

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

**SIGNALOMICS: UNSUPERVISED AND
GLOBAL STRATEGIES TO EXPLORE
MECHANISMS OF RADIORESISTANCE
IN LUNG CANCER CELLS**

Sara Ståhl



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*“A book made of
Steel...”*

*“For every complex problem
there is a simple answer...but it’s
wrong.”*

H.L Mencken

ABSTRACT

To combat lung cancer (LC) which is the number one cancer associated killer, a combination of surgery, chemotherapy, radiotherapy and targeted agents are currently used. The main subtype of LC, Non small cell lung cancer (NSCLC) unfortunately responds poorly to conventional radiotherapy (RT) for reasons yet only partly understood. Searching for the underlying mechanisms of RT-resistance has the potential to indentify signaling targets which can be used for radiosensitizing purposes. The overall aim of this thesis aims was to set up and use unsupervised global methods to gain novel insight of cellular signalling events responsible for the mechanisms controlling radioresistance in NSCLC cells and potentially find radiosensitizing targets. In **paper I** we studied differences in NSCLC cellular response between irradiation of conventional low Linear Energy Transfer (LET) photons and high LET accelerated particles, which in the latter case induced an apoptotic response. A shotgun MS-based proteomics approach was used to analyze global protein expression after low and high LET ionizing radiation (IR). By applying the novel pathway search engine (PSE) on protein expression data, a JNK-pathway was suggested as a key event in apoptotic response to high-LET IR in these cells. Both JNK as well as additional molecules of the JNK-pathway i.e. MKK4 and 14-3-3 α , were proven to be regulated. This study demonstrates that, in contrast to low LET IR, high LET IR can trigger activation of the JNK pathway, which in turn is critical for induction of apoptosis in these cells. In **paper II** genomic profiling was used to identify putative low LET IR sensitizing targets used by the staurosporin analogue PKC 412 in RT sensitization of NSCLC cells. Out of a several altered genes, suppressed *Ephrin B3* expression was selected and further studied. We found that siRNA-mediated silencing of *Ephrin B3* resulted in increased senescence, apoptosis and mitotic catastrophe and combination with IR also decreased IR-mediated G₂-arrest. In **paper III** and **IV** a phosphoproteomic method consisting of strong cat ion exchange (SCX) and TiO₂-based fractionation followed by nano-LC mass spectrometry analysis was set up and used to further study the role of Ephrin B3 (**Paper III**) and reveal signaling events which may further explain how high LET IR can overcome low LET IR resistance (**Paper IV**). In **paper III**, this method in combination to network analysis found and verified that silencing of Ephrin B3 in NSCLC cells results in loss of a specific phosphorylation on Ser 897 on the erythropoietin-producing hepatocellular receptortyrosine kinase class A2 (EphA2), a Eph kinase member shown to be upregulated in NSCLC patient tumor material. Moreover, inhibition of Ephrin B3 expression blocked phosphorylation on Ser 129 of Akt1, a kinase previously implicated in regulation of Ser 897 on EphA2. Our data also support a process in which a Heat shock protein 90 isoform (HSP90AA1) acts as a protector of EphA2, thereby saving it from degradation. In **paper IV** the phosphoproteomic profiling of low and high LET irradiated U-1810 cells and subsequent pathway analysis suggested that in addition to JNK, down regulation of Akt-mediated survival signaling as well as impaired t-RNA synthesis and eIF-mediated protein translation may contribute to high LET IR induced apoptosis. Hopefully, when further validated these findings may have the potential of becoming clinically useful for RT sensitizing strategies of NSCLC.

LIST OF PUBLICATIONS

- I. **Stahl S**, Fung E, Adams C, Lengqvist J, Mork B, Stenerlow B, Lewensohn R, Lehtio J, Zubarev RA, Viktorsson K. Proteomics and pathway analysis identifies JNK signaling as critical for high linear energy transfer radiation-induced apoptosis in non-small lung cancer cells. *Mol Cell Proteomics* 2009; **8**(5): 1117-29.
- II. **Stahl S**, Kaminiskyy V, Vaculova A, Efazat G, Rodriguez-Nieto S, Moshfegh A, Lewensohn R, Viktorsson K, Zhivotovsky B. Global gene analysis reveals Ephrin B3 as a potential radiosensitizing target in non-small cell lung carcinoma cells. *Manuscript*
- III. **Stahl S**, Mm Branca R, Efazat G, Ruzzene M, Zhivotovsky B, Lewensohn R, Viktorsson K, Lehtio J. Phosphoproteomic profiling of NSCLC cells reveals that ephrin B3 regulates pro-survival signaling through Akt1-mediated phosphorylation of the EphA2receptor. *J Proteome Res* 2011 Mar 17
- IV. **Stahl S**, Mm Branca R, Efazat G, Stenerlöw B, Lewensohn R, Lehtio J, Viktorsson K. Phosphoproteomic profiling to explore differences in cellular response between high and low LET irradiated Non small cell lung cancer cells. *Manuscript*

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LIST OF ABBREVIATIONS

LC	Lung cancer
NSCLC	Non small cell lung cancer
RT	Radiotherapy
LET	Linear Energy Transfer
PSA	Pathway search engine
IR	Ionizing radiation
SCX	Strong cat ion exchange
EphA2	Erythropoietin-producing hepatocellular receptortyrosine kinase class A2
SCLC	Small cell lung carcinoma
SBRT	Stereotactic body radiotherapy
LD	Limited disease
ED	Extensive disease
SSB	Single strand break
DSB	Double strand break
HR	Homologous recombination
NHEJ	Non-homologous end joining
EGFR	Epidermal growth factor receptor
IGF-1R	Insulin growth factor 1 receptor
TKIs	Thyrosin kinase inhibitors
PTM	Post translational modification
P-ser	Phosphorylated serine
MS	Mass spectrometry
2D- gel electrophoresis	Two-dimensional gel electrophoresis
SELDI	Surface enhanced lased desorption ionization
MALDI	Matrix associated laser desorption ionization
pI	Isoelectric point
IMAC	Immobilized metal affinity chromatography
TFA	Triflouracetic acid
ACN	Acetonitrile
nanoLC-ESI	Nano-liquidchromatography-electrospray ionization
DHB	2.5-dihydroxybenzoic acid
CFSE	Carboxyfluorescein succinimidyl ester
LTQ	Linear ion trap
FT	Fourier transform
CID	Collision induced dissociation
HCD	Higher energy collision dissociation
ECD	Electron transfer dissociation
IPA	Ingenuity pathway analysis

1 INTRODUCTION

1.1 WHAT IS CANCER AND WHY DOES IT KILL US?

Cancer is the name for a group of diseases in which the cells become abnormal and divide without control. Cancer cells may invade nearby tissues and they may also spread through the bloodstream and lymphatic system to other parts of the body. Eventually when the tumor is growing and/or further spread it becomes malignant and lethally affects the invaded organs. It can physically exercise significant pressure on vital organs and/or will use all energy and resources available which in the end is lethal to the body. One of the main problem when trying to stop cancer is that the tumor origin from ourselves and when trying to effectively kill and eradicate all cancer cells we are also killing our normal cells. It has emerged in the past years that tumors are more than just highly proliferating cancer cells. Instead, they are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another. In the tumor micro-environment - there are also recruited normal cells, which form the tumor-associated stroma, as they are believed to be active participants in tumor genesis rather than passive bystanders (1). The reason why normal cells are transformed into cancer cells is due to genomic instability and somatic mutations also known as oncogenic dependency of the cancer cells. However, the events occurring during the transformation process is complex to overlook and grasp. Yet they comprises eight biological capabilities; sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics and avoiding immune destruction. These capabilities constitute an organizing principle with the aim to provide a logical framework for understanding the remarkable diversity of neoplastic cells, namely the hallmarks of cancer (1). The transformation process and how it is regulated by, as well as how it is regulating the hallmarks depends in turn on the communication within and between cancer cells. Hence, to improve cancer therapy, it is crucial to understand the cancer and how it communicates – for that we need to understand the “language of cancer”. Thus, revealing cellular signaling events in the context of cancer is of outmost importance. Hopefully, this thesis can contribute a piece of the enormous puzzle that needs to be finished.

1.2 LUNG CANCER

Although lung cancer is not the most common tumor form, it is the major cause of cancer-related death in men and women and is annually responsible for 1.3 million deaths worldwide (2-3). The possibility for a patient diagnosed with lung cancer to survive more than five years is only 15% and at the late stage lung cancer only 5% (4). The most common cause of lung cancer is long-term exposure to tobacco smoke (figure 1) However, still as many as 15% of lung cancer cases occurs in nonsmokers and is then often attributed to a combination of genetic factors, radon gas, asbestos, and air pollution including secondhand smoke (5). The most common symptoms are shortness of breath, coughing and weight loss. The vast majority of primary lung cancers are carcinomas of the lung, derived from epithelial cells, where the main types are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). This distinction is important, because the treatment varies; NSCLC constitutes 80 % of lung cancer cases and is more often diagnosed earlier and can sometimes be treated with surgery but otherwise often responses poorly to conventional radiotherapy and chemotherapy (4), while SCLC initially responds better to chemotherapy and radiation but resistant clones unavoidably start to grow again and the disease becomes rapidly aggressive.



Figure 1: Cancer of the lung from a heavy smoker (*picture: from public domain Wikimedia commons*).

1.2.1 Lung cancer therapy today

Treatment of lung cancer depends on the clinical stage at diagnosis. For NSCLC the first hand choice is radical surgical resection of the primary tumor for both stage I and II, this is obtained by the removal of the lung lobe or sometimes the entire lung(6) (figure 1). In non-operable cases Stereotactic body radiotherapy (SBRT) is given with curative intent in stage I (7) and chemotherapy in stage II. In stage III chemotherapy and radiotherapy is given (8-9) whereas palliative chemotherapy or biological agents are used in stage IV. SCLC is only classified as limited disease (LD) or extensive disease (ED), in case of LD a combination of chemotherapy and radiotherapy is used (10) while only palliative chemotherapy is given in ED (11).

1.2.2 Radiotherapy

Radiotherapy (RT) or ionizing radiation (IR) used for therapeutic purposes mainly consists of X or γ -rays in which the radiation is delivered by photons and which is termed low Linear Energy Transfer (LET) which indicate that it induces few ionizations per the unit of length it traverse (given as keV/ μ m) (12-13). Photons deposit their energy in the matter they traverse e.g. in cellular membranes, cellular cytosol or the DNA. The release of energy results in fast moving electrons which subsequently causes ionizations or breakage of chemical bonds (12). Another type of radiation quality which in some cases are used for tumor therapeutic purposes is high LET accelerated particles such as charged particles of boron, carbon or neon ions which have an energy content of above 100 keV/ μ m as compared to low LET IR which don't exceed 1 keV/ μ m.

The critical cellular target of IR is the DNA as it contains the genetical material and if DNA is damaged by IR in an unreparable way genetic information may be lost resulting in either mutation or cell death. Both low and high LET IR may cause different types of DNA damages including DNA single and DNA double strand breaks (DNA SSBs and DNA DSBs respectively), DNA base damages, sugar damages as well as DNA-DNA cross links and DNA-protein cross links. For 1 Gy of low LET IR, it is estimated that it causes 1000 base damages, 1000 DNA SSBs and about 40 DNA DSB per nucleus it traverse (12). Yet the induced DNA DSBs are the most detrimental as they if not repaired properly will result in loss of genetic information. Indeed results have demonstrated that a direct correlation exists between the number of DNA DSB formed and loss of clonogenic survival in irradiated cells (14-15).

As indicated by low LET nomenclature γ -rays photons induces few direct ionizations which are randomly distributed within the DNA (16) (Fig.2). Instead most of the low LET IR- induced DNA DSBs is a result of hydroxyl radicals formed when fast moving electrons ionizes water (16) (Fig.2).

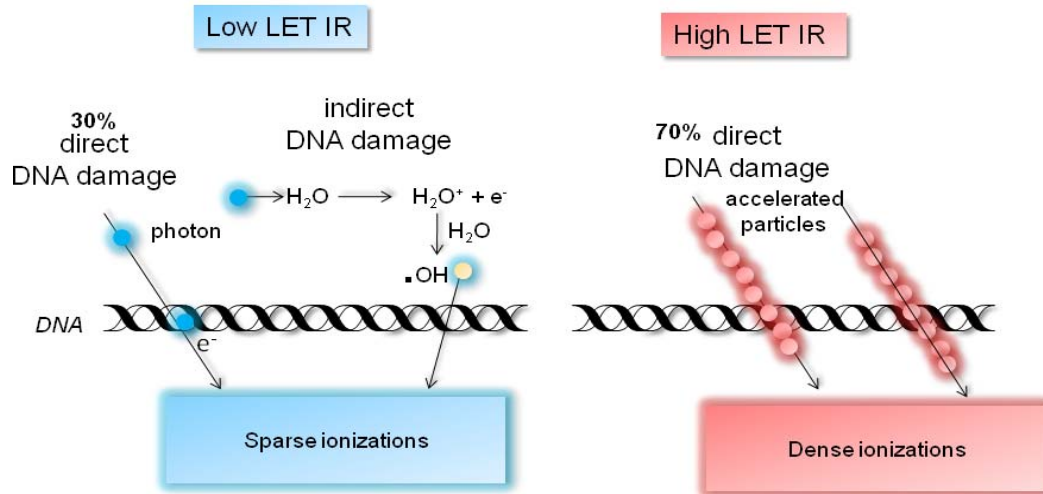


Figure 2: Ionization of low and high LET IR. Low LET photons mainly exert DNA DSB formation by indirect effects in which ionization of water produces hydroxyl radicals which subsequently cause DNA DSBs. In contrast high LET accelerated particles cause's dense ionizations along the tracks causing complex DNA DSBs to be formed. For details see text.

As solid tumors can outgrow their blood supply, causing a low-oxygen state known as hypoxia, a state which accordingly impairs the effect of low LET IR this may cause tumor cells to be as as much as 2 to 3 times more resistant to low LET IR-induced DNA DSBs than those in a normal oxygen environment (17). In contrast to low LET, high LET charged particles such as nitrogen, boron, carbon or neon ions (high LET IR) act mostly via direct energy transfer into the DNA causing a higher number of ionizations for a given dose, hence a higher amount of DNA DSBs and also DNA DSBs with greater complexity (18-19) (Fig.2). Moreover, as high LET IR can induce DNA DSB directly, it is likely less influenced by oxygen status of the tumour.

Molecular signaling circuits induced by IR

DNA DSBs are the most critical type of DNA damage as if left unrepaired may cause mutations or cell death. Hence, cells are equipped with “emergency systems” in the form of cellular signaling to handle the detection and repair of these DNA DBSs. Although both high and low LET induced DNA DSBs activate these signaling cascades some of the molecular components demonstrated to have a critical role in response to low LET IR are reported to be dispensable for efficiency of high LET IR. One example of such a component is the tumor suppressor p53 (20-22) and onother is the DNA-repair protein DNA-PK (23).

The simplified view is that IR-induced DNA damages trigger cell cycle arrest allowing DNA repair to take place and if the DNA damage is too severe, cell death is induced. Yet the molecules which are responsible for directing the cell towards repair of the DNA damage, towards survival or towards cell death i.e. the so called decision makers are not completely elucidated. Nevertheless, four principal signaling cascades can be seen activated in response to IR (Fig.3): (I) DNA DSB detectors, (II) cell cycle checkpoints regulators which halt the cell cycle, (III) DNA DSB repair pathways and (IV) cell death regulating proteins.

A number of different proteins are involved in recognition including BRCA1/2, the Mre11/Rad50/Nbs1 (MRN) complex and the Ataxia telangiectasia mutated (ATM) kinase (Fig.3). ATM is a member of the phosphatidylinositol-3-kinase (PI3K) family (24) and was cloned from patients with the autosomal recessive disorder ataxia telangiectasia (AT) whose cells were found to display genetic instability and increased radiosensitivity (25). ATM has been shown to reside in undamaged cells in an inactive dimer and in response to DNA DSBs, most likely as a consequence of chromatin structure changes, these dimers phosohorylate each other with the net outcome of active ATM monomers (26). The active monomers of ATM are then interacting with the Mre11/Rad50/Nbs1 (MRN) complex at the site of the DNA DSB allowing its full activation and capacity to phosphorylate different downstream substrates (27). Among the different substrates are the DNA DSB marker H2AX resulting in γ H2AX (28) and the Chk2, an important IR-activated G2 cell cycle checkpoint protein (29) (Fig.3).

