNA-PKcs phosphorylation at Ser2056 and Thr2609 the later which in turn regulated Akt phosphorylation [201].

Since Ephrin B3 and EphA2/EphA4 similar to EGFR and IGF-1R in **Paper IV** were found to intervene with DDR signaling and given that we found that EphA2 ablation *per se* and as a consequence of Ephrin B3 inhibition results in altered Akt phosphorylation (**Paper I-III**), it would be interesting to further elucidate if the phosphorylated Akt that we found in EphA2 immunocomplexes in NSCLC cells (**Paper III**) may in fact control phosphorylation of DNA-PKcs or other DDR components.

Blocking EGFR signaling has indeed been shown to sensitize tumor cells to RT as illustrated in cells with K-RAS mutation where a decreased DNA-repair and increased RT sensitivity upon pharmacological EGFR blockade was reported [204]. With respect to results in **Paper IV** it would therefore also be of interest to study the effect on EphA2 when blocking EGFR and PI3K signaling upon RT since additional blockage of EphA2 using for example a small kinase inhibitor could give rise to an even more pronounced sensitivity to RT in NSCLC cells. In addition to EphA2 it would be relevant to study if Ephrin A1 administration or blocking Ephrin B3 interaction with Ephs would be an even more potent way in context of EGFR blockade since both such strategies may directly target several Ephs or indirectly influence other RTKs such as IGF-1R.

DNA DSBs formation as a result of oncogenic stress have been associated with induction of senescence and reported to be suppressed upon ATM inhibition in a mouse tumor model resulting in increased invasiveness [205]. In **Paper I** we showed that inhibition of Ephrin B3 in combination with IR resulted in increased senescence cells and cellular arrest in G_0/G_1-phase of the cell cycle. Moreover, in **Paper IV** Ephrin B3 was shown to interact with pATM (S1981) and we found that this interaction increased after RT. Thus it would be interesting to analyze if forced overexpression of Ephrin B3 and/or EphA2/EphA4 also would lead to a decrease in pATM (S1981) and reduced senescence in RT-sensitive NSCLC as such evidence would strengthen the case of Ephrin B3/EphA2/EphA4 as RT sensitizing targets.

EphA2 has been shown to exert ligand-independent pro-oncogenic functions as a result of reduced EphrinA1 ligand expression and altered Akt activation [151]. Accordingly administration of Ephrin A1 has been reported to decrease invasiveness and tumorigenic potential [206, 207]. Moreover, EphA2 has been reported to be a substrate of Akt in different tumor forms including glioma and prostate cancer [150, 156]. Hence, in glioma cells addition of exogenous Ephrin A1 was reported to block EphA2 Ser897 and Akt Ser129 phosphorylation and impair migration and invasion [150]. In line with these reports we in **Paper II** we found EphA2 to be phosphorylated on Ser897 and Akt on Ser 129 only when Ephrin B3 expression was maintained in NSCLC cells indicating that Ephrin B3 engagement with EphA2 might trigger another signaling than those elicited upon Ephrin A1 binding. Moreover, treatment with exogenous Ephrin A1 was found to decrease phosphorylation of FAK and prevent cell migration [208] hence further supporting another function of Ephrin B3 with respect to EphA2.

Akt1 Ser129 phosphorylation was furthermore reported to be regulated by CK2 in Jurkat cells [195] and in turn activate the chaperone HSP90 [209] prohibiting EphA2 from proteasome- mediated degradation [196]. In **Paper II** we indeed showed that HSP90AA1 Ser263 phosphorylation was evident only in NSCLC with intact Ephrin B3 expression illustrating that such mechanism with respect to EphA2 may also exist in NSCLC cells.

In **Paper III** we found that Ephrin B3 is a ligand of EphA2 and reported that inhibition of either Ephrin B3 or EphA2 decreased proliferation, migration and invasion as well as EMT signaling in NSCLC cells. Moreover, we demonstrate that Ephrin B3 is concomitantly expressed with Ephrin A1 and EphA2 in both NSCLC cells *in vitro* and also in NSCLC clinical material. We may from our data speculate that Ephrin B3 binding is more potent when it comes to activation of the EphA2 receptor and therefore the inhibitory action of Ephrin A1 that previously has been described [150] will be circumvented.

