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BIASED SIGNALLING AT THE IGF-1R; PITFALLS AND POTENTIAL OF THE GRK/BETA-ARRESTIN SYSTEM IN CANCER THERAPEUTICS

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Biased Signalling at the IGF-1R;
Pitfalls and Potential of the GRK/ β -arrestin system in
Cancer Therapeutics

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Any cell carries within it the experiences of a billion years of experimentation. You cannot expect to explain so wise an old bird in a few simple words.”

– Max Delbrück, Geneticist, Nobel Prize in Physiology or Medicine 1969

*This exploration of cells, the smallest unit of biological life,
is dedicated to those who gave me theirs,
and the whole world ever since.*

*Mum and Dad,
this is for you.*

ABSTRACT

Embedded into the boundary between the living machinery and the external space, plasma membrane receptors allow cells to perceive their environment and elicit appropriate responses. Two of the largest classes of receptors are the G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). Distinct in their structural and functional characteristics, each lend themselves to important cellular operations. While crosstalk between members of either family is a general phenomenon, a growing body of evidence suggests there may be a more direct overlap. This thesis explores the insulin-like growth factor type 1 receptor (IGF-1R), as an intermediate between these two receptor families. Supporting growth and survival in many human cancers, the IGF-1R has long been considered an attractive therapeutic target. Despite the pre-clinical appeal and intense pharmaceutical development, disappointing clinical trials suggest that its potential has not been fulfilled. This thesis examines the true complexity of this receptor system, with particular focus on its use of GPCR components and how they contribute to the paradigm of *biased signalling*.

Study I categorizes the therapeutic relevance of biased signalling at the IGF-1R. Our results identify small molecule Nutlin-3 as strategy that synergizes with MEK inhibition, by co-targeting the p53 and IGF-1R, without biased signal activation. **Study II** set out to define the role of the β -arrestin 2 isoform at the IGF-1R, and in doing so identifies the mechanism controlling a balanced versus biased receptor conformation. The β -arrestin isoforms antagonize each other's function at the IGF-1R, imposing regulation on the receptor expression, signalling and crosstalk to p53. The position of β -arrestins, between an important mitogenic pathway and perhaps the ultimate tumour suppressor pathway, reveals potential for therapeutic gain. **Study III** develops strategies for targeting β -arrestin/GRK-biased agonism at the IGF-1R for cancer treatment, with focus on clinical applicability. This work provides the proof of concept for cross-targeting the IGF-1R through GRK 2 inhibition, and suggests clinical feasibility of such an approach by repurposing the widely used drug paroxetine. Acknowledging the clinical importance of biased signalling at the IGF-1R, **Study IV** aims to explore the utility of microRNAs as biomarkers to quantify signalling bias downstream of IGF-1R. MicroRNA array and IGF-1R mutation analysis identifies miR-106a as a candidate that can specify a β -arrestin biased IGF-1R signal that could be developed for patient stratification in anti-IGF-1R trials.

Altogether, our findings highlight the shortcomings of first line anti-IGF-1R strategies, and the overly simplistic models in use at the time. Armed with an appreciation of the true complexity, plasticity and interconnectivity of receptor systems, we have examined the therapeutic utility of the novel components GRK/ β -arrestin, and identified targets that may hold potential in unlocking the true potential of anti-IGF-1R.

LIST OF SCIENTIFIC PAPERS:

- I. Enhanced response of melanoma cells to MEK inhibitors following unbiased IGF-1R down-regulation.
Suleymanova N.*, **Crudden C.***, Worrall C., Dricu A., Girnita A., and Girnita L.
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 - II. Functional antagonism of β -arrestin isoforms balance IGF-1R expression and signalling with distinct cancer-related biological outcomes.
Suleymanova N., **Crudden C.**, Shibano T., Worrall C., Oprea I., Tica A., Calin G. A., Girnita A., and Girnita L.
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 - III. G protein-coupled receptor kinase 2 inhibition promotes unbiased insulin-like growth factor 1 receptor down-regulation and restrains malignant cell growth.
Crudden C., Shibano T., Song D., Serly J., Nedelcu D., Tica A., Calin G. A., Girnita A., and Girnita L.
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 - IV. Uncoupling signalling at IGF-1R identifies miR-106a as biomarker of cancer-promoting β -arrestin signalling.
Crudden C., Iulian O., Song, D., Redis R., Ivan C., Girnita A., Calin G. A., and Girnita L.
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- * Equal contribution

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- I. Blurring Boundaries: Receptor Tyrosine Kinases as functional G protein-coupled receptors.
Crudden C., Shibano T., Song D., Suleymanova N., Girnita A., and Girnita L.
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- II. Genome-wide screen for microRNAs reveals a role for miR-203 in melanoma metastasis.
Lohcharoenkal W., Das Mahapatra K., Pasquali L., **Crudden C.**, Kular L., Akkaya Ulum Y.Z., Zhang L., Xu Laden N., Girnita L., Jagodic M., Stähle M., Sonkoly E., and Pivarsci A.
Journal of Investigative Dermatology, 2018; 138(4): 882-892.
PMID: 29104160
- III. Unbalancing p53/Mdm2/IGF-1R axis by Mdm2 activation restrains the IGF-1-dependent invasive phenotype of skin melanoma.
Worrall C., Suleymanova N., **Crudden C.**, T-Drakensjö I., Candrea E., Nedelcu D., Takahashi S.I., Girnita L., and Girnita A.
Oncogene, 2017; 36(23): 3274-3286.
PMID: 28092675
- IV. When Phosphorylation encounters Ubiquitination; A balanced perspective on IGF-1R signalling.
Girnita L., Takahashi S.I., **Crudden C.**, Fukushima T., Worrall C., Furuta H., Yoshihara H., Hakuno F., and Girnita A.
Progress in Molecular Biology and Translational Science, 2016; 141: 277-311.
PMID: 27378760
- V. Differential regulation of IGF-1 and Insulin signalling by GRKs.
Girnita L., Girnita A., and **Crudden C.**
Methods in Pharmacology and Toxicology, 2016;
Book: G protein-coupled receptor kinases
DOI: 10.1007/978-1-4939-3798-1_7
- VI. Targeting the IGF-1R: The Tale of the Tortoise and the Hare.
Crudden C., Girnita A., and Girnita L.
Frontiers in Endocrinology, 2015; 6: 64.
PMID: 25964779
- VII. The dichotomy of the insulin-like growth factor 1 receptor: RTK and GPCR: friend or foe for cancer treatment?
Crudden C., Ilic M., Suleymanova N., Worrall C., Girnita A., and Girnita L.
Growth Hormone and IGF Research, 2015; 25(1): 2-12.
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LIST OF ABBREVIATIONS

| | |
|--------------|--|
| LUCA | Last universal common ancestor |
| ATP | Adenosine triphosphate |
| SH2 | Src Homology 2 |
| PTB | Phosphotyrosine-binding |
| GPCR | G protein-coupled receptor |
| GPS | GPCR proteolytic site |
| 7TMR | Seven transmembrane receptor |
| G protein | Guanine-nucleotide-binding regulatory proteins |
| AMP | Adenosine monophosphate |
| GAPS | GTPase-activating proteins |
| GRKs | G protein-coupled receptor kinase |
| β -arr | β -arrestin |
| RH | RGS homology |
| β_2 AR | β_2 -adrenergic receptor |
| RGS | Regulator of G protein Signalling |
| KO | Knock-out |
| CCR7 | C-C chemokine receptor type 7 |
| CCL19 | C-C chemokine ligand 19 |
| CCL21 | C-C chemokine ligand 21 |
| LPA | Lysophosphatidic acid |
| DAPI | 4',6-diamidino-2-phenylindole |
| RTK | Receptor tyrosine kinase |
| EGFR | Epidermal growth factor receptors (EGFR, ErbB and HER) |
| PDGFR | Platelet-derived growth factor receptor |
| FGFR | Fibroblast growth factor receptor |
| VEGFR | Vascular endothelial growth factor receptor |
| IR | Insulin receptor |
| IGF-1R | Insulin-like growth factor type 1 receptor |
| IGFs | Insulin-like growth factors |
| GH | Growth hormone |
| v-sis | Simian sarcoma virus oncogene |
| SV40T | Simian virus 40 T-antigen |
| v-src | Rous sarcoma virus oncogene |
| MAPK | Mitogen activated protein kinase |
| ERK1/2 | Extracellular signal-regulated kinases |
| PI3K | Phosphatidylinositol 3-kinase |
| Akt (PKB) | Protein kinase B |
| IGF-1 | Insulin-like growth factor 1 |
| IGF-2 | Insulin-like growth factor 2 |
| IGFBP | Insulin-like growth factor binding protein |
| IRR | Insulin-receptor-related receptor |
| A-loop | Activation-loop |

| | |
|------------------|---|
| Tyr | Tyrosine |
| IRS | Insulin receptor substrate |
| PTB | Phosphotyrosine binding |
| SH2 | Src homology 2 |
| SOS | Son of sevenless |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| PIP ₃ | Phosphatidylinositol 3,4,5-trisphosphate |
| PIP ₂ | Phosphatidylinositol 4,5-bisphosphate |
| PDK1 | 3-phosphoinositide-dependent kinase-1 |
| BAD | Bcl-2-associated death promoter protein |
| mTOR | Mammalian target of rapamycin |
| GSK-3 β | Glycogen synthase kinase-3 β |
| FOXO | Forkhead box O-class protein |
| MMPs | Matrix metallo-proteinases |
| Mdm2 | Mouse double minute 2 homolog |
| BRCA-1 | Breast cancer protein-1 |
| SAPKs | Stress-activated protein kinases |
| JNK | Jun N terminal kinase |
| RACK1 | Receptor for activated C kinase 1 |
| SOCS | Suppressor of cytokine signalling |
| FAK | Focal adhesion kinase |
| GEF | Guanine nucleotide exchange factor |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |
| WT-1 | Wilms tumour-1 |
| MEFs | Mouse embryonic fibroblasts |
| LS | Laron syndrome |
| CP | CP-751,871 (IGF-1R targeting antibody figitumumab) |
| SUMO | Small ubiquitin-like modifier |
| DAPI | 4',6-diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| PX | Paroxetine |
| qRT-PCR | Quantitative real time polymerase chain reaction |
| WB | Western blot |
| pIGF-1R | Phosphorylated IGF-1R |
| pAkt | Phosphorylated Akt |
| pERK | Phosphorylated ERK |
| C-terminal | Carboxyl-terminal |
| NF- κ B | Nuclear Factor- κ B |
| Poly-2-HEMA | Poly-2-hydroxethyl methacrylate |
| SSRI | Selective serotonin reuptake inhibitor |
| miRNA | MicroRNA |

1 INTRODUCTION

1.1 CELL SIGNALLING

The cell is often referred to as the smallest fundamental unit of biological life. Cells are the building blocks of every living thing on the planet today; every animal, plant and microorganism. And if we reverse in time and arrive somewhere around 3.2 billion years ago, we all share a single common ancestor - a cell called LUCA (Last Universal Common Ancestor) [1]. All living cells must be able to perceive their environments and adjust their behaviour accordingly. Early unicellular organisms needed to be able to sense and navigate towards nutrients, or sense and avoid toxins. Later, at the evolution of multi-cellular organisms (metazoa) there arose the added complexity of needing all cells to act in a coordinated and specialized manner, and hence the need to develop information transfer between cells. In organisms as extraordinarily complex as mammals, intercellular communication and signalling mediate a sophisticated web of growth, death, differentiation and metabolism, and the efficiency of such a network constitutes a major component of survival. Remarkably, from early single-celled organisms, the signalling pathways our cells use are fundamentally unchanged in their transduction components, only the variety of ligands, receptors, targets, and hence sophistication, has increased [1].

The plasma membrane of a cell is the interface between the living machinery and its environment. As such, this interface is a highly specialized organ, with distinctive properties to perceive and relay a wide variety of information. Diverse receptors anchored into the plasma membrane mediate execution of cellular responses by receiving information and managing its processing. As crucial components of normal cellular physiology, mutational changes in such systems often result in pathophysiological processes leading to disease. Whilst this thesis will focus on the role played by cellular signalling in the development and maintenance of human cancers, perturbations in signal pathways span virtually all known human diseases.

1.2 G PROTEIN-COUPLED RECEPTORS

The evolution from a unicellular ancestor to multicellular metazoans was one of the most important advances in the history of biological life. We can derive clues about how that transition took place by following the expression pattern changes of different intercellular molecules. Pre-metazoans did not need to maintain cell-to-cell communication, but were relatively sophisticated in their ability to sense and respond to the environment, therefore some key signalling pathways were already present in unicellular organisms. One of these

evolutionarily conserved pathways is the G protein-coupled receptor (GPCR) pathway. Genome survey and evolution reconstruction studies suggest that various components evolved independently through eukaryotic lineages, highlighting the modular nature of the GPCR system [2]. The full GPCR toolbox as we know it is not found in unicellular fungi, or plantae, but is found in the unicellular *chanoflagellates*, the closest known unicellular relative to metazoans, indicating that it pre-dates metazoan separation [3]. Expression patterns suggest that the transition to multi-cellularity conserved and amplified this system through massive receptor diversification to cope with new multicellular needs [2, 4].

In mammalian cells GPCRs are the largest class of cell surface receptors, and there are around 800 currently annotated in the human genome [5], commonly grouped into five or six families [6, 7]. Classical GPCRs consist of an extracellular N-terminal domain, a membrane-spanning domain and an intracellular C-terminal cytoplasmic tail. Due to the “serpent-like” section spanning the plasma membrane seven times, GPCRs are also sometimes referred to as serpentine receptors, heptahelical receptors or seven transmembrane receptors (7TMRs). They are functionally diverse, spanning nearly every physiological process in the human body – from nerve transmission and olfactory sensation to hormone signalling. This diversity is reflected in the repertoire of ligands they can respond to: including photons, neurotransmitters, odorants, lipids, proteins, amino acids, hormones and chemokines. Notably, GPCRs are critically important in pharmaceutical targeting, and are the targets of somewhere between one third [8], to as many as a half [9], of all currently marketed drugs. Due to their *druggability*, they are the focus of intense translational study in both academia and industry.

Despite the great diversity in receptors and ultimate function, mechanistically GPCRs are largely similar. Unlike other receptor families, GPCRs lack intrinsic catalytic activity, and instead transduce their signals by coupling to their namesake G proteins. Upon agonist binding, the receptor is stabilized into its active conformation, allowing recruitment and activation of heterotrimeric G proteins, which subsequently activate a wide range of secondary signalling.

1.2.1 G Proteins

Heterotrimeric guanine-nucleotide-binding regulatory proteins (G proteins) are intermediaries that relay information from ligand-bound GPCRs into secondary signals. G proteins are composed of α , β and γ subunits, and because the β and γ subunits are firmly linked, they are commonly referred to as one functional unit. Generally, G proteins are classified by their α subunit; subgroups include $G\alpha_s$ (stimulatory), $G\alpha_i$ (inhibitory), $G\alpha_q$ and $G\alpha_{12}$, each with various sub-group members. Isoform expression varies throughout the body - some are exclusive to cell types (e.g. $G\alpha_{olf}$ (olfaction) is a $G\alpha_s$ member found exclusively in olfactory neurons), and others are expressed ubiquitously (e.g. $G\alpha_q$ members $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and

$G\alpha_{16}$) [10]. The β and γ subunits also exist in various isoforms, there are 5 β and 12 γ encoded in the human genome. The β subunits share substantial sequence homology (80-90 %), whereas the γ show wide variability (20-80 %), and again show a mixed pattern of cell specific or ubiquitous expression [11]. To determine the role of any given protein in the cell, it is customary to manipulate its expression level and examine functional repercussions. Knocking out the gene that codes for each G protein isoform in a mouse model, gives rise to a panel of individuals each with a specific set of deficiencies and abnormalities, highlighting their wide-ranging physiological roles (explored in detail by Syrovatkina *et al.* [12]).

G proteins function as a molecular binary switch; when inactive the α , β and γ subunits are bound together and α is bound to a molecule of guanosine diphosphate (GDP) [13]. G protein switch activation starts with a ligand-induced conformational change in the GPCR that facilitates an interaction with its cognate G protein. GPCRs and G proteins are able to diffuse with the plasma membrane [12] and hence originally, it was postulated that the receptor-G protein interaction occurred through collision. There have also been examples described in which GPCR complexes are “pre-assembled” with G proteins in the absence of ligands [14, 15], however only agonist-GPCR binding causes the conformational changes required for G protein activation. The active GPCR functions as a guanine-nucleotide exchange factor (GEF) that facilitates the exchange of GDP to guanosine *tri*-phosphate (GTP) on the $G\alpha$ -subunit, leading to its dissociation from the $G\beta\gamma$ subunit [16]. Both the GTP-bound $G\alpha$ and the free $G\beta\gamma$ subunits are capable of initiating secondary signals through interaction with downstream effector proteins e.g. cyclic AMP, inositol triphosphate and calcium [12, 17, 18]. These effector molecules regulate the intracellular concentration of second-messenger molecules or ions that elicit the ultimate cellular responses to the receptor/agonist pairing. G protein activity is a highly amplified scheme - an activated GPCRs guanine nucleotide exchange activity lasts long enough to induce the dissociation of multiple G proteins, which amplify the signal further as each subunit is able to produce many secondary molecules. $G\alpha$ subunit signalling terminates by its intrinsic GTPase activity, that hydrolyses GTP back to GDP, facilitated by GTPase-activating proteins (GAPs). The $G\beta\gamma$ signalling is terminated by reassociation with $G\alpha$ GDP [12], thus completing the G protein cycle.

1.2.2 G Protein-Coupled Receptor Kinases

The cell must possess a negative feedback loop to quench the signalling response to persistent stimuli, and prevent overstimulation. Impairment of GPCR signalling is termed desensitization and the same mechanism is evident across the massive diversity of receptors. Feedback loops at GPCRs imbed the *off* mechanism into signal *on* activity. The dissociation of G protein subunits carries out two important roles; along with their signal transduction activity, increases in $G\beta\gamma$ concentration leads to the recruitment of G protein-coupled receptor kinases (GRKs). GRKs bind to and phosphorylate the activated receptor, and the process of

turning off is initiated [19, 20]. The first reports of activation-dependent phosphorylation of the rhodopsin receptor came in the 1970s [21, 22], soon followed by the description of the mediator – “opsin kinase” (now known as GRK 1) [23]. It had been observed that there was a loss in responsiveness of GPCR signalling following prolonged stimulation [24, 25], and later, that phosphorylation of the receptor was necessary for this deactivation [26]. This led to the hypothesis that these specific kinases were part of the negative feedback regulation of receptor signalling, later confirmed at many different GPCRs [25, 27, 28].

These serine/threonine protein kinases belong to a family now referred to as GRKs, within the AGC kinase group. GRKs are multi-domain proteins containing an N-terminal region specific to each family, a regulator of G protein signalling (RGS) homology domain, a serine/threonine protein kinase domain, and their C-terminal domain containing structural elements responsible for differential membrane targeting [4]. In humans there are seven GRK isoforms (GRK 1-7) that together regulate hundreds of GPCRs. GRK 1 and 7 are found exclusively in the retina, GRK 4 is predominantly found in the testis, while GRK 2, 3, 5 and 6 are expressed ubiquitously [29, 30]. Based upon sequence similarities, GRKs are often grouped into three subfamilies; GRK 1 family contains isoforms 1 and 7, GRK 2 family contains 2 and 3 and GRK 4 family contains 4, 5 and 6 [4]. GRK 2 and 3 share a C-terminal pleckstrin homology domain that controls PIP₂ and G protein subunit-mediated translocation of these kinases to the plasma membrane near to activated receptor substrates. GRK 4, 5 and 6 on the other hand, lack this domain and instead use direct PIP₂ binding and lipid modifications to reside primarily at the plasma membrane [30].

Knock-out (KO) mouse models support the functional diversity and sometimes overlapping function of the isoforms, and ultimately highlight that dysfunctional desensitization of GPCRs can have profound physiological impact. GRK 2 KO mice are embryonic lethal [31], with thin myocardium syndrome in embryos [32] and altered cardiac function in adult heterozygotes [33]. KO mice for the other GRK isoforms develop relatively normally, however without GRKs many of their GPCRs remain aberrantly sensitive to agonist challenge, evident as persistent or exaggerated responses. GRK 6 KO mice show a hypersensitivity to dopamine [34] and develop autoimmune disease [35]. GRK 3 KO have a lack of olfactory desensitization [36], altered M2 muscarinic airway regulation [37], and an altered κ -opioid receptor mediated tolerance in a spinal analgesia test [38]. GRK 4 KO on the other hand, shows no obvious phenotype (KOs reviewed in depth by Premont *et al.* [30]).

Mechanistically, GRKs are serine/threonine protein kinases that phosphorylate the intracellular loops and C-termini of agonist-bound GPCRs as their primary substrates. Following agonist-induced GPCR activation, GRK-mediated receptor phosphorylation rapidly impairs G protein signalling [20]. This occurs because the phosphorylation event promotes the recruitment of a family of proteins known as β -arrestins to the receptor, which physically interrupts the receptor-G protein coupling [19, 20]. Such phosphorylation dependent regulation gave rise to the development of a *barcode hypothesis* [29, 39]. By translating a specific receptor conformation into patterns of β -arrestin recruitment and

interaction, GRKs are said to establish a *barcode* across serine and threonine residues on the C-terminal tail, thus regulating functionality [40, 41].

Like the majority of protein mediators, GRKs exhibit multi-functionality, and their substrates extend past receptors [42]. Mutation analysis has shown that GRKs can also function outside of their kinase ability, for example a GRK 2 kinase-dead mutant can still suppress mGluR1/5 signalling via its RGS homology (RH) domain [43, 44]. Their expanding interactome means that GRKs are increasingly being recognized as important signalling mediators in their own right [29, 45-47].

1.2.3 β -arrestins

Following the discovery of GRKs as responsible for the phosphorylation events required to desensitize a GPCR, it was then understood that this event greatly increased the receptor affinity for a family of proteins that further blocked signalling [48, 49]. At the time, these proteins were referred to as S-antigen or 48-kDa protein but are now known as arrestins. Opened up by studies at the β_2 -adrenergic receptor (β_2 AR) [50] and then other GPCRs, this work set the ground for a 2-step GPCR desensitization hypothesis whereby a family of Ser-Thr protein kinases (GRKs) specifically phosphorylate ligand-activated GPCRs, creating binding sites for arrestins to complete a process termed *heterologous desensitization* [28, 51, 52]. There are four mammalian arrestin isoforms, equipped with remarkable diversity to interact with hundreds of GPCRs and downstream components. Arrestin 1 and 4 are exclusively expressed in retinal tissue, whereas non-visual isoforms arrestin 2 and 3 (also known as, and referred to herein as, β -arrestin 1 and 2 (β -arr 1 and 2)) show ubiquitous expression and therefore interact with a diverse array of GPCRs throughout the body [53]. Arrestins specifically bind to phosphorylated GPCRs, serving as a physical link to components of receptor internalization and signalling [54-56]. Differential affinities for the β -arr isoforms separate GPCRs into two major classes. Class A members such as the dopamine D1A receptor, μ -opioid receptor and β_2 adrenergic receptor, bind β -arr 2 with greater affinity than β -arr 1. Class B such as the angiotensin II type 1A receptor and the vasopressin V2 receptor, bind both β -arr isoforms with equal affinity [57].

Isoform specific KO models illustrate β -arr roles in the desensitization of many receptor systems, however it is noteworthy that with the exception of GRK 2 (embryonic lethal), GRK/arrestin KO mice appear grossly normal. Consistent with their roles, one must challenge these animals with receptor stimulation before their inabilities to dampen excessive signalling becomes evident [30]. β -arr 1 KO mice appear phenotypically normal, but have dysfunctional cardiac responses to β -adrenergic stimulation [58]. β -arr 2 KO mice show a disrupted morphine response [59, 60], reward and dopamine-mediated behaviours [61, 62], defective lymphocyte and neutrophil chemotaxis [63, 64], and altered bone mass/architecture [65, 66].

