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FROM MEMBRANE TO NUCLEUS: NEW ROLES AND FUNCTIONS OF SUMOYLATED IGF-1R AND EGFR

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Stockholm 2015

Published by Karolinska Institutet.
Printed by AJ E-print AB, Stockholm, Sweden

ISBN 978-91-7549-877-5

To my beloved Justin and Family

ABSTRACT

Cell surface receptor tyrosine kinases (RTKs) role in cell signaling have been studied for decades and their role in cancer progression are undisputable. The insulin-like growth factor 1 receptor, IGF-1R, has been demonstrated to play a critical part in tumorigenesis; downregulation of the IGF-1R in tumor xenografts results in complete tumor regression. Previously, RTK research has focused on the canonical signaling pathways activated by ligand binding at the plasma membrane. However, strong evidence keeps emerging that several RTKs have a second functionally mechanism, inside the cell nucleus, where the receptors reside after ligand stimulation. The aim of this thesis was to elucidate the function of recently discovered nuclear IGF-1R as well as to investigate its nuclear translocation pathway. Since it was previously shown that SUMOylation of the IGF-1R is essential for its nuclear translocation we also set out to investigate SUMO modification of the epidermal growth factor receptor (EGFR).

In paper I, we present a functional role for nuclear IGF-1R in gene transcription. Inside the nucleus, IGF-1R functions as a co-activator to LEF-1/TCF transcription factor. Nuclear IGF-1R enhances transcription of cyclin D1 and axin2, and we show that it is enriched in the *cyclin D1* promoter region. In the following study, paper II, we propose a pathway by which IGF-1R is transported into the nucleus. IGF-1R is transported along microtubules via the dynactin transportation complex, to the nuclear pore where it is transferred to importin-β which guides the receptor to the nuclear pore complex protein RanBP2, which further assists the receptor into the cell nucleus in a RanGTPase dependent manner. Inhibition or obstruction of any of these components results in a reduction in nuclear IGF-1R. Further, we suggest that RanBP2 is the SUMO E3 ligase in IGF-1R SUMOylation and we show that SUMO-1 modification of the receptor is also important for its stability. In paper III, we demonstrate that the EGFR is SUMOylated and propose five lysine residues as SUMO-1 targets which were identified by two different mass spectrometry strategies. One of these residues, lysine 37, came up as a suggested target in both mass spectrometry methods. EGFR mutated in this site – EGFR-K37R – causes a decrease in protein levels as well as transcriptional activity of *cyclin D1* and *c-myc*, two target genes of nuclear EGFR.

To summarize, our data shows (I) a pathway by which nuclear IGF-1R is being transported and the functional importance of nuclear IGF-1R as a co-activator in transcription and (II) that the EGFR is also SUMOylated and might play a role in its transcriptional activity. Together these results may unravel new mechanisms for IGF-1R and EGFR that have implications in carcinogenesis.

LIST OF SCIENTIFIC PAPERS

I. Warsito D, **Sjöström S**, Andersson S, Larsson O and Sehat B. Nuclear IGF1R is a transcriptional co-activator of LEF1/TCF. *EMBO Rep*. 2012 Mar 1;13(3):244-50.

II. **Packham S**, Warsito D, Lin Y, Sadi S, Karlsson R, Sehat B and Larsson O. Nuclear translocation of IGF-1R via $p150^{Glued}$ and an importin-β/RanBP2-dependent pathway in cancer cells. *Oncogene,* 2014 Jun 9; doi: 10.1038/onc.2014.165. [Epub ahead of print].

III. **Packham S***, Lin Y*, Zhao Z, Rutishauser D, Warsito D and Larsson O. Nuclearly localized epidermal growth factor receptor is SUMOylated. *Manuscript.*

*Authors contributed equally

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LIST OF ABBREVIATIONS

4E-BP1	elF4E binding protein 1
ADP	Adenosine diphosphate
Akt	Active human protein kinase
A-loop	activation loop
ALS	acid labile subunit
APC	Adenomatous polyposis coli
Arg	Arginine
ARM	Armadillo repeats
ATP	Adenosine triphosphate
BAD	The Bcl-2-associated death promoter protein
Bcl-2	B-cell lymphoma 2
BRCA	Breast cancer susceptibility gene
CBP	CREB-binding protein
CK1	Casein kinase 1α
c-Myc	Myelocytomatosis viral oncogene homologue
CtBP	C-terminal binding protein
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	Double-strand breaks
Dsh	Dishevelled phosphoprotein
DUB	De-ubiquitinating enzymes
EEA1	Early endosomal antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
elF4E	Eukaryotic translational initiation factor 4E
Elk	ETS domain-containing protein
ER	Endoplasmic reticulum
ErbB	Erythroblastic leukemia viral oncogene
Erk	Extracellular signal-regulated kinases
FAK	Focal adhesion kinase
FDA	U.S Food and Drug Administration
FG	Phenylalanine-glycine repeats
FGFR-1	Fibroblast growth factor receptor 1
FoxO	Forkhead box transcription factor
Fz	Frizzled receptor
GDP	Guanosine diphosphate
Grb2	Growth factor receptor-bound protein 2

GSK-3β	Glycogen synthase kinase 3-β
GTP	Guanosine triphosphate
HDAC4	Histone deacetylase 4
HER	Human epidermal growth factor receptor
HPV	Human papilloma virus
HSP70	Heat-shock protein 70
ICD	Intra-cellular domain
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2R	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding protein
INFS	Integrative nuclear FGFR-1 signaling
IR	Insulin receptor
INTERNET	Integral trafficking from the ER to the nuclear envelope transportation
IRS	Insulin receptor substrate
JAK	Janus kinase
JM	Juxtamembrane
KRAS	Kirsten rat sarcoma viral oncogene homologue
LEF1/TCF	Lymphoid enhancing factor/T-cell factor transcription factor
LRP	Low density lipoprotein-related protein
Lys	Lysine
M6P	Mannose 6-phosphate
MAPK	Mitogen-activated protein kinase
Mdm2	Mouse double minute 2 homologue
MEK	MAPK or ERK kinase
Met	Hepatocyte growth factor receptor
mRNA	messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cell
NLS	Nuclear localization signal
NPC	Nuclear pore complex
nRTK	Nuclear receptor tyrosine kinase
NSCLC	Non-small cell lung cancer
NTF2	Nuclear transport factor 2
Nup	Nucleoporin
OS	Overall survival
Pc2	Polycomb protein 2
PcG	Polycomb group
PCNA	Proliferation-cell nuclear antigen
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor

PDK	3´-phosphoinositide-dependent kinases
PFS	Progression free survival
PI3K	Phosphatidylinositol-3-kinases
PIAS	Protein inhibitor of activated STAT
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PSA	Prostate-specific antigen
PTB	Phosphotyrosine binding domain
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatases
Raf	Rapidly accelerated fibrosarcoma
Ran	Ras-related nuclear protein
RanBP2	Ran binding protein 2
RanGAP	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor
RCC	Renal cell carcinoma
RHA	RNA helicase A
RTK	Receptor tyrosine kinase
Ryk	Related to receptor tyrosine kinase
S6K1	Ribosomal protein S6 kinase beta-1
SAE1/2	SUMO-activating enzyme 1 and 2
SENP	SUMO-specific protease
SH2	Src homology 2-domain
SOS	Son of sevenless
Sp1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-like modifier
SV40	Simian virus 40
TDG	Thymine-DNA glycosylase
TrkA	Tropomyosin receptor kinase A
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless-related integration site

1. INTRODUCTION

1.1 CANCER

Cancer, a word that most of the population is familiar with. A word charged with emotions. Even my nine year old nephews have heard of it and they describe it as “a disease you can die from”. According to “Centers for Disease Control and Prevention”, Atlanta ,USA, each year 12.7 million people worldwide will learn that they have cancer and out of those 60% will die from the disease [1]. Many of these deaths can be prevented and as we live longer the incidence of cancer is increasing. In Sweden the risk of getting cancer during your lifetime is 30%.

1.1.1 Cellular imbalance

Cancer is not just one disease. There are hundreds of different groups and subgroups of cancer and it can occur in all different tissues of the body. They all have one thing in common; cancer is when cells are growing out of control. In adults, normal cells are under strict control of a variety of different cellular mechanisms. The cells have a finite life span and in general they only divide and multiply to replace old cells or to repair an injury. When this fine balance between cellular life and death is disrupted the cell can keep on dividing and multiplying and give rise to cancer cells.

Malignancy is caused by many different factors which can be dived into two sub-groups; inherited (genetic) or environmental origins. Inherited cancers are caused by germ-line mutations, whilst environmental factors give rise to somatic mutations, the latter one being more common. Examples of germ-line mutations are mutations in the *BRCA1* and *BRCA2* genes and the *APC* gene, which increase the risk for breast cancer and colon cancer respectively [2, 3]. Somatic mutations, on the other hand, arises from environmental factors such as ultraviolet exposure, tobacco, diet, alcohol and lack of physical activity, which is by far the more common cause for cancerogenesis [4].

For a normal somatic (adult) cell to transform into a cancer cell it requires multiple mutations. A single mutation alone is not enough as our cells have many sophisticated back-up systems which act to prevent any abnormal cell to continue growing and dividing. Thus, a cell requires several mutations in either so-called oncogenes and/or tumor suppressors to drive malignant growth. Proteins encoded by oncogenes control cellular growth pathways and since their normal function is crucial for a cell’s survival it is highly evolutionary conserved. A protein becomes an oncogene when it starts promoting a disproportionate and uncontrolled growth signaling. This occurs through different mechanism; (I) point-mutations in the oncogene which can give rise to a consecutively active protein, (II) gene amplification of the DNA leading to the protein product

being overexpressed and (III) chromosomal translocation causing the oncogene to be under the control of a promoter causing excessive transcription. Whilst oncogenes are giving the cells "a green light" for growth, tumor-suppressor genes act as the cells "brakes". Many cancer cells have lost their "brakes", i.e. loss-of-function in the tumor-suppressor genes which give rise to an oncogenic effect on the cells. Proteins encoded by tumor-suppressor genes can (I) control different stages of the cell cycle and can arrest the cell cycle at a specific step or inhibit cell proliferation, (II) detect DNA damage during cell cycle check-points (III) promote apoptosis (cell death), and (IV) repair DNA damage. Many cancers have either deletions or point-mutations in the tumor-suppressor genes causing a loss of protein or non-functional protein [5].

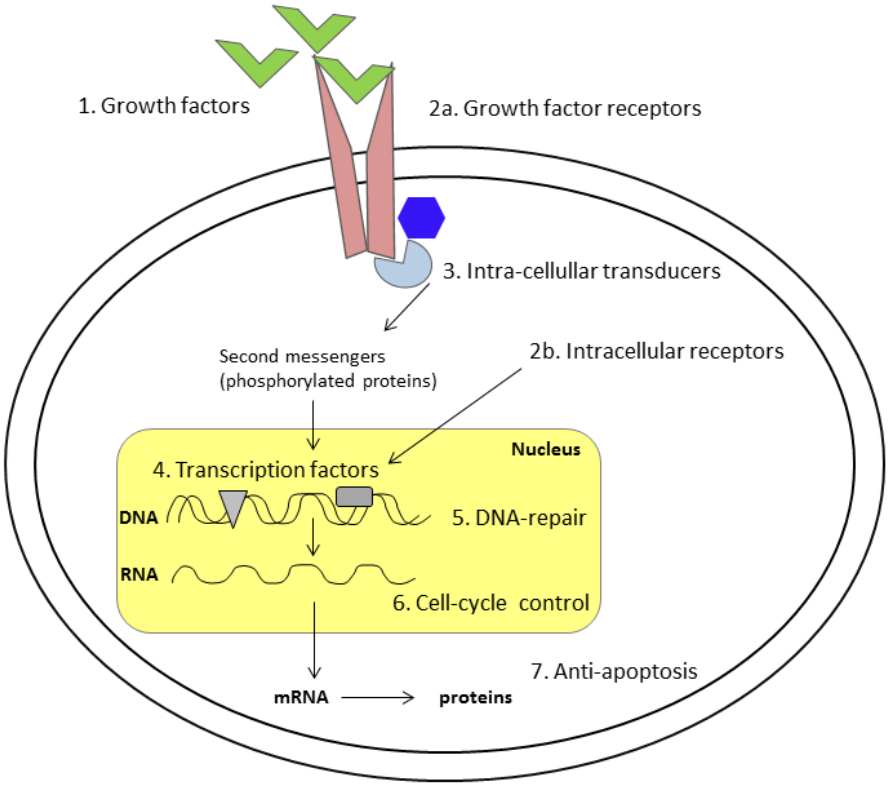

Figure 1. The different types of proteins that control cell proliferation. Aberrant or loss of function in any of these seven proteins; growth factors, growth factor receptors, intracellular transducers, transcription factors, DNA-repair proteins, cell-cycle control proteins and anti-apoptosis proteins can give rise to an oncogenic cell.

Even though the cellular genotype between cancer cells differs they all share the same phenotype which can be divided up into the following characteristics [6]:

1. Sustaining proliferative signaling
2. Evading growth suppressors
3. Activating invasion and metastasis
4. Enabling replicative immortality
5. Inducing angiogenesis
6. Resisting cell death
7. Evading immune destruction
8. Reprogramming of energy metabolism

This thesis is mainly focusing on the first characteristic (Figure 1), which will be discussed in more detail in the following sections.

1.1.2 Targeted cancer therapy

This thesis studies basic molecular mechanisms in cancer biology. The long term aim, reaching beyond this thesis, is that our findings will help in the discovery of new drug targets and that it will eventually lead to more efficient and specific cancer drugs with fewer adverse side effects.