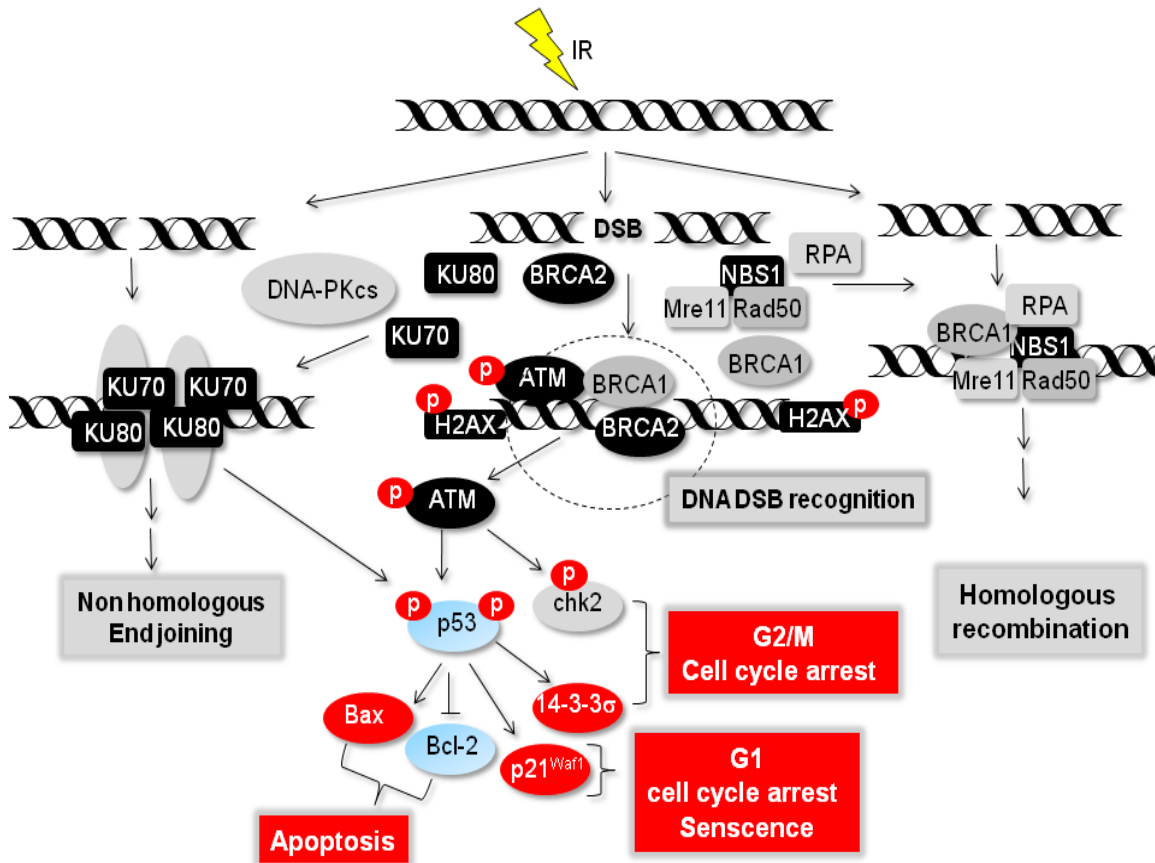


Figure 3: Molecular signaling circuits activated in response to radiation. For details see text.

With respect to IR-induced cell cycle regulation two principal responses may occur, i.e. activation of a G1 arrest or activation of the G2 checkpoint. The tumor suppressor protein p53 is an important regulator of both cell cycle responses after IR and is also instrumental for induction of senescence and apoptotic cell death (see below). In order to exert these tasks, p53 expression needs to be elevated as low levels are found in non stressed cells. In response to IR-induced DNA DSBs this is achieved by phosphorylation of p53 on different residues by ATM (30) DNA-PK(31) and Chk2 (32) thereby alleviating the association with the negative regulator Mdm-2 and resulting in increased p53 levels (reviewed in (33)).

p53 is instrumental in IR-mediated cell cycle control as it causes transcriptional activation of p21^{WAF1/Cip1}, an inhibitor of cyclin dependent kinases 4 and 6 (CDK4/6) (34) and as a net result the phosphorylation of retinoblastoma protein (Rb) is blocked and hence the release of E2F transcription factor driving S phase entry is inhibited. Importantly, in many tumors this IR-induced G1 arrest is lost as they display mutations in p53 which impairs its capacity to induce p21^{WAF1/Cip1} transcription. This is also true for LC in which p53 mutation is one of the most frequently found genetic alteration

identified in about 70% of all SCLC and in 50% of NSCLC cases respectively (35). Whereas the IR-induced G1-arrest mainly is controlled by p53 and hence frequently is lost in tumors, IR-induced G2/M cell cycle control is executed by p53-dependent and independent pathways in which the cyclin dependent kinase Cdc2, an essential kinase for mitotic entry, is the primarily regulated component but where its binding partner cyclin B is also interfered with (reviewed in (36)).

Apart from DNA DSB recognition and cell cycle arrest, the activation of the two complementary DNA DSB repair pathways homologous recombination (HR) and non-homologous end joining (NHEJ) (37) are of importance for the cellular response to IR. Whereas HR, which make use of the sister chromatid in repair of the DSB can act only in late S and G2 phase when such a template is available NHEJ may work also on DNA DSBs formed in different cell cycle phases (38). In short, the HR starts by recruitment of ATM and BRCA1/2 to the site of the DNA DSB forming the BASC complex which subsequently recruits Nbs1, Rad 50 and MRE11 into the MRN complex (39) (Fig.3). Thereafter RPA is binding to this complex and homology search between the sister chromatids occurs followed by strand invasion, DNA synthesis, and relegation of the DNA strand (39).

By activating and utilizing NHEJ, cells may efficiently repair IR-induced DNA DSBs in different cell cycle phases primarily in G0/G1 but in an error prone way as no error free DNA copy is used as template (40). The principal steps in NHEJ is the binding of the free DNA ends within the DNA DSBs by the ku heterodimers KU80/KU70 which results in the recruitment of the phosphatidylinositol-3-kinase (PI3K) family kinase DNA-PKcs (41-43). The critical role of NHEJ in determining cellular IR sensitivity is illustrated by rodent and human cells lacking either DNA-PKcs or KU-proteins all which display a degree of IR sensitivity relative to their parental cell lines (44-46, 47).

The fourth principal signaling cascades activated in response to IR is the triggering of different cell death processes within tumor cells which roughly can be divided into cell cycle independent and mitotic cell death which is induced in response to residual DNA DSBs and/or other DNA damages. The mode of cell death can in either case be apoptosis, autophagy, mitotic catastrophe followed by apoptosis, necrosis or senescence and which path the tumor cells choose is not entirely clear but may depend on tissue of origin, amount of and how severe the DNA damage is as well as of the signaling circuits functionally operating within irradiated cells (reviewed in (48)). Thus it is shown that thymocytes and cells of hematopoietic origin respond to IR with a rapid induction of apoptosis (49). In contrast, IR may in solid tumor cells primarily causes growth arrest (either in G1 or G2 dependent on p53 status) followed by at least in some cases mitotic catastrophe in which cells with unresolved DNA DSBs cannot divide resulting in giant cells which later either die by apoptosis or by necrosis.

The morphological hallmarks of apoptosis was described by Kerr, Wyllie and Currie in 1972 as plasma membrane blebbing, cell shrinkage, condensation/fragmentation of the chromatin, and disintegration of the cell into apoptotic bodies (50). It has through

extensive analysis become evident that all these characteristics are result of selective proteolysis of cell signaling, DNA-repair as well as structural proteins executed by a group of cystein-aspartate enzymes named caspases (51). Thus both the DNA DSB repair protein DNA-PK and the DNA SSB repair protein Poly (ADP-ribose) polymerase (PARP) are subjected to caspase-mediated cleavage, as is the caspase activated DNAase (CAD) which results in the characteristic DNA fragmentation of apoptosis (51-52).

The caspases are divided into initiator caspases i.e. caspase-2, -8, -9, and -10 and effector caspases i.e. caspase-3, -6, and -7 the former being activator of the later which subsequently carries out the selective substrate proteolysis. Two principal pathways may causes caspase activation either the extrinsic pathway in which death ligand binding to the a death receptor e.g. Fas ligand to the Fas receptor causes receptor oligomerization, adaptor protein recruitment and formation of a complex with pro-caspase-8 which then undergoes autoproteolytical cleavage into active caspase-8 which directly or indirectly can cause caspase-3 activation (53) (Fig.4)

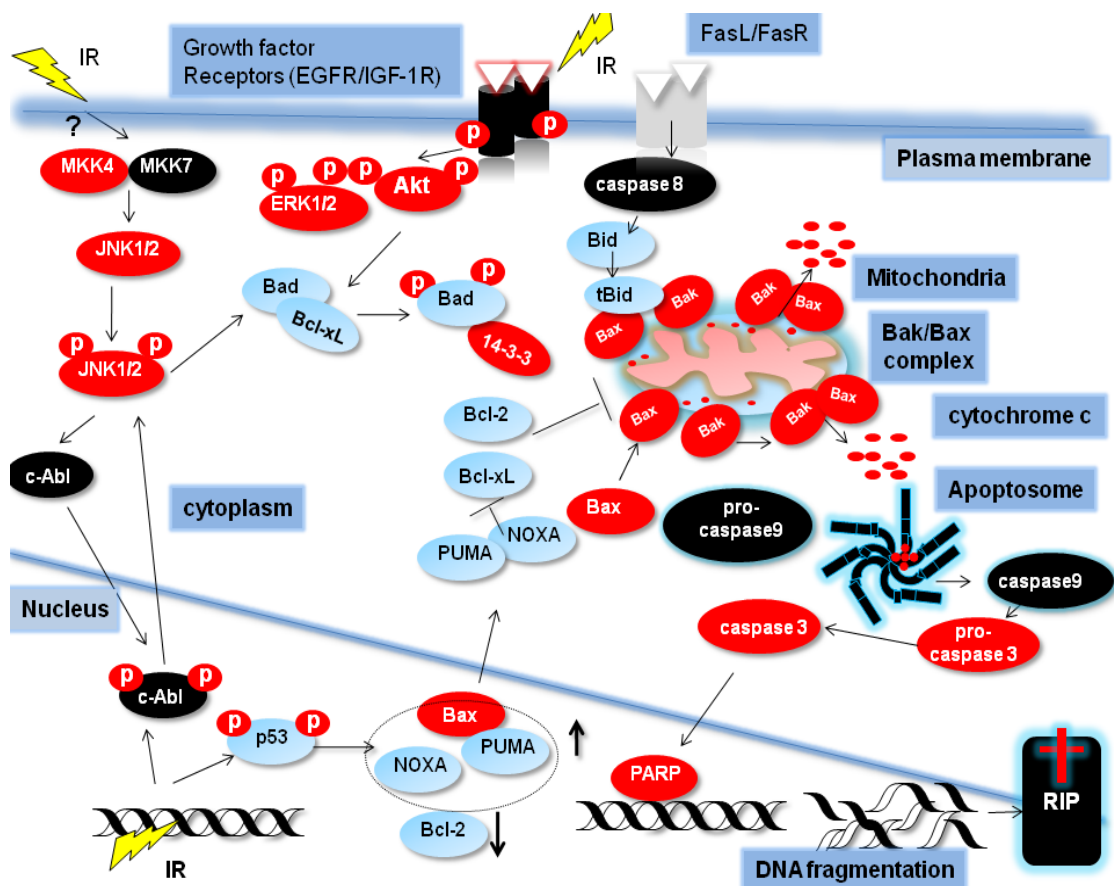


Figure 4: Apoptotic signaling events in response to IR. For details see text.

However, for IR-induced apoptosis multiple evidence points towards a critical role for the intrinsic activation of caspases i.e. via mitochondrial release of apoptogenic proteins e.g. cytochrome c and Smac/DIABLO from the inter membrane to the cytosol (54) (Fig.4). Within the cytosol the released cytochrome c forms a complex together with apoptosis protease-activating factor 1 (Apaf-1) and dATP, the apoptosome in which several procaspase-9 are brought in close proximity allowing their activation into caspase-9 (55-56). Subsequently caspase-9 triggers caspase-3 activation and the apoptotic hallmarks can be observed.

The release of cytochrome c from mitochondria which is critical for activation of the intrinsic pathway of apoptosis is highly controlled by among other the Bcl-2 family proteins. (Fig.4). The Bcl-2 family proteins comes in two principal flavors, an anti-apoptotic group exemplified by Bcl-xL and Bcl-2 and a proapoptotic group which consists of the multidomain proteins Bak and Bax and the BH3-only proteins exemplified by Bid, Bim, Bad, PUMA, and NOXA (57). The critical role for Bak and Bax but also their redundant function in DNA-damage induced apoptosis is illustrated by the impaired apoptotic response of mouse embryo fibroblasts (MEFS) with targeted deletion of Bak and Bax to the DNA DSB-inducing agent etoposide (58). Moreover, it has been demonstrated that in response to DNA damage, Bax undergo N-terminal conformation changes allowing its integration into the outer mitochondrial membrane (Fig.4) (59-60) and enabling it to form a multimeric homo- or heterocomplex with Bak allowing release of cytochrome c (61-62). Similarly, Bak, which already reside in the mitochondria, undergoes several N-terminal conformational changes allowing its multimerization and interaction with Bax (60, 62-65). The current view is that the antiapoptotic proteins Bcl-2 and Bcl-XL inhibits the multimerization of Bak and Bax and that the BH3-only proteins act by antagonizing this effect (57) (Fig.4). Moreover, the BH3-only proteins play a critical role as they function to integrate signals from both DNA-damage as well as from growth factor receptors onto Bak and Bax (57). Thus the tumor suppressor p53 which is activated by DNA DSB as described above, may not only induce transcriptional activation of Bax and repression of Bcl-2 but also induces transcription of the BH3 proteins NOXA (66) and PUMA (67-68) and thereby tilt the balance towards a pro-apoptotic response. Tumor cells which normally are characterized by high growth factor receptor signaling e.g. Epidermal growth factor receptor (EGFR) or Insulin growth factor 1 receptor (IGF-1R) may also have impaired apoptotic circuits as the BH3-only protein Bad when phosphorylated by the survival kinases Akt and MAPK ERK is blocked in its proapoptotic function by binding to 14-3-3 (69-70).

1.2.3 Radioresistance in lung cancer - the molecular perspective

Inherent resistance to chemo-and radiotherapy (CT/RT) is frequently observed among NSCLC patients and complete response to therapy is unusual. The underlying mechanism on the molecular level is likely a combination of several factors such as for example *(I)* deregulated growth factor signaling which may impede apoptotic response e.g. EGFR-mediated activation of Akt resulting in Bad inactivation, *(II)* increased DNA-repair capacity enabling cells to circumvent CT-or RT-induced DNA damages as well as *(III)* impaired function of cell death signaling pathways.

With respect to increased growth factor signaling and RT resistance of NSCLC, deregulation of EGFR signaling is of course of importance and EGFR signaling will be further discussed below in section 1.2.4. Yet another growth factor receptor which may be instrumental for RT resistance of NSCLC is Insulin growth factor 1 receptor (IGF-1R) as demonstrated by earlier findings from our group (71-73). Thus it was shown that in NSCLC U-1810 cells a combination of IGF-1R inhibition and RT caused synergy with respect to cell death and resulted in an increased G2 cell cycle accumulation (73). In yet other NSCLC cell lines IGF-1R inhibition and RT were reported to be additive with respect to cell death. Interestingly, it was also demonstrated that RT actually activated IGF-1R as early as 10 minutes after IR with a maximal activation effect 2 h post-irradiation and that IGF-1R in its active form bind and activate p38MAPK thus suggesting that growth factor receptor signaling may indeed most likely directly be activated by IR (71) which recently also was reported for EGFR (74). Importantly, blocking IGF-1R with small molecule kinase inhibitor was found to disrupt IGF1-R/p38MAPK complex, block MAPKp38 activity and to sensitize for RT-induced cell death illustrating that targeting growth factor receptors may overcome NSCLC RT resistance (71).