Mechanistically, following GRK phosphorylation β -arr binds to an agonist-activated receptor, an event that physically blocks further receptor-G protein interaction [67, 68]. In addition, β -arrestins serve as a physical link between the activated receptor and components of trafficking machinery. The binding of β -arrestins to GRK-phosphorylated receptors initiates receptor endocytosis with degradation, or into recycling endosomes for return to the cell surface in a competent form to receive a new signal through *resensitization* [69-72]. Although discovered and hence named for their signal *arresting* role, it is now well accepted that in addition to G protein signal cessation, β -arrestins themselves couple to various signal components, activating their own wave of (G protein independent) signalling. In various cellular contexts β -arrestins have been shown to link to the MAPK, PI3K, NF- κ B cascades by acting as a scaffold for complex formation [73-78]. A recent study has also uncovered the possibility of β -arrestins to sustain G protein signal through initiating the normal internalisation process but binding in a position where physical blocking of the G protein does not occur, thus prolonging the G protein signal [79]. Together, the data reinforces the key central role β -arrestin plays in the signal switch and final patterning. For example, signal *bias* can be translated downstream of a ligand/receptor pairing through differential GRK and β -arrestin recruitment. At the chemokine receptor type 7 (CCR7) there are two ligands that each facilitate different GRK/ β -arrestin involvement. The CC chemokine ligand 19 (CCL19) induces GPCR desensitization by GRK 3 and 6, whereas CC chemokine ligand 21 (CCL21) promotes β -arrestin signalling that relies solely on GRK 6 [80]. As such, the GRK/ β -arrestin system is increasingly establishing themselves as central controllers of receptor physiology, shaping aspects of signalling, desensitization, internalization and subcellular trafficking.

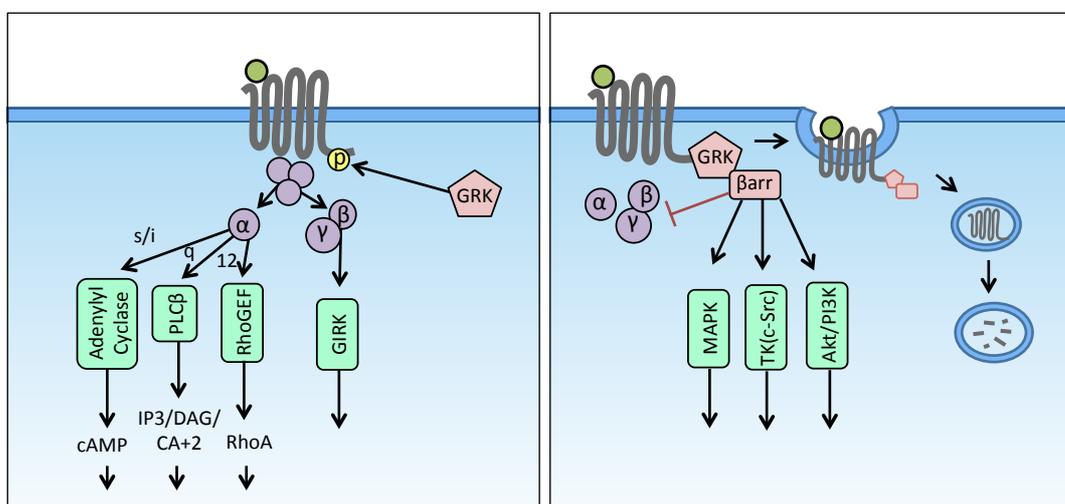


Figure 1: GPCR function. G protein-coupled receptors make use of three functional modules; G proteins, G protein-coupled receptor kinases (GRKs) and β -arrestins (β -arr). Ligand-induced conformational changes in the receptor allow it to act as a guanine nucleotide exchange factor, switching GDP to GTP on G proteins, allowing subunit (α , β and γ) dissociation and downstream signal transduction. Subunit dissociation then recruits GRKs to phosphorylate the receptor C-terminal tail, creating high affinity binding sites for β -arrestins. β -arrestins uncouple G proteins bringing their signal to an end, initiate receptor internalization and transduce a secondary (G protein-independent) signal wave.

1.2.4 Biased Signalling

Traditionally, receptors were believed to exist in the plasma membrane in an inactive *off* conformation, where the binding of the specific ligand changed the conformation of the receptor to an active or *on* position – which in turn, facilitated the binding of intracellular docking molecules. Originally identified as playing a role in desensitization and degradation, the GRK/ β -arrestin system was then shown to be able to initiate an intracellular signal independently of classical G proteins. As well as increasing the complexity of the GPCR signalling system, simultaneous research also opened up a new conceptual possibility; that of *multiple* distinct active conformations, spurring the development of the GPCR extended ternary complex model [81-85].

The initial *two-state* ternary complex model of GPCR function conceptualizes the receptor as an off/on switch, existing in either an agonist-empty or agonist-bound position [86]. Based upon this model, decades of pharmaceutical development created receptor-paired agonist/antagonists, giving rise to some of the most widely used clinical agents. The discovery that β -adrenergic receptors could exhibit two agonist affinity states challenged the model, and suggested additional complexity [87]. Subsequent work involving many GPCRs developed the idea such that receptors can exist in an equilibrium state between the active and inactive conformations [88]. *Full agonists* stabilize the active conformation, *partial agonists* have lower efficacy and hence pull the equilibrium to a lesser degree and a submaximal response, *antagonists* bind and produce no physiological response, and *inverse agonists* can actively reduce receptor-mediated activity [81, 82, 89].

This model was further developed to accommodate conformations beyond a two-state receptor conformation. Many GPCRs are able to stimulate different signalling pathways to different degrees [90-93], and hence a two-state model is insufficient to explain these spatially and temporally textured signals. The *multi-state* model provides the theoretical basis for these findings, and opened up the field of *biased signalling*, also sometimes referred to as biased agonism or functional selectivity [94, 95]. Biased signalling describes the ability of a receptor to be selectively activated in a *biased* response, as opposed to the simple activation/deactivation by *balanced* agonists/antagonists. This model explains the variations between G protein and β -arr signals emanating from a single GPCR. Following the discovery that β -arr mediates a signal cascade independent of G protein activity, came the appreciation could signals could selected in a biased manner by agonists [96]. Indeed, this idea has proven correct and the concept of biased agonism describes the receptor system whereby a specific ligand can induce one form of active receptor conformation, distinct from that encoded by another ligand, and each give rise to distinct signalling outcomes. At many GPCRs the signals generated by G proteins or β -arrs show distinct biochemical profiles and resultant physiological outcomes [97].

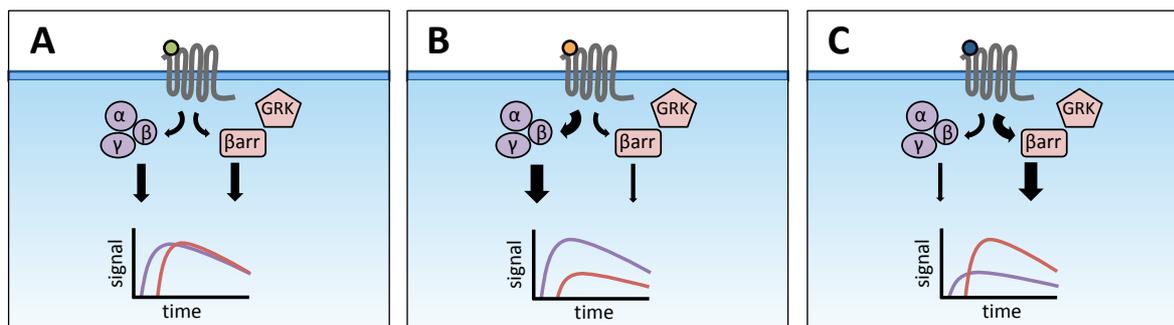


Figure 2: GPCR Biased Signalling. It was originally believed that a ligand-activated receptor conformation transduced all downstream pathways in a balanced manner (A). However, the model of GPCR activation had to be updated to accommodate observed experimental variations. Biased signalling describes the ability of a receptor active conformation to preference one or other signals. At GPCRs, this is evident as either a G protein-biased (B) or β -arr-biased (C) signal.

Given that GPCRs represent the majority of current drug targets, the complexity that this model dictates is therapeutically relevant, and opens up another degree of fine-tuning therapeutics to desired outcomes. Drug development shifts from a mere antagonist/agonist choice, to a continuum of signal-tuning through the use of biased agonists [96]. The development of biased agonists now represents a major drug discovery effort [98, 99].

1.3 RECEPTOR TYROSINE KINASES

An early tool for signal transduction in unicellular organisms was biochemical protein modifications, such as tyrosine phosphorylation. Tyrosine phosphorylation is the transferal of a phosphate group from an adenosine triphosphate (ATP) molecule to the amino acid tyrosine, by enzymes termed tyrosine kinases. This modification generates docking sites that promote specific interactions between a tyrosine-phosphorylated protein and another protein that contains a specific domain (such as SH2 or PTB), allowing proteins to regulate each other's function. These interactions serve as the basis for a signal to be passed along a chain of consecutive interactions. Choanoflagellates, introduced earlier as the unicellular organism possessing the full GPCR toolbox, also show primitive phospho-tyrosine based signalling [100, 101]. Importantly, these phospho-tyrosine events occurred in cytoplasmic proteins. *Receptor* tyrosine kinases (RTKs), i.e. tyrosine kinase enzymes embedded into the plasma membrane, emerge only later, perhaps through the fusion of a receptor gene and a protein tyrosine gene [101]. This anchoring event into the interface between the cell and the environment, allowed RTKs to relay information from the cell surface intracellularly to the nucleus. The difference in their expression patterns, in contrast to the cytoplasmic tyrosine kinases, suggests that these receptors allowed for exquisite specialization in function. They can receive and respond to environmental information, as well as support *inter-cellular*

communication, and hence played a critical part in the emergence and explosion of metazoan success and diversity [100, 102, 103].

In mammalian cells, RTKs are responsible for relaying signals into cellular action surrounding survival, differentiation, growth, migration and invasion. There are 58 RTKs coded in the human genome, commonly sub-categorized by shared sequence homology into 20 families [104]. RTKs share structural similarities; an extracellular ligand-binding domain at the N-terminal end and a cytoplasmic domain containing their tyrosine kinase ability at the C-terminal end, linked by a transmembrane domain. Their specific ligands are proteins secreted into the intercellular space or expressed on nearby cells. RTKs can be split into two major families based on manifestation in the membrane; either monomeric or dimeric. The majority of RTKs exist as monomeric receptors when inactive, including the epidermal growth factor receptors (EGFR/HER/ErbBs), fibroblast growth factor receptor (FGFR) and the vascular endothelial growth factor receptors (VEGFRs). When each of these receptors binds to their respective ligands, two monomeric receptors join together. Dimerization causes the two adjacent cytoplasmic domains to interact, and resultant molecular exchanges drive activation of the kinase domains of both receptors, which initiate signal transduction of downstream cascades [105]. Dimeric RTKs on the other hand, which include only the insulin receptor (IR) and the insulin-like growth factor type 1 receptor (IGF-1R), exist in the plasma membrane as preformed dimers. These receptors are composed of a pair of monomeric units, each composed of an α and β subunit, held together by disulphide bonds. When inactive, the molecular conformation of the two adjacent kinase domains keeps the basal kinase activity of the receptor low. Ligand binding drives conformational changes within the active site of the receptor, to induce trans-autophosphorylation of adjacent kinase domains that result in signal transduction.

1.3.1 RTK: Cancer Relevance

Cancer arises through a multistep acquisition of mutations, allowing a cancer cell to acquire common hallmarks including self-sufficiency in growth signals and resistance to apoptotic signals, yielding unlimited proliferative potential [106]. These mutations encode tumour-supportive genes called oncogenes [107], and the discovery of these oncogenes raised mechanistic questions – how could the resultant oncoproteins drive catastrophic deregulation of cellular growth? A vital clue came in the early 1980s when two independent research groups reported that a known viral oncogene v-sis (simian sarcoma virus oncogene) shared a high degree of sequence homology with the RTK platelet-derived growth factor receptor (PDGFR) [108, 109]. This discovery generated a spur of research uncovering the intimate relationship between RTKs and cancer development. Many of the receptors, their ligands and the components of their signal cascades turned out to be homologs of oncogenes such as gp55, bovine papilloma virus, SV40T antigen and v-src [110].

Since the fundamental characteristic of all cancers is chronic proliferation, it made logical sense that RTKs could be critical facilitators of survival and proliferative signalling. In the next decades, RTKs gained a great deal of pharmaceutical attention, and RTK targeting strategies have had many success stories [111]. Antibodies against the EGFR family receptor HER2 have shown great clinical utility in breast cancer [112, 113]. The antibody Imatinib, initially designed for the non-receptor tyrosine kinase oncogene BCR-ABL had unprecedented success in patients with chronic myelogenous leukaemia (CML) [114], and was later also recognized to block the activity of several RTKs such as Kit and PDGF, extending its clinical utility [115]. It is worth noting that many of these success stories involved cancers in which certain RTKs were consistently overexpressed or mutated, which is not always the case. During this intensive RTK research period, the Baserga research group reported compelling findings that fibroblasts derived from mouse embryos knockout for the gene encoding the RTK insulin-like growth factor type 1 receptor (IGF-1R) could not be transformed by numerous cellular oncogenes. Known oncogenes such as Ras, human polyomavirus, c-Src, oncogenic fusion proteins and overexpressed RTKs failed to transform these cells, unlike their wild-type counterparts, or after the IGF-1R had been reinserted [116-118], suggesting this particular RTK to be essential to oncogenic transformation.

1.3.2 The Insulin-like Growth Factor type 1 Receptor (IGF-1R)

The insulin-like growth factor type 1 receptor (IGF-1R) is typically classified as a widely expressed RTK, responsible for cellular mitogenic and anti-apoptotic responses to a higher-level endocrine growth hormone (GH) signal. Although nominally compared to the insulin receptor due to its later discovery, evolutionarily speaking it is likely to have evolved first, with the insulin receptor (IR) diverging into a largely metabolic role around the appearance of the first vertebrates [119]. A functional IGF-1R system is found in all vertebrate groups, and can be further traced back to an ancestral insulin-like signalling system near the dawn of metazoan evolution [120]. The evolution of the IR/IGF-1R network allowed a multi-cellular organism to coordinate appropriate responses to nutrient availability, coordinating a switch between nutrient conservation and growth [121, 122]. Although structurally similar ($\approx 70\%$ sequence homology) [123] and able to act in functionally redundant ways at supra-physiological ligand concentrations or in KO animal models, it is generally accepted that they are functionally distinct. The IR acts in a primarily metabolic role and the IGF-1R in a mitogenic/anti-apoptotic role [124].

Mouse models generated containing homozygous disruption of the IGF-1R gene present severe growth deficiency ($\approx 45\%$ of normal weight) and general organ hypoplasia, and die at birth of respiratory failure [125]. To study the post-natal role of the receptor, conditional KO models have been developed that generated approximately 40% fewer IGF-1 binding sites. These IGF-1R-dampened mice grew more slowly than wild-type littermates [126].

1.3.3 IGF-1R: Signal Transduction

The insulin-like growth factor (IGF) system comprises of plasma membrane-anchored receptors that translate an extracellular ligand into two main intracellular signalling pathways; the mitogen activated protein kinase (MAPK) cascade and the phosphatidylinositol 3-kinase (PI3K) cascade. These pathways culminate in the transcriptional activation of various anti-apoptotic, cell cycle progression, and cell motility components. Extracellularly, there are three classical ligands: insulin, IGF-1 and IGF-2, and unlike insulin which circulates free, growth factor availability is tightly controlled, held in circulation by IGF-binding proteins (IGFBPs) [127]. When required, the IGF-IGFBP complex is dismantled by proteases, releasing IGFs for biological action. IGFs were originally identified as liver-secreted serum factors [128], but have since been shown to be produced by most organs in an autocrine and/or paracrine fashion. Mice KO for the IGF-1 or IGF-2 genes show similar growth deficiencies (\approx 60% of normal birth weight) and while some die at birth, others can survive until adulthood [125, 129]. At the cell surface level, the major receptors within the family are the IGF-1R and the IR, but also present are the IGF-2R that largely acts as a decoy receptor with no kinase activity, the IR-related receptor (IRR) [130], and the most recently added IR/IGF-1R hybrid receptor [131, 132].

Unlike other RTKs, that exist as monomers and dimerize upon ligand binding, the IR and IGF-1R are already assembled as pre-formed dimers. According to the classical model, ligand binding induces a receptor conformational change that leads to its activation. Three important tyrosine (Tyr) residues are located in a region termed the activation loop (A-loop). When the receptor is in its inactive conformation, Tyr 1135 is bound in a cis position and acts as a blocking pseudosubstrate by occluding the ATP binding site and preventing substrate access [133]. In this position the basal catalytic activity of the receptor is kept very low. Conformation changes associated with agonist binding cause those A-loop tyrosines to be trans-phosphorylated by their dimeric partner, stabilizing the new catalytically optimized conformation [133, 134]. Consequently, auto-phosphorylation extends to tyrosine residues outside of the kinase domain, creating binding sites for signal modules such as Shc and IRS, which link the receptor to its main downstream cascades [135, 136].

The MAPK cascade is initiated when the docking proteins IRS and Shc bind to the juxtamembrane domain of the receptors through their phosphotyrosine binding (PTB) domains, and are themselves tyrosine-phosphorylated. Their phosphorylated tyrosine residues are then recognized by the next in line component Grb2, through its src homology 2 (SH2) domain [137]. Grb2 complexes with the Ras exchange factor son of sevenless (SOS), which can exchange GDP for GTP on Ras. Once active, Ras interacts with the serine/threonine kinase Rafs to activate mitogen-activated protein kinase kinases (MEKs), that go on to activate extracellular signal-regulated kinases (ERK1 and 2) through tyrosine and threonine

phosphorylation events. Activated ERK1 and 2 translocate to the nucleus where they bind and activate transcription factors such as Ets, Elk, and c-Fos, initiating the transcription of genes involved in cell cycle progression, proliferation and motility [138-143]. Furthermore, ERK1/2 can regulate transcriptional repression and chromatin remodelling [144], as well as function in the cytoplasm, controlling microtubule dynamics [145, 146].

The second main cascade is initiated when phosphatidylinositol 3-kinase (PI3K) interacts with IRS and the active receptor, causing it to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP₂). This event generates the messenger phosphatidylinositol 3,4,5-trisphosphate (PIP₃) at the membrane [147]. Next, 3-phosphoinositide-dependent kinase-1 (PDK1) and Akt bind to PIP₃ at the inner leaf of the membrane, and PDK1 phosphorylates Akt [148]. Activated Akt phosphorylates and inhibits a myriad of cellular substrates including the Bcl-2-associated death promoter (BAD) [149], caspase 9 [150], the pro-apoptotic effector protein glycogen synthase kinase-3 β (GSK-3 β), Forkhead box O-class protein (FOXO) and Bcl-2 [151]. Akt activity can stimulate mTOR that acts to regulate multiple RNAs and proteins involved in cell cycle progression [138, 152], and can activate matrix metalloproteinases (MMPs), involved in cell migration and invasion [153]. Akt phosphorylation also phosphorylates Mdm2, which allows the translocation of this E3 ubiquitin ligase to the nucleus where it decreases the transcriptional activity of p53 [154]. Altogether PI3K activity regulates critical cell processes such as protein synthesis and cell survival [134, 155, 156].

Signal transduction downstream of IGF-1R can extend past these two best-known cascades. The ligand-activated receptor can also activate the stress-activated protein kinase (SAPK) pathways, including those of Jun N terminal kinase (JNK) and p38, which regulate cell response to DNA damage. Grb10 has been shown to bind to the ligand-activated autophosphorylated tyrosine residues of the IGF-1R [157, 158], which appears to drive cell growth. In various cellular contexts many additional substrates are employed, including the adapter proteins CrkII and CrkL [159], RACK1 [160], focal adhesion kinase (FAK) [161], Syp [162], GTPase-activating-protein [163], and suppressor of cytokine signalling 2 (SOCS2) [164].

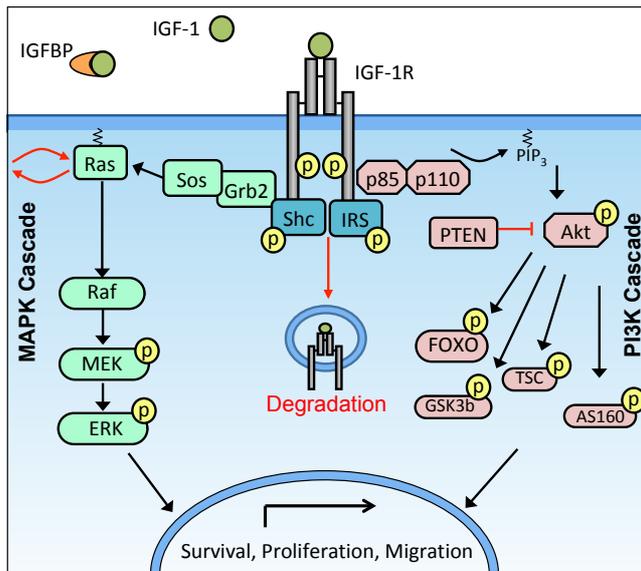


Figure 3: IGF-1R Signal Transduction.

The plasma membrane-anchored insulin-like growth factor type 1 receptor (IGF-1R) translates the extracellular ligand insulin-like growth factor 1 (IGF-1) into two main intracellular signalling pathways; the mitogen activated protein kinase (MAPK) cascade and the phosphatidylinositol 3-kinase (PI3K) cascade. These pathways culminate in cellular actions surrounding survival, proliferation and motility. MAPK and PI3K activity is balanced by negative feedback loops such as the Ras GTP/GDP molecular switch and PTEN respectively. Ligand-activated receptors are internalized and processed for degradation to avoid overstimulation.

1.3.4 IGF-1R: Signal Cessation

In terms of signal termination, the RTK system has multiple layers of feedback that maintain homeostasis across different temporal and spatial needs. Within minutes of agonist binding, phosphorylation cascades are counterbalanced and can be virtually eliminated. In addition, after several hours desensitization is augmented by receptor down-regulation through the endolysosomal network. And at yet later time points, receptor or signal component expression levels can be diminished through transcriptional control. The fact that many of these molecular antagonising mechanisms are disrupted or missing in cancer is illustrative of their oncogenic potential.

In an acute sense, several components directly antagonise the signal cascades of MAPK and PI3K. As an example, imbedded into the activity of the MAPK cascade is a molecular switch to return it to an inactive state: Ras is a small GTPase, which toggles between active (GTP-bound) and inactive (GDP-bound). In response to extracellular stimuli through RTKs, guanine nucleotide exchange factors (GEFs) catalyse the displacement of GDP, allowing GTP to replace it. Ras-GTP interacts with target proteins to initiate downstream signal activity. The cycle is then completed as Ras returns to its GDP-bound inactive state [165]. Discovered as an oncogene in the early 1980s [166], Ras is now understood to be one of the most important oncogenes in cancer [167]. Oncogenic Ras mutants are constitutively active, stuck in the *on* position, unable to process GTP. There are three mammalian Ras isoforms (HRas, KRas and NRas) and their expression patterns are tissue specific. Expression of mutant KRas in murine embryos causes extensive morphological aberrations causing embryonic lethality [168]. Some KRas mutants can survive until adulthood, but then develop

cancers such as lung adenomas and adenocarcinomas [169]. Models containing tissue-specific expression of mutant KRas report preneoplastic hyperplasias, adenomas and adenocarcinomas. And although they do not have a high frequency of metastasis by themselves, it is induced with high frequency in the presence of other cancer driver mutations such as p53 [170, 171], PTEN [172], wnt/ β -catenin [173] and Arf [174]. Furthermore, germline mutations in Ras cause a range of genetic disorders in which patients exhibit, among other defects including neurocognitive and cardiac abnormalities, a predisposition to many malignancies [175-177].