Surgery, chemotherapy and radiation are the "golden standards" for treating cancer and have been used for more than a hundred years [7, 8]. Traditional chemotherapy targets rapidly dividing cells, thereby affecting not only cancer cells but also, for example, cells in the intestine which leads to severe side effects. Targeted therapies on the other hand, refer to a new generation of cancer drugs designed to target specific functions of cancer cells. Targeted therapies include two classifications of drugs; small-molecules and monoclonal antibodies [9]. Imatinib mesylate (also known as Gleevec) is a small-molecule kinase inhibitor that has shown to be a clinical success in both chronic myeloid leukemia and gastrointestinal stromal tumors [10-12]. Cetuximab and trastuzumab are two FDA approved monoclonal antibodies for treatments against metastatic colorectal cancer and HER-positive breast cancer respectively [13, 14]. These new classes of drugs have proven to be very efficient for certain subgroups of cancer, however, it has been shown that when treating cancer with a single target the risk for acquired drug resistance increases [15]. However, there are more studies emerging presenting very promising results using combined therapies; either targeted therapies in combination with radiation or chemotherapy or using multiple targeted therapies to avoid resistance and to get a better response [16, 17].

1.2 RECEPTOR TYROSINE KINASES (RTKs)

Cell communication is controlled by molecular switches, i.e. proteins activate (or inactivate) other proteins which start a signaling cascade. Kinases are a group of enzymes that phosphorylates other proteins. A kinase transfers a phosphate group from adenosine triphosphate (ATP) to an amino acid of a protein substrate. The most common (and studied) amino acids that are phosphorylated at their hydroxyl group side chain are serine, threonine or tyrosine [18, 19].

Tyrosine kinases are further dived into two subgroups; receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases [20]. By sequencing the human genome 90 tyrosine kinase genes have been identified, and 58 of those are classified as RTKs, which are divided into 20 families, including receptors for insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). The remaining 38 non-receptor tyrosine kinases are grouped into 10 subfamilies and include, for example, Src, JAK and FAK [21]. Cell signaling through RTKs have been studied for decades and many essential signal transduction pathways have been identified. RTKs control cellular mechanisms such as proliferation and differentiation, metabolism, cell-cycle control and cell migration [22-24]. Via extracellular stimuli through RTK ligands, the cell transfers the signal through complicated signaling cascade pathways and in to nuclear events.

Structurally, RTKs consist of three segments; the extracellular fragment which contains the ligand binding domain, a transmembrane helix and the cytoplasmic fragment that covers the tyrosine kinase catalytic activity [20]. The cytoplasmic portion is further divided up into a regulator juxtamembrane (JM) domain, the tyrosine kinase domain and the carboxy (c)-terminal region [22]. The extracellular fragment of the receptors contains a variety of different domains including immunoglobulin-like domains, fibronectin type III-like domains, leucine-rich domains, cysteine-rich domains and EGF-like domains. Based on the composition of the extracellular domain they are divided up into the 20 families (Figure 2) [20].

With the exception of the insulin receptor (IR) family, RTKs are monomeric but dimerizes upon ligand binding. (The structure of the IR family will be covered in section 1.3). When ligand binds to the receptor, the receptor goes through a conformational change into its active state, either as a dimer or as an oligomer [22]. Once activated, the receptor *trans*-autophosphorylates its dimeric partner [25, 26]. This creates binding sites for proteins containing Src homology 2 (SH2) domain and phosphotyrosine binding (PTB) domain [27, 28], which is the starting point of the signaling cascades (see section 1.5).

Figure 2. Schematic representation of the 20 subfamilies of human RTKs. Structural domains in the extracellular regions are marked according to the key. The intracellular domains are shown as red rectangles.
Re-printed with permission from Elsevier [22].

Signal downregulation is controlled by receptor internalization (endocytosis) and protein tyrosine phosphatases (PTPs) [29, 30]. Internalized receptors are either targeted for degradation through the lysosome or the ubiquitin-directed proteasome or the receptors are re-cycled to the plasma membrane [30]. More than 100 PTPs genes have been identified and they function as enzymes which catalyze the de-phosphorylation and control the length and the duration of the response [31].

1.2.1 RTKs and cancer

When trying to identify new drug targets to combat cancer cell growth there are certain criteria that should be met. Workman and Kaye summarized it as follows [32]:

1. Frequency of genetic or epigenetic deregulation of the target or pathway in human cancer.
2. Demonstration in a model system that the target contributes to the malignant phenotype.
3. Evidence of the reversal of the malignant phenotype; for example by gene knockout.
4. Practical feasibility, tractability or drugability of the target.
5. Availability of a robust and efficient biological test to support the drug discovery program.
6. Ability to run a robust cost-effective high-throughput screen.
7. Availability of a structure-based drug design approach.

Based on these criteria, RTKs are optimal drug targets and today there are drugs targeting ErbB-2, EGFR, VEGFR. c-KIT, MET and PDGFR [33, 34].

In normal cells, RTK signaling is tightly regulated to keep cell growth under control. However, when this balance is perturbed and tyrosine kinase signaling is overexpressed, the cell starts to transform. Sequencing of the epidermal growth factor receptor (EGFR) revealed similarities with the oncogene v-ErbB [35]. This was one of the very first studies describing how cancer cells can be self-sufficient. Molecular dys-regulation of RTKs signaling, direct or in-direct, are causing cancer cells to be self-sufficient and are classified into three major groups;

- Ligand independent signaling: Mutations of the EGFR has been found in cancers such as gliomas, non-small cell lung carcinomas (NSCLC) and ovarian carcinomas, with the type III deletion mutant, EGFRvIII, being the most common. EGFRvIII is missing the ligand binding domain and is constitutively phosphorylated and activates downstream signaling cascades [36].

- Mutations: As mentioned, there are truncated variants of RTKs which cause ligand independent signaling. There also are mutations of the downstream signaling proteins, thereby having an in-direct impact of RTK signaling. One such example includes inactivation mutation of the tumor suppressor PTEN (a phosphatase that inhibits AKT activation) [37].

- Autocrine signaling: A third way for cancer cells to obtain self-sufficiency is through autocrine signaling which is common in cancer cells. For example, IGF-1 is strongly expressed in melanoma cells and by inhibiting IGF-1 with antibody the IGF-1 receptor

(IGF-1R) is deactivated followed by MAPK signaling inactivation. This blocks the melanoma cell proliferation and causes a net loss of melanoma cells [38].

1.3 THE INSULIN-LIKE GROWTH FACTOR (IGF) FAMILY

The insulin-like growth factor (IGF) system regulates fundamental biological mechanisms throughout fetal and childhood development. In adult life, it regulates metabolism, proliferation, differentiation and apoptotic protection. The IGF family comprises of three ligands (IGF-1, IGF-2 and insulin), three cell-surface receptors including the IGF-1R, the IGF-2R/mannose 6-phosphate (M6P) receptor and the insulin receptor (IR) and six high affinity IGF-binding proteins (IGFBP-1 to 6) (Figure 3) [39].

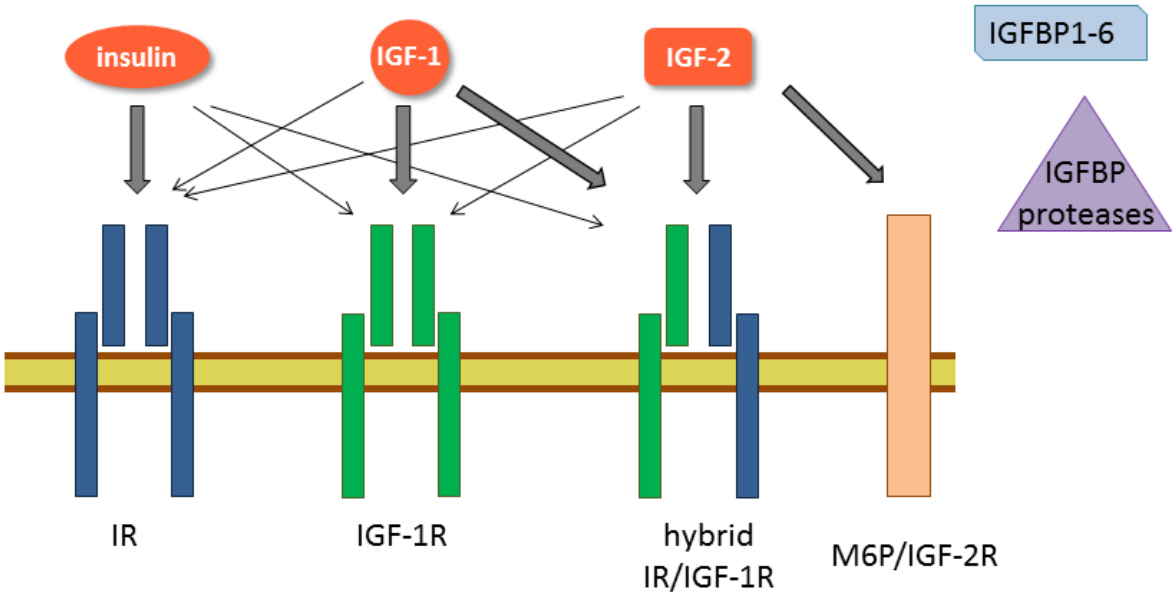

Figure 3. The IGF system with its ligands (insulin, IGF-1 and IGF-2), receptors (IR, IGF-1R, hybrid IR/IGF-1R and IGF-2R) and IGFBP1-6 with their proteases. Thick arrows and thin arrows indicate high and low ligand affinity binding.

The IGF-2R is monomeric and structurally distinct from the IGF-1R and IR, e.g., it lacks the tyrosine kinase domain and as a consequence it does not belong to the tyrosine kinase family. It is a multi-functional protein and binds both IGF-2 and M6P [40]. However, the receptor lacks signaling capacity. It has two main functions; (I) regulating M6P-containing lysosomal enzymes by trafficking them between the trans-golgi network to lysosomes and (II) regulating circulating IGF-2 by binding followed by internalization and lysosomal degradation [41, 42]. The receptors ability to regulate IGF-2 has proposed it to function as a tumor suppressor and IGF-2R mutations are found in human hepatocellular carcinomas [43].

Insulin and the IR are generally regarded to regulate glucose metabolism and growth in normal tissue, but lately there is an increased focus on IR's role in cancer progression [44]. The western lifestyle has led to increased occurrence in obesity and type 2 diabetes; two risk factors in malignant growth [45, 46]. The insulin receptor exists in two isoforms due to splicing of exon 11; IR-A and IR-B and it is the former isoform that has been suggested to play a role in cancerogenesis. IR-A, as opposed to IR-B, binds IGF-2 with high affinity. IR-A activation by insulin causes metabolic response, whilst IGF-2 dependent activation results mainly in mitogenic effects [47]. Overexpression of IR-A is reported in, for example, ovarian, hepatocellular and endometrial carcinoma [47-50]. Another emerging role of the IR-A in cancer progression is its ability to form a hybrid receptor together with IGF-1R [51, 52]. The hybrid receptor can be activated by IGF-1, IGF-2 and insulin [53].

1.3.1 Insulin-like growth factors

IGF-1 and IGF-2 are extremely potent mitogens and play a pivotal role in regulating cell proliferation, differentiation and apoptosis. They exert their signaling through endocrine/paracrine as well as autocrine pathways.

The IGF-1 is a 70 amino acid long peptide mainly produced by the liver in response to stimulation by growth hormone (GH). The structure of IGF-1 is 70% homologues to IGF-2 and 50% homologues to pro-insulin [54]. After birth, the IGF-1 serum levels increase slowly and peak at puberty and then decline with age. In adults, serum levels of IGF-1 range between 100-200 ng/ml [55]. Several studies propose that high serum levels of IGF-1 is a predictive factor in common cancers such as prostate, breast and colorectal cancers [56-59]. More recently, the link between IGF-1 and cancer was further established in an epidemiological study of 230 individuals with Laron syndrome, a form of dwarfism as a result of GH insensitivity, and as a consequence they suffer from IGF-1 deficiency. This study found that none of the 230 individuals worldwide developed cancer [60].

IGF-1 promotes cell division by stimulating cyclin D1 production and increasing DNA synthesis, which progresses the cell cycle from G_1 to S phase [61, 62]. In addition to IGF-1's mitogenic property it triggers an anti-apoptotic effect by increasing transcription of Bcl-xL protein and suppressing expression of Bax, which blocks the apoptotic pathway [63, 64].

The IGF-2 peptide consists of 67 amino acids and is produced by a variety of tissues. The IGF-2 production is still not understood, but it is independent of GH [65]. The serum levels of IGF-2 are higher than the ones of IGF-1, ranging between 400-600 ng/ml. It is believed that IGF-2 is a key regulator during embryonic and fetal growth [55]. It is reported that in adrenal cortical malignant tumors the IGF-2 levels are up to 10 times higher in malignant tumors compared to benign or normal gland [66].

1.3.2 Insulin-like growth factor binding proteins (IGFBPs) and proteases

There are six IGFBPs that bind IGFs with high affinity. All six proteins share approximately 35% sequence identity. Less than 1% of IGFs are circulating in serum in free form. Most of the circulating IGFs exist as a ternary complex together with mainly IGFBP-3 but also IGFBP-5 and the glycoprotein acid labile subunit (ALS) [67].

IGFBPs bind IGFs and through three different mechanisms they affect the IGF stimuli in the body; (I) increase IGFs half-life from 10 minutes up to 12 hours (II) IGFBPs function as a transport receptor for IGFs and distribute them to extravascular spaces (III) regulate the interaction with IGF-1R [54]. IGFBPs either inhibit or enhance IGFs binding to the IGF-1R. Phosphorylation of IGFBP at the plasma membrane increases IGFs association with IGF-1R [68, 69].

There are several studies suggesting that some IGFBPs affect target cells through an IGF-independent mechanism. IGFBP-3 has been reported to suppress tumor growth by blocking tumor angiogenesis [70] and it has been described to induce apoptosis independent of both p53 and IGF-receptor mediated pathways [71].

IGFBPs binding to IGFs are further regulated by numerous different IGFBP proteases. Prostate-specific antigen (PSA) is one serine protease that can cleave IGFBP-3 and IGFBP-5, making more IGF available to the cells [72, 73]. The complete regulation of IGFBP proteolysis is complex and not very well understood.