Moreover, the downstream targets of growth factors such as PI3K/Akt signaling has also been demonstrated to confer RT resistance as reviewed in (75) and shown in (76-77). Thus Brognard et al., showed that NSCLC often have constitutively activation of Akt with phosphorylation of S473 as a result of increased PI3K activity. Importantly by inhibiting PI3K using LY294002 an additive effect on IR-induced cytotoxicity as well as on IR-induced apoptosis was observed (76). Similarly our collaborators in the Zhivotovsky group demonstrated that targeting Akt1 as well as MAPK ERK-1 indeed sensitized for DNA DSB inducing treatments in some but not all NSCLC cell lines tested (77) thus further suggesting a role for Akt1 and at least in some cases, MAPK ERK in RT resistance of NSCLC.

In addition, our group previously showed that expression and activity of the DNA-repair enzyme DNA-PKcs as well as rejoining of IR-induced DNA DSBs may be correlated to intrinsic RT resistance of a panel of SCLC cell lines (78). Moreover, it has been demonstrated that inhibiting DNA repair capacity of NSCLC can sensitize for treatments inducing DNA DSB (79).

Last but not least, RT resistance mechanisms of LC may of course be a result of deregulated cell death pathway signaling including apoptotic signaling as demonstrated by our group and others previously (65, 77, 80-87) and further emphasized in this thesis (**Paper I, II and IV**) using a signalomics approach. Nevertheless, in part this deregulation may be a result of increased growth factor signaling as Raf, Akt as well as MAPK ERKs are known to regulate the BH3 only protein Bad and impeding its proapoptotic function (69-70). Yet results also suggest that failure of IR-induced apoptosis in LC may indeed be a result of Bak and Bax activation directly as well as of lack of SAPK JNK activation (65).

1.2.4 Targeted therapy in Lung Cancer

To circumvent resistance and improve survival rate in current clinical practice, there is an urgent need for more targeted and individualized therapies that impact the tumor-driving cellular pathways from multiple angles. The Epidermal growth factor receptor (EGFR) is overexpressed in NSCLC and has been extensively studied (88). Inhibitors of this receptor have recently reached the clinic but are only beneficial in a limited group of patients. Yet other signaling aberrations in NSCLC which have been explored for targeted therapy approaches include Insulin-like growth factor 1 receptor (IGF-1R), the mTOR pathway and the fusion protein EML4-ALK (89). The erythropoietin-producing hepatocellular (Eph) receptors and their corresponding ligands, ephrins have recently been acknowledged as a potential signaling network of importance in tumors in general and in NSCLC in particular. EphA2 is an upcoming candidate which together with the ligand ephrin A1 shows promising preclinical results as potential targets in NSCLC therapy.

Targeting EGFR

NSCLC is frequently associated with EGFR overexpression, which occurs in 40–80% of the patients (90-94) and a high EGFR copy number is found in 30–60 % of cases (95-96). EGFR has a role in activating two major pathways in solid tumors, the PI3K/Akt/mTOR pathway, and the RAS/RAF/MEK/MAPK pathway (Fig 5) (97). These signaling pathways are important in tumor cell growth, local invasion, angiogenesis, protein translation, and cell metabolism (98). Consequently, EGFR-blocking strategies have been enthusiastically investigated during the past decade.

While the development of first-generation EGFR tyrosin kinase inhibitors (TKIs) has been a major accomplishment in targeted NSCLC therapy, only a fraction of patients respond to Erlotinib or Gefitinib. Primary resistance is problematic, but acquired resistance, which occurs after an initial response, is also common, and may result from redundant or overlapping signaling pathways. In addition, none of the four large Phase III trials i.e. Iressa NSCLC Trial Assessing Combination Treatment [INTACT] 1, [INTACT] 2, Tarceva Lung Cancer Investigation [TALENT] and Tarceva Response in Conjugation with Paclitaxel and Carboplatin [TRIBUTE], which evaluated the addition of erlotinib or gefitinib to chemotherapy as first-line therapy in patients with NSCLC

reported a survival benefit with the EGFR TKI (99-102). (99-102). Recent molecular and clinical studies have revealed that EGFR-TKIs are mainly effective in tumors bearing specific *EGFR* mutations in the gene region coding for the ATP-binding domain, which corresponds also to the binding site of the TKIs. In addition, tumors treated with gefitinib or erlotinib usually develop an additional “resistance” gene mutation which makes EGFR insensitive to inhibition by these drugs. Next generation inhibitors of EGFR have been developed using novel strategies that may improve efficacy; these include irreversible inhibition, targeting more than one EGFR family member, and simultaneous targeting of EGFR and other tumorigenic pathways (88). However, it is obvious that more candidates for targeted therapies in NSCLC are highly warranted

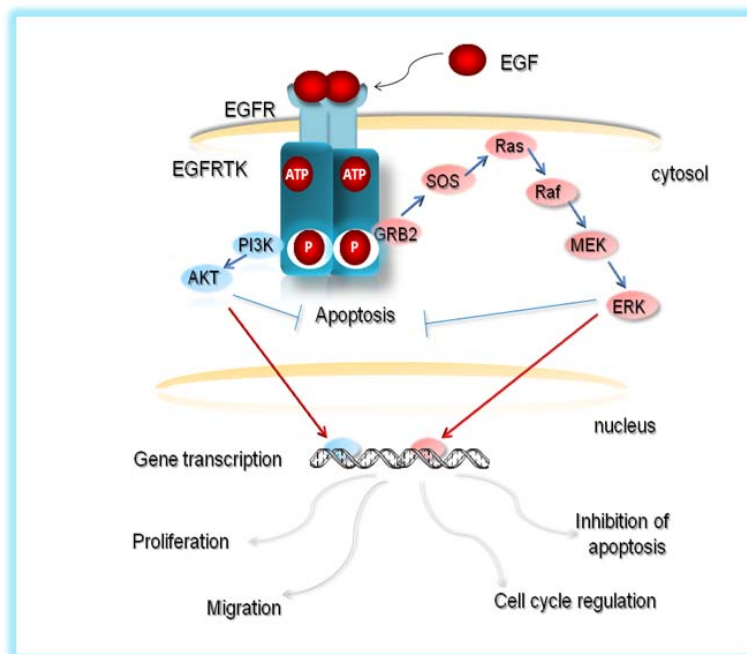


Figure 5: EGFR-signaling is targeted by inhibition in NSCLC-therapy.

The emerging role of EphA2 as a target for NSCLC therapy

Receptor tyrosine kinases functioning as proto-oncogenes often display increased activity and play an essential role in the cell signaling pathways in carcinogenesis (103-105). While several tyrosine kinase receptors like EGFR have been documented and targeted for their critical role/s in tumorigenesis (106), the cell-membrane-bound EphA2 receptor, a member of the erythropoietin-producing hepatocellular (Eph) tyrosine kinases receptor family, has generated great interest only in the last few years. The Eph family is the largest group among the tyrosine kinase receptor families and is comprised of the EphA (EphA1-10) or EphB (EphB1-6) subclasses of receptors classified with regard to their sequence homologies and their binding affinity for their ligands - the ephrins (107-109). The first member of Eph family was cloned from an erythropoietin-producing hepatocellular cancer cell line in 1987 and was named as EphA1 (110). A few years later, in 1990 EphA2 was identified by the screening of a human epithelial cDNA library (111) and it was established that the human EphA2 gene encodes a receptor tyrosine kinase of 976 amino acids with an apparent molecular weight of 130 kDa (112). The Eph family contains an extracellular conserved N-terminal ligand-binding domain followed by a cysteine rich domain with an EGF-like motif and two fibronectin type-III repeats. The extracellular motif is followed by a membrane-spanning region and a cytoplasmic region that encompasses a juxtamembrane region with a tyrosine kinase domain (113-116). EphA2 shows 25 - 35% sequence homologies with other Eph receptors, and the tyrosine residues are conserved within the juxtamembrane and kinase domain (113, 117).

The ligands for Eph receptors, the ephrins, are divided into two subclasses ephrinA (ephrinA1 - 6) and ephrinB (ephrinB1 - 3) (114-115). EphrinA members are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage while ephrinB members have a transmembrane and a cytoplasmic domain. Ephrin A1 is the most storied ligand of EphA2 and was found to be a ligand for the receptor based on its ability to bind the extracellular domain of the EphA2 receptor when tagged to an affinity column (118). EphrinA1 is a GPI-anchored protein with an apparent molecular weight of 28 kDa and shows 30 - 40% amino acid similarities with other ephrins (119-120). Eph-ephrin signaling functions like the classical tyrosine kinase receptor-mediated cell signaling where a cell bearing an Eph receptor upon binding to ephrin ligand transmits signals downstream known as forward signaling. In addition, intracellular signals in backward direction are also generated in ephrin-bearing cells known as reverse signaling. Accordingly, a contact between two communicating cells and a clustering of membrane-bound ephrin ligands is required for the Eph--ephrin bidirectional signaling (121-122). EphrinA1 organizes into micro-clusters upon association of an EphA2 receptor and actin cytoskeleton on the adjacent cell (123-124). The crystal structures of EphA2 extracellular domain also suggest that EphA2 localization at the cell-cell contacts depends on the clustering of EphA2, and a high concentration of EphA2 clustering independent of the ephrin ligand could impart a phenotype typical of a cancerous cell (125). The role of Eph-ephrin signaling has

been studied in great detail in the development of the nervous system and spans a wide range of functions such as the development of neuronal networks, axon guidance, formation and remodeling of synaptic connections, and nervous system repair (121). Properties harbored by developmental proteins are by their nature likely to be of importance also for cancer cells. Hence, understanding of EphA2 and how it is regulated especially in the context of its interplay with EGFR is most likely to be important when developing new strategies for NSCLC cancer therapeutics.

EphA2 have recently been found overexpressed in several cancer forms including lung. Hence, during the past two years, this receptor has gained lots of attention as an impending target for cancer therapy. In a retrospective NSCLC cohort, moderate to high immunostaining of EphA2 has been observed in the membrane and cytoplasm in more than 70% of the carcinomas(126). This increase is similar in adenocarcinoma, squamous cell carcinoma and large cell carcinomas. The highest level of EphA2 is observed in clinically advanced stages of disease, and the patients with increased EphA2 levels showed a poorer prognosis compared with patients with reduced EphA2 levels. Additionally, patients with brain metastasis of NSCLC displayed high levels of EphA2. EphA2 overexpression was also correlated with poor survival and a history of smoking (127).

Effects of suppressing EphA2 in cancer

To suppress EphA2 overexpression, sequence-specific silencing of EphA2 gene expression has been tested with many RNA interference (RNAi) approaches. In pancreatic adenocarcinoma-derived MIA PaCa2 cells, EphA2 expression was suppressed by RNAi, and it also inhibited tumor growth in a nude mice xenograft model (128). By weekly treatment with EphA2-specific siRNA, tumor growth was retarded by 70% in six weeks coincidently with an increase in caspase 3 activity and presence of apoptotic cells (128). In human glioma-derived U-251 cells, suppression of EphA2 by RNAi reduced cell proliferation and increased caspase 3 activity and apoptosis (129). EphA2 is also regulated by EGFR signaling in the EGFR overexpressing human head and neck carcinoma-derived cell line - HN5. In line with this, in the human epidermoid-derived cell line, A-431, also EphA2 levels are increased when EGFR is activated by EGF. In HN5 cells, suppression of EphA2 expression by RNAi significantly decreased EGF-induced migration in vitro wound healing assays. These results indicate that targeting EphA2 reduces EGFR-mediated cancer cell migration and strengthens the theory of a cross-talk between EphA2 and EGFR signaling (130-131). Together, these studies demonstrate RNAi-mediated EphA2 suppression as a viable approach targeting EphA2 overexpressing cancers.

The differential expression of EphA2 with substantially high levels in cancer cells compared with normal cells is documented in cell lines, and clinical specimens derived from cancer patients with several types of cancers indicate a generalized importance of EphA2 in carcinogenesis (132). The mechanisms by which EphA2 overexpression occurs and then contributes towards cancer phenotype are not entirely

clear and depend on the genomic context and cell type-specific function in different cancers. Nonetheless, the studies discussed above point towards the role of oncogenic signaling transduction pathways coupled with the contributions of crosstalk with other receptor tyrosine kinases. Importantly, the tyrosine phosphorylation of EphA2 resulting in its degradation and turnover has important functional consequences in tumor biology (132). EphA2 overexpression, functional alterations and gain of oncogenic function provide an opportunity to target EphA2 for therapeutic intervention. Thus, targeting EphA2 overexpression in preclinical studies of various cancer forms are already showing promising results and have so far been done e.g. by EphA2 antibodies, RNAi, and small-molecule inhibitors (132).

1.3 THE IMPORTANCE OF PROTEIN PHOSPHORYLATIONS

Protein phosphorylation is one of the most widespread regulatory mechanisms in nature. It is a key regulator of intracellular biological processes and at present, it is the most studied and best understood post translational modification (PTM) (133-134). Protein phosphorylation regulates cellular signaling and communication and is known to be involved in controlling diverse processes including metabolism, transcriptional, and translational regulation, degradation of proteins, proliferation, differentiation, and cell survival (105, 135). Phosphorylation events frequently initiate and propagate signal transduction pathways. It is a transient, reversible PTM that typically leads to changes in the conformation, activity, and interactions of a protein within a very short timeframe. Since the modification is reversible, changes in protein activity can be tightly controlled in response to cellular or environmental stimuli. Phosphorylation often occurs at multiple residues within a protein and in most cases by different protein kinases. Multiple sites of phosphorylation allow a protein to adapt several different functions, depending on which phosphorylation site becomes occupied. For example, phosphorylation at one particular amino acid may lead to conformational changes that in turn allow for the phosphorylation of different amino acid residues within the same protein, or it can prevent the phosphorylation of nearby amino acids through sterical hindrance. Phosphorylation is regulated by a complex interplay between specific protein kinases and protein phosphatases, which keep a strict temporal and spatial control of the phosphorylation and dephosphorylation events at specific sites in target proteins. In eukaryotes, phosphorylation takes place at serine, threonine, or tyrosine residues and genomic sequencing has revealed that 2–3% of all eukaryotic genes are likely to code for protein kinases (136) and in addition, gene annotation have predicted more than 100 human protein phosphatases (137). At present, no studies have been able to elucidate how many proteins are in fact regulated by phosphorylation, but it has been estimated that more than 50 percent of all proteins are phosphorylated during their lifetime (134), and that more than 100 000 phosphorylation sites may exist in the human proteome. Despite its ubiquitous role, phosphoproteins are generally present at relatively low abundance within the cell and phosphorylated forms of individual proteins also tend to be present at much lower ratios than their non-phosphorylated counterparts. Due to the central regulatory role phosphorylation plays in maintaining

the dynamics of a living cell, it is crucial to develop analytical methods and strategies for the characterization of phosphorylated peptides. However, low relative abundance of many phosphoproteins, low phosphorylation stoichiometry and the dynamic regulation of phosphoproteins put high demands on the analytical methods required for the studies of phosphoproteins. Sensitive, comprehensive and highly specific analytical strategies are needed in order to characterize a meaningful number of modified proteins, including those present at very low abundance.

