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MOLECULAR FUNCTION AND TARGETING OF β-ARRESTINS IN CANCER

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ABSTRACT

In the selection leading to cancer, cancer cells make use of the normal extracellular signaling to gain a growth advantage over normal cells. These signals are, in part, generated by plasma membrane receptors. G Protein Coupled Receptors (GPCRs) and Receptor Tyrosine Kinases (RTKs) are major transducer of signals across the plasma membrane. Each cell surface receptor family possesses unique structural characteristics and leads to specific signaling outcomes in the cell. However, there is extensive overlap in the signaling proteins and pathways used to produce these effects. Among them, β-arrestins, molecules previously considered to be associated exclusively with GPCRs are also involved in modulating signaling through a classical RTK, the insulin-like growth factor type 1 (IGF-1R). The overall objective of this thesis is to investigate the function and determine potential utility of the β-arrestins as molecular targets in cancer. This is based on the underlying hypothesis that the signaling complexes coordinated by β-arrestins and involving kinases and ubiquitin ligases contribute to tumorigenesis and the progression of cancer and could be targeted in therapies. Paper I identified the antimicrobial cathelicidin peptide LL-37 as a natural agonist for the IGF-1R. LL-37 binding to the receptor resulted in phosphorylation and ubiquitination of IGF-1R, and β-arrestin dependent signaling activation. This signaling activation was limited to the MAPK/ERK pathway without affecting the other main IGF-1R signaling pathway through PI3K/AKT, indicating that LL-37 may act as a β-arrestin biased agonist for the IGF-1R, sustaining the invasive phenotype. Paper II investigated the β-arrestin-IGF-1R binding mechanism and reveal the missing links that to functionally portray a prototypical RTK, the IGF-1R, as a GPCR: GRK dependent phosphorylation of IGF-1R serine residues as the underlying mechanism for β-arrestin binding. While highlighting the cross-talk between the IGF-1R and GPCR at the level of GRKs, this study identified the molecular basis of IGF-1R biased signaling to be dependent on β-arrestin/IGF-1R interaction controlled by GRKs. Paper III investigates the paradox of agonist-like IGF-1R downregulation following treatment with antagonist anti-IGF-1R antibodies. The results show that this process is governed by β-arrestin1 recruitment to the IGF-1R, initiating receptor ubiquitination and degradation. Yet, this β-arrestin1 recruitment to the IGF-1R initiates a wave of ERK signaling activation, demonstrated to have a protective role for the cancer cells. Paper IV reveals that β-arrestin1 mediated IGF-1R signalling is crucial for H-Ras induced transformation. The mechanism underlying this process is impaired intensity and spatial distribution of activated MAPK/ERK signalling in the absence of β-arrestin1.

In conclusion, this thesis demonstrates that β-arrestins play a central role in IGF-1R function, controlling ubiquitination/degradation of the receptor, and receptor signaling. This study, focusing on β-arrestins as central molecules in modulation of the intracellular signaling, may provide new clues in the search for new molecular-designed treatments of cancer.

Key words: IGF-1R, β-arrestins, GRKs, biased agonist, cancer therapy.
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To my beloved Tong and family

The grand essentials of happiness are: something to do, something to love, and something to hope for.

Allan K. Chalmers

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LIST OF ABBREVIATIONS

Abbreviation	Definition
Akt	Protein kinase B
A-loop	Activation loop of the receptor
ARF	Alternative reading frame (protein)
ATP	Adenosine triphosphate
Bad	Bcl associated death promoter
Bak	Bcl-2 Homologous Antagonist-Killer Protein
Bax	Bcl-2-Associated X Protein
Bcl	B-cell leukemia protein
β2AR	β2 adrenergic receptor
Cbl	Cellular product of cbl oncogene
CDK	Cyclin-dependent protein kinase
cDNA	Complementary DNA (DNA copy of mRNA)
DNA	Deoxyribonucleic acid
E2F	Transcription factor activating adenovirus E2 gene
EGF	Epithelial growth factor
ERK1 / 2	Extracellular signal-regulated kinase 1 / 2
FGF	Fibroblast growth factor
GH	Growth hormone
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
Grb10	Growth factor receptor-bound protein 10
GEF	Guanine nucleotide exchange factors
GTP	Guanosine triphosphate
GDP	nucleotide guanosine diphosphate
GRKs	G-protein coupled receptor kinase
GPCR	G-protein coupled receptor
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2	Insulin-like growth factor 2
IGF-2R	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding proteins
IR	Insulin receptor
IR-A/B	Insulin receptor isoform A/B
IRS1-4	Insulin receptor substrate 1-4
JAK	Janus protein tyrosine kinase
JNK	Jun N-terminal kinase

MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin (FK506 binding protein)
Mdm2	Murine double minute 2
MEK	MAP kinase kinase
mRNA	Messenger ribonucleic acid
Nedd4	Neuronal precursor cell-expressed developmentally downregulated
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PI3K	Phosphatidylinositol-3'-kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PyMT	Polyoma middle T antigene
Raf	Protein-serine/threonine kinase (encoded by the raf oncogene)
Ras	Human homologue of Rat sarcoma
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
Ser	Serine
SH	Src homology
Shc	Src homology and collagen
SHP	SH2-containing phosphatase
siRNA	Small interference RNA
shRNA	Small hairpin RNA
S0S	Son of the sevenless
Src	Protein encoded by src proto-oncogene
SV40	Simian virus 40
TK	Tyrosine kinase
V2R	Vasopresin-2 receptor
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 RECEPTOR SIGNALING AND CANCER

Cancer has become the most severe disease to affect human health. The character of this disease is uncontrolled growth which progresses to limitless expansion to other body tissues. In early studies, tumor transformation was considered to be solely driven by genetic mechanisms, with carcinogens inducing mutations in essential genes, leading to abnormal growth of normal cells. Today, tumor development is accepted to be a more complex process defined by at least six hallmarks (Hanahan and Weinberg 2000): a reduced dependence on exogenous growth factors, an acquired resistance to growth inhibitory signals, an ability to multiply indefinitely (immortalization), a reduced susceptibility to apoptosis; an ability to generate new blood vessels (angiogenesis), an acquisition of invasiveness and metastatic capability, an aptitude to evade elimination by the immune system and an acquisition of genomic instability (Hanahan and Weinberg 2000). Recently, followed expending research on cancer mechanistic concepts, two emerging hallmark capabilities: reprogramming of energy metabolism and evading immune destruction, are added to the list(Hanahan and Weinberg 2011).

In the selection leading to cancer, cancer cells make use of normal extracellular signaling systems for proliferation, migration and/or antiapoptosis to create a growth advantage over the normal cells. These signals are transmitted across the plasma membrane by receptors. These cell surface receptors can be divided into four classes: enzyme-linked receptors (receptor functions as an enzyme or is associated with one), G-protein coupled receptor (GPCR), intracellular hormone receptor and integrins. The majority of enzyme-linked receptors are receptor tyrosine kinases (RTKs), which are well-known regulators for a wide range of biological processes and also for tumorigenesis and metastasis The main focus of this thesis is to discuss the cross talk between RTKs and GPCRs.

1.2 GPCR

The G protein-coupled receptors (GPCRs) represent the largest (>2000) family of plasma membrane receptors and mediate a large proportion of physiological functions. GPCRs play critical roles in various physiological functions controlling the growth, proliferation, differentiation and death of multiple cell types. This enables GPCRs to be the target of significant therapeutic research and development and almost half of the drugs in clinical use today are targeting GPCRs. Aside from multiple regulatory roles in physiological functions, studies also indicate that GPCRs are important regulators for cancer development and metastasis (Balkwill, F, Semin. Cancer Biol , 2004), although the mechanism is as yet unclear.

The GPCRs are characterized by a structural motif of seven transmembrane - spanning regions across the cell membrane. Three families of proteins mediate the

function of the GPCR: the heterotrimeric G proteins, the G protein–coupled receptor kinases (GRKs) and the β-arrestins.

Agonist binding to the receptor activates heterotrimeric G proteins leading to canonical second-messenger signaling (Lefkowitz 1998). These activated receptors act as substrates for G protein-coupled receptor kinases (GRKs). The phosphorylation of activated receptors by GRKs promotes β-arrestins binding, which terminates further G protein signaling and causes receptor desensitization. The GRKs and β-arrestins were originally discovered as molecules that desensitize G protein–mediated signaling. However, over the past decade the formulation of GRKs and β-arrestins as simply a desensitization system has proven insufficient to explain many cellular events. In fact, β-arrestins have emerged as remarkably versatile adaptor molecules that regulate receptor endocytosis and also serve as signal transducers (Kim, Ahn et al. 2005). Thus, when β-arrestins terminate G-protein signals, they also initiate other signaling activation in the cell (Reiter and Lefkowitz 2006, DeWire, Ahn et al. 2007).

1.2.1 G proteins

Heterotrimeric G proteins which are coupled to GPCRs consist of three subunits: α, β and γ. They transduce signals from the plasma membrane to the cytosol through a cycle of guanine nucleotide exchange and hydrolysis. In basal conditions, Gα-GDP associates with Gβγ and together they bind the cytoplasmic tail of the GPCRs. Upon ligand activation, conformational changes occur within the GPCR activating its function as a guanine-nucleotide exchange factor (GEF) promoting the exchange of GDP for GTP on the Gα subunit. This process initiates the dissociation of Gα from the Gβγ heterodimer (Neves, Ram et al. 2002). Following dissociation, free Gα-GTP and Gβγ subunits interact with distinct effector proteins to transduce and amplify the signaling triggered by ligand binding to the receptor. The list of effectors interacting with Gα subunit include adenylyl cyclases (Simon, Strathmann et al. 1991, Sunahara and Taussig 2002) phospholipase C isoforms (Park, Gurney et al. 1993), phospholipase A2 and multiple calcium, potassium, sodium channels (Ikeda, Sunose et al. 1996), generating small-molecule second messengers to induce a range of biological outcomes. Additionally, free Gβγ dimers directly interact with multiple effectors including adenylyl cyclase, phosphoinositide 3-kinase, phospholipase C. The free Gβγ subunits also trigger the GRKs activating a feed-back mechanism, by targeting these enzymes to the activated receptor and subsequently terminating G-protein signaling (Pitcher, Payne et al. 1995, Smrcka 2008) .

1.2.2 GRKs

GRKs and β-arrestin mediate desensitization of GPCR (Freedman and Lefkowitz 1996, Shenoy and Lefkowitz 2005). This process occurs in two major steps. Firstly, agonist-occupied receptors are phosphorylated by GRKs on serine or threonine residues within the C terminus or third intracellular loop of GPCRs (Pitcher, Tesmer et al. 1999). The phosphorylated residues represent high-affinity binding sites for β-arrestins,

moving from the cytosol to the plasma membrane. The binding of β-arrestins inhibited G protein coupling (Daaka, Pitcher et al. 1997, Lefkowitz 1998, Ferguson 2001). The consequence of this process is the decrease of second messenger concentration, due to Gα uncoupling and increasing second messenger degradation (Perry, Baillie et al. 2002).

There are seven isoforms of GRKs (GRK1–GRK7) and they belong to the larger family of serine/threonine kinases. The GRKs are structurally related and considering the sequence homology, the members of the GRK family can be divided into three main groups: the visual GRK subfamily (GRK1 and GRK7) which are expressed in visual sensorial cells, the GRK2,GRK3 subfamily and the GRK4 subfamily (GRK4, GRK5 and GRK6) which are both ubiquitously expressed in a variety of tissues, with the exception of GRK4 whose expression is limited to testis cells (Penela, Ribas et al. 2003). GRK-mediated phosphorylation has been considered as a central process leading to β-arrestins binding and desensitization. However, the distinct GRK isoforms phosphorylate different GPCRs with different functional outcomes. By using small interfering RNAs (siRNA) technology, Kim and colleagues demonstrated that GRK2 and -3 were primarily responsible for angiotensin II 1a receptor (AT1aR) desensitization, β-arrestins recruitment and functional uncoupling in an agonist dependent manner (Kim, Ahn et al. 2005). Similar data is observed in the study of vasopressin 2 receptor (V2R) desensitization (Ren, Reiter et al. 2005). On the other hand, GRK5 and 6 have also been implicated in desensitization of several receptors *in vivo* and or *in vitro*. Simon and colleagues revealed that it is GRK6 which mediates β2-adrenergic receptor (β2AR) desensitization in uterine smooth muscle and neuroblastoma cells (Willets, Challiss et al. 2002, Simon, Robin et al. 2003). Moreover, GRK6 is responsible for D2-like dopamine receptors desensitization. Thus, the GRK isoforms responsible for mediating receptor desensitization can vary in distinct receptors or the tissues, emphasizing the notable diversity of the GRK regulatory system.

1.2.3 β-arrestins

In mammals, the arrestin family contains four members: two visual proteins arrestin 1 and arrestin 4; two non-visual arrestins 2 and 3 (also known β-arrestin 1 and β-arrestin 2). The expression of arrestin 1 and 4 is limited to the rod and cone photoreceptors, respectively and are specialized to bind to rhodopsin. β-arrestin1 and 2 are ubiquitously expressed in a variety of tissue. (Gurevich and Gurevich 2006)

1.2.3.1 Structure

Study of crystal structures of arrestins reveal that arrestins are elongated molecules with two domains (N-domain and C-domain) and an extended carboxy-terminal tail that makes a strong contact with the body of the C-domain (Hirsch, Schubert et al. 1999) The most distinctive feature of arrestin conformation is the polar core embedded between the N and C domains of the molecule. The main function of this unique polar

core is to regulate the affinity of arrestins binding to the phosphorylated receptor (McDowell, Nawrocki et al. 1993). Interestingly, both N- and C- domain contain elements to bind to activated receptors (Pulvermuller, Schroder et al. 2000). Furthermore, recent studies found out the distinct residues 49–90 in the N-domain and 237–268 in the C-domain of visual arrestins and homologous regions in arrestin2 are responsible for their receptor preference (Vishnivetskiy, Hosey et al. 2004).

1.2.3.2 Function of β-arrestins in GPCR signaling

Arrestins are soluble, predominantly cytoplasmic proteins. The initial discovery of β-arrestins in GPCR signaling was as an adaptor protein for the termination of G-protein-mediated signaling (receptor desensitization) following the β-arrestins binding to phosphorylated receptor. β-arrestins also interact with cytosolic effectors clathrin and the AP2 adaptor protein to trigger receptor internalization into clathrin-coated pits(Goodman, Krupnick et al. 1996, Lin, Krueger et al. 1997) leading to receptor degradation machinery. Recent studies show that β-arrestins function as signaling transducers promoting interactions with the receptor- β-arrestins complex of numerous signaling proteins including such as c-Src and JNK, linking GPCRs to a variety of signaling pathways (Lefkowitz and Shenoy 2005).

Almost all cell membrane receptors (including GPCRs) exhibit the common properties to internalize from plasma membrane to cytoplasm in response to ligands (Drake, Shenoy et al. 2006). Many well-characterized GPCRs utilize β-arrestins as endocytic adaptors via clathrin-coated pits (CCPs) (Freedman and Lefkowitz 1996, Goodman, Krupnick et al. 1998). Mechanistically, following receptor activation, β-arrestins bind to GRK-phosphorylated receptors and serve as adaptors, linking the receptors to elements of the endocytotic machinery, such as clathrin, the clathrin adaptor AP2 , the small G protein ADP-ribosylation factor 6 and its guanine nucleotide exchange factor ADP ribosylation factor nucleotide-binding site opener and N-ethylmaleimide-sensitive fusion protein (Laporte, Oakley et al. 1999, Ng, McDonald et al. 1999, Claing, Chen et al. 2001, Shenoy, Drake et al. 2006).

Many studies have confirmed the essential role of β-arrestins in GPCR internalization and endocytosis. Overexpression of β-arrestins promoted agonist-stimulated β2AR internalization (Ferguson, Downey et al. 1996). Agonist-stimulated internalization of heptahelical receptors were impaired in mouse embryonic fibroblast cells isolated from β-arrestin 1and 2 double knockout embryos (Shenoy, McDonald et al. 2001). Similar outcome were demonstrated in HEK293 cells by using small interfering RNA (siRNA) targeting β-arrestins (Wei, Ahn et al. 2003).

More recently, studies that characterized a direct interaction between β-arrestins and clathrin have increased insight into the mechanism of GPCR endocytosis. Mutagenesis and truncation analysis have shown that multiple residues in both N- and

C-terminal regions of β-arrs1 function to regulate basal interaction with clathrin (Harrington, Coon et al. 2010).

β-arrestins also interact with mouse double minute 2 protein (Mdm2), an E3 ubiquitin ligase, leading to ubiquitination of β-arrestins. The ubiquitination of β-arrestins controls the stability of the receptor/ β-arrestins complex, which in turn controls receptor endocytosis (Shenoy, McDonald et al. 2001). Overexpression of Mdm2 enhanced β-arrestin ubiquitination and stabilizes β-arrestins and its binding to the β2AR complex, supporting their colocalization in endosomes (Shenoy, Modi et al. 2009). Moreover, ubiquitinated β-arrestins exhibited enhanced clathrin binding compared to non-ubiquitinated β-arrestins. This indicated that the β-arrestins ubiquitination might serve as an efficient binding platform, which facilitates multiple interactions (Shenoy, Barak et al. 2007).

The differences in the ubiquitination of receptor-bound β-arrestins and the specific molecular character of a serine/threonine motif within the receptor C-tail, determine the stability of GPCR- β-arrestins complexes. Based on the stability of the receptor / β-arrestins complex, two classes (A and B) of endocytosis patterns were described for GPCRs (Shenoy and Lefkowitz 2003). For example, AT1aR or V2R forms tight complexes with ubiquitinated β-arrestins2 within endocytic vesicles. These complexes are also associated with sustained mitogen-activated protein kinase (MAPK) activity. Receptors that show this pattern of β-arrestins recruitment are defined as 'class B' receptors. By contrast, β2AR induces transient ubiquitination of β-arrestins2 and forms only transient receptor/arrestins complexes at the plasma membrane. Such receptors are referred as 'class A' receptors (Oakley, Laporte et al. 2000). Notably, replacing the β2AR carboxyl tail with that of either the AT1aR or the V2R, or alternatively fusing ubiquitinto the C terminus of β-arrestins 2 converts β2AR–β-arrestin complexes from class A to class B, with stable signalosomes (Shenoy and Lefkowitz 2005). Recently, deubiquitination of β-arrestin by the deubiquitinating enzyme ubiquitin-specific protease 33 (USP33) was proven as another essential process to determine stability of receptor and receptor- β-arrestins complex subcellular localization (Shenoy, Modi et al. 2009)

1.2.3.3 New roles of β-arrestins: activation of the intracellular signaling

Although β-arrestins proteins were discovered in the context of inhibition of receptor-activated signaling, it was recently discovered that they also initiate signaling waves from the receptors they 'desensitize'. The list of kinases and other signaling proteins that bind to β-arrestins isoforms include adapters for Src-family tyrosine kinases, ERK, JNK (c-Jun N-terminal kinase) and p38 MAPK modules.

The first report of this paradigm was described for β2AR , in which β-arrestins scaffold the tyrosine kinase c-Src to the agonist-activated receptor, leading to activation of extracellular signal-regulated kinase (ERK1/2) (Della Rocca, Maudsley et al. 1999),

This finding has subsequently led to the discovery of the new field of G protein-independent, β-arrestins -dependent signaling via GPCRs.