1.3.3 Structure and activation of the IGF-1R

The IGF-1R gene is located in chromosome 15 and contains 21 exons; exons 1-10 encoding the α-subunit of the receptor and the β-subunit is encoded by exons 11-21 [74, 75]. The IGF-1R is synthesized as a single-chain pro-receptor. With the assistance of chaperone proteins the pro-receptor is glycosylated, folded and dimerized. In the Golgi the pro-receptor is cleaved in the 30 amino acid signal peptide, containing the protease cleavage site, Arg-Lys-Arg-Arg, generating the extracellular α-subunit (130-135 kDa) and the transmembrane/cytoplasmic β-subunit (90-97 kDa) [74]. The subunits are linked with disulfide bonds between the α/β subunits and the α/α subunits in the mature $\alpha_2\beta_2$ heterotetramer receptor (Figure 4) [76].

Ligand binding of IGF-1 to the extracellular domain of the receptor triggers autophosphorylation of three tyrosine residues in the activation loop (a-loop); Tyr1131, Tyr1135 and Tyr1136 [77, 78]. The autophosphorylation of the receptor stabilizes the a-loop in a conformation that

facilitates catalysis [79]. Following autophosphorylation, Tyr950 in the JM domain is phosphorylated and act as docking site for signaling molecules including insulin receptor substrates 1 (IRS-1) and Shc. Lysine 1003 in the tyrosine kinase domain corresponds to the ATP-binding site [80, 81]. Mutations within the a-loop, Tyr950 or Lys1003 inhibit the IGF-1R's mitogenic and transformation ability, demonstrating that these residues are required for both transformation and proliferation [82-85].

The work presented in this thesis is a continuation of our group's previous finding that the IGF-1R is SUMOylated at three specific lysine residues, namely Lys1025, Lys1100 and Lys1120 and upon SUMOylation the receptor undergoes nuclear translocation [86].

The c-terminal tail of the receptor functions as a regulatory domain important in many IGF-1R signaling responses. Tyrosine residues 1250 and 1251 are together with histidine 1293 and lysine 1294 important in the anti-apoptotic response [87]. Mutations of tyrosine 1250-1251 and serine residues 1280-1283 affect the cell proliferation and the IGF-1R's transforming ability [88, 89]. It has also been proposed that phosphorylation of serine 1248 restrains the receptor's kinase activity [90].

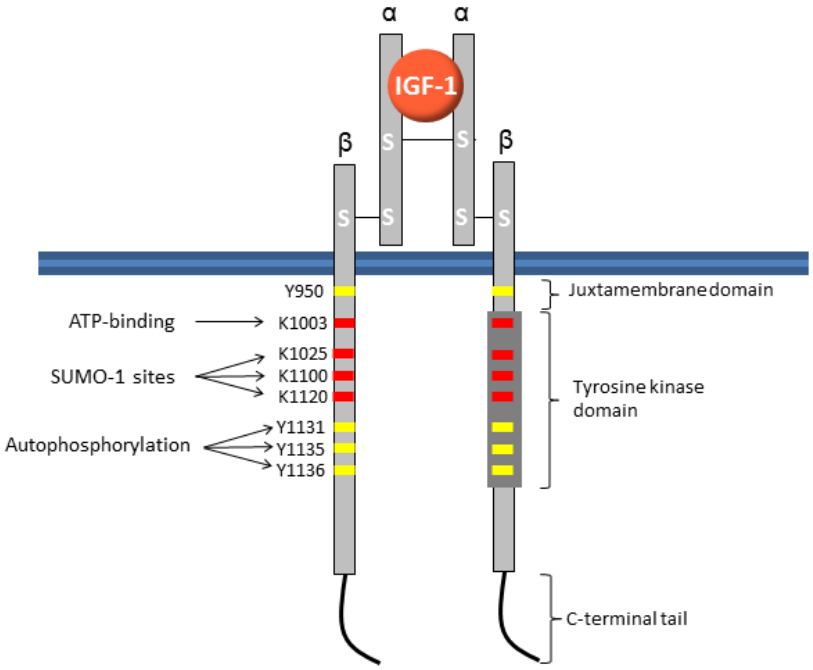

Figure 4. Schematic structure of IGF-1R with important domains and residues presented. S-S = disulfide bonds, Y = tyrosine, K = lysine.

1.3.4 IGF-1R as a target in cancer therapy

In 1993, Sell *et al.*, published a study showing that the IGF-1R is a pre-requisite for malignant growth. Mouse fibroblast embryo cells from the igf1r (-/-) knock-out, (R- cells), do not grow in serum free medium supplemented with platelet-derived growth factor, epidermal growth factor, and IGF-I, whilst the wild-type cells do. Further they provided evidence that R- cells grown in serum and stably transfected with the simian virus 40 (SV40) large T antigen or the Ha-ras, cannot be transformed [91, 92].

There are many clinical studies showing that most cancers overexpress IGF-1R [93, 94], but there are some exceptions, for example, a total loss of IGF-1R has been reported in prostate cancer bone marrow metastases [95] . Although IGF-1R's role in cancer progression is undisputed, it is important to remember that it is not considered to be an oncogene *per se*. Activation of IGF-1R by IGF-1 is alone not sufficient for transformation, but after an oncogenic event has occurred it is well established that the IGF-1R plays a key role in cell survival, progression, apoptotic protection and DNA repair [96-98].

Recently, the IGF-1R expression has been postulated to function as a predictive and prognostic biomarker in different cancers. A few examples are listed below:

- In a study consisting of 49 patients with surgically removed gastric cancer, of which 21 patients had lymph node metastases, they found that IGF-1R expression associates with lymph node metastasis, it correlates with worse prognosis and is an independent predictor of survival in patients with gastric cancer [99].
- In metastatic colorectal cancer, high expression of IGF-1R correlates with longer progressive free survival (PFS) in combination with cetuximab treatment, thereby suggesting a predictive role of IGF-1R for patients that will benefit from cetuximab [100].
- IGF-1R is a strong predictive marker of lack of response to radiotherapy in patients with locally advanced HPV16-positive cervical cancer [101].
- Vilmer *et al*., showed in a study concluded of 33 patients with advanced NSCLC, that high IGF-1R mRNA expression correlates with shorter PFS compared to the negative subgroup; 6.1 months vs 7.4 months [102].

The IGF-1R's expression in many different cancers and its ability to sustain tumor growth has made it an attractive pharmaceutical target. There are different techniques of targeting the IGF-1R mediated signaling; antibodies or by small-molecule inhibitors. Other methods that have been discussed for IGF-1R downregulation are the use of dominant-negative receptors or RNA interference/antisense, but due to limitation in drug administration these are yet not a feasible option today [81].

However, results from clinical studies have been disappointing. Small-molecule inhibitors are very efficient in targeting the IGF-1R *in vitro* and in xenograft tumor models but there is a problem of IR cross-reactivity and toxicity but several compounds are still under investigation [103, 104]. Monoclonal antibodies are designed to target the extracellular domain of the IGF-1R, thereby inhibiting IGF-1 binding and receptor activation. The receptor is instead internalized and downregulated. The effects of tumor regression is very prominent *in vitro* and the tolerance against antibodies in the human body are in general good [104]. However, it seems like it is only a subset of patients that benefit from antibody therapy and there is also a problem with patients developing resistance against the drug [105].

Proposed explanations to the discouraging results in the clinical trials with IGF-1R targeted drugs include (I) targeting IGF-1R with antibody in tumor lacking IRS-1 is inefficient [106]. Without IRS-1, IGF-1R signals differentiation response rather than a mitogenic response (II) Resistance through switching from IGF-1R dependency to EGFR dependency (and *vice versa*) [82, 107] (III) Resistance due to tumor heterogeneity, there are evidence of subpopulations of cancer cells in human tumors [108] (IV) Failure to target IGF-1R might also be as a consequence of mutations in the PI3K pathway making it constitutive active independently of IGF-1R activation [109] and (V) the presence of nuclear IGF-1R (which will be reviewed in section 1.8).

However, IGF-1R might still be a very useful target to combat cancer, but it might be more efficient and give better response in combination therapies rather than as a single agent.

1.4 THE ERBB FAMILY

The ErbB tyrosine kinase receptor family consists of four receptors. Stanley Cohen was the first researcher who described the epidermal growth factor receptor (EGFR), which is also referred to as ErbB-1/HER-1. Cohen identified the EGF, EGFR and its tyrosine kinase activity. The other three member consist of ErbB-2 (HER-2/Neu), ErbB-3, (HER-3) and ErbB-4 (HER-4). The ErbB name is derived for the avian erythroblastosis oncogene, which the human receptors are homologous to. The v-ErbB oncoprotein lacks the EGF ligand binding domain; mimicking activated EGFR, resulting in a constant growth signal to the cell.[110].

The four receptors, together with 13 polypeptide ligands, containing a conserved EGF domain, make up a complex signaling network (table 1) [111]. Receptor activation of the ErbB family includes both homo- and heterodimers. ErbB-2 lacks the ligand binding domain and to function it has to form a heterodimer with EGFR, ErbB-3 or ErbB-4. The ErbB-3 also relies on the other receptors since it has a defective tyrosine kinase domain [112, 113], although a recent study suggests that the ErbB-3 has some phosphorylation activity [114].

Table 1. Specificity of ErbB receptors and ligands. Re-printed with permission from Elsevier [111].

Ligand	Receptor			
	ErbB-1	ErbB-2	ErbB-3	ErbB-4
EGF	+	–	–	–
TGF-α	+	–	–	–
HB-EGF	+	–	–	+
Amphiregulin	+	–	–	–
Betacellulin	+	–	–	+
Epigen	+	–	–	–
Epiregulin	+	–	–	+
Neuregulin-1	–	–	+	+
Neuregulin-2	–	–	+	+
Neuregulin-3	–	–	–	+
Neuregulin-4	–	–	–	+

The ErbB signaling network is involved in numerous different biological processes. Null mutations of any of the ErbB genes are lethal – embryonic or perinatal – with defects in heart, skin, lung, gastrointestinal tract, brain and kidney [110]. Insufficient ErbB signaling through the ligand neuregulin-1 is found in neurodegenerative diseases such as Parkinson disease, schizophrenia and multiple sclerosis [115, 116], whilst overabundant signaling (due to receptor overexpression, mutations or autocrine signaling) is well documented in many different carcinomas [110, 117].

The ErbB network is very complex, and for the purpose and aim of this thesis I will now focus on the EGFR.

1.4.1 EGFR structure

The extracellular domain of the EGFR consists of two ligand binding domains (domains I and III) and two cysteine rich domains (domains II and IV) [20]. The activated receptor is autophosphorylated at six tyrosine residues in the c-terminal tail; Tyr1068, Tyr1148 and Tyr1173 are the major sites [118] and Tyr992[119], Tyr1045[120] and Tyr1086[121] are minor autophosphorylation sites. The kinase domain of EGFR is divided into two parts; the N-lobe and the C-lobe, and upon activation two monomeric receptors will dimerize asymmetrical, connecting the N-lobe of one receptor to the C-lobe of the other (Figure 5) [122]. Ligand activation of EGFR initiates signaling cascades of the ras/raf/MEK/MAPK pathway and the PI3K pathway.

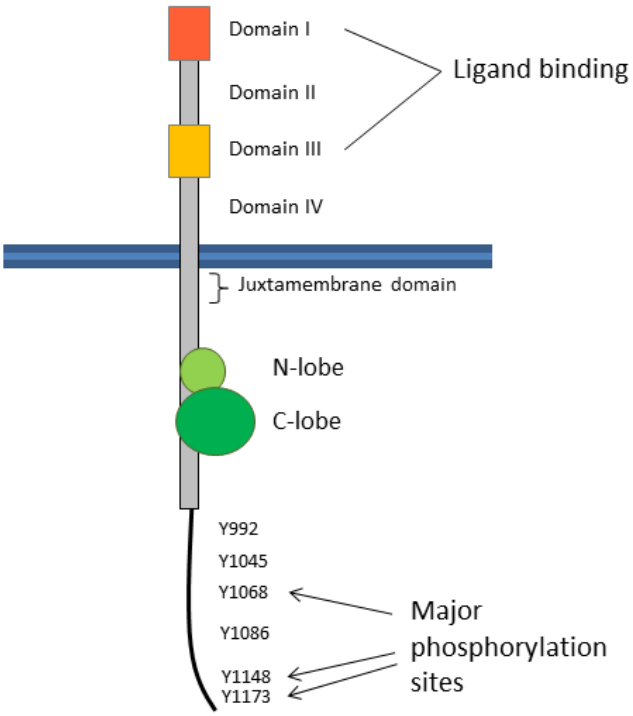

Figure 5. Schematic structure of EGFR with important domains and residues presented.

1.4.2 EGFR in cancer therapy

Several *in vitro* studies have shown that overexpression of EGFR induces transformation together with ligand [123, 124]. Today EGFR overexpression is established in many different cancers; lung cancer, breast cancer, colorectal cancer, gastric cancer, head and neck cancer, pancreatic cancer and glioblastoma. Overexpression of the EGFR is most commonly a result of gene amplification [125]. Other genetic variations of the EGFR in carcinoma are summarized in Table 2.

In a study containing 31 colorectal cancer patients, they identified that eight out of nine patients who responded to anti-EGFR treatment (cetuximab or panitumumab) had an increased EGFR gene copy number and that there is no correlation with mutations occurring in the EGFR catalytic domain (exons 18-21). This suggests that EGFR gene amplification is a good way to select patients which benefits from anti-EGFR treatment [126].

Table 2. Genetic alterations of the EGFR in human carcinoma. Re-printed with permission from Elsevier [125].