1.3.1 Protein phosphorylations and cancer

It is well known that dysregulation of kinase signaling pathways is commonly associated with various cancers (138). Aberrations in kinases have been reported in several cancers including gastrointestinal stromal tumors (139), lung cancer (140), haematologic malignancies (141), breast cancer (142), pancreatic cancer (143) and prostate cancer (144). This aberrant regulation may result from overexpression of kinases, mutations or defects in negative regulatory mechanisms, among others. Activated kinases can be specifically targeted using small molecule inhibitors. Examples of such targeted therapeutic approach employing small molecule kinase inhibitors have been previously reported in various cancers including chronic myelogenous leukemia (CML) (145-146), small cell lung cancer (147), breast cancer (148-149), non-small cell lung cancer (150) and melanomas (151). Personalized medicine in the context of cancer therapy is illustrated by the use of imatinib in CML and erlotinib in non-small cell lung cancer through blockade of Abl and EGFR, respectively. A number of proteomic approaches have been developed over the years to identify aberrantly activated kinases and their downstream substrates. Most often, but not always, phosphorylation is used as a surrogate for monitoring kinase activity in cells. Direct involvement of protein kinases in cancers came from the discovery of v-SRC oncogene (152). The corresponding oncoprotein was later purified and found to be a protein kinase that undergoes phosphorylation (153-154). Subsequent biochemical studies revealed that Src was a tyrosine kinase (155-156). In fact, the transforming proteins of a number of avian and mammalian retroviruses were later found to possess tyrosine kinase activity (157-158). Tyrosine phosphorylation of host cellular proteins by viral kinases and their apparent involvement in cellular transformation was established using Rous sarcoma and Abelson murine leukemia viruses (159-161). To date, mutations in kinases have been studied in a large variety of cancers (162-164) and, correspondingly, kinase inhibitors have emerged a major class of anti-cancer drugs that are either in clinical use or in various phases of clinical trials (165-166). In recent years, advances in selective enrichment of phosphoproteome have led to systematic characterization of aberrantly activated kinase signaling pathways in several cancers. Information from such studies is likely to contribute to further development in future targeted cancer therapies. Studies describing and using technologies for monitoring global phosphorylation events are handled in the section for phosphoproteomics in section 1.4.3.

1.4 GLOBAL METHODS TO STUDY CELL SIGNALING – THE OMICS ERA

Genomics, proteomics, phosphoproteomics and other omic-based approaches are now broadly used in biomedical research to facilitate the understanding of disease mechanisms and identification of molecular targets and biomarkers for therapeutic and diagnostic development. One important issue which differs between omics-based approaches and traditional research is the global and unsupervised strategies. In traditional research experiments there is already from the beginning a hypothesis involving the outcome of a few known players, this hypothesis is then tested if it is valid or not – the experiment is hypothesis driven. In contrast, in omics based strategies snap shots of large data sets are compared and the result is used to generate a hypothesis about what is ongoing in different situations and the players involved is presented by the experiment it self – the experiment is hypothesis generating.

Biomarkers are referred to as biological entities or characteristics that can be used to indicate the states of healthy or diseased cells, tissues, and individuals. Nowadays, biomarkers are mostly molecular makers, such as genes, proteins, metabolites, glycans, and other molecules, that can be used for disease diagnosis, prognosis, prediction of therapeutic responses, as well as therapeutic development (167-169). Over the past decade, high-throughput technologies, such as genomic microarrays, proteomics and metabolomics by mass spectrometry, have been used to generate large amount of data from single experiments that allow for global comparison of changes in molecular profiles that underlie particular cellular phenotypes. As a result, the omics-based approaches, coupled with computational and bioinformatics methods, provide unprecedented opportunities to speed up the biomarker discovery and now are widely used to facilitate diagnostic and therapeutic developments for many diseases and particularly in cancers (170-177). Potential biomarkers have been identified at various molecular levels, including genetic, mRNA, protein/peptide, as well as epigenetic (178-180), miRNA (181-182), glycans (183-186), and metabolites (169). For example, using DIGE-based proteomics potential biomarkers were identified to be useful for the diagnosis of metastatic prostate cancer (187), and a proteolytic fragment of alpha1-antitrypsin was identified as a potential diagnostic and prognostic marker for inflammatory breast cancer as well as a target for potential therapeutic intervention (188-189). In addition, microRNAs, such as miR-500, were identified as a potential diagnostic marker for hepatic cell carcinoma (190). Increasingly, pathway and network-based analyses are applied to Omics data to gain more insight into the underlying biological function and processes, such as cell signaling and metabolic pathways and gene regulatory networks (191-192). For example, 12 core signaling pathways were shown to be altered in human pancreatic cancers through genomic analyses (191). Network modeling linked breast cancer susceptibility to the centrosome dysfunction (193), and led to the identification of a proliferation/ differentiation switch in cellular networks of multicellular organisms (194). These approaches have led to a new trend in identifying biomarkers in recent years, namely, pathway and network-based biomarker discovery, which identify

panels of, instead of single, biomarkers for practical use in diagnostic and therapeutic developments (195-198). Protein networks have been shown to provide a powerful source of information for disease classification and to help in predicting disease causing genes (199-202). Network approaches have also been used for improving the prediction of cancer outcome (203-204), providing novel hypotheses for pathways involved in tumor progression (204), and exploring cancer associated genes (205). However, while the omics technologies and bioinformatics tools for analyzing omics data are rapidly advancing, the functional analysis and interpretation of the data remain challenging.

1.4.1 Genomics

Over recent years the sequencing of the whole genome of different organisms, including man, has provided the foundation for several powerful new biotechnologies offering insight into the molecular characteristics of the cell, the regulation of cellular activity, the response of the cell to its environment and cellular defects in disease states. Genomics/transcriptomics are based on the faithful transcription of DNA from the coding region of the gene into a complementary single-stranded mRNA molecule and subsequent translation into the protein. Application of these technologies were revolutionizing the biosciences in the early 2000's, (206). Functional genomics is being used to characterize the molecular events occurring in disease processes (207), to identify polymorphisms in genes that may be associated with increased (or decreased) risk of specific diseases (208) and to search for biomarkers of disease (209). DNA array technology allows the expression of thousands of genes to be analyzed simultaneously. This high-throughput approach is facilitating rapid advances in genomics and transcriptomics. The principle underlying the array technologies is hybridization between complementary single-stranded nucleic acid sequences exploiting the specific relationship between A and T, and G and C (210). The latest micro-arrays consist of densely-packed spots of single-strand cDNA or oligonucleotides complementary to the sequence of the gene of interest immobilized onto a substrate, e.g. glass or nitrocellulose. mRNA is extracted from cells, reverse-transcribed using fluorescently-labelled nucleotides and hybridized to the array. The array is laser-scanned and expression levels determined from the intensity of the fluorescence at each spot. The results are processed through a database and data mining tools used for analysis (211). Such experiments generate massive amounts of expression data, the interpretation of which is challenging and depend largely on the quality of genomic databases and of the software tools available. Good experimental design and careful attention to experimental protocols are critical to the success of micro-array experiments. Lately, the development of systematic approaches to identify somatic mutations has prompted exhaustive analyses of changes in cancer genomes, including copy-number changes (deletions and amplifications of DNA), rearrangements, small insertions and deletions, and point mutations (212). Recently, these efforts have culminated in the sequencing of complete genomes of human cancers, providing comprehensive catalogues of somatic mutations (213-214). These studies have yielded insights into the genes that contribute to cellular transformation (212).

1.4.2 Proteomics

The term ‘proteome’ was coined by Marc Wilkins and Keith Williams at the Center for Analytical Biotechnology of Macquarie University, Australia in 1995. The word is a combination of ‘protein’ and ‘genome,’ and may be defined as the protein molecules associated with a genome (215). Proteomics is the study of the various types of proteins and their interactions in a cell or other biological entity. It is the application of tools from different fields, as diverse as clinical medicine, molecular biology, mass spectrometry (MS) and bioinformatics to explore the localization, identification and characterization of protein, as well as to shape this information into new knowledge (215).

Proteomics, which can be defined as large scale analysis of the whole protein complement of e.g. cells and tissues, enables the unbiased comparison of different cellular states in biology and medicine at a “systems-wide” level. However, technological challenges associated with proteomics have long prevented its full potential. Two-dimensional (2D) gel electrophoresis was conceived more than 30 years ago (216). This technology has been useful for low-complexity protein mixtures but has still not matured into a comprehensive and accurate technology for complex proteomes. The introduction of high-sensitivity protein identification by MS was first promising also for 2D gel analysis, but in fact it revealed that the thousands of spots seen in the gel maps are actually variants of a few hundred of the most abundant proteins (217). A few years ago it also became clear that quantification of these proteins is far from accurate because of spot overlap (218). Accordingly, “biomarkers” found by these technologies tend to be the same regardless of the system under investigation (219). MS technology with low resolving power e.g. in the form of surface enhanced laser desorption ionization (SELDI), gained a lot of attention among clinicians a few years ago. However, from a mass-spectrometric point of view, SELDI boils down to simple matrix associated laser desorption ionization (MALDI) spectra of very complex mixtures and would be expected to only yield a subset of the most abundant low-mass peptides and protein fragments. Such species could still have proven sufficient to classify patient samples. Yet, identification of the peaks comprising the SELDI patterns, usually turned out to belong to the same nonspecific proteins unlikely to be directly associated with the disease. In contrast to the above approaches, which were discussed as promising proteomics technologies as late as a few years ago; MS-based proteomics has taken great strides in development (220). In particular, technological improvements in the last 5 years have dramatically increased the routine availability of extremely high-performance MS. In some cases, these technologies already existed but could only be applied in specialized situations by expert laboratories and at low throughput. Today, MS techniques with high accuracy can be applied in most proteomics contexts and comprehensive analysis of complex proteomes is best achieved by using high-resolution proteomics technologies. An important aspect of MS-based proteomics is the remarkable inroads of proteomics strategies into the analysis of phosphorylated proteins.

1.4.3 Phosphoproteomics

In the past, kinases and their activities were generally studied on an individual basis using biochemical approaches. However, technological advances in the recent past have led to development of several high-throughput strategies to study the phosphoproteome. High-throughput technologies for monitoring phosphorylation events include array-based technologies such as peptide arrays (221-224), reverse phase protein arrays (225), antibody arrays (226) and mass spectrometry (227-231).

The first technique for global analysis of phosphorylated proteins from complex protein samples was done by separating the proteins according to their pI and their molecular weight using 2-DE (232-236). The separated proteins can subsequently be visualized using standard protein stains (e.g., Coomassie blue and silver staining). By this method the post translationally modified proteins, such as phosphoproteins, can appear as multiple pI variants according to their modification states, since the addition or subtraction of a chemical group can subtly, but detectably, alter the pI of a protein. This is especially evident for multiply phosphorylated proteins. Phosphoproteins separated by gel electrophoresis can also be visualized by the use of a phosphospecific stain such as Pro-Q Diamond (237-241). However, as discussed above, 2D is having a tough time with highly complex samples. On the other hand, ideas originating from 2D by using narrow-pH-IPG-strips on the peptide level is showing excellent results and is promising also for phosphoproteomics (242-243). Alternatively, phosphoproteins can be radioactively labeled using ^{32}P or ^{33}P (237-238) and detected by autoradiography, which is the most sensitive method available, but may not be compatible with downstream analytical procedures such as MS. Radioactive labeling is possible both in vivo as well as in vitro. In in vivo studies, cells are incubated with ^{32}P , however, the presence of endogenous ATP pools within cells can interfere with the incorporation of the label and result in inefficient radioactive labeling (244). Furthermore, ^{32}P is toxic for many cells and will over time cause damage to the cell. In in vitro studies, proteins are incubated with specific kinases in the presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, and under appropriate conditions ^{32}P is incorporated at the phosphorylated amino acid residue. A major drawback of this method, however, is that kinases may phosphorylate target proteins promiscuously. The occurrence of promiscuous phosphorylation events is due to the unnaturally high concentration of kinase compared to the substrate concentration (134), and kinases that would not phosphorylate a particular protein in vivo might still induce phosphorylation in vitro (245).

Enrichment strategies prior to MS-analyses in phosphoproteomics

Phosphoproteins are nowadays mostly characterized using MS methods after proteolytic processing. However, phosphopeptides are notoriously difficult to analyze by MS, especially in the presence of non-modified peptides. This is mainly due to lower ionization efficiency of phosphopeptides resulting in lower signal intensities in the presence of nonphosphorylated peptide ions. Thus, efficient enrichment of the phosphorylated peptides prior to MS analysis will result in increased sensitivity and

more efficient characterization. There are various phosphoproteomic enrichment strategies to choose from and the most common is described below.

IMAC

Immobilized metal affinity chromatography (IMAC) is a widely used affinity based technique for enrichment of phosphopeptides prior to MS analysis. Metal ions (Fe^{3+} , Al^{3+} , Ga^{3+} , or Co^{2+}) are chelated to nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) coated beads, forming a stationary phase to which negatively charged phosphopeptides in a mobile phase can bind. The technique was initially used for the affinity-purification of proteins based on the interactions of histidine and cysteine residues with the IMAC resin (246-248). However, the binding of phosphoproteins and phosphoamino acids to metal ions, first demonstrated by Andersson and Porath in 1986 (249), gave the technique new potential. IMAC was further extended by Neville et al. to the enrichment of phosphopeptides obtained from proteolytically digested proteins (250), and the IMAC technique has since been used extensively for enrichment of phosphopeptides prior to MS analysis (251-256). One of the problems associated with the IMAC technique is the often high level of nonspecific binding when used for phosphopeptide enrichment of highly complex peptide samples. Nonphosphorylated peptides containing multiple acidic amino acid residues co-purify with the phosphopeptides, thereby reducing the selectivity of the method. This is a serious problem, due to the more efficient ionization of nonphosphorylated peptides in subsequent MS analyses resulting in the suppression of signals from phosphopeptides. Ficarro and co-workers circumvented this problem by derivatizing the carboxylic groups on acidic amino acid residues in peptides by O methyl esterification (257) and thereby improving phosphopeptide enrichment considerably (257-258). However, further experiments have shown that Omethyl esterification does not derivatize 100% of the available carboxylic acid groups, and that it can increase the complexity of the analysis due to signals originating from peptides with different degrees of O-methylation (259). Another approach to increase the efficiency of IMAC is to adjust the pH. Acidification of a sample prior to IMAC analysis protonates the carboxyl groups on the highly acidic amino acid residues and reduces nonspecific binding. In most studies, 0.1–0.25 M acetic acid (pH 2.7) were used as a loading buffer for IMAC, but it has been shown that the pKa value of phosphoric acid decreased to 1.1 upon methylation. The pKa values of phosphopeptides would therefore be expected to be significantly lower than that of phosphoric acid due to the organic environment provided by the surrounding amino acid residues. By decrease in the pH of the IMAC loading conditions further (below pH 1.9), more acidic peptides will become neutralized while phosphopeptides will retain their negative charge and their binding affinity toward the IMAC resin. Kokubu and co-workers showed that 0.1% trifluoroacetic acid (TFA), 50% ACN as IMAC loading buffer reduced the level of nonspecific binding and improved the specificity of the method toward phosphopeptides (260). Yet another approach to improve the performance of IMAC is to reduce the complexity of the peptide samples by prefractionation using methods such as IEF (261-262), Ion Exchange Chromatography (263) or hydrophilic interaction chromatography (HILIC) (264) prior to IMAC purification.

TiO₂

In the mid-2000s TiO₂ chromatography was introduced as an alternative to IMAC. In 2004, Pinkse and co-workers presented an online 2-D LC strategy for phosphopeptide analysis with spherical particles of titanium dioxide (Titansphere) as the first dimension and RP material as the second dimension. The peptide sample was loaded onto TiO₂ columns in 0.25 M acetic acid (pH 2.9), which promoted the binding of phosphopeptides. Unbound, nonphosphorylated peptides were trapped on the RP column, and subsequently eluted and analyzed using nanoLC-ESI-MS/MS. The phosphopeptides were eluted from the TiO₂ column using an alkaline buffer (pH 9.0), concentrated on the RP precolumn and analyzed using nanoLC-ESI-MS/MS (265). The strategy was tested on a 153 kDa homo-dimeric cGMP-dependent protein kinase, and eight phosphorylation sites were identified, including two novel sites(265). A protocol for phosphopeptide enrichment using TiO₂ has been developed by the protein research group in Odense. In this offline strategy peptide loading is performed at highly acidic loading conditions using various substituted organic acids including 2,5-dihydroxybenzoic acid (DHB), phthalic acid or glycolic acid to efficiently eliminate binding of acidic peptides to the TiO₂ resin (266-268). In addition, by increasing pH of the elution buffer from 9.0 to 11.3 (using ammonia solution), phosphopeptides are more efficiently eluted from the TiO₂ resin, resulting in increased sensitivity (267). TiO₂ chromatography provides a high selectivity toward phosphopeptides in the optimized loading buffer and the offline setup is simple, fast, and does not require expensive equipment. Furthermore, TiO₂ is extremely tolerant toward most buffers and salts used in biochemistry and cell biology laboratories (266).