Among β-arrestins -dependent signaling pathways, MAPK/ERK activation is the best characterized. Multiple approaches including mutant receptors, modified ligands, siRNA inhibition of β-arrestins and specific inhibitors of protein kinase A or C have all been used to prove the mechanisms leading to ERK activation by GPCRs (Shenoy and Lefkowitz 2003, Lefkowitz and Whalen 2004, Lefkowitz and Shenoy 2005). The ERK activation via β-arrestins has different dynamics from G protein-activated. β-arrestins -mediated ERK activation is slower to peak around after 5-10 min ligand stimulation and persists longer, around 30 min to 1 h and in most of the cases is localized in the cytosol. By contrast, G protein-mediated ERK activation is rapid, transient, and translocates to the nucleus, leading to transcriptional activation and proliferation. These very different characteristics strongly suggest distinct physiological outcomes activated by the two pathways.

Remarkably, different β-arrestins and GRK isoforms exhibit specialized, sometimes opposite functions on β-arrestins-dependent ERK activation. For the type 1A receptor (AT1AR), β-arrestin 2 is the activator of the β-arrestins -dependent ERK signaling, whereas β-arrestin 1 acts as an inhibitor (Ahn, Shenoy et al. 2004) for the protease-activated receptor 2, neurokinin-1 receptor, parathyroid hormone receptor and β2AR (Ge, Shenoy et al. 2004, Shenoy and Lefkowitz 2005, Gesty-Palmer, Chen et al. 2006), both β-arrestin 1 and 2 isoforms promote ERK activation. This complexity is further increase by the abilities of β-arrestin 1 and 2 to form homo- and heterodimers of β-arrestins; it has been suggested that some receptors use β-arrestin homodimers for signaling, whereas others require heterodimers (Storez, Scott et al. 2005).

GRK isoforms also impact on the ERK activation induced by β-arrestins. For example, GRK5 or -6 are required for β-arrestins -dependent ERK activation by AT1AR, V2R and β2AR (Kim, Ahn et al. 2005, Ren, Reiter et al. 2005, Shenoy and Lefkowitz 2005). Indeed, when either GRK5 or 6 were depleted using siRNA, the β-arrestins dependent ERK activation was completely abolished. Conversely, GRK2 and -3 tend to attenuate the β-arrestins -dependent ERK.

1.2.3.4 Other signaling activation

In addition to ERK activation, β-arrestins have the ability to scaffold various other key protein controling various signaling pathways. For example, β-arrestins are reported to scaffold AKT, PI3 kinase and phosphodiesterase 4 (PDE4) upon activation of the dopamine receptors (Beaulieu, J.M. et al. Cell, 2005). Additionally, β-arrestins interact with the inhibitor of nuclear factor κB (NF-κB) (IκB) resulting in IκB stabilization and eventual inhibition of the NF-κB activity (Witherow, Garrison et al. 2004). β-arrestins 1was also demonstrated to be involved in stress fiber formation induced by the small guanosine triphosphatase Ras (RhoA) pathway (Barnes, Reiter et al. 2005)

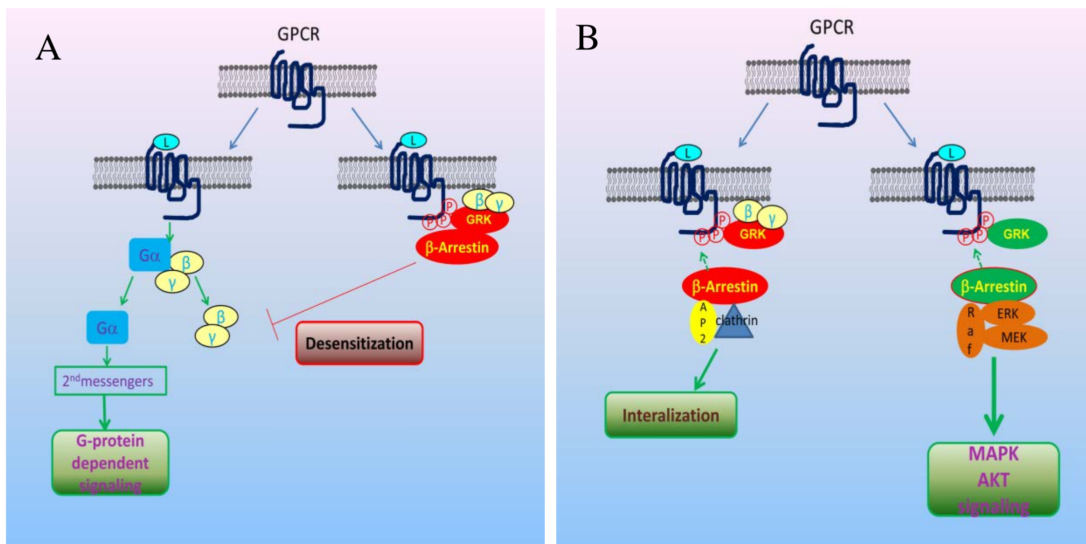

Fig 1. GPCR signaling activation. (**A**) G-protein dependent paradigm. Ligand binding stimulates heterotrimeric G protein diassociation and induces signaling activation. The receptor is phosphorylated by GRKs which recruit βarrestin binding. This GRK/βarrestin complex terminates G-protein signaling (desensitization). (**B**) β-arrestin dependent paradigm. β-arrestins not only induce desensitization of G protein–signaling but also mediate signaling.

1.2.3.5 β-arrestinestin Gene Knockouts

Considering the various roles of β-arrestins in the regulation and control of important receptors and signaling molecules, one would expect that genetic ablation of arrestins would have significant phenotypic consequences. β-arrestin1 knockout (−/−) mice display altered cardiac responsiveness to β-adrenergic receptor stimulation(Conner, Mathier et al. 1997). β-arrestin 2 knockout (−/−) mice demonstrate enhanced morphine analgesia(Bohn, Lefkowitz et al. 1999). However, a β-arrestin1/2 double knockout is embryonic lethal (Luttrell, Roudabush et al. 2001). Similarly, knockouts (−/−) of kurtz, the β-arrestin analog in Drosophila, display a broad lethal phase during embryogenesis (Roman, He et al. 2000).

As neither knockout displays such extreme abnormality, this implies that there is a functional redundancy between these two proteins. This hypothesis is supported by the fact that the two genes show extensive tissue expression overlap (Sterne-Marr, Gurevich et al. 1993) and functional redundancy in that they bind many receptors with comparable affinity (Gurevich and Benovic 1995).

1.2.4 β-arrestin biased signaling of GPCRs

Ligands bind to the relevant receptor with distinct affinity and exhibit intrinsic efficacy contributing to biological effects. Based on their affinity and efficacy, ligands of GPCR are classified into three classes: agonists (full or partial), inverse agonists (or antagonists) and neutral antagonists (Samama, Cotecchia et al. 1993). In this paradigm, full and partial agonists stabilize an active conformation, whereas inverse agonists stabilize an inactive one; by contrast, neutral antagonists do not affect the equilibrium between the two conformations. However, recently, substantial evidence challenges this concept. It has been show that the ability of ligands to selectively stabilize receptor conformations exhibit "imbalanced efficacies"(Kenakin 2002, Christopoulos, Christopoulos et al. 2003). For the GPCR it has been demonstrated that selected ligands can induce only G-protein signaling but no phosphorylation of GPCR with subsequent β-arrestin recruitment and receptor internalization (Whistler and von Zastrow 1998). On the other hand, few ligands have abilities to induce recruitment of β-arrestins and receptor internalization but fail to stimulate G-protein signaling. This indicates that G-protein activation is not sufficient and is not necessary for β-arrestins recruitment and activation(Wei, Ahn et al. 2003, Gesty-Palmer, Chen et al. 2006). Based on the above evidence the concept of biased agonism was developed where ligand stimulation stabilize only a subset of the receptor conformations and selectively activate a subset of intracellular signaling pathways (Reiter, Ahn et al. 2012).

The signaling activation, either balanced or biased, is a property of ligand-receptor interaction. Therefore, in addition to biased ligands, biased mutant receptor were created and use to prove the concept of "β-arrestins-biased" signaling. A biased mutant of AT1aR (DRY/AAY) defective in activating G-protein signaling, was reported to initiate β-arrestins ERK activation upon stimulation with either full agonist or β-arrestin biased agonist (Wei, Ahn et al. 2003). Furthermore, different GRKs isoforms have been reported to govern β-arrestins recruitment, thus controlling the balanced or biased signaling activation. For instance, type 1A receptor (AT1AR)(Kim, Ahn et al. 2005),V2R(Ren, Reiter et al. 2005) and β2AR(Shenoy, Drake et al. 2006) requires GRK5 and GRK6 for desentization, endocytosis and β-arrestins mediated signaling. Whereas, in V2R (Ren, Reiter et al. 2005) and H1 histamine receptor (Iwata, Luo et al. 2005), GRK2 negatively regulated receptor function. These results indicate that GRKs are important regulators of β-arrestins biased signaling of GPCR.

1.3 TYROSINE KINASE RECEPTORS

The receptors tyrosine kinases (RTKs) are a related family of about 60 cell surface receptors with similar structural and functional characteristics. RTKs are membrane proteins with an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. Excepting the dimer structure of the insulin receptor (IR) family, all known RTKs (e.g. EGFR, PDGFR) are expressed as monomers at the cell surface. The growth factors are unable to cross the cell membrane and they exert their effects via binding to cell surface receptors, most of them RTKs. Following interaction of polypeptide growth factors with their specific transmembrane receptors, a cascade of intracellular signals results in the activation or repression of various subsets of genes (Schlessinger 2000, Jimenez, Shvartsman et al. 2012). Although they are few compared with the large number of GPCRs, RTKs receive particular interest for targeted therapy in cancer. Among them, IGF-1R is considered as one of the most attractive target for cancer therapy. This is due to the essential role the IGF-1R plays in cancer development. IGF-1R is responsible for the transformation, proliferation and metastasis of malignant cells, and maintains the malignant phenotype (Baserga 1995, Baserga 2000, Girnita, Wang et al. 2000, Girnita, Girnita et al. 2003), (Baserga 1995, Baserga 2000, Yu and Rohan 2000, Baserga 2005).

1.3.1 IGF system

The insulin-like growth factor (IGF) system is critical for normal growth and development as well as for metabolism regulation. The IGF system is also implicated in several human diseases other than cancer, including diabetes, metabolic syndromes, endocrinology disorders, psoriasis and arteriosclerosis.

The IGF system consists of two ligands (IGF-1 and IGF-2), three cell membrane receptors (IGF-1R, IR and IGF-2R) and six high-affinity IGF binding proteins (IGFBP-1-6). Additionally, new members of the IGF family have been described, like the insulin receptor related receptor (IRR) (Dandekar, Wallach et al. 1998) (Zhang and Roth 1991) and the IGF-1R/IR hybrid receptor (Treadway, Morrison et al. 1989, Frattali, Treadway et al. 1992).

1.3.2 The IGF ligands

The IGF ligands, IGF-1 and IGF-2 are major growth factors, while insulin mainly regulates glucose uptake and cellular metabolism. IGF-1 and 2 are named for their primary structural homology to proinsulin and were isolated and sequenced in 1978 (Rinderknecht and Humbel 1978, Rinderknecht and Humbel 1978). IGF-1 and IGF-2 share a 62% homology in amino acid sequence, and there is a 40% homology between the IGFs and proinsulin (Furstenberger and Senn 2002). Most of the circulating IGF is produced by hepatocytes in response to growth hormone stimulation (Arany, Afford et al. 1994, Olivecrona, Hilding et al. 1999) and of which more than 90% are bound to

IGFBP-3. Multiple tissues are also able to synthesize IGFs , which can act locally in autocrine or paracrine loops, in addition to their endocrine modes (Cohen and Rosenfeld 1994).

Serum IGF-1 levels increase following the secretion from the liver to the bloodstream. In turn, increasing IGF-1 negatively regulates the hypothalamus and pituitary gland, reducing GH secretion from the anterior pituitary gland (Rosen and Pollak 1999).

Liver-specific disruption of the IGF-1 gene in mice did not affect growth, development and sexual maturation, assumed to be because the autocrine/paracrine IGF circuits are sufficient for normal growth and development (Werner, Shalita-Chesner et al. 2000). Unlike IGF-1 mRNA, IGF-2 mRNA levels in all tissues are high during late fetal and prenatal periods, and decline thereafter. In humans, however, IGF-2 can be detected in the circulation at adult stages (Yu and Rohan 2000).

Deletion of the paternally imprinted IGF-2 gene results in low fetal weight (Constancia, Hemberger et al. 2002). Both IGF-1 and IGF-2 can bind to IGF-1R. However, IGF-I binds with higher affinity to IGF-1R while IGF-2 binds to IGF-1R with lower affinity than IGF-1. IGF-II also binds to IGF- II R (Federici, Porzio et al. 1997, Le Roith 2003).

1.3.3 IGF binding proteins (IGFBPs)

Normally about 99% of IGF in circulation is bound to the IGF-binding proteins (IGFBPs) and less than 1% is free (Fuglsang, Lauszus et al. 2003). IGFBPs are the most important factor to regulate bioavailability and activity of IGFs in circulation and local tissue. This family includes sex proteins IGFBP1–6 which bind the IGFs with high affinities (Clemmons 1998, Rosenfeld, Hwa et al. 1999). The regulation function of IGFBPs to IGFs exerts two effects. One is to act as “transport proteins” and prolong the half-life of IGF-1and IGF-2 in circulation (Zapf 1995); on the other hand, they can also reduce the activity of IGFs by competitively inhibiting IGF binding to their receptors (Gockerman, Prevette et al. 1995, Baxter 2000). The capacity of binding affinity can be reduced when local proteases cleave IGFBPs into fragments and release IGF-1. The status of phosphorylation and the adhesion to cell surface can influence the binding affinities as well.

Among the IGFBPs, an early study reported that IGFBP-3 can regulate IGF-1 signaling by acting as a competitive inhibitor for IGF-1 (Collett-Solberg and Cohen 1996). On the other hand, IGFBP-3 is also reported to have an IGF-independent inhibitory effect on cell growth which is mediated through a specific cell membrane receptor (Firth, McDougall et al. 2002). In addition, in specific contexts, overexpression of IGFBPs (e.g. IGFBP-2 and IGFBP-5) is associated with increased, rather than decreased, IGF action; adverse effects on cancer prognosis (Pollak 2008).

1.3.4 IGF receptor

The IGF1R and IR are members of the tyrosine kinase family of membrane receptors. The IR exists in two splice variant isoforms: IR-B recognizes only insulin, but IR-A, which is the isoform most commonly expressed in tumors, recognizes all IGF system ligands (Belfiore, Frasca et al. 2009).

The human IGF-1R gene encodes for a single chain 180 kDa 1367 amino acid precursor. Cleavage of the precursor generates α and β subunits which are glycosylated and dimerized. These subunits, through disulfide bonds, form a tetramer (β-α-α-β) that is transported to the plasma membrane (Carlberg, Dricu et al. 1996, Jansson, Hallen et al. 1997, Wang, Xie et al. 1999). The two entirely extracellular α subunits contain the IGF binding site, while the two β subunits span the membrane and in the intracellular region contain the tyrosine kinase domain (Steele-Perkins, Turner et al. 1988). The α-subunit contains 706 amino acids and has L1 and L2 homologous domains containing cysteine-rich domains for ligand binding (Andersen, Kjeldsen et al. 1990, Gustafson and Rutter 1990, Kjeldsen, Andersen et al. 1991, Schumacher, Mosthaf et al. 1991, Zhang and Roth 1991). These regions are the most hydrophilic sequences of the extracellular domain and are likely to be exposed on the surface of this domain and function in defining ligand specificity (Ullrich, Gray et al. 1986). The β-subunit contains 627 amino acid residues and has three domains: the extracellular, transmembrane and intracellular domains. The extracellular domain of the β-subunit is 196 amino acids in length, while the transmembranous domain is 24 amino acids in length, located at position 906-929. The intracellular part of the β-subunit can be divided into a juxtamembrane, a tyrosine kinase (TK) and a C-terminal domain.

The TK domain exhibits the highest homology between the IGF-1R and IR (84%) and the juxtamembranous domain shares 61% homology, whereas the C-terminal domain shares only 44% (Ullrich, Gray et al. 1986). Within the TK domain a cluster of three tyrosine residues, located at position 1131, 1135 and 1136, is critical for receptor autophosphorylation (LeRoith, Werner et al. 1995).

Furthermore, tyrosine residue 950 is the binding site for insulin receptor substrates (IRS1-4) and a catalytic lysine in position 1003 are essential for ATP binding (Hanks, Quinn et al. 1988). Mutation analysis approaches facilitate understanding of the function of distinct residues in IGF-1R signaling. Mutation at Y950 preserves the mitogenic effect in response to IGF- 1 with lower level. Mutation at the lysine 1003 results in a non-functional receptor, whereas, mutations at the three tyrosine residues of the activation loop result in a completely inactive receptor (Baserga 2000).

The C-terminal domain (1229-1337) of IGF-1R is essential for IGF-1R signaling. However, its function in normal and malignant cells is not fully understood. Mutation analysis studies and recent data facilitate to understand the function of these residues in IGF-1R signaling transduction and transformation. C-terminal truncated IGF-1R does not affect IGF-1R tyrosine phosphorylation as well as the phosphorylation of IRS1 and AKT activation (Sehat, Andersson et al. 2008). However, C-terminal mutated IGF-1R decrease β-arrestin1 dependent ERK activation (Girnita, Shenoy et al. 2007, Zavros, Waghray et al. 2007) and inhibit IGF-1R ubiquitination (Sehat, Andersson et al. 2007), which suggest that C-terminal domain is critical for β-arrestin1 dependent signaling. On

the other hand, truncation at residue 1229 or 1245 maintains receptor's mitogenesis function in response to IGF-1 but such receptors are no longer transforming (Baserga, Hongo et al. 1997). Truncation of IGF-1R at residue 1290 has no effect on the mitogenicity or the transforming ability of the IGF-1R. These results indicate that the IGF-1R domain essential for transformation may be present between residues 1245 to 1290. Serine 1248 has been reported to be required for RACK1 binding (Kiely, Sant et al. 2002). Serines 1250/1251 restore IGF-1R mitogenesis function (Leahy, Lyons et al. 2004). while mutation at residues 1289-1294 is sufficient to abolish transforming ability of IGF-1R, however no information about the effect on signaling is reported (Baserga 2004).

As a consequence of high homology of IGF-1R and 1R, hybrid receptors can be formed by an insulin α/β hemireceptor and an IGF-1 α/β hemireceptor, in cells expressing both of them. These hybrid receptors have high affinity to bind IGF-1 and IGF-2 similar to IGF-1R but very low affinity to bind to insulin. The biological response elicited by these hybrid receptors can vary, depending on the ligands involved and the specific IR-A or IR-B isoforms (Pandini, Frasca et al. 2002).