Notably, the Ephs has in general different functions in different tumor types and the site of EphA2 seems to matter. In both prostate- and breast cancer EphA2 phosphorylation is necessary to for the oncogenic potential of EphA2 [157-159] whereas other studies state that EphA2 phosphorylation causes tumor suppression [161] or that EphA2 phosphorylation is not needed to cause kinase activity and tumorigenic potential [128, 160]. Moreover, it has been shown that binding of Ephrin A1 to EphA2 results in tyrosine phosphorylation and EphA2 downregulation [131], thus acting as a tumor suppressor. Given the findings from **Paper II** further analysis of EphA2 phosphorylation on particular sites should be carried out in NSCLC cells with either Ephrin A1 or Ephrin B3 binding in order to understand their role in NSCLC cell signaling.

Our data generate a hypothesis of alternative binding or engagement with the EphAs than used by Ephrin A1 as the opposite effects on cellular signaling is apparent. It has been shown that in addition to the normal forward signaling and Ephrin binding to Eph in *trans* at the same time another Ephrin can bind to the same Eph in *cis* [130]. This novel reports generates an expansion of our hypothesis that Ephrin B3 may in contrast to Ephrin A1 bind EphA2 in

both *cis* and *trans* thereby enabling a different cellular signaling pattern. One may speculate that Ephrin B3 binding is more potent than Ephrin A1 engagement and therefore the inhibitory action of Ephrin A1 that previously has been described [150] will be circumvented by Ephrin B3 binding resulting in activation of EphA2. Further studies on Ephrin B3 engagement with EphA2 using surface plasmon resonance (SPR) are therefore warranted.

In **Paper III** we found EphA2 to bind p38MAPK which has indeed been shown to be in complex with IGF-1R thereby acting as a pro-survival factor and a blocker of RT- induced cell death [87]. Hence, EphA2 also might when bound to Ephrin B3 exert pro-survival signaling via this route and given our findings in **Paper IV** where also EphA2 blockade was found to cause RT sensitization, our data may also suggest such a route of signaling to control NSCLC RT sensitivity. It would indeed therefore be interesting to analyze how the EphA2 complex looks like in RT sensitive NSCLC cells and to what extent they have active p38MAPK upon RT.

In **Paper III** we demonstrate that Ephrin B3 also bind EphA3, EphA4 and EphA5. The role of EphA3 in NSCLC proliferation or migration has not been analyzed whereas inhibition of EphA4 was reported to promote migration [132] and suppression of EphA5 to block cell survival in NSCLC [199]. The results on EphA5 as a driver of proliferation and a RT sensitizing target is in line with our findings on EphA2 presented in **Paper III-IV**. EphA4 is a dependence-receptor and in absence of its ligand Ephrin B3, EphA4 is cleaved and subsequently undergoes apoptosis [210]. It has been demonstrated that EphA4 mRNA expression is, in contrast to EphA2, associated with improved outcome in NSCLC [132]. Moreover, *in vitro* studies have shown that overexpression of EphA4 in NSCLC cells with low endogenous EphA4 expression decreases ERK expression and inhibits migration and invasion [132]. These results are somewhat in contrast to our results which demonstrate Ephrin B3 to be a ligand of EphA4 and given that we found that a blockade of EphA4 expression sensitized NSCLC cells to RT. As we did not studied the role of EphA4 with respect to migration in our system we cannot rule out that it is a cell type dependent effect in which the expression of other Ephrin ligands may play a role.

In NSCLC patient samples, EphA2 expression was positively associated with a smoking history and a high EphA2 score predicted poorer overall- and progression-free survival of NSCLC patients [145, 146]. EphA2 also showed correlation to activated EGFR where a higher level of expression was observed in tumors with K-RAS mutations [145]. In our patient material AC displayed higher levels of Ephrin B3 compared to SQ and with respect to EphA2 there was a tendency of higher expression in AC. As for Ephrin A1 the expression was high both in AC and in SQ. We did not in contrast to Brannan et al., [145] found that EphA2 was linked to poorer outcome. Our analyses of Ephrin B3 and EphA2 in NSCLC in **Paper III** show that it is important to analyze different histological subtypes of NSCLC separately and then use bigger patient material to allow for subgroup analyses with respect to patient survival and prognostic potential. It would also be interesting to perform profiling of different Eph and Ephrin ratios in the NSCLC specimen to further understand their potential