Combining SCX and downstream enrichment strategies

Strong cation exchange (SCX) was originally used for prefractionation of proteins and peptides, where positively charged groups (cations) interact with negatively charged functional groups on the SCX particles. In 2004, Beausoleil and co-workers introduced the use of SCX for phosphopeptide enrichment (263). Tryptic peptides were acidified in 5 mM KH₂PO₄, 30% ACN, pH 2.7. At this pH many tryptic peptides will have a charge state of 12 due to the charge at the C-terminal arginine or lysine as well as the charge on their N-terminus. Tryptic mono-phosphorylated peptides carrying the negative charge from a phosphate group would therefore have a net charge of 11 at pH 2.7. Phosphopeptides were therefore expected to display weakened binding to SCX material, and therefore elute before nonphosphorylated peptides with multiple positive charges. Multiply phosphorylated peptides would have net charges at 0, and would therefore not be retained by the SCX material. However, in this particular study, the authors did not examine the unbound peptides in the SCX flow-through. Bound tryptic peptides were separated by SCX chromatography using a gradient of KCl and finally an elution step using pH 7.0. The early eluting peptides were desalted and analyzed by RP LC-MS/MS. Their results confirmed a high level of phosphopeptides in the early fractions, which had been separated from nonphosphorylated peptides found in later fractions. Beausoleil et al. identified 2 000 phosphorylation sites from 8 mg nuclear extract of HeLa cell lysate using this approach (263). For large-scale phosphoproteomic

studies, SCX is most efficient as a prefractionation technique prior to phosphopeptide enrichment using either IMAC or TiO₂ chromatography. Trinidad and co-workers compared the enrichment efficiency of SCX as a prefractionation method prior to IMAC to both IMAC and SCX alone (263). They found that the combination of SCX and IMAC led to at least a three-fold increase in the number of phosphopeptides identified relative to either approach alone. In addition, a work by Villén et al. in which they fractionated tryptic digests from murine liver using SCX and enriched all 15 fractions for phosphopeptides using IMAC, showed that phosphopeptides were spread throughout all SCX fractions (269). Nevertheless, SCX greatly improves phosphopeptide recovery when used as prefractionation prior to subsequent phosphopeptide enrichment and robust protocols for such studies are now available to the scientific community (270). Using a combination of SCX and IMAC, Gruhler et al. performed a large-scale phosphoproteomic study on the yeast pheromone signaling pathway and identified and quantified more than 700 phosphopeptides (252). Another study from Olsen et al found 2 244 phosphorylated proteins in HeLa cells upon stimulation using EGF by combining SCX and TiO₂ chromatography (271). Anyhow, it should be noted that in both cases large amounts of starting material were used and it remains to be determined whether the combination of prefractionation and enrichment will be ideal for very low microgram levels of starting protein. Finally, the leading study with regard to phosphoproteomics also uses SCX in combination to IMAC and TiO₂ and was presented recently by Olsen and Mann, reporting the impressive number of more than 6000 phosphorylated proteins in their study describing phosphorylation events during mitosis (272).

Interpretation of phosphoproteomic experiments - the importance of sharing data

Even though phosphoproteomic studies really have started to pay off in numbers, the knowledge of the phosphorylated sites per se is neither sufficient to identify how signals are propagated into cells nor adequate to define the complexity of the intracellular networks. To fully appreciate the relevance of phosphoproteomic approaches it is essential to gain additional knowledge about the biological conditions under which the phosphorylation occurs, to identify the enzymes (kinases and phosphatases) that switch 'on and off' their substrates, and to understand the functional consequences that these modification events have on cellular processes. Since the future knowledge and exploitation of reversible phosphorylation relies on the accessibility of the data, it is of fundamental importance to develop and maintain public depositories to facilitate shared data retrieval. Bioinformatics resources which also incorporate substantial phosphorylation data are PHOSIDA (273-274) and phosphoPEP (275) which are specialized in annotation of large-scale experiments. Additional information on phosphorylation resources can be found at the Phospho.ELM link page (<http://phospho.embl.de/linkshtml>).

2 THE PRESTENT STUDY

2.1 AIMS

The general aim of this thesis was to set up and use unsupervised global methods to gain novel insight of cellular signaling events responsible for the mechanisms controlling radio resistance in NSCLC cells. The aim was further to use such information to generate hypothesis and find candidates that could be sent on to future pre-clinical evaluation in larger cohorts of cell lines and optimally also to future clinical intervention.

The specific aims were:

Paper I: To use quantitative proteomics and a novel pathway search engine (PSE) to find differences on the pathway level when NSCLC cells are irradiated with low – and high LET IR.

Paper II: To globally screen for genes of importance for the increased cell death observed in NSCLC cells when combining conventional IR and the Staurosporine analouge, PKC412. In addition, to further evaluate the cellular impact of such gen/genes.

Paper III: To set up a method for phosphoproteomic profiling of complex samples. Moreover, to use this method to explore signaling events in NSCLC cells controlled by ephrin B3 (ephrin B3 was a found to be of importance for mechanisms of radio resistance in **Paper II**).

Paper IV: To further explore the signaling question regarding differential apoptotic response to low and high LET IR (**Paper I**) in NSCLC cells by using the approach of the phosphoproteomic set up in **Paper III**.

2.2 MATERIAL AND METHODS

All material and methods used in paper I-IV are described in each paper and/or in their corresponding references. A brief description and comments are presented below.

2.2.1 U-1810 cells as a model system to study radioresistance mechanisms of NSCLC

In all of the studies (**Paper I - IV**) of this thesis, the NSCLC cell line U-1810 was used as model system to study mechanisms of resistance towards conventional low LET radiation. U-1810 cells were characterized by Berg et al at the department of Oncology at Uppsala Academic Hospital in Sweden (276). Accordingly, the "U" in U-1810 stands for Uppsala. U-1810 cells were derived from a patient with undifferentiated large cell carcinoma and are classified as adherent as they grow attached to the flasks. One way of characterize the radio sensitivity or how resistant a cell line is to radio therapy is to use clonogenic survival assay to calculate its SF2 value (277). The SF2 value is equal to the mean survival fraction after cells have been irradiated with 2Gy, where the value of 1.0 means that all cells have survived. U-1810 cells are deficient in p53 and show a high degree of radioresistance with a SF2 value of 0.88. Moreover, U-1810 cells could be stated to have a high proliferation rate relative to other lung cancer cell lines. Thus, it was considered as a good model system for method development and for studying mechanisms of radioresistance in NSCLC.

2.2.2 Low - and high LET irradiation

Low LET IR delivered as photons of gamma rays using a ^{60}Co source (LET= 3 KeV/ μm , absorbed dose 8 Gy) was used in **Paper I**, **Paper II** and **Paper IV** and is, as indicated in the section for radiotherapy in the introduction, the quality of radiation that is commonly used in the clinic for cancer therapy; hence it is characterized as conventional radiotherapy. Whenever we are referring to only IR without stating the quality of IR in the text of this thesis, we are referring to low LET IR. High LET IR was used in **Paper I** and **Paper IV** and in the form of accelerated nitrogen ions (LET=125 KeV/ μm , absorbed dose 4 Gy). The $^{14}\text{N}^{7+}$ ions were delivered with a mean energy of 20.9 MeV/nucleon from the Gustaf Werner synchrocyclotron at the Svedberg Laboratory, Uppsala University, Sweden.

2.2.3 RNA interference

Small RNA molecules called siRNA (short interference RNA) is short single stranded RNA oligo nucleotide strands consisting of 20-22 nucleotides, binding specifically to sequences of mRNA. As a result of the binding, the mRNA is rapidly digested and cannot be expressed. However, to achieve robust gene silencing, experimental conditions need to be carefully optimized for each gene/protein to be silenced and every different cell line to be used. The siRNA technique was used to silence ephrin B3 in **Paper II** and **Paper III**. Custom made ephrin B3 siRNA was purchased from Qiagen (Qiagen, Maryland, USA). One potential problem with siRNA technology is the off target effects in which a design siRNA in addition to inhibit its target also

blocks the expression of other proteins making the biological implementation of results difficult. To avoid this in our studies, we used the 5'-CCAGGAGTATAGCCCTAAT-3' sequence since it was previously described as unique relative to other Ephrin members (278). Transfections were performed according to the manufacturers' instructions. Briefly, 500,000 cells were seeded onto 10 cm dishes and transfected after 6 h using 100 nM siRNA.

2.2.4 Assays of cell fate

Growth

Cell division was assessed in **Paper II** using the CFSE labeling method. The CFSE is a cell-permeable molecule which is non-fluorescent until it is cleaved by intracellular esterases to yield the fluorescent carboxyfluorescein succinimidyl ester (CFSE), which is retained within the cells. During each cell division, the fluorescent label will be progressively diluted, allowing cell proliferation to be monitored by Fluorescence Associated Cell Sorting (FACS) (279).

Apoptosis and mitotic catastrophe

To assess if cells were dying from apoptosis or mitotic catastrophe the characteristics of nuclear morphology typical for each state was analyzed in **Paper II**. Cells were fixed and the nuclei were stained with mounting media containing 4,6'-diamidino-2-phenylindole (DAPI). The number of cells with nuclear morphology indicative of an apoptotic or mitotic cell was quantified using a fluorescent microscope. Another strategy to measure apoptosis was the solid-phase sandwich enzyme immunoassay M30-Apoptosense™ ELISA in **Paper IV**. In this assay, apoptosis is detected as caspase-mediated cleavage of cytokeratin 18 by the M30 monoclonal antibody which specifically recognize a neo epitope exposed in caspase-cleaved cytokeratin 18 (280). Also, Caspase-3 activity was examined in **Paper I** in intact cells using an antibody that recognizes active caspase-3 as an indication of apoptosis.

Senescence

NSCLC cells were analyzed in **Paper II** using a commercial Senescence Cells Histochemical Staining Kit where the cells are stained with X-gal-based staining mixture overnight at 37 °C. The percentage of blue-stained cells expressing β -galactosidase was then examined in a microscope.

2.2.5 Gene array

In **paper II**, the method for global analysis was on the level of expressed genes and data was acquired from gene array. The mRNA was isolated from 10 million U-1810 cells/ treatment and RNA purity and concentration were assessed using a NanoDrop ND-1000 UV-Vis Spectrophotometer. Gene expression analyses were performed using Affymetrix chip on the Affymetrix platform (Affymetrix Inc, CA, USA). The principle was to synthesize cDNA from the mRNA and then cRNA from the synthesized DNA. Samples are labeled with a fluorophore and if the nucleic acid sequences on the probe

of the array match the sample hybridization occur. It generates a signal that depends on the strength of the hybridization determined by the number of paired bases, thereby reflecting the degree of mRNA expression. The synthesis of cRNA, labeling and hybridization were performed according to the protocols provided by the manufacturer.

2.2.6 Enrichment of phosphorylated peptides using SCX-fractionation and TiO₂-magnetic beads

In **Paper III** and **IV** a phosphoproteomic approach was applied, thus requiring strategies to enrich for phosphorylated peptides before sending the samples to analysis in the mass spectrometer. This is required basically because of the low abundance of phosphorylated peptides compared to the non phosphorylated peptides. The enrichment of our complex samples was performed in two steps, where step one was SCX-fractionation inspired by the protocol of Villen et al., (270) where the peptides are fractionated depending on their net charge. Peptides with low positive net charge elute early. Each sample was manually injected into the loop; an Agilent 1200 LC system was used, complete with binary pump, diode array detector and fraction collector. Samples were fractionated into 12 fractions per run and identical fractions from the three runs per sample were then pooled before cleanup.

The second step of enrichment was performed by taking advantage of the affinity of the negative phosphate group/s to positive Titanium charges. After clean up, each fraction from SCX fractionation was resuspended in binding buffer (80 % ACN, 5 % TFA and 1M Glucolic Acid). TiO₂ magnetic beads *TiO₂ (Mag Sepharose™, GE Healthcare)* were used for enrichment of phosphopeptides. In this process, peptides are bound to the beads, beads are washed from non bounding peptides, the flowthrough is put on new beads for a second chance of binding before extensive washing steps and final elution steps in Amonia-based basic buffer (pH > 11). All wash steps and elutions were done by pipeting the solvents while the tubes where in a magnetic tube rack where beads were allowed to congregate at the magnet for one minute before collecting the supernatant, an approach that was convenient and highly simplifying the handling of the beads. More detailed aspects regarding global studies of protein phosphorylations are conversed in the section for discussion.

2.2.7 LTQ-FT mass spectrometry

For the mass spectrometry (MS) analysis in **Paper I**, a 7-Tesla hybrid linear ion trap Fourier transform mass spectrometer (LTQ FT) equipped with a Proxeon nanoelectrospray ion source was used. For separation of the peptides, a high performance liquid chromatography setup, Agilent 1100 nanoflow system, was applied. As analytical column, a 15 cm long fused silica emitter was used. The column was packed in house from a slurry of reverse phase material in methanol. The peptide solution was loaded onto the analytical column and for data acquisition unattended mode was used in which the mass spectrometer automatically alternated between a high resolution overview MS-scan and a lower resolution fragmentation scan (ECD followed by CAD) of the two most abundant peptides at any given moment.

2.2.8 LTQ - Orbitrap Velos mass spectrometry

For phosphoproteomic analyses in **Paper III** and **IV** each fraction from a sample enriched for phosphopeptides was injected into online HPLC-MS performed on a hybrid LTQ-Orbitrap Velos mass spectrometer. An Agilent HPLC 1200 system was used to provide the gradient for online reversed-phase nano-LC. The sample was injected into a C18 guard desalting column prior to a C18 picofrit column installed on to the nano electrospray ionisation source of the Orbitrap Velos instrument. Acquisition proceeded in ~3.5s scan cycles, starting by a single full scan MS, followed by two stages of data-dependent tandem MS: the top 5 ions from the full scan MS were selected firstly for collision induced dissociation (CID) with MS/MS detection in the ion trap, and finally for higher energy collision dissociation (HCD) with MS/MS detection in the orbitrap (FTMS). Additional experiments were performed using electron transfer dissociation (ETD), as an alternative fragmentation method for peptide ions

2.2.9 Data handling and validation

Subsequent to gene array analysis performed in **paper II**, the data were analyzed by the GeneSpring GX software (Agilent Technologies, CA, USA). Normalization of gene expression data was accomplished in two ways: per chip normalization and per gene normalization. For per chip normalization, all expression data on a chip were normalized to the 50th percentile of all values on that chip. For per gene normalization, the data for a given gene was normalized to the median expression level of that gene across all samples. The expression profiles from the three independent experiments were compared using ANOVA (parametric test, variances assumed equal) to identify genes that were differentially expressed between the different treatments. A few selected genes were validated on real time quantitative PCR (RT-qPCR) to confirm expression pattern from gene array analyses. Further analyses of gene ontology were also done using the GeneSpring GX software. For pathway analysis of proteomic data in **Paper I**, a novel pathway search engine (PSE) developed by the group of Roman Zubarev (281) was applied. This search engine allows identified proteins to connect to key nodes which receive a score due to its connectivity with the proteins. Top scoring key nodes is then mapped to corresponding genes which in turned are mapped on pathways in the Transpath data base. In **paper III and IV** Protein Center (PROXEON/Thermo Fisher Scientific) were used to handle and filter phosphorylated peptides and proteins as well as visualize phosphorylation sites. Ingenuity pathway analysis (Ingenuity Systems, Inc., Redwood city, California, USA) was used to generate hypothesis for biological interpretation by creating networks and map differentially phosphorylated proteins on canonical pathways. For validation of hypotheses generated from omics data and subsequent interpretation of the data using bioinformatic tools, orthogonal strategies using siRNA (described above), standard immunobased assays like western blots, immuno precipitation, ELISA and immunofluorescence analysis were used. In addition, for validation of biological function in **Paper I** pharmacological inhibitors of JNK and p38MAPK were applied to ascertain the role of these kinases in high LET IR-induced apoptotic signaling.