The most well-studied negative regulator of the PI3K cascade is PTEN (phosphatase and tensin homolog deleted on chromosome 10) [178, 179]. Mechanistically, PTEN is a lipid phosphatase antagonising the PI3K pathway by catalysing the conversion of PIP₂ back to PIP₃, thereby regulating PIP₃ mediated signalling [180]. In the same vein as Ras, the negative regulator PTEN is an important tumour suppressor – and it is also one of the most frequently mutated genes in cancers [181]. An estimated 50-80% of sporadic tumours, and 30-50% of breast, lung and colon tumours contain a mono-allelic PTEN mutation [181]. Complete loss of the PTEN gene is associated with advanced metastatic disease [182, 183]. Germline mutations of PTEN lead to syndromes characterized by an increased risk of many cancers, as well as developmental and neurological defects [184]. A panel of murine models with mutated PTEN demonstrate high cancer susceptibility and often embryonic lethality [185-188].

On an intermediate timescale, following activation, the ligand/receptor pairing must be removed from the cell surface and processed through degradation or recycling pathways, dependent upon the cellular need. The post-transcriptional modification aiding these processes is ubiquitination: the addition of the small molecular tag *ubiquitin* that signals movement through the endolysosomal sorting network, culminating in either recycling or degradation. This 3-step process involves firstly a ubiquitin-activating enzyme (E1), then a ubiquitin-conjugating enzyme (E2) and a final ubiquitin-protein ligase (E3) [189]. The IGF-1R itself has a complex set of post-activation fates. It can be internalised via clathrin or caveolin coated pits, it can be processed via both proteosomal and lysosomal pathways, and so far has been shown to be ubiquitinated by at four E3 ligases; Nedd4 [190], c-Cbl [191, 192], Mdm2 [193] and HRD1 [194]. On top of that, downstream of the receptor, many of the components of the subcellular signalling pathway itself are also subjected to ubiquitin-based processing for regulation or turnover (for an extensive review see Girnita *et al.* [195]). The endolysosomal system is an integral sorting platform for the plasma membrane, and controls much of the expression, turnover and hence function of this dynamic border area. As such, there is also evidence to suggest that this system is exploited in cancer settings, both to manage endocytosis and exocytosis, in order to aid transformed cell survival [196-199].

In the longer term, further signal suppression occurs through negative feedback of the transcription of critical IGF-1R axis components. For example, IGF-1 itself, along with many important tumour suppressor genes e.g. Wilms tumour-1, breast cancer protein-1 (BRCA-1),

and p53 negatively regulate novel IGF-1R production [200]. Most growth factors and many hormones on the other hand, stimulate its transcription [201]. As IGF-1R is sometimes referred to as a *progression* factor aiding cell cycle continuation, growth factor-mediated transcription synchronises the sequence of events from initiation, progression through to completion of a successful cell cycle.

1.3.5 IGF-1R: Cancer Relevance

With such a critical role in cellular fate, the IGF-1 system is heavily involved in normal physiology, as well as in many pathological states. An important set of experiments showed that mouse embryonic fibroblasts (MEFs) lacking the IGF-1R were refractory to transformation [116, 118, 202]. While not strictly adhering to the definition of an oncogene *per se*, these experiments solidified a key *enabling* role for the receptor in oncogenesis. As well as protecting against apoptosis and promoting proliferation, the IGF-1R has been shown to be intrinsically involved in anchorage-independent growth, tumour neovascularisation, migration and invasion [203, 204] - all supportive to the malignant phenotype.

Genetic disorders and syndromes often provide crucial functional information about a particular protein, and what happens in its absence/dysfunction. Whilst IGF-1R deletion is not observed in the human population, Laron syndrome (LS) is a disease characterised by dwarfism in which patients exhibit genetic mutations in the growth hormone (GH) receptor, resulting in the biochemical characteristic of high GH but low serum IGF-1 levels [205]. Interestingly, these patients are protected against the development of cancer, when compared to non-affected family controls [206]. Studies on immortalized lymphoblastoid cells derived from LS patients and healthy matched controls identified multiple differentially expressed genes and hence differentially activated signalling pathways, such as Jak-STAT and PI3K-Akt, controlling cell cycle and metabolism, [207]. Furthermore, changes could be reversed *ex vivo* with the addition of IGF-1 [208]. It has long been known that caloric restriction limits the growth of xenograft tumours in animal models, and more recently this protection has been shown to extend to spontaneous and chemical- or radiation-induced tumours in numerous animal models [209-213]. While the cancer-protective mechanism of caloric restriction is likely multi-factorial, the I/IGF system was hypothesized to be involved since it interlinks nutritional responses and cellular growth, and hence warranted exploration. Indeed, the cancer-protective effect of caloric restriction in animal models was reversed by infusing GH or IGF-1 [214-216]. Linking many crucial processes surrounding survival, metabolism, growth and important pathologies such as diabetes and cancer, there is quite an abundance of evidence supporting the role of insulin/IGF-1 in longevity. In animal models using *Caenorhabditis elegans*, *Drosophila melanogaster* and mice, various strategies to decrease GH/IGF-1 activity have been shown to enhance lifespan [217], whereas in humans the evidence is more varied [218]. However, in the offspring of centenarians longevity is linked

with IGF-1: the bioactivity of serum IGF-1 is significantly lower in the offspring of centenarians than age, gender and BMI matched offspring control groups [219]. Moreover, IGF-1 levels can be used to predict life expectancy in long-lived individuals [220].

The IGF-1R is heavily implicated in the molecular pathogenesis of many cancer types. In terms of protein expression, although there have been numerous reports of IGF-1R overexpression [221-223], this seems not always to be the case. Evidence of autocrine IGF-1 production have been found in sarcomas [224-228], melanomas [229], colon cancer [230], pancreatic cancer [231] and ovarian cancer [232], suggesting that enhanced IGF-1 system activity is an important metastatic-supportive loop. Inhibition strategies have been shown to perturb the growth of colon cancer [233], gastric cancer [234], pancreatic cancer [235], ovarian [232], melanoma [236-240] and prostate cancer cell lines [241]. In mouse xenograft mouse models various IGF-1R system targeting agents decrease the growth and/or invasion of many cancer cell lines, including those from colon [242], lung [243], osteosarcoma [244], breast [245], and prostate [246-248]. A comprehensive overview of the pre-clinical anti-IGF-1R data is given by Khandwala *et al.* [249].

In summary, several decades of research has produced a wide spectrum of basic, animal, clinical and epidemiological evidence demonstrating an association between the IGF system and neoplastic growth. Together, they emphasize that the IGF-1 axis plays a significant role in the initiation and advancement of many human cancers.

1.3.6 IGF-1R: Therapeutic Targeting

Given its clear fundamental role in many cancers, several research groups explored the possibility of targeting the IGF-1R in interventional strategies, and pre-clinical testing appeared promising [250-252]. Animal models given various anti-IGF-1R strategies halted or regressed tumour growth with very little toxicity [253, 254]. This fuelled great excitement and pharmaceutical interest, and the IGF-1R fast became one of the most intensively studied oncological targets. Very quickly, numerous targeting strategies developed independently all sharing an aim to inhibiting the kinase signalling downstream of the receptor: IGF-1 peptide analogues [241], IGF-1R blocking antibodies [255, 256] and small molecule tyrosine kinase inhibitors [257-259] were all brought forward to clinical trials [251].

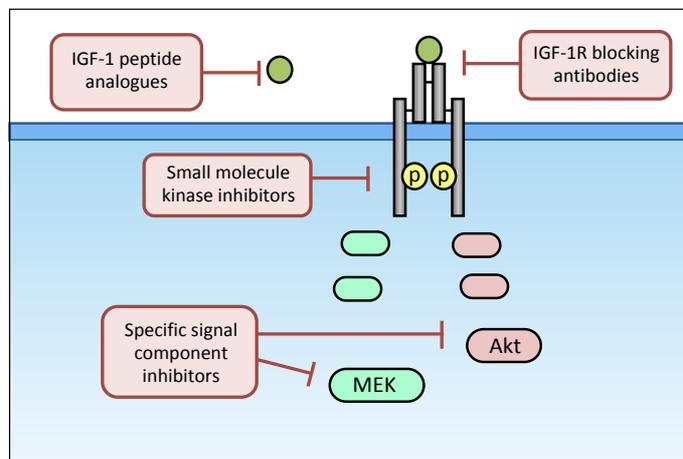
Despite their pre-clinical promise, the actual clinical results of most of these trials were largely disappointing [110]. Most regimes reported being well tolerated, however clinical response was limited to small patient populations (e.g. Ewing's sarcoma and non-small cell lung cancer), in many instances too few to maintain pharmaceutical interest [260]. Many reasons have been offered for the lack of promising results: including inadequate patient selection or stratification, constitutive activation of downstream components [261], selective pressure on IR/hybrid receptors to act in mitogenic ways once the IGF-1R was targeted, and

IGF-1R non-canonical signalling [110]. The importance of non-canonical signalling became apparent during post-trial mechanistic studies of the anti-IGF-1R antibody figitumumab (CP-751,871; CP). Designed through efforts to uncover IGF-1R antagonists, CP is a humanized monoclonal antibody that binds specifically to the receptor, blocking the binding of the natural agonist IGF-1 [262], and hence was brought forward to clinical trials [263-267]. However, later studies discovered that CP actually acts as a β -arr 1 biased agonist. Upon binding, it down-regulates the IGF-1R with parallel β -arr 1 signalling activity to the MAPK cascade [134, 268], and hence counteracts any cancer-curbing desired effects.

This finding was of paramount importance, as it highlighted non-canonical activation of the IGF-1R through a pharmaceutical agonist, which led to specific downstream signal profiles. The ability of an exogenous agent to activate an IGF-1R signal, uncovered the obvious shortcomings of agents designed and selected under a paradigm of IGF-1 only/kinase-only signalling. Subsequent research has provided additional evidence that many of the targeting strategies were premature due to their simplistic view of the IGF-1R system in use at the time [260, 269]. As cell signalling research develops, a more complex and network-like reality governs over step-by-step pathways, offering plasticity and hence resilience against mono-target approaches. Although the strived for research direction is bench-to-bedside, this story takes a turn here and brings IGF-1R targeting back from bedside-to-bench, to extend our knowledge around functional complexity, before attempting to design smarter second generation targeting strategies.

Figure 4: Targeting the IGF-1R.

Following the demonstration of the importance of the IGF-1R axis in cancer, many targeting strategies were developed. Although their mechanism of action and exact target differed, they shared the common aim of inhibiting the kinase ability of the receptor. In addition, various inhibitors of signal components of the downstream pathways have been developed, which could be used alone or in combination with IGF-1R targeting.



1.4 IGF-1R SYSTEM UPDATES

Many of the realizations following the return-to-bench years circled around unappreciated layers of complexity. Among such, was the immense crosstalk between various signal systems that offered plasticity and resilience to targeting. Whilst signal cascades are often depicted by box-to-box schematics, it has long been understood that tremendous crosstalk

occurs between cell surface receptor systems. Crosstalk (also known as transactivation) between RTK and GPCR systems was first described in an instance where the EGFR became tyrosine phosphorylated after stimulation with various GPCR agonists [270]. This led to the speculation that a ligand-independent mechanism could activate RTKs through intercellular crosstalk. Indeed, many additional studies have since shown examples of GPCR mediated RTK activation, including PDGF [271], FGFR [272], and Trk A [273]. A wide variety of mechanisms exist, including close-proximity platforms [274], the GPCR-dependent release of an RTK ligand [275], and GPCR-dependent activation of cytoplasmic tyrosine kinases such as Src and Pyk that induce RTK tyrosine phosphorylation [276, 277]. Conceptually, such crosstalk mitigates many of the inhibition strategies if cells can simply re-route component activation. However, a distinction from mere crosstalk arose from experiments suggesting that the RTK member IGF-1R directly utilized components of the GPCR toolbox, questioning the classical boundaries of receptor families. Such non-canonical components require a shift in our current operational models, with widespread therapeutic repercussions if we aim to design efficient targeting agents.

1.4.1 IGF-1R: GPCR Components

Despite classical pigeon-holing of the IGF-1R as a typical RTK, and hence separate to the GPCR family, experimental results began to throw this into question. Initial indications of a shared signalling toolbox came from studies showing that the IR and IGF-1R signals were sensitive to pertussis, a toxin that locks the α_i subunit of G proteins in their inactive state [278-280]. This idea was further verified by studies demonstrating the association of $G\alpha_i$ and $G\beta$ with the IGF-1R in mouse fibroblasts and rat neuronal cells [281, 282]. $G\alpha_i$ and $G\beta$ were present in IGF-1R immunoprecipitates, and upon IGF-1 stimulation $G\beta$ was released [281]. An independent line of investigation into the mechanism whereby Mdm2 ubiquitinates the IGF-1R, uncovered the fact that β -arrestin plays a remarkably similar role in IGF-1R desensitization as they do at GPCRs. Mdm2 and both β -arrestin isoforms (1 and 2) co-immunoprecipitated with the IGF-1R, enhancing the Mdm2 mediated ubiquitination of the receptor [283]. A dominant negative mutant β -arrestin 1 impaired IGF-1R internalization, whereas β -arrestin 1 overexpression increased it [284]. Since G proteins, and then β -arrestin, seemed to blur the boundaries between GPCR and RTK categories, this warranted exploration of the third GPCR functional element; GRKs. Indeed, GRK 2 and GRK 6 were shown to be responsible for mediating the IGF-1R- β -arrestin interaction via a conserved agonist-activated receptor phosphorylation mechanism [285]. There seems to be contrasting roles between GRK 2 and 6, whereby phosphorylation of serine residues on the receptor C-terminal tail by either isoform encodes a barcode for subcellular fate. Specifically, GRK 2 phosphorylation promotes transient β -arrestin binding and predominance for receptor recycling, whereas GRK 6 promotes a stable receptor/ β -arrestin interaction that leads to receptor complex degradation [285].

Initially suggested by the peculiar sensitivity of the IGF-1R to pertussis toxin, and fully explored by numerous studies since, it is now abundantly clear that the IGF-1R makes direct use of all GPCR signalling components; G proteins, GRKs and β -arrestins. In a wider context, there are many examples of other RTKs acting upstream of GPCR components [286]. EGF promotes the apparent association of $G\alpha_i$ with the EGFR [287], VEGFR utilises G proteins for cell migration [288, 289], and PDGFR-mediated MAPK stimulation is pertussis toxin sensitive [290, 291]. Furthermore, GRK 2 phosphorylates the IR, PDGFR and the EGFR, although its desensitizing effects seem to differ [292, 293], and β -arr 2 promotes the endocytosis of VEGFR following ligand binding [294]. Taken together, these findings suggest that other RTKs can interact with GPCR components. However, only IGF-1R has thus far been shown to directly utilise all functional GPCR components in the same manner as a GPCR would. As such, it would seem that the IGF-1R sits in a unique position between the receptor family groups, and if transactivation can be viewed as bringing the families into close proximity, the IGF-1R actually integrates the two signalling networks. This advocates for an updating of this receptors classification to an RTK/GPCR functional hybrid.

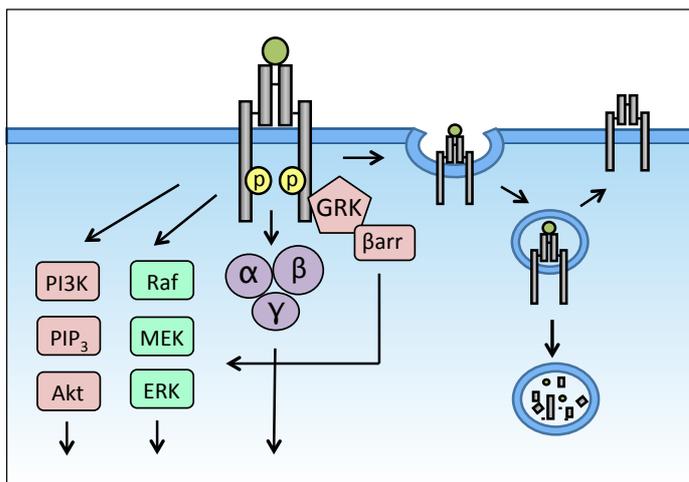


Figure 5: IGF-1R uses GPCR components. In addition to classical kinase action through the MAPK and PI3K cascades, the IGF-1R has been shown to use components of the GPCR toolbox. G proteins bind to and signal from a ligand-activated receptor. GRK 2 and 6 phosphorylation of the C-terminal tail generate binding sites for β -arr 1 and 2. The β -arrestins are involved in receptor internalization and subcellular processing (degradation/recycling), as well as generating a secondary signal towards the MAPK cascade.

1.4.2 IGF-1R: Biased Signalling

In parallel to the discovery path of the roles of β -arrestins at GPCRs, shortly after being recognized through Mdm2-ubiquitination of the IGF-1R, β -arr 1 was shown to mediate its own signalling downstream of an active IGF-1R, independent of the classical kinase cascade [295]. Such a multi-arm IGF-1R signal (kinase-dependent versus β -arrestin-dependent) presents the requirements to conform to the GPCR model of biased signalling, which describes the ability of an agonist/receptor pairing to preferentially activate a subset of downstream signal cascades. Thus, the new perspective in ligand-activated receptor action through biased signalling warranted investigation at RTKs to address whether this concept is universal.

Indeed, research revealed exactly that when exploring the mechanism of action of an IGF-1R blocking antibody. Designed as an antagonist for the IGF-1R, to bind and thereby hinder the natural IGF-1 ligand binding, figitumumab (CP-751,871; CP) was shown to actually act as a β -arr biased agonist [268]. The concomitant activation of receptor internalization and signalling offers yet another possible reason for the less than expected clinical results from anti-IGF-1R targeting trials. Later, the anti-microbial peptide LL-37 [296], the cyclolignan picropodophyllin (PPP) [297] and HASF - a natural protein released by mesenchymal stem cells [298], were all shown as novel ligands towards the IGF-1R that act in a β -arr 1 biased manner. These studies indicate that similar to GPCRs, the IGF-1R can also act in a biased manner, and hence an additional level of complexity must be taken into account in targeting approaches. Importantly, positioned between receptor internalization, desensitization, and signalling, the GRK/ β -arr system again identifies itself as critical in translating this bias.

1.4.3 IGF-1R: New Functional Classification

The intracellular tyrosine kinase domain has always meant that the IGF-1R was classified as a prototypical RTK, and as such, all targeting strategies thus far have aimed to inhibit its intrinsic kinase activity. However, in light of recent updates, it is evident that the IGF-1R can signal in ways separate to its classical kinase activity, and assumed blocking antibodies can actually act as biased agonists, circumventing the hypothesised inhibition of the receptor.

While examples of receptor family crosstalk have been known for quite some time, this example represents something quite separate. Crosstalk is typified by the GPCR-dependent increase in the activity of an RTK, or *vice versa*, and many examples span the GPCR/RTK boundaries. As an example, lysophosphatidic acid (LPA) acts as a GPCR agonist, yet also triggers EGFR activation - the mechanism believed to be via GPCR-release of an EGFR ligand [275]. Crucially, this process is still dependent on the kinase ability of the EGFR [299]. This is distinct from that which can occur at the IGF-1R, whereby this receptor is directly utilising GPCR components of G proteins, GRKs and β -arrests, and can activate a signal cascade in a kinase-domain-independent fashion. In fact, by all functional definitions, the IGF-1R has shown itself capable of classification as a functional GPCR;

- i) Ligand-binding activates signalling through heterotrimeric G proteins [278, 281]
- ii) GRKs phosphorylate serine residues on the activated receptor [285]
- iii) Creating binding sites for β -arrestins [283]
- iv) Causing desensitization of the signal [283],
- v) Initiation of a second β -arrestin-dependent signal [295]
- vi) Receptor endocytosis through recycling or degradation pathways [283].

As such, our group has proposed the idea that the IGF-1R should be regarded as an RTK/GPCR functional hybrid [134, 269], and until this paradigm shift is accepted by drug-developers, targeting strategies designed under a kinase-only paradigm will be insufficient and thus outsmarted by this network.

1.4.4 Redesigning IGF-1R Targeting Strategies

Since the first round of testing several targeting strategies, and near complete pharmaceutical abandonment, the IGF-1R has been brought back to the bench, to slowly revealed a much more complex multi-layered system than first considered. In addition to the typical phosphorylation control of the network, numerous other post-translational modifications such as ubiquitination [195] and SUMO-ylation (small ubiquitin-like modifiers) [300, 301] orchestrate the signal, and additional regulation layers are likely yet to be discovered. Second to regulatory layers, novel signalling players have been added; G proteins, GRKs and β -arrs. Originating in the field of GPCR research, the theory of biased signalling with multiple possibilities of activation, opens up the IGF-1R system for greater therapeutic exploitation. Going forward, the lessons learnt in the years following the unsuccessful clinical trials need to piece together an updated and more accurate depiction of the IGF-1R system.

To borrow a sentiment from many reviews of the field, “*Targeting the IGF-1R might not be as simple as just targeting the IGF-1R*” [260, 261, 302-304]. Initially designed targeting approaches of kinase inhibition or antibody-mediated blocking, lacked an appreciation of key capabilities to circumvent these approaches. However, it is plausible that strategies designed to not only recognise, but utilise these additional mechanisms could yield robust, whole-network effects capable of toppling the core pillars cancer cells rely on. The GPCR research field heralds the most successfully targeted drugs, therefore components of this system could hold potential to control the functional hybrid IGF-1R. Despite exponentially increasing signal complexity, the paradigm of biased signalling holds the promise of being able to fine-tune a designed therapeutic to an unprecedentedly detailed outcome [305].

1.5 THESIS AIMS

In light of the paradigm updates of the IGF-1R axis discussed herein, the overall aim of this thesis was to firstly continue to explore and characterise non-canonical components of the IGF-1R signalling system. And secondly, to investigate the utility of these previously unexplored components to target or track the IGF-1R in a cancer setting. Specifically, this overarching aim was broken down into the following project aims;

Study I: Investigate the therapeutic relevance of balanced versus β -arrestin biased IGF-1R targeting.

Study II: Characterize the role of the β -arrestin 2 isoform at the IGF-1R.

Study III: Investigate the clinical potential of targeting the GRK system to control β -arrestin involvement at the IGF-1R.

Study IV: Investigate microRNAs as clinical biomarkers to specifically measure β -arrestin biased signalling at the IGF-1R.

2 METHODOLOGICAL CONSIDERATIONS

The work comprising this thesis investigated the mechanism controlling balanced versus biased IGF-1R signalling, and whether its components could be developed for therapeutic gain. In order to accomplish this, the materials and methods of the four studies comprises of techniques, tools and strategies to; modulate signalling, measure such signals, and investigate their biological impact on cell phenotype.

2.1 CELL MODELS

The IGF-1R plays a central supportive role in the malignant conduct of many cancer cells, and hence an ever-present objective was the manipulation of such a system for anti-cancer strategies. As such, the four studies have employed several human cancer cell lines.

In **melanoma**, IGF-1R expression is correlated with disease progression [306, 307] and activity is driven by autocrine/paracrine stimulation [308, 309], attaining apoptosis prevention and proliferation [310]. In particular, the IGF-1R has been shown to play a crucial role in the metastatic mechanisms of melanoma [259, 311], and importantly, anti-IGF-1R therapies have shown some promising responses in advanced melanoma patients [312-316], a patient group usually refractory to treatment. Due to the chaotic plasticity afforded by melanomas' particularly high mutation load [317], it is likely that a synchronized multi-hit therapy will have utility in this disease setting. Studies into mechanisms of resistance suggest that IGF-1R co-targeting is a rationally validated approach [238, 318, 319]. Study I used a panel of melanoma cell lines to investigate the relevance of IGF-1R biased agonism in such a co-targeting regime.