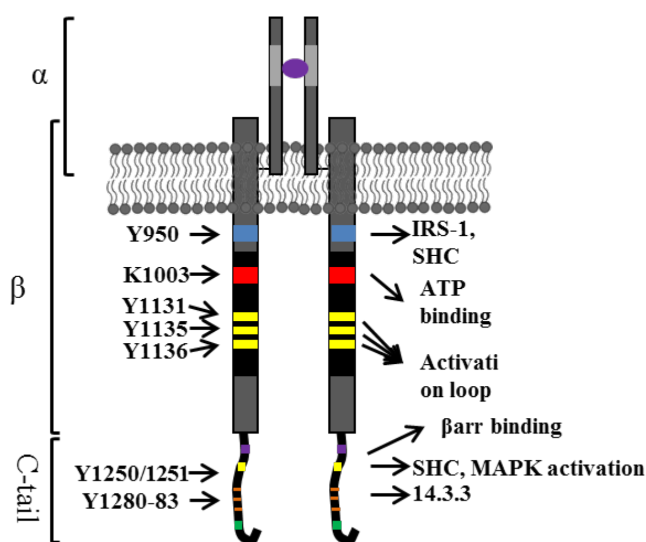

Fig2. Cartoon of IGF-1R structure.

1.3.5 Receptor activation and signaling

1.3.5.1 Phosphorylation

Phosphorylation of IGF-1R was considered to be the central process driving receptor signaling. IGF-1 binding to α-subunit of the IGF-1R causes receptor conformational changes which initiate auto-phosphorylation of the activation loop at tyrosines 1136, 1135 and 1131 in the intracellular β subunit (Favelyukis, Till et al. 2001). In unstimulated state, the activation loop (a-loop), containing the critical tyrosine (Y) residues 1131, 1135 and 1136, behaves as a pseudo-substrate that blocks the active site. Y1135 in the a-loop is bound in the active site preventing the substrate access and occluding the ATP binding site as well. After ligand binding, the three tyrosines of the a-loop are transphosphorylated by the dimeric subunit partner. Initial

phosphorylation of Y1135 induces stable structure followed by phosphorylation of Y1131 and Y1136. The catalytic kinase activity is increased due to opening of the a-loop (Favelyukis, Till et al. 2001). The conformation changes of the a-loop allow ATP binding and the access of substrate proteins.

1.3.5.2 Ras/Raf/MAPK

Ras is a single small GTPase molecule related in structure to the Gα subunit of heterotrimeric G proteins. G proteins possess the ability to switch the cycle of inactive nucleotide guanosine diphosphate (GDP) bound and active guanosine triphosphate (GTP) bound states. In basal conditions, Ras stays in the GDP bound status and is inactive, while upon extracellular stimuli, Ras binds to GTP in a signal emitting configuration. This signal is terminated when GTP is hydrolyzed by using its intrinsic GTPase-activating proteins and return back to inactive state. This cyclic GDP/GTP process is catalyzed by guanine nucleotide exchange factors (GEFs) (Vetter and Wittinghofer 2001).

In the context of IGF-1R signaling, following phosphorylation of the activated IGF-1R, Src Homology 2(SH2)-containing protein Src homology collagen(Shc) becomes activated by phosphorylation and recruits the growth factor receptor-binding protein 2 (Grb2). Grb2 interacts with the son of sevenless (SOS), a GEF at the plasma membrane, to stimulate GDP dissociation and its rapid replacement by GTP, whose intracellular concentration is much higher than that of GDP (Scheffzek and Ahmadian 2005). Once Ras binds GTP, the effector loop is able to interact with alternative signaling partners, termed Ras effectors. The most important effector is Raf kinase. Raf can phosphorylate serine/threonine residues. The interaction between Ras and Raf recruits the Raf kinases (A-RAF, B-RAF and C-RAF) to the plasma membrane(Chong, Vikis et al. 2003, Wellbrock, Karasarides et al. 2004) . Among the three Raf kinases, B-RAF binds best to Ras(Weber, Slupsky et al. 2000) and is the most active in phosphorylating MEK *in vitro* (Emuss, Garnett et al. 2005) and in transforming NIH3T3 cells in culture (Papin, Denouel-Galy et al. 1998).

The association of Raf with Ras results in configuration change of Raf kinase, which leads to the activation of a second kinase known as MEK as well as Raf . MEK phosphorylates and activates a mitogen-activated protein kinase (MAPK) which were originally termed "extracellular signal-regulated kinases" (ERKs). This signaling cascade is called MAPK pathway. MAPK pathways contain three-tiered kinase modules: ERK1 and ERK2 are at the bottom of this cascade and are considered as MAPKs. They are phosphorylated by a dual specificity kinase termed MAP2 kinase (MAPKK). Similarly, MAP3 kinase (MAPKKK), like Raf, is the kinase responsible for phosphorylation of MAP2 kinase which is activated at the membrane and initiates the cascade.

MAPK groups include 4 distinct cascades: 1) extracellular signal-regulated kinase 1 and 2 (ERK1/2); 2) c-Jun N-terminal kinase 1 to 3(JNK1-3); 3) p38MAPK α, β, γ, and δ (p38α-δ); and 4) ERK5 (also known as Big MAPK1). The ERK1/2 cascade transmits mostly mitogenic signals, whereas the p38 and JNK cascades transmit mainly stress signals. ERK5 seems to play a role in both mitogenic and stress-response processes.

Activation of ERK kinases have been identified to phosphorylate about 200 distinct substrates and regulate various ERK1/2 dependent cellular processes in cytoplasm, cytoskeleton and nucleus(Yoon and Seger 2006). Translocation of activated ERK to the nucleus has been proved to be the most essential function of MAPK signaling on the regulation of proliferation, differentiation, and oncogenic transformation. The main effectors of ERK activated in the nucleus are transcription factors. One of the best-studied ERK1/2-activated transcription factors is Elk1, an immediate early gene (IEG)(Marais, Wynne et al. 1993). The phosphorylation of Elk1 results in up-regulation and stabilization of the growth regulated gene c-Fos, which is important for proliferation and differentiation. Stabilization of c-Fos is achieved only when ERK1/2 activation is sustained long and strong enough and subsequent c-Fos significant expressed(Eferl and Wagner 2003).

ERK activation also enhances transcription of the cyclin *D1* gene and induces phosphorylation of the pRb which is tumor suppressor gene followed the release of the E2F-1 transcription factor to promote G_1 to S phase progression(Murphy, MacKeigan et al. 2004).

The mechanism of translocation of activated ERK to the nucleus is still unclear. The early studies demonstrated that molecules transfer into the nucleus through specialized nuclear pores (NPCs) which allow free diffusion of small molecules and proteins (up to 40 kDa) to the nucleus (Nigg 1997). Later data shows that ERK1/2 translocation seems to be mediated by binding to protein importin7(Lorenzen, Baker et al. 2001). Recent studies illustrated that the dissociation of two threonine and tyrosine residues of ERK allows the nuclear translocation signal (NTS) domain of ERK to interact with importin7 preceding nuclear translocation(Zehorai, Yao et al. 2010).

Aside from the regulation of cell proliferation in nucleus, ERK activation is also sustained in cytoplasm to induce distinct signaling activation. The Mnk1 kinase, a cytoplasmic substrate of ERK1 and ERK2, activates the translation initiation factor eIF4E, following the activation of cellular machinery responsible for protein synthesis. Additionally, ERK activation can be regulated by cytoskeletal elements. It was reported that the interaction of microtubule and actin filaments with ERK1/2 resulted in restricted nuclear entry of activated ERK1/2(Smith, Smedberg et al. 2004).

1.3.5.3 PI3K/AKT

Phosphorylation of tyrosine residues of the β subunit of IGF-1R recruits adaptors such as IRS family proteins, which bind to the regulatory p85 subunit of Phosphatidylinositol 3-kinases (PI3Ks) and initiate the activation of AKT.

IRS1 has three domains: an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding domain (PTB) and a C-terminal domain (Wang, Myers et al. 1993). PI3K are heterodimeric kinases that are composed of an 85 kDa regulatory (p85) and a 110 kDa catalytic subunit (p110). Interaction of p85 and IRS1 allow the activation of catalytic p110 subunits of PI3K. Activated PI3K phosphorylates the lipid

phosphatidyl-inositol bisphosphate (PIP2) to generate phosphatidyl-inositol trisphosphate (PIP3). These phospholipids function as ligands for pleckstrin-homology (PH) domain-containing proteins to the inner surface of the cell membrane. AKT/PKB is a serine threonine kinase which interacts with these phospholipids causing its translocation to the inner membrane and activation by the 3-phosphoinositide-dependent protein-kinases (PDKs). PDK1 phosphorylates AKT on threonine 308 in the a-loop of the kinase domain, while PDK2 phosphorylates serine 473 of AKT to regulate its efficiency of activation. The AKT signaling activation controls numerous cellular processes including cell proliferation and anti-apoptosis signals.

Phosphorylation of AKT activates mTORC1 signaling, which regulates protein synthesis by activating p70S6K and inactivating 4EBP1 (Inoki, Li et al. 2002). p70S6K is a positive effector to regulate protein synthesis and cell size(Ruvinsky and Meyuhas 2006). Upon phosphorylation by mTORC1, 4EBP1 dissociates from the cap-binding protein (eIF4E) and initiates the formation of the 4F translational initiation complex (eIF4F) complex to start the translation of the oncogenic cap-mRNAs, including MYC, HIF-1, cyclin-D1 and Bcl-XL(De Benedetti and Graff 2004). On the other hand, mTORC1 mediates negative feedback to AKT through IRS1degradation (Harrington, Findlay et al. 2004). mTORC2 has PDK2 activity and can phosphorylate AKT on Ser473. AKT could also activate NF-κB by regulating activity of IκB resulting in increased transcription of pro-survival genes.

Activation of AKT plays an essential role in anti-apoptosis via mitochondrial effectors. In the absence of Bad phosphorylation, cytochrome c is released from mitochondria by proapoptotic proteins such as Bax from the inhibitory control of Bcl2, and activates caspases 3 and 9 followed by degradation of DNA which occurs in the apoptosis process (Bouchard, Rouleau et al. 2003). When Bad is phosphorylated by activated AKT, Bcl2 dissociated from Bad-Bcl2 complex and inhibit Bax to release caspases exerting anti-apoptotic effect (Hanahan and Weinberg 2000). In addition to Bad, AKT activation also inhibits other pro-apoptotic proteins, including caspase 9, survival transcription factor CREB, glycogen synthase kinase- 3β(GSK-3β), as well as forkhead transcription factors (FOXO) (Vanhaesebroeck and Alessi 2000).

Another essential event involved in the anti-apoptosis function of activated AKT is related to Mdm2 regulation. Activated AKT can phosphorylate Mdm2 at serines 166 and 186 following mitogen-induced activation (Zhou, Liao et al. 2001). Phosphorylation on these sites is necessary for translocation of Mdm2 from the cytoplasm into the nucleus. Expression of constitutively active AKT promotes nuclear entry of Mdm2, diminishes cellular levels of p53 and decreases p53 transcriptional activity.

PI-3K/AKT activation is regulated by the phosphatase and tensin homolog (PTEN) signaling pathway. PTEN dephosphorylates the PIP2 and PIP3 second messengers on the 3'-position of the inositol ring of PI-3K leading to its inhibitory effect on AKT activation(Downes, Perera et al. 2007). Thus, PTEN acts as a potent regulator of IGF-1R signaling.

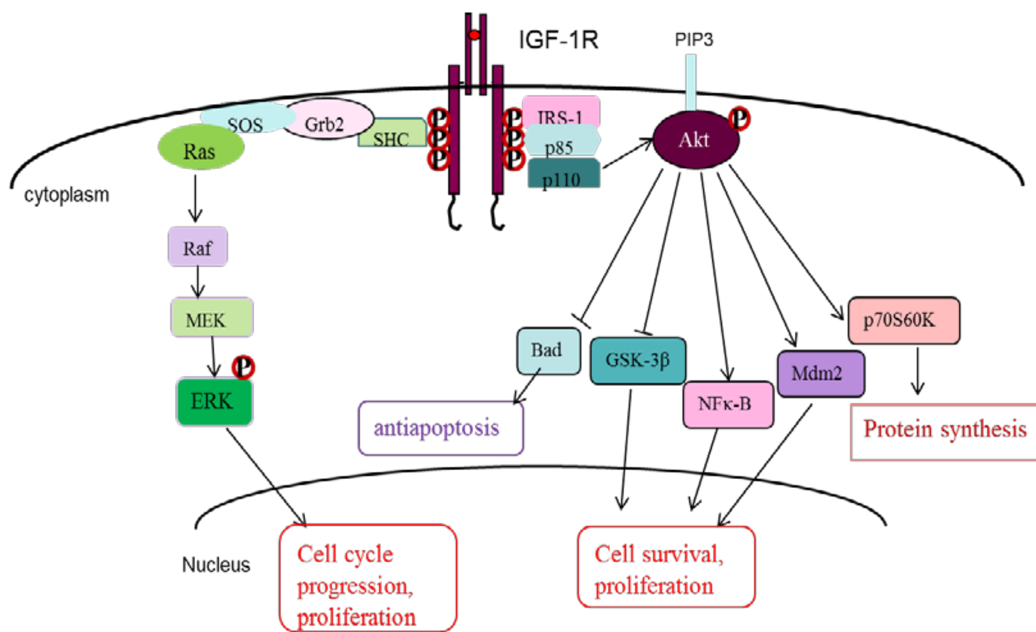

Fig3.Classical IGF-1R signaling.

1.3.6 Ubiquitination

IGF-1R is classified as an RTK and accordingly tyrosine phosphorylation was considered to be the central process governing IGF-1R signalling. However, during the last decade, we and others have challenged this view by demonstrating the involvement of ubiquitination in IGF-1R function (Girnita, Girnita et al. 2003).

Ubiquitination (or ubiquitylation) of proteins requires the action of three enzymes. The first one is ubiquitin-activating enzyme (E1) that binds the 76 amino acid protein ubiquitin(Ub) in an ATP dependent manner to generate a high energy E1-ubiquitin intermediate. The second one constitutes ubiquitin-conjugating enzyme (E2), acting as a carrier protein linking to E1 ubiquitin complex with its cysteine residue. The third, the ubiquitin ligase (E3) play a central role in ubiquitination. They recognize specific substrates and facilitates ubiquitin transfer from the E2 to the substrate resulting ultimately in the covalent attachment of Ub to the ε-amino group of a lysine (Lys) residue in the target protein (Bonifacino and Weissman 1998, Glickman and Ciechanover 2002). Two major classes of E3s have been identified: HECT (homologous to E6AP C-terminus) domain E3s form a catalytic ubiquitin intermediate on a cysteine residue before E2 transfer(Scheffner and Staub 2007). The second class of E3s, which contains RING-type (i.e. Mdm2, c-Cbl) facilitates the direct transfer of Ub from E2 onto the substrate (Fang, Jensen et al. 2000). RING E3 ubiquitin ligases play an essential role in the regulation of many biological processes and defects in some of them are involved in cancer development. Furthermore, some RING E3 ligases are frequently overexpressed in human cancers. Another subclass of RING ubiquitin ligases cullin RING ligases (CRLs) composed of multi-subunit E3 ligases E3 enzymes. CRLs are multicomponent E3 ligases composed of a RING domain-containing protein

(RBX1 or RBX2), a regulatory cullin, and a substrate-binding adaptor (Zimmerman, Schulman et al. 2010).

Different types of ubiquitination modification control the fate of substrate proteins. Mono- ubiquitination, in which a single ubiquitin molecule is attached to a single site on a protein, results in receptor endocystosis and signal transduction(Robzyk, Recht et al. 2000). In contrast, old or damaged cytosolic proteins are labeled with a poly-ubiquitin chain, in which several ubiquitin moieties are added to the target protein to form poly-ubiquitin chains, is recognized for degradation by the proteasome. In addition to these types of ubiquitination, different ubiquitin -linkage topologies are associated with diverse biological functions. For instance, Lys48-linked ubiquitin chains initiate targeted degradation by the 26S proteasome(Thrower, Hoffman et al. 2000, Petroski and Deshaies 2003, Rodrigo-Brenni, Foster et al. 2010). Lys63 or -linked ubiquitin chains may regulate signaling pathways (Deng, Wang et al. 2000, Kanayama, Seth et al. 2004), DNA repair(Spence, Sadis et al. 1995) and protein location(Hoege, Pfander et al. 2002). Recycling of ubiquitin is regulated by specific deubiquitinating enzymes (DUBs) that catalyze the removal of Ub-moieties from ubiquitinated proteins. DUBs as regulators for ubiquitination reactions are suggested to play important role in the regulation of various cellular processes, including sorting of membrane receptors (Clague and Urbe 2006).

Proteins modified by ubiquitination are degraded through both the proteasome and lysosomal pathways in mammalian cells (Hicke 1999). The 26S proteasome is the common form of proteasome and contains one 20S core particle structure and two 19S regulatory caps. Polyubiquitinated proteins are recognized by19S regulatory subunits and are transferred to the catalytic core of 20S core in an unfolded form resulting in degradation. Degradation of several mammalian receptors is impaired by inhibitors of the proteasome as well as by agents blocking the lysosomal degradation (Bonifacino and Weissman 1998, Glickman and Ciechanover 2002).

Three distinct E3 ligases, Nedd4; Mdm2 and Cb1, have been proven to be important for IGF-1R ubiquitination(Girnita, Girnita et al. 2003). Nedd4 is a member of the HECT domain E3 ligases and Nedd4 mediated IGF-1R ubiquitination requires the adaptor protein Grb10 for receptor internalization (Vecchione, Marchese et al. 2003). c-Cbl and Mdm2 are members of RING finger group of E3 ligases. In the case of Mdm2 mediated IGF-1R ubiquitination, β-arrestin1 is the adaptor protein to bring Mdm2 to activated receptor for ubiquitination (Girnita, Shenoy et al. 2005) and degradation (Girnita, Girnita et al. 2003).

1.3.7 Mdm2

Mdm2(murine double minute-2) is a well-known oncoprotein which suppresses p53 activity. In unstressed conditions, Mdm2 monoubiquitinates p53 leading to export of p53 from the nucleus to the cytosol followed by degradation by the proteasome. Overexpression of Mdm2 results in reduces of p53 level and activity(Kubbutat, Jones et al. 1997), whereas loss of Mdm2 induces apoptosis mediated by p53(de Rozieres, Maya et al. 2000). P53 transcriptionally activates many target genes, one of which is the Mdm2 gene. Because Mdm2 inhibits p53 activity, this forms a negative feedback

loop that tightly regulates p53 function. In turn, decreased p53 activity results in decreased Mdm2 to constitutive levels. Mdm2 can also ubiquitinate itself and induce its own degradation. Upon DNA damage, p53 is phosphorylated and acetylated to inhibit interactions with Mdm2 leading to cell cycle arrest through increased expression of tumor suppressor protein ARF. By contrast, ARF interacts with Mdm2, blocking Mdm2 shuttling between the nucleus and cytoplasm. Sequestration of Mdm2 in the nucleolus thus results in activation of p53 (Ganguli and Wasylyk 2003).