2.3 RESULTS

2.3.1 Paper I

Proteomics and Pathway Analysis Identifies JNK-signaling as Critical for High-LET Radiation-induced Apoptosis in Non-Small Lung Cancer Cells

The aim of this study was to, by an approach of quantitative proteomics, gain insight of which pathways that are critical for induction of apoptosis by high LET IR. The NSCLC cell line U-1810 which is highly resistant to conventional low LET IR and also has impaired low LET IR-induced apoptotic signaling (65) was shown as sensitive to high LET IR by induction of apoptosis. In response to high LET but not low LET IR, these cells showed a time-dependent increase in apoptotic morphology. Moreover a time-dependent increase in activation of caspase-3, one of the major executor caspases in apoptotic signaling, was observed after high LET IR. In accordance with previous results from our research group (65), little or no increase in caspase-3 activity was observed in response to low LET IR in these NSCLC cells. Thus, the U-1810 cell line could be considered as a suitable model system for the study.

We here employed an unsupervised shotgun proteomics approach where Fourier transform mass spectrometry coupled to nanoflow-liquid chromatography determined the identity and relative abundance of expressed proteins. The development in different global proteomic methods have increased the generation of large proteomic data sets. In order to extract the most critical signaling events from such large data sets in a non-biased way, computerized methods based on statistics are required. The Pathway search engine (PSE) has been developed as a novel tool intended to meet these requirements and its application was used for the first time in this study. Based on the proteomics data, PSE suggested the JNK-pathway as a key event in response to high-LET IR. In addition, the Fas-pathway was reported activated and the p38- pathway was found deactivated compared to untreated cells. Immuno-based analyses (western blot and immunofluorescence analysis) confirmed that high-LET IR caused an increase in phosphorylation of JNK. Also, more players of the JNK-pathway as it is defined in the TRANSPATH database, i.e. MKK4 and 14-3-3 α , were proven to be regulated. Moreover, pharmacological inhibition of JNK blocked high-LET IR induced apoptotic signaling as measured by decreased caspase-3 activation and apoptotic morphology. In contrast, neither an activation of p38 nor a role for p38 in high-LET IR induced apoptotic signaling was found. Thus PSE predictions were largely confirmed and it was proven that PSE could be useful as a hypothesis-generating tool.

2.3.2 Paper II

Global gene analysis reveals ephrin B3 as a major player in mechanisms controlling radioresistance in non-small cell lung carcinoma cells

The staurosporine analogue PKC 412, but not Ro 31-8220, had previously been shown to sensitize NSCLC cells to ionizing radiation (IR) (77, 83). These effect of combining PKC 412 and IR was indeed confirmed in this study as an increased caspase-3 activity was documented using PARP and cytokeratin 18 cleavage as endpoints. Interestingly

we also observed a striking change in the morphology of the cells as they become elongated, significantly larger and flattened. To reveal genes of importance for the radiosensitizing effect of PKC 412, a total gene profiling in response to co-administration of i) PKC 412 with IR and ii) Ro 31-8220 with IR, was implemented using the Affymetrix gene array platform. The death increasing combination of IR and PKC 412 specifically caused upregulation of 140 genes and down-regulation of 253 genes.

A few genes were selected and verified by real-time quantitative PCR. Out of these, suppressed ephrin B3 expression was suggested as a possible radiosensitizing mechanism of PKC 412. Silencing of ephrin B3 using siRNA resulted in a decrease in cell proliferation, induction of an elongated cell phenotype similar to the phenotype we had observed on cells treated with the combination of PKC 412 and IR as well as an increase in cell death, measured by quantifying PARP cleavage on western blot and by induction of apoptotic cell nuclear morphology. Moreover, silencing of ephrin B3 in combination with IR caused a decrease in IR-mediated G2 cell cycle arrest and induced cellular senescence illustrated by increased β -galactosidase staining as well as the characteristic flattened and elongated cellular phenotype in addition to increase of cells in the G0/G1 phase of the cell cycle. Using western blotting an inhibition of the MAPKs ERK and p38 phosphorylation and up regulation of p27 was observed. In conclusion, our non-supervised approach identified ephrin B3 as a putative signaling molecule involved in resistance to conventional radiotherapy.

2.3.3 Paper III

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

In paper II we identified ephrin B3 as a critical signaling molecule mediating radioresistance of NSCLC cells. Following up on these results, we in this paper aimed to understand how silencing of ephrin B3 influenced the phosphoproteome and thereby the signalome of these NSCLC cells. For this purpose ephrin B3 expression was silenced in the non small cell lung carcinoma cells U-1810 using siRNA. Cellular extracts were generated from these siRNA ephrin B3 silenced cells and non-targeting siRNA was used as control.

One of the main aims of this thesis was to set up a robust global method for analyses of the phosphoproteome of complex samples. The method was used in this project and we applied a phosphoproteomic approach to perform a global and unsupervised screen for changes in the phosphoproteome between U-1810 cells with or without silenced Ephrin B3. The workflow consisted of cell lysis, protein digestion, SCX-fractionation (as a first separation and phosphopeptide enrichment step) and finally, before mass spectrometry analysis, an enrichment of phosphorylated peptides using magnetic titanium dioxide beads.

In total, 1083 phosphorylated proteins were identified. Out of these, 150 and 66 proteins respectively were identified and characterized as only being phosphorylated in U-1810 cells treated with non target siRNA versus cells treated with siRNA for Ephrin

B3. Network analysis by the Ingenuity software was used to elucidate these data and a few candidates from the top network were selected for further studies. Interestingly, another ephrin-family member, i.e. the EphA2 receptor B3 together with activation of Focal adhesion kinase 1 (FAK1) was reported as a top candidates, activated only in the presence of Ephrin B3.

The EphA2 was phosphorylated on Ser 897 only in the presence of ephrin B3, a phosphorylation which is known to take place only in absence of the EphA2 ligand ephrin A1(282). This phosphorylation is generated by Akt1 (282) which in turn is activated by CK2 on Ser 129 (283). Also HSP90AA1 is activated by CK2 and has been reported to stabilize EphA2 and save it from degradation (284). These results reported in the literature fitted well with our data of the identified phosphorylation sites which only were phosphorylated in presence of ephrin B3. In line with such a hypothesis, we could also prove in this study that EphA2 actually not existed in these cells when ephrin B3 is absent, supporting its degradation when not chaperoned by HSP90AA1. By stepwise pathway walking and validation of phosphorylation sites by immune-based strategies, this signaling scenario was largely confirmed and our data support an ephrin B3/EphA2 survival pathway which potentially is critical for retaining resistance mechanisms in NSCLC cells.

2.3.4 Paper IV

Phosphoproteomic profiling to explore differences in cellular response between high and low LET irradiated Non small cell lung cancer cells

The present study is from a biological perspective a follow up on **Paper I** as we intended to further explore how the signalome differs in response to low vs. high LET IR. However, from a technical perspective study IV is rather a follow up on **Paper III**. Thus, we applied the phosphoproteomic approach for profiling, using basically the same method as applied in **Paper III**.

To confirm that our biological system from paper I was still valid i.e. to confirm that high LET IR induced apoptosis in the U-1810 cells, we checked activation of the proapoptotic BAK and BAX mitochondrial proteins. Indeed we could clearly detect BAK and BAX activation in response to high LET IR which was in marked contrast to the failure of activation found after low LET IR (65).

In this project, the depth of the identified phosphoproteome were even larger compared to our previous study (Paper II). In total, as much as 12012 phosphorylated peptides were identified. In addition 1781 other non-phosphorylated peptides also mapping to the same proteins as the phosphorylated peptides were identified. This corresponded to a total of 2314 unique phosphorylated proteins, thus, reaching numbers of identified phosphorylated proteins in the same range as world leading phosphoproteomic laboratories (265, 285). Out of those, in a first step of pathway analysis, only phosphorylated proteins that were detected in all three replicates corresponding to a certain treatment i.e. low or high LET IR but never detected in the comparative treatment were sent to pathway analysis (low LET IR: 242 phosphorylated proteins,

high LET IR:128 phosphorylated proteins). Using this strategy, IPA canonical pathway analysis suggested pathways describing assembly of RNA polymerase III complex and eIF-signaling only in response to low LET IR. After selection of these two pathways which both are implemented in protein synthesis, a more allowing step was added to pathway analysis. This second step was added as a strategy to take advantage of as much as possible of the information available from the data sets and thereby increase coverage of the two pathways of interest from step 1. Hence, the requirement for a phosphorylated protein to participate in the second part of pathway analysis was that it had to be significantly identified only in at least one of the three replicates corresponding to a certain treatment i.e. low or high LET IR but of course still never detected in any of the replicates of the comparative treatment. These requirements were fulfilled for 823 phosphorylated proteins in response to low LET IR and 431 phosphorylated proteins in response to high LET IR. By adding the second layer of data, the coverage of the pathways describing assembly of RNA polymerase III complex and eIF-signaling which were found activated only in response to low LET IR were increased and the data covered a majority of the circuits of these canonical pathways.

U-1810 cells are resistant to low LET IR but respond to high LET by induction of apoptosis. Thus, NSCLC cells irradiated by low LET IR most probably have higher proliferation rate than cells irradiated by high LET IR. As a consequence of higher proliferation rate, our finding of increased active protein synthesis which indeed is a requirement for speeding up proliferation could be considered as logic since such a property undoubtedly would be beneficial for NSCLC cells to resist conventional radiotherapy. However, the detailed regulations of the pathways operated by the specific phosphorylation sites and upstream kinases await further validation before more sophisticated conclusions can be drawn.

2.4 GENERAL DISCUSSION

2.4.1 Methodological and technical perspectives

Some aspects of using cell lines

The NSCLC cell line U-1810 is highly radioresistant (65, 77, 82-83, 286), which is further described in the method section. This cell line has been used as model system in all studies (**paper I, paper II, paper III and paper IV**) of this thesis, with the aim to gain novel insight of the mechanism that control radioresistance in NSCLC. When the final goal of a study is to improve lung cancer therapy one may of course ask why not all studies use clinical material or at least living animals like rats or mice. Is it really likely that what we can identify as critical signaling networks in tumor cell line can be translated into important signaling hubs driving tumors of lung cancer patients? It is of course true that it is beneficial to perform research for improved cancer therapeutics on clinical material or on animals carrying tumors. However, the advantages for using cell lines and perform preclinical studies are several. For example, it is important to realize that in science there is a lot of unknown parameters, leading to the fact that unexpected situations continuously happen. - This is true for all studies that require some kind of method development and not least in a field of research as young as proteomics and phosphoproteomics such as those included in this thesis. Other situations are when using unsecure tools, like for example assays that involves antibodies or siRNA's, there is no quality guarantee and the quality of such tools can vary even from batch to batch. When setting up and optimizing new methods it is likely that experiments will fail several times both because of unpredicted chemical and biological reactions as well as simple laborative mistakes. In these situations, the use of a cell line model that is easy to maintain is optimal. One other advantage is also that when the method finally is working and the reagents involved have been tested and quality secured, it is easy to set up several independent experiments to check the robustness of the method. This thesis uses omics methods and one critical step is to interpret the output data of the experiment. Also this step is usually much easier when using cell lines, since you avoid the obvious complexity of having different individuals which all have their individual genomes and proteomes which differs by definition. The existing bio banks of clinical material are due to years of hard work and are of course precious and extremely valuable in the work of finding new cancer therapeutics. Moreover, albeit there are biobanks of clinical tumor materials these are of much greater complexity as they contained interindividual changes in the proteome which might be a confounding factor when to discover the important signalling aberrations within a given tumor type. Therefore, it is important to establish robust methods first, preferably using cell lines and then animals, before running the experiments also on clinical material. One alternative strategy for biomarker discovery or for identification of targets with therapeutic potential could be to run omics methods first on cell lines and maybe also on animals to generate hypotheses, which are first validated on the same material and in the end, only perform hypothesis driven validation on clinical material. Thereby avoiding the use of large amounts of clinical material and also circumvents some of the difficulties with fluctuations among individuals.

Workflow for signalomics experiments

Taking the structure of all four projects in the thesis into account, a general workflow for signalomics can be created by gathering their common principles. The first step in this workflow was to define the samples to be compared and to prove that there were parameters that differs between them, thereby justifying the likelihood of finding differences also in the cellular signaling responsible for such events. In this thesis all the studies were using situations where a certain treatment forced the radioresistant U-1810 cells to somehow increase death while the control treatment did not. However, the same workflow can of course be used also for any other comparative situations. A schematic illustration of such general workflow for signalomics experiment is described in figure 7.

In Paper I we compared non-irradiated, low LET and high LET irradiated NSCLC cells, and the fact that high LET IR increased apoptotic signaling was proven by increased apoptotic morphology as well as increased caspase 3 activation. The comparison between low and high LET IR was made also in **Paper IV** and here the increase of apoptotic signaling in response to high LET IR was confirmed by increased activation of mitochondria by activation of the pro-apoptotic BAK and BAX proteins observed by two different methods. **In Paper II** the comparison was between genes that were specifically up and down regulated for samples treated with the combination of IR and RO-3182 and samples treated with IR and PKC 412. Hence, genes regulated in untreated samples or in samples only receiving IR or either of the PKC inhibitors alone were excluded during data filtering. The cellular effect of combining IR and PKC 412 were striking by a pronounced effect on cellular morphology as well as, PARP cleavage and CK18 cleavage. Finally, in **paper III**, we were comparing control cells and cells with silenced ephrin B3. This was due to results from **Paper II** and the cellular effects of silencing ephrin B3 were confirmed by the clear changes in cellular morphology having a similar appearance as in **Paper II**.

For sample preparation prior to proteomics experiment we were using a buffer based on strong concentrations of UREA for cell lysis, protease and phosphatase inhibitor tablets were always added, however, they were probably not necessary as the detergent will most probably immediately denature all proteases and phosphatases. For genomics experiment, cells were lysed in a commercial lysis buffer from Sigma Aldrich. Different amount of material were required depending on the analysis. For example about 10 million cells were needed for genomic experiments, while hundreds of millions of cells were used for the phosphoproteomic experiments. When proper and equal amounts of material for each comparable set of samples have been prepared different procedures follows. These are dependent of the purpose of the analysis e.g. extracting mRNA and synthesizing DNA and cRNA for genomics or digestion of proteins to peptides for shotgun proteomics, in case of phosphoproteomics also further fractionation steps and enriching strategies had to be done (see separate aspects of phosphoproteomics below).

Next, follows the step of omics data collection. In the genomics experiments in **Paper II** we used the Gene Array platform from Affymetrix to globally measure gene expression. For proteomics experiments in **Paper I** a LTQ-FT mass spectrometer were used and for phosphoproteomics experiments in **Paper III** and **IV** we used an Orbitrap Velos mass spectrometer. With regard to the phosphoproteomics experiment, several different types of mass spectrometers were tried out, however, only the Orbitrap Velos managed to identify a satisfying number of phosphorylated proteins. Yet, this is most likely not only due to the higher resolution per se, but to differences in fragmentation techniques and the increased detection sensitivity of this instrument.