as prognostic biomarkers in NSCLC. To get a bigger picture more parameters than patient survival should also be looked upon such as smoking history but also EGFR/K-RAS mutation status or IGF-1R expression as these all have been shown to influence EphA2 signaling [108, 145, 146]. As our *in vitro* data from NSCLC indicate that both Ephrin A1 and Ephrin B3 may compete for the same EphA2, and given that Ephrin B3 engagement results in EphA2 Ser897 phosphorylation, it would be of further value to study the Ephrin and Eph interaction *in situ* in NSCLC by using PLA. Finally, it will also be of importance to look into the different regions of the tumor specimen and sort out what Ephrins and Ephs operate in different rumor parts due to the tumor heterogeneity.

5 CONCLUSION AND FUTURE PERSPECTIVES

In this thesis I put evidence that Ephrin B3 alongside different Ephs control NSCLC cell proliferation, migration, invasion and RT response. I here would like to make some outlook on my findings in context of biomarkers and therapeutic avenues for NSCLC and other tumor types.

In NSCLC adenocarcinoma small molecules against EGFR mutations or towards the EML4-ALK fusion protein has resulted in a new era of precision cancer medicine strategies. However there are still some challenges such as the fact that these genetic aberrations of NSCLC are only found in 1-5% of all NSCLC cases and development of drug-resistance associated mutations or signaling aberrations constitute clinical problem [211, 212]. Despite the fact that new agents are being tested in clinic against the acquired EGFR resistance, there is expected that patients will eventually also develop acquired resistance to these agents [211]. Hence, there is a need to identify additional targets and novel ways to combat signaling aberrations of NSCLC and here I present some evidence of Ephrin B3 and associated Ephs in this respect.

In the constituting papers of my thesis it is demonstrated that Ephrin B3 and multiple Ephs control NSCLC proliferation, migration, invasion and response to RT illustrating that targeting Ephrin B3 for therapeutic purposes in NSCLC may hold potential alone or in combination with RT. As we in **Paper I** and **Paper III** found that ablation of Ephrin B3 or EphA2 expression decreased MAPK ERK, p38MAPK and Akt signaling, one may speculate that Ephrin B3 signaling via EphA2 or other Ephs are drivers of these pathways instrumental for controlling proliferation and invasion. Given that EphA2 and EGFR show functional interaction [208] and given that EGFR is known to control the MAPK/Akt signaling, such alternative pathway via Ephrin B3 and associated Ephs may drive resistance to treatment in EGFR mutated NSCLC cases. There are indeed multiple reports that support a role of EphA2 in erlotinib and gefitinib treatment response of NSCLC while a role of Ephrin B3 still remains to be established albeit our findings of their interaction point in such direction. Thus EphA2 has been found to be overexpressed in gefitinib resistant NSCLC cells and inhibition of EphA2 expression or treatment of such NSCLC cells with the multikinase inhibitor dasatinib, restored the sensitivity to gefitinib [208]. Moreover, Brannan et al., also showed that erlotinib treatment of responsive EGFR-mutant NSCLC cell lines decreased EphA2 expression [145, 146] and Koch et al., by phospho proteomic profiling demonstrated that EphA2 may drive EGFR resistance *in vitro* [208]. Recently an increased EphA2 expression level was reported in NSCLC upon erlotinib resistance [213, 214]. Thus targeting EphA2 by using a small molecule against the EphA2 kinase domain was reported to revert erlotinib resistance *in vivo* in mice causing a decreased EphA2 expression level [213]. However as EphA2 expression is reported to correlate with EGFR expression [145] targeting aberrant Ephrin B3 or EphA2 signaling will likely be a therapy that goes beyond EGFR-mutation

driven NSCLC. Based on our results showing that Ephrin B3 binds to multiple Ephs enabling migration and proliferation potential I propose that targeting the interaction of Ephrin B3 with Ephs would be more efficient than ablating EphA2 kinase as signaling through multiple receptors then could be blocked and the problem of poor kinase inhibitor selectively could be circumvented.