Mdm2 has been found to associate with certain cell surface receptors and regulate their functions (Shenoy, McDonald et al. 2001)). It was shown that under conditions when p53 was inhibited, Mdm2 was redistributed and bound to the IGF-1R (Girnita, Girnita et al. 2003). Mdm2 was proven to ubiquitinate the IGF-1R and degrade it in a proteasome-dependent manner , eventually leading to cell death (Girnita, Girnita et al. 2003). This action of Mdm2 explains earlier results that inhibition of wild-type p53 unexpectedly leads to downregulation of the IGF-1R (Girnita, Girnita et al. 2000). On the other hand, an increased distribution of Mdm2 to the cell nucleus to interact with p53 may indirectly increase the expression of IGF-1R since less cytoplasmic Mdm2 will be available to ubiquitinate and degrade the receptor. Reciprocally, the IGF-1 system has been shown to influence the activity of Mdm2. IGF-1 was demonstrated to regulate Mdm2 activity by inhibiting the association between $p19^{ARF}$ and Mdm2 in a p38 MAPK-dependent manner (Heron-Milhavet and LeRoith 2002). Thus, when IGF-1 was used to rescue the cells from UV-induced DNA damage, the p53 protein was degraded through the Mdm2-mediated pathway. Other studies indicate that expression of phosphorylated AKT increases Mdm2-mediated ubiquitination of p53 (Mayo and Donner 2001). The serum-induced increase in p53 ubiquitination was blocked by a PI3K inhibitor, suggesting that phosphorylated AKT enhances the ubiquitination-promoting function of Mdm2, determining reduction of p53 protein.

1.4 β-arrestin paradigm in IGF-1R

It has been shown that β-arrestin1 is acting as a scaffold protein for recruitment of the E3 ligase Mdm2 to the activated IGF-1R followed by receptor ubiquitination and degradation(Girnita, Shenoy et al. 2005). Co-immunoprecipitation studies discovered that IGF-1R, β-arrestin and Mdm2 complex were associated upon IGF-1 treatment. Furthermore, addition of β-arrestin1 *in vitro* to IGF-1R, E1, E2, and Mdm2 greatly enhances IGF-1R ubiquitination. On the other hand, depletion of β-arrestin1 by siRNA inhibit IGF-1R ubiquitination in melanoma cell lines. Consistently, the cells expressing dominant negative Mdm2 exhibit reduced IGF-1R ubiquitination (Girnita, Girnita et al. 2003) .This Mdm2/β-arrestin mediated IGF-1R ubiquitination machinery and its functional consequences are also supported by previous findings. For example, microinjection of a β-arrestin 1 antibody was observed to specifically inhibit IGF-1 mitogenic actions but had no effect on epidermal growth factor or insulin action(Dalle, Ricketts et al. 2001).

β-arrestins have been discovered to play important roles in the transduction of several signaling pathways stimulated by GPCRs (Lefkowitz 2004, Lefkowitz and Shenoy 2005). Moreover, some previous studies have suggested involvement of β-arrestin in MAPK signaling activation by the IGF-1R (Dalle, Ricketts et al. 2001).

Recent studies demonstrate that β-arrestin1 involvement in IGF-1-induced signaling to ERK1/2 is dependent on Mdm2 mediated β-arrestin1 ubiquitination (Girnita, Shenoy et al. 2005). In this process, β-arrestin1 was shown to bind to the C terminus of the IGF-1R and become ubiquitinated by the Mdm2 E3 ligase.

Taken together, β-arrestin1 plays a dual role in regulation of IGF-1R: ubiquitination and receptor down-regulation as well as IGF-1R/β-arrestin1 mediated signaling activation of the MAPK pathway, which is consisted with the dual regulatory role of β-arrestin1 in GPCR mediating desensitization and downstream MAPK signaling activation (Lefkowitz 2004, Lefkowitz and Shenoy 2005, Shenoy, Drake et al. 2006).

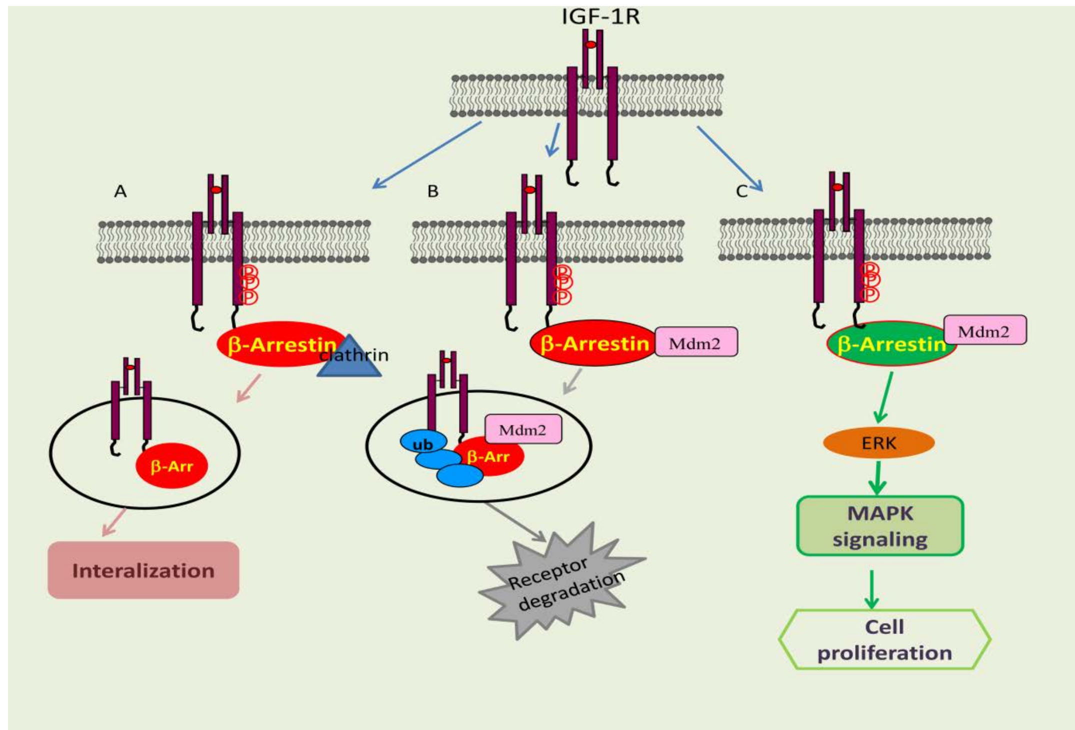

Fig4.βarrestin paradigm mediated IGF-1R signaling. A. βarrestin recruit to activated IGF-1R and induces receptor internalization in a clathrin coat manner. B. βarrestin binding recruits Mdm2 to the activated IGF-1R resultings in receptor ubiquitination and degradation. C. βarrestin- dependent ERK activation.

1.5 ROLE OF IGF-1R IN CANCER

1.5.1 IGF-1R and oncogenes

The IGF-1R gene is constitutively expressed in most cells. The IGF-1R promoter , which is CG-rich and lacks TATA and CCAAT elements, exhibits a high basal transcriptional activity. IGF-1R level is regulated by physiological conditions including nutritional factors (Olchovsky, Song et al. 1993), hormonal stimulation , the developmental stage(Zhou and Bondy 1992) ,and cellular factors including transcription factor (Werner and Roberts 2003), oncogenes (Kim, Park et al. 1996) and suppressor genes(Maor, Abramovitch et al. 2000) .

Tumor suppressors, including the breast and ovarian cancer susceptibility gene 1 (BRCA1), p53, and the Wilms' tumor protein-1 (WT1) are reported to control IGF-1R transcription level(Sarfstein, Maor et al. 2006). Among them, the p53 and IGFs axis connection attracts most interest from researchers. P53 acts as a tumor suppressor factor to suppress almost 90% IGF-1R promoter activity as well as endogenous IGF-1R mRNA levels (Prisco, Hongo et al. 1997). By contrast, mutant p53 clearly enhances IGF-1R gene expression (Idelman, Glaser et al. 2003). In addition, wild-type p53 reduce the IGF-1R phosphorylation in response to IGF-1, while mutant p53 increases receptor phosphorylation (Ohlsson, Kley et al. 1998).

WT1, a member of this family, has been shown to bind to the IGF-1R promoter and to suppress activity (Werner, Hernandezsanchez et al. 1995). Consistently, loss of WT1 activity in Wilms' tumour and related malignancies may result in transcriptional derepression of the IGF-1R gene(Gerald, Rosai et al. 1995). Pathologic fusion of the Ewing gene EWS to WT1 (t(11;22)(p13;q12)(EWS/WT1)) has been shown to abrogate the tumour suppressor function of WT1 and to generate an oncogenic chimeric protein capable of binding and activating the IGF-1R promoter (Karnieli, Werner et al. 1996).

Some of the oncogenes increasing the IGF-1R promoter activity can also affect IGF-1R action by nontranscriptional mechanisms. For instance, transformation of human cells by the Src oncogene of the Rous sarcoma virus results in constitutive phosphorylation of the receptor β-subunit to enhance receptor mediated signaling(Werner and Le Roith 2000).

1.5.2 Transformation

The involvement of the IGF-1R in malignant transformation was first observed in Mouse embryo fibroblasts (MEFs) derived from homozygous IGF-1R null mice (R- cells) (Sell, Rubini et al. 1993) . R- cells are resistant to transformation by several oncogenes, including the SV40 T antigen, activated H-Ras, bovine papillomavirus E5 protein, human papillomavirus E7 protein, Ewing's sarcoma fusion protein, activated c-Src and others (Baserga, Hongo et al. 1997). The only two proteins known so far to sustain oncogenic properties in the absence of IGF-1R is v-Src (Valentinis, Morrione et al. 1997) and constitutively active Gq13α (Liu, Blakesley et al. 1997). Cellular transformation and progression of several types of sarcoma, including rhabdomyosarcoma, synovial sarcoma, leiomyosarcoma, Ewing's sarcoma and osteosarcoma are influenced by IGF-1R (Rikhof, de Jong et al. 2009).

1.5.3 Proliferation and anti-apoptosis

IGF-1R mediated MAPK/PI3 K activation has been proven to support cancer progression through enhancement of mitogenesis or suppression of apoptosis(Baserga, Peruzzi et al. 2003).Promoting cell cycle and escaping from cell cycle arrest is the common character of tumor cells. IGF-1R mediated MAPK promotes Cyclin D1 expression while AKT activation prevents Cyclin D1 nuclear export and degradation by inhibiting GSK-3β activity (Diehl 2002). This cell cycle progression in breast cancer cells can be reduced by PI3K inhibitors. Activity of c-myc, a transcription gene

promoting survival, can be stimulated by activation of NF-κB followed by AKT activation (Datta, Brunet et al. 1999).

1.5.4 Migration and metastasis

Cancer metastasis is composed of multiple processes, including tumor cell adhesion, migration, extracellular matrix (ECM) proteolysis and invasion (Miyata Y, Prostate, 2003*)*. Accumulated studies support the IGF axis regulatory role in each of these processes both in vitro (LeBedis, Chen et al. 2002, Fizazi, Yang et al. 2003) and in vivo(Lopez and Hanahan 2002, Lay, B et al. 2005, Sakatani, Kaneda et al. 2005).

Proteolytic activities of the matrix metalloproteinase (MMP) system are essential for malignant cell migrated through the ECM and the basement membrane. The IGF system plays a role in the regulation of MMPs group (MMP-2, MMP-9 and MMP-14) and promotes tumor invasion. Overexpression or inhibition of IGF-IR modifies MMP-2 expression levels in a liver metastasis model (Yoon and Hurta 2001). IGF-1 dramatically increases MMP-9 activity and enhanced cell migration through vitronectin in breast cancer cell line(Zhang and Brodt 2003). This metastasis advantage driven by IGF-1R can be reduced by an IGF-1R inhibitor in uveal melanoma xenografted mice. The expression and activity of MMP-2 also decreases in uveal melanoma cells as well as in tumors (Girnita, All-Ericsson et al. 2006).

IGF-1 is reported to induce angiogenesis by stimulating the migration and morphological differentiation of endothelial cells (Lee, Bae et al. 2000). Another direct effect of IGF on angiogenesis is demonstrated in mice with vascular endothelial cells knockout IGF-1R or IR, in which remarkable reduction of retinal vessel formation is observed (Economou, Andersson et al. 2008). IGF-1 and insulin are both involved in regulation of vascular mediators VEGF (Grulich-Henn, Ritter et al. 2002).

1.6 TARGETING IGF-1R IN CANCER

Targeting IGF-1R and its signaling pathway has become an attractive strategy for anti- cancer therapy. Overall, several approaches have been developed to target the IGF-1R axis: small-molecule tyrosine kinase inhibitors (TKI), antibodies targeting IGF-1R and antibodies that target IGF ligands(Cohen, Baker et al. 2005, Goetsch, Gonzalez et al. 2005, Giles, Rizzieri et al. 2007, Haluska, Shaw et al. 2007, Higano 2007, Yin, Vreeland et al. 2007, Lacy, Alsina et al. 2008, Pollak 2008, Karp, Pollak et al. 2009, Ranchal, Gonzalez et al. 2009).

Table 1. Targeting IGF-1R strategies

Anti-IGF-1R antibodies					
product	Class	company	development	indication	ref
IMC-A12	fully human IgG1 mAb against IGF-1R extracellular domain	ImClone (New York)	Phase II	Head-and-neck cancer	(Barnes, et al. 2007)
R1507	fully human mAb against IGF-1R extracellular domain	Genmab (Copenhagen)/Roche (Basel)	Phase II	Solid tumors, including sarcoma	(Pappo, al. 2011)
MK-0646	humanized IgG1 antibody against IGF-1R extracellular domain	Merck	Phase II	Metastatic colon cancer	(Reidy-Lagunes, et al. 2012)
AMG 479	fully human mAb targeting IGF-1R extracellular domain	Amgen	Phase II	Advanced solid tumors	(Rosen, et al. 2012)
AVE1642	humanized IgG1 mAb against IGF-1R extracellular domain	Sanofi-Avensis	Phase II	Multiple myeloma	(Moreau, et al. 2011)
Anti-IGF-1R small molecule /Tyrosine kinase inhibitor					
BMS-536924	Small molecule TKI	Bristol-Myers Squibb	undergoing phase I		(Wittman et al. 2005)
OSI-906	Small molecule TKI	OSI pharmaceuticals	preclinical		(Mulvihil et al. 2009)
PPP	Small molecule TKI	Axelar	Phase I/II trial		(Ekman, et. Al. 2011)
BMS-754807	Small molecule TKI	Bristol Myers Squibb	preclinical		(Kolb, et al. 2011)

1.7 EWING'S SARCOMA

Ewing's sarcoma (ES) is the second most common malignant bone tumor in children and young people. This malignancy is characterized by rapid growth and a high potential to develop metastases.

The genetic feature of ES is the chromosomal translocation causing fusion of the EWS gene with the ETS family of transcription factors FLI1 gene. In normal conditions, EWS gene locus on chromosome 22 band q12 while FLI 1gene is located on chromosome 11 band q24 (Peter, Couturier et al. 1997), other *ETS* family members are also reported to combine with the *EWS* gene include *ERG* (chromosome 21), *ETV1* (chromosome 7), and *E1AF* (chromosome 17) (Balamuth and Womer 2010). The fusion of *EWS-FLI1* lacks a stable structure and contains a high proportion of disordered regions, which act as an aberrant transcription factor to alter several cellular processes(Erkizan, Uversky et al. 2010). Overall, the aspect of cellular regulation by *EWS-FLI1* include cell cycle regulation and DNA repair by affecting CDKs, p53 function, cell adhesion and migration via interaction with integrin, and heparin-binding proteins, regulate apoptotic genes (Hancock and Lessnick 2008).

The importance of IGF-1R and receptor signaling pathway to tumorgenesis in ES has been widely demonstrated (Scotlandi, Benini et al. 1998). Notably, IGF-1R expression is necessary for *EWS-FLI*-mediated transformation in MEFs (Toretsky, Kalebic et al. 1997).

2 MATERIALS AND METHODS

2.1 REAGENTS

Polyclonal antibodies against IGF-1R (H-60), β-arrestin1 (K-16) and GAPDH (FL-335) and Monoclonal antibodies against ubiquitin (P4D1), GRK2 (C-9), GRK6 (XX-4) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal antibodies against phosphorylated (p)AKT (S473), AKT, pERK1/2, ERK1/2, pIGF1R and IGF-1R were from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibody against phospho-serine and Dynabeads protein G were from Invitrogen (Carlsbad, CA, USA). Polyclonal antibody against flag and anti-flag M2-agarose were from Sigma (St. Louis, MO, USA). LL-37 (purity of 98%) was obtained from Polypeptide Laboratories, Hillero ̈ d, Denmark. Insulin like Growth Factor-1(IGF-1) was from Sigma (Sigma Aldrich, St Louis, MO, USA).

2.2 CELL CULTURE

The Human Embryonic Kidney 293T cell line (HEK 293T) was obtained from ATCC (via LGC Promochem, Boras, Sweden). HEK 293T was grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). BE and DFB, skin melanoma cells, were cultured in RPMI medium supplemented with 10% FBS and 1% PS. The mouse embryonic fibroblast (MEF), MEF cell with targeted disruption of IGF-1R gene (R-) and Δ1245 mouse cell lines (IGF-1R KO cells, stably transfected with IGF-1R with C terminus truncated at position 1245) were a kind gift from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA, USA) and are as previously described (Dews, Prisco et al. 2000). These cell lines were cultured in DMEM supplemented with 10% FBS and 1% PS in the presence of G-418 (Promega, WI, USA). Ewing's sarcoma cell lines SKES, RDES, CADO and A673 were obtained from ATCC (via LGC Promochem, Boras, Sweden). SKES was grown in McCoy's 5A medium supplemented with 10% (vol/vol) fetal bovine serum (FBS). RDES, CADO were cultured in RPMI medium supplemented with 10% (vol/vol) FBS. A673 and SKBR3 were grown in DMEM supplemented with 10% (vol/vol) FBS. MCF-7 and ZR75-1 human breast cancer cell lines, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

2.3 SMALL INTERFERING RNAS (SIRNAS) AND TRANSFECTION

Chemically synthesized, double-stranded siRNAs were purchased from Dharmacon, Inc. (Pierce, IL, USA). The siRNA sequences targeting endogenous GRKs are: GRK2, 5'-GGAAGAAUGUGGAGCUCAAtt-3'; GRK3, 5'-GCAGCAAGAAGUAACGGA Att-3'; GRK5, 5'-CGUCUACCGAGAUCUGAAAtt-3'; GRK6, 5'-GAGAAAAAGC GGAUCAAGAtt-3'. The siRNA sequence that was used to deplete endogenous βarrestin-1 levels was 50-AAAGCCUUCUGUGCUGAGAAC-30. A non-silencing RNA duplex (50-AAUUCUCCGAACGUGUCACGU-30), as the manufacturer indicated, was used as a control. The siRNA targeting human IGF-1R sequence 50-GCAGACACCUACAACAUCAUU-30 (Natalishvili, Axelson et al. 2009).

The cells were transfected at 40–50% confluency in 6-well plates, using DharmaFECT transfection reagent (Pierce) or Lipofectamine RNAiMAX (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.4 PLASMIDS AND TRANSFECTION

The plasmid encoding full length IGF1R-YFP was previously described (Blanquart, Gonzalez-Yanes et al. 2006). Various mutants were constructed from IGF1R-YFP plasmids using the QuikChange XL II kit (Agilent Technologies, CA, USA). βarrestin1-CFP plasmid was obtained from Dr. Carsten Hoffmann, Wuerzburg University, Germany (Hoffmann, Ziegler et al. 2008). The plasmids expressing human GRK2 and GRK6 (Kim, Ahn et al. 2005), the β-arrestin1-flag plasmid and GFP-β-arrestin1 (Shenoy and Lefkowitz 2003) were a kind gift from Dr. Robert J. Lefkowitz. Cells cultured at 90% confluency in 6-well plates were transfected with plasmids using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The plasmid expressing β-arrestin1-flag was a kind gift from Dr. Robert J.Lefkowitz (Duke University Medical Center/Howard Hughes Medical Institute, Durham, NC).

