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THE MANY FACES OF IGF-1R FROM CELL SURFACE TO THE NUCLEUS

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ABSTRACT

The IGF-1R is due to its implication in cancer an intensively studied subject of research. It is well known today that intracellular signals mediated by the IGF-1R play pivotal roles in tumorigenesis and cancer progression. However, the focus has been lying mainly on the classic canonical biochemical cascades originating from the cell surface receptor resulting in proliferation and sustained cell survival mediated by receptor activation and phosphorylation. The impact of other modifications, like ubiquitination, has just recently begun to be appreciated. In addition, the detection of nuclear IGF-1R in cancer cells implicates a novel role for the receptor in tumor biology.

In paper I, we focus on IGF-1R ubiquitination and analyze its role in receptor signaling and degradation. By using wild type and different mutated IGF-1R constructs, we identified functional sites and domains necessary for receptor ubiquitination. We show that ubiquitination requires receptor tyrosine kinase activity and that the C-terminal domain of IGF-1R is necessary for ubiquitination and ERK phosphorylation as well as for proteasomal degradation of the receptor. The second paper identifies c-Cbl as novel ubiquitin ligase for IGF-1R. We present the findings that c-Cbl mediates Lys48 polyubiquitination and internalization through lipid raft dependent endocytosis upon high dose IGF-1 stimulation. In paper III, we utilize a mutant form of IGF-1R which is autophosphorylation-deficient and demonstrate that FAK phosphorylates the receptor in an a-loop independent manner. Furthermore, FAK stabilizes IGF-1R protein levels and enables downstream signaling in cells expressing the mutant form of IGF-1R through MAPK/Erk and PI3K/Akt pathway. In paper IV we aimed to reveal the impact of nuclear IGF-1R on gene transcription. We show that, apart from its classical tyrosine kinase activity, IGF-1R binds to and activates the ß-catenin/LEF-1 transcription complex in the nucleus and increases protein levels of cyclin D1 and c-Myc. Taken together, our studies provide new aspects of IGF-1R modification and identified novel interaction partners. We also present an additional molecular mechanism by which IGF-1R may promote uncontrolled cell proliferation.

LIST OF PUBLICATIONS

I. Sehat B, **Andersson S**, Vasilcanu R, Girnita L, and Larsson O. Role of ubiquitination in IGF-1 receptor signaling and degradation. *PLoS ONE*, 2(4):e340, 2007.

II. Sehat B, **Andersson S**, Girnita L, and Larsson O. Identification of c-Cbl as a new ligase for IGF-1R with distinct roles to Mdm2 in receptor ubiquitination and endocytosis. *Cancer Research*, 68(14):5669-77, 2008.

III. **Andersson S**, D'Arcy P, Larsson O, and Sehat B. Focal Adhesion Kinase (FAK) activates and stabilizes Insulin-like Growth Factor-1 Receptor (IGF-1R). *Submitted 2009.*

IV. **Andersson S**, Warsito D, Larsson O, and Sehat B. IGF-1R interacts with components of the Wnt signaling pathway and activates gene transcription. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

A-loop	activation loop
AKT	protein kinase B
ATP	adenosine triphosphate
Cbl	casitas B-lineage lymphoma
ChIP	chromatin immunoprecipitation
DMEM	Dulbecco's modified essential medium
DNA	deoxyribonucleic acid
DUB	deubiquitination enzyme
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
ECM	extracellular matrix
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GF	growth factor
Grb	growth factor receptor-bound protein
GST	glutathione S-transferase
HECT	homology to E6 associated protein C-terminus
HEK	human embryonic kidney cells
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IR	insulin receptor
IRS	insulin receptor substrate
kDa	kilo Dalton
LEF	lymphoid enhancer factor

LyI	lysosome inhibitor
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
MEF	mouse embryonic fibroblast
Myc	myelocytomatosis
Nedd4	neural precursor cells-expressed developmentally down-regulated 4
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK	3-phosphoinositide-dependent protein kinase
PI	proteasome inhibitor
PI3K	phosphatidylinositol-3'-kinase
PIP3	phosphatidylinositol 3,4,5-triphosphate
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homolog deleted on chrom. 10
RING	really interesting new gene
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	src homology
Shc	src homology and collagen
siRNA	small interfering RNA
Src	protein encoded by Src proto-oncogene
SUMO	small ubiquitin-related modifier
TCF	T-cell factor
TF	transcription factor
TGF	transforming growth factor
TNF	tumor necrosis factor
Ub	ubiquitin
UBD	ubiquitin binding domain
UBP	ubiquitin binding protein
VEGF	vascular endothelial growth factor
wt	wild type

INTRODUCTION

Cancer

Cancer is a genetic disease, caused by a variety of mutations in germline or somatic cells of the human body. Before a tumor arises, several genetic and epigenetic alterations must be accumulated, abrogating the normal regulatory mechanisms for cell proliferation and survival which normally prevent neoplastic cell growth.

In the last decades many of the genes that play a central role in tumor development have been identified. These genes can be divided in proto-oncogenes and tumor suppressor genes. While the aberrant activation of oncogenes leads in most of all cases to increased signaling towards cell growth and anti-apoptosis; the latter are, when inactivated, unable to act as molecular breaks of uncontrolled cell division. The accumulation of additional mutations eventually leads to the progressive development of cancer. While the number of proto-oncogenes and tumor suppressor genes is vast, the physiological changes they cause can be rationed into six essential alterations that are necessary for cancer cell formation. They are known as the hallmarks of cancer as suggested by Hanahan and Weinberg in 2000. These physiologic changes of a cell are 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) evading apoptosis, 4) limitless replicative potential, 5) sustained angiogenesis and, 6) tissue invasion and metastasis (Hanahan and Weinberg 2000).

The main focus of this thesis lies on the first mentioned attribute of cancer cells, their self-sufficiency in growth signals. This is generally achieved by the activation of cell surface receptors transducing extracellular stimuli from the cell surface into the nucleus where they activate genes that cause cells to grow and divide. Today, there are numerous receptors known to be involved in cell signal transduction and their ability to interconnect with each other results in the high complexity of the system. Three major players involved in cancer cell signaling are of further importance for this thesis: receptor tyrosine kinases, integrin receptor signaling and the Wnt signaling pathway.

Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) belong to the class of transmembrane receptors with intrinsic protein-tyrosine kinase activity. RTKs transmit regulatory signals involved in cell proliferation and survival, migration and differentiation as well as in modulation of cell metabolism (Hunter 1998). Mutated or structurally altered RTKs play an important role in cellular transformation. These cell-surface receptors consist of the extracellular ligand binding domain, the transmembrane domain, the intracellular kinase domain and an intracellular regulatory domain. The twenty human RTK-subfamilies known today display structural varieties in their extracellular domain, like cysteine rich domains, leucine rich domains or immunoglobulin rich domains. The members of a subfamily are similar in structure but differ in tissue and ligand specificity. Fig.1 shows an overview of the human receptor tyrosine kinases.

RTK signaling requires ligand-induced receptor dimerization which results in the trans-phosphorylation of tyrosine residues in the dimeric receptor subunits, a process called auto-phosphorylation (Schlessinger 1988; Lemmon and Schlessinger 1994; Jiang, den Hertog et al. 1999). The phosphorylated residues function as docking sites for numerous adapter proteins which in turn can activate a number of signaling cascades including the MAPK- (mitogen-activated kinase), PI3K- (phosphatidylinositol-3 kinase), JAK- (Janus kinase), PLC-γ-(Phospholipase C- γ) and JNK- (Jun N-terminal kinase) signaling pathway. While RTK signaling covers a broad spectrum of biological responses, they play a major role in cancer as transmitters of mitogenic signals which mediate the subsequent transcription of target genes involved in cell growth and division. The aberrant activation of RTKs can occur through different mechanisms, all of which cause dysregulated signaling (Blume-Jensen and Hunter 2001). Overexpression and structural alterations of RTKs are associated with many human cancers. A well known example is the amplification of ErbB2 in breast carcinomas (Slamon and Pegram 2001). Point mutations or deletions, mainly in the kinase domain may also lead to increased kinase activity of RTKs (for example c-kit and PDGFRα in gastrointestinal stromal tumors (Penzel, Aulmann et al. 2005; Lasota, Stachura et al. 2006). Moreover, chromosomal translocations can generate fusion tyrosine kinases that are constitutively dimerized and activated like the tel-PDGFß receptor in chronic myelomonocytic leukemia (CMML) (Golub, Barker et al. 1994).