Current therapeutic strategies for EphA2 aims to target kinase signaling by mimicking Ephrin A1 ligand engagement using either small molecules, agonistic antibodies or by application of kinase inhibitors towards EphA2 kinase domain [108, 151, 213]. Indeed doxazosin, a small molecule agonist for EphA2 and EphA4 which act in a similar way as Ephrin A1, inhibit Akt and ERK kinase activities in an EphA2-dependent manner, resulting in EphA2 internalization and suppressed migration of prostate cancer, glioma cells and breast cancer [151]. However since tyrosine kinase inhibitors usually have poor specificity and since in the most cases Ephrin A1 ligand-dependent stimulation of Eph kinase activity is tumor suppressive, both these approaches may not be efficient [215]. Here targeting Ephrin B3 and EphA interaction may be a more promising strategy as we found this signaling to be pro-proliferative and to influence migration and invasion potential and also to go beyond EphA2 into other EphA receptors.

Deregulated EphA2 is reported in multiple tumor types and evidences indicate that targeting EphA2 signaling either alone or in combination with other agents may have a role in other tumor types than NSCLC. Thus EphA2 expression has been correlated to reduced overall survival in breast cancer [216] and inhibition of EphA2 was reported to restore sensitivity to trastuzumab [217]. Moreover, targeting of EphA2 in combination with the ER estrogen receptor α in breast cancer was reported to restore tamoxifen sensitivity [218]. In melanoma, EphA2 has been reported to be a mediator of vemurafenib resistance [219] and in colorectal cancer high EphA2 expression has been correlated with worse outcome in patients treated with cetuximab [220]. Given our findings of Ephrin B3 as an interactor of EphA2 it would be interesting to further explore as to what extent Ephrin B3 also could be used as a target for therapy in all these malignancies *per se* and in the context of other targeted therapies.

With respect to my findings on Ephrin B3 it would be highly interesting also to explore it for imaging purposes in PET (Positron Emission Tomography) imaging as a way to follow metastatic NSCLC and its response to therapy, such as RT. This is of particular interest since my work show Ephrin B3 and two of its interacting receptors EphA2 and EphA4 to contribute to RT resistance of NSCLC. PET labelled EphA2 antibody has been successfully used in monitoring breast cancer tumor growth [221], and PET labelled EphA5 was successfully described to monitor NSCLC growth, and to some extent RT response [199]. In our NSCLC panel I have shown that in contrast to Ephrin B3, the cells do not express all Ephs, and Ephrin B3 binds to several Ephs. Thus, the development of an Ephrin B3-directed PET labelled antibody could be interesting for observing RT response in NSCLC patients.

In conclusion, in this thesis I have put forward evidence that Ephrin B3 and its associated Ephs may offer a novel way to combat NSCLC and may be candidates for future precision or personalized therapy approach of this tumor malignancy.

6 ACKNOWLEDGEMENTS

First of all I would like to express my deepest gratitude to all the people that I have been working with or met during my PhD studies.

Dr Kristina Viktorsson, my main supervisor, thank you for seeing my potentials and giving me the opportunity to perform this PhD-project. You are very dedicated and enthusiastic to science and your work. Thank you for sharing your knowledge and for your guidance and encouragement, it has been very nice knowing that if I need, you will be there. Thanks for the scientific and non-scientific talks.

Prof Rolf Lewensohn, my co-supervisor and the head of the group, thank you for welcoming me to the group. Your dedication to science is extraordinary. I truly believe that the best place to be has been to working with you in your group. Your strong beliefs in translational research and the personalized cancer medicine concept show how passionate you are for contributing to improved cancer research and in better treatments for patients. You really want to make a change and I believe that you will.

Dr Petra Hååg, my co-supervisor, thank you for your positive and calm attitude and for always understanding. Your technical and methodological knowledge is outstanding. I really appreciate your friendly attitude, encouragement and for always being keen to help and gave advices. Thanks for your support whenever I needed it.

I would also like to thank all the co-authors and collaborators of the papers included in my thesis. Specially thanks to: **Professor Boris Zhivotovsky**, **Vitaliy Kaminskyy** and **Ali Moshfegh** for your input on the projects and **Sara Ståhl** for your great work in the first two projects.