Genetic alteration in EGFR	Ligand dependence
Gene amplification	+
N-terminal truncation (EGFRvI)	−
Deletion exons 14–15 (EGFRvII)	+
Deletion exons 2–7 (EGFRvIII)	
Deletion exons 25–27 (EGFRvIV)	+
C-terminal truncation (EGFRvV)	+
Tandem duplication exons 2–7	+
Tandem duplication exons 18–25	−
Tandem duplication exons 18–26	−
Small in frame deletion or point mutations in the kinase domain (exons 18–21)	+

Today there are six drugs approved by the FDA targeting the EGFR. Four of them which are small-molecule inhibitors targeting the receptor [110]:

- Afatinib: First-line treatment of NSCLC if patients have exon 19 or the exon 21 L858R mutation. Approved 2013.
- Erlotinib: First-line treatment with the same indications as afatinib or as a second-line treatment following chemotherapy or as a first-line treatment of pancreatic cancer in combination with gemcitabine. Approved 2004.
- Gefitinib: Second-line treatment of NSCLC after chemotherapy. Approved in 2005, but withdrawn in the United States due to lack of evidence that it prolonged survival, but still used in many different countries [127].
- Lapatinib: A dual inhibitor, which also targets ErbB-2. Second-line treatment in ErbB2-positive breast cancer in combination with chemotherapy or with letrozole in post-menopausal hormone receptor-positive breast cancer. Approved 2007.

Approved antibodies include the chimeric cetuximab and the human antibody panitumumab:

- Cetuximab: to be used in wild-type KRAS colorectal cancer in combination with chemotherapy or in head and neck cancers in combination with chemotherapy/radiation. Approved 2004.
- Panitumumab: Second-line treatment for metastatic colorectal cancer after cytotoxic therapies. Approved 2006.

Targeted therapies are still relatively new on the market and several ongoing clinical studies are evaluating new substances and the above mentioned molecules/antibodies to get their approval extended for other cancers or to be used in combination therapies.

1.5 SIGNALING PATHWAYS

As I have already mentioned, upon ligand binding of RTKs they become activated and send their survival, proliferation and anti-apoptotic signals through cytoplasmic signaling cascades. These signaling pathways are very important in understanding the tumorigenic effect RTKs have in cancer cells. My projects presented in this thesis do not cover these pathway as such, but I will here, very simplified and schematically cover the two major pathways activated by both the IGF-1R and the EGFR; the PI3K and MAPK pathways. I will focus on the IGF-1R line of activation, however in principal it works the same for EGFR activation, but at certain stages there are different adapter/scaffolding proteins or EGFR-specific substrates involved.

1.5.1 The PI3K/Akt pathway

The phosphatidylinositol 3-kinase (PI3K) pathway is involved in cell survival, proliferation, protein translation and glucose metabolism. Upon IGF-1R activation and phosphorylation of tyrosine 950, insulin receptor substrate proteins (IRS 1-4) binds to the receptor [80]. Phosphorylated IRS-1 at tyrosine residues 612 and 632 recruits the p85 regulatory subunit, followed by activation of the catalytic domain of the PI3 kinase, p110 [128]. Activation of PI3K results in an increase in phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 3,4-triphosphate (PIP2) and recruitment of the serine/threonine Akt kinase. The constitutively activated 3´-phosphoinositide-dependent kinases (PDK1 and 2) phosphorylate Akt at threonine 308 and serine 473 [129, 130].

Activated Akt is an important step in the PI3K signaling cascade; once it is phosphorylated it affects downstream signaling in a complex network. Akt inhibits apoptosis through phosphorylation of for example: (I) forkhead related transcription factors, such as FoxO. Phosphorylation inhibits FoxO's nuclear translocation and thereby its transcriptional activity of pro-apoptotic proteins such as Fas Ligand and Trail [131] (II) the Bcl-2 family member BAD and caspase 9, thereby suppressing apoptosis and promoting cell survival [132, 133] (III) the pro-apoptotic GSK-3β and (IV) Mdm2, which translocates into the nucleus and suppresses transcription of the tumor suppressor gene p53 and increase p53 degradation [134, 135].

Further, Akt activates proteins like mammalian target of rapamycin (mTOR) and NF-κβ. The mTOR kinase regulates protein synthesis and is thereby a major effector of cell growth and proliferation. Two downstream targets of mTOR are 4E-binding protein 1 (4E-BP1) and S6 kinase (S6K1) [136]. Phosphorylation of 4E-BP1, which is a repressor of translation, leads to its inhibition, thereby increasing protein translation of e.g. c-myc and cyclin D1, two proteins important in cell-cycle progression [23]. S6K1, a ribosomal protein, is activated upon mTOR phosphorylation and increase protein synthesis [137].

One of the effectors responsible for inactivation of the PI3K/Akt pathway is phosphatase and tensin homologue (PTEN). It negatively regulates PIP3 levels, causing PI3K/Akt inhibition. PTEN acts as a tumor suppressor and *PTEN* deletion is found in~40% of all prostate cancers [138] and more than 330 somatic PTEN mutations have been reported in primary tumors and metastasis [139]. As a result of deletion or loss of function of PTEN, RTK signaling through PI3K is strengthened. Schematic overview of PI3K/Akt signaling pathway is shown in Figure 6.

1.5.2 The MAPK pathway

The second major signaling pathway for the IGF-1R and EGFR is the mitogen-activated protein kinase (MAPK) pathway (also known as the extra-cellular signal-regulated kinase (ERK) pathway.) Tyrosine 950 of the IGF-1R also functions as a docking site for Shc adapter proteins [39, 140]. Grb2 is a small protein containing one SH2 domain and two SH3 domains and can interact with both IRS-1 and Shc via the SH2 domain [141]. Through the N-terminal SH3 domain Grb2 interacts with son of sevenless (SOS) as a preexisting complex in the cytosol. The recruitment of Grb2/SOS to Shc or IRS-1 makes it available for binding to the membrane associated GTPase Ras. Upon activation, Ras exchanges GDP for GTP and activates Raf [142], which is a key point in the cascade signaling. Raf activates MEK1/2 (MAP kinase kinase), which in turn activates ERK1/2 (MAP kinase) through phosphorylation and results in a cellular response that triggers cell proliferation and survival (Figure 6) [143, 144].

Activated ERKs translocate into the cell nucleus and phosphorylate transcription factors such as Elk1, Ets1 and Ets2, c-Myc, STAT-3 and Sp1 [145-149]. This results in an activation of transcription through recruitment of co-factors, or they promote transcription by relieving repressive mechanism, for example, activation of ERKs result in removal of small ubiquitin-like modifier (SUMO) from a second regulatory domain of Elk-1, allowing activation of transcription by Elk-1 [150].

There are substantial evidence of the importance of the MAPK/ERK signaling pathway in cancer progression, cell growth and metastasis. For example, constitutively activated mutants of Raf and MEK transform rodent fibroblasts [144] and B-Raf is commonly mutated in malignant melanoma [151]. Today many different inhibitors against Ras/Raf/MEK are under clinical investigations.

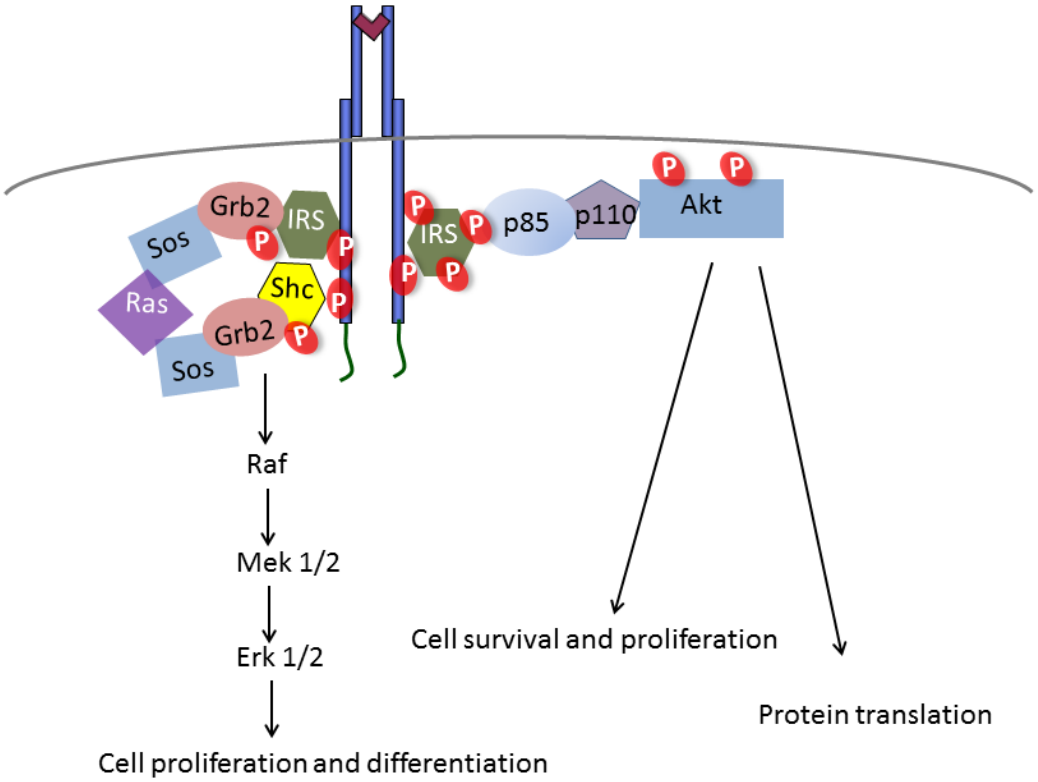

Figure 6. Schematic simplification of MAPK and PI3K signaling upon IGF-1R activation.

1.5.3 The Wnt/β-catenin signaling pathway

In the first project presented in the results section, we investigated IGF-1R's role as a potential co-activator of LEF1/TCF (lymphoid enhancing factor/T-cell factor) transcription factor. Therefore, I am introducing the Wnt/β-catenin signaling pathway.

The Wnt/β-catenin signaling is an evolutionary conserved pathway. Wnt was first discovered as segment polarity gene in *Drosophila melanogaster,* where its function is important in the formation of the body axis during embryonic development, and due to the knockout phenotype it was given the name *wingless* (Wg). Later the name was fused with the vertebrate homolog, integrated or Int-1 giving rise to new name Wnt (wingless-related integration site) [152].

There are several intra-cellular pathways stimulated by Wnt. The three main pathways are; the canonical Wnt/β-catenin pathway, the non-canonical planar cell polarity pathway and the non-canonical Wnt/calcium pathway [153]. Here I only present the canonical pathway which includes β-catenin.

In humans, 19 Wnt genes are identified and the genes are predicted to encode secreted proteins [154]. Wnt proteins are glycoproteins that bind to the extracellular domain of the Frizzled (Fz)

receptor family. The Fz receptors share homology with G-protein couple receptors and are seven-pass transmembrane proteins [155]. When there is no ligand bound to the receptor, cytoplasmic β-catenin is degraded by a protein complex consisting of Axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3 (GSK3). Casein kinase 1α (CK1) phosphorylates β-catenin within this complex followed by ubiquitination by β-Trcp, thereby targeting β-catenin for degradation by the proteasome [156]. Wnt and the Fz receptor require interaction with a co-receptor to become fully activated, namely with the low density lipoprotein-related protein 5/6 (LRP5 and LRP6) [157]. Once the two receptors are activated through Wnt binding, the cytoplasmic phosphoprotein Dishevelled (Dsh) directly interacts with Fz followed by Axin translocation to the LRP receptor. This results in accumulation of β-catenin, followed by its nuclear translocation [153, 157, 158]. Nuclear β-catenin forms an active transcriptional complex with LEF1/TCF by displacing the transcriptional inhibitor Groucho and CBP [159, 160] (Figure 7). Target genes of β-catenin/LEF1/TCF transcription complex include *C-MYC* [161], *AXIN2* [162] and *cyclin D1* [163].

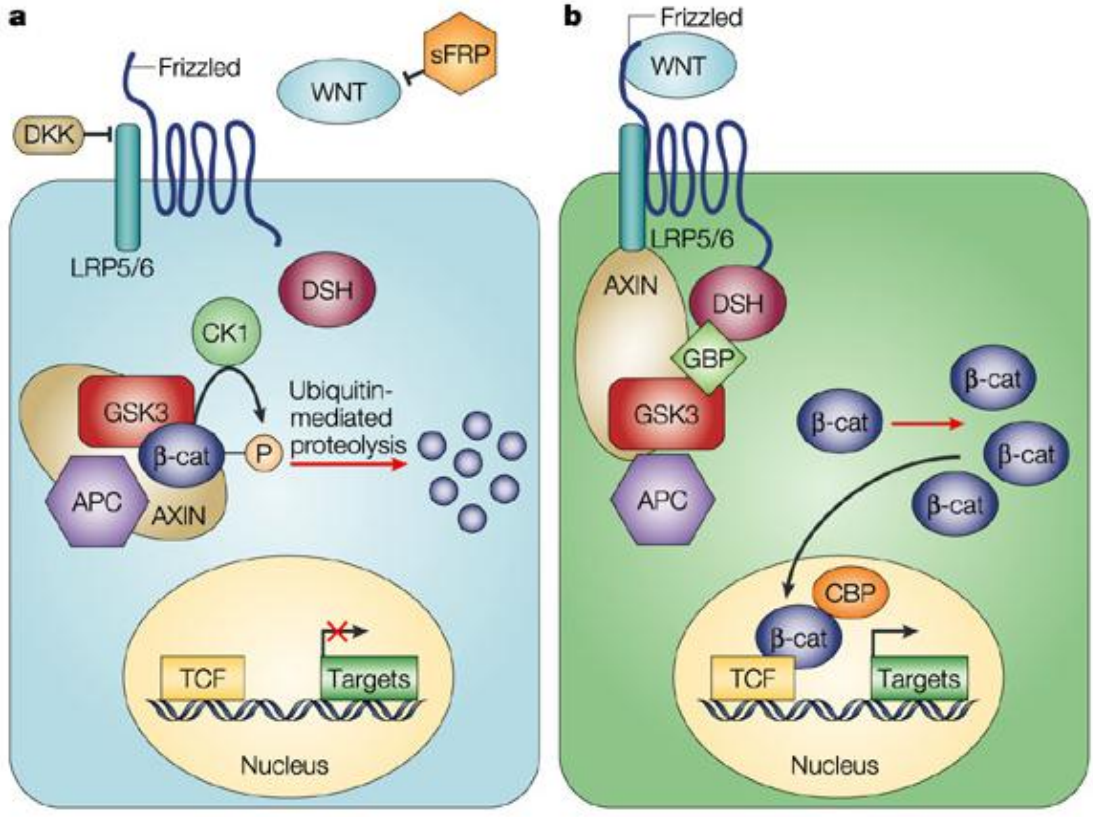

Figure 7. Wnt signaling pathway. (a) In the absence of active Wnt β-catenin is degraded. (b) Activated Wnt signaling causes β-catenin accumulation and its nuclear translocation. It induces transcription through binding to LEF1/TCF transcription factors. Re-printed with permission from Nature Publishing Group [164]

The Wnt/β-catenin transduction pathway is important for embryonic development [155, 165, 166] and is crucial for angiogenesis in the central nervous system [167]. Aberrant signaling associates with many different human diseases. A single amino acid substitution mutation in LRP5 causes an increase in bone density in e.g the jaw [168], whilst another LRP5 mutation instead causes a loss-of function and results in the autosomal recessive disorder osteoporosis-pseudoglioma syndrome and a decrease in bone mass [169]. Hyperactivation in the Wnt/β-catenin signaling pathway (as a result of mutations) is important in carcinogenesis. For example, truncations in APC result in Wnt activation, which increases cell proliferation and leads to adenomatous lesions. This give rise to familial adenomatous polyposis, an autosomal, dominantly inherited disease in which patients display hundreds or thousands of polyps in the colon and rectum [170, 171].