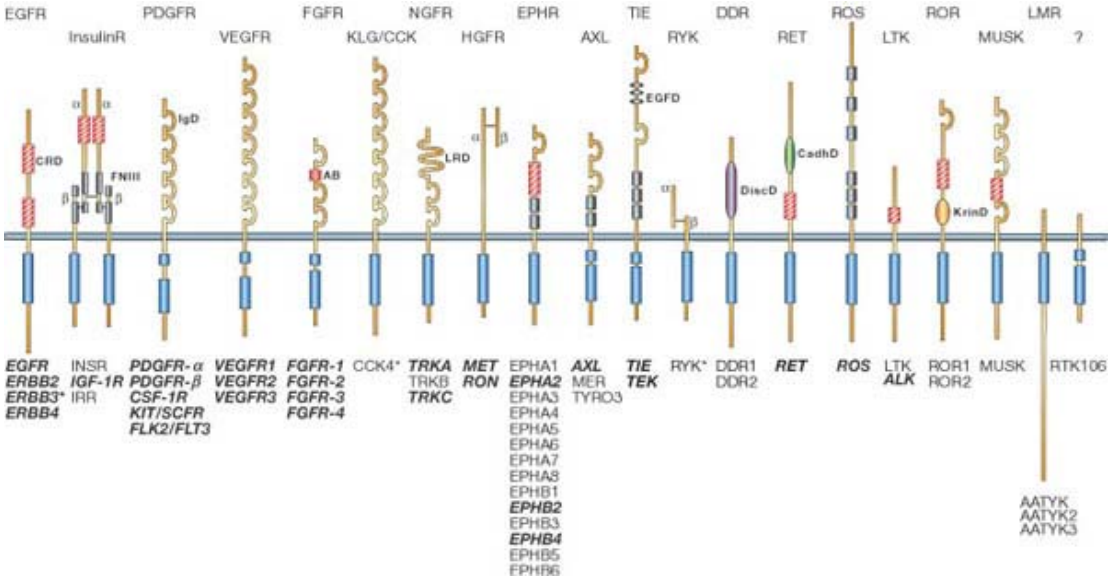

Figure 1: Human receptor protein tyrosine kinases

The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Abbreviations of the prototypic receptors: EGFR, epidermal growth factor receptor; InsR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR; vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; KLG/CCK, colon carcinoma kinase; NGFR, nerve growth factor receptor; HGFR, hepatocyte growth factor receptor, EphR, ephrin receptor; Axl, a Tyro3 PTK; TIE, tyrosine kinase receptor in endothelial cells; RYK, receptor related to tyrosine kinases; DDR, discoidin domain receptor; Ret, rearranged during transfection; ROS, RPTK expressed in some epithelial cell types; LTK, leukocyte tyrosine kinase; ROR, receptor orphan; MuSK, muscle-specific kinase; LMR, Lemur. Other abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols α and β denote distinct RPTK subunits. RPTK members in bold and italic type are implicated in human malignancies (see Table 1). An asterisk indicates that the member is devoid of intrinsic kinase activity. **(Blume-Jensen and Hunter; NATURE 2001, VOL 411)**

Integrin receptors and FAK

Integrin receptors are a family of at least 25 different cell surface receptors that mediate cell adhesion and regulate gene expression, cell growth, differentiation and survival (Schwartz, Schaller et al. 1995). The receptors are heterodimers of integrins, anchor-like proteins consisting of one of 16 different α- and one of 8 different ß-transmembrane subunits. The ectodomains of these receptors bind to extracellular matrix (ECM) components like collagen, laminin and fibronectin which serve as ligands for the receptors. While normal cells require attachment to ECM proteins in order to survive and proliferate, transformed cells acquired the ability to grow in anchorage-independent fashion. Integrins are regarded as signaling receptors since they share many of their signaling properties with growth factor receptors. Multiple interactions between growth factor signaling and integrin activation form a cooperative signaling network and integrin activation has been shown to exhibit synergistic effects on growth factor activity (Eliceiri, Klemke et al. 1998; DeMali, Balciunaite et al. 1999; Renshaw, Price et al. 1999; Assoian and Schwartz 2001; Juliano, Aplin et al. 2001). Unlike growth factors, their ligands are immobilized and binding of integrin receptors to their ligands provides a physical connection to the actin cytoskeleton through their cytoplasmic domains. Cell-adhesion through integrins is thought to contribute to tumorigenesis through trans-activation of growth factor receptors like platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), vascular-endothelial growth factor receptor (VEGFR), hepatocyte growth factor receptor (HGFR) and epidermal growth factor receptor (EGFR) (Schneller, Vuori et al. 1997; Giancotti and Ruoslahti 1999; Soldi, Mitola et al. 1999). Defective regulation of trans-activation, for example through the loss-of-function of gene products responsible for its limitation might be a possible mechanism for tumor formation (Schwartz and Ginsberg 2002). Moreover, several studies reported a cooperative interaction between integrin and IGF-1R mediated signaling in cancer (Brooks, Klemke et al. 1997; Zheng and Clemmons 1998).

The focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase expressed in all tissues and cell types which integrates signals from integrin- and growth factor activation. FAK is located at the site of integrin aggregation – the so-called focal adhesions– where it coordinates the adhesion dynamics and cell migration with survival signaling.

Integrin binding to ECM and clustering of integrins induce auto-phosphorylation (Tyr397) of FAK, which creates a high-affinity binding site for several SH2 containing proteins like Src and PI3K. Src phosphorylates FAK on Y576 and Y577 which makes the kinase fully active and additional phosphorylations create docking sites for e.g. Grb2 which activates the MAPK pathway (Schlaepfer and Hunter 1996). FAK activation provides a complex mechanism of action, transmitting adhesion-dependent and growth factor-dependent signals into the cells through a web of downstream signaling connections. Enhanced FAK signaling has been shown to lead to increased cell survival, proliferation and migration and its overexpression and activity are correlated with the invasive potential of tumors and poor patient prognosis (Owens, Xu et al. 1995; Cance, Harris et al. 2000; Recher, Ysebaert et al. 2004; Schlaepfer and Mitra 2004). The interaction of FAK with several RTKs suggests a mutual role for these kinases in tumorigenesis. Sieg et al. have shown that FAK associates with activated PDGFR and EGFR to promote cell migration (Sieg, Hauck et al. 2000) while recent studies provided evidence for the interaction of FAK and IGF-1R and demonstrated that their simultaneous inhibition results in synergistic anti-cancer effects (Liu, LaFortune et al. 2007; Liu, Bloom et al. 2008; Watanabe, Takaoka et al. 2008).

Wnt signaling and ß-catenin

The Wnt signaling pathway is highly conserved among species. Its ligands, the Wnt factors were identified through the discovery of the *Drosphila* mutant ***Wi****ngless*, a segment polarity gene important during larval development, and its mouse homolog ***int****-1*, a proto-oncogene activated by integration of the mouse mammary tumor virus in breast tumors (Nusslein-Volhard and Wieschaus 1980; Nusse and Varmus 1982). Wnt signaling is important for embryonic development and homeostatic self-renewal in a number of adult tissues, like the epithelium of small intestines, hair follicles or in the hematopoietic system (Cadigan and Nusse 1997; Reya, Duncan et al. 2003; Lowry, Blanpain et al. 2005; Reya and Clevers 2005) while somatic mutations in the pathway are associated with cancer like the hereditary cancer syndrome *familiar adenomatous polyposis coli* (FAP) (Rubinfeld, Souza et al. 1993; Su, Vogelstein et al. 1993). There are about 12 Wnt subfamilies with a total of 20 secreted Wnt proteins found in mammalians; the proteins are hydrophobic and poorly soluble due to glycosilation and palmityolation and are usually localized close to cell membranes.

Wnt signaling is initiated by binding of a Wnt ligand to a Frizzled receptor which belongs to a family of 10 receptors in humans and mice with seven transmembrane regions, sometimes called serpentine receptors (Bhanot, Brink et al. 1996). There are three different pathways that can be activated by Wnt stimulation, amongst them the canonical pathway which involves signaling trough ß-catenin is the most studied one. When Wnt signaling is inactive, the cytoplasmic enzyme glycogen synthase kinase-3ß (GSK3ß) is active and phosphorylates ß-catenin, targeting it for destruction via the ubiquitin- proteasome system (Aberle, Bauer et al. 1997).

ß-catenin is a cytoplasmic protein whose stability is regulated by the destruction complex consisting of the tumor suppressor proteins Axin, APC (ademomatous polyposis coli) and the kinase GSK3ß (Behrens, Jerchow et al. 1998; Itoh, Krupnik et al. 1998). Upon ligand binding, the Frizzled receptors interact with and control the phosphorylation of Dishevelled (Dsh), a cytoplasmic protein that acts upstream of GSK3ß and leads to phosphorylation of LRP (low-density lipoprotein receptor related protein). This creates a docking site for the scaffolding protein Axin which otherwise is bound to the destruction complex. The recruitment of Axin to the cell membrane results in the release of ß-catenin from the destruction complex. Free ß-catenin translocates to the nucleus where it displaces the transcriptional repressor Groucho from LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors and thereby activates the transcription of genes that drive cell proliferation. Important target genes of the Wnt pathway involved in cancer include c-Myc and cyclin D1 (Behrens, von Kries et al. 1996; Molenaar, van de Wetering et al. 1996; van de Wetering, Cavallo et al. 1997; He, Sparks et al. 1998; Tetsu and McCormick 1999). Apart from regulating gene transcription through the canonical Wnt pathway, ß-catenin is found as a component of cell-to-cell adhesions where it acts as binding partner for E-cadherin and α-catenin (Bienz 2005). Because ß-catenin bound to cadherin is highly stable, it is thought to be unavailable for canonical Wnt signaling. However, during epithelial-mesenchymal transition (EMT) of cells, ß-catenin becomes available for signaling due to the loss of E-cadherin. While EMT is a normal event during morphogenesis and wound healing, it is also associated to cancer progression by diminishing epithelial cell adhesion and facilitating cell invasiveness (Thiery 2002; Kang and Massague 2004).

The insulin-like growth factor system

The IGF system consists of the ligands IGF-1, IGF-2 and insulin, the cell surface receptors insulin-like growth factor 1 receptor (IGF-1R), insulin-like growth factor 2 receptor (IGF-2R) and the insulin-receptor (IR) together with the family of IGF-binding proteins (IGFBPs) and IGF-binding protein proteases (Baserga, Resnicoff et al. 1997; Pollak 2004). While the importance of insulin and IR in regulation of carbohydrate metabolism and growth is well established, their role in neoplastic tissue formation is an area of extensive investigation. Nevertheless, I will from now on focus solely on the remaining members of the IGF system.

Insulin-like growth factors

The polypeptide ligands IGF-1 and IGF-2 are potent growth factors which play a central role in growth and development of the organism as well as in tissue regulation and wound healing (Heald, Kaushal et al. 2006). IGF-1 and -2 are produced by the liver and delivered to responsive cells and tissues in an endocrine manner through circulation. IGF-1 is a potent anabolic and mitogenic hormone and its overexpression is linked to an increased risk for cancer (Hankinson, Willett et al. 1998; Ma, Pollak et al. 1999; Yu and Berkel 1999). It consists of 70 amino acids, composing the A and B domain which are connected by disulfide bonds, a linking c-peptide and a carboxy-terminal D-peptide. A carboxy-terminal E-domain is cleaved in the Golgi apparatus before its secretion as hormone (Daughaday and Rotwein 1989).