2.5 IMMUNOPRECIPITATION

To measure protein-protein interaction or receptor phosphorylation /ubiquitination, the cells were cultured in 6-well plates and lysed with 500 µl lysis buffer (110 mM KOAc, 0.5% (v/v) Triton X-100, 100 mM NaCl, buffering salts pH 7.4). The protein concentration was determined by bicinchoninic acid assay (Pierce, IL, USA). 1 µg antibody and 10 µl Dynabeads protein G (Invitrogen, CA, USA) were added to 500 µg of protein. After overnight incubation at 4°C, the immunoprecipitates were collected and dissolved in the sample buffer for SDS-PAGE.

2.6 SDS-PAGE AND WESTERN BLOTTING

Protein samples were dissolved in LDS sample buffer (Invitrogen, CA, USA). Samples were analyzed by SDS-PAGE with 4-12% Bis-Tris gel (Invitrogen, CA, USA). Following separation, the proteins were transferred to nitrocellulose membranes for 1 h at 100 V. Membranes were then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween 20 in Tris-buffered saline (TBS), pH 7.5 (TBS-T). Incubation with appropriate primary antibody was performed overnight at 4°C. After washing, the membrane was incubated with a horseradish peroxidase-labelled for 1 h. The detection was made with ECL substrate (Pierce, IL, USA) and exposure to x-ray film.

2.7 *IN VITRO* BINDING ASSAY

Cell lysates contained β arrestin1-flag was purified by anti-flag M2 agarose with overnight incubation at 4°C following PBS wash for 4 times. On the other hand, the cells transfected with IGF-1RYFP were lysed as described above. 500 µl lysate was added to the beads-β arr1-flag complex and incubated overnight at 4°C. The pellet was collected by centrifugation at 6,000 g for 1 min and washed three times with 1xTBS buffer in a spin cup column (Pierce, IL, USA), whereupon the pellet was dissolved in sample buffer for SDSPAGE. WB analysis probing with anti-IGF-1R identified IGF-1R YFP while flag detection was used as a loading control.

2.8 CONFOCAL MICROSCOPY

The cells were serum starved for 8 h before stimulation. After washing three times with PBS, the cells were fixed with 4% paraformaldehyde diluted in PBS containing

calcium and magnesium before confocal analysis. The cells were immunostained with appropriate primary antibody overnight and secondary ALEXA 594/488 (Invitrogen) for 1 h. For living cell image, the cells were maintained in HBS buffer (150 mM NaCl, 10 mM HEPES, 10 mM glucose, 2.5 mM KCl, 4 mM CaCl2, 2 mM MgCl2, pH 7.4) and placed on a stage top incubator with 37°C and 5% CO_2. Confocal experiments were performed on a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany).

2.9 FÖRSTER RESONANCE ENERGY TRANSFER (FRET)

Fluorescence resonance energy transfer (FRET) is a technique based on the following principles: when a suitable pair of fluorophores is brought into close proximity (1-10 Å) of one another, excitation of the donor-fluorophore results in a transfer of energy to the acceptor-fluorophore, resulting in an increase of the acceptor emission signal and a decrease in donor-emission. The most common pair of chromophore is yellow fluorescence protein (YFP) and cyan fluorescence protein (CFP). The FRET efficiency is measured and used to identify interactions between the labeled complexes. In our study, in order to investigate the interaction between mutant IGF-1R and βarrestin1, we induced mutant IGF-1R-YFP and βarrestin1-CFP plasmids into HEK 293T cells and measure the nonradiative energy transfer from a CFP donor to a YFP acceptor. To validate the FRET in single cells, we performed acceptor photobleaching(Dinant, van Royen et al. 2008)by using Leica TCS SP5 confocal microscope and FRET wizards in Leica Application Suite Advanced Fluorescence (LAS AF) software. To measure the kinetics of β-arr1 binding to the IGF-1R, the transfected cells were measured with FRET in cell populations via a Tecan Infinite M1000 monochromator based reader (Tecan, Switzerland)(Kraft, Olbrich et al. 2001).

2.10 CELL VIABILITY ASSAY

The cell proliferation can be acquired by measuring of DNA content or apoptotic markers by radioactive, luminescent or fluorescent assays. In our experiments, cell viability was measured by PrestoBlue Cell Viability Assay (Invitrogen, CA, USA), which acts as a cell viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells. The cell number was calculated by Countess® (Invitrogen, CA, USA) before plating cells to 96 well plate. The cells were cultured overnight in the absence of serum and then stimulate with ligands. Fluorescence was measured via Tecan Infinite M1000 monochromator based reader (Tecan, Switzerland).

3 AIMS OF THE STUDY

The specific aims of this thesis were:

- To investigate the mechanism of β-arrestin/IGF-1R interaction.
- To investigate the effects of β-arrestin/IGF-1R interaction on receptor expression and signaling.
- To investigate the roles of β-arrestin in tumorigenesis and to evaluate its potential utility as therapeutic target.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Identification of the cathelicidin peptide LL-37 as agonist for the type I insulin-like growth factor receptor.

Antimicrobial peptides, AMPs, are important components of the innate immune system in most living organisms since they exhibit broad antimicrobial function against bacteria, fungi, yeast and viruses. LL-37, a C terminal peptide cleaved from the human cathelicidin antimicrobial protein-18, is expressed in leukocytes and epithelial cells. LL-37 displays broad antimicrobial activity via targeting the microbial cell membrane. Over the last years, the repertoire of known biological functions for LL-37 expanded to include immunomodulation, apoptosis and carcinogenesis. Based on the hypothesis that LL-37 exerts its oncogenic effects through activation of specific signaling pathways, in this study we aimed to explore if and how LL-37 contributes to the signaling involved in tumor development.

Firstly, by using an ELISA approach, we investigated the interaction between LL-37 and growth factor receptors which are well known to be involved in tumor progression. Among several growth factor receptors tested, IGF-1R showed the highest affinity for LL-37, therefore we explored whether this interaction occurred in a cell system. By using such a system we could confirm the LL-37/IGF-1R interaction as well as the outcomes on IGF-1R dependent signaling activation. Using cell lines expressing endogenous or ones transgenically modified to overexpress IGF-1R, we confirmed that LL-37 binding triggers receptor tyrosine kinase phosphorylation in a dose dependent manner. Moreover, receptor phosphorylation induces downstream MAPK/ERK signaling activation. Intriguingly, LL-37 had no effect on AKT signaling, in contrast to IGF-1R signaling activation by IGF-1, which activates both pathways. By investigating the mechanism of this signaling dissociation, we found that the C-terminal domain of IGF-1R is required for LL-37 dependent ERK activation. As previous studies demonstrated that the C-terminus is important for β-arrestin1 recruitment to the IGF-1R and for IGF-1R ubiquitination in response to IGF-1 (Girnita *et al.*, 2005), we investigated the effects of LL-37 on IGF-1R/ β-arrestin1 association and receptor ubiquitination. The results demonstrated that following LL-37 stimulation IGF-1R ubiquitination is clearly increased. In addition, in response to LL-37, stable β-arrestin1/IGF-1R complexes were revealed by confocal microcopy. Conversely, when the β-arrestin1 level was downregulated by siRNA, LL-37 induced ERK phosphorylation was severely impaired. Thus, our data demonstrates that LL-37 binding to IGF-1R activates MAPK/ERK signaling and this process is dependent on β-arrestin1 recruitment and IGF-1R ubiquitination.

In the last set of experiments, we explored the biological outcomes of LL-37 induced IGF-1R signaling activation. In the absence of PI3K/AKT signaling, LL-37 induced ERK activation has a minor effect on cell proliferation, however it promotes cell migration and invasion. We further demonstrated that LL-37/IGF-1R/ β-arrestin1 /ERK specifically support the invasive phenotype since inhibition of any component of this axis completely abrogated the LL-37 and IGF-1 induced migration/invasion of breast cancer cells.

Main findings:

- This study identifies the first natural biased agonist for the IGF-1R (to our knowledge the first natural biased agonist for a RTK) and fully supports the concept of biasing signaling at the IGF-1R.
- The results indicate that a migratory/invasive phenotype could be promoted by peptides generated by an inflammatory process within the tumor surrounding tissue.

4.2 PAPER II

Selective recruitment of G protein-coupled receptor kinases (GRKs) controls signaling of the insulin-like growth factor 1 receptor

G Protein-Coupled Receptors (GPCR) and Receptor Tyrosine Kinases (RTK) are major transducers of signals across the plasma membrane. Each cell surface receptor family possesses unique structural characteristics and leads to specific signaling outcomes in the cell. However, there is extensive overlap in the signaling proteins and pathways used to produce these effects. Among them, β-arrestins, molecules previously considered to be associated exclusively with GPCRs, were also demonstrated to be involved in modulating signaling through a classical RTK, insulin-like growth factor type 1 (IGF-1R), with the same major outcomes as for GPCRs: they shut down the receptor and redirect signaling to MAPK/ERK pathway. Yet, the mechanism of β-arrestin1 recruitment is unclear. Using as a model the GPCR signaling, β-arrestins binding to activated receptors proceeds only after G protein-coupled receptor kinase (GRK) phosphorylation of distinct serine/threonine residues following, we investigated the regulatory roles GRKs on expression and function of the IGF-1R.

In the initial screening, the roles of the four widely expressed GRKs (2, 3, 5, and 6) on IGF-1R signaling were investigated by suppressing their expression with specific small interfering RNA (siRNA) and close monitoring of the dynamics of IGF-1 mediated activation of the two key downstream IGF-1R signaling pathways, the Ras/Raf/MEK/ERK pathway and the PI3K/AKT pathway. The results suggested that downregulation of GRK5/6 abolishes IGF-1-mediated ERK and AKT activation, whereas GRK2 inhibition increases ERK activation and partially inhibits AKT

signaling. Conversely, overexpression experiments demonstrated that GRK6 enhanced β-arrestin1–mediated ERK signaling and ligand induced IGF-1R degradation while overexpression of GRK2 decrease ERK activation and prevented receptor degradation.

Overall, these results indicated GRKs as mediators of β-arrestin recruitment to the IGF-1R therefore we functionally validated this process by demonstrating that GRKs co-immunoprecipitate with IGF-1R and phosphorylate serine residues within the C-terminal domain of the IGF-1R, in this way creating binding sites for β-arrestin1. GRK dependent β-arrestin1 recruitment to the IGF-1R was demonstrated in different experimental models, including confocal microscopy and co-immunoprecipitation. Following mutation analysis that identified and validated serines 1248 and 1291 as the major GRK serine phosphorylation sites on IGF- 1R, we investigated the functional outcomes of β-arrestin1 recruitment to these phosphorylated residues.

Mutation of the two identified residues demonstrated clear differences in behaviour that mirrored the alterations seen after manipulation of the GRKs expression. This unambiguous correspondence between the effects from specific GRKs inhibition/overexpression and mutation analysis of specific serine residues, advocates substrate specificity at the level of individual residues: GRK2 phosphorylates serine 1248 whilst GRK6 phosphorylates serine 1291. When the S1291D mutant mimicking S1291 phosphorylation was expressed, a prolonged β-arrestin/IGF-1R interaction was observed, similar to the pattern observed after GRK6 overexpression. Likewise, IGF-1R S1291D demonstrated a very high *in vitro* binding affinity for β-arrestin1 corresponding to the increased IGF-1R/β-arrestin co-immunoprecipitation from cells overexpressing GRK6. Finally, IGF-1R degradation was considerably increased by either GRK6 or S1291D mutation. In the opposite manner, phosphorylation of S1248 either by GRK2 overexpression or mimicking it by mutation to S1248D results in a rapid and transient β-arrestin/IGF-1R association preventing IGF-1R degradation.

In the context of cancer, the C-terminus of the IGF-1R has been extensively studied (Baserga 2000, Baserga 2005) therefore identification of GRK phosphorylated serines as β-arrestin1 binding sites within this domain deserves particular consideration. The IGF-1R C-terminus was proven essential for malignant transformation. Moreover, ectopic competitive expression of the C terminal domain is inhibitory to tumor cell survival (Hongo, Yumet et al. 1998). A receptor truncated at 1245 is mitogenic but no longer transforming, whilst truncation beyond 1311 has no effects on the transforming abilities of the IGF-1R. If an additional 10 amino acids were deleted started at 1291, the receptor lost its transforming abilities (Liu, Zong et al. 1993). Intriguingly, deletion of a further 20 amino acids (Δ1271) restored the transforming abilities which were lost again after further deletion of at least 21 AA (Δ1250). These findings are remarkable for the fact that mutations in this domain, although preserving the RTK activity, abrogate the receptor transforming abilities (Baserga 2005), clearly indicating that kinase activity is not enough to sustain or

maintain the malignant phenotype. These results also suggest that there are at least two domains (and/or signals originating from them), implicated in malignant transformation: one including serine 1291, narrowed to 1289-1294 and one including S1248 (1245-1271) involved in mediating both positive and negative signals (Baserga 2005). There is no information on the signaling originating from the 1289 – 1294 sequence while serine 1248 has been reported to be required for RACK1 binding which integrates IGF-1R and integrin signaling with positive effects on IGF-1R-mediated cell motility and proliferation, but inhibitory effects on cell survival (Kiely, Sant et al. 2002, Baserga 2005). Our results fully support this model of positive and negative signals, as we demonstrated that GRK6 dependent phosphorylation of S1291 prolonged ERK signaling at the cost of IGF-1R degradation whereas GRK2 through S1248 exerts protective effects on receptor degradation while limiting the extent of ERK phosphorylation. In addition, our results reveal two possible signaling pathways, originating from S1248 and /or S1291 of the IGF-1R that could contribute to the transforming potential of the C-terminus: G-protein signaling, desensitized by the GRK mediated β-arrestin binding and the second wave of β-arrestin signals for the S1291.

Main findings:

- Identification of the GRK2 and GRK6 as serine kinases for the IGF-1R.
- Identification of serine 1248 and 1291 as the major β-arrestin1 binding sites on IGF-1R.
- GRKs generate β-arrestin1 biased signaling at the IGF-1R: distinct phosphorylation patterns resulting in different functional activities of recruited β-arrestin1 to the IGF-1R.

4.3 PAPER III

β-arrestin biased agonism as a novel mechanism of action for the IGF-1R targeting antibodies in Ewing's sarcoma.

Over the last decades, IGF-1R has received particular attention as a key factor involved in the development and progression of human cancers. Thus, IGF-1R molecular targeted therapy has become an exciting approach for the treatment of the most aggressive forms of cancer, including breast, prostate, lung, melanoma and Ewing's sarcoma.

The IGF-1R is the target of small molecules and antibodies in current clinical trials and which have demonstrated some clinical efficacy in Ewing's sarcoma (ES). All anti-IGF-1R antibodies used in clinical trials were designed to prevent the ligand-receptor interaction; however they also induce receptor downregulation. The aim of

this study was to investigate the paradox of receptor downregulation by anti-IGF-1R antibodies in Ewing's sarcoma cell lines.

IGF-1R mediated downstream PI3K/AKT and Ras/MAPK/ERK signaling activation are the key pathways to aid tumor cells gaining a growth advantage. Firstly, we confirmed the functional activity of the IGF-1R and in 4 Ewing's sarcoma cell lines and investigated their sensitivity to anti-IGF-1R antibodies. Targeting IGF-1R by specific antibodies, results in an overall decrease in cell number in all tested ES cell lines. Intriguingly, the magnitude of this effect was essentially identical in the presence or absence of serum, evidence against a simple ligand blocking mechanism for the antibody. Since receptor downregulation has been described for all IGF-1R targeting antibodies, we also investigated the effect and the mechanism of the anti-IGF-1R antibodies induced receptor degradation. We confirmed that anti-IGF-1R antibodies induced rapid receptor degradation within 12 hours, with the antibodies being more efficient than IGF-1 at equivalent molar concentrations. By isolating the IGF-1R from cells treated or not with the targeting antibodies, we identified ubiquitination as the main process triggering receptor downregulation.

As previous studies demonstrated β-arrestin1 as a key molecule controlling agonist-induced IGF-1R ubiquitiantion, we tested whether β-arrestin1 is involved in the process of antibody-induced receptor degradation. Co-immunoprecipitation experiments demonstrated β-arrestin1 recruitment to the receptor following antibody treatment while the dependency of this process on IGF-1R/β-arrestin1 interaction was demonstrated in transgenically modified cell lines, expressing IGF-1R defective in binding β-arrestin1. Moreover, the crucial role of β-arrestin1 in mediating the effects of anti-IGF-1R antibodies was validated in β-arrestin1knock out cell lines, which were insensitive to antibody treatment regarding both cell proliferation and receptor downregulation.

It has been previously demonstrated that in addition to receptor degradation, β-arrestin1 binding to IGF-1R is sufficient to trigger activation of the MAPK/ERK signaling pathway, an effect which is not dependent on the IGF-1R kinase activity. Accordingly, we investigate the effects of anti-IGF-1R antibodies on signaling activation. Intriguingly, our results demonstrate that anti-IGF-1R antibodies can induce a clear activation of ERK signaling even in the absence of IGF-1R tyrosine phosphorylation or activation of AKT, a process mediated by β-arrestin1. Moreover, this signaling activation by the IGF-1R targeting antibody was demonstrated to have a protective role for the ES cells, pointing out this pathway as a possible target for a combination anti-cancer therapy. This hypothesis was tested and confirmed in experiments in which ES cells were treated with anti-IGF-1R antibodies in the presence or absence of MAPK inhibitors, indicating a strategy to improve responsiveness to anti-IGF-1R therapy, appropriate for clinical settings.

Main findings:

- In this study, we reveal the mechanism of action of anti- IGF-1R antibody induced receptor downregulation.
- We propose the concept of β-arrestin1 biased signaling at the IGF-1R and identify anti-IGF-1R antibodies as the first engineered biased agonists.
- We identified and validates a potential co-target, MAPK, to improve anti-IGF-1R antibody based therapy.

4.4 PAPER IV

β-arrestin1 mediated IGF-1R signalling in the Ras induced transformation of mammalian cells.

β-arrestin1, known primarily as a regulator of GPCR signaling, interacts with the IGF-1R and has been shown to be involved in IGF-1R signaling in cancer. β-arrestin has been shown to bring Mdm2, an E3 ubiquitin ligase, to the IGF-1R resulting in receptor ubiquitination and subsequent activation of the MAPK/ERK signalling cascade. Recently, this β-arrestin1 dependent IGF-1R signalling and degradation occur under the control of differential action of the GRKs.

The IGF-1R is well documented in various experimental models to be an important factor in cell transformation, tumour progression, protection from apoptosis and metastasis. In the absence of IGF-1R, most oncogenes, including activated Ras, are unable to induce malignant transformation, suggesting that some signalling pathways activated by IGF-1R are essential for transformation. In this study, we aimed to investigate the role of β-arrestin1 mediated signaling in oncogene induced transformation in mouse embryonic fibroblasts (MEFs).

MEFs and MEFs lacking β-arrestin1 (MEF KO) cells were stably transfected with powerful oncogenes like H-RasV12, PyMT or v-Src and cultured in serum free and anchorage independent conditions to assess cellular transformation. Results show that all oncogenes easily transform MEFs. However, in the absence of β-arrestin1, only PyMT and v-Src transfected MEF KO cells increased proliferation and colonies formation in soft agar, whereas H-RasV12 failed to transform MEF KO cells as demonstrated by lack of self-sufficiency for proliferation and no colony formation in soft agar. This suggests that β-arrestin1 is required for Ras mediated activation of one or several oncogenic pathways.