After collecting the data, during data analysis, there are several different bioinformatic tools that can be used. For genomics experiments, we applied Gene spring (**Paper II**). In **Paper III** and **IV**, Protein Center was used to sort phosphorylated peptides from non phosphorylated ones. In fact Protein Center was to our knowledge the only commercial software where modified proteins could easily be imported from Proteome Discoverer without any manual changes of the sequences, something that dramatically facilitated the data handling in **Paper III** and **IV**. Protein Center was further used to compare lists of phosphopeptides and phosphoproteins between replicates of the same sample as well as between different samples. Moreover, this tool also provided short information about functions for each protein and also presented known phosphorylation sites. Such information was convenient and very helpful to have while manually scrolling the lists to inspect the data. Also for visualization, Protein Center played an important role, as it provided pictures covering the total sequences of a protein but highlighting the parts covered by our data as well as indicating the modifications regardless if they were previously known or not.

In genomics and proteomics experiments, what one usually has is an enormous list with genes/proteins regulated by abundance or as in phosphoproteomics, by phosphorylations. The big question at present in proteomics lab worldwide, as well as in our lab - is how we should find the biological relevance of our samples from this list. In **Paper I**, we claim that PSE could be such a novel tool and in **Paper III** and **IV** network analysis and data mapping on canonical pathways using IPA were used to visualize and to create hypotheses regarding the biological interpretation. What is delivered from such software tools for global analysis available today can of course not be considered as the truth but might still be helpful to get ideas of the type of molecules and their possible areas of function. However, specific manual interpretation and knowledge of the field in the research question was used to fine tune the hypotheses. Such hypotheses were then validated by orthogonal methods like western blot (**Paper I, II, III**), IF and pharmacological inhibitors in combination with ELISA (**Paper I**), siRNA (**Paper II**) and in cases where commercial antibodies were not available, like in **Paper III**, when there were no specific antibodies for certain phosphorylation sites, immunoprecipitation was used to “fish out” the total protein. Westernblot was then run on precipitates and the membrane was blotted with a pan phospho – antibody.

Importantly, after generation and validation of the hypothesis, the hypothesis which now can be termed as a strong, can be used in a feedback loop to drive and expand the project. While we now have a starting point, like for example an interesting protein regulated in apoptotic cells, we are able to perform targeted analysis of the same global data and search for known interacting partners or partners of the same signaling cascades as the candidate protein. **In paper III**, this feedback approach was used for upstream pathway walking by pinpointing the upstream kinases generating the specific phosphorylation on a regulated substrate, which in this case was the phosphorylation on Ser 897 on the EphA2 receptor and its upstream kinase Akt1.

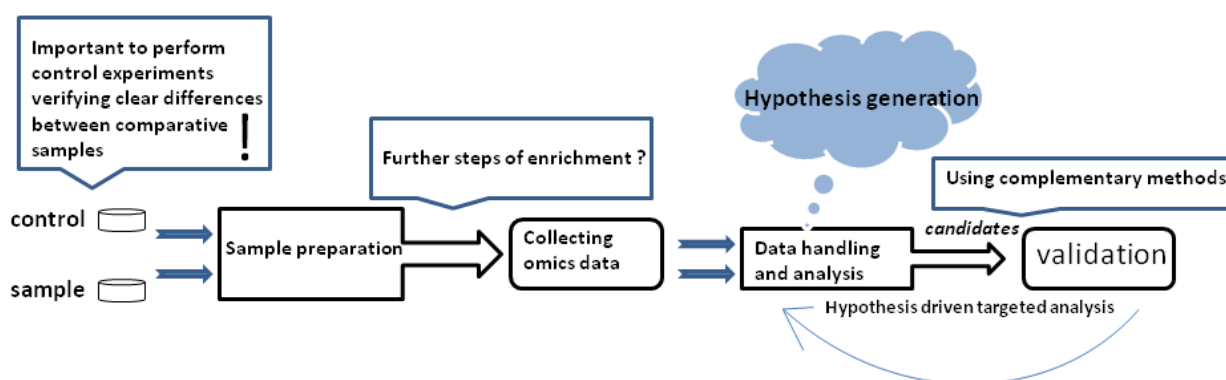


Figure 7: Workflow for signalomics experiments

Aspects of phosphoproteomics

Phosphoproteomics has already played and will most probably continue to play a significant role in our ability to understand molecular mechanisms that govern human cancers (287-290). Various technological platforms are now available for phosphoproteomic studies enabling us to address different aspects of tumor biology governed by phosphorylation-mediated signaling pathways (291).

Cellular proteins in humans are predominantly phosphorylated on serine/threonine residues with a smaller extent of phosphorylation occurring on tyrosine residues. However, the overall low abundance of phosphoproteins complicates identification and characterization of this sub-proteome using standard mass spectrometry based methods. In addition to the problem of low abundance, phosphorylated peptides are, due to their phospho-groups in general more acidic than mainstream peptides, thereby complicating ionization in positive ion mode of mass spectrometry (292-293). Moreover, trypsin, which is the most commonly used enzyme for digestion of proteins to peptides prior to mass spectrometry, has difficulties to cleave next to a phosphorylation, resulting in large peptides which in general have poor fly ability in a mass spectrometer due to their size. However, digestion can be improved by using an additional enzyme complementary to Trypsin, like for example Lysozyme C.

The challenges with global studies of phosphorylated peptides/proteins have spurred the development of molecular tools to preferentially enrich the phosphoproteome. Ferric ions was the first enrichment strategy and was exploited by biochemists to isolate phosphoproteins and phosphopeptides using immobilized metal affinity chromatography (IMAC)(249). Analogous to ferric ions, gallium (III) has also been shown to display affinity and selectivity to phosphopeptides (256). These enrichment strategies followed by mass spectrometry have convincingly proved to reveal the identity of the proteins and the sites of modification (260, 270, 294-303). One of the main aims of the current thesis was to set up a phopshoproteomic method; several experiments were performed using both IMAC-Fe and IMAC-Ga, off line coupled to MALDI-TOF/TOF. These experiments were somewhat successful for non-complex samples like α and β Casein, but always failed when applied to complex samples like total cell proteomes. In retrospect, we realized that the failure had little to do with choice of metal, but instead was an issue of extended pre-fractionation and qualities of the mass spectrometer. One of the other advances that particularly enhanced the ability to enrich serine/threonine phosphopeptides is the technique based on titanium dioxide (TiO_2) for selective enrichment of phosphorylated peptides (267, 285, 302, 304-306).

While setting up a method for our phosphoproteomic studies, before choosing which strategy to use for enrichment of phosphorylated peptides from complex samples prior to mass spectrometry, we performed a comparative study between a kit using the IMAC-Fe-based method and a kit based on the TiO_2 method. Their ability to enrich for phosphorylated peptides was analyzed by radiolabeling the phosphates with p32 and then measuring the p32-activity in the different phases. In our hands, as shown in figure 8 the TiO_2 -method proved to give slightly better results by showing higher affinity for the phosphopeptides as well as giving higher yield during elutions and was thus our method of choice.

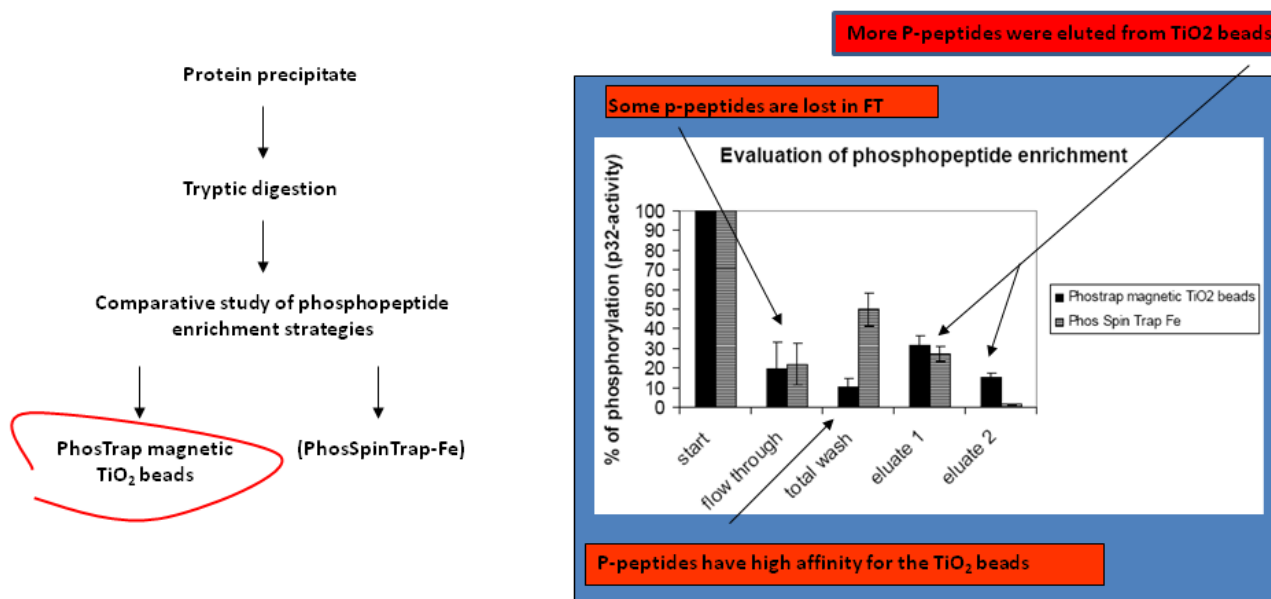


Figure 8: TiO_2 was the method we choose for enrichment of phosphorylated peptides.

As previously described in the section of Material and Methods, prefractionation using SCX and subsequent enrichment using TiO₂ before analysis by LTQ – Orbitrap Velos inspired by Villen et al (270) was set up for **Paper III** and was also further applied in **Paper IV**. By using SCX-based fractionation, peptides in the same fraction had a similar net charge; this is a great advantage when the next step is enrichment by TiO₂. TiO₂ has affinity for the phosphogroup but also for other highly electronegative structures and in a fraction where all peptides have similar net charge i.e. similar electronegativity, it will be more likely that phosphorylated peptides win the competition of binding TiO₂ compared to those who are non phosphorylated (267-268). This phenomenon is believed to be due to that the electronegative oxygen of the phosphogroups has stronger affinity to parts of the structure of the TiO₂ resin than the acidic residues and the C-terminals of the peptides. However, other structures of the TiO₂ resin instead binds strongly to peptide C terminals which is why strong aromatic acids like 2,4-dihydroxybenzoic acid (DHB) or Glucolic acid, which have even stronger affinity for such structures, are used as blockers of such non phosphorylated acidic-peptide residues when equilibrating the resin as well in the buffer while binding the sample (267-268, 300, 305).

In general, studies using phosphoproteomics have clearly taken us beyond looking at mutations or other genetic variations commonly observed in cancers and are providing us insights into functional consequences of these changes in conferring survival advantages to cancer cells. Such studies are already being used as the basis for determining therapeutic options (290, 307-308).

2.4.2 Biological perspectives

One advantage of using the same model system in all four studies was that we now in the end can collect everything we have learned from each study to get a more holistic picture of signaling events regarding resistant mechanism against conventional radiotherapy in this model. Moreover, by observing signs that are pointing in the same direction in different studies, the different studies are somehow used as controls for each other. One example is up regulation of p38 which was found both in Paper I and Paper II in response to low LET IR. Interestingly, we have some indications of active p38 also in Paper IV since we found a phosphorylation on Ser 611 on TFII δ in response to low LET IR. The predicted kinase of this site is p38 (<http://www.phosphosite.org>), but further validation experiments need to be done to find out if this is true also in our system. Moreover, the activation of p38 has been reported downstream of IGF-1R in a previous study of U-1810 cells from our group (71). Together, these four studies strongly suggest that active p38 mediated signaling is a mechanism used for protection against low LET IR in this model system.

Lessons about cellular signaling regarding the increased cell death observed in response to high LET IR in NSCLC cells resistant to low LET IR

Beginning with **Paper I** and **paper IV**, high LET IR was proven to circumvent resistance mechanisms observed in response to low LET IR by forcing induction of apoptosis, which was evident as increased apoptotic morphology, increased caspase 3 activation and activation of mitochondria through the pro-apoptotic BAK and BAX proteins. High LET IR has been proven beneficial for treatment of solid tumors and is today applied in the clinic to some extent (19, 309-315). However, at present high LET IR is not likely to become a clinical standard treatment in the same way as low LET IR because of the inconvenience of using the source as it requires a set up of large cyclotrons with limited beam times. Such sources are only present in a few places in the world, one being at the Svedberg laboratory in Uppsala. However, clinical benefits can also be achieved to a large extent by understanding the cellular signaling responsible for the improved tumor control in response to high LET compared to conventional radio therapy. Using such strategy may lead to identification of novel clinical targets which can be used to sensitize for conventional radiotherapy or possibly as markers to select patients particularly susceptible for high LET radiotherapy. The JNK-pathway was found to play a significant role upstream of the apoptotic signaling cascade in response to high LET IR, in particular involving MKK4, JNK and 14-3-3 σ . JNK signaling have been reported as a pro-apoptotic event in several studies (316) and as our data supported a pulsed early and late JNK response (fig 9) and we do see activation of MKK4 at 4h post high-LET IR this might indicate that MKK4 is involved in the late activation of JNK but cannot be responsible for the observed early JNK activation since it was already at 2h. When considering other signaling molecules involved, it is tempting to speculate that the non-receptor tyrosine kinase c-Abl is involved upstream of MKK4 as it has been shown to activate MKK4 and JNK via MEKK1-mediated signalling after conventional IR as well as other DNA damaging treatments (317-320). It was recently shown that an early initial JNK activation is required in order to trigger release of c-Abl from the adaptor protein 14-3-3, c-Abl is then directed into the cell nuclei which subsequently allow c-Abl and late JNK activation (321-323). This is in line with the observed reduction in 14-3-3 σ in U-1810 cells 4 hours after high-LET IR. Hence, the conditions fit to support that high-LET IR but not low-LET IR is capable of recycling c-Abl to the nucleus where c-Abl in turn activates JNK and initiate apoptosis in this system. Further studies of c-Abl activation and localization in response to high-LET IR will clarify if this is the case.

In addition to increased apoptotic signaling reported in **Paper I**, **Paper IV** is adding information about the perturbations in cellular signaling in response to high LET IR by suggesting that this quality of radiation interrupts protein synthesis. Hence, signaling pathways describing assembly of the RNA polymerase III complex responsible for expressing tRNA and eIF-signaling responsible for translation from mRNA to peptides sequences were found active in response to low LET IR, but suggested as deactivated in response to high LET IR. Interestingly, connections between JNK-signaling and eIF2-signaling have been previously reported, however it is not completely clarified

how JNK1 and JNK2 are controlling the regulations. One suggestion is that JNK2 is up regulated while eIF2-signalig is deactivated (324). Importantly, the requirement of JNK-signaling for inducing apoptosis in response to high LET IR in our model system could be confirmed also on the functional level, as a specific pharmacological inhibitor of JNK, SP600125 efficiently also inhibited apoptosis induced by high LET IR. Hence, one may speculate that over expressing JNK is a potential strategy to sensitize NSCLC cells to conventional radiotherapy.