The Wnt/β-catenin pathway has extensive crosstalk with RTKs, including both IGF-1R and EGFR signaling. Aberrant Wnt signaling associates with an increased risk of type 2 diabetes [172-174]. Palsgaard *et al.,* found that Wnt stimulation leads to phosphorylation of Akt, GSK3β, and ERK1/2 and that it is insulin/IGF-1 receptor dependent. LRP5 interacts with the insulin receptor and knockdown of LRP strongly decreases insulin induced phosphorylation of downstream mediators [175]. IGF stimulation results in phosphorylation of β-catenin, followed by disassociation from E-cadherin, a protein important in cell-cell adhesion [176, 177]. Disruption between β-catenin and E-cadherin is important in the initiation of epithelial-mesenchymal transition [176, 178]. IGF stimulation leads to a rapid nuclear translocation of β-catenin followed by an increase in transcription [177, 179]. Collectively, these studies add another multiplex level in understanding the IGF signaling.

1.6 SMALL UBIQUITIN-LIKE MODIFIER (SUMO)

The IGF-1R was the first RTK revealed to be SUMO modified. Since then it has also been demonstrated that the IR and the intra-cellular domain of ErbB-4 are SUMOylated [180, 181].

Phosphorylation and ubiquitin are two post-translational modifications that have been mentioned above and they rapidly send biological messages across the cell. A third post-translational modification that is being studied more and more is small ubiquitin-like modifier, SUMO. One reason to why this modification was discovered relatively late could be that there is only a small portion of substrate modified at any given time, usually around 1% [182]. It was originally found to modify proteins within the cell nucleus, but as more research has been carried out it has also been found to regulate mechanisms within other compartments of the cell.

SUMO proteins (also known under names such as Smt3p[183], PIC-1[184], GMP1[185], and sentrin [186]), have a mass of ~11 kDa and approximately 100 amino acids. SUMOs are highly

conserved within eukaryotes and expressed in all tissues and cells and are essential for cell viability [187] There are four SUMOs identified in mammals; SUMO-1, SUMO-2, SUMO-3 and SUMO-4.

SUMO-1 and ubiquitin share 18% amino acid sequence identity, but their 3D structures are closely related. However, their charge topology differs significantly suggesting the two proteins have different target substrates and modifying enzymes [188]. SUMO-2 and SUMO-3 share 97% identity with each other and are often referred to as SUMO-2/3. They only share 50% with SUMO-1 but 86% identity with SUMO-4 [189]. It has been suggested that SUMO-4 lacks the ability to form covalent isopeptide bonds with substrates [190].

There are a few differences observed between SUMO-1 and SUMO-2/3. Saitoh *et al.* showed that there are high amounts of unconjugated SUMO-2/3 in cells, whilst most SUMO-1 are conjugated. When exposing the cells to stress through acute temperature changes, the amount of conjugated SUMO-2/3 to high molecular mass proteins rapidly increase, whereas no change is observed in the amount SUMO-1 conjugated proteins [191]. Some proteins, like RanGAP1, are preferentially SUMOylated by SUMO-1 [191] and other proteins, like the human thymine-DNA glycosylase, is modified equally well by SUMO-1 and SUMO-2/3 [192]. Another difference between the two SUMO groups is that SUMO-2/3 contains an internal consensus site for SUMOylation in their N-terminal allowing SUMO chain formations [193]. Poly-SUMO chains are identified for proliferation-cell nuclear antigen (PCNA) [194], promyelocytic leukima protein (PML) [195] and histone deacetylase 4 (HDAC4) [193].

The SUMO consensus site is described as ΨKXE, where Ψ is a large hydrophobic amino acid; K is the modified lysine residue; X is any residue; and E is a glutamic acid. This sequence is very short and exists in many proteins, and it is unlikely that all those sites are SUMOylated [187]. Many SUMOylated proteins are indeed SUMO modified at different sites [196-199], for example, the three SUMO-1 sites identified in the IGF-1R do not have this consensus sequence [86].

1.6.1 SUMOylation ligases

The process of SUMO attaching to a target protein is referred to as SUMOylation and is homologous to the mechanism of ubiquitylation. It involves three enzymatic steps; activation, conjugation, and ligation (Figure 8);

- SUMO-activating enzyme (E1): This is an ATP dependent step, which results in a high-energy thioester bond between the E1 and a glycine residue in the c-terminal of SUMO. This step is achieved by the heterodimeric SUMO activating enzyme 1 and 2 (SAE1/SAE2), also known as Aos1/Uba2 [200].

- SUMO-conjugating enzyme (E2): After activation, SUMO is transferred to a cysteine residue on the E2 enzyme. Unlike the ubiquitin pathway, there is only one E2 enzyme known for SUMOylation; Ubc9 [201, 202]. SUMO and Ubc9 form a thioester intermediate through a conserved catalytic cysteine residue of Ubc9 and the c-terminal of SUMO [200].

- SUMO ligases (E3): In the last step, SUMO is transferred from Ubc9 to the substrate with the aid of an E3 ligase, which mainly serves to increase stability of the SUMO conjugation. An isopeptide bond forms between the glycine residue of SUMO and the target lysine residue of the substrate [203].

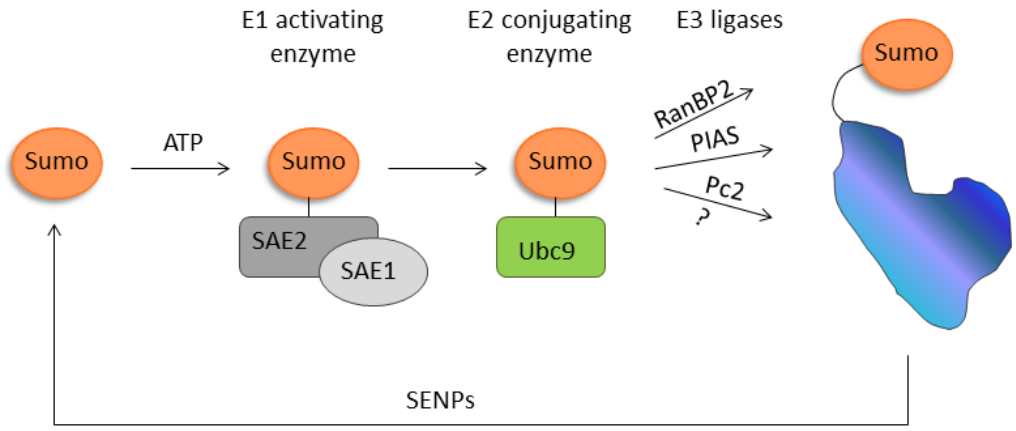

Figure 8. Schematic representation of the SUMOylation pathway. Mature SUMO is activated by SAE1/SAE2 in an ATP dependent manner. Subsequently, SUMO is transferred to the E2 conjugation enzyme Ubc9 and finally conjugated to the substrate by an E3 ligase. SUMO conjugation can be reversed by protease activity of SENPs thereby releasing free SUMO.

There are three distinct groups of SUMO E3 ligases [187, 203, 204]; (I) Members of the PIAS (protein inhibitor of activated STAT) family. Six mammalian PIAS proteins are identified; PIAS1, PIAS3, KChaP (splice variant of PIAS3), PIASxα, PIASxβ and PIASγ. PIAS proteins contain a SP-RING domain which binds directly to Ubc9 and essential for the SUMO E3-ligase activity [205, 206]. (II) The polycomb protein Pc2, which is a part of the large multimeric complex polycomb group (PcG). By recruiting Ubc9 and CtBP (c-terminal binding protein) to PcG bodies, CtBP will be SUMOylated [207] and (III) the Ran binding protein 2, RanBP2. This large protein is located at the cytoplasmic fibril of the nuclear pore complex and it is believed to SUMOylate certain proteins upon nuclear translocation. It SUMOylates RanGAP, and in the case of RanGAP it forms a stable trimeric complex between SUMO-RanGAP and Ubc9 [208].

1.6.2 SUMO-specific proteases (SENPs)

There are two main functions of SUMO-specific proteases; to mature newly synthesized SUMO by cleavage in the C-terminal tail and to make the SUMOylation reversible by removing SUMO from substrates. These enzymes are called ubiquitin-like proteases in yeast and sentrin-specific proteases (SENPs) in mammals [209]. Even though the SUMOylation process is very similar to the ubiquitylation, there are few similarities between SENPs and DUBs (de-ubiquitinating enzymes). SENPs appear to be more structural related to viral proteases [210]. Another difference is that there are >100 DUBs identified, but only seven SENPs; SENPs 1-3 and 5-7 [211].

Due to the low number of SENPs it is believed that the specificity of the SENPs is regulated by their sub-cellular localization. For example, SENP1 is mainly localized in the nucleus and is essential in mouse embryos for deSUMOylation of SUMO-1 [212, 213]. SENP2 binds RanBP2 in the nuclear pore and processes SUMO-2 more efficiently than SUMO-1 and SUMO-3 [214]. Further, SENP7 localizes in the nucleoplasm and functions as a SUMO-2/3 protease rather than SUMO-1 and it only regulates substrate cleavage and is unable to process SUMO pre-cursors [215].

1.6.3 SUMO targets and biological function

The biological functions of SUMOylated proteins described in the literature include facilitating nuclear translocation, DNA repair, suppress or activate transcription and stabilizing multi-protein complexes [187, 200].

There are several studies showing that SUMOylation affects subcellular trafficking of the target protein. In the case of the transcription factor Elk-1, it has been demonstrated that upon SUMOylation, Elk-1 translocates from the nucleus to the cytoplasm and thereby inhibits transcription [216]. Kishi *et al*. have described a similar event for SUMOylated Pdx1, but in reverse, i.e. the transcription factor Pdx1 will upon SUMO-1 modification translocate into the cell nucleus and activate transcription of the insulin gene [217]. The tumor suppressor p53 is another example where gene activation is enhanced upon SUMOylation [218].

SUMOylation of thymine-DNA glycosylase (TDG) plays a crucial role in the DNA repair. TDG initiates base excision repair, where uracil/thymine mismatches to guanine are repaired. Once the damage is repaired TDG needs to be SUMOylated in order to disassociate from the so called harmful abasic site [192]. SUMO modification plays an important role in PML nuclear bodies, where SUMOylation of PML links and stabilizes this heterogenetic group of proteins [187, 219].

1.7 CYTOPLASMIC-NUCLEAR SHUTTLING

The nuclear envelope, which separates the cytoplasm from the nucleoplasm, is a double membrane structure. In order to pass signals from the cytoplasm into the cell nucleus or for synthesized RNA molecules to leave the nucleus they have to pass this barrier, and they do so through the nuclear pore complexes (NPCs). Smaller molecules, such as water and nucleotides, are able to passively diffuse through the NPCs, whereas larger molecules, >40 kDa require active transportation [220].

1.7.1 The nuclear pore complex (NPC)

The NPC is one of the largest and most complex multi-protein assemblies in the eukaryotic cell. The NPCs make up aqueous channels which transport molecules from the cytoplasm into the nucleus and vice versa. The complex contains approximately 30 different evolutionary conserved proteins, referred to as nucleoporins (Nups) and each protein exists in multiple copies. In total, this multifaceted unit includes 500 – 1000 protein molecules [221]. By using electron microscopy the size of the NPC is ~150 nm long and 60-70 nm wide at the cytoplasmic and nuclear periphery and 45 nm wide in the central part of the complex [222].

The NPC structure is divided into different parts; cytoplasmic filaments, central channel, spoke complex, lumenal ring, cytoplasmic and nucleoplasmic rings and a nuclear basket (Figure 9) [223]. The cytoplasmic filaments consist of phenylalanine-glycine (FG) repeats that interact with transport receptor and are composed by the largest nucleoporin, RanBP2 (also known as Nup358) [224]. The "FG Nups" have shown to function as direct mediators in cytoplasmic-nuclear shuttling by serving as docking sites for transport receptors [225, 226].