Oncogene H-RasV12 once activated has the property to retain the GTP loading, resulting in prolonged activation of the downstream Raf, MEK1 and MAPK/ERK signalling cascade. By investigating the IGF-1 mediated ERK and PI3K/AKT signalling activation in H-RasV12 transfected KO cells we found that in KO cells ERK activation was impaired and diminished early (after 10 min) relative to MEF control cells transfected with the same oncogene.

As the Ras/Raf/MEK/ ERK cascade is initiated by the Ras-GTP loading, we measured the activated Ras levels in the presence or absence of β-arrestin1. Using Raf-beads as bait in a pull-down assay, we found that Ras activation in response to IGF-1 was impaired in the absence of β-arrestin1.

Translocation of activated ERK to nucleus has been proved to be the most essential function of MAPK signaling in the regulation of proliferation and oncogenic transformation.

Considering impaired ERK activation occurred in Ras transfected KO cells, and taking into consideration the β-arrestin1 function as scaffold of the MAPK pathway components, we also investigated the subcellular distribution of activated ERK. Using alternative experimental approaches including confocal microscopy and subcellular fractionation, we found that activated Ras oncogene induces clear nuclear translocation of activated ERK in response to IGF-1 in the MEF cells, whereas in absence of β-arrestin1, active ERK remains localized mostly at the membrane and does not translocate to the nucleus.

Main findings:

- This study indicates that β-arrestin1-dependent signalling of the IGF-1R is required for malignant transformation of cancer cells and could be targeted for cancer therapy.

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6 REFERENCES

Ahn, S., S. K. Shenoy, H. Wei and R. J. Lefkowitz (2004). "Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor." J Biol Chem **279**(34): 35518-35525.
Andersen, A. S., T. Kjeldsen, F. C. Wiberg, P. M. Christensen, J. S. Rasmussen, K. Norris, K. B. Moller and N. P. Moller (1990). "Changing the insulin receptor to possess insulin-like growth factor I ligand specificity." Biochemistry **29**(32): 7363-7366.
Arany, E., S. Afford, A. J. Strain, P. J. Winwood, M. J. Arthur and D. J. Hill (1994). "Differential cellular synthesis of insulin-like growth factor binding protein-1 (IGFBP-1) and IGFBP-3 within human liver." J Clin Endocrinol Metab **79**(6): 1871-1876.
Balamuth, N. J. and R. B. Womer (2010). "Ewing's sarcoma." Lancet Oncol **11**(2): 184-192.
Barnes, C. J., K. Ohshiro, S. K. Rayala, A. K. El-Naggar and R. Kumar (2007). "Insulin-like growth factor receptor as a therapeutic target in head and neck cancer." Clinical Cancer Research **13**(14): 4291-4299.
Barnes, W. G., E. Reiter, J. D. Violin, X. R. Ren, G. Milligan and R. J. Lefkowitz (2005). "beta-Arrestin 1 and Galphaq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation." J Biol Chem **280**(9): 8041-8050.
Baserga, R. (1995). "The insulin-like growth factor I receptor: a key to tumor growth?" Cancer Res **55**(2): 249-252.
Baserga, R. (2000). "The contradictions of the insulin-like growth factor 1 receptor." Oncogene **19**(49): 5574-5581.
Baserga, R. (2004). "Targeting the IGF-1 receptor: from rags to riches." Eur J Cancer **40**(14): 2013-2015.
Baserga, R. (2005). "The insulin-like growth factor-I receptor as a target for cancer therapy." Expert Opin Ther Targets **9**(4): 753-768.
Baserga, R., A. Hongo, M. Rubini, M. Prisco and B. Valentinis (1997). "The IGF-I receptor in cell growth, transformation and apoptosis." Biochim Biophys Acta **1332**(3): F105-126.
Baserga, R., A. Hongo, M. Rubini, M. Prisco and B. Valentinis (1997). "The IGF-I receptor in cell growth, transformation and apoptosis." Biochimica Et Biophysica Acta-Reviews On Cancer **1332**(3): F105-F126.
Baserga, R., F. Peruzzi and K. Reiss (2003). "The IGF-1 receptor in cancer biology." Int J Cancer **107**(6): 873-877.
Baxter, R. C. (2000). "Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities." Am J Physiol Endocrinol Metab **278**(6): E967-976.
Belfiore, A., F. Frasca, G. Pandini, L. Sciacca and R. Vigneri (2009). "Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease." Endocr Rev **30**(6): 586-623.
Blanquart, C., C. Gonzalez-Yanes and T. Issad (2006). "Monitoring the activation state of insulin/insulin-like growth factor-1 hybrid receptors using bioluminescence resonance energy transfer." Mol Pharmacol **70**(5): 1802-1811.
Bohn, L. M., R. J. Lefkowitz, R. R. Gainetdinov, K. Peppel, M. G. Caron and F. T. Lin (1999). "Enhanced morphine analgesia in mice lacking beta-arrestin 2." Science **286**(5449): 2495-2498.
Bonifacino, J. S. and A. M. Weissman (1998). "Ubiquitin and the control of protein fate in the secretory and endocytic pathways." Annu Rev Cell Dev Biol **14**: 19-57.
Bouchard, V. J., M. Rouleau and G. G. Poirier (2003). "PARP-1, a determinant of cell survival in response to DNA damage." Exp Hematol **31**(6): 446-454.
Carlberg, M., A. Dricu, H. Blegen, M. Wang, M. Hjertman, P. Zickert, A. Hoog and O. Larsson (1996). "Mevalonic acid is limiting for N-linked glycosylation and translocation of the insulin-like growth factor-1 receptor to the cell surface. Evidence for a new link between 3-hydroxy-3-methylglutaryl-coenzyme a reductase and cell growth." J Biol Chem **271**(29): 17453-17462.

Chong, H., H. G. Vikis and K. L. Guan (2003). "Mechanisms of regulating the Raf kinase family." Cell Signal **15**(5): 463-469.
Christopoulos, A., G. Christopoulos, M. Morfis, M. Udawela, M. Laburthe, A. Couvineau, K. Kuwasako, N. Tilakaratne and P. M. Sexton (2003). "Novel receptor partners and function of receptor activity-modifying proteins." J Biol Chem **278**(5): 3293-3297.
Clague, M. J. and S. Urbe (2006). "Endocytosis: the DUB version." Trends Cell Biol **16**(11): 551-559.
Claing, A., W. Chen, W. E. Miller, N. Vitale, J. Moss, R. T. Premont and R. J. Lefkowitz (2001). "beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis." J Biol Chem **276**(45): 42509-42513.
Clemmons, D. R. (1998). "Role of insulin-like growth factor binding proteins in controlling IGF actions." Mol Cell Endocrinol **140**(1-2): 19-24.
Cohen, B. D., D. A. Baker, C. Soderstrom, G. Tkalcevic, A. M. Rossi, P. E. Miller, M. W. Tengowski, F. Wang, A. Gualberto, J. S. Beebe and J. D. Moyer (2005). "Combination therapy enhances the inhibition of tumor growth with the fully human anti-type 1 insulin-like growth factor receptor monoclonal antibody CP-751,871." Clin Cancer Res **11**(5): 2063-2073.
Cohen, P. and R. G. Rosenfeld (1994). "Physiologic and clinical relevance of the insulin-like growth factor binding proteins." Curr Opin Pediatr **6**(4): 462-467.
Collett-Solberg, P. F. and P. Cohen (1996). "The role of the insulin-like growth factor binding proteins and the IGFBP proteases in modulating IGF action." Endocrinol Metab Clin North Am **25**(3): 591-614.
Conner, D. A., M. A. Mathier, R. M. Mortensen, M. Christe, S. F. Vatner, C. E. Seidman and J. G. Seidman (1997). "beta-Arrestin1 knockout mice appear normal but demonstrate altered cardiac responses to beta-adrenergic stimulation." Circ Res **81**(6): 1021-1026.
Constancia, M., M. Hemberger, J. Hughes, W. Dean, A. Ferguson-Smith, R. Fundele, F. Stewart, G. Kelsey, A. Fowden, C. Sibley and W. Reik (2002). "Placental-specific IGF-II is a major modulator of placental and fetal growth." Nature **417**(6892): 945-948.
Daaka, Y., J. A. Pitcher, M. Richardson, R. H. Stoffel, J. D. Robishaw and R. J. Lefkowitz (1997). "Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases." Proceedings of the National Academy of Sciences of the United States of America **94**(6): 2180-2185.
Dalle, S., W. Ricketts, T. Imamura, P. Vollenweider and J. M. Olefsky (2001). "Insulin and Insulin-like Growth Factor I Receptors Utilize Different G Protein Signaling Components." J. Biol. Chem. **276**(19): 15688-15695.
Dalle, S., W. Ricketts, T. Imamura, P. Vollenweider and J. M. Olefsky (2001). "Insulin and insulin-like growth factor I receptors utilize different G protein signaling components." J Biol Chem **276**(19): 15688-15695.
Dandekar, A. A., B. J. Wallach, A. Barthel and R. A. Roth (1998). "Comparison of the signaling abilities of the cytoplasmic domains of the insulin receptor and the insulin receptor-related receptor in 3T3-L1 adipocytes." Endocrinology **139**(8): 3578-3584.
Datta, S. R., A. Brunet and M. E. Greenberg (1999). "Cellular survival: a play in three Akts." Genes Dev **13**(22): 2905-2927.
De Benedetti, A. and J. R. Graff (2004). "eIF-4E expression and its role in malignancies and metastases." Oncogene **23**(18): 3189-3199.
de Rozieres, S., R. Maya, M. Oren and G. Lozano (2000). "The loss of mdm2 induces p53-mediated apoptosis." Oncogene **19**(13): 1691-1697.
Della Rocca, G. J., S. Maudsley, Y. Daaka, R. J. Lefkowitz and L. M. Luttrell (1999). "Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases." J Biol Chem **274**(20): 13978-13984.
Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart and Z. J. Chen (2000). "Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain." Cell **103**(2): 351-361.
DeWire, S. M., S. Ahn, R. J. Lefkowitz and S. K. Shenoy (2007). "Beta-arrestins and cell signaling." Annu Rev Physiol **69**: 483-510.

Dews, M., M. Prisco, F. Peruzzi, G. Romano, A. Morrione and R. Baserga (2000). "Domains of the insulin-like growth factor I receptor required for the activation of extracellular signal-regulated kinases." Endocrinology **141**(4): 1289-1300.
Diehl, J. A. (2002). "Cycling to cancer with cyclin D1." Cancer Biol Ther **1**(3): 226-231.
Dinant, C., M. E. van Royen, W. Vermeulen and A. B. Houtsmuller (2008). "Fluorescence resonance energy transfer of GFP and YFP by spectral imaging and quantitative acceptor photobleaching." J Microsc **231**(Pt 1): 97-104.
Downes, C. P., N. Perera, S. Ross and N. R. Leslie (2007). "Substrate specificity and acute regulation of the tumour suppressor phosphatase, PTEN." Biochem Soc Symp(74): 69-80.
Drake, M. T., S. K. Shenoy and R. J. Lefkowitz (2006). "Trafficking of G protein-coupled receptors." Circ Res **99**(6): 570-582.
Economou, M. A., S. Andersson, D. Vasilcanu, C. All-Ericsson, E. Menu, A. Girnita, L. Girnita, M. Axelson, S. Seregard and O. Larsson (2008). "Oral picropodophyllin (PPP) is well tolerated in vivo and inhibits IGF-1R expression and growth of uveal melanoma." Invest Ophthalmol Vis Sci **49**(6): 2337-2342.
Eferl, R. and E. F. Wagner (2003). "AP-1: a double-edged sword in tumorigenesis." Nat Rev Cancer **3**(11): 859-868.
Emuss, V., M. Garnett, C. Mason and R. Marais (2005). "Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF." Cancer Res **65**(21): 9719-9726.
Erkizan, H. V., V. N. Uversky and J. A. Toretsky (2010). "Oncogenic partnerships: EWS-FLI1 protein interactions initiate key pathways of Ewing's sarcoma." Clin Cancer Res **16**(16): 4077-4083.
Fang, S., J. P. Jensen, R. L. Ludwig, K. H. Vousden and A. M. Weissman (2000). "Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53." J Biol Chem **275**(12): 8945-8951.
Favelyukis, S., J. H. Till, S. R. Hubbard and W. T. Miller (2001). "Structure and autoregulation of the insulin-like growth factor 1 receptor kinase." Nat Struct Biol **8**(12): 1058-1063.
Federici, M., O. Porzio, L. Zucaro, A. Fusco, P. Borboni, D. Lauro and G. Sesti (1997). "Distribution of insulin/insulin-like growth factor-I hybrid receptors in human tissues." Mol Cell Endocrinol **129**(2): 121-126.
Ferguson, S. S. (2001). "Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling." Pharmacol Rev **53**(1): 1-24.
Ferguson, S. S., W. E. Downey, 3rd, A. M. Colapietro, L. S. Barak, L. Menard and M. G. Caron (1996). "Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization." Science **271**(5247): 363-366.
Firth, S. M., F. McDougall, A. J. McLachlan and R. C. Baxter (2002). "Impaired blockade of insulin-like growth factor I (IGF-I)-induced hypoglycemia by IGF binding protein-3 analog with reduced ternary complex-forming ability." Endocrinology **143**(5): 1669-1676.
Fizazi, K., J. Yang, S. Peleg, C. R. Sikes, E. L. Kreimann, D. Daliani, M. Olive, K. A. Raymond, T. J. Janus, C. J. Logothetis, G. Karsenty and N. M. Navone (2003). "Prostate cancer cells-osteoblast interaction shifts expression of growth/survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts." Clin Cancer Res **9**(7): 2587-2597.
Frattali, A. L., J. L. Treadway and J. E. Pessin (1992). "Insulin/IGF-1 hybrid receptors: implications for the dominant-negative phenotype in syndromes of insulin resistance." J Cell Biochem **48**(1): 43-50.
Freedman, N. J. and R. J. Lefkowitz (1996). "Desensitization of G protein-coupled receptors." Recent Prog Horm Res **51**: 319-351; discussion 352-313.
Fuglsang, J., F. Lauszus, A. Flyvbjerg and P. Ovesen (2003). "Human placental growth hormone, insulin-like growth factor I and -II, and insulin requirements during pregnancy in type 1 diabetes." J Clin Endocrinol Metab **88**(9): 4355-4361.
Furstenberger, G. and H. J. Senn (2002). "Insulin-like growth factors and cancer." Lancet Oncol **3**(5): 298-302.
Ganguli, G. and B. Wasylyk (2003). "p53-independent functions of MDM2." Mol Cancer Res **1**(14): 1027-1035.

Ge, L., S. K. Shenoy, R. J. Lefkowitz and K. DeFea (2004). "Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2." J Biol Chem **279**(53): 55419-55424.
Gerald, W. L., J. Rosai and L. Ladanyi (1995). "Characterization of the Genomic Breakpoint and Chimeric Transcripts in the Ews-Wt1 Gene Fusion of Desmoplastic Small Round-Cell Tumor." Proceedings of the National Academy of Sciences of the United States of America **92**(4): 1028-1032.
Gesty-Palmer, D., M. Chen, E. Reiter, S. Ahn, C. D. Nelson, S. Wang, A. E. Eckhardt, C. L. Cowan, R. F. Spurney, L. M. Luttrell and R. J. Lefkowitz (2006). "Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation." J Biol Chem **281**(16): 10856-10864.
Giles, F., D. Rizzieri, J. Karp, N. Vey, F. Ravandi, S. Faderl, K. D. Khan, G. Verhoef, P. Wijermans, A. Advani, G. Roboz, H. Kantarjian, S. F. Bilgrami, A. Ferrant, S. M. Daenen, V. Karsten, A. Cahill, M. Albitar, G. Mufti and S. O'Brien (2007). "Cloretazine (VNP40101M), a novel sulfonylhydrazine alkylating agent, in patients age 60 years or older with previously untreated acute myeloid leukemia." J Clin Oncol **25**(1): 25-31.
Girnita, A., C. All-Ericsson, M. A. Economou, K. Astrom, M. Axelson, S. Seregard, O. Larsson and L. Girnita (2006). "The insulin-like growth factor-I receptor inhibitor picropodophyllin causes tumor regression and attenuates mechanisms involved in invasion of uveal melanoma cells." Clin Cancer Res **12**(4): 1383-1391.
Girnita, L., A. Girnita, B. Brodin, Y. Xie, G. Nilsson, A. Dricu, J. Lundeberg, J. Wejde, A. Bartolazzi, K. G. Wiman and O. Larsson (2000). "Increased expression of insulin-like growth factor I receptor in malignant cells expressing aberrant p53: functional impact." Cancer Res **60**(18): 5278-5283.
Girnita, L., A. Girnita and O. Larsson (2003). "Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor." Proc Natl Acad Sci U S A **100**(14): 8247-8252.
Girnita, L., S. K. Shenoy, B. Sehat, R. Vasilcanu, A. Girnita, R. J. Lefkowitz and O. Larsson (2005). "{beta}-Arrestin Is Crucial for Ubiquitination and Down-regulation of the Insulin-like Growth Factor-1 Receptor by Acting as Adaptor for the MDM2 E3 Ligase." J. Biol. Chem. **280**(26): 24412-24419.
Girnita, L., S. K. Shenoy, B. Sehat, R. Vasilcanu, A. Girnita, R. J. Lefkowitz and O. Larsson (2005). "{beta}-Arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase." The Journal of biological chemistry **280**(26): 24412-24419.
Girnita, L., S. K. Shenoy, B. Sehat, R. Vasilcanu, D. Vasilcanu, A. Girnita, R. J. Lefkowitz and O. Larsson (2007). "Beta-arrestin and Mdm2 mediate IGF-1 receptor-stimulated ERK activation and cell cycle progression." J Biol Chem **282**(15): 11329-11338.
Girnita, L., M. Wang, Y. Xie, G. Nilsson, A. Dricu, J. Wejde and O. Larsson (2000). "Inhibition of N-linked glycosylation down-regulates insulin-like growth factor-1 receptor at the cell surface and kills Ewing's sarcoma cells: therapeutic implications." Anticancer Drug Des **15**(1): 67-72.
Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol Rev **82**(2): 373-428.
Gockerman, A., T. Prevette, J. I. Jones and D. R. Clemmons (1995). "Insulin-like growth factor (IGF)-binding proteins inhibit the smooth muscle cell migration responses to IGF-I and IGF-II." Endocrinology **136**(10): 4168-4173.
Goetsch, L., A. Gonzalez, O. Leger, A. Beck, P. J. Pauwels, J. F. Haeuw and N. Corvaia (2005). "A recombinant humanized anti-insulin-like growth factor receptor type I antibody (h7C10) enhances the antitumor activity of vinorelbine and anti-epidermal growth factor receptor therapy against human cancer xenografts." Int J Cancer **113**(2): 316-328.
Goodman, O. B., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen and J. L. Benovic (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-450.
Goodman, O. B., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen and J. L. Benovic (1998). "Role of arrestins in G-protein-coupled receptor endocytosis." Adv Pharmacol **42**: 429-433.