Especially thanks to my brothers and sisters in the lab who always made me laugh and had the best dancing moves at my wedding: **Therese Juntti** not only did I find one of my best friends I also found a sister and your encouragements and endless support has meant a lot, you always make me smile, **Hogir Salim** I really miss your tales and laughter, **Dali Zhong** for all the laughter and your big heart I miss you and **Claudia Selck** you are actually a sunflower with the most beautiful voice. We all complemented each other so well ☺

Thanks to my friends in the Lewensohn goup: **Lovisa Lundholm** you are the most warmhearted person I know and I miss your smile in the lab corridor, **Metka Novak** you are one of the kindest person I know and joined the group at the best time you are truly an angel, **Katarzyna Zielinska-Chomej**, I will miss our talks and laughter and all the fun we have had

together both in the lab and outside the lab, thanks for your support. **Liselotte Hälleberg**, for always being there for me and for supporting me no matter what. Thank you **Ana Zovko** for your positive attitude and friendship, **Elham-Yektaei-Karin** for your kindness and **Adam Sierakowiak** for all the nice talks.

Also thanks to **Lena Kanter Lewensohn**, **Christina von Gertten**, **Leif Stenke**, **Marianne Langéen**, **Birgitta Mörk**, **Maria Palmquist**, **Luigi de Petris**, **Simon Ekman**, **Anders Nilsson**, **Gabriela Prochazka**, **Elisa Basmaci**, **Teresa Holmlund**, **Susanne Becker** and **Sonja Gustavsson** for creating a nice working atmosphere.

I would also like to thank former and new people at the department: **Sanaz Attarha**, **Pedram Kharaziha**, **Alireza Azimi**, **Alexandros Arvanitis**, **Jelve Zendegani**, **Xinming Wang**, **Mustafa Ismail**, **Haythem Ismail**, **Espen Hesselberg**, **Pieter De Koning**, **Nadeem Akbar**, **Mahdi Mojallal**, **Jessica Tu Mojallal** and **Carl-Henrik Shah**.

The friends I made for life: **Dhifaf Sarhan** for your positive attitude, **Khairun Munna Nahar** for always understanding me, **Pinar Akçakaya** for your friendship and kind smiles and **Susanna Bächle** I really miss our meetings after work ☺

My dear "kusinsystrar", **Nina Mola**, **Samira Efazat** and **Sahar Efazat** thanks for all your support, love and encouragement during this journey. Also thanks to **Leila Mola** for telling me you will do it! By best friend and sister **Tina Saberi** you always ask me when I will get finish with the PhD, now I can tell you it is done, thanks for showing me what strength is.

My wonderful uncle **Alireza Moula** you are truly passionate about research and knowledge and always encourage and believe in me. Thanks also to other relatives for simply being there and making me happy namely my aunt **Mahnaz Moula** and uncle **Mohammadreza Pourian** and all other **Efazat** and **Movla** realtives.

In the end I would like to thank two people who believed in me but unfortunately is not a part of this world anymore. First thanks to khale **Ezzat** for always telling me to get good grades and get a Dr-title and also thanks to my dear father in law **MohammadAli** who always told me that I make him and his family proud and wanted to come to my dissertation party. You are here with me in spirit.

My dear "lillebror" **Faraz Efazat** and "lillasyster" **Elnaz Efazat** thanks for being with me during the whole journey and being proud of me no matter what, my lovely warmhearted and loving grandmother and "secondmother" **Sedighe Movla** you love me unconditionally and

always believe in me, I am who I am because of you. My dear parents, my mother **Haideh Movla** and father **Aria Efazat** thank you for your endless love and support during these years. I love you all!

At last but not least I would like to thank my husband, my best friend and soulmate **Habib Mohammadi**. From the beginning that I wanted to study research at the university you have believed in me and standing by my side. You have always encouraged me, giving me advice, listen to my talks about the work in good and bad days and in ups-and-downs. This is a long way and you have been with me all the time holding my hand and without you it would not have been easy completing the PhD-years, words cannot say how much your support means to me. I love you more than words can say!

My dear beautiful daughter **Kimia**, thank you for entering our lives. A look at you makes all the stress that comes along with a PhD and life to disappear. Mommy loves you very much and this is for you and your future siblings! ☺

The projects of this thesis was supported by: grants from the Swedish Cancer Society, the Stockholm County Council, the Swedish research Foundation, the Swedish Childhood Cancer Society, the Robert Lundberg Foundation, the European Union-Chemores, the Stockholm Cancer Society, the Swedish Radiation Safety Authority, Knut och Alice Wallenbergs Stiftelse, the Swdish National Board of Health and Wealfare and the Karolinska Institutet Research Funds.

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