Substantial evidence supports a central role of the IGF-1R axis in sarcomas. In **osteosarcomas**, polymorphisms of IGF-2R have been shown to be a risk factor, and given its negative regulation role, likely accounts for an increase in IGF-1R activity [320]. Study II and III employed the osteosarcoma cell lines U2OS and SAOS-2, which endogenously express high and low Mdm2 levels respectively, in order to examine not only IGF-1R perturbation but also the functional relevance of Mdm2. **Ewing's sarcoma** (ES) is a bone and soft tissue malignancy shown particularly reliant on the IGF-1R. ES predominantly arises in children and young adults, and is characterized by a specific chromosomal translocation that produces the EWS/ets family of genetic rearrangements [321]. This lone translocation has the ability to transform embryonic fibroblasts, however crucially, it does so only in the presence of the IGF-1R [226]. The protein product of this fusion has been shown to down-regulate IGF-BPs [322] and increase IGF-1 [323, 324], and autocrine IGF-1 loops have been shown in many ES studies [325]. The fact that ES patients were among the only to show clinical

response in early IGF-1R targeting trials [326, 327], reiterates the potential at stake. However, despite the wealth of evidence suggesting benefit, the optimal target and timing of agents to interfere with the IGF system in ES is still not clear [328]. In a malignancy that relies so heavily on the IGF-1R axis, any alterations its functionality are likely to manifest effects on cell viability. Therefore Study IV used a panel of ES cells to investigate the clinical utility of novel IGF-1R targeting strategies.

The non-malignant, but experimentally immortal, cell lines human embryonic kidney **HEK293T**, and mouse embryonic fibroblasts (**MEFs**) were employed throughout each study as models of protein expression control. HEK293T permit robust and reliable transient transfection, and thereby provide internal controls. MEFs derived from mouse models knockout for various components afforded a second model of protein elimination (see below).

2.2 MODULATION TOOLS

Every experimental pursuit depends on the ability to perturb a system intentionally, and then measure the effects of such perturbations. This thesis utilizes multiple strategies to perturb the IGF-1R axis, each instructive in their own right, yet were often used in combination to compliment each other's limitations.

Isoform specific **small interfering RNA (siRNA)** or **plasmids containing signal components** (β -arrs, GRKs, IGF-1R) were **transiently transfected** into cells in order to track knock-on effects. As a relatively straightforward and reliable technique, transfection was often used as a first-line exploration of effects. The disadvantage of this strategy is that alterations are short-lived and can differ in their efficiency across cell lines and replicates. Moreover, by targeting mRNA, the success of siRNA depends on the natural turnover of the protein of interest – silencing is much more efficient in the case of short-lived proteins. Transfection efficiency was controlled by western blot and/or qRT-PCR in all studies, using non-target siRNA or empty vector plasmids to control for the possible effects of the procedure alone. One must also be aware of the possibility that siRNA may cause some knockdown of closely related proteins and hence cause off-target effects [329-332]. While plasmid overexpression does not carry the same off-target risks, a drawback is the level of protein achieved. Final protein expression could be many-fold that of normal cellular ranges and this may impact stress pathway activation, or lead to protein interactions not usually evident at physiological levels [333].

While transient transfection allows for efficient short-term changes, siRNA achieved around 80% maximal knockdown. Therefore to validate results, we also used **MEFs derived from knockout models**. Genetic knockouts such as MEF KO β -arr 1 and MEF KO β -arr 2 are advantageous as they afford complete elimination of the protein of interest, and hence

provide a “clean” system. However, the main limitation of such a system is that these cells can develop ways to compensate and rely on other pathways for mitogenic requirements [333, 334], altering the way a cell would usually behave and masking effects that may be seen in shorter-term perturbations. MEFs derived from embryos lacking the IGF-1R (R-cells) were used in isolation and/or following the transfection of C-terminal tail truncated receptor (Study III) or phospho-mutants (Study IV) versions.

Manipulating the expression level of a protein in order to determine its roles is helpful in many ways, however it must also be recognized that this is a very crude manipulation. Knockdown or overexpression alters every one of the protein’s functions, and hence, specifics may be masked. Mutation analysis on the other hand, has the advantage of allowing modulation of one function at a time, allowing for a more meticulous interrogation of a multi-function protein. Exploring the concept of biased signalling, many GPCR mutants capable of carrying out only G protein or β -arr dependent signals have been successfully generated [335, 336]. In Study IV **IGF-1R mutation analysis** was used to alter the way the receptor is able to behave in the cellular environment. Aiming to uncouple the signalling arms emanating from an activated IGF-1R, we generated mutant versions of the receptor that could only signal through one or the other arm, in order to examine their relative influence. Mutating tyrosine/serine residues to alanine generated kinase-dead phospho-mutants, and mutating alanine to aspartic acid generated phospho-mimetics, both of which allowed control of the enzymatic function of this protein [337-339]. In all instances these mutant receptor transfections were compared to a parallel transfection of a wild-type receptor.

Targeting agents. Study I compiled a panel of compounds to compare balanced versus biased IGF-1R down-regulation. Small molecule Nutlin-3 inhibits the interaction between p53 and its negative regulator Mdm2, and has recently been shown to concomitantly down-regulate the IGF-1R with transient signal activation [240]. The IGF-1R targeting antibody Figitumumab (CP) was developed to antagonise the system, but it was later discovered to act as a β -arr 1 biased agonist [268]. siRNA towards the IGF-1R was included as an experimental strategy to down-regulate the receptor and all associated signalling in a balanced manner. The MEK inhibitor U1026 was used to assess possible synergism with IGF-1R targeting regimes. Study III made use of the GRK 2 inhibitor paroxetine (PX), used clinically as a selective serotonin reuptake inhibitor (SSRI) in the treatment of depression and anxiety-related disorders [340]. In this context, GRK 2 inhibition was used to cross-target the IGF-1R in malignant ES cells.

2.3 PROTEIN/RNA ANALYSIS

Despite the overlapping signal components that they signal to, the kinase and β -arr 1 arms downstream of the IGF-1R can be at least partially distinguished kinetically. Hence, these studies have used **western blot analysis** and the time-course dynamics of protein

phosphorylation to assess their relative contribution. Cells were serum-starved to bring extracellularly activated signal cascades to basal levels, and then stimulated with IGF-1 for various durations before lysis. A wild-type receptor generates a balanced cascade through both the MAPK and the PI3K pathways. Protein levels of phosphorylated (p) IGF-1R, pERK and pAkt (used as readouts of the MAPK and PI3K pathways respectively) peak at 5/10 mins after exogenous addition of IGF-1, and decrease slowly over the course of 60 mins. The β -arr 1 biased signal occurs later in time, and because it acts as a scaffold protein holding components together, it maintains MAPK activity longer. For this reason, throughout the studies we have used sustained pERK activity (expression level at 60 min as % of maximum), and the relative phosphorylation levels of the receptor, ERK and Akt, to attain information about the biased nature of the signal. To investigate the molecular interactions that gave rise to biased signals, protein interactions were analysed by **immunoprecipitation**. For validation of results, multiple independent western blots were quantified, combined and subjected to statistical analysis.

The “central dogma” of DNA-to-RNA-to-protein ignores the functions of RNA beyond protein blueprints. Among such, microRNAs (miRNA) are small non-coding RNAs that play important roles in protein expression, by specifically interfering with mRNA translation. The fact that their expression patterns are indicative of disease states, and that a notable amount can be found extracellularly in the circulation, presented the possibility of using them as clinical biomarkers [341-343]. In order to look for specific biomarkers for β -arr biased signalling Study IV carried out **microRNA array** analysis. **Quantitative real time polymerase chain reaction (qRT-PCR)** was used for analysis of RNA levels, both to validate the array results (Study IV), and to determine mRNA level changes following transient transfections (Study III and IV).

2.4 BIOLOGICAL EFFECTS

Activation of the IGF-1R promotes cellular responses surrounding anti-apoptosis, cell cycle progression and growth. As readout of functionality, techniques were used that examined this behaviour following experimental perturbations.

PrestoBlue cell viability assay employs a resazurin-based reagent, which is processed to a fluorescent variant by viable cells. Hence, using a spectro-photometer microplate reader and a standard curve of known cell number, experimental cell viability can be calculated. Cell viability effects were calculated in Studies I-III by comparing serum-starved, transfected, or drug-treated cells to IGF-1 stimulated, mock-transfected or solvent-only treated controls, respectively. One downside to a viability assay such as this is that its results are likely a mix of proliferation and apoptotic changes.

IGF-1R activation promotes the completion of a cell cycle, and hence **cell cycle distribution analysis** affords a closer look at the shorter-term impact of signal perturbation. In order to examine specific changes in the cell cycle, including ant halts, we used fluorescence activated cell sorting. Following experimental treatment, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) in order to determine their DNA content, and separated out into cell cycle stages.

Clonogenic assay is a long-term cell survival and proliferation assay based on a single cell's ability to produce progeny and grow into a colony [344]. This assay has a long history of use in examining the effects of ionizing radiation and chemical exposure on cancer cells [345]. The clonogenic assay enables an assessment of differences in reproductive potential following experimental procedures. In Study III, this assay was employed to compare the long-term effects of IGF-1R targeting agents CP and PX, and in Study IV it was used to follow genetic manipulations following siRNA towards β -arr 1 or mutant IGF-1R transfections.

The IGF-1R is required for the transformative event allowing cells to survive and proliferate in an anchorage-independent manner [116, 118]. In Study III we used an **anchorage-independent growth assay** using poly-2-hydroxethyl methacrylate (poly-2-HEMA) coated plates to prevent cell attachment. This experiment tested the cell's reliance on a functional IGF-1R system in such a stringent scenario, and how changes to this system manifest in survival and growth. There is a close correlation between cell growth on poly-2-HEMA coated plates and soft agar colony formation as a measurement of anchorage-independent growth in transformed cells [346], but this assay has the advantages of being simpler and easily quantitative when combined with the aforementioned PrestoBlue reagent.

A full description of the materials and methods used in each study can be found in each of the papers comprising the second part of this thesis.

3 OVERVIEW OF THE STUDIES

3.1 STUDY I: ENHANCED RESPONSE OF MELANOMA CELLS TO MEK INHIBITORS FOLLOWING UNBIASED IGF-1R DOWN-REGULATION

Background and Rationale:

Malignant melanoma is a highly aggressive disease with a grim prognosis, and increasing incidence worldwide. An important mutational event in its molecular pathophysiology is constitutive activation of the MAPK cascade [347]. Aberrant activity is most often caused by mutations in B-RAF or RAS genes, and hence warranted specific therapeutic targeting. Unfortunately, in many cases tumours initially responsive to MAPK inhibitors rapidly acquire resistance, by re-routing mitogenic signal requirements through alternative pathways [348]. Due to the fact that it often compensates for signals lost after therapeutic MAPK-inhibition, the insulin-like growth factor type 1 receptor (IGF-1R) stood out as a rational co-targeting approach [237, 238, 349]. However IGF-1R targeting is not straight forward, as conformational changes associated with its inhibition have been shown to preferentially activate the MAPK pathway, through a process known as biased signalling [268, 297, 298]. We explored the impact of IGF-1R biased signalling, on response to MAPK inhibition in melanoma cell lines spanning common mutational signatures.

Results and Conclusions:

Using a panel of skin melanoma cell lines spanning RAS/RAF and p53 mutational status, we demonstrated that all cell lines show dose-dependent sensitivity to the MEK inhibitor U1026, as measured by a PrestoBlue cell viability assay. Of note, the cells were more sensitive in serum-free media than in serum or IGF-1 supplemented conditions, supportive of the notion that they may be relying on growth factors for survival support.

We firstly categorised our targeting agents in terms of IGF-1R down-regulation, following expression levels by western blot after treatment. All three agents down-regulate the receptor through different mechanisms – siRNA through mRNA interference, Nutlin-3 through the redirection of the E3 ubiquitin ligase Mdm2 towards the receptor [240], and Figitumumab through binding extracellularly to the receptor and initiating down-regulation through β -arr 1 [268]. We verified that all three treatments decreased the expression of IGF-1R protein to a similar extent, with the only exception of Nutlin-3 in Mel28 (containing low Mdm2). Concomitant western blot analysis of the important tumour suppressor p53 showed that Nutlin-3-mediated receptor decline coincided with p53 accumulation, whereas levels were unchanged in all other regimes. In all cases, the receptor depletion was functionally significant, limiting the cell proliferative response to IGF-1 stimulation.

Previous reports have shown that some receptor conformations that drive down-regulation also engage signal modules and hence trigger downstream signalling. Therefore we next we explored the agonistic impact of these treatments on IGF-1R signalling activity. In line with expression, siRNA against the IGF-1R down-regulates all downstream signalling in a balanced manner. IGF-1R targeting by small molecule Nutlin-3 does parallel receptor degradation with some pERK1/2 activity but this is transient in nature. IGF-1R down-regulation by a targeted antibody (Figitumumab) on the other hand, coincides with a receptor conformation that is biased and maintains sustained MAPK activity, evident even if the receptor is stimulated with the natural (balanced) ligand IGF-1.

Despite efficient receptor down-regulation, the strategies each differ in their p53 and MAPK activity, and hence are likely different in their ultimate functionality. In order to assess possible synergy between the MEK inhibitor U1026 and IGF-1R targeting strategies, we followed cell viability after co-treatment regimes. Targeting the IGF-1R through both siRNA and Nutlin-3 synergize with the MEK1/2 inhibitor U0126, cell death is enhanced to a level beyond addition of each strategy alone. Following Figitumumab co-treatment no synergistic cell toxicity is evident, conceptually due to the signal activity counteracting U0126 MEK1/2 inhibition.

Our results indicate that IGF-1R down-regulation does offer an approach to increase sensitivity of melanoma cells to MAPK inhibition, but highlights that this co-targeting must acknowledge the paradigm of biased signalling at the IGF-1R. Only IGF-1R down-regulation strategies that do not induce a sustained biased signal synergize with MAPK inhibitors. Efficient recognition and control of biased agonism in co-targeting regimes can inform the design of improved therapies with more durable clinical responses.

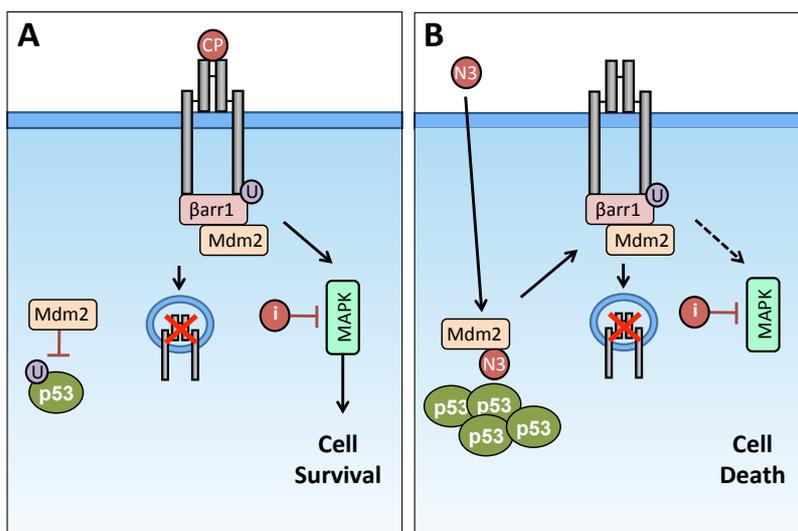


Figure 6: Unbiased IGF-1R down-regulation synergizes with MEK inhibition. (A) IGF-1R targeting antibody CP induces receptor down-regulation that coincides with β -arr 1 biased signalling, and hence counteracts MEK inhibition. (B) Targeting the IGF-1R through Nutlin-3 however, acts to redistribute the E3 ubiquitin ligase Mdm2 away from p53 and towards IGF-1R. The resultant p53 accumulation, IGF-1R depletion and transient MAPK activity, synergizes with MEK inhibition treatment causing malignant cell death.

3.2 STUDY II: FUNCTIONAL ANTAGONISM OF B-ARRESTIN ISOFORMS BALANCE IGF-1R EXPRESSION AND SIGNALLING WITH DISTINCT CANCER-RELATED BIOLOGICAL OUTCOMES

Background and Rationale:

β -arrestins (β -arrests) are central regulators of G protein-coupled receptor (GPCR) functions including signalling, desensitization, down-regulation and trafficking [67, 72, 350-352]. Recent evidence opened up new perspectives in the cancer-supportive insulin-like growth factor type 1 receptor (IGF-1R) function, by demonstrating that the β -arr system is also a key regulator of this receptor tyrosine kinase (RTK). The role of β -arr 1 at the IGF-1R is now well categorised: enhancing ligand-dependent degradation alongside initiating its own (kinase-independent) wave of MAPK/ERK signalling [283, 295]. However, apart from the fact that the β -arr 2 isoform binds to the receptor, little is currently known about its role. Sharing similar 3D conformations, the ubiquitously expressed β -arr isoforms 1 and 2 can play indistinguishable, separate or opposing roles in regulating GPCR function [57]. This project aimed to gain insight into the functional interplay between the β -arr isoforms at the IGF-1R, and the relevance of such interplay in cancer cell biology.

Results and Conclusions:

Control of β -arrestin expression was carried out using isoform specific siRNA and plasmid transfection, in addition to embryonic fibroblasts derived from isoform specific knock-out mouse models. Western blot analysed the impact of controlled β -arr expression on IGF-1R expression and signalling, results of which showed that β -arr 2 acts in an opposing manner to β -arr 1 by promoting the degradation of a ligand-unoccupied IGF-1R, but protecting against ligand-induced degradation. In terms of IGF-1 signal transduction, strategies that imbalance towards the β -arr 2 isoform limit the sustained MAPK activity associated with β -arr 1.

β -arr 1 binds to the C-terminal tail of a ligand-activated IGF-1R, recruits the E3 ubiquitin ligase Mdm2, leading to receptor ubiquitination and down-regulation. Expression and signalling experiments whilst modifying either the receptor C-terminal tail or Mdm2 levels, suggest that the differential β -arr 2 functions rely on this same sequence of events. Co-immunoprecipitation allowed us to define the interactions of either isoform with the receptor. Both isoforms interact with the IGF-1R as measured by co-immunoprecipitation, however the ligand-occupied receptor shows greater affinity and a more stable interaction with β -arr 1. Conversely, β -arr 2 shows greater affinity for the ligand-unoccupied receptor and this interaction is transient, disbanding completely in the presence of the ligand.

Using fluorescence activated cells sorting (FACS) and PrestoBlue cell viability assay we assessed the functional impact of β -arr isoforms on IGF-1 induced cell cycle progression and proliferation. In both U2OS and SAOS-2, imbalance towards the β -arr 1 isoform slightly

increased IGF-1 mediated cell cycle progression compared to control. Imbalance towards β -arr 2 yielded cells unresponsive to IGF-1 and considerably reduced cell number. The pattern of arrest observed in FACS separated the two cell lines; U2OS demonstrated a G1 and G2/M arrest, whereas SAOS-2 appear able to enter the cell cycle in response to IGF-1 but appear unable to finish, as illustrated by G2 phase arrest. A parallel cell viability assay shows that this coincides with a dramatically decreased SAOS-2 cell number; meaning these G2/M arrested cells are channelled into cell death routes.

Considering the possible mechanistic reasons, we tested p53 activity as one important difference between the cell lines. Results show that in the presence or preference of β -arr 1, p53 levels are kept at a basal low level. Preference of the β -arr 2 isoform on the other hand, appears to remove this inhibitory signal, and p53 levels accumulate in $-\beta$ -arr 1 and $+\beta$ -arr 2 conditions. As only U2OS contains functional p53, this goes at least part way to explain the cell cycle arrest patterns – reactivated p53 stops U2OS from entering the cell cycle, whereas SAOS-2 (mutant p53) enter, but without the mitogenic β -arr 1 signal are unable to complete – and vulnerable part-way through, undergo cell death.

Altogether, our results show that although both isoforms bind to IGF-1R, the ligand-occupied receptor has greater affinity for β -arr 1; this association is stable, sustains MAPK/ERK activity and suppresses p53. Conversely, β -arr 2 shows greater affinity for the ligand-unoccupied receptor; this interaction is transient in nature and occurs without signalling. Imbalance towards this isoform leads to a lack of responsiveness to IGF-1, cell cycle arrest and cell death. This study identifies the mechanism controlling balanced versus biased IGF-1R conformation, in the divergent affinities of IGF-1R towards each β -arrestin isoform. Our results demonstrate antagonism between the two β -arr isoforms in controlling IGF-1R expression and function, interplay that presents potential for anti-IGF-1R control in cancer therapeutics.

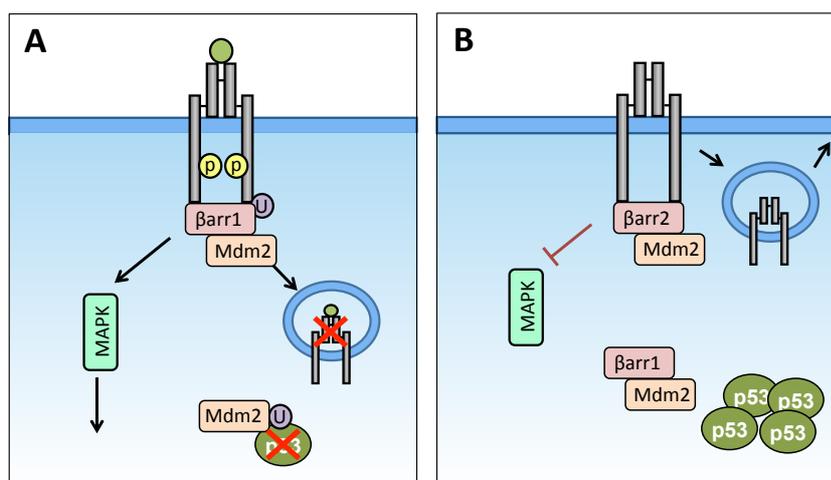


Figure 7: β -arrestin isoform antagonism. β -arrestin (β -arr) isoforms antagonize each other's function at the IGF-1R. (A) β -arr 1 binds to a ligand-activated receptor, driving receptor internalization and degradation, a MAPK signal and inhibition of p53. (B) β -arr 2 on the other hand, preferentially binds to a ligand-unoccupied receptor, mitigating any MAPK activity and p53 repression.

3.3 STUDY III: G PROTEIN-COUPLED RECEPTOR KINASE 2 INHIBITION PROMOTES UNBIASED INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR DOWN-REGULATION AND RESTRAINS MALIGNANT CELL GROWTH

Background and Rationale:

The capacity of a receptor to preferentially activate a subset of downstream signal cascades is termed biased signalling. First described for G protein-coupled receptor (GPCRs), this process is now recognised for receptor tyrosine kinases (RTKs) including the cancer relevant insulin-like growth factor-1 receptor (IGF-1R). Successful anti-IGF-1R therapy requires receptor removal from the cell surface, yet effectiveness is limited because this can be accompanied by protective β -arrestin (β -arr) biased MAPK activity. There is a need to develop anti-IGF-1R targeting strategies in cancer, which recognise and control β -arr biased agonism at the receptor [110, 195, 269]. As IGF-1R's ability to activate β -arrestins is dependent on G protein-coupled receptor kinases (GRKs) [285], we investigated the contrasting abilities between GRK 2 and 6 isoforms in promoting IGF-1R down-regulation with focus on clinical applicability.

Results and Conclusions:

To investigate the impact of GRK modulation, we employed small-interfering RNA (siRNA) technology and plasmid overexpression, combined with western blot to follow their impact on IGF-1R degradation and signalling. Transgenic modulation of either isoform demonstrates that GRK 6 inhibition and GRK 2 overexpression are receptor-protective, whereas GRK 2 inhibition and GRK 6 overexpression enhance IGF-1R degradation. In line with therapeutic requisite, GRK 2 inhibition and GRK 6 overexpression were taken forward to follow the concurrent signal effects. Both strategies degrade the receptor while sustaining biased MAPK/ERK activity in response to IGF-1 stimulation. These apparent contrasting effects (receptor degradation and signalling), still display functional consequences of blunted proliferation in both anchorage-dependent and -independent assays.