IGF-1 exerts a range of mitogenic and anti-apoptotic effects such as stimulation of protein-, glycogen- and DNA synthesis and cell cycle progression. It is produced mainly by the liver as a result of growth hormone (GH) stimulation, but to some degree even in bone, lung and muscle tissue. IGF-1 is frequently expressed by cancer cells and exerts endocrine, paracrine and autocrine effects. The levels of IGF-1 in humans rise from 20ng/ml during prenatal growth to 100-200ng/ml in adults and decline later in life (Cohen 2006). IGF-2, whose 67 amino acids share 62% sequence identity with IGF-1, is primarily important during fetal growth but also essential for development and function of organs such as the brain, liver and kidney. The expression levels of IGF-2 in humans are 2 to 6-fold higher than those of IGF-1.

While IGF-2 is involved in the progression of a variety of tumors, the expression of IGF-2 alone is not sufficient for malignant transformation (Yakar, Pennisi et al. 2005).

Insulin-like growth factor binding proteins (IGFBP)

The majority of IGF ligands does not circulate freely but is transported in the plasma bound to one of the six high affinity IGFBPs. IGFBPs bind IGFs with higher affinity than IGFRs and modulate the biological activity of circulating IGF ligands by protecting them from degradation, controlling their availability for receptor binding and influencing their direct interaction with the receptors (Jones and Clemmons 1995; Firth and Baxter 2002; Mohan and Baylink 2002). Approximately 80% of IGFs in serum are bound in a trimeric complex with IGFBP 3 and the abundant acid labile subunit (ALS) (Clemmons 1998). IGFBPs 1-4 have similar affinities for IGF-1 and IGF-2, while IGFBPs 5 and 6 bind IGF-2 with 10- and 100-fold greater affinity than IGF-1. IGFBPs are in turn controlled by various proteases, providing an additional level of regulation for IGF ligands (Rajah, Katz et al. 1995; Collett-Solberg and Cohen 1996).

Insulin-like growth factor receptors

Both IGF-1 and IGF-2 interact with the IGF-1R, a ubiquitously expressed trans-membrane tyrosine kinase involved in regulation of cell survival, proliferation, differentiation and motility. Since IGF-1R is the main subject of this thesis, it will be discussed in greater detail below. The IGF-2R, also known as mannose-6-phosphate (M6P) receptor differs structurally and functionally from the IGF-1R. The monomeric receptor binds IGF-2 with high affinity, regulating its availability through internalization and degradation. Although IGF-2R lacks intrinsic signaling capacity, it plays a major role in endocytosis and intracellular trafficking of M6P containing proteins.

IGF-1R

The IGF-1R is synthesized as a single polypeptide, glycosylated and proteolytically cleaved to yield a disulfide-linked tetrameric receptor composed of two identical α-subunits and two identical β-subunits which are arranged in the configuration α-β-β-α. The extracellular α-subunits (135kDa) contain the cystein-rich ligand-binding site and the β-subunits (90kDa) the transmembrane region, the intracellular tyrosine kinase (TK) domain and the C-terminal domain (LeRoith, Werner et al. 1995; Adams, Epa et al. 2000).

IGF-1R activation and signaling

Unlike other RTKs, where ligand binding initiates their dimerization, the IGF-1R is present as a tetramer at all times. Upon ligand binding to the α-subunits, the three tyrosine residues Tyr1135, Tyr1131 and Tyr 1136 of the activation-loop within the TK domain of the ß-subunit are trans-autophosphorylated (Kato, Faria et al. 1993; Favelyukis, Till et al. 2001), leading to conformational changes that allow substrate and ATP access. Increased intrinsic TK activity results in the phosphorylation of further tyrosine residues which serve as docking sites for various proteins mediating the signaling cascades induced by IGF-1 stimulation. Further important residues for the receptor function are Tyr950 which is the main binding site for the downstream signaling molecules IRS 1-4 and Shc, and the ATP binding site Lys1003. Both are critical for the proliferative and transforming capacities of IGF-1R (Gronborg, Wulff et al. 1993; Li, Ferber et al. 1994; Miura, Li et al. 1995; Dews, Prisco et al. 2000). Several residues within the C-terminal domain have been found to be required for the anti-apoptotic properties of IGF-1R (Tyr1250, Tyr1251, His1293 and Lys1294). Two serine residues (Ser1280 and Ser1283) compose the binding site for the 14-3-3 proteins which play a role in inhibiting apoptosis (Kato, Faria et al. 1993; Kato, Faria et al. 1994; Blakesley, Kalebic et al. 1996; Hongo, D'Ambrosio et al. 1996; O'Connor, Kauffmann-Zeh et al. 1997). For a schematic overview of IGF-1R see Figure 2.

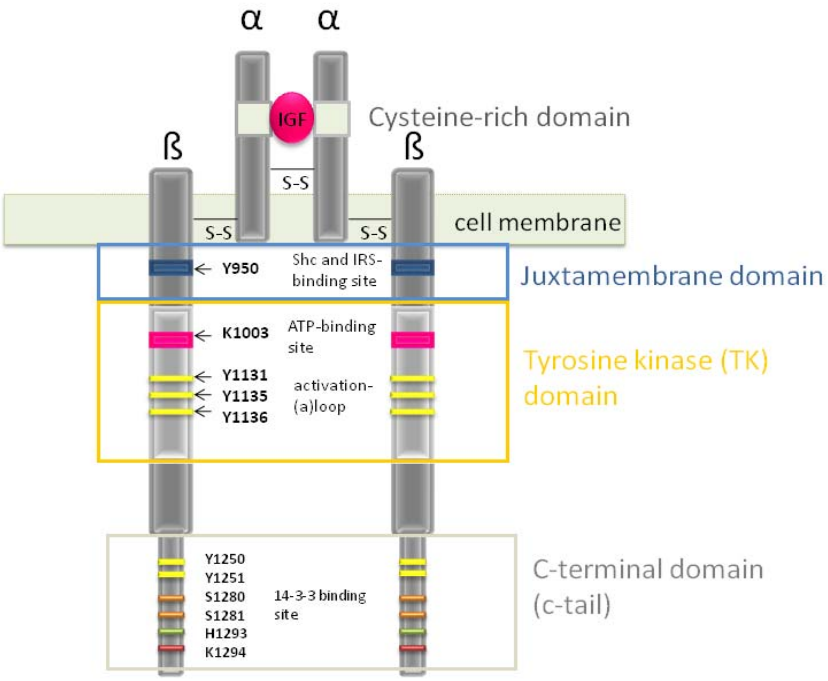

Figure 2: Schematic overview of IGF-1R structure including functionally important domains and residues. Y=tyrosine, K = lysine, S=serine, H=histidine, S-S =disulfide bonds

PI3K pathway

The activation of the phosphatidylinositol-3 kinase (PI3K) pathway requires the binding of insulin receptor substrate (IRS) proteins via their SH2 domains to Tyr 950 of the activated receptor. Upon binding, these proteins become tyrosine phosphorylated by the receptor and capable of binding various adapter proteins like Grb2, Nck, Fyn, Syp and the regulatory subunit (p85) of phosphoinositide 3'-kinase which binds to the catalytic subunit of PI3K. Activated PI3K generates the phospholipids PIP2 and PIP3 which in turn activate 3-phosphoinositide-dependent kinase (PDK) 1 and 2 and promote Akt translocation to the membrane. The serine/threonine kinase Akt becomes phosphorylated at Ser473 and Thr308 by PDK1 and 2 which results in its activation. Akt mediates the anti-apoptotic effects of the IGFs by phosphorylating and inhibiting several pro-apoptotic downstream targets like glycogen synthase kinase (GSK) 3, BAD, and caspase 9, as well as transcription factors like CREB (Cyclic AMP Response Element Binding Factor) and the FKHR (forkhead-related transcription factor) family.

Another target of Akt is Mdm2, which is phosphorylated Ser166 and Ser186, leading to its translocation into the nucleus where it decreases the levels of p53 (Mayo and Donner 2001). Furthermore, Akt participates in the activation of NFκB and mTOR, resulting in the transcription of genes which mediate cell survival and protein synthesis (Peterson, Ledgard et al. 1998). Akt activation is tightly regulated by the lipid phosphatases protein tyrosine phosphatase (PTEN) and 5' lipid phosphatase (Datta, Dudek et al. 1997; Kulik, Klippel et al. 1997; Navarro and Baserga 2001).

MAPK pathway

The recruitment of Shc to Tyr950 of the activated IGF-1R stimulates the Ras/Raf/mitogen activated protein (MAP) kinase pathway (Navarro and Baserga 2001). Shc serves as adapter for the SH2-domain containing signaling molecule Grb2. Grb2 is bound the guanine nucleotide releasing factor Sos via its SH3 domains, bringing it closely to the GTPase Ras which becomes activated through the exchange of GDP to GTP and hereby binds and activates the serine/threonine kinase Raf-1. Active Raf-1 phosphorylates the MAPK kinase MEK1/2 which phosphorylates the MAP kinase Erk1/2, thereby enabling it to translocate into the nucleus where it phosphorylates and activates a number of transcription factors necessary for cell growth and proliferation (e.g. STAT-1 and -3, Elk-1 and c-Myc) (Treisman 1996). Figure 3 represents the MAPK/Erk and PI3K/Akt signaling cascades activated by IGF-1.