Grulich-Henn, J., J. Ritter, S. Mesewinkel, U. Heinrich, M. Bettendorf and K. T. Preissner (2002). "Transport of insulin-like growth factor-I across endothelial cell monolayers and its binding to the subendothelial matrix." Exp Clin Endocrinol Diabetes **110**(2): 67-73.
Gurevich, E. V. and V. V. Gurevich (2006). "Arrestins: ubiquitous regulators of cellular signaling pathways." Genome Biol **7**(9): 236.
Gurevich, V. V. and J. L. Benovic (1995). "Visual arrestin binding to rhodopsin. Diverse functional roles of positively charged residues within the phosphorylation-recognition region of arrestin." J Biol Chem **270**(11): 6010-6016.
Gustafson, T. A. and W. J. Rutter (1990). "The cysteine-rich domains of the insulin and insulin-like growth factor I receptors are primary determinants of hormone binding specificity. Evidence from receptor chimeras." J Biol Chem **265**(30): 18663-18667.
Haluska, P., H. M. Shaw, G. N. Batzel, D. Yin, J. R. Molina, L. R. Molife, T. A. Yap, M. L. Roberts, A. Sharma, A. Gualberto, A. A. Adjei and J. S. de Bono (2007). "Phase I dose escalation study of the anti insulin-like growth factor-I receptor monoclonal antibody CP-751,871 in patients with refractory solid tumors." Clin Cancer Res **13**(19): 5834-5840.
Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.
Hancock, J. D. and S. L. Lessnick (2008). "A transcriptional profiling meta-analysis reveals a core EWS-FLI gene expression signature." Cell Cycle **7**(2): 250-256.
Hanks, S. K., A. M. Quinn and T. Hunter (1988). "The protein kinase family: conserved features and deduced phylogeny of the catalytic domains." Science **241**(4861): 42-52.
Harrington, E. K., D. J. Coon, M. F. Kern and K. K. Svoboda (2010). "PTH stimulated growth and decreased Col-X deposition are phosphotidylinositol-3,4,5 triphosphate kinase and mitogen activating protein kinase dependent in avian sterna." Anat Rec (Hoboken) **293**(2): 225-234.
Harrington, L. S., G. M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N. R. Leslie, S. Cheng, P. R. Shepherd, I. Gout, C. P. Downes and R. F. Lamb (2004). "The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins." J Cell Biol **166**(2): 213-223.
Heron-Milhavet, L. and D. LeRoith (2002). "Insulin-like growth factor I induces MDM2-dependent degradation of p53 via the p38 MAPK pathway in response to DNA damage." J Biol Chem **277**(18): 15600-15606.
Hicke, L. (1999). "Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels." Trends Cell Biol **9**(3): 107-112.
Higano, C. S. (2007). "Annual zoledronic acid: is less more?" J Clin Oncol **25**(9): 1026.
Hirsch, J. A., C. Schubert, V. V. Gurevich and P. B. Sigler (1999). "The 2.8 A crystal structure of visual arrestin: a model for arrestin's regulation." Cell **97**(2): 257-269.
Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis and S. Jentsch (2002). "RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO." Nature **419**(6903): 135-141.
Hoffmann, C., N. Ziegler, S. Reiner, C. Krasel and M. J. Lohse (2008). "Agonist-selective, receptor-specific interaction of human P2Y receptors with beta-arrestin-1 and -2." The Journal of biological chemistry **283**(45): 30933-30941.
Hongo, A., G. Yumet, M. Resnicoff, G. Romano, R. O'Connor and R. Baserga (1998). "Inhibition of tumorigenesis and induction of apoptosis in human tumor cells by the stable expression of a myristylated COOH terminus of the insulin-like growth factor I receptor." Cancer Res **58**(11): 2477-2484.
Idelman, G., T. Glaser, C. T. Roberts and H. Werner (2003). "WT1-p53 interactions in insulin-like growth factor-I receptor gene regulation." Journal of Biological Chemistry **278**(5): 3474-3482.
Ikeda, K., H. Sunose and T. Takasaka (1996). "Effects of protein kinase C on the Na(+)-H+ exchange in the cochlear outer hair cell." Acta Otolaryngol **116**(6): 828-832.
Inoki, K., Y. Li, T. Zhu, J. Wu and K. L. Guan (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." Nat Cell Biol **4**(9): 648-657.

Iwata, K., J. Luo, R. B. Penn and J. L. Benovic (2005). "Bimodal regulation of the human H1 histamine receptor by G protein-coupled receptor kinase 2." J Biol Chem **280**(3): 2197-2204.
Jansson, M., D. Hallen, H. Koho, G. Andersson, L. Berghard, J. Heidrich, E. Nyberg, M. Uhlen, J. Kordel and B. Nilsson (1997). "Characterization of ligand binding of a soluble human insulin-like growth factor I receptor variant suggests a ligand-induced conformational change." J Biol Chem **272**(13): 8189-8197.
Jimenez, G., S. Y. Shvartsman and Z. Paroush (2012). "The Capicua repressor - a general sensor of RTK signaling in development and disease." J Cell Sci **125**(Pt 6): 1383-1391.
Kanayama, A., R. B. Seth, L. Sun, C. K. Ea, M. Hong, A. Shaito, Y. H. Chiu, L. Deng and Z. J. Chen (2004). "TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains." Mol Cell **15**(4): 535-548.
Karnieli, E., H. Werner, F. J. Rauscher, L. E. Benjamin and D. LeRoith (1996). "The IGF-I receptor gene promoter is a molecular target for the Ewing's sarcoma Wilms' tumor 1 fusion protein." Journal of Biological Chemistry **271**(32): 19304-19309.
Karp, D. D., M. N. Pollak, R. B. Cohen, P. D. Eisenberg, P. Haluska, D. Yin, A. Lipton, L. Demers, K. Leitzel, M. L. Hixon, L. W. Terstappen, L. Garland, L. G. Paz-Ares, F. Cardenal, C. J. Langer and A. Gualberto (2009). "Safety, pharmacokinetics, and pharmacodynamics of the insulin-like growth factor type 1 receptor inhibitor figitumumab (CP-751,871) in combination with paclitaxel and carboplatin." J Thorac Oncol **4**(11): 1397-1403.
Kenakin, T. (2002). "Efficacy at G-protein-coupled receptors." Nat Rev Drug Discov **1**(2): 103-110.
Kiely, P. A., A. Sant and R. O'Connor (2002). "RACK1 is an insulin-like growth factor 1 (IGF-1) receptor-interacting protein that can regulate IGF-1-mediated Akt activation and protection from cell death." J Biol Chem **277**(25): 22581-22589.
Kim, J., S. Ahn, X. R. Ren, E. J. Whalen, E. Reiter, H. Wei and R. J. Lefkowitz (2005). "Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling." Proc Natl Acad Sci U S A **102**(5): 1442-1447.
Kim, J., S. Ahn, X. R. Ren, E. J. Whalen, E. Reiter, H. Wei and R. J. Lefkowitz (2005). "Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling." Proceedings of the National Academy of Sciences of the United States of America **102**(5): 1442-1447.
Kim, J. H., S. Y. Park, J. K. Jeong, S. G. Kang, W. S. Choi and B. J. Lee (1996). "Progesterone inhibits Pit-1 and prolactin gene expression but activates promoter activity of c-H-ras oncogene in the rat pituitary adenoma GH(3) cell." Mol Cells **6**(1): 73-78.
Kjeldsen, T., A. S. Andersen, F. C. Wiberg, J. S. Rasmussen, L. Schaffer, P. Balschmidt, K. B. Moller and N. P. Moller (1991). "The ligand specificities of the insulin receptor and the insulin-like growth factor I receptor reside in different regions of a common binding site." Proc Natl Acad Sci U S A **88**(10): 4404-4408.
Kolb, E. A., R. Gorlick, R. Lock, H. Carol, C. L. Morton, S. T. Keir, C. P. Reynolds, M. H. Kang, J. M. Maris, C. Billups, M. A. Smith and P. J. Houghton (2011). "Initial testing (stage 1) of the IGF-1 receptor inhibitor BMS-754807 by the pediatric preclinical testing program." Pediatr Blood Cancer **56**(4): 595-603.
Kraft, K., H. Olbrich, I. Majoul, M. Mack, A. Proudfoot and M. Oppermann (2001). "Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization." J Biol Chem **276**(37): 34408-34418.
Kubbutat, M. H., S. N. Jones and K. H. Vousden (1997). "Regulation of p53 stability by Mdm2." Nature **387**(6630): 299-303.
Lacy, M. Q., M. Alsina, R. Fonseca, M. L. Paccagnella, C. L. Melvin, D. Yin, A. Sharma, M. Enriquez Sarano, M. Pollak, S. Jagannath, P. Richardson and A. Gualberto (2008). "Phase I, pharmacokinetic and pharmacodynamic study of the anti-insulinlike growth factor type 1 Receptor monoclonal antibody CP-751,871 in patients with multiple myeloma." J Clin Oncol **26**(19): 3196-3203.
Laporte, S. A., R. H. Oakley, J. Zhang, J. A. Holt, S. S. Ferguson, M. G. Caron and L. S. Barak (1999). "The beta2-adrenergic receptor/betaarrestin complex recruits the

clathrin adaptor AP-2 during endocytosis." Proc Natl Acad Sci U S A **96**(7): 3712-3717.
Lay, D., L. G. B, H. Heid, K. Gorgas and W. W. Just (2005). "Binding and functions of ADP-ribosylation factor on mammalian and yeast peroxisomes." J Biol Chem **280**(41): 34489-34499.
Le Roith, D. (2003). "The insulin-like growth factor system." Exp Diabesity Res **4**(4): 205-212.
Leahy, M., A. Lyons, D. Krause and R. O'Connor (2004). "Impaired Shc, Ras, and MAPK activation but normal Akt activation in FL5.12 cells expressing an insulin-like growth factor I receptor mutated at tyrosines 1250 and 1251." J Biol Chem **279**(18): 18306-18313.
LeBedis, C., K. Chen, L. Fallavollita, T. Boutros and P. Brodt (2002). "Peripheral lymph node stromal cells can promote growth and tumorigenicity of breast carcinoma cells through the release of IGF-I and EGF." Int J Cancer **100**(1): 2-8.
Lee, O. H., S. K. Bae, M. H. Bae, Y. M. Lee, E. J. Moon, H. J. Cha, Y. G. Kwon and K. W. Kim (2000). "Identification of angiogenic properties of insulin-like growth factor II in in vitro angiogenesis models." Br J Cancer **82**(2): 385-391.
Lefkowitz, R. J. (1998). "G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization." The Journal of biological chemistry **273**(30): 18677-18680.
Lefkowitz, R. J. (1998). "G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization." J Biol Chem **273**(30): 18677-18680.
Lefkowitz, R. J. (2004). "Historical review: a brief history and personal retrospective of seven-transmembrane receptors." Trends in pharmacological sciences **25**(8): 413-422.
Lefkowitz, R. J. (2004). "Historical review: a brief history and personal retrospective of seven-transmembrane receptors." Trends Pharmacol Sci **25**(8): 413-422.
Lefkowitz, R. J. and S. K. Shenoy (2005). "Transduction of receptor signals by beta-arrestins." Science **308**(5721): 512-517.
Lefkowitz, R. J. and E. J. Whalen (2004). "beta-arrestins: traffic cops of cell signaling." Curr Opin Cell Biol **16**(2): 162-168.
LeRoith, D., H. Werner, D. Beitner-Johnson and C. T. Roberts, Jr. (1995). "Molecular and cellular aspects of the insulin-like growth factor I receptor." Endocr Rev **16**(2): 143-163.
Lin, F. T., K. M. Krueger, H. E. Kendall, Y. Daaka, Z. L. Fredericks, J. A. Pitcher and R. J. Lefkowitz (1997). "Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1." The Journal of biological chemistry **272**(49): 31051-31057.
Liu, D., C. S. Zong and L. H. Wang (1993). "Distinctive effects of the carboxyl-terminal sequence of the insulin-like growth factor I receptor on its signaling functions." J Virol **67**(11): 6835-6840.
Liu, J.-L., V. A. Blakesley, J. S. Gutkind and D. LeRoith (1997). "The Constitutively Active Mutant Galpha 13 Transforms Mouse Fibroblast Cells Deficient in Insulin-like Growth Factor-I Receptor." J. Biol. Chem. **272**(47): 29438-29441.
Lopez, T. and D. Hanahan (2002). "Elevated levels of IGF-1 receptor convey invasive and metastatic capability in a mouse model of pancreatic islet tumorigenesis." Cancer Cell **1**(4): 339-353.
Lorenzen, J. A., S. E. Baker, F. Denhez, M. B. Melnick, D. L. Brower and L. A. Perkins (2001). "Nuclear import of activated D-ERK by DIM-7, an importin family member encoded by the gene moleskin." Development **128**(8): 1403-1414.
Luttrell, L. M., F. L. Roudabush, E. W. Choy, W. E. Miller, M. E. Field, K. L. Pierce and R. J. Lefkowitz (2001). "Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds." Proc Natl Acad Sci U S A **98**(5): 2449-2454.
Maor, S. B., S. Abramovitch, M. R. Erdos, L. C. Brody and H. Werner (2000). "BRCA1 suppresses insulin-like growth factor-I receptor promoter activity: Potential interaction between BRCA1 and Sp1." Molecular Genetics and Metabolism **69**(2): 130-136.
Marais, R., J. Wynne and R. Treisman (1993). "The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain." Cell **73**(2): 381-393.

Mayo, L. D. and D. B. Donner (2001). "A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus." Proc Natl Acad Sci U S A **98**(20): 11598-11603.
McDowell, J. H., J. P. Nawrocki and P. A. Hargrave (1993). "Phosphorylation sites in bovine rhodopsin." Biochemistry **32**(18): 4968-4974.
Moreau, P., F. Cavallo, X. Leleu, C. Hulin, M. Amiot, G. Descamps, T. Facon, M. Boccadoro, D. Mignard and J. L. Harousseau (2011). "Phase I study of the anti insulin-like growth factor 1 receptor (IGF-1R) monoclonal antibody, AVE1642, as single agent and in combination with bortezomib in patients with relapsed multiple myeloma." Leukemia **25**(5): 872-874.
Mulvihill, M. J., A. Cooke, M. Rosenfeld-Franklin, E. Buck, K. Foreman, D. Landfair, M. O'Connor, C. Pirritt, Y. Sun, Y. Yao, L. D. Arnold, N. W. Gibson and Q. S. Ji (2009). "Discovery of OSI-906: a selective and orally efficacious dual inhibitor of the IGF-1 receptor and insulin receptor." Future Med Chem **1**(6): 1153-1171.
Murphy, L. O., J. P. MacKeigan and J. Blenis (2004). "A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration." Mol Cell Biol **24**(1): 144-153.
Natalishvili, N., M. Axelson, L. Girnita, O. Larsson and D. Vasilcanu (2009). "Aberrant intracellular IGF-1R beta-subunit makes receptor knockout cells (IGF1R-/-) susceptible to oncogenic transformation." Exp Cell Res **315**(8): 1458-1467.
Neves, S. R., P. T. Ram and R. Iyengar (2002). "G protein pathways." Science **296**(5573): 1636-1639.
Ng, G. Y., T. McDonald, T. Bonnert, M. Rigby, R. Heavens, P. Whiting, A. Chateauneuf, N. Coulombe, S. Kargman, T. Caskey, J. Evans, P. O'Neill G and Q. Liu (1999). "Cloning of a novel G-protein-coupled receptor GPR 51 resembling GABAB receptors expressed predominantly in nervous tissues and mapped proximal to the hereditary sensory neuropathy type 1 locus on chromosome 9." Genomics **56**(3): 288-295.
Nigg, E. A. (1997). "Nucleocytoplasmic transport: signals, mechanisms and regulation." Nature **386**(6627): 779-787.
Oakley, R. H., S. A. Laporte, J. A. Holt, M. G. Caron and L. S. Barak (2000). "Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors." J Biol Chem **275**(22): 17201-17210.
Ohlsson, C., N. Kley, H. Werner and D. LeRoith (1998). "p53 regulates insulin-like growth factor-I (IGF-I) receptor expression and IGF-I-induced tyrosine phosphorylation in an osteosarcoma cell line: Interaction between p53 and Sp1." Endocrinology **139**(3): 1101-1107.
Olchovsky, D., J. Song, M. C. Gelato, J. Sherwood, E. Spatola, J. F. Bruno and M. Berelowitz (1993). "Pituitary and hypothalamic insulin-like growth factor-I (IGF-I) and IGF-I receptor expression in food-deprived rats." Mol Cell Endocrinol **93**(2): 193-198.
Olivecrona, H., A. Hilding, C. Ekstrom, H. Barle, B. Nyberg, C. Moller, P. J. Delhanty, R. C. Baxter, B. Angelin, T. J. Ekstrom and M. Tally (1999). "Acute and short-term effects of growth hormone on insulin-like growth factors and their binding proteins: serum levels and hepatic messenger ribonucleic acid responses in humans." J Clin Endocrinol Metab **84**(2): 553-560.
Pandini, G., F. Frasca, R. Mineo, L. Sciacca, R. Vigneri and A. Belfiore (2002). "Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved." J Biol Chem **277**(42): 39684-39695.
Papin, C., A. Denouel-Galy, D. Laugier, G. Calothy and A. Eychene (1998). "Modulation of kinase activity and oncogenic properties by alternative splicing reveals a novel regulatory mechanism for B-Raf." J Biol Chem **273**(38): 24939-24947.
Pappo, A. S., S. R. Patel, J. Crowley, D. K. Reinke, K. P. Kuenkele, S. P. Chawla, G. C. Toner, R. G. Maki, P. A. Meyers, R. Chugh, K. N. Ganjoo, S. M. Schuetze, H. Juergens, M. G. Leahy, B. Geoerger, R. S. Benjamin, L. J. Helman and L. H. Baker (2011). "R1507, a monoclonal antibody to the insulin-like growth factor 1 receptor, in patients with recurrent or refractory Ewing sarcoma family of tumors: results of a phase II Sarcoma Alliance for Research through Collaboration study." J Clin Oncol **29**(34): 4541-4547.