Pharmacological GRK 2 inhibition by the clinically-approved serotonin reuptake inhibitor paroxetine (PX), recapitulated the effects of GRK 2 silencing, with dose and time-dependent IGF-1R down-regulation, but crucially in this instance without β -arr biased MAPK/ERK signalling. PX induced degradation relied on the presence of β -arr 1, and an IGF-1R C-terminal tail and hence a functional β -arr 1/IGF-1R interaction. When compared to a known β -arr 1 biased agonist, PX induced MAPK activity, evident only in Mdm2-overexpressing U2OS cells, was transient in nature and dispelled within minutes.

For the larger family of GPCRs, as well as for the IGF-1R, the GRK phosphorylation controlled receptor–arrestin interaction directs receptor fate, thus we investigated the mechanism of action of PX using coimmunoprecipitation. These studies reveal that PX

exploits the antagonism between β -arr isoforms in controlling IGF-1R activity. In conditions without exogenous addition of ligand, PX prevents the usual β -arr 2 binding and instead favours a β -arr 1 interaction, with ensuing Mdm2-mediated ubiquitination and degradation of IGF-1R, effects usually exclusive to the presence of the ligand.

Given the combined desired effects of PX- degrading the IGF-1R without signalling, we took this strategy forward to a malignant model reliant on this axis. In Ewing's sarcoma cells, PX recapitulates expression and signalling control. Previous studies have demonstrated the inefficiency of an IGF-1R β -arr 1 biased agonist in Ewing's sarcoma treatment [268], and hence we next sought to compare the two approaches. Cells receiving a single dose of PX and incubated long-term demonstrated a dose-dependent inhibition of their ability to produce progeny, even at the lowest and clinically relevant dose. CP, coinciding receptor down-regulation with MAPK activity, failed to produce significant effects on colony formation at any tested dose. This data demonstrates a clear benefit of targeting the receptor through a regime that does not sustain a biased signal.

This study provides the proof of concept for targeting the IGF-1R through the GRK/ β -arrestin system. Pharmacological translation validates a widely used drug PX as a selective GRK 2 inhibitor that could be used as a starting point for the rational design of more potent inhibitors for anti-IGF-1R cancer therapy.

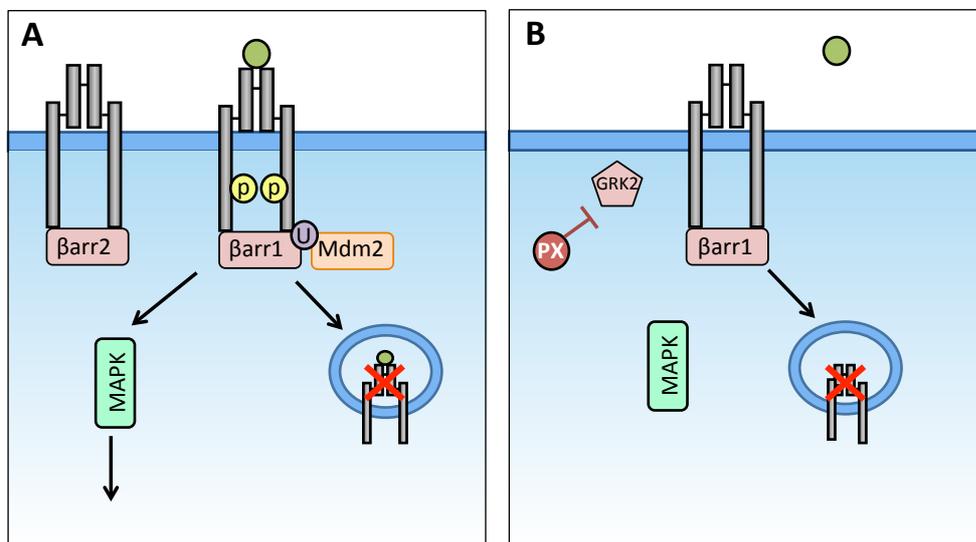


Figure 8: Targeting the IGF-1R axis through paroxetine. (A) Under normal physiological conditions, β -arr 2 binds to the ligand-unoccupied IGF-1R. Upon ligand binding, β -arr 2 is replaced by β -arr 1, with the dual outcome of receptor internalization with degradation, and MAPK activity. (B) Paroxetine acts by switching the affinity of the ligand-unoccupied receptor through GRK 2 inhibition. This preferences β -arr 1 binding and receptor degradation, but without any MAPK activity. Receptor depletion in a non-biased manner negatively impacts cancer cell growth and survival.

3.4 STUDY IV: UNCOUPLING SIGNALLING AT IGF-1R IDENTIFIES MIR-106A AS BIOMARKER OF CANCER-PROMOTING B-ARRESTIN SIGNALLING.

Background and Rationale:

Whilst substantial evidence exists supporting IGF-1R inhibition in many cancer types, strategies developed thus far have failed to live up to expectation in clinical trials [110, 203, 260, 269, 353]. One reason for the shortcomings is likely the cancer-protective β -arrestin (β -arr) signalling associated with IGF-1R antibody targeting [268]. As a result, biomarkers that can measure signal bias/activity are crucially important to enhance response to IGF-1R therapy. Relatively recently discovered and added to the regulatory machinery of cells, microRNAs (miRNAs) are small non-coding RNAs that adjust protein expression by interfering with mRNA translation [354]. As such, many studies have shown that miRNAs regulate, or are the targets of, important signalling pathways [355-357]. Given the differential kinetics and functions of signals downstream of the IGF-1R, we sought to investigate the ability of miRNAs to serve as biomarker indicators of IGF-1R biased signalling.

Results and Conclusions:

To initially distinguish a β -arr 1 biased signal from a canonical balanced signal, cells were transfected with specific siRNA against β -arr 1 and investigated by western blot for IGF-1 signalling, and functional impact by FACS analysis and a long-term clonogenic assay. Results suggest that the signalling emanating from IGF-1 stimulation is biphasic in terms of MAPK activity, and that β -arr 1 is responsible for sustained pERK activity. Functionally, this β -arr 1 biased signal is distinct in that it promotes cell cycle progression, and long-term survival and proliferation. These results disclosed that an IGF-1R β -arr 1 signal is not only temporally, but also functionally distinct in supporting aspects of the tumorigenic phenotype.

To examine whether specific miRNA expression profiles associate with either signal, cells were transfected with siRNA against β -arr 1, IGF-1 stimulated, and had their RNA extracted and subjected to miRNA array. Bioinformatic analysis compared the miRNA expression between the β -arr 1 conditions, and hierarchical clustering identified numerous candidate miRNAs specifically up- or down-regulated in si β -arr 1 compared to mock-transfected controls.

To validate whether any of these miRNAs were able to specify a β -arr 1 signal, we generated a panel of IGF-1R mutants containing specific residue phospho-mutant or phospho-mimetic changes that rendered the receptor capable of kinase and/or β -arr 1 signalling only. Using this more stringent system to uncouple kinase from β -arr 1 signalling at the IGF-1R, we assessed functional impact, and found that an IGF-1R mutant only able to signal through β -arr 1 (IGF-1R ADTM), was still able to initiate cell cycle progression in response to IGF-1, similar to a

wild-type control and in contrast to a silenced mutation of the same residue (AATM). Long-term, this β -arr 1 biased mutant thrived in a clonogenic assay, with a higher proliferative ability than either kinase-only or wild-type controls. The array had identified multiple miRNAs specifically associated with a β -arr 1 signal following IGF-1 stimulation. Using both the siRNA strategy and IGF-1R mutants controlling β -arr 1 interaction, qRT-PCR validated miR-106a as an indicator of a β -arr 1 biased signal.

Both siRNA silencing and interaction-controlled mutant IGF-1R transfections support the fact that a β -arr 1 signal drives tumour supportive changes in malignant cells. Candidate miRNAs identified in a screen, were verified by mutant receptors that identified of miR-106a as a candidate biomarker of a β -arr 1 signal downstream of the IGF-1R. Target analysis using numerous miRNA databases (Supplementary Table 1) supports the relevance of miR-106a in the mitogenic IGF-1R β -arr 1 biased signal, as numerous appropriate pathways, surrounding cell cycle progression and proliferation mechanisms are already described targets of this miRNA. There also now exist multiple studies linking miR-106a to cancer [358-364].

Given the potential of miRNAs in liquid biopsies, miR-106a or other candidate miRNAs may be able to predict β -arr 1 signalling in plasma/sera patient samples, as a molecular diagnostic tool for treatment response, disease relapse or to stratify patients into appropriate anti-IGF-1R trials.

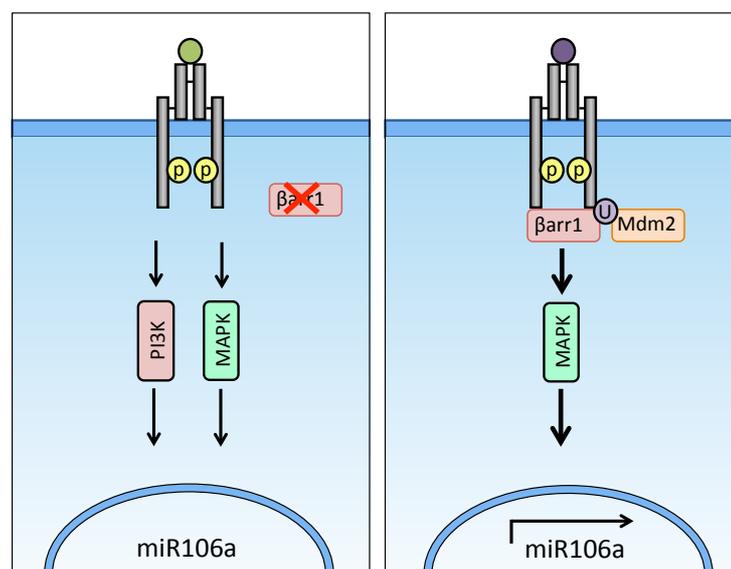


Figure 9: miR-106a identifies a β -arrestin 1 biased IGF-1R signal. Multiple signals can emanate from a ligand-activated IGF-1R. Strategies to uncouple classical kinase from a β -arr 1 biased signals, suggest that miR-106a may result from a β -arr 1 biased signal. Given the tumour supportive nature of this signal, this miRNA may hold potential to specify system bias and therefore serve as a biomarker for patient stratification or therapy decisions.

4 DISCUSSION

The IGF-1R targeting drive was forced into introspection following a run of failed clinical trials. However, an important sentiment echoed by Basu *et al.* is to make sure not to “*throw the baby out with the bathwater*” [365]. Disappointing trial results must be viewed with the wisdom of hindsight, and with an appreciation that critical components were missing in the models and ideas in use at the time. The extensive pre-clinical and epidemiological evidence supporting the importance of the IGF-1R axis in cancer cell biology remains valid.

The aim of this body of work was to continue the post-trial effort of categorizing the true complexity, and in particular the non-canonical regulation of this receptor system. Prior work from our group had opened up the possibility of biased signalling of the IGF-1R by exploring the mechanism of action of a targeting antibody [268]. Functionally, the puzzle was in the process of being pieced together to explain this phenomenon. It was already recognized that in addition to the classical RTK kinase-based activity, the IGF-1R made use of components of the GPCR machinery. IGF-1R signalling was sensitive to a G protein toxin [278], and throwing classical receptor families into question, G proteins physically interact with a ligand-activated IGF-1R [282]. Next, β -arrestins were shown to be crucial for IGF-1R down-regulation through Mdm2-mediated ubiquitination [283], and subsequently, to mediate their own wave of kinase-independent signalling [295]. The final GPCR *module* followed, with the demonstration that GRK phosphorylation controlled β -arrestin recruitment and interaction with the IGF-1R [285], and hence completed all functional requirements for the IGF-1R to be classified as a GPCR [134]. In light of this new framework, this thesis aimed to examine the therapeutic relevance of this RTK/GPCR hybrid concept.

In **Study I**, our results reinforce the idea that biased signalling is therapeutically relevant in anti-IGF-1R strategies and that it needs to be considered in drug development. In melanoma, the re-routing of MAPK activation following MEK inhibitor treatment often relies on the IGF-1R. Down-regulation of the receptor is warranted in order to stifle the survival signals emanating from its expression. However, down-regulation itself is interwoven with signal activation, and hence, must be manipulated delicately. Strategies that down-regulate the receptor in a manner that sustains β -arrestin 1 biased signalling, do not aid MEK inhibition, whereas balanced (unbiased) strategies such as siRNA or small molecule Nutlin-3, synergize in terms of melanoma cell cytotoxicity. This study identifies a strong triple target regime for melanoma treatment, whereby MEK inhibitor U0126 and small molecule Nutlin-3 synergize by means of dual mitogenic inhibition (MEK + IGF-1R) alongside p53 reactivation. In addition, the results of this study suggest that true IGF-1R co-targeting potential will likely be underestimated until drug development research at least recognises, if not actively employs, appropriate non-canonical components to control the receptor.

Study II set out to complete a missing piece of the puzzle; to investigate the role of the β -arrestin 2 isoform. Results indicate that the β -arrestin isoforms functionally antagonise each other at the

IGF-1R. In contrast to the known roles of β -arr 1, β -arr 2 binds to a ligand-unoccupied (inactive) receptor conformation, and antagonises the β -arr 1 effects of receptor degradation and signalling. Phenotypic changes led us to explore p53, and demonstrated that while a β -arr 1 signal restrains p53, this can be counteracted by β -arr 2. Strategies to imbalance towards the β -arr 2 isoform cause p53 accumulation and blunting of the IGF-1R signal. It is tempting to speculate that imbalance strategies either side of this partnership, positioned between a mitogenic receptor and a tumour suppressive pathway, hold strong potential for anti-cancer strategies. In revealing the isoform interplay, the data presented a four-component functional partnership whereby GRK 6/ β -arr 1 seems to be balanced by GRK 2/ β -arr 2.

An overarching theme of this thesis is the intricate interweaving of *off*-states and *on*-states. This choice does not operate as a binary switch that can be therapeutically pushed to one side. Instead, the components that mediate desensitization (including GRKs, β -arrestins, Mdm2) link to secondary signal activity, and hence any desensitization *push* also enhances the activity side too. While first line thinking may be that β -arr 1 enhances receptor degradation and therefore warrants hijacking, the final outcome is that it counter intuitively drives β -arr 1 mediated signalling [268, 283, 295]. Although balancing against β -arr 1 limits the signal, it also protects the receptor against down-regulation [366]. It was clear that there was potential in this system, yet created the conundrum of how best to target. In such a functionally integrated network, a clear target is difficult to deduce and the system requires careful fine-tuning.

Study III explored the utility of the upstream component – GRKs, to regulate β -arr involvement. As kinases, they represent apt drug targets [367-369,] and the barcode hypothesis [39, 370] supports their key role in dictating receptor fate. Hence, they may ultimately be better targets than the β -arrestins themselves. Experimentally, our results show that GRK 2/6 modulation are in line with β -arr 1/2 changes. While GRK 6 plasmid overexpression and siGRK 2 promote receptor degradation, they both coincide with sustained MAPK activity. This confronts us once more with the degradation = biased signal problem, bringing us conceptually back to square one. Except that, employing a pharmaceutical inhibitor to selectively control one function (the kinase ability [371]) leaves GRK 2 protein level unchanged, and yields a different outcome. GRK 2 inhibition by paroxetine (PX) presents a method whereby degradation does not have to coincide with signalling, by manipulating the natural IGF-1R ligand-dependency model. Immunoprecipitation verified that PX treatment in serum-free media promotes the association of the receptor with β -arr 1, driving receptor down-regulation: a sequence of events that is usually exclusive to ligand presence. In order to explain why this does not coincide with a biased signal, we propose the differences in GRK 2 expression level between siRNA and PX approaches. In a protein as multi-functional as GRKs or arrestins, the impact of whole cell expression changes disrupts many processes, and hence off-target effects cannot be contained [333]. PX reveals that in circumstances where only the relevant kinase ability of GRK 2 is perturbed, receptor degradation is maintained without evident biased signalling. There is data showing the many roles of GRK 2 outside of its kinase ability [45]. The model we propose is that ubiquitination substrate competition is maintained with PX, but not with siGRK 2, therefore having

different knock-on effects on other components. For example, Mdm2 is an E3 ubiquitin ligase for IGF-1R [283] and p53 [240], but also for β -arr 1 and GRK 2 [372], which implies that their expression is linked. If GRK 2 levels are depleted, such as with siRNA, Mdm2 is more readily available to ubiquitinate other substrates. Importantly, β -arr 1 ubiquitination aids scaffolding and allows it to form signalosome complexes [373, 374]. If GRK 2 levels are not depleted, and instead its kinase ability is selectively inhibited as in the case of PX, Mdm2 is perhaps not redistributed and β -arr 1 signalosomes are not favoured. Another of our studies highlights the therapeutic relevance of such substrate-level competition, in the mechanism of action of Nutlin-3. Designed to inhibit the interaction between p53 and Mdm2, this study demonstrated knock-on effects on the other Mdm2 substrate IGF-1R [240]. Unable to bind and degrade p53, Mdm2 redistribution increased the ubiquitination and hence degradation of the IGF-1R. There are likely many substrate-level, and other repercussions of removing the whole reservoir of GRK 2 from the cell, which may explain the differences in signalling between the two GRK 2 inhibition strategies.

The complex interactome of GRK 2 suggests that this kinase acts as a node in the signal transduction network of the cell [45, 375], further supported by the fact that it is the only GRK isoform in which KOs are embryonic lethal [32]. Many studies show that GRKs are good drug targets, in particular from studies that inhibit GRK 2 in cardiovascular disease [46, 376, 377]. Drawing parallels from Study III, studies have targeted the β -adrenergic receptor through GRK 2 inhibition [378], and as such it has been suggested as an effective drug target in preventing heart failure [379-381], hypertension [382, 383], and inflammation [384-387]. In malignancy, GRK 2 functionality has been reported as altered in granulose cell tumours [388], thyroid [389] and pancreatic cancers [390], and also associated with aberrant activation of the PI3K/Akt cascade [372]. Our study advocates for the use of GRK 2 inhibitors to control the IGF-1R in a cancer setting, and suggests that the clinically used SSRI PX could be used as a starting point for developing greater specificity analogues [377, 391].

The conceptual repercussion within the paradigm of biased signalling, is the appreciation of *intracellular bias*. Sometimes used interchangeably, the term *biased agonism* suggests the central role of the extracellular agonist in the ensuing bias. However, the PX example demonstrates intracellular actions that cause the receptor to act in a biased manner - either towards or away from degradation or signalling. While it may be novel for this receptor, it is not a new idea and sits amongst many examples in the GPCR field [392]. It does however encourage the continued development of the paradigm of receptor signalling. Once viewed as two-state switches, a recent review moves the field forward by referring to GPCRs as microprocessors [393]. In addition to agonists, biased signalling can be controlled by the receptor itself (biased receptor) or by the relative expression of intracellular transducers (biased system). The receptor can therefore be viewed as a microprocessor that integrates extracellular and intracellular stimuli into a distinct conformation, resulting in varied cellular responses. Study III identifies an intracellular modification (GRK 2 inhibition by PX) that can generate a biased system; an IGF-1R conformation that shows a preference for degradation over signalling.

In **Study IV**, we turned our focus to another line of clinical application. Whilst it should not be ruled out that microRNAs (miRNAs) might be useful targets themselves, one realm in which they have stood out is as clinical biomarkers [341, 342, 357, 394]. A growing awareness of the spectrum of activity around the IGF-1R, and the tumour-supportive nature of a β -arr 1 biased signal, presents a need to measure such variations. A biomarker would allow for patient stratification in anti-IGF-1R trials, as well as prognosis or resistance monitoring. Among numerous candidates under investigation, results already in hand suggest that miR-106a represents a viable candidate to identify β -arr 1 biased signalling at the IGF-1R. This candidate miRNA has already been linked to cancer in many studies [358-364], supportive of the role it appears to play following an IGF-1R β -arr 1 biased signal.

Given the importance of the IGF-1R in such a wide spectrum of cancer types and hence genetic mutational backgrounds, it is unlikely that one targeting approach will fit all contexts. Instead, an appreciation of the true complexity, methods to test and measure fluctuations/bias, and an arsenal of targeting agents will likely equip us to deal with different scenarios. Two of the disease models used within, melanoma and Ewing's sarcoma, are at other ends of the spectrum when cancers are ranked by mutation load [317]. Melanoma typically presents with one of the highest mutation loads of all cancer types, and hence the genome is often very unstable. In such circumstances, a multi-hit method such as the IGF-1R and MEK co-targeting in Study I, or the IGF-1R and p53 co-targeting by Nutlin-3 [303], may be required to outsmart network plasticity and hence resistance. In Ewing's sarcoma on the other hand, a sole genetic translocation event drives transformation. Such a scenario presents a disease Achilles' heel – where direct inhibition of the receptor system it relies on may be sufficient. In such a scenario, Study III identifies a strong IGF-1R cross-targeting strategy in GRK 2 inhibition, and suggests clinical feasibility by repurposing the widely used drug paroxetine. Study IV identifies a potential clinical biomarker in miR-106a to test or follow biased signalling at the IGF-1R, allowing patient stratification and therapeutic decision-making. Future studies are needed to shed further light on the intricacies of this system. Unknowns remain around the IGF-1R's exact use of G proteins, and how they fit into the kinase versus β -arr signal arms. It will be interesting to continue mutation analysis to further interrogate the GRK/ β -arr functional partnerships, and their molecular interactions and dynamics at the IGF-1R, such as has been done at GPCRs [333]. With many cancer types relying on this signal axis, either primarily or in progression or resistance mechanisms, the contribution of this work to our understanding of the IGF-1R system is twofold. Firstly, it puts forward an updated framework of IGF-1R function in using GPCR components. Secondly it identifies pitfalls and potential of this framework, providing knowledge and strength to the arsenal of anti-IGF-1R cancer therapeutics.

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6 REFERENCES

1. Gerhart, J., *1998 Warkany lecture: signaling pathways in development*. Teratology, 1999. **60**(4): p. 226-39.
2. de Mendoza, A., A. Sebe-Pedros, and I. Ruiz-Trillo, *The evolution of the GPCR signaling system in eukaryotes: modularity, conservation, and the transition to metazoan multicellularity*. Genome Biol Evol, 2014. **6**(3): p. 606-19.
3. King, N., C.T. Hittinger, and S.B. Carroll, *Evolution of key cell signaling and adhesion protein families predates animal origins*. Science, 2003. **301**(5631): p. 361-3.
4. Mushegian, A., V.V. Gurevich, and E.V. Gurevich, *The origin and evolution of G protein-coupled receptor kinases*. PLoS One, 2012. **7**(3): p. e33806.
5. Duc, N.M., H.R. Kim, and K.Y. Chung, *Structural mechanism of G protein activation by G protein-coupled receptor*. Eur J Pharmacol, 2015. **763**(Pt B): p. 214-22.
6. Kroeze, W.K., D.J. Sheffler, and B.L. Roth, *G-protein-coupled receptors at a glance*. Journal of Cell Science, 2003. **116**(24): p. 4867-4869.
7. Fredriksson, R., et al., *The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints*. Molecular Pharmacology, 2003. **63**(6): p. 1256-1272.
8. Robas, N., et al., *Maximizing serendipity: strategies for identifying ligands for orphan G-protein-coupled receptors*. Current Opinion in Pharmacology, 2003. **3**(2): p. 121-126.
9. Flower, D.R., *Modelling G-protein-coupled receptors for drug design*. Biochimica Et Biophysica Acta-Reviews on Biomembranes, 1999. **1422**(3): p. 207-234.
10. Baltoumas, F.A., M.C. Theodoropoulou, and S.J. Hamodrakas, *Interactions of the alpha-subunits of heterotrimeric G-proteins with GPCRs, effectors and RGS proteins: a critical review and analysis of interacting surfaces, conformational shifts, structural diversity and electrostatic potentials*. J Struct Biol, 2013. **182**(3): p. 209-18.
11. Gilman, A.G., *G-Proteins - Transducers of Receptor-Generated Signals*. Annual Review of Biochemistry, 1987. **56**: p. 615-649.
12. Syrovatkina, V., et al., *Regulation, Signaling, and Physiological Functions of G-Proteins*. J Mol Biol, 2016. **428**(19): p. 3850-68.
13. Oldham, W.M. and H.E. Hamm, *Heterotrimeric G protein activation by G-protein-coupled receptors*. Nat Rev Mol Cell Biol, 2008. **9**(1): p. 60-71.
14. Neubig, R.R., R.D. Gantzoz, and W.J. Thomsen, *Mechanism of agonist and antagonist binding to alpha 2 adrenergic receptors: evidence for a precoupled receptor-guanine nucleotide protein complex*. Biochemistry, 1988. **27**(7): p. 2374-84.
15. Strange, P.G., *Signaling mechanisms of GPCR ligands*. Curr Opin Drug Discov Devel, 2008. **11**(2): p. 196-202.
16. Lefkowitz, R.J., *Historical review: a brief history and personal retrospective of seven-transmembrane receptors*. Trends Pharmacol Sci, 2004. **25**(8): p. 413-22.