14-3-3 pathway

14-3-3 proteins belong to a highly conserved family of proteins which exist as homo- and heterodimers in all eukaryotic cells. These phosphoserine binding proteins interact with diverse cellular proteins and modulate the interactions between components of mitogenic and apoptotic signal transduction pathways and cell cycle control (Furlanetto, Dey et al. 1997; Baldin 2000). 14-3-3 proteins interact with the IGFIR and IRS-1 *in vivo* and play a role in a transformation pathway signaled by the IGFIR. (Craparo, Freund et al. 1997; Spence, Dey et al. 2003). The interaction with 14-3-3 proteins requires receptor kinase activity and phosphorylation of Ser1283 in the C-terminus of the receptor and results in the mitochondrial translocation of Raf.

This leads to phosphorylation and inactivation of the pro-apoptotic protein BAD and sustained cell viability (Peruzzi, Prisco et al. 1999).

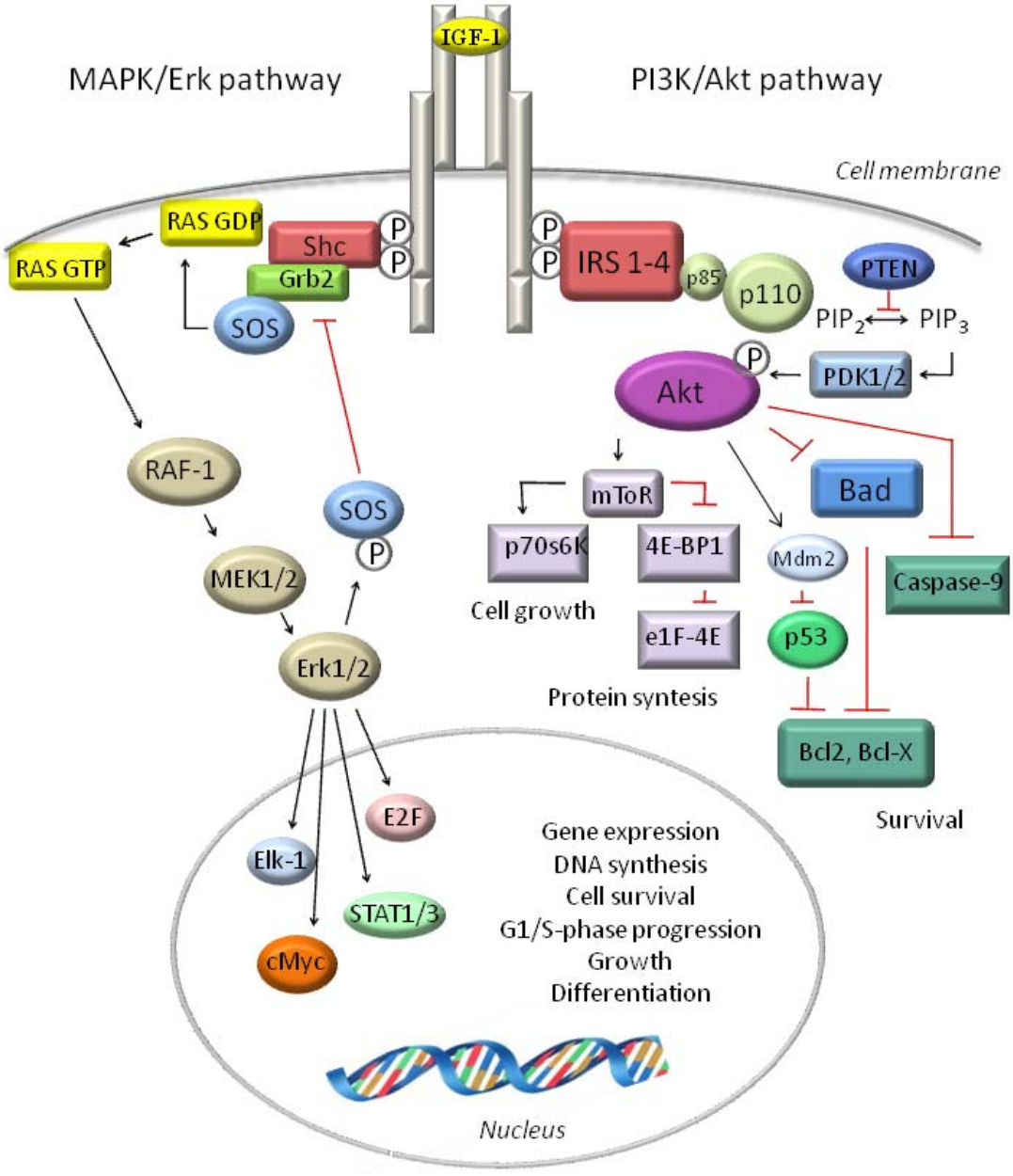

Figure 3: Simplified scheme of MAPK and PI3K signaling pathways upon IGF-1R activation

Internalization & degradation

While the function of IGF-1R as tyrosine kinase in PI3K and MAPK signaling is well understood, much less is known about the IGF-1R life cycle and degradation. In general, cell surface receptors are either recycled continuously, or, as for the IGF-1R, after ligand stimulation, a process called receptor mediated endocytosis (Chow, Condorelli et al. 1998). Several endocytic pathways are known to mediate internalization of cell surface receptors, some of which are dependent on ubiquitination of its target (Alexander 1998; Waterman and Yarden 2001). Eventually, the internalized receptors are recycled and transported back to the cell surface without the ligand or degraded in either lysosomes or proteasomes or in both. The role of endocytosis in regulating the signaling activity of receptors through internalization and downregulation is well known but the discovery that some receptors signal also from endosomes provided evidence that endocytosis is more than just a switch-off function for receptor activity (Attisano and Wrana 2002; Felberbaum-Corti and Gruenberg 2002).

Clathrin-dependent endocytosis

Ligand-induced receptor internalization is achieved through clathrin-coated pits, involving a number of adaptor and accessory proteins. The clathrin adaptor comlex 2 (AP-2) links the cargo to clathrin (Hirst and Robinson 1998) and binds phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2), which is required for the correct localization of clathrin and progression of endocytosis (Beck and Keen 1991; Zoncu, Perera et al. 2007), the GTPase dynamin promotes clathrin polymerization and mediates the detachment of the vesicle from the plasma membrane (Vallee, Herskovits et al. 1993; Altschuler, Barbas et al. 1998; McNiven, Cao et al. 2000). Thereafter, the vesicle sheds clathrin and fuses with a cytoplasmic vesicle to form the early endosome. Its maturation into the late endosome goes along with a reduction of internal pH and the accumulation of hydrolytic enzymes. Eventually, the invagination of the endosomal membrane leads to the formation of a specialized sorting compartment, the multivesicular body (MVB). It has been shown that not until here the EGFR is separated from its cytoplasmic transducers, attenuating its signaling activity (Miaczynska, Pelkmans et al. 2004).

Upon its fusion with the lysosome, the MVB releases its content for degradation by hydrolytic enzymes. It is important to mention that the endocytic pathway does not inevitably cause degradation but can also lead to the recycling of proteins back to the plasma membrane or other locations (Felder, Miller et al. 1990; French, Sudlow et al. 1994; Bao, Alroy et al. 2000).

Raft/caveolae-mediated endocytosis

This clathrin-independent endocytic pathway is reliant on lipid rafts. Rafts are cholesterol- and glycosphingolipid-rich membrane domains involved in the compartmentalization of various cellular processes (Conner and Schmid 2003; Pike 2006). Caveolae are flask-shaped invaginations of the plasma membrane and considered a subdomain of lipid rafts. Caveolin-1 is the major component of caveolae and essential for their formation. Several raft-dependent endocytosis pathways have been described, involving various cellular components like cholesterol and dynamin as well as regulators of the actin cytoskeleton (Pelkmans, Puntener et al. 2002; Kirkham and Parton 2005). Besides the internalization of membrane components, extracellular ligands, some viruses (Simian virus 40) and bacterial toxins, caveolae-mediated endocytosis has also been demonstrated for several tyrosine kinase receptors (Montesano, Roth et al. 1982; Rothberg, Ying et al. 1990; Parton, Joggerst et al. 1994; Schnitzer, Oh et al. 1994; Anderson, Chen et al. 1996; Carpenter 2000; Waugh, Minogue et al. 2001). The caveolar pathway of cell surface receptors is triggered by ligand binding to the receptor. Internalized receptors are placed in so-called caveosomes, preexisting membrane organelles with neutral pH that are distributed throughout the cytoplasm. From here, the cargo is either sorted to the Golgi complex or the endoplasmatic reticulum. The sequestration of the transforming growth factor beta receptor (TGFßR) and epidermal growth factor receptor (EGFR) to caveolae and their interaction with caveolin-1 is associated with the inhibition of their signaling capacity (Mineo, Gill et al. 1999; Park, Park et al. 2000; Di Guglielmo, Le Roy et al. 2003). Interestingly, Sigismund et al. showed that the EGFR is internalized through the clathrin-dependent endocytosis pathway upon low dose EGF stimulation but through the clathrin-independent route via lipid-rafts upon higher dosages of EGF. Further studies on endocytic pathways will lead to more detailed knowledge on how different trafficking routes modulate the fate and biological function of cell surface receptors.

Ubiquitination

Ubiquitin was discovered in the 1970s as a protein tag covalently attached to other proteins, marking them for destruction by a large multiprotein complex, the proteasome (Hershko, Ciechanover et al. 1980; Ciechanover, Finley et al. 1984). While its function by then was solely seen as the "kiss of death" eliminating dysfunctional proteins, today ubiquitination is known to be involved in numerous cellular processes such as protein internalization and trafficking, regulation of signaling, DNA repair, cell cycle and gene expression. The ubiquitin system consists of the small protein ubiquitin and three types of enzymes (E1, E2 and E3) that recognize its substrates and sequentially mediate its conjugation (Hershko 1983; Scheffner, Nuber et al. 1995).