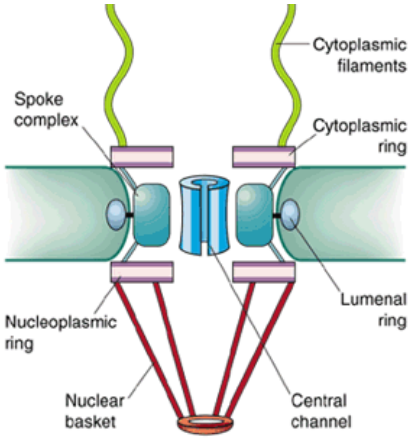

Figure 9. Schematic representation of the major structures in the nuclear pore complex. Re-printed with permission from Nature Publishing Group [223].

1.7.2 Transport through the NPC

Active transportation through the NPC requires several timely coordinated protein interactions. Transport receptors in humans are proteins belonging to the karyopherin family. Today there are more than 20 karyopherins identified in humans and they are subdivided into two groups: karyopherin α and karyopherin β [227]. Karyopherins involved in importing proteins to the nucleus are also known as importins and export karyopherins are known as exportins. From this point forward I will use this terminology.

Importins recognize proteins targeted for nuclear translocation by a nuclear localization signal (NLS). Importin-α was the first identified importin to be able to bind to a NLS. Importin-β forms a heterodimer with importin-α, but it has been demonstrated that importin-β can direct nuclear translocation without binding to the α-subunit [228-230]. The structure of importin-α is composed of ten armadillo (ARM) repeats, repetitive amino acid sequence of ~40 amino acids which forms three α-helices, and these ARM repeats recognize the NLS of cargo proteins [231]. Importin-β contains a similar repetitively structure named HEAT, which forms a superhelical coil. These repeats are flexible structures allowing recognition of many different cargos [227]. It is worth mentioning that it has been reported that proteins can undergo nuclear translocation without the aid of importins, e.g., β-catenin can associate – independently of transport receptors – with Nups followed by nuclear translocation [232].

There are different types of NLS sequences identified and they are classified as “classical NLS” or “non-classical NLS.” Examples of classical NLSs include the monopartite sequences PKKKRKV (found in the SV40 T antigen) and PAAKRVKLD (found in c-myc) and the bipartite sequence VKRPAATKKAGQAKKKKLD (found in nucleoplasmin) [231]. The classical NLSs contain either one or two clusters of basic residues. Further, by screening of peptide libraries against binding to importin-α, Kosugi *et al*. proposed six classes of NLS sequences with consensus motives (Table 3) [233]. In contrast, the non-classisical NLS sequence M9 (found in hnRNP) contains acidic amino acids [234, 235].

The core of the cytoplasmic-nuclear trafficking of molecules involves the RanGTPase system. Ran is a small GTPase of the Ras superfamily and it is essential for nuclear translocation. The Ran transportation cycle also includes Ran guanine nucleotide exchange factor (RanGEF) and Ran GTPase activating protein (RanGAP). On the cytoplasmic side, Ran is bound to RanGDP. Substrate bound to importin-α/β translocates into the nucleus through binding to RanBP1/2. Inside the nucleus RanGAP converts GDP to GTP, and RanGTP will bind to the importin/cargo complex, resulting in cargo release into the nucleus. RanGTP-importin complex shuttles back to the cytoplasmic side where RanGEF hydrolysis RanGTP and making importin available for a new transportation cycle. The cellular localization of RanGEF (cytoplasm) and RanGAP (nucleus) creates a concentration gradient of RanGDP and RanGTP making this shuttling

possible. A small RanGDP-binding protein, nuclear transport factor 2 (NTF2) contributes to maintaining this gradient by facilitating RanGDP import into the nucleus [236].

Table 3. Six classes of NLS sequences proposed by Kosugi *et al.* [233]. Sequence representation is as follows: (^DE), any amino acid except Asp or Glu; $X_{10–12}$, any 10–12 amino acids. Re-printed with permission from the American Society for Biochemistry and Molecular Biology.

NLS class	Consensus sequence
Class 1	KR(K/R)R, K(K/R)RK
Class 2	(P/R)*XX*KR(^DE)(K/R)
Class 3	KR*X*(W/F/Y)*XX*AF
Class 4	(R/P)*XX*KR(K/R)(^DE)
Class 5	LGKR(K/R)(W/F/Y)
Bipartite	KR$X_{10–12}$K(KR)(KR)
Bipartite	KR$X_{10–12}$K(KR)*X*(K/R)

1.8 NUCLEAR RTKs

In previous sections the traditional RTK signaling has been covered, i.e. ligand binding induces dimerization, followed by autophosphorylation allowing adaptor proteins to attach and activate the downstream signaling cascade. Even though it still might be considered somewhat controversial, there is more and more evidence emerging of a non-canonical RTK signaling pathway, namely the topic of this thesis; their ability to translocate into the cell nucleus. Today several RTKs have been reported to localize in the cell nucleus, e.g the ErbB receptor family [237-241], FGFR [242], IGF-1R [86], InR [243], Ryk [244], TrkA [245] and VEGFR2 [246].

1.8.1 Mechanisms for nuclear translocation

There have been different investigations and hypothesis presented trying to explain the mechanisms behind RTKs nuclear translocation. There are several studies demonstrating that RTKs undergo proteolytic cleavage creating a soluble intra-cellular domain (ICD) which translocates to the nucleus. Within the category of cleaved receptor, ErbB-4 is the most studied. ErbB-4 has several isoforms as a result of alternative splicing, two of these are the juxtamembrane isoforms JMa and JMb. Ligand stimulation of ErbB-4-JMa results in cleavage in the extracellular juxtamembrane region; resulting in a 80 kDa membrane-bound intra-cellular fragment, which is further processed by a γ-secretase complex, releasing a soluble ICD [247]. This ErbB-4-ICD fragment contains a NLS sequence that further mediates its import to the nucleus [248]. Other RTK fragments found in the nucleus due to splice variants include e.g., EGFR and ErbB-3 [249, 250].

However, the most controversial issue regarding nuclear RTKs is the discovery of intact receptors, holoreceptors, inside the nucleus. Seemingly, the biggest obstacle is how a membrane-bound receptor can translocate into the cell nucleus. Holoreceptors identified in the nucleus include EGFR, ErbB-2, ErbB-4, IGF-1R, and FGFR amongst others.

Nuclear translocations of the EGFR and ErbB-2 occur through a retrograde transportation [251-253]. Wang *et al*. suggested two alternative routes for the nuclear transport of RTKs [252];

1. The INTERNET pathway; **in**tegral **t**rafficking from the **ER** to the **n**uclear **e**nvelope **t**ransportation. This pathway involves ligand-induce internalization of the receptor which translocates to Golgi, followed by retrograde transportation to the endoplasmic reticulum (ER) via the translocon sec61β. The receptor transports to the inner nuclear membrane through movement along the ER/outer nuclear membrane via membrane-bound importin-β and through the NPC.

2. The INFS pathway; **i**ntegrative **n**uclear **F**GFR-1 **s**ignaling. The FGFR-1 has an atypical transmembrane domain containing short stretches of hydrophobic amino acids interrupted with hydrophilic amino acids. Upon stimulation it detaches from the plasma membrane and releases into the cytosol [254]. It translocates into the cell nucleus via soluble importin-β. However, the interaction to importin-β is unclear as FGFR-1 lacks a NLS sequence.

These two pathways were proposed after treating cells with digitonin, thereby washing away cytosolic proteins, and studying the cellular localization of EGFR/ErbB-2 and FGFR-1 [252]. The suggestion of membrane-bound EGFR and ErbB-2 translocation is further supported by studies showing that they are associated with the endosomal marker EEA1 (early endosomal antigen 1) in vicinity of the nuclear envelope or inside the nucleus [238, 255]. Additional reports have shown that nuclear EGFR transportation is associated with HSP70 and MUC-1, suggesting that those proteins could interact with the transmembrane domain of the EGFR [251, 256].

Further, one can speculate in a third pathway represented by ErbB-3 as its nuclear translocation is neither clathrin nor caveolin-dependent. Instead, nuclear ErbB-3 in prostate cancer cells is dependent on macropinocytosis; when treating the cells with inhibitors of macropinocytosis nuclear ErbB-3 transportation is inhibited [257].

Nuclear localization signal has been found in all of the receptors belonging to the ErbB family [247, 249, 255, 258] but not in e.g. the FGFR-1 and the IGF-1R. It is speculated that they enter the nucleus with proteins that do have a NLS. There have also been studies pointing to the existence of multi-partite NLS which would not be detected when running prediction programs based on only the protein sequence [259].

1.8.2 Biological functions

The biological functions of nRTKs are slowly starting to unravel as more investigations are being conducted. The EGFR is one of the most well studied receptors and in 2001, Lin *et al.* demonstrated nEGFR's role as a co-activator in gene transcription [237]. Several following studies confirmed this finding and nEGFR affects transcription of *cyclin D1* [237], *iNOS* [260], *COX-2* [261], *Aurora-A* [262], *c-Myc* [263], *B-Myb* [264], *thymidylate synthase* [265] and *BCRP* [266]. Transcriptions of these genes have further demonstrated to take place via nEGFR-binding to various transcription factors, such as STAT3/5, RNA helicase A (RHA) and E2F1 (Figure 10) [267, 268].

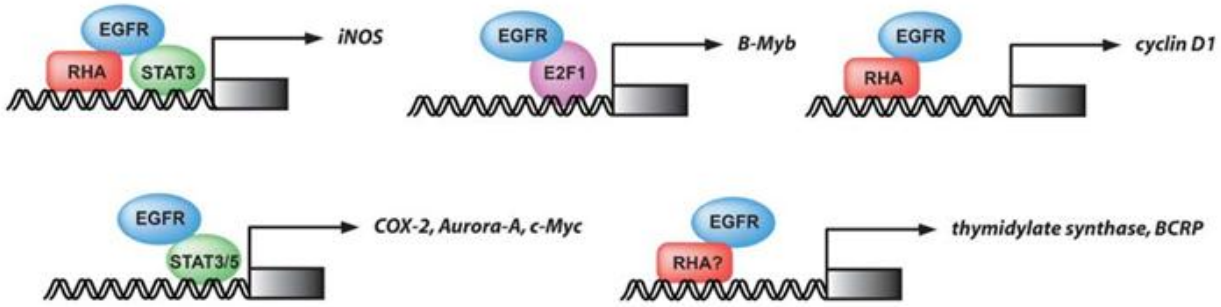

Figure 10. Schematic representation of nEGFR as a co-activator in gene transcription and its target genes [268]. Re-printed with permission from BioMed Central.

Further, nuclear EGFR is associated with DNA replication and repair through interactions with PCNA and DNA-PK. Proliferation cell nuclear antigen (PCNA) protein binds to DNA and plays a pivotal role in DNA replication and damage repair [269]. Nuclear EGFR phosphorylates PCNA at tyrosine 211, which increases PCNA's binding to chromatin and its stability and thereby increasing cell proliferation [270]. By using a specific PCNA phosphor-tyrosine 211 antibody a correlation between both nEGFR as well as poor overall survival is observed in primary breast cancer tumors [270]. The DNA-dependent protein kinase (DNA-PK) is a serine/threonine protein kinase and it is a crucial component of the DNA double-strand break (DSB) repair machinery. Dittmann *et al.* demonstrated that upon ionization radiation of bronchial carcinoma cells, EGFR rapidly translocates into the nucleus where it associates with DNA-PK followed by increased activity of DNA-PK. When blocking nEGFR by pretreatment of cells with cetuximab, the activity of DNA-PK as well as its ability to interact with DNA binding complexes decreases [271].

FGFR-1 is another receptor whose nuclear function has been studied relatively well. Several studies have presented nFGFR-1 as a mediator in neuronal differentiation, *in vivo*. During proliferation of brain neural stem/progenitor cells FGFR-1 is mainly located in the plasma membrane, but as the cells exit the cell cycle FGFR-1 accumulates in the nucleus. The receptor remains nuclear during differentiation and migration of the cells from the subventricular zone to

target brain regions [272-274]. This proposes a role of nFGFR-1 in neurogenesis and could potentially be a new target for neurodegenerative disorders.

The first study where nIGF-1R was identified demonstrated that the receptor associates with chromatin and that it could, as shown by reporter assays, increase transcription [86]. The following study presenting nIGF-1R was published just months later by Aleksic *et al.* and by immunoprecipitation and immunofluorescence they showed that nuclear IGF-1R associates with histone H3 and RNA polymerase II respectively [275]. Further, nIGF-1R binds to and induces *IGF-1R* gene promoter activity. Interestingly, the IR also binds to the *IGF-1R* gene promoter, but is suggested to inhibit IGF-1R transcriptional activity [181]. More recently, Zhang *et al.* demonstrated the importance of the IGF-1R SUMOylation in proliferation; the IGF-1R SUMO-1 mutant, unable to translocate into the nucleus, has a lower proliferation rate than the wild type receptor [276]. An interesting study published by Aslam *et al.* showed that rhabdomyosarcoma cells (derived from a transgenic mouse model) that are initially high in cell surface IGF-1R expression show greater nIGF-1R after just one cell cycle compared to cells with low cell surface IGF-1R. Further, it was demonstrated that high expression of nIGF-1R results in an increase tumorigenic phenotype to murine alveolar rhabdomyosarcoma cells [277].

My first project presented in this thesis aimed at further investigating the functions of nIGF-1R.

1.8.3 Clinical implications of nIGF-1R and nEGFR in cancer

The anti-IGF-1R therapy era started off with huge optimism, but as the results from many clinical trials were analyzed it has changed to being a great disappointment. However, in unselected patient groups there are subgroups that strongly benefit from anti-IGF-1R therapy and today researchers urge for finding clinical biomarkers to identify those patient groups, alternatively starting to use anti-IGF-1R in combination with other cancer drugs [105, 278]. There are studies suggesting that nIGF-1R might be a good biomarker for identifying those patients.