17. Cabrera-Vera, T.M., et al., *Insights into G protein structure, function, and regulation*. *Endocr Rev*, 2003. **24**(6): p. 765-81.
18. Oldham, W.M. and H.E. Hamm, *Structural basis of function in heterotrimeric G proteins*. *Q Rev Biophys*, 2006. **39**(2): p. 117-66.
19. Reiter, E. and R.J. Lefkowitz, *GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling*. *Trends Endocrinol Metab*, 2006. **17**(4): p. 159-65.
20. Pitcher, J.A., N.J. Freedman, and R.J. Lefkowitz, *G protein-coupled receptor kinases*. *Annu Rev Biochem*, 1998. **67**: p. 653-92.
21. Kuhn, H. and W.J. Dreyer, *Light dependent phosphorylation of rhodopsin by ATP*. *FEBS Lett*, 1972. **20**(1): p. 1-6.
22. Bownds, D., et al., *Phosphorylation of frog photoreceptor membranes induced by light*. *Nat New Biol*, 1972. **237**(73): p. 125-7.
23. Weller, M., N. Virmaux, and P. Mandel, *Light-stimulated phosphorylation of rhodopsin in the retina: the presence of a protein kinase that is specific for photobleached rhodopsin*. *Proc Natl Acad Sci U S A*, 1975. **72**(1): p. 381-5.
24. Stadel, J.M., et al., *Catecholamine-Induced Desensitization of Turkey Erythrocyte Adenylate-Cyclase - Structural Alterations in the Beta-Adrenergic-Receptor Revealed by Photoaffinity-Labeling*. *Journal of Biological Chemistry*, 1982. **257**(16): p. 9242-9245.
25. Stadel, J.M., et al., *Catecholamine-Induced Desensitization of Turkey Erythrocyte Adenylate-Cyclase Is Associated with Phosphorylation of the Beta-Adrenergic-Receptor*. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 1983. **80**(11): p. 3173-3177.
26. Liebman, P.A. and E.N. Pugh, Jr., *ATP mediates rapid reversal of cyclic GMP phosphodiesterase activation in visual receptor membranes*. *Nature*, 1980. **287**(5784): p. 734-6.
27. Sibley, D.R., et al., *Homologous Desensitization of Adenylate-Cyclase Is Associated with Phosphorylation of the Beta-Adrenergic-Receptor*. *Journal of Biological Chemistry*, 1985. **260**(7): p. 3883-3886.
28. Carman, C.V. and J.L. Benovic, *G-protein-coupled receptors: turn-ons and turn-offs*. *Current Opinion in Neurobiology*, 1998. **8**(3): p. 335-344.
29. Watari, K., M. Nakaya, and H. Kurose, *Multiple functions of G protein-coupled receptor kinases*. *J Mol Signal*, 2014. **9**(1): p. 1.
30. Premont, R.T. and R.R. Gainetdinov, *Physiological roles of G protein-coupled receptor kinases and arrestins*. *Annu Rev Physiol*, 2007. **69**: p. 511-34.
31. Matkovich, S.J., et al., *Cardiac-specific ablation of GRK2 re-defines its roles in heart development and beta-adrenergic signaling*. *Circulation*, 2006. **114**(18): p. 159-159.
32. Jaber, M., et al., *Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function*. *Proc Natl Acad Sci U S A*, 1996. **93**(23): p. 12974-9.
33. Rockman, H.A., et al., *Control of myocardial contractile function by the level of beta-adrenergic receptor kinase 1 in gene-targeted mice*. *J Biol Chem*, 1998. **273**(29): p. 18180-4.

34. Gainetdinov, R.R., et al., *Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice*. *Neuron*, 2003. **38**(2): p. 291-303.
35. Nakaya, M., et al., *GRK6 deficiency in mice causes autoimmune disease due to impaired apoptotic cell clearance*. *Nature Communications*, 2013. **4**.
36. Peppel, K., et al., *G protein-coupled receptor kinase 3 (GRK3) gene disruption leads to loss of odorant receptor desensitization*. *J Biol Chem*, 1997. **272**(41): p. 25425-8.
37. Walker, J.K., et al., *Altered airway and cardiac responses in mice lacking G protein-coupled receptor kinase 3*. *Am J Physiol*, 1999. **276**(4 Pt 2): p. R1214-21.
38. Xu, M., et al., *Neuropathic pain activates the endogenous kappa opioid system in mouse spinal cord and induces opioid receptor tolerance*. *J Neurosci*, 2004. **24**(19): p. 4576-84.
39. Nobles, K.N., et al., *Distinct phosphorylation sites on the beta(2)-adrenergic receptor establish a barcode that encodes differential functions of beta-arrestin*. *Sci Signal*, 2011. **4**(185): p. ra51.
40. Lefkowitz, R.J., *Arrestins come of age: a personal historical perspective*. *Prog Mol Biol Transl Sci*, 2013. **118**: p. 3-18.
41. Butcher, A.J., et al., *Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code*. *J Biol Chem*, 2011. **286**(13): p. 11506-18.
42. Gurevich, E.V., et al., *G protein-coupled receptor kinases: more than just kinases and not only for GPCRs*. *Pharmacol Ther*, 2012. **133**(1): p. 40-69.
43. Day, P.W., P.B. Wedegaertner, and J.L. Benovic, *Analysis of G-protein-coupled receptor kinase RGS homology domains*. *Methods Enzymol*, 2004. **390**: p. 295-310.
44. Sterne-Marr, R., et al., *G protein-coupled receptor Kinase 2/G alpha q/11 interaction. A novel surface on a regulator of G protein signaling homology domain for binding G alpha subunits*. *J Biol Chem*, 2003. **278**(8): p. 6050-8.
45. Penela, P., et al., *The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets*. *British Journal of Pharmacology*, 2010. **160**(4): p. 821-832.
46. Hullmann, J., et al., *The expanding GRK interactome: Implications in cardiovascular disease and potential for therapeutic development*. *Pharmacol Res*, 2016. **110**: p. 52-64.
47. Nogues, L., et al., *G protein-coupled receptor kinases (GRKs) in tumorigenesis and cancer progression: GPCR regulators and signaling hubs*. *Semin Cancer Biol*, 2017.
48. Wilden, U., S.W. Hall, and H. Kuhn, *Phosphodiesterase Activation by Photoexcited Rhodopsin Is Quenched When Rhodopsin Is Phosphorylated and Binds the Intrinsic 48-Kda Protein of Rod Outer Segments*. *Proceedings of the National Academy of Sciences of the United States of America*, 1986. **83**(5): p. 1174-1178.
49. Wilden, U., et al., *Rapid affinity purification of retinal arrestin (48 kDa protein) via its light-dependent binding to phosphorylated rhodopsin*. *FEBS Lett*, 1986. **207**(2): p. 292-5.
50. Benovic, J.L., et al., *Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the*

- retinal protein arrestin (48-kDa protein)*. Proc Natl Acad Sci U S A, 1987. **84**(24): p. 8879-82.
51. Gurevich, V.V. and E.V. Gurevich, *The structural basis of arrestin-mediated regulation of G-protein-coupled receptors*. Pharmacol Ther, 2006. **110**(3): p. 465-502.
 52. Gurevich, V.V. and E.V. Gurevich, *The molecular acrobatics of arrestin activation*. Trends Pharmacol Sci, 2004. **25**(2): p. 105-11.
 53. Gurevich, V.V. and E.V. Gurevich, *Structural determinants of arrestin functions*. Prog Mol Biol Transl Sci, 2013. **118**: p. 57-92.
 54. Kohout, T.A. and R.J. Lefkowitz, *Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization*. Mol Pharmacol, 2003. **63**(1): p. 9-18.
 55. Hirsch, J.A., et al., *The 2.8 Å crystal structure of visual arrestin: a model for arrestin's regulation*. Cell, 1999. **97**(2): p. 257-69.
 56. Vishnivetskiy, S.A., et al., *Mapping the arrestin-receptor interface. Structural elements responsible for receptor specificity of arrestin proteins*. J Biol Chem, 2004. **279**(2): p. 1262-8.
 57. Oakley, R.H., et al., *Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors*. Journal of Biological Chemistry, 2000. **275**(22): p. 17201-17210.
 58. Conner, D.A., et al., *beta-Arrestin1 knockout mice appear normal but demonstrate altered cardiac responses to beta-adrenergic stimulation*. Circ Res, 1997. **81**(6): p. 1021-6.
 59. Bohn, L.M., et al., *Enhanced morphine analgesia in mice lacking beta-arrestin 2*. Science, 1999. **286**(5449): p. 2495-8.
 60. Bohn, L.M., R.J. Lefkowitz, and M.G. Caron, *Differential mechanisms of morphine antinociceptive tolerance revealed in beta arrestin-2 knock-out mice*. Journal of Neuroscience, 2002. **22**(23): p. 10494-10500.
 61. Bohn, L.M., et al., *Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice*. J Neurosci, 2003. **23**(32): p. 10265-73.
 62. Beaulieu, J.M., et al., *An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior*. Cell, 2005. **122**(2): p. 261-73.
 63. Fong, A.M., et al., *Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice*. Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7478-83.
 64. Su, Y., et al., *Altered CXCR2 signaling in beta-arrestin-2-deficient mouse models*. J Immunol, 2005. **175**(8): p. 5396-402.
 65. Bouxsein, M.L., et al., *beta-Arrestin2 regulates the differential response of cortical and trabecular bone to intermittent PTH in female mice*. J Bone Miner Res, 2005. **20**(4): p. 635-43.
 66. Ferrari, S.L., et al., *Bone response to intermittent parathyroid hormone is altered in mice null for beta-arrestin2*. Endocrinology, 2005. **146**(4): p. 1854-1862.
 67. Lefkowitz, R.J. and E.J. Whalen, *beta-arrestins: traffic cops of cell signaling*. Current Opinion in Cell Biology, 2004. **16**(2): p. 162-168.

68. Shenoy, S.K. and R.J. Lefkowitz, *beta-Arrestin-mediated receptor trafficking and signal transduction*. Trends Pharmacol Sci, 2011. **32**(9): p. 521-33.
69. Vonzastrow, M. and B.K. Kobilka, *Ligand-Regulated Internalization and Recycling of Human Beta-2-Adrenergic Receptors between the Plasma-Membrane and Endosomes Containing Transferrin Receptors*. Journal of Biological Chemistry, 1992. **267**(5): p. 3530-3538.
70. Pitcher, J.A., et al., *The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity*. Proc Natl Acad Sci U S A, 1995. **92**(18): p. 8343-7.
71. Ferguson, S.S., et al., *Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization*. Science, 1996. **271**(5247): p. 363-6.
72. Gurevich, V.V. and E.V. Gurevich, *Arrestins: Critical Players in Trafficking of Many GPCRs*. Prog Mol Biol Transl Sci, 2015. **132**: p. 1-14.
73. Gao, H., et al., *Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways*. Mol Cell, 2004. **14**(3): p. 303-17.
74. Shenoy, S.K., et al., *beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor*. J Biol Chem, 2006. **281**(2): p. 1261-73.
75. Luttrell, L.M., et al., *Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes*. Science, 1999. **283**(5402): p. 655-61.
76. Ahn, S., et al., *{beta}-Arrestin-2 Mediates Anti-apoptotic Signaling through Regulation of BAD Phosphorylation*. J Biol Chem, 2009. **284**(13): p. 8855-65.
77. Kendall, R.T., et al., *Arrestin-dependent angiotensin AT1 receptor signaling regulates Akt and mTor-mediated protein synthesis*. J Biol Chem, 2014. **289**(38): p. 26155-66.
78. Coffa, S., et al., *A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding*. Biochemistry, 2011. **50**(32): p. 6951-8.
79. Thomsen, A.R., et al., *GPCR-G Protein-beta-Arrestin Super-Complex Mediates Sustained G Protein Signaling*. Cell, 2016. **166**(4): p. 907-19.
80. Zidar, D.A., et al., *Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands*. Proc Natl Acad Sci U S A, 2009. **106**(24): p. 9649-54.
81. Lefkowitz, R.J., et al., *Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins*. Trends Pharmacol Sci, 1993. **14**(8): p. 303-7.
82. Kenakin, T., *Pharmacological proteus?* Trends Pharmacol Sci, 1995. **16**(8): p. 256-8.
83. Costa, T. and A. Herz, *Antagonists with Negative Intrinsic Activity at Delta-Opioid Receptors Coupled to Gtp-Binding Proteins*. Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(19): p. 7321-7325.
84. Samama, P., et al., *A Mutation-Induced Activated State of the Beta(2)-Adrenergic Receptor - Extending the Ternary Complex Model*. Journal of Biological Chemistry, 1993. **268**(7): p. 4625-4636.
85. Reiter, E., et al., *Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors*. Annu Rev Pharmacol Toxicol, 2012. **52**: p. 179-97.

86. Karlin, A., *On the application of "a plausible model" of allosteric proteins to the receptor for acetylcholine.* J Theor Biol, 1967. **16**(2): p. 306-20.
87. De Lean, A., J.M. Stadel, and R.J. Lefkowitz, *A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor.* J Biol Chem, 1980. **255**(15): p. 7108-17.
88. Samama, P., et al., *A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model.* J Biol Chem, 1993. **268**(7): p. 4625-36.
89. Maudsley, S., B. Martin, and L.M. Luttrell, *The origins of diversity and specificity in G protein-coupled receptor signaling.* J Pharmacol Exp Ther, 2005. **314**(2): p. 485-94.
90. Berg, K.A., et al., *Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus.* Mol Pharmacol, 1998. **54**(1): p. 94-104.
91. Spengler, D., et al., *Differential signal transduction by five splice variants of the PACAP receptor.* Nature, 1993. **365**(6442): p. 170-5.
92. Meller, E., et al., *Comparative effects of receptor inactivation, 17 beta-estradiol and pertussis toxin on dopaminergic inhibition of prolactin secretion in vitro.* J Pharmacol Exp Ther, 1992. **263**(2): p. 462-9.
93. Sagan, S., et al., *Further delineation of the two binding sites (R*(n)) associated with tachykinin neurokinin-1 receptors using [3-Prolinomethionine(11)]SP analogues.* J Biol Chem, 1999. **274**(34): p. 23770-6.
94. Kenakin, T., *Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity.* Trends Pharmacol Sci, 1995. **16**(6): p. 188-92.
95. Kenakin, T., *Agonist-receptor efficacy. II. Agonist trafficking of receptor signals.* Trends Pharmacol Sci, 1995. **16**(7): p. 232-8.
96. Violin, J.D. and R.J. Lefkowitz, *Beta-arrestin-biased ligands at seven-transmembrane receptors.* Trends Pharmacol Sci, 2007. **28**(8): p. 416-22.
97. Wei, H., et al., *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, 2003. **100**(19): p. 10782-7.
98. Whalen, E.J., S. Rajagopal, and R.J. Lefkowitz, *Therapeutic potential of beta-arrestin- and G protein-biased agonists.* Trends Mol Med, 2011. **17**(3): p. 126-39.
99. Violin, J.D., et al., *Biased ligands at G-protein-coupled receptors: promise and progress.* Trends Pharmacol Sci, 2014. **35**(7): p. 308-16.
100. King, N., et al., *The genome of the choanoflagellate Monosiga brevicollis and the origin of metazoans.* Nature, 2008. **451**(7180): p. 783-8.
101. Hunter, T., *The genesis of tyrosine phosphorylation.* Cold Spring Harb Perspect Biol, 2014. **6**(5): p. a020644.
102. Suga, H., et al., *Genomic Survey of Premetazoans Shows Deep Conservation of Cytoplasmic Tyrosine Kinases and Multiple Radiations of Receptor Tyrosine Kinases.* Science Signaling, 2012. **5**(222).

103. Manning, G., et al., *The protist, Monosiga brevicollis, has a tyrosine kinase signaling network more elaborate and diverse than found in any known metazoan*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(28): p. 9674-9679.
104. Lemmon, M.A. and J. Schlessinger, *Cell Signaling by Receptor Tyrosine Kinases*. Cell, 2010. **141**(7): p. 1117-1134.
105. Ullrich, A. and J. Schlessinger, *Signal transduction by receptors with tyrosine kinase activity*. Cell, 1990. **61**(2): p. 203-12.
106. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. Cell, 2000. **100**(1): p. 57-70.
107. Weinstein, I.B., *Cancer. Addiction to oncogenes--the Achilles heel of cancer*. Science, 2002. **297**(5578): p. 63-4.
108. Waterfield, M.D., et al., *Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus*. Nature, 1983. **304**(5921): p. 35-9.
109. Doolittle, R.F., et al., *Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor*. Science, 1983. **221**(4607): p. 275-7.
110. Crudden, C., A. Girnita, and L. Girnita, *Targeting the IGF-1R: The Tale of the Tortoise and the Hare*. Front Endocrinol (Lausanne), 2015. **6**: p. 64.
111. Baselga, J., *Targeting tyrosine kinases in cancer: the second wave*. Science, 2006. **312**(5777): p. 1175-8.
112. Romond, E.H., et al., *Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer*. N Engl J Med, 2005. **353**(16): p. 1673-84.
113. Piccart-Gebhart, M.J., et al., *Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer*. N Engl J Med, 2005. **353**(16): p. 1659-72.
114. An, X., et al., *BCR-ABL tyrosine kinase inhibitors in the treatment of Philadelphia chromosome positive chronic myeloid leukemia: a review*. Leuk Res, 2010. **34**(10): p. 1255-68.
115. Heinrich, M.C., et al., *Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor*. J Clin Oncol, 2003. **21**(23): p. 4342-9.
116. Sell, C., et al., *Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts*. Mol Cell Biol, 1994. **14**(6): p. 3604-12.
117. Reiss, K., et al., *The insulin-like growth factor I receptor is required for the proliferation of hemopoietic cells*. Oncogene, 1992. **7**(11): p. 2243-8.
118. Sell, C., et al., *Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor*. Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11217-21.
119. Wood, A.W., C. Duan, and H.A. Bern, *Insulin-like growth factor signaling in fish*. Int Rev Cytol, 2005. **243**: p. 215-85.

120. Skorokhod, A., et al., *Origin of insulin receptor-like tyrosine kinases in marine sponges*. Biol Bull, 1999. **197**(2): p. 198-206.
121. Savage, M.O., *Insulin-like growth factors, nutrition and growth*. World Rev Nutr Diet, 2013. **106**: p. 52-9.
122. Giovannucci, E., *Nutrition, insulin, insulin-like growth factors and cancer*. Horm Metab Res, 2003. **35**(11-12): p. 694-704.
123. Ullrich, A., et al., *Insulin-Like Growth Factor-I Receptor Primary Structure - Comparison with Insulin-Receptor Suggests Structural Determinants That Define Functional Specificity*. Embo Journal, 1986. **5**(10): p. 2503-2512.
124. Werner, H., D. Weinstein, and I. Bentov, *Similarities and differences between insulin and IGF-I: structures, receptors, and signalling pathways*. Arch Physiol Biochem, 2008. **114**(1): p. 17-22.
125. Liu, J.P., et al., *Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type I IGF receptor (Igf1r)*. Cell, 1993. **75**(1): p. 59-72.
126. Holzenberger, M., et al., *A targeted partial invalidation of the insulin-like growth factor I receptor gene in mice causes a postnatal growth deficit*. Endocrinology, 2000. **141**(7): p. 2557-66.
127. Bach, L.A., S.J. Headey, and R.S. Norton, *IGF-binding proteins - the pieces are failing into place*. Trends in Endocrinology and Metabolism, 2005. **16**(5): p. 228-234.
128. Salmon, W.D., Jr. and W.H. Daughaday, *A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro*. J Lab Clin Med, 1957. **49**(6): p. 825-36.
129. Baker, J., et al., *Role of insulin-like growth factors in embryonic and postnatal growth*. Cell, 1993. **75**(1): p. 73-82.
130. Petrenko, A.G., et al., *Insulin receptor-related receptor as an extracellular pH sensor involved in the regulation of acid-base balance*. Biochimica Et Biophysica Acta-Proteins and Proteomics, 2013. **1834**(10): p. 2170-2175.
131. Bailyes, E.M., et al., *Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting*. Biochemical Journal, 1997. **327**: p. 209-215.
132. Belfiore, A., et al., *Insulin Receptor Isoforms and Insulin Receptor/Insulin-Like Growth Factor Receptor Hybrids in Physiology and Disease*. Endocrine Reviews, 2009. **30**(6): p. 586-623.
133. Favellyukis, S., et al., *Structure and autoregulation of the insulin-like growth factor I receptor kinase*. Nature Structural Biology, 2001. **8**(12): p. 1058-1063.
134. Girnita, L., et al., *Something old, something new and something borrowed: emerging paradigm of insulin-like growth factor type I receptor (IGF-1R) signaling regulation*. Cell Mol Life Sci, 2014. **71**(13): p. 2403-27.
135. Craparo, A., T.J. Oneill, and T.A. Gustafson, *Non-Sh2 Domains within Insulin-Receptor Substrate-1 and Shc Mediate Their Phosphotyrosine-Dependent Interaction with the Npety Motif of the Insulin-Like Growth-Factor-I Receptor*. Journal of Biological Chemistry, 1995. **270**(26): p. 15639-15643.

136. Fukushima, T., et al., *Phosphatidylinositol 3-Kinase (PI3K) Activity Bound to Insulin-like Growth Factor-I (IGF-I) Receptor, which Is Continuously Sustained by IGF-I Stimulation, Is Required for IGF-I-induced Cell Proliferation*. Journal of Biological Chemistry, 2012. **287**(35): p. 29713-29721.
137. Skolnik, E.Y., et al., *The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling*. EMBO J, 1993. **12**(5): p. 1929-36.
138. Dupont, J., et al., *The insulin-like growth factor axis in cell cycle progression*. Horm Metab Res, 2003. **35**(11-12): p. 740-50.
139. Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells*. Cell Res, 2002. **12**(1): p. 9-18.
140. Roskoski, R., Jr., *ERK1/2 MAP kinases: structure, function, and regulation*. Pharmacol Res, 2012. **66**(2): p. 105-43.
141. Peeper, D.S., et al., *Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein (vol. 386, pg 177, 1997)*. Nature, 1997. **386**(6624): p. 521-521.
142. Bates, S., et al., *p14ARF links the tumour suppressors RB and p53*. Nature, 1998. **395**(6698): p. 124-5.
143. Sherr, C.J. and J.D. Weber, *The ARF/p53 pathway*. Current Opinion in Genetics & Development, 2000. **10**(1): p. 94-99.
144. Plotnikov, A., et al., *The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation*. Biochim Biophys Acta, 2011. **1813**(9): p. 1619-33.
145. Walsh, S., S.S. Margolis, and S. Kornbluth, *Phosphorylation of the cyclin b1 cytoplasmic retention sequence by mitogen-activated protein kinase and Plx*. Mol Cancer Res, 2003. **1**(4): p. 280-9.
146. Strausfeld, U., et al., *Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein*. Nature, 1991. **351**(6323): p. 242-5.
147. Shepherd, P.R., D.J. Withers, and K. Siddle, *Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling*. Biochem J, 1998. **333** (Pt 3): p. 471-90.
148. Alessi, D.R., et al., *Mechanism of activation of protein kinase B by insulin and IGF-I*. EMBO J, 1996. **15**(23): p. 6541-51.
149. delPeso, L., et al., *Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt*. Science, 1997. **278**(5338): p. 687-689.
150. Cardone, M.H., et al., *Regulation of cell death protease caspase-9 by phosphorylation*. Science, 1998. **282**(5392): p. 1318-21.
151. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream*. Cell, 2007. **129**(7): p. 1261-74.
152. Tee, A.R. and J. Blenis, *mTOR, translational control and human disease*. Seminars in Cell & Developmental Biology, 2005. **16**(1): p. 29-37.