Ubiquitin

Ubiquitin is a small globular peptide of 76 amino acids which is present in all eukaryotic cells and highly conserved between single-cell eukaryotes and mammalians. Its covalent attachment to other proteins is called ubiquitination or ubiquitylation. Due to its globular structure and a large number of hydrogen bonds, ubiquitin is highly stable and insensitive to extreme temperature and pH. There is only a small pool of free ubiquitin in the cell; the majority is conjugated to other proteins in form of mono-, multi- or polyubiquitination.

E1 ubiquitin-activating enzymes

E1s are so-called ubiquitin-activating enzymes that form a thioester bond between their active site cysteine and the carboxy-terminal glycine of ubiquitin in an ATP-dependent process (Haas, Warms et al. 1982).

E2 ubiquitin-conjugating enzymes

This group of ubiquitin-conjugating enzymes, also known as ubiquitin carriers (UBCs), mediates the transfer of ubiquitin from the E1s through an ATP-dependent trans-thiolation reaction. At least 25 mammalian members of the E2 family are known today.

E3 ubiquitin ligases

The ubiquitin protein ligases finally catalyze the transfer of ubiquitin from the E2 enzyme to the substrate, creating an isopeptide bond between the ε–amino group of a lysine residue on the target protein and the C-terminal glycine of ubiquitin. E3s are a large group of enzymes of which two different families with conserved protein domains exist: the HECT- (homologous to E6-AP carboxyl terminus) domain E3 ligases and the RING-domain E3 ligases (Huibregtse, Scheffner et al. 1995; Joazeiro and Weissman 2000). The first mentioned build thioester-intermediates with ubiquitin before they transfer it to the target, whereas the latter mediate ubiquitination by acting as an adapter, bringing substrate and ubiquitin in close proximity to each other. RING-domain E3s are able to regulate their own activity by auto-ubiquitination. The oncoproteins Mdm2 and c-Cbl are examples of well-known E3 ligases of the RING-domain family (Joazeiro, Wing et al. 1999; Waterman, Levkowitz et al. 1999; Fang, Jensen et al. 2000; Honda and Yasuda 2000). The E3s, alone or together with the ubiquitin-conjugating enzyme determine the high substrate specificities.

Ubiquitin-like modifiers (UBLs)

Cellular proteins can be modified by a number of small polypeptides, commonly named ubiquitin-like modifiers (UBLs) which mark them for a variety of metabolic fates. Like ubiquitin, they form an isopeptide bond with the ε–amino group of a lysine residue of the target protein and require E1-like enzymes for conjugation. Some of the UBLs share sequence homology with ubiquitin, like the small-ubiquitin related modifier-1 (SUMO-1), a 12kDa protein which is the best characterized among them. SUMOylation of proteins is involved in nuclear transport, gene regulation, apoptosis and protein stability (Jentsch and Pyrowolakis 2000; Muller, Ledl et al. 2004).

Mono- multi- and polyubiquitination

The covalent binding of a single ubiquitin is called monoubiquitination, the attachment of several ubiquitin monomers to one substrate multiubiquitination.

Monoubiquitination is mainly involved in non-proteolytic functions such as endocytic trafficking, DNA replication and repair (Haglund, Sigismund et al. 2003; Sigismund, Polo et al. 2004). The attachment of several mono-ubiquitins has been shown to be important for internalization and lysosomal degradation of IGF-1R, EGFR and PDGFR (Haglund, Sigismund et al. 2003; Huang, Kirkpatrick et al. 2006; Monami, Emiliozzi et al. 2008). Polyubiquitination creates structurally different ubiquitin chains with distinct functions. This type of modification is achieved by the formation of an ubiquitin chain between the C-terminus of one ubiquitin and one of the seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63) of the next ubiquitin. Polyubiquitination requires sequential E1-E2 and E2-E3 interactions since E2s cannot be reactivated by E1 while it is bound to E3.

Proteins with Lys48 linked ubiquitin chains are primarily targeted for proteasomal degradation; however it has also been reported to be involved in endosomal trafficking and non-proteolytic degradation of the hepatocyte growth factor receptor Met (Carter, Urbe et al. 2004). Polyubiquitin chains linked though Lys63 on the other hand are associated with functional activation like endocytosis, cell signaling and DNA repair (Spence, Sadis et al. 1995; Hoege, Pfander et al. 2002; Pickart and Eddins 2004; Laine and Ronai 2005). Whereas Lys29 polyubiquitination has been shown to result in proteasomal degradation of a special set of substrates, the UFDs (Ubiquitin Fusion Degradation substrates) (Johnson, Ma et al. 1995; Lindsten, de Vrij et al. 2002), the function of Lys6 polyubiquitination is to inhibit protein degradation (Shang, Deng et al. 2005). The purpose of Lys11 degradation *in vivo* is still unknown. A special case of polyubiquitination is the conjugation of the first ubiquitin to the α-amino group at the N-terminus of the substrate, therefore called N-terminal ubiquitination. So far, it has been found only in a small number of proteins, mediating proteasomal degradation (Breitschopf, Bengal et al. 1998).

Deubiquitination

Like phosphorylation, ubiquitination is a reversible process. A total of 95 thioester-proteases are thought to function as deubiquitinating enzymes (DUBs) in humans (Nijman, Huang et al. 2005). Their large number and diversity reflects their specificity for substrates and diverse regulatory functions (Amerik and Hochstrasser 2004).

DUBs are divided in ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin-specific processing enzymes (UBPs). The first mentioned remove small carboxy-terminal fusion proteins from ubiquitin; UBPs preferentially remove ubiquitin from larger proteins and cleave polyubiquitin chains at the proteasome (Chung and Baek 1999; Wilkinson 2000). Deubiquitinating enzymes fulfill diverse functions in the regulation of ubiquitin-mediated pathways. Besides their crucial role in the regulation of ubiquitin-dependent signaling, these enzymes are responsible for processing inactive ubiquitin precursors, proofreading ubiquitin-protein conjugates, recycling ubiquitin moieties and keeping the 26S proteasome free of inhibitory ubiquitin chains (Baek 2003; Kim, Park et al. 2003).

Ubiquitin-dependent degradation

Proteins with Lys48 linked ubiquitin chains are recognized by the 26S proteasome, a 2.4 megadalton complex consisting of a catalytic core protease (the 20S core) and a regulatory component, the 19S cap or PA700 (Pickart and Cohen 2004). The 20S core has the form of a cylindrical chamber consisting of two identical outer and two identical inner rings (Groll, Ditzel et al. 1997). The inner rings contain the peptidolytic active sites. The 19S caps recognize the substrates and also contain DUBs and multiple ATPases. The ATP-dependent interaction with the 20S core promotes the access of the protein substrates to the proteolytic chamber. The proteins are unfolded and deubiquitinated and further digested into oligopeptides or amino acid residues (Hershko, Ciechanover et al. 1980).

About 80% of mammalian proteins are degraded by the proteasome, among them many whose expression has to be tightly regulated, e.g. proteins involved in cell growth, like c-Myc, p53 and certain cyclins (Glotzer, Murray et al. 1991; Clurman, Sheaff et al. 1996; Diehl, Zindy et al. 1997; Flinn, Busch et al. 1998; Vogelstein, Lane et al. 2000). Proteasomal degradation is not limited to ubiquitinated cytosolic and nuclear proteins, it also functions as quality control system by targeting misfolded proteins that are retained within the endoplasmatic reticulum (Amerik and Hochstrasser 2004; Ardley, Hung et al. 2005). To meet the varying cellular requirements, the proteasome is a highly dynamic structure; both the nature and the magnitude of its function can be altered in response to changes in physiological demands (Xie and Varshavsky 2001; Leggett, Hanna et al. 2002; Glickman and Raveh 2005; Lecker, Goldberg et al. 2006).