The first study investigating the clinical relevance of nIGF-1R demonstrates that nuclear IGF-1R is present in many different cancers including renal cell carcinoma (RCC), prostate cancer, adenocarcinomas and pancreatic cancers. The nIGF-1R expression in 195 clear cell RCCs were investigated and nIGF-1R was detected in 94 samples and high levels of nIGF-1R correlates with shorter survival [275].

Another study included 16 patients diagnosed with soft tissue sarcomas, Ewing sarcoma or osteosarcoma and treated with IGF-1R antibody. Tumor samples were analyzed by immunohistochemistry for IGF-1R expression and subcellular localization. In nine samples, exclusive nIGF-1R was observed, three samples showed nuclear and cytoplasmic IGF-1R

staining and the remaining four samples showed exclusive cytoplasmic IGF-1R localization. Patients with exclusive nuclear staining had a significant superior PFS (10.1 - months vs. 1.6 months) and OS (28.3 months vs. 6.6 months) as compared to the group with nuclear and cytoplasmic or cytoplasmic IGF-1R, suggesting that nIGF-1R might be a biomarker for sarcoma patients that will benefit from IGF-1R antibody treatment [279]. Another study included 88 patients with synovial sarcomas were all of them underwent surgery, 56% adjuvant radiotherapy and 65% adjuvant chemotherapy. Nuclear IGF-1R was identified by immunohistochemistry in 21 patients. Five patients had exclusive nIGF-1R staining and 16 patients had both cytoplasmic and nuclear IGF-1R staining. The analysis showed that nIGF-1R expression was negatively correlated with OS; the 5-year OS was 63% for patients with positive nIGF-1R expression and 73% in negative patients ($p = 0.05$) [280].

It has also been suggested that nIGF-1R might play a role in gefitinib resistance. Bodzin *et al*. demonstrated in the gefitinib-resistant cell line hepatocellular carcinoma Mahlavu cells, that there is a strong increase in both IGF-1R activation as well as nuclear translocation upon gefitinib treatment [281].

Several groups have investigated the correlation between nuclear EGFR and the clinical outcome in many different cancers. High levels of nEGFR are associated with poor OS and clinical aggressiveness in e.g., breast cancer, ovarian cancer, gallbladder cancer and NSCLC [282-286].

2. AIMS OF THESIS

The general aims of this thesis were to investigate the functional effect of nuclear IGF-1R and its mechanism for nuclear translocation. We also wanted to explore if other RTKs are SUMOylated and decided to focus on the epidermal growth factor receptor.

More specifically, the aims of the three projects presented in this thesis are:

I. To investigate the role of nuclear IGF-1R as a co-activator of LEF-1/TCF transcription in the Wnt/β-catenin signaling pathway.

II. To uncover the mechanism by which IGF-1R is translocated into the nucleus.

III. To elucidate EGFR SUMOylation and what the function of SUMO-1 modified EGFR is.

3. RESULTS AND DISCUSSION

3.1 PAPER I

Nuclear IGF-1R is a transcriptional co-activator of LEF-1/TCF

When our group first discovered nuclear IGF-1R (nIGF-1R) it was revealed that nIGF-1R binds to putative enhancer regions of genomic DNA and drives transcription as shown by gene reporter assays [86]. IGF-1 and Wnt signaling have both demonstrated to result in nuclear translocation of β-catenin, which in turn binds to transcription factors of the lymphoid enhancer factor/T cell factor/ (LEF/TCF) family [177]. Based on these studies we hypothesized that nuclear IGF-1R has a more direct impact on LEF-1 transcription.

We first demonstrated that IGF-1R binds to both β-catenin and LEF-1 in the cell nucleus in the following three cell lines; DFB (melanoma), H1299 (NSCLC) and HeLa (cervical). It has previously been described that nIGF-1R is dependent on IGF-1 stimulation and we found that this is also the case for the IGF-1R—LEF-1 association.

Stimulation with IGF-1 causes nuclear translocation of IRS-1, which is required for IGF-1-mediated nuclear translocation of β-catenin [287]. Next we addressed whether or not IGF-1R—LEF-1 association is dependent on IRS-1. The nuclear translocation of IGF-1R had previously been speculated to be dependent of IRS-1, seeing as IGF-1R lacks a conserved NLS, whilst IRS-1 has two conserved NLS sites [288]. To assess the importance of IRS-1 we used the mouse fibroblasts cell line, R-ΔIRS1, which expresses human IGF-1R with a mutation in the IRS-1 binding site (Y950F). The R-ΔIRS1 cells were subjected to subcellular fractionation and our results show that the cells express nuclear IGF-1R; demonstrating that nuclear IGF-1R is independent of IRS-1. In addition, the IGF-1R-ΔIRS1 mutant associates with LEF-1 as determined by immunoprecipitation experiments and based on those results we concluded that IGF1R-LEF1 binding is independent of IRS-1. Upon Wnt/IGF activation, β-catenin translocates into the nucleus and creates an active transcriptional complex with LEF1/TCF by displacing the transcriptional inhibitor Groucho and CBP by binding to the N-terminal of LEF-1 [160]. To assess if β-catenin is required for IGF-1R—LEF-1 association we transfected H1299 cells with mock, Myc-wt-LEF-1 and Myc-Δβ-cat-LEF-1, the latter being a LEF-1 mutant lacking the β-catenin binding site. Surprisingly, this mutant did not affect IGF-1R—LEF-1 association, neither was it affected by β-catenin knockdown nor overexpression.

Next we sought to investigate if IGF-1R can activate *cyclin D1* and *axin2*; two target genes of LEF-1. To do so, we used the IGF-1R triple sumo mutant (TSM), mutated in lysine residues 1025, 1100 and 1120. TSM impairs IGF-1R SUMOylation and the receptors ability to translocate into the nucleus, but it does not affect the canonical IGF signaling through PI3K and MAPK pathways [86]. SKUT-1 cells – lacking endogenous IGF-1R – were transfected with mock, IGF-

1R or TSM and the mutant does not associate with LEF-1. IGF-1R and LEF-1 associations are also solely nuclear as visualized by *in situ* PLA. By using promoter-luciferase *cyclin D1* and *axin2* reporter constructs in SKUT-1 cells, transfected with IGF-1R or TSM, we found that upon IGF-1R overexpression the activity of *cyclin D1* and *axin2* promoters increase by 17% and 22% respectively, compared to mock. However, in cells overexpressing TSM, the c*yclin D1* and *axin2* promoter activity decreases with 7% and 20%, respectively, compared to mock transfections. This data suggests that nIGF-1R increases LEF-1 downstream activity. We also observed an increase at the protein level of cyclin D1 and axin2 when transfecting H1299 cells with IGF-1R compared to mock and TSM transfections.

Finally, we showed that nIGF-1R binds to genomic *cyclin D1* promoter. This was demonstrated by chromatin IP (ChIP)-qPCR. Chromatin fragments from DFB and HeLa cell lysates were pulled down with IGF-1R antibody and analyzed by qPCR to detect genomic regions containing the TCF/LEF-1 binding element of the *cyclin D1* promoter. The binding of IGF-1R to LEF-1-binding site in the *cyclin D1* promoter is nearly six-fold higher in DFB and 12-fold higher in HeLa cells compared with a non-specific control region. Further, H1299 cells were transfected with empty vector, IGF-1R or TSM, followed by ChIP-qPCR. IGF-1R transfected cells have a 120-fold increase in LEF-1-binding site in the *cyclin D1* promoter compared to empty vector and TSM transfected cells. This suggests that nuclear IGF-1R binds to the LEF-1 binding site of the *cyclin D1* promoter.

Our data suggests that upon nuclear translocation of the IGF-1R, the receptor binds to the LEF-1 transcription factor in the nucleus, leading to elevated protein levels of cyclin D1 and axin2. This study proposes an additional function of the IGF-1R, besides its classical tyrosine kinase activity.

3.2 PAPER II

Nuclear translocation of IGF-1R via p150^{Glued} and an importin-β/RanBP2-dependent pathway in cancer cells

This study aimed at unravelling the transportation mechanism of the IGF-1R; how the receptor travels from the plasma membrane, across the nuclear envelop and further into the nucleus.

Previously, it was revealed that nuclear IGF-1R is dependent on clathrin-mediated endocytosis, rather than caveolin-raft endocytosis [275]. We hypothesised that the IGF-1R is transported as a membrane-bound entity via vesicle-mediated transportation. Firstly, we found that the EEA1 protein, important in clathrin-dependent internalization, is co-localized with the IGF-1R, both in the cytoplasm and inside the cell nucleus, suggesting that the receptor might exist in the nucleus in a vesicle formation derived from the endosomal compartment, or alternatively, that the endosomes have fused with the nuclear membrane.

Knowing that microtubules (MT) are considered to be the "highways" for long distance vesicular transportation, we explored the possibility that the IGF-1R is retrogradely transported inside the cell along microtubules. MT-structures were disrupted by the drug colchicine, followed by subcellular fractionation. Colchicine treated cells do not show nuclear accumulation of IGF-1R after IGF-1 stimulation. Next we investigated if MT-associated translocation is mediated through the dynactin complex, a multi-subunit complex which interacts with EEA1-positive vesicle [289]. Indeed, the largest subunit of dynactin, $p150^{Glued}$, associates with IGF-1R in *in situ* PLA as well as immunoprecipitations. When we disrupted dynactin by either $p150^{Glued}$ knockdown with siRNA or overexpressing dynamitin (also a member of dynactin and well documented to inhibit dynein/dynactin transportation if overexpressed in cells), there is a significant decrease in nIGF-1R by nearly 50% compared to control transfected cells. This data indicates that IGF-1R is transported to the nucleus via dynactin-mediated transfer along microtubule.

As mentioned in the introduction, for several RTKs it has already been established that their nuclear translocations are dependent on importin-β interaction. This is also the case for IGF-1R. First we presented that IGF-1R and importin-β associates and that it occurs after interacting with dynactin. This was based on the results showing that the IGF-1R—importin-β association diminishes after dynamitin overexpression. In line with previous reports we demonstrated a decrease in nIGF-1R after importin-β siRNA knockdown. This result was further confirmed by overexpression of the Ran mutant, RanQ69L, which inhibits the essential GTP hydrolysis in importin-β-mediated nuclear translocation.

SUMO-1 modification of the IGF-1R is a prerequisite for its nuclear translocation. Next we addressed the role of SUMOylation in more detail. We decided to focus on the nucleoporin RanBP2, one of the known SUMO E3 ligases that interacts with importin-β. A study from Giri *et al.* has demonstrated that RanBP2 is involved in the nuclear translocation of ErbB-2 [238]. Using *in situ* PLA we demonstrated that RanBP2 and IGF-1R co-localize around the nuclear brim. These results were confirmed by immunoprecipitation, where after RanBP2 pull-down, the sizes of the IGF-1R bands are equal to the size of SUMOylated IGF-1R. To explore if RanBP2 is a potential SUMO E3 ligase for IGF-1R, cells were transfected with the E3-domain of RanBP2. Indeed, the IGF-1R SUMOylation increases, but does not affect nuclear IGF-1R, which could be due to that the RanBP2E3 protein no longer localizes at the nuclear brim, but in the cytoplasm. RanBP2 knockdown by siRNA results in a strong reduction in nIGF-1R; 70% less nIGF-1R in knockdown cells compared to control.

Several studies have reported that SUMO modifications affect protein stability. To investigate the stability of SUMOylated IGF-1R we used the IGF-1R SUMO mutant, TSM. Wild-type IGF-1R and TSM were transfected in IGF-1R deficient SKUT-1 cells, followed by cycloheximide treatment to block newly synthesized protein. In fact, TSM is degraded faster than wild-type receptor. To further relate SUMO-1's role in IGF-1R stability with RanBP2 as the E3 ligase, cells

were treated with cycloheximide after knockdown of RanBP2. In RanBP2 knockdown cells the IGF-1R is degraded dramatically faster.

Knockdown of RanBP2 gives a greater effect on nIGF-1R, than knockdown of either dynactin-mediated transportation or importin-β. It could of course be due to better silencing efficiency, or it could be because RanBP2 has a dual role in IGF-1R translocation; it mediates transportation through the NPC, as well as stabilizes the receptor. It can also be speculated that IGF-1R SUMOylation might function as a sorting mechanism; SUMO-1 modified receptors will be targeted for nuclear import, whilst un-SUMOylated receptors will be targeted for degradation. Future studies should investigate if TSM is ubiquitylated to a higher degree than the wild-type receptor.

Further, we demonstrated that the RanBP2—IGF-1R association is dependent on importin-β. Knockdown of importin-β or overexpression of the importin-β-IA/YA, a mutant which previously has been shown to impede importin-β — RanBP2 association, results in a decrease in RanBP2—IGF-1R association. Finally, we showed that nIGF-1R pathway is IGF-1 dependent. After 30 minutes of IGF-1 stimulation there is a maximum association between IGF-1R — $p150^{Glued}$ and between IGF-1R— importin-β. Interestingly, no change was observed between IGF-1R—RanBP2 association; this interaction is stable even after serum starvation. Thus, this data suggests that IGF-1R bound to RanBP2 might act as an intra-cellular reserve.

In summary, the suggested pathway presented in this work is as follows; Plasma membrane-bound IGF-1R internalizes in EEA1-positive vesicles upon ligand stimulation. The vesicle-bound IGF-1R transports to the nuclear brim along microtubules via dynactin complex. Importin-β further transfers IGF-1R to RanBP2 which mediates transportation through the NPC (Figure 11).

It still needs to be elucidated how importin-β binds to the IGF-1R seeing as it lacks a conserved NLS. It could still be a direct interaction through a non-canonical NLS sequence, or the association occurs through a yet undefined protein. It is plausible that the proposed pathway is also utilizing the so-called INTERNET pathway, involving retrograde transportation to Golgi and the endoplasmic reticulum, via the translocon sec61β. The nIGF-1R pathway has many similarities to the one for EGFR and ErbB-2, e.g., they are interacting with EEA1 and importin-β. It has also been reported that the ER can itself slide along microtubules, and this was visualized by following the sec61β translocon [290]. These microtubules were also nocodazole-resistant due to acetylation, which is very interesting as our group have struggled to see a difference in nIGF-1R after nocodazole treatment (unpublished data). Further studies need to be carried out to connect the IGF-1R pathway with the ER-dependent pathway via sec61β.