Park, E. A., A. L. Gurney, S. E. Nizielski, P. Hakimi, Z. Cao, A. Moorman and R. W. Hanson (1993). "Relative roles of CCAAT/enhancer-binding protein beta and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP)." The Journal of biological chemistry **268**(1): 613-619.
Penela, P., C. Ribas and F. Mayor, Jr. (2003). "Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases." Cell Signal **15**(11): 973-981.
Perry, S. J., G. S. Baillie, T. A. Kohout, I. McPhee, M. M. Magiera, K. L. Ang, W. E. Miller, A. J. McLean, M. Conti, M. D. Houslay and R. J. Lefkowitz (2002). "Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins." Science **298**(5594): 834-836.
Peter, M., J. Couturier, H. Pacquement, J. Michon, G. Thomas, H. Magdelenat and O. Delattre (1997). "A new member of the ETS family fused to EWS in Ewing tumors." Oncogene **14**(10): 1159-1164.
Petroski, M. D. and R. J. Deshaies (2003). "Context of multiubiquitin chain attachment influences the rate of Sic1 degradation." Mol Cell **11**(6): 1435-1444.
Pitcher, J. A., E. S. Payne, C. Csortos, A. A. DePaoli-Roach and R. J. Lefkowitz (1995). "The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity." Proceedings of the National Academy of Sciences of the United States of America **92**(18): 8343-8347.
Pitcher, J. A., J. J. Tesmer, J. L. Freeman, W. D. Capel, W. C. Stone and R. J. Lefkowitz (1999). "Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases." The Journal of biological chemistry **274**(49): 34531-34534.
Pollak, M. (2008). "Insulin and insulin-like growth factor signalling in neoplasia." Nature Reviews Cancer **8**(12): 915-928.
Pollak, M. (2008). "Targeting insulin and insulin-like growth factor signalling in oncology." Curr Opin Pharmacol **8**(4): 384-392.
Prisco, M., A. Hongo, M. G. Rizzo, A. Sacchi and R. Baserga (1997). "The insulin-like growth factor I receptor as a physiologically relevant target of p53 in apoptosis caused by interleukin-3 withdrawal." Molecular and Cellular Biology **17**(3): 1084-1092.
Pulvermuller, A., K. Schroder, T. Fischer and K. P. Hofmann (2000). "Interactions of metarhodopsin II. Arrestin peptides compete with arrestin and transducin." The Journal of biological chemistry **275**(48): 37679-37685.
Ranchal, I., R. Gonzalez, R. I. Bello, G. Ferrin, A. B. Hidalgo, C. I. Linares, P. Aguilar-Melero, S. Gonzalez-Rubio, P. Barrera, T. Marchal, K. I. Nakayama, M. de la Mata and J. Muntane (2009). "The reduction of cell death and proliferation by p27(Kip1) minimizes DNA damage in an experimental model of genotoxicity." Int J Cancer **125**(10): 2270-2280.
Reidy-Lagunes, D. L., E. Vakiani, M. F. Segal, E. M. Hollywood, L. H. Tang, D. B. Solit, M. C. Pietanza, M. Capanu and L. B. Saltz (2012). "A phase 2 study of the insulin-like growth factor-1 receptor inhibitor MK-0646 in patients with metastatic, well-differentiated neuroendocrine tumors." Cancer.
Reiter, E., S. Ahn, A. K. Shukla and R. J. Lefkowitz (2012). "Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors." Annu Rev Pharmacol Toxicol **52**: 179-197.
Reiter, E. and R. J. Lefkowitz (2006). "GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling." Trends Endocrinol Metab **17**(4): 159-165.
Ren, X. R., E. Reiter, S. Ahn, J. Kim, W. Chen and R. J. Lefkowitz (2005). "Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor." Proceedings of the National Academy of Sciences of the United States of America **102**(5): 1448-1453.
Ren, X. R., E. Reiter, S. Ahn, J. Kim, W. Chen and R. J. Lefkowitz (2005). "Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor." Proc Natl Acad Sci U S A **102**(5): 1448-1453.
Rikhof, B., S. de Jong, A. J. H. Suurmeijer, C. M. Winette and T. A. van der Graaf (2009). "The insulin-like growth factor system and sarcomas." The Journal of Pathology **217**(4): 469-482.

Rinderknecht, E. and R. E. Humbel (1978). "The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin." J Biol Chem **253**(8): 2769-2776.
Rinderknecht, E. and R. E. Humbel (1978). "Primary structure of human insulin-like growth factor II." FEBS Lett **89**(2): 283-286.
Robzyk, K., J. Recht and M. A. Osley (2000). "Rad6-dependent ubiquitination of histone H2B in yeast." Science **287**(5452): 501-504.
Rodrigo-Brenni, M. C., S. A. Foster and D. O. Morgan (2010). "Catalysis of lysine 48-specific ubiquitin chain assembly by residues in E2 and ubiquitin." Mol Cell **39**(4): 548-559.
Roman, G., J. He and R. L. Davis (2000). "kurtz, a novel nonvisual arrestin, is an essential neural gene in Drosophila." Genetics **155**(3): 1281-1295.
Rosen, C. J. and M. Pollak (1999). "Circulating IGF-I: New Perspectives for a New Century." Trends Endocrinol Metab **10**(4): 136-141.
Rosen, L. S., I. Puzanov, G. Friberg, E. Chan, Y. C. Hwang, H. Deng, J. Gilbert, D. Mahalingam, I. McCaffery, S. A. Michael, A. C. Mita, M. M. Mita, M. Mulay, P. Shubhakar, M. Zhu and J. Sarantopoulos (2012). "Safety and pharmacokinetics of ganitumab (AMG 479) combined with sorafenib, panitumumab, erlotinib, or gemcitabine in patients with advanced solid tumors." Clin Cancer Res **18**(12): 3414-3427.
Rosenfeld, R. G., V. Hwa, L. Wilson, A. Lopez-Bermejo, C. Buckway, C. Burren, W. K. Choi, G. Devi, A. Ingermann, D. Graham, G. Minniti, A. Spagnoli and Y. Oh (1999). "The insulin-like growth factor binding protein superfamily: new perspectives." Pediatrics **104**(4 Pt 2): 1018-1021.
Ruvinsky, I. and O. Meyuhas (2006). "Ribosomal protein S6 phosphorylation: from protein synthesis to cell size." Trends Biochem Sci **31**(6): 342-348.
Sakatani, T., A. Kaneda, C. A. Iacobuzio-Donahue, M. G. Carter, S. de Boom Witzel, H. Okano, M. S. Ko, R. Ohlsson, D. L. Longo and A. P. Feinberg (2005). "Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice." Science **307**(5717): 1976-1978.
Samama, P., S. Cotecchia, T. Costa and R. J. Lefkowitz (1993). "A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model." J Biol Chem **268**(7): 4625-4636.
Sarfstein, R., S. Maor, N. Reizner, S. Abramovitch and H. Werner (2006). "Transcriptional regulation of the insulin-like growth factor-I receptor gene in breast cancer." Mol Cell Endocrinol **252**(1-2): 241-246.
Scheffner, M. and O. Staub (2007). "HECT E3s and human disease." BMC Biochem **8 Suppl 1**: S6.
Scheffzek, K. and M. Ahmadian (2005). "GTPase activating proteins: structural and functional insights 18 years after discovery." Cellular and Molecular Life Sciences (CMLS) **62**(24): 3014-3038.
Schlessinger, J. (2000). "Cell signaling by receptor tyrosine kinases." Cell **103**(2): 211-225.
Schumacher, R., L. Mosthaf, J. Schlessinger, D. Brandenburg and A. Ullrich (1991). "Insulin and insulin-like growth factor-1 binding specificity is determined by distinct regions of their cognate receptors." J Biol Chem **266**(29): 19288-19295.
Scotlandi, K., S. Benini, P. Nanni, P. L. Lollini, G. Nicoletti, L. Landuzzi, M. Serra, M. C. Manara, P. Picci and N. Baldini (1998). "Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice." Cancer Research **58**(18): 4127-4131.
Sehat, B., S. Andersson, L. Girnita and O. Larsson (2008). "Identification of c-Cbl as a new ligase for insulin-like growth factor-I receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis." Cancer research **68**(14): 5669-5677.
Sehat, B., S. Andersson, R. Vasilcanu, L. Girnita and O. Larsson (2007). "Role of Ubiquitination in IGF-1 Receptor Signaling and Degradation." PLoS One **2**(4).
Sell, C., M. Rubini, R. Rubin, J. P. Liu, A. Efstratiadis and R. Baserga (1993). "Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor." Proceedings of the National Academy of Sciences of the United States of America **90**(23): 11217-11221.

Shenoy, S. K., L. S. Barak, K. Xiao, S. Ahn, M. Berthouze, A. K. Shukla, L. M. Luttrell and R. J. Lefkowitz (2007). "Ubiquitination of beta-arrestin links seven-transmembrane receptor endocytosis and ERK activation." J Biol Chem **282**(40): 29549-29562.

Shenoy, S. K., M. T. Drake, C. D. Nelson, D. A. Houtz, K. Xiao, S. Madabushi, E. Reiter, R. T. Premont, O. Lichtarge and R. J. Lefkowitz (2006). "beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor." J Biol Chem **281**(2): 1261-1273.

Shenoy, S. K., M. T. Drake, C. D. Nelson, D. A. Houtz, K. Xiao, S. Madabushi, E. Reiter, R. T. Premont, O. Lichtarge and R. J. Lefkowitz (2006). "beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor." The Journal of biological chemistry **281**(2): 1261-1273.

Shenoy, S. K. and R. J. Lefkowitz (2003). "Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination." J Biol Chem **278**(16): 14498-14506.

Shenoy, S. K. and R. J. Lefkowitz (2003). "Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination." The Journal of biological chemistry **278**(16): 14498-14506.

Shenoy, S. K. and R. J. Lefkowitz (2005). "Angiotensin II-stimulated signaling through G proteins and beta-arrestin." Sci STKE **2005**(311): cm14.

Shenoy, S. K. and R. J. Lefkowitz (2005). "Receptor-specific ubiquitination of beta-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes." J Biol Chem **280**(15): 15315-15324.

Shenoy, S. K. and R. J. Lefkowitz (2005). "Seven-transmembrane receptor signaling through beta-arrestin." Sci STKE **2005**(308): cm10.

Shenoy, S. K., P. H. McDonald, T. A. Kohout and R. J. Lefkowitz (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." Science **294**(5545): 1307-1313.

Shenoy, S. K., A. S. Modi, A. K. Shukla, K. Xiao, M. Berthouze, S. Ahn, K. D. Wilkinson, W. E. Miller and R. J. Lefkowitz (2009). "Beta-arrestin-dependent signaling and trafficking of 7-transmembrane receptors is reciprocally regulated by the deubiquitinase USP33 and the E3 ligase Mdm2." Proc Natl Acad Sci U S A **106**(16): 6650-6655.

Simon, M. I., M. P. Strathmann and N. Gautam (1991). "Diversity of G proteins in signal transduction." Science **252**(5007): 802-808.

Simon, V., M. T. Robin, C. Legrand and J. Cohen-Tannoudji (2003). "Endogenous G protein-coupled receptor kinase 6 triggers homologous beta-adrenergic receptor desensitization in primary uterine smooth muscle cells." Endocrinology **144**(7): 3058-3066.

Smith, E. R., J. L. Smedberg, M. E. Rula and X. X. Xu (2004). "Regulation of Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endoderm differentiation of embryonic carcinoma and stem cells." J Cell Biol **164**(5): 689-699.

Smrcka, A. V. (2008). "G protein betagamma subunits: central mediators of G protein-coupled receptor signaling." Cell Mol Life Sci **65**(14): 2191-2214.

Spence, J., S. Sadis, A. L. Haas and D. Finley (1995). "A ubiquitin mutant with specific defects in DNA repair and multiubiquitination." Mol Cell Biol **15**(3): 1265-1273.

Steele-Perkins, G., J. Turner, J. C. Edman, J. Hari, S. B. Pierce, C. Stover, W. J. Rutter and R. A. Roth (1988). "Expression and characterization of a functional human insulin-like growth factor I receptor." J Biol Chem **263**(23): 11486-11492.

Sterne-Marr, R., V. V. Gurevich, P. Goldsmith, R. C. Bodine, C. Sanders, L. A. Donoso and J. L. Benovic (1993). "Polypeptide variants of beta-arrestin and arrestin3." J Biol Chem **268**(21): 15640-15648.

Storez, H., M. G. Scott, H. Issafras, A. Burtey, A. Benmerah, O. Muntaner, T. Piolot, M. Tramier, M. Coppey-Moisan, M. Bouvier, C. Labbe-Jullie and S. Marullo (2005). "Homo- and hetero-oligomerization of beta-arrestins in living cells." J Biol Chem **280**(48): 40210-40215.

Sunahara, R. K. and R. Taussig (2002). "Isoforms of mammalian adenylyl cyclase: multiplicities of signaling." Mol Interv **2**(3): 168-184.

Thrower, J. S., L. Hoffman, M. Rechsteiner and C. M. Pickart (2000). "Recognition of the polyubiquitin proteolytic signal." Embo J **19**(1): 94-102.
Toretsky, J. A., T. Kalebic, V. Blakesley, D. LeRoith and L. J. Helman (1997). "The insulin-like growth factor-I receptor is required for EWS/FLI-1 transformation of fibroblasts." Journal of Biological Chemistry **272**(49): 30822-30827.
Treadway, J. L., B. D. Morrison, I. D. Goldfine and J. E. Pessin (1989). "Assembly of insulin/insulin-like growth factor-1 hybrid receptors in vitro." J Biol Chem **264**(36): 21450-21453.
Ullrich, A., A. Gray, A. W. Tam, T. Yang-Feng, M. Tsubokawa, C. Collins, W. Henzel, T. Le Bon, S. Kathuria, E. Chen and et al. (1986). "Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity." Embo J **5**(10): 2503-2512.
Valentinis, B., A. Morrione, S. J. Taylor and R. Baserga (1997). "Insulin-like growth factor I receptor signaling in transformation by src oncogenes." Mol. Cell. Biol. **17**(7): 3744-3754.
Vanhaesebroeck, B. and D. R. Alessi (2000). "The PI3K-PDK1 connection: more than just a road to PKB." Biochem J **346 Pt 3**: 561-576.
Vecchione, A., A. Marchese, P. Henry, D. Rotin and A. Morrione (2003). "The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor." Mol Cell Biol **23**(9): 3363-3372.
Vetter, I. R. and A. Wittinghofer (2001). "The guanine nucleotide-binding switch in three dimensions." Science **294**(5545): 1299-1304.
Vishnivetskiy, S. A., M. M. Hosey, J. L. Benovic and V. V. Gurevich (2004). "Mapping the arrestin-receptor interface. Structural elements responsible for receptor specificity of arrestin proteins." The Journal of biological chemistry **279**(2): 1262-1268.
Wang, L. M., M. G. Myers, Jr., X. J. Sun, S. A. Aaronson, M. White and J. H. Pierce (1993). "IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells." Science **261**(5128): 1591-1594.
Wang, M., Y. Xie, L. Girnita, G. Nilsson, A. Dricu, J. Wejde and O. Larsson (1999). "Regulatory role of mevalonate and N-linked glycosylation in proliferation and expression of the EWS/FLI-1 fusion protein in Ewing's sarcoma cells." Exp Cell Res **246**(1): 38-46.
Weber, C. K., J. R. Slupsky, C. Herrmann, M. Schuler, U. R. Rapp and C. Block (2000). "Mitogenic signaling of Ras is regulated by differential interaction with Raf isozymes." Oncogene **19**(2): 169-176.
Wei, H., S. Ahn, S. K. Shenoy, S. S. Karnik, L. Hunyady, L. M. Luttrell and R. J. Lefkowitz (2003). "Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2." Proc Natl Acad Sci U S A **100**(19): 10782-10787.
Wellbrock, C., M. Karasarides and R. Marais (2004). "The RAF proteins take centre stage." Nat Rev Mol Cell Biol **5**(11): 875-885.
Werner, H., C. Hernandezsanchez, E. Karnieli and D. Leroith (1995). "The Regulation of Igf-I Receptor Gene-Expression." International Journal of Biochemistry & Cell Biology **27**(10): 987-994.
Werner, H. and D. Le Roith (2000). "New concepts in regulation and function of the insulin-like growth factors: implications for understanding normal growth and neoplasia." Cell Mol Life Sci **57**(6): 932-942.
Werner, H. and C. T. Roberts, Jr. (2003). "The IGFI receptor gene: a molecular target for disrupted transcription factors." Genes Chromosomes Cancer **36**(2): 113-120.
Werner, H., M. Shalita-Chesner, S. Abramovitch, G. Idelman, L. Shaharabani-Gargir and T. Glaser (2000). "Regulation of the insulin-like growth factor-I receptor gene by oncogenes and antioncogenes: implications in human cancer." Mol Genet Metab **71**(1-2): 315-320.
Whistler, J. L. and M. von Zastrow (1998). "Morphine-activated opioid receptors elude desensitization by beta-arrestin." Proc Natl Acad Sci U S A **95**(17): 9914-9919.
Willets, J. M., R. A. Challiss and S. R. Nahorski (2002). "Endogenous G protein-coupled receptor kinase 6 Regulates M3 muscarinic acetylcholine receptor phosphorylation and desensitization in human SH-SY5Y neuroblastoma cells." J Biol Chem **277**(18): 15523-15529.

Witherow, D. S., T. R. Garrison, W. E. Miller and R. J. Lefkowitz (2004). "beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha." Proc Natl Acad Sci U S A **101**(23): 8603-8607.
Wittman, M., J. Carboni, R. Attar, B. Balasubramanian, P. Balimane, P. Brassil, F. Beaulieu, C. Chang, W. Clarke, J. Dell, J. Eummer, D. Frennesson, M. Gottardis, A. Greer, S. Hansel, W. Hurlburt, B. Jacobson, S. Krishnananthan, F. Y. Lee, A. Li, T. A. Lin, P. Liu, C. Ouellet, X. Sang, M. G. Saulnier, K. Stoffan, Y. Sun, U. Velaparthi, H. Wong, Z. Yang, K. Zimmermann, M. Zoeckler and D. Vyas (2005). "Discovery of a (1H-benzoimidazol-2-yl)-1H-pyridin-2-one (BMS-536924) inhibitor of insulin-like growth factor I receptor kinase with in vivo antitumor activity." J Med Chem **48**(18): 5639-5643.
Yin, D., F. Vreeland, L. J. Schaaf, R. Millham, B. A. Duncan and A. Sharma (2007). "Clinical pharmacodynamic effects of the growth hormone receptor antagonist pegvisomant: implications for cancer therapy." Clin Cancer Res **13**(3): 1000-1009.
Yoon, A. and R. A. Hurta (2001). "Insulin like growth factor-1 selectively regulates the expression of matrix metalloproteinase-2 in malignant H-ras transformed cells." Mol Cell Biochem **223**(1-2): 1-6.
Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." Growth Factors **24**(1): 21-44.
Yu, H. and T. Rohan (2000). "Role of the insulin-like growth factor family in cancer development and progression." J Natl Cancer Inst **92**(18): 1472-1489.
Zapf, J. (1995). "Insulinlike growth factor binding proteins and tumor hypoglycemia." Trends Endocrinol Metab **6**(2): 37-42.
Zavros, Y., M. Waghray, A. Tessier, L. Bai, A. Todisco, L. G. D, L. C. Samuelson, A. Dlugosz and J. L. Merchant (2007). "Reduced pepsin A processing of sonic hedgehog in parietal cells precedes gastric atrophy and transformation." J Biol Chem **282**(46): 33265-33274.
Zehorai, E., Z. Yao, A. Plotnikov and R. Seger (2010). "The subcellular localization of MEK and ERK--a novel nuclear translocation signal (NTS) paves a way to the nucleus." Mol Cell Endocrinol **314**(2): 213-220.
Zhang, B. and R. A. Roth (1991). "Binding properties of chimeric insulin receptors containing the cysteine-rich domain of either the insulin-like growth factor I receptor or the insulin receptor related receptor." Biochemistry **30**(21): 5113-5117.
Zhang, D. and P. Brodt (2003). "Type 1 insulin-like growth factor regulates MT1-MMP synthesis and tumor invasion via PI 3-kinase/Akt signaling." Oncogene **22**(7): 974-982.
Zhou, B. P., Y. Liao, W. Xia, Y. Zou, B. Spohn and M. C. Hung (2001). "HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation." Nat Cell Biol **3**(11): 973-982.
Zhou, J. and C. Bondy (1992). "Insulin-Like Growth Factor-Ii and Its Binding-Proteins in Placental Development." Endocrinology **131**(3): 1230-1240.
Zimmerman, E. S., B. A. Schulman and N. Zheng (2010). "Structural assembly of cullin-RING ubiquitin ligase complexes." Curr Opin Struct Biol **20**(6): 714-721.