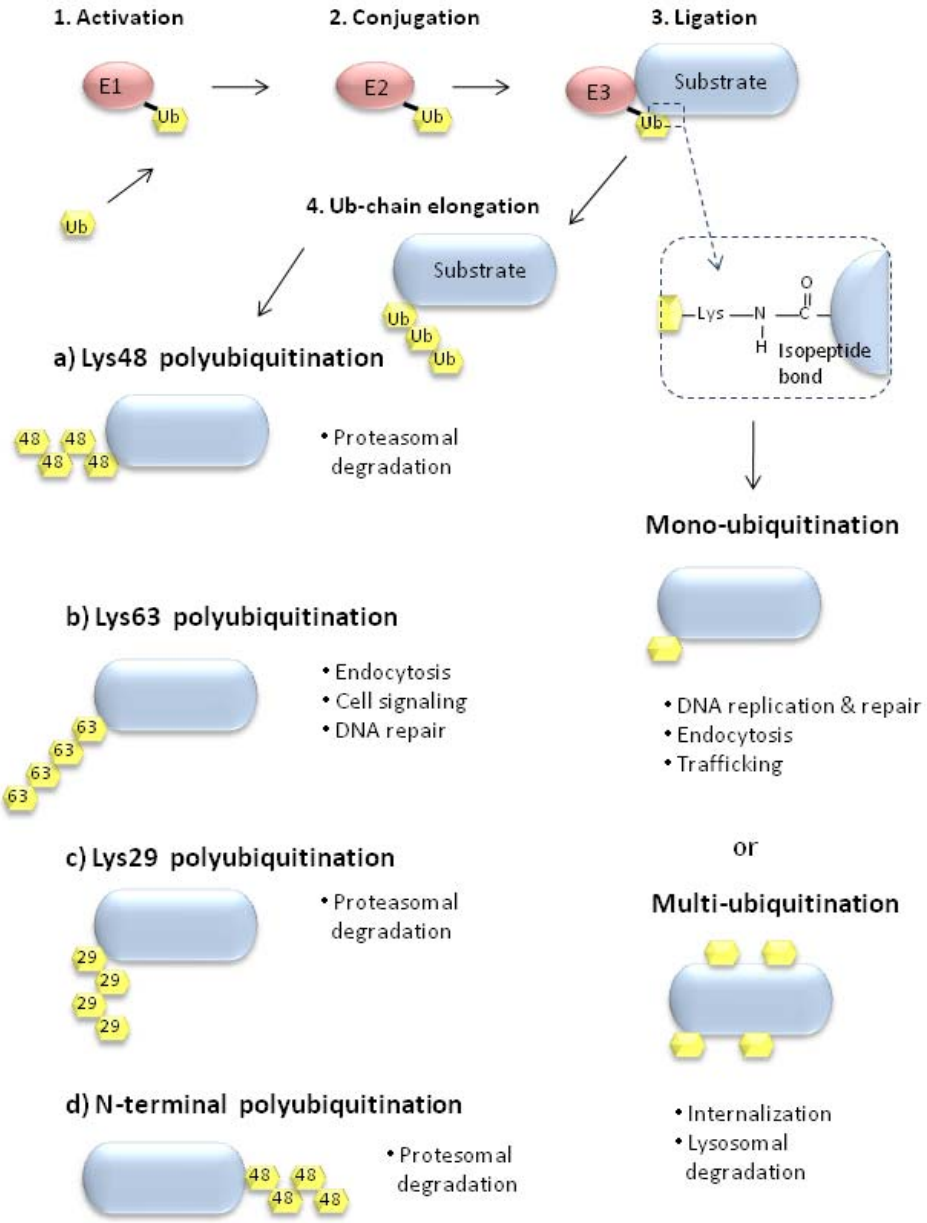

Figure 4: Overview over the different variants of ubiquitination and their functional impacts.

IGF-1R ubiquitination

IGF-1R ubiquitination has been shown to be mediated by two different ubiquitin E3 ligases: Mdm2, with ß-arrestin as adapter protein (Girnita, Girnita et al. 2003) and Nedd4 together with its adapter Grb10 (Vecchione, Marchese et al. 2003).

Mdm2 and ß-arrestin

The oncoprotein Mdm2 (murine double minute 2), a RING domain E3 ligase was discovered in a spontaneously transformed murine fibroblast cell line (Cahilly-Snyder, Yang-Feng et al. 1987). Its human homolog is frequently overexpressed in various tumors (Bueso-Ramos, Yang et al. 1993; Leach, Tokino et al. 1993; Reifenberger, Liu et al. 1993; Teoh, Urashima et al. 1997). Mdm2 regulates the activity of the tumor suppressor p53 by mediating its ubiquitination and proteasomal degradation in a negative auto-regulatory feedback loop (Fang, Jensen et al. 2000; Woods and Vousden 2001). Together with p53, Mdm2 shuttles from the nucleus to the cytoplasm (Roth, Dobbelstein et al. 1998) where p53 is degraded in cytoplasmic proteasomes. Besides its role in p53 degradation, Mdm2 has also been shown to increase the degradation of other proteins, like beta-arrestin and the beta 2 adrenergic receptor (Shenoy, McDonald et al. 2001). Our group has shown that Mdm2 also acts as E3 ligase for IGF-1R ubiquitination leading to proteasomal degradation of the receptor (Girnita, Girnita et al. 2003). While the levels of cytosolic Mdm2 depend on its nuclear interaction with p53, the PI3K/Akt signaling pathway and its own degradation rate, the availability of cytosolic Mdm2 seems to directly affect ubiquitination and hence downregulation of the IGF-1R.

Arrestins were originally identified as negative regulators of seven-transmembrane receptor (7TMR) function. Whereas the expression of arrestin 1 and 4 is limited to cone and photoreceptor cells in the retina, ß-arrestin 1 and 2 are ubiquitously expressed cytosolic proteins involved in clathrin-dependent internalization and sequestration of G-protein-coupled receptors (GPCRs). Today, ß-arrestins are regarded as multi-functional adapters involved in the regulation of various signaling molecules.

Besides its ability to activate several downstream signaling kinases like c-Src, and MAPK (Luttrell, Ferguson et al. 1999; DeFea, Zalevsky et al. 2000), ß-arrestin 1 interacts with nuclear proteins, modulates gene transcription (Witherow, Garrison et al. 2004; Kobayashi, Narita et al. 2005; Wang, Lu et al. 2006) and plays a role in cellular migration and metastasis (Buchanan, Gorden et al. 2006). Lin et al. demonstrated that ß-arrestins bind ligand-stimulated IGF-1R and promote receptor internalization via clathrin-coated vesicles (Lin, Daaka et al. 1998). Previous work from our group identified ß- arrestin 1 as adaptor for Mdm2 in IGF-1R ubiquitination, mediating ligand-induced Erk activation and cell cycle progression together with Mdm2 (Girnita, Shenoy et al. 2005; Girnita, Shenoy et al. 2007).

Nedd4 and Grb10

Nedd4 (neural precursor cells-expressed developmentally downregulated 4) is a HECT domain ubiquitin E3 ligase that was identified in a screen for developmentally downregulated genes in the early embryonic mouse central nervous system, hence its name (Kanwar, Kumar et al. 2002). In total, there are nine members of the Nedd4-like E3 ligase encoded in the human genome, all with structural similarity; a C2 domain at the N-terminus and a homologous to E6AP carboxyl terminus-domain at their C-terminus (Huibregtse, Scheffner et al. 1995). All Nedd4–like proteins regulate ubiquitin-mediated functions, like proteasomal or lysosomal degradation, protein trafficking and nuclear translocation. Furthermore, they play a role in signaling pathways activated by TGFß, EGF, IGF, VEGF, and TNFα (Rotin, Staub et al. 2000; Harvey, Dinudom et al. 2001; Ingham, Gish et al. 2004). Nedd4, in complex with Grb10 mediates ligand-dependent ubiquitination of the IGF-1R in Grb10 overexpressing cells whereas the simultaneous overexpression of a catalytically inactive form of Nedd4 abolishes IGF-1R ubiquitination in these cells (Vecchione, Marchese et al. 2003).

The adaptor protein Grb10 (growth factor receptor-bound protein 10) was found to interact with several RTKs like EGFR, IGF-1R, IR and PDGFR (Dey, Frick et al. 1996; Hansen, Svensson et al. 1996; Morrione, Valentinis et al. 1996; O'Neill, Rose et al. 1996; Dong, Farris et al. 1997; Frantz, Giorgetti-Peraldi et al. 1997).

Vecchione et al. showed that it serves as adapter protein building a bridge between Nedd4 and the IGF-1R (Vecchione, Marchese et al. 2003). Grb10 expresses a highly conserved src-homology 2 (SH2) domain in the carboxy-terminal region and several putative binding sites for src-homology 3 (SH3) -domain containing proteins. The BPS (Between Pleckstrin homology- and SH2 domain) domain is important for the interaction with activated IGF-1R (Morrione 2003). The binding to IGF-1R is phosphotyrosine dependent (Morrione 2000) while its association with Nedd4 is constitutive and independent of phosphotyrosine (Morrione, Plant et al. 1999).

The role of IGF-1R in cancer

The IGF-1R is known plays a crucial role in the development and progression of human cancer by promoting cell growth and survival of malignant cells. Moreover, IGF-1R has been shown to be important for anchorage-independent growth of tumor cells, another hallmark of cancer and a prerequisite for metastasis (Baserga 1995; Baserga, Resnicoff et al. 1997; Werner and Le Roith 1997).

While many RTKs acquire their oncogenic potential not only by overexpression but also by mutations which results in ligand-independent constitutive signaling, mutant forms of IGF-1R in cancer have not been detected. However, overexpression of IGF-1R is observed in many human malignancies (Ouban, Muraca et al. 2003), often correlating with bad prognosis for the patient (Parker, Cheville et al. 2002; Spentzos, Cannistra et al. 2007). The regulation of IGF-1R gene expression depends on the function of several oncogenes and tumor suppressor genes. p53 for instance inhibits IGF-1R expression (Werner and LeRoith 1996), while the expression of a mutant form of p53 results in elevated IGF-1R protein levels in cancer cells (Girnita, Girnita et al. 2000). Many tumors express even altered levels of IGF-1R ligands or binding proteins. Additionally, overexpression of insulin receptor isoform A (IRA), the fetal form of insulin receptor, and hybrid receptors between IGF-1R and IRA is found in a subset of tumors. Remarkably, hybrid receptors can be activated by IGF-1, IGF-2 and Insulin which ultimately broadens the spectrum of mitogenic signaling (Belfiore 2007).

The IGF-1R is regarded as a prominent target for anti-cancer therapy and the downregulation of its activity has been shown to inhibit the growth of many types of human tumor cells. Various strategies to inhibit IGF-1R function have been investigated during the past years. The expression of a dominant negative IGF-1R in Ewing's sarcoma cells leads to increased apoptosis and decreased tumorigenesis (Scotlandi, Avnet et al. 2002). Results from the use of monoclonal antibodies in order to mediate receptor downregulation have been encouraging in cancer cell lines (Hailey, Maxwell et al. 2002; Sachdev, Li et al. 2003) and cancer xenografts, especially as combination therapy (Goetsch, Gonzalez et al. 2005; Cohen 2006). Another promising attempt to inhibit IGF-1R kinase activity is the use of small molecules like the cyclolignan picropodophyllin (PPP) (Girnita, Girnita et al. 2004), of which some are currently tested in clinical trials.