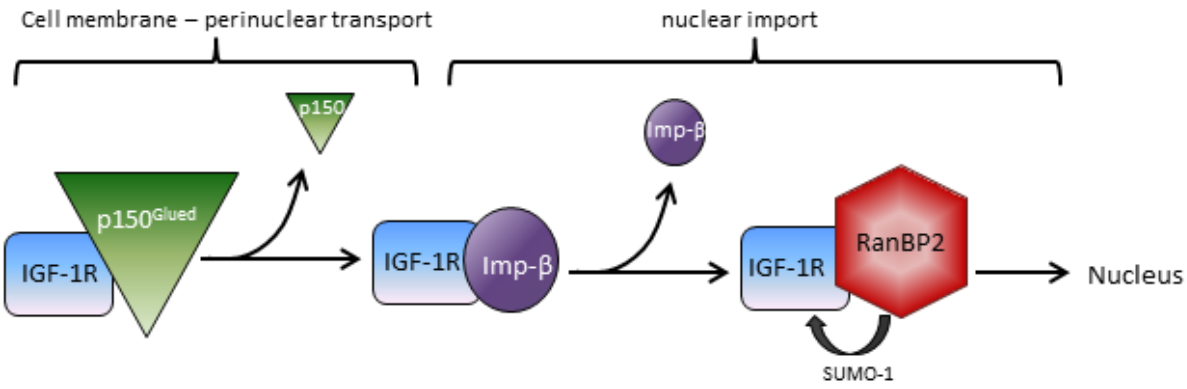

Figure 11. Schematic representation of nuclear translocation of IGF-1R.

3.3 PAPER III

Nuclearly localized epidermal growth factor receptor is SUMOylated

After the initial study showing that the IGF-1R is SUMOylated, it has been reported that the IR and the ErbB-4 are also SUMO modified. This project aimed at investigating EGFR SUMOylation and its potential function.

Here, as the first group, we demonstrated that the EGFR is indeed SUMO-1 modified in three tested cell lines; H1299 (NSCLC), HCT116 (colon cancer) and HeLa (cervical cancer). This interaction is mainly nuclear, in contrary to the IGF-1R SUMOylation which is perinuclear, indicating that it is likely that the function of EGFR SUMOylation is different compared to the one reported for IGF-1R.

The EGFR contains 66 lysine residues, all of which could be a potential target for SUMO-1. To be able to study the functional importance of EGFR SUMOylation we performed multiple mass spectrometry analyzes in order to identify which lysine residue/s is/are SUMOylated. We used the porcine aortic endothelia (PAE) cell line, which does not express endogenous EGFR, ErbB-2 and ErbB-3. PAE cells were transfected with EGFR and SUMO-1. SUMO-1 generates a 19 amino acid side chain when attached to the receptor. In the first analysis, we used MALDI-TOF peptide mass fingerprints and identified four peptides that are SUMO-1 modified, corresponding to lysine residues 37, 129, 399 and 949 of the EGFR. However, this method is restricted to only identifying peptides smaller than 5000 Da. In addition, we used liquid chromatography tandem mass spectrometry, and a T95R mutation on SUMO-1 was introduced, allowing us to get a specific glycine-glycine signature on the SUMOylated peptides. This method revealed one additional lysine residue; K328 as well as confirming lysine 37.

Five EGFR-constructs were created using site-directed mutagenesis; generating lysine to arginine mutants in the suggested sites above. These five single mutants do not block EGFR SUMOylation, which is to be expected, since the remaining four un-mutated lysine residues can still be targeted by SUMO-1. This result tells us that the EGFR is either mono-SUMOylated, but when only mutating one residue at a time, the receptor will be SUMOylated in a compensatory manner in any of the other four sites, or that the EGFR is modified by several SUMO-1 proteins at the same time. For future studies, all five identified residues need to be mutated.

Since, only one lysine came up as a hit between the two mass spectrometry methods we decided to focus on that one; i.e. lysine 37. EGFR-K37R mutant did not alter the EGFR downstream activation by EGF. Neither did EGFR-K37R influence the stability of the receptor nor its capability to undergo nuclear translocation. As the EGFR SUMOylation is only visualized in the nucleus in endogenous expressing cells, it is very likely that the SUMOylation occurs first after nuclear translocation. Thereby, it would not be dependent on RanBP2 as the SUMO E3 ligase as in the case of the IGF-1R. In fact, we cannot detect EGFR-RanBP2 association by immunoprecipitation, but with several of the PIAS E3 ligases (data not shown).

To address if EGFR-K37R has any functional outcome of nEGFR-mediated responses, we decided to investigate if the mutant affects *c-Myc* and *cyclin D1* expression; two genes whose transcription is induced by nEGFR. PAE cells were transiently transfected with mock, EGFR or EGFR-K37R. Both c-Myc and cyclin D1 protein expressions are significantly reduced in the EGFR-K37R mutant compared to wild-type receptor. Further, by performing luciferase-promoter assays we revealed that the *c-Myc* promoter activity, as well as the *cyclin D1* promoter activity, are significantly reduced in the mutant compared to wild-type EGFR.

Even though there are several studies reporting that SUMO-1 modification can increase transcription, it is more commonly shown to function as a repressive mechanism. It could be that the K37R mutants permit stronger SUMOylation by any of the remaining four suggested residues, thereby reducing transcriptional activity or that it is in fact SUMOylation of lysine 37 that induces transcription. Future studies should investigate if EGFR-K37R affects the binding to the transcription factors STAT3 and RNA helicase A, which are demonstrated to associate with nEGFR-dependent transcription of *c-Myc* and *cyclin D1* respectively. Nuclear EGFR is associated with DNA repair and it would be interesting to investigate its relation to EGFR SUMOylation.

3.4 GENERAL DISCUSSION AND CONCLUDING REMARKS

Initially, the IGF-1R targeted therapy started out very promising; showing solid anti-tumor effects *in vitro* as well as in animal models, but as the clinical trials took off and the result from the patient outcome returned, the enthusiasm was replaced with disappointment. Today, research groups are trying to identify biomarkers to predict patient groups that will respond to anti-IGF-1R therapy. To isolate new biomarkers, we have to go back to the lab bench and proceed with basic research to unravel the yet undefined mechanisms in IGF-1R cellular response. This thesis aims at just that. This group has previously identified nuclear IGF-1R and based on research carried out by several other groups, nIGF-1R has a significant role in cancer therapy; both as a prognostic factor, but it also has diagnostic value. Therefore it is important to understand the basic underlying functions and mechanisms of nuclear IGF-1R, which are presented in project I and II and the results can be summarized as follows:

- Nuclear IGF-1R associates with the LEF-1/TCF transcription factor and acts as co-activator and induces transcription of genes such as *Axin2* and *cyclin D1*, two genes shown to be important in cancer biology. In addition, IGF-1R—LEF-1 interaction is independent of β-catenin and IRS-1.

- Upon IGF-1 stimulation IGF-1R undergoes nuclear translocation. This transportation is dependent on transport along microtubules through binding to the dynactin complex. Its nuclear import across NPC is mediated through importin-β/RanBP2. IGF-1R SUMOylation is also important for the stability of the receptor.

With regards to EGFR SUMOylation, this is just an initial study and more thorough studies need to be carried out in the quest of fully understanding the mechanism and cellular response of SUMO-1 modified EGFR. Although this study does not provide a complete picture of the EGFR SUMOylation it tells us that:

- EGFR SUMOylation is mainly nuclear and five targeted lysine residues are proposed; K37, K129, K328, K399 and K949. By blocking SUMOylation of one site, K37, it affects EGFR-dependent transcription of *c-Myc* and *cyclin D1*.

These three projects together imply that SUMOylation of RTKs might be a very important modification that influences their recently identified nuclear functions. Future direction is to identify the clinical importance of IGF-1R and EGFR SUMOylation. Could RTK SUMOylation be a new biomarker? Could SUMOylation be the missing link in finding patients responsive to anti-IGF-1R therapy? Could identified SUMO sites be potential drug targets? We will only have the answers by continuing to investigate the role of SUMOylation in RTKs.

4. ACKNOWLEDGEMENTS

Six years ago I decided I wanted a challenge and joined the Karolinska Institute's PhD program. It has been a rollercoaster, with high and lows, from weeks when nothing worked in the lab to arousing joy when your hypothesis is verified by a tiny little protein band. I could not have done it without the help and support of so many wonderful people in my life. I especially want to thank the following people:

My supervisor, Professor **Olle Larsson**, for taking a chance and hiring me on the spot, without really knowing my lab skills! I still remember the very first email you sent me. I was in a hostel in Florence, Italy, and you told me about the exciting new finding of nuclear IGF-1R and asked me if I would be interested in joining your group. You have given me the opportunity to develop into an independent researcher and I am truly grateful for that!

Bita, my co-supervisor, for introducing me to the field when I joined the lab and discussing and explaining the little details I could not get my head around. It was an adventure going to Canada and spend three months with you! In particular, I want to thank you for the great support during the periods of absent experimental results and you told me it would eventually work out – and it did!

My co-supervisor, **Yingbo**, you have been great with handling technical details in my projects and always taking the time to explain the science behind it. You also impress me with your skill in mental arithmetic!

I want to thank my labmates; **Dudi**, for a fantastic collaboration during these years! I would still have another couple of years to go if it wasn't for you. I have never met anybody as hardworking as you! **Margherita**, come back, I miss you every day!! You were a refreshing little whirlwind in our lab and honestly, you are one of the craziest (in the crazy good category) people I have ever met! Puss min lilla potatiskompis! ☺ **Ahmed,** is it five years we've been in the same lab? Hard to tell, when you are working your wacky night hours! I admire your dedication and positive energy; you will get far with those qualities! **Maria**, it was always a pleasure having you around during your research periods. Keeping my fingers crossed for some amazing staining results to come! **Sara L**, I really appreciated your company in the lab.

Professor **Eiman**, I am glad you came to our lab and sharing your life experiences with me! I wished you would have stayed at Karolinska!

Also, thanks to all the master **students in the lab** that have come and gone during the years.

To my collaboration partners in my projects; **Sara S**, for your hard work with the live imaging. You are one of the sweetest girls I have worked with. **Roger**, for your valuable input in our article. **Dorothea**, for your expertise in mass spectrometry!

To the members of the **Farnebo group**. **Sofia,** for being a sincere friend. **Hanif,** I am so happy I didn't scare you off from research and that you came back as the next door lab neighbor! **Elisabeth,** it's empty without you around. **Christos,** for being a funny and honest guy. **Soniya** for always taking the time to ask how things are going. Also, thanks to the latest additions to the group; **Stefanie**, **Chiara** (aka pretty face) and **Dominika.**

The **Klas Wiman group**, for letting me join your social activities and being great lunch companions. **Cinzia** for handing me the left-over cakes, **Lidi**, **Qiang** and **Mei** for including me in your hot pot nights, **Emarn**, for getting the dance floor started at department parties, **Susanne**, for helping me out with tricky ordering procedures, **Fredrik**, your enthusiasm is really catching, **Sofi** and **Julie** for being excellent office buddies and **Sophia**, for giving me a great wasabi story to tell my friends!

All other, past and present, members of CCK that crossed my way during these years.

Till mina underbaraste Umetjejer, **Helena, Hanna, Therese, Sara, Åsa, Linn, Caroline** och **Frida**. Våra tjejhelger, som numera bytts ut från partyhelger, till myshelger med småbarn, ger mig alltid en sådan energikick! Jag är otroligt tacksam att vi får till våra träffa någon gång per år! Extra tack till Helena, du finns *alltid* där för mig, och är den bästa vän man kan tänka sig!

Mina fina Göteborgs-tjejer, **Ulrika** och **Anna**. Ullis, tack för att du alltid hörde av dig och såg till att det var dags för en dejt när jag isolerade mig i labbet och för att du introducerade mig till Anna!! Ser så sjukt mycket framemot vår London weekend!

Beatrice, vi har helt klart spenderat mer tid ifrån varandra än med varandra sen vi gick skola i Arvidsjaur, men ändå känns det lika enkelt, avslappnat och självklart när vi ses. Du är en genuin vän och man blir alltid glad av få vara med dig!

To my Australian family, **Kathryn** and **Colin**, for constantly showing your support and helping us out in any way you can. **Alison, Damian, Jessica, Isabelle, Michelle, Matt** and **Hendrix**, for always giving us a great time in Sydney and I wished we lived a lot closer to each other!

Till mina syskon, **Jonas,** tack för att du underhåller mig med din smarta humor (som jag inte alltid hänger med i) och djupa diskussioner. **Jeanette**, för våra vinkvällar när jag kommer på besök. **Thony,** för att du ställer upp med skjuts/hämtning till tåget. Och givetvis tack till **Fredrik, Max, Liam, Erika** och **Tilde**!

Till mina föräldrar, **Doris** och **Stefan**, för att ni alltid ställer upp och jag skulle inte vara den jag är idag om det inte vore för er! **Pappa**, för våra naturvetenskapliga diskussioner (vann jag nu? ;) och **mamma** för att du säger ifrån när det är dags att släppa jobbet och göra något annat emellanåt ☺

Finally, I've reached the two most important people in my life. **Justin**, I can't even begin to thank you enough! Every single day, you are there for me and not once did you complain that our weekend plans often included a stop at Karolinska! To **Alex**, your cheeky smile can make any day the best day of the year! I love you, jag älskar er, more than you can ever imagine!

This thesis was supported by the Swedish Cancer Foundation, the Swedish Research Council, the Cancer Society in Stockholm, the Swedish Children Cancer Society, the King Gustaf V's research foundation, the Stockholm County Council and the Karolinska Institute.

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