153. Zhang, D., et al., *Dual regulation of MMP-2 expression by the type 1 insulin-like growth factor receptor: the phosphatidylinositol 3-kinase/Akt and Raf/ERK pathways transmit opposing signals.* J Biol Chem, 2004. **279**(19): p. 19683-90.
154. Mayo, L.D. and D.B. Donner, *A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11598-603.
155. Blume-Jensen, P. and T. Hunter, *Oncogenic kinase signalling.* Nature, 2001. **411**(6835): p. 355-365.
156. Manning, B.D. and A. Toker, *AKT/PKB Signaling: Navigating the Network.* Cell, 2017. **169**(3): p. 381-405.
157. Morrione, A., et al., *Grb10: A new substrate of the insulin-like growth factor I receptor.* Cancer Res, 1996. **56**(14): p. 3165-7.
158. Wang, J., et al., *Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis.* Mol Cell Biol, 1999. **19**(9): p. 6217-28.
159. Beitner-Johnson, D. and D. LeRoith, *Insulin-like growth factor-I stimulates tyrosine phosphorylation of endogenous c-Crk.* J Biol Chem, 1995. **270**(10): p. 5187-90.
160. Hermanto, U., et al., *RACK1, an insulin-like growth factor I (IGF-I) receptor-interacting protein, modulates IGF-I-dependent integrin signaling and promotes cell spreading and contact with extracellular matrix.* Mol Cell Biol, 2002. **22**(7): p. 2345-65.
161. Baron, V., et al., *p125Fak focal adhesion kinase is a substrate for the insulin and insulin-like growth factor-I tyrosine kinase receptors.* J Biol Chem, 1998. **273**(12): p. 7162-8.
162. Sepp-Lorenzino, L., *Structure and function of the insulin-like growth factor I receptor.* Breast Cancer Res Treat, 1998. **47**(3): p. 235-53.
163. Seely, B.L., et al., *Localization of the insulin-like growth factor I receptor binding sites for the SH2 domain proteins p85, Syp, and GTPase activating protein.* J Biol Chem, 1995. **270**(32): p. 19151-7.
164. Dey, B.R., et al., *Interaction of human suppressor of cytokine signaling (SOCS)-2 with the insulin-like growth factor-I receptor.* J Biol Chem, 1998. **273**(37): p. 24095-101.
165. Bar-Sagi, D. and A. Hall, *Ras and Rho GTPases: a family reunion.* Cell, 2000. **103**(2): p. 227-38.
166. Der, C.J., T.G. Krontiris, and G.M. Cooper, *Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses.* Proc Natl Acad Sci U S A, 1982. **79**(11): p. 3637-40.
167. Ledford, H., *Cancer: The Ras renaissance.* Nature, 2015. **520**(7547): p. 278-80.
168. Tuveson, D.A., et al., *Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects.* Cancer Cell, 2004. **5**(4): p. 375-87.
169. Guerra, C., et al., *Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context.* Cancer Cell, 2003. **4**(2): p. 111-20.

170. O'Dell, M.R., et al., *Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma*. *Cancer Res*, 2012. **72**(6): p. 1557-67.
171. Jackson, E.L., et al., *The differential effects of mutant p53 alleles on advanced murine lung cancer*. *Cancer Res*, 2005. **65**(22): p. 10280-8.
172. Hill, R., et al., *PTEN loss accelerates KrasG12D-induced pancreatic cancer development*. *Cancer Res*, 2010. **70**(18): p. 7114-24.
173. Pacheco-Pinedo, E.C., et al., *Wnt/beta-catenin signaling accelerates mouse lung tumorigenesis by imposing an embryonic distal progenitor phenotype on lung epithelium*. *J Clin Invest*, 2011. **121**(5): p. 1935-45.
174. Bardeesy, N., et al., *Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse*. *Proc Natl Acad Sci U S A*, 2006. **103**(15): p. 5947-52.
175. Aoki, Y. and Y. Matsubara, *Ras/MAPK syndromes and childhood hematological diseases*. *Int J Hematol*, 2013. **97**(1): p. 30-6.
176. Aoki, Y., et al., *The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders*. *Hum Mutat*, 2008. **29**(8): p. 992-1006.
177. Tidyman, W.E. and K.A. Rauen, *The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation*. *Curr Opin Genet Dev*, 2009. **19**(3): p. 230-6.
178. Maehama, T. and J.E. Dixon, *PTEN: a tumour suppressor that functions as a phospholipid phosphatase*. *Trends in Cell Biology*, 1999. **9**(4): p. 125-128.
179. Paez, J. and W.R. Sellers, *PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling*. *Cancer Treat Res*, 2003. **115**: p. 145-67.
180. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate*. *J Biol Chem*, 1998. **273**(22): p. 13375-8.
181. Salmena, L., A. Carracedo, and P.P. Pandolfi, *Tenets of PTEN tumor suppression*. *Cell*, 2008. **133**(3): p. 403-14.
182. Ali, I.U., L.M. Schriml, and M. Dean, *Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity*. *J Natl Cancer Inst*, 1999. **91**(22): p. 1922-32.
183. Saal, L.H., et al., *Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair*. *Nat Genet*, 2008. **40**(1): p. 102-7.
184. Hobert, J.A. and C. Eng, *PTEN hamartoma tumor syndrome: an overview*. *Genet Med*, 2009. **11**(10): p. 687-94.
185. Di Cristofano, A., et al., *Pten is essential for embryonic development and tumour suppression*. *Nature Genetics*, 1998. **19**(4): p. 348-355.
186. Podsypanina, K., et al., *Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems*. *Proceedings of the National Academy of Sciences of the United States of America*, 1999. **96**(4): p. 1563-1568.
187. Suzuki, A., et al., *High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice*. *Current Biology*, 1998. **8**(21): p. 1169-1178.

188. Trotman, L.C., et al., *Pten dose dictates cancer progression in the prostate*. Plos Biology, 2003. **1**(3): p. 385-396.
189. Pickart, C.M., *Mechanisms underlying ubiquitination*. Annual Review of Biochemistry, 2001. **70**: p. 503-533.
190. Zhang, Y.P., et al., *NEDD4 ubiquitin ligase is a putative oncogene in endometrial cancer that activates IGF-1R/PI3K/Akt signaling*. Gynecologic Oncology, 2015. **139**(1): p. 127-133.
191. Sehat, B., et al., *Identification of c-Cbl as a new ligase for insulin-like growth factor-1 receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis*. Cancer Res, 2008. **68**(14): p. 5669-77.
192. Nakao, R., et al., *Ubiquitin Ligase Cbl-b Is a Negative Regulator for Insulin-Like Growth Factor 1 Signaling during Muscle Atrophy Caused by Unloading*. Molecular and Cellular Biology, 2009. **29**(17): p. 4798-4811.
193. Girnita, L., A. Girnita, and O. Larsson, *Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor*. Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8247-52.
194. Xu, Y.M., et al., *HRD1 suppresses the growth and metastasis of breast cancer cells by promoting IGF-1R degradation*. Oncotarget, 2015. **6**(40): p. 42854-67.
195. Girnita, L., et al., *Chapter Seven - When Phosphorylation Encounters Ubiquitination: A Balanced Perspective on IGF-1R Signaling*. Prog Mol Biol Transl Sci, 2016. **141**: p. 277-311.
196. Safaei, R., et al., *Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells*. Mol Cancer Ther, 2005. **4**(10): p. 1595-604.
197. Johnson, I.R., et al., *Altered endosome biogenesis in prostate cancer has biomarker potential*. Mol Cancer Res, 2014. **12**(12): p. 1851-62.
198. Dozynkiewicz, M.A., et al., *Rab25 and CLIC3 collaborate to promote integrin recycling from late endosomes/lysosomes and drive cancer progression*. Dev Cell, 2012. **22**(1): p. 131-45.
199. Kern, U., et al., *Lysosomal protein turnover contributes to the acquisition of TGFbeta-1 induced invasive properties of mammary cancer cells*. Mol Cancer, 2015. **14**: p. 39.
200. Werner, H. and R. Sarfstein, *Transcriptional and epigenetic control of IGF1R gene expression: implications in metabolism and cancer*. Growth Horm IGF Res, 2014. **24**(4): p. 112-8.
201. Yu, H. and T. Rohan, *Role of the insulin-like growth factor family in cancer development and progression*. J Natl Cancer Inst, 2000. **92**(18): p. 1472-89.
202. Coppola, D., et al., *A functional insulin-like growth factor 1 receptor is required for the mitogenic and transforming activities of the epidermal growth factor receptor*. Mol Cell Biol, 1994. **14**(7): p. 4588-95.
203. Baserga, R., F. Peruzzi, and K. Reiss, *The IGF-1 receptor in cancer biology*. Int J Cancer, 2003. **107**(6): p. 873-7.

204. Girnita, A., et al., *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, 2006. **12**(4): p. 1383-91.
205. Janecka, A., M. Kolodziej-Rzepa, and B. Biesaga, *Clinical and Molecular Features of Laron Syndrome, A Genetic Disorder Protecting from Cancer*. In Vivo, 2016. **30**(4): p. 375-81.
206. Laron, Z., *Lessons from 50 Years of Study of Laron Syndrome*. Endocr Pract, 2015. **21**(12): p. 1395-402.
207. Lapkina-Gendler, L., et al., *Identification of signaling pathways associated with cancer protection in Laron syndrome*. Endocr Relat Cancer, 2016. **23**(5): p. 399-410.
208. Guevara-Aguirre, J. and A.L. Rosenbloom, *Obesity, diabetes and cancer: insight into the relationship from a cohort with growth hormone receptor deficiency*. Diabetologia, 2015. **58**(1): p. 37-42.
209. Tannenbaum, A. and H. Silverstone, *The Influence of the Degree of Caloric Restriction on the Formation of Skin Tumors and Hepatomas in Mice*. Cancer Research, 1949. **9**(12): p. 724-727.
210. Thompson, H.J., Z. Zhu, and W. Jiang, *Dietary energy restriction in breast cancer prevention*. J Mammary Gland Biol Neoplasia, 2003. **8**(1): p. 133-42.
211. Cheney, K.E., et al., *The effect of dietary restriction of varying duration on survival, tumor patterns, immune function, and body temperature in B10C3F1 female mice*. J Gerontol, 1983. **38**(4): p. 420-30.
212. Dean, W., *The Retardation of Aging and Diseases of Aging by Dietary Restriction - Weindruch, R, Walford, Rl*. Journal of the American Geriatrics Society, 1990. **38**(6): p. 736-736.
213. Shimokawa, I., et al., *Influence of dietary components on occurrence of and mortality due to neoplasms in male F344 rats*. Aging-Clinical and Experimental Research, 1996. **8**(4): p. 254-262.
214. Tomas, F.M., et al., *Effects of insulin and insulin-like growth factors on protein and energy metabolism in tumour-bearing rats*. Biochem J, 1994. **301** (Pt 3): p. 769-75.
215. Hursting, S.D., et al., *The growth hormone: insulin-like growth factor I axis is a mediator of diet restriction-induced inhibition of mononuclear cell leukemia in Fischer rats*. Cancer Res, 1993. **53**(12): p. 2750-7.
216. Dunn, S.E., et al., *Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice*. Cancer Research, 1997. **57**(21): p. 4667-4672.
217. van Heemst, D., *Insulin, IGF-1 and longevity*. Aging Dis, 2010. **1**(2): p. 147-57.
218. Janssen, J.A. and S.W. Lamberts, *Igf-I and longevity*. Horm Res, 2004. **62 Suppl 3**: p. 104-9.
219. Vitale, G., et al., *Low circulating IGF-I bioactivity is associated with human longevity: findings in centenarians' offspring*. Aging (Albany NY), 2012. **4**(9): p. 580-9.
220. Milman, S., et al., *Low insulin-like growth factor-1 level predicts survival in humans with exceptional longevity*. Aging Cell, 2014. **13**(4): p. 769-71.

221. Merrill, M.J. and N.A. Edwards, *Insulin-like growth factor-I receptors in human glial tumors*. J Clin Endocrinol Metab, 1990. **71**(1): p. 199-209.
222. Gammeltoft, S., et al., *Expression of two types of receptor for insulin-like growth factors in human malignant glioma*. Cancer Res, 1988. **48**(5): p. 1233-7.
223. Guo, Y.S., et al., *Characterization of insulinlike growth factor I receptors in human colon cancer*. Gastroenterology, 1992. **102**(4 Pt 1): p. 1101-8.
224. Scotlandi, K., et al., *Expression of insulin-like growth factor system components in Ewing's sarcoma and their association with survival*. Eur J Cancer, 2011. **47**(8): p. 1258-66.
225. Cao, L., et al., *Addiction to elevated insulin-like growth factor I receptor and initial modulation of the AKT pathway define the responsiveness of rhabdomyosarcoma to the targeting antibody*. Cancer Res, 2008. **68**(19): p. 8039-48.
226. Toretsky, J.A., et al., *The insulin-like growth factor-I receptor is required for EWS/FLI-1 transformation of fibroblasts*. J Biol Chem, 1997. **272**(49): p. 30822-7.
227. Wang, W., et al., *Insulin-like growth factor II and PAX3-FKHR cooperate in the oncogenesis of rhabdomyosarcoma*. Cancer Res, 1998. **58**(19): p. 4426-33.
228. Morrison, K.B., et al., *ETV6-NTRK3 transformation requires insulin-like growth factor I receptor signaling and is associated with constitutive IRS-1 tyrosine phosphorylation*. Oncogene, 2002. **21**(37): p. 5684-5695.
229. Rodeck, U., et al., *Metastatic but not primary melanoma cell lines grow in vitro independently of exogenous growth factors*. Int J Cancer, 1987. **40**(5): p. 687-90.
230. Culouscou, J.M., et al., *Simultaneous production of IGF-I and EGF competing growth factors by HT-29 human colon cancer line*. Int J Cancer, 1987. **40**(5): p. 646-52.
231. Ohmura, E., et al., *Insulin-like growth factor I and transforming growth factor alpha as autocrine growth factors in human pancreatic cancer cell growth*. Cancer Res, 1990. **50**(1): p. 103-7.
232. Yee, D., et al., *Expression of insulin-like growth factor I, its binding proteins, and its receptor in ovarian cancer*. Cancer Res, 1991. **51**(19): p. 5107-12.
233. Lahm, H., et al., *Blockade of the insulin-like growth-factor-I receptor inhibits growth of human colorectal cancer cells: evidence of a functional IGF-II-mediated autocrine loop*. Int J Cancer, 1994. **58**(3): p. 452-9.
234. Durrant, L.G., et al., *Co-stimulation of gastrointestinal tumour cell growth by gastrin, transforming growth factor alpha and insulin like growth factor-I*. Br J Cancer, 1991. **63**(1): p. 67-70.
235. Bergmann, U., et al., *Insulin-like growth factor I overexpression in human pancreatic cancer: evidence for autocrine and paracrine roles*. Cancer Res, 1995. **55**(10): p. 2007-11.
236. Macaulay, V.M., et al., *Downregulation of the type I insulin-like growth factor receptor in mouse melanoma cells is associated with enhanced radiosensitivity and impaired activation of Atm kinase*. Oncogene, 2001. **20**(30): p. 4029-4040.

237. Yeh, A.H., E.A. Bohula, and V.M. Macaulay, *Human melanoma cells expressing V600E B-RAF are susceptible to IGF1R targeting by small interfering RNAs*. *Oncogene*, 2006. **25**(50): p. 6574-6581.
238. Herkert, B., et al., *Maximizing the Efficacy of MAPK-Targeted Treatment in PTENLOF/BRAF(MUT) Melanoma through PI3K and IGF1R Inhibition*. *Cancer Research*, 2016. **76**(2): p. 390-402.
239. Suleymanova, N., et al., *Enhanced response of melanoma cells to MEK inhibitors following unbiased IGF-1R down-regulation*. *Oncotarget*, 2017. **8**(47): p. 82256-82267.
240. Worrall, C., et al., *Unbalancing p53/Mdm2/IGF-1R axis by Mdm2 activation restrains the IGF-1-dependent invasive phenotype of skin melanoma*. *Oncogene*, 2017.
241. Pietrzkowski, Z., et al., *Inhibition of growth of prostatic cancer cell lines by peptide analogues of insulin-like growth factor 1*. *Cancer Res*, 1993. **53**(5): p. 1102-6.
242. Smith, J.P. and T.E. Solomon, *Effects of gastrin, proglumide, and somatostatin on growth of human colon cancer*. *Gastroenterology*, 1988. **95**(6): p. 1541-8.
243. Pinski, J., et al., *Effects of somatostatin analogue RC-160 and bombesin/gastrin-releasing peptide antagonists on the growth of human small-cell and non-small-cell lung carcinomas in nude mice*. *Br J Cancer*, 1994. **70**(5): p. 886-92.
244. Pinski, J., et al., *Somatostatin analog RC-160 inhibits the growth of human osteosarcomas in nude mice*. *Int J Cancer*, 1996. **65**(6): p. 870-4.
245. Yang, X.F., et al., *Reduced growth of human breast cancer xenografts in hosts homozygous for the lit mutation*. *Cancer Res*, 1996. **56**(7): p. 1509-11.
246. Ofer, P., et al., *Both IGF1R and INSR Knockdown Exert Antitumorigenic Effects in Prostate Cancer In Vitro and In Vivo*. *Mol Endocrinol*, 2015. **29**(12): p. 1694-707.
247. Burfeind, P., et al., *Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo*. *Proc Natl Acad Sci U S A*, 1996. **93**(14): p. 7263-8.
248. Jungwirth, A., et al., *Inhibition of in vivo proliferation of androgen-independent prostate cancers by an antagonist of growth hormone-releasing hormone*. *Br J Cancer*, 1997. **75**(11): p. 1585-92.
249. Khandwala, H.M., et al., *The effects of insulin-like growth factors on tumorigenesis and neoplastic growth*. *Endocr Rev*, 2000. **21**(3): p. 215-44.
250. Yuen, J.S.P. and V.M. Macaulay, *Targeting the type I insulin-like growth factor receptor as a treatment for cancer*. *Expert Opinion on Therapeutic Targets*, 2008. **12**(5): p. 589-603.
251. Gualberto, A. and M. Pollak, *Emerging role of insulin-like growth factor receptor inhibitors in oncology: early clinical trial results and future directions*. *Oncogene*, 2009. **28**(34): p. 3009-21.
252. Tognon, C.E. and P.H.B. Sorensen, *Targeting the insulin-like growth factor 1 receptor (IGF1R) signaling pathway for cancer therapy*. *Expert Opinion on Therapeutic Targets*, 2012. **16**(1): p. 33-48.

253. Resnicoff, M., et al., *Growth inhibition of human melanoma cells in nude mice by antisense strategies to the type 1 insulin-like growth factor receptor*. *Cancer Res*, 1994. **54**(18): p. 4848-50.
254. Resnicoff, M., et al., *Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors*. *Cancer Res*, 1994. **54**(8): p. 2218-22.
255. Rohlik, Q.T., et al., *An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF-7 cells in tissue culture*. *Biochem Biophys Res Commun*, 1987. **149**(1): p. 276-81.
256. Kalebic, T., M. Tsokos, and L.J. Helman, *In vivo treatment with antibody against IGF-1 receptor suppresses growth of human rhabdomyosarcoma and down-regulates p34cdc2*. *Cancer Res*, 1994. **54**(21): p. 5531-4.
257. Buck, E. and M. Mulvihill, *Small molecule inhibitors of the IGF-1R/IR axis for the treatment of cancer*. *Expert Opin Investig Drugs*, 2011. **20**(5): p. 605-21.
258. Girnita, A., et al., *Cyclolignans as inhibitors of the insulin-like growth factor-1 receptor and malignant cell growth*. *Cancer Res*, 2004. **64**(1): p. 236-42.
259. Economou, M.A., et al., *Oral picropodophyllin (PPP) is well tolerated in vivo and inhibits IGF-1R expression and growth of uveal melanoma*. *Invest Ophthalmol Vis Sci*, 2008. **49**(6): p. 2337-42.
260. Chen, H.X. and E. Sharon, *IGF-1R as an anti-cancer target--trials and tribulations*. *Chin J Cancer*, 2013. **32**(5): p. 242-52.
261. Baserga, R., *The decline and fall of the IGF-1 receptor*. *J Cell Physiol*, 2013. **228**(4): p. 675-9.
262. Cohen, B.D., et al., *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*, 2005. **11**(5): p. 2063-73.
263. Becerra, C.R., et al., *Figitumumab in patients with refractory metastatic colorectal cancer previously treated with standard therapies: a nonrandomized, open-label, phase II trial*. *Cancer Chemother Pharmacol*, 2014. **73**(4): p. 695-702.
264. Di Maio, M. and G.V. Scagliotti, *The lesson learned from figitumumab clinical program and the hope for better results in squamous lung cancer*. *Transl Lung Cancer Res*, 2015. **4**(1): p. 15-7.
265. Yin, D., et al., *Pharmacokinetics and pharmacodynamics of figitumumab, a monoclonal antibody targeting the insulin-like growth factor 1 receptor, in healthy participants*. *J Clin Pharmacol*, 2013. **53**(1): p. 21-8.
266. de Bono, J.S., et al., *Phase II randomized study of figitumumab plus docetaxel and docetaxel alone with crossover for metastatic castration-resistant prostate cancer*. *Clin Cancer Res*, 2014. **20**(7): p. 1925-34.
267. Olmos, D., et al., *Safety, pharmacokinetics, and preliminary activity of the anti-IGF-1R antibody figitumumab (CP-751,871) in patients with sarcoma and Ewing's sarcoma: a phase I expansion cohort study*. *Lancet Oncol*, 2010. **11**(2): p. 129-35.
268. Zheng, H., et al., *beta-Arrestin-biased agonism as the central mechanism of action for insulin-like growth factor 1 receptor-targeting antibodies in Ewing's sarcoma*. *Proc Natl Acad Sci U S A*, 2012. **109**(50): p. 20620-5.