While the MAPK and PI3K pathways and their proliferative and anti-apoptotic effects upon IGF-1R activation are well known, the implication of IGF-1R in various other cellular signaling systems is still not fully understood. However, a series of studies provide evidence for cross-talk between IGF-1R and other receptors and their signaling pathways, such as EGFR (Adams, McKern et al. 2004; Saxena, Taliaferro-Smith et al. 2008), nuclear steroid receptors (Dupont, Karas et al. 2000) or G-protein coupled receptors (Dalle, Ricketts et al. 2001). These interactions may contribute to compensatory effects in receptor signaling. Furthermore, the IGF-1R has been shown to influence integrins and cell adhesion; its activity stimulates the migration of epithelial colonic cells by altering integrin localization and disrupting the association of E-cadherin and ß-catenin with the cytoskeleton (Andre, Rigot et al. 1999). Several studies have shown that upon IGF-1 stimulation, ß-catenin dissociates from E-cadherin and translocates to the nucleus where it activates the transcription of proliferative genes (Eastman and Grosschedl 1999; Playford, Bicknell et al. 2000; Morali, Delmas et al. 2001; Satyamoorthy, Li et al. 2001) Thus, various nodal points integrate signals between the IGF-1R and other signaling pathways in cancer cells and contribute to the complexity of the system.

Nuclear RTKs

The recent discovery of nuclear IGF-1R by our group (Sehat, Tofigh et al. 2009) provides further support for a multifunctional role of IGF-1R. While the nuclear existence of several other RTKs including EGFR, HER-2,-3, and -4, FGFR, NGFR and VEGFR has been observed previously, its functional relevance is not fully understood yet. However, there is evidence that nuclear EGFR and FGFR contain the intrinsic ability to enhance gene transcription. The FGFR1 has been found to localize to the nuclear matrix and splicing-rich speckles, which suggests a role in gene transcription and splicing (Maher 1996; Somanathan, Stachowiak et al. 2003), and the nuclear import of FGFR is has been shown to be associated with proliferation (Reilly and Maher 2001). Nuclear EGFR physically binds to AT-rich DNA sequences in the promoter region of cyclin D1, inducing its transcription and subsequent cell cycle progression (Lin, Makino et al. 2001). Furthermore, nuclear EGFR has also been shown to bind to the promoter regions of iNOS and B-Myb (Lo, Hsu et al. 2005; Hanada, Lo et al. 2006). Interestingly, all transcriptional targets of EGFR identified so far are involved in tumorigenesis, tumor proliferation and progression. Hence, nuclear EGFR has been suggested as a prognostic indicator for poor clinical outcome (Lo, Xia et al. 2005; Psyrri, Yu et al. 2005).

The isolation of full-length receptors from cell nuclei (Maher 1996; Stachowiak, Maher et al. 1996; Stachowiak, Maher et al. 1997; Lin, Makino et al. 2001; Reilly and Maher 2001) and studies on biotinylated FGFR demonstrated that nuclear receptors originate from the cell surface (Olsnes, Klingenberg et al. 2003). Consequently, the question is through which mechanism the nuclear translocation of these plasma membrane proteins occurs. Since RTKs seem to exist as non-membrane bound receptors in the nucleus, it is speculated that cells utilize a general mechanism for extracting transmembrane receptors prior to their passage through the nuclear pore complex (NPC). However, such mechanism has not yet been identified. Until now, nuclear localization signals (NLS) have been found in EGFR but not FGFR (Lin, Makino et al. 2001; Offterdinger, Schofer et al. 2002). Conversely, several cell surface receptors are capable of interacting with nuclear transport receptors, like importins and exportins, and thus enter and exit the cell nucleus, respectively (Maher 1996; Reilly and Maher 2001; Giri, Ali-Seyed et al. 2005).

The nuclear translocation of RTKs has been shown to occur in both ligand-dependent and -independent manner (Dittmann, Mayer et al. 2005; Massie and Mills 2006). Receptor internalization into the endocytic pathway is the initiating step for the subsequent nuclear localization of RTKs through lipid raft and/or clathrin-dependent endocytosis (Citores, Wesche et al. 1999; Citores, Khnykin et al. 2001; Bryant and Stow 2005).

Nuclear IGF-1R was demonstrated to bind to DNA enhancer regions in the nucleus which suggests a role in gene regulation. Besides ligand stimulation, the IGF-1R requires SUMOylation, a special post-translational modification, for its nuclear import (Sehat, Tofigh et al. 2009). Therefore it is tempting to speculate that SUMOylation might represent a general mechanism for the nuclear import of transmembrane receptors. The data from Sehat et al. revealed a novel pathway in IGF-1R function additionally to the well-established canonical signaling pathway from the cell membrane. Although it is thought to participate in transcriptional regulation, the specific physiological function of nuclear IGF-1R remains to be elucidated. Further investigation of the multiple roles of IGF-1R from the cell surface to the nucleus is necessary in order to fully appreciate its function in cancer development.

AIMS OF THESIS

This work was conducted in order to identify novel interaction partners of IGF-1R and to further investigate its multiple roles in cancer development.

The specific aims of this study were:

- **Paper I:** To address the relationship between IGF-1R phosphorylation and ubiquitination and their effects on IGF-1R function.

- **Paper II:** To further investigate Mdm2 dependent ubiquitination of IGF1R and to identify a novel E3 ubiquitin ligase that leads to polyubiquitination of IGF-1R in the absence of Mdm2.

- **Paper III:** To identify the role of focal adhesion kinase (FAK) in IGF-1R function.

- **Paper IV:** To investigate the role of nuclear IGF-1R and to analyze if nuclear IGF-1R effects the transcriptional activity of Wnt target genes.

RESULTS AND DISCUSSION

Paper I

Role of ubiquitination in IGF-1 receptor signaling and degradation

The IGF-1R undergoes several modifications upon ligand stimulation. While the role of phosphorylation in receptor activation is well established, much less is known about the impact of ubiquitination on IGF-1R function. In this paper, we used wild type and different mutated IGF-1R constructs to identify functional sites and domains necessary for receptor ubiquitination. We also investigated whether ubiquitination is involved in the control of IGF-1R signaling and degradation. Several reports have shown that transmembrane receptor tyrosine kinases are degraded through the lysosomal and/or proteasomal pathway (Beguinot, Lyall et al. 1984; Hershko and Ciechanover 1998; Nawaz, Lonard et al. 1999), but the mechanism by which the IGF-1R is downregulated has not yet been confirmed.

Ubiquitination makes the IGF-1R a potential target for proteasomal degradation. In order to investigate if lysosomes or proteasomes are responsible for its degradation, we used specific lysosome- and proteasome inhibitors (LyI and PI). Our results show that the majority of IGF-1R undergoes lysosomal degradation. However, inhibition of proteasomal degradation by the PI epoxomycin also reduced IGF-1R degradation. This led us to the conclusion, that the proteolytic pathway is at least partially involved in receptor downregulation. The finding, that the C-terminal truncated IGF-1R mutant, that does not undergo ubiquitination, was exclusively degraded through the lysosomal pathway supports this observation. We found IGF-1R autophosphorylation to be an absolute requirement for receptor ubiquitination since mutants without kinase activity were not ubiquitinated. Furthermore, the C-terminus seems to be the site where IGF-1R ubiquitination occurs. In the absence of the C-terminal domain, IGF-1 induced receptor ubiquitination was undetectable in spite of maintained autophosphorylation.

Additionally, in cells expressing the C-terminal truncated version of the receptor, no ERK activation could be observed, whereas cells with impaired IGF-1R kinase activity exhibited both receptor ubiquitination and ERK phosphorylation, but not Akt phosphorylation. Therefore, ERK activation seems to be dependent on ubiquitination, while Akt activation is likely to require full kinase activity. Interestingly, the IGF-1R mutants with impaired PI3K/Akt signaling were degraded mainly by proteasomes. While the reason for this is unknown, one could speculate that Akt activation has a positive effect on lysosomal degradation, since IGF-1R constructs in cells with Akt phosphorylation were mainly degraded by the lysosomal pathway. Another explanation could be the involvement of Akt activity in nuclear translocation of Mdm2 (Harris and Levine 2005). Abolished Akt phosphorylation may lead to an increase in cytoplasmic Mdm2, hereby increasing its activity in IGF-1R ubiquitination and proteasomal degradation.

Paper II

Identification of c-Cbl as a New Ligase for Insulin-like Growth Factor-1 Receptor with Distinct Roles from Mdm2 in Receptor Ubiquitination and Endocytosis

Mdm2 and Nedd4 were identified previously as E3 ligases mediating IGF-1R ubiquitination. We aimed to analyze the role of Mdm2 in receptor ubiquitination in greater detail by using two osteosarcoma cell lines, one expressing very high levels of Mdm2 (U2OS) and the other one exhibiting very low Mdm2 expression due to p53 deficiency (SAOS2). Surprisingly, we could detect polyubiquitinated IGF-1R even in Mdm2 deficient cells. Since Nedd4 is known to mediate multiubiquitination rather than polyubiquitination, a third E3 ligase seemed likely to be involved in IGF-1R polyubiquitination. c-Cbl appeared to be a promising candidate since it is a known E3 ligase also for other RTKs, like EGFR and PDGFR (Levkowitz, Waterman et al. 1998; Miyake, Lupher et al. 1998; Haglund, Di Fiore et al. 2003). We could demonstrate c-Cbl mediated IGF-1R ubiquitination in an *in vitro* ubiquitination assay and co-immunoprecipitated c-Cbl and IGF-1R under basal conditions in U2OS, SAOS2 and HEK293 cells. Interestingly, SAOS2 cells were polyubiquitinated upon stimulation with higher doses of IGF-1 (50-100ng/ml) whereas polyubiquitination in U2OS cells occurred after low-dose IGF-1stimulation (5-50ng).