Lessons about survival signaling controlled by members of the Eph/ephrin family in resistant prone NSCLC cells

By the unsupervised screening of regulated genes in **Paper II**, it appeared that the developmental protein ephrin B3 was down regulated in NSCLC cells receiving combinational treatment by IR and the staurosporin analogue PKC 412. We found the regulation of ephrinB3 highly interesting since other members of the Eph/ephrin family have during the past few years emerged as important in oncogenic transformation in several cancer forms including lung cancer (325). However, the member, ephrin B3 have not gained much attention in such matter before. Previous studies from the group of Boris Zhivotovsky had highlighted the radiosensitizing effects of PKC412 in U-1810 cells and also observed an increased induction of mitotic catastrophe (77, 83). We also observed stress factors on the cells while using such treatment combination, as the cells decreased in number and changed their cellular morphology. In addition, these treatments also resulted in increased apoptotic signalling as cleavage of cytokeratin 18 and PARP. Hence, our data from the gene array experiments generated the hypothesis that one mechanism which U-1810 cells use to protect themselves from the effects of conventional radiotherapy is to keep high levels of ephrin B3 in the system. To confirm our hypothesis and further elucidate the role of ephrin B3 in NSCLC cells, we silenced the expression of this gene. The effect of the silencing was unexpectedly striking, as we observed clear visible changes in the cellular morphology, very similar to those observed on the cells when they were treated with the combination of IR and PKC 412. We could also confirm increased PARP cleavage and increased apoptotic nuclear morphology and also some increase in nuclear morphology typical for cells going through mitotic catastrophe. When observing these effects, we become curious in if we could further boost the effects by irradiating the NSCLC cells after silencing ephrin B3. Doing so, it was evident that both nuclear morphologies typical for apoptosis and mitotic catastrophe increased significantly and cell growth was clearly interrupted. The cellular morphology which now was highly differentiated, with flattened, transparent and highly elongated cells that were at least 10-fold the size of normal NSCLC cells, is typical for cells which are going in to the state of senescence (326). Indeed, this appeared to be the case as cells with silenced ephrin B3 were staining blue in β -galactosidase (β -gal) - assay. This effect was even clearer when cells with silenced ephrinB3 were further treated with IR and as much as 40% of the cells showed high β -gal-staining. Knowing that senescent cells are arrested in G0/ G1(326) we performed studies of the cellular distribution of the cell cycle. As expected, cells with silenced ephrin B3 arrested in G1, but in contrast irradiated cells appeared to shortly get stacked

in G2/M having trouble to get through mitosis properly, however at least a part of the culture did not arrest for long but started to proliferate again. Interestingly, this was not the case when the irradiated cells also had lost expression of ephrin B3, thus only a small part of the cells were now left in G2/M while the majority of the cells accumulated in G1 where they arrested and became senescent. Thus our data suggests a role of ephrin B3 as a driver, forcing the NSCLC cells to cycle also when they were having lesions in their DNA from irradiation that ideally would induce cell death. The results from **Paper II** encouraged us to continue to search for important players in signaling events controlled by ephrin B3. Consequently, we took on a phosphoproteomic approach in **Paper III** and searched unsupervised for differences in the overall profiles of phosphorylated proteins in cells with present or absent ephrin B3. More than 2000 phosphorylated protein were identified in this study and 150 of them were specifically phosphorylated only in presence of ephrin B3. One of the top regulated active networks mapped from these 150 proteins included another member from the Eph/ephrin family, the Eph A2 receptor. Only recently, during the past two years, the field of Eph A2 as a target for cancer therapy has exploded, since Eph A2 have been discovered over expressed in several cancer forms, including lung cancer (127, 327-332). When ephrin B3 was present, we detected a phosphorylation on Ser 897 on this receptor, a site reported as phosphorylated by Akt1 only when Eph A2 is bound by its ligand, ephrin A1 (282). Total cellular levels of ephrin A1 was not changed by the presence or absence of the ephrin B3 ligand in our model. Nevertheless, our data suggested that ephrin B3 somehow inhibits the binding of ephrin A1 to the Eph A2 receptor in the U-1810 cells. Interestingly, we also found Akt1 to be phosphorylated on Ser 129, only in presence of ephrin B3. As this site is known to be specifically phosphorylated by the serine/threonine kinase CK2 in Jurkat cells (283), it is interesting to speculate that this might be the case also in our model. CK2 is a kinase reported to have elevated levels in tumors and being high in signaling controlling hierarchy by having direct influence of perhaps as much as 20% of the so far identified phosphorylations sites (333), indeed it was found to be highly expressed also in our model system. HSP90 is another target of CK2 (334) which in fact protects EphA2 from proteosomal degradation (284, 335). - When realizing this, we went through our data again specifically searching for this chaperon protein. In accordance with this hypothesis, we did find a phosphorylated form of HSP90AA1 only in presence of ephrin B3. In addition more than 40% of the sequence was identified on HSP90AA1 in presence of ephrin B3 while no peptides from HSP90AA1 were detected when ephrin B3 was silenced. As the function of HSP90 is to protect proteins from degradation and EphA2 has been reported previously as a client of HSP90 (284, 335), our data strongly support such a mechanism also in our system, hence during validation experiments it appeared that silencing ephrin B3 did not only led to dephosphorylation of EphA2, it actually led to degradation of the total protein. In turn, the binding of ephrin A1 to EphA2 is known to cause tyrosine phosphorylation on the receptor and thereby mediate its down regulation (116). Thus one may speculate that - in absence of ephrin B3 this might also be the case in our system, as the phosphorylation on the Tyrosine is probably not detected with our method. Ephrin A1 is known as a tumor suppressor and has functions which involve repression of oncogenic signaling through receptor

Tyrosine phosphorylation further leading to attenuating growth factor induced activation of Ras. However, in the case of present ephrin B3 ligand, even though we still detected basal levels of ephrin A1 in our system, ephrin A1 is not properly binding the EphA2 receptor, thus EphA2 is losing its kinase capacity and instead act as a substrate to Akt1(282). A contradictory aspect of the non proliferative effects when ephrin A1 binds Eph A2 in tumor cells is that it appears to be the opposite in angiogenesis where ephrin A1 binding EphA2 instead stimulates migration (336-341). Yet, this phenomenon further states the importance to improve the knowledge of differential signaling via specific phosphorylation sites of signaling proteins rather than only focusing on protein identities as this might explain why interactions between the same proteins can mean different things in different situations.

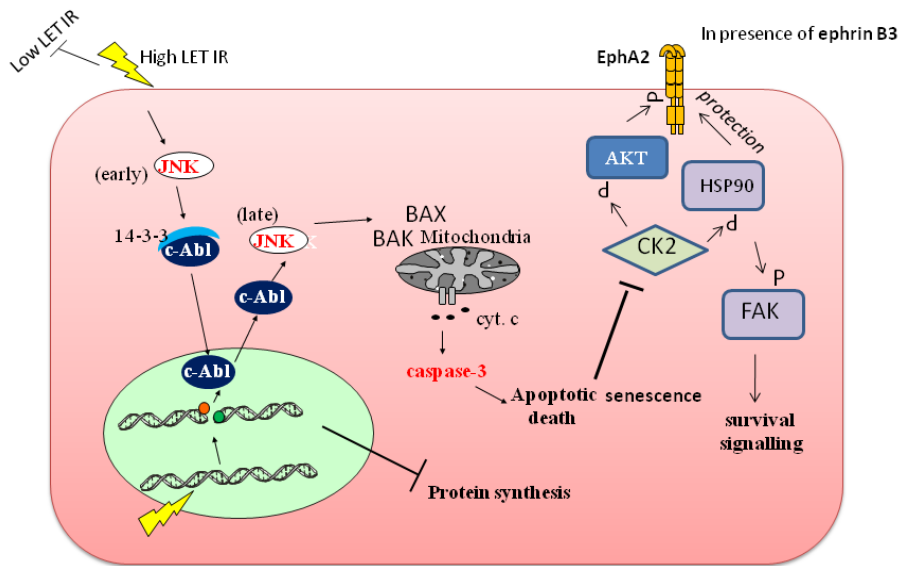


Figure 9: Potential mechanisms of radioresistance in NSCLC. Failure of proapoptotic activation of JNK, overactive protein synthesis as well as ephrin B3/EphA2 mediated activation of survival mechanisms which force cells to cycle instead of becoming senescent even though they are carrying damages in their DNA, are all mechanisms that may contribute to the radioresistant phenotype of NSCLC cells.

2.5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The goals with this thesis were to use global methods for signaling studies in NSCLC cells. In particular, the main goal was to set up a platform for global assessment of phosphorylated proteins in complex samples. Such a platform was indeed established and was used in the two last papers of this thesis. From a biological and preclinical perspective, the aim was to gain novel insight of the cellular mechanisms that are responsible for the radioresistant phenotype expressed by NSCLC cells. Paper I and IV cover NSCLC cellular response to low and high LET IR while Paper II and III deal with the role of ephrin B3 – mediated signaling and how it affects radiosensitivity in NSCLC. The general conclusions are:

✓ *Experimental design:*

When performing global studies in cellular signaling, it is of high importance to plan the workflow as much as possible before starting. One way is to use the template workflow for signalomics, described in the section of Discussion *Workflow for signalomics experiments*. Hence, check that you have the prerequisite for each step. Note that the principle of “Garbage in gives garbage out” is valid for this workflow. Step 1 is crucial in this matter and one should be sure to define the control and comparative sample in a way that secure clear effects in the sample. For example, a certain treatment clearly induces measurable cell death in the sample. Thus, the last step, validation using orthogonal methods, can be tricky anyway but will be impossible if the input samples are not reflecting the signaling question.

✓ *Phosphoproteomics*

Our platform for globally identifying phosphorylated proteins includes enriching for the phosphoproteome using SCX-fractionation and affinity based binding to magnetic TiO₂ beads before nano-LC online to mass spectrometry based analysis. When analyzing complex samples like whole cellular proteomes, it requires a fast mass spectrometer with high sensitivity and proper fragmentation techniques e.g. HCD and ETD. Hence, Orbitrap Velos (Thermo Fisher Scientific) is a good choice. We could identify more than 2000 phosphorylated proteins from NSCLC cells and the enriched fraction contained 70-90% phosphorylated peptides compared to non phosphorylated ones. This numbers have similar quality as world leading laboratories for phosphoproteomics (265, 285) .

✓ *Radioresistance mechanisms of NSCLC cells*

Regarding our studies in response to low and high LET IR, we could conclude that one mechanisms hindering response to conventional low LET IR in NSCLC cells is failure of inducing apoptosis by pro-apoptotic JNK-signaling and subsequent activation of mitochondria by BAK and BAX. In addition, we

also report up regulation of pro-survival p38 MAPK signaling. Furthermore, our data suggest that one way these cells use to resist low LET radiotherapy is to continually synthesize new proteins, illustrated in our studies by activation of the assembly of RNA-polymerase III complex and eIF2-signaling.

In **Paper II**, we found that the developmental protein ephrin B3 was down regulated when combining PKC 412 and radiotherapy. A treatment which indeed increased cell death compared to single treatment of either PKC 412 or IR. NSCLC cells treated with the combination PKC 412 and IR showed pronounced changes in morphology and a similar morphology of enlarged, flattened and elongated cells was evident also when we silenced ephrin B3. We could conclude that NSCLC cells deficient of ephrin B3 either died (partly from mitotic catastrophe in G2/M) or actually went in to a senescent state, arrested in G1. In addition, as a follow up from **Paper II**, in **Paper III** - we propose a survival pathway, where ephrin B3 regulates P- Ser 129 on Akt1 and P- Ser 897 on Eph A2 in a signaling circuit that most probably contributes to the radioresistant phenotype of U-1810 cells. The mechanisms described in this section may all be clinically relevant for treatment of NSCLC and deserves to be further investigated.

2.5.1 Future perspectives

All studies in this thesis are preclinical and each of them has generated candidates which may be used for future targeted therapy in NSCLC either alone or as sensitizer for conventional radiotherapy. However, the way is still long before any of these targets can actually reach the clinic.

Perhaps the most interesting part of this thesis is the novel finding of ephrin B3 as a regulator NSCLC radiotherapy resistance and as a regulator of the phosphorylation on Ser 897 on EphA2. Ephrin B3 has not been connected to radiosensitivity before and is not reported as one of the ligands of EphA2. Yet we have not revealed if ephrin B3 is binding the EphA2 receptor directly or if the regulation is indirect. We only know that the original ligand ephrin A1, most probably does not bind if ephrin B3 is present in the cells. The details about the molecular interplay in the relationship between ephrin B3, ephrin A1 and EphA2 probably play important roles for the grade of radiosensitivity of these cells and would be interesting as follow up study. Inhibitors of EphA2 are already under investigation and the field of this receptor as a target for cancer therapy is a highly vibrating and its regulation in NSCLC is of highest interest. Validation of the regulation of phosphorylation on Ser 897 in a larger cohort of clinical material would be highly interesting. A problem when it comes to validation of many novel phosphorylation sites is the lack of specific antibodies; this is the case also for phosphorylation of Ser 897 on EphA2. Under such circumstances, the use of selected reaction monitoring (SRM)-mass spectrometry (342) for validation would be ideal and definitely an interesting future experiment to run.

The protocol used to analyze phosphorylated proteins have been set up for cell lines, however when creating this protocol, in the back of our mind, the plan was to use the protocol also for clinical material in the future. Hence, the basic of the protocol is already compatible for clinical material, but further optimization is required to inhibit dephosphorylation of the sample during collection from the clinic. Moreover, optimization is needed also in the matter of down scaling volumes needed in the step of SCX-fractionation to reduce starting amount of material but still keep high levels of identified phosphorylated proteins. Such protocol is also dependent on the development in the field of mass spectrometers, this field have developed extremely fast during the past 5 years and it is not impossible that we soon can measure even very low amounts of phosphorylated peptides which will reduce the amount of start material needed and ultimately benefit a translation of our pre-clinical protocol to a clinical protocol for phosphoproteomics.

Surgery, chemo – and radiotherapy have been the major treatment strategy for lung cancer ever since the 70's. However, targeted therapies mainly in the form of kinase inhibitors are now in clinical use and directing the way towards more personalized treatment forms ultimately giving improved efficacy and fewer side effects. Kinases are important for cellular signaling in cancer which is the reason why they are targeted in cancer therapy today, but one should not forget that they are important also for cellular signaling in healthy cells. A kinase may generate phosphorylation of hundreds of sites on different substrates which explains the high impact on major functions also in healthy cells. In addition, it is difficult to design specific kinase inhibitors, as many kinases are similar in their active site, which even further reduces selectivity and specificity, meaning that we still have very little control of which signaling pathways that we are manipulating with these drugs. Indeed, severe side effects are reported also for several of the kinase inhibitors (343-346). However, some results of such drugs are promising and this development of cancer treatment is definitely a step in the right direction. Nevertheless, an alternative way to gain even higher control of which signaling pathways that are affected in the cell would be to target the actual site of phosphorylation on the substrate instead of the active site on the kinase. This is even more beneficial if the site of phosphorylation on the substrate is generated specifically from one kinase, which is the case in most situations. However, even in cases when a phosphorylation site has several upstream kinases, the site will still, most likely be more specific than a pleiotropic kinase. A model for potential target points on phosphorylated substrates identified in paper III is presented in figure 10.

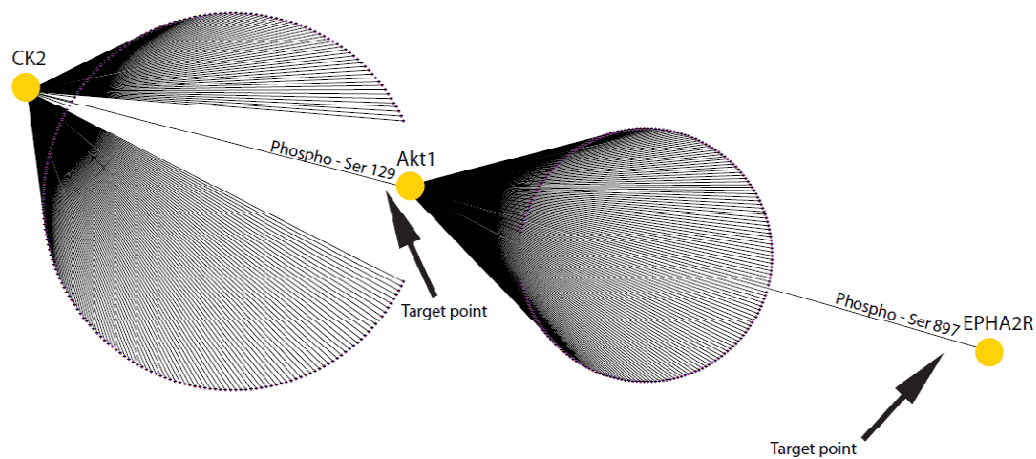


Figure 10: Model of potential target sites in NSCLC. Most targeted therapies today inhibit the active site on a kinase (yellow dots). Thus, hundreds of substrates (small black dots) will be affected. If we instead target specific phosphorylation sites (a part of a small black dot) in the future, we may have improved control on which specific signaling path that is manipulated (here illustrated by the straight line reaching from CK2 via Akt to EphA2).

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