269. Crudden, C., et al., *The dichotomy of the Insulin-like growth factor 1 receptor: RTK and GPCR: friend or foe for cancer treatment?* Growth Horm IGF Res, 2015. **25**(1): p. 2-12.
270. Daub, H., et al., *Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors.* Nature, 1996. **379**(6565): p. 557-60.
271. Kruk, J.S., et al., *5-HT(1A) receptors transactivate the platelet-derived growth factor receptor type beta in neuronal cells.* Cell Signal, 2013. **25**(1): p. 133-43.
272. Shah, B.H. and K.J. Catt, *GPCR-mediated transactivation of RTKs in the CNS: mechanisms and consequences.* Trends Neurosci, 2004. **27**(1): p. 48-53.
273. Rajagopal, R. and M.V. Chao, *A role for Fyn in Trk receptor transactivation by G-protein-coupled receptor signaling.* Mol Cell Neurosci, 2006. **33**(1): p. 36-46.
274. Pyne, N.J. and S. Pyne, *Receptor tyrosine kinase-G-protein-coupled receptor signalling platforms: out of the shadow?* Trends in Pharmacological Sciences, 2011. **32**(8): p. 443-450.
275. Prenzel, N., et al., *EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF.* Nature, 1999. **402**(6764): p. 884-8.
276. Biscardi, J.S., et al., *c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function.* J Biol Chem, 1999. **274**(12): p. 8335-43.
277. Keely, S.J., S.O. Calandrella, and K.E. Barrett, *Carbachol-stimulated transactivation of epidermal growth factor receptor and mitogen-activated protein kinase in T(84) cells is mediated by intracellular Ca²⁺, PYK-2, and p60(src).* J Biol Chem, 2000. **275**(17): p. 12619-25.
278. Luttrell, L.M., et al., *G beta gamma subunits mediate mitogen-activated protein kinase activation by the tyrosine kinase insulin-like growth factor 1 receptor.* J Biol Chem, 1995. **270**(28): p. 16495-8.
279. Luttrell, L.M., et al., *Pertussis toxin treatment attenuates some effects of insulin in BC3H-1 murine myocytes.* J Biol Chem, 1988. **263**(13): p. 6134-41.
280. Luttrell, L., et al., *A pertussis toxin-sensitive G-protein mediates some aspects of insulin action in BC3H-1 murine myocytes.* J Biol Chem, 1990. **265**(28): p. 16873-9.
281. Hallak, H., et al., *Association of heterotrimeric G(i) with the insulin-like growth factor-1 receptor - Release of G(beta gamma) subunits upon receptor activation.* Journal of Biological Chemistry, 2000. **275**(4): p. 2255-2258.
282. Dalle, S., et al., *Insulin and insulin-like growth factor I receptors utilize different G protein signaling components.* J Biol Chem, 2001. **276**(19): p. 15688-95.
283. Girmita, L., et al., *{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, 2005. **280**(26): p. 24412-9.
284. Lin, F.T., Y. Daaka, and R.J. Lefkowitz, *beta-arrestins regulate mitogenic signaling and clathrin-mediated endocytosis of the insulin-like growth factor I receptor.* Journal of Biological Chemistry, 1998. **273**(48): p. 31640-31643.

285. Zheng, H., et al., *Selective recruitment of G protein-coupled receptor kinases (GRKs) controls signaling of the insulin-like growth factor 1 receptor*. Proc Natl Acad Sci U S A, 2012. **109**(18): p. 7055-60.
286. Waters, C., S. Pyne, and N.J. Pyne, *The role of G-protein coupled receptors and associated proteins in receptor tyrosine kinase signal transduction*. Seminars in Cell & Developmental Biology, 2004. **15**(3): p. 309-323.
287. Piiper, A., D. StryjekKaminska, and S. Zeuzem, *Epidermal growth factor activates phospholipase C-gamma(1) via G(i1-2) proteins in isolated pancreatic acinar membranes*. American Journal of Physiology-Gastrointestinal and Liver Physiology, 1997. **272**(5): p. G1276-G1284.
288. Zeng, H., D. Zhao, and D. Mukhopadhyay, *KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA*. J Biol Chem, 2002. **277**(48): p. 46791-8.
289. Zeng, H.Y., et al., *Heterotrimeric G alpha(q)/G alpha(11) proteins function upstream of vascular endothelial growth factor (VEGF) receptor-2 (KDR) phosphorylation in vascular permeability factor/VEGF signaling*. Journal of Biological Chemistry, 2003. **278**(23): p. 20738-20745.
290. Conway, A.M., et al., *Platelet-derived-growth-factor stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle: role of pertussis-toxin-sensitive G-proteins, c-Src tyrosine kinases and phosphoinositide 3-kinase*. Biochem J, 1999. **337** (Pt 2): p. 171-7.
291. Alderton, F., et al., *Tethering of the platelet-derived growth factor ss receptor to G-protein-coupled receptors - A novel platform for integrative signaling by these receptor classes in mammalian cells*. Journal of Biological Chemistry, 2001. **276**(30): p. 28578-28585.
292. Usui, I., et al., *GRK2 is an endogenous protein inhibitor of the insulin signaling pathway for glucose transport stimulation*. EMBO J, 2004. **23**(14): p. 2821-9.
293. Freedman, N.J., et al., *Phosphorylation of the platelet-derived growth factor receptor-beta and epidermal growth factor receptor by G protein-coupled receptor kinase-2 - Mechanisms for selectivity of desensitization*. Journal of Biological Chemistry, 2002. **277**(50): p. 48261-48269.
294. Gavard, J. and J.S. Gutkind, *VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin*. Nature Cell Biology, 2006. **8**(11): p. 1223-U17.
295. Girnita, L., et al., *Beta-arrestin and Mdm2 mediate IGF-1 receptor-stimulated ERK activation and cell cycle progression*. J Biol Chem, 2007. **282**(15): p. 11329-38.
296. Girnita, A., et al., *Identification of the cathelicidin peptide LL-37 as agonist for the type I insulin-like growth factor receptor*. Oncogene, 2012. **31**(3): p. 352-65.
297. Vasilcanu, R., et al., *Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor: potential mechanistic involvement of Mdm2 and beta-arrestin1*. Oncogene, 2008. **27**(11): p. 1629-38.
298. Bareja, A., et al., *Understanding the mechanism of bias signaling of the insulin-like growth factor 1 receptor: Effects of LL37 and HASF*. Cell Signal, 2018. **46**: p. 113-119.

299. Daub, H., et al., *Signal characteristics of G protein-transactivated EGF receptor*. EMBO J, 1997. **16**(23): p. 7032-44.
300. Deng, H., et al., *Over-accumulation of nuclear IGF-1 receptor in tumor cells requires elevated expression of the receptor and the SUMO-conjugating enzyme Ubc9*. Biochem Biophys Res Commun, 2011. **404**(2): p. 667-71.
301. Sehat, B., et al., *SUMOylation mediates the nuclear translocation and signaling of the IGF-1 receptor*. Sci Signal, 2010. **3**(108): p. ra10.
302. Scotlandi, K. and A. Belfiore, *Targeting the Insulin-Like Growth Factor (IGF) System Is Not as Simple as Just Targeting the Type 1 IGF Receptor*. Am Soc Clin Oncol Educ Book, 2012: p. 599-604.
303. Worrall, C., et al., *Novel mechanisms of regulation of IGF-1R action: functional and therapeutic implications*. Pediatr Endocrinol Rev, 2013. **10**(4): p. 473-84.
304. Pollak, M., *The insulin and insulin-like growth factor receptor family in neoplasia: an update*. Nature Reviews Cancer, 2012. **12**(3): p. 159-169.
305. Galandrin, S., G. Oligny-Longpre, and M. Bouvier, *The evasive nature of drug efficacy: implications for drug discovery*. Trends Pharmacol Sci, 2007. **28**(8): p. 423-30.
306. Kanter-Lewensohn, L., et al., *Expression of insulin-like growth factor-1 receptor (IGF-1R) and p27Kip1 in melanocytic tumors: a potential regulatory role of IGF-1 pathway in distribution of p27Kip1 between different cyclins*. Growth Factors, 2000. **17**(3): p. 193-202.
307. Lee, J.T., P. Brafford, and M. Herlyn, *Unraveling the mysteries of IGF-1 signaling in melanoma*. J Invest Dermatol, 2008. **128**(6): p. 1358-60.
308. Molhoek, K.R., et al., *Comprehensive analysis of receptor tyrosine kinase activation in human melanomas reveals autocrine signaling through IGF-1R*. Melanoma Research, 2011. **21**(4): p. 274-284.
309. Satyamoorthy, K., et al., *Insulin-like growth factor-1 induces survival and growth of biologically early melanoma cells through both the mitogen-activated protein kinase and beta-catenin pathways*. Cancer Res, 2001. **61**(19): p. 7318-24.
310. Karasic, T.B., T.K. Hei, and V.N. Ivanov, *Disruption of IGF-1R signaling increases TRAIL-induced apoptosis: A new potential therapy for the treatment of melanoma*. Experimental Cell Research, 2010. **316**(12): p. 1994-2007.
311. Neudauer, C.L. and J.B. McCarthy, *Insulin-like growth factor I-stimulated melanoma cell migration requires phosphoinositide 3-kinase but not extracellular-regulated kinase activation*. Experimental Cell Research, 2003. **286**(1): p. 128-137.
312. Puzanov, I., et al., *A Phase I Study of Continuous Oral Dosing of OSI-906, a Dual Inhibitor of Insulin-Like Growth Factor-1 and Insulin Receptors, in Patients with Advanced Solid Tumors*. Clinical Cancer Research, 2015. **21**(4): p. 701-711.
313. Mahadevan, D., et al., *Phase 1b study of safety, tolerability and efficacy of R1507, a monoclonal antibody to IGF-1R in combination with multiple standard oncology regimens in patients with advanced solid malignancies*. Cancer Chemotherapy and Pharmacology, 2014. **73**(3): p. 467-473.
314. Macaulay, V.M., et al., *Phase I study of humanized monoclonal antibody AVE1642 directed against the type 1 insulin-like growth factor receptor (IGF-1R), administered*

- in combination with anticancer therapies to patients with advanced solid tumors. Annals of Oncology, 2013. 24(3): p. 784-791.*
315. Kanter-Lewensohn, L., et al., *Expression of the insulin-like growth factor-1 receptor and its anti-apoptotic effect in malignant melanoma: a potential therapeutic target. Melanoma Research, 1998. 8(5): p. 389-397.*
 316. Vasilcanu, D., et al., *The insulin-like growth factor-1 receptor inhibitor PPP produces only very limited resistance in tumor cells exposed to long-term selection. Oncogene, 2006. 25(22): p. 3186-95.*
 317. Chalmers, Z.R., et al., *Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med, 2017. 9(1): p. 34.*
 318. Villanueva, J., et al., *Acquired Resistance to BRAF Inhibitors Mediated by a RAF Kinase Switch in Melanoma Can Be Overcome by Cotargeting MEK and IGF-1R/PI3K. Cancer Cell, 2010. 18(6): p. 683-695.*
 319. Obenauf, A.C., et al., *Therapy-induced tumour secretomes promote resistance and tumour progression. Nature, 2015. 520(7547): p. 368-72.*
 320. Savage, S.A., et al., *Analysis of genes critical for growth regulation identifies Insulin-like Growth Factor 2 Receptor variations with possible functional significance as risk factors for osteosarcoma. Cancer Epidemiol Biomarkers Prev, 2007. 16(8): p. 1667-74.*
 321. Delattre, O., et al., *The Ewing family of tumors--a subgroup of small-round-cell tumors defined by specific chimeric transcripts. N Engl J Med, 1994. 331(5): p. 294-9.*
 322. Prieur, A., et al., *EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. Mol Cell Biol, 2004. 24(16): p. 7275-83.*
 323. Riggi, N., et al., *Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. Cancer Res, 2005. 65(24): p. 11459-68.*
 324. Riggi, N. and I. Stamenkovic, *The Biology of Ewing sarcoma. Cancer Lett, 2007. 254(1): p. 1-10.*
 325. Scotlandi, K., et al., *Insulin-like growth factor I receptor-mediated circuit in Ewing's sarcoma/peripheral neuroectodermal tumor: a possible therapeutic target. Cancer Res, 1996. 56(20): p. 4570-4.*
 326. Pappo, A.S., et al., *A phase 2 trial of R1507, a monoclonal antibody to the insulin-like growth factor-1 receptor (IGF-1R), in patients with recurrent or refractory rhabdomyosarcoma, osteosarcoma, synovial sarcoma, and other soft tissue sarcomas: Results of a Sarcoma Alliance for Research Through Collaboration study. Cancer, 2014.*
 327. Pappo, A.S., et al., *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, 2011. 29(34): p. 4541-7.*
 328. van de Luijngaarden, A.C., et al., *Prognostic and therapeutic relevance of the IGF pathway in Ewing's sarcoma patients. Target Oncol, 2013. 8(4): p. 253-60.*

329. Manteniotis, S., et al., *Comprehensive RNA-Seq expression analysis of sensory ganglia with a focus on ion channels and GPCRs in Trigeminal ganglia*. PLoS One, 2013. **8**(11): p. e79523.
330. Pronin, A., et al., *Expression of olfactory signaling genes in the eye*. PLoS One, 2014. **9**(4): p. e96435.
331. Yu, Y., et al., *A comparative analysis of liver transcriptome suggests divergent liver function among human, mouse and rat*. Genomics, 2010. **96**(5): p. 281-9.
332. Jonchere, V. and D. Bennett, *Validating RNAi phenotypes in Drosophila using a synthetic RNAi-resistant transgene*. PLoS One, 2013. **8**(8): p. e70489.
333. Gurevich, V.V. and E.V. Gurevich, *Analyzing the roles of multi-functional proteins in cells: The case of arrestins and GRKs*. Crit Rev Biochem Mol Biol, 2015. **50**(5): p. 440-52.
334. Natalishvili, N., et al., *Aberrant intracellular IGF-1R beta-subunit makes receptor knockout cells (IGF1R^{-/-}) susceptible to oncogenic transformation*. Exp Cell Res, 2009. **315**(8): p. 1458-67.
335. Nakajima, K. and J. Wess, *Design and functional characterization of a novel, arrestin-biased designer G protein-coupled receptor*. Mol Pharmacol, 2012. **82**(4): p. 575-82.
336. Peterson, S.M., et al., *Elucidation of G-protein and beta-arrestin functional selectivity at the dopamine D2 receptor*. Proc Natl Acad Sci U S A, 2015. **112**(22): p. 7097-102.
337. Taylor, S.S., et al., *Pseudokinases from a structural perspective*. Biochem Soc Trans, 2013. **41**(4): p. 981-6.
338. Gibbs, C.S. and M.J. Zoller, *Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions*. J Biol Chem, 1991. **266**(14): p. 8923-31.
339. Ohno, S., et al., *A point mutation at the putative ATP-binding site of protein kinase C alpha abolishes the kinase activity and renders it down-regulation-insensitive. A molecular link between autophosphorylation and down-regulation*. J Biol Chem, 1990. **265**(11): p. 6296-300.
340. Nutt, D.J., et al., *Mechanisms of action of selective serotonin reuptake inhibitors in the treatment of psychiatric disorders*. European Neuropsychopharmacology, 1999. **9**: p. S81-S86.
341. Kim, Y.K., *Extracellular microRNAs as Biomarkers in Human Disease*. Chonnam Med J, 2015. **51**(2): p. 51-7.
342. Lan, H., et al., *MicroRNAs as potential biomarkers in cancer: opportunities and challenges*. Biomed Res Int, 2015. **2015**: p. 125094.
343. Cortez, M.A., J.W. Welsh, and G.A. Calin, *Circulating microRNAs as noninvasive biomarkers in breast cancer*. Recent Results Cancer Res, 2012. **195**: p. 151-61.
344. Rafahi, H., et al., *Clonogenic assay: adherent cells*. J Vis Exp, 2011(49).
345. Puck, T.T. and P.I. Marcus, *Action of x-rays on mammalian cells*. J Exp Med, 1956. **103**(5): p. 653-66.

346. Fukazawa, H., S. Mizuno, and Y. Uehara, *A microplate assay for quantitation of anchorage-independent growth of transformed cells*. *Anal Biochem*, 1995. **228**(1): p. 83-90.
347. Shtivelman, E., et al., *Pathways and therapeutic targets in melanoma*. *Oncotarget*, 2014. **5**(7): p. 1701-52.
348. Wellbrock, C., *MAPK pathway inhibition in melanoma: resistance three ways*. *Biochem Soc Trans*, 2014. **42**(4): p. 727-32.
349. Nazarian, R., et al., *Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation*. *Nature*, 2010. **468**(7326): p. 973-7.
350. DeWire, S.M., et al., *Beta-arrestins and cell signaling*. *Annu Rev Physiol*, 2007. **69**: p. 483-510.
351. McDonald, P.H. and R.J. Lefkowitz, *Beta-Arrestins: new roles in regulating heptahelical receptors' functions*. *Cell Signal*, 2001. **13**(10): p. 683-9.
352. Luttrell, L.M. and D. Gesty-Palmer, *Beyond desensitization: physiological relevance of arrestin-dependent signaling*. *Pharmacol Rev*, 2010. **62**(2): p. 305-30.
353. Larsson, O., A. Girnita, and L. Girnita, *Role of insulin-like growth factor 1 receptor signalling in cancer*. *Br J Cancer*, 2005. **92**(12): p. 2097-101.
354. Fischer, S.E., *RNA Interference and MicroRNA-Mediated Silencing*. *Curr Protoc Mol Biol*, 2015. **112**: p. 26 1 1-5.
355. Ghosh, A.K., et al., *Aberrant regulation of pVHL levels by microRNA promotes the HIF/VEGF axis in CLL B cells*. *Blood*, 2009. **113**(22): p. 5568-74.
356. Calin, G.A. and C.M. Croce, *Chronic lymphocytic leukemia: interplay between noncoding RNAs and protein-coding genes*. *Blood*, 2009. **114**(23): p. 4761-70.
357. Li, J., et al., *MicroRNAs as novel biological targets for detection and regulation*. *Chem Soc Rev*, 2014. **43**(2): p. 506-17.
358. Hao, H., et al., *Diagnostic and prognostic value of miR-106a in colorectal cancer*. *Oncotarget*, 2017. **8**(3): p. 5038-5047.
359. Tusong, H., et al., *Functional analysis of serum microRNAs miR-21 and miR-106a in renal cell carcinoma*. *Cancer Biomark*, 2017. **18**(1): p. 79-85.
360. Edatt, L., et al., *MicroRNA106a regulates matrix metalloprotease 9 in a sirtuin-1 dependent mechanism*. *J Cell Physiol*, 2018. **233**(1): p. 238-248.
361. Li, X., et al., *MicroRNA-106a promotes cell migration and invasion by targeting tissue inhibitor of matrix metalloproteinase 2 in cervical cancer*. *Oncol Rep*, 2017. **38**(3): p. 1774-1782.
362. Chen, L., et al., *MicroRNA-106a regulates phosphatase and tensin homologue expression and promotes the proliferation and invasion of ovarian cancer cells*. *Oncol Rep*, 2016. **36**(4): p. 2135-41.
363. Pan, Y.J., et al., *MiR-106a: Promising biomarker for cancer*. *Bioorg Med Chem Lett*, 2016. **26**(22): p. 5373-5377.
364. Koppers, D.A., et al., *The miR-106a~363(Xpcl1) miRNA cluster induces murine T cell lymphoma despite transcriptional activation of the p27(Kip1) cell cycle inhibitor*. *Oncotarget*, 2017. **8**(31): p. 50680-50691.

365. Basu, B., D. Olmos, and J.S. de Bono, *Targeting IGF-1R: throwing out the baby with the bathwater?* Br J Cancer, 2011. **104**(1): p. 1-3.
366. Suleymanova, N., et al., *Functional antagonism of beta-arrestin isoforms balance IGF-1R expression and signalling with distinct cancer-related biological outcomes.* Oncogene, 2017.
367. Rask-Andersen, M., et al., *Advances in kinase targeting: current clinical use and clinical trials.* Trends Pharmacol Sci, 2014. **35**(11): p. 604-20.
368. Drake, J.M., J.K. Lee, and O.N. Witte, *Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer.* Mol Cell Biol, 2014. **34**(10): p. 1722-32.
369. Cohen, P. and D.R. Alessi, *Kinase drug discovery--what's next in the field?* ACS Chem Biol, 2013. **8**(1): p. 96-104.
370. Liggett, S.B., *Phosphorylation barcoding as a mechanism of directing GPCR signaling.* Sci Signal, 2011. **4**(185): p. pe36.
371. Thal, D.M., et al., *Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility.* ACS Chem Biol, 2012. **7**(11): p. 1830-9.
372. Salcedo, A., F. Mayor, and P. Penela, *Mdm2 is involved in the ubiquitination and degradation of G-protein-coupled receptor kinase 2.* Embo Journal, 2006. **25**(20): p. 4752-4762.
373. Kommaddi, R.P. and S.K. Shenoy, *Arrestins and protein ubiquitination.* Prog Mol Biol Transl Sci, 2013. **118**: p. 175-204.
374. Jean-Charles, P.Y., V. Rajiv, and S.K. Shenoy, *Ubiquitin-Related Roles of beta-Arrestins in Endocytic Trafficking and Signal Transduction.* J Cell Physiol, 2016. **231**(10): p. 2071-80.
375. Komolov, K.E. and J.L. Benovic, *G protein-coupled receptor kinases: Past, present and future.* Cell Signal, 2018. **41**: p. 17-24.
376. Homan, K.T., et al., *Crystal Structure of G Protein-coupled Receptor Kinase 5 in Complex with a Rationally Designed Inhibitor.* J Biol Chem, 2015. **290**(34): p. 20649-59.
377. Waldschmidt, H.V., et al., *Structure-Based Design of Highly Selective and Potent G Protein-Coupled Receptor Kinase 2 Inhibitors Based on Paroxetine.* Journal of Medicinal Chemistry, 2017. **60**(7): p. 3052-3069.
378. Rengo, G., et al., *Targeting the beta-adrenergic receptor system through G-protein-coupled receptor kinase 2: a new paradigm for therapy and prognostic evaluation in heart failure: from bench to bedside.* Circ Heart Fail, 2012. **5**(3): p. 385-91.
379. Huang, Z.M., et al., *GRK2 in the heart: a GPCR kinase and beyond.* Antioxid Redox Signal, 2014. **21**(14): p. 2032-43.
380. Rengo, G., et al., *Impact of diabetes mellitus on lymphocyte GRK2 protein levels in patients with heart failure.* Eur J Clin Invest, 2015. **45**(2): p. 187-95.
381. Dorn, G.W., 2nd, *GRK mythology: G-protein receptor kinases in cardiovascular disease.* J Mol Med (Berl), 2009. **87**(5): p. 455-63.

382. Santulli, G., B. Trimarco, and G. Iaccarino, *G-protein-coupled receptor kinase 2 and hypertension: molecular insights and pathophysiological mechanisms*. High Blood Press Cardiovasc Prev, 2013. **20**(1): p. 5-12.
383. Eckhart, A.D., et al., *Vascular-targeted overexpression of G protein-coupled receptor kinase-2 in transgenic mice attenuates beta-adrenergic receptor signaling and increases resting blood pressure*. Mol Pharmacol, 2002. **61**(4): p. 749-58.
384. Han, C.C., et al., *Regulatory effects of GRK2 on GPCRs and non-GPCRs and possible use as a drug target*. International Journal of Molecular Medicine, 2016. **38**(4): p. 987-994.
385. Bax, M., et al., *Genetics of rheumatoid arthritis: what have we learned?* Immunogenetics, 2011. **63**(8): p. 459-66.
386. Bartok, B. and G.S. Firestein, *Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis*. Immunol Rev, 2010. **233**(1): p. 233-55.
387. Chen, J.Y., et al., *Paeoniflorin inhibits proliferation of fibroblast-like synoviocytes through suppressing G-protein-coupled receptor kinase 2*. Planta Med, 2012. **78**(7): p. 665-71.
388. King, D.W., et al., *Differential expression of GRK isoforms in nonmalignant and malignant human granulosa cells*. Endocrine, 2003. **22**(2): p. 135-42.
389. Metaye, T., et al., *Expression and activity of g protein-coupled receptor kinases in differentiated thyroid carcinoma*. J Clin Endocrinol Metab, 2002. **87**(7): p. 3279-86.
390. Prowatke, I., et al., *Expression analysis of imbalanced genes in prostate carcinoma using tissue microarrays*. Br J Cancer, 2007. **96**(1): p. 82-8.
391. Homan, K.T., et al., *Structural and functional analysis of g protein-coupled receptor kinase inhibition by paroxetine and a rationally designed analog*. Mol Pharmacol, 2014. **85**(2): p. 237-48.
392. Shukla, A.K., *Biasing GPCR signaling from inside*. Sci Signal, 2014. **7**(310): p. pe3.
393. Smith, J.S., R.J. Lefkowitz, and S. Rajagopal, *Biased signalling: from simple switches to allosteric microprocessors*. Nat Rev Drug Discov, 2018.
394. Volinia, S., et al., *A microRNA expression signature of human solid tumors defines cancer gene targets*. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(7): p. 2257-2261.