This suggests that c-Cbl mediated IGF-1R ubiquitination requires high-dose IGF-1 stimulation, whereas Mdm2 ubiquitinates the receptor after stimulation with low-dose IGF-1. Overexpression of one ligase and inhibition of the other ligase via siRNA or DN constructs in HEK293 cells confirmed this observation. Consistent with this, we found that co-localization of IGF-1R and Mdm2 occurred after 5ng/ml IGF-1 stimulation, whereas the association of c-Cbl and IGF-1R appeared mainly after stimulation with 100ng/ml IGF-1. Since simultaneous overexpression of both Mdm2 and c-Cbl resulted in IGF-1R ubiquitination after both low- and high-dose IGF-1 stimulation, the question if Mdm2 and c-Cbl are cooperators or competitors for IGF-1R ubiquitination remains to be answered.

Whereas both c-Cbl and Mdm2 were shown to bind to and ubiquitinate the IGF-1R upon ligand stimulation, we found that the two ligases differ in ubiquitin lysine specificity and trigger the receptor into different endocytic pathways. By performing a set of *in vitro* reactions using mutated ubiquitin, we determined that Mdm2 causes Lys63 polyubiquitination, whereas c-Cbl uses Lys48 for polyubiquitin chain formation. Since the type of ubiquitination plays an important role in receptor internalization and endosomal sorting (Schnell and Hicke 2003), we also wanted to investigate if Mdm2 and c-Cbl mediate different endocytic pathways. We analyzed co-localization of IGF-1R with markers for different endocytic pathways (phospho-caveolin and EEA-1) and found that Mdm2-ubiquitinated IGF-1R is internalized through the clathrin pathway while c-Cbl-ubiquitinated receptors are endocytosed via the clathrin-independent pathway mediated by rafts-caveolae. Whether it is the direct association to one or the other ligase that decides which internalization pathway is used or the type of polyubiquitination (Lys48 vs. Lys63) itself remains to be determined.

Paper III

Focal Adhesion Kinase (FAK) activates and stabilizes Insulin-like Growth Factor-1 Receptor (IGF-1R)

This paper started with the surprising observation that the mutagenic disruption of the three core tyrosine residues Y1135, Y1131 and Y1136 in the activation loop of the IGF-1R does not prevent the phosphorylation of other receptor tyrosine residues upon IGF-1 stimulation. Because the mutant construct is unable to undergo autophosphorylation, we assumed that another kinase must be involved in the a-loop independent phosphorylation of IGF-1R. Among the pool of cytosolic non-receptor tyrosine kinases, we considered focal adhesion kinase (FAK) to be a promising candidate. Liu et al. have recently shown that FAK and IGF-1R associate with each other in pancreatic cancer cells and two other reports demonstrated that their simultaneous inhibition results in synergistic anti-cancer effects (Liu, LaFortune et al. 2007; Liu, Bloom et al. 2008; Watanabe, Takaoka et al. 2008).

Firstly, we confirmed the association between FAK and IGF-1R in IGF-1R knock-out mouse embryonic fibroblasts (R-), transiently transfected with wild type (R-wt) or triple mutated IGF-1R (R-TM), as well as in the human melanoma cell line DFB. In order to investigate the effect of FAK on IGF-1R phosphorylation, we used wt and FAK -/- mouse embryonic fibroblasts (MEF) overexpressing WT (MEF wt) or TM IGF-1R (MEF TM) constructs. After 10min of IGF-1 stimulation, we found IGF-1R to be phosphorylated in all cell lines except for FAK-/- cells transfected with TM IGF-1R. This was unexpected, since TM IGF-1R was previously shown to exhibit severely reduced ligand-induced autophosphorylation upon 5min ligand stimulation (Kato, 1994). Hence, FAK has the ability of phosphorylating the IGF-1R independently of the a-loop domain. Having demonstrated that FAK enables IGF-1R phosphorylation, we wanted to investigate how FAK affects IGF-1R downstream signaling. We analyzed ERK and AKT phosphorylation as a consequence of MAPK and PI3K activity upon IGF-1 stimulation. As expected, we found reduced levels of both Akt and ERK phosphorylation in FAK-/- cells overexpressing TM IGF-1R compared to FAK positive cells.

Additionally, we showed that FAK inactivation leads to increased IGF-1R downregulation by culturing R-wt and R-TM cells for up to 48h under non-adherent conditions. The total levels of IGF-1R decreased in both cell lines; however this effect was much stronger in R-TM cells, suggesting that the stability of the mutant form of IGF-1R is more dependent on FAK activity than the WT construct. We confirmed this observation in DFB cells, where we determined the IGF-1R levels by cycloheximide (CHX) chase upon siRNA mediated downregulation of FAK. To date, we do not know if it is the direct association between the two kinases that causes the stabilizing effect on IGF-1R protein levels, or if FAK mediated phosphorylation causes changes in IGF-1R conformation which protect it from degradation. Another explanation might be that without FAK, the TM receptor is recognized as nonfunctional and therefore degraded at higher extend than wt IGF-1R.

Paper IV

IGF-1R interacts with components of the Wnt signaling pathway and affects gene transcription.

The identification of nuclear IGF-1R by our group indicated a novel role for IGF-1R besides the well-known signaling properties as transmembrane receptor through MAPK/Erk and the PI3K/Akt pathway. For the first time, IGF-1R was shown to bind to genomic DNA and its overexpression was found to enhance the transcription of yet unknown genes.

In this paper we wanted to analyze the role of IGF-1R as transcriptional activator in the nucleus with regard to its possible interactions with factors of the Wnt signaling pathway. Firstly, we identified the interaction between IGF-1R with beta-catenin in both cell membrane and nucleus and LEF-1 in the nucleus by subcellular fractionation and immunoprecipitation. Additionally, we demonstrate the positive effect of IGF-1R overexpression on TCF/LEF activity and cyclin D1 promoter activity in IGF-1R negative SKUT-1cells via luciferase reporter assay.

Since it is known, that IGF-1 regulates LEF/TCF-dependent transcription through PI3K activation (Desbois-Mouthon, Cadoret et al. 2001), the challenge was to determine if the increase in LEF/TCF activity is triggered by IGF-1-dependent signaling of the receptor or by nuclear translocation of IGF-1R and its association with LEF-1. For this purpose, we used the SUMOylation-deficient IGF-1R plasmid (TSM IGF-1R) which does not translocate to the nucleus as control. Duolink in situ proximity ligation assay confirmed that wt IGF-1R associates with endogenous LEF-1 in the nucleus of IGF-1R deficient SKUT-1 cells whereas TSM IGF-1R does not. Upon wt IGF-1R transfection in SKUT-1 cells, we found TCF/LEF activity to be increased by approximately 65% compared to mock transfection, whereas the expression of TSM IGF-1R reduced the activity by 15%. Furthermore, the activity of the full-length human cyclin D1 promoter linked to a luciferase reporter gene was increased by 35% after wt IGF-1R transfection compared to mock transfection and reduced by 65% upon TSM IGF-1R transfection. Having demonstrated its physical interaction with LEF-1 and the impact on TCF/LEF promoter activity, the next question was if nuclear IGF-1R *in vivo* binds to the same DNA site as LEF-1.

In order to answer this question, we investigated the DNA binding sites of nuclear IGF-1R in complex with LEF-1 transcription factor by chromatin-IP (ChIP) analysis. We first demonstrated binding of nuclear IGF-1R to the LEF-1 binding consensus sequence in the cyclin D1 promoter in two human cancer cell lines (DFB and HeLa). Next, we wanted to investigate if the overexpression of wt IGF-1R results in increased binding to the LEF-1 consensus sequence. We chose to perform this experiment in the human non-small cell lung carcinoma cell line H1299 which has been shown to be more easily transfected. Our results demonstrate that the DNA site occupancy is increased almost 120-fold by the expression of wt IGF-1R but not by TSM IGF-1R overexpression. In order to evaluate whether this binding induces changes in protein expression of cyclin D1 and c-Myc, we performed western blot analysis after overexpression of wt IGF-1R and TSM IGF-1R and found that wt IGF-1R increases protein levels of both cyclin D1 and c-Myc while TSM IGF-1R decreases it. Thus, we suggest that increased expression of cyclin D1 and c-Myc in wt IGF-1R transfected cells does result from transcriptional activation caused by nuclear IGF-1R.

Conclusions

The work presented in this thesis provides further aspects of IGF-1R function, the identification of novel IGF-1R interaction partners and the functional impact of some of its post-translational modifications. The results can be summarized as follows:

I. Ubiquitination plays an important role in IGF-1R signaling and degradation and requires receptor tyrosine kinase activity. The C-terminal domain of IGF-1R is necessary for ubiquitination and ERK phosphorylation as well as for proteasomal degradation of the receptor.

II. Both Mdm2 and c-Cbl are ligases associating with and polyubiquitinating the IGF-1R in response to Igf-1 stimulation. Stimulation of IGF-1R with low doses of IGF-1 leads to Mdm2-induced ubiquitination, whereas c-Cbl ubiquitinates the receptor in the presence of high concentrations of IGF-1. The ligases differ in their responsiveness to ligand stimulation and ubiquitin lysine residue specificity for linkage of the polyubiquitin chains and mediate different endocytotic pathways.

III. Focal adhesion kinase (FAK) is associated with IGF-1R and causes a-loop independent phosphorylation of the receptor. Besides activation, FAK also stabilizes the IGF-1R and influences its downstream signaling.

IV. Apart from its classical tyrosine kinase activity, IGF-1R binds to and activates the ß-catenin/LEF-1 complex in the nucleus which leads to elevated levels of cyclin D1 and c-Myc. This might represent an additional molecular mechanism by which IGF-1R promotes uncontrolled cell proliferation and contributes to neoplastic transformation of cells.

In order to fully understand the roles of IGF-1R in cancer, further studies revealing further details of the functional connection between IGF-1R and its interaction partners, the impact of its modifications and its function in the nucleus need to be conducted. Hopefully, their accomplishment will add to the knowledge necessary for the development of new strategies against cancer.

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